Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 9726–9729, November 1991
Biochemistry

A peptide mimetic of calcium

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Communicated by George K. Davis, July 29, 1991

ABSTRACT **Proteins of the troponin superfamily use homologous amino acid sequences as binding sites for Ca^{2+} and seem to have evolved from an ancestral Ca^{2+} binding site. We have utilized this ancestral sequence to construct a peptide (Ca^{2+}-like peptide) with inverted hydropathy to the calcium-coordinating region of this protein. This synthetic peptide acted like Ca^{2+} in that (*i*) it increased the calmodulin-dependent hydrolysis of cAMP by phosphodiesterase, (*ii*) it interacted with EDTA, and (*iii*) it enhanced contraction of urinary bladder smooth muscle *in vitro*. Unlike Ca^{2+}, the peptide's effects were destroyed by acid hydrolysis. These findings demonstrate the synthesis of a peptide that can substitute for Ca^{2+} and may have considerable utility for the study of Ca^{2+}-regulated pathways and possible therapeutic value as a pharmacologic agent.**

Calcium–protein complexes are important second messengers that regulate muscle excitation–contraction coupling, hormonal secretion, and neuronal signal integration. Ligands that interact with calcium sites on proteins offer the opportunity to modify these activities. To design a peptide ligand for Ca^{2+}-coordinating sites, we have relied on two observations. (*i*) Ca^{2+}-coordinating sites on calmodulin (CAM), troponin C, and other members of the troponin superfamily have marked amino acid sequence homology (1). In fact, genes that code for these sites are believed to have evolved from an ancestral gene that codes for a single Ca^{2+}-binding domain (2). (*ii*) A number of polypeptides that have inverted hydropathy profiles can have affinity for each other (for review, see ref. 3). Thus we synthesized a peptide with an opposite hydropathy to the calcium-coordinating regions of the ancestral calcium-binding site (Table 1) and then tested the peptide for Ca-like activation of CAM.

MATERIALS AND METHODS

Peptide Synthesis. The 842-Da octapeptide (Ca^{2+}-like peptide, CALP) was synthesized in our laboratory by *t*-butoxycarbonyl chemistry and at Advanced ChemTech by fluoren-9-ylmethoxycarbonyl chemistry. The peptide was purified to homogeneity by HPLC and the sequence was verified in the Protein Core Facility, University of Alabama, Birmingham. Both peptide preparations were equally active.

CAM-Stimulated Phosphodiesterase (PDE) Assay. To minimize background calcium concentrations, the following procedures were followed. Assays were performed in 12 × 75 mm polypropylene tubes; all plastic solution bottles were washed in 0.25 M HCl and rinsed repeatedly with deionized water (obtained using a Milli-Q water system, Millipore, from which the water consistently had a resistance of 18 MΩ); reaction buffers and dimethyl sulfoxide were stirred with Chelex resin (50 g/liter) for 1 h; CAM solutions were dialyzed four times against 10% (wt/vol) Chelex in reaction buffer (9) at 4°C for 12 h. Dialysis tubing (Spectra/por 1, Spectrum Medical Industries) was boiled in 2% (wt/vol) sodium bicarbonate and washed in Milli-Q water prior to use. Bovine brain CAM, prepared using a fluphenazine-Sepharose affinity column as described (8), was a gift from R. W. Wallace (Boehringer Ingelheim, Ridgefield, CT). Assays were performed with cAMP, sodium salt (ICN); *Crotalus* toxin, EGTA, and bovine brain PDE (Sigma); tritiated cAMP, ammonium salt (Amersham); and AG 1X2 anion-exchange resin (200–400 mesh, chloride form) and Chelex 100 resin (100–200 mesh, sodium form; Bio-Rad).

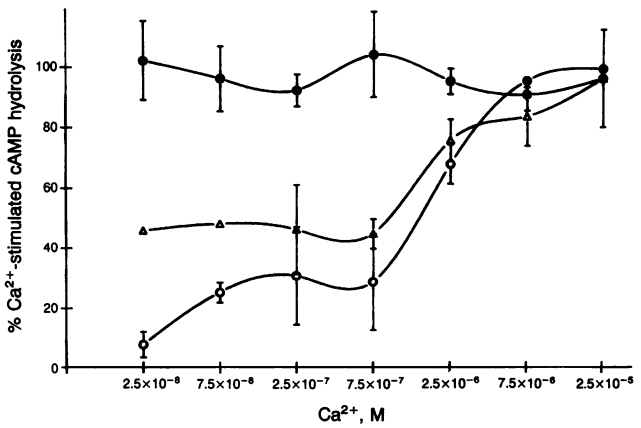

FIG. 1. Effect of CALP on Ca^{2+}-stimulated CAM-dependent cAMP hydrolysis. PDE reactions were performed in the presence of 0 (○), 1 μM (△), or 0.1 mM (●) CALP and various amounts of Ca^{2+}. Values were standardized to the amount of cAMP hydrolysis in the presence of 2.5 × 10^{-5} M Ca^{2+} and each point is the mean ± SD (n = 5).

All reagents were dissolved in the reaction buffer; PDE was dissolved in 50% (vol/vol) glycerol. Reaction tubes containing 10.0 μl of 1.0 μM CAM, 20.0 μl of 1.0 μM PDE, 25.0 μl $CaCl_2$, and 25.0 μl of CALP were preincubated at 30°C for 10 min. The PDE reaction was performed as described (9) and was initiated by addition of 20.0 μl of 4 mM tritiated cAMP (containing 100,000 cpm). Reactions proceeded at 30°C for 20 min by which time 3 ± 0.6% of the added cAMP was hydrolyzed in the absence of CAM. The percent cAMP hydrolysis was corrected for background PDE activity by the formula: % cAMP hydrolysis = (experimental − zero PDE activity in cpm)/(total cpm). Zero PDE activity was measured in tubes heated to 100°C for 1 min prior to addition of cAMP.

Circular Dichroism (CD). The ellipticity of the peptide was followed with a Jasco J41C spectropolarimeter (Jasco, Easton, MD). Waterjacketed thermostated quartz cuvettes with path lengths of 1 cm were obtained from Hellma (Forest Hills, NY). Peptides were solubilized in H_2O at 1 mM (pH 3.5). The

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Abbreviations: CALP, Ca^{2+}-like peptide; CAM, calmodulin; PDE, phosphodiesterase; ACh, acetylcholine.

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Table 1. Amino acid sequences of the ancestral calcium-binding site and CALP

Ancestral Ca^{2+} binding site	Asp*	Glu	Asp*	Gly	Asp*	Gly	Asn*	Ile
Hydropathy score for the ancestral site	−3.5	−3.5	−3.5	−0.4	−3.5	−0.4	−3.5	+4.5
Hydropathy score for CALP	+4.2	+1.8	+4.5	−0.7	+4.2	+3.7	+4.2	−3.9
CALP	Val	Ala	Ile	Thr	Val	Leu	Val	Lys

Based on eukaryotic codon usage (4, 5), a nucleotide sequence was constructed for the Ca^{2+} coordinating amino acids of the ancestral Ca^{2+}-binding site (6). This nucleotide sequence was then used to derive a complementary sequence to which amino acids were assigned (3). This peptide (CALP) was synthesized in our laboratory and at Advanced ChemTech. Hydropathic scores are as described (7) and calcium-coordinating positions are denoted (*).

spectra were taken at 22°C in 20 mM Tris·HCl, pH 7.2/20% trifluoroethanol and the final concentration of peptide was 0.1 mM. EDTA, Ca^{2+}, Na^+, and K^+ were prepared at 200 mM stock solution in Tris buffer (pH 7.2) and added in a maximal volume of 10 μl. The values of the CD spectra are expressed as the mean ellipticity per residue, which has the dimensions of degrees per cm^2 per dmol. Data were analyzed according to the reference spectra of Chang *et al.* (10).

Smooth Muscle Contraction. Adult mongrel dogs (20 ± 3 kg) were anesthetized with intravenous pentobarbital (30 mg/kg) and their urinary bladders were excised. The bladders were perfused through an intravesicular cannula with a canine physiological solution containing 145 mM Na, 4.2 mM K, 1.27 mM Ca, 0.7 mM Mg, 124 mM Cl, 0.7 mM SO_4, 25 mM HCO_3, and 2.4 mM PO_4. The solution was at pH 7.4 and the P_{O_2} was >500 mmHg. Bladders were pinned to the floor of a 50-ml chamber and the sphincter region was tied by a 5.0 suture to a Grass FT3 transducer (isometric). After a 1-h equilibration at 25°C, mechanical responses to acetylcholine (ACh) (1×10^{-6} M) were measured. The effects of CALP (1.2×10^{-6} M) on resting and ACh-induced tension development were also assessed. ACh and CALP were added to the bladder lumen.

RESULTS

Biochemical Activity of CALP. We initially determined whether the synthetic peptide could substitute for Ca^{2+} in the activation of CAM. CAM activation was measured by a stimulation of PDE. The CALP increased CAM-stimulated PDE activity in a dose-related fashion with maximal stimulation at 1×10^{-4} M peptide (Fig. 1). This was not due to a direct effect of CALP on PDE since the peptide (1×10^{-6} M) caused only a 0.2% increase in cAMP hydrolysis in the absence of CAM. Furthermore, the V_{max} of PDE in the assay was independent of the CALP concentration. Lineweaver–Burk plots of the relationship between Ca^{2+} and CALP showed that both curves had identical *y*-axis intercepts indicating that they were competing for the same binding site on CAM (Fig. 2).

We next tested whether, like Ca^{2+}, a chelating agent (EGTA) could affect the free CALP concentration. In the presence of EGTA, CALP concentrations up to 1×10^{-4} M did not increase CAM-dependent PDE-mediated cAMP hydrolysis (compare Figs. 1 and 3). The data are consistent with an interaction between CALP and EGTA. CD spectroscopy provided more direct evidence for such an interaction between the peptide and a chelating agent. In the presence of 20% trifluoroethanol [20 mM Tris·HCl (pH 7.2)], CALP is mostly a random coil with a small degree of α-helix, as indicated by a double minimum near 218 and 204 nm in the spectrum (Fig. 4). EDTA caused a transition to a more β form, as indicated by a minimum at 215 nm and a maximum at 202 nm. This transition was partially reversed by Ca^{2+} (Fig. 4) but not Na^+ or K^+ (data not shown). This result strongly suggests a direct interaction between CALP and EDTA. A similar, but smaller, effect was observed with EGTA (data not shown).

Although the Ca^{2+} reversibility of the EDTA-mediated CD spectral change of CALP argues strongly against Ca^{2+} contamination, two experiments were performed to assure that the CAM-stimulated PDE activity was enhanced by the peptide rather than trace Ca^{2+}. (*i*) A sample of CALP was subjected to atomic absorption spectrophotometry, which showed <50 ng of Ca^{2+} per mg of peptide. Thus, at the

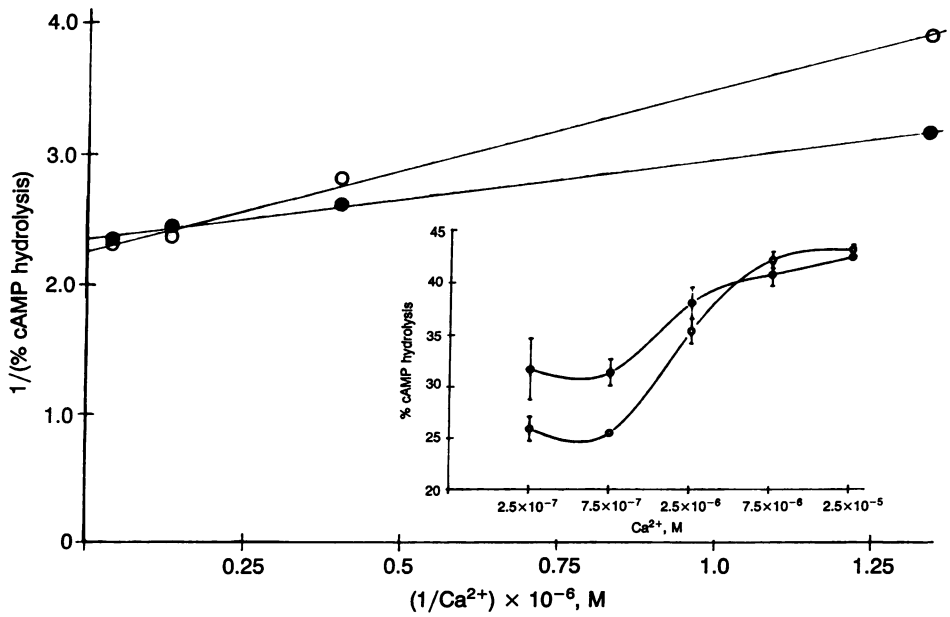

FIG. 2. Lineweaver–Burk plots of Ca^{2+} and CALP-stimulated CAM-dependent cAMP hydrolysis. Conditions are as in Fig. 1 and plots are based on the data in the *Inset*. Values represent the amount of cAMP hydrolyzed in the presence of 1×10^{-6} M CALP and the indicated concentration of Ca^{2+}. Each point is the mean ± SD ($n = 5$).

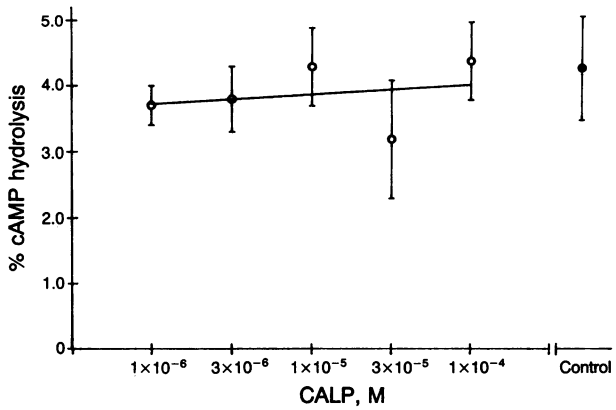

FIG. 3. Dose–response curve of CALP in the presence of EGTA. CAM, cAMP, and PDE were incubated with various amounts of CALP (1×10^{-6} to 1×10^{-4} M), Ca^{2+} (5×10^{-8} M) and EGTA (100 mM). Data points are means ± 1 SD ($n = 5$). This curve is typical of six experiments performed in the presence of Ca^{2+}/EGTA buffer. ○, CALP; ●, no CALP.

threshold concentration of the CALP effect (3×10^{-7} M, Fig. 5), the maximal Ca^{2+} concentration (3.63×10^{-10} M) is well below that which affects the assay (Fig. 1). Even at 1×10^{-4} M CALP, the maximal Ca^{2+} concentration was 1.21×10^{-7} M which had a negligible effect on the assay. The second experiment showed that, unlike Ca^{2+}, acid hydrolysis (4 h at 100°C in 1 M HCl) destroyed the PDE effect at all concentrations tested (Fig. 5).

Biological Effect of CALP. To determine whether the interaction of CALP with Ca^{2+} binding sites would have biological consequences, we assessed the effect of the peptide on smooth muscle force development. CALP (1.2×10^{-6} M) increased resting and ACh-stimulated tension in canine urinary bladder smooth muscle (Table 2). Fig. 6 shows the Ca^{2+}-like effect of CALP on bladder tension and ACh responses.

DISCUSSION

These studies have demonstrated that an octapeptide (CALP) designed to interact with proteins of the troponin superfamily can replace free Ca^{2+} in its effects on CAM-stimulated PDE activity, muscle tension, and ability to interact with chelating agents. Conditions of CAM/PDE activation (7.8 mM $MgCl_2$, pH 7.0) were chosen so that Ca^{2+}-dependent stimulation occurred at Ca^{2+} concentrations that were significantly

Table 2. Effects of 1.2×10^{-6} M CALP on resting and ACh-induced contraction of canine urinary bladder

	Resting tension, g	ACh-induced total tension, g	Specific ACh-induced tension, g
Control	10.52 ± 0.74	22.71 ± 1.08	12.19 ± 0.60
CALP	17.37 ± 1.03	41.25 ± 2.46	23.88 ± 1.23
% change	+65	+82	+96
P value	<0.05	<0.01	<0.01

Specific ACh-induced tension is the difference between the total ACh-induced tension and the resting tension. Data represent the mean ± SEM ($n = 8$). *P* value is from a paired differences *t* test.

above background (11). Under these conditions, the results with enzyme activation were consistent with those of Haiech *et al.* (11) and demonstrated both free Ca^{2+} and CALP have activation constants in the micromolar range. Our data strongly suggest CALP interacts with CAM at a Ca^{2+} binding domain. Additionally, the results of our studies show that CALP directly interacts with EDTA in a Ca^{2+}-competitive manner. Both of these results suggest that the binding of CALP to CAM is directly involved in CAM-activated PDE activity. Alternatively, a similar result might be obtained if CALP interaction with a site on CAM increased the affinity of other CAM sites for Ca^{2+}. Although this cannot be ruled out, considering the low Ca^{2+} concentration, this mechanism seems unlikely. In either event, however, PDE would be CAM-activated. The CALP effects on canine urinary bladder are a second Ca^{2+}-like effect mediated through CAM since tension development in smooth muscle is directly related to Ca^{2+}-sensitive CAM-dependent phosphorylation of myosin light chain kinase (12). The CALP was delivered to the muscle tissue by addition to the extracellular solution and its effective concentration was closer to the normal range of intracellular free Ca^{2+} (1×10^{-7} to 1×10^{-5} M; ref. 13) than it was to that of the extracellular free Ca^{2+} (1×10^{-7} M). Thus to reach an effective intracellular concentration, the peptide would most likely have to be taken into the cell. Further studies will be required to determine whether CALP *per se* diffuses into the cell and is intrinsically active or whether it facilitates the diffusion of free Ca^{2+}. In this regard, Callewaert *et al.* (14) have demonstrated differential modulation of Ca currents in cardiac and neuronal tissues by a rat brain-derived peptide. This peptide, however, is quite different

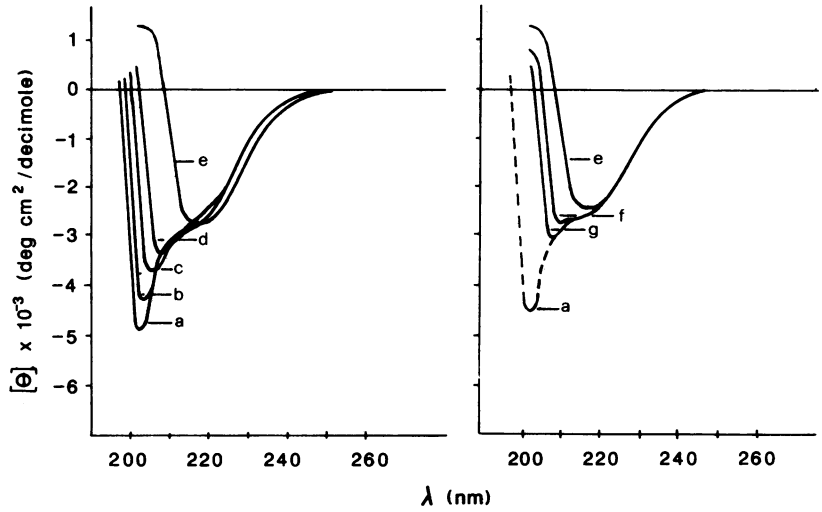

FIG. 4. CD spectra of CALP in the presence and absence of EDTA. Curves: a–g, 0.1 mM CALP. (*Left*) Curves: a, no EDTA; b, 0.5 mM EDTA; c, 1.0 mM EDTA; d, 1.5 mM EDTA; e, 2.0 mM EDTA. (*Right*) Curves: f, 2 mM EDTA and 1 mM Ca^{2+}; g, 2 mM EDTA and 2 mM Ca^{2+}. EDTA alone gave a baseline reading.

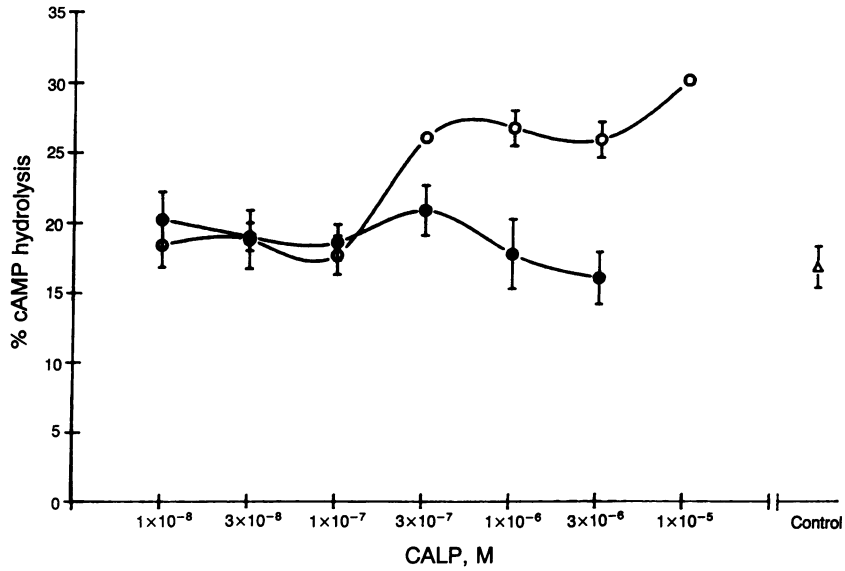

FIG. 5. Effect of acid hydrolysis on CALP activity. CALP was denatured in 1 M HCl for 4 h at 100°C. The pH was restored to 7 by the addition of 1 M NaOH. Equal amounts of NaOH and HCl were added to the nondenatured and control samples. CAM-dependent PDE activity was measured in the presence of various concentrations of CALP and 5×10^{-8} M Ca^{2+}. Data points are the mean ± SD ($n = 5$). △, No CALP; ●, denatured CALP; ○, active CALP.

from the CALP in terms of amino acid composition. In the future, the ability to design such small peptides to modulate Ca^{2+} or other ion fluxes or compete for Ca^{2+} or other ion binding sites may have implications for physiological regulatory mechanisms and utility as pharmacologic agents.

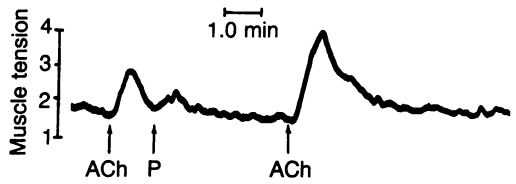

FIG. 6. Canine urinary bladder smooth muscle tension response to CALP. This force transducer recording was produced by a longitudinally stretched urinary bladder. ACh (1×10^{-6} M) was added to the bladder lumen and 1 min later CALP (P) (1.2×10^{-6} M) was added. At the indicated time, smooth muscle was again stimulated with ACh (1×10^{-6} M). Muscle tension is presented as g × 10^{-1}; time scale was 1.0 min/cm.

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