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REVIEW

Regulation of AMP-activated protein kinase by natural and synthetic activators

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Abstract The AMP-activated protein kinase (AMPK) is a sensor of cellular energy status that is almost universally expressed in eukaryotic cells. While it appears to have evolved in single-celled eukaryotes to regulate energy balance in a cell-autonomous manner, during the evolution of multicellular animals its role has become adapted so that it also regulates energy balance at the whole body level, by responding to hormones that act primarily on the hypothalamus. AMPK monitors energy balance at the cellular level by sensing the ratios of AMP/ATP and ADP/ATP, and recent structural analyses of the AMPK heterotrimer that have provided insight into the complex mechanisms for these effects will be discussed. Given the central importance of energy balance in diseases that are major causes of morbidity or death in humans, such as type 2 diabetes, cancer and inflammatory disorders, there has been a major drive to develop pharmacological activators of AMPK. Many such activators have been described, and the various mechanisms by which these activate AMPK will be discussed. A particularly large class of AMPK activators are natural products of plants derived from traditional herbal medicines. While the mechanism by which most of these activate AMPK has not yet been addressed, I will argue that many of them may be defensive compounds produced by plants to deter infection by pathogens or grazing by insects or herbivores, and that many of them will turn out to be inhibitors of mitochondrial function.

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1. Introduction

The 5'-adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a sensor of cellular energy that helps to maintain energy balance at both the cellular and whole body levels[1–4]. Since type 2 diabetes, which affects 5%−10% of the world population, can be regarded as a disorder of energy balance caused by overnutrition, there has been much interest in AMPK as a drug target. It is also becoming apparent that two other major causes of human death and morbidity, *i.e.*, cancer and inflammatory disease, can be viewed as metabolic derangements. Thus, tumor cells and cells, involved in inflammation, tend to display a glycolytic phenotype (termed the Warburg effect or aerobic glycolysis), whereas quiescent cells and cells involved in the resolution of inflammatory responses tend to utilize oxidative metabolism[5]. Since AMPK inhibits cell growth and proliferation, and also promotes the more glucose-sparing and energy-efficient mitochondrial oxidative metabolism rather than glycolysis, interest in the system as a drug target in the fields of cancer and inflammatory disease has been steadily increasing.

Following its initial definition by our group in the late 1980s[6–8], over 9000 papers have been published on the AMPK system, and it is not possible to give a full coverage of the field in a single article. In this review I will focus on its structure and evolution, its regulation by metabolites, and its modulation by synthetic compounds that are being developed as pharmacological AMPK activators and by natural products that are being tested as medicines.

2. AMPK—subunit structure and evolution

AMPK appears to exist in almost all eukaryotic species as heterotrimeric complexes comprising a catalytic α subunit and regulatory β and γ subunits. In humans and other mammals, the α subunits are encoded by two genes (*PRKAA1*/*PRKAA2*, encoding $\alpha1/\alpha2$), the β subunits by two (*PRKAB1*/*PRKAB2*, encoding $\beta1/\beta2$) and the γ subunits by three (*PRKAG1*/*PRKAG2*/*PRKAG3*, encoding $\gamma1/\gamma2/\gamma3$). All twelve combinations of α, β and γ subunit isoforms are able to form heterotrimeric complexes when co-expressed, although certain combinations appear to be favored *in vivo*[9]. Genes encoding orthologs of AMPK-α, -β and -γ subunits are readily found in all eukaryotes where genome sequences have been completed. The one known exception to this is the microsporidian *Encephalitozoon cuniculi*, an obligate intracellular parasite that lives inside other mammalian cells including those of humans, and which has no free-living form other than metabolically inert spores[10]. While a genuine eukaryote, *E. cuniculi* has an extremely small genome encoding only 29 conventional protein kinase catalytic subunits, and lacks genes encoding the α, β and γ subunits of AMPK[11]. It does contain genes encoding the enzymes required for a complete glycolytic pathway[10], but lacks adenosine-triphosphate (ATP)-generating mitochondria although having mitochondrial remnants termed mitosomes[12]. Interestingly, *E. cuniculi* expresses unusual transmembrane ATP/adenosine diphosphate (ADP) translocases, some of which appear to be located in the plasma membrane[13]. The implication of this is that the organism may utilize these translocases to "steal" ATP from the host cell in exchange for ADP. *E. cuniculi* may therefore have been able to afford to lose genes encoding AMPK, because its host cell does express the kinase and can regulate energy homeostasis on its behalf.

Given that AMPK is found in essentially all present day eukaryotes, it seems likely that it evolved soon after the development of the first eukaryote. It is widely believed that the key event that led to the first eukaryotic cell was the endosymbiotic acquisition by an archaeal host cell of aerobic bacteria, which eventually became mitochondria. One can speculate that the host cell would have needed a system to monitor the output of their newly acquired oxidative organelles, and to regulate the ability of those organelles to supply ATP according to the demands of the host. AMPK fits the bill to be such a system: for example, in the budding yeast *Saccharomyces cerevisiae* the AMPK ortholog is not required for growth by the fermentative metabolism (*i.e.*, glycolysis) that is utilized in high glucose, but is required for the switch to oxidative metabolism that occurs when glucose run low[14]. Similarly, mitochondrial biogenesis is one of the key downstream effects of AMPK activation in mammalian cells[15–17].

Most energy-requiring processes in eukaryotic cells are driven, either directly or indirectly, by hydrolysis of ATP to ADP, and it is possible to draw an analogy between these nucleotides and the chemicals in a rechargeable battery. A high ratio of ATP to ADP is equivalent to a fully charged battery, while if this ratio is falling the cellular battery is becoming flat. Extending this analogy, AMPK can be regarded as the biological equivalent of the system within a cellphone or laptop computer that monitors the battery charge. As discussed in more detail in Section 3, it is activated by increasing ratios of AMP/ATP and ADP/ATP. An increase in either ratio signifies falling cellular energy, but if the reversible reaction catalyzed by adenylate kinase ($2ADP \leftrightarrow ATP + AMP$) is at equilibrium (as seems to be the case in most eukaryotic cells) it is easy to show that the AMP/ATP ratio will vary as the square of the ADP/ATP ratio[18], making the former a much more sensitive signal of falling energy status than the latter. A full description of the downstream targets for AMPK is beyond the scope of this article, and readers interested in that aspect should consult other reviews (*e.g.*, Ref. 19). However, once activated by energy stress, AMPK attempts to restore cellular energy homeostasis by activating catabolic pathways that generate ATP, while switching off ATP-consuming processes not essential to short-term cell survival, including almost all anabolic pathways. Although AMPK almost certainly arose in single-celled eukaryotes as a cell-autonomous mediator of energy balance, it is intriguing that role of the system seems to have become adapted during the evolution of multicellular eukaryotes so that it also regulates energy balance at the whole body level. It does this particularly by mediating effects of hormones acting on the hypothalamus of the brain that control energy intake (*i.e.*, feeding) and energy expenditure[1–4].

3. Canonical regulation by phosphorylation and by adenine nucleotides

AMPK is normally only significantly active after phosphorylation of a conserved threonine residue within the activation loop of the kinase domain on the α subunit. This threonine residue is usually referred to as Thr172 due to its position in the rat $\alpha2$ subunit where originally identified[20], although the precise numbering may differ in other isoforms and species. Following a long search, the primary upstream kinase that phosphorylates Thr172 *in vivo* was shown to be a heterotrimeric complex between the tumor suppressor kinase liver kinase B1 (LKB1), the pseudokinase STE20-related adaptor (STRAD) and the scaffold protein mouse protein 25 (MO25)[21–23]. This complex appears to be constitutively active in that its activity is not regulated under situations of energy stress when AMPK is activated in an LKB1-dependent manner[24,25]. Nevertheless, binding of AMP to AMPK can regulate both the phosphorylation of Thr172 by LKB1, and its dephosphorylation (see below). Almost as soon as it was found that LKB1 was the primary upstream kinase, it was realized that there was some phosphorylation of Thr172 even in tumor cells that had lost LKB1, and this was traced to the calmodulin-dependent protein kinase, calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ)[26–28]. This provides an alternate Ca^{2+}-mediated upstream pathway for AMPK activation, which mediates effects of hormones and mediators acting through G_q/G_{11}-coupled receptors that trigger release of Ca^{2+} from intracellular stores *via* the second messenger inositol-1,4,5-trisphosphate (IP_3)[29]. Such hormones include thrombin acting on endothelial cells *via* the protease-activated receptor[30], and ghrelin acting on hypothalamic neurons *via* the glutathione reductase 1 (GSHR1) receptor[31]. Thr172 can also be phosphorylated, and AMPK activated, in intact cells by the protein kinase transforming growth factor-β-activated kinase-1 (TAK1)[32,33], although the physiological relevance of that mechanism currently remains unclear.

Allosteric activation of the phosphorylated kinase by 5′-AMP was originally demonstrated in 1980[34] (before AMPK acquired its current name), but in the early 1990s it was shown that AMP binding to AMPK not only caused allosteric activation but also promoted its net phosphorylation at Thr172[35]. It is now clear that AMP binding has three effects on AMPK[36] that activate the system in a synergistic manner, making the final response very sensitive to even small changes in AMP:

(i) promotion of phosphorylation by LKB1, but not CaMKKβ (although this selectivity for LKB1 has been disputed[37]);
(ii) protection against dephosphorylation of Thr172 by protein phosphatases; and
(iii) allosteric activation of the phosphorylated kinase.

Of these three effects, it has been reported that mechanisms (i)[37] and (ii)[38] are also mimicked by binding of ADP. Given that ADP is present in unstressed cells at concentrations ten times higher than AMP, and that allosteric activation (which is only caused by AMP binding) is often reported as being small in magnitude (<2-fold), this led to proposals that ADP rather than AMP might be the crucial activator of AMPK[37–39]. However, our group[36] reported that while mechanism (ii) can indeed be caused by binding of ADP, AMP is about 10-fold more potent. Moreover, using a native preparation of mammalian AMPK rather than a bacterially expressed complex, allosteric activation by AMP can be substantial (>10-fold), even in the presence of concentrations of ATP that are $1-2$ orders of magnitude higher and within the physiological range (5 mmol/L)[36]. Thus, while ADP may contribute to activation, we would argue that AMP remains the primary regulator of AMPK.

4. Pharmacological activators of AMPK

Since the realization in the late 1990s that activation of AMPK might be useful for treatment of type 2 diabetes[40], numerous pharmacological activators have been developed. Based on their mechanism of action, they can be divided into four classes that are discussed in Sections 4.1–4.4.

4.1. *Activators that act indirectly by inhibiting cellular ATP synthesis*

Since depletion of ATP always causes increases in AMP and ADP, AMPK is activated by any compound that inhibits ATP synthesis. In cells that are primarily using glycolysis to generate ATP (as in most rapidly proliferating cells), AMPK is activated by inhibitors of glycolysis such as 2-deoxyglucose[41]. A much larger class of activators, some of which are shown in Fig. 1A, are those that inhibit mitochondrial ATP synthesis by inhibiting the respiratory chain at Complex I (*e.g.*, metformin or phenformin[42,43]) or Complex III (*e.g.*, antimycin A[44]), or that inhibit Complex V, the mitochondrial F1 ATP synthase (*e.g.*, oligomycin or resveratrol[41,45]). All of these agents will increase cellular ADP/ATP and/or AMP/ATP ratios, although correlations between such ratios and changes in AMPK activity do not prove on their own that activation by AMP or ADP is the sole mechanism. The best method to confirm this is to use a cell line expressing an AMP/ADP-insensitive mutant of AMPK such as the R531G mutation in $\gamma2$[41] or the equivalent R299G mutation in $\gamma1$[46]. Any agent that activates AMPK solely by increasing the cellular levels of AMP or ADP will fail to activate such mutants. Further discrimination can be obtained by measuring cellular oxygen uptake and acidification of the medium using an extracellular flux analyzer. Compounds that inhibit mitochondrial function should inhibit oxygen uptake, while those that inhibit glycolysis should reduce lactate output and hence extracellular acidification. For example, the compound PT-1, which was originally proposed to act by direct binding to AMPK[47], was recently shown using these methods to act instead by inhibiting the respiratory chain[46].

In the last few years well over 100 natural products or extracts derived from plants, many of which are used in traditional Asian medicines, have been reported to activate AMPK. These are considered in more detail in Section 7. However, it is worth stating here that several of them, including berberine[41,48] and arctigenin[49], appear to activate AMPK by inhibiting the mitochondrial respiratory chain, as does galegine, a natural product from the medicinal plant *Galega officinalis* from which metformin and phenformin were derived[41,50] (Fig. 1A). At least one potent synthetic compound, derived from a high-throughput screen designed to detect compounds that activate AMPK in cell-based assays, has also been shown to activate AMPK by inhibiting Complex I of the respiratory chain[51].

4.2. *Pro-drugs that are converted into AMP analogs inside cells*

It is clear that the regulatory adenine nucleotide-binding sites on the γ subunits of AMPK, which are discussed in more detail below, require the presence of negatively charged phosphate groups on bound nucleotides, and it therefore may be difficult to develop cell-permeable AMP analogs that bind these sites. However, a related approach is to develop pro-drugs that are cell permeable but are converted following their uptake into AMP analogs by cellular enzymes. In fact, 5-aminoimidazole-4-carboxamide ribonucleoside, the first pharmacological AMPK activator to be developed[49,50], works by this mechanism. This compound is often referred to as AICAR and I adopt this usage below, although this can cause confusion because researchers in the field of nucleotide metabolism use the same acronym to describe the phosphorylated ribotide form, which I will refer to instead as ZMP (AICAR monophosphate). AICAR is an adenosine analog that is taken up into cells by adenosine transporters[52] and phosphorylated by intracellular adenosine kinase into ZMP (Fig. 1B). ZMP is an AMP analog that binds to AMPK at the same sites as AMP[53] and mimics all of the effects of AMP on the

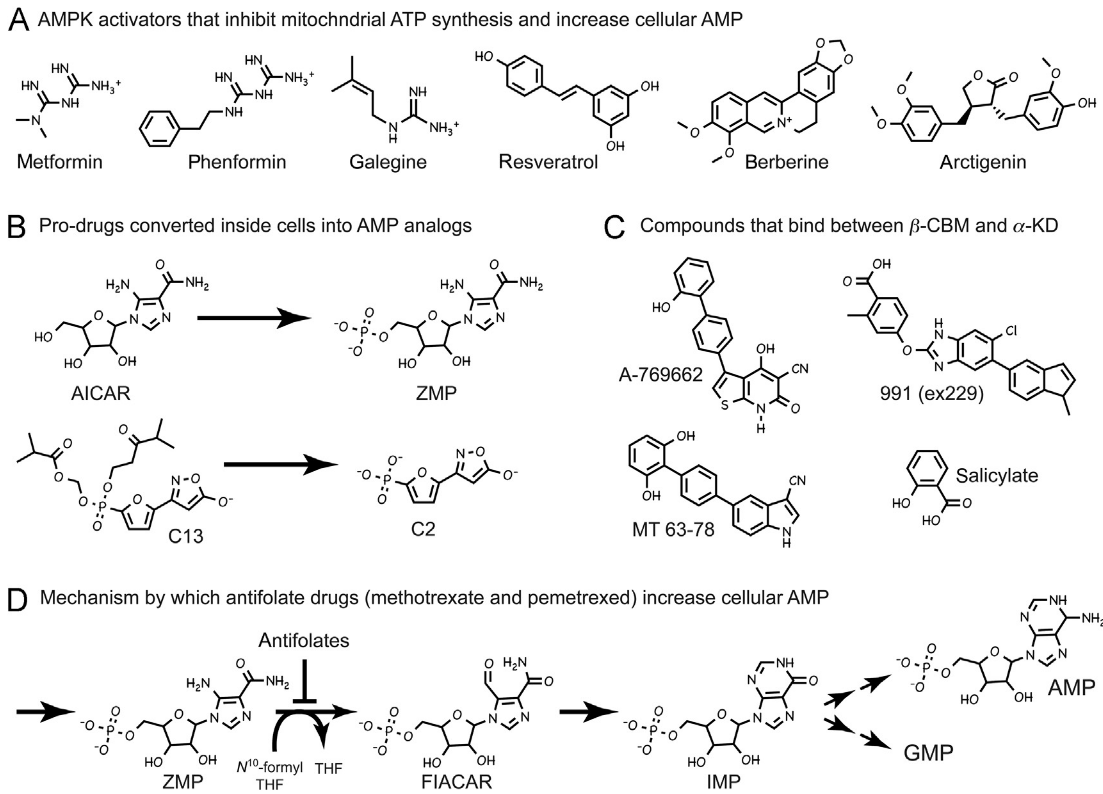

Figure 1 Structures of AMPK-activating compounds that act *via*: (A) inhibiting mitochondrial ATP synthesis; (B) pro-drugs converted to active agents inside cells, as shown; and (C) direct activators. (D) shows the mechanism by which antifolate drugs activate AMPK by causing accumulation of ZMP, an intermediate in the synthesis of the purine nucleotides inosine monophosphate (IMP), AMP and guanosine monophosphate (GMP).

AMPK system[54]. In fact, ZMP has low potency compared with AMP[54], but AICAR nevertheless activates AMPK in most primary cells and tissues because AICAR is rapidly converted to ZMP, which is then metabolized much more slowly. ZMP therefore accumulates within many cells to concentrations within the millimolar range (even higher than the external AICAR concentration), which is necessary for it to activate AMPK. It is important to note that ZMP is a natural intermediate in purine nucleotide synthesis, and some immortalized cell lines have a high rate of purine synthesis such that ZMP does not accumulate in response to extracellular AICAR, and AMPK is therefore not activated. Interestingly, however, antifolate drugs that are used to treat cancer, or inflammatory disorders such as rheumatoid arthritis, inhibit the transformylase that catalyzes the first step in the metabolism of ZMP to purine nucleotides, thus causing accumulation of ZMP (Fig. 1D). For example, the antifolate methotrexate dramatically sensitizes cells to the activating effects of AICAR[55], while pemetrexed can activate AMPK even in the absence of exogenous AICAR[56].

Recently, a synthetic compound that activates AMPK by a pro-drug mechanism has been developed. C13 is a phosphonate diester that is taken up into cells and converted by cellular esterases into C2 (Fig. 1B), an AMP analog that is 2 − 3 orders of magnitude more potent as an allosteric activator of AMPK than AMP, and 4 orders of magnitude more potent than ZMP[57]. Another major advantage of C13 over AICAR is that C2, unlike ZMP, does not modulate other AMP-sensitive enzymes such as glycogen phosphorylase, phosphofructokinase or fructose-1,6-bisphosphatase[58]. C2 is, however, selective for AMPK complexes containing the α1 rather than the α2 isoform[58], an interesting finding that is considered in more detail in Section 5.3 below. Finally, 3'-deoxyadenosine (cordycepin) is a bioactive compound derived from the fungus *Cordyceps militaris,* which is an analog of adenosine lacking oxygen on the 3' position of the ribose ring. Although it has been shown to activate AMPK in intact cells and to bind directly to the AMPK-γ subunit[59,60], it is perhaps more likely that the true activator is cordycepin-5'-monophosphate generated from cordycepin within the cell.

4.3. Allosteric activators that bind directly to AMPK at sites distinct from the AMP sites

The first compound in this class was A-769662 (Fig. 1C), developed by Abbott laboratories from a high throughput screen searching for allosteric activators of purified AMPK. Although it has poor oral availability, when administered by intraperitoneal injection it was found to have favorable effects on the metabolism of an insulin-resistant animal model, the *ob*/*ob* mouse[61]. A-769662 did not increase cellular ADP/ATP or AMP/ATP ratios[61], still activated AMPK in cells expressing an AMP-insensitive mutant[41], and did not displace AMP from its binding sites on the γ subunit[62], suggesting that it bound at a different site from AMP even though, like AMP, it caused both allosteric activation and protection against Thr172 dephosphorylation[62,63]. A-769662 is also selective for activation of β1 rather than β2 complexes[64], and its effects are abolished by an S108A mutation in β1 that prevents the autophosphorylation of that serine residue[63], suggesting that the binding site involved the β subunit. As discussed in Section 5.2 below, the binding site has now been identified by structural biology to be a cleft located between the N-lobe of the kinase domain on the α subunit and the carbohydrate-binding module on the β-subunit. Another, more potent activator that binds at this site, 991[65] (also known as ex229[66]), has emerged from high-throughput screens (Fig. 1C). Like A-769662, this compound shows some selectivity for β1 complexes although it will activate β2 complexes at higher concentrations. A third compound, MT 63-78[67] (Fig. 1C), also shows selectivity for β1 complexes and may therefore bind this site, although this has not yet been formally demonstrated. None of these compounds has yet entered clinical trials. However, it should be noted that, of these compounds, only A-769662 has been available for a prolonged period, and enthusiasm for its entry into clinical trials may have been dampened in part by poor oral availability[61] and in part by the occurrence of AMPK-independent, "off-target" effects[68].

A key question regarding the binding site for A-769662 is whether it binds any naturally occurring ligands. One natural product derived from plants that does bind to this site is salicylate[69], which has been used as a medicinal compound by humans since ancient times[70]. Acetyl salicylic acid (ASA or aspirin), which is broken down to salicylate within minutes of its adsorption into the bloodstream, is a synthetic derivative developed in the 1890s as a less irritating formulation to deliver salicylate orally. Aspirin is a potent inhibitor of the cyclo-oxygenases[71] (COX1 and COX2) that catalyze the key initial steps in the biosynthesis of prostaglandins and other eicosanoids; irreversible inhibition of synthesis of the eicosanoid thromboxane A2 in platelets is the mechanism by which it inhibits platelet aggregation and hence blood clotting[72]. However, since aspirin and salicylate have equal potency as anti-inflammatory agents, yet salicylate is a very poor COX inhibitor, it remains unclear whether all of the anti-inflammatory actions of aspirin can be attributed to COX inhibition[73]. In 2012 we reported that salicylate, but not aspirin, activated AMPK[69]. Like A-769662, salicylate is a poor activator of β2 complexes and its effect were abolished by an S108A mutation in β1, so it seemed likely that it bound to the same site as A-769662[69], a proposal recently confirmed by a crystal structure of the human $\alpha1\beta1\gamma1$ complex with bound iodosalicylate[74]. When salicylate or A-769662 were injected into wild type mice, they promoted a more rapid switch from carbohydrate to fat oxidation on food withdrawal, as would be expected for an AMPK activator that triggered phosphorylation and inactivation of both isoforms of acetyl-CoA carboxylase (ACC1 and ACC2) and hence caused a rapid switch from fat synthesis to fat oxidation. However, these effects were lost in AMPK-β1 knockout mice; since salicylate and A-769662 do not activate β2-containing complexes, this provided strong evidence that these metabolic effects were mediated by AMPK[69].

When AMP and A-769662 are added to AMPK together, they cause a synergistic allosteric activation even of "naïve" AMPK complexes that are not phosphorylated on Thr172, although prior autophosphorylation of Ser108 (or a phosphomimetic S108E mutation) is required for a maximal effect, as well as for a maximal response to A-769662 alone[75]. Synergism between these activating sites may also be relevant in intact cells, because metformin (which increases cellular AMP) and salicylate act synergistically to activate AMPK and inhibit fat synthesis in isolated mouse and human hepatocytes—while little AMPK activation was observed with metformin or salicylate on their own at concentrations (100 μmol/L and $300-500$ μmol/L, respectively) observed in human plasma following normal doses, significant effects were observed when they were given together[69]. There were also additive effects of low doses of metformin and salicylate *in vivo* to activate AMPK in livers of high-fat fed mice, accompanied by reduced liver triglycerides and increased hepatic insulin sensitivity[69].

4.4. *Oxidative stress*

It was reported in 2001 that oxidative stress produced by hydrogen peroxide increased Thr172 phosphorylation and activated AMPK; this was accompanied by increases in AMP/ATP ratios, suggesting that the effect might be AMP-dependent (*i.e.*, the mechanism described in Section 4.1)[76]. More recently, Zmijewski et al.[77] used glucose oxidase to generate H_2O_2 from glucose present in the medium—this appears to be a better model for physiological oxidative stress, because it generates a constant low level of H_2O_2 in the medium (<20 μmol/L) rather than a transient spike of much higher concentrations that is obtained by adding H_2O_2 directly[78]. Zmijewski et al. reported that glucose oxidase treatment of HEK-293 cells did not cause decreases in ATP levels, and presented evidence that AMPK activation was caused instead by oxidation of two conserved cysteine residues (Cys299 and Cys304) present in the auto-inhibitory domain of the α subunit (see Section 5.1). However, our group[78] reported that glucose oxidase treatment did increase AMP/ATP ratios in the same cell line, and that AMPK activation was largely abolished in HEK-293 cells expressing the AMP-insensitive R531G mutant of γ2. While this suggested that the effect was primarily AMP-dependent, there was a small residual effect observed with the R531G mutant that might be explained by the mechanism described by Zmijewski et al.[77]. More recently, Shao et al.[79] reported that AMPK was inactivated rather than activated by oxidative stress in primary cardiomyocytes, and that this was prevented by thioredoxin. Inactivation was traced to oxidation of two cysteine residues within the kinase domain of AMPK (Cys130 and Cys174), distinct from those whose oxidation was proposed by Zmijewski et al.[77] to cause activation of AMPK. Cys174 is almost adjacent to Thr172, and unmodified cysteine residues at these positions were shown to be necessary for activation by LKB1. Shao et al.[79] suggested that the activation of AMPK caused by oxidative stress in HEK-293 cells[77,78] may occur because higher levels of anti-oxidant enzymes in this immortalized cell line may prevent the inactivation that they observed in primary cardiomyocytes.

4.5. *Why do different pharmacological activators of AMPK have different effects?*

Some of the pharmacological activators of AMPK discussed above have been used as medicines by humans for decades (metformin), centuries (berberine) or even millennia (salicylate). Why are their pharmacological effects so different? One potential explanation is pharmacokinetics—for example, metformin is a cation with poor cell permeability, and it requires expression of transporters of the organic cation transporter (OCT) family, such as OCT1, for cellular uptake. Because OCT1 is highly expressed in hepatocytes, 24% of an intravenous dose of metformin was found in the liver of wild type mice ten minutes after injection, compared with $<1\%$ in *Oct1*$^{-/-}$ knockout mice[80]. Thus, the effects of metformin *in vivo* are likely to be restricted to the liver, whereas other compounds will also activate AMPK in other organs or cell types. In addition, since metformin activates AMPK indirectly by inhibiting the respiratory chain and thus increasing cellular AMP and ADP[41], it is likely that it has many "off-target" or AMPK-independent effects; indeed the acute effects of metformin on hepatic glucose production[81], as opposed to its longer-term effects on hepatic insulin sensitivity[82], appear to be independent of AMPK. Similarly, although salicylate does bind directly to AMPK, being a particularly small molecule it is unlikely to bind to any target with high affinity, and it almost certainly has several AMPK-independent effects. Acetyl salicylate (aspirin) is, of course, already known to inhibit cyclo-oxygenases and hence prostanoid biosynthesis, although salicylate itself is a relatively poor cyclo-oxygenase inhibitor[83]. The different pharmacological effects of these AMPK activators may therefore be due to a combination of different pharmacokinetics, and distinct AMPK-independent effects.

5. Domain architecture and structure of AMPK

5.1. The α subunits

Each AMPK-α subunit contains at the N-terminus a typical eukaryotic kinase domain, with a conventional small N-lobe consisting mainly of β-sheets, followed by the larger C-lobe consisting mainly of α-helices. In the most recent crystal structures[38,65,74,84], such as that shown in Fig. 2, the kinase had been crystallized in the presence of the non-specific, ATP-competitive kinase inhibitor staurosporine, and as expected this was located in the ATP-binding cleft between the N- and C-lobes. The critical phosphorylation site, Thr172, is located in the "activation loop", a sequence region that must be phosphorylated in many kinases before they become active. Most of the crystal structures of AMPK were obtained with Thr172 phosphorylated and the activation loop was well ordered, although in at least one structure in the unphosphorylated state the activation loop was partially disordered[84]. The α subunit kinase domain (α-KD) is immediately followed by the auto-inhibitory domain (α-AID), so-called because bacterially expressed α-KD:α-AID constructs are about 10-fold less active than constructs containing the α-KD only, even when both have been phosphorylated on Thr172[62,85]. There is now good evidence that the α-AID inhibits the α-KD when AMP is not bound to the γ subunit, thus explaining the 10-fold allosteric activation by AMP. Crystal structures of α-KD:α-AID constructs from the AMPK ortholog from the fission yeast *Schizosaccharomyces pombe*[86], and more recently from humans[84], show that in this low activity state the α-AID, a bundle of three short α-helices, binds to the α-KD on the opposite surface to the catalytic cleft, with the α3 helix of the α-AID interacting with the N-lobe and the hinge between the N- and C-lobes (Fig. 3A). By comparing many structures of kinase domains in active and inactive conformations, it has been found that four hydrophobic residues termed the "regulatory spine" are universally aligned in active conformations, indicating that the active site is correctly disposed for activity, but that these residues are out of alignment in inactive conformations[87]. In the structures of the inactive α-KD:α-AID constructs of AMPK, the four residues that form the "regulatory spine" (Leu68 and Leu79 from the N-lobe, and His137 and Phe158 from the C-lobe) are not aligned (Fig. 3A). By contrast, in all structures of AMPK heterotrimers in active states, which are phosphorylated on Thr172 and have AMP bound to the γ subunit (see below), the α-AID has undergone a rotation such that helix α3 now interacts primarily with the γ subunit rather than with the N-lobe of the α-KD. At the same time the α-KD switches to

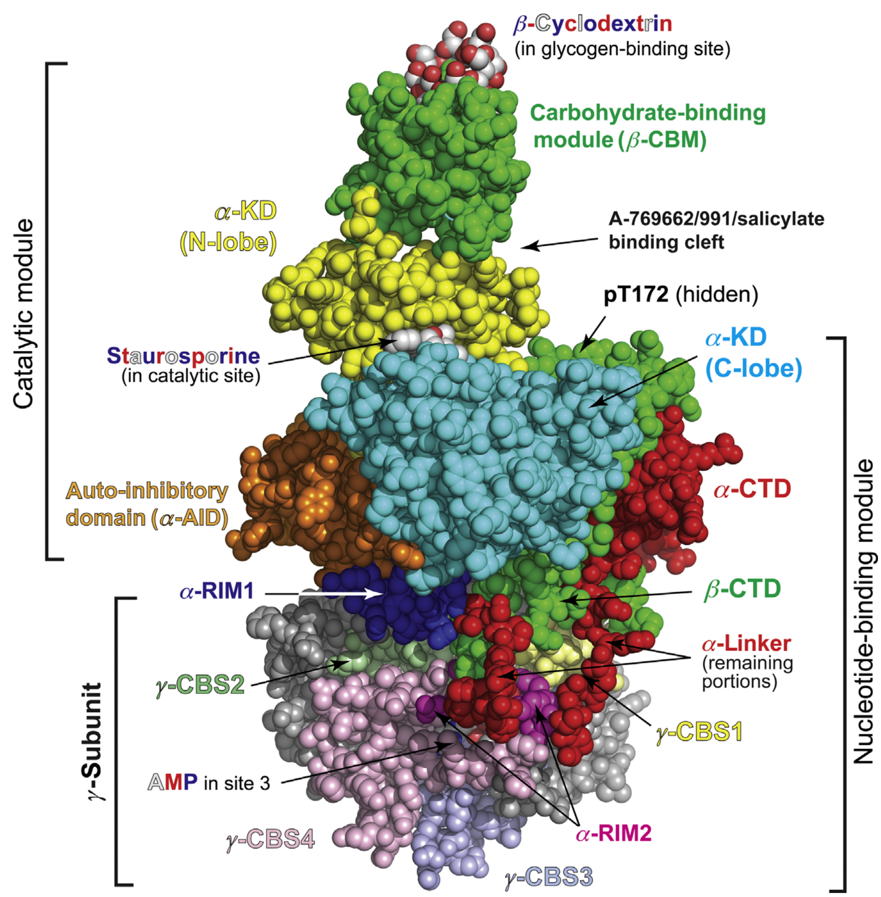

Figure 2 Structure of complete $\alpha1\beta2\gamma1$ heterotrimer of AMPK. The model was created with MacPyMol using PDB file 4RER[84]. All molecules are shown in "sphere view", omitting hydrogen atoms. Domains of the heterotrimer are color coded and labeled as decribed in the text, whereas ancillary ligands (β-cyclodextrin, staurosporine and AMP) are shown with carbon atoms in light gray, oxygen in red and nitrogen in blue. AMP in site 3 is just visible beneath α-RIM2, while AMP in sites 1 and 4 are located around the other side of the γ1 subunit.

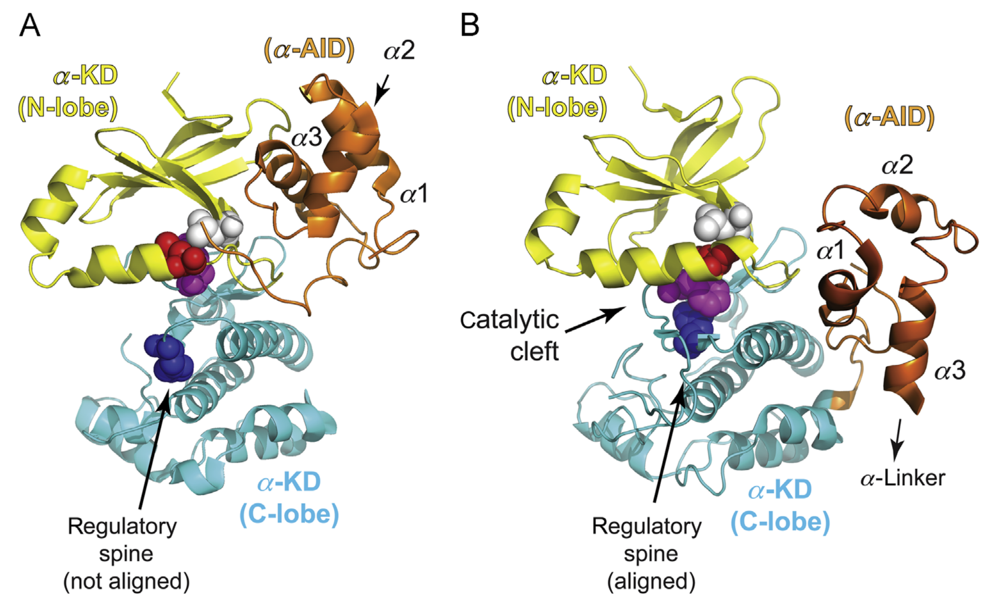

Figure 3 Two views of the kinase and auto-inhibitory domains of the α subunit (α-KD:α-AID) in inactive (A) and active (B) conformations. Note the major rotation of the α-AID relative to the α-KD between the two models; in (A), α-AID helix α3 interacts mainly with the α-KD small lobe (and with the hinge between the small and large lobes), but in (B) it interacts mainly with the γ subunit (not shown) instead. Note also that the four side chains of the "regulatory spine" (in white, red, magenta and blue) are out of alignment in (A) but are stacked in alignment in (B), indicating an active conformation[87]. The models were created with MacPyMol using PDB files 4RED (A) and 4RER (B)[84], and are shown in "cartoon" view except for the four residues that form the "regulatory spine" which are in "sphere" view. The view in (A) is of a structure derived from a construct containing only the α-KD and α-AID of human AMPK-α1. The structure crystallized as a dimer, and the α-KD shown is from one molecule while the α-AID shown is from the other molecule within the dimer. Nevertheless, the α-KD:α-AID construct behaved as a monomer in solution[84], and the structure is very similar to that of an α-KD:α-AID from *S. pombe*[86], where the arrangement of the α-AID and α-KD from the same molecule were very similar to that shown here. The view in (B) is of the same structure shown in Fig. 2, but only the α-KD and α-AID are shown.

an active conformation, where the four residues of the regulatory spine are now stacked in alignment (Fig. 3B).

The α-AID is connected to the α subunit C-terminal domain (α-CTD) by the α-linker, a region of extended polypeptide that wraps around one face of the γ subunit (Fig. 2) and is crucial in the mechanism for activation by AMP (discussed in more detail in Section 5.3). The α-CTD is a small globular domain that forms the interface with the C-terminal domain of the β subunit. An interesting feature of the α-CTD is that it ends in both the α1 and α2 isoforms with well-defined nuclear export sequences, although these have only been shown to be functional in the case of α2[88]. Both isoforms also contain serine/threonine-rich sequences of about 50 residues that we term the ST loops[89], which are discussed in Section 6 below.

5.2. *The β subunits*

When β subunit sequences are compared across isoforms and species, they contain two conserved regions, a central carbohydrate-binding module (β-CBM) and the C-terminal domain (β-CTD). The latter is a small compact domain that interacts with the α-CTD, and also contributes to an intrasubunit β-sheet containing two strands from the β-CTD and one from the N-terminus of γ. This architecture for assembling the three subunits is highly conserved throughout eukaryotes, from budding yeast[90] to fission yeast[91] and humans[92]. The β-CTD can be considered to form the core of the heterotrimeric AMPK complex, bridging the α and γ subunits.

The β-CBM is interesting because it is a member of the CBM48 family of carbohydrate-binding modules, non-catalytic domains usually found in enzymes that metabolize α1→6 linkages in carbohydrates, such as glycogen-branching enzymes and isoamylases[93]. The β-CBM causes a proportion of cellular AMPK to bind to glycogen particles[94,95], particularly in the case of the β2 isoform[94] whose CBM appears to have a higher affinity for glycogen than that in β1[96]. The carbohydrate-binding site is well defined, since crystal structures of isolated β-CBMs and a heterotrimeric α1β2γ1 complex (Fig. 2) have been solved in the presence of β-cyclodextrin, a circular heptasaccharide of α1→4-linked glucose units[84,97,98]. Until recently it had been unclear why only a proportion of AMPK in the cell is bound to glycogen, especially in skeletal muscle where β2 is the main β subunit isoform and where glycogen content can be very high. However, a recent paper shows that activated AMPK can autophosphorylate at Thr148 located within the β-CBM of β1[99], a residue known to be directly involved in the carbohydrate-binding site[97]. Phosphorylation at Thr148 prevents AMPK from binding to glycogen, although AMPK already bound to glycogen appears to be protected against autophosphorylation at this site[99].

CBMs are present within the subunits of all eukaryotic AMPK orthologs, although higher plant orthologs contain unusual "βγ" subunits that contain a CBM fused at the N-terminus of a γ subunit, as well as more conventional β subunits with central CBMs[100]. The universal occurrence of CBMs within AMPK orthologs suggest that they have key physiological functions, although these remain incompletely understood. Since both the skeletal muscle (GYS1)[101,102] and liver (GYS2)[103] isoforms of glycogen synthase are physiological targets that are inactivated after phosphorylation by AMPK, one function may be to co-localize AMPK with this glycogen-bound substrate. It has also been suggested that the β-CBM may allow AMPK to sense the structural state of glycogen and regulate glycogen synthesis according to the status of glycogen stores[104,105], although further work is required to confirm that hypothesis.

Despite the uncertain role of glycogen binding, another function of the β-CBM has become clear with exciting findings that a cleft between it and the N-lobe of the α-KD form the binding site for A-769662, 991 and salicylate[65,74]. This cleft forms between the surface of the β-CBM opposite to the known carbohydrate-binding site, and the surface of the KD N-lobe opposite to the catalytic site. In the structure shown in Fig. 2, where the cleft was unoccupied, an electrostatic interaction between Lys29 and Lys31 from the N-lobe and the phosphate group on Ser108 of the β-CTD appeared to stabilize the interaction between the two domains[84]. In other structures, the side chain of Lys29 interacts with the carboxylate group at one end of 991, while the side chains of Lys29 and Lys31 are involved with the interaction with A-769662[65,74]. These findings help to explain the requirement for autophosphorylation of Ser108 for full activation by A-769662[75]. Based on a crystal soaked with iodosalicylate, salicylates also appear to bind in this site, although the resolution was not sufficient to analyze the detailed molecular interactions[74].

5.3. *The γ subunits*

The γ subunits contain at their C-terminal end four tandem repeats, termed cystathionine-beta-sythase 1 (CBS1) through CBS4, of a sequence motif of around 60 residues known as a CBS repeat. First recognized by bio-informatic analysis[106], CBS repeats also occur in a small number of other proteins in the human genome. Most CBS-containing proteins have only two repeats that assemble into a structure known as a Bateman domain, with the cleft between the repeats often binding regulatory ligands containing adenosine, such as ATP or S-adenosyl methionine[107]. The γ subunits of AMPK and its orthologs are unusual in that they contain four repeats, thus generating two Bateman domains formed by CBS1/CBS2 and CBS3/CBS4 respectively. These assemble in a head-to-head manner to form a disc-like shape, with one CBS repeat in each quadrant of the disk; these are color-coded in Fig. 2, although much of CBS1, CBS2 and CBS3 are hidden in the view shown. This arrangement generates four pseudosymmetrical clefts in the center where ligands might bind, two accessible from one side of the disc and two from the other. Isolated γ subunits were originally reported to competitively bind just two molecules of AMP or ATP[107], but when the core of the AMPK heterotrimer was crystallized in the presence of AMP, it was found to have three molecules of AMP bound in sites 1, 3 and 4 (the sites are numbered by convention according to the CBS repeat bearing an aspartate side chain that interacts with the ribose ring of the nucleotide; site 2 lacks an aspartate and appears to be unused). In the view shown in Fig. 2, part of a molecule of AMP is just visible in site 3, while sites 1 and 4 are hidden around the back of the γ subunit. Soaking of ATP into crystals made with AMP displaced AMP by ATP in sites 1 and 3, but not 4, leading to the idea that site 4 contains a permanently bound, "non-exchangeable" AMP[92] and perhaps explaining why only two sites were detected in the original binding studies[107]. However, when another group crystallized the core complex with ATP (as opposed to soaking ATP into crystals made with AMP), they found that ATP was bound at sites 1 and 4, while site 3 was empty[108].

The extended α-linker that connects the AID to the α-CTD (see Section 5.1) can be seen from the viewpoint of Fig. 2 to wrap around the front face of the γ subunit. One conserved region within this linker termed α-regulatory subunit interacting motif-1 (α-RIM1) interacts with the unused site 2, while another (α-RIM2) interacts with site 3. A highly conserved glutamate in α-RIM2 (Glu364 in human α1) interacts with Arg70 and Lys170 in γ1, which in turn interact with the phosphate group of AMP bound in site 3. This AMP- and site 3-dependent interaction between the γ subunit and the α-linker is proposed to cause the AID to move away from its inhibitory position behind the N-lobe of the kinase domain (Fig. 3A) into the position shown in Fig. 3B, thus explaining allosteric activation by AMP. If binding of ATP at site 3 did not allow the interaction with α-RIM2, this would also explain how ATP antagonizes activation by AMP. A variety of evidence now strongly supports this model:

(1) Mutations in both α-RIM1 and α-RIM2 expected to reduce interaction with the γ subunit (including mutation of Glu364), or their replacement by a shorter artificial linker, abolished allosteric activation by AMP[74,109].
(2) Singlet oxygen-mediated luminescence energy transfer (AlphaScreen) assays, which can monitor changes in the distance between donor and acceptor probes, were used to analyze interactions between a core $\alpha1\beta2\gamma1$ heterotrimer (consisting of just the α- and β-CTDs and full length γ1) and a construct containing the AID, α-RIM1 and α-RIM2 from α1. Addition of AMP increased the interaction, whereas ATP decreased it.
(3) As mentioned in Section 4.2, the AMP analog C2 is rather selective for α1 complexes, with which it causes both allosteric activation and protection against dephosphorylation of Thr172. However, both effects of C2 could be transferred to α2 complexes merely by replacing α-RIM2 and the remainder of the α-linker from α2 with the equivalent region from α1. These results emphasize the importance of the α-linker in the dual mechanisms of activation by this AMP analog.

This model was also supported by AlphaScreen assays in which the donor and acceptor probes were attached to the N-termini of the α and γ subunits in a complete heterotrimer[84]. Addition of AMP yielded changes indicating that the probes moved together, suggesting the formation of a more compact conformation for the heterotrimer in the presence of AMP, as suggested by previous results obtained by small angle X-ray scattering in solution[110]. On the other hand, addition of ATP caused the probes to move apart, indicating a less compact conformation. This is consistent with the idea that the α-linker dissociates from the γ subunit in the inactive conformation in the presence of ATP, allowing the whole heterotrimer to adopt a more extended structure in which the AID interacts with and inhibits the kinase domain. These AlphaScreen assays also allowed the concentration dependence of the effects of AMP and ATP on these conformational changes to be measured, independently of their binding at the catalytic site. The results showed that the half-maximal effect (EC_{50}) for the effect of AMP (measured in the absence of ATP) occurred at 0.95 μmol/L, whereas the EC_{50} for the effect of ATP was at 0.85 mmol/L, almost 1000-fold higher[84]. For comparison (although it is not possible to measure allosteric activation in the absence of ATP) the estimated EC_{50} values for allosteric activation of γ1 complexes by AMP in the presence of 0.2, 1 and 5 mmol/L ATP were 5.3, 22 and 140 μmol/L, showing that increasing concentrations of ATP compete with AMP at the γ subunit sites[36].

5.4. *Remaining challenges in understanding regulation by adenine nucleotides*

Although the various crystal structures obtained over the last few years have yielded considerable insight into the mechanism of regulation by adenine nucleotides, several questions remain. One is the role of binding of AMP and other nucleotides at sites 1 and 4, especially given the evidence discussed in Section 5.3 that binding at site 3 recruits the α-RIM1 motif, which is crucial both for allosteric activation and for protection against dephosphorylation. Interestingly, mutation to alanine of any one of the three aspartate residues that bind the ribose rings of nucleotides at sites 1, 3 and 4 abolishes both allosteric activation and promotion of Thr172 phosphorylation[111]. The three nucleotide binding sites are located close together at the center of the γ subunit, and side chains of highly conserved basic residues from the γ subunit interact with phosphate groups of nucleotides in more than one site. For example, the side chains of His151 and His298 in human $\gamma1$ interact with phosphate groups of AMP in both sites 1 and 4[92]. It therefore seems very likely that binding of nucleotides at these three sites will show mutual dependencies on each other, either positive or negative. Along these lines, the group who crystallized the core complex in the presence of ATP suggested that the mode of binding of ATP at site 4 would preclude binding of AMP (or any other nucleotide) at site 3. Thus, AMP may have to be bound at site 4 (and possibly also at site 1) before it binds at the crucial site 3.

Another question not full answered is how binding of AMP inhibits Thr172 dephosphorylation. Since the lack of ability of the AMP analog C2 to protect against dephosphorylation of $\alpha2$ complexes can be restored by replacing α-RIM2 of $\alpha2$ (which binds site 3 when AMP is bound) with the equivalent region from $\alpha1$, it appears that it is binding of AMP at site 3 that is crucial for the effect. However, unlike allosteric activation by AMP, which does not require the presence of the β-CBM[94], protection against Thr172 dephosphorylation by AMP does require it[65], although the reasons for this are poorly understood.

Another puzzle is why ADP binding should provide protection against dephosphorylation of Thr172[38] yet does not, like AMP binding, cause allosteric activation. This would be hard to explain if the effects of ADP and AMP were due to binding at the same site. However, studies of the budding yeast ortholog of AMPK suggest that the γ subunit SNF4 is not required for the response to glucose starvation[112], and that binding of ADP to the catalytic site on the kinase domain, rather than to the γ subunit, may be responsible for its ability to protect against dephosphorylation of the site equivalent to Thr172[113]. In the same study, it was reported that binding of the kinase inhibitor staurosporine (which binds at the catalytic site[38]) to either the budding yeast or mammalian kinases provides protection against Thr172 dephosphorylation. Thus, it is possible that AMP and ADP protect against dephosphorylation by binding at different sites.

A final question that has not yet been illuminated by the structural studies concerns how phosphorylation of Thr172 by LKB1, but not CaMKKβ, is promoted by binding of AMP[36]. A radical proposal to explain this, which has been developed by Lin and colleagues[114,115] at Xiamen University, is that AMP binding to AMPK causes it to co-localize with LKB1 due to their mutual interactions with the scaffold protein axin, which in turn binds to late endosomal/lysosomal adaptor and MAPK and mTOR activator (LAMTOR1) at the surface of the lysosome. However, promotion by AMP of Thr172 phosphorylation by LKB1 can be observed on reconstitution of highly purified LKB1 and AMPK[36], suggesting that the effect does not strictly require any of these additional components.

6. Non-canonical regulation by phosphorylation of the ST loop and other sites

The hormone insulin represents a signal that nutrients are available, with those nutrients (glucose, amino acids and fats) either directly triggering insulin release from the β cells of the pancreas, or doing so indirectly *via* release of incretins such as glucagon-like peptide-1 from the small intestine. Insulin then stimulates target cells to take up these nutrients and convert them to their storage forms of glycogen, triglycerides and proteins. Insulin-like growth factor-1 (IGF1), which acts *via* a signaling pathway closely related to that of insulin, is a growth factor that promotes biosynthesis and hence cell growth. Since AMPK is generally switched on under the opposite circumstances to insulin and IGF1 (lack of nutrients or energy) it is not surprising that the AKT/PKB (protein kinase B) pathway, the principal signaling pathway downstream of insulin and IGF1, should antagonize the AMPK pathway. In 2006 it was reported that AKT phosphorylated rat AMPK-$\alpha1$ at Ser485 (equivalent to Ser487 in humans, with human numbering being used below, with the exception of Thr172). Evidence was presented that prior phosphorylation at Ser487 by AKT reduced subsequent phosphorylation at Thr172 and consequent activation by LKB1, and that this mechanism explained how prior treatment of perfused rat heart with insulin reduced AMPK activation during subsequent ischemia[116].

Ser487 occurs within a region of around $50-55$ residues in the AMPK-α subunits that we now term the "ST loop". This is a serine/threonine rich region that is present in α-CTDs in all vertebrates and nematodes, but not in orthologs from insects, plants, fungi or protozoa. In all crystal structures of mammalian complexes containing an α-CTD, the ST loop was either not resolved, suggesting that it is disordered within the crystals (perhaps because it is not phosphorylated during bacterial expression), or had been replaced by a short artificial spacer in the construct crystallized, because it was thought that it might hinder crystallization. In these structures the ST loop therefore appears as a gap between the end of penultimate β-strand and the start of the last α-helix in the α-CTD. My group[89] has recently confirmed that AKT efficiently phosphorylates Ser487 on AMPK-$\alpha1$, although the equivalent residue on AMPK-$\alpha2$, Ser491, is an extremely poor substrate for AKT—it is therefore important not to simply assume that the regulation of $\alpha1$ and $\alpha2$ by phosphorylation in this region will be identical. In fact, Ser491 on $\alpha2$ is efficiently autophosphorylated by AMPK itself, and becomes phosphorylated in intact cells when AMPK, rather than AKT, is activated. By generating HEK-293 cells expressing wild type or mutant $\alpha1$, we showed that prior activation of AKT using IGF1 inhibited subsequent Thr172 phosphorylation and AMPK-$\alpha1$ activation in response to A-769662, and that this was blocked by a specific AKT inhibitor or by mutation of Ser487 to alanine. We also showed that the effect of Ser487 phosphorylation by AKT to inhibit subsequent phosphorylation at Thr172 on AMPK-$\alpha1$ was identical using either LKB1 or CaMKKβ as the upstream kinase, suggesting that the mechanism may involve a simple physical occlusion of Thr172. Consistent with this, mutation of three basic residues in the α-C helix of the N-lobe, which are conserved in all vertebrate AMPK-α subunits but not in closely related kinases, abolished the inhibitory effect of AKT even though Ser487 was still phosphorylated. This suggested that the ST loop interacts with the α-C helix following its phosphorylation, thus reducing access to Thr172[89].

ST loops also appear to be phosphorylated by other kinases. Hurley et al.[117] reported that Ser487/491 on AMPK-$\alpha1$ or -$\alpha2$ (isoform not specified) was phosphorylated in response to cyclic AMP elevation in INS1 cells, a pancreatic β cell line, while a recombinant AMPK-$\alpha1$ peptide was phosphorylated in cell-free assays at Ser487 by cyclic AMP-dependent protein kinase (PKA).

Complicating this story, however, the effects of cyclic AMP-elevation were abolished in *CaMKKβ*-null mouse embryo fibroblasts, and CaMKKβ was inactivated by cyclic AMP-elevating agents, suggesting that effects in intact cells were mediated by modulation of CaMKKβ, rather than AMPK[117]. Using a bacterially expressed α1β1γ1 complex, PKA has been reported to phosphorylate not only Ser487 but also Ser499 and Ser175, and it was proposed that this limited AMPK activation, and hence inhibition of lipolysis, when PKA was activated in white adipocytes[118]. Like Ser487, Ser499 is located in the ST loop, but Ser175 is immediately adjacent to Thr174, the residue equivalent to Thr172 in human α1. Based on analysis of various mutations, the authors[118] suggested that it was phosphorylation at Ser175 rather than Ser487 or Ser499 that blocked subsequent AMPK activation. A puzzling feature is why they did not observe any effects on subsequent Thr172 phosphorylation when Ser487 was phosphorylated by PKA, even though two other groups[89,116] have shown that there is a marked effect when Ser487 is phosphorylated by AKT. Finally, it has been reported that two residues in the ST loop just upstream of Ser487, *i.e.,* Thr481 and Ser477, are phosphorylated by glycogen synthase kinase 3 (GSK3) when Ser487 has been phosphorylated[119]. GSK3 often phosphorylates serine or threonine side chains 4 residues N-terminal to a "priming" phosphoamino acid, although the spacing between Ser487 and Thr481 is six rather than four residues. It was proposed that phosphorylation of Ser477 and Thr481 inhibited net Thr172 phosphorylation by promoting its dephosphorylation. While these observations are interesting, the physiological rationale underlying inhibition of AMPK by GSK3 is difficult to grasp, because both GSK3 isoforms (α and β) are inactivated by phosphorylation by AKT, and because GSK3 usually acts to inhibit rather than promote anabolic pathways, similar to AMPK but opposite to AKT.

7. Regulation of AMPK by natural products used in traditional medicines

As mentioned in Section 4.1, over the last few years more than 100 different natural products have been shown to activate AMPK; a list of these, which is almost certainly not comprehensive, is shown in Table 1[41,44,49,50,59,60,69,120–249]. Although many of them can be classed as polyphenols, their structures are very varied. The majority are products of plants used in herbal remedies, particularly in traditional Asian medicine. The mechanism by which most of them activate AMPK is unknown, and a puzzling feature is why so many natural plant products should all be AMPK activators. One clue is that among the small number of these activators where the mechanism has been established (given at the top of the list in Table 1), most are inhibitors of mitochondrial ATP synthesis, either by inhibiting Complex I of the respiratory chain, or by inhibiting the ATP synthase (Complex V). Most of the natural plant products that activate AMPK appear to be secondary metabolites*, i.e.*, they are not required for plant growth, development or reproduction, and a reasonable working hypothesis is that many of them are molecules produced by plants to deter infection by pathogens, or grazing by insect or other herbivorous animals, to whom these molecules are toxic. In support of this idea, resveratrol is known to be produced by grapes in response to fungal infection[250], while *Galega officinalis,* the source of galegine from which metformin and phenformin were derived, is classified as a noxious weed in the USA because it is poisonous to herbivorous animals (reflected in one of the common names for *Galega officinalis*, Goat's Rue).

Why should plants produce inhibitors of mitochondrial function as defensive chemicals? The respiratory chain and the ATP synthase contain five large hydrophobic multiprotein complexes, with Complex I containing no less than 44 protein subunits, while the ATP synthase has at least 14. It seems probable that many different hydrophobic, xenobiotic compounds might find a binding site in one or more of these complexes that would inhibit their function. Many secondary metabolites of plants are stored in the cell vacuole (equivalent to the lysosome of animal cells), and are therefore kept away from their own mitochondria. The production of mitochondrial poisons might therefore be a useful general approach for plants to produce compounds that would deter infection or grazing. However, in line with the aphorism of Paracelsus that "the dose makes the poison", lower doses of these compounds that are not sufficient to fully inhibit mitochondrial function might still have useful therapeutic effects by activating AMPK.

It is also interesting to note that the barbiturate drug, phenobarbital, activates AMPK in an AMP-dependent manner by inhibiting the respiratory chain[41]. In hepatocytes, AMPK activation is required for phenobarbital to induce expression of genes (*e.g.*, *CYP2B6*) encoding enzymes of the cytochrome P450 (CYP) family, *via* the constitutively active/androstane receptor, constitutive active/androstane receptor (CAR)[251,252]. Some classes of CYP enzymes (especially the CYP1/CYP2/CYP3 families) catalyze the initial steps in metabolism of drugs and other hydrophobic xenobiotics, making them more soluble for excretion. Plant products that are defensive agents inhibiting mitochondrial ATP synthesis would activate AMPK, and induction of CYP enzymes by AMPK might then be a good general way for the animal to mount a response to deal with potential poisoning by these xenobiotics.

8. Conclusions and perspectives

Most indications for drugs targeting AMPK suggest that activators rather than inhibitors would be therapeutically beneficial. In general, development of activators is probably more difficult than development of inhibitors, but the fact that there are already many known activators of AMPK, acting by three or four different mechanisms, shows that this goal is reachable. Many of the activators already known are natural plant products, or derivatives of natural products, that originate from traditional medicines. Two of these, metformin and salicylate, are already among the most successful and widely used drugs of all time, although the extent to which their therapeutic effects are mediated by AMPK is still being debated. Of the many natural plant products whose mechanism of activation of AMPK has not yet been elucidated, my suspicion is that most of them will turn out to be compounds used by plants for defensive purposes, most of which are likely to activate AMPK indirectly by inhibiting mitochondrial ATP synthesis. In such cases, the question must always be asked whether the new agent is more effective than metformin, and whether it has fewer side effects. However, there may also be some direct activators among the long list of natural products in Table 1, and this is certainly an avenue worth pursuing. Of the known binding sites on AMPK where ligand binding can cause activation, the A-769662/salicylate-binding site is perhaps the easiest to target for drug development, although the AMP-binding sites can also be targeted by pro-drugs such as AICAR or C13. It will be fascinating to see whether the current effort to develop novel AMPK activators will result in any clinically useful drugs over the next few years.

Table 1 Partial list of natural products (mostly from plants) that have been reported to activate AMPK in intact cells or *in vivo*. Although a single source species is usually listed, most of the compounds are probably also produced by related species. The author compiled this list but has not read all of the papers cited as thoroughly as other papers discussed in this review. ?, unkown.

Natural product	Source	Mechanism	Ref.
Antimycin A	*Streptomyces* (bacteria)	Inhibits Complex III	44
Apoptolidins A/C	*Nocardiopsis spp.* (bacteria)	Inhibits ATP synthase	120
Arctigenin	*Arctium lappa*	Inhibits Complex I	49,121
Berberine	*Berberis spp.*, other plants	Inhibits Complex I	41,122
Cordycepin (3′-deoxyadenosine)	*Cordyceps militaris* (fungus)	Converted to AMP analog?	59,60,123
Galegine	*Galega officinalis*	Inhibits Complex I	50
Oligomycin	*Streptomyces* (bacteria)	Inhibits F1 ATP synthase	41
Quercetin	Many plants	Inhibits Complex I	124
Resveratrol	Grapes, red wine	Inhibits ATP synthase	41,125,126
Salicylate	*Salix alba* (willow), other plants	Binds to A-769662 site	69
Alternol	*Alternaria alternata*	?	127
Anthocyanin fraction	Purple sweet potato	?	128
Anthocyanin fraction	Korean black bean	?	129
Apigenin	*Matricaria chamomilla*	?	130
Artemisinin	*Artemisia annua*	?	131
Aspalathin	*Aspalathus linearis*	?	132
Bavachalcone	*Psoralea corylifolia*	?	133
Caffeic acid	All plants	?	134
Caffeic acid, phenethyl and phenylpropyl esters	All plants	?	135
Celastrol	Many plants	?	136
Chalcones	Various plants	?	137
Chitosan	Crustaceans	?	138
Chrysin	*Passiflora caerulea*	?	139
Cucurbitane triterpenoids	*Siraitia grosvenorii*	?	140
Curcumin	*Curcuma longa*	?	141,142
Cyanidin	*Daucus carota* (black carrot)	?	143
Dehydrozingerone	*Zingiber officinale* (ginger)	?	144
Delphinidin-3-glucoside	Many plants	?	145
14-Deoxyandrographolide	*Andrographis paniculata*	?	146
Dihydromyricetin	*Ampelopsis grossedentata*	?	147
2-(2,4-Dihydroxyphenyl)-5-(*E*)-propenylbenzofuran	*Krameria lappacea*	?	148
Emodin	*Rheum emodi*	?	149–151
ENERGI-F704	Bamboo	?	152,153
Epigallocatechin gallate	*Camellia sinensis*	?	124,154
Ergostatrien-3β-ol	*Antrodia camphorata*	?	155
Eugenol	Clove oil, nutmeg, cinnamon, basil	?	156
Fargesin	*Magnolia spp.*	?	157
Foenumoside B	*Lysimachia foenum-graecum*	?	158
Fucoidan	Brown seaweeds	?	159
Fungal extract	*Clitocybe nuda*	?	160,161
Gallic acid	Many plants	?	162
Geraniol	Rose/palmarosa/citronella oils	?	163
GGEx18	Traditional Korean medicine	?	164
6-Gingerol	*Zingiber officinale* (ginger)	?	165–167
Ginsenosides	*Panax ginseng*	?	168 – 172
Glabridin	*Glycyrrhiza glabra*	?	173,174
Green tea extract	*Camellia sinensis*	?	175,176
Hispidulin	*Saussurea involucrate*	?	177–179
Honokiol	*Magnolia grandiflora*	?	180,181
Hugan Qingzhi tablet	Chinese herbal medicine	?	182
Indazole-type alkaloids	*Nigella sativa*	?	183
Isoquercitrin	Many plants	?	184
Isorhamnetin	*Tagetes lucida*	?	185
Jinlida granule	Chinese herbal medicine	?	186
Jinqi formula	*Coptidis rhizome/Astragali rhadix/Lonicerae japonicae*	?	187
Karanjin	*Pongamia pinnata*	?	188
Kazinol C	*Broussonetia kazinoki*	?	189

Table 1 (*continued*)

Natural product	Source	Mechanism	Ref.
Licochalcone	*Glycyrrhiza glabra* (licorice)	?	190
Lindenenyl acetate	*Lindera strychnifolia*	?	191
Luteolin	Many plants	?	124
Malvidin	*Daucus carota* (black carrot)	?	143
Mangiferin	*Iris unguicularis*	?	192–195
Methyl cinnamate	*Zanthoxylum armatum*	?	196
4-*O*-methylhonokiol analog	*Magnolia grandiflora*	?	197
2-Methyl-7-hydroxymethyl-1,4-naphthoquinone	*Pyrola rotundifolia*	?	198
Monascin/ankaflavin	*Monascus pilosus* (a fungus)	?	199
Monascuspiloin	*Monascus pilosus* (a fungus)	?	200
Naringin	*Citrus x paradisi*	?	201
Nectrandin B	*Myristica fragrans* (nutmeg)	?	202–204
Octaphlorethol A	*Ishige foliacea* (a brown alga)	?	205
Oleanolic acid	Many plants	?	206
Osthole	*Cnidium monnieri*	?	207,208
Parthenolide	*Tanacetum parthenium* (feverfew)	?	209
Persimmon tannin	*Diospyros kaki* (persimmon)	?	210
Petasin	*Petasites spp.*	?	211
Piperlongumine	*Piper longum*	?	212
Plant extract	*Boesenbergia pandurata*	?	213
Plant extract	*Cirsium japonicum*	?	193
Plant extract	*Houttuynia cordata*	?	214
Plant extract	*Impatiens balsamina*	?	215
Plant extract	*Lycium barbarum*	?	216
Plant extract	*Malva verticillata*	?	217
Plant extract	*Remotiflori radix*	?	218
Plant extract	*Rhus verniciflua Stokes*	?	219
Plant extract	*Scutellaria baicalensis*	?	220
Plant extract	*Sechium edule*	?	221
Plant extract	*Taraxacum mongolicum*	?	222
Plant extract	*Theobroma cacao* (cocoa)	?	223
Plant extract	*Viola mandshurica*	?	224
Plant extract	*Vitis thunbergii*	?	225
Pomolic acid	*Chrysobalanus icaco*	?	226
Pterostilbene	Grapes, other fruits	?	227
Puerarin	*Radix puerariae*	?	228
ReishiMax	*Ganoderma lucidum*	?	229
Rhizochalin (aglycone)	*Rhizochalina incrustata* (a sponge)	?	230
S-methylmethionine sulfonium chloride	Many plants	?	231
Salidroside	*Rhodiola rosea*	?	232
Saponins	*Rubus parvifolius*	?	233
Scopoletin	*Scopolia spp.*	?	234
Soybean peptides	*Glycine max (soybean)*	?	235
Sulforaphane	*Brassica oleracea*	?	236
Tangeretin	*Citrus tangerine* (tangerine)	?	237
Tanshinone IIA	*Salvia miltiorrhiza*	?	238,239
Theaflavins	*Camellia sinensis* (tea)	?	240
Theasinsensins	*Camellia sinensis* (tea)	?	241
Thymoquinone	*Nigella sativa*	?	242
Tiliroside	Rose hips, strawberry, raspberry	?	243
Tormentic acid	*Eriobotrya japonica*	?	244
Trans-cinnamic acid	Cinnamon	?	245
Triterpenoid saponins	*Stauntonia chinensis*	?	246
Ursolic acid	*Mirabilis jalapa*, other plants	?	247,248
Xanthigen	*Punica granatum*	?	249

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