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Calcineurin: A Member of a Family of Calmodulin-Stimulated Protein Phosphatases (41905)

ALLAN S. MANALAN, MARIE H. KRINKS, AND CLAUDE B. KLEE

Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

Abstract. Calcineurin, a major calmodulin-binding protein of brain, is a heterodimer composed of a 61,000 M_r calmodulin-binding subunit, calcineurin A, and a 19,000 M_r Ca^{2+}-binding subunit, calcineurin B. The discovery of a calmodulin-regulated protein phosphatase in rabbit skeletal muscle with a similar subunit structure led to the identification of calcineurin as a protein phosphatase (AA Stewart, TS Ingebritsen, A Manalan, CB Klee, P Cohen (1982) FEBS Lett **137**:80–84). Using rabbit polyclonal antibodies to bovine brain calcineurin, both subunits of calcineurin can be identified in crude homogenates of bovine brain by an immunoblotting technique. In crude homogenates of bovine skeletal and cardiac muscle, a 59,000–61,000 M_r doublet and a 15,000 M_r species (the electrophoretic mobility of calcineurin B) are also detected by this technique. The cross-reactivity of these species with antibodies to brain calcineurin indicates antigenic similarity between the muscle proteins and calcineurin, and suggests the existence of a family of structurally related calmodulin-stimulated protein phosphatases. Like calcineurin, the 61,000 M_r subunits in skeletal and cardiac muscle bind calmodulin and are detected in crude tissue extracts by ^{125}I-calmodulin gel overlay. Thus, both the ^{125}I-calmodulin gel overlay method and the immunoblotting technique are useful in screening crude preparations, in which detection of calmodulin-stimulated protein phosphatase activity may be complicated by the many phosphatases present.

Calcineurin, a major calmodulin-binding protein of brain (1), was initially identified as a heat labile inhibitor of calmodulin-stimulated cyclic nucleotide phosphodiesterase (2, 3). Because of its Ca^{2+}-binding properties (1) and its predominantly neural localization (4), the protein was named calcineurin (1). The discovery of a Ca^{2+}-stimulated protein phosphatase with a similar subunit structure (5, 6) led to the identification of calcineurin as a protein phosphatase (6).

Calmodulin-stimulated protein phosphatase activity copurifies with calcineurin (7). Phosphatase activity cosediments with calcineurin in glycerol gradients in the presence of Ca^{2+} and calmodulin ($s_{20,w}$ = 5.0 S), in the presence of EGTA ($s_{20,w}$ = 4.5 S), and after limited tryptic digestion ($s_{20,w}$ = 4.3 S) (8). Recently, calcineurin and its associated protein phosphatase activity have been shown to bind to a substrate affinity column (thiophosphorylated myosin P-light chain Sepharose) in the presence of Ca^{2+}, with specific elution by EGTA (9).

Calmodulin-regulated phosphatases have now been detected in several tissues (10), and have been purified to homogeneity from skeletal muscle (5) and brain (2, 11, 12). A similar protein has also been isolated from bovine heart, where it is present in relatively low amounts (13, 14). This group of phosphatases, the protein phosphatases 2B, has been defined on the basis of characteristic substrate specificity, stimulation by Ca^{2+} and calmodulin, and inhibition by phenothiazines (5, 15).

The many sources of protein phosphatase activity found in cells complicate the direct identification of calmodulin-stimulated protein phosphatases in tissue homogenates. However, by correlating results of ^{125}I-calmodulin gel overlay studies with results of an immunoblotting technique using polyclonal antibodies to bovine brain calcineurin, members of the family of calmodulin-stimulated protein phosphatases can be identified in crude extracts of bovine skeletal and cardiac muscle.

Methods. Calcineurin was purified from bovine brain as previously described (7). Calmodulin was purified from bovine testes by a modification of previously published procedures (16, 17). Calmodulin was ^{125}I iodinated as described (7). Preparation and characterization of rabbit polyclonal antibodies to bovine brain calcineurin was performed

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as described (18). SDS–slab gel electrophoresis was performed in linear gradients of acrylamide by the method of Laemmli (19). ^{125}I-Calmodulin gel overlay was performed as described (7). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose paper and detection of immunoreactive species were performed by modification (18) of previously published methods (20, 21).

Tissue homogenates were prepared from bovine brain, cardiac muscle, skeletal muscle, and gastric smooth muscle as described (18). Soluble extracts of muscle were prepared in media containing either 0.5 *M* NaCl (high salt) or 10 m*M* CHAPS (3-[3-cholamidopropyldimethylammonio] - 1 - propanesulfonate) for comparison by ^{125}I-calmodulin gel overlay.

Results. Calcineurin is a heterodimer, composed of a 61,000 M_r calmodulin-binding subunit, calcineurin A, and a 19,000 M_r Ca^{2+}-binding subunit, calcineurin B (1). The use of an immunoblotting technique for identification of calcineurin in a crude homogenate of bovine brain is shown in Fig. 1. The polyclonal anti-calcineurin antibodies specifically recognize only calcineurin A and calcineurin B. In homogenates of bovine skeletal or cardiac muscle, a doublet with electrophoretic mobility similar to that of calcineurin A is labeled. In addition, a low-molecular-weight species is labeled in homogenates of cardiac and skeletal muscle (Fig. 1). When these homogenates are compared by SDS–gel electrophoresis, the mobility of the low-molecular-weight immunoreactive species in skeletal and cardiac muscle is identical to that of calcineurin B. The detection of these species in muscle indicates some degree of antigenic similarity between these proteins and calcineurin. Cross-reactivity of both members of the 59,000–61,000 M_r doublet in skeletal and cardiac muscle suggests that both are antigenically related to calcineurin. Based on available evidence, it seems likely that the doublet observed in muscle arises as a result of limited proteolysis. All other proteins visualized by immunoblot using this technique are also observed in the absence of anti-calcineurin antibody, and represent direct binding of the biotin—avidin peroxidase reagent used to develop the images.

The amount of protein cross-reacting with anti-calcineurin antibodies is higher in brain than in skeletal or cardiac muscle. No cross-reacting protein is detected in homogenates of bovine gastric smooth muscle (data not shown). Consistent with the reported experience in purification of these proteins (2, 5, 7, 11–14), these results suggest that the amount of calcineurin is higher in brain than in muscle. Alternatively, the cross-reacting species in skeletal and cardiac muscle may exhibit some structural differences, and therefore the antibodies may have a lower affinity for these proteins.

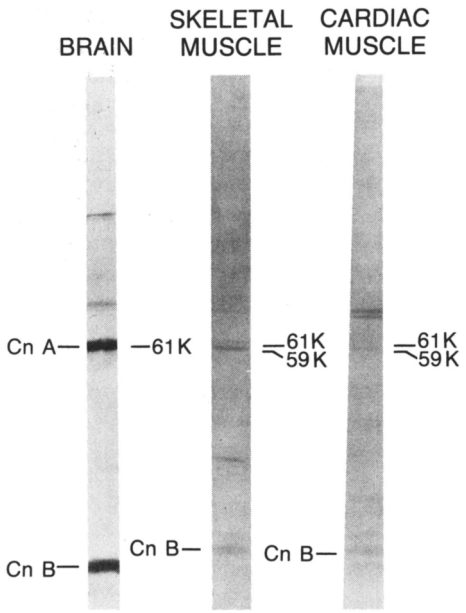

FIG. 1. Identification of immunoreactive proteins in tissue homogenates by immunoblot using polyclonal rabbit antibody to bovine brain calcineurin. In each lane, homogenate representing 0.15 mg tissue wet wt was subjected to SDS–gel electrophoresis in a 5–10% linear gradient of acrylamide for subsequent electrophoretic transfer to nitrocellulose paper. Anti-calcineurin antibodies specifically labeled calcineurin A, calcineurin B, and homologous cross-reacting species in skeletal and cardiac muscle. All other labeled proteins were observed in the absence of anti-calcineurin antibodies (see text).

Calcineurin A can interact with calmodulin even after SDS–gel electrophoresis and can thus be identified in crude homogenates of bovine brain using an ^{125}I-calmodulin gel overlay technique (7). With this method, a 61,000 M_r protein is labeled by ^{125}I-calmodulin in crude extracts of bovine cardiac and

skeletal muscle (Fig. 2). More of this 61,000 M_r protein is identified by ^{125}I-calmodulin gel overlay in skeletal than in cardiac muscle, in agreement with the results obtained with the immunoblotting technique. Again, no analogous protein is detected in crude extracts of bovine gastric smooth muscle (data not shown). The effectiveness of either 0.5 *M* NaCl (high salt) or the zwitterionic detergent CHAPS (10 m*M*) in extracting the calcineurin-like proteins from muscle is also compared (Fig. 2). In skeletal muscle, more of the 61,000 M_r protein is labeled after extraction in 0.5 *M* NaCl, a condition which also promotes the extraction of myosin. The myosin, which is greatly overloaded on the gel, is faintly labeled by ^{125}I-calmodulin (Fig. 2). Although the pattern of extraction suggests that these calcineurin-like proteins are cytosolic, immunohistochemical studies are required to define their subcellular distribution in muscle.

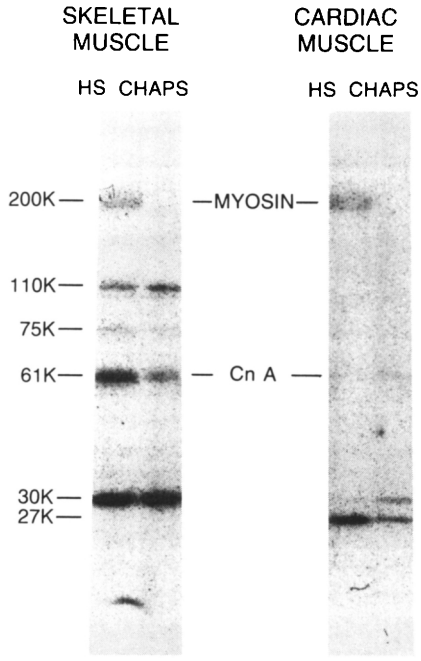

FIG. 2. Analysis of crude extracts of bovine skeletal and cardiac muscle by the ^{125}I-calmodulin gel overlay method. Soluble tissue extracts were prepared in the presence of 0.5 *M* NaCl (high salt, HS) or 10 m*M* CHAPS (CHAPS). Tissue extracts corresponding to 1.5 mg tissue wet wt were subjected to SDS–gel electrophoresis in a 5–15% linear gradient of acrylamide for identification of calmodulin-binding proteins by ^{125}I-calmodulin gel overlay.

Discussion. Although the physiologic role of calcineurin is unknown, the protein is present in high concentration in brain (2, 4, 11, 12), suggesting a potentially significant regulatory function in neural processes. The calcineurin-like proteins which have been identified in skeletal and cardiac muscle demonstrate immunologic cross-reactivity with polyclonal antibodies to the brain enzyme. These results support the existence of a family of structurally related protein phosphatases, which have been classified as protein phosphatases 2-B by functional criteria (5, 15). The purified enzyme dephosphorylates the α subunit of phosphorylase kinase, protein phosphatase inhibitor 1, smooth muscle myosin light chains (5, 15), and R_{II}, the regulatory subunit of type II cAMP-dependent protein kinase (7, 22). The V_{max} for these substrates is between 0.4 and 2.0 μmol/min/mg (5, 22). Other substrates, such as histones IIa, VS, and the β subunit of phosphorylase kinase, are dephosphorylated at a rate one tenth to one thousandth that of protein phosphatase inhibitor 1 (15). Like other protein phosphatases, the calmodulin-regulated enzyme has been shown to dephosphorylate *p*-nitrophenylphosphate and free phosphotyrosine (23). However, there is no evidence that this enzyme exhibits specificity for phosphotyrosyl residues in proteins (D. K. Werth, personal communication). Thus, in evaluating the role of calmodulin-regulated protein phosphatases in muscle, attention is directed toward the four substrates which are known to be dephosphorylated at a substantial rate. In the case of phosphorylase kinase, cAMP-dependent phosphorylation of the α subunit has no effect on enzyme activity (24), and the role of dephosphorylation of this subunit is unknown. Whereas isolated smooth muscle myosin P-light chains are dephosphorylated, intact myosin is not dephosphorylated by calcineurin at a significant rate (D. R. Hathaway, personal communication). Furthermore, the regulatory significance of myosin light-chain phosphorylation in skeletal and cardiac muscle has not yet been clarified. Autophosphorylation of R_{II} has been shown to decrease the rate of reassociation of the catalytic and regulatory subunits of cAMP-dependent protein kinase (25).

Dephosphorylation of R_{II} by calcineurin would, in principle, increase the rate of binding of catalytic subunits, resulting in earlier termination of the effects of cAMP. Thus, it is conceivable that the calmodulin-stimulated protein phosphatases play a role in attenuating responses to cAMP. Consistent with this idea, protein phosphatase inhibitor 1 is phosphorylated by cAMP-dependent protein kinase, and dephosphorylated by calmodulin-stimulated protein phosphatases. Protein phosphatase inhibitor 1, in the phosphorylated state, is a potent inhibitor of protein phosphatase 1 (26, 27). The dephosphorylation of inhibitor 1 by calcineurin could be a mechanism for activating protein phosphatase 1, thus linking the control of glycogen metabolism to muscle contraction. Protein phosphatase 1 is the major enzyme in skeletal muscle that dephosphorylates glycogen phosphorylase, the β subunit of phosphorylase kinase, and glycogen synthase (10). Thus, activation of protein phosphatase 1 should stimulate the rate at which glycogen is resynthesized when contraction ceases (5).

Recent evidence from studies of the Na^+/Ca^{2+} exchange mechanism in cardiac sarcolemma indicates that the rate of cation exchange may be regulated by protein phosphorylation (28). The data suggest that this regulation may involve a calmodulin-stimulated protein phosphatase (28). The site of phosphorylation responsible for the observed pattern of regulation is not known. However, these results emphasize the possibility that some physiologically relevant substrates for the family of calmodulin-stimulated phosphatases remain to be identified.

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