THE DISTRIBUTION OF THE Ca^{++}-DEPENDENT PROTEIN ACTIVATOR OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE IN INVERTEBRATES

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Summary: Animal species representative of major phyla were found to contain activating factors similar to the Ca^{++} binding protein activator of 3':5' - cyclic nucleotide phosphodiesterase isolated from bovine heart. These factors are heat stable, non-dialysable and susceptible to proteolysis. All activate bovine heart cyclic nucleotide phosphodiesterase to a comparable extent and all activations are Ca^{++} dependent and reversible. The results suggest a ubiquitous distribution and fundamental importance for this Ca^{++} binding protein.

INTRODUCTION

A Ca^{++} activated cyclic nucleotide phosphodiesterase has been found in many mammalian tissues (1,2). The enzyme activity is known to be mediated by a protein activator which has been purified to homogeniety and identified as Ca^{++} binding protein (3-8). The mechanism of this Ca^{++} activation has been shown to be similar to that proposed for the Ca^{++} activation of actomyosin ATPase (9) which is also modulated by the Ca^{++} binding protein: troponin-C (10). Because of similarities in physical and chemical properties it has been suggested that the protein activator and troponin-C, the Ca^{++} binding component of troponin, may have a common evolutionary origin. (9). Recently several other Ca^{++} binding proteins have been suggested to be evolutionarily related to troponin-C (11 - 14).

From extensive comparative studies, Lehman et al. (15) have suggested that troponin has a restricted distribution in the animal kingdom and lower forms of animals, such as Mollusca and Brachipoda do

not contain this Ca^{++} binding protein. In contrast, results from the present study suggest that the protein activator of cyclic nucleotide phosphodiesterase is ubiquitous in the animal kingdom. The conservation of the activator activity throughout evolution suggests that this Ca^{++} binding protein serves a fundamental function in animal cells.

MATERIALS AND METHODS

Bovine heart protein activator and activator-deficient cyclic nucleotide phosphodiesterase were prepared and assayed as previously described. One unit of activator was defined as the amount which is required to give 50% stimulation of the enzyme (5). Extracts of animal species were prepared by homogenization with a polytron for 1 min. in 5 volumes 0.04M Tris, pH 7.5. After homogenization, samples were centrifuged at 10,000 x g for thirty minutes and supernatant fractions were dialysed overnight against two changes of 4 liters of 0.04M Tris, pH 7.5. The extracts were then heated at 95 - 100°C for 6 minutes; precipitated protein was removed by centrifugation and analysis was performed on the supernatant.

RESULTS AND DISCUSSIONS

Cheung and Kakiuchi and their co-workers (2, 16) have examined a variety of mammalian tissues and found that they all contain a specific protein activator of cyclic nucleotide phosphodiesterase. In the present study, the possible occurrence of similar activation factors in lower forms of animals was investigated. A number of animal species in several major phyla were chosen (Table I). All the supernatant fractions analyzed were found capable of activating the activator-deficient bovine heart enzyme. The results suggest that all animals contain heat-stable and non-dialysable activators for cyclic nucleotide phosphodiesterase. Mammalian protein activators of phosphodiesterase have been shown to be heat-stable proteins (3).

Table I: Activating Factors in Crude Animal Extracts

ANIMAL			ACTIVATOR ACTIVITY	
COMMON NAME	GENUS	PHYLUM	TOTAL ACTIVITY unit/g tissue	SPECIFIC ACTIVITY units/mg protein
SEA ANEMONE	METRIDIUM	CNIDARIA	813	51
CLAM	ANODONTA	MOLLUSCA	500	27
SNAIL	AMPULLARIA	MOLLUSCA	1259	41
EARTH WORM	LUMBRICUS	ANNELIDIA	2376	110
ROUND WORM	ASCARIS	NEMATHELMINTHES	400	10
BLUE CRAB	CALLINECTES	ARTHROPODA	704	11
MEAL WORM	TENEBRIA	ARTHROPODA	960	20
STAR FISH	ASTERIAS	ECHINODERMA	2205	161
SPONGE	EUSPONGIA	PORIFERA	930	133
BEEF HEART	BOSS	CHORDATA	45	2

For all samples the dependence of enzyme activation upon the activator concentration was determined. Representative examples of these activation curves are presented in Fig. 1. The activation curves are similar to that of enzyme activation by the purified bovine heart protein activator and assume the form of a sigmoidal ligand saturation curve suggesting homotropic cooperativity in the enzyme activation. The activator-deficient bovine heart cyclic nucleotide phosphodiesterase is activated by these various samples to approximately the same maximal enzyme activity. Furthermore, activations appear to depend on the presence of free Ca^{++}, since the chelating agent ethylene-glycol bis

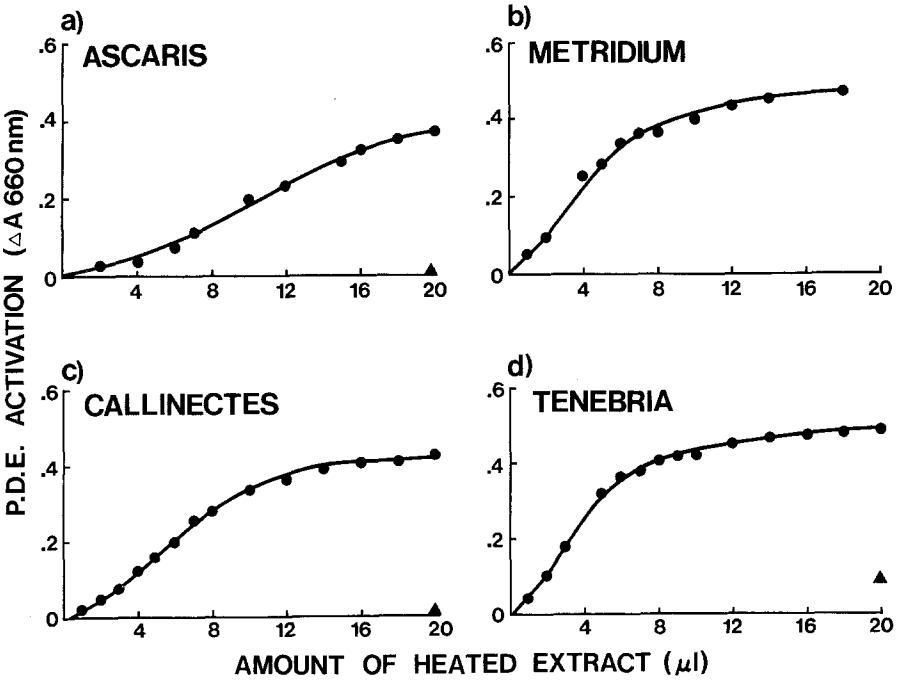

Figure 1. Activation of cyclic nucleotide phosphodiesterase: Bovine heart phosphodiesterase was assayed in the presence (▲) or absence (●) of 300 μM EGTA with various amounts of the heat-treated and dialyzed homogenate supernatants of: (A), Ascaris, the round worm; (B), Metridium, the sea anemone, (C), Callinectes, the blue crab and (D), Tenebria, the meal worm. Enzyme activation is expressed as the difference in activity between the activated and the control sample (sample with no added homogenates).

(β-aminoethyl-ether)-N, N' - tetraacetic acid (EGTA) at 300 μM completely blocks these activations.

In addition to its activation by the Ca^{++} dependent protein activator, mammalian cyclic nucleotide phosphodiesterase may also be activated by limited proteolysis (3). To exclude the possibility that activation of the bovine heart enzyme by animal extracts is due to proteolysis, the reversibility of enzyme activations was tested by adding EGTA to the reaction mixture to chelate free Ca^{++} ions. Typical results are given in Fig. 2. The activation of the enzyme by the added

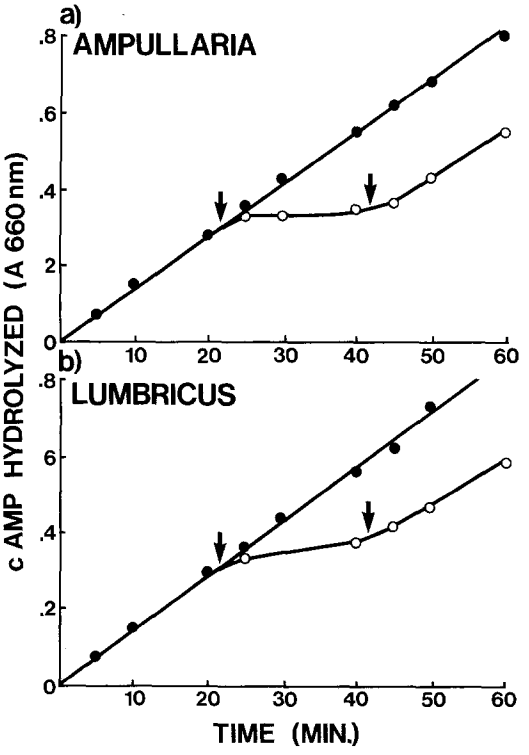

Figure 2. Reversibility of the phosphodiesterase activation: time course of bovine heart phosphodiesterase reactions in the presence of 10μM Ca^{++} and 50 μl of the heated and dialyzed extracts of (A) Ampullaria, the mystery snail and (B) Lumbricus, the earthworm. For each experiment, two identical incubation mixtures were prepared; one (O) was adjusted to 0.48 mM EGTA at 22 min. and to 0.8 mM Ca^{++} at 42 min., the other (●) was maintained as a control. As various intervals after initiation of the reaction, aliquots were removed and analyzed for phosphate production.

extracts can be effectively suppressed by addition of EGTA. When Ca^{++} ions in excess of EGTA are introduced into the reaction mixtures, the enhanced rates of enzyme reactions are re-established. The results indicate that activations of bovine heart cyclic nucleotide phosphodiesterase by the animal extracts are reversible and therefore, not due to proteolysis of the enzyme.

An estimate of the relative amounts of activator in the different species examined was obtained. Table 1 shows that all the lower animals contain high levels of the activator activity. The amounts of the activator activity in all these animals are at least one order of magnitude higher than that found in the bovine heart. Paradoxically, these animals contain much less cyclic AMP phosphodiesterase activity than bovine heart tissue. In several of these extracts, cyclic AMP phosphodiesterase activities are too low to be determined accurately. In cases where accurate determination of enzyme activity is possible, EGTA was found to cause 5 to 20% inhibition of the enzyme. Although, this low level of EGTA inhibition may suggest that most of the non-mammalian cyclic AMP phosphodiesterase is not regulated by Ca^{++}, the possibility that Ca^{++} dependence of the enzyme becomes pronounced under different assay conditions cannot be excluded. In the case of Tenebria molitor and Anodonta grandis cyclic GMP phosphodiesterase activity in the extracts was also measured and again found to be much lower than that in bovine heart.

To establish that activating factors found in the lower animals are indeed proteins, the susceptibility of the activator activity to proteolysis was examined. Mammalian protein activators are rapidly inactivated by trypsin, especially in the presence of EGTA (17). Activator activity in all the animal extracts examined was destroyed after one hour of incubation with trypsin (0.1 mg/ml) at 30°, pH 7.5 in the presence of 0.1mM EGTA. In control samples

where no trypsin was added or the addition of trypsin inhibitor preceeded the addition of trypsin, no loss in activator activity was detected.

In conclusion, all animal species examined in this study contain the protein activator of cyclic nucleotide phosphodiesterase thus suggesting that it is a very primitive protein. It seems that the protein activator is suitable for phylogenetic study since it is widely distributed and its activity may be readily measured. The fact that activators from various organisms are capable of activating bovine heart phosphodiesterase to the same extent suggests that the structure and function of this protein is conserved to a large extent. In this context it should be pointed out that activation of cyclic nucleotide phosphodeisterase may not be the sole function of this protein. Brostrom et al. (18) have recently shown that this protein activator also mediates the Ca^{++} activation of adenyl cyclase. In any case, the wide occurrence of this Ca^{++} dependent regulatory protein suggests that the regulation of cyclic nucleotide metabolism by Ca^{++} is of fundamental importance in the animal kingdom.

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