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## **SIGNAL TRANSDUCTION AND PROTEIN PHOSPHORYLATION**

*Research in our group focuses on the role of protein phosphorylation in the control of cell function, with special emphasis on the control of metabolism by nutrients, hormones and various stresses. As a model system, we studied 6-phosphofructo-2-kinase (PFK-2)/fructose-2,6-bisphosphatase (FBPase-2) and the control of its activity by various protein kinases. This bifunctional enzyme catalyzes the synthesis and degradation of fructose 2,6-bisphosphate, a potent stimulator of glycolysis that was discovered in this Institute by Van Schaftingen, Hue and Hers in 1980. Our previous work on the regulation of heart PFK-2 activity by phosphorylation led to the study of the insulin and AMP-activated protein kinase (AMPK) signalling cascades, which are now our main research interests.*

### **INSULIN SIGNALLING**

*L. Toussaint, N. Hussain, D. Vertommen, L. Maisin, M.-A. Gueuning, L. Hue, M.H. Rider, in collaboration with B. Hemmings, Basel*

#### **Stimulation of heart glycolysis and adipose tissue lipogenesis by insulin**

Insulin stimulates heart glycolysis by increasing glucose transport and by activating PFK-2. This in turn leads to a rise in fructose 2,6-bisphosphate (ref. 1). The recombinant heart PFK-2 isozyme is a substrate of protein

kinases of the insulin signalling pathways, such as protein kinase B (PKB), also known as Akt, which is believed to mediate most metabolic effects of insulin. Our previous studies suggested that PFK-2 activation by insulin was dependent on PDK1, a protein kinase upstream of insulin-stimulated protein kinases (Fig. 1). We examined the role of SGK3 (serum- and glucocorticoid-regulated protein kinase-3) in insulin-stimulated PFK-2 activation. Co-transfection of HEK 293T cells with SGK3 siRNA did not affect PFK-2 activation, suggesting that this protein kinase is not required for PFK-2 activation by insulin. Therefore, we re-evaluated the role of PKB. In HEK293 cells co-transfected with heart PFK-2 and total PKB siRNA, insulin-induced PFK-2 activation

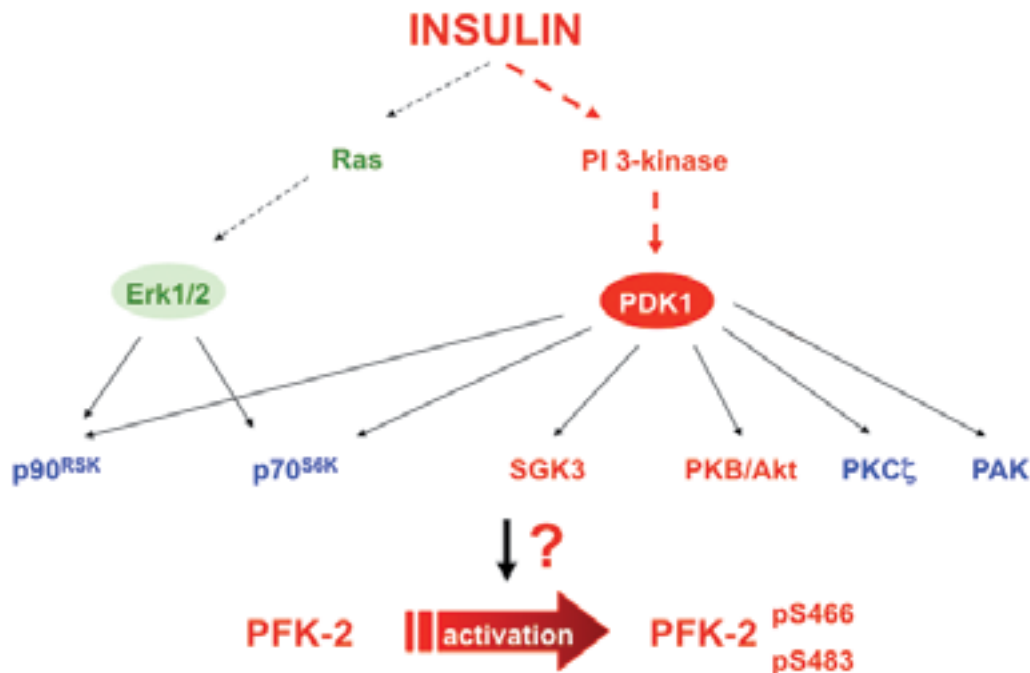


Fig. 1. Protein kinases of signalling pathways that converge on heart PFK-2.

was abrogated. Also in cardiomyocytes treated with the Akti-1/2 PKB $\alpha/\beta$ -selective inhibitor, PFK-2 activation by insulin was prevented. Our results with PKB $\beta$ -knockout mice indicated that this isoform is not required for heart PFK-2 activation by insulin. Moreover, PKB $\alpha$  silencing using the siRNA approach indicated that this PKB isoform is likely to be responsible for heart PFK-2 activation by insulin (ref. 2).

A well-known effect of insulin is the stimulation of adipose tissue lipogenesis, mediated by pyruvate dehydrogenase (PDH) activation via dephosphorylation, and acetyl-CoA carboxylase (ACC) activation. In rat adipocytes incubated with fructose as carbohydrate substrate (to circumvent effects of insulin on glucose uptake), insulin induced lipogenesis was completely abolished by the Akti-1/2 inhibitor suggesting a key role for PKB $\alpha/\beta$  in the control of this pathway by insulin. Insulin-induced PDH dephosphorylation/activation

persisted in the presence of Akti-1/2, indicating that PKB isoforms are not involved in this effect of the hormone. However, ACC Ser79 dephosphorylation by insulin was completely reversed by Akti-1/2. Therefore, a key point of control of the lipogenic pathway by insulin is the dephosphorylation of ACC mediated by PKB, which we showed previously to antagonize AMPK (ref. 3) responsible for ACC Ser79 phosphorylation (see below).

## AMP-ACTIVATED PROTEIN KINASE

*B. Sid, L. Miranda, L. Bultot, Y. Liu, C. Plaideau, Y.-C. Lai, L. D. Vertommen, N. Hussain, R. Jacobs, Hue, M.H. Rider, in collaboration with S. Carpentier and P. Courtoy, de Duve Institute, S. Horman, UCL, Brussels, K. Sakamoto, Dundee, J. Jenssen, Oslo, K. Storey, Ottawa and B. Violette, Paris*

AMP-activated protein kinase (AMPK) acts as a sensor of cellular energy status. AMPK is activated by an increase in the AMP/ATP ratio as occurs during hypoxia or after ATP depletion with oligomycin. In certain cells, AMPK can also be activated by 5-aminoimidazole-4-carboxamide (AICA)-riboside, which enters cells to be phosphorylated to ZMP, an analogue of AMP, or by the A769662 Abbott compound. Full AMPK activation requires phosphorylation of the  $\alpha$ -catalytic subunits at Thr172 by upstream kinases, either LKB1 (the Peutz-Jeghers protein) or calmodulin-dependent protein kinase kinase- $\beta$  (CaMKK $\beta$ ). The role of AMPK in the cell is to maintain ATP by stimulating ATP-producing pathways and inhibiting energy-consuming biosynthetic pathways. For example phosphorylation of ACC, the best-known substrate of AMPK, inhibits energy consuming lipogenesis. We contributed to the field by discovering new substrates of AMPK and mechanisms of upstream regulation. We demonstrated that the activation of PFK-2

by AMPK participates in the stimulation of heart glycolysis by ischaemia. We also showed that phosphorylation-induced inactivation of elongation factor 2 (eEF2) by AMPK partly explains the inhibition of protein synthesis by anoxia (ref. 4). Lastly, we demonstrated that PKB-induced phosphorylation of the AMPK catalytic  $\alpha/\beta$  subunits at Ser485/491 in response to insulin antagonizes AMPK activation by LKB1 (ref. 3).

### AMPK activation in diapausing insects as a means for surviving energy stress

Winter survival for many insects depends on cold hardiness adaptations as well as entry into a hypometabolic diapause state that minimizes energy expenditure. We investigated whether AMPK could be involved in this adaptation in larvae of two cold hardy insects, *Eurosta*

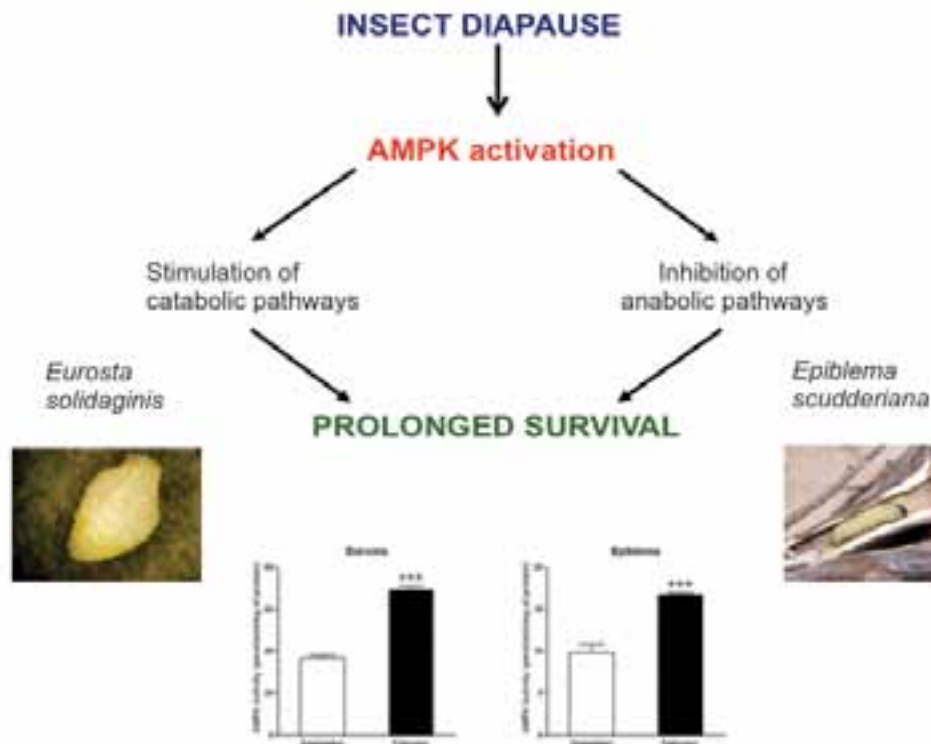


Fig. 2. Role of AMPK to prolong survival during insect diapause.

*solidaginis* that is freeze tolerant and *Epiblema scudderiana* that uses a freeze avoidance strategy. AMPK activity was almost 2-fold higher in midwinter larvae (February) compared with animals collected in September and accordingly phosphorylation of ACC increased more than 3-fold in both species during midwinter which would strongly suppress lipogenesis. Overall, our study suggests a role for AMPK in minimizing anabolic processes to decrease energy expenditure during insect diapause (Fig. 2; ref. 5).

### Control of ion transport by AMPK

We studied whether the mechanism of increased Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup> (NKCC1) co-transporter activity by osmotic shrinkage involved AMPK activation. AMPK was found to phosphorylate a recombinant GST-dogfish (1-260) NKCC1 fragment at Ser38 and Ser214, corresponding to Ser77 and Ser242 in human NKCC1, respectively. Incubation of human erythrocytes with A769662 AMPK activator increased Ser242 NKCC1 phosphorylation but did not stimulate <sup>86</sup>Rb<sup>+</sup> uptake. Under hypertonic conditions in human red blood cells (RBCs) incubated with 0.3 M sucrose, NKCC1 activity increased as measured by bumetanide-sensitive <sup>86</sup>Rb<sup>+</sup> uptake and AMPK was activated. However, there was no effect of AMPK $\alpha$ 1 deletion in mouse RBCs on the increased rate of <sup>86</sup>Rb<sup>+</sup> uptake induced by hyperosmolarity. AMPK activation by osmotic shrinkage of mouse RBCs was abrogated by the STO-609 CaMKK $\beta$  inhibitor, but incubation with STO-609 did not affect the increase in <sup>86</sup>Rb<sup>+</sup> uptake induced by hyperosmolarity. Osmotic shrinkage of human and mouse RBCs led to activation loop phosphorylation of the STE20/SPS1-related proline/alanine-rich kinase (SPAK) at Thr233, which was accompanied by phosphorylation of NKCC1 at Thr203/207/212, one of which (Thr207) is responsible for co-transporter activation. Therefore, phosphorylation-induced activation of NKCC1 by osmotic shrinkage does not involve AMPK and is likely due to

SPAK activation (Fig. 3, ref. 6).

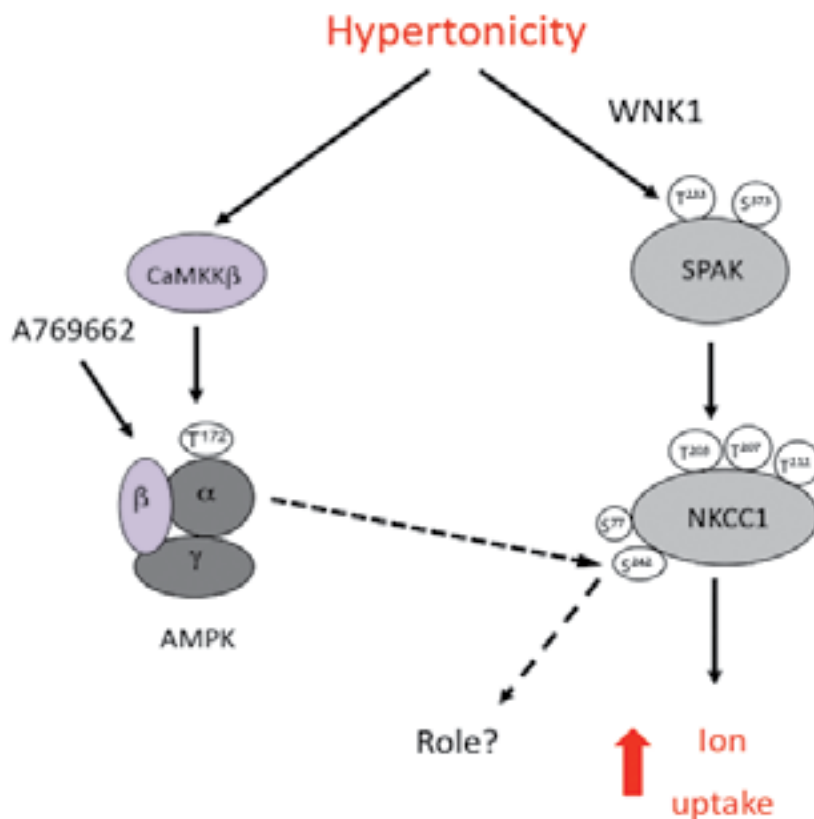
### Role of AMPK in the control of cytoskeletal actin organization

AMPK function is now recognized to extend beyond metabolic regulation to control cell division, cell polarity and cell migration, all of which depend on the actin cytoskeleton. We studied the effects of A769662 on cytoskeletal organization and signalling in epithelial Madin-Darby canine kidney (MDCK) cells. AMPK activation induced disassembly of stress fibers and accumulation of cortical F-actin. In parallel, Rho-kinase downstream targets, namely myosin regulatory light chain (MLC) and cofilin, were phosphorylated. These effects resembled the morphological changes in MDCK cells exposed to hyperosmotic shock, which led to Ca<sup>2+</sup>-dependent AMPK activation via CaMKK $\beta$ . Indeed, hypertonicity-induced AMPK activation was markedly reduced by the STO-609 CaMKK $\beta$  inhibitor, as was the increase in MLC and cofilin phosphorylation. We propose that AMPK links osmotic stress to the reorganization of the actin cytoskeleton (ref. 7).

### MASS SPECTROMETRY

*D. Vertommen, G. Herinckx, M. Rider in collaboration with C. Sindic, UCL and J.-F. Collet, UCL*

The development of mass spectrometry facilities within our laboratory has been an enormous asset to our group and institution. Since the acquisition of our first electrospray mass spectrometer in 1997, the application of mass spectrometry techniques to protein identification, identification of sites of covalent modification and quantification of changes in protein expression has led to well over 30 joint publications. In our own research, it has been paramount in identifying new phosphorylation sites. In 2009 we updated our electrospray machine to the Finnigan LTQ linear ion trap



**Fig. 3.** Cell shrinkage by sucrose treatment in RBCs leads to activation of  $\alpha$ 1-AMPK and SPAK via the CaMKK $\beta$  and WNK (with-no-lysine kinase)-1 pathways, respectively. SPAK activation correlates with NKCC1 Thr203/207/212 phosphorylation and is likely responsible for its activation and increased  $^{86}\text{Rb}^+$  uptake induced by hyperosmolarity. AMPK activation by hyperosmolarity does not affect NKCC1 activity. Treatment with A769662 activates AMPK in RBCs and increases Ser242 NKCC1 phosphorylation, whose function is at present unknown.

equipped with electron-induced transfer dissociation (ETD) fragmentation and we are now part of the UCL proteomics platform MASS-PROT, open to the scientific community of the de Duve Institute and UCL and for outside collaborations.

We are continuing our efforts to develop new techniques for the mass spectrometric analysis of proteins. These include a 2D-LC/MS label-free proteomics approach for differential protein expression studies and phosphoproteomics to identify new AMPK substrates, involving hydrophilic interaction chromatography (HILIC) followed by enrichment of phosphopeptides by immobilized metal affinity chromatography (IMAC) and analysis by LC-MS (reviewed in ref. 8). We are also using

14-3-3 pull-downs of extracts from electrically stimulated skeletal muscles to identify new AMPK targets.

We pursue our collaboration with other laboratories within our university and with groups outside and abroad. For example with C. Sindic, the screening of glycoproteins from sera and cerebro-spinal fluid of patients with neurodegenerative diseases is under study to discover new biomarkers. In collaboration with the group of J.-F. Collet, we investigated the mechanisms of outer membrane biogenesis in *E. coli* (refs. 9, 10)

## SELECTED PUBLICATIONS

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