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Characterization of calmodulin-dependent cyclic nucleotide phosphodiesterase isoenzymes

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Calmodulin-dependent phosphodiesterase (CaMPDE) is one of the key enzymes involved in the complex interactions which occur between the cyclic-nucleotide and Ca^{2+} second-messenger systems. Calmodulin-dependent phosphodiesterase exists in different isoenzymic forms, which exhibit distinct molecular and/or catalytic properties. The kinetic properties suggest that the 63 kDa brain isoenzyme is distinct from the brain 60 kDa and heart and lung CaMPDE isoenzymes. The CaMPDE isoenzymes of 60 kDa from brain, heart and lung are regulated by calmodulin, but the affinities for calmodulin are different. At identical concentrations of calmodulin, the bovine heart CaMPDE isoenzyme is stimulated at a much lower Ca^{2+} concentration than the bovine brain or lung isoenzymes. The bovine lung CaMPDE isoenzyme contains calmodulin as a tightly bound subunit, so that a change in calmodulin concentration had no effect on the $[Ca^{2+}]$-dependence of activation of this isoenzyme. These observations are consistent with the notion that differential regulation by calmodulin and Ca^{2+} is an important function of these isoenzymes, which provide fine-tuning mechanisms for calmodulin action.

INTRODUCTION

Kakiuchi and Yamazaki [1] originally demonstrated the existence of a Ca^{2+}-stimulated cyclic nucleotide phosphodiesterase in rat brain. In addition, they discovered an endogenous brain protein factor which could enhance the Ca^{2+}-sensitivity of the enzyme [2]. It was subsequently established [3] that the protein factor was identical with the protein activator of cyclic nucleotide phosphodiesterase (later called calmodulin), which was originally discovered by Cheung [4,5], and that stimulation of the phosphodiesterase required the simultaneous presence of both Ca^{2+} and calmodulin [3,6]. The activity of calmodulin-dependent phosphodiesterase (CaMPDE) was found to be widely distributed in mammalian tissues and other eukaryotes [7–10]. CaMPDE is one of the most intensively studied and best characterized phosphodiesterases. Initially, it was thought that a single form of the enzyme existed in all tissues [8]; however, over the years it has become clear that CaMPDE exists as tissue-specific isoenzymes [11–17]. Four CaMPDE isoenzymes have been purified to near-homogeneity from bovine tissue in this laboratory [12–14]. They are designated according to tissue origin and subunit molecular mass as brain 63 kDa, brain 60 kDa, heart and lung PDE isoenzymes. Bovine brain 60 kDa, bovine heart and bovine lung CaMPDE isoenzymes are almost identical in terms of immunological properties, but they are differentially activated by calmodulin [12–14]. In this study, we examined the kinetic properties of different CaMPDE isoenzymes. We also show that they differ in affinity for calmodulin, which accounts for differences in Ca^{2+} sensitivity.

MATERIALS AND METHODS

Materials

Cyclic AMP, cyclic GMP and snake venom 5′-nucleotidase were from Sigma. Cyclic [^{3}H]AMP and cyclic [^{3}H]GMP were obtained from DuPont Canada, Mississauga, Ont., Canada. General laboratory chemicals were obtained from Sigma Chemical Co., Fisher and BDH. Bovine brain calmodulin was purified as described by Sharma [18]. Bovine brain 60 kDa and 63 kDa and the heart and lung CaMPDE isoenzymes were purified to near homogeneity as described previously [12–14]. The SDS/PAGE patterns and schematic illustration of various CaMPDE isoenzymes are presented in Figure 1.

Methods

Assay of phosphodiesterase

CaMPDE activity was measured at pH 7.5 and 30 °C by one of two methods. In the first method [19], the substrate concentrations used were in the millimolar range. This method involved the conversion of the reaction product 5′-AMP into the nucleoside and P_i by 5′-nucleotidase, followed by the spectrophotometric determination of phosphate at 660 nm. The reaction mixture contained 40 mM Tris/HCl, 5 mM magnesium acetate, pH 7.5, 0.5 unit of 5′-nucleotidase, 1.2 mM cyclic AMP and other components as described in Figure legends in a total volume of 0.9 ml. Reactions were carried out for 30 min. The second assay method was carried out as described by Thompson et al. [20], with cyclic [^{3}H]nucleotides. The reaction conditions were the same as those of the first method, except that 0.4 ml of mixture was used and the concentration of the nucleotides (substrates) was much lower. This assay method was used for the kinetic studies. All kinetic determinations were performed in triplicate. One unit of phosphodiesterase is defined as the amount of enzyme which, when fully activated, hydrolyses 1 μmol of cyclic nucleotide/min at 30 °C.

Other methods

Protein concentration was determined by the method of Bradford [21], with BSA as a standard. The concentrations of free Ca^{2+} in the EGTA-buffered solution were calculated as described previously [22].

Abbreviation used: CaMPDE, calmodulin-dependent phosphodiesterase.

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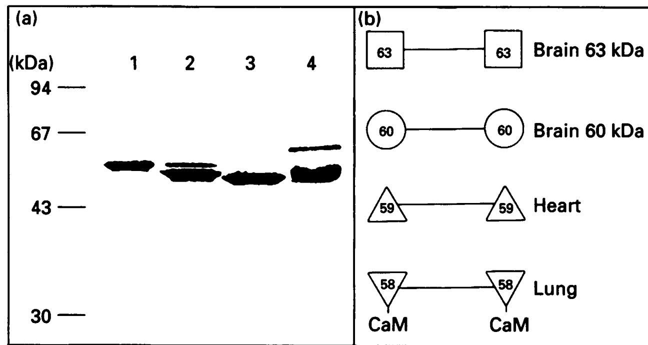

Figure 1 Purified CaMPDE isoenzymes from bovine tissues

(**a**) SDS/PAGE pattern of purified CaMPDE isoenzymes. Lanes: 1, brain 63 kDa isoenzyme; 2, brain 60 kDa isoenzyme; 3, heart isoenzyme; 4, lung CaMPDE isoenzyme. (**b**) Schematic illustration of CaMPDE isoenzymes. The 'kDa' values are for molecular-mass markers.

Table 1 Comparison of kinetic properties of CaMPDE isoenzymes from bovine tissues

CaMPDE isoenzymes were assayed by the procedure of Thompson et al. [20] by using cyclic [^{3}H]nucleotide as described in the Materials and methods section at 60 ng/ml calmodulin in the presence of 0.1 mM Ca^{2+}.

	K_m (μM)		$V_{max.}$ (μmol/min per mg)	
Isoenzyme	Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP
Brain 63 kDa	12.0	1.2	10.0	30.0
Brain 60 kDa	35.0	2.7	166.0	93.0
Heart	40.0	3.2	133.0	44.0
Lung	42.0	2.8	82.0	47.0

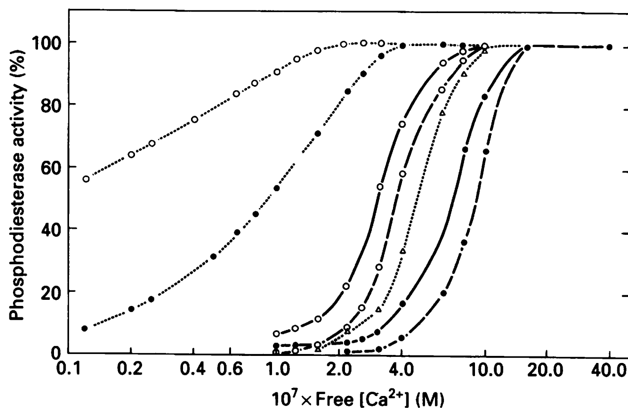

Figure 2 Activation of CaMPDE isoenzymes by Ca^{2+} at various levels of calmodulin

Phosphodiesterase assays were carried out by the procedure of Sharma and Wang [19] as described in the Materials and methods section at various concentrations of calmodulin (0.04 μM, △; 1 μM, ●; 10 μM, ○) with brain 63 kDa CaMPDE isoenzyme (– – –), brain 60 kDa CaMPDE isoenzyme (——) and heart CaMPDE isoenzyme (· · · ·).

Table 2 Ca^{2+} activation of CaMPDE isoenzymes at various concentrations of calmodulin

CaMPDE activities were determined as described in Figure 2. The Ca^{2+} concentration is defined as the amount required for half-maximal activation: nd, not done. These Ca^{2+} concentrations were calculated graphically as shown in Figure 2.

		[Ca^{2+}] (μM) required for half-maximal activation		
[Calmodulin] (μM)	CaMPDE isoenzyme...	Heart	Brain 60 kDa	Brain 63 kDa
0.04		0.50	nd	nd
1.00		0.08	0.90	0.70
10.0		0.01	0.35	0.30

RESULTS

Comparison of kinetic properties of various CaMPDE isoenzymes

Kinetic properties of the purified CaMPDE isoenzymes from various bovine tissues were examined with both cyclic AMP and cyclic GMP as substrates. Table 1 shows that both bovine brain CaMPDE isoenzymes (63 kDa and 60 kDa), bovine heart and bovine lung isoenzymes have a higher affinity towards cyclic GMP than towards cyclic AMP. The brain 60 kDa, heart and lung isoenzymes have very similar kinetic properties, whereas the brain 63 kDa CaMPDE isoenzyme has a 2–3-fold higher affinity for both substrates (cyclic AMP and cyclic GMP) and a higher $V_{max.}$ for cyclic GMP than for cyclic AMP. These results indicate that the brain 63 kDa CaMPDE isoenzyme is kinetically distinct from the other three CaMPDE isoenzymes.

Differential regulation of CaMPDE isoenzymes

Although bovine brain 60 kDa, heart and lung CaMPDE isoenzymes are almost identical in terms of immunological properties, they are differentially activated by calmodulin [12,23,24]. It may be possible that the differential affinity for calmodulin may reflect subtle differences in Ca^{2+} activation of these CaMPDE isoenzymes. The [Ca^{2+}]-dependence of the various CaMPDE isoenzymes was therefore examined at saturating levels of calmodulin (Figure 2). Figure 2 shows that Ca^{2+} and calmodulin interact synergistically in activation of both the brain and heart CaMPDE isoenzymes: when the calmodulin concentration was increased, the Ca^{2+} concentration required for half-maximal activation decreased. At saturating levels of calmodulin, the heart CaMPDE isoenzyme was half-maximally activated at $\simeq$ 10-fold lower Ca^{2+} concentration than the brain 63 kDa or 60 kDa CaMPDE isoenzymes: at 1 μM and 10 μM calmodulin, bovine heart CaMPDE required 0.08 μM and 0.01 μM Ca^{2+}, and brain CaMPDE required 0.9 μM and 0.3 μM Ca^{2+} respectively (Table 2). The results suggest that the bovine heart CaMPDE isoenzyme is stimulated at a much lower Ca^{2+} concentration than the bovine brain 60 kDa CaMPDE isoenzyme at an identical calmodulin concentration. Determination of the free Ca^{2+} concentration may not be very accurate at very low Ca^{2+} concentrations *in vitro*, so the Ca^{2+}-sensitivity of the bovine heart CaMPDE isoenzyme was also studied at 0.04 μM calmodulin. The results show that half-maximal stimulation of the heart CaMPDE isoenzyme occurred at 0.5 μM Ca^{2+} (Table 2). This result further suggests that the bovine heart isoenzyme is stimulated by much lower Ca^{2+} concentrations than are the other CaMPDE isoenzymes. Since bovine lung CaMPDE isoenzyme contains calmodulin as a subunit, the concentration-dependence of Ca^{2+} ac-

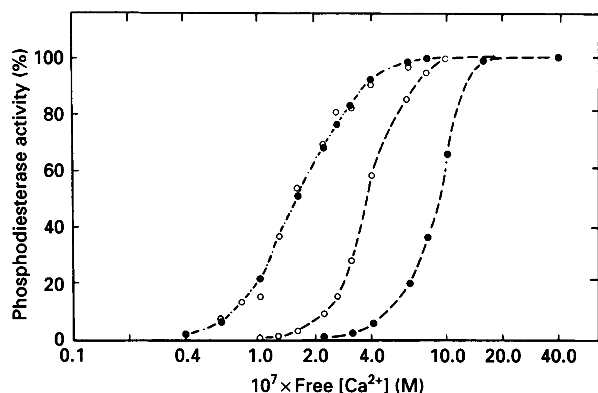

Figure 3 Effect of calmodulin on Ca^{2+}-dependent activation of lung CaMPDE isoenzyme

Phosphodiesterase assays were carried out as described in Figure 2. Calmodulin concentrations were 1 μM (●) and 10 μM (○), with bovine brain 60 kDa CaMPDE isoenzyme (– – – –) and bovine lung CaMPDE isoenzyme (–·–·–).

tivation of the lung enzyme was also carried out at two different concentrations of calmodulin. Figure 3 shows that there was no change in Ca^{2+}-sensitivity at different concentrations of calmodulin, indicating that the bovine lung CaMPDE isoenzyme does not undergo Ca^{2+}-dependent reversible association with calmodulin. Therefore this study indicates that bovine lung CaMPDE isoenzyme is distinct from bovine brain 63 kDa and 60 kDa and heart CaMPDE isoenzymes.

DISCUSSION

Several CaMPDE isoenzymes with different molecular masses, catalytic and regulatory properties have been purified to homogeneity and extensively characterized [11,12,25–30]. We have demonstrated that the brain 63 kDa CaMPDE isoenzyme is kinetically different from the brain 60 kDa, heart and lung CaMPDE isoenzymes (Table 1). Although brain 60 kDa, heart and lung CaMPDE isoenzymes are almost identical in terms of immunological properties, they are differentially activated by calmodulin [12,23,24]. The interaction of Ca^{2+}, calmodulin and CaMPDE in a calmodulin-stimulated reaction is the interdependence of Ca^{2+}-binding to calmodulin and the association of calmodulin and the enzyme. We have observed that, at an identical calmodulin concentration, the bovine heart CaMPDE isoenzyme is stimulated at much lower Ca^{2+} concentrations than are the bovine brain 60 kDa and 63 kDa isoenzymes. Furthermore, synergistic interactions between Ca^{2+} and calmodulin in the activation of the isoenzyme were also observed (Figure 2). Such synergistic interactions have been repeatedly shown for various calmodulin-dependent enzymes, including CaMPDE [31]. Although the physiological significance of the observed differential Ca^{2+}-sensitivity of the CaMPDE isoenzymes is not known (Table 2), it is noteworthy that the calmodulin concentration in mammalian heart is approx. 10 times lower than that in mammalian brain [32]. These results may suggest that the differential Ca^{2+} affinity of the tissue-specific isoenzymes is a mechanism by which the calmodulin regulatory reactions are adapted to the respective tissues. Similarly, the pig brain CaMPDE has been shown to have a lower affinity for calmodulin than the isoenzyme from pig artery [33]. Activation of the bovine lung CaMPDE isoenzyme by Ca^{2+} was unaffected by addition of exogeneous calmodulin (Figure 3), suggesting that this isoenzyme does not undergo Ca^{2+}-dependent reversible association with calmodulin; lung CaMPDE contains calmodulin as a tightly bound subunit [13]. The lung isoenzyme is not inhibited by calmodulin antagonists such as trifluoperazine or other calmodulin-binding proteins such as calmodulin-dependent phosphatase [13]. At present, the significance of calmodulin as a tightly bound subunit is not known; however, these results suggested that the tightly bound calmodulin in lung CaMPDE is not subject to competition by other calmodulin-dependent proteins after an increase in intracellular free Ca^{2+} concentration upon stimulation of the cell. Very recently, we have also demonstrated that brain 60 kDa and heart CaMPDE isoenzymes are inhibited by ginsenosides, but the brain 63 kDa CaMPDE isoenzyme is not [34]. The inhibition of CaMPDE by ginsenosides was overcome by increasing the concentration of calmodulin, suggesting that ginsenosides act specifically and reversibly to block the action of calmodulin.

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