Stimulation of tyrosine phosphorylation of rat brain membrane proteins by calmodulin

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Calmodulin stimulates the alkali-resistant phosphorylation of peptides of 50 and 58–60 kDa in rat brain membrane. Phosphoamino acid analysis indicated a calmodulin stimulated increase of phosphotyrosine in these peptides. Calmodulin also stimulated the phosphorylation of these peptides at serine and threonine residues. This suggests the involvement of the calmodulin regulatory system in the effects of tyrosine protein kinases.

Calmodulin *Protein* *Tyrosine phosphorylation* *Brain*

1. INTRODUCTION

Tyrosine protein kinases were originally discovered to be associated with the gene products of certain transforming viruses [1–3]. Normal cells also contain tyrosine protein kinases such as the cellular homologs of v-src [1] and v-fps/fes [4] gene products. Receptors for insulin [5,6], insulin-like growth factor I [7], epidermal growth factor [8,9] and platelet-derived growth factor [10,11] have also been shown to possess ligand-stimulated tyrosine protein kinase activity. These observations suggest that tyrosine phosphorylation in protein is important in the regulation of growth. The regulatory properties of most of these enzymes are not known.

Calmodulin, a Ca^{2+}-dependent regulator has been implicated in various metabolic and physiological processes. Recently, it has been found that calcineurin, the calmodulin-stimulated phosphatase, is capable of catalyzing the dephosphorylation of phosphotyrosyl proteins [12,13]. In this study we present evidence to suggest that calmodulin stimulates the phosphorylation of tyrosine residues on proteins in rat brain membranes.

2. MATERIALS AND METHODS

Purification of calmodulin [14] and preparation of calmodulin-Sepharose 4B gel [15] were as previously described. Synaptic membrane from rat brain was prepared by the method of Schulman and Greengard [16] with an additional wash of the membrane in 10 mM EDTA, 5 mM EGTA buffer. Membrane phosphorylation was performed at 30°C in a reaction mixture (50 μl) containing 25 mM Hepes (pH 7.5), 20 mM $MnCl_2$, 12 μM $ZnCl_2$, 0.1% (w/v) Nonidet P-40, 3.5 mM *p*-nitrophenyl phosphate, 2.9 μM calmodulin and 2.5 μM [γ-^{32}P]ATP (30–70 Ci/mmol). After preincubation for 1 min, the reaction was initiated by the addition of [γ-^{32}P]ATP and terminated after 15 s by the addition of electrophoresis sample buffer containing 0.125 mM Tris-HCl (pH 6.8), 4% SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol and 0.01% bromophenol blue and immediate boiling for 3 min. The solubilized proteins were fractionated by SDS-PAGE on 10% (w/v) acrylamide gels according to Laemmli [17]. Dried gels were autoradiographed at −80°C using X-Omat AR film with X-Omat intensifying screens. Alkaline treatment of gels was carried out

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with 1 N KOH at 56°C for 1 h [18]. For phosphoamino acid analysis, peptides were electrophoretically eluted from untreated gels, precipitated with 15% trichloroacetic acid; and then partially hydrolyzed in 6 N HCl at 110°C for 1 h. The phosphoamino acids were separated by thin layer electrophoresis at pH 3.5 [1]. Comparison of the autoradiograph with the ninhydrin staining pattern of added phosphoamino acid standards allowed identification of the radioactive phosphoamino acids.

3. RESULTS AND DISCUSSION

Synaptic membranes from rat brain were incubated with [γ-^{32}P]ATP in the presence of calmodulin or the calmodulin-inhibitor, Compound 48/80 [19] (fig.1). Autoradiography following SDS-PAGE revealed a number of phosphopeptides whose phosphorylation was markedly enhanced by calmodulin (fig.1A). Treatment of gel in an alkaline solution prior to autoradiography eliminates most of the radioactive bands (fig.1B). Among the remaining radioactive bands, two phosphopeptides of apparent molecular masses 58–60 and 50 kDa showed a calmodulin-stimulated increase in phosphorylation. On the other hand, Compound 48/80 caused a slight suppression of the phosphorylation level. Since phosphotyrosine is generally resistant to alkali-treatment while phosphoserine and phosphothreonine are more alkali-labile [18], the results suggest that calmodulin stimulates tyrosine phosphorylation of the 58–60 and 50 kDa rat brain membrane proteins.

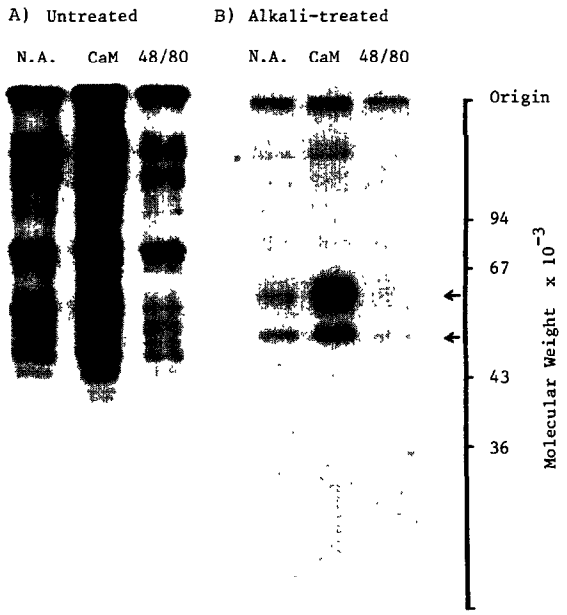

Fig.1. Autoradiogram of untreated and alkali-treated gels of rat brain membrane proteins. Membrane was incubated with [γ-^{32}P]ATP (2.5 μM) and either calmodulin (CaM, 2.9 μM) or Compound 48/80 (100 μg/ml). The membrane proteins were subjected to SDS-PAGE and were either left untreated or treated with 1 N KOH for 1 h at 56°C prior to autoradiography. Approximate M_r-values are indicated in the margin. Arrows indicate the 58–60 and 50 kDa peptides. N.A. indicates no addition.

The 58–60 and 50 kDa phosphopeptides were electrophoretically eluted from the gels. Recovery was approx. 90% for both peptides (table 1). The phosphoamino acids of the eluted peptides were analyzed (fig.2). Both phosphopeptides were found to contain phosphotyrosine, phosphothreonine and phosphoserine. Phosphopeptides from the sample phosphorylated in the presence of calmodulin appeared to contain higher amounts of all 3 phosphoamino acids. The radioactive phosphoamino acids were scraped from the plates and counted (table 2). Calmodulin stimulation of tyrosine phosphorylation was evident. A portion of the eluted peptides were subjected to alkali treatment prior to phosphoamino acid analysis. The results showed calmodulin-stimulated tyrosine phosphorylation of both peptides and that the phosphothreonine residues were partially resistant to alkali (table 2).

The calmodulin-stimulated phosphorylation of the 58–60 and 50 kDa peptides resembles the autophosphorylation of the α- and β-subunits of calmodulin-stimulated synapsin I kinase [20–22], suggesting that this calmodulin-dependent kinase may be a tyrosine protein kinase substrate. However, the possibility that the phosphotyrosine-phosphopeptides are minor peptides that co-migrate on SDS-PAGE with the phosphoserine- and phosphotyrosine-peptides cannot be ruled out. The mechanism of the calmodulin stimulation of tyrosine phosphorylation remains to be investigated. It is possible that calmodulin stimulates the tyrosine protein kinase or that calmodulin-binding to the substrate causes conformational

Table 1

Recovery of eluted peptides

Phosphopeptide	Addition	cpm in gel slices	cpm eluted	Recovery (%)	cpm remaining in gel
58–60 kDa	None	39600	34681	88	996
	CaM	93080	83820	90	2282
	Comp.48/80	27856	24014	86	931
50 kDa	None	27384	25546	93	447
	CaM	44100	39776	90	612
	Comp.48/80	21762	18686	86	346

Phosphopeptides separated by SDS-PAGE were eluted from gel pieces by embedding in 2% agarose, 0.125 M Tris-HCl (pH 6.8). Peptides were eluted electrophoretically at 4 mA/tube using 50 mM Tris, 0.38 M glycine, 0.1% SDS (pH 8.3) electrode buffer and counted by their Cerenkov radiation

changes which make it a better substrate for the tyrosine kinase.

Other interrelationships between the calmodulin regulatory system and the tyrosine protein kinase regulatory systems have been found. Calmodulin is phosphorylated by purified pp60src [23] and the insulin receptor kinase [24]. Calmodulin-dependent protein kinase is phosphorylated by the insulin receptor kinase [24]. Tyrosine phosphorylation of the β-estradiol receptor is stimulated by

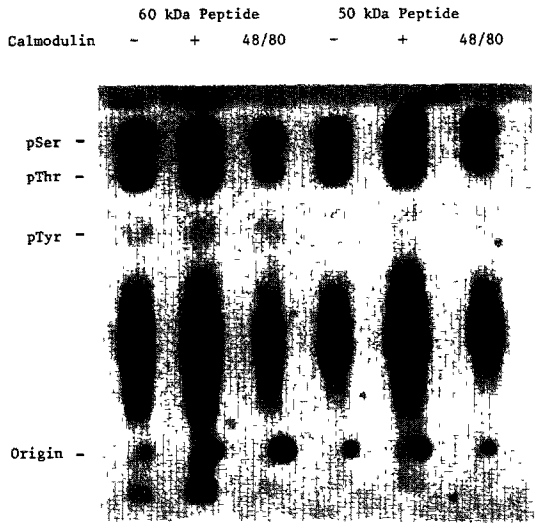

Fig.2. Autoradiogram of the phosphoamino acids of 58–60 and 50 kDa peptides. Peptides were electrophoretically eluted from gels after SDS-PAGE, precipitated with trichloroacetic acid, hydrolyzed and the phosphoamino acids were separated by thin layer electrophoresis in pH 3.5 buffer [1]. Phosphoamino acids, identified by ninhydrin staining of added controls, are indicated in the margin.

Table 2

Phosphoamino acid analysis of eluted phosphopeptides without (a) and with (b) alkali treatment

Peptide	Addition	pSer	pThr	pTyr
(a) Trichloroacetic acid-precipitated sample				
58–60 kDa	None	402	170	36
	CaM	650	370	56
	Comp.48/80	254	94	28
50 kDa	None	192	125	22
	CaM	319	248	35
	Comp.48/80	118	81	18
(b) Alkali-treated sample				
58–60 kDa	None		356	130
	CaM		1530	363
	Comp.48/80		244	128
50 kDa	None		358	68
	CaM		694	104
	Comp.48/80		235	58

Values are cpm of radioactive spots scraped off cellulose plates and are the average of duplicate initial phosphorylations. 20% of the eluted peptide sample was trichloroacetic acid precipitated and the rest was treated with alkali

calmodulin [25] and the EGF receptor-kinase which is autophosphorylated is dephosphorylated by calcineurin [12]. These indicate that the calmodulin regulatory system may modulate some of the effects of tyrosine protein kinases.

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