Extracellular adenosine activates AMP-dependent protein kinase (AMPK)

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Summary

Adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a heterotrimeric complex that senses intracellular energy status and exerts rapid regulation on energy-demanding and -consuming metabolic pathways. Although alterations in the intracellular adenosine nucleotide pool are traditionally assumed to be the consequence of changes in energy metabolism, in this study we have addressed the question of whether extracellular adenosine contributes to AMPK regulation. In the intestinal rat epithelial cell line IEC-6, addition of adenosine rapidly increases AMP intracellular concentrations and upregulates α1AMPK, thus promoting phosphorylation of its downstream target acetyl-CoA carboxylase (ACC). The effect of adenosine on AMPK signaling is completely blocked by transducing IEC-6 cells with an adenoviral vector expressing a mutated α1 subunit, resulting in a dominant-negative effect on endogenous AMPK activity. These effects are blocked by 5′-iodotubercidine (5′-ITU), an inhibitor of adenosine kinase. Moreover, inhibition of adenosine transport through the concentrative adenosine plasma membrane transporter CNT2 with formycin B results in the blockade of adenosine-mediated AMPK signaling. Extracellular adenosine is equally able to activate AMPK and promote ACC phosphorylation in liver parenchymal cell models in a manner that is also inhibited by 5′-ITU. In summary, this study shows that adenosine, when added at physiological concentrations, activates AMPK and promotes ACC phosphorylation. Adenosine must be transported and phosphorylated to exert its action. Thus, nucleoside transporters might be novel players in the complex regulation of AMPK and energy metabolism.

Key words: Adenosine, AMPK, Transport, CNT2, ACC

Introduction

The adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a heterotrimeric complex that is directly implicated in the control of energy metabolism both at the cellular and whole-organ level (Carling, 2004; Hardie, 2003; Hardie and Carling, 1997; Rutter et al., 2003). AMPK activates energy-producing pathways and downregulates energy-consuming metabolic processes (Hardie and Carling, 1997). It promotes glucose transport and fatty acid oxidation in the skeletal muscle and is a putative target for the treatment of type II diabetes (Rutter et al., 2003). The role of AMPK in inter-organ metabolic regulation is illustrated by the fact that adipocyte-secreted hormones, such as adiponectin (Ad), exert their action on skeletal muscle and liver through AMPK activation, thus leading to the stimulation of glucose utilization and fatty acid oxidation, as well as to a reduction of gluconeogenesis (Yamauchi et al., 2002). Whole-body nutrient homeostasis is also regulated by changes in AMPK activity in the brain. Expression of a dominant-negative AMPK in the hypothalamus reduces food intake, whereas a constitutively active kinase triggers the opposite effect (Minokoshi et al., 2004).

The biochemical mechanisms responsible for the increase in AMPK activity and phosphorylation of its downstream targets have been comprehensively reviewed (Carling, 2004; Hardie, 2003; Rutter et al., 2003) and essentially involve two interacting processes: allosteric activation and changes in the phosphorylation state of the enzyme. AMP binds to the kinase and allosterically activates it (Hardie, 1999); moreover, it makes the enzyme a better substrate for the upstream kinases (AMPKK) (Hawley et al., 1995). AMP interaction with AMPK also seems to turn this kinase into a poorer substrate for phosphatases (Davies et al., 1995). Phosphorylation of AMPK occurs at Thr172 of the catalytic α subunit (Hawley et al., 1996; Stein et al., 2000). Two isoforms with different tissue distribution have been identified for the α subunit: α1 and α2 (Stapleton et al., 1996). Some evidence suggests that the two isoforms might exhibit different regulatory properties (Salt et al., 1998).

Efforts to identify upstream AMPKKs have recently resulted in the characterization of the first AMPKK – the tumor suppressor LKB-1 (Hawley et al., 2003; Woods et al., 2003). The LKB-1 kinase is involved in determining epithelial polarity (for reviews, see Baas et al., 2004; Spicer and Ashworth, 2004), as well as modulating other important cell functions such as apoptosis (Shaw et al., 2004).

From the evidence discussed above, it appears likely that slight changes in intracellular AMP concentrations will modulate AMPK activity in a synergistic manner. Although alterations in the intracellular adenosine nucleotide pool are

traditionally assumed to be the consequence of changes in energy metabolism (e.g. those derived from oxidative stress), the possibility that extracellular adenosine contributes directly to AMPK activation has not been previously addressed. Nevertheless recent work from Saitoh et al. demonstrated that adenosine induces apoptosis in gastric cancer cells, apparently through the activation of AMPK, in a way that appears to depend on extracellular adenosine availability (Saitoh et al., 2004). Unfortunately, these experiments were performed using nucleoside concentrations in the mM range, which are at least two orders of magnitude higher than those expected in body fluids and might be even toxic.

Adenosine is taken up into mammalian cells by equilibrative (ENT) and concentrative (CNT) transporters (for reviews, see Baldwin et al., 2004; Gray et al., 2004; Pastor-Anglada et al., 2004). In particular, ENT1 and CNT2 are responsible for this process, since the expression of both transporters is widespread, and they show an apparent K_m for adenosine in the physiological range (40 μM and 8 μM for ENT1 and CNT2, respectively) (Wang et al., 1997; Ward et al., 2000). Nevertheless, CNT-type carriers are Na^+-dependent concentrative transporters, with either one Na^+ or two Na^+ molecules per nucleoside being translocated (Larrayoz et al., 2004; Ritzel et al., 2001; Smith et al., 2004). This makes CNT2 a better candidate to mediate adenosine transport and thereby contribute to the intracellular AMP pool, although a role for ENT1 cannot be ruled out. Moreover, we have recently demonstrated that CNT2 function can be transiently stimulated by purinergic activation of A1 receptors, through a mechanism that is mediated by K_{ATP} channel opening (Duflot et al., 2004). Since this particular type of K^+ channel is indeed a sensor of changes in the adenosine nucleotide pool, as is AMPK, extracellular adenosine, and in particular its uptake through CNT2, might contribute to AMPK regulation.

In this study we have addressed the question of whether extracellular adenosine is a suitable activator of AMPK. We demonstrate that adenosine transport and phosphorylation are required for the activation of AMPK and the modulation of its downstream target acetyl-CoA carboxylase (ACC) in the rat intestinal epithelial cell line IEC-6, as well as in liver parenchymal and FAO cells. These epithelial cell models show significant CNT2 expression, and partial inhibition of CNT2 function without modifying ENT-type transport activity results in the blockade of the adenosine-mediated activation of the AMPK pathway. This suggests that the highly regulated CNT2 transporter is a novel target of AMPK-mediated signaling.

Results

Adenosine activates AMPK in IEC-6 cells

To determine whether adenosine can activate AMPK, the nucleoside was added to the culture medium of IEC-6 cells at a concentration of 10 μM. Ten minutes after adenosine addition, AMPK activity was measured following immunoprecipitation of the kinase using either anti-α1AMPK or anti-α2AMPK antibodies. As shown in Fig. 1A, most of the AMPK activity in IEC-6 cells was associated with the α1-containing heterotrimer. Adenosine upregulated AMPK activity by nearly twofold. As a control for this experiment, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), a known pharmacological activator of AMPK, was added to the culture medium at a concentration (500 μM) known to exert maximal AMPK

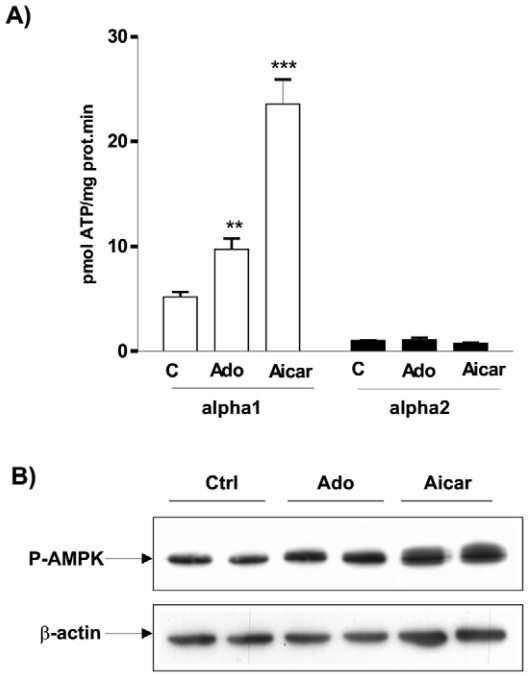

Fig. 1. Adenosine activates AMPK in IEC-6 cells. (A) IEC-6 cells were incubated either in the presence or absence of 10 μM adenosine (Ado) or 500 μM AICAR (Aicar) for 10 minutes and 1 hour, respectively; C, control. AMPK complexes were isolated from cell lysates by immunoprecipitation with antibodies to either anti-α1AMPK (open bars) or anti-α2AMPK antibody (solid bars). α1AMPK and α2AMPK complex activities were measured by phosphorylation of AMARA peptide. Activities shown are the mean and s.e. of five and three independent experiments for α1AMPK and α2AMPK, respectively, with each assayed in duplicate. The statistical significance of the adenosine and AICAR effects was established by Student's *t*-test (**P<0.01; ***P<0.001). (B) Cells were treated with either adenosine or AICAR or control (Ctrl), as detailed above. Equal amounts of protein (40 μg) were loaded to allow direct comparisons of phosphorylated AMPK (P-AMPK) levels. β-actin was used as a loading control. A representative western blot is shown.

activation, and AMPK activity was measured 60 minutes after the addition of the drug. As for adenosine, activation was only evident for the predominant α1-containing AMPK. The increase in kinase activity triggered by adenosine and AICAR was closely correlated with the phosphorylation state of Thr172 (Fig. 1B).

To define better the conditions required for adenosine to activate AMPK, a time-course (Fig. 2A) and a dose-response analysis (Fig. 2B) of this effect was performed. As shown in Fig. 2A, adenosine rapidly affected AMPK phosphorylation (already evident 1 minute after nucleoside addition) and the effect was relatively sustained (the kinase still showed increased phosphorylation of Thr172 30 minutes after adenosine addition). Moreover, the effect was dose dependent, with a maximum at 10 μM. The lowest adenosine concentrations assayed (1 μM) significantly increased AMPK phosphorylation. A similar pattern was obtained when α1AMPK activity was monitored after treatment of IEC-6 cells with increasing concentrations of adenosine and the $K_{0.5}$ value of the adenosine effect was 0.47 μM (Fig. 2C). Thus, the effect

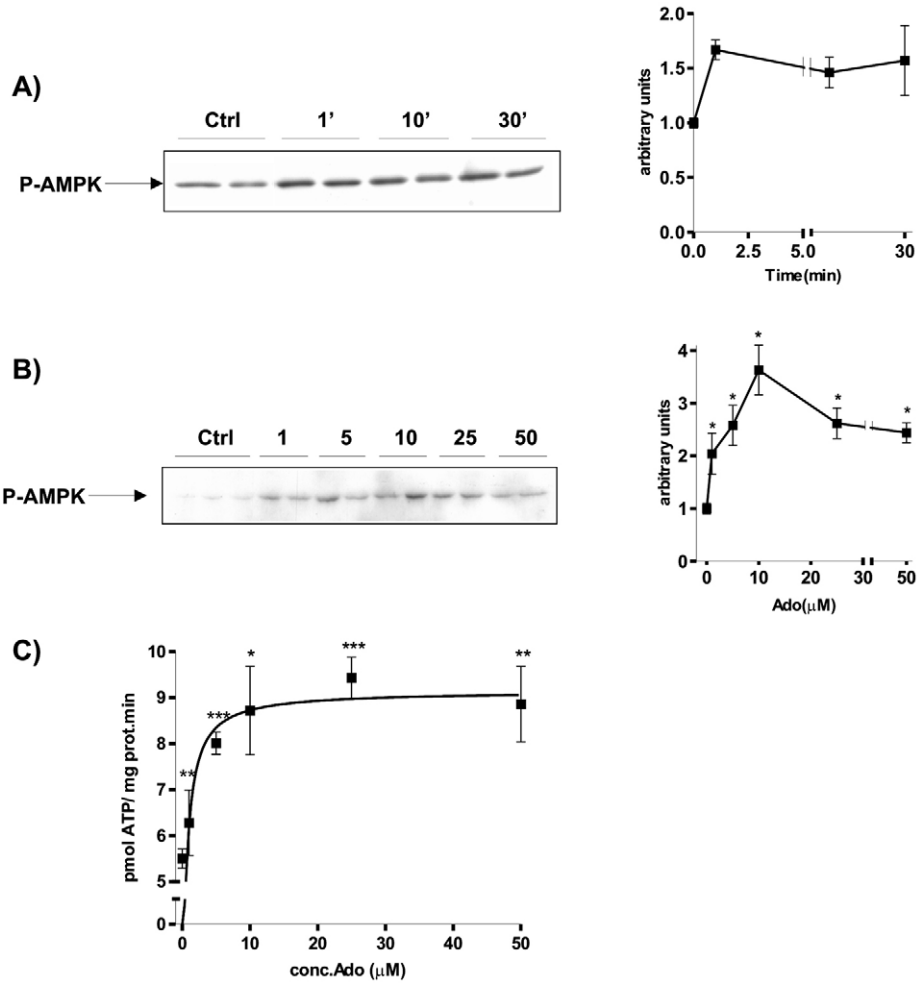

Fig. 2. Dose dependence of the adenosine effect on AMPK phosphorylation and activity. (A) Phosphorylated AMPK (P-AMPK) protein levels were monitored by western blot at the indicated times after the addition of 10 μM adenosine. (B) The dose dependence of the adenosine effect was assessed after treatment for 10 minutes at the adenosine concentrations shown. Representative western blots are shown. The magnitude of the induction triggered in four independent experiments was quantified densitometrically. Results (mean ± s.e.m.) are shown as arbitrary units normalized to control values (non-treated cells). (C) Endogenous α1AMPK activity was measured 10 minutes after the addition of adenosine at the indicated concentrations. Non-linear fitting of the data was used for the estimation of the $K_{0.5}$ value. Results are the mean and s.e. from four independent experiments. The statistical significance of the adenosine effect was assessed by analysis of variance (P<0.05) combined with the Student's t-test: *P<0.05; **P<0.01; ***P<0.001. Ado, adenosine; Ctrl, control.

triggered by this nucleoside is physiologically relevant, since it occurs within the range of adenosine concentrations found in body fluids. Adenosine in peripheral blood has been reported to occur at concentrations of 0.3-1 μM (Lasley et al., 1998; Pasini et al., 1996; Saadjian et al., 2002); however, in the intestinal lumen and the portal vein it is likely to achieve higher levels. Moreover, it is accepted that adenosine can also be produced by ectonucleotidase action on nucleotides, thus leading to high local nucleoside concentrations (Che et al., 1997; Roman and Fitz, 1999). To confirm that extracellular adenosine induces an increase in AMP intracellular concentration, the adenine nucleotide content was measured at different times after addition of 10 μM adenosine. Considering a mean volume of IEC-6 cells of 2.5×10^{-6} μl, as determined by flow cytometry, basal AMP concentrations reported here were found to be 0.24 mM, in the range of what has been previously reported (Lopez et al., 2003; Noma, 2005). As shown in Table 1, adenosine treatment resulted in an immediate and transient (from 30 seconds to 1 minute after nucleoside addition) twofold increase in total AMP intracellular levels that coincides with the beginning of AMPK activation. The other adenine nucleotides, adenosine diphosphate (ADP) and adenosine triphosphate (ATP), remained practically unchanged in the conditions assayed. This indicates that changes in extracellular adenosine concentrations modulate the adenine nucleotide pool.

Adenosine triggers the phosphorylation of AMPK downstream targets

To determine whether the activation of AMPK triggered by adenosine could indeed be translated into known downstream targets of the kinase, we analyzed ACC phosphorylation in IEC-6 cells that had been treated either with 10 μM adenosine

Table 1. Nucleotide concentrations of IEC-6 cells after adenosine treatment

	AMP (nmol/10^7cell)	ADP (nmol/10^7cell)	ATP (nmol/10^7cell)
No inhibitor			
Control	6.11±0.57	9.01±0.49	26.86±0.74
Ado 30 seconds	15.29±2.18*	9.14±1.87	25.05±3.08
Ado 1 minute	12.17±1.47*	7.68±1.35	21.70±1.63
Ado 10 minutes	9.65±1.72	5.92±1.22	23.59±1.63
Ado 60 minutes	8.43±0.32	5.68±1.09*	31.85±2.66
5′-ITU			
Control	9.08±0.14	4.76±0.61*	20.07±1.34*
Ado 30 seconds	9.32±1.03	6.35±0.27	22.41±2.88
Ado 1 minute	6.46±1.25†	5.94±0.96	25.54±3.27

Cells were incubated with 10 μM adenosine (Ado) in the absence or presence of 5′-iodotubercidine (5′-ITU) for the indicated times. Results are expressed in nmol/10^7 cells as mean ± s.e.m. from three experiments. The statistical significance was assessed by Student's-t test: *P<0.05; †P<0.05 inhibitor vs no inhibitor.

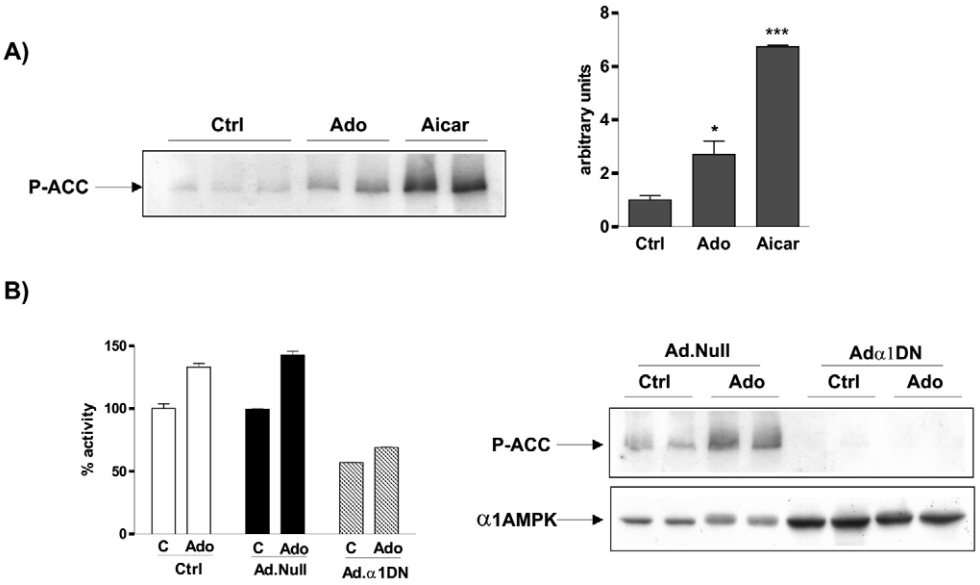

Fig. 3. Adenosine promotes phosphorylation of the AMPK downstream target acetyl-CoA carboxylase (ACC) in IEC-6 cells. (A) Western blot analysis of phosphorylated ACC (P-ACC) was performed after treatment for 10 minutes and 60 minutes with 10 μM adenosine (Ado) and 500 μM AICAR (Aicar), respectively; Ctrl, control. A representative immunoblot is shown. The densitometric analysis corresponds to the mean ± s.e.m. of three independent experiments. The statistical significance was assessed by Student's *t*-test: **P*<0.05; ****P*<0.001. (B) Cells were infected with either Ad.Null or Ad.α1DN adenoviral vectors (30 pfu/cells). At 24 hours post-infection, cells were incubated for 10 minutes either in the presence (Ado) or the absence (C) of 10 μM adenosine. Total AMPK activity was measured in cell lysates, without prior immunoprecipitation, using the AMARA peptide assay and ACC regulation was monitored by western blot under the same conditions. As a control for infection efficiency, total α1AMPK subunit protein was measured by western blot in both Ad.Null- and Ad.α1DN-infected cultures.

for 10 minutes or with 500 μM AICAR for 60 minutes. As shown in Fig. 3A, both adenosine and AICAR, the latter used again at maximal concentrations, caused increased phosphorylation of ACC. To demonstrate further that this effect was mediated by AMPK, IEC-6 cells were transduced with an adenoviral vector expressing a mutated α1AMPK subunit. This results in a dominant-negative effect on endogenous AMPK subunits, thereby also significantly inhibiting basal activity and phosphorylation of the downstream target ACC (Fig. 3B). Non-transduced cells and cells infected with a null vector still retained the ability to respond to adenosine in terms of activation of AMPK (Fig. 3B, left panel). Accordingly, adenosine was able to promote ACC phosphorylation in cells transduced with the null vector, whereas phosphorylation of the enzyme was significantly decreased and no further increase was evident after adenosine treatment in IEC-6 cells infected with the dominant-negative mutant (Fig. 3B, right panel). Western blot analysis of total α1 subunit protein revealed that adenoviral transduction with the mutant indeed resulted in a much higher protein abundance than its corresponding null vector used as a control.

Adenosine phosphorylation is required for AMPK activation

Adenosine is an autocoid that exerts a variety of effects through several types of membrane adenosine receptors. Thus, the mere addition of adenosine to the culture medium does not provide any insight into whether the activation of AMPK is the result of a purinergic effect triggered by this nucleoside. To rule out this possibility, adenosine phosphorylation to yield AMP was blocked with 5′-iodotubercidin (5′-ITU), a known inhibitor of adenosine kinase. As shown in Fig. 4A, the increase in α1AMPK activity triggered by adenosine was significantly blocked if cells had been pretreated for 30 minutes with 0.2 μM 5′-ITU. This adenosine kinase inhibitor was equally able to inhibit AMPK and ACC phosphorylation (Fig. 4B). In fact, the inhibition of adenosine kinase prevented the increase in intracellular AMP concentrations after the addition of adenosine (Table 1). Pretreatment with 0.2 μM 5′-ITU resulted in a marked decrease in ADP concentrations and, to a much lesser extent, in ATP levels (Table 1), probably as a compensatory mechanism associated with the inhibition of adenosine phosphorylation (Table 1). This adenosine kinase inhibitor also blocked, as expected, AICAR-triggered AMPK phosphorylation (Fig. 4C). Moreover, inosine, a metabolite generated from adenosine by deamination through adenosine deaminase, did not significantly modify AMPK activity (not shown). Thus, adenosine metabolism and, more precisely, generation of AMP from extracellular adenosine is required for AMPK activation.

Adenosine transport is required for AMPK activation

At this point we wondered whether adenosine transport contributes to AMPK activation, and in particular whether selected transporters play significant roles in this effect. As introduced above, the concentrative high-affinity CNT transporters, and in particular CNT2, are good candidates for having a role in adenosine-triggered AMPK activation. Thus, we determined whether IEC-6 cells express CNT proteins. As shown in Fig. 5, the high-affinity adenosine transporter CNT2 is highly expressed in this cell line. CNT2 mRNA is detected in IEC-6 cells, as well as in rat small intestine (Fig. 5A). Since CNT2 is relatively abundant in hepatocytes, its mRNA was also easily detected by RT-PCR in liver, in the rat hepatoma cell line FAO and in primary cultures of rat hepatocytes (Fig. 5A). Accordingly, CNT2 protein was also found in these three cell types (IEC-6, FAO and hepatocytes), in both crude extracts and membrane fractions, in which significant enrichments of this transporter were found (Fig. 5B). This correlated with a Na^+-dependent adenosine transport activity, which was much higher in IEC-6 than in FAO cells or

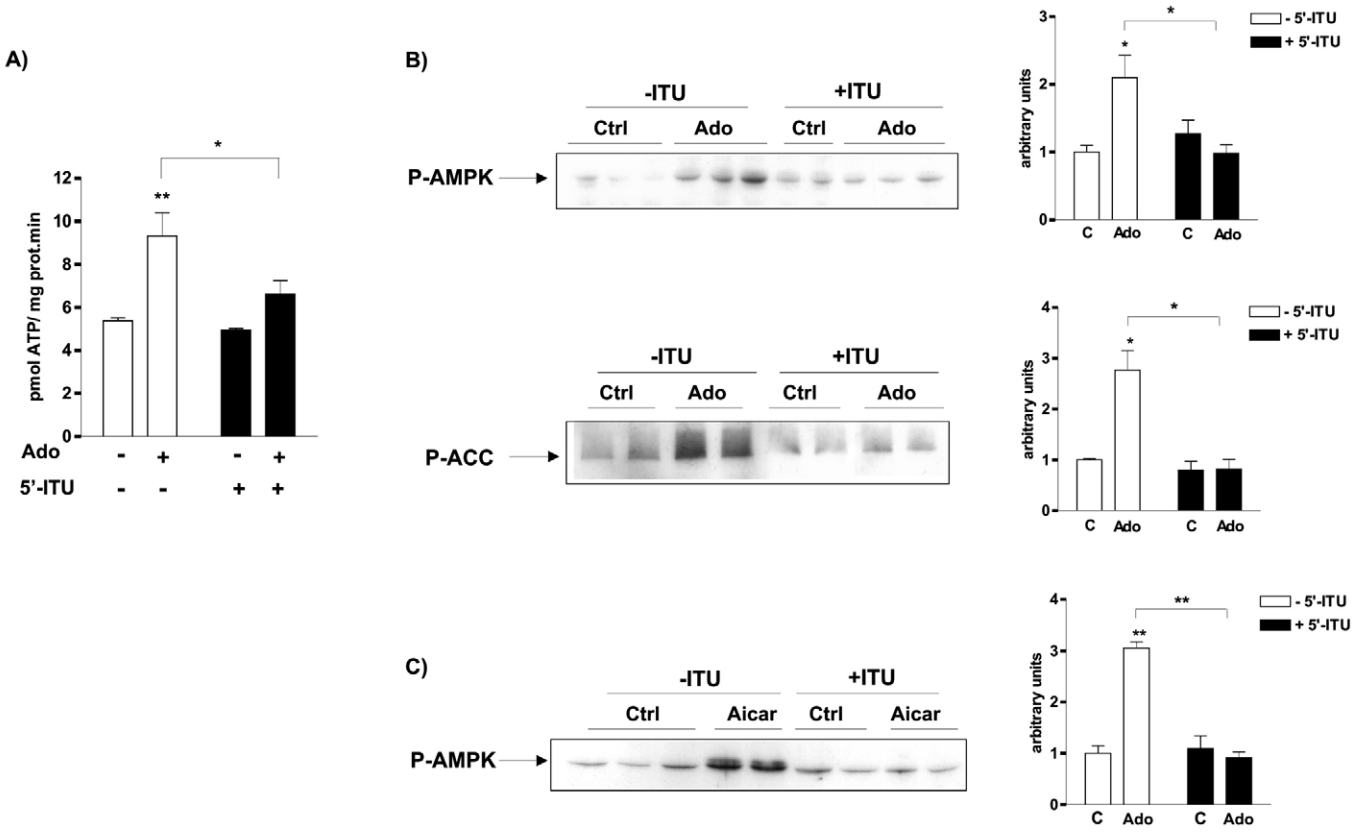

Fig. 4. Adenosine phosphorylation is required for AMPK activation in IEC-6 cells. The effect of adenosine kinase inhibition on the increase in AMPK activity triggered by adenosine (Ado) was monitored after pre-incubating the cells with 0.2 μM 5′-iodotubercidin (5′-ITU) for 30 minutes. (A) Endogenous α1AMPK activity (mean ± s.e.m.) from four independent experiments. (B) Two representative western blots of phosphorylated AMPK (P-AMPK) and phosphorylated ACC (P-ACC), and corresponding densitometric analysis (means ± s.e.m.). (C) The effect of 5′-ITU on AICAR-triggered AMPK stimulation was determined. A representative western blot and corresponding densitometric analysis is shown. The statistical significance was assessed by Student's *t*-test: A: **P*<0.05, ***P*<0.01; B: **P*<0.05; C: ***P*<0.01. Ctrl or C, control.

hepatocytes (Fig. 5C). Almost all adenosine transport measured under these conditions was inhibited by high concentrations of guanosine, but could not be inhibited by cytidine, suggesting that this transport activity was accounted for exclusively by CNT2 (data not shown).

Although there are no specific blockers of CNT2-mediated substrate translocation, CNT2 function can be cis-inhibited by the nucleoside analog formycin B, a high-affinity CNT2 substrate that is poorly metabolized. Thus, this drug could be a suitable tool with which to determine whether CNT2-mediated transport contributes significantly to AMPK

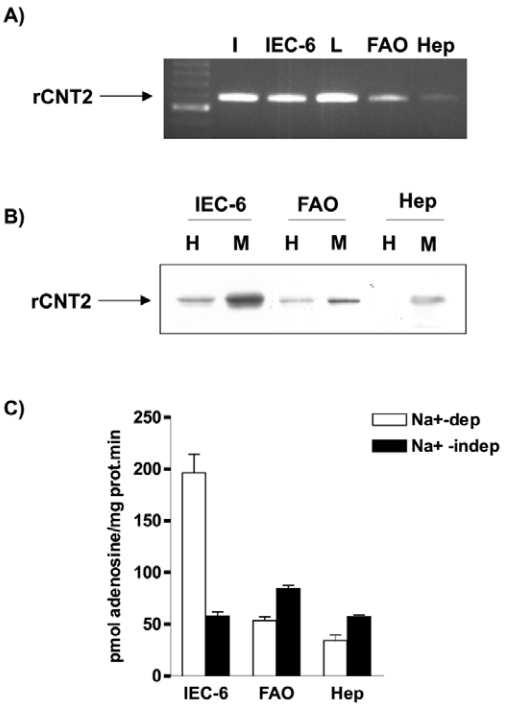

Fig. 5. Basal CNT2 expression and activity in intestinal and liver cells. (A) Reverse transcriptase (RT)-PCR was performed on RNA from intestinal mucosa (I), IEC-6 cells, liver (L), FAO cells and rat liver parenchymal cells (Hep). (B) CNT2 protein expression was analyzed in crude extracts (H) and total membrane (M) fractions from IEC-6 cells, FAO cells and rat hepatocytes (Hep) by western blot. (C) CNT2-mediated uptake (open bars) was measured, as indicated in the Materials and Methods, using 1 μM adenosine as a substrate and 1 minute incubation time. Na^+-independent adenosine uptake (solid bars) corresponds to the sum of ENT1- and ENT2-related transport activities, plus putative binding. Results are the mean ± s.e.m. of four experiments.

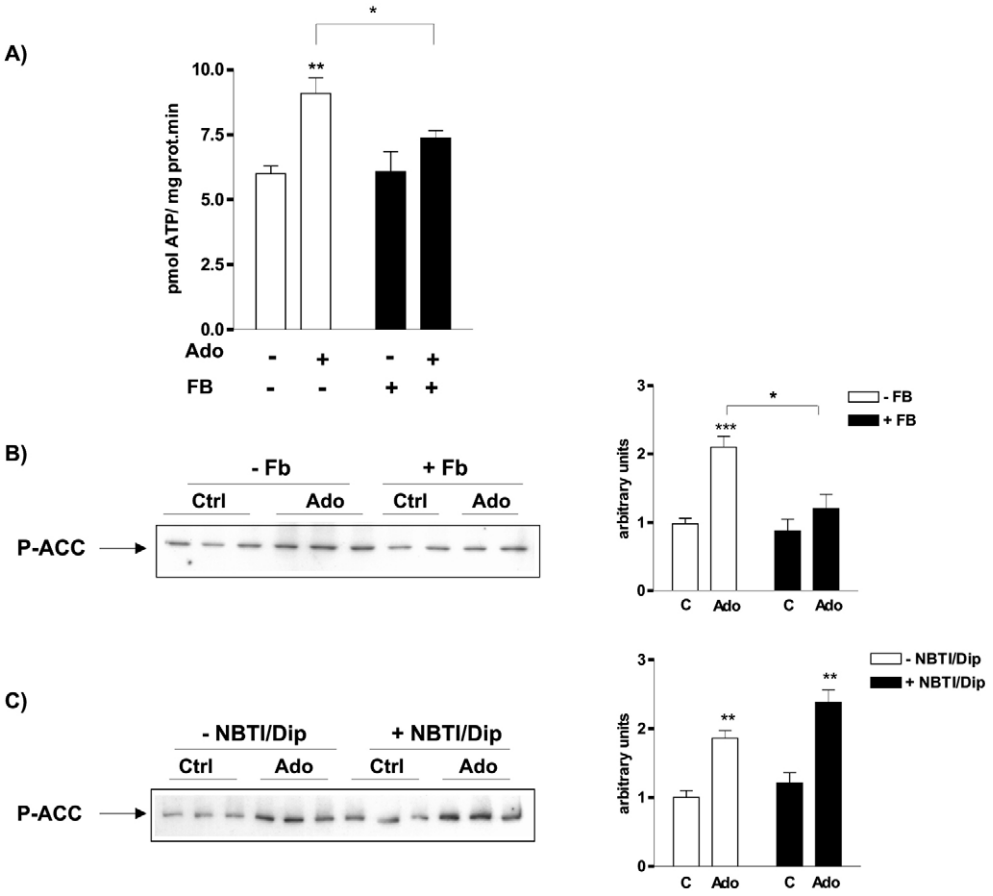

Fig. 6. CNT2-mediated adenosine transport is required for AMPK activation in IEC-6 cells. Cells were treated with 1 μM adenosine (Ado), either in the absence or presence of 100 μM formycin B (FB) for 10 minutes, and then processed as indicated previously. (A) α1AMPK activity (mean ± s.e.m.) from four independent experiments. (B) A representative western blot of phosphorylated ACC (P-ACC) and corresponding densitometric analysis of the gels (mean ± s.e.m.). (C) Cells were incubated with 1 μM adenosine, either in the absence or the presence of 1 μM NBTI and 1 μM dipyridamole (Dip) for 10 minutes. A representative western blot of phosphorylated ACC and the corresponding densitometric analysis (mean ± s.e.m.) is shown. The statistical significance was assessed by Student's *t*-test: A: **P*<0.05, ***P*<0.01; B: **P*<0.05, ****P*<0.001; C: ***P*<0.01. Ctrl or C, control.

activation. To highlight the role of the high-affinity adenosine transporter CNT2, experiments were performed using 1 μM adenosine. The Na^+-dependent component of adenosine transport at this substrate concentration was inhibited by nearly 65% when formycin B was added at a concentration of 100 μM (data not shown). Under these conditions, the Na^+-independent component of adenosine transport, which was mediated by ENT-type transporters, was insensitive to formycin B inhibition (data not shown). As shown previously, the treatment of IEC-6 cells with 1 μM adenosine for 10 minutes was able consistently and significantly to increase α1AMPK activity (Fig. 6A) and promote the phosphorylation of its downstream target ACC (Fig. 6B). When the same experiment was performed in the presence of 100 μM formycin B, both the increase in AMPK activity and the phosphorylation of ACC were blocked.

The putative role of ENT-type transporters on the adenosine-triggered activation of AMPK was analyzed in a complementary manner by incubating IEC-6 cells with 1 μM S-(4-nitrobenzyl)-6-thioinosine (NBTI) and 1 μM dipyridamole, prior to adenosine addition to the culture medium. Under these conditions, the ENT-related transport function was inhibited by nearly 80%. Higher concentrations of dipyridamole slightly modified CNT2-related transport activity (data not shown). Inhibition of ENT-type transporters did not at all modify either adenosine-induced AMPK phosphorylation (data not shown) or phosphorylation of the downstream target ACC (Fig. 6C).

Adenosine is able to activate AMPK and phosphorylate its downstream target ACC in rat liver cells

Once the role of extracellular adenosine in AMPK activation was established in the rat intestinal cell line IEC-6, we wondered whether this effect could also be reproduced in liver cells, in which AMPK plays a key role in modulating energy metabolism. Incubation of FAO cells with 10 μM adenosine and 500 μM AICAR for 30 and 60 minutes, respectively, significantly upregulated α1AMPK activity (data not shown). This resulted in phosphorylation of the downstream target ACC (Fig. 7A). This increase in AMPK and ACC phosphorylation can be blocked by pre-incubating the cells with 5′-ITU (Fig. 8), showing that adenosine phosphorylation is also required in FAO cells for AMPK activation. To assess the physiological relevance of this phenomenon further, the same experiment was performed in primary cultures of rat hepatocytes (Fig. 7B), with similar results: extracellular adenosine similarly activated AMPK and induced the phosphorylation of ACC.

Discussion

This study shows that extracellular adenosine activates AMPK in the epithelial intestinal cell line IEC-6 through a mechanism that requires adenosine transport and results in an increase in the AMP intracellular pool. Moreover, AMPK activation is to some extent dependent on the α1-subunit-containing complexes, which are predominant in IEC-6 cells. This effect is rapid, transient and dose dependent, with an apparent $K_{0.5}$ value of approximately 0.48 μM. Thus, this effect is

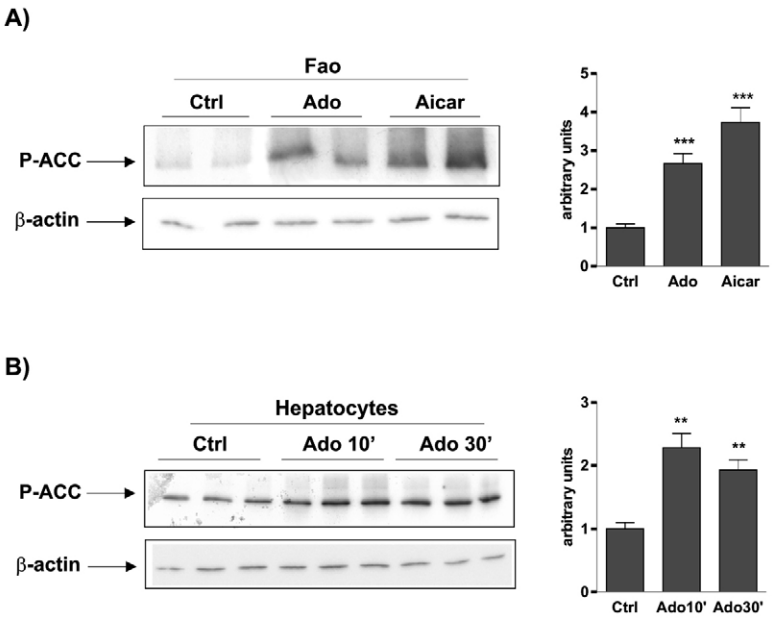

Fig. 7. Adenosine activates AMPK and phosphorylates its downstream target in the hepatoma cell line FAO and primary culture of rat hepatocytes. (A) FAO cells (Fao) were treated with either 10 μM adenosine (Ado) or 500 μM AICAR (Aicar) for 30 minutes and 60 minutes, respectively. A representative western blot of the phosphorylated form of ACC (P-ACC) and densitometric analysis of four independent experiments are shown. (B) Primary cultures of rat hepatocytes were incubated in the presence of 10 μM adenosine and protein extracts were analyzed at the indicated times for ACC phosphorylation. A western blot and the corresponding densitometric analysis of three independent experiments are shown. β-actin is used as a loading control. The statistical significance was assessed by Student's *t*-test: A: ***P<0.001; B: **P<0.01. Ctrl, control.

physiologically relevant: first, the adenosine concentrations used are not pharmacological; second, it also triggers the phosphorylation of the downstream AMPK target ACC; and third, it is blocked when IEC-6 cells are transduced with an adenoviral vector expressing a dominant-negative isoform of the α1AMPK subunit. Moreover, the magnitude of the effect triggered by adenosine is in the range of what has been previously reported for the physiological activation of AMPK by adiponectin in adipocytes (Wu et al., 2003)

Although it could be argued that extracellular adenosine modulates AMPK through purinergic activation, this is unlikely in our cell system. In fact, inhibition of adenosine transport and phosphorylation should have resulted in increased extracellular adenosine concentrations and enhanced availability to P1 receptors, and thus further activation of AMPK would be expected, instead of the blockade of the adenosine-induced AMPK response reported here. In our system, AMPK stimulation and consequently ACC modulation is a result of adenosine transport and conversion into AMP, promoting an increase in the AMP intracellular pool.

The IEC-6 cell line shows significant ENT-type transport activity but, interestingly, retains high levels of CNT2 protein and mRNA, which, in turn, results in high CNT2-mediated adenosine uptake. CNT-related transport activity and CNT2

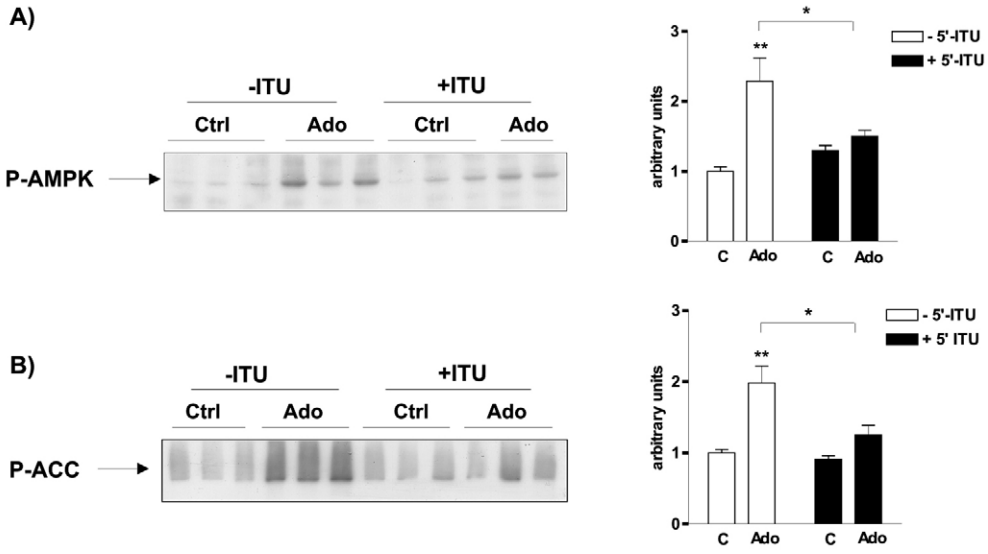

Fig. 8. Adenosine phosphorylation is necessary for AMPK and ACC phosphorylation in FAO cells. The effect of adenosine kinase inhibition on the increase in AMPK and ACC phosphorylation triggered by adenosine (Ado) was monitored after pre-incubating the cells with 0.2 μM 5′-iodotubercidin (ITU) for 30 minutes. Two representative western blots of phosphorylated AMPK (P-AMPK) (A) and phosphorylated ACC (P-ACC) (B), and corresponding densitometric analysis (means ± s.e.m.) of six independent experiments are shown. Statistical significance was assessed by Student's *t*-test: **P<0.01; *P<0.05. Ctrl or C, control.

protein have been identified in human and rat brush border intestinal membrane vesicles (Patil and Unadkat, 1997; Valdes et al., 2000). Moreover, in stably transfected renal cell lines, tagged CNT1 and CNT2 proteins are inserted at the apical domain of the plasma membrane (Lai et al., 2002; Mangravite et al., 2001). We know that CNT2 expression does not respond to mitogenic signals in IEC-6 cells, although it is transcriptionally regulated by agents such as glucocorticoids that are implicated in the development of the differentiated enterocyte phenotype (Aymerich et al., 2004). Thus, at first sight it appears that the major role of CNT2 would be nucleoside absorption. However, in this study, we have shown that partial inhibition of CNT2 function results in a significant blockade of the AMPK pathway. As previously indicated, no specific blockers of CNT2 function are available and, at least in our hands and with this cell model, only partial inhibition of CNT2 can be achieved if no effect on ENT-mediated adenosine transport is envisaged. Using 1 μM adenosine, a consistent and significant increase (50% above basal values) in AMPK activity was observed. The actual in situ activation of AMPK is difficult to determine, since the differential allosteric effects of variable concentrations of AMP reaching the enzyme in vivo are ruled out when performing the kinase assays in the presence of saturating concentrations of AMP. Nevertheless, the addition of 1 μM adenosine resulted in enhanced ACC phosphorylation and this pathway was almost completely blocked when CNT2 function was inhibited by only 65%. Thus, besides absorption, CNT2 also contributes to AMPK signaling. Since enterocytes show nutritionally regulated de novo lipogenesis (Haidari et al., 2002), it is tempting to speculate that increased adenosine availability in the lumen, associated with food intake, might modulate energy-demanding pathways in the intestinal epithelia by activating AMPK and its downstream targets.

The putative role of ENT-type transporters in adenosine-mediated activation of the AMPK pathway has also been addressed by inhibiting ENT-related transport function. As for CNT2, total inhibition was not achieved without slightly affecting CNT2-related activity. Nevertheless, conversely to what was found for CNT2, 80% inhibition of ENT-related adenosine uptake was not enough to block adenosine-triggered AMPK and ACC phosphorylation. Overall, these data provide further evidence for CNT2 being a key mediator of AMPK activation triggered by extracellular adenosine. This observation does not rule out the possibility that cell types lacking CNT-related transport activity might rely upon ENT-type transporters to mediate the effects of extracellular adenosine.

In contrast to the initial view that CNT-type transporter expression would be restricted to absorptive epithelia (principally intestinal and renal epithelial cells), CNT2 protein distribution has been shown to be widespread (Valdes et al., 2000). Its function in liver is regulated by purinergic activation of A1R through a mechanism in which K_{ATP} channels are implicated (Duflot et al., 2004). This was the first indirect evidence in support of the putative interaction between adenosine transport and the energy status of the cell. It is for this reason that we attempted to determine whether the activation of AMPK triggered by extracellular adenosine could also be detected in cell types such as hepatocytes, in which the regulatory role of AMPK on energy metabolism has been better documented. In this study, a similar response to that found in the intestinal cell line IEC-6 was observed both in the rat hepatoma cell line FAO and in primary cultures of rat hepatocytes. In FAO cells, the increase in AMPK phosphorylation triggered by the addition of adenosine was equally blocked by the adenosine kinase inhibitor 5′-ITU, suggesting that transport and phosphorylation are also required for AMPK to be activated by extracellular adenosine in liver cells. A major effect of AMPK activation in hepatocytes is the inhibition of gluconeogenesis, lipogenesis and cholesterol synthesis (Carling, 2004; Hardie and Carling, 1997) and we have shown here that transport of adenosine leads to ACC phosphorylation through AMPK activation, which is consistent with an inhibition of lipogenic flux. A significant body of literature has investigated the role of adenosine on hepatic metabolic functions (e.g. Carabaza et al., 1990; Lund et al., 1975). In some cases, the adenosine concentrations used were far from the expected physiological range and concomitant purinergic responses could not be ruled out. Nevertheless, we anticipate that a re-evaluation of adenosine effects on energy metabolism, taking into consideration the role of transport processes at physiological adenosine concentrations, will help to discriminate better between receptor- and transporter-mediated processes.

In summary, this study shows that physiological concentrations of adenosine activate AMPK through a mechanism that requires its transport into the cell and subsequent phosphorylation, resulting in phosphorylation of the downstream AMPK target ACC. This effect is mostly dependent on the high-affinity concentrative adenosine transporter CNT2. Thus, CNT2 function might not only respond to the requirement for nucleoside salvage but might also play a role in cell signaling. This plasma membrane transporter might therefore be considered a novel player in the complex regulation of AMPK and energy metabolism.

Materials and Methods

Reagents

Adenosine, formycin B, S-(4-nitrobenzyl)-6-thioinosine (NBTI) and dipyridamole were purchased from Sigma Chemicals. 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) was from Toronto Research Chemicals. [2-^{3}H]adenosine (21 Ci/mmol) and [γ-^{32}P]ATP were from Amersham. 5′-iodotubercidine (5′-ITU) was from Biomol Research Labs. All other reagents were of analytical grade.

Liver parenchymal, FAO and IEC-6 cells

Hepatocytes were isolated from male Wistar rats (200-220 g body wt) using a modified protocol based on the classical collagenase perfusion method, as previously described (del Santo et al., 1998). The livers were perfused anterogradely with Hanks' salt solution containing 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.33 mM Na_2HPO_4 $2H_2O$, 136.4 mM NaCl, 4.2 mM $NaHCO_3$ and 0.5 mM EGTA (pH 7.3). This buffer was used to wash off the blood, before a second buffer comprising 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.98 mM $MgCl_2$ $6H_2O$, 0.81 mM $MgSO_4$ $7H_2O$, 136.8 mM NaCl, 1.33 mM NaH_2PO_4 $2H_2O$, 5 mM $CaCl_2$ $2H_2O$, 5.5 mM D-glucose and 20 mM HEPES (pH 7.4) supplemented with collagenase (Sigma) at a final concentration of 0.05 mg/ml, was used to perfuse the liver again. Disaggregated cells were washed in the same buffer (without collagenase) supplemented with 1% bovine serum albumin (BSA; fraction V, fatty acid free). Isolated hepatocytes were then seeded at a density of 1×10^{4} in Earle's E-199 medium supplemented with 2% fetal calf serum (FCS) and a mixture of antibiotics (100 U/ml penicillin G, 0.1 mg/ml streptomycin and 0.25 μg/ml fungizone). This medium was then replaced with Earle's E-199 medium containing 0.5% BSA instead of FCS. All studies using primary cultures were performed 15 hours after seeding.

The rat hepatoma cell line FAO and the rat intestinal epithelial cell line IEC-6 were purchased from the American Type Culture Collection. FAO and IEC-6 cells are relatively differentiated cells that retain significant CNT2-mediated Na^{+}-dependent activity (del Santo et al., 1998). FAO cells were grown in Coon's F12 medium supplemented with 10% calf serum and a mixture of antibiotics (100 U/ml penicillin G, 0.1 mg/ml streptomycin and 0.25 μg/ml fungizone). IEC-6 cells were

routinely cultured in high-glucose DMEM supplemented with 5% FCS, 1 mM sodium pyruvate, 4 mM glutamine, 0.1 U/ml bovine insulin and the same mixture of antibiotics as described above. Before monitoring the effects of different treatments on activity and AMPK assays, primary cultures and cell lines were incubated in a serum-free BSA-supplemented medium for 2-3 hours.

Adenoviral infection of IEC-6 cells

IEC-6 cells were densely plated and grown to confluence in 60 cm dishes. To suppress AMPK activity, cells were infected with Ad.α1DN, a recombinant adenovirus harboring cDNA encoding α1AMPK containing a mutation that alters aspartic acid residue 157 to alanine (Woods et al., 2000). An adenovirus whose expression cassette contains the major late promoter but not the exogenous gene was used to infect cells as a control. Adenovirus stocks were diluted in serum-free medium to give a multiplicity of infection of 30, and were incubated with the cells for 3 hours at 37°C. An equal volume of 5% FCS in medium was then added to the cells, which were incubated for a further period of 24 hours at 37°C.

Western blot analysis

Anti-phospho-AMPK and anti-phospho-ACC were purchased from Cell Signaling Technology and used at a dilution 1/1000. The anti-CNT2 antibody, which had been characterized previously (Valdes et al., 2000), was used at a dilution of 1/1000. The antibody against the α1AMPK subunit, which recognizes both the endogenous and heterologously expressed protein, has also been characterized previously (Woods et al., 1996) and was used at a dilution of 1/5000. This antibody was a kind gift from G. Hardie (University of Dundee, Dundee, Scotland, UK).

Cell lysates were prepared from IEC-6, FAO and hepatocyte monolayers grown in 10 cm culture dishes by treatment with a buffer containing 25 mM Hepes (pH 7.4), 250 mM sucrose, 2 mM EDTA and a mixture of protease inhibitors (Complete MINI; Roche). The cell suspension was homogenized on ice by passing it 20 times through a 25-gauge needle and cell debris were removed by centrifugation at 4000 ***g*** for 15 minutes. Total membrane fractions were obtained after ultracentrifugation at 200,000 ***g*** for 1 hour at 4°C.

For analysis of AMPK and ACC phosphorylation, cells were immediately scraped off into lysis buffer A (1% Triton X-100 in 50 mM Hepes pH 7.4 at 4°C, containing 50 mM NaF, 5 mM NaPPi, 1 mM EDTA, 1 mM EGTA, 10% glycerol and the same mixture of protease inhibitors).

The protein content of these samples was measured using the Bradford assay (Bio-Rad Laboratories) and 10-40 μg protein aliquots were used for PAGE separation and further transfer onto Immobilon-P membrane filters (Millipore), as described (Valdes et al., 2000). Antibody binding was detected with a horseradish peroxidase (HRP)-conjugated secondary antibody using the ECL detection system (Amersham Pharmacia Biotech). Protein loading was routinely confirmed with an antibody against β-actin (Sigma-Aldrich). Densitometric analysis was carried out using Phoretix 1D Software. Semiquantitative analysis was carried out by calculating the densitometry ratios versus β-actin.

Immunoprecipitation and AMPK assays

The antibody against the α1AMPK subunit used for western blot analysis and the antibody against the α2AMPK isoform, which were kind gifts of G. Hardie, were used for immunoprecipitation of AMPK complexes. Endogenous AMPK was immunoprecipitated using 500 μg protein from cell lysates by incubation for 2 hours at 4°C with either anti-α1 or anti-α2 antibody bound to protein G-Sepharose. Immune complexes were collected by centrifugation at 8000 ***g*** for 1 minute and were washed extensively with cold buffer A. AMPK activity in the resulting immune complexes was determined by the incorporation of ^{32}P from [γ-^{32}P]ATP into the AMARA synthetic peptide (AMARAASAAASARRR) (Barnes et al., 2002). The assay reagents (buffer A, supplemented with 200 μM ATP, 200 μM AMP, 5 mM $MgCl_2$, 200 μM AMARA and [γ-^{32}P]ATP) were added and the mixture was incubated with shaking for 15 minutes at 30°C. Aliquots (20 μl) of the reaction were spotted onto P81 paper (Whatman) and unreacted ATP was removed with 1% (v/v) phosphoric acid. Radioactivity was measured by liquid scintillation counting.

Nucleoside transport activity measurements

Uptake measurements were performed with 1 μM tritiated adenosine in the presence of either 137 mM NaCl or 137 mM choline chloride. The uptake medium also contained 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 10 mM Hepes (pH 7.4). Incubation was stopped after incubation for 1 minute by washing monolayers three times in 2 ml of a cold buffer comprising 137 mM NaCl and 10 mM Hepes (pH 7.4). Cells were then dissolved in 100 μl of 100 mM NaOH, 0.5% Triton X-100, and aliquots were taken for protein determination (Bio-Rad Laboratories) and radioactivity measurements.

Analysis of intracellular adenine nucleotides by HPLC

10^7 cells were used for nucleotide analysis. After incubation, culture medium was removed by aspiration, followed by immediate addition of 300 μl of ice-cold 10% perchloric acid. The culture dish was sealed tightly with parafilm and cooled at –80°C. Cell lysates were thawed on ice, scrapped off the wells thoroughly, and transferred to 1.5 ml microfuge tubes. Samples were centrifuged at 12,000 ***g*** at 4°C for 10 minutes. The supernatant was separated from the pellet, neutralized with 5.0 M potassium carbonate and filtered with Ultrafree-MC Centrifugal Filter Units (NMWL 10,000) (Millipore) by centrifugation at 12,000 ***g*** for 15 minutes at 4°C. Neutralized extracts were frozen and stored at –80°C for high-performance liquid chromatography (HPLC) analysis. A 20 μl sample was injected onto a Excel 120 ODS B 3 μm reverse phase column (20×0.46 cm) using a gradient of Buffer 1 (0.1 M KH_2PO_4, 0.5 mM TBA pH 6.4) and Buffer 2 (70% Buffer 1 and 30% methanol) at a flow rate of 0.6 ml/minute. A linear gradient was developed over 30 minutes at 0% Buffer B to 100% Buffer 2. An Alliance 2695 Waters high-pressure liquid chromatograph equipped with a PDA Waters 2996 detector was used. The various peaks in the extracts were identified by comparison for retention times with known external standards and the relative absorbance at 260 nm. AMP, ADP and ATP were quantified in the extracts. The results are expressed as nmol/10^7 cells.

Reverse transcription and RT-PCR

1 μg of total RNA was used for cDNA synthesis using the TaqMan Reverse Transcription System (Applied Biosystems), according to the manufacturer's instructions. Oligonucleotides employed for CNT2 amplification were the following: forward primer 776-799 (GCTCAAAGGCCAGAGCAGCTGATC) and reverse primer 1442-1445 (CAGCTTCACTCCCTCCTTGCTCTT). RT-PCR was run for 28 cycles at 94°C (2 minutes), 58°C (1 minute) and 72°C (3 minutes), followed by a final extension at 72°C (15 minutes) in a Thermocycler (Applied Biosystems). The polymerase used was TaqDNA polymerase (Promega).

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