STIMULATION OF TYROSINE PHOSPHORYLATION OF RAT BRAIN MEMBRANE PROTEINS BY CALMODULIN

BY

DENNIS FRANKLIN MICHIEL

A thesis

submitted to the University of Manitoba

in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

in

Department of Biochemistry

University of Manitoba

Winnipeg, Manitoba, 1985

ISBN Ø-315-33574-2

STIMULATION OF TYROSINE PHOSPHORYLATION OF RAT BRAIN MEMBRANE PROTEINS BY CALMODULIN

BY

DENNIS MICHIEL

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

ACKNOWLEDGEMENT

I would like to thank Dr. J.H. Wang for his continued support and supervision during the course of this work. I am grateful that I had the opportunity to study in his lab.

Many people in his lab have helped me in many ways, special thanks to Drs. R.K. Sharma and K. Adachi, Mr. E. Wirch and Mrs. A.-M. Adachi.

I would also like to thank Therese for her valuable contributions.

Finally, I thank the Alberta Heritage Foundation for Medical Research for the funding they generously provided.

ABSTRACT

While the actions of calcium are not mediated by a universal mechanism, many are a result of the activation of calmodulin. The effects of calmodulin are due in part to the activation of protein kinases and phosphoprotein phosphatase(s) and the subsequent phosphorylation/dephosphorylation of regulatory enzymes. Calcineurin, a calmodulin-dependent phosphoprotein phosphatase, exhibits some of the characteristics of phosphotyrosyl-protein phosphatases, such as its ability to hydrolyse free phosphotyrosine and p-nitrophenylphosphate and its inhibition by Zn^{2+}.

Brain membranes were examined for potential phosphotyrosyl proteins which could be substrates for calcineurin. Rat brain membrane phosphoproteins, separated by SDS-PAGE, were subjected to alkali treatment. Alkali resistance has been used as an indicator of possible phosphotyrosyl-proteins since phosphotyrosine is more resistant to alkali hydrolysis than phosphoserine and phosphothreonine. Two peptides of Mr 50 kDa and 60 kDa were found to be markedly alkali-resistant. These phosphopeptides could be dephosphorylated by calcineurin and this dephosphorylation could be blocked by p-nitrophenylphosphate. The phosphorylation of these peptides could be stimulated by calmodulin and inhibited by the calmodulin inhibitor Compound 48/80. Elution of the peptides from the gel and phosphoamino acid analysis showed that the peptides did contain phosphotyrosine as well as phosphoserine and phosphothreonine (which were at levels 10 to 20 fold higher than phosphotyrosine). The amount of phosphotyrosine was increased 1 to 2 fold when the phosphorylation reaction contained calmodulin. Calmodulin stimulated serine and threonine phosphorylation 2 to 3 fold.

The putative peptide substrates for tyrosine phosphorylation were solubilized from a brain crude membrane preparation and partially purified by chromatography on AffiGel Blue and calmodulin-Sepharose 4B. The two peptides copurified and exhibited calmodulin-stimulated phosphorylation suggesting that the two peptides may be subunits of a calmodulin-dependent protein kinase which undergoes autophosphorylation. Calmodulin-stimulated phosphorylation of the purified peptides was on serine and threonine residues. Phosphotyrosine was faintly detectable in the 60 kDa peptide. It is an interesting possibility that these peptides which may be a calmodulin-stimulated serine-, threonine-protein kinase may also be regulated by a phosphotyrosine protein kinase. Experiments using angiotensin II as a substrate failed to show calmodulin-stimulated tyrosine protein kinase in synaptic membrane however there appeared to be calmodulin-stimulated activity in solubilized fractions. It is possible that the observed stimulation by calmodulin of the tyrosine phosphorylation of the 60 kDa and 50 kDa peptides in synaptic membrane is through a substrate effect.

In summary this thesis shows that calmodulin may be involved in the regulation of tyrosine phosphorylation/dephosphorylation of two brain peptides which may be a calmodulin-stimulated protein kinase. The findings of this work form a basis for future studies on the involvement of calmodulin in the function of tyrosine protein kinases.

ABBREVIATIONS

ATP	adenosine triphosphate
CaM	calmodulin
cpm	counts per minute
cyclic AMP	adenosine 3',5'-cyclic monophosphate
DEAE-cellulose	diethylaminoethyl-cellulose
DMSO	dimethylsulfoxide
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol bis (beta aminoethyl ether)-N,N,N',N'-tetraacetic acid
Hepes	4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid
LC20	phosphorylatable 20,000-dalton ligh chain of myosin
MLCK	myosin light chain kinase
NP40	nonidet P-40
OAc	acetate
PMSF	phenylmethylsulfonyl fluoride
PNPP	p-nitrophenyl phosphate
Pi	phosphate (inorganic)
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TLE	thin layer electrophoresis
Tris	tris (hydroxymethyl) aminoethane

CONTENTS

ACKNOWLEDGEMENT	ii
ABSTRACT	iii
ABBREVIATIONS	v
CONTENTS	vi
LIST OF FIGURES	viii
LIST OF TABLES	x

Chapter	page
I. INTRODUCTION	1
II. LITERATURE REVIEW	4
Introduction	4
Mechanism of Calmodulin Activation	6
The Calmodulin Regulatory System	10
Multifunctional Calmodulin-dependent Protein Kinase	12
Myosin Light Chain Kinase	13
Calcineurin	16
Tyrosine Protein Kinases	18
EGF Receptor Kinase	22
III. EXPERIMENTAL PROCEDURES	30
Materials	30
Chemicals	30
Preparation of Proteins	31
Calcineurin and Calmodulin	31
Calmodulin-Sepharose 4B Affinity Gel	31
Methods	31
Protein Concentration	31
Preparation of Synaptic Membrane	31
Tyrosine Protein Kinase Assay	32
Endogenous Phosphorylation	33
SDS-Polyacrylamide Gel Electrophoresis	34
Autoradiography of SDS Gels	35
Alkali Treatment of Polyacrylamide Gels	35
Electroelution of Proteins from Gels	35
Phosphoamino Acid Analysis	36

IV. Results 38

Alkali Resistance of Brain Phosphopeptides 38
Effect of DMSO on Brain Peptide Phosphorylation 43
Effect of Calmodulin and Calcineurin on Peptide Phosphorylation 44
Comparison of Brain Phosphopeptides with Phosphopeptides of Membranes with Known Tyrosine Protein Kinase Activity 47
Effect of Phosphatase Inhibitors on Calmodulin-stimulated Phosphorylation 48
Inhibition of Calcineurin Phosphatase Activity by PNPP 51
Calcium-Calmodulin Stimulation of Phosphorylation and Nickel Stimulation of Calcineurin Phosphatase Activity 56
Immunoblot Detection of Calcineurin In Rat Brain Synaptic Membrane 59
Dependence of Peptide Phosphorylation on Calmodulin Concentration 59
Effect of EGF and Insulin on Alkali-Resistant Phosphopeptides 64
Phosphoamino Acid Analysis of the 60-kDa and 50-kDa Phosphopeptides from Rat Brain Synaptic Membrane 67
Preparation of the 50-kDa and 60-kDa Phosphorylation Substrates 78
Membrane Preparation and Solubilization 78
AffiGel Blue Chromatography 79
Calmodulin-Sepharose 4B Affinity Chromatography 79
Copurification of 50-kDa Peptide, 60-kDa Peptide and Calmodulin-stimulated Protein Kinase Activity 84
Phosphoprotein pp74 from Chicken Brain 96

V. DISCUSSION 102

VI. CONCLUSIONS 111

REFERENCES 113

LIST OF FIGURES

Figure		Page
1.	Mechanism of Enzyme Activation by Calmodulin	7
2.	Alkali Resistance of Phosphopeptides from Rat, Chicken and Bovine Brain	39
3.	Effect of DMSO on the Phosphorylation of Brain Peptides and Resistance to Alkali	41
4.	Effect of Calmodulin and Calcineurin on Synaptic Membrane Phosphorylation	45
5.	Comparison of Synaptic Membrane Phosphorylation and Alkali Treatment with Membranes Containing Tyrosine Protein Kinase Activity	49
6.	Effect of Vanadate, PNPP and Calcineurin on Membrane Phosphorylation	52
7.	PNPP Blocks the Dephosphorylation by Calcineurin	54
8.	Dephosphorylation of Synaptic Membrane Phosphopeptides by Ni^{2+}.Calcineurin	57
9.	Immunoblot Detection of Calcineurin in Rat Brain Synaptic Membrane	60
10.	Dependence of the Phosphorylation of the 50 kDa and 60 kDa Peptides of Brain Synaptic Membrane on Calmodulin	62
11.	Effect of Insulin and EGF on Alkali-Resistant Phosphopeptides of Rat Brain Synaptic Membrane	65
12.	Rat Brain Synaptic Membrane Phosphorylation for Phosphoamino Acid Analysis	68
13.	Phosphoamino Acid Analysis of 50 kDa and 60 kDa Phosphopeptides after TCA Precipitation	70
14.	Phosphoamino Acid Analysis of 50 kDa and 60 kDa Phosphopeptides after Alkali Treatment	73
15.	Separation of Solubilized Proteins by AffiGel Blue Chromatography	80
16.	Calmodulin-Sepharose 4B Affinity Chromatography	82
17.	Endogenous Protein Kinase Activity in Solubilization from Rat Brain	85

18. Endogenous Protein Kinase Activity in Chromatography Fractions from AffiGel Blue and Calmodulin-Sepharose 4B Affinity Chromatographies 87

19. Phosphoamino Acid Analysis of Calmodulin-Binding Phosphopeptides after TCA Precipitation 89

20. Phosphoamino Acid Analysis of Calmodulin-Binding Phosphopeptides after Alkali Treatment 94

21. Chicken Brain Membrane pp74 from AffiGel Blue 97

22. Phosphoamino Acid Analysis of Phosphopeptides from AffiGel Blue KCl Fraction of Solubilized Chicken Brain Membrane 99

23. Phosphorylation of pp74 in the Calmodulin-Binding Fraction from Solubilized Chicken Brain Membrane 101

LIST OF TABLES

Table		Page
1.	Some Substrates for Tyrosine Protein Kinases	26
2.	Phosphoamino Acid Analysis of 60 kDa and 50 kDa Phosphopeptides from Rat Brain Synaptic Membrane	75
3.	Recovery of Radioactivity During Electroelution and Alkali Hydrolysis	76
4.	Partial Purification of the 60 kDa Phosphopeptide from Rat Brain Crude Membrane	91
5.	Partial Purification of the 50 kDa Phosphopeptide from Rat Brain Crude Membrane	92

CHAPTER I

INTRODUCTION

The importance of intracellular calcium ions as second messengers in bioregulation was first emphasized by Rasmussen (1970). Normally Ca^{2+} levels are extremely low intracellularly (10^{-8} to 10^{-7} M) and high extracellularly (10^{-3} M). Several "activators" of the cell such as hormones, mutagens, depolarizing currents, drugs and toxins cause a transient increase in cytosolic Ca^{2+} which is rapidly removed by membrane-bound calcium pumps. Several calcium binding proteins are involved in transducing this transient Ca^{2+} signal into physiological effects. These have been termed the calcium modulated proteins and they act as intracellular calcium receptors. These proteins have an affinity for calcium in the micromolar range and, since the intracellular level of Mg^{2+} is approximately 1 mM, they must show a selectivity for Ca^{2+} over Mg^{2+} by a factor of 10^3. Generally these calcium modulated proteins are low molecular weight proteins with an acidic isoelectric point. Among these proteins a great deal of sequence homology has been noted especially in the calcium-binding structure. This structure has been termed the E-F hand after the calcium binding loop of parvalbumin (Moews & Kretsinger, 1975; Kretsinger, 1976; Kretsinger, 1980). The homology is probably indicative of structural elements underlying their mechanism of response to calcium. This group of proteins containing the E-F hand calcium binding structure is sometimes referred to as the Troponin C superfamily.

With the widespread physiological effects of calcium it is suprising that the function of many of the calcium binding proteins is unknown. Only two Ca^{2+} binding proteins, calmodulin and troponin C, appear to have unequivocally established functions. Troponin C functions specifically as the subunit of the troponin complex which binds calcium and confers calcium sensitivity to the contraction process in striated muscle. Calmodulin appears to have a general Ca^{2+} mediating role. It is ubiquitous in eukaryotes and its structure has been highy conserved throughout evolution (Waisman et al., 1975). Calmodulin has been implicated in the regulation of many enzymes and in many different physiological processes (for reviews see Van Eldik et al., 1982; Levine & Dalgarno, 1983; Klee and Vanaman, 1982; Klee et al., 1980; Wang & Waisman, 1979; Cheung, 1980a; Cheung, 1980b; Means & Dedman, 1980; Watterson & Vincenzi, 1980).

Calmodulin was first discovered (Cheung, 1970; Cheung, 1971; Kakiuchi & Yamakazi, 1970) as an activator of cyclic nucleotide phosphodiesterase. Kakiuchi and Yamakazi (1970) found that cyclic nucleotide phosphodiesterase was stimulated by calcium and that addition of the activator could increase the calcium sensitivity. Teo and Wang (1973) subsequently showed that the activating factor was the calcium binding protein which conferred calcium sensitivity to cyclic nucleotide phosphodiesterase. Brostrom et al., (1975, 1976) showed that calcium/calmodulin also stimulated adenylate cyclase from brain and from glial tumor cells which led to the examination of several calcium sensitive systems for calmodulin effect. In many systems calmodulin was responsible for conferring calcium sensitivity and in 1978 Cheung et al., (1978) proposed that the ubiquitous protein activator be called calmodulin. Calmodulin

has many target proteins responsible for its cellular effects. In each mammalian tissue the set of target proteins will determine the specificity of Ca^{2+} action. Although some of these target proteins are enzymes with direct involvement in metabolic and other cellular processes, several calmodulin-binding proteins are protein kinases which exert their regulatory actions by phosphorylating other cellular proteins. In addition, at least one protein phosphatase, called calcineurin, that is regulated by calmodulin, has been demonstrated. Some of the calmodulin- stimulated protein kinases have a known function, such as myosin light chain kinase (Dabrowska et al., 1977; Adelstein et al., 1978), phosphorylase kinase (Cohen, 1978), glycogen synthase kinase (Ahmad et al., 1982, Payne & Solderling, 1980). In other cases calmodulin-dependent phosphorylation has been shown but the enzyme function of the phosphorylation target is not known.

This study was initiated to examine calmodulin-stimulated protein phosphorylation in brain. When calcineurin was shown to be a calmodulin-dependent phosphoprotein phosphatase with activity towards p-nitrophenyl phosphate and free phosphotyrosine, brain membrane was examined for phosphotyrosine-containing proteins and the effect of calmodulin on tyrosine phosphorylation was examined.

CHAPTER II

LITERATURE REVIEW

2.1 Introduction

Over 100 years have passed since Ringer (1883) first presented evidence that calcium ions were involved in the contraction of frog heart muscle. Over 30 years ago Heilbrun and Wiercinski (1947) showed that injection of calcium into a muscle fiber caused contraction. Since then calcium has been shown to be required for a variety of cellular functions including contractile and secretory processes (Pires et al., 1974; Adelstein & Conti, 1975; Dabrowska & Hartshorne, 1978), membrane ATPases (MacLennan & Holland, 1976; Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1977), cyclic nucleotide metabolism (Ho et al., 1972; Lin et al., 1974; Bradham et al., 1979; Brostrom et al., 1975) protein phosphorylation and dephosphorylation (Adelstein & Conti, 1975; Cohen et al., 1978, Srivastava et al., 1979; DeLorenzo, 1976; Krueger et al., 1977), regulation of mitosis (Berridge, 1975), microtubule assembly/disassembly (Snyder & McIntosh, 1976; Marcum et al., 1978) and proteolysis. For calcium to have these effects a calcium-binding protein must be present to transduce the Ca^{2+} signal into physiological effects. The first calcium-mediated protein to be shown was troponin (Ebashi & Kodama, 1965) which acts as the Ca^{2+}-dependent stimulator of contraction in striated muscle. Ca^{2+} binds to troponin C, one of the subunits of troponin, causing a conformational change which alters the relationship of troponin to actin. This change permits the actin to interact with myosin to produce actin-activated ATPase activity and contraction. Several other calcium-

binding proteins have been identified including parvalbumin but their functions are unknown. Calmodulin is the only other calcium-binding protein with unequivocally established functions. While troponin C is localized in striated muscle, calmodulin is present in most tissues and appears to have a general Ca^{2+} mediating role. Calmodulin is ubiquitous in eukaryotes (Waisman et al., 1975; Drabikowski et al., 1978) and its structure has been highly conserved throughout evolution (Goodman et al., 1979). Calmodulin has been implicated in the regulation of many proteins and in many different physiological processes. Calmodulin was discovered by W Y Cheung (Cheung, 1970a & b) and by Kakiuchi and Yamazaki (1970) as a protein activator of cyclic nucleotide phosphodiesterase (PDE). Kakiuchi and Yamazaki (1970) reported that PDE activity was stimulated by calcium and that addition of the protein activator increased calcium sensitivity. Teo and Wang (1973) showed that it was the protein activator which was the calcium-binding protein responsible for the calcium sensitivity of PDE.

Among the calcium-binding proteins a great deal of sequence homology has been noted especially in the calcium-binding structure termed the E-F hand, named after the calcium-binding loop found in parvalbumin (Moews & Kretsinger, 1975; Kretsinger, 1975; Kretsinger, 1980. This structure is formed by two helices, the E-helix and the F-helix, connected by a peptide loop that chelates the calcium ion. The sequence homology is probably indicative of the structural elements underlying their mechanism of response to calcium.

2.2 Mechanism of Calmodulin Activation

The helices by the Ca^{2+}-binding loops contain hydrophobic residues oriented on one side. When calcium binds a reorientation of the helices is triggered (Levine and Dalgarno, 1983), exposing several hydrophobic side chains and creating a hydrophobic binding domain. The Ca^{2+}-induced formation of a hydrophobic region was confirmed by fluorescent probe analysis (Tanaka & Hidaka, 1980; Laporte et al., 1980). This hydrophobic region participates in calmodulin-protein and calmodulin-drug interactions. Antipsychotic phenothiazine drugs bind to this hydrophobic domain preventing activated calmodulin from interacting with the target enzymes (Prozialeck, 1983).

Enzyme activation by calmodulin was originally viewed as a two-step process (Wang et al., 1975; Wolff & Brostrom, 1979) Figure 1a. In the first step calmodulin binds Ca^{2+} causing a conformational change to the activated form of calmodulin. This activated complex then binds to the target enzyme causing a conformation change, leading to activation of the target enzyme. When multiple Ca^{2+}-binding and Ca^{2+}-induced calmodulin-enzyme interactions are considered, a more global scheme for the mechanism of enzyme activation may be proposed as in Figure 1b (Chau et al., 1982; Wang et al., 1983). Several investigators (Blumenthal & Stull, 1980; Cox et al., 1981; Burger et al., 1983; Huang et al., 1980) have carried out studies exploring the nature of multiple Ca^{2+} binding and energy coupling. Steady state kinetic analysis of the activation of phosphodiesterase by calcium and calmodulin has led to the suggestion that the enzyme activation requires the binding of at least three and most likely all four calcium ions per calmodulin (Chau et al., 1982). Using a specific Ca^{2+} indicator,

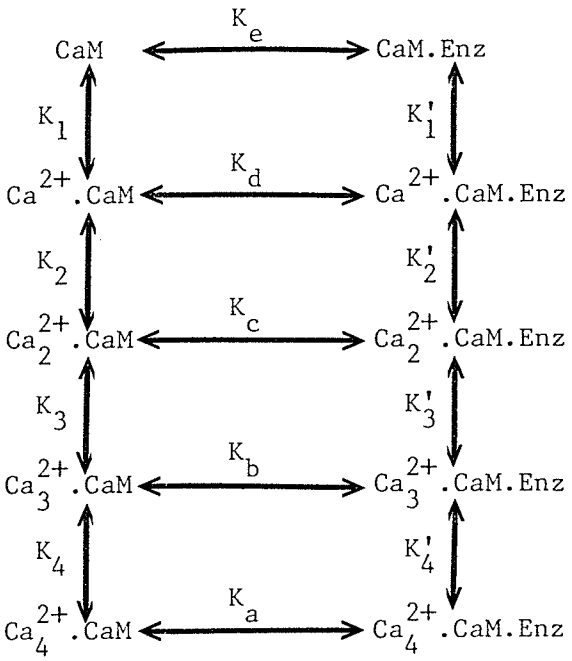
Mechanism of Enzyme Activation by Calmodulin[a]

A)

$$Ca^{2+} + CaM \longleftrightarrow Ca^{2+}.CaM \longleftrightarrow Ca^{2+}.CaM^{*}$$

$$Ca^{2+}.CaM^{*} + Enz \longleftrightarrow Ca^{2+}.CaM^{*}.Enz \longleftrightarrow Ca^{2+}.CaM^{*}.Enz^{*}$$

B)



[a]Modified from Wang et al. 1983. * denotes activated form.

Figure 1

1,2-bis-(O-aminophenoxy-ethane-N,N,N',N'-tetraacetic acid (Tsien, 1980), they found that the half life of the most rapidly dissociating Ca^{2+} from the Ca^{2+} Cm-PDE complex was found to be in the range of 150 to 200 msec. Using a rapid chemical quenching method to measure enzyme activity, it was found that the rate constant for inactivation of the fully activated phosphodiesterase by EGTA was 4.5 sec^{-1} which corresponds to a half life of 154 msec. Since this value is about the same as the value for the most rapidly dissociating Ca^{2+}, it supports the hypothesis that four Ca^{2+} per calmodulin are required to maintain the enzyme in its active state. Steady state kinetic studies of the activation of skeletal myosin light chain kinase have shown that four Ca^{2+} per calmodulin are also required for enzyme activation (Blumenthal & Stull, 1980). On the other hand, Cox and co-workers (Cox et al., 1981; Burger et al., 1983) have studied the kinetics of activation of phosphodiesterase, phosphorylase kinase and Ca^{2+}/Mg^{2+} ATPase by Ca^{2+} and calmodulin, and concluded from their results that activation depends on the binding of three Ca^{2+} per calmodulin. Since kinetic studies involve certain assumptions which are difficult to test experimentally, the discrepancies in the different studies are not totally unexpected.

As has been thoroughly discussed by Huang et al. (1981) there are several advantages associated with the multiple Ca^{2+}-binding. Analysis of the energy coupling between Ca^{2+} binding and Ca^{2+}-induced protein- protein interaction revealed that multiple Ca^{2+} binding is essential for the reversible protein association system. The dissociation constants for calmodulin and most of the target enzymes in the absence and presence of saturating levels of Ca^{2+} are $>10^{-5}$ and 10^{-9}-10^{-10} respectively. Thus, the binding of Ca^{2+} to calmodulin

enzymes. Since in most cells, Ca^{2+} concentrations fluctuate between 10^{-7} to 10^{-5} M, multiple Ca^{2+} binding to calmodulin is essential for protein-protein interactions. As is shown in Figure 1b, with the binding of four Ca^{2+}, each Ca^{2+} having an affinity toward the protein complex about one to two orders of magnitude higher than that for calmodulin alone, the affinity of calmodulin toward the enzyme can be increased more than 100,000 fold by Ca^{2+}.

In addition to being essential for the regulation via reversible protein-protein interaction, multiple Ca^{2+} binding may have other regulatory advantages. For example, it provides a highly cooperative activation of the enzymes by Ca^{2+} and it may also give more flexibility to the regulatory system. Thus different enzymes may be regulated by different forms of the calcium-calmodulin complexes.

Although most of the calmodulin-binding proteins are regulated by calmodulin according to the mechanism of Figure 1b, some alternate methods have been shown. Some Ca^{2+}-regulated enzymes may contain calmodulin as a subunit. Calmodulin is an integral subunit of phosphorylase kinase and remains bound to the enzyme even in the presence of EGTA (Cohen et al., 1978). Calmodulin has been shown to bind to other proteins in the presence of EGTA (Hooper & Kelly, 1984). Calmodulin also binds to a novel bovine brain protein (Andreasen et al., 1983) in the presence of EGTA but does not bind in the presence of calcium. This would indicate that in some cases calmodulin activation would not be by the mechanism in Figure 1b.

Because this thesis is primarily concerned with the interrelationship of calmodulin and tyrosine phosphorylation in the rat brain, the calmodulin regulatory system (including the multifunctional calmodulin-dependent protein

kinase, myosin light chain kinase and calcineurin, the calmodulin-dependent phosphoprotein phosphatase) and tyrosine protein kinases will be reviewed in some detail.

2.3 The Calmodulin Regulatory System

Cyclic AMP and calcium are two major second messengers in the cell. The effects of cyclic AMP in eukaryotes are mediated by only one mechanism, the activation of cyclic AMP-dependent protein kinase and the subsequent phophorylation of target enzymes. Two forms of cyclic AMP-dependent protein kinase are known but they have identical catalytic subunits and phosphorylate the same substrate proteins. The effects of cyclic AMP are determine by the protein substrates present in various tissues. Unlike cyclic AMP, the effects of calcium are not mediated by a universal mechanism; however, many are a result of the activation of calmodulin. The effects of calmodulin are in part due to the activation of protein kinases and phosphoprotein phosphatase(s) and the subsequent phosphorylation/dephosphorylate of target regulatory enzymes. The cyclic AMP regulatory system and the calmodulin regulatory system are interrelated at several levels (Pallen et al., 1985). They affect the amount of the other second messenger (Ca^{2+} calmodulin stimulates adenylate cyclase and cyclic nucleotide phosphodiesterase while cyclic AMP affects Ca^{2+} uptake) and they often act on the same target enzymes (the calmodulin binding proteins myosin light chain kinase and phosphorylase kinase are phosphorylated by cyclic AMP-dependent protein kinase) or they affect the phosphorylation level of the same target enzymes (synapsin I is phosphorylated by cyclic AMP-dependent protein kinase and calmodulin-stimulated protein kinase and is dephosphorylated

by calcineurin, a calmodulin-dependent phosphatase). Such a multi-level interrelationship allows these regulatory systems to enhance or oppose some or all of the effects of the other regulatory system and to have different effects in different tissues.

Calmodulin has been referred to as the major intracellular receptor for calcium in all non-muscle and smooth muscle cells (Means & Dedman, 1980). One important mechanism of action of calmodulin is the phosphorylation/dephosphorylation of target regulatory enzymes through the action of calmodulin-dependent protein kinases and phosphoprotein phosphatase(s). Calmodulin-stimulated phosphorylation of various cellular proteins has been demonstrated. These proteins include phospholamban (LePeuch et al., 1979), phosphorylase b (Cohen et al., 1978), myosin light chain (Dabrowska & Hartshorne, 1978), fodrin (Sobue et al., 1980), tubulin (Burke & DeLorenzo, 1981), tyrosine hydroxylase (Yamauchi & Fujisawa, 1980), tryptophan hydroxylase (Yamauchi & Fujisawa, 1979), brain protein (Iwasa et al., 1983) and synapsin I (Kennedy & Greengard, 1981; Schulman & Greengard, 1978). Specific protein kinases for some of these reactions, such as phosphorylase kinase (Cohen et al., 1978) and myosin light chain kinase (Dabrowska & Hartshorne, 1978) have been purified and found to phosphorylate a limited number of proteins. Myosin light chain kinase only phosphorylates the light chain of myosin. Phosphorylase kinase only phosphorylates itself, phosphorylase b and glycogen synthase D. Some of the other phosphorylations may be due to a general multifunctional calmodulin-dependent protein kinase (McGuinness et al., 1983). For example, the synapsin I kinase II from brain and glycogen synthase kinase from skeletal

muscle have similar substrate and phosphorylation site specificities, similar phosphopeptide map after proteolysis and show immunological cross reactivity.

2.3.1 Multifunctional Calmodulin-Dependent Protein Kinase

Schulman and Greengard (1978 a & b; Schulman, 1982) found calcium-dependent protein kinase activity in a wide variety of rat tissues. Each tissue showed a distinct patterm of protein phosphorylation reflecting the regulation of different processes. The kinase contains autophosphorylatable subunits of 50 kDa and 58/60 kDa but a monoclonal antibody has been produced that recognizes both subunits (Ouimet et al., 1984) and the ratio of the subunits is not constant in all regions of the brain (Walaas et al., 1983a, b). Recently similarities between the calmodulin-dependent protein kinases that have been isolated by different groups have been noted (Iwasa et al., 1983; Woodgett et al., 1984; McGuinness et al., 1983) suggesting that these may be isoenzymes of a multifunctional calmodulin-dependent protein kinase (McGuinness et al., 1983). When calmodulin-dependent glycogen synthase kinase purified from rabbit skeletal muscle was compared to soluble synapsin I kinase II from rat brain they were found to be very similar in several respects including substrate and site specificity, immunological cross-reactivity and phosphopeptide mapping following limited proteolysis. Glycogen synthase in skeletal muscle is phosphorylated on at least seven serine residues by five different protein kinases. Both the calmodulin-dependent glycogen synthase kinase and synapsin I kinase II phosphorylate the same sites on glycogen synthase (sites 2 and 1b) at a rate of 2 > 1b. On SDS-PAGE glycogen synthase kinase has subunits of 54 kDa and 58/59 kDa and synapsin I kinase II has subunits of 50 kDa and 58/60 kDa which

can be autophosphorylated when incubated with Mg^{2+}-ATP, Ca^{2+} and calmodulin.

A monoclonal antibody that reacts with the bands of synapsin I kinase II was found to cross react with the bands of glycogen synthase kinase. Proteolysis and phosphopeptide mapping showed the 58 kDa phosphopeptide of synapsin kinase to contain several phosphopeptides in common with the 54- and 58/59 kDa bands of glycogen synthase kinase although some differences were noticed wth the 50 kDa subunit. The calmodulin-dependent trypotophan monooxygenase kinase is probably the same enzyme as synapsin I kinase II (Woodgett et al., 1984) as is the enzyme of Iwasa et al. (1983), the calmodulin-dependent MAP-2 protein kinase (Schulman, 1984) and the muclear matrix-associated calmodulin-dependent protein kinase (Sahyoun et al., 1984a,b, 1985). These enzymes may represent a class of Ca^{2+}, calmodulin-dependent protein kinases.

2.3.2 Myosin Light Chain Kinase

Although the components of the contractile apparatus of striated muscle, smooth muscle and nonmuscle cells are similar, the mechanisms that regulate contraction are believed to be different. Unlike striated muscle where troponin and tropomyosin are responsible for primary regulation (Huxley, 1972; Ebashi, 1976), in smooth muscle the calcium dependent phosphorylation of the 20,000-dalton light chain (LC20) is a prerequisite for actin activation of actomyosin MgATPase activity (Adelstein & Eisenberg, 1980; Walsh & Hartshorne, 1982). Phosphorylation of LC20 was first shown by Perrie et al., 1972, 1973. Myosin light chain kinase (MLCK), the enzyme that catalyzes the phosphorylation of LC20, was first isolated from skeletal muscle (Pires et al.,

1974) and has since been shown to be present in cardiac muscle (Frearson & Perry, 1975), smooth muscle (Dabrowska et al., 1977; Adelstein et al., 1978; Vallet et al., 1981) and in nonmuscle cells such as platelets (Daniel & Adelstein, 1976), macrophages (Trotter & Adelstein, 1979), brain (Dabrowska & Hartshorne, 1978) and fibroblasts (Muhlrad & Oplatka, 1977; Yerna et al., 1979). MLCK is highly specific for serine-19 of LC20 (Maita et al., 1981a), a phosphorylation site surrounded by a highly conserved sequence of amino acids (Perrie et al., 1973; Jakes et al., 1976; Matsuda et al., 1977; Maita et al., 1981a) Myosin from smooth muscle and nonmuscle cells cannot be actin-activated unless this serine is phosphorylated (Sellers et al., 1981; Maita et al., 1981b). The P-light chain of vertebrate skeletal muscle myosin is also phosphorylated by MLCK but the function is unknown.

MLCK is activated by Ca^{2+}-calmodulin and is inactive in the absence of calmodulin or at Ca^{2+} concentrations found in the resting cell (Adelstein & Eisenberg, 1980). The Ca^{2+} dependence of LC20 phosphorylation was first noted by Sobieszek (1977a,b) and Bremel et al. (1977) who suggested this may be the mechanism regulating the actin-myosin interaction. The requirement for calmodulin was first demonstrated for chicken gizzard MLCK (Dabrowska et al., 1977; Dabrowska & Hartshorne, 1978) and was subsequently shown in turkey gizzard (Adelstein et al., 1978), skeletal muscle (Yazawa & Yagi, 1977; Barylko et al., 1978), platelets (Dabrowska & Hartshorne, 1978), brain (Dabrowska & Hartshorne, 1978), cardiac muscle (Walsh et al., 1979), fibroblasts (Yerna et al., 1979) and aorta (Vallet et al., 1981). The active species of MLCK contains kinase and calmodulin in a 1:1 molar ratio (Adelstein & Klee, 1981) with all four divalent metal-binding sites occupied by calcium (Blumenthal & Stull, 1980; Stull

et al., 1982). MLCK is very susceptible to proteolysis to lower molecular weight forms which retain catalytic activity (Pires & Perry, 1977) but may be Ca^{2+}-calmodulin independent. The Ca^{2+}-calmodulin independent MLCK from platelets (Daniel & Adelstein, 1976) was found to be due to proteolysis which removed the calmodulin-binding site during the preparation (Hathaway & Adelstin, 1979). Several molecular weights have been reported for MLCK due to either the presence of isozymes or proteolysis (Hathaway & Adelstein, 1979; Walsh et al., 1980a; Adachi et al., 1983). It has been reported to be 105 kDa in chicken gizzard (Dabrowska et al., 1977; Mrwa & Hartshorne, 1980) but recently a 136 kDa MLCK was detected using a monoclonal antibody (Adachi et al., 1983; Ngai et al., 1984); 125-136 kDa in turkey gizzard (Adelstein et al., 1978; Adelstein & Klee, 1981; Adachi et al., 1983); 77-90 kDa or 155 kDa in skeletal muscle (Pires & Perry, 1977; Yazawa & Yagi, 1978) and 85 kDa, 94 kDa or 155 kDa in cardiac muscle (Walsh et al., 1979; Wolf & Hofman, 1980; Walsh & Guilleux, 1981).

In gizzard, activation by Ca^{2+} may be confined to its effect though MLCK (Small & Sobieszek, 1977; Chacko, 1981; Sherry et al., 1978) but this may not be the case in other smooth muscles where Ca^{2+} can cause a further stimulation of actin-dependent ATPase when added to phosphorylated myosin (Chacko et al., 1977; Chacko & Rosenfeld, 1982) suggesting that other regulatory mechanisms such as the leiotonin system (Hirata et al., 1977; Ebashi et al., 1982) may also have an effect on activity.

Ca^{2+}-calmodulin can also modify the phosphorylation and regulation of MLCK by cyclic AMP-dependent protein kinase (Adelstein, 1982). Some of the lower molecular weight forms of MLCK are not substrates for cyclic

AMP-dependent phosphorylation but the higher molecular weight forms are (Walsh & Hartshorne, 1982). In the absence of calmodulin, cyclic AMP-dependent protein kinase can phosphorylate two sites on smooth muscle MLCK (Adelstein et al., 1978; Conti & Adelstein, 1981). The two sites are close together as limited tryptic digestion releases a 22 kDa peptide with both phosphorylation sites. If MLCK is phosphorylated in the presence of calmodulin only one site is phosphorylated. Phosphorylation of both sites decreases the affinity of MLCK for calmodulin and dephosphorylation restores the calmodulin-binding and thus full enzyme activity. The diphosphorylated form of MLCK is less active than the monophosphorylated or unphosphorylated form implicating the site that is blocked by calmodulin as the important site in decreasing calmodulin binding (Conti & Adelstein, 1981). Smooth muscle phosphatase-1 (Pato & Adelstein, 1983) will dephosphorylate both sites on MLCK in the absence of calmodulin. In the presence of calmodulin only one site is dephosphorylated. Interestingly the site that is dephosphorylated when calmodulin is bound to MLCK is the site whose phosphorylation by cyclic AMP is blocked by calmodulin. This suggests that calmodulin exhibits an alternative mechanism for regulating target enzymes beside direct Ca^{2+}-dependent activation. Calmodulin may cause conformational changes in target enzymes that can modify their susceptibility to regulation by other regulatory processes such as phosphorylation.

2.3.3 Calcineurin

Calcineurin was first detected as a heat-labile factor that could inhibit the activation of cyclic nucleotide phosphodiesterase by virtue of its binding to calmodulin (Wang & Desai, 1977). It was shown to be a major Ca^{2+}-binding and calmodulin-binding protein in brain extracts (Klee & Krinks, 1978). It was shown

to consist of two subunits, termed A with a molecular weight of approximately 61 kDa and B with a molecular weight of 19 kDa (by amino acid sequence, Aitken et al., 1984, but has an apparent Mr of 15 kDa on SDS gels). The A subunit binds calmodulin in a Ca^{2+}-dependent manner and is responsible for catalytic activity. The B subunit contains four Ca^{2+}-binding sites and shows sequence homology with calmodulin and troponin C (Aitken et al., 1984). It has been demonstrated that calcineurin is a calmodulin-stimulated phosphoprotein phosphatase (Stewart et al., 1982). It was originally thought that calcineurin was specific for nervous tissue (Klee et al., 1979). Radioimmunoassay (Wallace et al., 1980) confirmed that brain had a high level of calcineurin while heart, skeletal muscle, liver and other tissues had a low level of calcineurin. Using a phosphatase assay that was specific for Ca^{2+}-calmodulin stimulated activity it was found (Ingebritsen et al., 1983) that levels in skeletal muscle were higher than brain while liver contained about half as much as brain (skeletal muscle, brain and liver contained 8.1, 6.1 and 3.4 units of phosphoprotein phosphatase 2B/g wet weight). It may be that calcineurin is an isozyme that belongs to a class of calmodulin-stimulated phosphoprotein phosphatases (protein phosphatase-2B). It was initially thought that calcineurin had a narrow substrate specificity and only dephosphorylated the alpha-subunit of phosphorylase kinase, inhibitor-1 and myosin light chains (Ingebritsen & Cohen, 1983). It is now known to dephosphorylate a large number of compounds (Pallen & Wang, 1985) including phosphoseryl- and phosphothreonyl-proteins (Stewart et al., 1982, 1983; King et al., 1984), phosphotyrosyl proteins including the EGF receptor (Pallen CJ, Valentine KA, Wang JH, Hollenberg MD, manuscript in submission), phosphotyrosyl-casein and phosphotyrosyl histone (Chernoff et al., 1984) and

some nonprotein phosphocompounds. Calcineurin dephosphorylates free phosphotyrosine while it has low activity towards free phosphoserine or phosphothreonine. Calcineurin also hydrolyzes p-nitrophenyl phosphate which is structurally similar to phosphotyrosine (Pallen & Wang, 1983). Phosphoprotein phosphatase from rabbit muscle, which hydrolyzes phosphoserine residues much faster than phosphotyrosine, has little activity towards p-nitrophenyl phosphate (Swarup et al., 1981). The ability to hydrolyze p-nitrophenyl phosphate appears to be a property of phosphotyrosyl-protein phosphatases (Chernoff et al., 1983; Leis & Kaplan, 1982; Cobb & Rosen, 1984). The activity of calcineurin is inhibited by vanadate and zinc (Tallant & Cheung, 1984). Calcineurin appears to have some of the characteristics of phosphotyrosyl-protein phosphatases (Swarup et al., 1982; Brautigan et al., 1981; Gallis et al., 1981; Horlein et al., 1982). Little is known, however, about the physiological substrates for calcineurin.

This suggested to us that there could be phosphotyrosyl proteins in brain that could act as substrates for calcineurin and further that this would indicate the presence of tyrosine specific protein kinase activity in brain, an intriguing possibility since tyrosine protein kinases are generally associated with growth effects and brain is a relatively nonproliferative tissue.

2.4 Tyrosine Protein Kinases

Phosphorylation of proteins at tyrosine residues is a relatively new discovery. While most normal cells contain phosphotyrosine, some virally-transformed cells contain up to tenfold higher amounts of phosphotyrosine (Eckhart et al., 1979; Hunter & Sefton, 1980). In 1979 Eckhart et al. reported that immunoprecipitates from polyoma virus-infected cells and antipolyoma

tumor antiserum contained a novel activity that could phosphorylate the 60,000 dalton large tumor antigen of polyoma virus on a tyrosine residue. Later Hunter and Sefton (1980) examined the protein kinase activity that is associated with $pp60^{src}$, the transforming gene product of the src gene of Rous sarcoma virus (RSV). Using an immunoprecipitated $pp60^{src}$ which phosphorylates the immunoglobulin heavy chain (Levinson et al., 1978; Rubsamen et al., 1979; Collett & Erikson, 1978) they found the site of phosphorylation was a tyrosine residue. Hunter and Sefton (1980) also showed that the closely related cellular homogue of viral $pp60^{src}$ (Rohrschneider et al., 1979) present in all vertebrate cells, also possessed tyrosine kinase activity. Autophosphorylation of $pp60^{src}$ occurred at a tyrosine residue. $pp60^{src}$ also contains a serine residue in the NH_2-terminal half of the molecule which is also a site of phosphorylation (Collett et al., 1979a)

Since then six of the proteins (or oncogene products) that cause viral transformation have been shown to possess tyrosine protein kinase activity; v-src (Hunter & Sefton, 1980; Collett et al., 1980; Levinson et al., 1980), v-yes (Kawai et al., 1980), v-fgr (Naharro et al., 1983, 1984), v-fps/fes (Pawson et al., 1980; Barbacid et al., 1980; Feldman et al., 1980; Neil et al., 1981), v-abl (Witte et al., 1980) and v-ros (Feldman et al., 1982).

There is a great deal of homology in the domain responsible for kinase activity, homology with each other and homology with the catalytic subunit of cAMP-dependent protein kinase (Beemon, 1981; Barker & Dayhoff, 1982; Kitamura et al., 1982; Hampe et al., 1982; Shibuya & Hanafusa, 1982; Reddy et al., 1983; Privalsky et al., 1984).

Normal cells contain genes (Proto-oncogenes) homologous to the viral transforming genes shown by the binding of cDNA probes (Stehelin et al., 1976; Spector et al., 1978a). The sequence homology between the homologues and the viral transforming proteins is high especially in the catalytic domain. Two of these normal cell homologous proteins have been shown to have tyrosine protein kinase activity, the product of c-src ($pp60^{c\text{-}src}$, Hunter & Sefton, 1980; Collett et al., 1978; Oppermann et al., 1979) and the product of c-fps/fes (NCP98, Mathey-Prevot et al., 1982). The product of c-abl ($NCP\text{-}150^{c\text{-}abl}$) has been isolate but no tyrosine protein kinase activity was demonstrated (Ponticelli et al., 1982) although an altered form in a leukemia cell line does have tyrosine protein kinase activity (Konopka et al., 1984).

The viruses are believed to have acquired the oncogene from the host cell by a recombination between the genome of the infecting retrovirus and the host (Bishop, 1981; Bishop, 1983; Duesberg, 1983). Many of the tumors that are not caused by viruses may be due to activation of cellular proto-oncogenes (Tabin et al., , 1982). Slamon et al. (1984) examined 20 different types of human tumors using hybridization of mRNA to v-onc-specific probes. They found that more than one c-onc was expressed in all the tumors examined and higher levels of oncogene expression occurred in tumor vs. normal tissue in some cases. Src, fes and abl were expressed in some tumors while erbB and yes were not observed in any of the 54 tumors examined.

Carcinogens may convert proto-oncogenes into oncogenes by causing increased levels of normal c-onc gene product (dosage hypothesis), expression of activity at the wrong time or in the wrong type of cell or by causing structural changes which would produce a structurally aberrant gene product with possible

loss of regulation sites or modified activity. There is some evidence that all these mechanisms are in fact plausible explanations for different cases (Hunter, 1984).

While both $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$ have tyrosine protein kinase activity they have structural differences especially in the COOH terminal. Residues 519-538 of $pp60^{c\text{-}src}$ are completely deleted in $pp60^{v\text{-}src}$ whose residues 519-530 come from a sequence about one kilobase further downstream (Takeya & Hanafusa, 1983; Swanstrom et al., 1983; Wyke, 1983). This altered structure may be the reason that elevated levels of $pp60^{c\text{-}src}$ do not promote transformation (Parker et al., 1984). This suggests that $pp60^{c\text{-}src}$ may be susceptible to regulation to which $pp60^{v\text{-}src}$ is not subject because of its altered structure. The middle tumor antigen of polyoma virus appears to have no protein kinase activity (Schaffhausen et al., 1982) but it is phosphorylated and has an associated protein kinase activity due to its association with $pp60^{c\text{-}src}$ (Courtneidge & Smith, 1983, 1984). Bolen et al. (1984) found that the association of middle T antigen with $pp60^{c\text{-}src}$ caused a severalfold increase in tyrosine kinase activity. $pp60^{c\text{-}src}$ may require an activator protein for expression of activity that has been missed.

Tyrosine phosphorylation also appears to occur in some cases of receptor-mediated phosphorylation and cell activation. Tyrosine protein kinase has been shown to be an activity associated with the epidermal growth factor (EGF) receptor (Ushiro & Cohen, 1980; Cohen et al., 1980; Carpenter et al., 1978), the platelet-derived growth factor (PDGF) receptor (Ek et al., 1982; Ek & Heldin, 1982), the insulin receptor (Kasuga et al., , 1982b, Petruzelli et al.,

1982, 1984; Cobb & Rosen, 1984) and the insulin-like growth factor I (somatomedin C) receptor (Jacobs et al., 1983).

The first step in hormone action is the binding of the hormone to specific cellular receptors. The next step is a transduction of the hormone signal across the plasma membrane to the post receptor level. Phosphorylation may be part of the mechanism for transduction of the receptor signal for some hormones.

2.4.1 EGF Receptor Kinase

EGF is a 6045 dalton peptide and binding of EGF to its receptor stimulate proliferation of a wide variety of cells. For recent reviews of EGF and the EGF receptor kinase refer to Carpenter (1983), Carpenter & Cohen (1979) and Soderquist & Carpenter (1983). One of the first events triggered by EGF binding is the activation of a tyrosine protein kinase which phosphorylates a number of cellular proteins including the EGF receptor itself (Carpenter et al., 1978; Cohen et al., 1980; Ushiro & Cohen, 1980; Hunter & Cooper, 1981). The endogenous EGF receptor is 170,000 daltons (Cohen et al., 1982) which can be converted to a 150,000 dalton form by a Ca^{2+}-dependent neutral protease (Gates & King, 1982). While the binding of EGF is similar for the two forms, the tyrosine protein kinase activity of the 170 kDa form is five to ten times greater in terms of EGF stimulated autophosphorylation. Carlin & Knowles (1982) reported that both forms from A431 cells contain phosphotyrosine and phosphothreonine but only the 170 kDa form contains phosphoserine. EGF-stimulated receptor phosphorylation decreases with receptor down regulation (Fernandez-Pol, 1981). The EGF receptor, the EGF-stimulated tyrosine protein kinase and the autophosphorylatable sites appear to be parts of the same polypeptide (Buhrow et al., 1982, 1983; Cohen et al., 1982; Cohen et

al., 1980). Unlike some receptors, the EGF receptor is not recycled after internalization so the internalization and degradation process may play a role in the translocation of the tyrosine protein kinase. EGF binding to A431 cells causes a rapid increase in phosphorylation (Carpenter et al., 1978, 1979). In these cells EGF inhibits proliferation (Gill & Lazar, 1981; Barnes, 1982). Some variants of A431 cells that are resistant to the inhibitory effects of EGF also show decreased EGF-stimulated tyrosine protein kinase activity (Buss et al., 1982) a further correlation of tyrosine phosphorylation and growth effects.

Other proteins are also phosphorylated including an 80 kDa protein, a 22.5 kDa protein (King et al., 1980) and the 36 kDa protein which is also phosphorylated by vital transformation (Hunter & Cooper, 1981). Histone, protamine (Carpenter et al., 1979), tubulin (Cohen et al., 1982), myosin light chain (Gallis et al., 1983) and the IgG heavy chain of the antibody to $pp60^{v\text{-}src}$ (Chinkers & Cohen, 1981; Kudlow et al., 1981) will serve as substrates for the EGF receptor/kinase, as will several small peptides including analogs of the site of tyrosine autophosphorylation in $pp60^{src}$ (Pike et al., 1982; Erneux et al., 1983) and an analog of gastrin 22-30 (Baldwin et al., 1982). Phosphorylation of the src peptide suggests that the tyrosine kinases may be able to interact with each other to regulate their activity. This suggested interrelationship with other systems may indeed be important. PDGF has been shown to inhibit EGF binding (Bowen-Pope et al., 1983). Cyclic AMP plus purified cyclic AMP-dependent protein kinase or purified catalytic subunit phosphorylates the solubilized EGF receptor stimulating tyrosine kinase activity threefold without changing the level of tyrosine phosphorylation of the receptor (Ghosh-Dastidar & Fox, 1984). Segawa and Ito (1982, 1983) found that EGF could stimulate tyrosine

phosphorylation of the middle T antigen of polyoma virus, an effect that could either be direct or through an effect on $pp60^{c\text{-}src}$ which is tightly associated with a subfraction of middle T antigen (Courtneidge & Smith, 1983). Gastrin-17 is also phosphorylated by EGF-stimulated tyrosine kinase (Baldwin, 1982, 1983a) which is interesting since gastrin has sequence homology with polyoma virus middle T antigen and acts as a growth factor for cells of the intestinal tract (Baldwin, 1982)

Harvey and Kirsten sarcoma viruses transforming proteins have threonine kinase activity and can autophosphorylate themselves. EGF-stimulated tyrosine kinase can phosphorylate a peptide analog of the autophosphorylation site on a tyrosine residue (Baldwin et al., 1983b). This has not been shown in vivo but it suggests a possible connection between tyrosine and threonine phosphorylation in transformation. When transformed by RNA tumor viruses, some cell lines lose EGF binding (Todaro et al., 1976) due to the production of polypeptide transforming growth factors which bind to the EGF receptors and whose biological effects can be blocked by anti-EGF receptor antibodies which block EGF binding (Carpenter et al., 1983). The product of the erbB gene of avian erythroblastosis virus shows sequence homology with several retroviral tyrosine protein kinases (Privalsky et al., 1984). Downward et al. (1984) sequenced several peptide fragments of the EGF receptor and found that six of the peptides were almost identical to sections of the erbB sequence (Yamamoto et al., 1983). Several other peptides showed no homology, possibly because they were from the EGF-binding domain of the receptor. This suggests that erbB may have arisen from the kinase domain of the EGF receptor. It is possible that this viral oncogene causes transformation by a constant expression of EGF

receptor-like activity. The similar response of cells to growth factors such as EGF and insulin may be due to similar substrate specificities of their receptor kinases. Pike et al. (1984) showed that purified receptors for insulin and EGF from solubilized placenta membranes had similar substrate specificities, had a preference for Mn^{2+} over Mg^{2+} and showed a strong selectivity for ATP over GTP. The involvement of the tyrosine protein kinase activity of the insulin and EGF receptors in the mechanism of action of these polypeptide growth factors and the steps between ligand binding to the receptor and the resulting stimulation of DNA synthesis in the nucleus have yet to be elucidated (Carpenter, 1983).

Most of the tyrosine protein kinases that have been reported are associated with transformation (uncontrolled growth) by viruses or are associated with a normal growth-regulating process (e.g. EGF or PDGF). This leads to the hypothesis that tyrosine phosphorylation is one of the mechanisms controlling growth and some transforming viruses use this mechanism to cause uncontrolled growth. There are several ways that the viral oncogene could cause transformation while the cellular homologous gene (proto-oncogene) does not. The higher levels of tyrosine phosphorylation may be due to a higher quantity of the transforming protein produced, expression of transforming protein activity in cells which don't show homolog expression or structural changes may have occurred which modify the activity of the gene product increasing its ability to transform cells. If indeed tyrosine phosphorylation is an important modification it will be shown by the substrates that are phosphorylated by the tyrosine protein kinase (Table 1).

Table 1

Some Substrates for Tyrosine Protein Kinases[a]

Mr	Identity	Tyrosine Protein Kinase	Reference
170 kDa	EGF receptor	Insulin receptor	Pike et al., 1984
130 kDa	Vinculin	$pp60^{v\text{-}src}$	Sefton et al., 1981
120 kDa	Liver EGF receptor?	EGF receptor	Ehrhart et al., 1981
95 kDa	Insulin receptor	EGF receptor	Pike et al., 1984
95 kDa	Band 3	Endogenous	Dekowski et al., 1983
81 kDa	?	EGF receptor	Hunter&Cooper 1981
		ST-FeSV	Hunter&Cooper 1983
50 kDa	Tyrosine Protein Kinase	$pp60^{v\text{-}src}$ $p105^{gagfps}$ $p90^{gagyes}$	Hunter&Sefton 1980 Hunter&Cooper 1984
46 kDa	Enolase	$pp60^{v\text{-}src}$	Cooper&Hunter 1983 Cooper et al., 1984
45/42 kDa	?	EGF receptor	Cooper et al., 1982
		PDGF receptor $pp60^{v\text{-}src}$	Martinez et al., 1982
36 kDa	Ca^{2+} modulated	EGF receptor $pp60^{v\text{-}src}$	Fava&Cohen 1984 Erikson&Erikson, 1980
35 kDa	Lactate dehydrogenase	$pp60^{v\text{-}src}$	same as 46 kDa
28/29 kDa	Phosphoglucerate mutase	$pp60^{v\text{-}src}$	same as 46 kDa

There are several problems that arise when the substrates of TPK are examined. One problem is that although only a few substrates have been identified, as yet is has been impossible to correlate their phosphorylation with some change in function that could result in growth effects. Three of the substrates, phosphoglycerate mutase, enolase and lactate dehydrogenase, are glycolytic enzymes which are phosphorylated on tyrosine in RSV-transformed cells (Cooper Hunter, 1983; Cooper et al., 1984); however, only 1% to 10% is phosphorylated, no obvious changes in the net rate of the steps catalyzed by these enzymes was noted and none of these enzymes is usually considered to be rate limiting for glycolysis.

Substrates appear to be phosphorylated poorly in vivo, typically less than 10% of the substrate is phosphorylated at a tyrosine residue. Vinculin, for example, is a substrate for $pp60^{src}$. It is present in adhesion plaques and may serve to link the microfilament bundles and the plasma membrane. Since cells change shape upon transformation, going from a flattened shape to a more rounded shape and also show less surface adhesion, vinculin would be a prime candidate for mediating these effects; however, in these virally transformed cells only 1% of the vinculin is phosphorylated on tyrosine, probably too few proteins to produce this effect. Some RSV mutants have been produced which show some of the phenotypic changes associated with transformation but it doesn't correlate with vinculin phosphorylation.

Transformation appears to be associated with proteins that have tyrosine protein kinase activity. While it is possible that the transforming proteins have other activities such as the activity towards glycerol or phosphatidylinositol that have been noted, it is probably the tyrosine protein kinase activity that is

responsible for the growth or transformation effects. One suggestion is that other protein kinases and other regulatory systems are involved with the tyrosine protein kinases. In vitro the insulin receptor has tyrosine protein kinase activity which phosphorylates the receptor itself. In vivo receptor phosphorylation also occurs on threonine and serine residues and insulin stimulates actin phosphorylation but at a serine residue (Kasuga et al., 1982). This suggests that some of the growth and transformation effects of tyrosine protein kinases may be mediated by other protein kinases which are not specific for tyrosine.

In normal cells the phosphorylated residues are approximately 90% phosphoserine, 10% phosphothreonine and less than 0.1% phosphotyrosine (Sefton et al., 1980). With phosphotyrosine such a minor component quantitatively, it was necessary to use techniques to enhance or distinguish phosphotyrosine from phosphoserine and phosphothreonine. Since phosphoserine and phosphothreonine are more susceptible to alkaline hydrolysis, it was possible to separate the ^{32}P-labeled proteins by SDS-PAGE followed by an alkali treatment of the gel to enrich for phosphotyrosyl proteins (Cooper & Hunter, 1981; Cheng & Chen, 1981). The putative phosphotyrosyl-proteins can then be identified by comparison of the autoradiogram of the treated and untreated gels. Unfortunately, this procedure does not give conclusive results. Because of the local environment of the phosphate group, some phophotyrosyl residues are not resistant to alkali and some phosphothreonyl residues are resistant (Cooper et al., 1983). The identity of the phosphorylated residue has to be confirmed by hydrolysis of the peptide and analysis of the release phosphoamino acids.

Chromatography of solubilized proteins on Affi-Gel Blue was used to enrich for protein kinases and remove protein phosphatases. Affi-Gel Blue contains the dye Cibacron Blue F_3GA which exhibits specific binding for proteins with a nucleotide binding site. Gel matrix incorporating this dye has been shown to have an affinity for kinases such as cyclic AMP-dependent protein kinase and cyclic GMP-dependent protein kinase (Witt & Roskoski, 1975; Kobayashi & Fang, 1976) but does not bind calcineurin (Sharma et al., 1983). It is possible that some protein kinase substrates could be removed by this procedure but usually the most prominent substrates for tyrosine protein kinases in membranes have been the tyrosine kinases themselves (autophosphorylation).

CHAPTER III

EXPERIMENTAL PROCEDURES

3.1 MATERIALS

3.1.1 Chemicals

[γ-^{32}P]ATP (3000 Ci/mol) was purchased from Amersham Radiochemicals. Phosphoserine, phosphothreonine, phosphotyrosine, PMSF, PNPP, sodium orthovanadate, compound 48/80, glyceraldehyde-3-phosphate dehydrogenase, and ninhydrin spray reagent were from Sigma. [Val^5]-Angiotensin II was from Peninsula Laboratories. AffiGel Blue and the SDS-PAGE reagents were from BioRad. Standards for calculating apparent molecular weights, phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and alpha-lactalbumin (14,400), were from Pharmacia. Calcineurin (alpha subunit; 61,000) and glyceraldehyde-3-phosphate dehydrogenase (36,000) were added to complete the molecular weight standards. The UltEmit marker was from New England Nuclear. XOmat AR film and cellulose-coated Chromogram Sheets were from Kodak. SpeedVac apparatus and the thin layer electrophoresis apparatus were from Savant.

3.1.2 Preparation of proteins

3.1.2.1 Calcineurin and calmodulin

Bovine brain calcineurin was purified by the method of Sharma et al. (1983). Bovine brain calmodulin was prepared at the same time by washing the DEAE-cellulose column with 0.5 M NaCl buffer. The crude calmodulin fraction was then purified using hydrophobic interaction chromatography (Gopalakrishna & Anderson, 1982). Concentration was determined by using E_{278nm} = 9.8 for calcineurin (Sharma et al., 1979) and E_{278nm} = 2.0 for calmodulin. The calcineurin and calmodulin used in this study were generously provided by Dr. R.K. Sharma.

3.1.2.2 Calmodulin-Sepharose 4B affinity gel

Calmodulin-Sepharose 4B affinity gel was prepared according to the divinylsulfone activation method of Sairam and Porath (1976) as modified by Sharma et al. (1983).

3.2 METHODS

3.2.1 Protein concentration

Protein concentrations were determined by the dye-binding method of Bradford (1976) using bovine serum albumin as standards. Bovine serum albumin concentration was determined using the extinction coefficient $E_{280\ nm}$ = 6.60.

3.2.2 Preparation of synaptic membrane

The method used was a modification of the procedure used by Schulman and Greengard (1978a). All procedures were carried out at 4°C or on ice. All solutions contained 0.3 mM PMSF made fresh from a 0.15M PMSF solution in ethanol. Two male Sprague Dawley rats (~250g each) were decapitated. The

cortices were removed and homogenized in 10 volumes of ice-cold 0.32 M sucrose, 5 mM Tris-HCl (pH 7.4) containing 0.5 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.3 mM PMSF using 10 strokes of a motorized pestle in a 55 ml Potter Elvjehm homogenizer (0.125 mm clearance). The homogenate was centrifuged for 10 min at 1000 x g. The supernatant was saved, while the pellet was rehomogenized in 10 volumes of sucrose buffer and recentrifuged for 10 min at 1000 x g. The supernatants were combined and recentrifuged for 10 min at 1000 x g. The pellet was discarded and the supernatant was centrifuged at 12,500 x g for 15 min. The pellet was resuspended in 5 volumes of sucrose buffer and recentrifued for 15 min at 12,500 x g. The pellet is subjected to osmotic shock by resuspension and homogenization in 20 volumes of ice-cold 0.3 mM PMSF. This suspension was stirred in the cold room for 30 min followed by centrifugation for 30 min at 20,000 x g. To remove some of the endogenous calmodulin, the pellet was generally resuspended in 0.32 M sucrose, 5 mM Tris (pH 7.4), 10 mM EDTA, 5 mM EGTA, 0.3 mM PMSF and recentrifuged for 30 min at 20,000 x g. The pellet is resuspended in 0.32 M sucrose, 5 mM Tris HCl (pH 7.4), 0.3 mM PMSF. Aliquots of synaptic membrane were stored at -80°C.

3.2.3 Tyrosine protein kinase assay

Several different peptides can serve as substrates for tyrosine protein kinases (Hunter, 1982; Casnellie et al., 1982b; Swarup et al., 1983; Wong & Goldberg, 1983a). Usually these are synthetic peptides homologous to the tyrosine phosphorylation site of $pp60^{src}$. Some are naturally occurring peptides such as angiotensin II which was shown to be phosphorylated on a tyrosine residue by $pp60^{src}$ and a liver tyrosine protein kinase (Wong & Goldberg, 1983a, b, 1984a, b). The reaction measures the incorporation of [^{32}P]phosphate from

[^{32}P]ATP into the peptide substrate Val^5-angiotensin II. The reaction mixture (final volume 50 µl contained 20 mM Hepes (pH 7.4), 5 mM $MnCl_2$, 12 mM $MgCl_2$, 100 µM Na_3VO_4 ± 100 ug/ml Compound 48/80, ± 50 ug/ml calmodulin, 19 mM PNPP, ± 20 mM Val^5-angiotensin II. The reaction was initiated by the addition of ATP to a final concentration of 15 µM (specific activity 12 mCi/umole). After 20 min at 0°C the reaction was stopped by the addition of 150 µl of 3.3% trichloroacetic acid and 10 µl of 20 mg/ml bovine serum albumin. The protein was precipitated by centrifugation leaving the soluble phosphopeptide in solution. The supernatant was spotted on squares of Whatman P81 paper (2 x 50 µl) samples and washed four times in 0.5% phosphoric acid and finally in acetone. The filter papers were dried and counted in 10 ml of ACS (New England Nuclear). A series of controls containing no peptide were run in case of endogenous peptide phosphorylation.

3.2.4 Endogenous phosphorylation

Incorporation of [^{32}P]phosphate into endogenous substrate proteins was performed under several different conditions which are described in the figure legends. Generally two main phosphorylation procedures with various additions were used. In procedure A) endogenous phosphorylation was performed at 30°C in a reaction mixture (final volume 50 µl) containing 25 mM HEPES (pH 7.5), 20 mM $MnCl_2$, 12 µM $ZnCl_2$, 0.1% (w/v) Nonidet P40, 1.25 mg/ml PNPP ± 50 ug/ml calmodulin, 1 mg/ml membrane protein and 2.5 µM [γ-^{32}P]ATP (30-70 Ci/mmole). After preincubation for 1 min the reaction was initiated by the addition of [γ-^{32}P]ATP and terminated after 15 sec.

In procedure B) endogenous phosphorylation was performed at 0°C in a reaction mixture (final volume 50 µl) containing 20 mM HEPES (pH 7.5), 5 mM

$MnCl_2$, 12 mM $MgCl_2$, 100 µM $NaVO_5$, 7 mg/ml PNPP, ± 50 ug/ml calmodulin, 15 µM [γ-^{32}P]ATP (12 Ci/mmole) and various amounts of membrane or solubilized protein (0.4-80 µg protein). After preincubation for 10 min, the reaction was intitiated by the addition of [γ-^{32}P]ATP and terminated after 20 min.

Both procedures A) and B) were terminated by the addition of 50 µl of electrophoresis sample buffer containing 0.125 M Tris HCl (pH 6.8), 4% SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanopl and 0.01 % Bromphenol Blue and immedate boiling for 3 min. Samples were then subjected to SDS-PAGE.

3.2.5 SDS-polyacrylamide gel electrophoresis

The solubilized proteins were fractionated by SDS-PAGE according to the procedure of Laemmli (1970). The resolving gel contained 10% (w/v) acrylamide, 0.27% (w/v) bis-acrylamide, 0.37 M Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 0.033% (w/v) ammonium persulfate, 0.05% (v/v) N,N,N',N'-tetramethylethylenediamene. The stacking gel contained 3% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide, 0.125 Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate and 0.05% (v/v) N,N,N',N'-tetramethylethylenediamene. The electrode buffer contained 0.05 M Tris, 0.38 M glycine and 0.1% w/v SDS (pH 8.3). Electrophoresis was performed at 18 mA (constant current) per 0.75 mm-thick slab gel. Run times were generally about 4 hr. The resolving gel was stained for 8-16 hr with 0.125% (w/v) Coomassie Blue R-250, 50% (v/v) denatured alcohol containing 10% (v/v) acetic acid and then diffusion-destained in 50% (v/v) denatured alcohol containing 10% (v/v) acetic acid for 1 hr followed by several changes of 5% (v/v) denatured alcohol containing 7% (v/v) acetic acid.

3.2.6 Autoradiography of SDS gels

SDS gels were shaken in a solution of 7% (v/v) acetic acid, 2% (v/v)glycerol and then dried on Whatman 4 MM papers in a LKB gel drier under vacuum. Autoradiography was carried out at -80°C using X-Omat AR film in Kodak cassettes equipped with X-Omat intensifying screens. An UltEmit marker was used to provide exact reference points on the autoradiograph.

3.2.7 Alkali treatment of polyacrylamide gels

A destained gel was rinsed with water for 5 min, then placed in a solution of 1 N KOH (500 ml/gel) in a square glass pan. The KOH solution was preheated to 56°C. The gel was incubated at 56°C for 1h or occasionally 2h with regular shaking. The gel was then rinsed with water and then put into a solution of 7% (v/v) acetic acid, 5% (v/v) denatured alcohol. After 30 min the solution was changed to 7% (w/v) acetic acid, 2% (w/v) glycerol. After a further 15 to 30 min the gel was dried and autoradiographed. This is essentially the same as the procedure used by Cooper & Hunter (1981a) and Cheng & Chen (1981).

3.2.8 Electroelution of protein from gels

Gel slices (containing phosphorylated protein) were cut out from a stained-and-destained gel and thoroughly equilibrated in 0.125 M Tris HCl (pH 6.8), 10 mM 2-mercaptoethanol overnight with constant rotation. The equilibrated gel slices were cut into small pieces and placed into a glass tube over a supporting gel of 2% (w/v) agarose, 0.125 M Tris HCl (pH 6.8). The gel pieces were then embedded in 2% (w/v) agarose, 0.125 M Tris HCl (pH 6.8). The tubes were filled to within 1.5 cm of the top with agarose solution. After polymerization was complete the tube were inserted into the upper chamber of

a tube gel electrophoresis apparatus with the chamber in an inverted position. The gap at the top of the tubes was filled with 0.125 M Tris HCl (pH 6.8) and sealed with dialysis membrane and pieces of tygon tubing were slipped over the ends to secure the dialysis membrane. The upper chamber was then placed into the electrophoresis apparatus and electrophoretic elution was performed at 4mA/tube for 6 to 7 hr using 50 mM Tris, 0.38 M Glycine (pH8.3), 0.1% SDS for electrode buffer. Proteins eluted from the gel were trapped by the dialysis membrane and could be recovered from the buffer solution. Recovery was usually about 95%.

3.2.9 Phosphoamino acid analysis

If desired the eluted proteins were incubated for 1 or 2 hr in 1 N KOH at 56°C followed by dialysis. Samples were lyophilized then taken up in 6 M HCl. The samples were incubated in Reactivials at 110°C. The HCl was removed using a Savant SpeedVac. The hydrolysate was resuspended in 30 µl H_2O and aliquots were applied to a 20 x 20 cm precoated cellulose thin-layer sheet (Kodak Chromagram). 1 µl of a solution of 50 mM phosphoserine, phosphothreonine and phosphotyrosine was spotted with each sample. Phosphoamino acids were separated as described by Hunter & Sefton (1980). Thin layer electrophoresis was performed in a Savant TLE Apparatus in a pH 3.5 buffer consisting of pyridine/glacial acetic acid/water (10 : 100 : 1890, v/v/v) at 1000 V for 50 min. The sheet was then dried, sprayed with ninhydrin reagent and put in an oven at 100°C for 10 min to allow visualization of the phosphoamino acid standards. The sheet was then exposed for autoradiography with Kodak XOmat AR film at -80°C using intensifying screens. Comparison of the autoradiograph with the ninhydrin staining pattern allowed identification of the radioactive

phosphoamino acids. Radioactive spots were scraped from the sheet and their radioactivity determined by their Cerenkov radiation.

CHAPTER IV

RESULTS

4.1 Alkali Resistance of Brain Phosphopeptides

Initially, resistance to alkaline hydrolysis was used as an indicator of possible phosphotyrosyl-proteins. Membrane fractions and Affi-Gel Blue-kinase enriched fractions from chicken, rat, guinea pig and cow brain were phosphorylated and examined for alkali-resistant phosphopeptides which could represent autophosphorylation of tyrosine protein kinase or substrates for tyrosine protein kinases. Phosphorylation was performed in a reaction mixture containing Mn^{2+}, Zn^{2+} and PNPP. Several of the known tyrosine kinases show higher activity in the presence of Mn^{2+} instead of Mg^{2+} and can use micromolar concentrations of ATP (Cooper et al., 1983; Richert et al., 1982; Feldman et al., 1982). Zn^{2+} was included as phosphotyrosyl-protein phosphatases appear to be effectively inhibited by micromolar concentrations of Zn^{2+} (Brautigan et al., 1981; Gallis et al., 1981). As PNPP is a substrate for calcineurin and for some phosphotyrosyl-protein phosphatases (Leis & Kaplan, 1982; Swarup et al., 1981; Wong & Goldberg, 1983a; Cobb & Rosen, 1984), it was included in the reaction mixture to inhibit endogenous phosphatase activity. These reaction conditions (Mn^{2+}, Zn^{2+}, PNPP and low ATP concentrations) are used to accentuate tyrosine phosphorylation. Figure 2 and Figure 3a show the effect of the alkali treatment on (^{32}P)-labelled brain phosphopeptides that had been separated by SDS-PAGE. At this time, the presence of (^{32}P) phosphopeptides stable to alkali

Figure 2. Alkali Resistance of Phosphopeptides from Rat, Chicken and Bovine Brain.

Autoradiograph of alkali treated and untreated gels of kinase enriched preparations from rat brain (Lanes A, E), chicken brain (B, F) or bovine brain (C, D, G, H). "Kinase enriched" preparations were made by centrifugation of brain homogenate at 900xg for 10 min. to remove cell debris. The supernatant was then centrifuged at 150,000xg for 40 min. to obtain a crude membrane fraction which was solubilized with 1 % (w/v) Triton X-100, 10 % (w/v) glycerol and applied to an AffiGel Blue column. After extensive washing, binding proteins ("kinase enriched fraction") were eluted with 1.5 M KCl. Two fractions from bovine brain were analysed. Proteins were phosphorylated for 3 min at 30°C in a reaction mixture (100 µl) containing 90 µg protein, 25 mM Hepes (pH 7.4), 12.5 mM $MnCl_2$, 25 µM Zn OAc, 3 mM PNPP and 5 µM [γ-^{32}P]- ATP (specific activity 12 mCi/mmole). The reaction was stopped by the addition of SDS sample buffer and boiling for 3 min. 17 µg of protein per well were electrophoresed. Duplicate gel was treated with 1N NaOH at 50°C for 1 hr. Lanes A - D are the untreated gel, lanes E - H are the corresponding lanes of the alkali-treated gel.

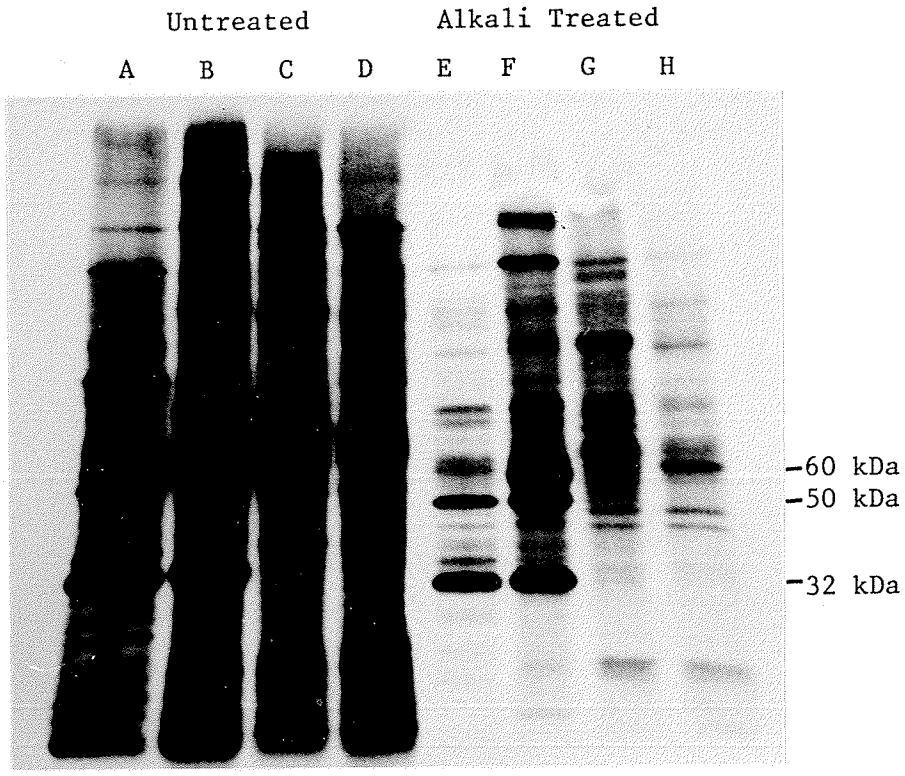
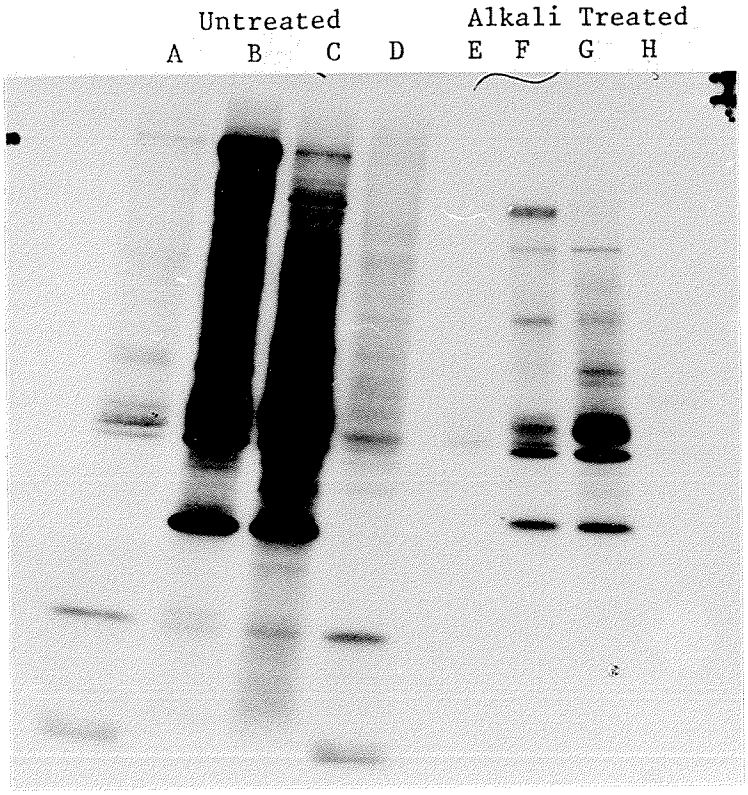


Figure 2

Figure 3. Effect of DMSO on the Phosphorylation of Brain Peptides and Resistance to Alkali

Autoradiograph shows the effect of the presence of DMSO on protein phosphorylation. A crude chicken brain membrane preparation was obtained as in figure 2. A kinase enriched fraction was prepared from the crude membrane (as in figure 2) and 2 fractions of this preparation were examined. Synaptic membrane was prepared from Guinea pig as in Methods. Crude membrane Chicken brain membrane (A, E, I, M), guinea pig synaptic membrane (D, H, L, P) or two fractions of a kinase enriched AffiGel Blue preparation from chicken brain (B, F, J, N and C, G, K, O) were phosphorylated in a reaction mixture (100 µl) containing 100 µg protein, 25 mM Hepes (pH 7.4), 13.5 mM $MgCl_2$, 13.5 µM ZnOAc, 3 mM PNPP, 5 µM [γ-^{32}P]ATP (specific activity 12 mCi/mmole) $\pm$ 20% (v/v) DMSO. Autoradiograph A shows the phosphopeptides (Lanes A-D) and the alkali-resistant phosphopeptides (E-H) obtained when the phosphorylation is performed in the absence of DMSO. Autoradiograph B shows the corresponding phosphopeptides (I-L) and alkali-resistant phosphopeptides (M-P) obtained when 20% DMSO is included in the phosphorylation reaction.

A) Without DMSO



B) With DMSO

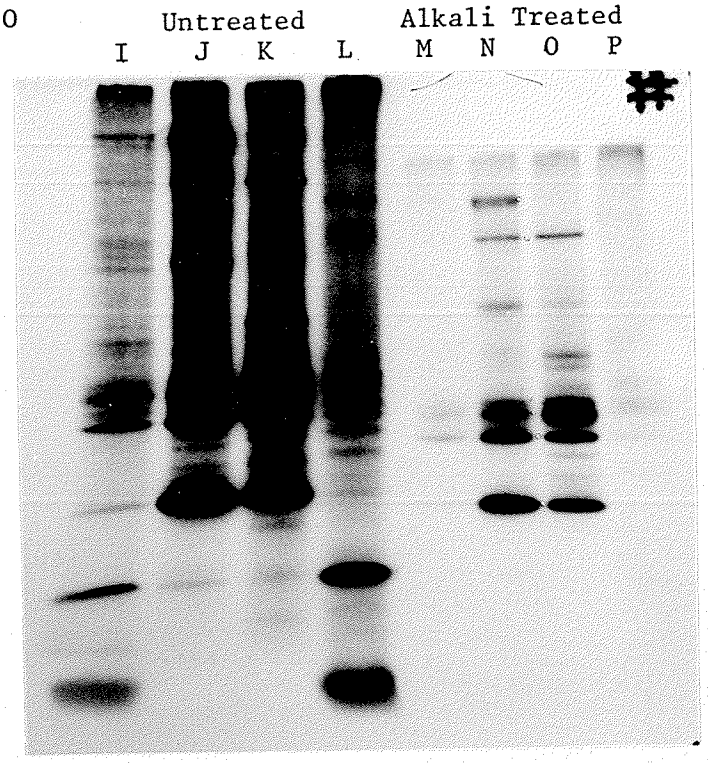


Figure 3

treatment was taken to be an indication of the presence of phosphotyrosyl-proteins and tyrosine protein kinase activity. Later results showed that some phosphothreonyl residues were resistant as well.

4.2 Effect of DMSO on Brain Peptide Phosphorylation

Dimethyl sulfoxide (DMSO) is a highly polar solvent which can stimulate differentiation of certain cell lines such as Friend murine erythroleukemia cells (Friend & Friedman, 1978) and others (Collins et al., 1978; Kimbi et al., 1976; Takasaki & Leive, 1982; Higgins & Borenfreund, 1980). DMSO can stimulate the tyrosine protein kinase activity of the EGF receptor in membranes (Rubin & Earp, 1983a) and even after solubilization (Rubin & Earp, 1983b) indicating that the solvent can act directly on the receptor protein to stimulate phosphorylation. DMSO may be able to stimulate other tyrosine protein kinases since it stimulates tyrosine phosphorylation in Friend murine erythroleukemia cells which do not show EGF stimulated phosphorylation (Earp et al., 1983). The effects of DMSO on the phosphorylation of chicken and guinea pig brain membrane and kinase enriched fractions of chicken brain were examined (Figure 3). Figure 3b shows the phosphopeptide pattern obtained when 20% DMSO is included in the reaction mixture. The inclusion of DMSO in the phosphorylation mixture causes an increase in the phosphorylation of the chicken and guinea pig membrane proteins before and after alkaline hydrolysis. The kinase enriched fractions show an increase in the alkali-resistant phosphorylation of the 60-, 50- and 32-kDa phosphopeptides. These experiments suggested that there are tyrosine protein kinases present in brain although later results showed some phosphorylation residues are also resistant to alkali. Some of the alkali resistant

phosphopeptides may be due to autophosphorylation of the tyrosine protein kinases themselves.

For this preliminary survey fractions from rat, guinea pig, chicken and cow brain were used. For later experiments only guinea pig and rat brain (which gave similar phosphopeptide patterns) were used since they could be homogenized immediately while use of chicken and cow brain involved transportation time before homogenization which allowed proteolysis to occur.

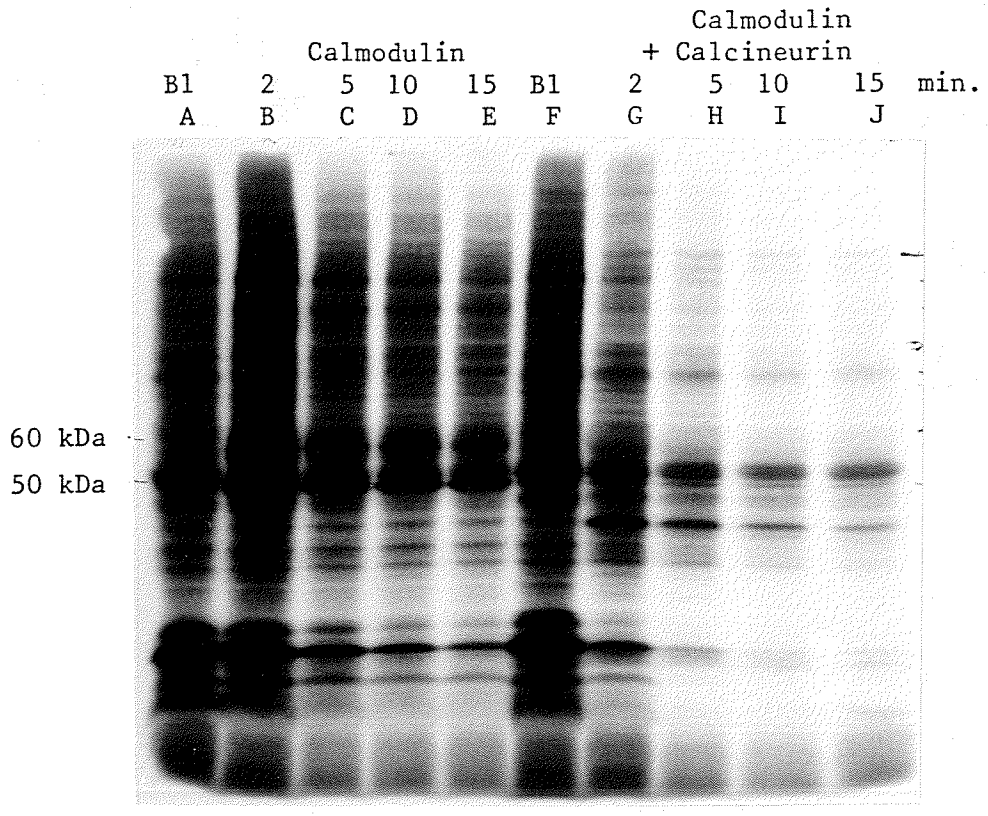
4.3 Effect of Calmodulin and Calcineurin on Peptide Phosphorylation

Synaptic membrane from guinea pig brain was examined for alkali resistant phosphopeptides which could serve as substrates for calcineurin. Synaptic membrane was phosphorylated at 0°C, at which temperature calcineurin has little activity, then the membranes were incubated at 30°C in the presence of calcineurin and/or calmodulin. With the Mn^{2+} and calmodulin concentrations used the calcineurin would be rapidly activated. A control incubation was done with Mn^{2+} and calmodulin and no phosphatase inhibitors were added. Samples were removed over a time course of 15 min and the reaction stopped by the addition of SDS sample buffer and boiling for 3 min. The autoradiogram of the untreated gel (Figure 4a) shows that calcineurin was active towards most of the phosphopeptides present. Also calmodulin in the control reaction appeared to stimulate the phosphorylation. It is also evident that there is phosphatase activity present in the guinea pig synaptic membrane so that maximum phosphorylation occurred at 2 min with calmodulin, after which a gradual dephosphorylation occurred. The autoradiogram of the alkali-treated gel (Figure 4b) shows two phosphopeptides with Mr of 50- and 60-kDa are markedly alkali resistant. The phosphorylation of these two peptides was

Figure 4. Effect of Calmodulin and Calcineurin on Synaptic Membrane Phosphorylation

Autoradiograph of synaptic membrane which was phosphorylated in a reaction mixture (50 µl) containing 100 µg of protein, 20 mM Hepes (pH 7.5), 10 mM $MnCl_2$, 7 µM [γ-^{32}P]ATP (specific activity 30 Ci/mol) with calmodulin (5 µg) (Lanes B-E) or calmodulin (5 µg) plus calcineurin (20 µg) (Lanes G-J). Reaction was for 10 min at 0°C followed by an incubation at 30°C for 2, 5, 10 or 15 min. Control samples (A, F) had no addition and reaction was stopped after the 10 min, 0°C incubation.

A) Untreated Gel



B) Alkali Treated Gel

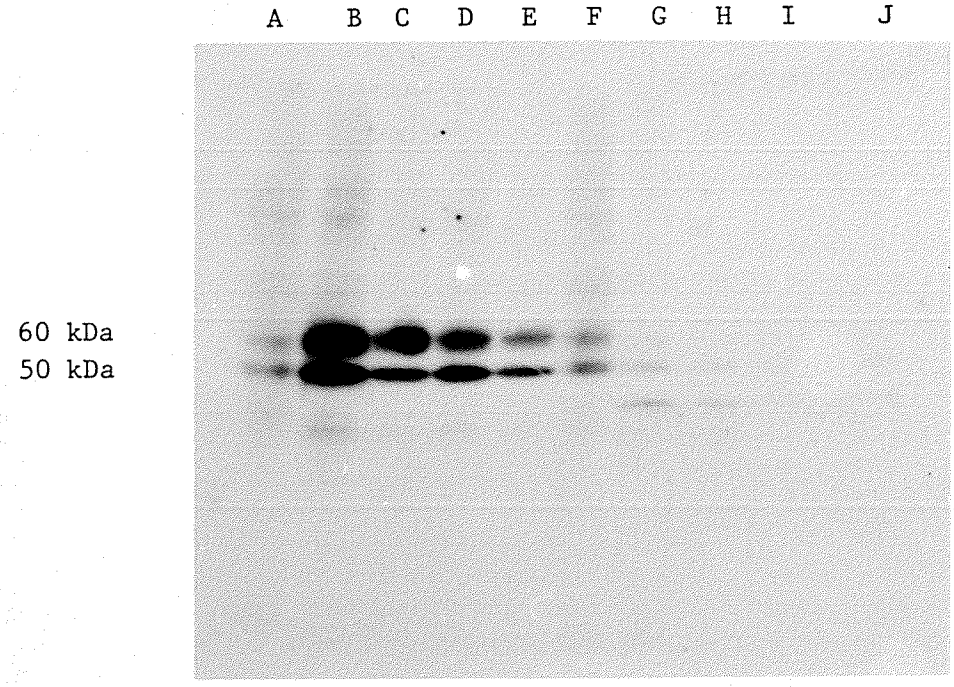


Figure 4

markedly stimulated in the calmodulin control reactions and show a gradual dephosphorylation over the 15 minute time course at 30°C. Calcineurin appears to be very effective towards these two alkali-resistant phosphopeptides. Most of the other phosphopeptides were dephosphorylated by the alkali treatment. The two alkali resistant bands could be phosphotyrosyl-proteins indicating that calcineurin could indeed have phosphotyrosyl-protein phosphatase activity and further that calmodulin could be involved in increasing the tyrosine phosphorylation of these peptides.

4.4 Comparison of Brain Phosphopeptides with Phosphopeptides of Membranes with Known Tyrosine Protein Kinase Activity

Rat spleen membrane and membrane from SR-NRK cells were used as control preparations which had already been shown to have phosphotyrosyl proteins. Spleen has been shown to contain higher tyrosine protein kinase activity than other rat tissues using a synthetic peptide substrate to measure tyrosine phosphorylation (Swarup et al., 1983). Low concentrations of Nonidet P-40, a nonionic detergent, stimulated tyrosine kinase activity in many tissue membranes including brain, lung and spleen. Two phosphopeptides in spleen membrane of Mr 53 kDa and 56kDa were stable to alkali treatment and were shown to contain phosphotyrosine. This tyrosine protein kinase activity could be due to T and B lymphocytes in spleen. Earp et al. (1984) found tyrosine phosphorylation of 55-kDa and 61-kDa peptides in B-lymphocyte membranes and 58-kDa and 64-kDa peptides in T-lymphocyte membrane which was increased by Triton X-100, a nonionic detergent. Harrison et al. (1984) reported phosphorylation of 56-kDa and 60-kDa peptides in B lymphocytes and a 58-kDa peptides in T lymphocytes which may be autophosphorylation of the tyrosine protein kinase (Casnellie et al., 1983).

SR-NRK cells are a cell line of Schmidt-Ruppin strain of avian sarcoma virus-transformed rat kidney fibroblasts (Courneidge et al., 1980; Levinson et al., 1980). These cells contain $pp60^{v\text{-}src}$ associated with the plasma membrane. Antiserum to $pp60^{v\text{-}src}$ recognized two proteins in these membranes with Mr of 60 kDa and 52 kDa. The 52-kDa is a proteolytic product of $pp60^{src}$.

The effects of Nonidet P-40 (NP40) and calmodulin on the phosphorylation of rat brain synaptic membrane proteins was examined. Control phosphorylationswith $Mn^{2+}Mg^{2+}$, $Zn^{2+}Mn^{2+}Mg^{2+}$ or $Zn^{2+}Mn^{2+}$ appeared to be virtually the same. Spleen membrane and membrane from SR-NRK cells were also phosphorylated with and without NP40 (Figure 5). NP40 stimulated the alkali resistant phosphorylation of four peptides of Mr 53, 55, 57 and 59 kDa in spleen membranes. NP40 appeared to decrease the phosphorylation of the major alkali resistant phosphopeptide (Mr = 58 kDa) in the membranes from SR-NRK cells but had little effect on the 50 kDa alkali resistant phosphopeptide. Nonidet P40 increased the phosphorylation of several peptides in synaptic membrane including two bands of 60 and 50 kDa that were stimulated by calmodulin. The 60 and 50 kDa phosphopeptides were markedly alkali resistant in both the NP40- and the calmodulin-stimulated samples. This experiment showed that the calmodulin stimulated alkali resistant phosphopeptides behaved in a fashion similar to known phosphotyrosyl proteins from SR-NRK cell membranes and spleen membranes.

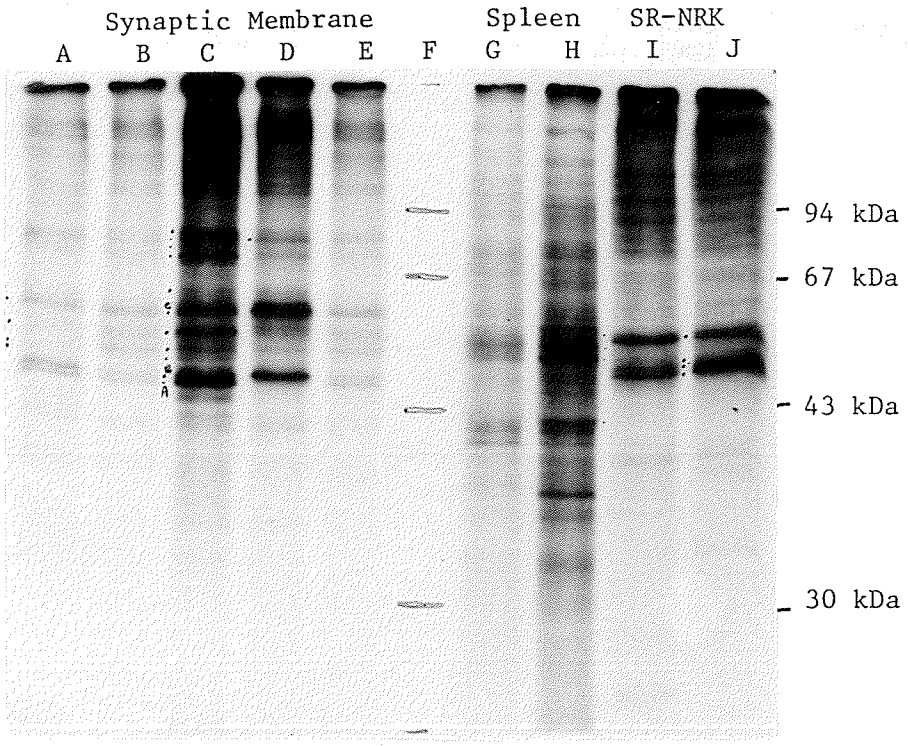
4.5 Effect of Phosphatase Inhibitors on Calmodulin-Stimulated Phosphorylation

Micromolar concentrations of vanadate have been shown to inhibit phosphotyrosine phosphatases (Wong & Goldberg, 1983; Leis & Kaplan, 1982;

Figure 5. Comparison of Synaptic Membrane Phosphorylation and Alkali Treatment with Membranes Containing Tyrosine Protein Kinase Activity

Autoradiograph of rat brain synaptic membrane (A-E), a 30,000 x g membrane pellet from rat spleen (G,H) and a 30,000 x g membrane pellet from SR-NRK cells (I, J) which were phosphorylated in a reaction mixture (100 µl final volume) containing 100 µg of protein (30 µg of SR-NRK membrane protein), 25 mm Hepes (pH 7.4), 24 mM $MnCl_2$, ± 10 mM $MgCl_2$, ± 12 µM ZnOAc, ± 0.1% (v/v) NP-40, ± 50 µg/ml calmodulin, ± 3 mM PNPP ± 2.5 µM [γ-^{32}P]ATP (specific activity 300 Ci/mole). Synaptic membranes were phosphorylated with Mn^{2+},Mg^{2+}(A); Zn^{2+},Mn^{2+},Mg^{2+} (B); Zn^{2+},Mn^{2+},Mg^{2+} NP-40 (C); Zn^{2+},Mn^{2+},Mg^{2+}, calmodulin (D); or Zn^{2+},Mn^{2+} (E) present. Lane F contained molecular weight markers. Spleen membrane were phosphorylated with Zn^{2+},Mn^{2+},Mg^{2+} present and without (G) or with NP-40 (H). SR-NRK membranes were phosphorylated with Zn^{2+},Mn^{2+},Mg^{2+} present and without (I) or with NP-40 (J). Low molecular weight proteins were run off the gel to help resolve the proteins with Mr 40-70 kDa.

A) Untreated Gel



B) Alkali Treated Gel

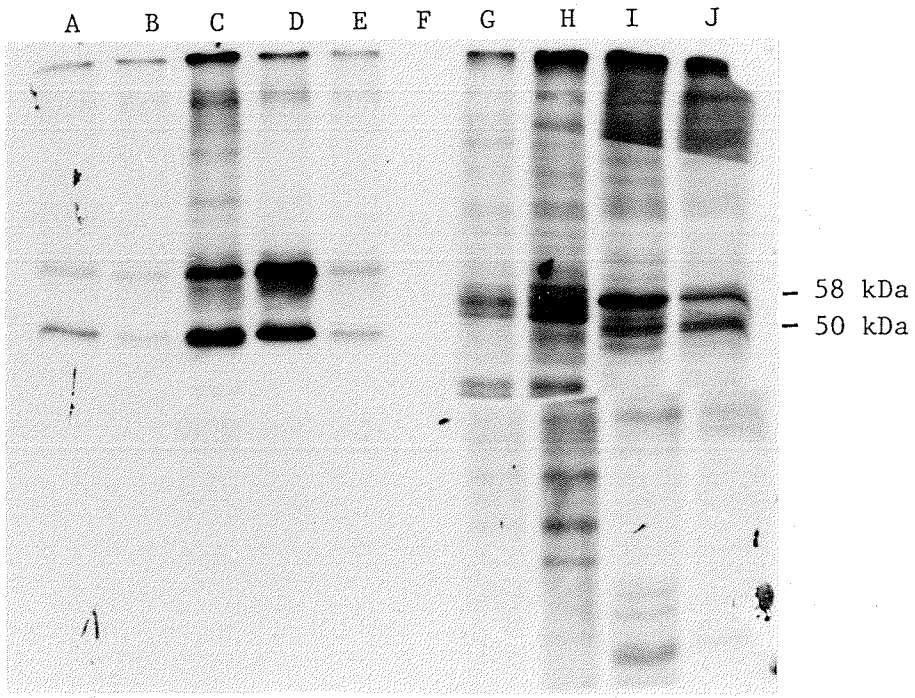


Figure 5

Swarup et al., 1982a & b). Because of its structural similarity to phosphotyrosine, p-nitrophenyl phosphate is a substrate for phosphotyrosine phosphatases and so can act as a competitive inhibitor (Wong & Goldberg, 1983). Figure 6 shows the effect of vanadate (30 μM) and p-nitrophenyl phosphate (3 mM) on calmodulin-stimulated phosphorylation. Vanadate appeared to have no effect on the calmodulin-stimulated phosphorylation under these conditions while p-nitrophenyl phosphate increased the phosphorylation of the 50- and 60-kDa bands both with and without alkali treatment. In the absence of vanadate and p-nitrophenyl phosphate calcineurin was an effective phosphatase towards the 50-kDa and 6-kDa phosphopeptides. PNPP appears to block endogenous phosphatase activity.

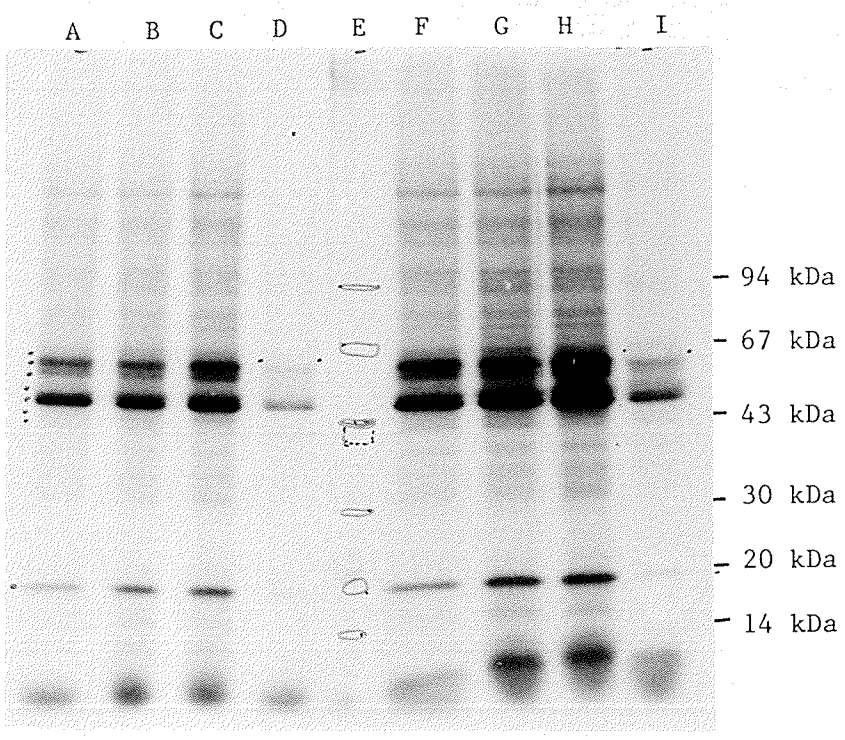
4.6 Inhibition of Calcineurin Phosphatase Activity by PNPP

Calcineurin is a calmodulin-binding protein. The effect of calcineurin on the membrane phosphopeptides could be due to its binding to calmodulin and inhibition of the calmodulin-stimulated activity or its phosphatase activity. Lanes F, G and H of Figure 7 show that the lower phosphorylation levels are due to calcineurin's phosphatase activity. Lanes F and G show that calcineurin causes a decrease in the level of calmodulin-stimulated phosphorylation before and after alkali treatment. Lane H shows that inclusion of PNPP with calcineurin in the reaction completely blocks calcineurin's action. Since PNPP would inhibit calcineurin's phosphatase activity but would have no effect on its interaction with calmodulin, this indicates that calcineurin is dephosphorylating these phosphopeptides. Cyclic AMP-dependent protein kinase did not cause the formation of any alkali-resistant phosphopeptides (Lane B, Figure 7).

Figure 6. Effect of Vanadate, PNPP and Calcineurin on Membrane Phosphorylation

Autoradiograph of guinea pig cortical synaptic membrane which was phosphorylated in a reaction mixture (final volume 100 µl) containing 240 µg membrane protein, 20 mM Hepes (pH 7.5), 5 mM $MnCl_2$, 60 µg/ml calmodulin, 5 µM [γ-^{32}P]ATP (specific activity 80 Ci/mole) for 2 min at 30°C. Additions were none (lanes A, F), 30 µM Na_3VO_4 (lanes B, G), 3 mM PNPP (lanes C, H) and 100 µg/ml calcineurin (lanes D, I). 12 µg protein per lane were run in lanes A-D and 24 µg protein per lane in lanes F-I. Lane E contained molecular weight markers in the untreated gel.

A) Untreated Gel



B) Alkali Treated Gel

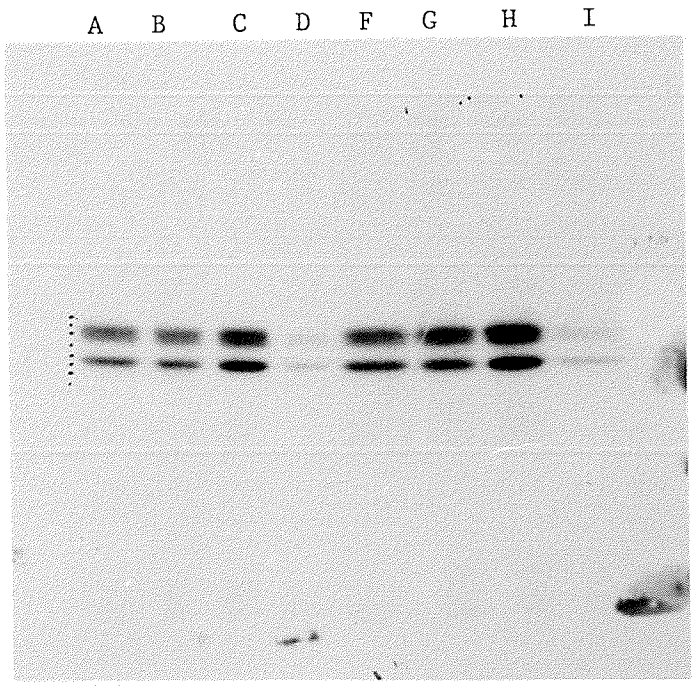
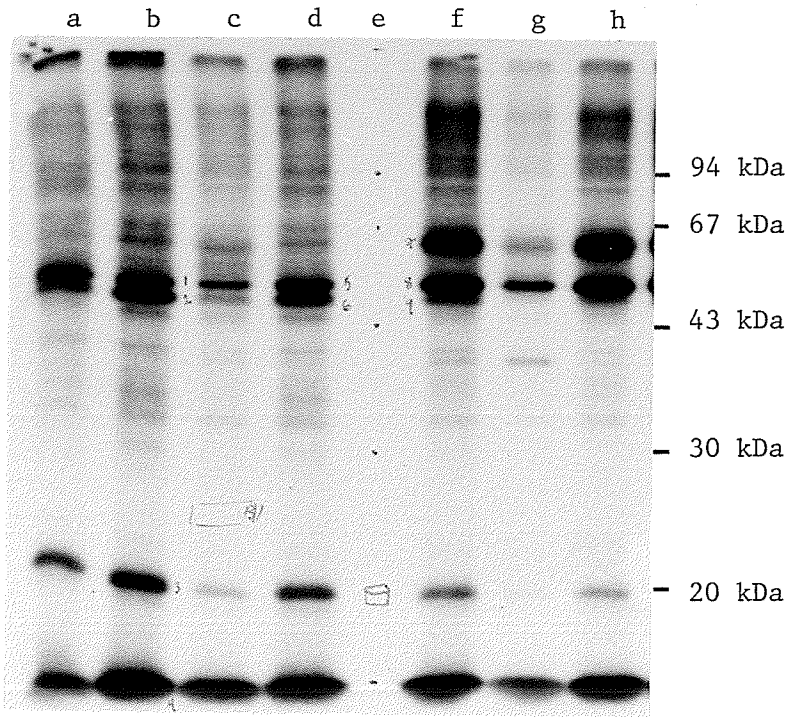


Figure 6

Figure 7. PNPP Blocks the Dephosphorylation by Calcineurin

Autoradiograph of guinea pig synaptic membrane which was phosphorylated in a reaction mixture (final volume 100 µl) containing 20 mM Hepes (pH 7.5), 5 mM MnC_2, 10 µM [γ-^{32}P]ATP (specific activity 80 Ci/mmole) with the following additions: (a) 5 mM $MgCl_2$, 5 mM EGTA; (b) 5 mM $MgCl_2$, 5 mM EGTA, 29 µg/ml cyclic AMP-dependent protein kinase catalytic subunit; (c) 5 mM $MgCl_2$, 5 mM EGTA, 29 µg/ml cyclic AMP-dependent protein kinase catalytic subunit, 280 µg/ml calcineurin; (d) 5 mM EGTA, 3 mM PNPP; (f) 120 µg/ml calmodulin, 3 mM PNPP; (g) 120 µg/ml calmodulin, 140 µg/ml calcineurin; (h) 120 µg/ml calmodulin, 140 µg/ml calcineurin, 6 mM PNPP.

A) Untreated Gel



B) Alkali Treated Gel

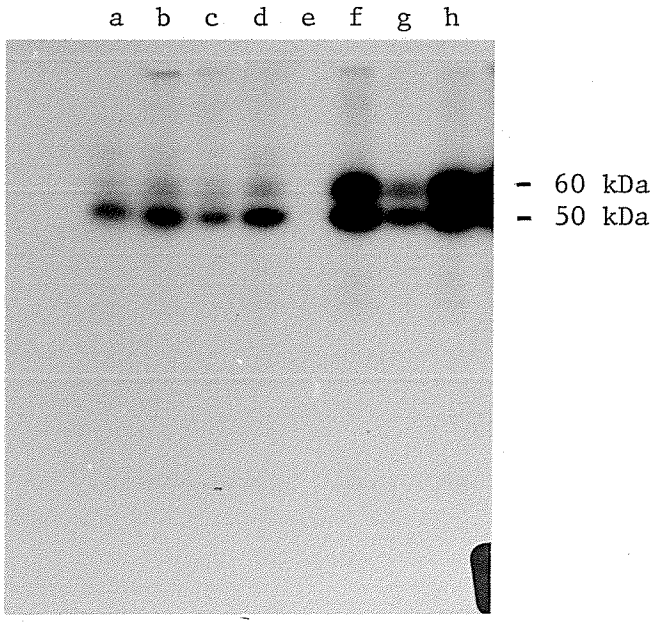


Figure 7

4.7 Calcium-Calmodulin Stimulation of Phosphorylation and Nickel Stimulation of Calcineurin Phosphatase Activity

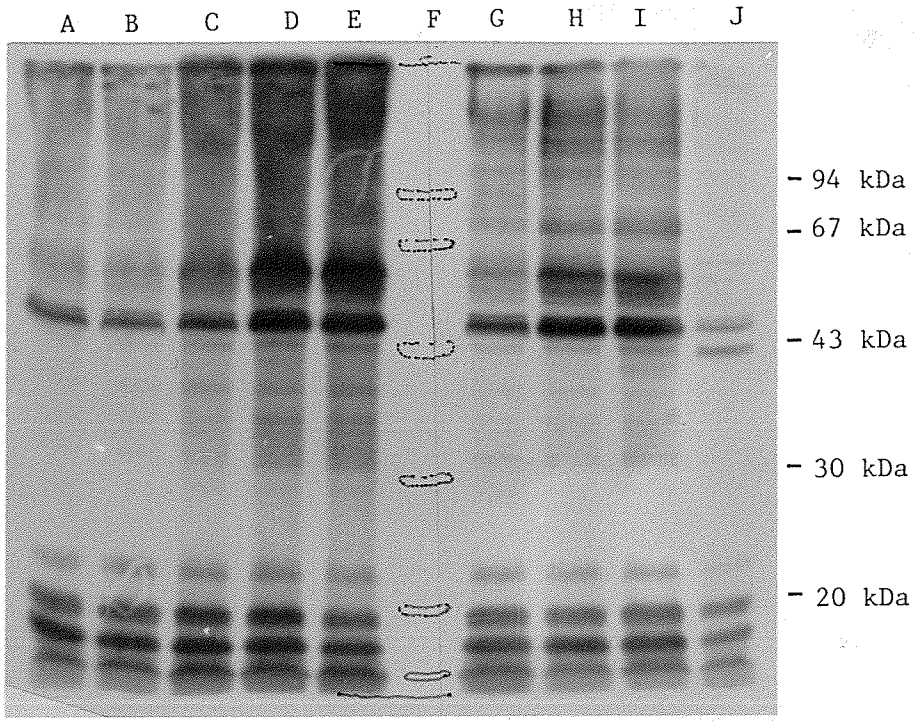
Mn^{2+} is a convenient divalent cation to use in the phosphorylation reaction because it is the preferred metal cofactor for some tyrosine protein kinases, it is also an effective activator of calmodulin and it is an effective activator of calcineurin activity and calmodulin-stimulated calcineurin activity. Ca^{2+} is, however, regarded as the *in vivo* activator of calmodulin. Replacement of Mn^{2+} in the reaction mixture with 1 mM Ca^{2+} and 7.2 mM Mg^{2+} showed that Ca^{2+}-calmodulin could also stimulate the alkali-resistant phosphorylation of the 60-kDa and 50-kDa peptides (Lanes G, H, Figure 8 vs. Lanes C, D). In several preparations the 60-kDa peptide has been thicker than the 50-kDa peptide on autoradiography. It is evident from Figure 8 that part of the phosphorylation can be attributed to a 58-kDa phosphopeptide which may be a proteolytic product of the 60-kDa band.

While Mn^{2+} is a better activator of calcineurin phosphatase activity than Ca^{2+}, Ni^{2+} was found to be an even more potent activator of calcineurin than Mn^{2+} for certain substrates (Pallen & Wang, 1983). Inclusion of Ni^{2+} in the reaction mixture had no effect on the calmodulin-stimulated phosphorylation when Zn^{2+} and PNPP were also included (Lane I, Figure 8). It was, however, effective in stimulating calcineurin phosphatase activity (Lane J, Figure 8) and appeared to be better than Mn^{2+} (Lane G, Figure 7) although no direct comparison was made. This showed that Ca^{2+}, Mg^{2+} could activate the calmodulin-stimulated phosphorylation of the 50- and 60-kDa peptides at the alkali-sensitive and alkali-resistant sites just as Mn^{2+} alone did. Ni^{2+} appeared

Figure 8. Dephosphorylation of Synaptic Membrane Phosphopeptides by Ni^{2+} Calcineurin.

Autoradiograph of rat brain synaptic membrane which was phosphorylated in a reaction mixture (final volume 100 µl) containing 24 mM Hepes (pH 7.4), 12 µM $ZnCl_2$, 3 mM PNPP, 6 µM [γ-^{32}P]ATP (specific activity 2.5 Ci/mmol) and 385 µg of synaptic membrane protein with the following additions: Lane A, 7.2 mM $Mg(OAc)_2$, 0.6 mM EGTA; Lane B, 7.2 mM $Mg(OAc)_2$; Lane C, 24 mM $MnCl_2$; Lane D, 24 mM $MnCl_2$, 65 µg/ml calmodulin; Lane E, 24 mM $MnCl_2$, 65 µg/ml calmodulin, 0.1% (w/v) Nonidet-P 40; Lane G, 7.2 mM $Mg(OAc)_2$, 1 mM $CaCl_2$; Lane H, 7.2 mM $Mg(OAc)_2$, 1 mM $CaCl_2$, 65 µg/ml calmodulin; Lane I, 7.2 mM $Mg(OAc)_2$, 1 mM $CaCl_2$, 65 µg/ml calmodulin, 1 mMn $NiCl_2$. For Lane J phosphorylation was carried out with 7.2 mM $Mg(OAc)_2$, 1 mM $CaCl_2$, 65 µg/ml calmodulin, 1 mM $NiCl_2$ and 140 µg/ml calcineurin with no Zn^{2+} or PNPP to block phosphatase activity.

A) Untreated Gel



B) Alkali Treated Gel

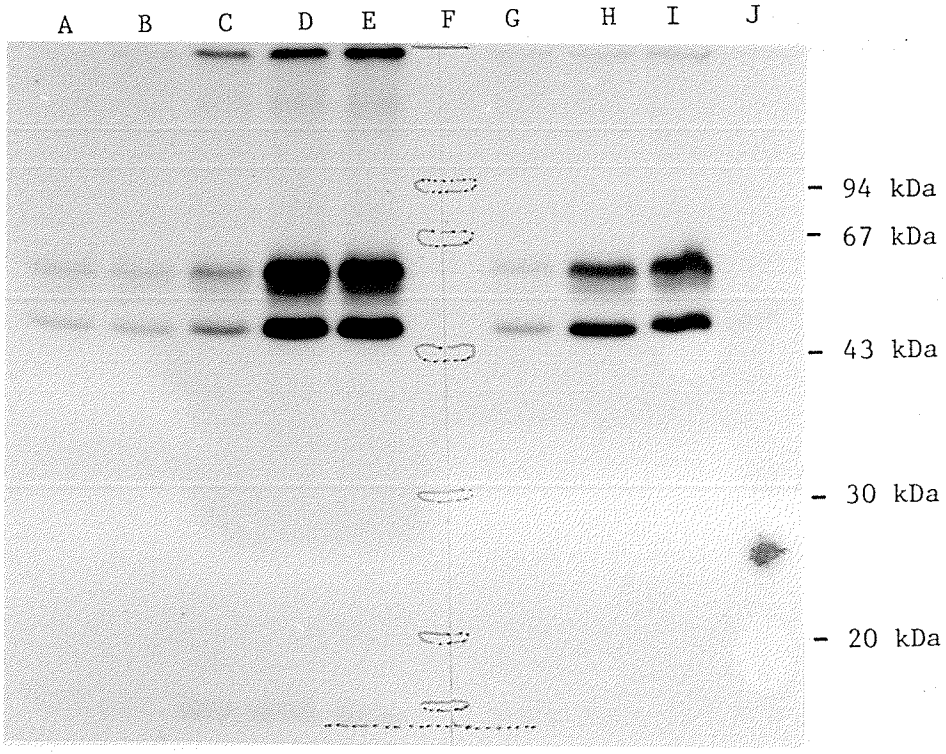


Figure 8

to activate calcineurin phosphatase activity towards these sites more potently than Mn^{2+}.

4.8 Immunoblot Detection of Calcineurin in Rat Brain Synaptic Membrane

A sample of rat brain synaptic membrane proteins was separated by SDS-PAGE. The proteins were then transferred electrophoretically to nitrocellulose (Towbin et al., 1979). Non-specific binding was blocked with bovine serum albumin and the nitrocellulose filter was incubated with monoclonal antibody specific for the B subunit of calcineurin (VA1). Following the addition of goat anti-mouse IgG-alkaline phosphatase conjugate, bound antibody was detected under UV light by the hydrolysis of 4-methylumbelliferyl phosphate. This procedure showed that calcineurin was present in the synaptic membrane preparation used in these experiments (Figure 9). Others have shown that calcineurin is found in both the soluble and particulate fractions of brain (Tallant et al., 1983). Endogenous calcineurin could contribute significantly to the endogenous phosphatase activity.

4.9 Dependence of Peptide Phosphorylation on Calmodulin Concentration

Under the standard phosphorylation conditions (see "Experimental Procedures", Procedure B) phosphorylation of the 50-kDa and 60-kDa peptides of rat brain synaptic membrane was dependent on calmodulin concentration (Figure 10). Phosphorylation of the 50-kDa and 60-kDa peptides occurred even in the absence of added calmodulin; however, this was partially blocked by the addition of the calmodulin-specific inhibitor, Compund 48/80 (100 µg/ml). Phosphorylation in the presence of added calmodulin was 2 to 3 fold higher than

Figure 9. Immunoblot Detection of Calcineurin in Rat Brain Synaptic Membrane

Samples of calcineurin (5 µg, lane a) or rat brain synaptic membrane (20 µg, lane c) were electrophoresed in a 12% PAG. Proteins were electroblotted onto nitrocellulose (Towbin et al., 1978). Nitrocellulose was incubated monoclonal antibody VA1 which is specific for the B subunit of calcineurin and goat anti-mouse IgG-alkaline phosphatase conjugate. Antibody bound to calcineurin was detected under UV light by the hydrolysis of 4-methylumbelliferyl phosphate by the alkaline phosphatase.

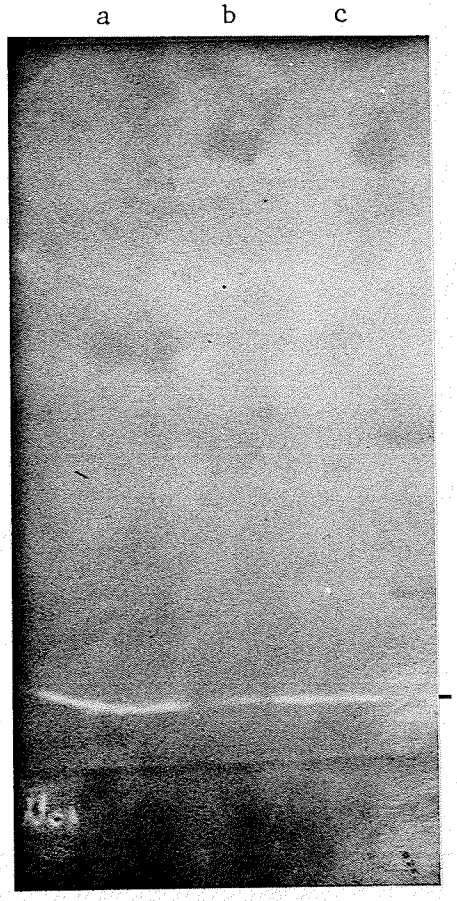
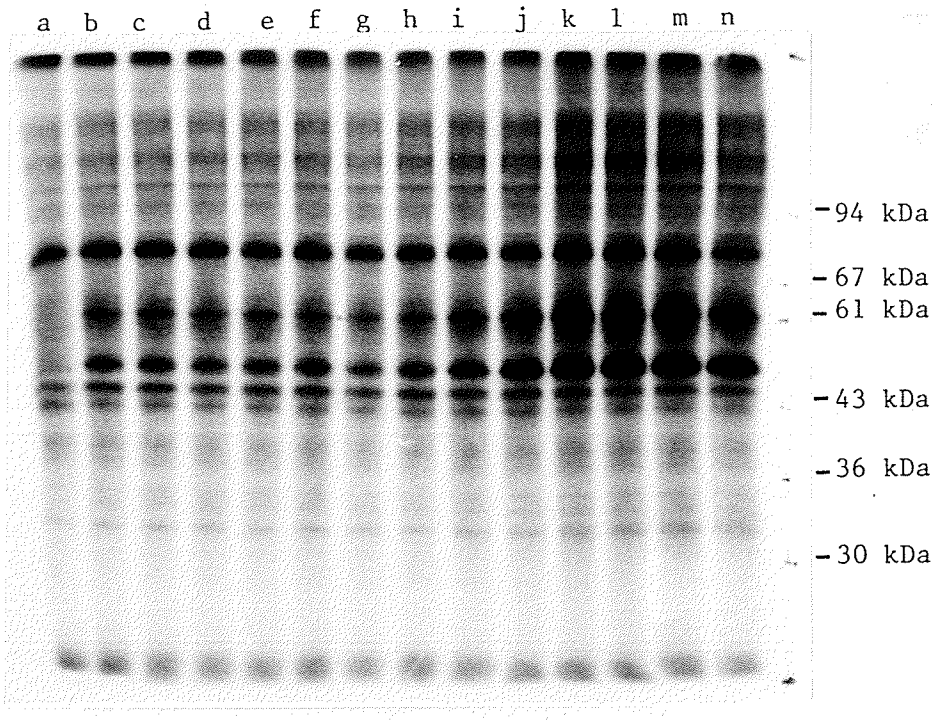


Figure 9

Figure 10. Dependence of the Phosphorylation of the 50 kDa and 60 kDa Peptides of Brain Synaptic Membrane on Calmodulin

Autoradiograph of rat brain synaptic membrane phosphorylated using Method B in the presence of 100 µg/ml of Compound 48/80 (lane a) or 0, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, 30, 40 50 µg/ml calmodulin (lanes b-n, respectively). Phosphorylation was quantitated (by scanning the autoradiographs from Figure 10A and 10B) using a LKB laser densitometer. The densitometer measures absorbance versus position on the autoradiograph which is integrated to give the relative amount of phosphorylation. Exposure time of the autoradiographs (A and B) were identical and so can be compared. Integration values for the untreated gel are on the left hand margin while those for the alkali treated gel are on the right hand margin.

A) Untreated Gel



B) Alkali Treared Gel

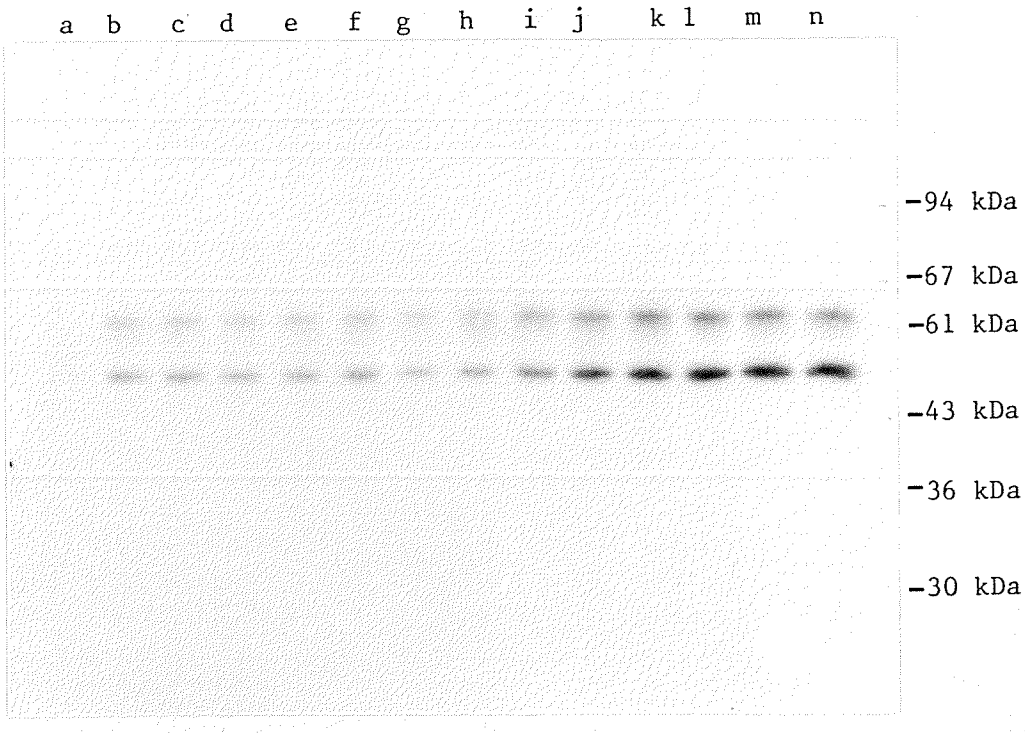
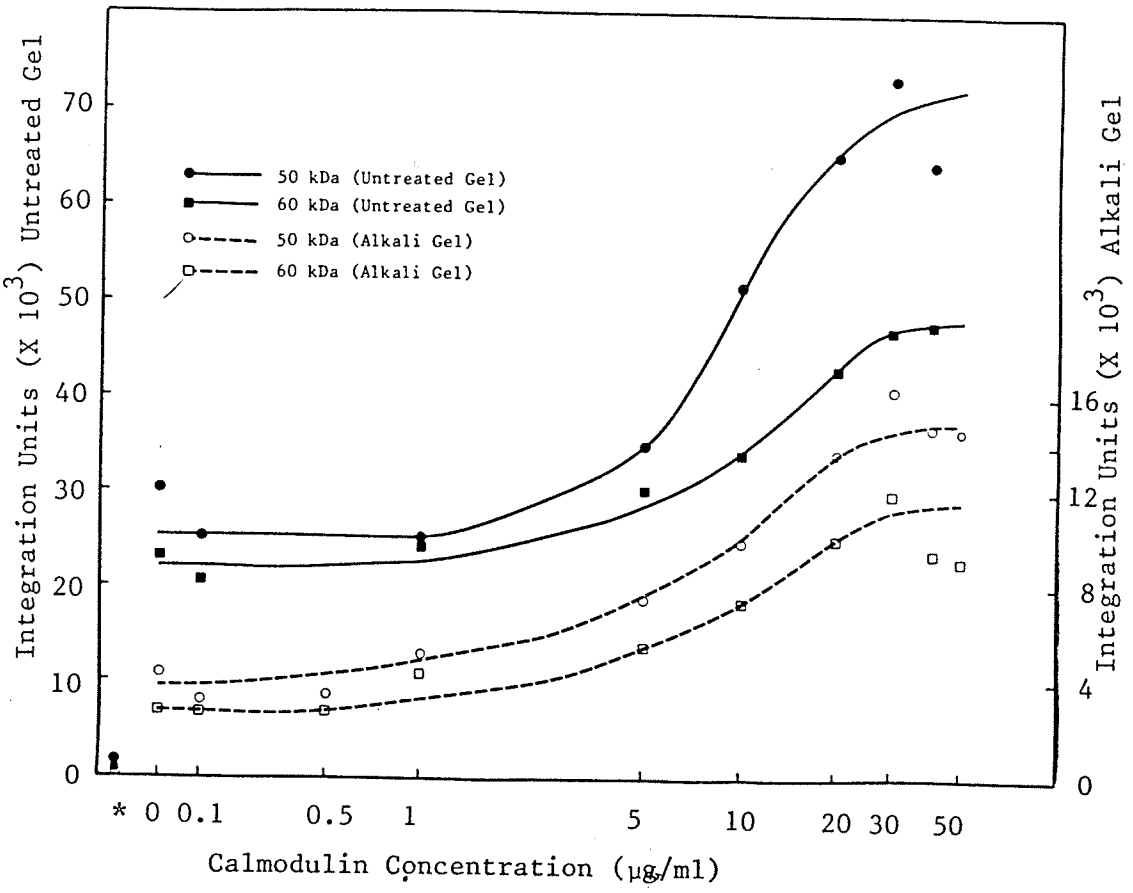


Figure 10



* Values for 100 µg/ml of Compound 48/80.

Figure 10 C

phosphorylation without added calmodulin. Half-maximal activation of peptide phosphorylation occurred at 0.4 to 0.5 μM calmodulin. The alkali-treated gel gave similar results with respect to calmodulin dependence.

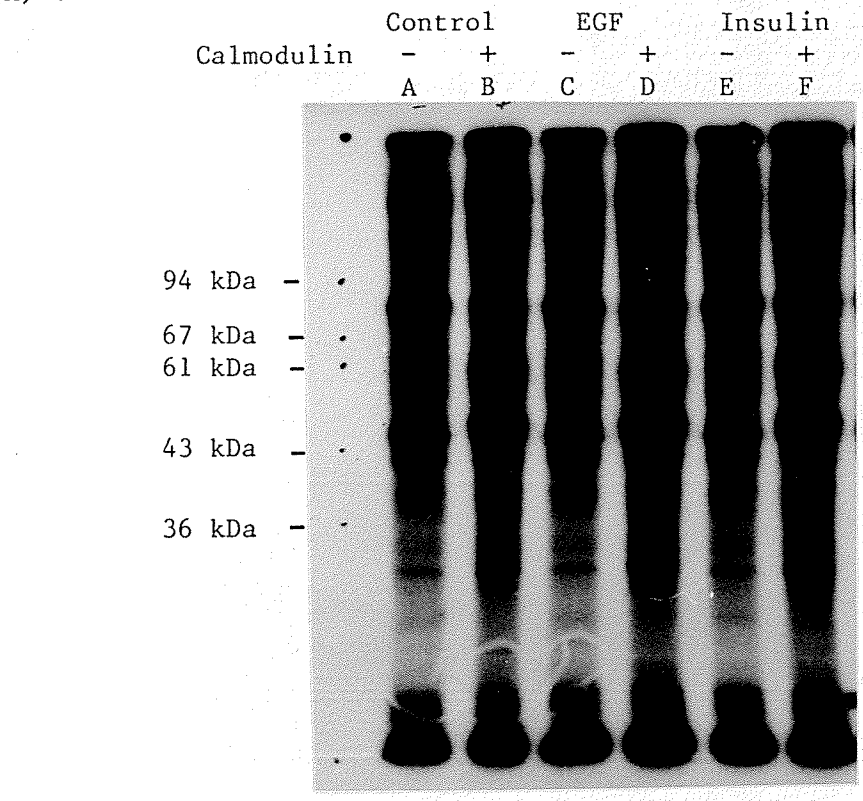
4.10 Effect of EGF and Insulin on Alkali-Resistant Phosphorylation

The receptors for insulin and EGF possess tyrosine protein kinase activity which is stimulated by their respective ligand (Ushiro & Cohen, 1980; Carpenter, 1983; Kasuga et al., 1982a, b), EGF- and insulin-stimulated tyrosine protein kinase activity results in the phosphorylation of the receptor itself, 170 kDa EGF receptor and the 95-kDa subunit of the insulin receptor (Hunter & Cooper, 1981; Kasuga et al., 1982a, b). Since it was possible that the tyrosine phosphorylation of the brain peptides was catalyzed by one or both of these receptor/tyrosine kinases, the effect of insulin and EGF on the phosphorylation of rat synaptic membrane peptides was examined. Figure 11 shows that neither insulin nor EGF had effect on peptide phosphorylation either in the presence or absence of calmodulin, as judged by the autoradiographs of the untreated or alkali-treated gels. No alkali-resistant phosphopeptides corresponding to the EGF receptor (170 kDa) or the 95-kDa subunit of the insulin receptor were detectable (even with extended exposure time). It may be that the levels of these receptor/kinases in the membrane were low and that the lack of effect is due to low receptor/kinase activity. These receptor/kinases may be able to phosphorylate the 60-kDa and 50-kDa peptides but their lack of effect here suggests that the tyrosine kinase activity responsible for the phosphorylation of the brain phosphopeptides was not due to the insulin or EGF receptor/tyrosine kinases.

Figure 11. Effect of Insulin and EGF on Alkali-Resistant Phosphopeptides of Rat Brain Synaptic Membrane

Autoradiograph of rat brain synaptic membrane phosphorylated in a reaction mixture (final volume 100 µl) containing 20 mM Hepes (pH 7.4), 5 mM Mn^2, 12 mM Mg^2, 10 µM Na_3VO_4, 19 mM PNPP, ± 100 µg/ml Compound 48/80, ± 50 µg/ml calmodulin, ± 2000 nM EGF, ± 300 nM insulin. Phosphorylation was carried out in the presence of Compound 48/80 (A, C, E) or calmodulin (B, D, F) with either no addition (control A, B) or with addition of EGF (C, D) or insulin (E, F).

A) Untreated Gel



B) Alkali Treated Gel

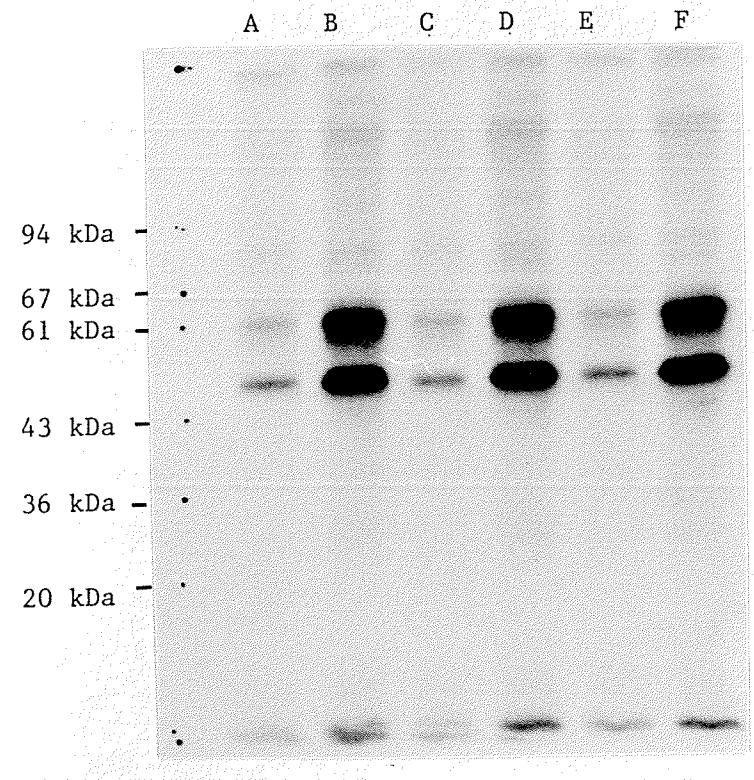


Figure 11

4.11 Phosphoamino Acid Analysis of the 60-kDa and 50-kDa Phosphopeptides from Rat Brain Synaptic Membrane

Although incubation of SDS polyacrylamide gels in alkali has been used in many studies as an indicator for phosphotyrosyl-proteins (Cooper et al., 1983; Swarup et al., 1983; Dasgupta et al., 1984) it is necessary to confirm the identity of the phosphoamino acid. To do this the peptide can be eluted from the SDS gel and partially hydrolyzed in 6N HCl. The phosphoamino acids can then be separated by thin layer electrophoresis at pH 3.5 on cellulose thin layer plates.

Rat brain synaptic membrane was phosphorylated using the conditions in the legend to Figure 12. Three conditions were examined, no addition, 50 µg/ml of calmodulin and 100 µg/ml of Compound 48/80, an inhibitor that appears to be specific for calmodulin stimulation (Gietzen, 1983).

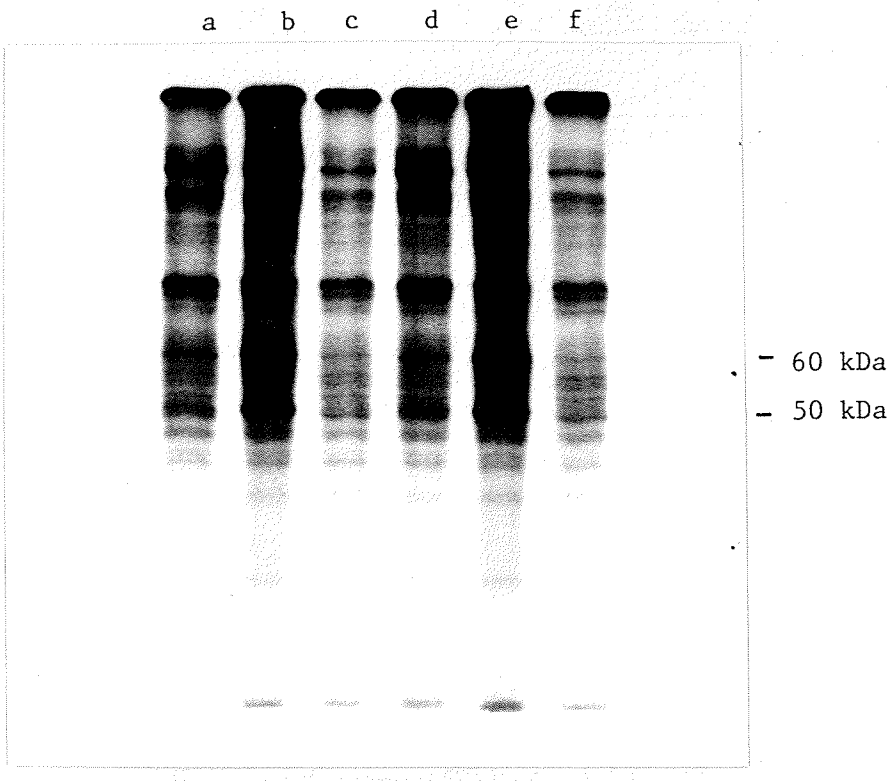
Membrane proteins were solubilized with sample buffer and separated by SDS-PAGE. One gel was dried for autoradiography (Fig 12a) and one gel was treated with alkali and then dried for autoradiography (Figure 12b). The position of the 60-kDa and 50-kDa phosphopeptides was determined and gel slices containing the specific bands were removed from gels that had been stained and destained but not dried or treated with alkali. The proteins were eluted from the gel slices using the agarose-tube gel method described in "Experimental Procedures". Recovery was usually 95 to 100%. A portion of the sample was treated with trichloroacetic acid. The precipitated protein was washed with acetone and dried. The peptides were then partially hydrolyzed with 6N HCl and the phosphoamino acids separated by thin layer electrophoresis (Figure 13). The rest of the sample was subjected to alkali hydrolysis in 1N KOH at 56°C for 1

Figure 12. Rat Brain Synaptic Membrane Phosphorylation for Phosphoamino Acid Analysis.

Autoradiograph of rat brain synaptic membrane phosphorylated for 15 sec at 30°C in a reaction mixture (final volume 100 µl) containing 24 mM Hepes (pH 7.4). 24 mM Mn^{2+}, 12 µM Zn^{2+}, 0.1% NP-40, 3 mM PNPP, 2.5 µM [γ-^{32}P]ATP (specific activity 600 Ci/mol), $\pm$ 50 µg/ml calmodulin, $\pm$ 100 µg/ml Compound 48/80.

Reaction mixture had no additions (lanes a, d), calmodulin (lanes b, e), or Compound 48/80 (lanes c, f). 50 kDa and 60 kDa phosphopeptides were eluted for phosphoamino acid analysis.

A) Untreated Gel



B) Alkali Treated Gel

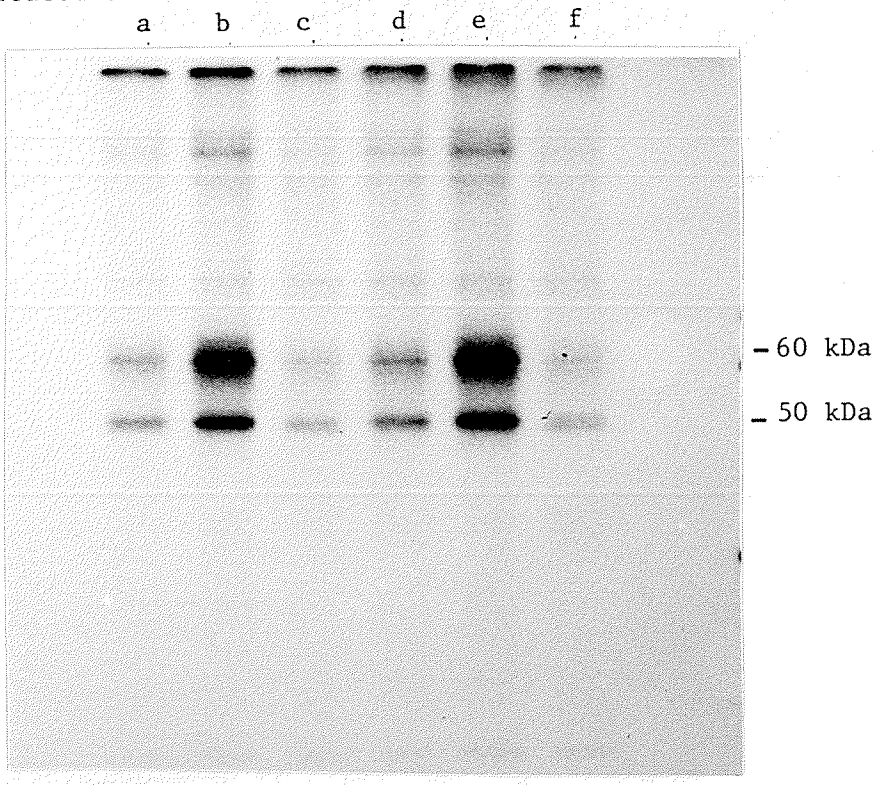


Figure 12

Figure 13. Phosphoamino Acid Analysis of 50 kDa and 60 kDa Phosphopeptides after TCA Precipitation

Autoradiograph of phosphoamino acids separated by thin layer electrophoresis. 50 kDa and 60 kDa were electrophoretically eluted from gel pieces (Figure 12). A portion was precipitated with trichloroacetic acid and hydrolysed with 6N HCl. Dried phosphoamino acids were taken up in a solution of phosphoserine (pS), phosphothreonine (pT) and phosphotyrosine (pY) and phosphoamino acids were separated by thin layer electrophoresis at pH 3.5. Phosphoamino acids were located by staining with ninhydrin and autoradiography. Lanes a, b, c are the 60 kDa phosphopeptide; lanes d, e, f are the 50 kDa phosphopeptide. Additions are none (a, d), calmodulin (b, e) or Compound 48/80. Phosphoserine, phosphothreonine and phosphotyrosine were identified by ninhydrin staining of added standards. The spots between the origin and phosphotyrosine are incompletely hydrolyzed peptides.

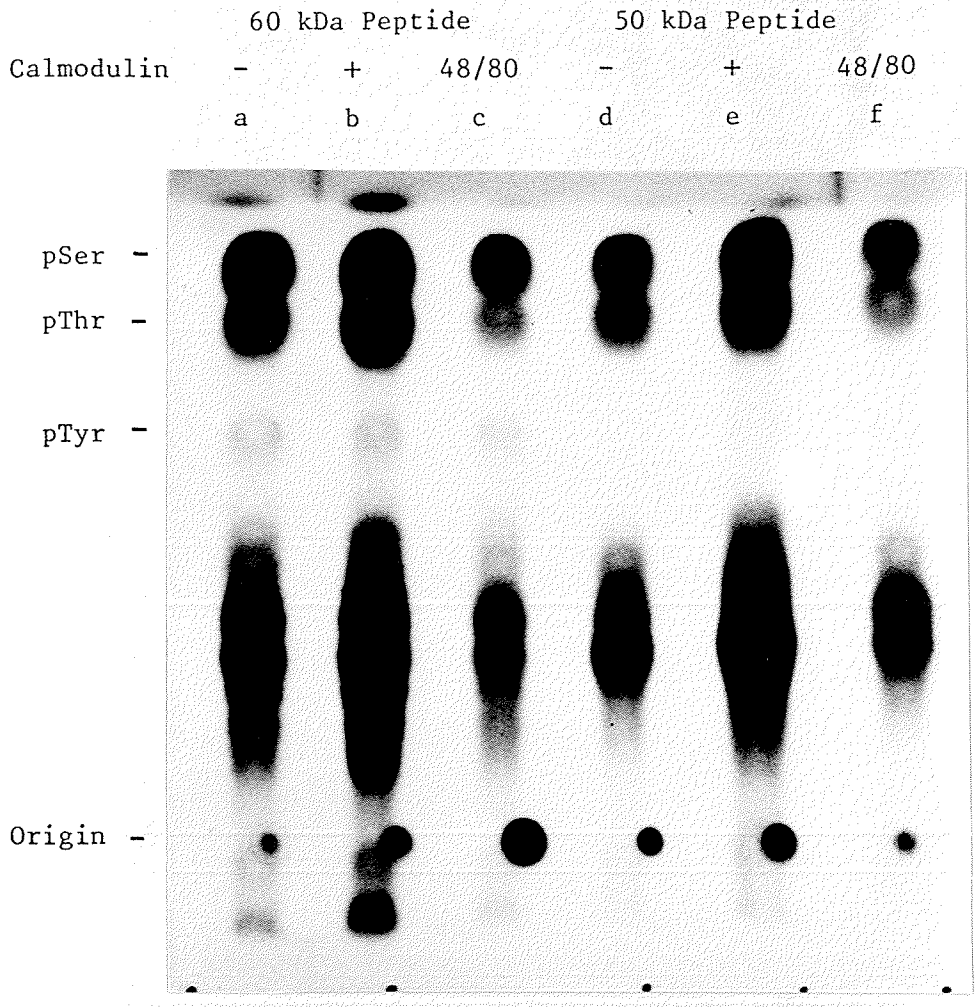

60 kDa Peptide
50 kDa Peptide
Calmodulin
-
+
48/80
-
+
48/80
a
b
c
d
e
f
pSer
pThr
pTyr
Origin

Figure 13

hr. The samples were dialyzed to remove salt and lyophilized. These samples were then partially hydrolyzed with 6N HCl and the phosphoamino acids separated by thin layer electrophoresis (Figure 14). By the radioactivity of the cut out gel slices (Table 3) it was determined that calmodulin stimulated the phosphorylation of the 60-kDa band 3.3 fold, and phosphorylation of the 50 kDa band 2.0 fold over the phosphorylation in the presence of Compound 48/80. Values after alkali hydrolysis were 4.6 fold and 3.1 fold for the 60-kDa and 50-kDa peptides respectively. It was evident that phosphotyrosine was present in both the 60-kDa and 50 kDa peptides in detectable amounts without alkali treatment (Figure 13). When cellulose containing the phosphoamino acids were scraped from the plates, collected and the radioactivity determined (Table 2) it was found that 5 to 8% of the radioactivity (as a percentage of the total radioactive phosphoamino acids recovered) was phosphotyrosine while 70 to 68% of the radioactivity in the 60-kDa peptide was phosphoserine (53 to 57% in the 50-kDa peptide) and 25 to 34% was phosphothreonine (37 to 41% in the 50-kDa peptide). The presence of calmodulin resulted in the stimulation of the phosphorylation of all three phosphoamino acids in both polypeptides. Stimulation of tyrosine phosphorylation was 2.0 fold for the 60-kDa peptide and 1.9 fold for the 50-kDa peptide when compared with phosphorylation in the presence of Compound 48/80.

Alkali treatment of the eluted peptides resulted in the removal of from 70% to 80% of the phosphate from the phosphopeptides (Table 2b). The calmodulin stimulated phosphorylation appeared to be slightly more resistant to the alkali treatment. The phosphoserine in the 50-kDa and 60-kDa phosphopeptide was almost completely hydrolyzed, phosphothreonine was

Figure 14. Phosphoamino Acid Analysis of 50 kDa and 60 kDa Phosphopeptides After Alkali Treatment

Autoradiograph of phosphoamino acids separated by thin layer electrophoresis. Electrophoretically eluted peptides were treated for 1 hr at 56°C in 1N KOH. KOH was removed by dialysis and the peptides were hydrolysed in 6N HCl and treated as described in Figure 13. Lanes a, b, c are the 60 kDa phosphopeptide; lanes d, e, f are the 50 kDa phosphopeptide. Additions are none (a, d), calmodulin (b, e) or Compound 48/80 (c, f).

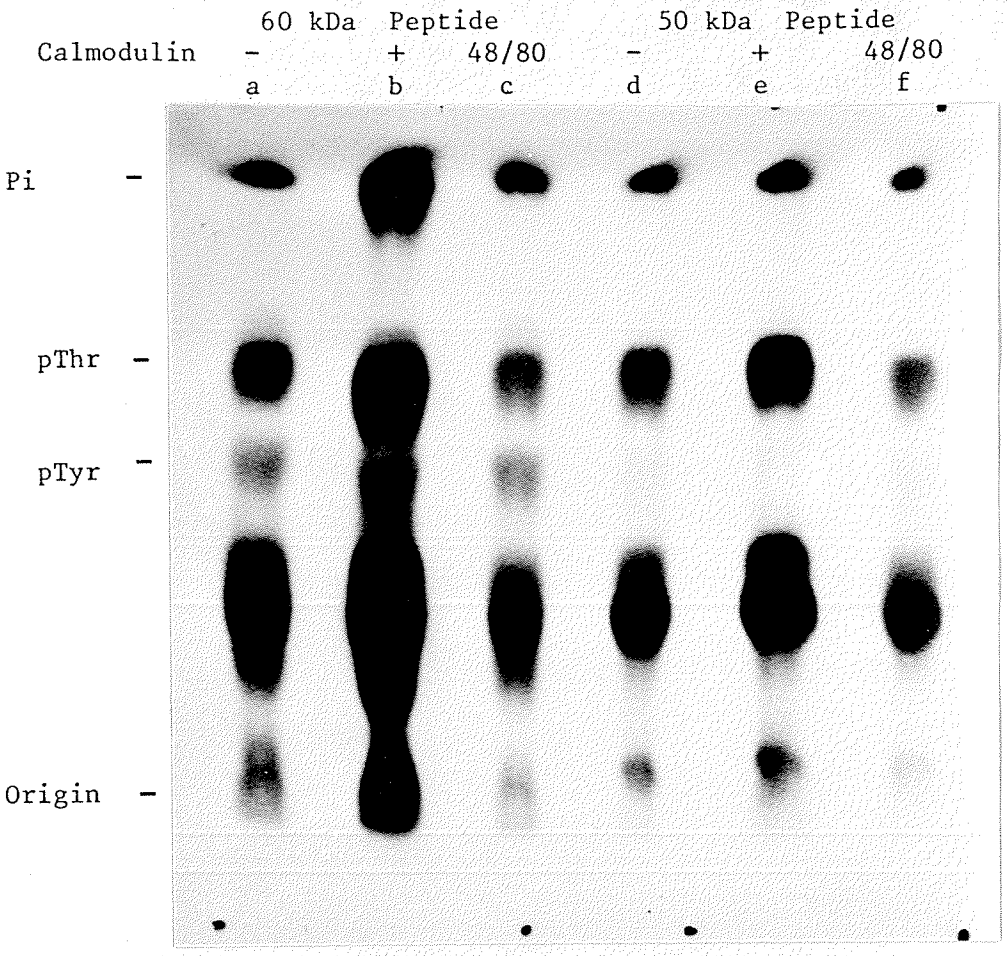

60 kDa Peptide
50 kDa Peptide
Calmodulin
-
+
48/80
-
+
48/80
a
b
c
d
e
f
Pi
pThr
pTyr
Origin

Figure 14

Table 2

Phosphoamino Acid Analysis of 60 kDa and 50 kDa Phosphopeptides from Rat Brain Synaptic Membrane

a) TCA-Precipitated Sample[b]

	60 kDa peptide			50 kDa peptide		
Additions	None	CaM	48/80	None	CaM	48/80
Phosphoamino Acid						
Phosphoserine	402[a]	650	254	192	319	118
Phosphothreonine	170	370	94	125	248	81
Phosphotyrosine	36	56	28	22	35	18

b) Alkali-treated Sample[c]

	60 kDa peptide			50 kDa peptide		
Additions	None	CaM	48/80	None	CaM	48/80
Phosphoamino Acid						
Phosphothreonine	356	1530	244	358	694	235
Phosphotyrosine	130	363	128	68	104	58

a 20% of the eluted sample was precipitated with TCA, the rest was used for the alkali treatment. Values are cpm of radioactive spots scraped off cellulose plates and counted by liquid scintillation spectrometry. Average of duplicate samples.

b Autoradiographed for Figure 13.

c Autoradiographed for Figure 14.

Table 3

Recovery of Radioactivity During Electroelution and Alkali Hydrolysis

A) Electroelution[a]

Additions	Phosphopeptide	CPM in Gel Slices	CPM Eluted	Recovery (%)	CPM Remaining in Gel
None	60 kDa	39600	32253	88	926
	50 kDa	27384	23758	93	416
Calmodulin	60 kDa	93080	77953	90	2122
	50 kDa	44100	36992	90	569
Compound 48/80	60 kDa	27856	22333	86	866
	50 kDa	21762	17378	86	322

B) Alkali Hydrolysis[b]

Additions	Phosphopeptide	CPM Before Alkali	CPM After Alkali	Recovery (%)
None	60 kDa	26770	5067	23
	50 kDa	19719	4101	25
Calmodulin	60 kDa	64701	16869	32
	50 kDa	30703	7433	30
Compound 48/80	60 kDa	18536	3651	24
	50 kDa	14424	2418	20

a CPM values are the average of two samples. Recovery was calculated using a decay factor of 0.93 for 36 hr.

b CPM values are the average of two samples. Recovery was calculated using a decay factory of 0.82.

partially dephosphorylated (approximately 40% hydrolyzed) while the phosphotyrosine appeared to be resistant (Table 2, Figures 13 and 14). Stimulation of tyrosine phosphorylation was 2.8 fold for the 60-kDa peptide and 1.8 fold for the 50-kDa peptide after alkali treatment. Repeated experiments have shown that calmodulin can increase the tyrosine phosphorylation of the 60-kDa and 50-kDa peptides twofold. Exact quantification of the phosphoamino acids is impossible with present techniques. The major problem lies with the differential stabilities of the phosphoamino acids. Phosphotyrosine is not stable to strong acid. According to Martensen & Levine (1983) conditions for complete hydrolysis of proteins (20 min in 3N HCl at 155°) would hydrolyze over 80% of the phosphotyrosine. Cooper et al. (1983) estimated that only 25% of the protein phosphotyrosyl residues are recoverable as phosphotyrosine after 2 hr at 110°C in 5.7N HCl. It is also evident from this experiment that some of the alkali resistant phosphopeptide can be attributed to phosphothreonine. We have found that various modifications to the assay mixture such as using high ATP concentration, longer phosphorylation time and inclusion of EGTA in the reaction mixture decrease the amount of phosphotyrosine or increase the amount of phosphothreonine enough to make the phosphotyrosine undetectable with respect to the phosphothreonine remaining after alkali treatment.

The use of SDS-PAGE as a method for separation of the phosphopeptides for phosphoamino acid analysis depends on an efficient method for elution of the separated peptide bands from the polyacrylamide gel. To accurately identify the peptides of interest we wanted the gels to be stained. Proteins have been recovered from gel slices by homogenization of the gel slices followed by diffusion into buffer or by electrophoretic elution. The diffusion technique usually gives low recovery while the electrophoretic elution techniques usually

require special equipment and work best with unfixed, unstained gels (Wu et al., 1982). The system we developed allows the elution of peptides from stained gels using a tube gel electrophoresis apparatus and the discontinuous buffer system of Laemmli (1970) (see "Experimental Procedures" for details of the method used). Recovery of the eluted protein was usually about 90% of the original radioactivity of the gel slices, with 2 to 3% of the radioactivity remaining bound to the gel (Table 3).

Experiments were undertaken to solubilize and partially purify the endogenous substrates of calmodulin-stimulated phosphorylation.

4.12 Preparation of the 50-kDa and 60-kDa Phosphorylation Substrates

A typical preparation from 9 g of rat brain is outlined as follows:

4.12.1 Membrane Preparation and Solubilization

All steps were performed at 0 to 4°C. Cortex and cerebellum (approx. 1.4 g) were removed from six (~250 g weight) male Sprague Dawley rats and homogenized immediately by eight up-and-down strokes of a motorized Teflon/glass Potter Elvejehm tissue grinder (0.125 mm clearance) in 10 volumes of hypotonic buffer A (5 mM Tris HCl (pH7.5), 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.3 mM PMSF). The PMSF was added from a fresh 0.15 M solution in ethanol. The homogenate was centrifuged at 900 x g for 10 min in a Beckman JA20 rotor to remove large debris. The pellet was rehomogenized in 10 volumes of buffer A and recentrifuged. The supernatants were combined and recentrifuged at 100,000 x g for 1 hr in a Beckman 70Ti rotor to obtain a crude membrane fraction. The membrane pellet was rehomogenized in 50 mM Tris HCl (pH 7.5), 5% (w/v) glycerol, 10 mM KCl, 1 mM EDTA, 1 mM 2-mercaptoethanol

containing 1% (w/v) Triton X-100. The suspension was stirred for 1 hr at 4°C and then centrifuged at 125,000 x g for 1 hr. The insoluble pellet was taken up in 50 mM Tris HCl (pH 7.5), 10 mM KCl, 1 mM EDTA, 1 mM 2-mercaptoethanol.

4.12.2 AffiGel Blue Chromatography

The supernatant fraction was applied to an AffiGel Blue column (2.5 x 13 cm) (Figure 15) previously equilibrated with buffer B (50 mM Tris HCl (pH 7.5), 5% (w/v) glycerol, 10 mM KCl, 1 mM EDTA, 1 mM 2-mercaptoethanol containing 0.1% (w/v) Triton X-100). The column was washed with three column volumes of buffer B. The binding proteins were eluted batchwise with 50 mM Tris HCl (pH 7.5), 5% (w/v) glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.1% (w/v) Triton X-100 containing 1.5 M KCl. The eluted protein fraction was pooled and examined for calmodulin-stimulated endogenous phosphorylation using SDS-PAGE and autoradiography. Fractions 71 to 79 were pooled and dialyzed overnight against 1.5 l of buffer C (50 mM Tris HCl (pH 7.5), 1 mM $MgCl_2$, 0.25 mM $CaCl_2$, 1 mM 2-mercaptoethanol, 0.2% (w/v) Triton X-100).

4.12.3 Calmodulin-Sepharose 4B Affinity Chromatography

After dialysis, the pooled fraction was applied to a calmodulin-Sepharose 4B column (1.5 x 4 cm) (Figure 16) previously equilibrated with buffer C. The column was washed with several bed volumes of buffer C containing 0.2 M NaCl. The calmodulin-binding proteins were eluted with 50 mM Tris HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.1% (w/v) Triton X-100 containing 0.2 M NaCl and 2 mM EGTA.

Figure 15. Separation of Solubilized Proteins by AffiGel Blue Chromatography

100,000 x g supernatant after solubilization (136 mg) was fractionated on an AffiGel Blue column (13 x 2.5 cm). Eluent was assayed for protein concentration using the Bradford method (Bradford, 1976). Column was washed with 200 ml of equilibration buffer starting at fractionation number 30. Elution buffer was changed at fraction 54 to contain 1.5 M KCl to elute the binding proteins. Fractions 21-45 were pooled (AffiGel Blue BT - peak A) and fractions 71-79 pooled (peak B, 21 mg) and dialysed (AffiGel Blue - KCl D) and assayed for endogenous protein kinase activity using SDS-PAGE. Peak A is the breakthrough fraction. Peak B is the binding protein fraction.

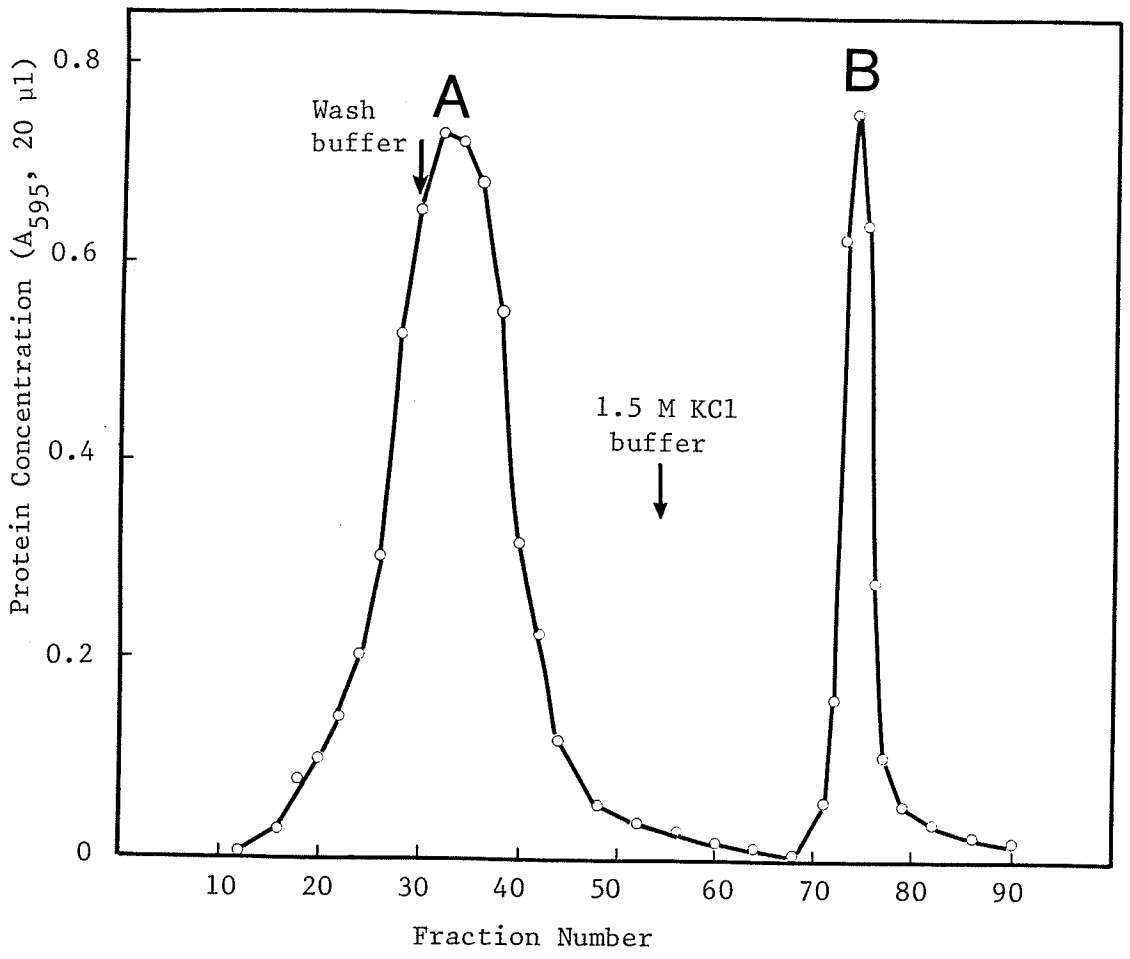

Protein Concentration (A_{595}, 20 µl)
0.8
0.6
0.4
0.2
0
Wash buffer
A
B
1.5 M KCl buffer
10
20
30
40
50
60
70
80
90
Fraction Number

Figure 15

Figure 16. Calmodulin-Sepharose 4B Affinity Chromatography

Purification of calmodulin binding proteins from the AffiGel Blue-binding proteins by calmodulin-Sepharose 4B affinity column. Peak B after dialysis (AffiGel Blue-KCl D) was fractionated on a calmodulin-Sepharose 4B affinity column (4 x 1.5 cm). Eluent was assayed for protein concentration (Bradford, 1976). Elution buffer was changed at fraction 24 to contain 0.2 M NaCl and at fraction 66 to contain 0.2 M NaCl, 2.0 mM EGTA with no calcium. 300 drops per fraction were collected up to fraction 66 where it was changed to 100 drops per fraction. Fractions 8-36 were pooled (CaM-BT, peak C, 14 mg) and fractions 79-82 were pooled (CaM-EGTA, Peak D, 270 µg) and assayed for endogenous protein kinase activity using SDS-PAGE. Peak C is the breakthrough fraction. Peak D is the calmodulin-binding protein fraction.

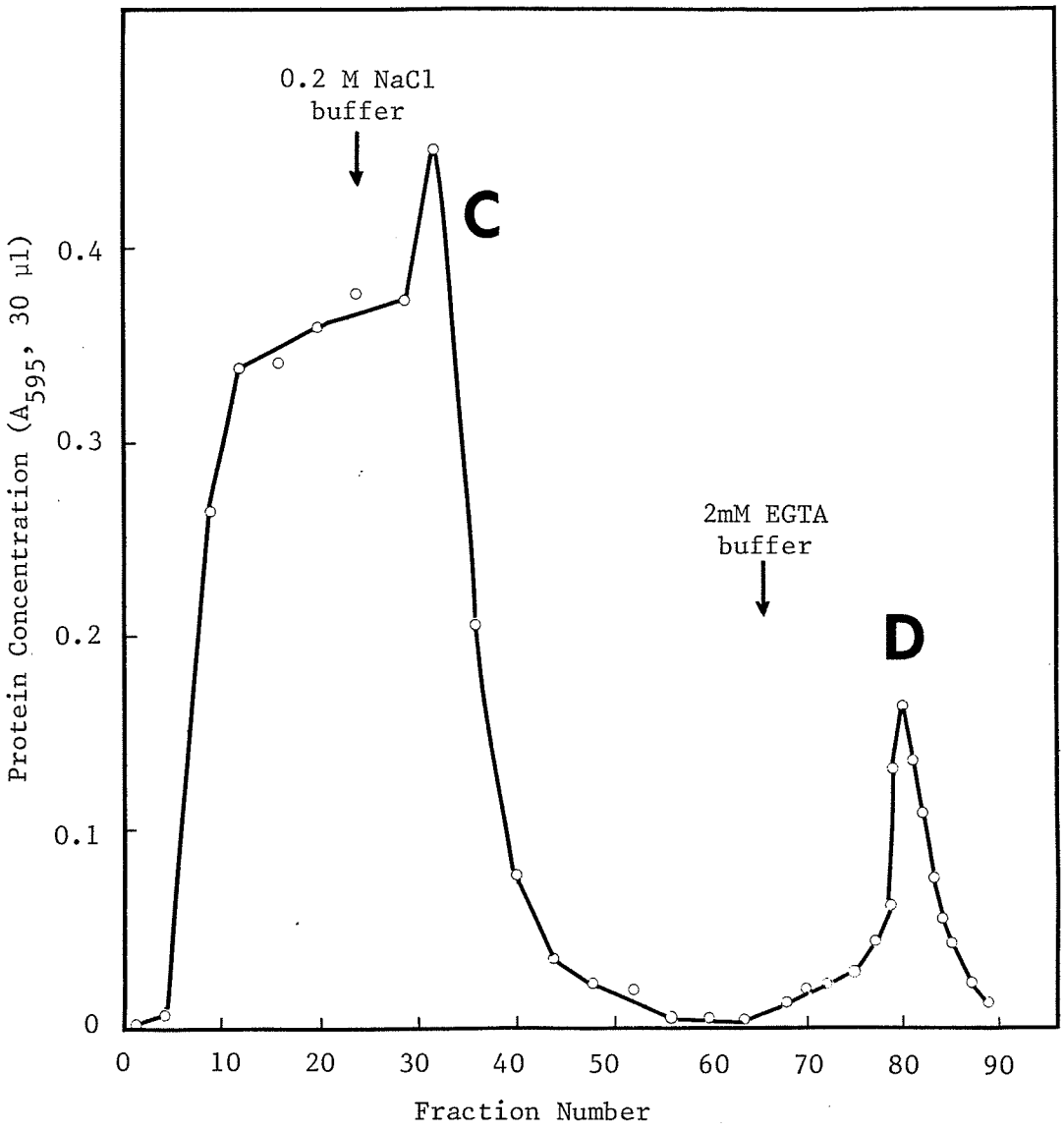

0.2 M NaCl
buffer
C
2mM EGTA
buffer
D
Protein Concentration (A_{595}, 30 µl)
0
0.1
0.2
0.3
0.4
0
10
20
30
40
50
60
70
80
90
Fraction Number

Figure 16

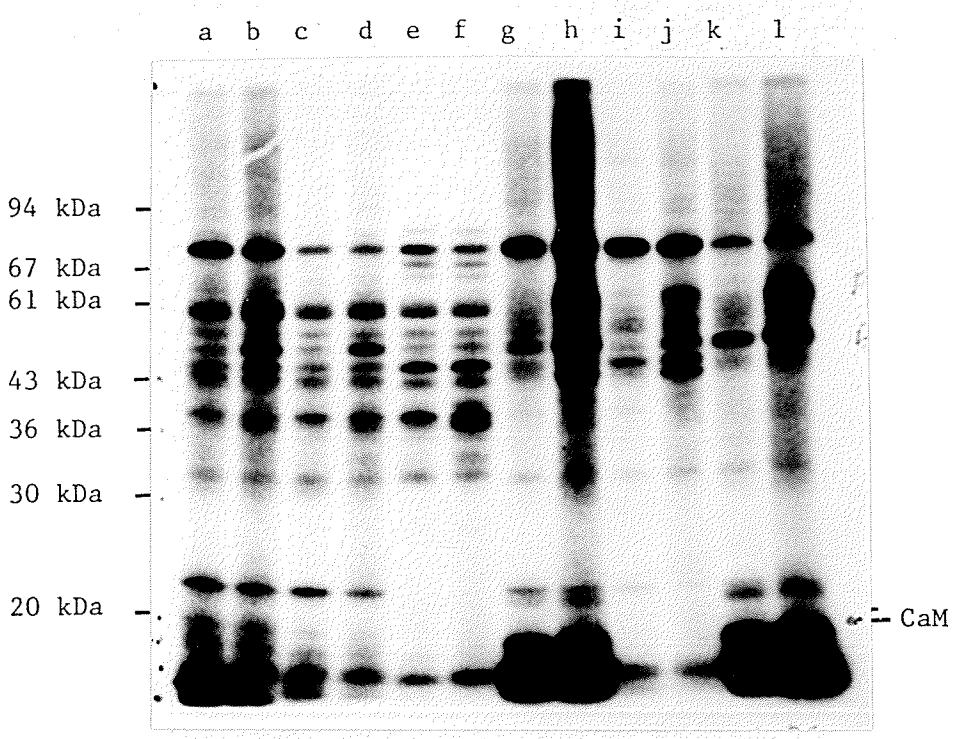
4.12.4 Copurification of 50-kDa Peptide, 60-kDa Peptide and Calmodulin-Stimulated Protein Kinase Activity

45 to 70% of the calmodulin-stimulated activity which phosphorylates the 50- and 60-kDa peptides was extracted from a 100,000 x g pellet of crude rat brain membranes using 1% (w/v) Triton X-100. Fractionation on AffiGel Blue and calmodulin-Sepharose 4B resulted in a copurification of the 50-kDa and 60-kDa peptides and the calmodulin-stimulated protein kinase which phosphorylates them. The 50-v) Triton X-100. Fractionation on AffiGel Blue and calmodulin-Sepharon-Sepharose 4B affinity chromatography. This band co-migrated with the radioactive phosphopeptide detected by autoradiography (Figures 17 and 18). The 60-kDa phosphopeptide was visible but either stains poorly with Coomassie blue or was very heavily phosphorylated compared to the 50-kDa band. Addition of the breakthrough fraction to the EGTA fraction caused no change in the phosphorylation pattern before or after alkali treatment. The copurification of the calmodulin-stimulated protein kinase activity with the 50-kDa and 60-kDa substrates for phosphorylation suggests that these peptides may be subunits of the calmodulin-stimulated protein kinase which undergoes autophosphorylation (Kuret & Schulman, 1985). The activity as judged by the calmodulin-stimulated phosphorylation of the 50-kDa and 60-kDa peptides represents a 90 to 105 fold purification from the homogenate (Tables 4 and 5). Phosphoamino acid analysis was performed on peptide eluted from SDS-polyacrylamide gels. Hydrolysis of TCA precipitated peptides showed that serine and threonine were the predominant sites of phosphorylation in the purified preparation (Figure 19). In the absence of calmodulin the 50-kDa peptide was phosphorylated mainly on threonine with phosphoserine detectable

Figure 17. Endogenous Protein Kinase Activity in Solubilization from Rat Brain

Autoradiograph of solubilization fractions phosphorylated in a reaction mixture containing 20 mM Hepes (pH 7.4), 5 mM $MnCl_2$, 12 mM $MgCl_2$, 100 µM Na_3VO_4, ± 100 µg/ml Compound 48/80, 50 µg/ml calmodulin, 7 mg/ml PNPP, 15 µM [γ-^{32}P]ATP (12 Ci/mmole) for 20 min at 0°C. Reaction was stopped by the addition of SDS-solubilization buffer and boiled for three minutes. Samples were subjected to SDS-PAGE. Duplicate gel was treated for 1 hr in 1N KOH at 56°C. Fractions were homogenate (a, b), 1000 x g supernatant (c, d), 100,000 x g supernatant (e, f), 100,000 x g pellet (g, h), Triton X-100 solubilized supernatant (i, j), Triton X-100 insoluble pellet (k, l). A) Untreated gel. B) Alkali treated gel. C) Protein stained gel.

A) Untreated Gel



B) Alkali Treated Gel

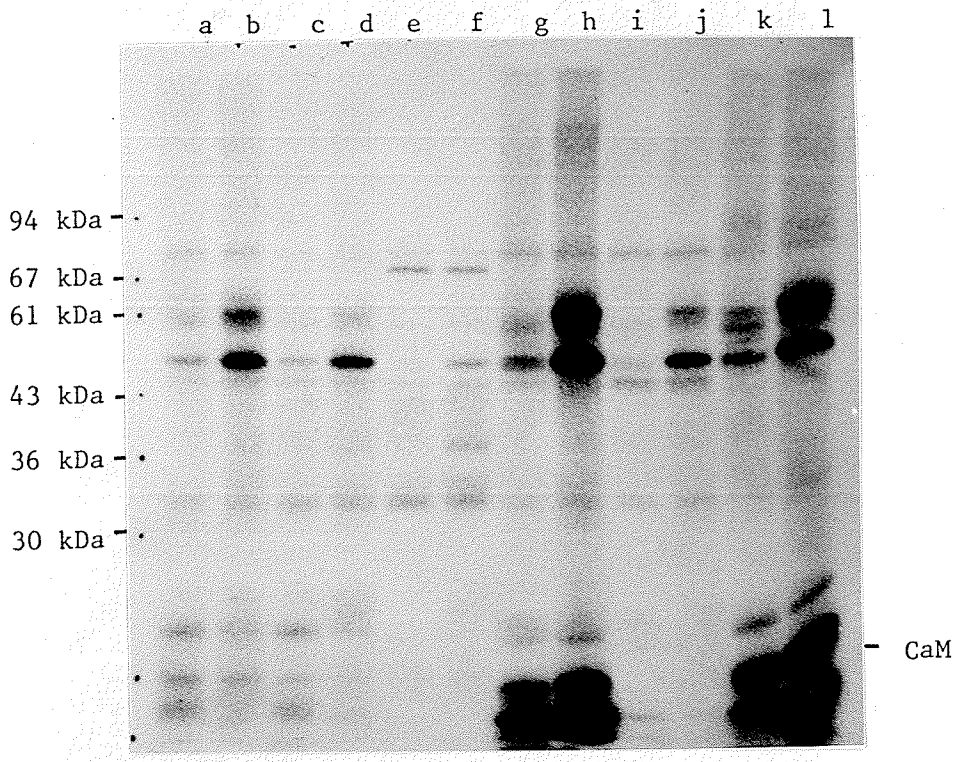


Figure 17

C) Protein Stain of Solubilization Fractions

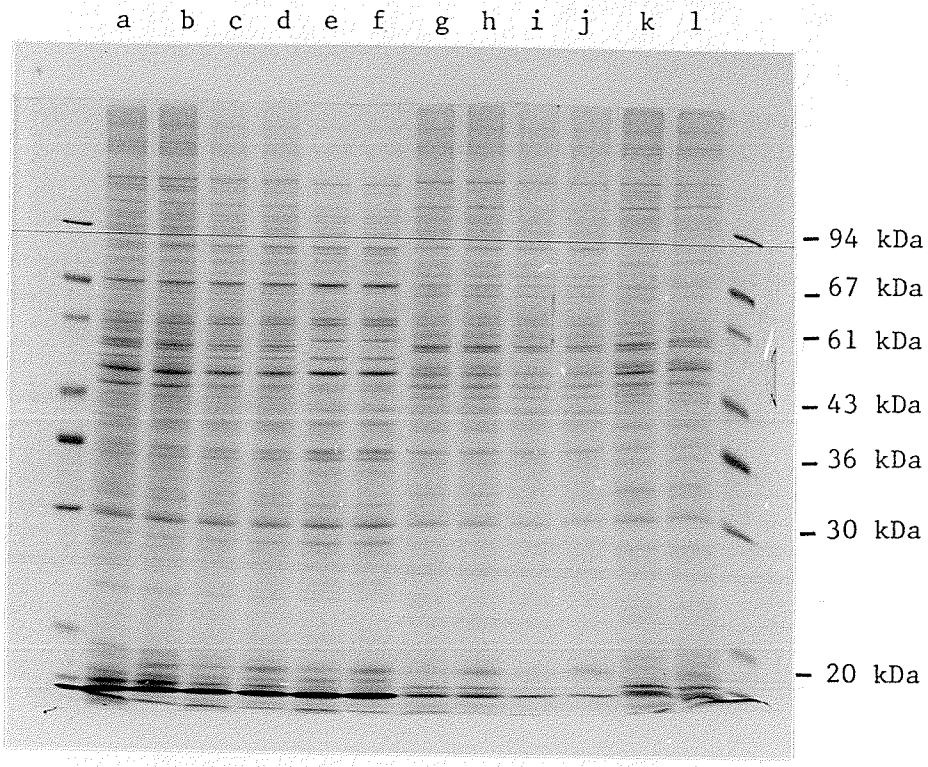
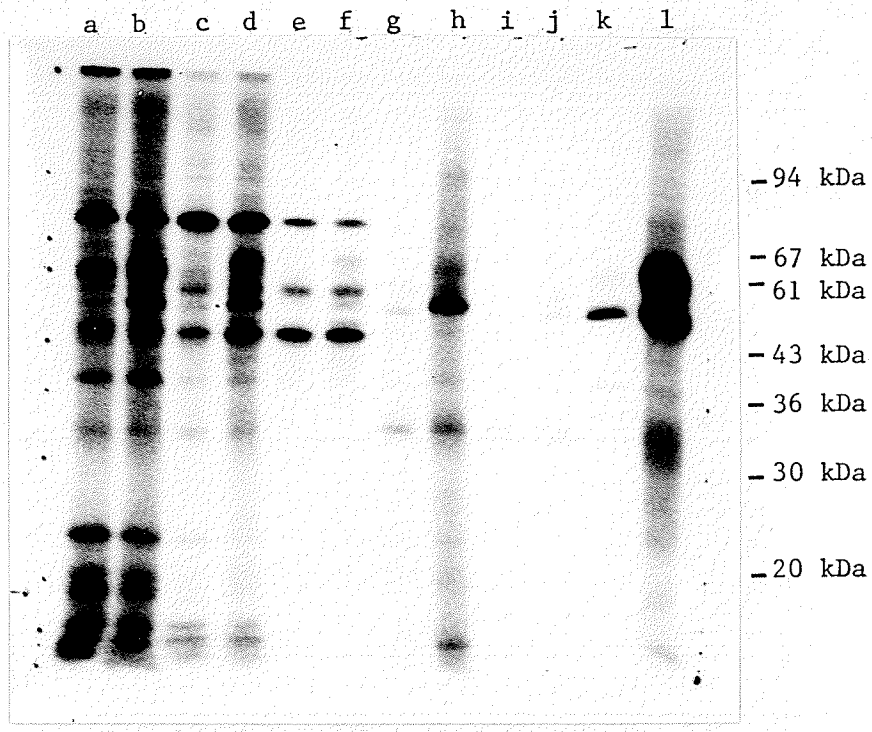


Figure 17 (C)

Figure 18. Endogenous Protein Kinase Activity in Chromatography Fractions from AffiGel Blue and Calmodulin-Sepharose 4B Affinity Chromatographies

Autoradiograph of homogenate, Triton X-100 solubilized supernatant and peak fractions from AffiGel Blue and calmodulin-Sepharose 4B fractions phosphorylated and run on SDS-PAGE as described in the previous figure. Fractions were homogenate (a, b), Triton X-100, solubilized supernatant (c, d), AffiGel Blue breakthrough - peak A (e, f), AffiGel Blue KCl eluted dialysed - peak B (g, h), calmodulin-Sepharose 4B EGTA wash (calmodulin-binding fraction) (k, l). A) Untreated gel. B) Alkali treated gel. C) Protein stained gel.

A) Untreated Gel



B) Alkali Treated Gel

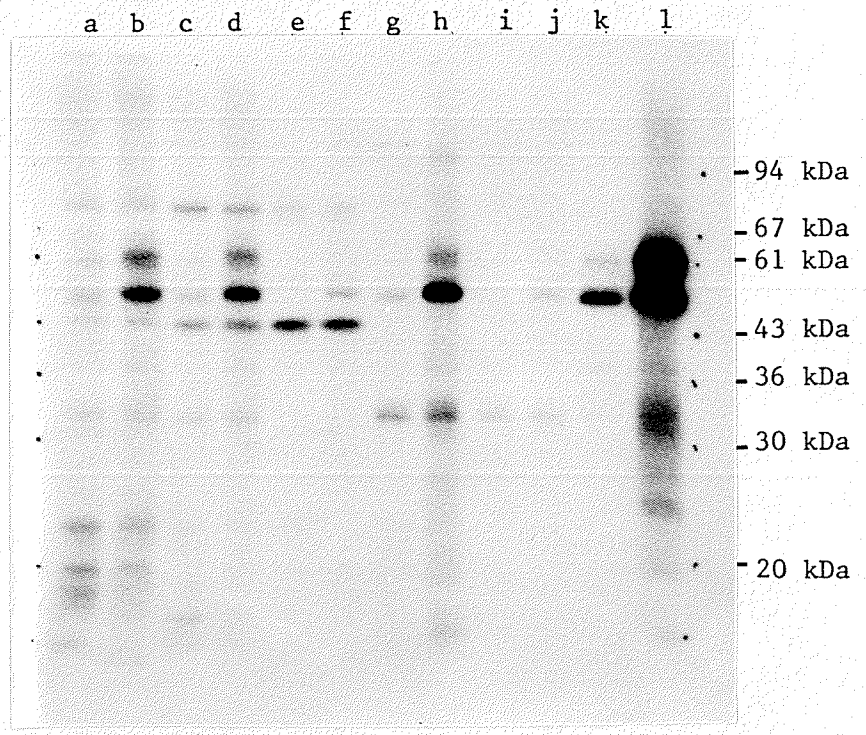


Figure 18

C) Protein Stain of Chromatography Fractions

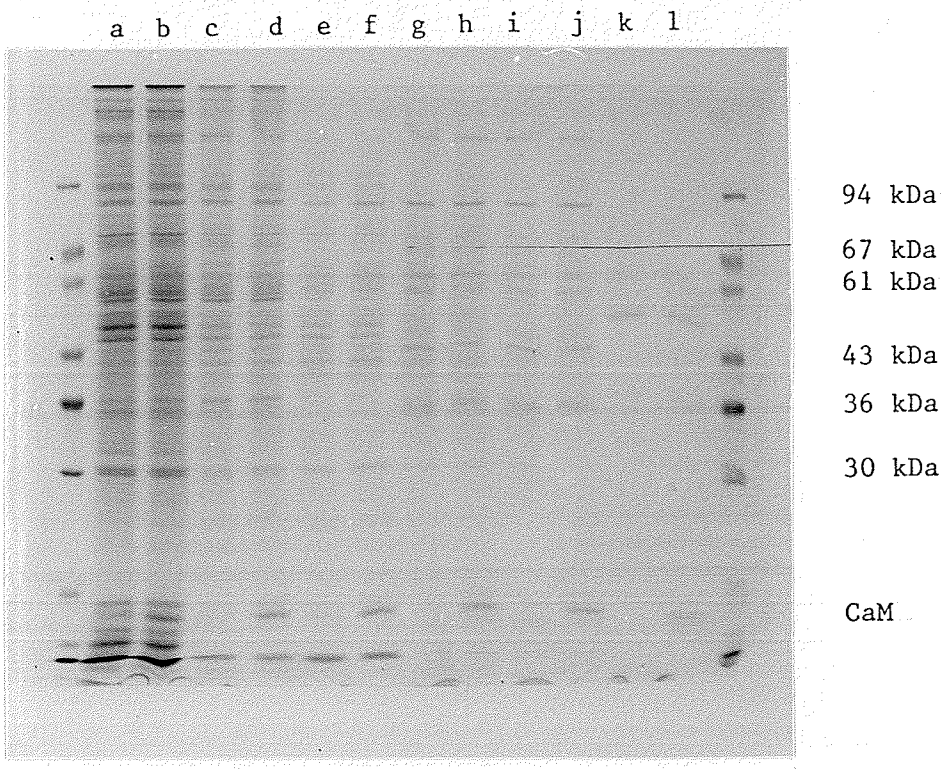


Figure 18 (C)

Figure 19. Phosphoamino Acid Analysis of Calmodulin-Binding Phosphopeptides After TCA Precipitation

Autoradiograph of phosphoamino acids separated by thin layer electrophoresis. Calmodulin binding proteins from rat brain membrane were purified as shown in figure 18. This fraction was phosphorylated using method B and the phosphopeptides separated by SDS-PAGE. 50 kDa and 60 kDa phosphopeptides were eluted from the gel and precipitated with TCA. Phosphopeptides were hydrolyzed in 6N HCl for 1 hr and separated by thin layer electophoresis as described for figure 13. Phosphorylation of the 60 kDa peptide was in the absence (lane a) or presence (b) of calmodulin. Phosphorylation of the 50 kDa peptide was in the absence (c) or presence (d) of calmodulin.

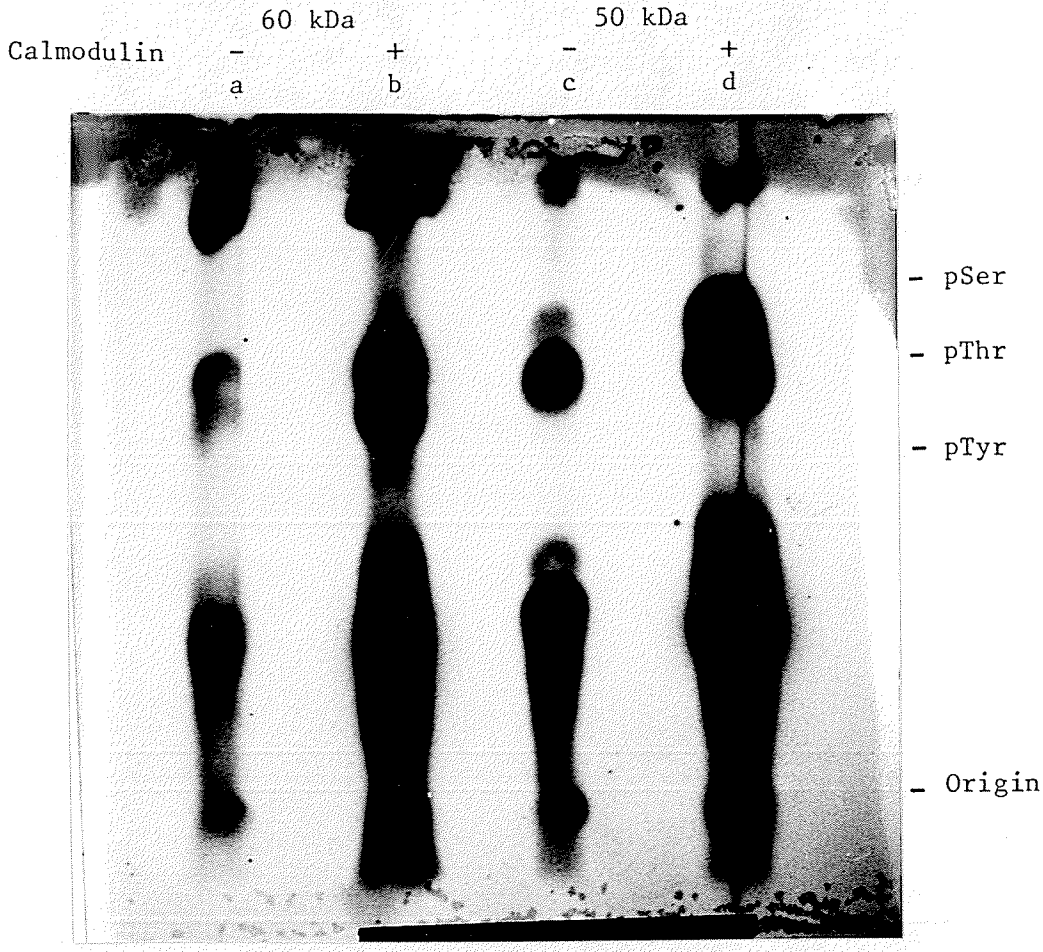


Figure 19

Table 4

Partial Purification of the 60-kDa Phosphopeptide from Rat Brain Crude Membrane

	Step	Total Activity cpm/20 min ($\times 10^3$)	Total Protein mg	Specific Activity[a] cpm/µg/20 min assay	Purification -fold	Recovery[b] %
1	Homogenate	22140	369	60	1	
2a	100,000 x g pellet	36154	194	186	3.1	100
2b	Detergent extract of 100,000 x g pellet	24780	140	177	2.9	68
3a	AffiGel Blue Breakthrough	0	78	0	0	0
3b	AffiGel Blue KCl dialyzed	8148	21	388	6.5	22
4a	Calmodulin-Sepharose 4B Breakthrough	1176	14	84	1.4	5.3
4b	Calmodulin-Sepharose 4B EGTA wash	1898	0.3	6325	105	5.2

a Specific calmodulin stimulated activity is expressed as cpm/ g/20 min assay at 0°C. Specific activity of [γ-^{32}P]ATP at time was 5240 cpm/pmole.

b Recovery is expressed as percentage of the activity in the 100,000 x g pellet.

Table 5

Partial Purification of the 50 kDa Phosphopeptide from Rat Brain Crude Membrane

	Step	Total Activity cpm/20 min ($\times 10^3$)	Total Protein mg	Specific Activity[a] cpm/µg/20 min assay	Purification -fold	Recovery[b] %
1	Homogenate	16974	369	46	1	
2a	100,000 x g pellet	15460	194	80	1.7	100
2b	Detergent extract of 100,000 x g pellet	7000	140	50	1.1	45
3a	AffiGel Blue Breakthrough	234	78	3	0	0
3b	AffiGel Blue KCl dialyzed	7560	21	360	7.8	49
4a	Calmodulin-Sepharose 4B Breakthrough	1176	14	84	1.8	7.6
4b	Calmodulin-Sepharose 4B EGTA wash	1252	0.3	4174	91	8.1

a Specific calmodulin stimulated activity is expressed as cpm/g/20 min assay at 0°C. Specific activity of [γ-^{32}P]ATP at time was 5240 cpm/pmole.

b Recovery is expressed as percentage of the activity in the 100,000 x g pellet.

as well. Calmodulin-stimulated phosphorylation occurred on both serine and threonine residues. In the absence of calmodulin the 60-kDa peptide was phosphorylated on serine and threonine at detectable levels. Calmodulin-stimulated phosphorylation of the 60-kDa peptide occurred at both serine and threonine residues as well. Phosphotyrosine was not detectable.

Hydrolysis of alkali-treated phosphopeptides showed that phosphothreonine was the major alkali-resistant phosphoamino acid although some phosphoserine was detectable as well (Figure 20). Tyrosine phosphorylation was detectable in the 60-kDa peptide phosphorylated in the absence of calmodulin. Phosphorylation of tyrosine in the presence of calmodulin was not visible in this sample.

The breakthrough fraction and the EGTA wash from the calmodulin affinity column were assayed for phosphotyrosine kinase activity using Val^5-angiotensin II which is a substrate for the tyrosine protein kinases associated with several avian sarcoma viruses, the EGF receptor/kinase and cellular tyrosine protein from rat liver (Wong & Goldberg, 1983a, 1984a). Activity in the absence of calmodulin was indistinguishable from background (no added peptide). Activity in the presence of calmodulin in the breakthrough fraction was 24.7 pmol/min/mg protein and 10.3 pmol/min/mg protein. Over 95% of the activity was present in the breakthrough fraction. Calmodulin may contribute to stabilizing the enzyme as activity in the absence of calmodulin was indistinguishable from the control reaction which contained no added Val^5-angiotensin II.

Very recently it was discovered that the purified Rous sarcoma virus src tyrosine kinase could phosphorylate calmodulin (Fukami & Lipmann, 1985). Alkali resistant phosphorylation of a peptide that co-migrates with calmodulin is

Figure 20. Phosphoamino Acid Analysis of Calmodulin-Binding Phosphopeptides After Alkali Treatment

Autoradiograph of phosphoamino acids separated by thin layer electrophoresis. 60 kDa and 50 kDa phosphopeptides were eluted from the phosphorylated calmodulin-binding fraction, as in figure 19, treated with alkali, hydrolyzed and the phosphoamino acids separated as in figure 14. Lanes a, b are without calmodulin, lanes c, d are with calmodulin. Lanes a, c are the 60 kDa phosphopeptide, lanes b, d are the 50 kDa phosphopeptide.

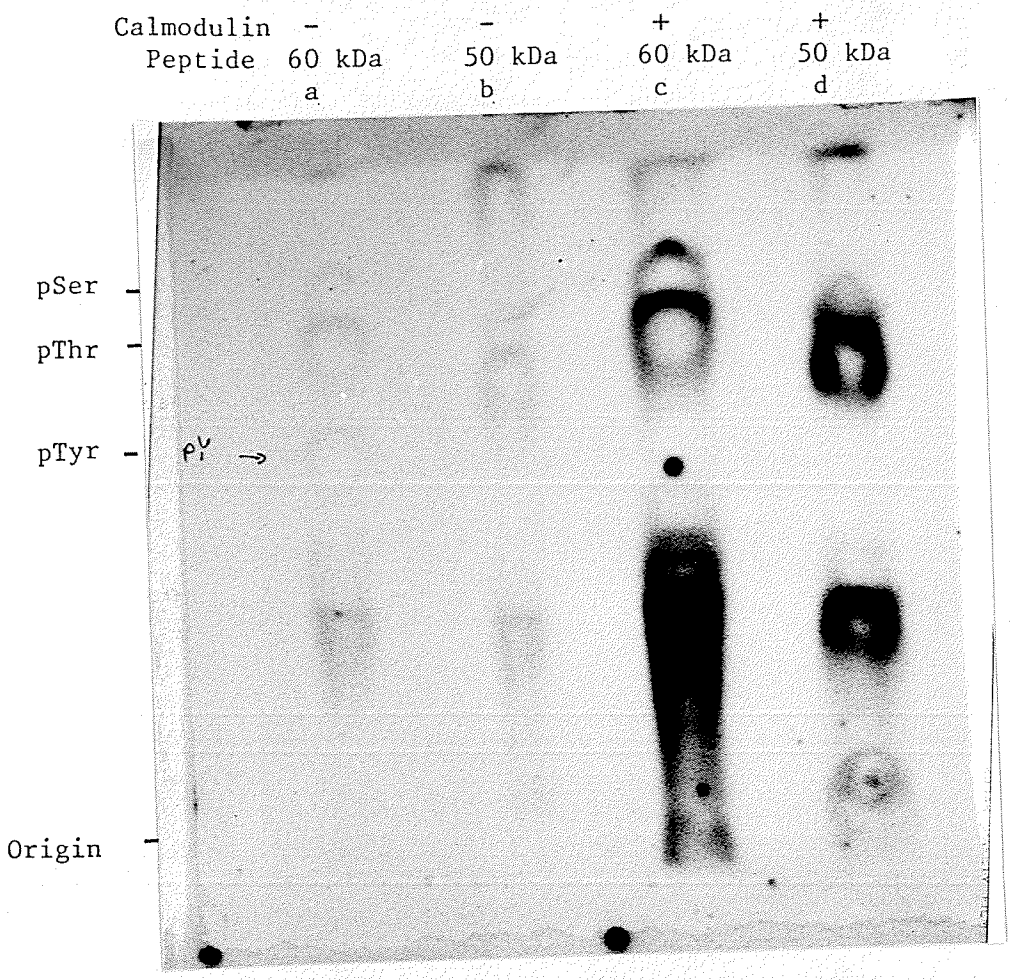

Calmodulin - - + +
Peptide 60 kDa 50 kDa 60 kDa 50 kDa
a b c d
pSer
pThr
pTyr
ρ_i^V →
Origin

Figure 20

present in the insoluble pellet fraction obtained after Triton X-100 extraction (Figure 17). The activity is absent in the supernatant fraction and the following fractions. If this does represent tyrosine phosphorylation of calmodulin then it may indicate that most of the endogenous tyrosine protein kinase was not solubilized.

4.13 Phosphoprotein pp74 from Chicken Brain

The same purification procedure was performed on membranes from chicken brains. In the KCl eluent from the AffiGel Blue column three major phosphopeptide bands were present. Only pp74 was dependent on calmodulin and resistant to alkali treatment (Figure 21). The three phosphopeptide bands were eluted from the gel and phosphoamino acid analysis performed. Only the lowest band contained phosphotyrosine; however, no effect on the tyrosine phosphorylation by calmodulin was present. Calmodulin stimulated phosphorylation of pp74 was found to be at threonine and serine residues without calmodulin. Calmodulin stimulated phosphorylation of pp74 was 13-fold. Another peptide of molecular weight 58-kDa was also phosphorylated in a calmodulin stimulated manner. Calmodulin-stimulated phosphorylation of a 74-kDa peptide present in a calmodulin-binding fraction has not yet been reported. This may represent a new substrate for calmodulin-stimulated protein kinase. Several peptides (over 20) are distinguishable by Coomassie blue staining in the EGTA wash fraction (Figure 23). The calmodulin-stimulated protein kinase which phosphorylates pp74 was not identified.

Figure 21. Chicken Brain Membrane pp74 From AffiGel Blue

Autoradiograph of breakthrough fraction (lanes a, b) and KCl eluted fraction (lanes c, d) from AffiGel Blue of solubilized chicken brain membrane phosphorylated (method B) in the absence (lanes a, d) or presence (lanes b, c) of calmodulin (50 µg/ml).

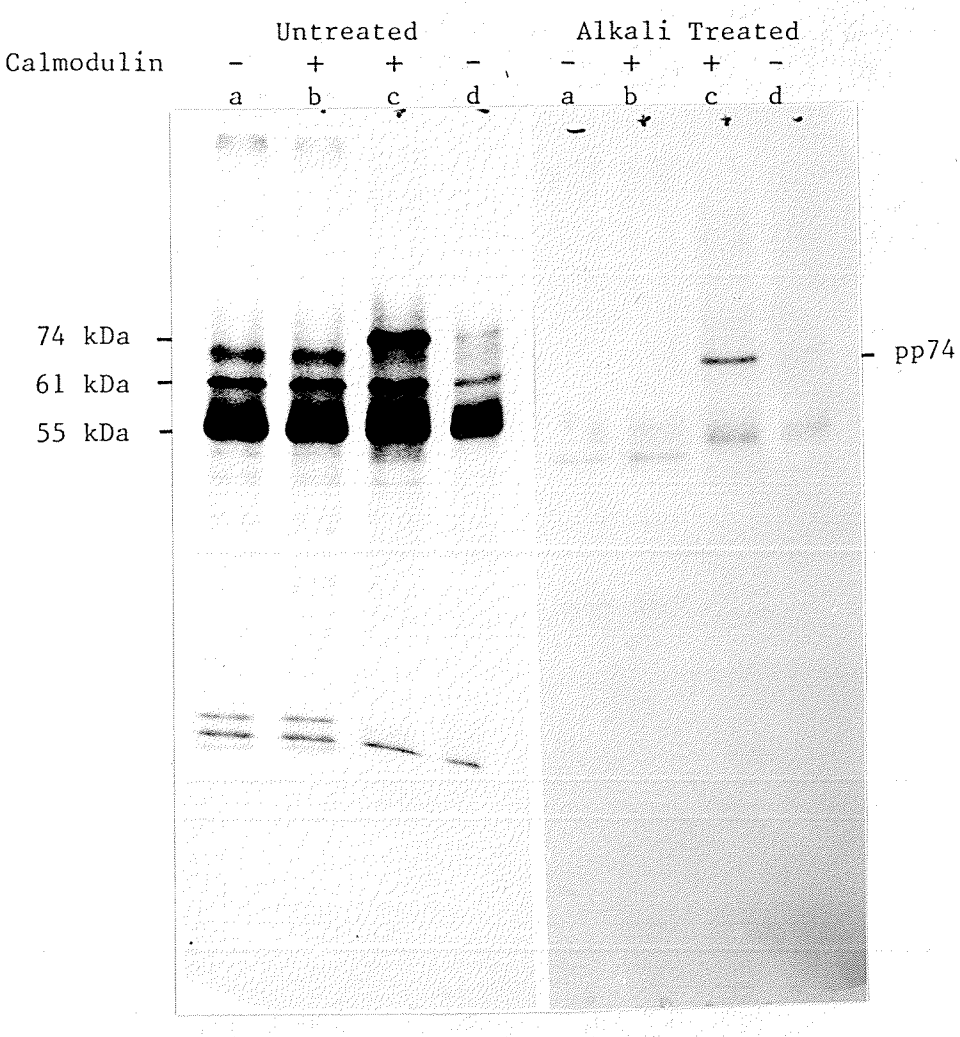

Untreated
Alkali Treated
Calmodulin
- + + - - + + -
a b c d a b c d
74 kDa
61 kDa
55 kDa
pp74

Figure 21

Figure 22. Phosphoamino Acid Analysis of Phosphopeptides from AffiGel Blue KCl Fraction of Solubilized Chicken Brain Membrane

Autoradiograph of phosphoamino acids separated by thin layer electrophoresis phosphopeptides were eluted from gel, TCA precipitated, hydrolyzed with HCl and separated by thin layer electrophoresis at pH 3.5 as already described. 74 kDa phosphopeptide (lanes a, b), 61 kDa phosphopeptide (lanes c, d), 55 kDa phosphopeptide (lanes e, f).

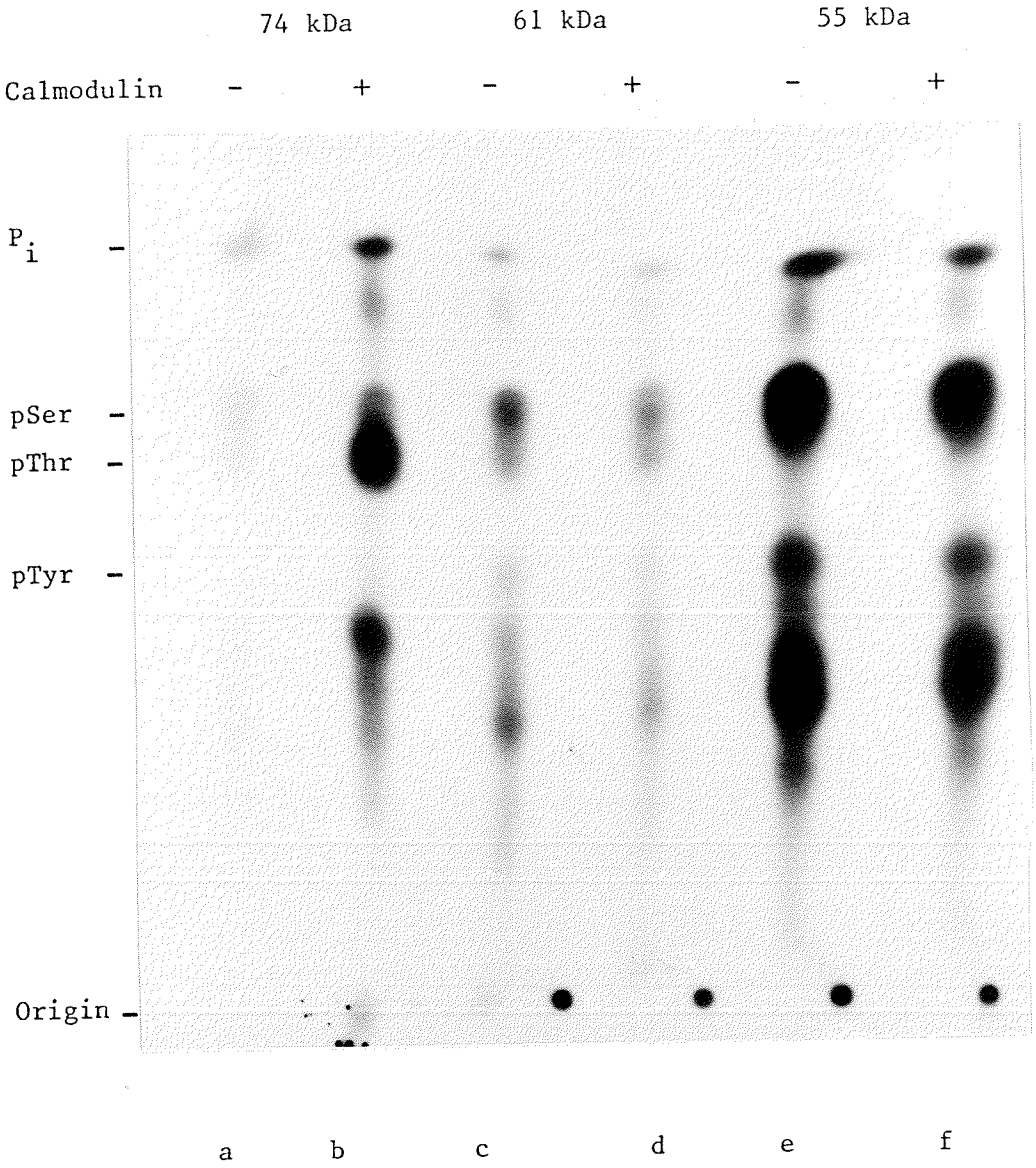
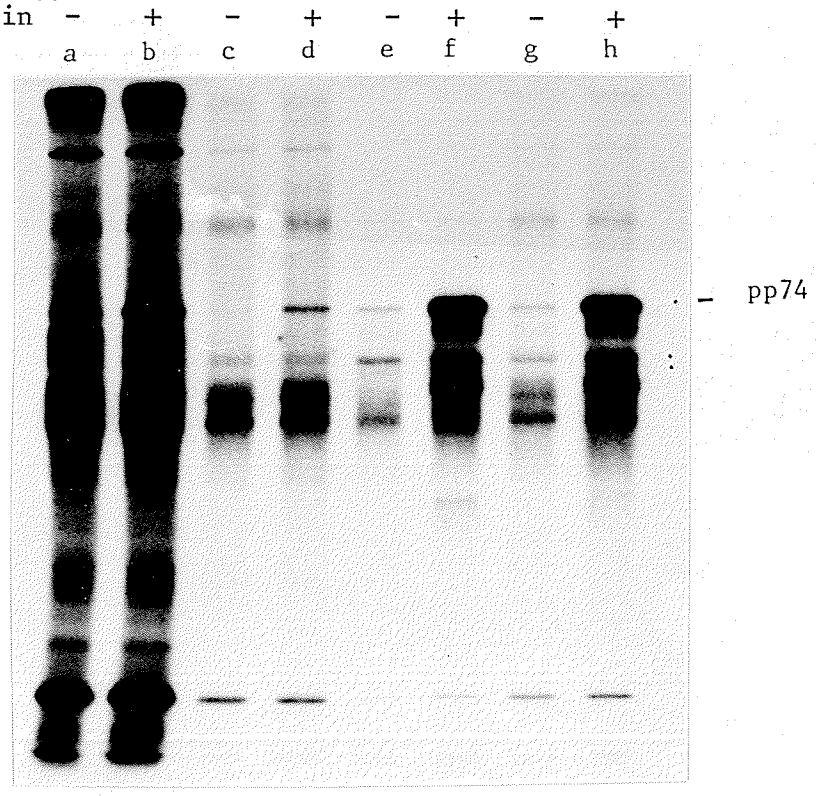

74 kDa
61 kDa
55 kDa
Calmodulin - + - + - +
Pi
pSer
pThr
pTyr
Origin
a b c d e f

Figure 22

Figure 23. Phosphorylation of pp74 in the Calmodulin-Binding Fraction from Solubilized Chicken Brain Membrane

pp74 was further purified by affinity chromatography on calmodulin-Sepharose 4B AffiGel Blue KCl fraction (lanes a, b), calmodulin-Sepharose 4B fractions (lanes c-h), breakthrough (lanes c, d), EGTA (binding) fraction (lanes e, f), 1:1 mixture of breakthrough and EGTA fractions (lanes g, h).

A) Untreated Gel



B) Alkali Treated Gel

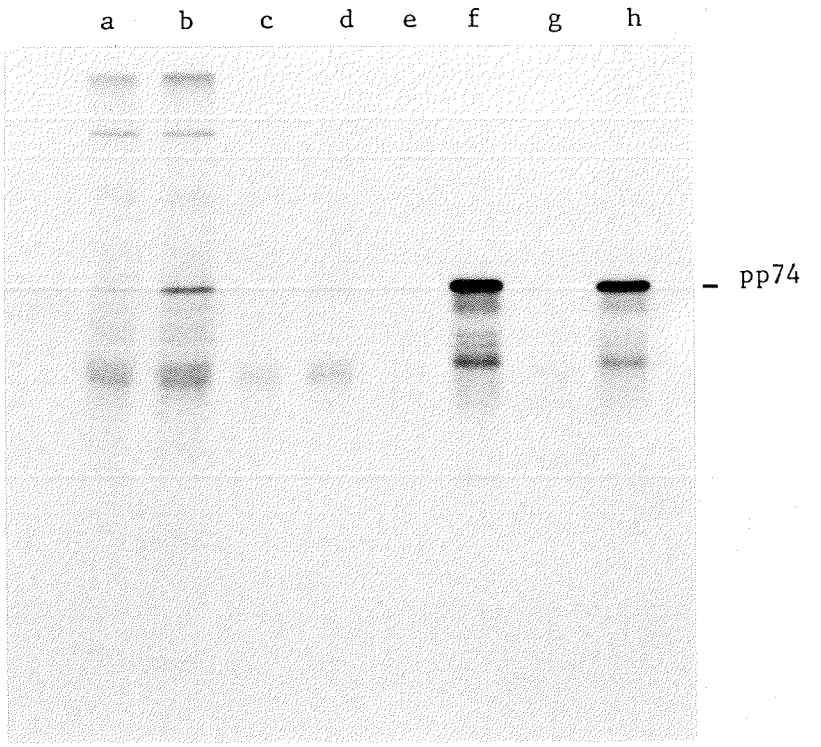


Figure 23

C) Protein Stain of Chicken Brain Fractions

(C) Calmodulin Sepharose 4B Breakthrough Fraction
(E) Calmodulin Sepharose 4B EGTA (Binding) Fraction

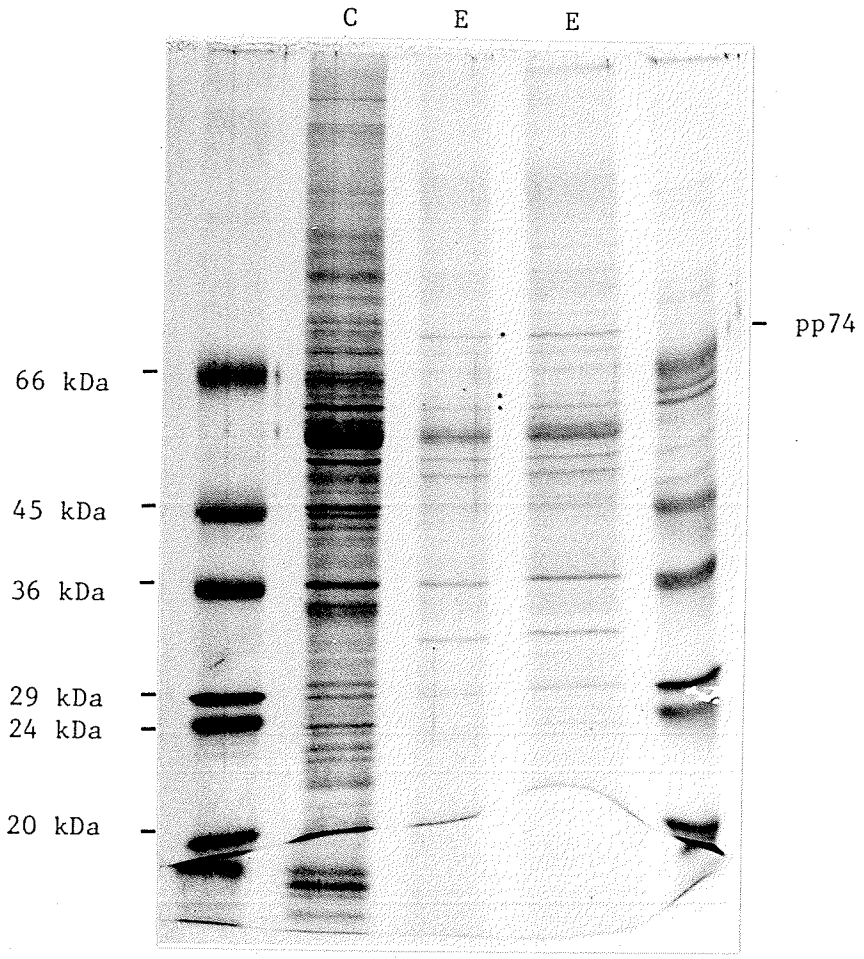


Figure 23 (C)

CHAPTER V

DISCUSSION

Some growth factors and acutely transforming viruses cause an increase in tyrosine phosphorylation. This has led to the hypothesis that tyrosine phosphorylation is one of mechanisms controlling growth, a mechanism which the transforming vituses use to cause uncontrolled growth. Tyrosine protein kinases were discovered due to their autophosphorylation activity. Little is known about their endogenous substrates, modulation of a physiological function by tyrosine phosphorylation or factors which regulate tyrosine protein kinase activity.

The calmodulin regulatory system is an important system for mediating the intracellular effects of calcium. Calmodulin has been shown to be important in the regulation of many physiological processes (Klee & Vanaman, 1982). Calmodulin is known to act directly on some proteins, such as cyclic nucleotide phosphodiesterase, to affect physiological processes. Calmodulin also acts indirectly on regulatory proteins through the regulation of protein phosphorylation by the actions of calmodulin-stimulated phosphoprotein phosphatases (Stewart et al., 1982).

Calcineurin, a calmodulin-binding protein (Wang & Desai, 1976), was shown to be a calmodulin-stimulated phosphoprotein phosphatase (Stewart et al., 1982). Pallen & Wang (1983) found that calcineurin could dephosphorylate free phosphotyrosine and the structurally similar PNPP but not free phosphothreonine or phosphoserine. This suggestd that calcineurin possessed phosphotyrosyl-

phosphatase activity and that the calmodulin regulatory system could have an effect on the tyrosine protein kinase system.

Since calcineurin is high in brain this tissue was examined for phosphotyrosyl proteins. Membranes from rat, Guinea pig, chicken and cow grains were examined for alkali-resistant phosphopeptides. Resistance to alkali hydrolysis has been used as an indication of phosphotyrosine in peptides which have been separated by SDS-PAGE (CARPENTER et al., 1983). Several alkali-resistant phosphopeptides were noted in the brain membrane fractions.

Synaptic membranes from rat and Guinea pig brain were examined for the effects of calmodulin and calcineurin on alkali-resistant phosphopeptides. Calmodulin was found to stimulate the phosphorylation of a 50-kDa alkali resistant phosphopeptide and a 60-kDa alkali resistant phosphopeptide. On SDS-polyacrylamide gels the 60-kDa phosphopeptide runs as a broad band and in some cases sis resolved into a doublet with a major (60-kDa) and a minor (58-kDa) band but these will be referred to collectively as the 60-kDa phosphopeptide.

These peptides were eluted from the gels and either precipitated with TCA and subjected to partial hydrolysis or treated with alkali followed by partial hydrolysis. Phosphoamino acid analysis indicated that phosphotyrosine was present in both the 50-kDa and 60-kDa phosphopeptides and that calmodulin stimulated tyrosine phosphorylation approximately 20-fold for the 60-kDa peptide and 1.9-fold for the 50-kDa peptide. The majority of the phosphoamino acid recovered from the two phosphopeptides was phosphoserine (approximately 65% of the 60-kDa peptide and 55% of the 50-kDa peptide). Phosphothreonine was also present (approximately 30% of th60-kDa peptide and 40% of the 50-kDa

peptide). Only 5 to 10% of the total phosphoamino acids recovered was phosphotyrosine.

Treatment of the eluted peptides with alkali resulted in the hydrolysis of 70 to 80% of the phosphate esters. Phosphoserine was almost completely resistant to hydrolysis. Only 40% of the phosphothreonine residues were hydrolyzed. Since there is much more phosphothreonine present this indicates that many of the alkali resistant phosphopeptides present in the brain samples could be due to phosphothreonyl proteins not phosphotyrosyl proteins. However, in this case 5 to 10% of the phosphoamino acids in the 50- and 60-kDa phosphopeptides is phosphotyrosine.

Insulin and EGF were found to have no effect on the phosphorylation of the 50-kDa and 60-kDa peptides in synaptic membrane in the absence or presence of calmodulin; however, this could be due to a lack of the receptor/kinase in the synaptic membrane preparation as no ligand-stimulated autophosphorylation of the receptors was noted.

These results indicate that calmodulin stimulates the phosphorylation of the 50-kDa and 60-kDa peptides at serine, threonine and tyrosine residues.

Calcineurin, the calmodulin-dependent phosphoprotein phosphatase, was found to dephosphorylate the 50-kDa and the 60-kDa phosphopeptides at both alkali sensitive and alkali resistant sites. Calcineurin also dephosphorylated many of the other phosphopeptides present in synaptic membrane. Dephosphorylation of these phosphopeptides could be blocked by addition of PNPP, a substrate for calcineurin and an inhibitor of phosphotyrosyl-protein phosphatases. Synaptic membranes contained endogenous phosphatase activity which could dephosphorylate the phosphopeptides. The endogenous phosphatase

activity may be due to calcineurin as calcineurin was detected in immunoblots of synaptic membrane using monoclonal antibody VA1, which is specific for the beta subunit of calcineurin. Calcineurin has been shown to catalyze the dephosphorylation of several phosphoseryl- and phosphothreonyl-proteins including the alpha-subunit of phosphorylase kinase, inhibitor-1, myosin light chain, phosphohistone, phosphocasein synapsin-1, G-substrate and DARPP-32 (Stewart et al., 1982; Blumenthal & Krebs, 1983; Tallant & Cheung, 1984; King et al., 1984). Calcineurin has also been shown to possess phosphatase activity towards phosphotyrosyl-proteins including phosphotyrosyl-casein, phosphotyrosyl-histone and the autophosphorylated EGF receptor (Chernoff et al., 1984; Pallen et al., 1985).

The results suggest that calmodulin may be involved in both the phosphorylation and the dephosphorylation of the 50- and 60-kDa peptides. These events may be temporally separated in that rising Ca^{2+} levels may stimulate the calmodulin-stimulated protein kinase followed by phosphorylation of the 50- and 60-kDa peptides. Further increase in Ca^{2+} may activate calcineurin followed by dephosphorylation of the 50- and 60-kDa peptides. A smilar sequence is thought to occur in cyclic nucleotide metabolism since calmodulin can stimulate adenylate cyclase and cyclic AMP formation and calmodulin also stimulates cyclic nucleotide phosphodiesterase and cyclic AMP degradation.

The identity of the 50-kDa and 60-kDa peptides has not been determined; however, the pattern of calmodulin-stimulated, alkali-resistant phosphorylation closely resembles the phosphorylation pattern obtained by the calmodulin-stimulated autophosphorylation of a cytosolic calmodulin-stimulated

protein kinase of brain (Kennedy et al., 1983). This calmodulin-stimulated protein kinase is found in several subcellular fractions of brain (Sahyoun et al., 1985) and has been called by several names, including calmodulin-dependent protein kinase II (Yamauchi & Fujisawa, 1980) and synapsin I kinase II (Kennedy et al., 1983). A cytosolic form of the enzyme has been purified (Bennett et al., 1983). the 50-kDa and 60-kDa subunits of the cytosolic calmodulin-dependent protein kinase have been shown to bind calmodulin and to undergo in situ autophosphorylation in renatured SDS-polyacrylamide gels (Kuret & Schulman, 1985). The brain enzymes may represent isozymic forms of a multifunctional calmodulin-dependent protein kinase (McGuinness et al., 1983). The calmodulin-dependent glycogen synthase kinase from skeletal muscle may be another form (Woodgett et al., 1984).

The 50- and 60-kDapeptides were extracted from a crude rat brain membrane using 1% Triton X100. The peptides werre further purified by affinity chromatography on AffiGel Blue and calmodulin-Sepharose 4B. This showed that the 50- and 60-kDa peptides were calmodulin-binding proteins. The purified 50- and 60-kDapeptides underwent calmodulin-stimulated protein kinase which copurified with the 50- and 60-kDa peptides. The activity, as judged by the calmodulin-stimulated phosphorylation of the 50-kDa and 60-kDa peptides, represented a 90- to 105-fold purification from the homogenate. Phosphoamino acid analysis showed that the phosphorylation of both peptides was almost exclusively at serine and threonine residues. After KOH hydrolysis, phosphothreonine was the major phosphoamino acid present with small amount of phosphoserine present in both peptides. After alkali hydrolysis phosphotyrosine

was faintly detectable in the 60-kDa phosphopeptide using prolonged autoradiography.

Preliminary experiments using Val^5-angiotensin II as a substrate for tyrosine protein kinase activity (Wong & Goldberg, 1983a) were performed. Although the experiments are difficult to interpret due to high control values (in the absence of Val^5-angiotensin II) it appeared that no tyrosine protein kinase activity was measurable in either the breakthrough or the EGTA wash fractions from the calmodulin-Sepharose 4B affinity column. In the presence of calmodulin, there appeared to be tyrosine protein kinase activity detectable in both fractions with 95% of the activity present in the breakthrough fraction. The results appear to suggest the presence of calmodulin-stimulated tyrosine protein kinase activity; however, this activity would be expected to bind to the calmodulin column and not be present in the breakthrough fraction.

Calmodulin itself appears to be a substrate for tyrosin protein kinase. Fukami & Lipmann (1985) found that purified $pp60^{src}$ could phosphorylate calmodulin. Haring et al. (1985) found that the insulin receptor could phosphorylate calmodulin. Most gels were run for one to two hours after the dye front was eluted to give better resolution of the 50- to 60-kDa peptides which would mean that the small proteins like calmodulin would be run off the gels. This was not done for Figure 17. In this autoradiograph alkali-resistant phosphorylation of a peptide that co-migrates with calmodulin is present in the insoluble pellet fraction obtained after Triton X100 extraction. Phosphorylation of this peptide appears to be increased with the addition of calmodulin to the reaction mixture. It is not yet known if this represents phosphorylation of calmodulin on a tyrosine residue but it is tempting to speculate that if it does,

it is an indication that the tyrosine protein kinase may not have been solubilized since this band is absent in the phosphorylation of the supernatant fraction.

The data presented suggests that tyrosine protein kinases can interact with the calmodulin regulatory system. Two peptides were phosphorylated in synaptic membrane by a calmodulin-stimulated protein kinase. It appears likely that the peptides are subunits of the calmodulin-stimulated protein kinase as they copurify with the kinase. Calmodulin appears to stimulate the tyrosine phosphorylation of these peptides in synaptic membrane; however, the calmodulin-stimulated tyrosine phosphorylation of peptides in synaptic membrane which comigrate with the 50-kDa and 60-kDa phosphopeptides cannot be discounted yet. To show that these are the peptides phosphorylated will require reconstitution of a system with purified 50-kDa and 60-kDa peptides and purifid brain tyrosine protein kinase. The mechanism of action of calmodulin stimulation of tyrosine phosphorylation is a matter of speculation. Calmodulin does not stimulate tyrosine protein kinase activity in rat brain synaptic membrane when Val^5-angiotensin II is used as a substrate to measure activity. This suggests that calmodulin may increase the tyrosine phosphorylation by its binding to the substrate through a substrate effect similar to the effect that calmodulin binding to myosin light chain kinase has on MLCK phosphorylation by cyclic AMP-dependent protein kinase (Conti & Adelstein, 1981) and dephosphorylation by smooth muscle phosphatase-1 (Pato & Adelstein, 1983). Calmodulin has been reported to stimulate a tyrosine protein kinase which phosphorylates the 17 β-estradiol receptor (Migliaccio et al., 1984); however, their data supports the claim that calmodulin stimulates tyrosine phosphorylation of the 17 β-estradiol

receptor. The effect may be through calmodulin stimulation of the tyrosine protein kinase but an effect through binding of calmodulin to the 17 ß-estradiol receptor could also be possible. The recent discovery that calmodulin itself could be a substrate for the Rous sarcoma virus tyrosine kinase (Fukami & Lipmann, 1985) indicates that these regulatory systems may be interrelated at more than one level, similar to the interrelationship of the cyclic AMP and the calmodulin regulatory systems. The phosphorylation of calmodulin and the possible phosphorylation of the calmodulin-stimulated protein kinase may be a clue to the mechanism for the physiological effects of tyrosine protein kinases. Because the tyrosine phosphorylation is a minor amount of the total phosphorylation of the 50-kDa and 60-kDa peptides, the possibility that it may be due to minor peptides that comigrate on SDS-PAGE cannot be discounted. However, if it can be confirmed that a calmodulin-dependent protein kinase can be phosphorylated by a tyrosine protein kinase, then it may be possible that some of the physiological effects of tyrosine kinases on growth are due to changes in the activity of calmodulin-dependent protein kinases. Haring et al. (1985) found similar results. The calmodulin-dependent glycogen synthase kinase from rabbit liver was phosphorylated at tyrosine by the insulin receptor; however, no stimulation of tyrosine phosphorylation by calmodulin was found.

A novel protein, pp74, was partially purified from chicken brain by affinity chromatography on AffiGel Blue and calmodulin-Sepharose 4B. This peptide was a calmodulin binding protein and was phosphorylated in calmodulin-stimulated fashion indicating that the calmodulin-dependent protein kinase which phosphorylates pp74 is also a calmodulin binding protein. Either the substrate pp74 and the protein kinase are both calmodulin-binding proteins

or the pp74 may represent autophosphorylation of a subunit of a calmodulin-stimulated protein kinase. The only known case of a substrate distinct from the protein kinase itself and the protein kinase which phosphorylates it both binding to calmodulin is fodrin and fodrin protein kinase (Sobue et al., 1982). pp74 was phosphorylated on threonine; however, it was resistant to alkali treatment. Again, this shows that much of the alkali-resistant phosphopeptides may be due to resistant phosphothreonine residues, not phosphotyrosine.

CHAPTER VI

CONCLUSIONS

1. In rat brain synaptic membrane, calmodulin stimulates the phosphorylation of two peptides (50-kDa and 60-kDa). The phosphopeptides were resistant to alkali which was due in part to the presence of phosphotyrosine and in part to phosphothreonine. Calmodulin stimulated the phosphorylation of both peptides at phosphoserine, phosphothreonine and phosphotyrosine residues. The mechanism of stimulation of tyrosine phosphorylation remains to be elucidated but it may be through an effect on the substrate.

2. These two peptides are dephosphorylated by calcineurin at both the alkali-labile and the alkali-resistant phosphorylation sites, indicating that calcineurin can dephosphorylate phosphotyrosyl residues as well as phosphoserine and phosphothreonine. Endogenous calcineurin was present (in synaptic membrane) and endogenous phosphatase activity was noted.

3. Solubilization and partial purification by chromatography on AffiGel Blue and calmodulin-Sepharose 4B affinity columns resulted in the purification of the 50-kDa and 60-kDa peptides and the calmodulin-stimulated protein kinase which phosphorylates them on serine and threonine. Their copurification indicates that they probably represent autophosphorylation of the subunits of a particulate form of a general multifunctional calmodulin-dependent protein kinase. A small amount of phosphotyrosine was detected in the 60-kDa phosphopeptide. This may be due to a small amount of tyrosine protein kinase in the EGTA wash of the calmodulin column as over 95% of the tyrosine protein kinase activity was in

the breakthrough fraction and less than 5% was in the calmodulin-binding fraction.

4. It is possible that the calmodulin-regulatory system and the tyrosine protein kinase regulatory system(s) may be interrelated at several levels similar to the interrelation of the calmodulin system and the cyclic AMP system.

a) Calmodulin is phosphorylated at a tyrosine residue by $pp60^{v\text{-}src}$ tyrosine kinase (Fukami & Lipmann, 1985).

b) Calmodulin may stimulate the tyrosine protein kinase which phosphorylates the 17 beta-estradiol receptor (Migliaccio et al., 1984).

c) Calcineurin, a calmodulin-stimulated phosphoprotein phosphatase, can dephosphorylate sites of tyrosine phosphorylation (this study and Chernoff et al., 1984; Pallen et al., in submission).

d) The 50-kDa and 60-kDa subunits of a calmodulin-stimulated protein kinase may be phosphorylated on tyrosine residues in a calmodulin-stimulated manner. The mechanism of calmodulin-stimulated phosphorylation remains to be elucidated but it may be through a substrate effect.

5. A new substrate for calmodulin-stimulated phosphorylation was discovered in chicken brain. The peptide had a molecular weight of 74,000 Da by SDS-PAGE. It was phosphorylated at a threonine residue which was resistant to alkali. Phosphorylation was stimulated 13-fold by calmodulin. Both the substrate (pp74) and the calmodulin-stimulated protein kinase were solubilized from chicken brain and copurified through AffiGel Blue and calmodulin-Sepharose 4B affinity chromatography.

REFERENCES

Adachi, K., Carruthers, C.A., Walsh, M.P. (1983) Biochem. Biophys. Res. Commun. 115, 855-863.

Adelstein, R.S. (1982) Cell 30, 349-350.

Adelstein, R.S., Conti, M.A. (1975) Nature 256, 597-598.

Adelstein, R.S., Conti, M.A., Hathaway, D.R., Klee, C.B. (1978) J. Biol. Chem. 253, 8347-8350.

Adelstein, R.S., Eisenberg, E. (1980) Annu. Rev. Biochem. 49, 921-956.

Adelstein, R.S., Klee, C.B. (1981) J. Biol. Chem. 256, 7501-7509.

Adelstein, R.S., de Lanerolle, P., Sellers, J.R., Pato, M.D., Conti, M.A. (1982) in "Calmodulin and Intracellular Ca^{++} Receptors" (S. Kakiuchi,H. Hidaka & A.R. Means, ed.) Plenum Press, New York, pp. 313-331.

Ahmad, Z., DePaoli-Roach, A.A., Roach, P.J. (1982) J. Biol. Chem. 257, 8348-8355.

Aitken, A., Klee, C.B., Stewart, A.A., Tonks, N.K., Cohen, P. (1983) in "Calcium-Binding Proteins 1983" Elsevier Science Publishers, pp. 113-119.

Aitken, A., Klee, C.B., Cohen, P. (1984) Eur. J. Biochem. 39, 663-671.

Andreasen, T.J., Luetje, C.W., Heideman, W., Storm, D.R. (1983) Biochemistry 22, 4615-4618.

Baldwin, G.S., Burgess, A.W., Kemp, B.E. (1982) Biochem. Biophys. Res. Commun. 109, 656-663.

Baldwin, G.S., Knesel, J., Moncton, J.M. (1983a) Nature 301, 435-437.

Baldwin, G.S., Stanley, I.J., Nice, E.C. (1983b) FEBS Lett. 153, 257-261.

Barbacid, M., Beemon, K., Devare, S.G. (1980) Proc. Natl. Acad. Sci. (U.S.A.) 77, 5158-5162.

Barker, W.C., Dayhoff, M.O. (1982) Proc. Natl. Acad. Sci. (U.S.A.) 79, 2836-2839.

Barnes, D.W. (1982) J. Cell. Biol. 93, 1-4.

Barylko, B., Kuznicki, J., Drabikowski, W. (1978) FEBS Lett 90, 301-304.

Beemon, K. (1981) Cell 24, 145-153.

Bennett, M.K., Erondu, N.E., Kennedy, M.B. (1983) J. Biol. Chem. 258, 12735-12744.

Berridge, M.J. (1975) Adv. Cyclic Nucleotide Res. 6, 1-8.

Bishop, J.M. (1981) Cell 23, 5-6.

Bishop, J.M. (1983) Annu. Rev. Biochem. 52, 301-354.

Blumenthal, D.K., Stull, J.T. (1980) Biochemistry 19, 5608-5614.

Blumenthal, D.K., Krebs, E.G. (1983) Biophys. J. 41, 409a.

Bolen, J.B., Thiele, C.J., Israel, M.A., Yonemoto, W., Lipsich, L.A., Brugge,J.S. (1984) Cell 38, 767-777.

Bowen-Pope, D.F., Dicorleto, P.E., Ross, R. (1983) J. Cell. Biol. 96, 679-683.

Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.

Bradham, L.S., Holt, D.A., Sims, M. (1970) Biochim. Biophys. Acta 201, 250-260.

Brautigan, D.L., Bornstein, P., Gallis, B. (1981) J. Biol. Chem. 256, 6519-6522.

Bremel, R.D., Sobieszek, A., Small, J.V. (1977) in "The Biochemistry of Smooth Muscle" (N.L. Stephens, ed.) University Park Press, Baltimore, Md., pp. 533-549.

Brostrom, C.O., Hunkeler, F.L., Krebs, E.G. (1971) J. Biol. Chem. 246, 1961-1967.

Brostrom, C.O., Huang, Y.C., Breckenridge, B.M., Wolff, D.J. (1975) Proc. Natl. Acad. Sci. (U.S.A.) 72, 64-68.

Brostrom, M.A., Brostrom, C.O., Breckenridge, B.M., Wolff, D.J. (1976) J. Biol. Chem. 251, 4744-4750.

Buhrow, S.A., Cohen, S., Staros, J.V. (1982) J. Biol. Chem. 257, 4019-4022.

Buhrow, S.A., Cohen, S., Garbers, D.L., Staros, J.V. (1983) J. Biol. Chem. 258, 7824-7827.

Burger, D., Stein, E.A., Cox, J.A. (1983) J. Biol. Chem. 258, 14733-14739.

Burke, B.E., DeLorenzo, R.J. (1981) Proc. Natl. Acad. Sci. (U.S.A.) 78, 991-995.

Buss, J.E., Kudlow, J.E., Lazar, C.S., Gill, G.N. (1982) Proc. Natl. Acad. Sci. (U.S.A.) 79, 2574-2578.

Carlin, C.R., Knowles, B.B. (1982) Proc. Natl. Acad. Sci. (U.S.A.) 79, 5026-5030.

Carpenter, G. (1983) Molec. Cell. Endocrinol. 31, 1-19.

Carpenter, G., King, L.Jr., Cohen, S. (1978) Nature 276, 409-410.

Carpenter, G., Cohen, S. (1979) Annu. Rev. Biochem. 48, 193-216.

Carpenter, G., King, L.E.Jr., Cohen, S. (1979) J. Biol. Chem. 254, 4884-4891.

Carpenter, G., Stoscheck, C.M., Preston, Y.A., DeLarco, J.E. (1983) Proc. Natl. Acad. Sci. (U.S.A.) 80, 5627-5630.

Casnellie, J.E., Harrison, M.L., Hellstrom, K.E., Krebs, E.G. (1982a) J. Biol. Chem. 257, 13877-13879.

Casnellie, J.E., Harrison, M.L.,Pike, L.J., Hellstrom, K.E., Krebs, E.G. (1982b) Proc. Natl. Acad. Sci. (U.S.A.) 79, 282-286.

Casnellie, J.E., Harrison, M.L., Hellstrom, K.E., Krebs, E.G. (1983) J. Biol. Chem. 258, 10738-10742.

Chacko, S. (1981) Biochemistry 20, 702-707.

Chacko, S., Conti, M.A., Adelstein, R.S. (1977) Proc. Natl. Acad. Sci. (U.S.A.) 74, 129-133.

Chacko, S., Rosenfeld, A. (1982) Proc. Natl. Acad. Sci. (U.S.A.) 79, 292-296.

Chau, V., Huang, C.Y., Chock, P.B., Wang, J.H., Sharma, R.K. (1982) in "Calmodulin and Intracellular Ca^{++} Receptors" (S. Kakiuchi, H, Hidaka & A.R. Means, eds.) Plenum Press, New York, pp. 199-217.

Cheng, Y.-S.E., Chen, L.B. (1981) Proc. Natl. Acad. Sci. (U.S.A.) 78, 2388-2392.

Chernoff, J., Sells, M.A., Li, H.C. (1984) Biochem. Biophys. Res. Commun. 121, 141-148.

Cheung, W.Y. (1970) Biochem. Biophys. Res. Commun. 38, 533-538.

Cheung, W.Y. (1971) J. Biol. Chem. 246, 2859-2869.

Cheung, W.Y. (1980a) Science 207, 19-27.

Cheung, W.Y. (ed.) (1980b) "Calcium and Cell Regulation Vol. 1, Calmodulin", Academic Press, New York.

Cheung, W.Y., Lynch, T.J., Wallace, R.W. (1978) Adv. Cyclic Nucleotide Res. 9, 233-251.

Chinkers, M., Cohen, S. (1981) Nature 296, 613-620.

Cobb, M.H., Rosen, O.M. (1983) J. Biol. Chem. 258, 12472-12481.

Cobb, M.H., Rosen, O.M. (1984) Biochim. Biophys. Acta 738, 1-8.

Cohen, P. (1974) Biochem. Soc. Symp. 39, 51-73.

Cohen, P. (1978) Curr. Topics Cell. Regul. 14, 117-196.

Cohen, P. (1980) Eur. J. Biochem. 111, 563-574.

Cohen, P., Burchell, A., Foulkes, J.G., Cohen, P.T.W., Vanaman, T.C., Nairn, A.C. (1978) FEBS Lett. 92, 287-293.

Cohen, P., Picton, C., Klee, C.B. (1979) FEBS Lett. 104, 25-30.

Cohen, S., Carpenter, G., King, L.Jr. (1980) J. Biol. Chem. 255, 4834-4842.

Cohen, S., Ushiro, H., Stoscheck, C., Chinkers, M. (1982) J. Biol. Chem. 257, 1523-1531.

Cole, H.A., Grand, R.J.A., Perry, S.V. (1982) Biochem. J. 206, 319-328.

Collett, M.S., Erikson, R.L. (1978) Proc. Natl. Acad. Sci. (U.S.A.) 75, 2021-2024.

Collett, M.S., Erikson, E., Erikson, R.L. (1979) J. Virol. 29, 770-781.

Collett, M.S., Purchio, A.F., Erikson, R.L. (1980) Nature 285, 167-169.

Collins, J.H. (1976) Nature 259, 699-700.

Collins, S.J., Ruscetti, F.W., Gallagher, R.E., Gallo, R.C. (1978) Proc. Natl. Acad. Sci. (U.S.A.) 75, 2458-2462.

Conti, M.A., Adelstein, R.S. (1981) J. Biol. Chem. 256, 3178-3181.

Cooper, J.A., Hunter, T. (1981a) Molec. Cell. Biol. 1, 165-178.

Cooper, J.A., Hunter, T. (1981b) Molec. Cell. Biol. 1, 394-407.

Cooper, J.A., Bowen-Pope, D., Raines, E., Ross, R., Hunter, T. (1982) Cell 31, 263-273.

Cooper, J.A., Hunter, T. (1983) J. Biol. Chem. 258, 1108-1115.

Cooper, J.A., Sefton, B.M., Hunter, T. (1983) Meth. Enzymol. 99, 387-402.

Cooper, J.A., Esch, F.S., Taylor, S.S., Hunter, T. (1984) J. Biol. Chem. 259, 7835-7841.

Courtneidge, S.A., Levinson, A.D., Bishop, J.M. (1980) Proc. Natl. Acad. Sci. (U.S.A.) 77, 3783-3787.

Courtneidge, S.A., Smith, A.E. (1983) Nature 303, 435-440.

Courtneidge, S.A., Smith, A.E. (1984) EMBO J. 3, 585-591.

Cox, D.E., Edelstrom, R.D. (1982) J. Biol. Chem. 257, 12728-12733.

Cox, J.A., Malnoe, A., Stein, E.A. (1981) J. Biol. Chem. 256, 3218-3222.

Dabrowska, R., Aromatorio, D., Sherry, J.M.F., Hartshorne, D.J. (1977) Biochem. Biophys. Res. Commun. 78, 1263-1272.

Dabrowska, R., Hartshorne, D.J. (1978) Biochem. Biophys. Res. Commun. 85, 1352-1359.

Daniel, J.L., Adelstein, R.S. (1976) Biochemistry 15, 2370-2377.

Dasgupta, J.D., Swarup, G., Garbers, D.L. (1984) Adv. Cyclic Nucleotide Protein Phosphorylation Res. 17, 461-470.

Dekowski, S.A., Rybicki, A., Drickamer, K. (1983) J. Biol. Chem. 258, 2750-2753.

DeLorenzo, R.J. (1976) Biochem. Biophys. Res. Commun. 71, 590-597.

DePaoli-Roach, A.A., Roach, P.J., Larner, J. (1979) J. Biol. Chem. 254, 4212-4219.

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J., Waterfeild, M.D. (1984) Natu83) Biochem. Biophys. Res. Commun. 112, 413-418.

Earp, H.S., Austin, K.S., Buessow, S.C., Dy, R.C., Gillespie, G.Y. (1984) Proc. Natl. Acad. Sci. (U.S.A.) 81, 2347-2351.

Ebashi, S. (1976) Annu. Rev. Physiol. 38, 293-313.

Ebashi, S., Kodama, A. (1965) J. Biochem. 58, 107-108.

Ebashi, S., Nonomura, Y., Hirata, M. (1982) in "Calmodulin and Intracellular Ca^{++} Receptors" (S. Kakiuchi, H. Hidaka & A.R. Means, eds.) Plenum Press, New York, pp. 189-197.

Eckhart, W., Hutchinson, M.A., Hunter, T. (1979) Cell 18, 925-933.

Ehrhart, J.C., Creuzet, C., Rollet, E., Loeb, J. (1981) Biochem. Biophys. Res. Commun. 102, 602-609.

Ek, B., Heldin, C.-H. (1982) J. Biol. Chem. 257, 486-492.

Ek, B., Westermark, B., Wasteson, A., Heldin, C.-H. (1982) Nature 295, 419-420.

Erikson, E., Erikson, R.L. (1980) Cell 21, 829-836.

Erneux, C., Cohen, S., Garbers, D.L. (1983) J. Biol. Chem. 258, 4137-4142.

Fava, R.A., Cohen, S. (1984) J. Biol. Chem. 259, 2636-2645.

Feldman, R.A., Hanafusa, T., Hanafusa, H. (1980) Cell 21, 757-766.

Feldman, R.A., Wang, L.-H., Hanafusa, H., Balduzzi, P. (1982) J. Virol. 42, 228-236.

Fernandez-Pol, J.A. (1981) J. Biol. Chem. 256, 9742-9749.

Foulkes, J.G., Erikson, E., Erikson, R.L. (1983) J. Biol. Chem. 258, 431-438.

Frearson, N., Perry, S.V. (1975) Biochem. J. 151, 99-107.

Friend, C., Freedman, H. (1978) Biochem. Pharmacol. 27, 1309-1313.

Fukami, Y., Lipmann, F. (1985) Proc. Natl. Acad. Sci. (U.S.A.) 82, 321-324.

Gacon, P., Gisselbrecht, S., Piau, J.P., Boissel, J.P., Tolle, J., Fischer, S. (1982) EMBO J. 1, 1579-1582.

Gallis, B., Bornstein, P., Brautigan, D.L. (1981) Proc. Natl. Acad. Sci. (U.S.A.) 78, 6689-6693.

Gallis, B., Edelman, A.M., Casnellie, J.E., Krebs, E.G. (1983) J. Biol. Chem. 258, 13089-13093.

Gates, R.E., King, L.E.Jr. (1982) Molec. Cell. Endocrinol. 27, 263-276.

Ghosh-Dastidar, P., Fox, C.F. (1984) J. Biol. Chem. 259, 3864-3869.

Gietzen, K., Adamezyk-Engelmann, P., Wuthrich, A., Konstantinova, A., Bader, H. (1983) Biochim. Biophys. Acta 736, 109-118.

Gill, G.N., Lazar, C.S. (1981) Nature 293, 305-307.

Goodman, M., Pechere, J.-F., Haiech, J., Demaille, J.G. (1979) J. Molec. Evol. 13, 331-352.

Golpalakrishna, R., Anderson, W.B. (1982) Biochem. Biophys. Res. Commun. 104, 830-836.

Gopinath, R.M., Vincenzi, F.F. (1977) Biochem. Biophys. Res. Commun. 77, 1203-1209.

Grand, R.J.A., Shenolikar, S., Cohen, P. (1981) Eur. J. Biochem. 113, 359-367.

Hampe, A., Laprevotte, I., Galibert, F., Fedele, L.A., Sherr, C.J. (1982) Cell 30, 775-785.

Harrison, M.L., Low, P.S., Geahlen, R.L. (1984) J. Biol. Chem. 259, 9348-9350.

Hartshorne, D.J., Walsh, M.P., Persechini, A. (1982) in "Calmodulin and Intracellular Ca^{++} Receptors" (S. Kakiuchi, H. Hidaka & A.R. Means, eds.) Plenum Press, New York, pp. 239-253.

Hathaway, D.R., Adelstein, R.S. (1979) Proc. Natl. Acad. Sci. (U.S.A.) 76, 1653-1657.

Hathaway, D.R., Eaton, C.R., Adelstein, R.S. (1981a) Nature 291, 252-254.

Hathaway,D.R., Adelstein, R.S., Klee, C.B. (1981b) J. Biol. Chem. 256, 8183-8189.

Heilbrunn, L.V., Wiercinski, F.J. (1947) J. Cell. Comp. Physiol. 29, 15-32.

Higgins, P.J., Borenfreund, E. (1980) Biochim. Biophys. Acta 610, 174-180.

Hirata, M., Mikawa, T. Nonomura, Y., Ebashi, S. (1977) J. Biochem. (Tokyo) 82, 1793-1796.

Ho, H.C., Wirch, E., Stevens, F.C., Wang, J.H. (1977) J. Biol. Chem. 252, 43-50.

Hooper, J.E., Kelly, R.B. (1984) J. Biol. Chem. 259, 148-153.

Horlein, D., Gallis, B., Brautigan, D.L., Bornstein, P. (1982) Biochemistry 21, 5577-5584.

Huang, C.Y., Chau, V., Chock, P.B., Wang, J.H., Sharma, R.K. (1981) Proc. Natl. Acad. Sci. (U.S.A.) 78, 871-875.

Hunter, T. (1982) J. Biol. Chem. 257, 4843-4848.

Hunter, T. (1984) JNCI 73, 773-786.

Hunter, T., Sefton, B.M. (1980) Proc. Natl. Acad. Sci. (U.S.A.) 77, 1311-1315.

Hunter, T., Cooper, J.A. (1981) Cell 24, 741-752.

Hunter, T., Cooper, J.A. (1983) Prog. Nucleic Acid Res. Mol. Biol. 29, 221-232.

Hunter, T., Cooper, J.A. (1984) Adv. Cyclic Nucleotide Protein Phosphorylation Res. 17, 443-455.

Huxley, H.E. (1972) Cold Spring Harb. Symp. 37, 361-376.

Ingebritsen, T.S., Stewart, A.A., Cohen, P. (1983) Eur. J. Biochem. 132, 297-307.

Ingebritsen, T.S., Cohen, P. (1983) Science 221, 331-338.

Iwasa, T., Fukunaga, K., Yamamoto, H., Tanaka, E., Miyamoto, E. (1983) FEBS Lett. 161, 28-32.

Jacobs, S., Kull, F.C.Jr., Earp, H.S., Svoboda, M.E., Van Wyk, J.J., Cuatrecasas, P. (1983) J. Biol. Chem. 258, 9581-9584.

Jakes, R., Northrop, F., Kendrick-Jones, J. (1976) FEBS Lett. 70, 229-234.

Jarrett, H.W., Penniston, J.T. (1977) Biochem. Biophys. Res. Commun. 77, 1210-1216.

Jennissen, H.P., Heilmeyer, L.M.G. (1974) FEBS Lett. 42, 77-80.

Kakiuchi, S., Yamazaki, R. (1970) Biochem. Biophys. Res. Commun. 41, 1104-1110.

Kasuga, M., Karlsson, F.A., Kahn, C.R. (1982a) Science 215, 185-187.

Kasuga, M., Zick, Y., Blithe, D.L., Crettaz, M., Kahn, C.R. (1982b) Nature 298, 667-679.

Kawai, S., Yoshida, M., Segawa, K., Sugiyama, H., Ishizaki, R., Toyoshima, K. (1980) Proc. Natl. Acad. Sci. (U.S.A.) 77, 6199-6203.

Kennedy, M.B., Greengard, P. (1981) Proc. Natl. Acad. Sci. (U.S.A.) 78, 1293-1297.

Kennedy, M.B., Bennett, M.K., Erondu, N.E. (1983) Proc. Natl. Acad. Sci. (U.S.A.) 80, 7357-7361.

Kennedy, M.B., McGuinness, T., Greengard, P. (1983) J. Neurosci. 3, 818-831.

Kimhi, Y., Palfrey, C., Spector, I., Barak, Y., Littauer, U.Z. (1976) Proc. Natl. Acad. Sci. (U.S.A.) 73, 462-466.

King, L.E.Jr., Carpenter, G., Cohen, S. (1980) Biochemistry 19, 1524-1528.

King, M.M., Huang, C.Y., Chock, P.B., Nairn, A.C., Hemmings, H.C.Jr., Chan, K.-F.J., Greengard, P. (1984) J. Biol. Chem. 259, 8080-8083.

Kitamura, N., Kitamura, A., Toyoshima, K., Hirayama, Y., Yoshida, M. (1982) Nature 297, 205-208.

Klee, C.B., Krinks, M.H. (1978) Biochemistry 17, 120-126.

Klee, C.B., Crouch, T.H., Krinks, M.H. (1979) Proc. Natl. Acad. Sci. (U.S.A.) 76, 6270-6273.

Klee, C.B., Crouch, T.H., Richman, P.G. (1980) Annu. Rev. Biochem. 49, 489-515.

Klee, C.B., Vanaman, T.C. (1982) Adv. Prot. Chem. 35, 213-321.

Kobayashi, R., Fang, V.S. (1976) Biochem. Biophys. Res. Commun. 69, 1080-1087.

Konopka, J.B., Watanabe, S.M., Witte, O.N. (1984) Cell 37, 1035-1042.

Kretsinger, R.H. (1976) Annu. Rev. Biochem. 45, 239-266.

Kretsinger, R.H. (1980) CRC Crit. Rev. Biochem. 8, 119-174.

Krueger, B.K., Forn, J., Greengard, P. (1977) J. Biol. Chem. 252, 2764-2773.

Kudlow, J.E., Buss, J., Gill, G.N. (1981) Nature 290, 519-521.

Laemmli, U.K. (1970) Nature 227, 680-685.

Lastick, S.M., McConkey, E.H. (1980) Biochem. Biophys. Res. Commun. 95, 917-923.

Leis, J.F., Kaplan, N.O. (1982) Proc. Natl. Acad. Sci. (U.S.A.) 79, 6507-6511.

LePeuch, C.J., Haiech, J., Demaille, J.G. (1979) Biochemistry 18, 5150-5157.

Levine, B.A., Dalgarno, D.C. (1983) Biochim. Biophys. Acta 726, 187-204.

Levinson, A.D., Opperman, H., Levintow, L., Varmus, H.E., Bishop, J.M. (1978) Cell 15, 561-572.

Levinson, A.D., Oppermann, H., Varmus, H.E., Bishop, J.M. (1980) J. Biol. Chem. 255, 11973-11980.

Lin, Y.M., Lin, Y.P., Cheung, W.Y. (1974) J. Biol. Chem. 249, 4943-4954.

MacLennan, D.H., Holland, P.C. (1976) in "The Enzymes of Biological Membranes" (A. Martonosi, ed.) Vol. 3, Plenum Press, New York, pp. 221-259.

Maita, T., Umegane, T., Matsuda, G. (1981a) Eur. J. Biochem. 114, 45-49.

Maita, T., Chen, J.I., Matsuda, G. (1981b) Eur. J. Biochem. 117, 417-424.

Malencik, D.A., Fischer, E.H. (1982) in "Calcium and Cell Function Vol.3" (W.Y. Cheung, ed.) Academic Press, New York, pp. 161-188.

Marcum, J.M., Dedman, J.R., Brinkley, B.R., Means, A.R. (1978) Proc. Natl. Acad. Sci. (U.S.A.) 75, 3771-3775.

Marston, S.B., Trevett, R.M., Walters, M. (1980) Biochem. J. 185, 355-365.

Martensen, T.M., Levine, R.L. (1983) Meth. Enzymol. 99, 402-405.

Martinez, R., Nakamura, K.D., Weber, M.J. (1982) Molec. Cell. Biol. 2, 653-665.

Martin-Perez, J., Thomas, G. (1983) Proc. Natl. Acad. Sci. (U.S.A.) 80, 926-930.

Mathey-Prevot, B., Hanafusa, H., Kawai, S. (1982) Cell 28, 897-906.

Matsuda, G. Suzuyama, Y., Maita, T., Umegane, T. (1977) FEBS Lett. 84, 53-56.

McGuinness, T.L., Lai, Y., Greengard, P., Woodgett, J.R., Cohen, P. (1983) FEBS Lett. 163, 329-334.

Means, A.R., Dedman, J.R. (1980) Nature, 285, 73-77.

Means, A.R., Tash, J.S., Chafouleas, J.G. (1982) Physiol. Rev. 62, 1-39.

Migliaccio, A., Rotondi, A., Auricchio, F. (1984) Proc. Natl. Acad. Sci. (U.S.A.) 81, 5921-5925.

Moews, P.C., Kretsinger, R.H. (1975) J. Molec. Biol. 91, 201-228.

Mrwa, U., Hartshorne, D.J. (1980) Fed. Proc. 39, 1564-1568.

Muhlrad, A., Oplatka, A. (1977) FEBS Lett. 77, 37-40.

Naharro, G., Dunn, C.Y., Robbins, K.C. (1983) Virology 125, 502-507.

Naharro, G., Robbins, K.C., Reddy, E.P. (1984) Science 223, 63-66.

Neil, J.C., Ghysdael, J., Vogt, P.K. (1981) Virology 109, 223-228.

Ngai, P.K., Carruthers, C.A., Walsh, M.P. (1984) Biochem. J. 218, 863-870.

Oppermann, H., Levinson, A.D., Varmus, H.E., Levintow, L., Bishop, J.M. (1979) Proc. Natl. Acad. Sci. (U.S.A.) 76, 1804-1808.

Ouimet, C.C., McGuinness, T.L., Greengard, P. (1984) Proc. Natl. Acad. Sci. (U.S.A.) 81, 5604-5608.

Pallen, C.J., Wang, J.H. (1983) J. Biol. Chem. 258, 8550-8553.

Pallen, C.J., Wang, J.H. (1985) Arch. Biochem. Biophys. 237, 281-291.

Pallen, C.J., Sharma, R.K., Wang, J.H. (1985) BioEssays 2, 113-117.

Parker, R.C., Varmus, H.E., Bishop, J.M. (1984) Cell 37, 131-139.

Pato, M.D., Adelstein, R.S. (1983) J. Biol. Chem. 258, 7047-7054.

Pawson, T., Guyden, J., King, T.-H., Radke, K., Gilmore, T., Martin, G.S. (1980) Cell 22, 767-776.

Payne, M.E., Soderling, T.R. (1980) J. Biol. Chem. 255, 8054-8056.

Perrie, W.T., Smillie, L.B., Perry, S.V. (1972) Biochem. J. 128, 105-106.

Perrie, W.T., Smillie, L.B., Perry, S.V. (1973) Biochem. J. 135, 151-164.

Petruzelli, L.M., Ganguly, S., Smith, C.J., Cobb, M.H., Rubin, C.S., Rosen, O.M. (1982) Proc. Natl. Acad. Sci. (U.S.A.) 79, 6792-6796.

Petruzzelli, L., Herrera, R., Rosen, O.M. (1984) Proc. Natl. Acad. Sci. (U.S.A.) 81, 3327-3331.

Picton, C., Klee, C.B., Cohen, P. (1980) Eur. J, Biochem. 111. 553-561.

Pike, L.J., Gallis, B., Casnellie, J.E., Bornstein, P., Krebs, E.G. (1982) Proc. Natl. Acad. Sci. (U.S.A.) 79, 1443-1447.

Pike, L.J., Kuenzel, E.A., Casnellie, J.E., Krebs, E.G. (1984) J. Biol. Chem. 259, 9913-9921.

Pires, E.M.V., Perry, S.V., Thomas, M.A.W. (1974) FEBS Lett. 41, 292-296.

Pires, E.M.V., Perry, S.V. (1977) Biochem. J. 167, 137-146.

Ponticelli, A.S., Whitlock, C.A., Rosenberg, N., Witte, O.N. (1982) Cell 29, 953-960.

Privalsky, M.L., Ralston, R., Bishop, J.M. (1984) Proc. Natl. Acad. Sci. (U.S.A.) 81, 704-707.

Prozialeck, W.C. (1983) Ann. Rep. Med. Chem. 18, 203-212.

Rasmussen, H. (1970) Science 170, 404-412.

Reddy, E.P., Smith, M.J., Srinivasan, A. (1983) Proc. Natl. Acad. Sci. (U.S.A.) 80, 3623-3627.

Reynolds, F.H.Jr., Van de Ven, W.J.M., Stephenson, J.R. (1980) J. Biol. Chem. 255, 11040-11047.

Richert, N.D., Blithe, D.L., Pastan, I. (1982) J. Biol. Chem. 257, 7143-7150.

Ringer, S. (1883) J. Physiol. (London) 4, 29-42.

Roach, P.J., DePaoli-Roach, A.A., Larner, J. (1978) J. Cyclic Nucleotide Res. 4, 245-257.

Rohrshneider, L.R., Eisenman, R.N., Leitch, C.R. (1979) Proc. Natl. Acad. Sci. (U.S.A.) 76, 4479-4483.

Rubin, R.A., Earp, H.S. (1983a) Science 219, 60-63.

Rubin, R.A., Earp, H.S. (1983b) J. Biol. Chem. 258, 5177-5182.

Rubsamen, H., Friis, R.R., Bauer, H. (1979) Proc. Natl. Acad. Sci. (U.S.A.) 76, 967-971.

Sahyoun, N., LeVine, H.III, Bronson, D., Cuatrecasas, P. (1984a) J. Biol. Chem. 259, 9341-9344.

Sahyoun, N., LeVine, H.III, Cuatrecasas, P. (1984b) Proc. Natl. Acad. Sci. (U.S.A.) 81, 4311-4315.

Sahyoun, N., LeVine, H.III, Bronson, D., Siegel-Greenstein, F., Cuatrecasas, P. (1985) J. Biol. Chem. 260, 1230-1237.

Sairam, M.R., Porath, J. (1976) Biochem. Biophys. Res. Commun. 69, 190-196.

Schaffhausen, B.S., Haimanti, D., Arakere, G., Benjamin, T.L. (1982) Molec. Cell. Biol. 2, 1187-1198.

Schulman, H. (1982) in Handbook of Experimental Pharmacology Vol. 58/I (J.A. Nathanson & J.W. Kebabian, eds.) Springer-Verlag, Berlin, pp. 425-478.

Schulman, H. (1984) J. Cell. Biol. 99, 11-19.

Schulman, H., Greengard, P. (1978a) Proc. Natl. Acad. Sci. (U.S.A.) 75, 5432-5436.

Schulman, H., Greengard, P. (1978b) Nature 271, 478-479.

Sefton, B.M., Hunter, T., Beemon, K., Eckhart, W. (1980) Cell 20, 807-816.

Sefton, B.M., Hunter, T., Ball, E.H., Singer, S.J. (1981) Cell 24, 165-174.

Segawa, K., Ito, Y. (1982) Proc. Natl. Acad. Sci. (U.S.A.) 79, 6812-6816.

Segawa, K., Ito, Y. (1983) Nature 304, 742-744.

Sellers, J.R., Pato, M.D., Adelstein, R.S. (1981) Biophys. J. 33, 278a.

Sharma, R.K., Taylor, W.A., Wang, J.A. (1983) Meth. Enzymol. 102, 210-219.

Sharma, R.K., Desai, R., Waisman, D.M., Wang, J.H. (1979) J. Biol. Chem. 254, 4276-4282.

Shenolikar, S., Cohen, P.T.W., Cohen, P. Nairn, A.C., Perry, S.V. (1979) Eur. J. Biochem. 100, 329-337.

Sherry, J.M.F., Gorecka, A., Oksoy, M.O., Dabrowska, R., Hartshorne, D.J. (1978) Biochemistry 17, 4411-4418.

Shibuya, M., Hanafusa, H. (1982) Cell 30, 787-795.

Shields, S.M., Vernon, P.J., Kelly, P.T. (1984) J. Neurochem. 43, 1599-1609.

Silver, P.J., Holroyde, M.J., Solaro, R.J., DiSalvo, J. (1981) Biochim. Biophys. Acta 674, 65-70.

Singh, T.J., Wang, J.H. (1977) J. Biol. Chem. 252, 625-632.

Slamon, D.J., DeKernion, J.B., Verma, I.M., Cline, M.J. (1984) Science 224, 256-262.

Small, J.V., Sobieszek, A. (1977) J. Cell Sci. 23, 243-268.

Smith, C.J., Wejksnora, P.J., Warner, J.R., Rubin, C.S., Rosen, O.M. (1979) Proc. Natl. Acad. Sci. (U.S.A.) 76, 2725-2729.

Snyder, J.A., McIntosh, J.R. (1976) Annu. Rev. Biochem. 45, 699-720.

Sobieszek, A. (1977a) in "The Biochemistry of Smooth Muscle" (N.L. Stephens, ed.) University Park Press, Baltimore, pp. 413-443.

Sobieszek, A. (1977b) Eur. J. Biochem. 73, 477-483.

Sobue, K., Fujita, M., Muramoto, Y., Kakiuchi, S. (1980) Biochem. Int. 1, 561-566.

Sobue, K., Kanda, K., Yamagami, K., Kakiuchi, S. (1982) Biomed. Res. 3, 561-570.

Soderling, T.R., Sheorain, V.A., Ericsson, L.H. (1979) FEBS Lett. 106, 181-184.

Soderquist, A.M., Carpenter, G. (1983) Federation Proc. 42, 2615-2620.

Spector, D.H., Smith, K., Padgett, T., McCombe, P., Roulland-Dussoix, D., Moscovici, C., Varmus, H.E., Bishop, J.M. (1978) Cell 13, 371-379.

Srivastava, A.K., Waisman, D.M., Brostrom, C.O., Soderling, T.R. (1979) J. Biol. Chem. 254, 583-586.

Stehelin, D., Varmus, H.E., Bishop, J.M. (1976) Nature 260, 170-173.

Stewart, A.A., Ingebritsen, T.S., Manalan, A., Klee, C.B., Cohen, P. (1982) FEBS Lett. 137, 80-84.

Stull, J.T., Blumenthal, D.K., Botterman, B.R., Klug, G.A., Manning, D.R., Silver, P.J. (1982) in "Calmodulin and Intracellular Ca^{++} Receptors" (S. Kakiuchi, H. Hidaka & A.R. Means, eds.) Plenum Press, New York, pp. 219-238.

Swanstrom, R., Parker, R.C., Varmus, H.E., Bishop, J.M. (1983) Proc. Natl. Acad. Sci. (U.S.A.) 80, 2519-2523.

Swarup, G., Cohen, S., Garbers, D.L. (1981) J. Biol. Chem. 256, 8197-8201.

Swarup, G., Speeg, K.V.Jr., Cohen, S., Garbers, D.L. (1982) J. Biol. Chem. 257, 7298-7301.

Swarup, G., Dasgupta, J.D., Garbers, D.L. (1983) J. Biol. Chem. 258, 10341-10347.

Tabin, C.J., Bradley, S.M., Bargmann, C.I., Weinberg, R.A., Papageorge, A.G., Scolnick, D.M., Dhar, E., Lowy, D.R., Chang, E.H. (1982) Nature 300, 143-149.

Takasaki, S., Leive, L. (1982) Proc. Natl. Acad. Sci. (U.S.A.) 79, 4980-4984.

Takeya, T., Hanafusa, H. (1983) Cell 32, 881-890.

Tallant, E.A., Cheung, W.Y. (1984) Arch. Biochem. Biophys. 232, 269-279.

Tallant, E.A., Wallace, R.W., Cheung, W.Y. (1983) Meth. Enzymol. 102, 244-256.

Teo, T.S., Wang, J.H. (1973) J. Biol. Chem. 248, 5950-5955.

Thompson, S.T., Cass, K.H., Stellwagen, E. (1975) Proc. Natl. Acad. Sci. (U.S.A.) 72, 669-672.

Todaro, G.J., DeLarco, J.E., Cohen, S. (1976) Nature 264, 26-31.

Towbin, H., Staehelin, T., Gordon, J. (1979) Proc. Natl. Acad. Sci. (U.S.A.) 76, 4350-4354.

Trotter, J.A., Adelstein, R.S. (1979) J. Biol. Chem. 254, 8781-8785.

Tsien, K.Y. (1980) Biochemistry 19, 2396-2404.

Ushiro, H., Cohen, S. (1980) J. Biol. Chem. 255, 8363-8365.

Vallet, B., Molla, A., Demaille, J.G. (1981) Biochim. Biophys. Acta 674, 256-264.

Van Eldik, L.J., Zendegui, J.G., Marshak, D.R., Watterson, D.M. (1982) Int. Rev. Cytol. 77, 1-61.

Waisman, D., Stevens, F.C., Wang, J.H. (1975) Biochem. Biophys. Res. Commun. 65, 975-982.

Walaas, S.I., Nairn, A.C., Greengard, P. (1983a) J. Neurosci. 3, 291-301.

Walaas, S.I., Nairn, A.C., Greengard, P. (1983b) J. Neurosci. 3, 302-311.

Wallace, R.W., Tallant, E.A., Cheung, W.Y. (1980) Biochemistry 19, 1831-1837.

Walsh, D.A., Perkins, J.P., Brostrom, C.O., Ho, E.S., Krebs, E.G. (1971) J. Biol. Chem. 246, 1968-1976.

Walsh, M.P., Vallet, B., Autric, F., Demaille, J.G. (1979) J. Biol. Chem. 254, 12136-12144.

Walsh, M.P., Vallet, B., Cavadore, J.C., Demaille, J.G. (1980) J. Biol. Chem. 255, 335-337.

Walsh, M.P., Guilleux, J.-C. (1981) Adv. Cyclic Nucleotide Res. 14, 375-390.

Walsh. M.P., Hartshorne, D.J. (1982) in "Calcium and Cell Function Vol. 3" (W.Y. Cheung, ed.) Academic Press, pp. 223-269.

Wang, J.H., Teo, T.S., Ho, H.S., Stevens, F.C. (1975) Adv. Cyclic Nucleotide Res. 5, 179-194.

Wang, J.H., Desai, R. (1976) Biochem. Biophys. Res. Commun. 72, 926-937.

Wang, J.H., Desai, R. (1977) J. Biol. Chem. 252, 4175-4184.

Wang, J.H., Waisman, D.M. (1979) Curr. Topics Cell. Regul. 15, 47-107.

Wang, K.C., Mutus, B., Sharma, R.K., Lam, H.-Y.P., Wang, J.H. (1983) Can. J. Biochem. Cell Biol. 61, 911-920.

Watterson, D.M., Vincenzi, F.F., (eds.) (1980) "Calmodulin and Cell Function" Ann. N.Y. Acad. Sci. 356, New York Academy of Sciences, New York.

Weeds, A.G., McLachlan, A.D. (1974) Nature 252, 646-649.

Witt, J.J., Roskoski, R. (1975) Biochemistry 14, 4503-4507.

Witte, O.N., Dasgupta, A., Baltimore, D. (1980) Nature 283, 826-831.

Wolf, H., Hofman, F. (1980) Proc. Natl. Acad. Sci. (U.S.A.) 77, 5852-5855.

Wolff, D.J., Brostrom, C.O. (1979) Adv. Cyclic Nucleotide Res. 11, 27-88.

Wong, T.W., Goldberg, A.R. (1983a) J. Biol. Chem. 258, 1022-1025.

Wong, T.W., Goldberg, A.R. (1983b) Proc. Natl. Acad. Sci. (U.S.A.) 80, 2529-2533.

Wong, T.W., Goldberg, A.R. (1984a) J. Biol. Chem. 259, 8505-8512.

Wong, T.W., Goldberg, A.R. (1984b) J. Biol. Chem. 259, 3127-3131.

Woodgett, J.R., Cohen, P., Yamauchi, T., Fujisawa, H. (1984) FEBS Lett. 163, 329-334.

Wu, R.S., Stedman, J.D., West, M.H.P., Pantazis, P., Bonner, W.M. (1982) Anal. Biochem. 124, 264-271.

Wyke, J. (1983) Nature 304, 491-492.

Yamamoto, T., Nishida, T., Miyajima, N., Kawai, S., Ooi,T., Toyoshima, K. (1983) Cell 35, 71-78.

Yamauchi, T., Fujisawa, H. (1979) Biochem. Biophys. Res. Commun. 90, 28-35.

Yamauchi, T., Fujisawa, H. (1980) Biochem. Int. 1, 98-104.

Yazawa, M., Yagi, K. (1977) J. Biochem. (Tokyo) 82, 287-289.

Yazawa, M., Yagi, K. (1978) J. Biochem. (Tokyo) 84, 1259-1265.

Yazawa, M., Kuwayama, H., Yagi, K. (1978) J. Biochem. (Tokyo) 84, 1253-1258.

Yerna, M.J., Dabrowska, R., Hartshorne, D.J., Goldman, R.D. (1979) Proc. Natl. Acad. Sci. (U.S.A.) 76, 184-188.