

# Cell Volume and Insulin Signaling

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Perturbations of cell hydration as provoked by changes in ambient osmolarity or under isoosmotic conditions by hormones, second messengers, intracellular substrate accumulation, or reactive oxygen intermediates critically contribute to the physiological regulation of cell function. In general an increase in cell hydration stimulates anabolic metabolism and proliferation and provides cytoprotection, whereas cellular dehydration leads to a catabolic situation and sensitizes cells to apoptotic stimuli. Insulin produces cell swelling by inducing a net K<sup>+</sup> and Na<sup>+</sup> accumulation inside the cell, which results from a concerted activation of Na<sup>+</sup>/H<sup>+</sup> exchange, Na<sup>+</sup>/K<sup>+</sup>/2Cl<sup>-</sup> symport, and the Na<sup>+</sup>/K<sup>+</sup>-ATPase. In the liver, insulin-induced cell swelling is critical for stimulation of glycogen and protein synthesis as well as inhibition of autophagic proteolysis. These insulin effects can largely be mimicked by hypoosmotic cell swelling, pointing to a role of cell swelling as a trigger of signal transduction. This article discusses insulin-induced signal transduction upstream of swelling and introduces the hypothesis that cell swelling as a signal amplifier represents an essential component in insulin signaling, which contributes to the full response to insulin at the level of signal transduction and function. Cellular dehydration impairs insulin signaling and may be a major cause of insulin resistance, which develops in systemic hyperosmolarity, nutrient deprivation, uremia, oxidative challenges, and unbalanced production of insulin-counteracting hormones. Hydration changes affect cell functions at multiple levels (such as transcriptom, proteom, phosphoproteom, and the metabolom) and a systems biological approach may allow us to develop a more holistic view on the hydration dependence of insulin signaling in the future.

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## I. Introduction

Insulin is the most important hormone that shifts cellular metabolism toward an anabolic state (Kahn, 1994). One prominent effect of insulin is the postprandial maintenance of glucose homeostasis. One underlying mechanism is the upregulation of glucose uptake in skeletal muscle and adipose tissue due to the insertion of intracellularly stored glucose transporting peptides into the plasma membrane. Other insulin effects on metabolism include increased amino acid uptake, stimulation of glycolysis, as well as synthesis of glycogen, proteins, fatty acids, and triglycerides. Insulin stimulates DNA synthesis and cell proliferation and exerts antiapoptotic effects at least in some cell types. Simultaneously, insulin inhibits catabolic pathways such as gluconeogenesis and glycogenolysis, as well as the degradation of lipids and proteins. Important physiological antagonists of insulin are glucagon and catecholamines, which trigger a catabolic metabolism.

Binding of insulin to its receptor triggers multiple integrated signaling pathways mediating the pleiotropic effects of the hormone (Avruch, 1998; Taha and Klip, 1999; Nystrom and Quon, 1999; Virkamaki *et al.*, 1999; Elchebly *et al.*, 2000; Cheng *et al.*, 2002; Lizcano and Alessi, 2002). Protein kinases and phosphatases are important players in cellular signal transduction. Much effort has been devoted to the identification of specific signaling pathways, which link the activated insulin/insulin receptor complex to the distinct cellular responses. The pathways identified are also addressed by many other factors and some of them induce metabolic changes different from those induced by insulin. Understanding how signal specificity is achieved is currently a major topic in signal transduction research. The components that constitute the signal transduction pathway represent one aspect. Other factors determining the specific outcome of a signal transduction pathway include cross-talk with other pathways, the cell-type-specific signaling background, and the organization of signaling modules within the cellular matrix. From a system biological point of view intracellular signal components are organized as a complex network rather than forming linear pathways.

About 10 years ago it was found that cell hydration changes critically contribute to the control and regulation of cell function under physiological conditions (Häussinger and Lang, 1991, 1992; later reviewed in Häussinger *et al.*, 1994; Häussinger and Schliess, 1995; McManus *et al.*, 1995; Häussinger, 1996a,b; Lang *et al.*, 1998; Kultz and Burg, 1998). In the liver, insulin stimulates cellular retention of  $K^+$ , associated with an osmotic water flux into the hepatocyte, which results in an increase in cell hydration, i.e., cell swelling. Insulin-induced swelling mediates inhibition of autophagic proteolysis as well as stimulation of protein and glycogen synthesis. Effects of insulin on proteolysis and glycogen and protein synthesis can be largely mimicked by liver cell swelling induced by either hypoosmolarity or concentrative uptake of amino acids such as glutamine. Glucagon reverses insulin-induced cell swelling and thereby antagonizes metabolic insulin effects.

The same is true if the insulin-treated liver is exposed to hyperosmolarity. These observations were consistent with the clinical experience that dehydration of insulin target tissues is associated with catabolism and insulin resistance and that correction of dehydration by adequate fluid supply improves the sensitivity of diabetic patients to therapeutically applied insulin (Waldhäusl, 1992; Häussinger *et al.*, 1993a).

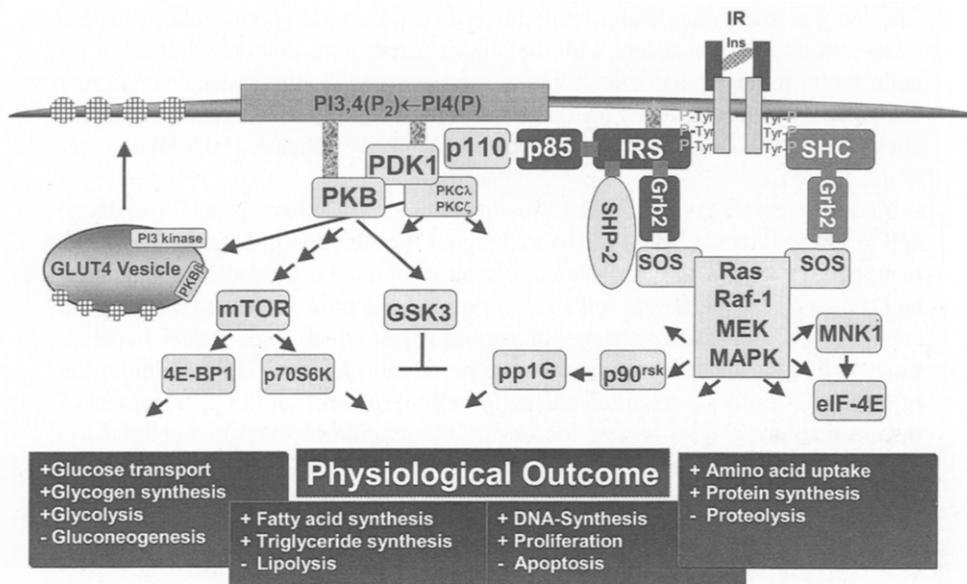
Recent interest focuses on the following questions: (1) How is insulin-induced cell swelling integrated into the overall signal transduction network activated by insulin? In other words: which signaling components act upstream of K<sup>+</sup> retention and cell swelling and how is cell swelling sensed and transduced into downstream signaling via covalent protein modifications? (2) At which level(s) does hyperosmotic dehydration impair insulin-induced signal transduction? (3) Is the inhibition of insulin-induced K<sup>+</sup> retention and cell swelling relevant for the development of insulin resistance? This review focuses on the relationship between cellular hydration, insulin signaling, and the sensitivity of cell metabolism toward regulation by insulin.

## **II. Insulin Signaling**

Much information is available regarding insulin-induced signaling cascades, which include differential changes in protein Ser/Thr and Tyr phosphorylation and the assembly of signaling modules by alterations of protein–protein interactions. Excellent recent reviews have summarized current knowledge about the insulin-responsive signaling network (e.g., Avruch, 1998; Taha and Klip, 1999; Nystrom and Quon, 1999; Virkamaki *et al.*, 1999; Elchebly *et al.*, 2000; Cheng *et al.*, 2002; Lizcano and Alessi, 2002). Here a brief overview is given about the signaling molecules most important in the context of this article (Fig. 1).

### **A. Insulin Receptor and Insulin Receptor Substrates**

Activation of the insulin receptor (IR) upon insulin binding initiates insulin signaling. The IR is a receptor tyrosine kinase comprising two extracellular  $\alpha$ -subunits and two transmembrane  $\beta$ -subunits (Fig. 1). Binding of insulin to the extracellular  $\alpha$ -subunits induces a rapid conformational change of the receptor that releases the intracellular tyrosine kinase activity of the  $\beta$ -subunits. This leads to *trans-autophosphorylation* of the  $\beta$ -subunits at different tyrosine residues and allows recruitment and multiple tyrosine phosphorylation of substrates such as the insulin receptor substrate-1 (IRS-1) and Shc. Tyrosine-phosphorylated substrates of the IR serve as interfaces between the receptor and various signaling proteins, which contain src homology-2 (SH2) domains. Binding of SH2 proteins to IR



**FIG. 1** Insulin-responsive signaling molecules. The scheme illustrates only a part of the complex signaling network activated by insulin. Extracellular binding of insulin (ins) to its receptor (IR) activates the intracellularly located IR-tyrosine kinase. Autophosphorylation of tyrosine residues generates docking sites for proteins with protein tyrosine binding domains such as the IRS proteins and Shc. In addition IRS proteins contact the plasma membrane by their pleckstrin domains. Following tyrosine phosphorylation by the IR kinase, IRS protein and Shc serve as adaptors for proteins recognizing phosphotyrosine residues by their SH2 domains. Both IRS and Shc can bind the growth factor receptor binding protein Grb2, which is associated with the guanine nucleotide exchange factor SOS. Activation of SOS leads to the exchange of GDP to GTP bound to Ras. This triggers activation of the Ras/Raf pathway toward the extracellular signal-regulated kinases Erk-1/Erk-2. Similar signaling modules are involved in activation of the p38- and JNK-type MAP-kinases (not shown in the figure). Via activation of the MAP-kinase-interacting kinase MNK1, Erk-1/Erk-2 lead to phosphorylation of the eukaryotic initiation factor 4E (eIF-4E) involved in regulation of translation. Via MAP-kinase-activated kinase 1 (MAPKAP1 = p90<sup>rsk</sup>) Erks were suggested earlier to be involved in activation of the glycogen-bound protein phosphatase PP1G, which dephosphorylates and thereby activates the glycogen synthetase. Another pathway that branches off from the receptor substrates is the PtdIns-3-kinase pathway, which plays a major role in mediating metabolic insulin effects. Docking of the regulatory subunit p85 via SH2 domain recruits and activates the PtdIns-3-kinase catalytic subunit p110. PtdIns-3-kinase activity leads to phosphorylation of phosphatidylinositol thereby generating docking sites for phosphoinositide-dependent kinases such as PDK1. Probably PDK1 is of importance for recruitment to the plasma membrane and activation of PKB and the atypical PKCs  $\zeta$  and  $\lambda$ . PKB and the PKC  $\zeta/\lambda$  are essential for the targeting of glucose transporter GLUT4-bearing vesicles to the plasma membranes, leading to insertion of additional GLUT4 molecules into the plasma membrane. Inactivation of the glycogen synthase kinase-3 (GSK-3) by phosphorylation stimulates glycogen synthesis. Activation of the mammalian target of rapamycin (mTOR) leads to activation of the p70S6 kinase and phosphorylation of the eIF-4E-binding protein-1 (4E-BP1), which are both involved in regulation of protein synthesis. The physiological outcome of insulin action is determined by the whole insulin-responsive network but also cell type- and environment-specific features of the cellular proteome and metabolism.

substrates stimulates enzymatic activities and juxtaposes heterogeneous signaling molecules close together to generate a composite signal. Finally, the receptor substrate/SH2 protein complexes are free to propagate the signals independent from the insulin/IR complex, which may be internalized. A detailed survey on insulin receptor activation and the differential involvement of insulin receptor substrates in insulin signaling has been given (Yenush and White, 1997; White, 1997, 1998; Hubbard, 1999; Virkamaki *et al.*, 1999).

## B. MAP-Kinase Pathways

Mitogen-activated protein kinases (MAPK) are important signaling components in regulation of cell growth and differentiation and the cellular response to environmental stress (Kyriakis and Avruch, 2001; Waskiewicz and Cooper, 1995; Robinson and Cobb, 1997; Elion, 1998; Kultz, 1998; Widmann *et al.*, 1999). The extracellular signal-regulated kinases Erk-1 and Erk-2 are primarily responsive to mitogens and growth factors. Insulin signaling toward Erk-1/Erk-2 involves engagement of adaptor proteins such as the growth factor receptor binding protein-2 (Grb-2) and the tyrosine phosphatase SHP2 by the tyrosine-phosphorylated Shc and/or IRS proteins (Fig. 1). Grb-2 is associated with the guanine nucleotide exchange factor SOS, which catalyzes the transition of inactive GDP-Ras into the active GTP-Ras. GTP-Ras associates with and activates the Ser/Thr kinase Raf-1, which phosphorylates and activates the MAP-kinase kinase MEK. MEK represents a dual specificity kinase, which activates Erk-1/Erk-2 by phosphorylation on both Thr and Tyr within a specific Thr-Glu-Tyr motif localized in the activation loop. The exact contribution of Erks in producing insulin effects is not yet clear as discussed some years ago (Denton and Tavare, 1995). Erk-1/Erk-2 are linked to the glycogen-bound form of the protein phosphatase 1 (PP1G) via activation of the p90 ribosomal S6 kinase-2 (p90rsk2, Fig. 1), thereby establishing a potential connection of the Erk pathway to activation of glycogen synthase. Via phosphorylation of the eukaryotic initiation factor 4E (eIF-4E) by the MAP-kinase interacting kinase MNK1 the Erks could be involved in regulation of translation (Waskiewicz *et al.*, 1997; Wang *et al.*, 1998; Knauf *et al.*, 2001) (Fig. 1). Insulin-induced Erk activation can contribute to the regulation of expression of specific genes, e.g., coding for 5-aminolevulinate synthase (Scassa *et al.*, 2001) or c-fos (Denton and Tavare, 1995; Xi *et al.*, 1997; Sale *et al.*, 1999) as well as to an increased DNA synthesis (Xi *et al.*, 1997; Dixon *et al.*, 1999). In addition, some evidence points to a contribution of Erk-1/Erk-2 to the choleric insulin effect in liver (Häussinger *et al.*, 2000).

Also MAP-kinases of the c-Jun-N-terminal kinase (JNK) and the p38 type, which were originally described as highly responsive to stress and cytokines, are activated by insulin (Häussinger *et al.*, 1999; Igarashi *et al.*, 2000; Fukunaga *et al.*, 2000). JNKs participate in insulin-induced glycogen synthase activation in skeletal

muscle (Moxham *et al.*, 1996) and play a role in AP-1 activation by insulin (Miller *et al.*, 1996). Activation of p38 by insulin mediates inhibition of hepatic proteolysis (Häussinger *et al.*, 1999) and participates in the regulation of glucose transport in 3T3-L1 adipocytes and L6 myotubes (Sweeney *et al.*, 1999; Somwar *et al.*, 2001; Fujishiro *et al.*, 2001; Huang *et al.*, 2002). Activation of both Erks and p38 is essential for activation of the transcription factor ATF2 by insulin (Ouwens *et al.*, 2002).

MAP-kinase phosphatases of dual specificity (MKPs) such as the MKP-1 substantially contribute to the termination of growth factor-induced MAP-kinase signals by dephosphorylation of the Thr/Tyr residues critical for activation (Keyse, 1995; Camps *et al.*, 2000). MKP-1 induction by insulin leads to MAP-kinase inhibition within a feedback loop: activation of MAP-kinases by insulin induces MKP-1 expression, which in turn inactivates the MAP-kinases (Begum *et al.*, 1998; Byon *et al.*, 2001). The transience of MAP-kinase signals is important for the nature of the physiological response to insulin. Thus, regulation of cell survival and proliferation by insulin critically depend on JNK inhibition by MKP-1 (Desbois-Mouthon *et al.*, 2000). Some studies suggest that strong and sustained activation of JNKs and p38 plays a role in the development of insulin resistance (Tomlinson, 1999; Blair *et al.*, 1999; Begum and Ragolia, 2000; Aguirre *et al.*, 2000; Igarashi *et al.*, 2000).

### C. Ptdins-3-Kinase Pathway

The role of the Ptdins-3-kinase in insulin signaling was reviewed extensively by Alessi and Downes (1998), Chan *et al.* (1999), and Vanhaesebroeck and Alessi (2000). Docking of the Ptdins-3-kinase-regulatory subunit p85 to IRS leads to binding and activation of the p110 catalytic subunit (Fig. 1). Ptdins-3-kinase activation belongs to the earliest insulin responses and leads to an increase in D3-phosphorylated phosphoinositides. These lipids are crucial for the recruitment of protein kinase B (PKB, also termed Akt) to the plasma membrane. Here, PKB localizes in close vicinity to the phosphoinositide-dependent kinases (PDK1/2), which may perform phosphorylation at Thr-308 and Ser-473 critical for activation of the PKB. Ptdins-3-kinase, PKB, and PDKs mediate activation of the p70 S6 kinase and phosphorylation of the eIF-4E-binding proteins (4E-BPs) via activation of the mammalian target of rapamycin (mTOR, Fig. 1). Another Ptdins-3-kinase and PDK-dependent signaling branch leads to activation of the atypical PKCs  $\zeta$  and  $\lambda$  by insulin (Fig. 1).

Ptdins-3-kinase-dependent signal transduction events play a key role in regulating cellular metabolism by insulin. Thus, insulin-stimulated glucose uptake by translocation of glucose transporters (GLUT4) from intracellular pools to the plasma membrane depends on Ptdins-3-kinase, PKB, and PKC  $\zeta/\lambda$  activity (Fig. 1). Glycogen synthesis is stimulated by activation of the glycogen synthase due to dephosphorylation of the enzyme. This is largely caused by glycogen synthase

kinase-3 (GSK-3) inactivation via GSK-3 phosphorylation by the PKB (Fig. 1). Protein translation is positively regulated by insulin via 4E-BP1 phosphorylation, which induces release of 4E-BP1 from eIF-4E, thereby facilitating translational initiation. Finally, insulin-induced inhibition of hepatic gluconeogenesis by repression of phosphoenolpyruvate carboxykinase (PEPCK) expression depends on PtdIns-3-kinase (Sutherland *et al.*, 1998; Kotani *et al.*, 1999; Lochhead *et al.*, 2001).

### III. Cell Volume Signaling

Alterations of cell volume induced by either anisoosmotic environments or under the influence of hormones, concentrative amino acid uptake, and oxidative stress represent an independent signal contributing to the regulation of cell function and gene expression (Häussinger and Lang, 1991, 1992; Häussinger *et al.*, 1994; Häussinger and Schliess, 1995; McManus *et al.*, 1995; Häussinger, 1996a,b; Lang *et al.*, 1998; Kultz and Burg, 1998; Fig. 2).

Changes in cell hydration within a narrow, physiological range markedly affect carbohydrate and protein metabolism as well as hepatic bile flow. Cell swelling induced by cellular uptake of ions and amino acids is important for completion of the cell cycle (Kim *et al.*, 2001a; Shen *et al.*, 2001a,b; Panet *et al.*, 1986, 1994; Berman *et al.*, 1995; Bussolati *et al.*, 1996; Panet and Atlan, 2000; Bildin *et al.*, 2000; Amsler *et al.*, 1985; Paris and Pouyssegur, 1986) and is accordingly involved in liver regeneration (Freeman *et al.*, 1999, 2002). In addition, the cellular susceptibility to chemical and thermal damage critically depends on the cellular hydration state (Saha *et al.*, 1992; Wettstein and Häussinger, 1997; Wettstein *et al.*, 1998; Kurz *et al.*, 1998; Schliess *et al.*, 1999; Lang *et al.*, 2000a; Lordnejad *et al.*, 2001; Reinehr *et al.*, 2002; Schliess and Häussinger, 2002). In general, cell swelling exerts growth factor-like effects and provides cytoprotection, whereas cell shrinkage supports a catabolic situation and increases cellular stress sensitivity.

The regulation of cell function by cell hydration requires structures, which sense hydration changes (“osmosensing”) and intracellular signaling pathways toward effector sites (“osmosignaling”). There is growing knowledge about the cellular signal transduction components, which are activated/inactivated by hypoosmotic cell swelling and hyperosmotic cell shrinkage, respectively (Häussinger and Schliess, 1999; Weiergräber and Häussinger, 2000). However, little is known about the integration of “osmosignaling” in the overall context of signal transduction, which is activated by hormones or substrates simultaneously with cell volume changes.

The following summarizes current knowledge about the role of isoosmotic cell swelling induced by insulin in mediating insulin effects on cellular metabolism and gene expression. Information is provided about the mechanisms underlying insulin-induced swelling and the potential contribution of cell swelling to the

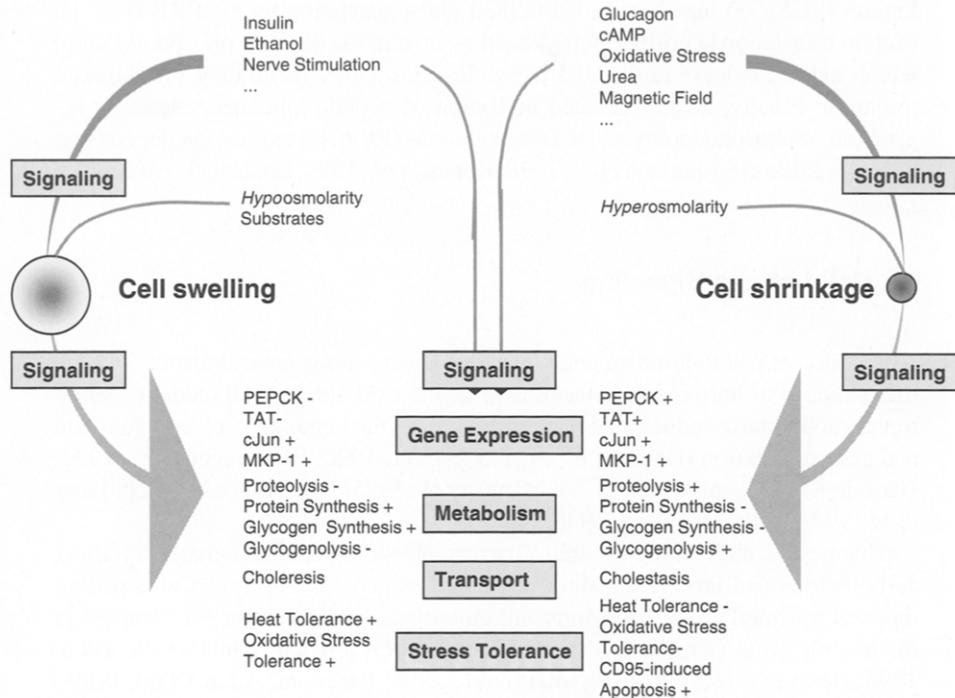


FIG. 2 Cell hydration and liver cell function. Alterations of the hepatocellular hydration states can be produced via signal transduction-dependent activation of solute transport systems, e.g., by hormones, second messenger molecules, ethanol, nerve stimulation, oxidative stress, urea, and magnetic fields as well as by physical means by ambient anisosmolarity and concentrative substrate uptake. Cell hydration changes are independent triggers of signal transduction processes involved in the regulation of cellular performances at multiple levels such as gene expression, metabolism, transport, and stress tolerance. Signal transduction induced by cell swelling or cell shrinkage, respectively, integrates together with cell volume-independent signaling events to the full activation pattern of signaling molecules, which is one determinant of the physiological outcome.

network of signaling molecules addressed by insulin is discussed. To illustrate the full potential of cell swelling as a determinant in regulation of cell function the effects of hypoosmotic cell swelling on signal transduction and cell function need also be considered.

#### A. Hypoosmotic Cell Volume Signaling

Hypoosmotic challenge is used for studies on the mechanisms of regulatory volume decrease (RVD). These mechanisms have been extensively reviewed (Graf *et al.*,

1988; Chamberlin and Strange, 1989; Boyer *et al.*, 1992; Parker, 1993; Burg, 1995; Graf and Häussinger, 1996; Breit *et al.*, 1998; Hoffmann, 2000). Hypoosmolarity triggers the extrusion of osmotically active compounds, which is accomplished by the activation of transport systems in the plasma membrane. In rat hepatocytes hypoosmolarity stimulates the release of cellular  $K^+$ ,  $Cl^-$ , and  $HCO_3^-$  (Lang *et al.*, 1989; Haddad and Graf, 1989; Häussinger *et al.*, 1990a; Haddad *et al.*, 1991) and of organic osmolytes such as betaine and taurine (Warskulat *et al.*, 1997; Wettstein *et al.*, 1998). It is important to note that even after completion of RVD cells are left in a slightly swollen “hydration state” as long as the hypoosmotic challenge continues. Thus, the initial cell volume is not completely restored and the intracellular osmolyte composition remains altered.

Progress has been made in the identification of signaling processes in linking cell swelling to functional consequences (Kultz and Burg, 1998; Häussinger and Schliess, 1999; Weiergräber and Häussinger, 2000). In the following we refer to current findings about “osmosensing” and the key pathways involved in mediation of metabolic effects.

## 1. Sensing of Cell Swelling

The mechanisms of “osmosensing” in mammalian cells are far from being understood (Loomis *et al.*, 1997; Weiergräber and Häussinger, 2000; Hamill and Martinac, 2001). In bacteria, plants, and fungi two component histidine kinases are involved in “osmosensing” and subsequent adaption of intracellular metabolism to adverse osmotic conditions (Wurgler-Murphy and Saito, 1997). Although the preparation of a protein fraction enriched in histidine kinase activity from rat liver has been reported (Motojima and Goto, 1994) the molecular identity of this activity is unknown. In *Lactococcus lactis* an ABC transport system for betaine/glycine (OpuA) simultaneously acts as osmosensor and regulator of cellular hydration, because its transport activity is inhibited under hypoosmotic conditions (van der Heide *et al.*, 2001). In liver, however, hypoosmolarity stimulates ABC transport activity through the multidrug resistance protein 2 Mrp2 (Kubitz *et al.*, 1997) and the bile salt export pump Bsep (Schmitt *et al.*, 2001) and this is preceded by activation of signal transduction (see below). Potential “sensors” of swelling in mammalian cells are the integrin system, the cytoskeleton, the caveolae, the plasma membrane lipid bilayer, the endoplasmic reticulum, and the cytoplasm.

Integrins are a family of cell surface receptors for extracellular matrix (ECM) proteins. At so called “focal adhesions,” clusters of integrins bind outside the cell to ECM proteins and inside the cell to certain cytoplasmic proteins, which in turn interact with different signal transduction components and are linked to the actin cytoskeleton (Aplin *et al.*, 1998; Bourdreau and Jones, 1999; Geiger and Bershadsky, 2001). According to the tensegrity concept (Wang *et al.*, 1993; Ingber, 1997; Chicurel *et al.*, 1998) integrins act as “mechanoreceptors” that allow transmission of mechanical forces to the cytoskeleton. Mechanotransduction, i.e.,

the conversion of a mechanical stimulus into covalent modifications of signaling components, may occur at multiple locations inside the cell through force-induced rearrangements within a tensionally integrated cytoskeleton.

Evidence accumulates that hypoosmotic cell swelling may affect integrin-mediated cell matrix interactions, thereby initiating signal transduction. Thus, hypoosmotic stimulation of glycogen synthesis in primary cultures of rat skeletal muscle cells is sensitive to the integrin-binding peptide GRGDSP and to cytochalasin D, leading to the suggestion that hypoosmotic perturbations of integrin-matrix interactions and the actin cytoskeleton are components of a cell volume sensory mechanism (Low *et al.*, 1997a). In perfused rat liver the hypoosmotic stimulation of the nonreceptor tyrosine kinase src and p38 as well as inhibition of autophagic proteolysis are largely abolished by the GRGDSP peptide (S. vom Dahl, F. Schliess, R. Reissmann, F. Dombrowski, O. H. Weiergräber, and D. Häussinger, unpublished). However, proteolysis inhibition by hypoosmolarity is apparently not mediated by the actin cytoskeleton. Instead, proteolysis inhibition by liver cell swelling is sensitive to colchicine and thus depends on intact microtubular structures (vom Dahl *et al.*, 1995, 2001a). Consistent with an engagement of focal adhesions in "osmosensing" hypoosmolarity stimulates tyrosine phosphorylation of the p125 focal adhesion kinase in HepG2 cells (Kim *et al.*, 2001b), intestine 407 cells (Tilly *et al.*, 1996a), and cardiomyocytes (Sadoshima *et al.*, 1996). Disruption of the actin cytoskeleton in HepG2 cells prevents hypoosmotic phosphorylation of p125 focal adhesion kinase and PKB as well as nuclear translocation of AP-1 (Kim *et al.*, 2001b), suggesting that actin reorganization is essential for hypoosmotic activation of at least some signaling pathways.

The caveolae are another candidate structure involved in sensing of cell swelling and mechanical challenges. Caveolae are cholesterol-enriched microdomains of the plasma membrane that have recently been implicated in the organization of signal transduction modules near the plasma membrane (Okamoto *et al.*, 1998; Schlegel *et al.*, 1998). Caveolin, a principal protein of caveolae, regulates hypoosmotic activation of volume-regulatory anion channels in endothelial cells (Trouet *et al.*, 1999, 2001). In addition, caveolin plays a role in generating the Erk response to fluid shear stress in bovine aortic endothelial cells (Park *et al.*, 2000). Further work is required to define the role of caveolae in cell volume signaling.

Another candidate for osmosensing could be the endoplasmic reticulum (ER): in astrocytes hypoosmolarity induced a phospholipase C-independent  $\text{Ca}^{2+}$  release from stores, which are also mobilized by extracellular ATP (Schliess *et al.*, 1996; Fischer *et al.*, 1997). It was suggested, that the extent of hypoosmolarity-induced store depletion and consecutive capacitative  $\text{Ca}^{2+}$  entry is primarily determined by the extent of cytoplasmic dilution, possibly sensed by  $\text{IP}_3$  receptors localized at the ER (Fischer *et al.*, 1997). Alterations in cell volume may also impact on both protein folding and protein concentration in the ER cisternae and thereby trigger signaling events typical for the "unfolded protein response" or the "ER overload response," respectively (Pahl, 1999). Interestingly, hypoosmotic swelling rapidly inhibits ER

to Golgi transport in mammalian cells (Lee and Linstedt, 1999). However, direct evidence for a role of the ER in cell volume sensing has yet to be presented.

Some studies suggested a swelling-induced release of signaling metabolites, which trigger signal transduction. In HTC hepatoma cells hypoosmolarity induced an ATP release into the extracellular space and this stimulates a volume-regulatory Cl<sup>-</sup> efflux subsequent to autocrine P2 receptor- and PtdIns-3-kinase activation (Feranchak *et al.*, 1998). In intestine 407 cells extracellularly released ATP triggers activation of Erk-1/Erk-2, but not the volume-regulatory chloride release under hypoosmotic conditions (van der Wijk *et al.*, 1999). Further, arachidonic acid metabolites are candidates to initiate hypoosmotic signaling. In Ehrlich acites tumor cells hypoosmolarity leads to translocation of PLA<sub>2α</sub> into the nucleus and the arachidonic acid released into the extracellular space originates from the nucleus (Pedersen *et al.*, 2000). Consistent with a function of PLA<sub>2α</sub> as a primary osmosensor, this translocation occurs in the absence of intracellular Ca<sup>2+</sup> elevation and is insensitive to inhibition of Erks, p38, tyrosine kinases, PKC, or disruption of F-actin (Pedersen *et al.*, 2000). Arachidonic acid metabolites stimulate in Ehrlich cells volume-regulatory ion fluxes in an autocrine fashion (Thoroe *et al.*, 1997). Interestingly, generation of membrane stretch by hypoosmotic swelling of large unilamellar vesicles is already sufficient to activate PLA<sub>2</sub> (Lehtonen and Kinnunen, 1995) and it seems conceivable that a hypoosmotic reduction of lateral membrane packaging of lipids due to the creation of membrane stretch represents another mechanism involved in osmosensing and signaling (Kinnunen, 2000).

Finally, cell volume sensing may be performed by changes in macromolecular crowding due to cytoplasmic dilution (Minton *et al.*, 1995; Burg, 2000). However, direct evidence for a role in signal transduction in mammalian cells is still lacking. Taken together, different structures may be involved in the sensing of hypoosmotic volume changes, intracellular osmolyte composition, or membrane stretch and its transformation into chemically based signal transduction. Probably, as in bacteria, plants, and fungi, multiple osmosensing mechanisms also exist in mammalian cells and it may be difficult to dissect their relative contributions. Although it seems attractive to consider mechanical challenges of integrin-matrix connections and the cytoskeleton to be the osmosensing mechanism, one should take into account that the integrin dependence of hypoosmotic signaling could also be a consequence of intracellularly generated signals ("inside out signaling") and, likewise, rearrangements of cytoskeletal components may represent a consequence of osmosignaling rather than its initiation (Gotoh *et al.*, 1991; Tilly *et al.*, 1996b; Guay *et al.*, 1997).

## 2. MAP-Kinase Pathways

An activation of the extracellular signal-regulated MAP-kinases Erk-1 and Erk-2 in response to hypoosmotic cell swelling is found in most cell types. Hypoosmotic activation of Erks was observed in a number of cells and tissues including hepatocytes and hepatoma cells (Schliess *et al.*, 1995; Noé *et al.*, 1996; Wiese *et al.*, 1998;

Kim *et al.*, 2000), perfused rat liver (Häussinger *et al.*, 1999; Kurz *et al.*, 2001; vom Dahl *et al.*, 2001b), astrocytes (Schliess *et al.*, 1996; Crepel *et al.*, 1998), and C6 glioma cells (Sinning *et al.*, 1997), liver macrophages (Kupffer cells) (Bode *et al.*, 1998), RAW 264.7 mouse macrophages (Warskulat *et al.*, 1998), pancreatic acinar cells (Han *et al.*, 1997), human intestine 407 cells (van der Wijk *et al.*, 1998, 1999), cardiac myocytes (Sadoshima *et al.*, 1996), adipocytes (Ritchie *et al.*, 2001), human cervical cancer cells (Shen *et al.*, 2001c), as well as in renal epithelial A6 cells (Niisato *et al.*, 1999). However, cell type-specific differences exist with respect to the hypoosmotic signaling upstream of Erk-1/Erk-2. For example, the hypoosmolarity-induced Erk activation in astrocytes requires an influx of extracellular  $\text{Ca}^{2+}$  (Schliess *et al.*, 1996), whereas the hypoosmotic Erk activation in hepatocytes, hepatoma cells (Schreiber and Häussinger, 1995; Schliess *et al.*, 1995), and cardiac myocytes (Sadoshima *et al.*, 1996) is  $\text{Ca}^{2+}$  independent, but is sensitive to pertussis toxin, suggestive of an involvement of G-proteins. Also genistein-sensitive tyrosine kinases are involved in the hypoosmotic Erk activation in hepatocytes, hepatoma cells (Schliess *et al.*, 1995; Noé *et al.*, 1996), cardiac myocytes (Sadoshima *et al.*, 1996), and adipocytes (Ritchie *et al.*, 2001), but not in astrocytes and C6 glioma cells (Schliess *et al.*, 1996; Sinning *et al.*, 1997). Hypoosmotic Erk activation in human intestine 407 cells occurs via Ras/Raf but is independent of PtdIns-3-kinase, PKC, and p21(rho) (van der Wijk *et al.*, 1998).

Apart from Erk-1/Erk-2 MAP-kinases of the JNK or p38 type, respectively, are also activated by hypoosmolarity in intestine 407 cells (Tilly *et al.*, 1996b), cardiomyocytes (Sadoshima *et al.*, 1996), hepatocytes (Kurz *et al.*, 1998; Häussinger *et al.*, 1999; Kim *et al.*, 2000), hepatoma cells (Wiese *et al.*, 1998; Kim *et al.*, 2000), perfused rat liver (Häussinger *et al.*, 1999; Kurz *et al.*, 2001; vom Dahl *et al.*, 2001b), human cervical cancer cells (Shen *et al.*, 2001c), and A6 cells (Niisato *et al.*, 1999).

MAP-kinase activation by hypoosmolarity is involved in the regulation of cell functions distinct from volume-regulatory processes. Hypoosmotic Erk activation in cardiac myocytes mediates the induction of c-fos via phosphorylation of p62TCF/Elk-1, which binds to the serum response element of the c-fos promoter (Sadoshima *et al.*, 1996). In the liver, both Erk-1/Erk-2 and p38 are involved in the hypoosmotic stimulation of canalicular bile acid transport (Noé *et al.*, 1996; Kurz *et al.*, 2001). Hypoosmotic Erk activation in liver involves pertussis toxin, genistein, erbstatin, and PD098059-sensitive steps and these inhibitors also abolish the stimulation of bile acid secretion by cell swelling (Noé *et al.*, 1996; Schliess *et al.*, 1998). In addition, hypoosmotic choleresis is abolished by the p38 inhibitor SB203580 but was insensitive to inhibition of PtdIns-3-kinase by wortmannin (Noé *et al.*, 1996; Kurz *et al.*, 2001). Disruption of microtubules uncouples cell swelling from the choleric response, but does not affect hypoosmotic activation of Erks and p38 (Häussinger *et al.*, 1993b; Schliess *et al.*, 1998; vom Dahl *et al.*, 2001a). This suggests that microtubule-associated proteins are downstream targets of Erks and p38. Possibly hypoosmotic signaling via Erks and p38 converges

upstream of the microtubular changes. An osmolarity-dependent insertion of bile salt transporter-bearing vesicles into the canalicular membrane underlies the increased taurocholate excretion in response to hypoosmolarity (Bruck *et al.*, 1992; Häussinger *et al.*, 1992, 1993b), and an osmodependent rapid insertion/retrieval has been shown recently by confocal laser scanning microscopy for the canalicular transport ATPases Mrp2 and Bsep (Kubitz *et al.*, 1997; Schmitt *et al.*, 2001). In adipocytes, hypoosmotic activation of Erks and p38 mediates an increase in glutamine uptake, which is sensitive to PD098059, SB203580, and genistein but not to wortmannin (Ritchie *et al.*, 2001). Interestingly this inhibitor profile resembles that found for blocking hypoosmotic effects on canalicular transport in the liver (see above).

Hypoosmotic swelling effectively inhibits autophagic proteolysis (Häussinger *et al.*, 1990b, 1991; vom Dahl *et al.*, 1991a,b; Hallbrucker *et al.*, 1991a,b; Meijer *et al.*, 1993; vom Dahl and Häussinger, 1995). Hypoosmotic inhibition of proteolysis strongly depends on p38 activation as shown by inhibition of the antiproteolytic effect in the presence of SB203580 (Häussinger *et al.*, 1999). Neither PD098059, pertussis or cholera toxin, nor genistein and erbstatin affect the antiproteolytic effect of hypoosmolarity. This suggests that the Erk pathway, which mediates the choleretic response to hypoosmolarity, is not involved in proteolysis regulation by cell swelling (Häussinger *et al.*, 1999). Hypoosmotic proteolysis inhibition is also insensitive to blockade of the PtdIns-3-kinase pathway (Häussinger *et al.*, 1999).

The inhibitors mentioned above do not reduce the hypoosmolarity-induced increase in intracellular hydration (vom Dahl *et al.*, 2001b). Thus, inhibition of the respective signaling elements uncouples biological effects from the hypoosmotic cell volume increase. Inhibition of the p38 even significantly increases hypoosmotic cell swelling in perfused rat liver by attenuating the volume-regulatory K<sup>+</sup> release, indicating an involvement of the p38 in RVD (vom Dahl *et al.*, 2001b). A role of p38 in RVD was also reported for cultured rat hepatocytes (Feranchak *et al.*, 2001).

### 3. PtdIns-3-Kinase Pathway

Hypoosmotic PtdIns-3-kinase activation is observed in cultured rat hepatocytes (Krause *et al.*, 1996; Feranchak *et al.*, 1998; Webster *et al.*, 2000), perfused rat liver (vom Dahl *et al.*, 2001b), HepG2 cells (Kim *et al.*, 2001c), and primary culture of rat skeletal muscle (Low *et al.*, 1996). In addition, activation of PKB by hypoosmotic swelling was shown in perfused rat liver (vom Dahl *et al.*, 2001b), cultured rat hepatocytes (Webster *et al.*, 2000), and HepG2 cells (Kim *et al.*, 2001c). Hypoosmolarity also activates p70S6 kinase in rat hepatocytes (Krause *et al.*, 1996; Webster *et al.*, 2000).

Hypoosmotic PtdIns-3-kinase and PKB activation mediate increased basolateral taurocholate uptake by the sodium-dependent taurocholate transport protein Ntcp

via the insertion of additional Ntcp molecules into the basolateral compartment of the plasma membrane (Webster *et al.*, 2000). This process is not sensitive to inhibition of the p70S6 kinase or the Erk pathway, respectively (Webster *et al.*, 2000). The Ptdins-3-kinase, but not p70 S6-kinase or Erk-1/Erk-2, is involved in the stimulation of glycogen and fatty acid synthesis by hypoosmotic swelling in hepatocytes (Krause *et al.*, 1996). In rat skeletal muscle hypoosmotic stimulation of glutamine uptake is mediated by Ptdins-3-kinase in a cholera- and pertussis toxin-sensitive manner (Low *et al.*, 1997b). It was excluded that the release of autocrine factors is involved in activation of glutamine uptake by hypoosmotic swelling (Low *et al.*, 1997b).

Ptdins-3-kinase may provide a link between cell swelling and growth in human hepatoma cells. In HepG2 cells, a brief hypoosmotic exposure stimulates proliferation via a Ptdins-3-kinase- and PKB-dependent activation of activator protein-1 (Kim *et al.*, 2001c).

Like insulin, hypoosmolarity decreases expression levels of the PEPCK mRNA in H4IIE rat hepatoma cells, cultured rat hepatocytes, and the perfused rat liver (Newsome *et al.*, 1994; Warskulat *et al.*, 1996; Quillard *et al.*, 1998). However, hypoosmotic repression of PEPCK mRNA expression is insensitive to inhibitors of Ptdins-3-kinase (Warskulat *et al.*, 1996), indicating that the upstream signaling events in part differ from that activated by insulin.

## B. Mechanisms of Insulin-Induced Cell Swelling

Ion fluxes across the plasma membrane represent an early cellular response elicited by growth factors (Rozengurt, 1986; Häussinger and Lang, 1992). Insulin administration causes a rapid drop of plasma  $K^+$  concentration and insulin deficiency is associated with depletion of intracellular  $[K^+]$  and an increase in plasma  $[K^+]$  (Moore, 1983). Splanchnic tissues account for about 70% of the initial decline in plasma  $[K^+]$  when insulin is given under euglycemic conditions (DeFronzo *et al.*, 1980).

Insulin-induced cell swelling results from a net increase of electrolyte and/or organic osmolyte concentrations inside the cell. Transport systems, which are activated by hyperosmolarity for regulatory volume increase (RVI), are activated by insulin, thereby producing cell swelling under isoosmotic conditions. For example, in rat hepatocytes insulin stimulates a net import of  $K^+$  (Berg and Iversen, 1976; Fig. 3). This is due to the concerted activation of an amiloride-sensitive  $Na^+/H^+$  exchange (Haimovici *et al.*, 1994; Sauvage *et al.*, 2000) and a bumetanide-sensitive  $Na^+/K^+/2Cl^-$  symport (Panet *et al.*, 1986) via the sodium–potassium–chloride cotransporter NKCC1 (Schliess *et al.*, 2002). Activation of the  $Na^+/K^+$ -ATPase, possibly triggered by increase in intracellular  $[Na^+]$ , exchanges  $3Na^+$  against  $2K^+$  and additionally contributes to elevated intracellular  $K^+$  concentration. (Gelehrter

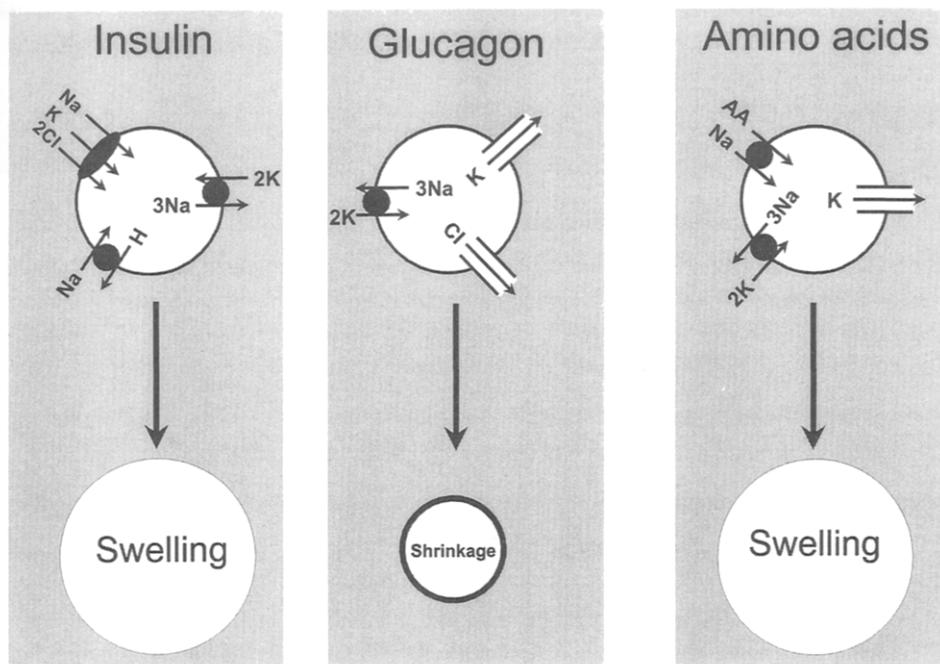
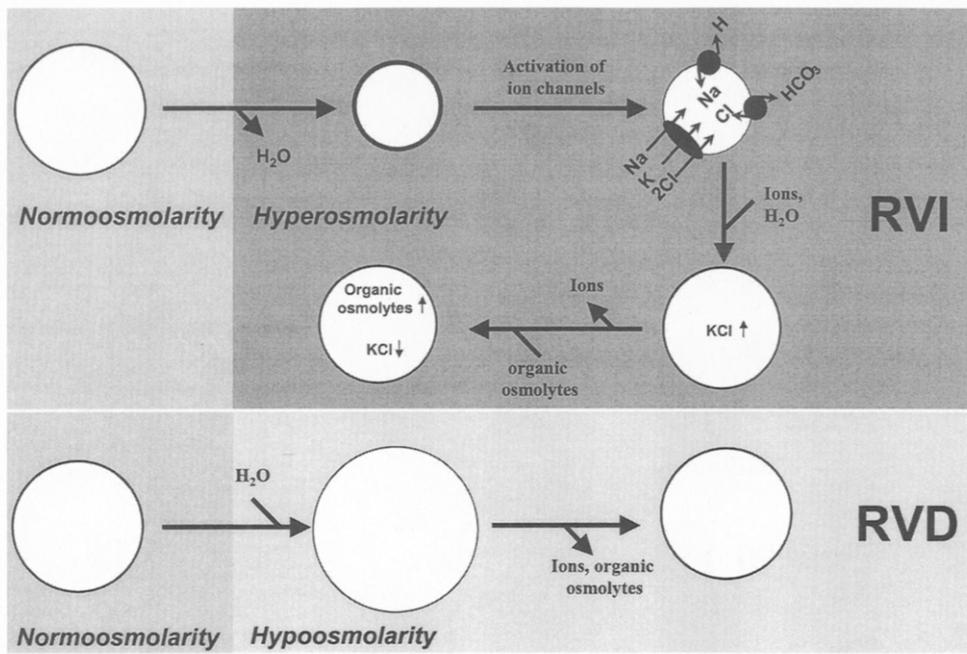


FIG. 3 Mechanisms of isoosmotic cell volume changes by insulin, glucagon, and amino acids. Insulin-induced cell swelling is due to a concerted activation of  $\text{Na}^+/\text{H}^+$  exchange,  $\text{Na}^+/\text{K}^+/2\text{Cl}^-$  symport, and  $\text{Na}^+/\text{K}^+$ -ATPase, resulting in a net retention of  $\text{K}^+$ . Glucagon-induced shrinkage is due to a net  $\text{K}^+$  release by opening of  $\text{K}^+$  and  $\text{Cl}^-$  channels and activation of  $\text{Na}^+/\text{K}^+$ -ATPase. Excessive cell swelling due to intracellular substrate (e.g., glutamine) accumulation is prevented by volume-regulatory  $\text{K}^+$  release and activation of  $\text{Na}^+/\text{K}^+$ -ATPase.

*et al.*, 1984; Fig. 3). This intracellular  $\text{K}^+$  and  $\text{Na}^+$  accumulation is the major basis for insulin-induced hepatocyte swelling. The above-mentioned transport processes are also activated in the liver by hyperosmolarity (Fig. 4) and hyperosmotic activation of  $\text{Na}^+/\text{H}^+$  exchange,  $\text{Na}^+/\text{K}^+/2\text{Cl}^-$  symport, an epithelial  $\text{Na}^+$  channel ENaC, and the  $\text{Na}^+/\text{K}^+$ -ATPase account for RVI (Wehner and Tinell, 1998, 2000; Heinzinger *et al.*, 2001; Schliess *et al.*, 2002; Haddad and Graf, 1989; Haddad *et al.*, 1989; Häussinger *et al.*, 1990a).

Similar transport processes produce insulin-induced swelling in other cell types including rat adipocytes (Sargent *et al.*, 1995; Arsenis, 1995; Su *et al.*, 1998; Ritchie *et al.*, 2001), rat skeletal muscle cells (Low *et al.*, 1996; Incerpi *et al.*, 1997; McDowell *et al.*, 1998), fetal rat type II pneumocytes (Marunaka *et al.*, 1996, 1999), fibroblasts (Vara and Rozengurt, 1985; Amsler *et al.*, 1985; Longo, 1996; Sweeney *et al.*, 1998; Sweeney and Klip, 1998), and A6 renal epithelia cells (Shintani and Marunaka, 1996; Marunaka *et al.*, 1998; Record *et al.*, 1996, 1998).



**FIG. 4** Anisoosmotic cell volume regulation. Hyperosmotic shrinkage induces a regulatory volume increase (RVI) and hypoosmotic swelling induces a regulatory volume decrease (RVD). At a short-term time scale RVI is performed by electrolyte uptake via activation of ion transporters such as the  $\text{Na}^+/\text{H}^+$  exchanger, the  $\text{Na}^+/\text{K}^+/2\text{Cl}^-$  cotransporter (NKCC1), and the  $\text{HCO}_3^-/\text{Cl}^-$  exchanger. This induces almost a net accumulation of KCl within the hyperosmotically challenged cell leading to osmotic water influx and cell swelling. At a long-term time scale the increased intracellular ionic strength exerts proteotoxic effects and is not compatible with normal protein function. Long-term adaption is performed by an isoosmotic exchange of ions against low-molecular organic, so-called “compatible” osmolytes such as betaine, taurine, myo-inositol, sorbitol, or glycerophosphorylcholine. Betaine, taurine, and myo-inositol accumulate within the cell by increased expression of their respective transport systems. Sorbitol accumulates due to increased synthesis by upregulation of aldose reductase expression and glycerophosphorylcholine due to a decreased degradation by the respective phosphodiesterase.

Activation of electrolyte and amino acid transport potentially contributing to cell swelling by insulin is mediated by the activation of signal transduction cascades. In perfused rat liver the inhibition of the PtdIns-3-kinase by wortmannin or LY294002, respectively, inhibits both insulin-induced  $\text{K}^+$  uptake and cell swelling, whereas the PKC inhibitor Gö6850 at low concentrations, which do not affect the atypical PKCs, is ineffective (Schliess *et al.*, 2001). Also in nonhepatic cells PtdIns-3-kinase seems to play a major role in stimulating transport processes by insulin. Wortmannin and LY294002 inhibit overall insulin-induced  $\text{K}^+$  uptake in 3T3-L1 fibroblasts and it was shown that activation of both the  $\text{Na}^+/\text{K}^+/2\text{Cl}^-$  symport and the  $\text{Na}^+/\text{K}^+$ -ATPase by insulin was sensitive to the PtdIns-3-kinase inhibitors

(Sweeney *et al.*, 1998; Sweeney and Klip, 1998). In addition, Gö6850 at high concentrations attenuates K<sup>+</sup> retention by insulin, suggesting an involvement of the atypical PKCs  $\zeta/\lambda$  (Sweeney *et al.*, 1998).

Inhibition of p70S6 kinase or Erk activation by rapamycin or PD098059, respectively, had no effect on insulin-induced K<sup>+</sup> retention (Sweeney *et al.*, 1998). Likewise, insulin-stimulated amino acid transport via transport system A in L6 rat skeletal muscle cells and 3T3L1 adipocytes is mediated by Ptdins-3-kinase, but not by the Erk pathway and the p70S6 kinase (Su *et al.*, 1998; McDowell *et al.*, 1998). However, Erk-1/Erk-2 may be important for the rapid activation of the high-affinity ASCT2 glutamine transporter by insulin in rat adipocytes (Ritchie *et al.*, 2001). Ptdins-3-kinase mediates insulin-stimulated sodium transport in A6 cells, whereas participation of the p70 S6 kinase was excluded (Record *et al.*, 1998). Tyrosine kinase inhibitors (erbstatin analogue and genistein) were reported to abolish stimulation of Na<sup>+</sup>/H<sup>+</sup> exchange by insulin in skeletal muscle cells (Incerpi *et al.*, 1997). Insulin-induced swelling of fetal rat type II pneumocytes due to activation of Na<sup>+</sup>/K<sup>+</sup>/2Cl<sup>-</sup> symport and Na<sup>+</sup> influx via ENaC depends on the presence of extracellular Ca<sup>2+</sup> (Marunaka *et al.*, 1999). The tyrosine kinase inhibitor laverdustin A partly inhibits insulin-induced Na<sup>+</sup>/K<sup>+</sup>/2Cl<sup>-</sup> symport whereas ENaC activation remains unaffected (Marunaka *et al.*, 1996, 1999).

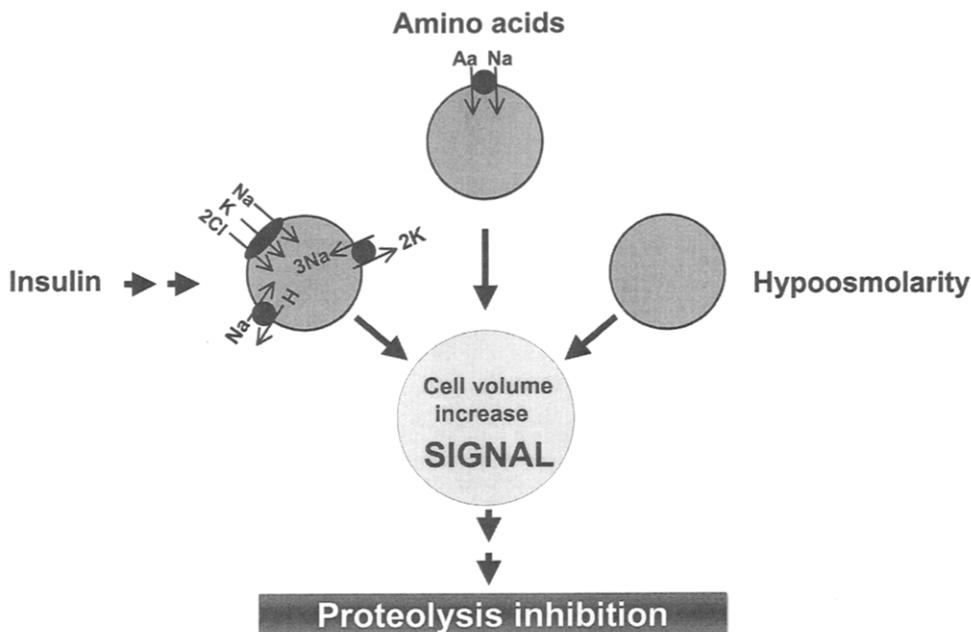
This compilation shows that current knowledge about insulin signaling leading to intracellular solute accumulation and cell swelling is by far not complete. Whereas many studies suggest a major role of Ptdins-3-kinase in stimulating transport and cell swelling by insulin, other signaling elements (e.g., the tyrosine kinases sensitive to the different tyrosine kinase inhibitors) remain to be identified. Cell type-specific differences may exist and within one cell system differential signaling pathways may address the different transport systems.

### C. Insulin-Induced Cell Swelling: A Signaling Amplifier?

As outlined in Section III.A, stimulation of cellular solute uptake accounts for insulin-induced cell swelling. Ion movements across the plasma membrane affect not only cell volume but also plasma membrane potential and intracellular pH. Thus, not only cell swelling, but also changes in membrane potential or pH<sub>i</sub> could be involved in mediating insulin effects. However, at least in the liver the contribution of cell swelling to insulin-induced signal transduction dominates. Insulin induces plasma membrane depolarization (Wondergem, 1983) and may alkalinize the cytosol (Graf and Häussinger, 1996), whereas hypoosmotic swelling causes plasma membrane hyperpolarization and cytosolic acidification (Busch *et al.*, 1994; Schreiber and Häussinger, 1995). In spite of this, hypoosmotic swelling mimics metabolic insulin effects such as stimulation of glycogen synthesis (Meijer *et al.*, 1992; Peak *et al.*, 1992; al Habori *et al.*, 1992; Krause *et al.*, 1996), protein synthesis (Stoll *et al.*, 1992; Grant *et al.*, 2000),

and inhibition of proteolysis (Häussinger *et al.*, 1990b, 1991; vom Dahl *et al.*, 1991a,b; Hallbrucker *et al.*, 1991a,b; Meijer *et al.*, 1993; vom Dahl and Häussinger, 1995).

A close relationship exists between cell hydration and the proteolytic activity in perfused rat liver, regardless of whether cell volume is modified by amino acids, hormones, ethanol, hypoosmolarity, transport inhibitors, or bile acids (vom Dahl and Häussinger, 1996, 1998). Inhibition of proteolysis by cell swelling is due to an inhibition of the formation of autophagic vacuoles (Häussinger *et al.*, 1999; vom Dahl *et al.*, 2002a). Equipotent hepatocyte swelling by either insulin or hypoosmotic exposure inhibits proteolysis to a similar extent, suggesting that the effect of insulin on proteolysis is transmitted by insulin-induced cell swelling (Fig. 5). Therefore, hepatic proteolysis was chosen as readout for assessment of the role of isoosmotic cell swelling by insulin, glutamine, and ethanol in signal transduction toward proteolysis inhibition (Häussinger *et al.*, 1999). Insulin,

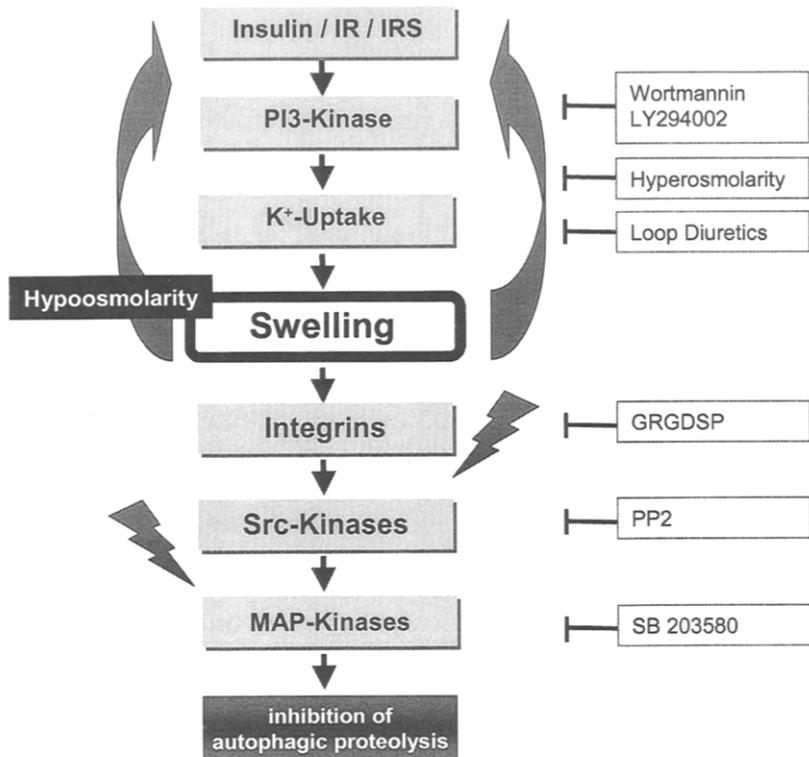


**FIG. 5** Cell swelling is a major component of insulin-induced signal transduction toward regulation of hepatic proteolysis. Insulin-induced cell swelling due to a concerted activation of  $\text{Na}^+/\text{H}^+$  exchange,  $\text{Na}^+/\text{K}^+/2\text{Cl}^-$  symport, and  $\text{Na}^+/\text{K}^+$ -ATPase, resulting in a net retention of  $\text{K}^+$ , is a major signal mediating the antiproteolytic insulin effect, which can be completely mimicked by hypoosmotic cell swelling or by cell swelling induced via concentrated amino acid (Aa) uptake. The cell volume increase is sensed by the cell probably via perturbations of integrin-matrix interactions and is converted in signal transduction, which is based on chemical reactions.

glutamine, and ethanol activate the p38 MAP-kinase in perfused rat liver. Specific inhibition of the p38 abrogates the antiproteolytic, but not the cell volume effects induced by insulin, glutamine, and ethanol. Thus, inhibition of the p38 uncouples the increase in cell hydration from proteolysis inhibition. On the other hand, inhibition of proteolysis by phenylalanine and asparagine, which have no effect on liver cell volume, is insensitive to inhibition of the p38. Thus, the p38 MAP-kinase seems to mediate specifically the antiproteolytic effect in response to cell swelling.

To examine the role of isoosmotic cell swelling for p38 activation, the inducibility of p38 activity by insulin was tested under conditions that are known to impair insulin-induced K<sup>+</sup> retention and cell swelling (Schliess *et al.*, 2001). NKCC1 inhibition by bumetanide impairs insulin-induced K<sup>+</sup> retention, cell swelling, p38 activation, and proteolysis inhibition. However, bumetanide does not impair insulin-induced IR $\beta$  and IRS1 Tyr phosphorylation and was without effect on p38 activation and proteolysis inhibition by hypoosmotic swelling (vom Dahl and Häussinger, 1998; Schliess *et al.*, 2001). As outlined in Section III.B, activation of Ptdins-3-kinase by insulin mediates K<sup>+</sup> retention and cell swelling by insulin. Consistent with a contribution of insulin-induced cell swelling to p38 activation, the Ptdins-3-kinase inhibitors wortmannin and LY294002 abolish the p38 activation by insulin in perfused rat liver. However, the hypoosmotic proteolysis inhibition is insensitive to Ptdin-3-kinase inhibitors. The PKC inhibitor Gö6850 at a concentration that did not inhibit K<sup>+</sup> retention and cell swelling by insulin was also ineffective in impairing p38 activation by insulin. Similarly, activation of Erk-type MAP-kinases by insulin is dependent on insulin-induced cell swelling in perfused rat liver (Schliess *et al.*, 2001). The data are consistent with a role of cell swelling as an amplifier of insulin signaling toward p38 and Erks in perfused rat liver (Fig. 6). Keeping in mind that hypoosmotic cell swelling by itself can activate Ptdins-3-kinase in hepatocytes and perfused rat liver (Section III.A.3) leads to the suggestion that insulin induces cell swelling via Ptdins-3-kinase activation, which in turn may amplify Ptdins-3-kinase activation and cell swelling. In line with this, inhibition of cell swelling produces hepatic insulin resistance.

Cell swelling was also considered to contribute to insulin signaling in adipocytes. In these cells swelling may feed forward the activation of Erk-1/Erk-2 and glutamine transport via the ASCT2 protein by insulin (Ritchie *et al.*, 2001). Amplification of insulin signaling by cell swelling requires structures that are able to sense hydration changes even under isoosmotic conditions. Current evidence suggests that the integrin system participates in this, because the integrin-antagonistic peptide GRGDSP fully inhibits the antiproteolytic effect of insulin in perfused rat liver, but not insulin-induced K<sup>+</sup> retention and cell swelling, respectively (F. Schliess, R. Reissmann, S. vom Dahl, and D. Häussinger, unpublished results). Thus, integrin-matrix or integrin-cell contacts are involved in sensing insulin-induced cell swelling. The aim of current investigations is to examine the involvement of integrins in the activation of signaling components by insulin.



**FIG. 6** Insulin-induced cell swelling acts as a signaling amplifier within the overall context of insulin signaling. This scheme outlines our current working hypothesis regarding the integration of cell swelling into insulin-responsive signal transduction. Insulin induces a PtdIns-3-kinase-mediated intracellular net K<sup>+</sup> accumulation and cell swelling. Cell swelling, which can also be instituted by ambient hypoosmolarity, is sensed probably by the cellular integrins and converted via src activation into chemical signaling leading to activation of the MAP-kinases Erk-1/Erk-2 and p38. Inhibition of the PtdIns-3-kinase by wortmannin or LY294002 blocks K<sup>+</sup> uptake, cell swelling, MAP-kinase activation, and proteolysis inhibition in response to insulin. The PtdIns-3-kinase inhibitors are without effect on proteolysis inhibition by hypoosmolarity. Hyperosmolarity blocks insulin-induced K<sup>+</sup> uptake, cell swelling, MAP-kinase activation, and proteolysis inhibition. Hyperosmolarity may interfere with signaling components downstream of the PtdIns-3-kinase and/or pre-stimulate the insulin-sensitive transport systems in order to perform RVI, thereby inhibiting further stimulation of K<sup>+</sup> import by insulin. Loop diuretics (bumetanide, furosemide), which block the NKCC1, impair insulin-induced K<sup>+</sup> uptake, cell swelling, MAP-kinase activation, and proteolysis inhibition but are without effect on MAP-kinase activation and proteolysis inhibition by hypoosmolarity. The integrin antagonistic peptide GRGDSP, the Src inhibitor PP2, and the p38 inhibitor SB203580 block proteolysis inhibition induced by both hypoosmolarity and insulin, respectively. This indicates that cell swelling induced by either hypoosmolarity or insulin activates a signaling path common to hypoosmotic and insulin signaling toward proteolysis inhibition. The scheme includes the possibility of swelling-independent input at the levels of src and MAP-kinases. Because hypoosmolarity activates the PtdIns-3-kinase, which acts upstream of cell swelling in the case of insulin signaling, it is conceivable that insulin-induced cell swelling feeds into the PtdIns-3-kinase activation thereby augmenting K<sup>+</sup> uptake, cell swelling, and insulin signaling within a feed forward regulatory loop.

#### IV. Cell Hydration and Insulin Resistance

Evidence has accumulated that hyperosmotic dehydration of insulin target tissues represents an additional factor contributing to insulin resistance. Thus endocrine and metabolic disturbances in severely diabetic patients are in part reversible after adequate rehydration therapy. In addition, rehydration improves the patient's sensitivity to low doses of therapeutically applied insulin (Page *et al.*, 1974; Waldhäusl *et al.*, 1979). In contrast, an artificial systemic hyperosmolarity induces insulin resistance. In healthy subjects an increase in serum osmolarity by about 8–10% (i.e., by about 25 mosmol/liter) due to infusion of mannitol for only 2–4 hr is sufficient to induce a significant reduction of insulin-mediated glucose metabolism and this cannot be explained by changes in plasma levels for insulin antagonists such as cortisol, growth hormone, or catecholamines (Bratusch-Marrain and DeFronzo, 1983). This impairment of insulin action was mainly due to a reduced glucose utilization by adipose tissue and skeletal muscle, whereas at least under conditions of mild hyperosmolarity, hepatic glucose production remains largely unchanged (Bratusch-Marrain and DeFronzo, 1983). These findings demonstrate that glucose disposal by peripheral tissues *in vivo* is sensitive to small increases in serum osmolarity, suggestive of a role of cell hydration for determining cellular insulin sensitivity.

Cellular dehydration, i.e., cell shrinkage, not only occurs in response to ambient hyperosmolarity, but also in response to glucagon, high urea concentrations, and reactive oxygen intermediates. In perfused liver, glucagon and its second messenger cAMP lead to cell shrinkage by simultaneous activation of the  $\text{Na}^+/\text{K}^+$ -ATPase and opening of  $\text{Ba}^{2+}$  and quinidine-sensitive  $\text{K}^+$  channels (Hallbrucker *et al.*, 1991a,b; vom Dahl *et al.*, 1991c; Fig. 3). Insulin-induced liver cell swelling is counteracted by further addition of glucagon, which thereby antagonizes the antiproteolytic action of insulin. Although urea equilibrates across the hepatocyte plasma membrane, it nevertheless induces cell shrinkage by activating  $\text{K}^+$  channels, thereby promoting a net  $\text{K}^+$  efflux, which resembles that observed during RVD after hypoosmotic exposure (Hallbrucker *et al.*, 1994). Likewise, reactive oxygen intermediates induce a net  $\text{K}^+$  release and cell shrinkage (Saha *et al.*, 1993; Hallbrucker *et al.*, 1993). Hyperglucagonemia, uremia, and oxidative stress are recognized as playing a role in the development of insulin resistance (DeFronzo *et al.*, 1981; Bratusch-Marrain, 1983; Waldhäusl, 1992; Schmidt and Stern, 2000) and it seems conceivable that cellular dehydration represents a “common denominator” that integrates at last some signal transduction events leading to the impairment of insulin signaling under such conditions. Potential molecular mechanisms underlying insulin resistance caused by intracellular dehydration are discussed in the following. First, some apparently insulin-like effects of hyperosmolarity are considered. Then the focus is directed to the interaction of hyperosmolarity-induced dehydration with signal transduction and metabolic changes triggered by insulin.

## A. Insulin-Like Effects of Hyperosmotic Shrinkage

Although hyperosmotic shrinkage in general triggers a metabolic pattern opposite to that induced by hypoosmotic swelling or insulin, there are some apparently insulin-like effects of hyperosmolarity, namely the stimulation of cellular uptake of electrolytes and glucose. In this context comparison to hyperosmotic signal transduction is made. For a more comprehensive review on the latter topic the reader is referred to Kultz and Burg (1998), Häussinger and Schliess (1999), and Tian *et al.* (2000).

### 1. Regulatory Volume Increase

Hyperosmotic shrinkage triggers solute uptake leading to a regulatory volume increase (RVI, Fig. 4; Boyer *et al.*, 1992; Burg, 1995; Graf and Häussinger, 1996; Breit *et al.*, 1998; Hoffmann, 2000; Graf *et al.*, 1988; Chamberlin and Strange, 1989; Parker, 1993). RVI essentially contributes to cell survival under hyperosmotic conditions. Cells unable to perform RVI undergo apoptosis when hyperosmotically challenged (Bortner and Cidlowski, 1996, 2000).

RVI occurs due to hyperosmotic activation of transport systems, which are in part identical to the transport proteins activated by insulin to produce cell swelling (see Section III.B). In some cell types hyperosmotic MAP-kinase activation can be linked to the activation of transport proteins involved in RVI. In line with this, inhibition of the p38 prevents short-term volume regulation in the rat medullary thick ascending limb of Henle (Roger *et al.*, 1999) and hyperosmotically activated JNKs directly phosphorylate NKCC1 *in vivo* (Klein *et al.*, 1999). On the other hand, MAP-kinases do not participate in the hyperosmotic activation of the  $\text{Na}^+/\text{H}^+$  exchanger NHE1 (Gillis *et al.*, 2001). Hyperosmotic p38 activation leads to upregulation of betaine- and myo-inositol accumulating transport systems in human blood monocytes and macrophages (Denkert *et al.*, 1998) and in Madin-Darby canine kidney (MDCK) cells (Sheikh-Hamad *et al.*, 1998) and Hsp70 expression in MDCK cells (Sheikh-Hamad *et al.*, 1998) and thus allows adaption to hyperosmolarity.

In HepG2 cells p38 mediates the hyperosmotic expression of the serum and glucocorticoid-dependent serine/threonine kinase (h-sgk) (Waldegg *et al.*, 2000; Bell *et al.*, 2000), which probably participates in cell volume regulation by phosphorylating the ENaC (Wagner *et al.*, 2001) and the NKCC1 (Lang *et al.*, 2000b) (compare to Section III.B). Interestingly, h-sgk expression is upregulated by high glucose in 3T3 fibroblasts and is markedly enhanced in diabetic nephropathy, with particularly high expression in mesangial cells, interstitial cells, and cells in thick ascending limbs of Henle's loop and distal tubules (Lang *et al.*, 2000b). It is conceivable that diabetic upregulation of h-sgk reflects in part chronic dehydration and may help to counteract cell shrinkage by activating volume-regulatory electrolyte transport.

Severe hyperosmolarity (700 mM sorbitol or 500 mM NaCl, respectively) was reported to induce tyrosine phosphorylation of the insulin receptor subunit IR $\beta$  and the receptor substrates IRS1 and Shc in IR-overexpressing Chinese hamster ovary (CHO) cells (compare to Section II.A and Fig. 1) (Ouwens *et al.*, 2001). However, IR phosphorylation seems not to be a general prerequisite to induce hyperosmotic signal transduction. First, whereas IRS1 and Shc tyrosine phosphorylation by hyperosmolarity depend on IR tyrosine kinase activity in the IR expressing CHO cells, the hyperosmotic activation of p38 and JNK is IR independent (Ouwens *et al.*, 2001). Second, hyperosmotic induction of IR tyrosine phosphorylation does not occur in all cell types (Chen *et al.*, 1999; Schliess *et al.*, 2001). Third, IR $\beta$  and the IRS1 tyrosine phosphorylation by insulin strongly activate Ptdins-3-kinase and PKB, whereas hyperosmolarity does not activate Ptdins-3-kinase (R. M. Lordnejad, C. Schäfer, D. Graf, D. Häussinger, and F. Schliess, unpublished) or increase phosphorylation of PKB (Meier *et al.*, 1998; Chen *et al.*, 1997, 1999; Parrott and Templeton, 1999). This points to principal differences in signal transduction patterns activated by insulin and hyperosmolarity, respectively.

## 2. Hyperosmotic Stimulation of Glucose Uptake

One major insulin effect is the stimulation of glucose uptake via the glucose transport protein GLUT4 in muscle and fat tissue (Kahn, 1994). In the basal state GLUT4 slowly cycles between the plasma membrane and one or more vesicular compartments inside the cell. Insulin increases the rate of GLUT4 vesicle exocytosis and simultaneously decreases the rate of endocytotic internalization of the transporter, resulting in a net increase of GLUT4 on the cell surface (Pessin *et al.*, 1999). Ptdins-3-kinase, PDK-1, PKB, and the atypical PKCs  $\zeta/\lambda$  as well as not yet identified signal elements mediate insulin-induced GLUT4 translocation to the plasma membrane (Taha and Klip, 1999) (Fig. 1). Stimulation of GLUT4 transport activity by insulin requires additional activation of another signaling branch involving the p38 MAP-kinase (Sweeney *et al.*, 1999; Barros *et al.*, 2001) (see Section II.B).

Like insulin, hyperosmolarity stimulates glucose transport into muscle and fat tissue (Kuzuya *et al.*, 1965; Clausen, 1968), which may support energy production to perform the ATP consuming RVI process. Glucose uptake by 3T3L1 adipocytes exposed to 600 mM sorbitol is about 50% of that induced by 100 nM insulin (Chen *et al.*, 1997). This correlates well with a hyperosmotic GLUT4 translocation into the plasma membrane, which is likewise about 50% compared to that induced by insulin (Chen *et al.*, 1997). More recently it was shown that further increasing osmolarity (up to 800 mM sorbitol) stimulates glucose uptake comparable to that inducible by insulin and it was concluded that the pool of GLUT4 that can be mobilized by hyperosmolarity is similar in size to the insulin-sensitive pool (Barros *et al.*, 2001). However, signal transduction underlying GLUT4 mobilization by hyperosmolarity differs in some aspects from that underlying the insulin effect. Hyperosmotic stimulation of GLUT4 translocation and glucose uptake is

independent of PtdIns-3-kinase activity (Chen *et al.*, 1997) and is not sensitive to inhibition of the p38 MAP-kinase (Barros *et al.*, 2001). A recent study delineated a signaling pathway essential for hyperosmotic stimulation of GLUT4 translocation and glucose uptake in rat adipocytes, L6 myotubes, and 3T3L1 adipocytes (Sajan *et al.*, 2002). Sorbitol activates the Erk pathway through stimulation of the non-receptor tyrosine kinase PYK2. Activation of the PYK2/SOS/Ras/Raf/MEK/Erk pathway is required for activation of phospholipase D, which in turn via production of phosphatidic acid activates atypical PKCs  $\zeta/\lambda$  and finally GLUT4 translocation (Sajan *et al.*, 2002). Activation of PKC  $\zeta/\lambda$  is also essential for GLUT4 translocation induced by insulin (Fig. 1, see Section II.C). Thus the insulin-activated PtdIns-3-kinase/PDK-1 branch and the sorbitol-activated PYK2/Erk/PLD branch converge at the level of atypical PKCs and it was speculated (Sajan *et al.*, 2002) that atypical PKCs may serve as common terminal activators of GLUT4 translocation under different conditions.

Studies with a rat epithelial cell line expressing GLUT1, but not GLUT4, revealed that glucose uptake via GLUT1 can be stimulated by hyperosmolarity (Barros *et al.*, 2001; Barnes *et al.*, 2002). Hyperosmolarity induces GLUT1 activity by apparently increasing accessibility of GLUT1 to its substrate without changing the amount of GLUT1 at the cell surface (Barnes *et al.*, 2002). Neither the PtdIns-3-kinase or the p38 (Barros *et al.*, 2001) nor conventional or novel PKCs (Barnes *et al.*, 2002) mediate hyperosmotic stimulation of glucose transport via GLUT1. Instead the AMP-activated protein kinase, which senses changes in cellular energy charge (Hardie and Hawley, 2001) and which is activated by hyperosmolarity but not insulin, is involved in hyperosmotic signaling toward GLUT1 (Barnes *et al.*, 2002).

Taken together, insulin and hyperosmolarity stimulate cellular glucose uptake via GLUT4 and GLUT1. The underlying signal transduction mechanisms, however, are different. Stimulation of the AMP-activated protein kinase by hyperosmolarity reflects a tendency toward decreased ATP production and/or increased ATP consumption under hyperosmotic conditions. The acute increase in glucose uptake may be part of the catabolic response to hyperosmolarity and serves to maintain vital metabolic processes in order to ensure short-term survival of the cell. As mentioned above, only severe hyperosmolarity (800 mM sorbitol) stimulates a glucose uptake in the order of magnitude similar to that induced by insulin. However, except for the kidney, insulin target tissues are not exposed to such a high osmolarity, and even under pathophysiological conditions hyperosmotic dehydration may stimulate glucose uptake to a much lesser extent than physiological concentrations of insulin in the absence of insulin resistance.

## B. Hyperosmotic Insulin Resistance

Although severe hyperosmolarity can induce some insulin-like effects on the stimulation of glucose transport (see Section IV.A), small increases in systemic

osmolarity are sufficient to impair peripheral glucose utilization *in vivo*. A hyperosmolarity-induced insulin resistance was also observed in *in vitro* studies with isolated tissues and cell culture models. Basal, insulin-independent glucose uptake by epididymal fat pads increases with hyperosmotic NaCl but not urea, whereas both hyperosmotic NaCl and urea impair insulin-induced glucose uptake, resulting in a net decrease of glucose uptake by hyperosmolarity in the presence of insulin (Komjati *et al.*, 1988). Likewise, insulin-stimulated glucose uptake by rat hemidiaphragms (Komjati *et al.*, 1989) and isolated rat adipocytes (Clausen, 1968) is suppressed by hyperosmolarity (Komjati *et al.*, 1989). Interestingly, in rat hepatocytes an increase of ambient osmolarity by already 10 mosmol/liter significantly impairs insulin-independent glucose uptake (Komjati *et al.*, 1989). This was attributed to impaired intracellular enzymatic activity rather than to the transport rate of glucose into the cell.

The aim of more recent studies was to identify components of the insulin-responsive signaling network that are sensitive to hyperosmolarity. The inhibition of insulin-induced glucose uptake, GLUT4 translocation into the plasma membrane, glycogen synthesis, and lipogenesis in 3T3L1 adipocytes is related to a hyperosmotic inactivation of PKB (Meier *et al.*, 1998) and a hyperosmotic inhibition of insulin-induced PKB activation (Chen *et al.*, 1997). Hyperosmotic inactivation of PKB occurs by dephosphorylation at Thr-308 and Ser-473 mediated by a calyculin A- and okadaic acid-sensitive PP2A-type Ser/Thr phosphatase (Chen *et al.*, 1997; Meier *et al.*, 1998). In line with this, hyperosmolarity is without effect on signaling components upstream of the PKB. Indeed, insulin-induced tyrosine phosphorylation of IR $\beta$  and IRS1, respectively, and also the insulin-induced association of the PtdIns-3-kinase regulatory subunit p85 to IRS1 and PtdIns-3-kinase activation are insensitive to hyperosmolarity in 3T3L1 adipocytes (Chen *et al.*, 1997). On the other hand, hyperosmotic inhibition of PKB activation by insulin is reflected by a hyperosmolarity-induced insensitivity to insulin of the p70S6 kinase (Chen *et al.*, 1997), which is a downstream target of the PKB (Fig. 1, see also Section II.C).

In other experimental systems [monkey kidney CV1 cells (Parrott and Templeton, 1999) and H4IIE rat hepatoma cells (M. R. Lornejad-Schäfe, C. Schäfer, D. Graf, D. Häussinger, and F. Schliess, unpublished)] hyperosmolarity impairs p70S6 kinase activation without affecting PKB activity. Calyculin A, but not okadaic acid, prevents hyperosmotic inhibition of the p70S6 kinase in CV1 cells. In H4IIE cells a panel of phosphatase inhibitors including calyculin A and vanadate was unable to revert the hyperosmotic impairment of p70S6 kinase phosphorylation by insulin. One may speculate that hyperosmolarity interferes with p70S6 kinase activation by acting at the level of mTOR, a kinase located upstream of p70S6 kinase (see Fig. 1 and Section II.C). Recent data suggest that the mTOR pathway is influenced by the intracellular concentration of ATP and that mTOR itself is an ATP sensor (Dennis *et al.*, 2001). Keeping in mind that hyperosmolarity activates the AMP-activated protein kinase (see Section IV.A.2), a hyperosmotically induced drop in ATP/AMP could be sensed by mTOR and thus mediate hyperosmotic effects on

insulin sensitivity. Hyperosmotic inhibition of mTOR signaling would be consistent with the finding that hyperosmolarity also reduces phosphorylation levels of the 4E-BP1 (Parrott and Templeton, 1999), another downstream target of mTOR (see Fig. 1 and Section II.C).

The interference of hyperosmotic dehydration with insulin signaling was also studied in the perfused rat liver. As outlined in Section III.C, insulin-induced cell swelling via activation of  $\text{Na}^+/\text{H}^+$  exchange,  $\text{Na}^+/\text{K}^+/2\text{Cl}^-$  symport, and  $\text{Na}^+/\text{K}^+$ -ATPase mediates the antiproteolytic insulin effect via activation of p38 MAP-kinase. When insulin is infused during hyperosmotic perfusion, proteolysis inhibition by insulin is almost completely abolished (Schliess *et al.*, 2001). In addition, hyperosmolarity blocks insulin-induced  $\text{K}^+$  uptake and cell swelling and reduces sensitivity of p38 to stimulation by insulin, but is without effect on insulin-induced tyrosine phosphorylation of  $\text{IR}\beta$  or IRS1, respectively (Schliess *et al.*, 2001). Hyperosmotic impairment of insulin-induced cell swelling and proteolysis inhibition may be due to the inactivation of a signaling component downstream of the PtdIns-3-kinase. Alternatively, hyperosmotic activation of the  $\text{Na}^+/\text{H}^+$  exchange and the NKCC1 in order to perform RVI may prevent further stimulation of these ion transport systems by insulin. The findings strongly suggest that inhibition of insulin-induced cell swelling is another mechanism underlying insulin resistance.

Hyperosmotic shrinkage may participate in mediating insulin resistance observed in the presence of high extracellular glucose levels. Chronic activation of the p38 MAP-kinase was suggested to play a role in the development of insulin resistance under hyperglycemic conditions (Tomlinson, 1999) and it was examined whether hyperosmotic dehydration could be involved. In aortic smooth muscle cells both hyperglycemia and mannitol activate p38, however, the mechanisms are different. Whereas moderate hyperglycemia activates p38 in a PKC-dependent manner, activation of p38 by mannitol is PKC independent. The PKC-independent path responsive to hyperosmotic shrinkage comes into play only at extremely high glucose levels (Igarashi *et al.*, 1999). In another study with vascular smooth muscle cells high glucose was identified as inhibiting MKP-1 induction by insulin and this inhibition was due to p38 activation by glucose (Begum and Ragolia, 2000). Substitution of elevated glucose by mannitol only slightly (about 35%) reduces MKP-1 induction by insulin (Begum and Ragolia, 2000). It is suggested that signaling components independent of glucose-induced dehydration dominate in developing insulin resistance at least in the case of mild hyperglycemia.

Amino acids were recently identified as interacting with insulin-dependent signal transduction in a complex manner (van Sluijters *et al.*, 2000). Amino acids activate p70S6 kinase and 4E-BP1 in synergism with insulin but simultaneously antagonize tyrosine phosphorylation of IRS1 and IRS2 and binding of Grb2 and p85 to these receptor substrates (Patti *et al.*, 1998). Resistance of the p70S6 kinase and the 4E-BP1 to stimulation by insulin was observed upon amino acid starvation of CHO cells (Hara *et al.*, 1998). Interestingly, amino acid starvation of H4IIIE

rat hepatoma cells was ineffective in blocking insulin-induced phosphorylation of p70S6 kinase and 4E-BP1 and this was assigned to the intracellular provision of amino acids due to stimulation of autophagic proteolysis (Shigemitsu *et al.*, 1999). Inhibition of autophagic proteolysis abolishes the phosphorylation of p70S6 kinase and 4E-BP1 by insulin in the amino acid-starved H4IIE cells (Shigemitsu *et al.*, 1999). Removal of extracellular amino acids induces progressive cell shrinkage, which is reversible upon readdition of amino acids (Franchi *et al.*, 1999). It is attractive to speculate that dynamic changes in cell hydration by alterations in intracellular amino acid concentration play a role in modulation of insulin sensitivity by amino acids. In this context it is of interest to note that livers from starved rats are insulin resistant with respect to stimulation of K<sup>+</sup> uptake and proteolysis inhibition (Hallbrucker *et al.*, 1991a), similar to the livers from fed rats perfused hyperosmotically (see above). Thus inhibition of insulin-induced cell swelling may contribute to insulin resistance under conditions of nutrient starvation and catabolism.

## V. Concluding Remarks

Solute transport across the plasma membrane was recognized as a very early cellular response to growth factors including insulin. During the past 15 years, evidence accumulated that growth factor-activated transport processes increase solute concentration inside the cell, which provokes an increase in intracellular hydration, i.e., cell volume. Mainly from studies with the perfused rat liver and hepatocyte cultures, but increasingly also from studies with other cell types, it became clear that cell swelling serves as an additional trigger of signal transduction. Swelling-induced signaling not only mediates cell volume-regulatory osmolyte fluxes but in addition is involved in regulation of cell functions at different levels such as gene expression, metabolic pathways, cytosolic and endosomal pH, hepatobiliary transport, and stress tolerance. For methodological reasons in many studies hypoosmotic exposure of cells and tissues was the experimental setting to evaluate the potential of cell swelling for triggering cell functions. Agents such as insulin that induce isoosmotic swelling additionally trigger signal transduction independent from cell volume alterations and one has to quantify the contribution of cell swelling in generating a particular insulin effect.

However, in the case of hepatic proteolysis a close relationship exists between the cell hydration change and the corresponding inhibition of proteolysis, regardless of how cell volume is modified. Thus, with respect to proteolysis control, insulin signaling virtually completely integrates at the level of cell swelling. This allows the identification of signaling steps largely dependent on the cell volume increase. Like hypoosmotic swelling, isoosmotic swelling by insulin triggers MAP-kinase activation and thereby mediates proteolysis inhibition (Figs. 5 and 6). We postulate that cell swelling serves as a signal amplifier, which is essential for generation of a full

response of the insulin-sensitive signaling network. Hyperosmotic dehydration and nutrient starvation inhibit insulin-induced  $K^+$  uptake and cell swelling in perfused rat liver and are known to induce insulin resistance in target tissues, such as liver, adipose tissue, and skeletal muscle, further supporting the view that cell swelling importantly contributes to insulin signaling. Cell shrinkage may be one component involved in producing insulin resistance by insulin-antagonistic hormones, hypernatremia, hyperglycemia, uremia, and oxidative challenge. Although cell shrinkage interferes with insulin-responsive signaling components located downstream of the PtdIns-3-kinase, the precise cross-talk between dehydration-induced signaling and insulin signaling needs to be defined.

The physiological outcome produced by the combined action of several hormones can frequently not be deduced by adding the individual compound effects. For example, in liver cells there is a marked redundancy regarding the signal transduction molecules sensitive to hypoosmolarity and insulin on the one hand and hyperosmolarity and glucagon on the other (see references cited in Sections II and II.B and Spector *et al.*, 1997; Schliess *et al.*, 2000). Although insulin and glucagon regulate cell volume and protein and carbohydrate metabolism in opposite directions, glucagon increases DNA synthesis stimulated by insulin in hepatocytes (Kimura and Ogihara, 1997) and is one important factor in liver regeneration (Hwang *et al.*, 1993). Both hypoosmotic swelling and cAMP-induced hepatocyte shrinkage activate PKB and thereby increase sinusoidal bile acid uptake via Ntcp (Webster and Anwer, 1999). Another example is that hydrogen peroxide on the one hand produces cell shrinkage, which antagonizes insulin action (see Section IV), but on the other contributes as a signaling metabolite to insulin signaling (Mahadev *et al.*, 2001). These few examples suggest that the relative contributions of "cell volume-dependent and -independent signaling" toward metabolic responses may vary.

The cellular hydration state affects cell functions at multiple levels such as signal transduction, transport, metabolism, pH regulation, hormone sensitivity, and stress tolerance. From a system biological point of view these levels represent particular projections of a highly complex cellular response. The multifarious and interwoven effect patterns indicate the limitations of simple path models to describe hydration-dependent cell functions. In the future the dynamics of physiological process patterns and their shift toward pathophysiology have to be modeled with respect to the heterogeneous experimental findings. Cell structures, module functions, and the physiological performance of the whole organ have to be seen as a coherent system. Therefore the comprehensive and complex experimental results generated by the different experimental approaches could be judged as mutually validating or contradicting subfindings. Gene expression patterns (transcriptome, proteome), posttranslational modifications (e.g., the phosphoproteome), as well as protein localization, compartmentation, targeting, and modular organization are of functional relevance. Additionally, cellular metabolism depends on the concentrations of metabolites and regulatory molecules ("metabolome") and environmental

factors such as temperature and osmolarity. One vision of system biology is to generate mathematical cell models integrating signal transduction, metabolism, and gene expression to allow quantitative and reliable prediction of the cellular response to different stimuli (Kitano, 2002).

Kinetic models such as deduced from "Metabolic Control Analysis" (Fell, 1992; Bowden, 1999; Cascante *et al.*, 2002), which was in particular applied to systemic analysis of hepatic metabolism (e.g., Groen *et al.*, 1983; Soboll *et al.*, 1998), may be the method of choice for quantification of control of hepatobiliary transport or autophagic proteolysis by cellular hydration changes in the intact organ model. However, an integral experimental and technological approach including analysis at the levels of transcriptome, proteom/phosphoproteom, and transport as well as the space-temporal resolution of protein movements and protein–protein interactions at the living cells (topological and interaction proteomics) in connection with a powerful integrated information management system will help to obtain a more holistic view on the hydration dependence of cellular performances.

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