Vol. 208, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 8, 1995 Pages 280-286

PITUITARY ADENYLATE CYCLASE ACTIVATING POLYPEPTIDE IS A POTENT CALMODULIN INHIBITOR

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Received January 19, 1995

Summary: We report here that pituitary adenylate cyclase activating polypeptide (PACAP38), a new 38-residue neuropeptide of the secretin/glucagon family, is a potent inhibitor of calmodulin in vitro in the activation of bovine brain calmodulin-dependent cyclic nucleotide phosphodiesterase. The concentration of PACAP38 for half-maximal inhibition of the phosphodiesterase is 15 nM, one of the lowest for known calmodulin inhibitors. In the presence of Ca^{2+}, PACAP38 binds strongly to calmodulin in a 1:1 ratio with a dissociation constant of about 28 nM. The binding is not dissociated by 4 M urea. In the absence of Ca^{2+} the binding is at random and can be dissociated by 4 M urea. Studies with PACAP38 derivatives show that the carboxyl half of the PACAP38 molecule is essential for the inhibition of calmodulin.

Pituitary adenylate cyclase activating polypeptide (PACAP38) and its truncated form PACAP27, which has the first 27 amino-acid residues of PACAP38, cause an elevation of intracellular cAMP in pituitary cells with equal potency (1,2). This has been attributed to the activation of adenylate cyclase after the PACAPs have bound to cell surface receptors. Both PACAP38 and PACAP27 are thought to come from the same gene product (3). Other important signaling systems such as inositol phosphate/diacylglycerol system, intracellular calcium and ion channels are also found to be PACAP-activatable.

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Abbreviations: cAMP, adenosine 3':5'-cyclic monophosphate; EGTA, ethylene glycol bis(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid; K_d, dissociation constant; CaM, calmodulin; IC_{50}, concentration of PACAP38 or derivatives required to produce a half-maximal inhibition of the phosphodiesterase.

0006-291X/95 $5.00

Calmodulin is a major intracellular calcium receptor in eukaryotic cells. It controls the activities of more than twenty enzymes in response to changes in calcium concentration in the cell (4). Current studies suggest that calmodulin-binding sites usually contain amphiphilic helices (5). In view of the calmodulin-binding property of some neuropeptides (6), and the involvement of PACAP38 in cAMP metabolism, we have begun to investigate a possible link between PACAP38 and calmodulin. In this paper we show that PACAP38 and some of its derivatives can bind to calmodulin and inhibit the calmodulin in the activation of bovine brain calmodulin-dependent cyclic nucleotide phosphodiesterase.

Materials and Methods

Materials: Bovine brain calmodulin, 5'-nucleotidase from snake venom, cAMP, malachite green and most chemicals were obtained from Sigma Chemical Company. Bradford protein assay solution was from Bio-Rad. PACAP38 and derivatives were obtained from Bachem California and Peninsula Laboratories, inc. Chemicals used were of reagent grade. Bovine brain calmodulin-dependent cyclic nucleotide phosphodiesterase was partially purified according to Wallace et al. (7)

Methods: The assay for the calmodulin-dependent cyclic nucleotide phosphodiesterase was done according to Sharma and Wang (8), scaled down to one-tenth as described in Leung et al. (9). In this assay the phosphodiesterase changes the cAMP to 5'-AMP and then the 5'-nucleotidase changes the 5'-AMP to adenosine and phosphate. The amount of phosphate was measured by the malachite green method, using wavelength 610 nm (10). Thus, absorbance at 610 represents the amount of phosphate formed and, directly, the enzyme activity. The final reaction volume was 90 μl, containing 0.0015 unit bovine brain calmodulin-dependent cyclic nucleotide phosphodiesterase, 2 units (=1.08 pmol) bovine brain calmodulin, 0.03 units 5'-nucleotidase, 40 mM Tris-HCl, 40 mM imidazole, 5 mM Mg acetate, and 0.5 mM $CaCl_2$, pH 7.5. PACAP38 or its derivatives was incubated with the reaction mixture for 1 minute at 30°C before the addition of cAMP to a final concentration of 1.2 mM to start the enzyme reaction. After 30 minutes at 30°C the reaction was stopped by the addition of 910 μl water and the amount of phosphate was measured.

The procedures for native gel electrophoresis were as in (11). Bovine brain calmodulin was incubated with various amounts of PACAP38 at room temperature for 1 hour in 100 mM Tris-HCl pH 7.2, 2 mM $CaCl_2$ or EGTA, with or without 4 M urea. Incubation volume was 30 μl. Then, 10 μl of 50% glycerol with 0.01% bromophenol blue was added to each incubation mixture and each mixture was applied to the gel. The slab gels was of 12.5% acrylamide, with 0.375 M Tris-HCl, pH 8.8, with or without 4 M urea, in the presence of 2 mM $CaCl_2$ or EGTA. After electrophoresis, the gel was stained with Coomassie Blue to reveal the protein bands. Calmodulin concentrations were determined by the Bradford method (12), using bovine serum albumin as a standard, and by UV absorbance at 276 nm, and then compared with manufacturer's measurement. The concentrations of PACAP38 and derivatives were determined by the manufacturer.

Results and Discussion

Figure 1 (●) shows the inhibition of the calmodulin-dependent phosphodiesterase by PACAP38, with a half-maximal inhibition at 15 nM PACAP38. At 100 nM of PACAP38, the inhibition is almost complete, with the activity of the phosphodiesterase falling back to the basal level. This inhibition can be reversed by the addition of excess calmodulin (Fig. 2). The same maximum of activation was reached when enough calmodulin was added. These results show that PACAP38 is inhibiting the calmodulin only in the assay. PACAP38 does not affect the phosphodiesterase itself as the basal activity of the enzyme was not inhibited (Fig.1 (o)).

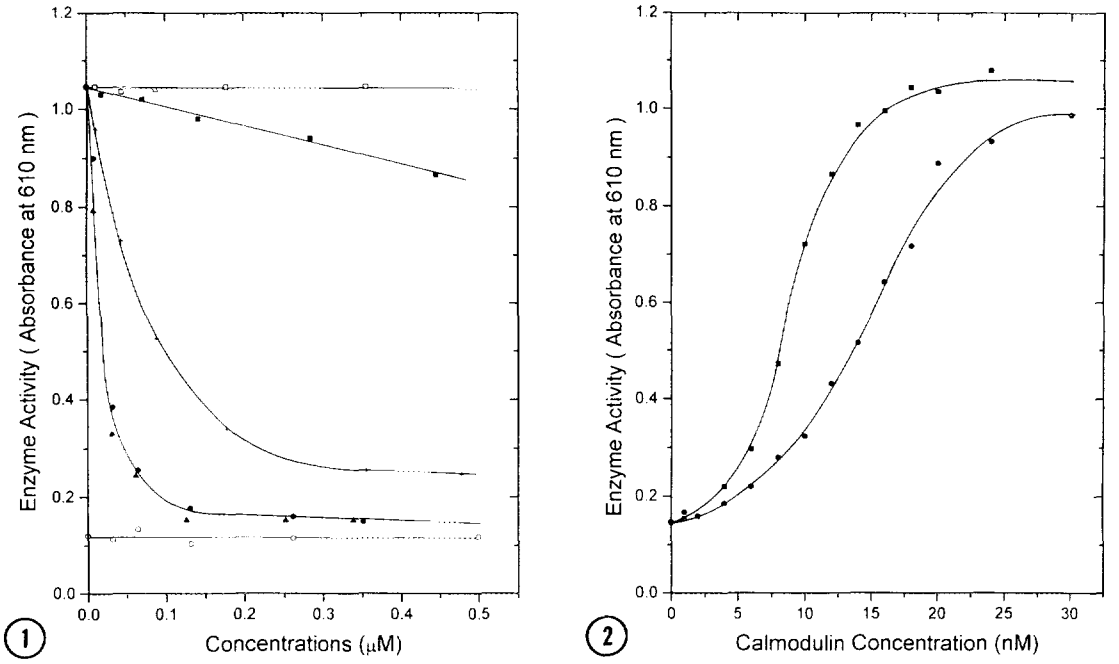

Fig. 1. Inhibition of calmodulin by PACAP38 and derivatives, measured by the loss of the ability to stimulate calmodulin-dependent cyclic nucleotide phosphodiesterase.

● PACAP38, ▲ PACAP(6-38), + PACAP(16-38), ■ PACAP27, □ PACAP(28-38), o basal activity with PACAP38 or derivatives. Basal activity is the activity of the phosphodiesterase in the absence of calmodulin. Enzyme activity above the basal level is due to calmodulin activation. Half-maximal inhibition is the inhibition of total calmodulin activity by 50%. IC_{50} for PACAP27 was determined from a separate inhibition curve (not shown) with a higher range of PACAP27 concentrations.

Fig. 2. Reversal of PACAP38 inhibition by the addition of excess bovine brain calmodulin.

The assay conditions were as in Fig. 1 except that varying amounts of bovine brain calmodulin were used. ■ no PACAP38, ● with 22.2 nM PACAP38 in the assay.

In order to show a direct binding between PACAP38 and calmodulin we use non-denaturing polyacrylamide gel electrophoresis (11). Fig. 3A shows that PACAP38 binds directly to calmodulin to form a PACAP38-calmodulin complex which moves slower than calmodulin. This slower mobility is probably due to the bigger size of the complex and to the fact that the complex is less electronegative than the calmodulin, as PACAP38 is highly positively charged at the pH used. Only one distinct complex band is seen from low (1:6) to high (4:1) PACAP38:calmodulin molar ratios (Fig. 3A, lanes 2-6), showing that one calmodulin molecule binds only one PACAP38 molecule. This conclusion is supported by the result that most of the calmodulin are complexed at the molar ratio of 1:1 (Fig. 3A, lane 4). In the absence of Ca^{2+}, the binding between PACAP38 and calmodulin is not distinct (Fig. 3B). At low concentrations of PACAP38 (Fig. 3B, lanes 2,3) no visible complex is formed. At 1:1 molar ratio (Fig. 3B, lane 4) a faint complex band with high molecular weight smearing is visible. At higher PACAP38 concentrations (Fig. 3B, lanes 5,6) there are a lot of smearing

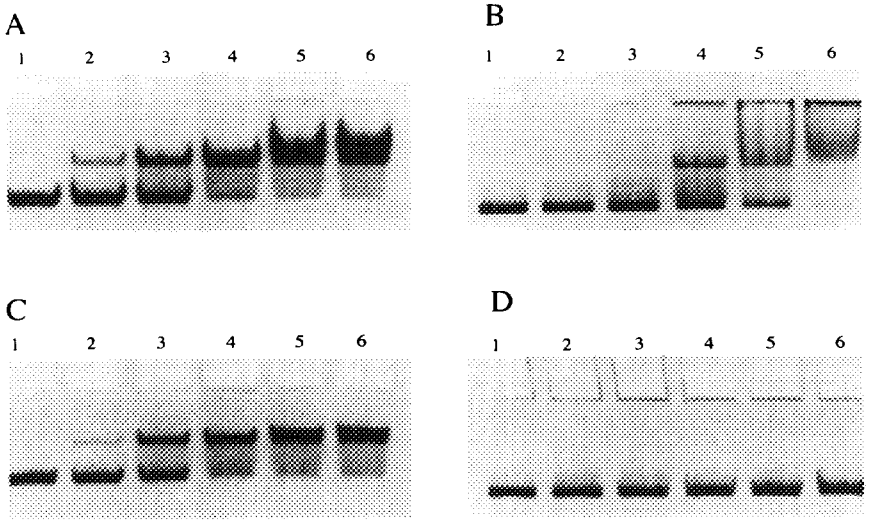

Fig. 3. Demonstration of PACAP38 binding to calmodulin by native gel electrophoresis.

A and C (2 mM $CaCl_2$), B and D (2 mM EGTA), A and B (no urea), C and D (4 M urea). All lanes contain 306 pmole of calmodulin. Lane 1 is a calmodulin control in which the calmodulin was not incubated with PACAP38. In lanes 2-6, the calmodulin was incubated with 50, 150, 300, 600, 1200 pmole PACAP38, giving a PACAP38:calmodulin molar ratio of 1:6, 1:2, 1:1, 2:1, 4:1, respectively.

with no distinct complex band, suggesting that PACAP38 binds to the calmodulin in a random manner. The binding in the absence of Ca^{2+} is dissociated by 4 M urea (compare Fig. 3D & 3B). In the presence of Ca^{2+}, the binding is not dissociated by 4 M urea. These results show that the binding of PACAP38 to calmodulin is much more specific in the presence than in the absence of Ca^{2+} because the complex is formed at a lower PACAP38 concentration with a constant 1:1 stoichiometry, and is very stable.

To estimate the K_d between PACAP38 and calmodulin in the presence of Ca^{2+} we apply the uncompetitive inhibition model in enzyme studies (13) to the PACAP38-calmodulin interaction. In this model, calmodulin (CaM) takes the part of the enzyme, Ca^{2+} the substrate, $CaM.Ca^{2+}$ the enzyme-substrate complex, and PACAP38 the uncompetitive inhibitor. The model is represented as follows:

$$\begin{array}{ccccc} CaM + Ca^{2+} & \rightleftharpoons & CaM.Ca^{2+} & \longrightarrow & CaM\ activity \\ & & + & & \\ & & PACAP38 & & \\ & & \Updownarrow K_d & & \\ & & CaM.Ca^{2+}.PACAP38 & & \end{array}$$

CaM activity is measured by the amount of activation of the phosphodiesterase. This uncompetitive model predicts that a Dixon plot of $(CaM\ activity)^{-1}$ versus inhibitor concentrations would give parallel lines with various concentrations of Ca^{2+} (13). The results in Fig. 4 show that at high Ca^{2+} concentrations the lines are parallel, suggesting under these conditions the uncompetitive model can be used. This model gives a K_d of 28 nM, the lowest of the secretin/glucagon family (14).

We also tested various derivatives of PACAP38 for their ability to inhibit calmodulin in the phosphodiesterase assay. The results are shown in Fig. 1. PACAP27 gives an IC_{50} of 1.6 μM, about 100 fold less potent than PACAP38 in inhibiting calmodulin. This shows that residues 28-38 of PACAP38

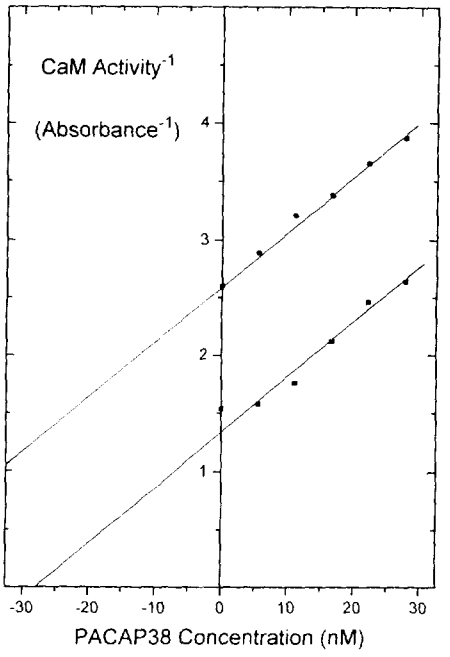

Fig. 4. Dixon plot of the calmodulin activity with different concentrations of PACAP38 and Ca^{2+}.

The assay conditions were as those used in Fig. 1 except with the $CaCl_2$ concentration. CaM activity is the activity of the phosphodiesterase in the presence of calmodulin minus the basal activity of the phosphodiesterase, at each PACAP38 concentration. The phosphodiesterase activity was measured as absorbance at 610 nm. ● 0.15 mM Ca^{2+}, ▪ 1.0 mM Ca^{2+}. The 1.0 mM Ca^{2+} concentration is a calcium-saturating condition because 0.2 mM Ca^{2+} also gave the same curve. The x-axis intercept at this calcium-saturating condition gives $-K_d$.

is essential for the potent inhibition of calmodulin. However, the eleven-residue fragment itself, PACAP(28-38), has no effect on calmodulin at all, showing that fragment 28-38 is essential only in the whole molecule. PACAP(6-38), which does not have residues 1 to 5, has the same inhibitory potency as PACAP38, showing that the first five residues in the PACAP38 molecule are not involved in calmodulin inhibition. PACAP(16-38), which has residues 1 to 15 deleted from PACAP38, gives an IC_{50} of 75 nM, about one-fifth as potent as PACAP38. Together these PACAP38 derivatives show that the C-terminal half of PACAP38 is more important than the N-terminal half in inhibiting calmodulin. The solution structure of PACAP38 is known (3,15). It does not show any amphiphilic helix, suggesting that the binding of PACAP38 to calmodulin is not by an amphiphilic-helix mechanism.

The possibility that PACAP molecules do interact with calmodulin in the cells needs to be investigated. The finding that vasoactive intestinal peptide, a peptide related to PACAP38, binds to calmodulin in guinea pig lung membrane (16,17) gives some support to this possibility.

Acknowledgments: We thank Dr. K.L. Yu for bringing PACAP38 to our attention, Wallace S.H. Chick for help in the preparation of the figures, and Dr. J.H. Wang for comments on the manuscript. This work is supported by the Research Grants Council of Hong Kong.

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