AMP-activated protein kinase is activated by low glucose in cell lines derived from pancreatic β cells, and may regulate insulin release

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The role of the AMP-activated protein kinase (AMPK) cascade in the glucose-sensitive pancreatic β cell lines HIT-T15 and INS-1 was addressed. In both cell types, removal of glucose leads to a > 5-fold activation of AMPK activity. Activation of AMPK was due to phosphorylation, since the effect was reversed by protein phosphatase treatment of the extracts, and was restored by re-addition of MgATP and the purified upstream kinase. When the effects of different concentrations of medium glucose were examined, insulin secretion and AMPK activity were inversely related, and varied over the same concentration range. The activation in response to glucose removal appeared to be due to changes in the concentration of the known regulators of the cascade, i.e. AMP and ATP, since AMPK activation was associated with a large increase in the cellular AMP/ATP ratio, and the two parameters varied over the same range of glucose concentrations. In late-passage HIT-T15 cells that had lost the glucose-dependent insulin secretion response, both AMPK activity and the AMP/ATP ratio also became insensitive to the extracellular glucose concentration. Treatment of INS-1 cells, but not HIT-T15 cells, with AICA riboside (5-aminoimidazole-4-carboxamide riboside) results in accumulation of the ribotide, ZMP (AICA riboside monophosphate), and activation of AMPK. AICA riboside treatment of INS-1 cells, and of isolated rat islets, had both inhibitory and stimulatory effects on insulin secretion. These results show that in β cell lines the AMP-activated protein kinase, like its yeast homologue the SNF1 complex, can respond to the level of glucose in the medium, and may be involved in regulating insulin release.

INTRODUCTION

The AMP-activated protein kinase (AMPK) is the mammalian homologue of the *Saccharomyces cerevisiae* SNF1 protein kinase [1,2]. Both the mammalian and yeast kinases exist as high-molecular-mass complexes comprising a catalytic α subunit and non-catalytic β and γ subunits; in yeast these are encoded by the *SNF1*, *SIP1/SIP2/GAL83* and *SNF4* genes respectively [2]. Functional *SNF1* and *SNF4* genes are essential for de-repression of genes which are repressed by high medium glucose and, consistent with this role, the kinase activity of the SNF1 complex is dramatically activated by phosphorylation when glucose is removed from the medium of yeast [3,4]. The SNF1 kinase therefore appears to be a primary factor in the response of yeast cells to the availability of glucose in the medium.

As yet, AMPK has not been shown to be regulated by glucose in mammalian cells, and instead the kinase has been found to be activated by environmental stresses which cause depletion of ATP. Such stresses include high medium fructose, heat shock and the trichloroacetic acid cycle inhibitor arsenite in isolated hepatocytes [5,6], exercise and electrical stimulation in skeletal muscle [7–9], and ischaemia in heart muscle [10]. Depletion of ATP in cells is always accompanied by elevation of AMP, due to displacement of the adenylate kinase reaction ($2ADP \leftrightarrow ATP + AMP$). AMPK is activated by high AMP (and low ATP) concentrations, due to multiple mechanisms acting both on the intrinsic activity of the kinase and on its phosphorylation and activation by an upstream kinase, AMPK kinase (AMPKK) [11–13]. The signals that switch on the SNF1 kinase in yeast deprived of glucose remain unclear, although activation of SNF1 is accompanied by large increases in cellular AMP and decreases in ATP [4].

With some exceptions, such as neurons and hepatocytes, the level of glucose in the extracellular medium is normally of less significance for mammalian cells than it is for yeast. Endocrine mechanisms, in which insulin secretion plays a crucial role, ensure that plasma glucose concentrations remain relatively constant even when an organism is subject to starvation. In addition (unlike yeast growing in exponential phase), most mammalian cells store glycogen, which acts as a short-term buffer of variations in the supply of glucose. However, one mammalian cell type which does respond to the level of glucose in the medium is the pancreatic β cell responsible for insulin secretion. These cells do not store glycogen, and express high-K_m forms of both glucose transporters (GLUT2) and hexokinase (glucokinase), so that their glucose metabolism responds to minute-by-minute fluctuations in the supply of glucose [14,15]. High glucose concentrations stimulate insulin release, and a current hypothesis to explain this proposes that increased glucose metabolism causes increases in the cellular ATP/ADP ratio. This activates ATP-sensitive K^+ channels, causing depolarization of the plasma membrane, opening of voltage-gated Ca^{2+} channels, elevation of cytoplasmic Ca^{2+} and consequent exocytosis of secretory granules [16,17]. While this model has received widespread acceptance, some components have not been conclusively demonstrated, and in any case this mechanism would not rule out the existence of ancillary systems that also regulate insulin release.

Implicit in this model is the idea that ATP, or the ATP/ADP ratio, is the signal that activates insulin release. Since AMPK is activated by depletion of ATP, it seemed possible that the kinase might be involved in regulating β cell function in response to changing glucose concentrations. There is already substantial evidence that reversible protein phosphorylation plays an im-

Abbreviations used: AMPK, AMP-activated protein kinase; AMPKK, AMPK kinase; ACC, acetyl-CoA carboxylase; PP2A, protein phosphatase-2A catalytic subunit; SAMS, the synthetic peptide HMRSAMSGLHLVKRR; AMARA, the synthetic peptide AMARAASAAALARRR; DTT, dithiothreitol; PEG, poly(ethylene glycol) 6000; AICA, 5-aminoimidazole-4-carboxamide; ZMP, AICA riboside monophosphate.

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portant role in regulating stimulus–secretion coupling in these cells [18]. A number of protein kinases have been implicated in the potentiation of glucose-induced insulin secretion by hormones and neurotransmitters, although their activation in the absence of glucose is not sufficient to stimulate insulin secretion [19–23]. There are already some observations which indicate indirectly that AMPK might be regulated in response to glucose in β cells. Incubation of HIT-T15 cells, or perifusion of rat islets, with high glucose concentrations leads to elevation of intracellular malonyl-CoA levels [24,25]. Malonyl-CoA is the product of acetyl-CoA carboxylase (ACC), one of the well established targets for AMPK, and Louis and Witters showed that glucose treatment of HIT-T15 cells activated ACC [26]. The effect survived purification of the enzyme (indicating covalent modification), and resulted from an increase in V_{max} and a decrease in the K_a for the allosteric activator, citrate. Since phosphorylation of ACC by AMPK has the opposite effects [27], this was consistent with the idea that AMPK was being inhibited by high glucose, leading to dephosphorylation of ACC.

We have recently shown that both the α1 and α2 isoforms of the AMPK catalytic subunit are expressed in the rat pancreatic β cell line INS-1 [28]. In the present paper we address the question of whether AMPK is regulated by the extracellular glucose concentration in INS-1 cells, and in the hamster β cell line HIT-T15. We were particularly interested in the possibility that AMPK may form part of a novel signalling pathway linking alterations in extracellular glucose concentration to insulin secretion.

EXPERIMENTAL

Materials

Protein A–agarose was from Sigma. Rabbit anti-(AMPK β subunit) antibody was a gift from David Carling (Imperial College, London, U.K.). AMPKK was purified from rat liver as far as the Mono Q step [29]. Protein phosphatase-2A catalytic subunit (PP2A) was prepared from bovine heart using a procedure described for rabbit muscle [30]. The SAMS peptide (HMRSAMSGLHLVKRR) [31] and the AMARA peptide (AMARAASAAALARRR) [32] were synthesized as described previously.

Cell culture

INS-1 cells [33] were grown in RPMI 1640 supplemented with 10 % (v/v) heat-inactivated foetal calf serum, 10 mM Hepes, 2 mM glutamine, 1 mM pyruvate, 50 μM β-mercaptoethanol, 100 units/ml penicillin and 100 μg/ml streptomycin. HIT-T15 cells [34] were grown in RPMI 1640 supplemented with 10 % (v/v) foetal calf serum, 25 mM Hepes, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. Both cell lines were maintained at 37 °C in 95 % air/5 % CO_2.

Cells were seeded in 10 cm dishes at a density of 3×10^6 HIT-T15 cells or 1×10^6 INS-1 cells/dish, and were incubated for 4–5 days prior to experiment. The medium was removed and cells were preincubated in 5 ml of KRH buffer [119 mM NaCl, 20 mM Hepes, pH 7.4, 5 mM $NaHCO_3$, 4.75 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.1 % (w/v) BSA] containing 10 mM glucose at 37 °C for 60 min. The medium was removed and replaced with 5 ml of KRH buffer with or without glucose or test substances at 37 °C for various times.

Enzyme and protein assays

For assay of AMPK, the medium was removed and 1.0 ml of ice-cold lysis buffer [50 mM Tris/HCl, pH 7.4 at 4 °C, 250 mM mannitol, 50 mM NaF, 1 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 0.1 mM benzamidine, 0.1 mM PMSF, 5 μg/ml soybean trypsin inhibitor, 1 % (v/v) Triton X-100] was added. The cells were scraped off with a cell scraper and the lysate transferred to a microcentrifuge tube. Extracts were vortex-mixed and centrifuged (14000 ***g***; 3 min; 4 °C). INS-1 cell extracts were taken for assay at this stage. For HIT-T15 cells, 2.5–5.0 % poly(ethylene glycol) 6000 (PEG) precipitates were prepared and resuspended in assay buffer prior to assay. SAMS peptide kinase(s) were assayed in crude cell lysates or resuspended PEG precipitates using a variation of the method of Davies et al. [31] as follows. Reaction mixtures containing 5 μl of assay buffer (62.5 mM Na Hepes, pH 7.0, 62.5 mM NaCl, 62.5 mM NaF, 1.25 mM sodium pyrophosphate, 1.25 mM EDTA, 1.25 mM EGTA, 1 mM DTT, 0.1 mM benzamidine, 0.1 mM PMSF, 5 μg/ml soybean trypsin inhibitor), 5 μl of 1 mM AMP in assay buffer, 5 μl of 1 mM substrate peptide in assay buffer and 5 μl of PEG precipitate or cell extract resuspended in assay buffer were prepared on ice, and the reaction was initiated by the addition of 5 μl of [γ-^{32}P]ATP solution {1 mM [γ-^{32}P]ATP (250–500 c.p.m./pmol), 25 mM $MgCl_2$}. Assays were then conducted as described previously [31]. In extracts of HIT-T15 cells, the PEG precipitation procedure gave quantitative recovery of AMPK and resulted in a higher incorporation of phosphate above the blank, so was used routinely. In extracts of INS-1 cells, the PEG precipitation procedure did not give quantitative recovery of AMPK, and the kinase was assayed in the crude lysate instead.

PP2A and AMPKK were assayed as described previously [29,30]. Units of AMPK and PP2A activity are nmol of phosphate transferred or removed per min at 30 °C. Protein concentration was determined by the method of Bradford [35].

Nucleotide extraction and analysis

The medium was removed and 0.5 ml of 5 % (w/v) ice-cold $HClO_4$ was added to the cells. The cells were scraped off with a cell scraper and transferred to a microcentrifuge tube. Precipitated protein was removed by centrifugation (14000 ***g***; 3 min). $HClO_4$ was extracted, and nucleotides were analysed by anion-exchange HPLC as described previously [6].

Insulin radioimmunoassay in medium of cultured cells

The cell medium was collected and centrifuged (1000 ***g***; 4 min; 4 °C). The supernatant (200 μl) was then assayed for insulin using an ^{125}I-insulin radioimmunoassay kit (ICN), according to the manufacturer's instructions.

Dephosphorylation and re-activation of AMPK

HIT-T15 cell extracts were prepared as described above, except that the NaF and sodium pyrophosphate in the lysis buffer were replaced with 50 mM NaCl to allow dephosphorylation. Dephosphorylation was carried out in a total volume of 250 μl containing cell extract and PP2A (7.5 units/ml, final). The mixture was incubated at 30 °C for 20 min before further dephosphorylation was prevented by the addition of okadaic acid to 100 nM (final). Then 3 vol. of this dephosphorylated extract was added to 1 vol. of re-activation buffer (50 mM Na Hepes, pH 7.4, 1 mM DTT, 0.02 % Brij-35) supplemented with ATP, $MgCl_2$ and AMP (final concentrations 200 μM, 5 mM and 100 μM respectively) and purified AMPKK (5000 units/ml). Controls were carried out in the absence of AMPKK.

Immunoprecipitation of AMPK

Rabbit anti-(AMPK β subunit) antibody was pre-bound to 20 μl of 25 % (w/v) Protein A–agarose in IP buffer [50 mM Tris/HCl, pH 7.4 at 4 °C, 150 mM NaCl, 50 mM NaF, 1 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM benzamidine, 0.1 mM PMSF, 5 μg/ml soybean trypsin inhibitor, 1 % (v/v) Triton X-100] by incubation for 1 h at 4 °C on a roller mixer. The mixture was then centrifuged (14000 ***g***; 30 s; 4 °C) and the pellet was washed with 5 × 1 ml of IP buffer. Cell extract was diluted in IP buffer and added to the Protein A–agarose pellet, and incubated on the roller mixer for 2 h at 4 °C. The mixture was then centrifuged (14000 ***g***; 30 s; 4 °C) and the immunodepleted supernatant was assayed for AMPK activity.

Isolation, incubation and insulin assays of rat islets

Islets of Langerhans were prepared by collagenase digestion of pancreases from male Wistar rats [36]. Batches of five islets were incubated in 0.6 ml of a modified Krebs bicarbonate medium containing 20 mM Hepes, pH 7.4, and 2 mg/ml BSA [37] for 30 or 60 min at 37 °C. Portions of incubation medium were collected and stored at −20 °C in 50 mM phosphate buffer, pH 7.4, containing 6 mM merthiolate and 1 mg/ml BSA, until assayed by radioimmunoassay as described previously [38].

RESULTS

Glucose removal activates AMPK in HIT-T15 and INS-1 cells

We have previously shown by Western blotting that the α1, α2, β and γ subunits of AMPK are present in both HIT-T15 and INS-1 cells ([28]; I. P. Salt, unpublished work). Using the SAMS kinase assay [31], it was possible to measure AMPK activity in both cell lines, and to show that the activity fluctuated in response to changes in the concentration of glucose in the medium. Transfer of early-passage HIT-T15 cells from medium containing 10 mM glucose to glucose-free medium provoked a rapid, sustained, 2-fold activation of peptide kinase activity, which was rapidly reversed by the re-introduction of 10 mM glucose to the medium (Figure 1). The true effect of glucose removal on AMPK activity was actually greater than 2-fold, because immunodepletion of the extracts with anti-(AMPK β subunit) antibody showed that a substantial proportion (75 %) of the basal peptide kinase activity, but only a small proportion (25 %) of the glucose-sensitive activity, was not precipitated by the antibody, and therefore appears to be due to protein kinases other than AMPK. The true basal AMPK activity was low and difficult to measure accurately, but glucose removal caused at least 5-fold activation of true AMPK activity. Very similar results were obtained in INS-1 cells. The peptide kinase activity rose from 0.1–0.2 to 0.5–0.6 nmol/min per mg within 30 min of glucose removal, and rapidly reverted to basal values on re-addition of 10 mM glucose (results not shown; but see Figure 2).

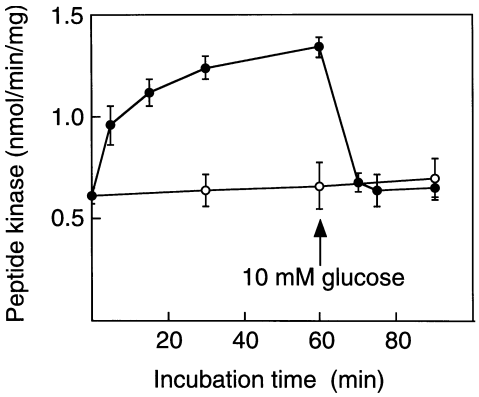

Figure 1 Effect of removing glucose from the extracellular medium of HIT-T15 cells

Cells were incubated in 10 mM glucose (○) or glucose-free medium (●) for 60 min. At the point shown by the arrow, glucose (10 mM) was re-introduced into the medium of the glucose-free cells. Extracts were prepared at various times and assayed for AMPK activity. The results are means ± S.E.M. for three separate experiments.

Figure 2 shows the effect of different concentrations of glucose on peptide kinase activity and insulin secretion in HIT-T15 cells (upper panel) and INS-1 cells (lower panel). In both cases, AMPK activity and insulin secretion were inversely related, with the concentrations having half-maximal effects on insulin release being marginally greater than those having half-maximal effects on AMPK inhibition. The EC_{50} values for insulin release and the IC_{50} values for AMPK inhibition were 2.8 ± 0.9 and 0.7 ± 0.2 mM (HIT-T15) and 4.6 ± 1.4 and 2.8 ± 0.9 mM (INS-1) respectively (means ± S.E.M.; see Figure 2 legend). The HIT-T15 cells were affected by lower glucose concentrations for both parameters.

It is well known that HIT-T15 cells lose the glucose sensitivity of insulin release after a high number of passages in culture [39]. By contrast, Louis and Witters [26] claimed that the glucose effect on ACC activity was retained in late-passage HIT cells. However, Figure 3 shows that, after more than 100 passages in culture (when the glucose-sensitive insulin release had been lost; results not shown), AMPK was no longer activated by glucose deprivation, although the response was normal in cells that had undergone only 74 passages. The high-passage cells could still respond to other AMPK-activating stimuli, since 500 μM arsenite activated the kinase by 2–3-fold within 15 min (results not shown).

Glucose removal activates AMPK by increased phosphorylation

To investigate whether the activation of AMPK by glucose removal was due to reversible phosphorylation, cell extracts were prepared from control or glucose-deprived HIT-T15 cells and assayed for AMPK activity during incubation with PP2A and subsequent incubation with MgATP and AMPKK. Figure 4 shows that the peptide kinase activity in extracts of glucose-deprived cells was 2-fold higher than that in control extracts, as before (for reasons explained in [29], we used the AMARA peptide as substrate in these assays, which is why the specific activities are slightly higher than those in Figures 1–3). Treatment with PP2A completely abolished the difference, by reducing the activity in extracts from glucose-deprived cells without affecting the activity in control extracts. This effect of PP2A was blocked by 100 nM okadaic acid (results not shown). Figure 4 also shows that, if okadaic acid was added to the PP2A-treated extracts at 20 min together with MgATP and AMPKK, a re-activation was seen in both extracts, reaching a plateau at an activity similar to that present in the initial extract of glucose-deprived cells.

Glucose removal increases cellular AMP/ATP and ADP/ATP ratios

To determine whether the activation of AMPK in glucose-deprived cells was associated with an alteration in the cellular AMP/ATP or ADP/ATP ratio, the adenine nucleotide content of early-passage HIT-T15 cells was examined using an HPLC method [6] after incubation of cells in different glucose concen-

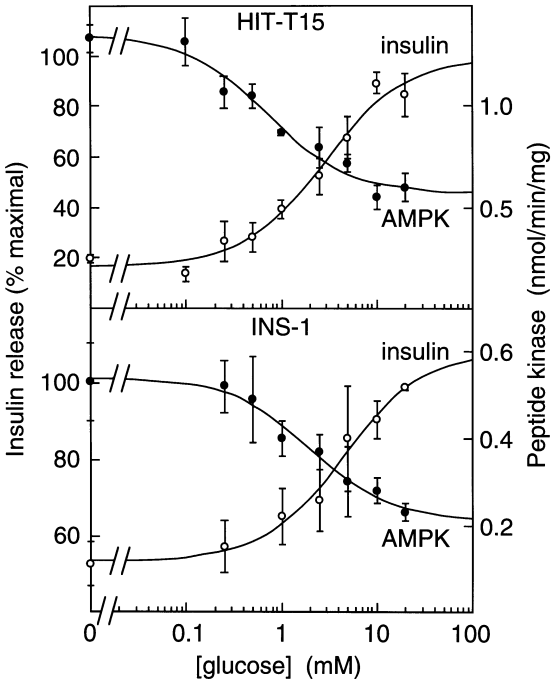

Figure 2 Effects of extracellular glucose concentration on AMPK activity (●) and insulin secretion (○) in HIT-T15 cells (upper panel) and INS-1 cells (lower panel)

Cells were incubated in medium containing various glucose concentrations for 60 min. Cell extracts were prepared and assayed for AMPK activity. The results are means ± S.E.M. for three (AMPK activity), eight (insulin; HIT-T15 cells) or four (insulin; INS-1 cells) experiments. The AMPK activity data were fitted to the equation:

$$\text{Activity} = \text{Basal} \times I - \{(\text{Basal} \times I - \text{Basal}) \times [\text{glucose}]\}/(IC_{50} + [\text{glucose}])$$

where Basal is the activity in saturating glucose and I = (activity in zero glucose)/(activity in saturating glucose). For each experiment, insulin release was measured in units/mg of protein and expressed as a percentage of the maximal release, which in some experiments with HIT-T15 cells occurred at 10 mM rather than 20 mM glucose. Insulin secretion data were fitted to the equation:

$$\text{Insulin release} = \text{Basal} + \{(\text{Maximal} \times [\text{glucose}])/(EC_{50} + [\text{glucose}])\}$$

where Basal is secretion in zero glucose and Maximal is secretion in saturating glucose. The continuous lines are theoretical curves derived from the fits; the values derived for the parameters EC_{50} and IC_{50} are quoted in the text.

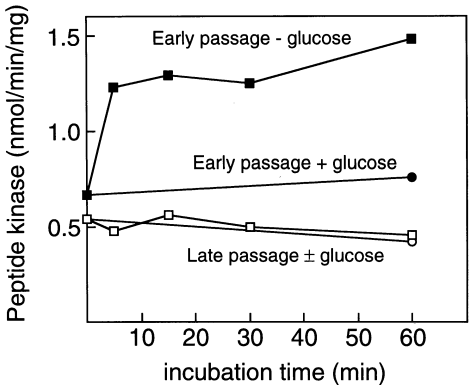

Figure 3 Effect of removing glucose (■, □) from the extracellular medium of late-passage (101 passages; □, ○) or early-passage (74 passages; ■, ●) HIT-T15 cells

Cell extracts were prepared at various times after removal of glucose and assayed for AMPK activity. Control cells (○, ●) were maintained in medium containing 10 mM glucose throughout. This experiment was repeated three times with cells at similar passage numbers, and very similar results were obtained.

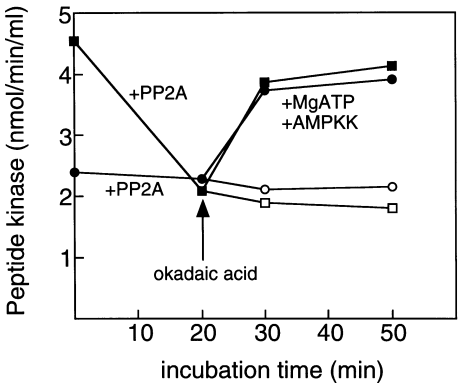

Figure 4 Effect of glucose removal on the phosphorylation state of AMPK in HIT-T15 cells

Extracts were prepared from cells incubated for 60 min in the presence (●, ○) or absence (■, □) of 10 mM glucose. Extracts were incubated in the presence of PP2A for 20 min. Dephosphorylation was terminated by the addition of okadaic acid. After 20 min the dephosphorylated AMPK was incubated with MgATP either with (●, ■) or without (○, □) purified AMPKK. Aliquots were removed at intervals and assayed for AMPK activity using the AMARA peptide. See the Experimental section for details. Very similar results were obtained in one other experiment.

trations (Figure 5). The ADP/ATP ratio increased by about 2-fold as the glucose concentration in the medium was decreased from 1 to 0.1 mM. The AMP concentration was too low to measure accurately in cells incubated in > 1 mM glucose, but as the glucose concentration fell from 1 to 0.1 mM a clear increase in the AMP/ATP ratio could be measured. The ADP/ATP ratio increased over broadly the same range of glucose concentrations as did AMPK activity (cf. Figure 2), although the IC_{50} was a little lower for the effect on the ADP/ATP ratio than for the effect on AMPK activity (0.2 ± 0.03 mM compared with 0.7 ± 0.2 mM). The results were similar in INS-1 cells (not shown). However, in late-passage HIT-T15 cells no alterations in adenine nucleotide ratios could be detected in response to glucose removal (results not shown).

Effect of 5-aminoimidazole-4-carboxamide (AICA) riboside on insulin secretion in INS-1 cells

AICA riboside is an adenosine analogue which is taken up into cells and converted by adenosine kinase into a phosphorylated form, AICA riboside monophosphate (ZMP) [40,41]. ZMP mimics the effects of AMP on both the allosteric activation and the phosphorylation of AMPK [41,42]. In order to address a possible effect of AMPK activation on insulin secretion, we incubated the two cell lines with AICA riboside in the presence of 10 mM glucose.

In HIT-T15 cells, incubation with AICA riboside did not affect AMPK activity or insulin secretion, nor did it cause accumulation of ZMP within the cells as judged by HPLC analysis (results not shown). The reasons why AICA riboside is ineffective in these cells are not certain, but we suspect that ZMP may be metabolized rapidly and does not accumulate to levels sufficient to activate AMPK. In INS-1 cells, however, incubation with AICA riboside led to accumulation of ZMP within the cells that was maximal by 30 min, and was associated with a 2-fold

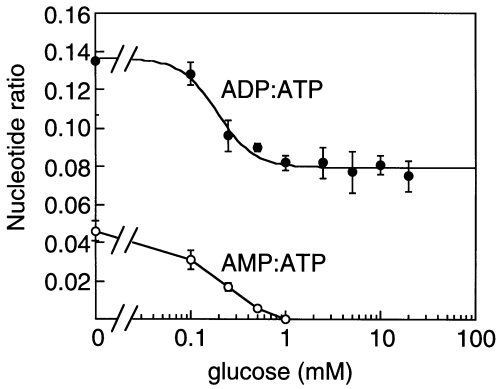

Figure 5 Effect of extracellular glucose concentration on HIT-T15 cell AMP/ATP ratio (○) and ADP/ATP ratio (●)

Cells were incubated in medium containing the indicated glucose concentration for 60 min. Cell extracts were analysed for adenine nucleotide content by anion-exchange HPLC. At glucose concentrations of > 1 mM, the AMP level was too low to allow an accurate measure of the AMP/ATP ratio. Results are expressed as means ± S.E.M. for three experiments. Data for the ADP/ATP ratio were fitted to the equation:

$$ADP/ATP = Basal \times I - \{(Basal \times I - Basal) \times [glucose]\}/(IC_{50} + [glucose])$$

where Basal is the ratio in saturating glucose and I = (ratio in zero glucose)/(ratio in saturating glucose).

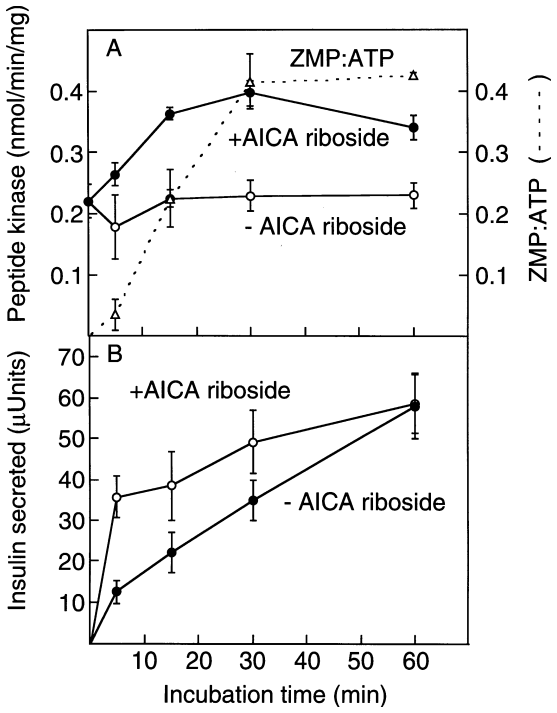

Figure 6 Effect of 1 mM AICA riboside on (A) AMPK activity and ZMP/ATP ratio, and (B) insulin secretion in INS-1 cells

(**A**) Cell extracts were prepared after various periods of incubation in the presence (●) or absence (○) of 1 mM AICA riboside and assayed for AMPK activity. The ZMP/ATP ratio (△; broken line) was determined after anion-exchange HPLC of neutralized $HClO_4$ extracts of cells incubated with AICA riboside. (**B**) Cells were incubated for various times in the presence (○) or absence (●) of 1 mM AICA riboside, and the medium was assayed for total insulin content. All results are means ± S.E.M. for three experiments.

increase in peptide kinase activity (Figure 6A). Surprisingly, AICA riboside caused an initial stimulation of insulin release within 5 min of addition, but over the period between 15 and 60 min, when AMPK activation was evident, there was a 53 % inhibition of insulin secretion (Figure 6B).

Table 1 Insulin secretion from isolated rat islets

Islets were incubated with or without 1 mM AICA riboside for 30 or 60 min, and insulin release was measured by radioimmunoassay. Results are means ± S.E.M. for 12 observations; *effects significant at the $P < 0.05$ level.

	Insulin release (μ-units/h per islet)	
Medium glucose (mM)	Control	AICA riboside
3.3	10.1 ± 2.3	27.2 ± 6.9*
5.5	25.1 ± 5.2	57.7 ± 10.4*
16.7	170 ± 36	84.7 ± 15.9*

Effect of AICA riboside on insulin secretion in rat islets

To investigate the effects of AMPK activation on insulin secretion in a more physiological system, we also examined the effects of AICA riboside on insulin release from isolated rat islets of Langerhans (Table 1). The nucleoside stimulated insulin release at low, submaximal concentrations of glucose, but inhibited release by 50 % at a saturating glucose concentration (16.7 mM).

DISCUSSION

The most significant findings of this study were that, in cell lines derived from hamster and rat pancreatic β cells, AMPK activity was regulated by the concentration of glucose in the medium. Although it has previously been reported that glucose regulates the activity of ACC, a known downstream target of AMPK [26], in HIT cells, our study provides the first direct evidence that glucose regulates AMPK itself. Our study also provides evidence as to the mechanism of activation by glucose removal. The effect is due to increased phosphorylation of AMPK, as shown by the following evidence. (1) The effect survives homogenization of the cells and partial purification by PEG precipitation; an allosteric effect would be unlikely to be preserved during these manipulations. (2) The effect of glucose deprivation is reversed by treating extracts with homogeneous protein phosphatase (Figure 4). (3) The low basal activity in extracts of cells maintained in high glucose can be increased to values similar to those in extracts of glucose-deprived cells by incubation with MgATP and the upstream kinase AMPKK (Figure 4).

In studies using purified cell-free systems, we have shown that AMP stimulates phosphorylation of AMPK by AMPKK, as well as causing direct allosteric activation, and that high ATP concentrations antagonize these effects [11–13]. The results presented here support the idea that increases in the cellular AMP/ATP ratio are responsible for the activation of AMPK on glucose removal from HIT-T15 and INS-1 cells. Although AMP was difficult to measure in cells incubated in glucose concentrations above 1 mM, there was a clear rise in the AMP/ATP ratio when medium glucose was decreased from 1 to 0.1 mM. The ADP/ATP ratio increased by 2-fold over the same range of glucose concentrations (Figure 5). Assuming that adenylate kinase is catalysing a near-equilibrium reaction, one would expect the AMP/ATP ratio to vary approximately as the square of the ADP/ATP ratio [1]. The nucleotide ratios and AMPK activity varied over a broadly similar range of glucose concentrations (cf. Figures 2 and 5), although the IC_{50} for the effect on the ADP/ATP ratio (0.2 mM) was slightly lower than that for

the effect on AMPK activity (0.7 mM). However, there is no reason why there should be a simple linear relationship between these two parameters, and in fact in other experiments we have shown that the relationship between the concentration of activating nucleotide and AMPK activity is sigmoidal (I. P. Salt and D. G. Hardie, unpublished work).

Taking all of these findings together, we propose that incubation of pancreatic β cell lines in low concentrations of glucose, which are not saturating for the glucose transporter and/or the expressed isoform of hexokinase, results in a reduction of glucose metabolism. Since β cells do not store significant reserves of carbon (e.g. glycogen), this leads to a decrease in ATP and a consequent increase in the ADP/ATP ratio, which is amplified by adenylate kinase into an even larger change in the AMP/ATP ratio. This in turn promotes phosphorylation of AMPK by AMPKK, via the multiple mechanisms we have previously elucidated [11–13]. The targets for activated AMPK in β cells are currently poorly defined, but ACC is likely to be one target. This would explain why re-addition of 10 mM glucose to HIT cells that had been deprived of glucose causes an activation of ACC which is stable to purification [26], and why glucose and other fuels cause increases in the malonyl-CoA content in HIT cells [24] and perifused rat islets [25]. Prentki et al. have proposed a model in which an increase in malonyl-CoA concentration is one of the signals that trigger insulin secretion [43]. Our results provide a mechanism by which high glucose causes increases in malonyl-CoA, but do not directly address the question of whether malonyl-CoA is a signal for insulin secretion *per se*. It seems possible to us that depression of the level of malonyl-CoA after activation of AMPK may merely be a mechanism to increase fatty acid oxidation at times when glucose is limiting, as has already been proposed in skeletal muscle [44]. Indeed, elevation of malonyl-CoA in HIT cells incubated with glucose is associated with a decrease in fatty acid oxidation [24].

In order to address whether activation of AMPK has an effect on insulin secretion itself, either via modulation of malonyl-CoA or by some other mechanism, we needed a specific method to activate AMPK even in the presence of high glucose. Incubation with AICA riboside provides such a method: the nucleoside is taken up into cells and phosphorylated to the ribotide, ZMP. In most cells ZMP accumulates to high levels with no effects on AMP, ADP or ATP concentrations [40,41], and ZMP is an AMP analogue which mimics all of the effects of AMP on the AMPK cascade [41]. There have been two previous studies of the effects of AICA riboside on insulin secretion [45,46], but the results were contradictory, and in neither case was it confirmed that ZMP was accumulating inside the cells, or that the riboside was causing activation of AMPK. Zhang and Kim [45] reported that 1 mM AICA riboside caused a marked inhibition of insulin secretion in INS-1 cells, while Akkan and Malaisse [46] reported that it stimulated insulin secretion in isolated rat islets or perfused pancreas.

We found both stimulatory and inhibitory effects of AICA riboside, depending on the conditions. With INS-1 cells incubated in 10 mM glucose, AICA riboside stimulated insulin release at early time points (5 min), and it also stimulated insulin release in rat islets in low glucose concentrations. Although the mechanisms for these effects remain unclear, we suspect that they are not AMPK-mediated. In the INS-1 cell experiments, accumulation of ZMP and activation of AMPK was barely significant in the 5 min time point where the effect on insulin release was seen. In the rat islet experiments, one might expect that AMPK would already have been fully activated by the low glucose concentration, and that AICA riboside would have no additional effect. We believe that the inhibitory effects on insulin release at later time points in high glucose concentrations, in both INS-1 cells and rat islets, are much more likely to be mediated by AMPK activation, although the target(s) of the kinase responsible for this effect remain unknown.

As with any pharmacological method, one has to carefully consider the specificity of AICA riboside as an AMPK activator. Its intracellular derivative, ZMP, can be regarded as an AMP analogue, and has been reported to inhibit gluconeogenesis by inhibiting fructose-1,6-bisphosphatase [47], and to activate glycogenolysis by activating phosphorylase [48]. However, neither of these pathways is likely to be operative in β cells. Since AICA riboside can be regarded as an adenosine analogue, another possibility was that the riboside acted externally by binding to adenosine receptors. Rat and mouse islets express A_1-type adenosine receptors, activation of which inhibits insulin release by lowering cAMP [49–52]. However, AICA riboside does not act as an agonist or antagonist at A_1 receptors in rat brain synaptosomes (D. Budd and D. G. Nicholls, personal communication). The finding that AICA riboside did not inhibit insulin release in HIT-T15 cells also strongly suggests that the effect is a consequence of the intracellular accumulation of ZMP, rather than an extracellular effect of AICA riboside itself.

Clearly, more specific methods for manipulating AMPK activity are required, and various molecular biological approaches towards this end are currently under development. However, we believe our results with AICA riboside do provide initial evidence that activation of AMPK in low glucose concentrations contributes to the low insulin release under these conditions. The downstream target(s) of AMPK responsible for this effect remains unknown. Finally, our hypothesis does not exclude the simultaneous occurrence of the widely accepted mechanism whereby high ATP levels in cells maintained in high concentrations of glucose inhibit the opening of ATP-sensitive K^+ channels.

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