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A MULTIFUNCTIONAL CYCLIC NUCLEOTIDE- AND Ca^{2+}-INDEPENDENT PROTEIN KINASE FROM RABBIT SKELETAL MUSCLE

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SUMMARY: A cyclic nucleotide- and Ca^{2+}-independent protein kinase, initially identified as a glycogen synthase kinase (Itarte, E. and Huang, K.-P. (1979) J. Biol. Chem. 254, 4052-4057), was also found to phosphorylate phosphorylase kinase and troponin from skeletal muscle as well as myosin light chain and myosin light chain kinase from both smooth and skeletal muscles. With the exception of myosin light chain from skeletal muscle, all the above-mentioned proteins are also substrates for the multifunctional cAMP-dependent protein kinase. The results suggest that this cyclic nucleotide- and Ca^{2+}-independent protein kinase, like cAMP-dependent protein kinase, may have multiple cellular functions.

Protein kinases which are activated by cyclic nucleotides (1), Ca^{2+} and calmodulin (2), and Ca^{2+} and phospholipid (3) have been isolated and characterized. Various studies have established that these kinases play important roles in regulating different cellular functions. By contrast, the physiological significance of the cyclic nucleotide- and Ca^{2+}-independent protein kinases remains obscure. This class of kinase, some of which were initially isolated as casein kinases, have since been shown to phosphorylate glycogen synthase (4-10), initiation factors (11), non-histone chromatin proteins (12), and troponin T (13). However, it is not clear whether a kinase isolated from a particular tissue can phosphorylate multiple substrates.

In the present study we find that a rabbit skeletal muscle cyclic nucleotide- and Ca^{2+}-independent protein kinase, initially identified as a glycogen synthase kinase (CK-1), also phosphorylates many other proteins. Phosphorylase kinase and troponin from skeletal muscle as well as myosin light chain and myosin light chain kinase from both smooth and skeletal muscles are all substrates. Since all these proteins, except myosin light chain from

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skeletal muscle, are also phosphorylated by the well-characterized cAMP-dependent protein kinase (A-kinase), we propose that CK-1 is a multifunctional protein kinase which may regulate many diverse functions as does the A-kinase.

MATERIALS AND METHODS: Nonactivated phosphorylase kinase (14), A-kinase (15), CK-1 (7), myosin light chain (16) and light chain kinase (17), and glycogen synthase (18) were prepared from rabbit skeletal muscle as previously described. Myosin light chain and light chain kinase (19) from smooth muscle were generous gifts from Drs. M. Pato and R.S. Adelstein, NIH. Troponin from rabbit skeletal muscle (20) was kindly supplied by Drs. J.M. Chalovich and E. Eisenberg, NIH. Myosin light chain preparations from smooth and skeletal muscles were mixtures of the phosphorylatable and nonphosphorylatable light chains. The phosphorylatable 20,000-dalton (smooth muscle) and 18,500-dalton (skeletal muscle) light chains comprised 76% and 43%, respectively, of the mixtures. The various protein substrates were phosphorylated in a reaction mixture (0.05 ml) that contained protein substrates, 0.2 mg/ml; dithiothreitol, 2.5 mM; EGTA, 0.5 mM; KF, 5 mM; magnesium acetate, 2 mM; [γ-^{32}P]ATP, 0.2 mM; Tris-HCl buffer (pH 7.4), 25 mM; and either 4 milliunits of A-kinase or 39 milliunits of CK-1. The reaction mixtures also contained 5 μM cAMP when A-kinase was used. The reactions were initiated by the addition of [γ-^{32}P]ATP. Aliquots of the reaction mixture were taken at timed intervals for the measurement of ^{32}P incorporation according to the published method (21). One unit of A-kinase and CK-1 is defined as the amount of enzyme catalyzing the incorporation of 1 nmol of phosphate from [γ-^{32}P]ATP into 4 mg/ml of histone IIA and casein, respectively, per min at 30°. The activity of myosin light chain kinase from either smooth or skeletal muscle was assayed using the respective light chain preparations as outlined previously (22). Electrophoesis of ^{32}P-labeled samples was carried out in 7.5% to 20% gradient slab gels containing 0.1% sodium dodecyl sulfate as described previously (23). The destained gels were exposed to film for autoradiography. Protein concentrations were determined by the method of Bradford (24).

RESULTS AND DISCUSSION

We found that several proteins that are known to be phosphorylated by A-kinase are also substrates for CK-1 when the reactions are carried out under the same conditions (Fig. 1). As expected phosphorylase kinase (14), glycogen synthase (15), and troponin (25) from skeletal muscle as well as the 20,000-dalton myosin light chain (26) and myosin light chain kinase (22, 27) from smooth muscle are readily phosphorylated by A-kinase. In addition to these proteins the 18,500-dalton myosin light chain from skeletal muscle is also a substrate of CK-1 (lane 8), but not of A-kinase (lane 16). A 22,000-dalton protein present as a trace contaminant in the skeletal muscle myosin light chain preparation is also phosphorylated by CK-1. It should be noted that in the absence of CK-1 or A-kinase both phosphorylase kinase (lane 1) and myosin light chain kinase (lane 9) undergo slight autophosphorylation. Endogenous

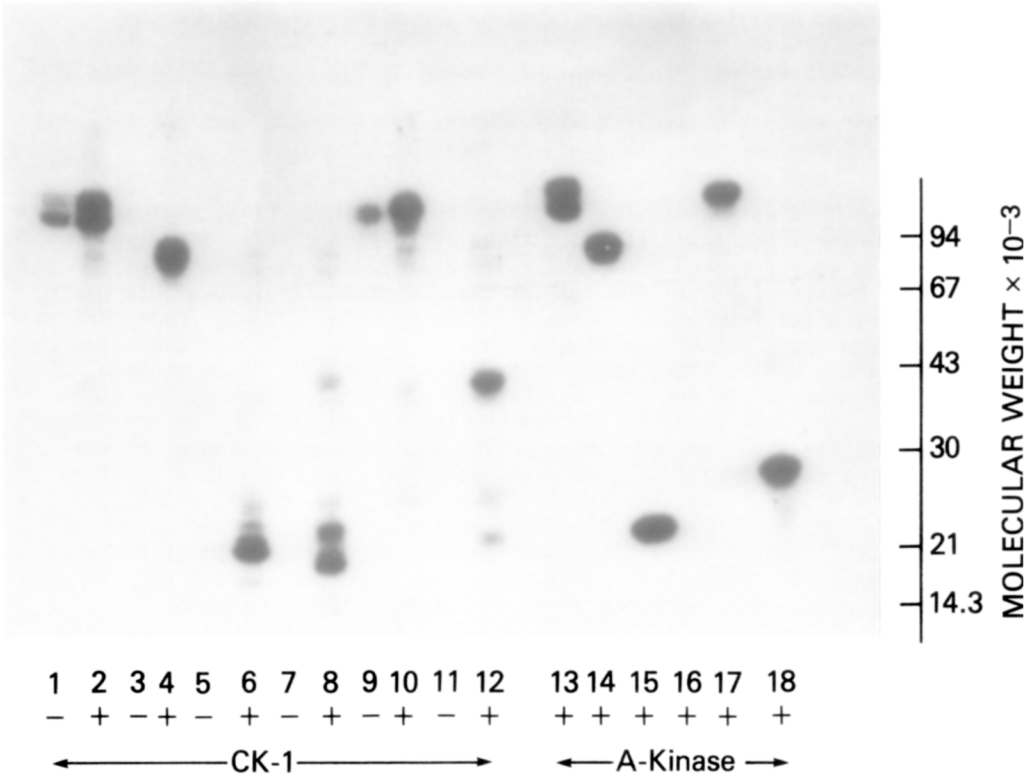

Fig. 1. Polyacrylamide gel electrophoresis of various proteins phosphorylated by CK-1 or A-kinase. These proteins were phosphorylated by CK-1 (lanes 1 to 12) or A-kinase (lanes 13 to 18) for 40 min. Aliquots of the phosphorylated proteins were analyzed by SDS-gel electrophoresis and autoradiography. The proteins are phosphorylase kinase (lanes 1, 2, 13); glycogen synthase (lanes 3, 4, 14); smooth muscle myosin light chain (lanes 5, 6, 15); skeletal muscle myosin light chain (lanes 7, 8, 16); myosin light chain kinase (lanes 9, 10, 17); and troponin (lanes 11, 12, 18). Lanes 1, 3, 5, 7, 9, and 11 represent phosphorylation of the substrates in the absence of added kinase.

phosphorylation of glycogen synthase (lane 3), smooth muscle (lane 5) and skeletal muscle (lane 7) myosin light chains, and troponin (lane 11) are negligible. Like the A-kinase, CK-1 phosphorylated both α and β subunits of phosphorylase kinase (lanes 2 and 13). However, CK-1 and A-kinase phosphorylate different subunits of the troponin complex (lanes 12 and 18). Previous studies (25) have shown that only troponin I of the complex is phosphorylated by A-kinase, as shown here (lane 18). With CK-1 as catalyst troponin T, but not troponin I, is phosphorylated (lane 12).

The time course of phosphorylation of the various proteins by A-kinase (Fig. 2) and CK-1 (Fig. 3) are shown. After 40 min of incubation phosphate incorporations into phosphorylase kinase catalyzed by A-kinase and CK-1 are

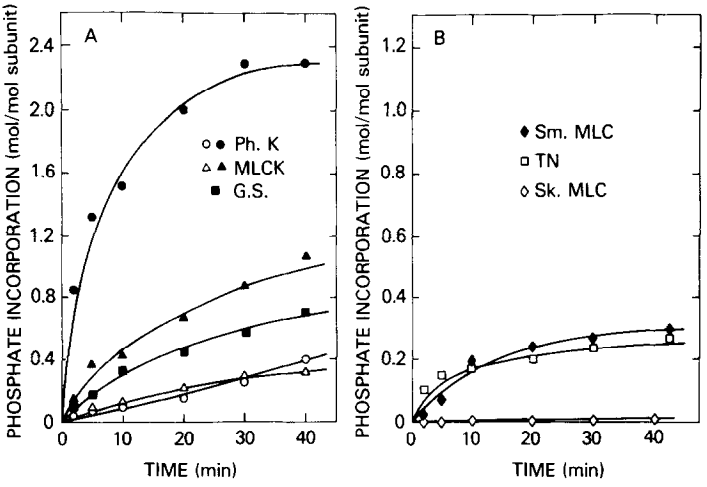

Fig. 2. Phosphorylation of the various muscle proteins by A-kinase. The reactions were carried out under standard assay conditions. In panel A, phosphorylase kinase (-●-), smooth muscle myosin light chain kinase (-▲-), and glycogen synthase (-■-) were phosphorylated by A-kinase. The autophosphorylation of phosphorylase kinase (-○-) and myosin light chain kinase (-△-) were carried out in the absence of A-kinase and cAMP. In panel B, smooth muscle myosin light chain (-♦-), troponin (-□-), and skeletal muscle myosin light chain (-◇-) were phosphorylated by A-kinase. The activities with smooth muscle myosin light chain and troponin are expressed as the phosphate incorporation into 20,000-dalton and troponin-I components, respectively.

2.3 and 3.4 mol per monomer, respectively. A-kinase and CK-1 catalyze the incorporations of 1.3 and 1.7 mol into the α subunit and 1.0 and 1.7 mol into the β subunit, respectively. Phosphorylation of phosphorylase kinase by A-

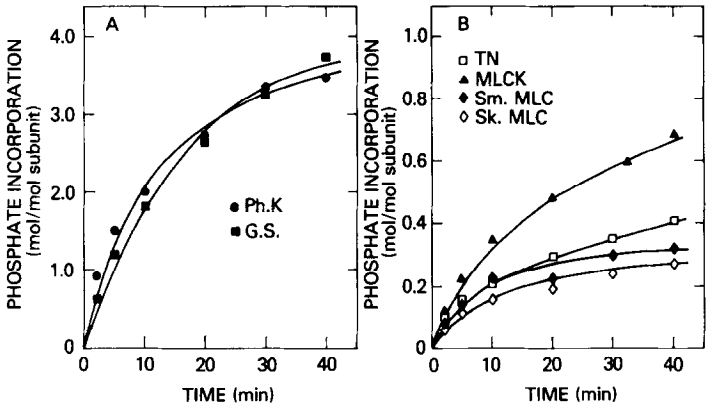

Fig. 3. Phosphorylation of the various muscle proteins by CK-1. In panel A, phosphorylase kinase (-●-) and glycogen synthase (-■-) were phosphorylated by CK-1. In panel B, troponin (-□-), myosin light chain kinase (-▲-), smooth muscle myosin light chain (-♦-), and skeletal muscle myosin light chain (-◇-) were phosphoryalted by CK-1. The activities with troponin, smooth muscle myosin light chain, and skeletal muscle myosin light chain are expressed as phosphate incorporation into troponin T, 20,000-dalton light chain, and 18,500-dalton light chain, respectively.

kinase and CK-1 result in 25- and 17-fold activation, respectively (not shown). The extents of phosphorylation of the α and β subunits by A-kinase are similar to that observed previously (14). However, these subunits are phosphorylated to higher levels by CK-1 than by A-kinase. In separate experiments we observed that greater than 3 mol phosphate is incorporated into each of the α and β subunits after prolonged incubation. Earlier studies (28, 29) have established that the α subunit can be phosphorylated to greater than 1 mol phosphate per subunit. It is the first time, however, that the β subunit has been shown to incorporate more than one phosphate.

Glycogen synthase is phosphorylated to different extents by A-kinase and CK-1 (Figs. 2 and 3). Under the assay conditions, 0.7 and 3.7 mol phosphate are incorporated into the synthase after 40 min of incubation with A-kinase and CK-1, respectively. Previous studies from this laboratory have shown that up to 1.5 mol and greater than 4 mol phosphate, respectively, were incorporated into the synthase after phosphorylation by A-kinase and CK-1.

Myosin light chain kinase from smooth muscle was shown to be a substrate for A-kinase (22, 27). In this study we found that it is also a substrate for CK-1. After 40 min of incubation phosphate incorporations catalyzed by A-kinase and CK-1 are 1.1 and 0.7 mol, respectively. An incorporation of 0.3 mol phosphate was contributed by autophosphorylation of myosin light chain kinase (Fig. 2). After prolonged incubation CK-1 and A-kinase catalyze the incorporation of 1.3 and 2 mol phosphate, respectively. The binding of calmodulin by the light chain kinase was shown to mask one of the two sites phosphorylated by A-kinase (27). In similar experiments we found a 30-40% inhibition of phosphorylation by A-kinase when 1 μM calmodulin was added to the phosphorylation mixture (the light chain kinase was present at 1 μM). No inhibition by calmodulin was observed for the CK-1-catalyzed reaction. Phosphorylation of the light chain kinase by A-kinase was shown to cause a 20-fold increase in the K_a for calmodulin (27). Our preliminary results indicate no significant change in the K_a for calmodulin after phosphorylation of the light chain kinase by CK-1.

Phosphorylation of troponin and the myosin light chains occur at approximately the same rate and to the same extent when either A-kinase or CK-1 is the catalyst (Figs. 2 and 3). Both troponin I and smooth muscle light chain incorporate approximately 0.3 mol phosphate when A-kinase is used (Fig. 2). The skeletal muscle light chain is not a substrate for A-kinase as was demonstrated in Fig. 1. Less than stoichiometric phosphorylation of both smooth muscle light chain (26) and skeletal muscle troponin (25) have been observed before. It is possible that these substrates are already partially phosphorylated. We did not dephosphorylate these proteins before using them as substrates. Native myosin from smooth muscle, unlike the isolated light chains, is not a substrate for CK-1 (not shown) or A-kinase (30).

In addition to the substrates described in Fig. 1, we found that CK-1 also phosphorylates myosin light chain kinase from skeletal muscle (Fig. 4A). Compared to its smooth muscle counterpart, the skeletal muscle enzyme seems to be a better substrate for CK-1 than A-kinase (Fig. 4B). After 40 min incubation, CK-1 and A-kinase catalyze the incorporation of 1.3 and 0.2 mol phosphate per subunit, respectively, into the skeletal muscle light chain kinase. The incorporations into the smooth muscle enzyme under similar conditions by CK-1 and A-kinase were 0.7 and 1.1 mol, respectively (Figs. 2 and 3). No change in the myosin light chain kinase activity was detected after phosphorylation by CK-1 (Fig. 4B). A similar observation has recently been reported for the phosphorylation of this light chain kinase by A-kinase (31).

The results presented in this report demonstrate that CK-1, a cyclic nucleotide- and Ca^{2+}-independent protein kinase, indeed phosphorylates many other muscle proteins in addition to glycogen synthase. The differences between CK-1 and A-kinase have been documented with respect to their phosphorylation of glycogen synthase (8, 32, 33) as well as their specificities toward troponin and skeletal muscle myosin light chain presented here. The differences in the sites of phosphorylation between the various proteins commonly phosphorylated by both kinases need further investigation.

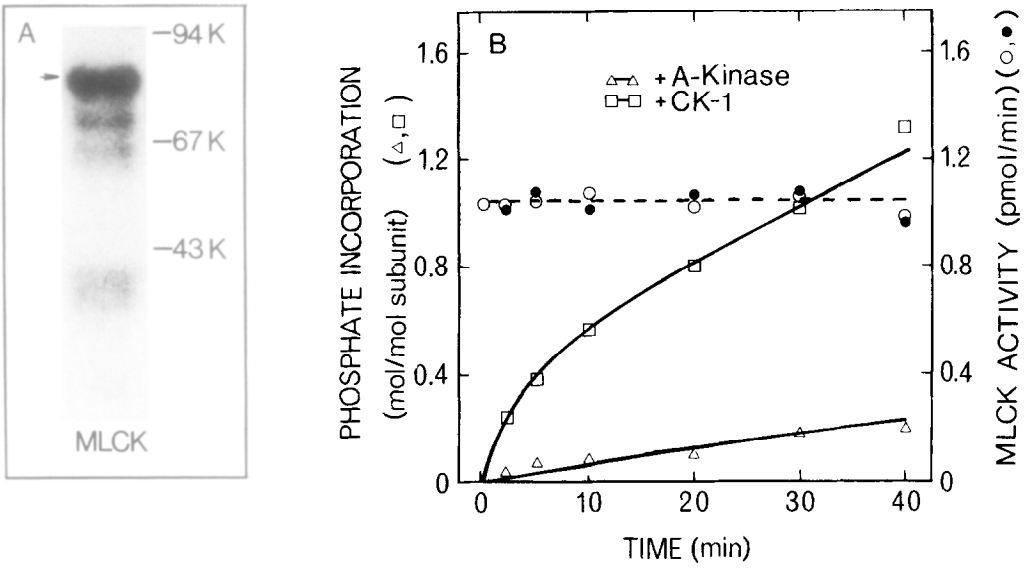

Fig. 4. Phosphorylation of skeletal muscle myosin light chain kinase by CK-1 and A-kinase. A, Myosin light chain kinase (0.10 mg/ml) from rabbit skeletal muscle was phosphorylated by CK-1 for 40 min and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. No phosphorylation was observed in the absence of CK-1. B, Time course of phosphorylation of the light chain kinase by CK-1 (-□-) and A-kinase (-△-). At the different times aliquots were removed from the CK-1-catalyzed rection and assayed for myosin light chain kinase activity (-●-). A sample of the enzyme incubated in the absence of ATP and CK-1 (or A-kinase) was also assayed for comparison (-o-).

CK-1 appears to have a broad substrate specificity; however, muscle phosphorylase b and phosphofructokinase are not phosphorylated by CK-1. Although we have not made an exhaustive survey of possible substrate for CK-1, the results presented here do imply the potential importance of this enzyme in the regulatory processes.

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