AMP-activated protein kinase (AMPK) and Ca\(^{2+}\)/calmodulin (CaM)-dependent protein kinase I (CaMKI) are protein kinases that are regulated both by allosteric activation (AMP and Ca\(^{2+}\)/CaM, respectively) and by phosphorylation by upstream protein kinases (AMPK kinase (AMPKK) and CaMKI kinase (CaMKIK), respectively). We now report that AMPKK can activate CaMKI and, conversely, CaMKIK can activate AMPK. CaMKIK is 68-fold more effective at activating CaMKI than AMPK, while AMPKK is 17-fold more effective at activating AMPK than CaMKI. Our results suggest that CaMKIK and AMPKK are distinct enzymes dedicated to their respective kinase targets but with some overlap in their substrate specificities. The availability of alternative substrates for AMPKK and CaMKIK allowed the unequivocal demonstration that AMP and Ca\(^{2+}\)/calmodulin promote the activation of AMPK and CaMKI, respectively, via three independent mechanisms: 1) direct activation of AMPK and CaMKI, 2) activation of AMPKK and CaMKIK, and 3) by binding to AMPK and CaMKI, inducing exposure of their phosphorylation sites. Since AMP and Ca\(^{2+}\)/calmodulin each has a triple effect in its respective system, in vivo, the two systems would be expected to be exquisitely sensitive to changes in concentration of their respective activating ligands.

The AMP-activated protein kinase (AMPK),\(^1\) is believed to be involved in protecting cells against ATP depletion due to environmental stress by inactivating several key biosynthetic enzymes (1, 2). 5′-AMP allosterically activates AMPK (up to 5-fold) (3, 4), but also promotes the phosphorylation and activation (up to 50-fold) of AMPK by an upstream protein kinase, AMP-activated protein kinase kinase (AMPKK) (5, 6). Since the two effects multiply, this mechanism ensures a sensitive activation of the system when AMP rises. This happens when ATP is depleted due to displacement of the adenylate kinase reaction (7). We have proposed that the effect of AMP on AMPK phosphorylation is, at least in part, substrate-mediated, i.e., that binding of the nucleotide to AMPK makes it a better substrate for AMPKK. The evidence was that AMP analogues, which either mimic or antagonize the allosteric effect of AMP on AMPK activity, have the same effects on the phosphorylation of AMPK (6). However, as no alternative substrates for AMPKK were available, it was difficult to exclude the possibility that AMPKK was directly stimulated by AMP. Calmodulin-dependent protein kinase-I (CaMKI) is one of a family of Ca\(^{2+}\)/calmodulin-dependent protein kinases (CaM kinases), which also includes CaM kinases II and IV, elongation factor-2 kinase, myosin light chain kinases, and phosphorylase kinase (for review, see Refs. 8 and 9). Two isofoms of CaMKI, CaMKIa and CaMKIb, have been separately purified from rat brain and characterized (10, 11). They are similar to purified bovine brain CaMKI (12) and expressed rat and human CaMKI (13, 14) in size, subunit structure, and substrate specificity. CaMKIa was isolated from rat brain in a relatively inactive, apparently nonphosphorylated state, but its activity was enhanced >10-fold by a protein activator. By contrast, CaMKIb was isolated from rat brain in an active, phosphorylated state but could be inactivated by treatment with purified protein phosphatase-2A (10, 11). The a and b forms of CaMKI may therefore represent dephospho- and phospho-forms respectively, of the same gene product. The activator of CaMKIa has been purified and characterized from pig brain (15). It is a protein kinase that phosphorylates and activates (up to 50-fold) purified rat brain CaMKIa or expressed human CaMKI (14–16). We will refer here to purified rat brain CaMKIa as CaMKI, and CaMKIa activator as CaMKI kinase (CaMKIK). Intriguingly, the phosphorylation of CaMKI by CaMKIK requires Ca\(^{2+}\)/calmodulin (10, 14, 15). For reasons analogous to those described above for AMPK, it has been difficult to determine whether the effect of Ca\(^{2+}\)/calmodulin is on CaMKIa itself or on CaMKIK, especially since both bind to calmodulin-Sepharose in a Ca\(^{2+}\)-dependent manner (11, 15).

CaMKI and AMPK therefore display one striking similarity in that the activating ligand (Ca\(^{2+}\)/calmodulin or AMP respectively) has a dual effect, causing both direct activation of the downstream protein kinase, and promotion of its phosphorylation and activation by the upstream kinase. A further similarity lies in their substrate specificities, since both CaMKI and AMPK recognize the consensus \(\Phi X R X X(S/T)X X X\Phi\), where...
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SAMPLE TEXT

AMPK is a hydrophobic residue (17–19). Furthermore, although AMPK is not regulated by Ca²⁺/calmodulin, the sequence of its kinase domain (and that of its yeast homologue, Snf1p) is closely related to those of members of the calmodulin-dependent protein kinase subfamily (20, 21). These observations raised the possibility that AMPKK and CaMKIK could be similar or even identical, enzymes. We report here that CaMKIK and AMPKK are distinct, with each preferring its respective kinase target. However their abilities to phosphorylate and activate the heterologous kinases allowed us to dissect the mechanisms of action of the activating ligands, AMP and Ca²⁺/calmodulin, in these two protein kinase cascades.

EXPERIMENTAL PROCEDURES

Materials—ATP and calmodulin were from Boehringer Mannheim; [γ-32P]ATP and Hyperfilm MP were from Amersham International, Bucks, UK; okadaic acid was from Life Technologies, Paisley, UK; AMP and arginine-bound 5'-nucleotidase from Corvallis adamaninae were from Sigma, Dorset, UK. All other chemical reagents were of analytic grade from BDH, Poole, Dorset, UK. The catalytic subunit of protein phosphatase-2A (PP2A) was purified from bovine heart as described previously for rabbit muscle (22). AMPK was purified from rat liver as a by-product of the AMPK preparation. Briefly, protein precipitating between 6 and 10% polyethylene glycol was further purified by chromatography on DEAE-Sepharose, blue-Sepharose, and Q-Sepharose, and active fractions were concentrated using a Centrifcon-30 (Amicon). CaMKI (Ia form) was purified from rat brain (11), and CaMKII (α,β,γ; all from pig brain (15)). AMPK antisera was raised in rabbits against the peptide PGLKHPHERMPPLI (residues 361–374 of the catalytic subunit) (20).

Kinase Assays—AMPK and CaMKII were assayed using the peptide AMARAASAALARRR (19) by a procedure described previously (23). One unit of AMPK or CaMKII was that amount that catalyzed the incorporation of 1 nmol of phosphate into the peptide at 30 °C. AMPK was assayed by its ability to activate dephosphorylated AMPK. AMPK (2000 units/ml) in buffer A (50 mM Na-Hepes, pH 7.0, 1 mM dithiothreitol, 0.02% (w/v) Brij-35) was inactivated by ~80% by treatment at 30 °C with PP2A (10 μM/ml). PP2A was then inhibited by the addition of okadaic acid to a final concentration of 100 nM. Three volumes of dephosphorylated AMPK were added to 1 volume of buffer A containing ATP, MgCl₂, and AMP to give final concentrations in the AMPK assay of 200 μM, 5 mM, and 100 μM, respectively. Three volumes of this mixture were then added to 1 volume of AMPK in buffer A, incubated for 25 min at 30 °C, and 5-μl aliquots were assayed in 25 μl of AMPK assays as described above. One unit of AMPK is defined as that amount that increases the activity of the dephosphorylated AMPK by 1 unit/ml (calculated at the dilution of the original stock AMPK) in 1 min.

Activation of AMPK/CaMKI—AMPK (dephosphorylated as described above) or CaMKI (purified in the dephosphorylated state), were activated by incubation with either AMPK or CaMKI in the presence of MgATP (200 μM) and either 1 mM CaCl₂, 1 μM calmodulin or 100 μM AMP as indicated. Agarose-bound snake venom 5'-nucleotidase (62 milliunits/ml) was included in all incubations that did not contain AMP to remove endogenous AMP. Preparations of AMPK and AMPKK were also pretreated with 5'-nucleotidase-agarose (62 milliunits/ml) for 10 min at 30 °C, the nucleotidase-agarose being removed by centrifugation (14,000 × g for 2 min) prior to the experiment.

As were occurred the final step, AMPK was purified from rat liver using a Molecular Dynamics Phosphor imager.

RESULTS

Activation of CaMKI by AMPKK—Fig. 1 shows that AMPKK activated CaMKI in the presence of MgATP. In the absence of AMPKK, the activity of CaMKI was undetectable and did not increase on incubation with MgATP either in the presence (not shown) or absence (open circles) of AMP and/or Ca²⁺/calmodu-

2 S. A. Hawley, personal communication.

3 J. M. Corton, J. G. Gillespie, S. A. Hawley, and D. G. Hardie, submitted for publication.

4 W. Wilson, unpublished results.
Under these conditions, the rate of activation of AMPK was described under "Experimental Procedures," for 20 min with 0.033 μM MgATP either with (filled symbols) or without (open symbols) CaMKIK (0.07 μg/ml). Where indicated, AMP and/or Ca2+/calmodulin were also added (concentrations and symbols as for Fig. 1). AMP and/or Ca2+/calmodulin had no effect in the absence of CaMKIK (not shown).

Reactivation of AMPK by CaMKIK—CaMKIK (6 units/ml) was dephosphorylated using the catalytic subunit of PP2A as described under "Experimental Procedures." At the point shown by the arrow, okadaic acid was added to inhibit PP2A, with MgATP either with (filled symbols) or without (open symbols) CaMKIK (0.055 μg/ml). Where indicated, AMP and/or Ca2+/calmodulin were also added (concentrations and symbols as for Fig. 1). AMP and/or Ca2+/calmodulin had no effect in the absence of CaMKIK (not shown).

Reactivation of dephosphorylated AMPK by CaMKIK. AMPK (6 units/ml) was dephosphorylated using the catalytic subunit of PP2A as described under "Experimental Procedures." At the point shown by the arrow, okadaic acid was added to inhibit PP2A, with MgATP either with (filled symbols) or without (open symbols) CaMKIK (0.07 μg/ml). Where indicated, AMP and/or Ca2+/calmodulin were also added (concentrations and symbols as for Fig. 1). AMP and/or Ca2+/calmodulin had no effect in the absence of CaMKIK (not shown).

Activation of AMPK by AMPKK. AMPK (6 units/ml) was dephosphorylated using the catalytic subunit of PP2A as described under "Experimental Procedures." At the point shown by the arrow, okadaic acid was added to inhibit PP2A, together with MgATP either with (filled symbols) or without (open symbols) AMPKK (1600 units/ml). In some incubations, AMP and/or Ca2+/calmodulin were also added (concentrations and symbols as for Fig. 1). AMP and/or Ca2+/calmodulin had no effect in the absence of AMPKK (not shown).

Reactivation of AMPK by AMPKK. AMPK (6 units/ml) was dephosphorylated using the catalytic subunit of PP2A as described under "Experimental Procedures." At the point shown by the arrow, okadaic acid was added to inhibit PP2A, with MgATP either with (filled symbols) or without (open symbols) CaMKIK (0.055 μg/ml). Where indicated, AMP and/or Ca2+/calmodulin were also added (concentrations and symbols as for Fig. 1). AMP and/or Ca2+/calmodulin had no effect in the absence of CaMKIK (not shown).

Phosphorylation of CaMKI by AMPKK and CaMKIK—CaMKIK (0.33 μg/ml) was incubated with MgATP as described under "Experimental Procedures," in the presence (filled symbols) and absence (open symbols) of CaMKIK (0.055 μg/ml). In some incubations, AMP and/or Ca2+/calmodulin were also added (concentrations and symbols as for Fig. 1). The time-independent increase in activity in the presence of CaMKIK (compare open and filled circles) is due to activation occurring during the subsequent CaMKI assay, which contains Ca2+/calmodulin. AMP and/or Ca2+/calmodulin had no effect in the absence of CaMKIK (not shown).
phosphorylation, the incubation time and concentrations of AMPK or CaMKIK were chosen such that the greatest activation observed under any condition was 33% of maximal activation obtained with AMPKK. When AMPK was incubated alone with [γ-32P]ATP in the presence of Ca2+/calmodulin, there was no significant autophosphorylation of p63 (lane 8). There was a low level of autophosphorylation when AMP was also added (lane 7), but this was not accompanied by activation of the kinase, indicating that the phosphorylation occurred at nonactivating sites. When AMPK was incubated with AMP, Ca2+/calmodulin, and sufficient AMPKK to give 16% activation, the phosphorylation of p63 (lane 1) was significantly higher (1.4-fold) than the relevant autophosphorylation control (lane 7). Both activation and phosphorylation of p63 were higher in the absence of Ca2+/calmodulin (compare lane 2 with lane 1), consistent with the observation in Fig. 5 that Ca2+/calmodulin inhibits activation of AMPK by AMPKK. When AMPK was incubated with CaMKIK in the absence of activating ligand, little phosphorylation of p63 or activation of AMPK was observed (lane 3). When Ca2+/calmodulin was also added, both phosphorylation of p63 and activation (4% maximal) were slightly stimulated (lane 4). AMP on its own caused a slightly greater degree of activation of AMPK by CaMKIK (7% maximal), correlating with markedly higher phosphorylation of p63 (lane 5). The greatest effects of CaMKIK on activation of AMPK (33% maximal) and p63 phosphorylation (lane 6, 1.9-fold above autophosphorylation control, lane 7) were obtained in the presence of both AMP and Ca2+/calmodulin. From the results of Figs. 2 and 7, it may be concluded that maximal phosphorylation and activation of AMPK by CaMKIK requires both AMP and Ca2+/calmodulin.

Collectively, the results of Figs. 1–7 are consistent with a model in which AMP and Ca2+/calmodulin, promote the phosphorylation and activation of AMPK and CaMKIK by both substrate- and enzyme-directed effects. In the activation of CaMKI by AMPKK, Ca2+/calmodulin binds to the former (exposing a phosphorylation site), and AMP binds to the latter (directly enhancing its activity). In the activation of AMPK by CaMKIK, AMP binds to the former (exposing a phosphorylation site) and Ca2+/calmodulin binds to the latter (directly enhancing its activity). In the homologous reactions, a single allosteric activator (either Ca2+/calmodulin or AMP depending on the system) is sufficient for both substrate- and enzyme-directed effects.

AMPKK and CaMKIK Are Distinct Enzymes—The use of the heterologous reactions to probe the activation mechanisms is based on AMPKK and CaMKIK being distinct enzymes but with some cross-reactivity toward the kinase targets. We also considered two alternative explanations, the first that CaMKIK and AMPKK are identical, i.e. that a common protein kinase regulates both CaMKIK and AMPK, and the second that the preparations of CaMKIK and AMPKK might be cross-contaminated. The first alternative explanation is ruled out by the following observations. 1) CaMKIK and AMPKK are highly selective toward their respective kinase targets. This was quantified by determining the preference of each kinase for the two kinase substrates. We expressed the activity of the two preparations as the percentage of maximal activation (i.e. of that achieved with maximal amounts of the homologous kinase)/min/μl of preparation. The kinase kinase preparations were diluted such that a linear increase in activity was obtained with time. For CaMKIK, the activity was 24.4% of maximum/min/μl using CaMKI as substrate and 0.36% of maximum/min/μl with AMPK as substrate. For AMPKK, the activity was 13.4% of maximum/min/μl with AMPK as substrate and 0.78% of maximum/min/μl with CaMKIK as substrate. CaMKIK was therefore 68-fold more active toward CaMKI than AMPK, whereas AMPKK was 17-fold more active toward AMPK than CaMKIK. 2) CaMKIK (15) but not AMPKK (data not shown) bound to calmodulin-Sepharose in a Ca2+-dependent manner. 3) The allosteric activators of AMPKK and CaMKIK are different. This is most clearly seen when the two kinase kinases are compared using a single kinase target and an allosteric activator that does not bind to the kinase target itself. Activation of CaMKI by AMPKK (Fig. 1), but not by CaMKIK (Fig. 4), was stimulated by AMP. And, activation of AMPK by CaMKIK (Fig. 2), but not by AMPKK (Fig. 5), was stimulated by Ca2+/calmodulin. Thus, AMPKK is stimulated by AMP but not Ca2+/calmodulin, while CaMKIK is stimulated by Ca2+/calmodulin, but not AMP.

The second alternative explanation, that the preparations of AMPKK and CaMKIK were cross-contaminated, is ruled out for the following reasons. 1) CaMKIK was purified by Ca2+-dependent affinity chromatography on calmodulin-Sepharose (15), to which AMPKK does not bind. 2) During the purification of AMPKK, CaMKI-activating activity exactly concomitantly with AMPK-activating activity on the last two column steps, Q-Sepharose, and Mg2+ gradient elution from Mono-Q, making it very unlikely that the minor CaMKI-activating activity detected was due to residual unresolved CaMKIK but indicating rather that this activity was an intrinsic property of AMPKK.
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**Fig. 8. Triple effects of AMP and Ca\(^{2+}\)/calmodulin (Ca\(^{2+}\)/CaM) on the AMPK and CaMKI protein kinase cascades.** The heavy arrows indicate the activating ligands have three effects: 1) direct allosteric activation of the downstream protein kinase; 2) binding to the downstream protein kinase and making it a better substrate for the upstream protein kinase; 3) direct activation of the upstream protein kinase. Existing evidence suggests that activation of AMPK is reversed by protein phosphatase 2C (PP2C) (25, 26). PP means that the protein phosphatase responsible for dephosphorylation of CaMKI is not known, although CaMKI can be deactivated in vitro by the catalytic subunit of PP2A (10).

**DISCUSSION**

As shown by these results, AMPKK and CaMKIK are kinase kinases each with some capacity to phosphorylate and activate the respective heterologous kinase target. Given that the heterologous reactions are catalyzed at a small fraction of the rate of the homologous reactions, these cross-reactivities may not be physiologically significant, although this is difficult to exclude at present. The cross-reactivities are explainable by assuming that AMPKK and CaMKIK are representatives of a related family of kinase kinases, which recognizes similar phosphorylation site sequences. In the case of CaMKI, this site has been recently identified as Thr-177 located in the “activation loop” of the catalytic domain (14). Efforts are currently underway to identify the activating phosphorylation site in AMPK.

The studies described here have also identified for the first time an alternative substrate for AMPKK, and for CaMKIK, the first instance of an alternative substrate regulated by a distinct activating ligand, since although CaMKIK does phosphorylate and activate CaMKIV (27), the latter is also Ca\(^{2+}\)/calmodulin-regulated. The availability of alternative substrates regulated by distinct activating ligands has allowed us, by analysis of the heterologous systems, to demonstrate that in these two protein kinase cascades, the regulatory molecules (Ca\(^{2+}\)/calmodulin and AMP) both have three independent effects (schematically illustrated in Fig. 8).

The first role described for Ca\(^{2+}\)/calmodulin in the regulation of CaMKI activity was that of a direct activator (10–12). The mechanism of this activation involves relief of intrastereic autoinhibition via identified calmodulin-binding and autoinhibitory domains (14). In addition to this classic role, Ca\(^{2+}\)/calmodulin is also required for the phosphorylation and activation of CaMKI by CaMKIK (Refs. 10, 14, and 15; see also Fig. 4). We show here that this latter role itself occurs through two distinct effects. Since phosphorylation and activation of AMPK by CaMKIK was stimulated by Ca\(^{2+}\)/calmodulin (Figs. 2 and 7), CaMKIK is itself a Ca\(^{2+}\)/calmodulin-stimulated enzyme. And since phosphorylation and activation of CaMKI by AMPKK was stimulated by Ca\(^{2+}\)/calmodulin (Figs. 1 and 6), the latter is also exerting an effect at the level of the substrate (CaMKI), to expose the site of phosphorylation. These results are supported by experiments with expressed native and mutated forms of human CaMKI in that mimicking calmodulin-binding by C-terminal truncation of the calmodulin-binding/autoinhibitory domain of CaMKI promoted its phosphorylation by CaMKIK, and phosphorylation of the calmodulin-independent CaMKI mutant by CaMKIK was still accelerated by Ca\(^{2+}\)/calmodulin (14).

Similarly, AMP both directly activates AMPK and promotes its phosphorylation by AMPKK (Refs. 5 and 6; see also Fig. 5). We show here that AMP both exerts a role itself occurs through two distinct effects. Since phosphorylation and activation of CaMKI by AMPKK was stimulated by AMP (Figs. 1 and 6), AMPKK is itself an AMP-activated protein kinase. AMP also stimulates the phosphorylation of several polypeptides in the AMPKK preparation (data not shown), further supporting the idea that AMPKK is an AMP-activated protein kinase. And since phosphorylation and activation of AMPK by CaMKIK was also stimulated by AMP (Fig. 2, 7), the latter is also exerting an effect at the level of the substrate (AMPK), to expose the site of phosphorylation.

Since CaMKIK is Ca\(^{2+}\)/calmodulin-activated, we determined the concentration of calmodulin that gave half-maximal CaMKIK activation. The value obtained, 15 ± 4 nM, is within the range found for other calmodulin-activated enzymes (8, 9). Interestingly, CaMKIK appears to have a significant basal activity in the absence of Ca\(^{2+}\)/calmodulin (Figs. 2 and 3) and thus differs from most other calmodulin-activated enzymes, which are essentially completely dependent on calmodulin. We also consistently find that ~25% of CaMKI does not bind to calmodulin-Sepharose (data not shown; Ref. 15). One explanation is that CaMKIK exists in an equilibrium between active and inactive conformations, and calmodulin binds with high affinity only to the latter, converting it to the active conformation. Our results also demonstrate that AMPKK is itself an AMP-activated protein kinase. However, because it was necessary to add 5’-nucleotidase to the minus AMP controls to observe the effects of AMP, it was not possible to determine the concentration dependence of AMPKK on AMP.

These findings that in the AMPK and CaMKI systems both the upstream and downstream protein kinases are regulated by the same activating ligand is not without precedent, since cyclin-dependent kinase-activating kinase, the protein kinase that phosphorylates the activating threonine of cyclin-dependent protein kinases, is itself a cyclin-dependent protein kinase (28–30). As in our systems, binding of the activating ligand (cyclin) to the downstream protein kinase stimulates both the activity of the downstream kinase and its phosphorylation by the upstream protein kinase (31). The cyclin-dependent kinase-activating kinase/cyclin-dependent protein kinase system differs in that the cyclin proteins that activate the upstream and downstream protein kinases are distinct (32–34). We are not aware of a precedent where the identical activating ligand (AMP or Ca\(^{2+}\)/calmodulin) has three effects on a protein kinase cascade. We propose that the triple actions of AMP and Ca\(^{2+}\)/calmodulin on their respective systems would make these systems respond in an exquisitely sensitive manner to small changes in the concentrations of the activating ligand. Although the allosteric regulators differ between the two systems, our study demonstrates a remarkable analogy between CaMKI and AMPK. We have already shown another striking similarity, in that the substrate sequence motifs recognized by these two protein kinases are very similar (17–19). These similarities in specificity, and in recognition by the upstream protein kinases presumably reflect the fact that the kinase domains of rat CaMKI and rat AMPK (13, 20) are 40% identical in sequence and phylogenetic analysis of kinase domain sequences places both within the same protein kinase subfamily.
(21). On the other hand, the two kinases are unrelated outside of the kinase domain, which is presumably where the binding sites for the distinct activating ligands are located.

Okuno et al. (35) and Tokumitsu et al. (36, 37) have recently reported that calmodulin-dependent protein kinase IV (CaMKIV) is also regulated in a Ca2+/calmodulin-dependent manner by an activator protein kinase (CaMKII kinase). In this case, it is not clear whether the effect of Ca2+/calmodulin to promote CaMKIV phosphorylation is due to binding to CaMKIV kinase, to CaMKIV, or both, although it is intriguing that CaMKII kinase, like CaMKIK, binds to calmodulin-Sepharse (36, 37). Moreover, CaMKIK also activates CaMKIV and does so by phosphorylating the equivalent threonine residue (Thr-196) to that phosphorylated in CaMKI (Thr-177) (14, 27). The precise relationship between CaMKII kinase and CaMKIK remains to be established.

It is also interesting to compare AMPK and CaMKI with calmodulin-dependent protein kinase II (CaMKII), which is regulated by autophosphorylation on Thr-172. Autophosphorylation of the latter is an intersubunit reaction occurring between neighboring subunits of the multimeric enzyme. Ca2+/calmodulin appears to play a role in CaMKII phosphorylation analogous to its role in CaMKI (and AMPK) phosphorylation in that it is required not only to initially activate the CaMKII subunit, which is acting as the kinase, but also must be bound to the neighboring subunit in order for it to act as a substrate (38). An important difference between CaMKII and CaMKI is that phosphorylation occurs at nonequivalent sites (14). Another major difference is that autophosphorylation of CaMKII on Thr-172 converts it from an active and Ca2+/calmodulin-dependent form to a Ca2+/calmodulin-independent form, whereas phosphorylation of CaMKI by CaMKII converts it from an inactive form to an active and Ca2+/calmodulin-dependent form (10, 15). CaMKIV incorporates both forms of regulation, since its phosphorylation by CaMKII or CaMKIV kinase activates and imparts partial Ca2+/calmodulin-independence (27, 35, 36).

In conclusion, studies described here indicate that the apparent relatedness of the calmodulin-dependent protein kinase kinases described to date extend beyond the immediate calmodulin-dependent protein kinase subfamily to that regulated by a distinct allosteric activator (AMP). This relationship is seen both in the ability of AMPKK and CaMKIK to cross-regulate the heterologous downstream kinases, as well as in the remarkable similarity of the roles of the allosteric activators (AMP and Ca2+/calmodulin) in the activation process. Despite our findings that the upstream protein kinases have a limited ability to activate the alternative downstream protein kinases in vitro, we suspect that they are likely to be dedicated to their respective downstream protein kinases in vivo, and would thus constitute elements in separate signal transduction cascades akin for example, to the MAP kinase kinase/MAP kinase signaling module. For both the AMP-regulated and Ca2+/calmodulin-regulated pathways, an important question for future consideration is the quantitative significance of the direct activation of the upstream protein kinases by the respective allosteric activators, relative to the dual effects of the activators on the downstream protein kinases. Studies of analogues that differentially activate or inhibit the upstream and downstream protein kinases or of mutant kinase kinases or kinases that do not respond to the activators will be necessary to address these questions.

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