AMPK

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AMPK – a cellular energy sensor primarily regulated by AMP

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Abbreviations: AMPK, AMP-activated protein kinase;

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AMP-activated protein kinase (AMPK) was discovered by two groups independently in 1973, in the form of partially purified kinase activities that phosphorylated and inactivated acetyl-CoA carboxylase (ACC) [1] and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (HMGR) [2], two enzymes that have key regulatory roles in the pathways of fatty acid and sterol/isoprenoid synthesis, respectively. The ACC kinase was shown to be allosterically activated by 5'-AMP [3], while the HMGR kinase was shown to be activated by phosphorylation by a distinct upstream kinase [4], and subsequently (like the ACC kinase) to be allosterically activated by AMP [5]. Finally, evidence was obtained that all of these effects could be explained by a single protein kinase [6], which was consequently renamed AMP-activated protein kinase or AMPK [7, 8].

Structure of AMPK and regulation by phosphorylation

AMPK is now known to occur in essentially all eukaryotes as heterotrimeric complexes comprising a catalytic α subunit and regulatory β and γ subunits (Fig. 1), with multiple isoforms of each subunit being encoded by distinct genes in mammals [9, 10]. The α subunits contain conventional serine/threonine kinase domains at the N-terminus, which are only significantly active when phosphorylated at a conserved threonine residue within the "activation loop" (this residue is usually referred to as Thr172 due to its position in the original rat α2 sequence [11], although the exact numbering may vary in different isoforms and species). The kinase domain is followed by an auto-inhibitory domain that appears to inhibit the phosphorylated kinase domain in the absence of the β and γ subunits [12], then by an extended linker region that leads to the globular C-terminal domain [13]. The latter associates with the C-terminal domain of the β subunit, which is in turn linked to the γ subunit via a β-sheet containing two strands from β and one from γ. The β subunits also contain a conserved carbohydrate-binding module (CBM), one function of which in mammals is to cause binding of AMPK to glycogen particles [14, 15]. Following the β-sheet strand that links them to the β subunit, the γ subunits contain four tandem repeats of a sequence motif known as a CBS repeat [16], which form the binding sites for the regulatory nucleotides AMP, ADP and ATP. It was originally thought that there were two binding sites [17], but it is now clear that the two pairs of CBS repeats assemble in a pseudosymmetric "head-to-head" manner to generate four binding clefts, although only three appear to be utilized for nucleotide binding (one between CBS1 and CBS2 and two between CBS3 and CBS4) [13, 18, 19].

The major kinase responsible for phosphorylation of Thr172, and thus for activation of AMPK, is a complex containing the tumour suppressor, LKB1 [20, 21]. Although the LKB1 complex appears to be constitutively active [22], binding of AMP to AMPK not only causes allosteric activation, but also makes AMPK a better substrate for the upstream kinase (i.e. LKB1) [23], and a worse substrate for protein phosphatases [24], thus promoting net phosphorylation and activation. Thr172 can also be phosphorylated by calmodulin-activated protein kinase kinases (CaMKKs, especially CaMKKβ) [25-27], thus providing a Ca^{2+}-dependent pathway for AMPK activation. This alternate pathway can occur in the absence of any changes in adenine nucleotides, although changes in cellular AMP can amplify the effect of the Ca^{2+}-activated pathway due to the protective effect of AMP on Th172 dephosphorylation [28].

Regulation of AMPK by AMP, ADP and ATP

The major role of the AMPK system is in the maintenance of cellular energy homeostasis. Thus, AMPK is activated by stresses that deplete cellular ATP and increase the ADP:ATP ratio [29]. Once activated, the kinase acts to restore energy homeostasis by switching on alternate catabolic pathways that generate ATP, while switching off anabolic pathways and other processes that consume ATP [9, 10]. We will discuss the roles of ADP and ATP in more detail below, but it is worth considering at this point why it might make sense for AMP to a key regulator. The level of AMP in the cell appears to be largely determined by the activity of the enzyme adenylate kinase [30], which catalyzes the reversible inter-conversion of the three adenine nucleotides (2ADP ↔ ATP + AMP); this reaction has an equilibrium constant close to one. Adenylate kinase appears to be a very active enzyme in essentially all eukaryotic cells, such that its mass action ratio

([ATP].[AMP]/[ADP]^2), appears to lie close to the equilibrium value. If this reaction is at equilibrium, it is easy to show [31] that the cellular AMP:ATP ratio will vary as the square of the ADP:ATP ratio, and that the increases in cellular AMP that occur when ATP:ADP ratio falls will be much larger than the fall in ATP or the increase in ADP. This is illustrated in Fig. 2, which shows results for the estimated changes in ATP, ADP and AMP when human (G361) cells were incubated with berberine, a natural product used in traditional Chinese medicine that is an inhibitor of complex I of the respiratory chain [32, 33]. Cellular ADP and ATP were estimated by perchloric acid extraction followed by capillary electrophoresis; AMP is unfortunately too low to reliably measure by this method, but its concentration was calculated by assuming that the adenylate kinase reaction was at equilibrium [34]. This analysis showed that the decrease in cellular ATP induced by berberine, while statistically significant, was quite small (from 4.5 to 3.8 mM). The increase in ADP was larger (2.3-fold, from 430 to 980 μM), but the largest change of all was in AMP (a 6.5-fold increase, from 42 to 270 μM). Thus, it would make sense for a system that responds to cellular energy status to primarily respond to AMP, the only potential difficulty being in the ability of the system to detect changes in AMP in the presence of much higher concentrations of ADP and ATP.

As discussed in the previous section, it was shown nearly twenty years ago that binding of AMP to AMPK not only caused allosteric activation but also promoted phosphorylation [23], and inhibited dephosphorylation [24], of Thr172. These three effects of AMP all act in the same direction, potentially generating a sensitive system in which there is a large degree of activation in response to a small change in the initial activating signal [35]. However, more recently this primary role for AMP in the regulation of the AMPK system has been questioned. In particular, it was reported that the effect of AMP both to inhibit Thr172 dephosphorylation [13], and to enhance Thr172 phosphorylation [36], could be mimicked by ADP. Since ADP is normally present at ten-fold higher concentrations than AMP (e.g. Fig. 2), it was argued that ADP, rather than AMP, might be the primary regulator of net Thr172 phosphorylation. There is general agreement that allosteric activation is caused only by AMP and not by ADP, but it was also argued that the allosteric mechanism might not be significant under physiological conditions [37, 38]. There appear to be two main reasons for these doubts. Firstly, the affinities for binding of AMP, ADP and ATP to the γ subunit, which were estimated indirectly by competition with binding of a fluorescent ATP analogue, were found to be quite similar [13]. This makes it hard to see how AMP could effectively compete, when its cellular concentration is typically around one or two orders of magnitude lower, respectively, than those of ADP and ATP (see Fig. 2). The second reason is that allosteric activation measured in cell-free assays is often reported to be quite modest (typically <2-fold) [39, 40], yet the effect of phosphorylation at Thr172 can be very large (>100-fold) [40], casting doubt on the quantitative significance of the former mechanism. However, as discussed in the next section, recent studies in our laboratory have suggested that these views are incorrect.

The empire strikes back – AMP as the key regulator of AMPK

We will now discuss in turn the three distinct mechanisms by which AMPK is regulated by adenine nucleotides, focusing particularly on the role of AMP.

AMP and ADP binding both protect against dephosphorylation of Thr172

We have confirmed that binding of ADP, as well as AMP, protects AMPK against dephosphorylation by phosphatases such as PP2Cα [34]. However, we find that AMP is about 10-fold more potent than ADP in mediating this effect; this also appeared to be the case, although not specifically pointed out, in the previous results [13]. To confirm that the effect of ADP was not due to breakdown of ADP to AMP during the assay [40], we showed that the effect of AMP was abolished by addition of the 5'-nucleotidase CD73 (which hydrolyses AMP to adenosine and phosphate) but that the effect of ADP (which is not metabolized by CD73) was almost unaffected.

When the dephosphorylation assays were conducted in the absence of ATP, the concentrations of AMP and ADP causing half-maximal effects (EC_{50}) were at 2.6 and 23 μM respectively. However, in intact cells ATP is present at around 5 mM, and since it competes with AMP and ADP for

binding to the γ subunit, it should reduce the effects of low concentrations of AMP and ADP. This was indeed the case, with the apparent EC_{50} for AMP and ADP increasing by nearly two orders of magnitude to 200 μM and 1.6 mM respectively; note, however, that the almost 10-fold higher potency of AMP compared with ADP was retained. When the changes in AMP and ADP observed in berberine-treated G361 cells (Fig. 2) were superimposed on the curves obtained at 5 mM ATP, it was evident that the change in AMP would produce a 3.3-fold increase in Thr172 phosphorylation, while the change in ADP would produce only a 1.8-fold increase [34]. Thus, while both nucleotides might contribute to the observed increases in Thr172 phosphorylation in response to the energy stress induced by berberine, AMP may make the more important contribution.

Only binding of AMP promotes phosphorylation of Thr172 by LKB1

Our laboratory originally reported that AMP binding to AMPK promoted phosphorylation of Thr172 by LKB1, but not CaMKKβ [25]. However, it was recently reported that binding of AMP promoted phosphorylation by both LKB1 and CaMKKβ, while ADP also promoted phosphorylation by CaMKKβ, as long as the β subunits of the complex were N-myristoylated [36, 41]. We have re-investigated this using native rat liver AMPK, which is already N-myristoylated, and have confirmed our original findings that AMP promoted phosphorylation only by LKB1, and not by CaMKKβ. We were also unable to detect any effects of ADP on the rate of phosphorylation by either kinase, using short (10 minute) incubations. We did observe small effects of ADP if the incubation time was extended to 20 minutes or more, but this effect was due to generation of AMP from ADP during prolonged assays, because it was blocked by addition of CD73. AMP caused a maximum stimulation of the rate of phosphorylation by 2.8-fold, with a half-maximal effect (measured at 200 μM ATP) of 160 μM. Thus, the effect of AMP to promote phosphorylation by LKB1 appears to require higher concentrations than its effects on dephosphorylation. This suggests that it is caused by binding to a different site, although which of the three sites on the γ subunit is responsible for this effect, or indeed for the other effects of AMP, remains unclear.

Allosteric activation by AMP is significant in cell-free assays and in intact cells

To address whether AMP can compete with high intracellular concentrations of ATP to cause allosteric activation of AMPK, we initially studied purified rat liver AMPK in cell-free assays [34]. Using the standard assay concentration of 200 μM ATP, AMP caused activation at very low concentrations (half-maximal effect at 5 μM) and then started to inhibit at concentrations above 100 μM. The latter was due to competition with ATP at the catalytic site on the α subunit, because an almost identical inhibition (but without any activation) was observed when isolated α subunit kinase domains were assayed under the same conditions. When the assays were conducted at the more physiological ATP concentration of 5 mM, the activating effect of AMP now required higher concentrations (half-maximal effect 140 μM), and the inhibition was only observed at non-physiological AMP concentrations above 1 mM. Strikingly, however, the degree of activation was higher than before (13-fold, versus 5.5-fold at 200 μM ATP). We suspect that previous failures to observe a large degree of allosteric activation [39, 40] were due to use of: (i) bacterially expressed AMPK, which appears to be less AMP-dependent than native mammalian AMPK, perhaps due to differences in covalent modification; (ii) long incubation times, which can result in generation of AMP during the assay, thus increasing the basal activity. The latter is more of a problem when low ATP concentrations are used, because the kinase is then activated by lower AMP concentrations.

Using our new assay format containing 5 mM ATP, AMP was able to cause a large allosteric activation over a concentration range (50-500 μM) that was from one to two orders of magnitude lower than that the ATP concentration. Using the kinetic parameters estimated from our cell-free assays, the increase in AMP caused by berberine treatment in G361 cells (Fig. 2) would be predicted to cause a 2.3-fold increase in AMPK activity due to the allosteric effect alone.

To address whether allosteric activation was indeed significant in intact cells [34], we first used the LKB1-null G361 (human melanoma) cell line, where a mitochondrial inhibitor such as berberine does not promote Thr172 phosphorylation (or cause AMPK activation measured in an immunoprecipitate) because of the lack of LKB1 to provide a high basal phosphorylation of

Thr172. Increases in both of these parameters could be observed using the Ca^{2+} ionophore A23187 (which activates the CaMKKβ pathway) but, strikingly, the phosphorylation of the downstream target ACC was greater in response to berberine than in response to A23187. The obvious explanation for this is that berberine causes allosteric activation of AMPK by AMP, which is not detectable in an immunoprecipitate kinase assay, whereas A23187 works only by increasing Thr172 phosphorylation. As a further proof of this, we utilized AMPK knockout mouse embryo fibroblasts in which both isoforms of the AMPK catalytic subunit had been eliminated by gene targeting [42]. In these cells there is no detectable phosphorylation of either AMPK or ACC in response to either A23187 or berberine. However, when wild type AMPK-α1 was co-expressed with β2 and γ1 in these cells, both A23187 and berberine promoted AMPK activation (measured in an immunoprecipitate) and phosphorylation of the AMPK site on ACC. However, although A23817 had a larger effect on apparent AMPK activity than berberine, berberine had the larger effect on ACC phosphorylation. This can be explained by the additional allosteric activation of AMPK induced by berberine. More convincing still, we co-expressed with β2 and γ1 the partially phospho-mimetic T172D mutant of AMPK-α1, which retains allosteric activation by AMP [43]. In cells expressing the T172D mutant there was a detectable AMPK activity measured in immunoprecipitates, but this was not increased by A23187 or berberine because the mutant cannot be phosphorylated at Thr172. Despite this, the phosphorylation of ACC increased markedly (>2-fold) in response to berberine, but not A23187. This can only have been due to the allosteric activation of AMPK caused by the berberine-induced increase in AMP. Finally, despite the fact that phosphorylation of Thr172 by upstream kinases can cause >100-fold activation when fully dephosphorylated AMPK complexes are incubated with upstream kinases and ATP in cell-free assays, we showed that the effects caused by changes in Thr172 phosphorylation in intact cells are generally much more modest than this [34]. In HEK-293 cells, which express LKB1, the stoichiometry of basal Thr172 phosphorylation was estimated to be around 25%, and this increased by only 1.5-fold in response to A23187 and 2-fold in response to berberine. In G361 cells, where the basal phosphorylation was much lower (4%) due to the lack of LKB1 and the low basal activity of CaMKKβ, the increase obtained on activating CaMKKβ with A23187 was still only 4-fold, to a final value of 16%. Thus, the changes in Thr172 phosphorylation that occur in intact cells, where Thr172 phosphatases are also active, are much smaller than those that can be generated in cell-free assays by incubation of recombinant AMPK with upstream kinases, when phosphatases are of course absent.

Conclusions and perspectives

Recent findings from other laboratories suggesting that ADP promotes phosphorylation and inhibits dephosphorylation of AMPK [13, 36], led to proposals [37, 38] that ADP, rather than AMP, is the critical intracellular signal that causes activation of AMPK during energy stress. It was also proposed [37, 38] that allosteric activation by AMP may not be significant in intact cells, compared with the apparently much larger effects on Thr172 phosphorylation. However, our re-evaluation of these questions have clearly shown that:

1) binding of both AMP and ADP to the γ subunit protects against dephosphorylation of Thr172 by protein phosphatases, although AMP is almost 10-fold more potent than ADP, and is likely to make a larger contribution to the overall effect of energy stress observed in intact cells;
2) only binding of AMP promotes Thr172 phosphorylation, and then only by LKB1; this effect also requires higher AMP concentrations than the effect on dephosphorylation, so it is not clear how much it might contribute to the overall effect of a mild energy stress in intact cells;
3) in cell-free assays AMP is able to compete effectively with normal physiological concentrations of ATP to cause allosteric activation, and the allosteric effect also appears to make an important contribution to the overall effect on phosphorylation of a downstream target (ACC) in intact cells.

In summary, we would argue that AMP remains the primary regulator of AMPK in vivo (see Fig. 3 for a summary of the mechanisms), and that the name AMP-activated protein kinase remains entirely appropriate.

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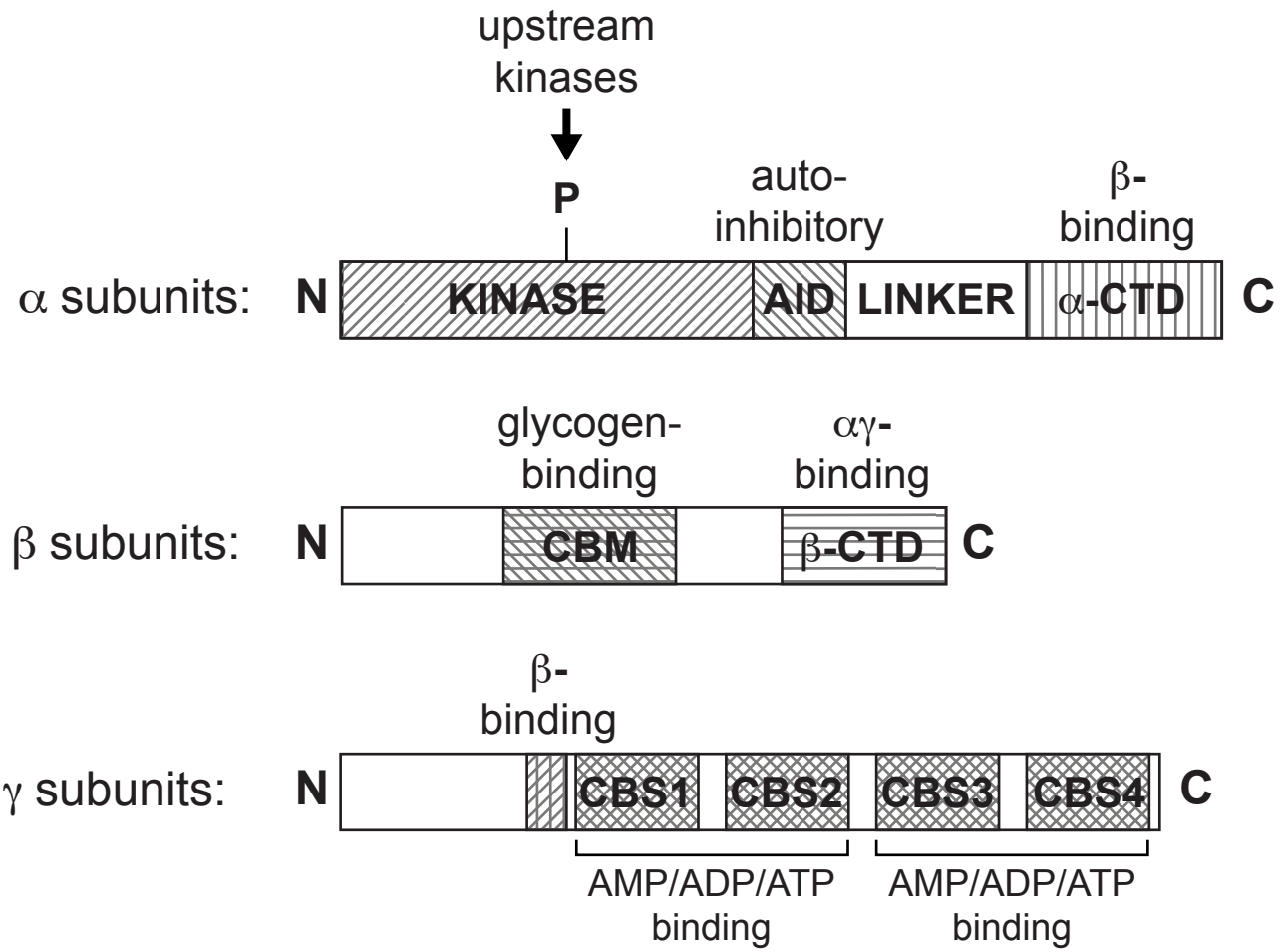
FIGURE LEGENDS:

Figure 1: Domain structure of the α, β and γ subunits of AMPK heterotrimers, drawn approximately to scale. KEY: AID, auto-inhibitory domain; α/β-CTD, α/β C-terminal domains; CBSx, CBS repeat, numbered in order from N-terminus.

Figure 2: Changes in ATP, ADP and AMP induced by treatment of G361 cells with berberine (100 μM, 1 hr). ATP and ADP were estimated by capillary electrophoresis, and AMP calculated by assuming that the adenylate kinase reaction was at equilibrium. Statistically significant differences from control without berberine ($P < 0.001$) are shown.

Figure 3: Triple mechanism by which AMP (and ADP) contribute to AMPK activation. (1) binding of AMP to AMPK promotes Thr172 phosphorylation by LKB1, but not CaMKKβ; (2) binding of AMP (and ADP at higher concentrations) inhibits Thr172 dephosphorylation; (3) binding of AMP causes allosteric activation. All three effects of AMP (and the single effect of ADP) are antagonized by binding of ATP.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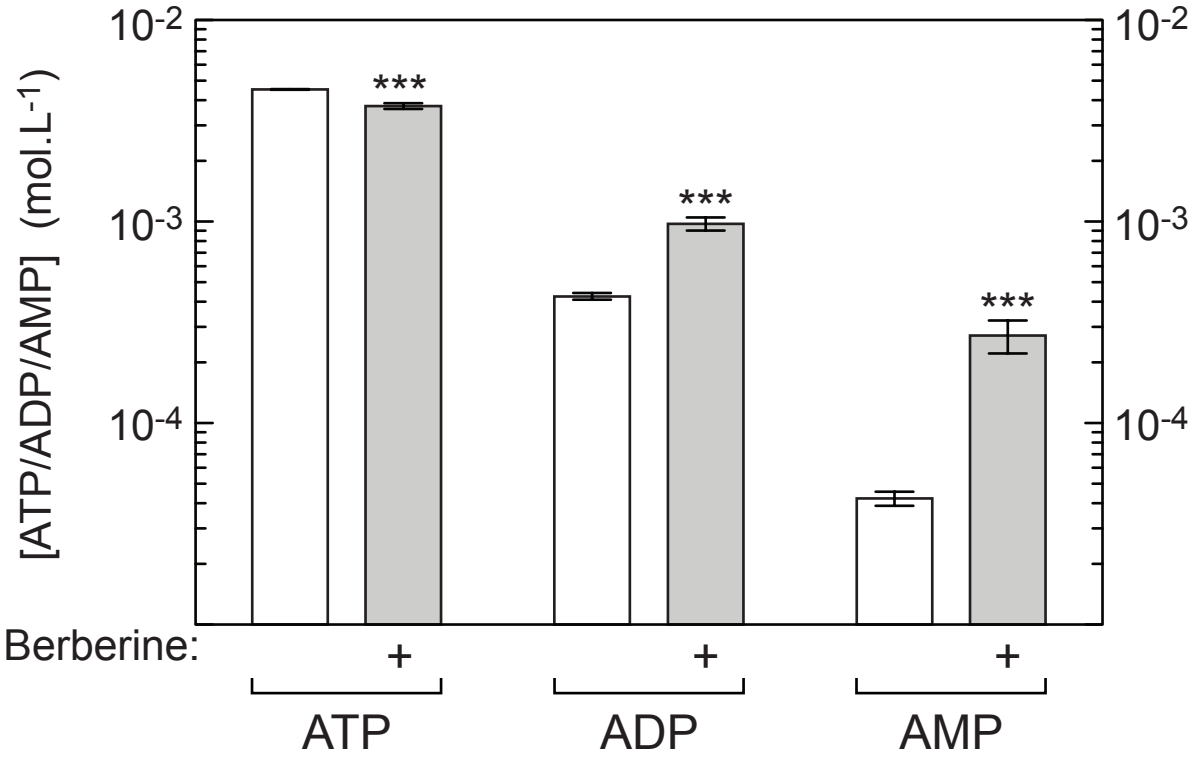
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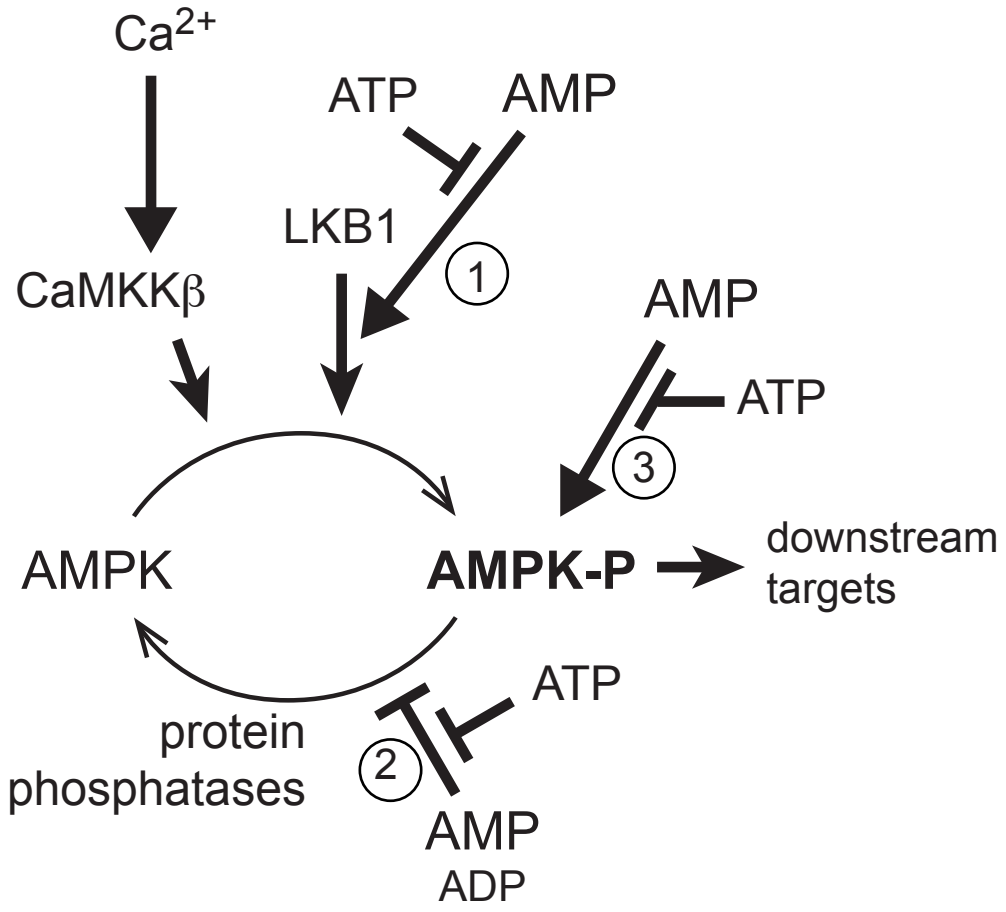
Berberine:
+
+
+
ATP
ADP
AMP


Ca2+
ATP
AMP
LKB1
1
CaMKKβ
AMP
ATP
3
AMPK
AMPK-P
downstream
targets
ATP
protein
phosphatases
2
AMP
ADP