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PHOSPHORYLATION OF CYTOSKELETAL PROTEINS BY PROTEIN KINASE C

by

David William Litchfield

Department of Biochemistry

Submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
University of Western Ontario
London, Ontario
August, 1987

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ABSTRACT

Protein kinase C is the Ca^{2+}/phospholipid-dependent enzyme that serves as the receptor for, and is directly activated by, the tumour-promoting phorbol esters. To examine the involvement of protein kinase C in the regulation of the organization or function of the actin-containing microfilaments, the activity of the enzyme towards two distinct groups of proteins that are thought to be involved in microfilament regulation has been investigated. For these studies, protein kinase C was partially purified from bovine brain or was more extensively purified from rat brain. Two proteins that are localized in certain areas of microfilament-membrane attachment (focal contacts), vinculin and talin, were identified as *in vitro* substrates for protein kinase C. Purified protein kinase C also phosphorylated chicken gizzard myosin light chain kinase and different forms of caldesmon, proteins that are involved in the regulation of contractile events. Chicken gizzard caldesmon and chicken liver caldesmon$_{72}$ as well as two forms of bovine liver caldesmon (caldesmon$_{150}$ and caldesmon$_{77}$) were all *in vitro* substrates for protein kinase C. The sites of phosphorylation of the substrate proteins were examined by phosphopeptide mapping and phosphoamino acid analysis. Phosphorylation of chicken gizzard caldesmon by protein kinase C partially abolished its inhibitory activity towards the actin-activated ATPase of skeletal muscle myosin and diminished associations between caldesmon and actin.

Treatment of intact human platelets with 12-O-tetradecanoyl-phorbol-13-acetate (TPA), a tumour-promoter that activates protein

kinase C in living cells, stimulated phosphorylation of talin and caldesmon$_{77}$. The phosphate content of talin was elevated by 44%, but the apparent stoichiometry of phosphorylation was low. In contrast, the phosphate content of caldesmon$_{77}$ increased approximately 4-fold. Moreover, the phosphopeptides that appeared in response to TPA treatment had the same migration pattern as the two major phosphopeptides of bovine liver caldesmon$_{77}$ phosphorylated in vitro.

The results of this study imply that protein kinase C, by phosphorylating focal contact proteins or proteins involved in the control of contractile events, may have a role in microfilament regulation.

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TO CHERYL AND MY PARENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

ATP	Adenosine 5'-triphosphate
$Caldesmon_{72}$	72,000 dalton form of chicken liver caldesmon
$Caldesmon_{77}$	77,000 dalton form of bovine liver caldesmon
$Caldesmon_{150}$	150,000 dalton form of bovine liver caldesmon
cAMP	Adenosine 3',5'-cyclic monophosphate
cGMP	Guanosine 3',5'-cyclic monophosphate
DEAE cellulose	Diethylaminoethyl cellulose
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol bis (2-aminoethyl ether)-N,N,N',N'-tetra-acetic acid
F-actin	Filamentous actin
G-actin	Monomeric globular actin
Mes	2-(N-morpholino) ethanesulfonic acid
Mr	Molecular weight
P20	20,000 dalton regulatory light chain of myosin
P36	36,000 dalton phospholipid and actin-binding protein
P47	47,000 dalton 5'-phosphomonoesterase specific for inositol trisphosphate
P235	235,000 dalton platelet form of talin
4α-PDD	4α-Phorbol 12,13-didecanoate
Pi	Inorganic phosphate
PMSF	Phenylmethylsulfonyl fluoride
$pp60^{v-src}$	The transforming protein of Rous sarcoma virus
Protein kinase C	The Ca^{2+}/phospholipid-dependent protein kinase

NOMENCLATURE	(Continued)
RSV	Rous sarcoma virus
SDS	Sodium dodecyl sulfate
STI	Soybean trypsin inhibitor
TLCK	Nα-p-Tosyl-L-lysine chloromethyl ketone
TPA	12-O-tetradecanoylphorbol-13-acetate
TPCK	N-Tosyl-L-phenylalanine chloromethyl ketone
Tris	Tris (hydroxymethyl) aminomethane

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CHAPTER 1

INTRODUCTION

Actin is one of the most abundant protein components of all eukaryotic cells (reviewed by Korn, 1982; Pollard and Cooper, 1986). This highly conserved protein was originally identified in skeletal muscle where it exists in highly organized filamentous arrays (Huxley, 1963). In association with myosin filaments and a number of accessory proteins, the actin filaments are a major constituent of the contractile apparatus of muscle. Actin and myosin filaments are also integral components of the contractile apparatus of smooth muscle, although the accessory proteins are not all the same as in skeletal muscle (reviewed by Marston and Smith, 1985). Actin was later identified, and then purified, from non-muscle cells (Ishikawa et al., 1969; Hatano and Oosawa, 1966). In non-muscle cells, actin exists in two distinct forms, a globular monomeric form (G-actin) and in filaments with a diameter of 4-7 nm (F-actin). Together with the microtubules (diameter 25 nm) and intermediate filaments (diameter 10 nm), the actin-containing microfilaments comprise the fibrous network of proteins known as the cytoskeleton (Weatherbee, 1981; Birchmeier, 1984).

Although the cytoskeleton has a significant structural role in living cells, this three-dimensional lattice is also a dynamic network that plays an essential role in a number of vital cellular processes. In view of the involvement of actin in muscle contraction, it has been suggested that actin is involved in

contractile events in non-muscle cells (reviewed by Adelstein, 1982). The actin-containing microfilaments may also be involved in morphological events such as the maintenance of cell shape and adhesion, and in cell motility. The postulated involvement of microfilaments in maintenance of cell shape and adhesion is based on the observation that large bundles of actin filaments, known as stress fibres, clearly have attachments to the plasma membrane in those regions where cultured cells are closest and most tightly attached to the substratum (Birchmeier, 1981; Burridge, 1981). Moreover, in cells that are subjected to high shear stresses, proliferation of stress fibres occurs (Franke et al., 1984). The involvement of actin in motile events has been implied by the demonstration that they can be arrested by cytochalasin B, a drug which disrupts microfilament organization (Wessells et al., 1971).

1.1 Regulation of Microfilament Organization and Function

The involvement of actin in cellular processes in non-muscle cells is dependent on its subcellular organization and polymerization state. In the presence of Mg^{2+} and ATP and under the proper ionic conditions, purified actin will spontaneously self-assemble (Pardee and Spudich, 1982). In living cells however, there are a number of actin-associated proteins (reviewed by Pollard and Cooper, 1986; Stossel et al., 1985; Weeds, 1982; Schliwa, 1981) that can regulate the conversions of actin between its monomeric and filamentous states. Actin filament length is also under the control of filament-severing proteins or other proteins that can cap the ends of a filament preventing further polymerization. In addition, there are a

number of proteins that are involved in the organization of the actin microfilaments. Among these proteins are the crosslinking or bundling proteins that organize F-actin into extended three-dimensional networks, and those proteins that are involved in the attachment of actin filaments to other cellular structures such as the plasma membrane (Mangeat and Burridge, 1984; Geiger, 1983). The latter group of proteins can be subdivided into two distinct categories on the basis of the actin-to-membrane linkages that they promote. Actin can be attached to the plasma membrane by side-to-membrane linkers and by end-to-membrane linkers. Clearly, there are many actin-associated proteins which must be considered to be important regulators of actin and its role in cellular processes.

The dynamic nature of the actin microfilaments is emphasized by the rapid reorganizations that they undergo. In motile cells in culture, the actin filaments are continually being reorganized and few stress fibres are evident (Burridge, 1981). It is only when these cells become less motile and form strong adhesions with the substratum that the stress fibres gain prominence. However, when these cells are stimulated to divide, drastic reorganizations of the microfilaments occur. In mitotic cells, the disassembly of the stress fibres is followed by the appearance of actin filaments in a contractile ring that is involved in the process of cytokinesis. Loss of stress fibres has also been observed when quiescent fibroblasts are stimulated with platelet-derived growth factor (Herman and Pledger, 1985). Furthermore, the expression of a number of cytoskeletal genes has been shown to be under the control of cell

shape and the existence of cell-cell contacts (Ben Ze'ev, 1986; Ungar et al., 1986). From these observations, it is obvious that alterations in the organization of the microfilaments accompany a number of normal cellular events.

1.2 Microfilament Alterations in Transformed Cells

In addition to the cytoskeletal alterations that occur under normal circumstances, alterations in the distribution and organization of the actin microfilaments also accompanies cell transformation (reviewed by Ben Ze'ev, 1985; Vasiliev et al., 1985; Boschek et al., 1981; Wang and Goldberg, 1976). In transformed cells, the stress fibres are disrupted and the cells attain a rounded morphology. The transformed cells also exhibit altered growth characteristics since they are no longer subject to contact inhibition and are capable of anchorage independent growth. The relationship between the alterations in microfilament distribution and cell morphology and those changes in growth characteristics is not known. However, the patterns of gene expression may be influenced by these alterations in cell shape and cytoskeletal organization (Ben Ze'ev, 1986; Vasiliev, 1985).

The transforming proteins of several tumour viruses such as $pp60^{v\text{-}src}$, the transforming protein of Rous sarcoma virus are known to be protein-tyrosine kinases (reviewed by Hunter and Cooper, 1985; Bishop, 1985). To explain the cytoskeletal effects that accompany transformation, it has been suggested that the phosphorylation of regulatory elements of the cytoskeleton by $pp60^{v\text{-}src}$ can affect their distribution or functional properties. Although certain cytoskeletal

proteins have been identified as substrates for $pp60^{v-src}$, the molecular mechanisms responsible for the cytoskeletal alterations seen in transformed cells remain poorly characterized (Cooper and Hunter, 1983).

1.3 The Tumour-Promoting Phorbol Esters

A number of properties of transformed cells can be mimicked when cultured cells are treated with compounds known as the tumour-promoting phorbol esters (Weinstein et al., 1979; Weinstein and Wigler, 1977). These compounds, the most potent of which is 12-O-tetradecanoylphorbol-13-acetate (TPA), were originally defined on the basis of their role in the multi-stage induction of experimental carcinogenesis (reviewed by Weinstein, 1981). The phorbol esters exhibited no carcinogenic activity when administered in isolation; however when applied following pretreatment with a known carcinogen, the active phorbol esters profoundly enhanced tumour development. When added to cells in culture, the pleiotropic effects of the phorbol esters are elicited without prior treatment with carcinogenic compounds.

In addition to the tumour-promoting phorbol esters, there are a number of phorbol esters that do not function as tumour promoters. The relationships between phorbol ester structure and activity have been extensively studied (reviewed by Ashendel, 1985). In general, the most active phorbol esters were those such as TPA that were esterified at the 12 and 13 positions of the parent phorbol. Phorbol monoesters and phorbol itself were biologically inactive.

In cultured cells, the tumour-promoting phorbol esters elicit a

variety of biological effects relating to cellular growth and differentiation characteristics (reviewed by Weinstein, 1981; Weinstein et al., 1979). Along with these changes, phorbol esters induce a number of alterations in the distribution and function of cytoskeletal components. Phorbol ester-treated cells attain a rounded morphology and have altered adhesive characteristics that are reminiscent of those observed in transformed cells (Rifkin et al., 1979; Parkinson and Emmerson, 1982; Schliwa et al., 1984). These morphological and adhesive changes are accompanied by reorganizations of cytoskeletal elements, most prominently the microfilaments. Furthermore, additional effects of the phorbol esters on the microfilament assemblies are implied by the demonstration that these compounds stimulate contractile behaviour in smooth muscle cells (Rasmussen et al., 1984; Park and Rasmussen, 1985; Park and Rasmussen; 1986).

1.4 Protein Kinase C: The Phorbol Ester Receptor

As a preliminary step in elucidating the molecular mechanisms of phorbol ester action, it is necessary to gain an understanding of how these compounds bind to and/or enter living cells (reviewed by Ashendel, 1985). The phorbol esters are lipophilic compounds that interact with membrane phospholipids. In addition to these non-specific interactions, the biologically active phorbol esters were shown to have high affinity protein binding sites. Purification and characterization of the phorbol ester receptor revealed that it was actually protein kinase C, the calcium/phospholipid-dependent protein kinase (reviewed by Ashendel, 1985; Kikkawa et al., 1984; Parker et

al., 1984; Kikkawa et al., 1983b; Niedel et al., 1983).

Prior to its identification as the receptor for the tumour-promoting phorbol esters, protein kinase C had been the subject of considerable interest (reviewed by Nishizuka, 1986; Nishizuka, 1984a; Nishizuka, 1984b). The enzyme is certainly widely distributed and has been detected in a variety of tissues and species (Kikkawa et al., 1983a; Kuo et al., 1980). Particularly high levels of the enzyme are present in platelets and in brain (Kikkawa et al., 1983a). This cyclic nucleotide-independent enzyme was originally identified as a serine/threonine-specific, protease-activated protein kinase in brain (Takai et al., 1977; Inoue et al., 1977). The intact protein was then shown to be activated by membrane phospholipids in the presence of calcium, with phosphatidylserine being the most effective phospholipid activator of the enzyme in vitro (Takai et al., 1979a; Takai et al., 1979b). However, the optimal concentration of calcium that was required for activation of the enzyme was somewhat dependent on the components of the lipid fractions used for its activation. This observation led to the exciting discovery that diacylglycerol markedly increases the affinity of protein kinase C for calcium (Takai et al., 1979c; Kishimoto et al., 1980). In the presence of phosphatidylserine without diacylglycerol, half-maximal activation occurs at a calcium concentration of approximately 1×10^{-4} M. However, in the presence of diacylglycerol and phosphatidylserine, half-maximal activation of the enzyme occurs at calcium concentrations in the micromolar range, within the limits of physiological calcium concentrations (Brown et al., 1984).

The observation that protein kinase C is activated by diacylglycerol is highly significant because it establishes a connection between the activation of protein kinase C and the receptor-mediated hydrolysis of phosphatidylinositols that was originally described by Hokin and Hokin (1953). In recent years, an important role in signal transduction has emerged for the hydrolysis of phosphatidylinositols and its phosphorylated products, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate (reviewed by Berridge, 1987; Hirasawa and Nishizuka, 1985; Berridge. 1984).

In most mammalian systems, the phosphatidylinositols represent less than 10% of total membrane phospholipids. Furthermore, the polyphosphoinositides characteristically represent only 10-20% of the total cellular phosphoinositides (Majerus et al., 1986). When a target cell is activated by an appropriate stimulus, the phosphatidylinositol 4,5-bisphosphate is selectively hydrolysed by a specific phosphodiesterase producing two hydrolysis products that serve as second messengers (Berridge, 1987; Bell, 1986; Berridge, 1984). The products of this hydrolysis are diacylglycerol, which activates protein kinase C, and inositol trisphosphate which serves to release calcium from intracellular stores presumably in the endoplasmic reticulum. There is an extensive list of hormones, neurotransmitters, mitogens and growth factors that have been shown to elicit polyphosphatidylinositol breakdown following interaction with their cell surface receptors. For these compounds, protein kinase C is undoubtedly involved in mediating at least some of their

physiological effects.

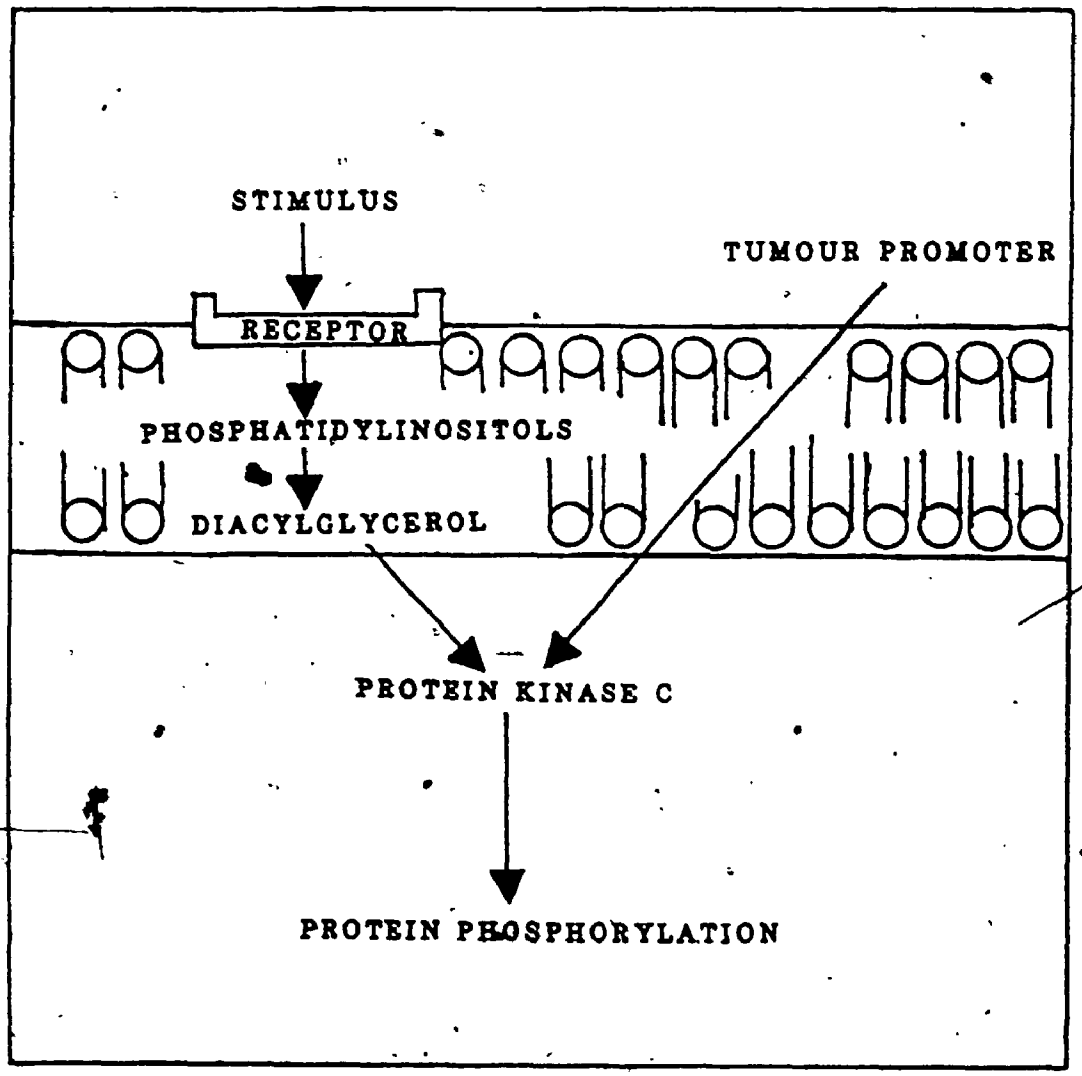
As was discussed previously, protein kinase C serves as the receptor for the tumour-promoting phorbol esters (reviewed by Ashendel, 1985; Kikkawa et al., 1984) and these compounds can directly activate protein kinase C in vitro and in intact cells (Castagna et al., 1982). The tumour-promoting phorbol esters activate protein kinase C in vitro by increasing the affinity of the enzyme for calcium. Structural similarities between phorbol esters and diacylglycerols suggests that the phorbol esters act as analogs of diacylglycerols (reviewed by Ashendel, 1985). Indeed, synthetic diacylglycerols that can activate protein kinase C in intact cells compete with tumour-promoting phorbol esters for their high affinity binding sites (Sharkey et al., 1984). In addition, TPA and diacylglycerols are deacylated by the same liver enzyme (Mentlein, 1986). The activation of protein kinase C by tumour promoters, or by diacylglycerols, is schematically depicted in figure 1.1.

Protein kinase C exists in two forms in most cells, a soluble form and a membrane-associated form (Kraft and Anderson, 1983; Wolf et al., 1985b). In the absence of protein kinase C activators, interconversions between the two forms can be controlled by the level of available calcium. When cells are treated with TPA, synthetic diacylglycerols, or with compounds that stimulate diacylglycerol production, the proportion of the enzyme that is membrane associated is significantly increased (Kraft and Anderson, 1983; Thomas et al., 1987). This translocation of the enzyme to the plasma membrane is associated with activation of the enzyme and subsequent

FIGURE 1.1

SCHEMATIC REPRESENTATION OF PROTEIN KINASE C ACTIVATION.

Protein kinase C is directly activated by membrane-soluble tumour-promoting phorbol esters and by diacylglycerols that are generated by the receptor-mediated hydrolysis of phosphatidylinositols which occurs within the plasma membrane. Through the phosphorylation of substrate proteins, protein kinase C mediates the biological effects of the tumour-promoting phorbol esters and those stimuli that elicit diacylglycerol production.


STIMULUS
TUMOUR PROMOTER
RECEPTOR
PHOSPHATIDYLINOSITOLS
DIACYLGLYCEROL
PROTEIN KINASE C
PROTEIN PHOSPHORYLATION

phosphorylation of substrate proteins. Although the activation of protein kinase C by tumour-promoting phorbol esters and by diacylglycerols which are generated by the breakdown of phosphoinositides are very similar there is one significant difference. Since diacylglycerols are rapidly metabolized in intact cells, the activation of protein kinase C by receptor-mediated phosphoinositide hydrolysis is transient. In contrast, the tumour-promoting phorbol esters are not readily metabolized in most mammalian cells with the result that enzyme activation may be persistent. Furthermore, prolonged treatment of living cells with TPA results in the disappearance of protein kinase C (Melloni et al., 1986; Tapley and Murray, 1984; Tapley and Murray, 1985). It is not as yet known whether either the persistent activation or disappearance of protein kinase C in phorbol ester-treated cells is related to any of the biological activities of these compounds.

In view of its activation by the tumour-promoting phorbol esters and its involvement in receptor-mediated responses, protein kinase C has been the subject of extensive investigation. The enzyme has been purified to homogeneity from a number of different tissues and demonstrated to be composed of a single polypeptide chain with molecular weight in the range of 77,000 to 85,000 (Kikkawa et al., 1982; Wise et al., 1982; Schatzman et al., 1983; Parker et al; 1984). This polypeptide can be divided into two distinct domains, a regulatory lipid-binding domain (Lee and Bell, 1986) and a catalytic domain (Kishimoto et al., 1983). It is the lipid-binding domain that promotes the calcium-mediated associations of protein kinase C with

the plasma membrane. By partial proteolysis *in vitro*, the catalytic domain can be separated from the regulatory domain producing a protein kinase that is fully active in the absence of phospholipid and calcium. Whether or not the proteolytic activation of protein kinase C has a role in physiological processes is still a matter of considerable controversy (Murray *et al.*, 1987). There have been reports that a catalytically active fragment of protein kinase C can be isolated from intact cells that have been treated with TPA (Melloni *et al.*, 1986; Tapley and Murray, 1985). In addition, the catalytic fragment has been implicated in various reactions associated with the activation of neutrophils (Melloni *et al.*, 1985; Pontremoli *et al.*, 1986). However, there is also a report in which the investigators failed to detect proteolytic fragments of protein kinase C using immunological techniques (Woodgett and Hunter, 1987a).

In attempts to elucidate the molecular pathways of signal transduction that involve protein kinase C, considerable effort has been directed towards the identification of the physiological substrates for this kinase. On the basis of experiments that have been conducted *in vitro* and in intact cells, a constantly growing list of substrates for protein kinase C has been obtained (reviewed by Nishizuka, 1986). Included on this list are a number of membrane or membrane-associated proteins that function as cell surface receptors or transporters for a variety of molecules, a number of metabolic enzymes and a number of cytoskeletal or contractile proteins. There are also a number of proteins of unknown identity or function that are known to be substrates for protein kinase C in

intact cells. In the majority of cases, the physiological significance of protein kinase C-mediated phosphorylation events remains to be characterized.

1.5 Objectives

The overall objectives of research in this laboratory have been to elucidate the molecular events involved in the regulation of microfilament dynamics. In particular, it was the aim of this study to identify and characterize the molecular events that are responsible for the alterations in cell morphology and microfilament organization and function that occur following the treatment of cells with tumour-promoting phorbol esters. More specifically I have been investigating the activity of the tumour-promoter receptor, protein kinase C, towards proteins that are thought to be involved in the regulation of actin filament organization and function.

On the basis of their regulatory potential, two distinct groups of microfilament-associated proteins have been the focus of investigation. The proteins that are responsible for the attachment of the termini of the actin-containing stress fibres to the plasma membrane represent one point of control. The second group of proteins under examination are those proteins that are involved in the regulation of contractile events. In the former group, the focal contact proteins vinculin and talin were selected as likely candidates for the action of protein kinase C. In the latter group, the two calmodulin-binding proteins myosin light chain kinase and caldesmon were the subject of examination.

1.5.1 Focal Contact Proteins

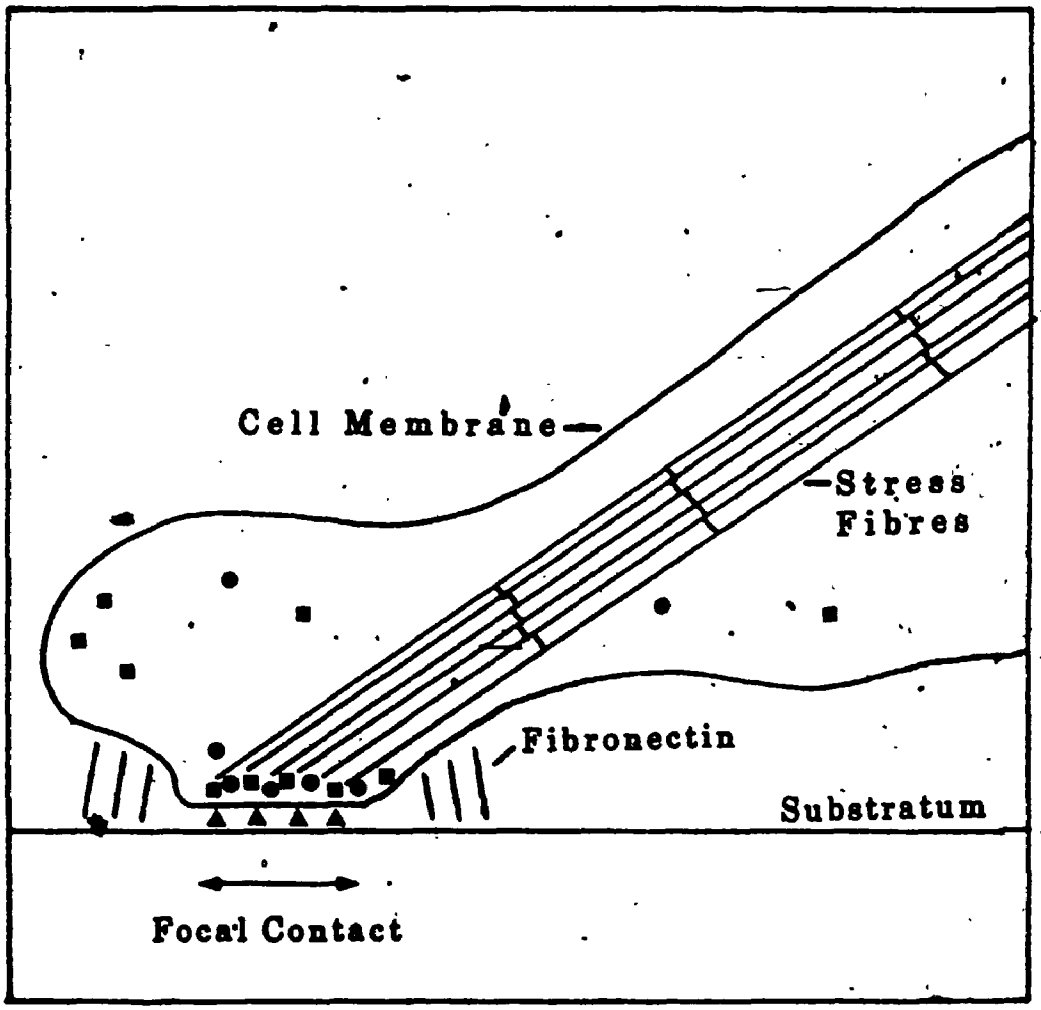
In cultured cells, the focal contacts are the regions of closest contact and tightest adhesion between the cells and the substratum (Birchmeier, 1981; Chen and Singer, 1982; Geiger, 1983). In well-spread cultured cells, these specialized adhesive organelles which contain the membrane attachment points of the stress fibre termini are oriented parallel to the direction of cell movement. A schematic representation of a focal contact is illustrated in figure 1.2.

Interest in focal contacts was stimulated by the observations that they become disrupted and decrease in number when cultured cells are treated with tumour-promoting phorbol esters (Schliwa et al., 1984; Herman et al., 1986), with growth factors such as platelet-derived growth factor (Herman and Pledger, 1985; Herman et al., 1986) or when cells become transformed (David-Pfeuty and Singer, 1980). Thus, it seems that alterations within the focal contact may be somehow involved with the additional cytoskeletal and morphological alterations that are seen upon cell transformation or following treatment with the active phorbol esters or platelet-derived growth factor. Furthermore, patterns of gene expression have been shown to be dependent on cell shape and the existence of cell contacts (Ben Ze'ev, 1986; Ungar et al., 1986). Therefore, it is plausible to suggest that alterations in the distribution or integrity of focal contacts may be partially responsible for the altered patterns of gene expression that are observed in transformed cells or in cells that have been treated with growth factors or phorbol esters. To gain a better understanding of the role of focal contact alterations

FIGURE 1.2

SCHEMATIC REPRESENTATION OF A FOCAL CONTACT OF CULTURED CELLS.

Focal contacts are the regions of cultured cells where the cell is most closely, and tightly, associated with the plasma membrane. Vinculin (●) and talin (■) are localized within these specialized regions of the cell where the termini of microfilament bundles (stress fibres) are attached to the plasma membrane. Unidentified proteins (▲) are involved in the anchorage of the focal contact to the substratum.


Cell Membrane
Stress Fibres
Fibronectin
Substratum
Focal Contact

in the control of cellular processes, it is clearly of interest to study the molecular mechanisms involved in focal contact regulation.

The observation that tumour-promoting phorbol esters induce alterations in the integrity and distribution of focal contacts suggests that certain components of these adhesive organelles may be substrates for protein kinase C (Schliwa et al., 1984; Herman et al., 1986). Among the proteins that have been localized to this region of the cell are vinculin (Geiger et al., 1980) and talin (Burridge and Connell, 1983a; Burridge and Connell, 1983b). Although their precise organization within the focal contact has not been fully characterized, it is presumed that these proteins are involved in the anchorage of stress fibres to the plasma membrane. Vinculin is a 130,000 dalton protein that was originally purified from chicken gizzard smooth muscle (Geiger, 1979; Feramisco and Burridge, 1980). In addition to its localization in focal contacts, vinculin is distributed diffusely throughout the cytoplasm. Whether or not vinculin interacts directly with F-actin is still a matter of controversy. Although initially demonstrated to be an actin-binding protein (Wilkins and Lin, 1982), this activity was later attributed to a contaminant in the vinculin preparation (Evans et al., 1984). Vinculin became the subject of considerable interest when it was identified as a substrate for $pp60^{v-src}$, the transforming protein of Rous sarcoma virus (Sefton et al., 1981). However, the possible role of this phosphorylation event in mediating cytoskeletal alterations is not yet defined (Rohrschneider and Rosok, 1983; Antler et al., 1985). On the basis of its localization near the plasma membrane,

vinculin is also a likely target for protein kinase C.

As was the case for vinculin, talin was purified initially from chicken gizzard smooth muscle (Burridge et al., 1982). The studies of Burridge and Connell (1983a, 1983b) demonstrated that this 215,000 dalton protein is localized in focal contacts and also in ruffling membranes. The former distribution is reminiscent of vinculin and led to the discovery that vinculin and talin bind to each other with high affinity (Burridge and Mangeat, 1984). Thus, talin and vinculin may be two components of a complex that anchors actin filaments to the plasma membrane. It should not be concluded however that all cell contacts contain both vinculin and talin (Heath, 1986). For example, talin is absent from a number of cell-cell contacts that contain vinculin. Nevertheless, the colocalization of talin with vinculin in focal contacts implies that the former protein may also be an available target for the action of protein kinase C. Although the involvement of phosphorylation in regulating the properties of talin has not been characterized, the protein has been shown to be a phosphoprotein and is a substrate for $pp60^{v\text{-}src}$ in RSV-transformed cells (Pasquale et al., 1986). Furthermore talin and talin/vinculin complexes have recently been shown to interact with the purified transmembrane fibronectin receptor in vitro (Horwitz et al., 1986). This observation clearly supports the suggestion that talin and vinculin are components of actin-to-membrane linkages and implies that they have the potential to control the assembly or disassembly of these linkages.

1.5.2 Proteins Involved in the Control of Contractile Events

In addition to effects on the organization of the microfilaments, biologically active phorbol esters induce alterations in their functional properties. In particular, smooth muscle can be induced to undergo a slow, sustained contraction when treated with TPA (Rasmussen et al., 1984; Park and Rasmussen, 1985; Park and Rasmussen, 1986). As a result, it is of interest to examine the activity of protein kinase C towards proteins that play a role in the regulation of contractile activity.

In association with myosin filaments and a number of accessory proteins, the actin-containing microfilaments make up the contractile apparatus. Some of the actin-binding accessory proteins can regulate contractile events by mediating the interactions between actin and myosin filaments that are responsible for contraction (Marston and Smith, 1985). In smooth muscle, and presumably in non-muscle cells, contractile events are under the control of free calcium concentrations (reviewed by Adelstein, 1982; Marston and Smith, 1985). In these cells, the effects of calcium are transmitted by calmodulin, the ubiquitous calcium-binding protein (reviewed by Klee et al., 1980). In the presence of calcium, calmodulin activates myosin light chain kinase which phosphorylates the 20,000 dalton regulatory light chain of myosin (reviewed by Adelstein, 1982; Kamm and Stull, 1985; Sellers and Adelstein, 1987). This event promotes interactions between actin and myosin filaments which stimulates the actin-activated myosin ATPase, a biochemical event that is correlated with contraction (Adelstein and Conti, 1975; Sellers et al., 1981).

Myosin light chain kinase (Mr 130,000) has been purified from gizzard smooth muscle (Adelstein and Klee, 1981), and also from a number of non-muscle tissues including blood platelets and brain (reviewed by Stull et al., 1986). In addition to its activation by calmodulin, the activity of myosin light chain kinase is under another level of control. The enzyme can be phosphorylated by cAMP-dependent protein kinase on two sites in the absence of calmodulin, with the result that the affinity of myosin light chain kinase for calmodulin is diminished (Adelstein et al., 1978; Conti and Adelstein, 1981). In the presence of calmodulin, phosphorylation occurs on only one site and there is no apparent effect on the properties of the enzyme. In view of its involvement in contractile regulation, we sought to examine the possibility that myosin light chain kinase may also be phosphorylated by protein kinase C.

On the basis of recent observations, a second calmodulin-binding protein has been implicated in the regulation of contractile events (Kakiuchi and Sobue, 1983; Marston and Smith, 1985). Caldesmon is an actin-binding protein that was originally purified from chicken gizzard smooth muscle on the basis of its calmodulin-binding ability (Sobue et al., 1981). The binding of caldesmon to F-actin can be partially reversed by the presence of calcium/calmodulin (Sobue et al., 1982; Kakiuchi and Sobue, 1983). Caldesmon has no known enzymatic activity, but has been shown to inhibit the actin-activated myosin ATPase in vitro (Ngai and Walsh, 1984; Ngai and Walsh, 1985; Marston and Lehman, 1985; Dabrowska et al., 1985; Clark et al., 1986; Lash et al, 1986; Smith et al., 1987). Furthermore, the protein

enhances interactions between actin and heavy meromyosin (Lash et al., 1986), and may be able to bundle actin filaments (Bretscher, 1984). On the basis of these observations, a role for caldesmon in the control of contractile events has been proposed.

In addition to the direct effects due to the binding of calmodulin to caldesmon, calmodulin exerts indirect effects on the properties of caldesmon. Caldesmon is phosphorylated by a calmodulin-dependent protein kinase that is obtained as a contaminant of some types of caldesmon preparation (Ngai and Walsh, 1984; Ngai and Walsh, 1985; Lash et al., 1986). The role of this phosphorylation is still somewhat unclear, although Ngai and Walsh (1984, 1987) have demonstrated that phosphorylated caldesmon does not inhibit the actin-activated ATPase of smooth muscle myosin. Our interest in caldesmon was as a potential substrate for protein kinase C.

Although it was originally purified from chicken gizzard smooth muscle as a 150,000 dalton protein, caldesmon was subsequently identified in a wide variety of cell and tissue types by immunological techniques (Owada et al., 1984; Bretscher and Lynch, 1985). The surprising finding of these studies was that on the basis of molecular weight there are two distinct classes of caldesmon, one class with Mr 140-150,000 and the other with Mr 70-80,000. Despite the large molecular weight differences, the two classes of caldesmon demonstrated similar properties (reviewed by Bretcher, 1986). Both caldesmon species are heat stable, bind to both F-actin and calmodulin and exert effects on the actin-activated ATPase of myosin (Ngai and Walsh, 1984; Onji et al., 1987). Since it seems likely

that both caldesmon forms play similar roles in cytoskeletal or contractile regulation, one of the aims of this study was to examine the phosphorylation of both caldesmon species by protein kinase C.

1.6 Experimental Approach

The investigation of the phosphorylation of cytoskeletal proteins by protein kinase C progressed in three distinct phases. In the first phase, we sought to characterize the phosphorylation of the potential regulators of cytoskeletal organization and function by purified protein kinase C. To achieve this aim, it was first necessary to establish protocols for the reliable purification of protein kinase C. Focal contact proteins (vinculin and talin, Chapter 2) and contractile regulators (myosin light chain kinase and various forms of caldesmon, Chapter 3) were subsequently identified as *in vitro* substrates for protein kinase C and the sites of phosphorylation examined.

To demonstrate that a phosphorylation reaction is of physiological importance it is not sufficient to simply present evidence that the reaction occurs *in vitro*, since a number of reactions that can occur using purified components in a non-physiological setting do not occur in living cells (Krebs and Beavo, 1979). Of the proteins that we had identified as *in vitro* substrates for protein kinase C, vinculin was the only one that was known to be a substrate for protein kinase C in living cells as well (Werth and Pastan, 1984). Thus, in the second phase of our investigations, we sought to examine in living cells the phosphorylation of the other proteins (talin, myosin light chain kinase and caldesmon) that we had

identified as *in vitro* substrates for protein kinase C. For these studies, intact human platelets were used. In this experimental system, the phosphorylations of platelet caldesmon and the platelet form of talin by protein kinase C were demonstrated and characterized (Chapter 4). Our inability to conveniently isolate myosin light chain kinase precluded an analysis of its phosphorylation in living cells.

The demonstration that phosphorylation can alter the localization or function of a protein is another advance towards establishing a physiological role for the phosphorylation event. Thus, to extend the results of our previous analyses, the effects of phosphorylation on some of the functional properties of caldesmon were investigated (Chapter 5). The objective of this final phase of the study was to gain more of an understanding of how the biologically active phorbol esters exert effects on the organization and function of cytoskeletal componenents.

CHAPTER 2

PHOSPHORYLATION OF FOCAL CONTACT PROTEINS BY PURIFIED PROTEIN KINASE C

2.1 INTRODUCTION

The focal contact of a cultured cell is the region of closest and tightest association between the plasma membrane and the substratum (Birchmeier, 1981; Geiger, 1983). Focal contacts are principally responsible for the adhesion of many cell types to culture dishes. This contact also contains the membrane attachment site for the actin microfilament bundles known as stress fibres (Geiger et al., 1980; Burridge et al., 1982). As a result, components of the focal contact are considered to be potential mediators of events which result in changes in cell morphology or adhesion.

When cells are treated with tumour-promoting phorbol esters (Rifkin et al., 1979; Scliwa et al., 1984), growth factors such as platelet-derived growth factor (Herman et al., 1986; Herman and Pledger, 1985) or become virally transformed (reviewed by Hanafusa, 1977), they undergo a series of biochemical and morphological alterations. These alterations are accompanied by cytoskeletal reorganizations which include rearrangements of the stress fibres and changes in the organization and distribution of focal contacts (David-Pfeuty and Singer, 1980; Schliwa et al., 1984; Herman and Pledger, 1985). Since these results imply that events within the focal contacts may be responsible for, or at least involved in,

some of the alterations in biochemical or morphological properties of cultured cells, it is clearly of interest to examine the molecular mechanisms by which focal contacts are regulated.

Many, if not all, of the effects of the tumour-promoting phorbol esters on cultured cells are transmitted by their receptor, protein kinase C (reviewed by Ashendel, 1985; Nishizuka, 1986; Pasti et al., 1986). Therefore, the objective of the work described in this chapter was to examine the activity of protein kinase C towards components of the focal contact that may mediate the morphological or biochemical alterations that are induced by the phorbol esters. Two focal contact phosphoproteins, vinculin and talin, were the focus of investigation (reviewed by Mangeat and Burridge, 1984; Geiger, 1983).

Although the physiological roles of vinculin and talin remain poorly understood, a number of interesting observations with the two proteins have been made. In addition to the interactions which have been observed between vinculin and talin (Burridge and Mangeat, 1984), interactions between talin or talin/vinculin complexes and the transmembrane fibronectin receptor have been discovered (Horwitz et al., 1986). On the basis of these results, and from studies on their subcellular distribution (Geiger, 1979; Geiger et al., 1980; Burridge and Connell, 1983a; Burridge and Connell, 1983b), vinculin and talin are thought to be involved in transmembrane linkages between stress fibres and the extracellular matrix. Interestingly, the two proteins have been identified as substrates for $pp60^{v-src}$, the transforming protein of Rous sarcoma virus (Sefton et al., 1981; Pasquale et al., 1986). The effects of phosphorylation are not yet known, however.

As a first step towards elucidating the mechanisms involved in the phorbol ester-mediated alteration in cytoskeletal organization and cell morphology, the results of this chapter demonstrate that vinculin and talin are phosphorylated by protein kinase C in vitro.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Blue Sepharose, phenyl-Sepharose, DEAE-Sephadex (A25) and Sephacryl S-300 were obtained from Pharmacia Fine Chemicals and DEAE-cellulose (DE52) and phosphocellulose (P11) from Whatman. Radiochemicals were obtained from New England Nuclear. Histones (III-S and IIa-S), trypsin (treated with diphenylcarbamyl chloride), and the catalytic subunit of cAMP-dependent protein kinase were from Sigma. Phosphatidylserine and diolein were from Serdary Research Laboratories and Staphylococcus Aureus V8 protease from Miles Scientific. Heat-killed formalin-fixed Staphylococcus Aureus was purchased from Boehringer Mannheim. Cellulose coated (0.1 mm) thin layer chromatography plates (10 cm X 10 cm) for peptide mapping and phosphoamino acid analysis were from Merck. Other reagents and chemicals were of analytical grade.

2.2.2 Purification of Protein Kinase C from Bovine Brain

Protein kinase C was purified from fresh bovine brain by modification of the methods of Kikkawa et al. (1982) and Wise et al. (1982). Fresh bovine brain (400 g) was homogenized in 3 volumes of buffer A (20 mM Tris-Cl, pH 7.5, 2 mM EDTA, 50 mM 2-mercaptoethanol) at 4°C. The homogenate was centrifuged for 40 minutes at 14,000 X g

and the supernatant filtered through glass wool prior to 20-45% ammonium sulphate fractionation. The 20-45% ammonium sulphate pellet was dissolved in buffer A and then extensively dialyzed against the same buffer. The dialyzate was subjected to DEAE-cellulose chromatography. Active fractions from this column were further purified using blue Sepharose and phenyl-Sepharose columns. Protein kinase activity was dependent on both phosphatidylserine and calcium. The enzyme was stored at 4°C and was used within one week of isolation.

2.2.3 Phosphorylation Assays

Protein kinase C was assayed for 5 minutes at 30°C using histone (types III-S, 0.2 mg/ml) as substrate. The reaction was conducted in a volume of 0.1 ml containing 20 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 0.01 mM ATP (specific activity 150-250 cpm/pmol) and either phosphatidylserine (0.05 mg/ml) and $CaCl_2$ (0.5 mM) or EGTA (0.5 mM). Histone phosphorylation was monitored by precipitation of the reaction mixture using ice-cold trichloroacetic acid as described by Corbin and Reimann (1974). One unit of protein kinase C is defined as the amount of enzyme required to transfer 1 nmol of phosphate per min into histone (type III-S) at 30°C under these conditions.

2.2.4 Affinity Labelling of Protein Kinase C Preparation with [^{14}C]-Fluorosulfonylbenzoyl Adenosine

Protein kinase C, purified as described above (section 2.2.2), was loaded on a 1 ml DEAE-cellulose (DE52) column pre-equilibrated with 20 mM Tris-Cl, pH 7.5, 2.0 mM EDTA, 50 mM 2-mercaptoethanol. The column was then extensively washed with 20 mM Tris-Cl, pH 7.5 to

remove EDTA and 2-mercaptoethanol. Protein kinase C was eluted from the column in the absence of 2-mercaptoethanol and EDTA with 0.1 M NaCl in 20 mM Tris-Cl, pH 7.5.

An aliquot (0.06 ml) of [^{14}C]-fluorosulfonylbenzoyl adenosine (0.44 mM) in 95% ethanol was dried under N_2 as described by Buhrow et al. (1982) and then resuspended in 0.012 ml of dimethylsulfoxide in preparation for reaction with the protein kinase C preparation.

The protein kinase C preparation was incubated at room temperature with [^{14}C]-fluorosulfonylbenzoyl adenosine (0.044 mM final concentration) for one hour in the presence or absence of 2 mM ATP in a reaction mixture containing phosphatidylserine (0.05 mg/ml) and $CaCl_2$ (0.5 mM). The reaction was terminated by boiling in sample buffer for SDS-polyacrylamide gel electrophoresis and the samples were run on an 8% gel. The gel was treated with Enhance, dried and autoradiographed at -70°C for 14 days.

2.2.5 Purification of Vinculin and Talin

Vinculin was purified from fresh chicken gizzards essentially by the method of Feramisco and Burridge (1980). Following ammonium sulphate fractionation as described by Feramisco and Burridge (1980), the ammonium sulphate pellet was dissolved in buffer (10 mM Tris-acetate, pH 7.5, 0.1 mM EDTA, 10 mM 2-mercaptoethanol) and extensively dialyzed against the same buffer prior to chromatography on DEAE-cellulose (DE52). After chromatography on DEAE-cellulose, the vinculin-containing fractions were identified by SDS-polyacrylamide gel electrophoresis, pooled and subjected to chromatography on a second DEAE-cellulose (DEAE A25) column.

Talin was purified essentially by the method of Burridge and Connell (1983b). Following DEAE-cellulose (DE52) and phosphocellulose (P11) columns, the talin was chromatographed on Sephacryl S-300. Talin was identified by SDS-polyacrylamide gel electrophoresis.

2.2.6 Phosphorylation of Vinculin and Talin

Vinculin (0.1 mg/ml) and talin (0.1 mg/ml) were phosphorylated at 30°C in reaction mixtures of 0.05 ml containing 0.01 units of protein kinase C (Litchfield and Ball, 1986). The reactions were terminated by boiling in SDS-polyacrylamide gel sample buffer (Laemmli, 1970) and the reaction mixtures subjected to SDS-polyacrylamide gel electrophoresis. The vinculin or talin bands were excised from the gel, and digested overnight at 70°C with 30% H_2O_2 so that ^{32}P incorporation could be determined by scintillation counting.

2.2.7 Immunoprecipitation

Vinculin and talin (5 µg) were immunoprecipitated essentially as described by Sefton et al. (1978) from phosphorylation reaction mixtures by the addition of affinity-purified rabbit antibodies (5 µg) prepared against chicken gizzard vinculin or talin. After incubation for 1 hour at 4°C, heat-killed Staphylococcus Aureus (0.01 ml of 10% v/v) in immunoprecipitation buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 20 mM phosphate, pH 7.4) containing 0.3% bovine serum albumin was added. Following incubation for 15 minutes at 4°C, the Staphylococcus Aureus was pelleted, washed twice with immunoprecipitation buffer and once with 20 mM Tris-Cl, pH 7.5. To solubilize the immunoprecipitated

proteins, the Staphylococcus Aureus was resuspended and boiled in sample buffer for SDS-polyacrylamide gel electrophoresis. Samples were then electrophoresed on SDS-polyacrylamide gels which were stained with Coomassie Blue and then dried and autoradiographed for visualization of the phosphorylated proteins.

2.2.8 Phosphopeptide Mapping

Proteins were labeled with ^{32}P as described above (section 2.2.6) and electrophoresed on SDS-polyacrylamide gels. The ^{32}P-labeled protein band was excised from the gel and incubated in 50 mM ammonium bicarbonate containing trypsin or Staphylococcus Aureus V8 protease (0.05 mg/ml) for 22 hours at 37°C in a siliconized test tube (12 X 75 mm). Complete digestion was ensured by transferring the supernatant to a new siliconized test tube containing more protease (5 µg) and incubating for 2 hours at 37°C. The solution was then frozen and extensively lyophilized before the phosphopeptides were separated in two dimensions on thin layer chromatography plates by the method of Elder et al. (1977). The peptides were subjected to electrophoresis at 1000 volts in pH 1.9 buffer (acetic acid: 88% formic acid: H_2O; 15: 5: 80) and then ascending chromatography (1-butanol: pyridine: acetic acid: H_2O; 32.5: 25: 5: 20). Phosphopeptides were visualized by autoradiography.

2.2.9 Phosphoamino Acid Analysis

Phosphoamino acid analysis was done essentially by the method of Cooper et al. (1983). Briefly, phosphopeptides were prepared and lyophilized as described above and were then subjected to partial hydrolysis with 5.7N HCl for 1 hour at 110°C. After drying, the

hydrolysate was dissolved in 0.01 ml of pH 1.9 buffer (2.5% formic acid (88% stock), 7.8% glacial acetic acid) containing unlabeled phosphoamino acids (phosphoserine, phosphothreonine, phosphotyrosine at 0.15 mg/ml each) and an aliquot (0.003 ml) applied to thin layer chromatography plates. Following electrophoresis towards the anode at 1250 volts for 25 minutes, the samples were electrophoresed in a second dimension at pH 3.5 (0.5% pyridine, 5.0% glacial acetic acid) at 1250 volts for 15 minutes. The plates were then dried, stained with ninhydrin (0.2% in acetone) to visualize standard phosphoamino acids and autoradiographed to detect the ^{32}P-labeled phosphoamino acids.

2.2.10 Protein Determinations

Protein determinations were done by the method of Bradford (1976) using commercial reagent (Biorad) and bovine immunoglobulin as protein standard.

2.2.11 SDS-Polyacrylamide Gel Electrophoresis and Autoradiography

SDS-polyacrylamide gel electrophoresis was performed using the buffer system of Laemmli (1970). Molecular weight markers were as follows: myosin heavy chain (200,000); β-galactosidase (116,000); phosphorylase b (92,000); bovine serum albumin (67,000); immunoglobulin heavy chain (50,000); actin (43,000). Autoradiography was done with Kodak X-Omat AR film at -70°C.

2.2.12 Preparation of Calcium Buffer Solutions

Protein kinase C reaction mixtures of a desired free calcium concentration were prepared using EGTA buffers according to the method of Bartfai (1979).

2.3 RESULTS

2.3.1 Purification of Protein Kinase C from Bovine Brain

As an initial step in investigating the role of protein kinase C in the regulation of cytoskeletal components, a protocol was devised to purify this enzyme from fresh bovine brain. After three column chromatographic steps, the active fractions of protein kinase C contained 2 major protein bands as shown on the Coomassie blue stained SDS-polyacrylamide gel (figure 2.1, lane 4). The upper band (molecular weight of 82,000) is of similar size to that previously reported for protein kinase C (Kikkawa et al., 1982; Wise et al., 1982). A major contaminant (molecular weight of 67,000) is also visible. This contaminant was also found by others (Parker et al., 1984).

A typical purification table (table 2.1) for bovine brain protein kinase C reveals that the enzyme is obtained in low yield (approximately 5%), a result common to a number of published purifications (Kikkawa et al., 1982; Wise et al., 1982; Parker et al., 1984). Protein kinase C activity could not be reliably assayed in the initial brain extract since the extract contains a number of contaminating protein kinase activities (Kikkawa et al., 1983a). The specific activity of the purified enzyme (94.2 nmol/min/mg) is similar to that obtained for protein kinase C purified from bovine heart (Wise et al., 1982) and porcine spleen (Schatzman et al., 1983) but is more than 10-fold lower than that reported for rat brain protein kinase C (Kikkawa et al., 1982) and nearly 50-fold lower than that subsequently reported for bovine brain protein kinase C (Parker

FIGURE 2.1

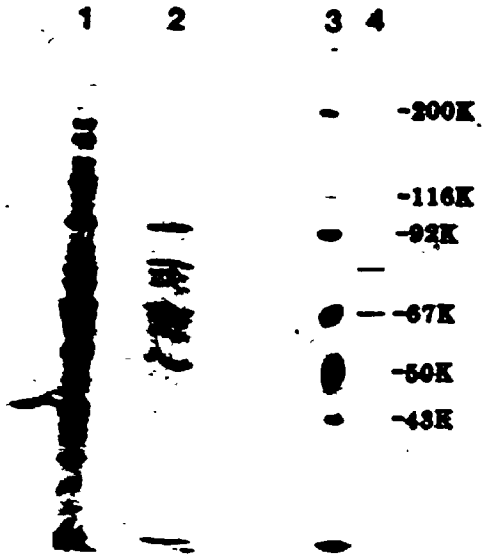
SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF FRACTIONS FROM BOVINE BRAIN PROTEIN KINASE C PURIFICATION.

Samples were run on a 10% gel and visualized by staining with Coomassie blue. Lane 1, DEAE-cellulose pool; lane 2, Blue Sepharose pool; lane 4, phenyl-Sepharose pool. Molecular weight markers shown in lane 3 are as follows: myosin heavy chain, 200,000; β-galactosidase, 116,000; phosphorylase b, 92,000; bovine serum albumin, 67,000; immunoglobulin heavy chain, 50,000; actin, 43,000.

FIGURE 2.2

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS AND AUTORADIOGRAPHY OF PROTEIN KINASE C.

Protein kinase C purified through phenyl-Sepharose chromatography was incubated with [γ-^{32}P]ATP in the presence of phosphatidylserine and calcium (lane 2) or in the presence of EGTA (lane 1). Samples were run on a 10% gel which was stained with Coomassie blue (panel A) prior to autoradiography (panel B).


1
2
3
4
-200K
-116K
-92K
-67K
-50K
-43K

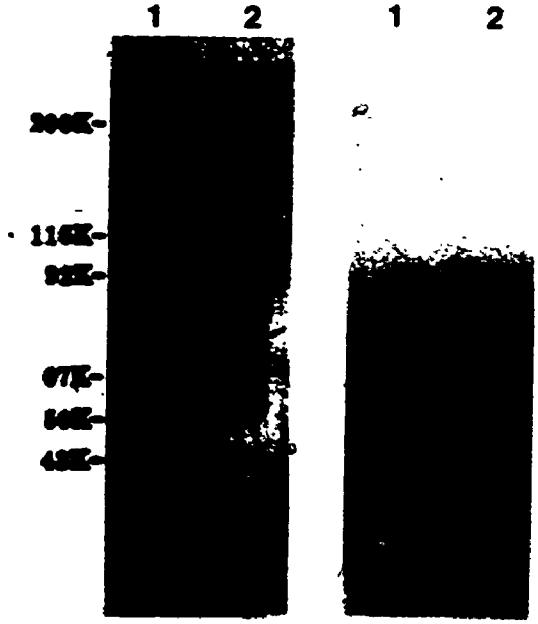

1
2
1
2
200K-
116K-
92K-
67K-
50K-
43K-

TABLE 2.1

PURIFICATION OF BOVINE BRAIN PROTEIN KINASE C.

Step	Protein	Total Activity	Specific Activity	Yield	Purification
	mg	units	units/mg	%	-fold
DEAE-cellulose	742	1,085	1.46	100	1
Blue Sepharose	15.8	71.2	4.51	6.6	3.1
Phenyl Sepharose (pool)	0,85	52.4	61.65	4.8	42.2
Phenyl Sepharose (peak fraction)	0.24	22.6	94.17	2.1	64.5

Aliquots of fractions from different stages during the purification of protein kinase C were assayed as described in Materials and Methods. One unit of protein kinase C activity is defined as the amount of enzyme required to transfer 1 nmol of phosphate per minute at 30°C into histone III-S under the conditions defined in Materials and Methods.

et al., 1984).

2.3.2 Characterization of Bovine Brain Protein Kinase C

2.3.2.1 Histone Specificity

Since the specific activity of our bovine brain protein kinase C was low in comparison to other protein kinase C preparations (Kikkawa et al., 1982; Parker et al., 1984) a number of experiments were conducted to establish the authenticity of the purified enzyme. Utilizing different histone fractions as substrate, protein kinase C exhibited nearly 4-fold higher activity towards histone III-S than histone IIa-S (data not shown). In addition, the dependency of protein kinase C on phosphatidylserine and calcium was nearly absolute.

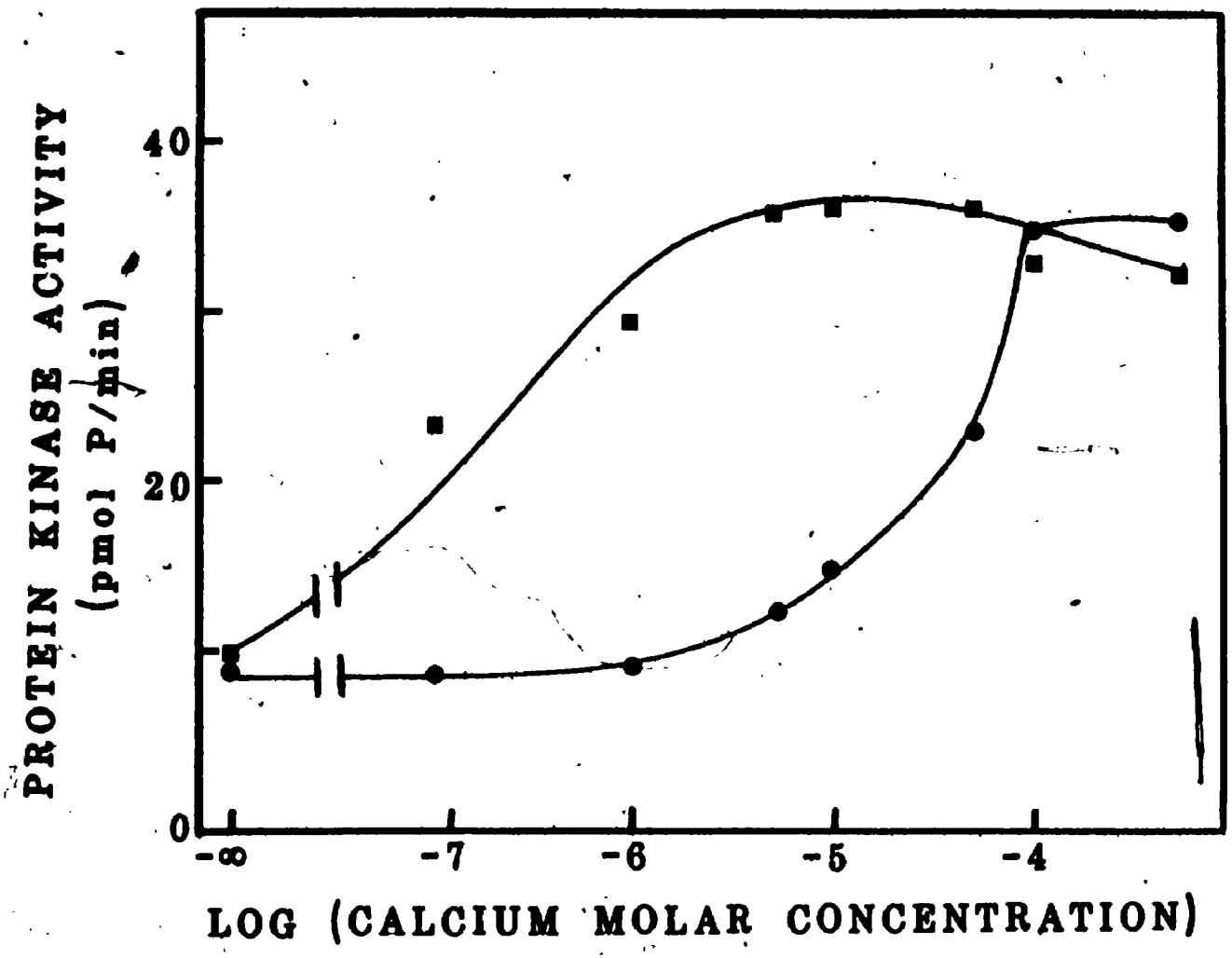
2.3.2.2 Phosphorylation of Proteins in Protein Kinase C Preparation

As is the case with a number of protein kinases, protein kinase C is known to be autophosphorylated in the presence of phospholipid and calcium (Kikkawa et al., 1982; Le Peuch et al., 1983; Huang et al., 1986a; Mochly-Rosen and Koshland, 1987). Bovine brain protein kinase C was incubated with [γ-^{32}P]ATP (figure 2.2) in the presence of phosphatidylserine and calcium (lane 2) or EGTA (lane 1). The autoradiogram (panel B) shows that the two major bands with molecular weights 82,000 and 67,000 that are visible on the Coomassie blue stained SDS-polyacrylamide gel (panel A) are both phosphorylated only in the presence of phosphatidylserine and calcium. This result is consistent with the 82,000 dalton protein either being protein kinase C or a substrate for the enzyme.

FIGURE 2.3

ACTIVATION OF PROTEIN KINASE C BY DIACYLGLYCEROL.

Protein kinase C was assayed as described in Materials and Methods in the presence of phosphatidylserine (20 µg/ml) and the indicated amount of calcium. Assays were conducted in the presence (■) or absence (●) of diolein (1 µg/ml). The results represent the average of duplicate samples and are typical of those obtained in two separate experiments.


PROTEIN KINASE ACTIVITY
(pmol P/min)
40
20
0
-8
-7
-6
-5
-4
LOG (CALCIUM MOLAR CONCENTRATION)

2.3.2.3 Activation by Diacylglycerol and TPA

The activity of protein kinase C is markedly stimulated by diacylglycerol in the presence of micromolar concentrations of calcium (Takai et al., 1979c; Kishimoto et al., 1980; Kaibuchi et al., 1981). The activation of bovine brain protein kinase C by the addition of diolein (1 µg/ml) is demonstrated in figure 2.3. As evidenced by this figure, half-maximal activation of protein kinase C occured at a calcium concentration of approximately 5×10^{-4}M in the absence of diolein and at a calcium concentration of less that 10^{-6}M in the presence of diolein. This significant activation of protein kinase activity by diacylglycerol at physiological calcium concentrations is a unique property of protein kinase C (Nishizuka, 1986; Bell, 1986). Protein kinase C is also known to be activated by tumour-promoting phorbol esters such as TPA in vitro and in vivo (Castagna et al., 1982). With our bovine brain enzyme, TPA at concentrations of 5-20 ng/ml exhibited nearly 2-fold activation of protein kinase activity in the presence of phosphatidylserine (0.05 mg/ml) and 0.5 mM calcium (results not shown).

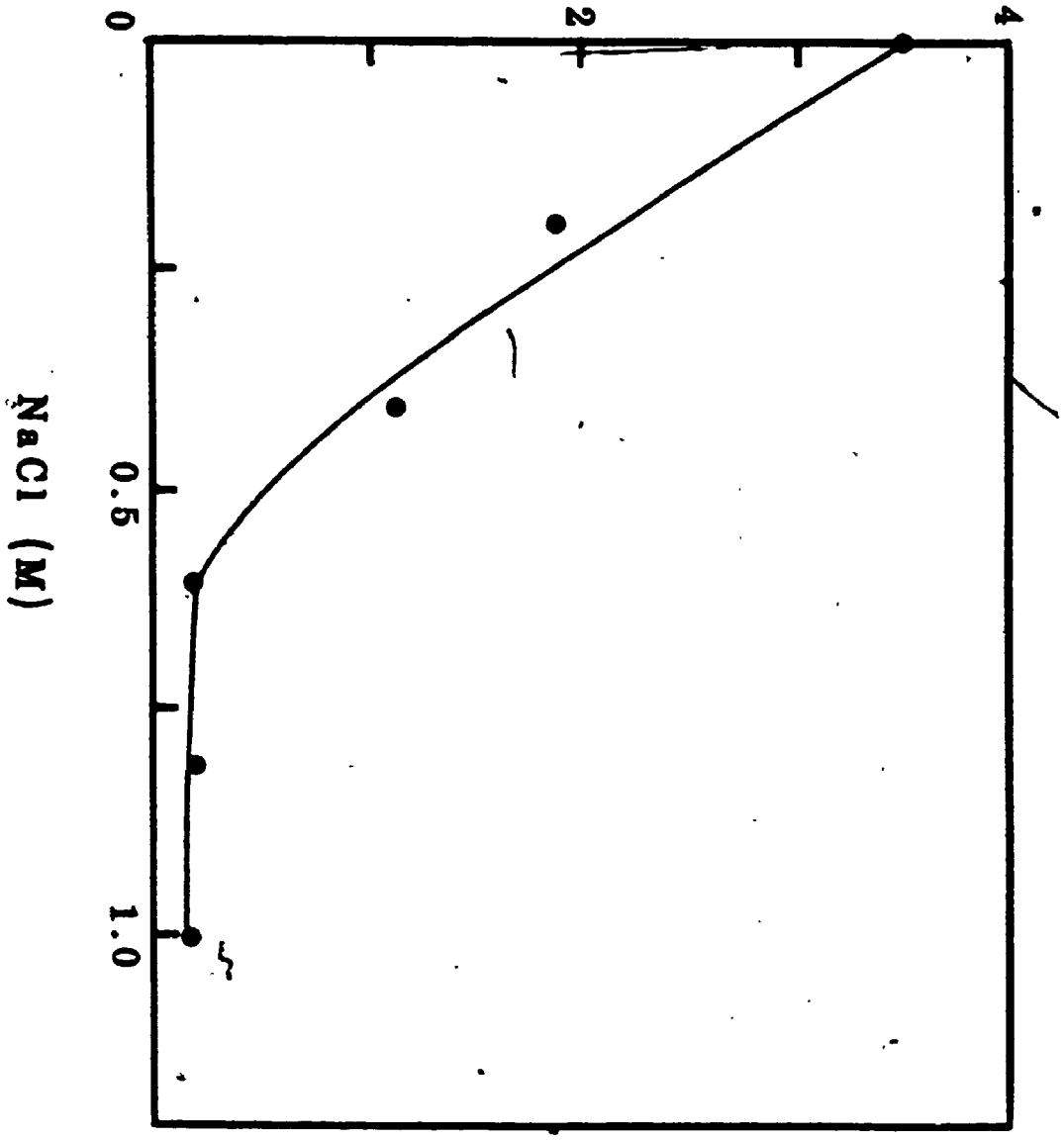
2.3.2.4 Effects of Salt and pH on Protein Kinase C Activity

Protein kinase C was purified from bovine brain for use in the in vitro phosphorylation of purified or partially-purified cytoskeletal proteins. Since these proteins would be purified by a variety of different protocols, the constituents of the solutions that they were in would be somewhat variable. Therefore, protein kinase C activity was measured as a function of sodium chloride concentration. As seen in figure 2.4, half-maximal inhibition of

FIGURE 2.4

INHIBITION OF PROTEIN KINASE C BY SODIUM CHLORIDE.

Protein kinase C was assayed as described in Materials and Methods in the presence of the indicated amount of sodium chloride (final concentration). The results represent the average of duplicate samples.


PROTEIN KINASE ACTIVITY
(pmol P/min)
0
2
4
NaCl (M)
0.5
1.0

protein kinase C activity occurs at a salt concentration of 0.2 M and complete inhibition occurs at a salt concentration of 0.6 M.

Protein kinase C activity is quite tolerant of variations in pH exhibiting near maximal activity in a pH range of 6-8 (results not shown). Background activity of protein kinase C in the absence of phospholipid and calcium was however markedly increased when assays were conducted above pH 7, confirming the previous findings of Wise et al. (1982).

2.3.2.5 Affinity Labelling of Bovine Brain Protein Kinase C by [^{14}C]-Fluorosulfonylbenzoyl adenosine

The ATP analog fluorosulfonylbenzoyl adenosine has been utilized to modify the ATP binding site of a variety of different protein kinases (Taylor et al., 1983; Bühow et al., 1982). As shown in figure 2.5, this alkylating agent specifically labeled a single band in our bovine brain protein kinase C preparation in the presence of phosphatidylserine and calcium. Labeling of the band occurs only in the absence of ATP (lane 2) and is blocked by the addition of 2 mM ATP as expected (lane 1). The labeled band has a molecular weight of approximately 80,000 which is very similar to that previously reported for protein kinase C (Kikkawa et al., 1982; Wise et al., 1982). However, the molecular weight of this band is not quite identical to that of the Mr 82,000 major upper band seen in our protein kinase C preparation. The difference in molecular weight suggests that the Mr 80,000 protein may be a distinct protein from the Mr 82,000 protein, or that the Mr 82,000 migrates as a protein with Mr 80,000 following covalent modification with

FIGURE 2.5

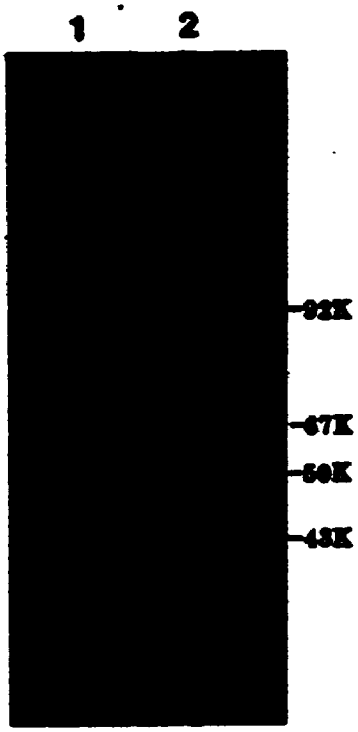
AFFINITY LABELLING OF PROTEIN KINASE C.

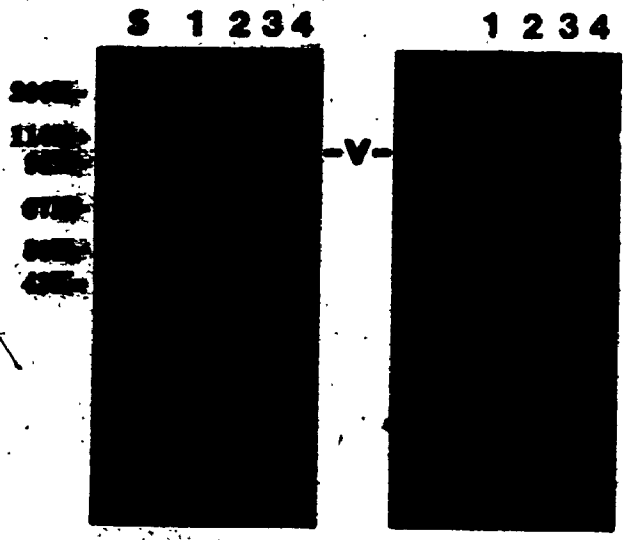
Protein kinase C was purified as described in Materials and Methods and concentrated on a 1 ml DEAE-cellulose in the absence of mercaptoethanol. Samples of the enzyme were incubated with [^{14}C]fluorosulfonylbenzoyl adenosine in the presence (lane 1) or absence (lane 2) of 2 mM ATP and subjected to electrophoresis on 10% SDS-polyacrylamide gels prior to autoradiography. Molecular weight markers are as follows: phosphorylase b, 92,000; bovine serum albumin, 67,000; immunoglobulin heavy chain, 50,000; actin, 43,000.

FIGURE 2.6

PHOSPHORYLATION OF VINCULIN BY PROTEIN KINASE C.

Vinculin (0.1 mg/ml) was phosphorylated by protein kinase C as described in Materials and Methods for 20 minutes at 30°C. The reaction mixtures were electrophoresed on a 10% SDS-polyacrylamide gel which was stained with Coomassie blue (panel A) prior to autoradiography (panel B). Lane 1, 0.5 mM $CaCl_2$ and phosphatidylserine (0.05 mg/ml); lane 2, 0.5 mM EGTA and phosphatidylserine (0.05 mg/ml); lane 3, 0.5 mM $CaCl_2$; lane 4 0.5 mM EGTA. Molecular weight markers are marked (S).


1
2
92K
67K
50K
43K


S 1 2 3 4
1 2 3 4
V

fluorosulfonylbenzoyl adenosine. Although the results of this experiment fail to prove that the Mr 82,000 protein seen on SDS-polyacrylamide gels (figure 2.1, figure 2.2) is protein kinase C, the preparation contains only one major ATP binding protein. Therefore, it seems apparent that the protein kinase C is free of other ATP binding proteins.

2.3.3 Phosphorylation of Vinculin by Protein Kinase C

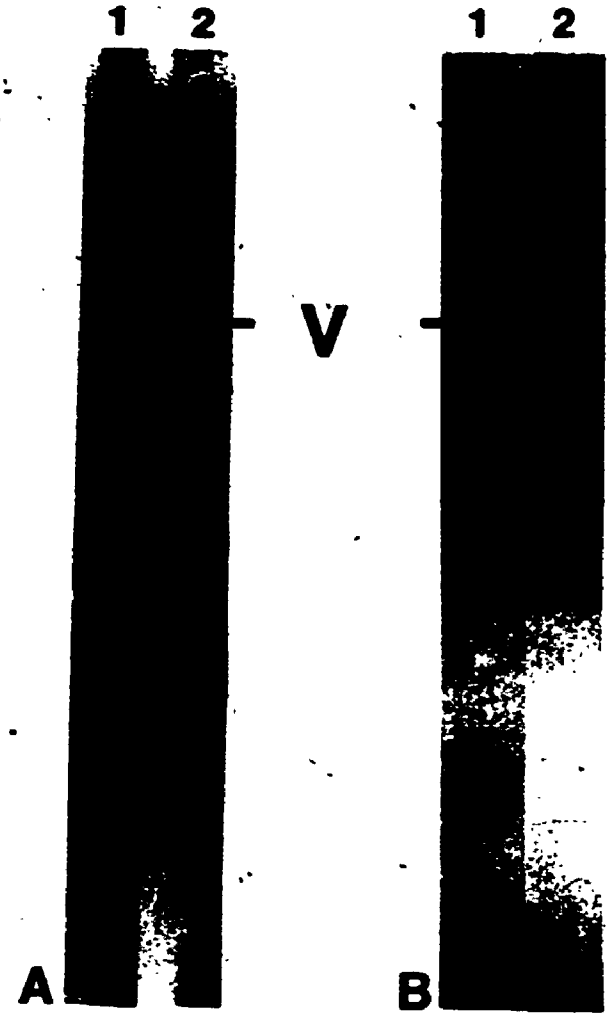
As seen in figure 2.6, chicken gizzard vinculin is phosphorylated by bovine brain protein kinase C in the presence of phosphatidylserine and calcium (panel B, lane 1). This observation confirms the finding of others (Werth et al., 1983; Kawamoto and Hidaka, 1984) who also demonstrated the phosphorylation of chicken gizzard vinculin by protein kinase C. Phosphorylation of vinculin does not occur in the presence of EGTA with (lane 2) or without phosphatidylserine (lane 4) although a low level of phosphorylation is evident in the presence of calcium without added phosphatidylserine (lane 3). Under similar conditions, vinculin was not phosphorylated to any significant extent by the catalytic subunit of cAMP-dependent protein kinase (results not shown).

To verify that the phosphorylated protein is vinculin and not a minor contaminant, the phosphorylation reaction mixture was subjected to immunoprecipitation using affinity-purified anti-vinculin antibodies (figure 2.7). The phosphorylated protein with the same electrophoretic mobility as vinculin is immunoprecipitated with affinity-purified anti-vinculin antibodies (lane 1) while no phosphoproteins are precipitated with antibodies from a non-immune

FIGURE 2.7

IMMUNOPRECIPITATION OF PHOSPHORYLATED VINCULIN.

Vinculin was phosphorylated and immunoprecipitated with affinity purified rabbit anti-vinculin immunoglobulin (lane 1) or with immunoglobulin (5 µg) from a non-immune rabbit (lane 2). The immunoprecipitated fractions were electrophoresed on an 8% SDS-polyacrylamide gel and stained with Coomassie blue (panel A) prior to autoradiography (panel B). The position of vinculin is marked (-V-).


1
2
1
2
V
A
B

rabbit. These results confirm that vinculin is indeed a substrate for protein kinase *in vitro*.

The time dependent incorporation of phosphate into vinculin is illustrated in figure 2.8. During an initial rapid phase, vinculin phosphorylation reaches a level of 0.2 mol phosphate/mol protein. This rapid phosphorylation is followed by slower phosphorylation resulting in incorporation to a level of 0.38 mol phosphate/mol protein. In the absence of phosphatidylserine and calcium, vinculin phosphorylation is insignificant. By comparison, phosphate incorporations of approximately 0.5 mol phosphate/mol vinculin and 0.08 mol phosphate/mol vinculin were achieved by Werth *et al.* (1983) and Kawamoto and Hidaka (1984) respectively.

2.3.4 Two-dimensional Peptide Mapping of Phosphorylated Vinculin

Two-dimensional peptide mapping was utilized to determine the number of sites on which vinculin was phosphorylated by protein kinase C. Examination of figure 2.9 reveals that vinculin contains two major phosphate acceptor sites in addition to one minor phosphorylation site. These results confirm the findings of Werth *et al.* (1983) who demonstrated that vinculin is phosphorylated on two major sites by protein kinase C. A curious feature of the vinculin phosphopeptide map is that all three sites of phosphorylation have the same electrophoretic mobility.

2.3.5 Phosphorylation of Talin by Protein Kinase C

In addition to vinculin, talin is another focal contact phosphoprotein that is a potential substrate for protein kinase C. Examination of figure 2.10 reveals that when chicken gizzard talin

FIGURE 2.8

TIME DEPENDENCE OF VINCULIN PHOSPHORYLATION.

Vinculin was incubated with protein kinase C in the presence of phosphatidylserine and calcium (●) or EGTA (○). At the times indicated, aliquots were removed and immediately boiled in sample buffer for SDS-polyacrylamide gel electrophoresis. Phosphate incorporation was determined as described in Materials and Methods. The results are typical of those obtained in five separate experiments.

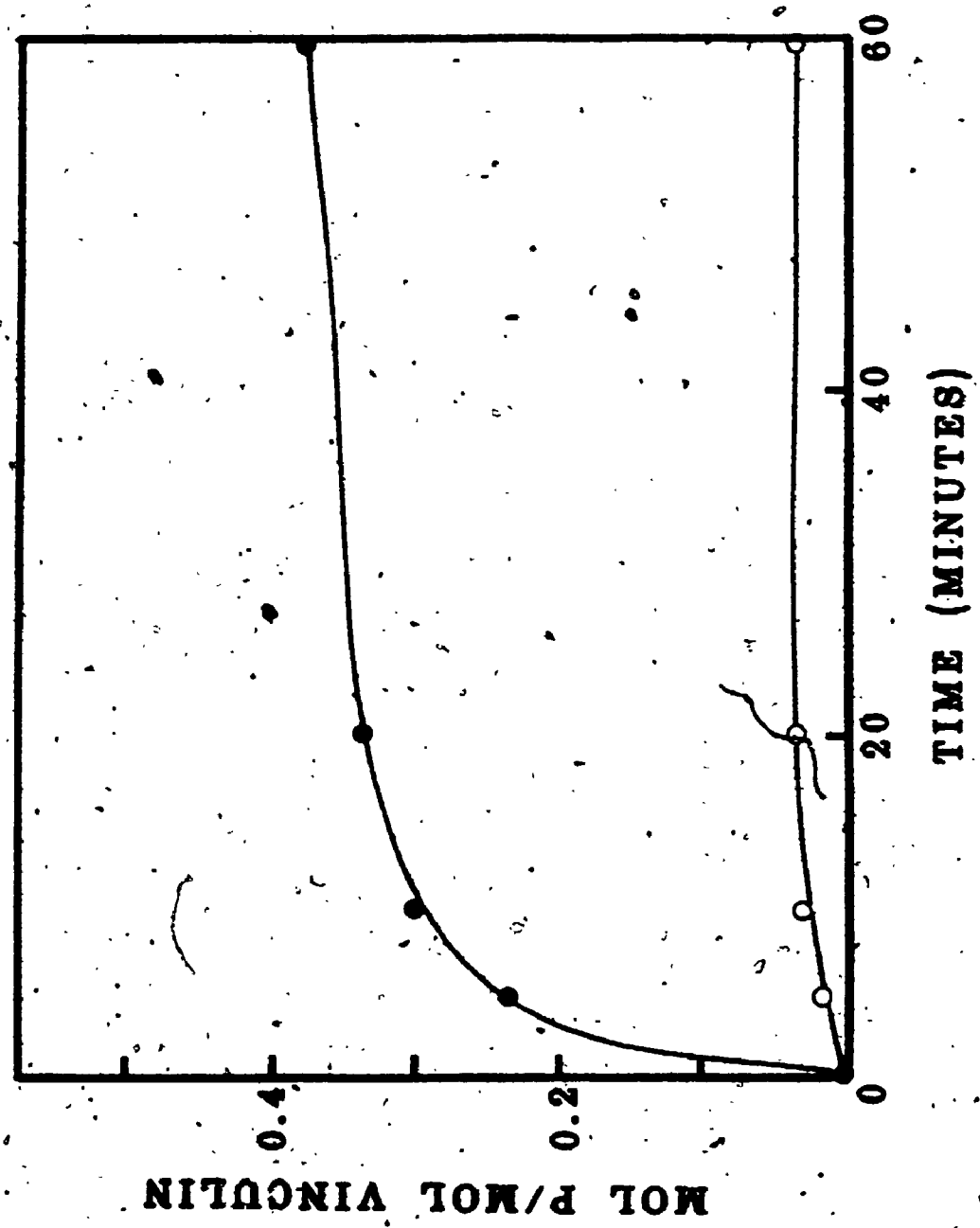

0.4
0.2
0
20
40
60
MOL P/MOL VINCULIN
TIME (MINUTES)

FIGURE 2.9

TWO-DIMENSIONAL TRYPTIC PHOSPHOPEPTIDE MAP OF VINCULIN PHOSPHORYLATED BY PROTEIN KINASE C.

After SDS-polyacrylamide gel electrophoresis, the phosphorylated vinculin band was excised and extensively digested with trypsin. The peptides were applied at the origin (O) and subjected to electrophoresis (horizontal dimension with cathode at right) at pH 1.9 before ascending chromatography (vertical dimension). Phosphopeptides were visualized by autoradiography.

was incubated with protein kinase C in the presence of phosphatidylserine and calcium, a single phosphorylated band corresponding in molecular weight to talin was observed (lane 4). In the absence of calcium or phosphatidylserine, talin phosphorylation was drastically reduced (lanes 1-3), although a slight reaction is evident when only phosphatidylserine was present (lane 3). Talin was not phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (results not shown).

To confirm that the phosphorylated protein was talin, the talin phosphorylation reaction mixture was immunoprecipitated with affinity-purified anti-talin antibodies (figure 2.11). The phosphorylated band was immunoprecipitated by anti-talin antibodies (lane 2), but was not precipitated by antibodies from non-immune serum (lane 1). These results demonstrate that the phosphorylated band is talin and not a minor contaminant.

The time dependent phosphorylation of talin is illustrated in figure 2.12. In an initial rapid phase of phosphorylation, talin reached a level of 0.5 mol phosphate/mol protein. This rapid phosphorylation was followed by more gradual phosphorylation with talin reaching a level of approximately 0.8 mol phosphate/mol protein after one hour. In the absence of phosphatidylserine and calcium, there was no measurable phosphorylation of talin.

2.3.6 Two-dimensional Peptide Mapping and Phosphoamino Acid Analysis of Talin

To investigate the sites of phosphorylation in talin, phosphopeptides generated by extensive proteolysis with trypsin

FIGURE 2.10

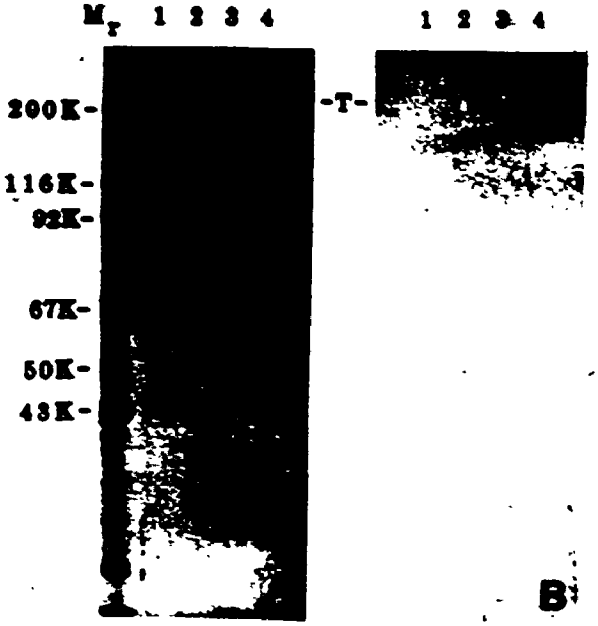
PHOSPHORYLATION OF TALIN BY PROTEIN KINASE C.

Talin (0.1 mg/ml) was phosphorylated by protein kinase C as described in Materials and Methods for 20 minutes at 30°C. The reaction mixtures were electrophoresed on an 8% SDS-polyacrylamide gel which was stained with Coomassie blue (panel A) prior to autoradiography (panel B). Lane 1, 0.5 mM EGTA; lane 2, 0.5 mM $CaCl_2$; lane 3, 0.5 mM EGTA and phosphatidylserine (0.05 mg/ml); lane 4, 0.5 mM $CaCl_2$ and phosphatidylserine (0.05 mg/ml). Molecular weight markers are marked (M_r).

FIGURE 2.11

IMMUNOPRECIPITATION OF PHOSPHORYLATED TALIN.

Talin was phosphorylated and immunoprecipitated with affinity purified rabbit anti-talin immunoglobulin (lane 2) or with immunoglobulin (5 µg) from a non-immune rabbit (lane 1). The immunoprecipitated fractions were electrophoresed on an 8% SDS-polyacrylamide gel and stained with Coomassie blue (panel A) prior to autoradiography (panel B). The position of talin is marked (-T-).

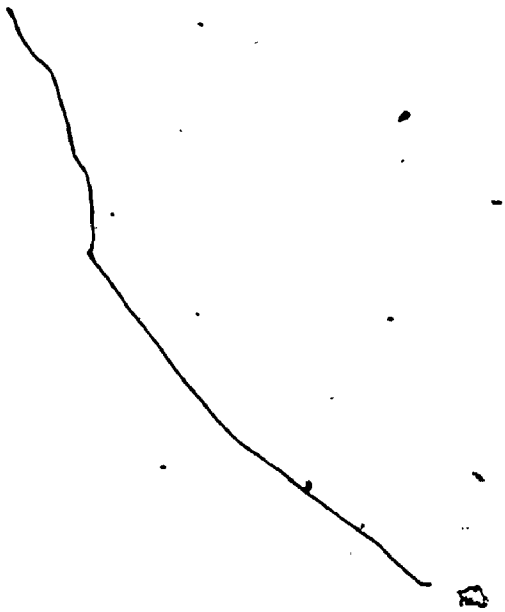

M_r 1 2 3 4
1 2 3 4
200K-
116K-
92K-
67K-
50K-
43K-
-T-
B

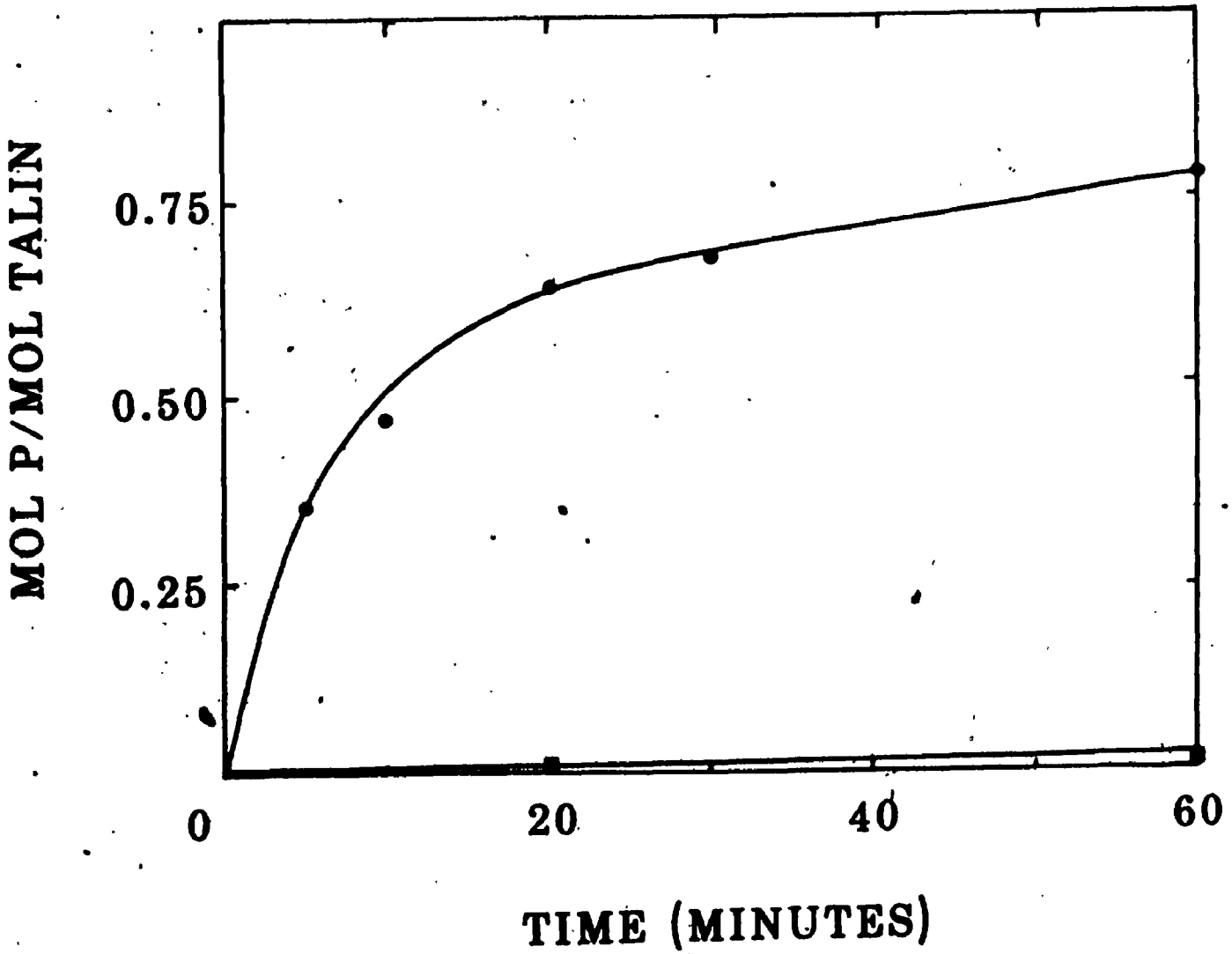

1 2
1 2
-T-
A
B

FIGURE 2.12

TIME DEPENDENCE OF TALIN PHOSPHORYLATION.

Talin was incubated with protein kinase C in the presence of phosphatidylserine and calcium (●) or EGTA (■). At the times indicated, aliquots were removed and immediately boiled in sample buffer for SDS-polyacrylamide gel electrophoresis. Phosphate incorporation was determined as described in Materials and Methods. The results are typical of those obtained in seven separate experiments.




MOL P/MOL TALIN
0.75
0.50
0.25
0
20
40
60
TIME (MINUTES)

(figure 2.13, panel A) or V8 protease (figure 2.13, panel B), were subjected to two-dimensional mapping. From these two-dimensional maps, it was apparent that talin contains multiple phosphorylation sites. Four major phosphopeptides and a number of minor phosphopeptides were obtained using each protease. Phosphoamino acid analysis of talin (figure 2.13, panel C) supported the finding that talin is phosphorylated at multiple sites, since phosphoserine and phosphothreonine were both detected. As expected from the specificity of protein kinase C, no phosphotyrosine was detected. All of the major tryptic phosphopeptides of talin contained phosphothreonine (results not shown). Although absent from the major spots, phosphoserine was detected in one of the minor spots.

2.3.7 Phosphorylation of Vinculin and Talin in Mixtures of the Two Proteins

Talin and vinculin are two focal proteins known to interact with each other (Burridge and Mangeat, 1984). As an initial step in examining the role of vinculin and/or talin phosphorylation on their interactions, the two proteins were incubated together in the presence of protein kinase C. Examination of table 2.2 reveals that talin phosphorylation is not altered in the presence of vinculin, whereas vinculin phosphorylation is diminished by approximately 40% in the presence of talin. The degree of inhibition of vinculin phosphorylation by talin is almost identical whether the phosphorylation reaction is allowed to proceed for 10 minutes or for 1 hour.

To extend the results of table 2.2, the relative incorporation

FIGURE 2.13

TWO-DIMENSIONAL PROTEOLYTIC PHOSPHOPEPTIDE MAPS AND PHOSPHOAMINO ACID ANALYSIS OF TALIN PHOSPHORYLATED BY PROTEIN KINASE C.

After SDS-polyacrylamide gel electrophoresis, the phosphorylated talin band was excised and extensively digested with trypsin (A) or V8 protease (B). The peptides were applied at the origin (O) and subjected to electrophoresis (horizontal dimension with cathode at right) at pH 1.9 before ascending chromatography (vertical dimension). Phosphopeptides were visualized by autoradiography. For phosphoamino acid analysis (C), the phoshopeptides were partially hydroyzed as described in Materials and Methods and applied to the thin layer plate at the origin (O). The samples were then subjected to electrophoresis at pH 1.9 (horizontal dimension with anode at left) and at pH 3.5 (vertical dimension with anode at top) prior to autoradiography. Standard phosphoamino acids - phosphoserine, (PS); phosphothreonine, (PT); phosphotyrosine (PY) - were visualized by ninhydrin staining.

TABLE 2.2

EFFECTS OF TALIN ON VINCULIN PHOSPHORYLATION.

Time	Vinculin	Vinculin (with talin)	Talin	Talin (with vinculin)
(minutes)		(% phosphorylation)		
10	100	57	100	99
60	100	59	100	113

Vinculin (0.1 mg/ml) and/or talin (0.1 mg/ml) were phosphorylated as indicated in Materials and Methods. In reactions containing both proteins, the reaction mixtures were incubated for 15 minutes at 30°C prior to commencement of the kinase reaction by the addition of ATP. The values represent the average of duplicate determinations and are typical of those obtained in three separate experiments.

of phosphate into each of the three vinculin phosphopeptides (see figure 2.9) was analysed when vinculin was phosphorylated in the presence, or absence, of talin (table 2.3). The vinculin phosphopeptide that was most significantly affected by inclusion of talin in the phosphorylation reaction mixture is the minor phosphopeptide (figure 2.9, phosphopeptide 3). In the presence of talin, the relative phosphate incorporation of phosphopeptide 3 is diminished by more than 70% at the 10 minute time point and by more than 50% after 1 hour. In contrast, the relative ratio of radioactive phosphate in phosphopeptides 1 and 2 remains rather invariant. Absolute measurements of phosphate incorporation were not possible since recovery of radioactive phosphopeptides during the different steps of the procedure is not quantitative.

2.4 DISCUSSION

Protein kinase C exists in two forms within the cell: an inactive soluble form and an active form which is tightly associated with the plasma membrane (Wolf et al, 1985b; Kraft and Anderson, 1983; Thomas et al., 1987). The localization of the active form of the protein kinase would place the enzyme in close proximity to membrane-associated structures such as focal contacts. As a result, it is of interest to examine the activity of purified protein kinase C on components of the focal contact which may serve as regulators of cytoskeletal organization or function.

The first objective of our studies was to purify protein kinase C for the purpose of conducting in vitro phosphorylation experiments.

TABLE 2.3

RELATIVE PHOSPHATE CONTENT OF VINCULIN PHOSPHOPEPTIDES.

Time	Phosphopeptide 1	Phosphopeptide 2	Phosphopeptide 3
	(% of total phosphate content of vinculin)		
10	55.3	33.1	11.6
10 (with talin)	65.4	31.6	3.1
60	56.5	25.5	18.0
60 (with talin)	63.7	28.0	8.3

Vinculin which was phosphorylated in the presence, or absence of talin, was subjected to phosphopeptide mapping. After visualization of the phosphopeptides by autoradiography, they were individually eluted from the thin layer plates with buffer (acetic acid: 88% formic acid: H_2O; 15:5:80) prior to scintillation counting. Phosphopeptides are numbered according to figure 2.9. The values represent the average of duplicate determinations.

Towards this aim, we succeeded in purifying protein kinase C from bovine brain using three column chromatographic steps. Although we could not unequivocally identify a band on SDS-polyacrylamide gels as protein kinase C, a number of experimental results confirmed that the purified enzyme was free of other contaminating protein kinases. The enzyme exhibited a marked specificity towards the histone III-S fraction over the histone IIa-S fraction and using the former as substrate exhibited nearly absolute (greater than 50-fold) dependence on phosphatidylserine and calcium. The histone kinase activity of the enzyme was also activated by addition of unsaturated diacylglycerol or TPA, as expected (Takai et al., 1979c; Kishimoto et al., 1980; Kaibuchi et al., 1981; Castagna et al., 1982). The maximal protein kinase C activity measured was however the same in the presence or absence of diacylglycerol in contrast to the results of Kishimoto et al, (1980) obtained with rat brain protein kinase C. In that study, maximal activity in the presence of diolein was significantly greater than in the absence of diolein. The reason for this discrepancy is not known. Using the alkylating ATP analog fluorosulfonylbenzoyl adenosine, a single band of molecular weight 80,000 was labeled, demonstrating that the preparation is free of contamination by other ATP binding proteins including protein kinases.

The specific activity of our purified bovine brain protein kinase C was quite low; approximately 50-fold lower than that subsequently reported by Parker et al. (1984). The lower specific activity that we obtained may be due to differences in the assay

conditions used. Parker et al., (1984) also utilized more purification steps. It is unclear how our preparation compares to that of Walsh et al. (1984) who also purified protein kinase C from bovine brain, since the specific activity of their purified enzyme was not reported. In any event, despite the low specific activity of our protein kinase C preparation, it exhibited the specificity and phospholipid/calcium dependency characteristics of the homogenous enzyme and was free of other kinase activities.

The next objective of this study was to examine the phosphorylation of the focal contact proteins vinculin and talin by protein kinase C. Our findings demonstrated the multisite phosphorylation of both proteins. Although in each instance less than stoichiometric incorporation of phosphate was achieved, our results do compare favourably to the findings of others who have examined the phosphorylation of vinculin by protein kinase C (Werth et al., 1983; Kawamoto and Hidaka, 1984). In our studies, vinculin was phosphorylated to a level approaching 0.4 mol phosphate/mol protein whereas incorporation of 0.5 mol phosphate/mol vinculin was achieved by Werth et al. (1983). Kawamoto and Hidaka (1984) achieved phosphate incorporation of less than 0.1 mol phosphate/mol vinculin using human platelet protein kinase C. It must also be noted that we did observe considerable variation in the phosphate incorporation of different preparations of vinculin and to a lesser extent in different preparations of talin. In fact, some preparations of vinculin were barely phosphorylated to detectable levels. It is possible that different preparations of vinculin or talin contained

different levels of bound phosphate as isolated. Indeed, vinculin and talin are both labeled with radioactive phosphate when isolated from ^{32}P-labeled cells (Sefton et al., 1981; Burridge and Connell, 1983b). However, we have not done any direct measurement of the phosphate content of the isolated proteins. Alternatively, the proteins as isolated may be contaminated with variable amounts of effectors, either activators or inhibitors, of the phosphorylation reaction. This possibility has not been explored further.

Phosphopeptide mapping reveals two major sites of phosphorylation for vinculin and four major sites for talin. Under some circumstances, the number of sites determined by tryptic peptide mapping can be erroneous as a result of the inability of trypsin to act as an exopeptidase (Hunter et al., 1984). This problem is relevant to our studies since basic residues have been shown to be important for protein kinase C substrate selectivity (House et al., 1987; Turner et al., 1985). In the case of vinculin, Werth et al., 1983) had shown that one of the major phosphopeptides contained phosphoserine and the other phosphothreonine clearly demonstrating that the two sites were distinct. However, in the case of talin, there was some concern that several overlapping peptides may have been generated by partial tryptic cleavage of one phosphorylation site. In an attempt to avoid this potential problem, talin phosphopeptides were generated by digestion with V8 protease of Staphylococcus Aureus, a protease which cleaves at acidic residues unlike trypsin which cleaves at basic residues (Drapeau, 1977). As was the case with tryptic peptides of talin, two-dimensional mapping

of V8 phosphopeptides revealed 4 major sites of phosphorylation. Since phosphoserine and phosphothreonine were both detected in talin, it was evident that the protein must contain a minimum of two phosphorylation sites.

The observation that vinculin and talin are phosphorylated in vitro by protein kinase C suggests that these reactions may be important in the control of focal contact organization and cell morphology. A key development in the advancement of this idea would be an elucidation of the effects of phosphorylation on the functional properties of the two proteins. Since the proteins do not have any known enzymatic activities, assays of their function are restricted to examinations of their binding properties and subcellular distributions (Burridge and Mangeat, 1984). As a preliminary step towards examining the effects of phosphorylation on talin and vinculin, the two proteins were mixed together before the phosphorylation reaction. Talin phosphorylation is unaltered in the presence of vinculin, whereas vinculin phosphorylation is significantly diminished in the presence of talin. This result suggests that one or more of the phosphorylation sites of vinculin may be located within the region of its talin binding site so that interactions between the two proteins selectively diminish phosphorylation. In examining phosphopeptide maps of vinculin which was phosphorylated in the presence of talin, a decrease in phosphorylation is most significant for the relatively minor phosphorylation site of vinculin. Although no certain conclusions can be drawn at this time, it is certainly appealing to postulate

that prior phosphorylation of vinculin, particularly at its relatively minor site, may play a role in regulating the interactions of vinculin with talin. It will clearly be of interest to direct future efforts towards an analysis of the effects of phosphorylation on the other known binding activities of these two key focal contact components.

CHAPTER 3

PHOSPHORYLATION OF CONTRACTILE REGULATORS BY PROTEIN KINASE C

3.1 INTRODUCTION

In smooth muscle, the tumour-promoting phorbol esters can stimulate contractile activity (Rasmussen et al., 1984; Park and Rasmussen, 1985; Park and Rasmussen, 1986), implying that protein kinase C may be involved in contractile regulation. Therefore, it is of interest to examine the activity of protein kinase C towards those proteins that play a role in the modulation of contractile events in smooth muscle and non-muscle cells. Two candidate proteins are the calmodulin-binding proteins myosin light chain kinase and caldesmon.

Myosin light chain kinase is a calmodulin-dependent enzyme responsible for phosphorylation of the regulatory light chain of myosin, a reaction which is essential for contraction in smooth muscle and non-muscle cells (reviewed by Kamm and Stull, 1985; Adelstein, 1982). In addition to calmodulin, phosphorylation has been implicated as a potential regulator of this Mr 130,000 enzyme (Adelstein et al, 1978; Conti and Adelstein, 1980). Phosphorylation of myosin light chain kinase by the cAMP-dependent protein kinase in the absence of calmodulin occurs at two sites with the result that phosphorylated myosin light chain kinase requires a much higher level of calmodulin to attain half-maximal activation (Conti and Adelstein, 1981). In the presence of calmodulin however, phosphorylation occurs on only one site with no apparent alteration in activity. A role for phosphorylation in the regulation of myosin light chain kinase is

also implicated by the demonstrations that the enzyme is a phosphoprotein in living cells (de Lanerolle et al., 1984) and that it can be phosphorylated by cGMP-dependent protein kinase (Nishikawa et al., 1984a).

Caldesmon was originally discovered in smooth muscle as a major calmodulin- and actin-binding protein (Sobue et al., 1981). The interactions between caldesmon and calmodulin or actin are controlled by the concentration of free calcium (Sobue et al., 1982; Kakiuchi and Sobue, 1983). Caldesmon binds to F-actin in the absence of calcium, but when free calcium concentrations are raised to the micromolar range caldesmon is partially displaced from actin filaments by the calcium/calmodulin complex. Caldesmon also exerts an inhibitory effect on the actin-activated myosin ATPase (Ngai and Walsh, 1984; Marston and Lehman, 1985; Smith et al., 1987), and enhances interactions between actin and heavy meremyosin (Lash et al., 1986). On the basis of these observations, caldesmon is implicated as a regulator of contractile events.

In addition to direct control by calcium/calmodulin, caldesmon has been identified as a substrate for a calmodulin-dependent protein kinase (Ngai and Walsh, 1984; Ngai and Walsh, 1987; Lash et al., 1986). The effects of phosphorylation are somewhat controversial (Lash et al., 1986) but may abolish the inhibitory effects on the actin-activated myosin ATPase (Ngai and Walsh, 1984; Ngai and Walsh, 1987).

Although caldesmon was initially discovered in smooth muscle, polypeptides with similar functional properties and immunological

cross-reactivity were subsequently observed in a number of tissue and cell types (Bretscher and Lynch, 1985; Owada et al., 1984). These studies identified two apparent categories of caldesmon molecules, both of which exhibited heat stability and binding to actin or calmodulin (reviewed by Bretscher, 1986). On the basis of molecular weight, caldesmon molecules fall into distinct classes with Mr 70-80,000 or Mr 140-150,000. To distinguish between different caldesmon species, the protein name reflects its molecular weight (i.e. caldesmon with Mr 77,000 is caldesmon$_{77}$) with the exception of the chicken gizzard protein which will be referred to simply as caldesmon.

The objective of the work described in this chapter was to study the activity of protein kinase C towards those proteins that are involved in cytoskeletal regulation through their control of contractile events. The phosphorylations of chicken gizzard myosin light chain kinase and various forms of the calmodulin-binding protein caldesmon were the focus of investigation. From avian sources, chicken gizzard caldesmon (Mr 150,000) and chicken liver caldesmon$_{72}$ were examined. Studies on the phosphorylation of mammalian caldesmons were conducted using caldesmon$_{150}$ and caldesmon$_{77}$ purified fr[redacted]bovine liver.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Phosphocellulose paper (P81) was obtained from Whatman and S-Sepharose from Pharmacia Fine Chemicals. Leupeptin was obtained from

Institut Armand Frappier and TLCK, TPCK, PMSF and soybean trypsin inhibitor (STI) were from Sigma. Nitrocellulose paper (0.2 µm pore size) was obtained from Schleicher and Schuell. All other reagents were as described in Materials and Methods of Chapter 2 or were of analytical grade.

3.2.2 Purification of Protein Kinase C from Rat Brain

Protein kinase C was purified from fresh rat brain by the method of Wolf et al. (1985a). Protein kinase activity was dependent on phosphatidylserine and calcium and by SDS-polyacrylamide gel electrophoresis the protein was near homogeneity. To enhance the reliability of this procedure, a number of modifications were subsequently made. Briefly, 5 grams of rat brain were homogenized with a Polytron (setting 3.5 for 45 seconds) in buffer A (20 mM Tris-Cl, pH 7.6, 10 mM Dithiothreitol) containing 1.0 mM $CaCl_2$ and leupeptin (0.05 mg/ml). The homogenate was centrifuged (40,000 X g for 15 minutes) and the pellet was washed twice with buffer A containing 0.1 mM $CaCl_2$ and leupeptin (0.05 mg/ml). Protein kinase C was eluted from the particulate fraction by suspension in buffer A containing 5.0 mM EGTA and 2.0 mM EDTA and stirring at 4°C for 1 hour. The suspension was then subjected to centrifugation at 140,000 X g for 1 hour and the supernatant was applied to a 10 ml DEAE-cellulose (DE52) column pre-equilibrated with buffer B (20 mM Tris-Cl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol). Protein kinase C was eluted from the column with a linear gradient of 0-0.3 M NaCl in buffer B. Active fractions were pooled, made 1.5 M with respect to NaCl by addition of 1/2 volume of 4.5 M NaCl, and were loaded on a

0.5 ml phenyl-Sepharose column pre-equilibrated with buffer B containing 1.5 M NaCl. The column was subsequently washed with buffer B containing 1.5 M NaCl, 1.0 M NaCl and 0.5 M NaCl before protein kinase C was eluted with salt-free buffer B. The enzyme was collected into siliconized test tubes and stored at 4°C in the presence of polyethylene glycol 20,000 (1 mg/ml) and ethylene glycol (25%) where it retained activity for at least 2 months.

3.2.3 Phosphorylation Assays

Protein kinase C was assayed for 3 minutes at 30°C using histone (type III-S, 0.2 mg/ml) as substrate. The reaction was conducted in a volume of 0.1 ml containing 20 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 0.01 mM ATP (specific activity 150-250 cpm/pmol) and either phosphatidylserine (50 µg/ml), diolein (1 µg/ml), and $CaCl_2$ (0.5 mM) or EGTA (0.5 mM). Histone phosphorylation was monitored as described by Corbin and Reimann (1974) or by spotting an aliquot of the reaction mixture (80 ul) on Whatman P81 phosphocellulose paper according to the method of Roskoski (1983). One unit of protein kinase C is defined as the amount of enzyme required to transfer 1 nmol of phosphate per min into histone III-S at 30°C under the described conditions.

3.2.4 Purification of Myosin Light Chain Kinase

Chicken gizzard myosin light chain kinase was purified by Dr. E.H. Ball using modification of the method of Adelstein and Klee (1981). Following 25-60% ammonium sulphate fractionation, the protein was further purified using DEAE-cellulose (DE52), and blue Sepharose column chromatography and calmodulin-Sepharose affinity

chromatography.

3.2.5 Purification of Calmodulin

Calmodulin was purified from bovine brain by Dr. E.H. Ball essentially by the method of Gopalakrishna and Anderson (1982).

3.2.6 Purification of the Inhibitor of cAMP-Dependent Protein Kinase

The heat stable inhibitor of cAMP-dependent protein kinase was purified from fresh bovine skeletal muscle by the method of Schlender et al. (1983). Briefly, ground muscle (800 g) was homogenized in 2 volumes of 4 mM EDTA, pH 6.8 and centrifuged at 10,000 X g for 35 minutes at 4°C. Following heat treatment and trichloroacetic acid precipitation, the precipitate was collected by centrifugation, dissolved in 1M K_2HPO_4 and dialyzed against 2 changes of distilled water and then 50 mM Tris-Cl, pH 7.5, 1 mM EDTA (buffer D). The dialysate was clarified by centrifugation and applied to a 5 ml DEAE-cellulose column. The column was washed extensively with buffer D and the protein kinase inhibitor was eluted with 0.1M NaCl in buffer D. The inhibitor was assayed on its ability to inhibit the histone (type IIa-S) kinase activity of the catalytic subunit of cAMP-dependent protein kinase. Active fractions were stored in 0.05 ml aliquots at -70°C.

3.2.7 Purification of Chicken Gizzard Caldesmon by Denaturing Methods

Caldesmon was purified from chicken gizzards by modification of the denaturing method of Bretscher (1984). After heat treatment, the protein was purified by column chromatography using DEAE-cellulose (DE52) and then phosphocellulose (P11) columns. Caldesmon was identified on the basis of its molecular weight on SDS-polyacrylamide

gels, and exhibited binding to calmodulin or F-actin.

3.2.8 Purification of Chicken Gizzard Caldesmon by Non-denaturing Methods

Caldesmon was purified from chicken gizzards by non-denaturing methods using modification of the methods of Ngai and Walsh (1984) and Lash et al. (1986). Following homogenization of ground gizzards with buffer H (20 mM Tris-Cl, pH 7.5, 40 mM NaCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EGTA) containing 0.05% Triton X-100 and protease inhibitors (0.1 mM PMSF, 0.1 mM TPCK, 0.1 mM TLCK and 0.01 mM leupeptin), the homogenate was centrifuged at 17,000 X g for 15 minutes at 4°C. The pellet was washed twice in the absence of Triton X-100 and was then extracted with buffer E (40 mM Tris-Cl, pH 7.5, 60 mM NaCl, 25 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EGTA). Caldesmon was then purified using column chromatography on DEAE-cellulose (DE52) and S-Sepharose columns and was identified by its migration on SDS-polyactylamide gels.

3.2.9 Purification of $Caldesmon_{72}$ from Chicken Liver

Fresh chicken liver (50 grams) was obtained following sacrifice of one chicken. The liver was rinsed with distilled water containing 0.2 mM PMSF and homogenized with a Waring blender in 6 volumes of extraction buffer (50 mM imidazole, pH 6.9, 0.3 M KCl, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.2 mM PMSF, leupeptin (1 µg/ml) and STI (10 µg/ml)). The homogenate was then heated to 90°C in a boiling water bath and allowed to remain at 90°C for 5 minutes. After cooling to 4°C, the homogenate was centrifuged for 25 minutes at 12,000 X g. To the supernatant, ammonium sulphate was added to 30% and the

suspension was stirred for 1 hour. The pellet was removed by centrifugation and the supernatant was made 50% with respect to ammonium sulphate. After stirring for 1 hour, the 30-50% ammonium sulphate pellet was collected by centrifugation, dissolved in a minimum volume of TEM buffer (10 mM Tris-acetate, pH 7.5, 1 mM EGTA, 1 mM 2-mercaptoethanol) and extensively dialyzed against TEM buffer. The dialyzed sample was loaded on a 10 ml phosphocellulose (P11) column pre-equilibrated with TEM buffer. Protein was eluted from the column with a linear gradient of 0-0.5 M NaCl in TEM buffer. Caldesmon$_{72}$-containing fractions were identified by SDS-polyacrylamide gel electrophoresis, pooled, and made 1.5 M with respect to NaCl. This pool was applied to 10 ml phenyl-Sepharose column in TEM buffer containing 1.5 M NaCl. Caldesmon$_{72}$ was eluted from the column with salt-free TEM buffer and was applied to a 10 ml DEAE-cellulose (DE52) column in TEM buffer. Caldesmon$_{72}$ was eluted from the column with a 0-0.3 M NaCl gradient in TEM buffer.

3.2.10 Purification of Caldesmon$_{77}$ and Caldesmon$_{150}$ from bovine liver

Caldesmon$_{77}$ and caldesmon$_{150}$ were purified from 200 grams of fresh bovine liver as described above. Column chromatography was done on 20 ml DEAE-cellulose, phenyl-Sepharose and phosphocellulose columns. Caldesmon-containing fractions were free of contaminating protein kinase or phosphatase activities.

3.2.11 Phosphorylation of Caldesmon and Caldesmon$_{77}$

The *in vitro* phosphorylation of proteins was performed as described for vinculin or talin in Materials and Methods of Chapter

2. To monitor the extent of caldesmon or caldesmon-77 phosphorylation, the assay mixtures were subjected to precipitation with trichloroacetic acid as described by Corbin and Reimann (1974) or were spotted on phosphocellulose (P81) paper as described by Roskoski (1983).

3.2.12 Microphosphate Determination

The phosphate content of chicken gizzard caldesmon which had been purified by denaturing methods was determined by the method of Buss and Stull (1983). For these determinations, 1 nmol of purified caldesmon (0.15 mg) was used. The final volume of the assay mixture (0.2 ml) was transferred to 96-well microtitre plates and absorbance readings at 740 nm were obtained using an automated ELISA reader. A typical phosphate standard curve obtained using this procedure is illustrated in figure 3.1.

3.2.13 Western Blotting

Western blotting was done by the method of Towbin et al. (1979) with modifications. Following SDS-polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose in buffer (25 mM Tris-Cl, pH 8.3, 192 mM glycine) containing 20% methanol and 0.1% SDS at 110 volts for 90 minutes using a Biorad Transblot Cell. The nitrocellulose filters were blocked by incubation at 37°C for 2 hours with 4% casein in Tris-buffered saline (20 mM Tris-Cl, pH 7.4, 0.15 M NaCl). Antibody (2 µg/Lane) was added directly to the blocking solution and was incubated with the blot overnight at room temperature. Bound antibody was detected with ^{125}I-Protein A which was visualised by autoradiography.

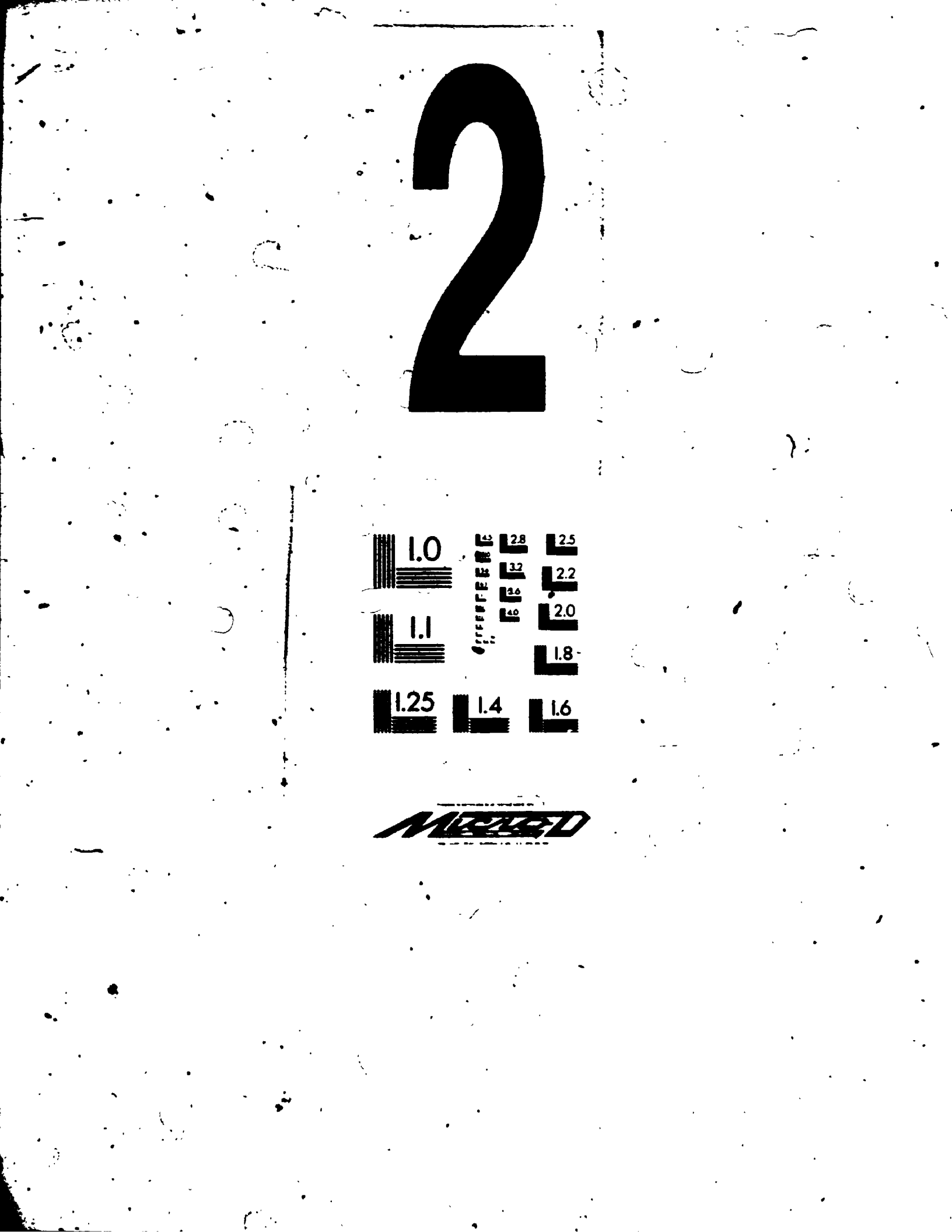
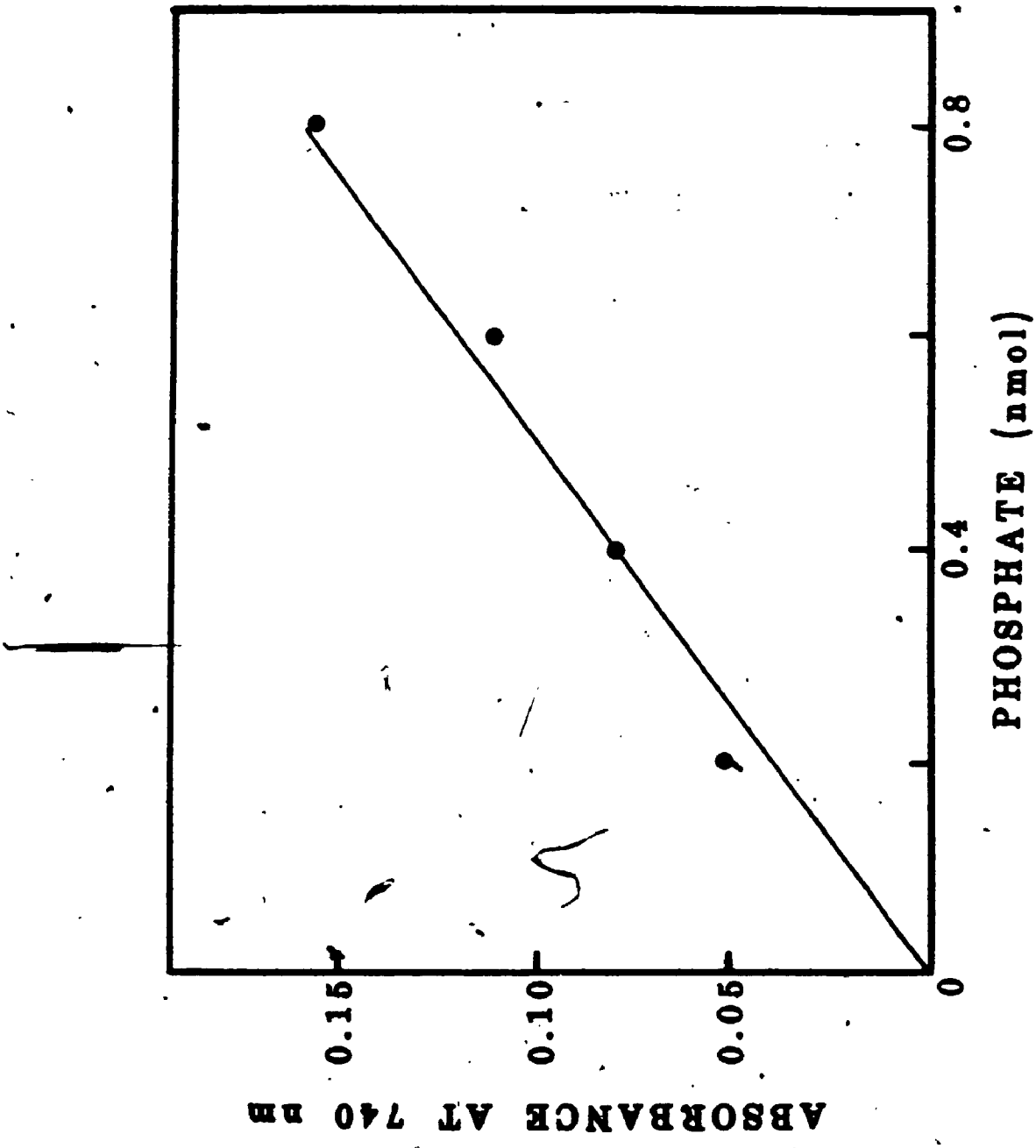

2
1.0
1.1
1.25
1.4
1.6
2.8
3.2
3.6
2.5
2.2
2.0
1.8

FIGURE 3.1

PHOSPHATE STANDARD CURVE OBTAINED WITH MICROPHOSPHATE ASSAY.

Standard amounts of phosphate (0.2 - 0.8 nmol) were measured in the microphosphate assay as described in Materials and Methods. Phosphate determinations of caldesmon were done with appropriate amounts of caldesmon to ensure that the measured quantities of phosphate did not exceed the linear range of the assay. The standard curve is typical of that obtained in several separate experiments.


ABSORBANCE AT 740 nm
0.15
0.10
0.05
0
0.4
0.8
PHOSPHATE (nmol)

3.2.14 Other Procedures

Protein determinations (except protein kinase C) were done as described by Hartree (1972) using bovine serum albumin as standard. Protein determinations of protein kinase C samples which contained high concentrations of dithiothreitol were done by the method of Bradford (1976). Other procedures were as outlined in Materials and Methods in Chapter 2.

3.3 RESULTS

3.3.1 Purification of Protein Kinase C from Rat Brain

As a first step in investigating how protein kinase C can exert effects on cytoskeletal components that are involved in contractile regulation, protein kinase C was purified from rat brain. Purification of protein kinase C from rat brain superceded the purification of the enzyme from bovine brain (described in Chapter 2) for a number of reasons. Methods were published (Wolf et al., 1984; Wolf et al., 1985a) by which protein kinase C could be purified from the brains of only a few rats yielding a preparation of nearly homogeneous protein kinase C within about 10 hours. By their reports, the enzyme had high specific activity (over 700 nmol/min/mg) and was stable for months in the presence of 25% ethylene glycol and 0.1% polyethylene glycol (20,000). Although our initial purifications using the method of Wolf et al. (1985a) were successful, we found that modifications to the procedure resulted in more reliable yields. A subsequent publication (House et al., 1987) has demonstrated that similar modifications have also been successful

in other laboratories.

As seen in Table 3.1, our modifications to the procedure of Wolf et al. (1985a) result in the purification within 24 hours of approximately 0.13 mg of protein kinase C from the brains of 3 rats (approximately 5 grams). The specific activity of the preparation (470 nmol/min/mg) is 5-fold higher than that obtained in our purification of bovine brain protein kinase C. When subjected to SDS-polyacrylamide gel electrophoresis, (figure 3.2) the enzyme purified through phenyl-Sepharose column chromatography (lane 6) can be seen as the major band with molecular weight of approximately 80,000. In some preparations protein kinase C could be seen as a doublet of protein bands. Recent studies by Woodgett and Hunter (1987a, 1987b) have demonstrated that this doublet represents two distinct forms of protein kinase C which are present in brain. By densitometric scanning of Coomassie blue stained gels, protein kinase C is approximately 50% pure at this stage. The enzyme typically exhibited at least 20-fold dependency on phosphatidylserine and calcium, and in some instances the dependency was nearly absolute. As was found by Wolf et al. (1985a), the enzyme could be stored for several weeks at 4°C in the presence of 25% ethylene glycol and 0.1% polyethylene glycol.

Protein kinase C from rat brain could be autophosphorylated when incubated with [γ-^{32}P]ATP in the presence of phosphatidylserine and calcium (figure 3.3, lane 1). This property of protein kinase C has been used as one criterion by which to identify the enzyme (Wolf et al., 1984). In addition to the autophosphorylated band of protein

TABLE 3.1

PURIFICATION OF RAT BRAIN PROTEIN KINASE C.

Step	Protein	Total Activity	Specific Activity	Yield	Purification
	mg	units	units/mg	%	-fold
EGTA/EDTA extract	9.8	123.3	12.6	100	1
DEAE-cellulose	1.8	111.7	61.4	90.6	4.9
Phenyl Sepharose	0.13	59.3	470	48.1	37.3

Aliquots of different fractions obtained during the purification of protein kinase C were assayed as described in Materials and Methods. One unit of protein kinase C activity is defined as the amount of enzyme required to transfer 1 nmol of phosphate per minute into histone III-S at 30°C under the conditions defined in Materials and Methods.

FIGURE 3.2

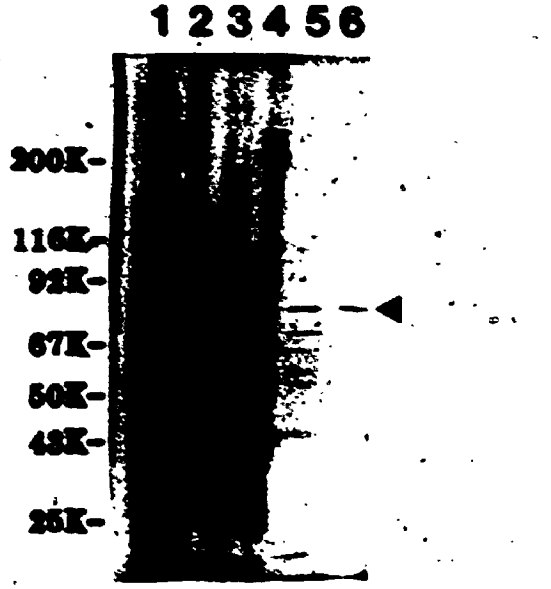
SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF FRACTIONS FROM PURIFICATION OF RAT BRAIN PROTEIN KINASE C.

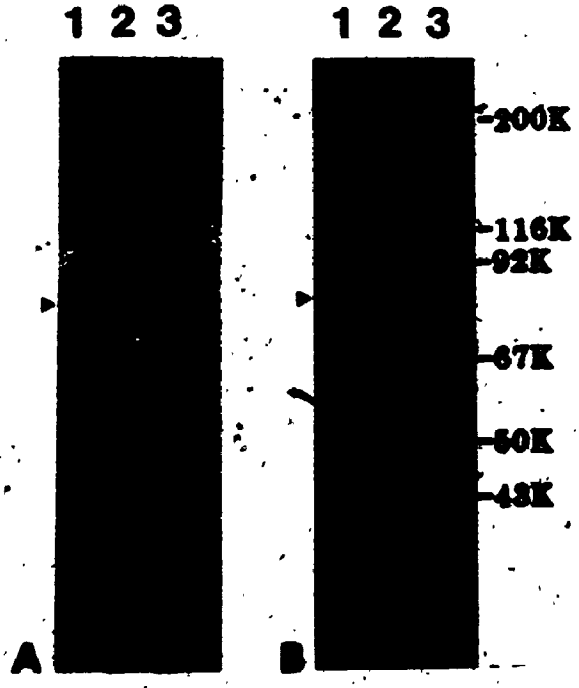
Samples from different steps during the purification of protein kinase C were run on a 6-15% SDS-polyacrylamide gel which was stained with Coomassie blue. Lane 1, 1.0 mM calcium extract; lane 2, 0.2 mM calcium extract; lane 3, EDTA/EGTA extract; lane 5, DEAE-cellulose pool; lane 6, phenyl-Sepharose pool. The position of protein kinase C is marked with an arrowhead. Molecular weight markers (lane 4) are as follows: myosin heavy chain, 200,000; β-galactosidase, 116,000; phosphorylase b, 92,000; bovine serum albumin, 67,000; immunoglobulin heavy chain, 50,000; actin, 43,000; immunoglobulin light chain, 25,000.

FIGURE 3.3

AUTOPHOSPHORYLATION OF RAT BRAIN PROTEIN KINASE C.

Protein kinase C purified through phenyl-Sepharose was incubated with [γ-^{32}P]ATP in the presence of phosphatidylserine and calcium (lane 1) or EGTA (lane 2). Samples were run on an 8% SDS-polyacrylamide gel which were stained with Coomassie Blue (panel A) and then autoradiographed (panel B). Molecular weight markers were run in lane 3.


1 2 3 4 5 6
200K
116K
92K
67K
50K
43K
26K


1 2 3
1 2 3
200K
116K
92K
67K
50K
43K
A
B

kinase C, the autoradiogram shows an additional phosphoprotein of low molecular weight (approximately 30,000). This band may be a phosphorylated fragment of protein kinase C (Huang et al., 1986a) although we have not made any attempt to determine its identity.

Although rigid comparisons between the substrate specificities of protein kinase from rat brain and bovine brain have not been done, we have no reason to believe that differences exist. Phosphopeptides maps of proteins phosphorylated by protein kinase C from the two different species do not show any differences (data not shown).

3.3.2 Phosphorylation of Myosin Light Chain Kinase by Protein Kinase C.

In figure 3.4, the phosphorylation of chicken gizzard myosin light chain kinase by protein kinase C (lanes 1-4) and by the catalytic subunit of cAMP-dependent protein kinase (lanes 9,10) is shown. It is also evident from this autoradiogram (lanes 5-8) that myosin light chain kinase phosphorylation occurs in the absence of exogenously added protein kinase. It is unclear whether this reaction is the result of autophosphorylation or contaminating protein kinase activity. The phosphorylation of myosin light chain kinase by the catalytic subunit of cAMP-dependent protein kinase (lane 9) is diminished by the addition of calmodulin (lane 10). This decrease in phosphorylation has been shown to result from the ability of bound calmodulin to prevent phosphorylation at a specific site on the myosin light chain kinase molecule (Conti and Adelstein, 1981). It can be seen from figure 3.4 that phosphorylation of myosin light chain kinase by protein kinase C in the presence of

FIGURE 3.4

PHOSPHORYLATION OF MYOSIN LIGHT CHAIN KINASE BY PROTEIN KINASE C AND THE CATALYTIC SUBUNIT OF cAMP-DEPENDENT PROTEIN KINASE.

Myosin light chain kinase (0.1 mg/ml) was incubated with protein kinase C (lanes 1-4, marked by PKC), with the catalytic subunit of cAMP-dependent protein kinase (lanes 9,10, marked by PKA) or without exogenous protein kinase (lanes 5-8) as described in Materials and Methods. The reaction mixtures were run on an 8% SDS-polyacrylamide gel which was dried and autoradiographed. Phosphorylation reactions were conducted in the presence (lanes 3,4,7,8,10; marked with c) or absence (lanes 1,2,5,6,9) of calmodulin. All phosphorylation reactions were conducted in the presence of phosphatidylserine and calcium with the exception of lanes 2,4,6,8 which contained EGTA instead. The position of myosin light chain kinase is marked with an arrowhead.


1 2 3 4 5 6 7 8 9 10
c c c
PKA

phosphatidylserine and calcium (lane 1) can also be decreased by the addition of calmodulin (lane 3). These results were subsequently confirmed in two laboratories using turkey gizzard myosin light chain kinase (Ikebe et al., 1985; Nishikawa et al., 1985). Considerable phosphorylation of myosin light chain kinase is evident in the absence of phosphatidylserine and calcium (lanes 2 and 4). However, since similar levels of phosphorylation are seen in lanes 6 and 8, it is likely that this phosphorylation is the result of protein kinase activity contaminanting the myosin light chain kinase preparation.

The phosphorylation of myosin light chain kinase without added protein kinase was inhibited by approximately 75% in the presence of calmodulin (figure 3.4, lane 7). No significant effect was however seen by the addition of the heat-stable inhibitor of cAMP-dependent protein kinase (results not shown). These results have been confirmed and extended by Foyt and Means (1985) who also observed 75% inhibition of this apparent autophosphorylation reaction by calmodulin.

Attempts to examine the time dependent phosphorylation of myosin light chain kinase by protein kinase C were initially hindered by the high levels of phosphorylation seen in the absence of exogenously added protein kinase. Further attempts were not made after the reports of Ikebe et al., (1985) and Nishikawa et al., (1985) were published.

3.3.3 Two-dimensional Peptide Mapping of Phosphorylated Myosin Light Chain Kinase

To compare the sites of phosphorylation on myosin light chain

FIGURE 3.5

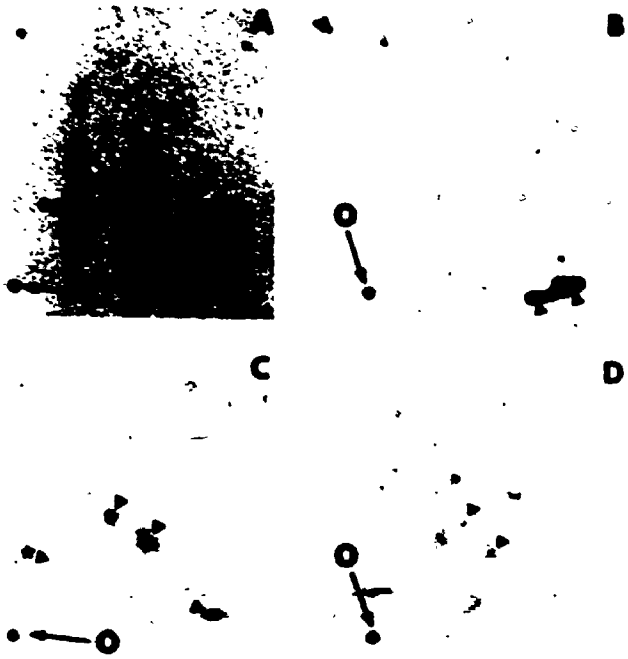
TWO-DIMENSIONAL TRYPTIC PHOSPHOPEPTIDE MAPS OF MYOSIN LIGHT CHAIN KINASE.

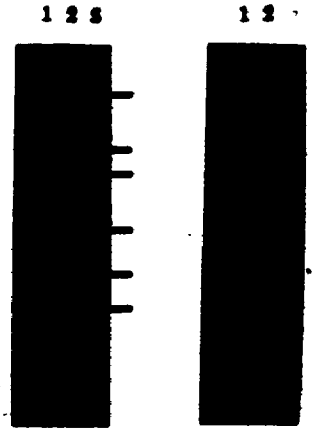
Myosin light chain kinase was phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (panels A,B) or by protein kinase C (panels C,D) in the presence (panels B,D) or absence (panels A,C) of calmodulin. Following SDS-polyacrylamide gel electrophoresis, tryptic phosphopeptides of phosphorylated myosin light chain kinase were prepared and separated on thin layer plates as previously described. Those phosphopeptides that are present only in the absence of calmodulin (panels A,C) are marked with open arrowheads (△). The other phosphopeptides that are present even when calmodulin is included in the phosphorylation mixture are marked with solid arrowheads (▲). One site marked with an asterisk (panels A,C) may be phosphorylated by either protein kinase C or the catalytic subunit of cAMP-dependent protein kinase.

FIGURE 3.6

PHOSPHORYLATION OF CHICKEN GIZZARD CALDESMON BY PROTEIN KINASE C.

Chicken gizzard caldesmon (0.1 mg/ml) was incubated with [γ-^{32}P]ATP in the presence (lane 1) or absence (lane 2) of protein kinase C. The reaction mixtures were run on an 8% SDS-polyacrylamide gel which was stained with Coomassie blue (panel A) prior to autoradiography (panel B). Molecular weight markers (lane s) from top to bottom are as follows: myosin heavy chain, 200,000; β-galactosidase, 116,000; phosphorylase b, 92,000; bovine serum albumin, 67,000; immunoglobulin heavy chain, 50,000; actin, 43,000.


A
B
C
D


1 2 3
1 2

kinase by protein kinase C and by the catalytic subunit of cAMP-dependent protein kinase, myosin light chain kinase was phosphorylated with each kinase, digested extensively with trypsin and the phosphopeptides separated in two dimensions. Peptide maps obtained following phosphorylation of myosin light chain kinase with the catalytic subunit of cAMP-dependent protein kinase (figure 3.5, panels A,B) reveal that calmodulin blocks phosphorylation at one major site and one minor site (marked with open arrows on panel A). Two additional sites (marked with solid arrows on panels A and B) are not blocked in the presence of calmodulin. These results confirm the findings of others with the exception that myosin light chain kinase is generally believed to contain only two major sites of phosphorylation when phosphorylated with the cAMP-dependent protein kinase (Conti and Adelstein, 1981; Nishikawa et al., 1985). It is possible that the additional sites that we observe are actually minor sites of phosphorylation, or are derived by incomplete tryptic digestion as discussed in chapter 2. Alternatively, the results may reflect species differences, since their findings were obtained using turkey gizzard and our results using chicken gizzard myosin light chain kinase. However, no data was obtained to resolve these issues.

The objective of the peptide mapping studies was to determine whether the sites on myosin light chain kinase which were phosphorylated by protein kinase C were distinct from those phosphorylated by cAMP-dependent protein kinase. Examination of the relevant peptide maps in figure 3.5 reveals this to be the case for a

number of the protein kinase C sites. Two sites phosphorylated by protein kinase C which appear to be distinct from any of the sites phosphorylated by cAMP-dependent protein kinase (marked with solid arrows on panels C and D) are not blocked by calmodulin. In the absence of calmodulin, there are two additional sites phosphoryated by protein kinase C (marked with open arrows on panel C) which both have similar migration patterns to phosphopeptides in panel A. However, since only one of these sites (marked with asterisk in panels A,C) is blocked by calmodulin when myosin light chain kinase is phosphorylated with cAMP-dependent protein kinase, it seems likely that this is the only site that can be phosphorylated by the two kinases. Thus, we can conclude from phosphopeptide mapping that myosin light chain kinase is phosphorylated by protein kinase C on three sites which are distinct from, and one which may be common to, those sites phosphorylated by cAMP-dependent protein kinase. Once again, this time with protein kinase C, our phosphopeptide maps show four sites of phosphorylation, compared with two seen by Nishikawa et al., (1985). As before, the reason for these discrepancies has not been determined.

3.3.4 Phosphorylation of Chicken Gizzard Caldesmon by Protein Kinase C

The phosphorylation of chicken gizzard caldesmon by bovine brain protein kinase C in the presence of phosphatidylserine and calcium is demonstrated in figure 3.6 (lane 1). In the absence of exogenously added protein kinase (lane 2), caldesmon is not phosphorylated, demonstrating the absence of contaminating protein kinase activity.

With the catalytic subunit of cAMP-dependent protein kinase, caldesmon is barely phosphorylated to detectable levels.

The time dependent phosphorylation of caldesmon by protein kinase C (figure 3.7) reveals a maximum incorporation of 2.3 mol phosphate/mol caldesmon after 2 hours in the presence of phosphatidylserine and calcium. Caldesmon phosphorylation occurs in two apparent phases; an initial rapid phase of phosphorylation results in incorporation of approximately 1.3 mol phosphate/mol caldesmon, and is followed by a slower phase of phosphorylation to the observed level of 2.3 mol phosphate/mol protein. In the absence of phosphatidylserine and calcium, phosphorylation is minimal. The phosphorylation of caldesmon by protein kinase C has also been observed by Umekawa and Hidaka (1985) who saw significantly higher levels of phosphorylation (approximately 3.85 mol phosphate/mol caldesmon).

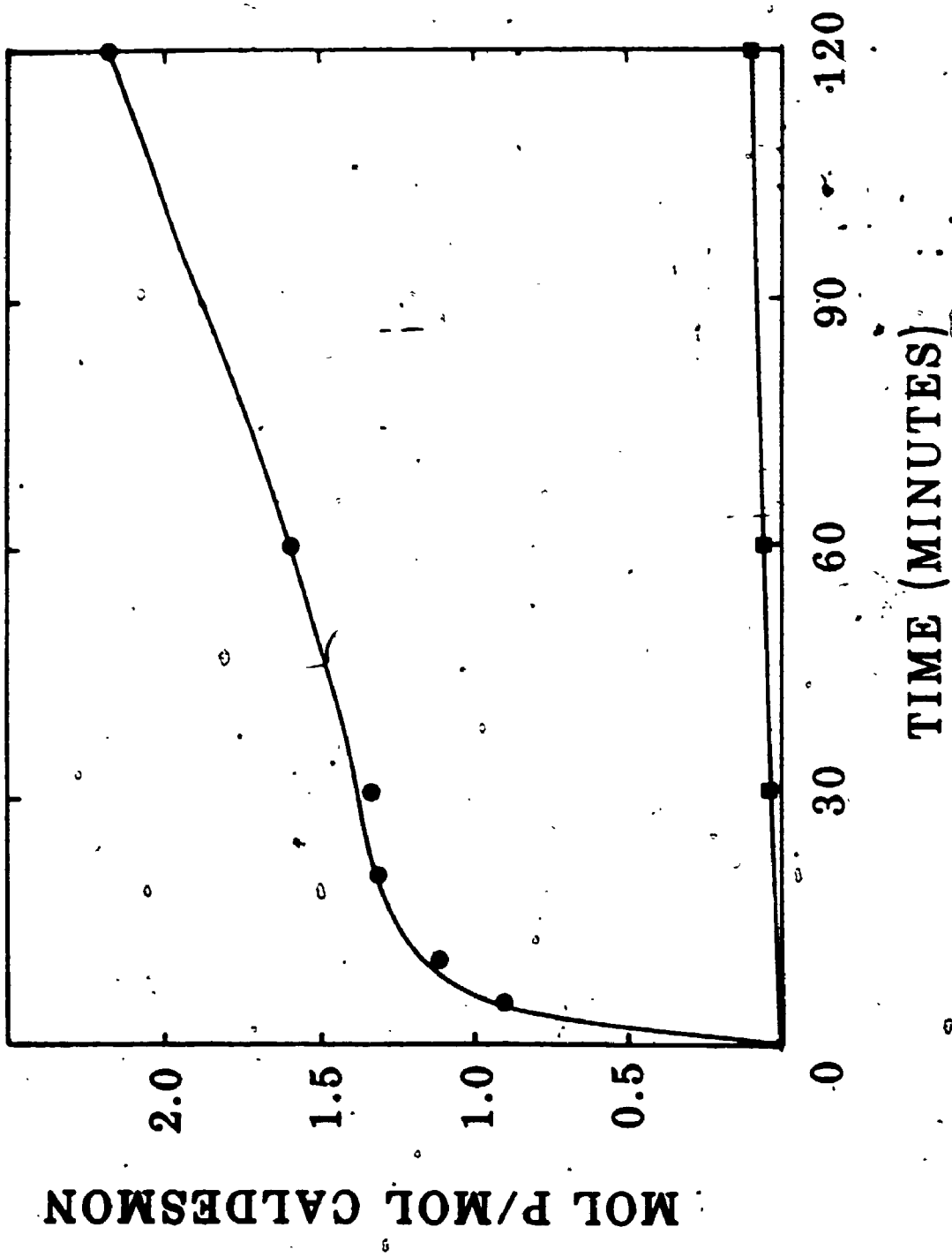
The results outlined in the preceding paragraphs were obtained using protein kinase C from bovine brain. Essentially identical results have also been obtained using rat brain protein kinase C (results not shown).

The phosphate content of purified caldesmon was assessed using the malachite green microphosphate assay of Buss and Stull (1983). A linear range of standard phosphate amounts of 0.2 - 0.8 nmol was established (see Figure 3.1). Phosphate determinations were done using 1 nmol (0.15 mg) of two different preparations of chicken gizzard caldesmon with the result that phosphate contents of 0.26 mol phosphate/mol caldesmon and 0.21 mol phosphate/mol caldesmon were

FIGURE 3.7

TIME DEPENDENCE OF PHOSPHORYLATION OF CALDESMON BY PROTEIN KINASE C.

Caldesmon was incubated with protein kinase C in the presence of phosphatidylserine and calcium (●) or EGTA (■). At the indicated times, aliquots of the reaction mixture were removed and phosphate incorporation determined by trichloroacetic acid precipitation. The values shown are typical of those obtained in nine separate experiments.


MOL P/MOL CALDESMON
2.0
1.5
1.0
0.5
0
30
60
90
120
TIME (MINUTES)

measured. However, the measured values were at the low end of the linear range of standard values and duplicate variation was as high as 20%. Nevertheless, the results demonstrate that purified caldesmon does contain a significant amount of covalently bound phosphate. The measured quantities are not however high enough to explain differences between our phosphate incorporation results and those of Umekawa and Hidaka (1985).

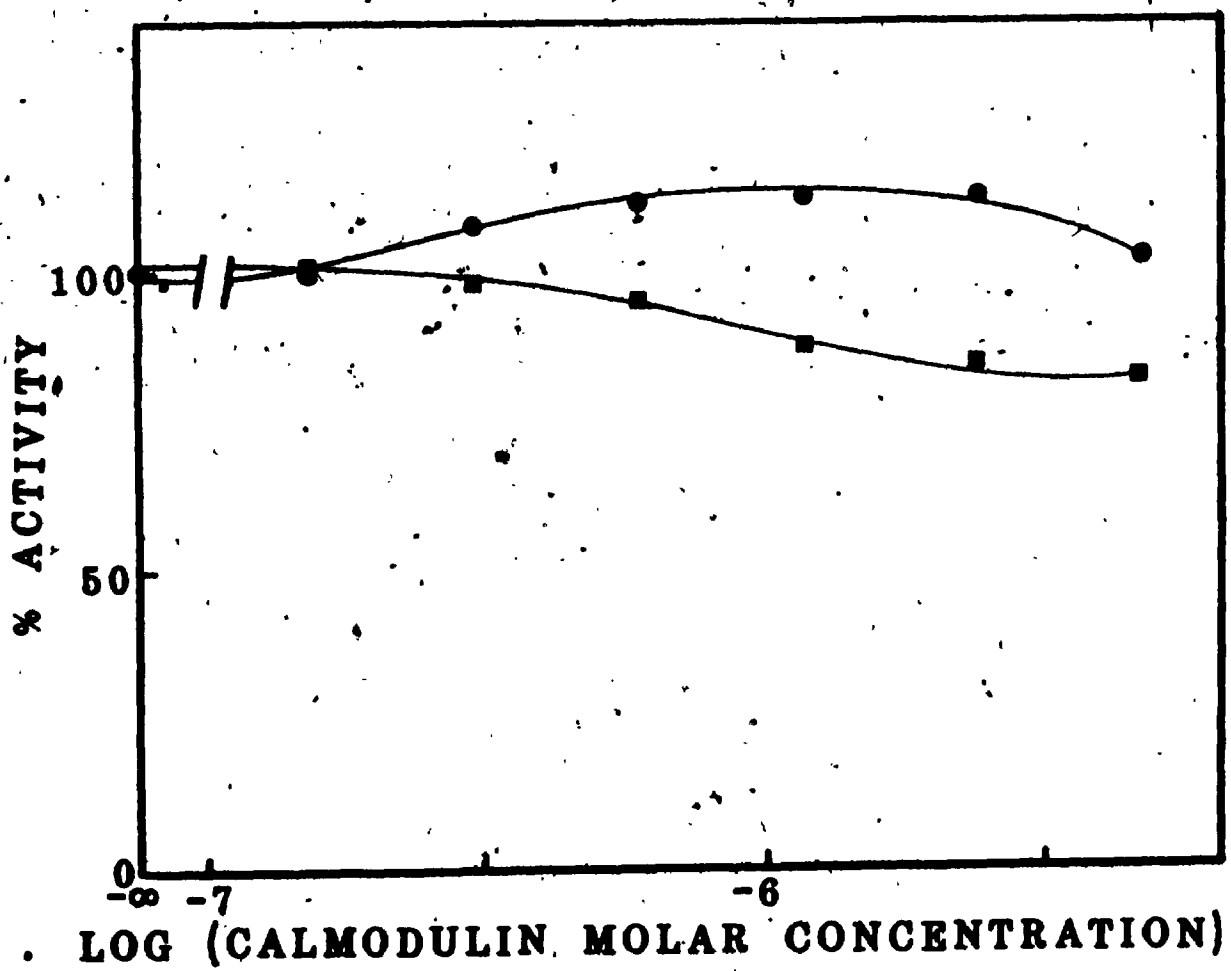
3.3.5 Inhibition of Caldesmon Phosphorylation by Calmodulin

Caldesmon was originally purified on the basis of its calmodulin-binding (Sobue et al., 1981), hence the effect of calmodulin on its phosphorylation is of interest. In addition, Umekawa and Hidaka (1985) demonstrated that calmodulin (at a molar ratio of 6 calmodulin: 1 caldesmon) diminished phosphorylation of caldesmon to a level of 2.75 mol phosphate/mol caldesmon after 2 hours (inhibition of approximately 25%). To more completely characterize this inhibition, we have examined the phosphorylation of caldesmon as a function of calmodulin concentration (figure 3.8). Maximal inhibition (approximately 20%) of caldesmon phosphorylation was observed at a calmodulin concentration of 4.8×10^{-6} M (molar ratio of 7.2 calmodulin:1 caldesmon). Interpolation from figure 3.8 suggests that half-maximal inhibition occurs at a calmodulin concentration of approximately 1×10^{-6} M (molar ratio of 1.5 calmodulin:1 caldesmon). To demonstrate that inhibition is not a result of interactions with the enzyme, histone kinase activity was also measured in the presence of calmodulin. Figure 3.8 clearly shows that histone kinase activity was not inhibited by calmodulin,

FIGURE 3.8

EFFECT OF CALMODULIN ON PHOSPHORYLATION OF CALDESMON AND HISTONE III-S BY PROTEIN KINASE C.

Caldesmon (■) and histone III-S (●) were incubated as described in Materials and Methods with protein kinase C in the presence of phosphatidylserine and calcium and the indicated amount of calmodulin. The extent of phosphorylation was assayed using precipitation with trichloroacetic acid. The values represent the average of duplicate samples and are typical of those obtained in two separate experiments.


100
50
0
% ACTIVITY
-∞
-7
-6
LOG (CALMODULIN MOLAR CONCENTRATION)

and was in fact activated slightly at some concentrations.

3.3.6 Phosphorylation of Caldesmon by Contaminating Calmodulin-Dependent Protein Kinase Activity

The results presented in previous sections were obtained using caldesmon which had been purified by heat treatment and did not contain any contaminating protein kinase activity (Bretscher, 1984). Caldesmon has also been purified by non-denaturing methods resulting in a preparation containing contaminating calmodulin-dependent caldesmon kinase activity (Ngai and Walsh, 1984; Lash et al., 1986). Figure 3.9 illustrates the phosphorylation of caldesmon which was purified using non-denaturing methods by protein kinase C in the presence of phosphatidylserine and calcium (lane 1) and by addition of calcium/calmodulin to the caldesmon preparation (lane 3). Caldesmon is not phosphorylated by the calmodulin-dependent kinase in the absence of calcium (lane 4).

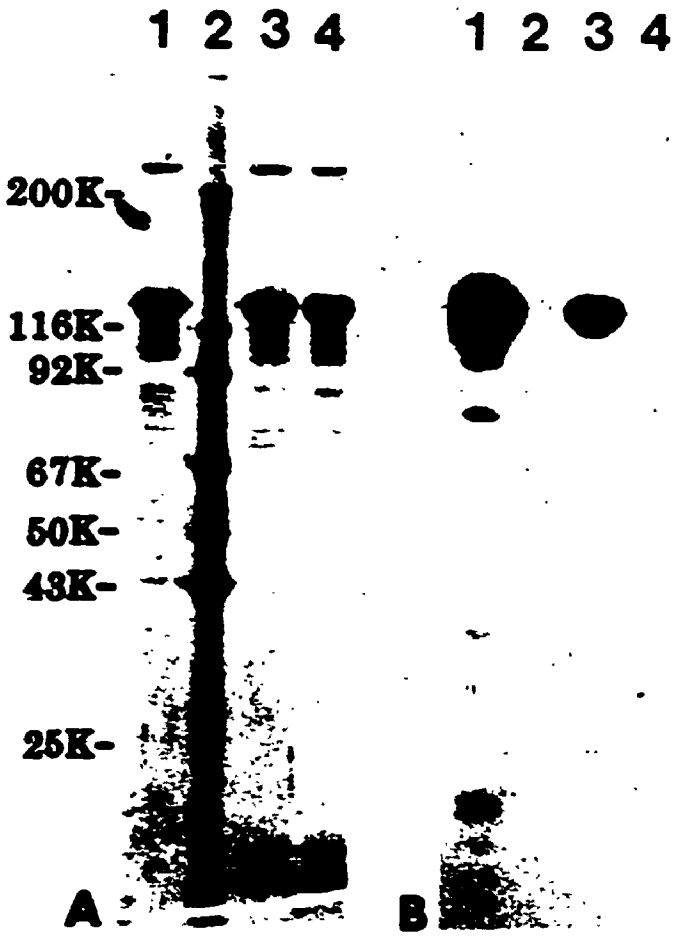
3.3.7 Peptide Mapping and Phosphoamino Acid Analysis of Phosphorylated Caldesmon

Caldesmon, purified by denaturing methods, was phosphorylated by protein kinase C in the presence or absence of calmodulin, digested extensively with trypsin and the resultant phosphopeptides separated in two dimensions (figure 3.10). Caldesmon, phosphorylated in the absence of calmodulin, contains three major phosphopeptides (panel A, marked with solid arrowheads) and a number of minor phosphopeptides. In the presence of calmodulin (panel B), caldesmon phosphorylation is inhibited (see figure 3.8), but there is no apparent alteration in phosphopeptide maps. This observation suggests that inhibition of

FIGURE 3.9

PHOSPHORYLATION OF CALDESMON BY PROTEIN KINASE C AND BY AN ENDOGENOUS CALMODULIN-DEPENDENT PROTEIN KINASE.

Caldesmon (0.3 mg/ml) was purified by non-denaturing methods as described in Materials and Methods and was phosphorylated prior to electrophoresis on a 6-15% SDS-polyacrylamide gel which was stained with Coomassie blue (panel A) prior to autoradiography (panel B). Lane 1, caldesmon with addition of phosphatidylserine, calcium and protein kinase C; lane 3, caldesmon with addition of calcium and calmodulin; lane 4, caldesmon with addition of EGTA and calmodulin. Molecular weight markers were run in lane 2.


1 2 3 4
1 2 3 4
200K-
116K-
92K-
67K-
50K-
43K-
25K-
A
B

caldesmon phosphorylation is not the result of selective inhibition of specific sites. Phosphopeptides are darker in panel B as a result of a longer exposure than was used for the phosphopeptide map shown in panel A.

Caldesmon, purified by non-denaturing methods, was phosphorylated by the contaminating calmodulin-dependent caldesmon kinase and shown by phosphopeptide mapping to have three apparent phosphorylation sites (panel C, marked with open arrowheads). A phosphopeptide map (panel D) obtained by mixing phosphopeptides obtained by phosphorylation with the two different protein kinases clearly demonstrates that the sites phosphorylated in each case are distinct.

Phosphoamino acid analysis (figure 3.11) shows that phosphorylation of caldesmon by protein kinase C (panel A) or by the calmodulin-dependent caldesmon kinase (panel B) occurs exclusively on serine residues.

3.3.8 Purification and Phosphorylation of Chicken Liver Caldesmon$_{72}$

To determine whether or not the lower molecular weight form of caldesmon is a substrate for protein kinase C, caldesmon$_{72}$ was purified from fresh chicken liver. By adaptation of the denaturing method of Bretscher (1984), caldesmon$_{72}$ was rapidly purified using heat treatment, ammonium sulphate fractionation and three column chromatographic steps. The purification of the protein was monitored by SDS-polyacrylamide gel electrophoresis (figure 3.12). The Mr 72,000 band can be visualized prior to phosphocellulose chromatography (lane L) and is progressively purified by

FIGURE 3.10

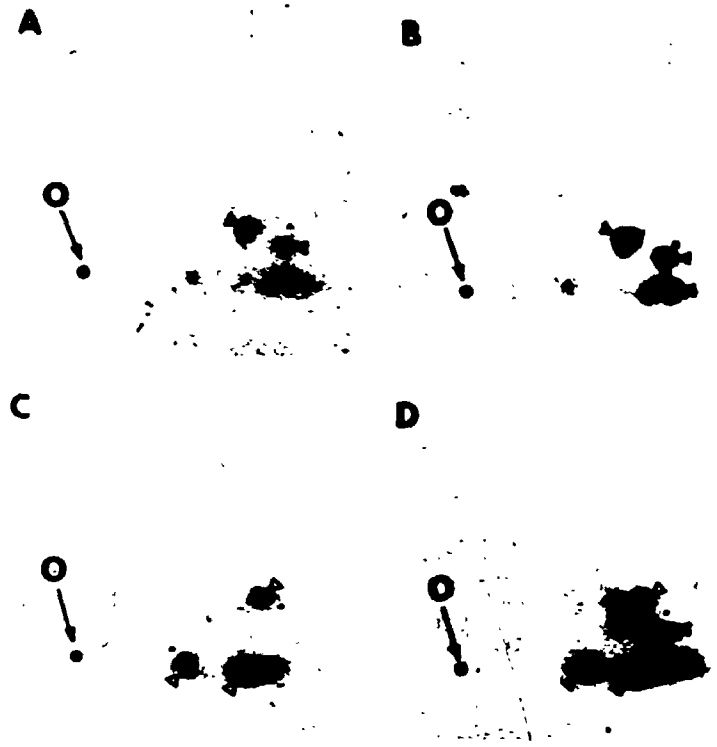
TWO-DIMENSIONAL TRYPTIC PHOSPHOPEPTIDE MAPPING OF CALDESMON PHOSPHORYLATED BY PROTEIN KINASE C AND BY AN ENDOGENOUS CALMODULIN-DEPENDENT PROTEIN KINASE.

Following SDS-polyacrylamide gel electrophoresis of phosphorylated caldesmon, caldesmon phosphopeptides were prepared and separated on thin layer plates as previously described. Panel A, caldesmon phosphorylated by protein kinase C; panel B, caldesmon phosphorylated by protein kinase C in the presence of calmodulin; panel C, caldesmon phosphorylated by endogenous calmodulin-dependent protein kinase; panel D, mix of phosphopeptides used for panels A and C. The major phosphopeptides obtained by phosphorylation with protein kinase C are marked with solid arrowheads. The major phosphopeptides obtained by phosphorylation with endogenous calmodulin-dependent protein kinase are marked with open arrowheads.

FIGURE 3.11

PHOSPHOAMINO ACID ANALYSIS OF PHOSPHORYLATED CALDESMON.

Caldesmon was phosphorylated with protein kinase C (panel A) and with endogenous calmodulin-dependent protein kinase (panel B). Phosphopeptides were subjected to partial hydrolysis as described in Materials and Methods. The hydrolysis products were applied at the origin (O), electrophoresed at pH 1.9 (horizontal dimension with anode at left) and at pH 3.5 (vertical dimension with anode at top) prior to visualization by autoradiography. Standard phosphoamino acids - phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y) - were visualized by ninhydrin staining.


A
B
C
D


S
T
Y
A

phosphocellulose (lane P) and DEAE-cellulose (lane D) chromatography. Caldesmon$_{72}$ purified through DEAE-cellulose (lane D) is not homogeneous, but is the major band (marked with an arrowhead).

To verify that the Mr 72,000 band is a form of caldesmon, samples from each step in the purification were subjected to Western blotting and probed with affinity-purified rabbit antibodies prepared against chicken gizzard caldesmon (figure 3.13). In addition, a sample of fresh chicken liver which was homogenized in sample buffer for SDS-gel electrophoresis was subjected to the same procedure (lane 7). The only major band which can be seen in the fresh liver is a band with Mr 72,000. The absence of any significant higher molecular weight bands suggests that the Mr 72,000 band is the intact physiological form of the protein. Each of the samples obtained during the purification of caldesmon$_{72}$ (lanes 1-5) contains the same major band and variable quantities of lower molecular weight bands. Since the lower molecular weight bands are less evident in the fresh liver sample, it is likely that they are proteolytic fragments of caldesmon$_{72}$ that were generated during the purification. For comparison purposes, a sample of caldesmon$_{77}$ purified from bovine liver (to be discussed later) was also subjected to Western blotting (lane 6) and demonstrates that the bovine protein has a slightly higher molecular weight than does the avian protein.

The phosphorylation of chicken liver caldesmon$_{72}$ by protein kinase C in the presence of phosphatidylserine and calcium is demonstrated in figure 3.14 (lane 1). As a positive control, the phosphorylation of chicken gizzard caldesmon by protein kinase C in

FIGURE 3.12

PURIFICATION OF CALDESMON$_{72}$ FROM FRESH CHICKEN LIVER.

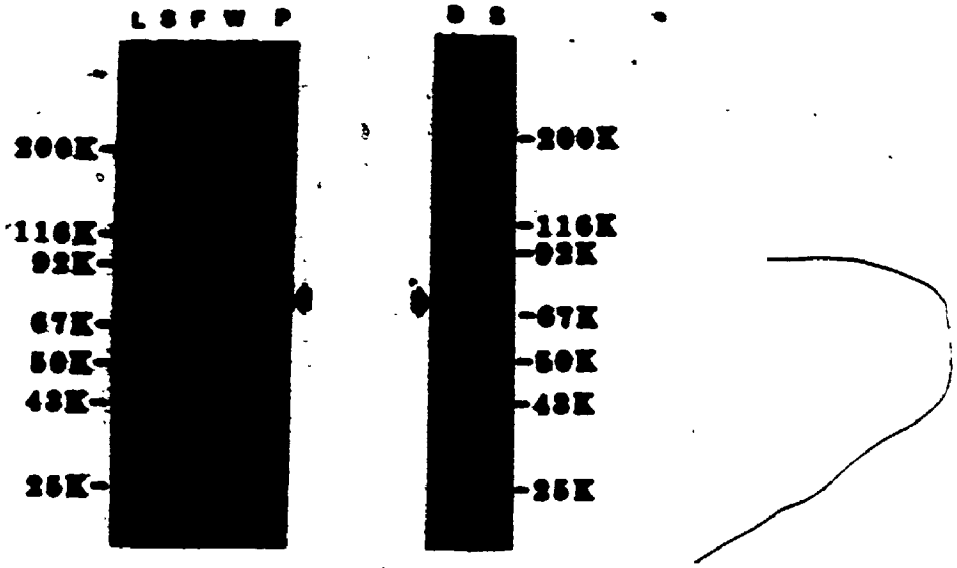
Samples of different fractions obtained during the purification of caldesmon$_{72}$ from fresh chicken liver were electrophoresed on 6-15% SDS-polyacrylamide gels and stained with Coomassie blue. Heat-treated liver extract was subjected to ammonium sulphate precipitation, phosphocellulose chromatography, phenyl-Sepharose chromatography and DEAE-cellulose chromatography. The position of caldesmon$_{72}$ is marked by an arrow.

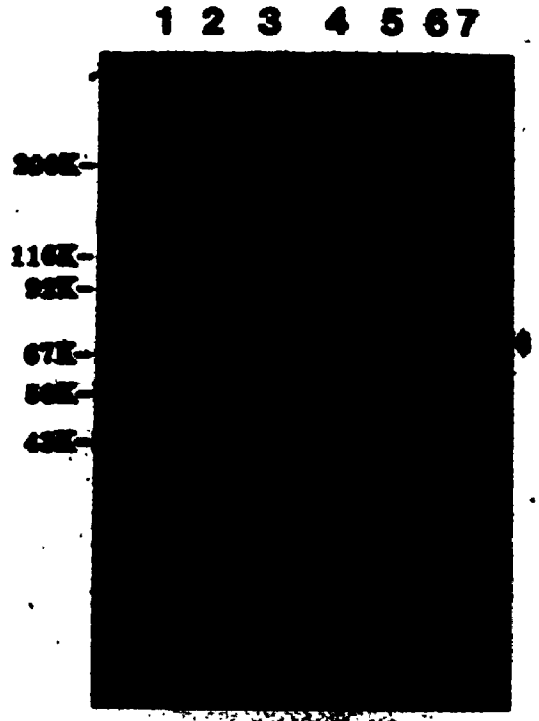
L - sample loaded on phosphocellulose
F - flow through from phosphocellulose
W - salt free wash from phosphocellulose
P - caldesmon$_{72}$ peak from phosphocellulose
D - caldesmon$_{72}$ peak from DEAE-cellulose
S - molecular weight standards

FIGURE 3.13

WESTERN BLOT OF FRACTIONS OBTAINED DURING CHICKEN LIVER CALDESMON$_{72}$ PURIFICATION WITH AFFINITY-PURIFIED ANTI-CALDESMON ANTIBODIES.

Samples of protein obtained during the purification of caldesmon$_{72}$ were electrophoresed on a 6-15% SDS-polyacrylamide gel and transferred to nitrocellulose. The transferred proteins were probed with affinity-purified anti-caldesmon antibodies. Lane 1, heat extract of chicken liver; lane 2, 30-50% ammonium sulphate pellet; lane 3, phosphocellulose pool; lane 4, phenyl-Sepharose pool; lane 5, DEAE-cellulose peak; lane 6, bovine liver caldesmon$_{77}$; lane 7, fresh chicken liver homogenized in SDS-gel sample buffer.


L S F W P
200K
116K
92K
67K
50K
43K
25K
D S
200K
116K
92K
67K
50K
43K
25K


1 2 3 4 5 6 7
200K
116K
92K
67K
50K
43K

the presence of phosphatidylserine and calcium is also illustrated (lane 5). Caldesmon$_{72}$ and chicken gizzard caldesmon are both phosphorylated to a low level in the presence of phosphatidylserine and EGTA (lanes 2 and 6 respectively). In the absence of phosphatidylserine, with calcium (lanes 3 and 7) or with EGTA (lanes 4 and 8) neither protein is significantly phosphorylated.

Insufficient quantities of caldesmon$_{72}$ (approximately 0.5 mg/ 50 grams liver) were obtained to examine the time dependent phosphorylation of this protein.

3.3.9 Peptide Mapping and Phosphoamino Acid Analysis of Phosphorylated Caldesmon$_{72}$

When phosphopeptides obtained from phosphorylated caldesmon$_{72}$ are separated in two dimensions (figure 3.15, panel A), the resultant map appears to be a subset of that obtained from chicken gizzard caldesmon (figure 3.15, panel B). In fact, the two maps appear to have 4 phosphopeptides in common (marked with solid arrowheads) while there is one additional phosphopeptide (marked with open arrowhead) on the chicken gizzard caldesmon phosphopeptide map. These results suggest that despite the large difference in their molecular weights, the two proteins contain conserved sequences near the sites of phosphorylation.

Phosphoamino acid analysis (figure 3.15, panel C) shows that caldesmon$_{72}$ is phosphorylated exclusively on serine residues, as was the case with chicken gizzard caldesmon.

FIGURE 3.14

PHOSPHORYLATION OF CHICKEN GIZZARD CALDESMON AND CHICKEN LIVER CALDESMON$_{72}$ BY PROTEIN KINASE C.

Chicken gizzard caldesmon (lanes 5-8) and chicken liver caldesmon$_{72}$ (lanes 1-4) were incubated with protein kinase C, electrophoresed on a 6-15% SDS-polyacrylamide gel which was stained with Coomassie blue (panel A) and then autoradiographed (panel B). Lanes 1 and 5, phosphatidylserine (0.05 mg/ml) and 0.5 mM $CaCl_2$; lanes 2 and 6, phosphatidylserine (0.05 mg/ml) and 0.5 mM EGTA; lanes 3 and 7, 0.5 mM $CaCl_2$; lanes 4 and 8, 0.5 mM EGTA; lane S, molecular weight standards. The position of chicken gizzard caldesmon is marked with an open arrowhead and the position of chicken liver caldesmon$_{72}$ is marked with a solid arrowhead.

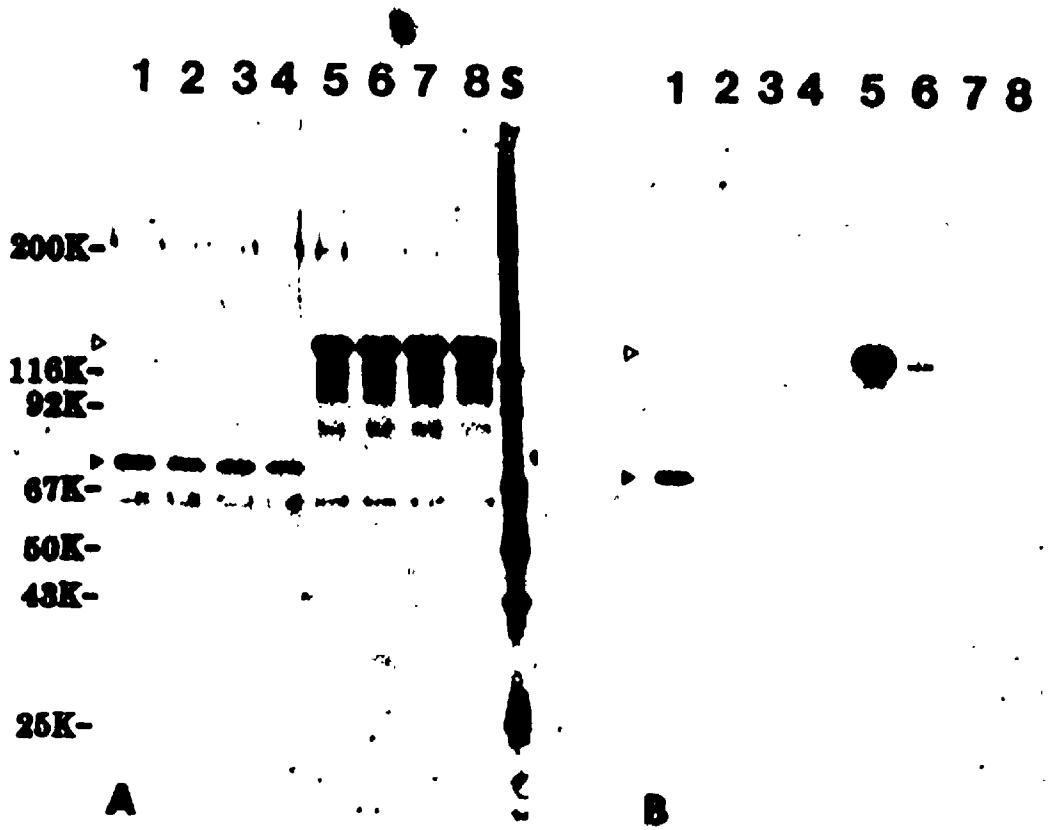
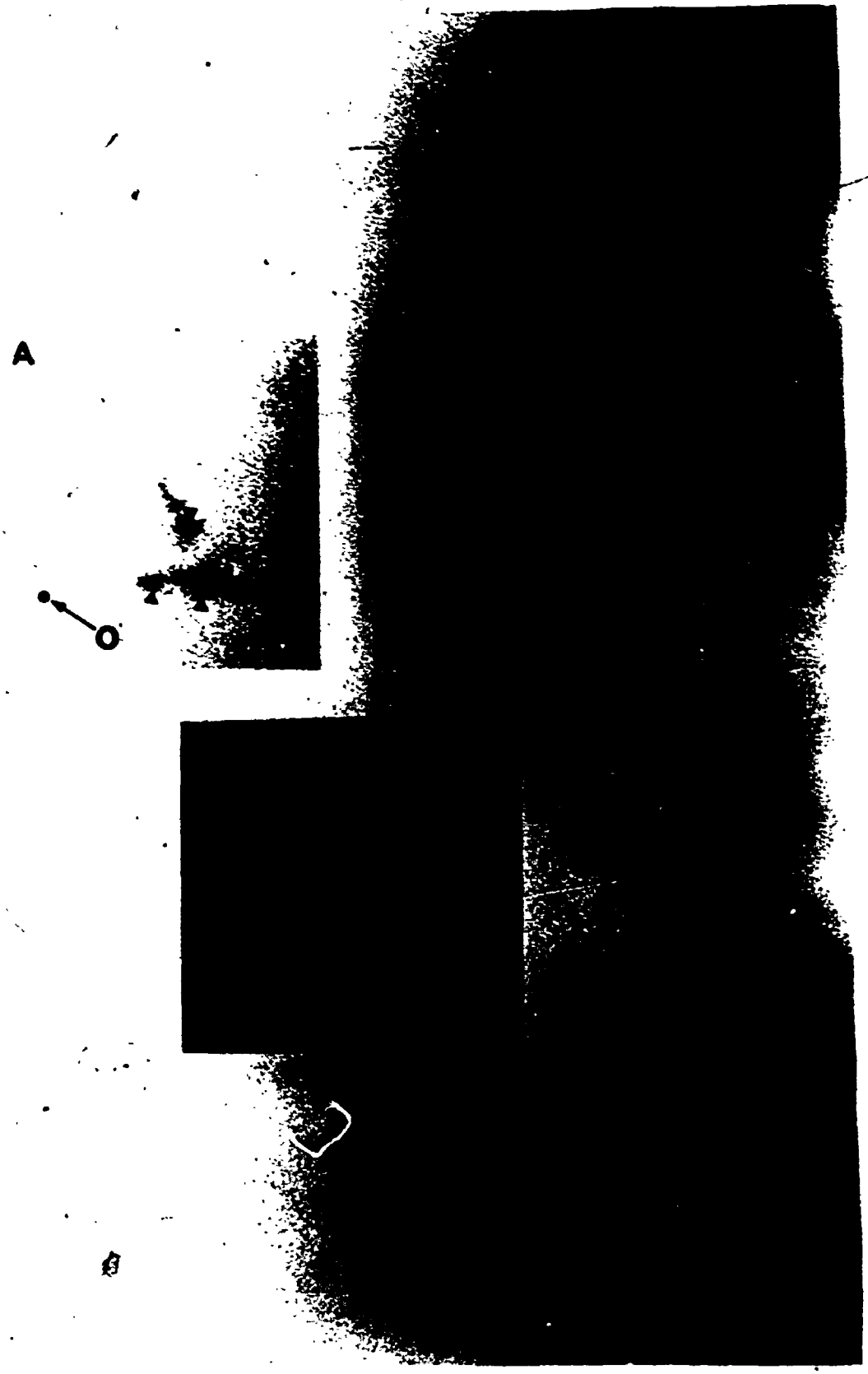

1 2 3 4 5 6 7 8 S
1 2 3 4 5 6 7 8
200K-
116K-
92K-
67K-
50K-
43K-
25K-
A
B

FIGURE 3.15

TWO-DIMENSIONAL PHOSPHOPEPTIDE MAPPING AND PHOSPHOAMINO ACID ANALYSIS OF PHOSPHORYLATED CHICKEN LIVER CALDESMON$_{72}$.

Following SDS-polyacrylamide gel electrophoresis of phosphorylated chicken liver caldesmon$_{72}$, tryptic phosphopeptides were prepared and separated on thin layer plates as previously described. For phosphoamino acid analysis, the tryptic phosphopeptides were subjected to partial acid hydrolysis prior to two dimensional electrophoresis on thin layer plates. Phosphoamino acid standards - phosphoserine (S), phosphothreonine (T), phosphotyrosine (Y) - were visualized by ninhydrin staining. Panel A, phosphopeptide map of chicken liver caldesmon$_{72}$; panel B, phosphopeptide map of chicken gizzard caldesmon; panel C; phosphoamino acid analysis of chicken liver caldesmon$_{72}$. Phosphopeptides which are common to both forms of caldesmon are marked with solid arrows. One phosphopeptide is found only on chicken gizzard caldesmon and is marked with an open arrow (panel B).


A
O

3.3.10 Purification and Phosphorylation of Two Different Forms of Bovine Liver Caldesmon

Yields of chicken liver caldesmon$_{72}$ (discussed in section 3.3.8) were too low to examine its time dependent phosphorylation. Thus, bovine liver was selected as the tissue from which larger quantities of the lower molecular weight form of caldesmon could be isolated. Caldesmon, purified from a mammalian source, would also be useful for comparisons with caldesmon phosphorylation in intact mammalian cells (to be discussed in following chapter). Utilizing the same protocol as for chicken liver caldesmon$_{72}$, bovine liver caldesmon$_{77}$ and a lesser quantity of caldesmon$_{150}$ were obtained. The purity of the protein samples obtained at each stage of the purification was assessed by SDS-polyacrylamide gel electrophoresis (figure 3.16, panel A). The existence of caldesmon species in each of these samples was confirmed on a Western blot which was probed with affinity-purified antibodies prepared in rabbits against chicken gizzard caldesmon (figure 3.16, panel B). The existence of significant quantities of caldesmon$_{77}$ in the initial heat extract of bovine liver (lane 1) suggests that this is an important physiological form of the protein that is not simply a proteoytic fragment of the larger caldesmon$_{150}$ species generated during the purification.

The phosphorylation of both bovine liver forms of caldesmon by purified rat brain protein kinase C is illustrated in figure 3.17. Caldesmon$_{77}$ (lanes 1,2) and caldesmon$_{150}$ (lanes 3,4) are phosphorylated in the presence of phosphatidylserine and calcium

FIGURE 3.16

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN BLOTTING OF DIFFERENT FRACTIONS OBTAINED DURING THE PURIFICATION OF BOVINE LIVER CALDESMON.

Samples obtained during the purification of bovine liver caldesmon were subjected to 6-15% SDS-polyacrylamide gel electrophoresis (panel A) and were also transferred to nitrocellulose and probed with affinity-purified anti-caldesmon antibodies (panel B). Lane 1, heat extract of fresh bovine liver; lane 2, 30-50% ammonium sulphate pellet; lane 3, early portion of phosphocellulose pool; lane 4, late portion of phosphocellulose pool; lane 5, phenyl-Sepharose pool; lane 6, DEAE-cellulose gradient fraction 14; lane 7, DEAE-cellulose gradient fraction 18; lane 8, DEAE-cellulose gradient fraction 24; lanes marked S, molecular weight markers. The position of caldesmon$_{150}$ is marked with an open arrow and the position of caldesmon$_{77}$ is marked with a solid arrow.

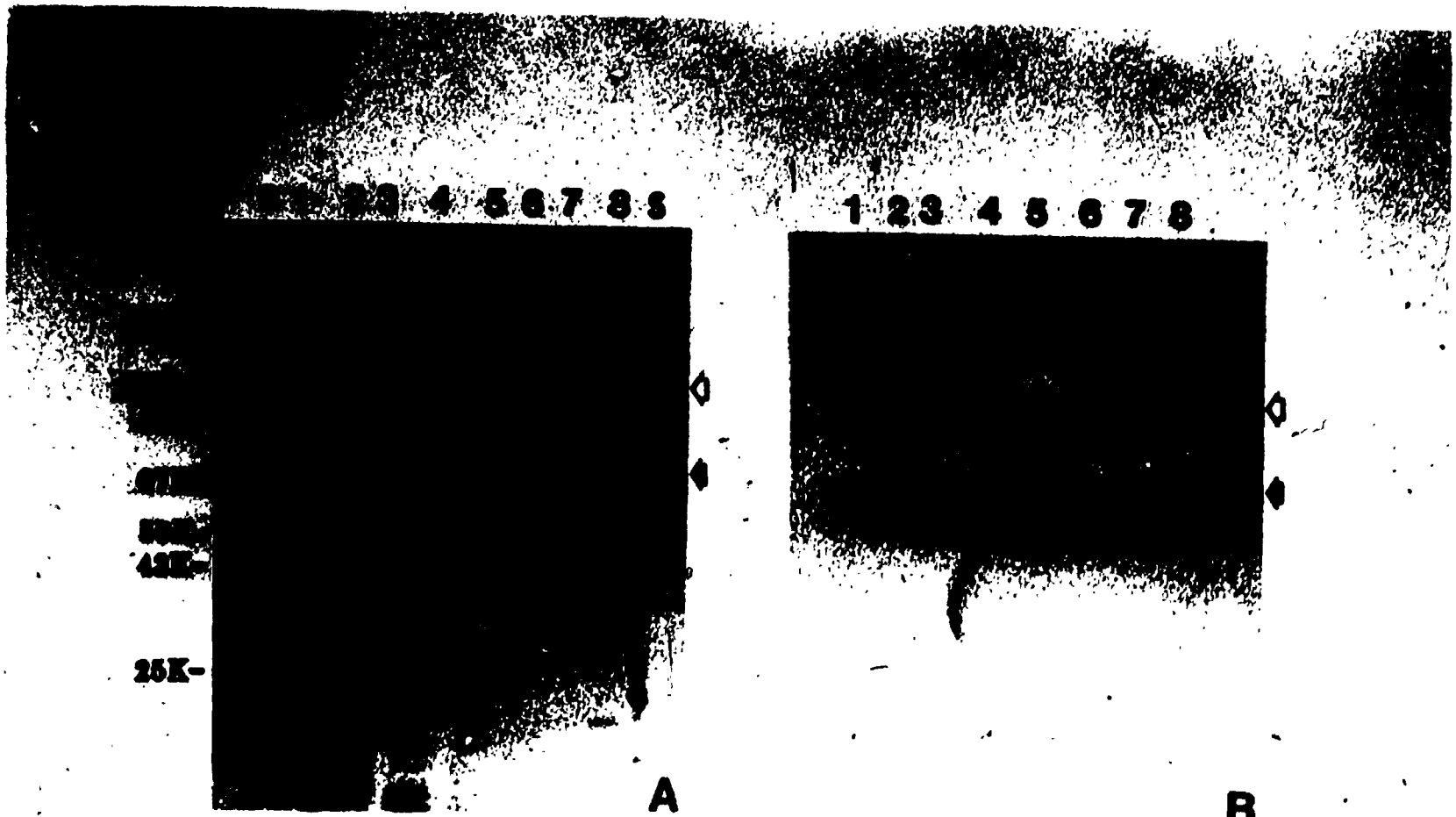
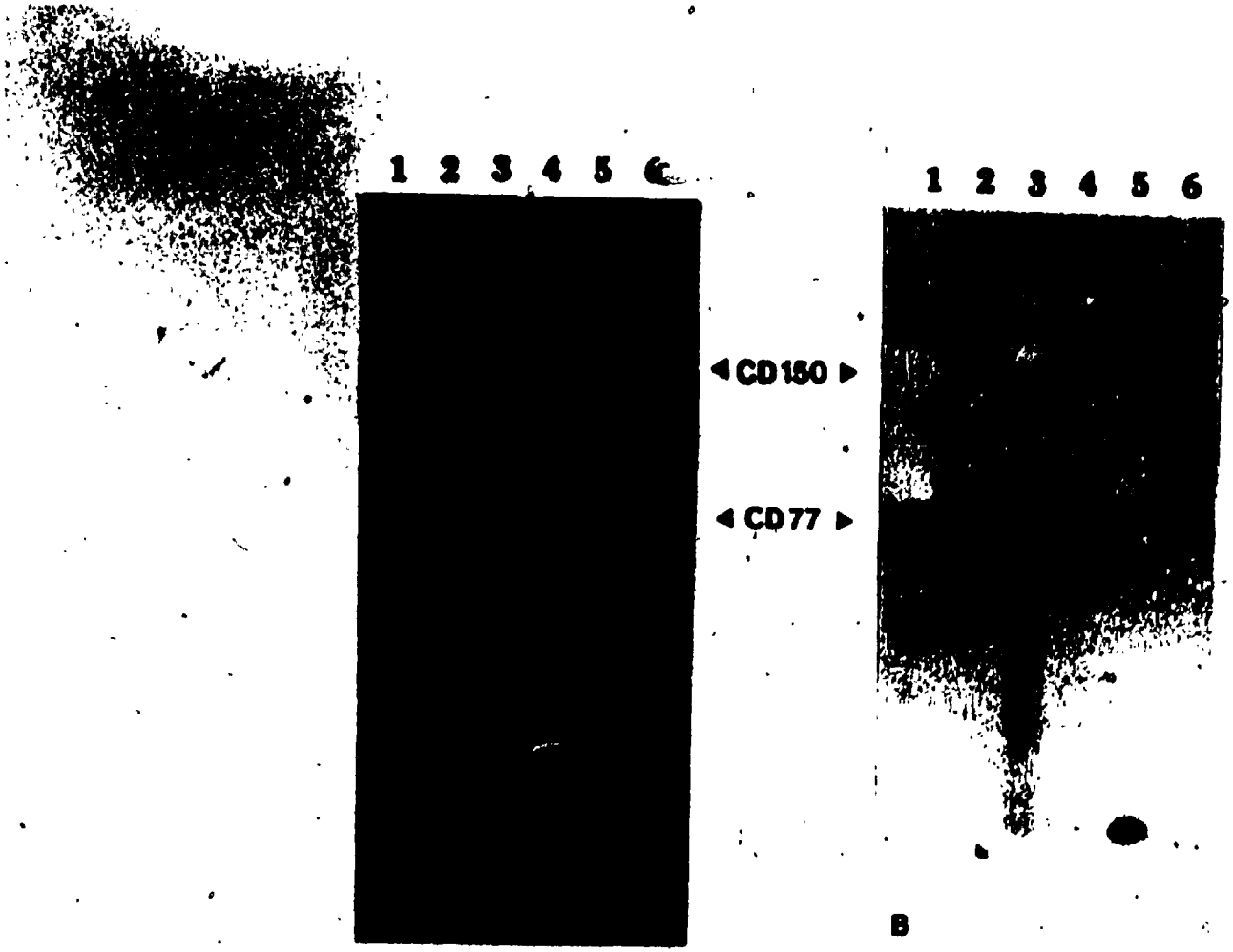

1 2 3 4 5 6 7 8
45K-
25K-
A
1 2 3 4 5 6 7 8
B

FIGURE 3.17

PHOSPHORYLATION OF DIFFERENT FORMS OF BOVINE LIVER CALDESMON BY PROTEIN KINASE C.

Caldesmon$_{77}$ (lanes 1,2) or caldesmon$_{150}$ (lanes 3,4) were incubated with protein kinase C in the presence of phosphatidylserine and calcium (lanes 1,3,5) or in the presence of EGTA (lanes 2,4,6). Protein kinase C in the absence of caldesmon$_{150}$ or caldesmon$_{77}$ is also shown (lanes 5,6). The reaction mixtures were run on an 8% SDS-polyacrylamide gel which was stained with Coomassie blue (panel A) prior to autoradiography (panel B).


1 2 3 4 5 6
CD150
CD77
1 2 3 4 5 6
B

(lanes 1,3) but not in the absence of these activators (lanes 2,4). A faint band of protein kinase C, running slightly behind caldesmon$_{77}$ with approximate molecular weight of 80,000 is also visible on the Coomassie blue stained SDS-polyacrylamide gel (panel A, lanes 1-6) and on the accompanying autoradiogram (panel B, lanes 1,3,5). For comparison with the phosphorylated caldesmon$_{77}$ band, autophosphorylated protein kinase C is shown in the absence of phosphorylated caldesmon (lane 5). The autophosphorylation of protein kinase C was previously described (section 3.3.1).

The time dependent phosphorylation of caldesmon$_{77}$ by protein kinase C is illustrated in figure 3.18. In the presence of phosphatidylserine and calcium, caldesmon$_{77}$ phosphorylation reaches a level slightly exceeding one mol phosphate/mol protein. Phosphorylation is minimal in the absence of activators. Time dependent phosphorylation of caldesmon$_{150}$ from bovine liver was not studied since very low levels of the protein were obtained in the purification.

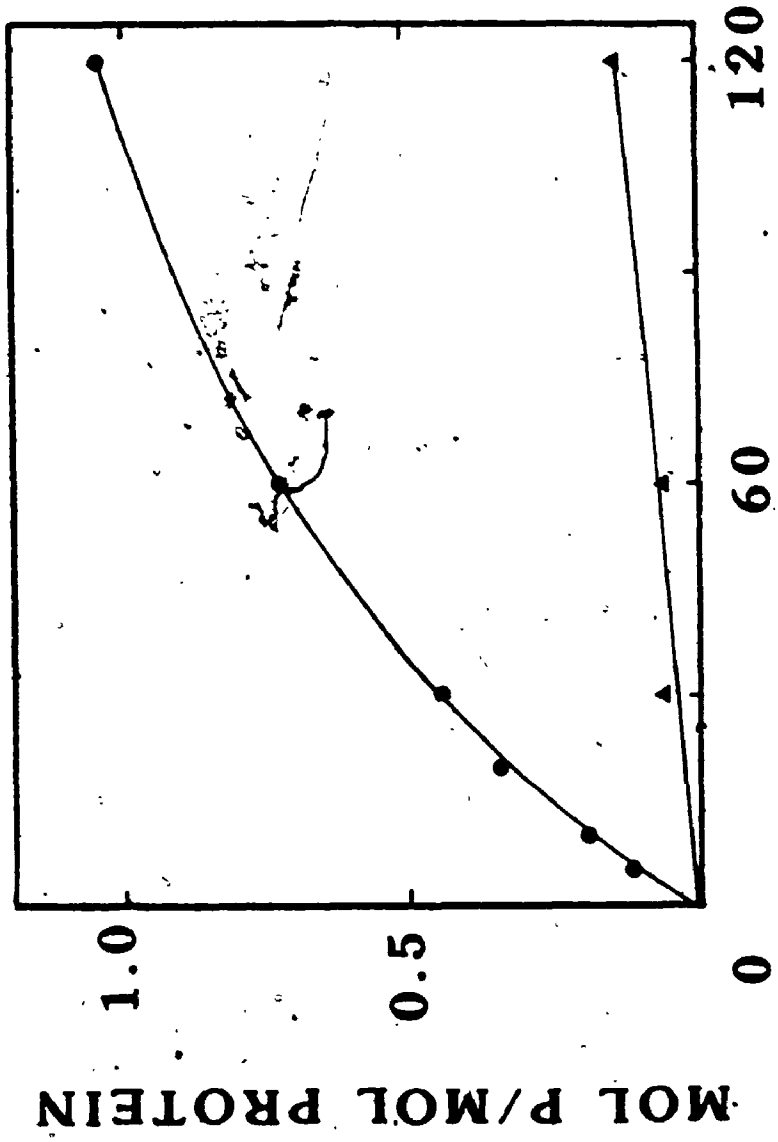
3.3.11 Phosphopeptide Mapping and Phosphoamino Acid Analysis of Phosphorylated Bovine Liver Caldesmon$_{77}$ and Caldesmon$_{150}$

Phosphopeptide mapping (figure 3.19) demonstrates that caldesmon$_{77}$ (panel A) and caldesmon$_{150}$ (panel B) are both phosphorylated at multiple sites. Phosphopeptide maps of the two proteins reveal that they have the same sites of phosphorylation since the two major sites and several minor sites have the same migration patterns on both maps. These results confirm our previous findings with two chicken forms of caldesmon; that is, the two

FIGURE 3.18

TIME DEPENDENCE OF BOVINE LIVER CALDESMON$_{77}$ PHOSPHORYLATION.

Bovine liver caldesmon$_{77}$ was incubated with protein kinase C in the presence of phosphatidylserine and calcium (●) or EGTA (▲) as described in Materials and Methods. At the indicated times, aliquots of the reaction mixture were withdrawn and the phosphate incorporation was determined. The results are typical of those obtained in two separate experiments.


MOL P/MOL PROTEIN
1.0
0.5
0
120
60
TIME (MINUTES)

different classes of caldesmon molecules have considerable structural identity.

Phosphoamino acid analysis shows that phosphorylation of caldesmon$_{77}$ (panel C) and caldesmon$_{150}$ (panel D) occurs on serine residues, as is the case with avian forms of caldesmon.

To demonstrate that phosphopeptide maps of caldesmon$_{77}$ were not contaminated with phosphopeptides of protein kinase C, the autophosphorylated enzyme was subjected to phosphopeptide mapping (figure 3.20). The resultant map is clearly distinct from the caldesmon$_{77}$ map and reveals that protein kinase C contains multiple sites of phosphorylation. This multisite autophosphorylation of protein kinase C has been well documented (Woodgett and Hunter, 1987b; Huang et al., 1986a).

3.4 DISCUSSION

In smooth muscle cells, activators of protein kinase C have been shown to induce various phases of the contractile response (Rasmussen et al., 1984; Park and Rasmussen, 1986; Inagaki et al., 1987). A key step in the elucidation of the molecular mechanisms of these events is to examine the activity of this enzyme towards proteins that are involved in the regulation of the contractile apparatus.

In the preceding chapter, the purification of protein kinase C from bovine brain was detailed. Although the bovine brain enzyme preparation was useful for the in vitro phosphorylation of cytoskeletal proteins, the enzyme had relatively low specific activity and very limited stability. In an effort to improve the

FIGURE 3.19

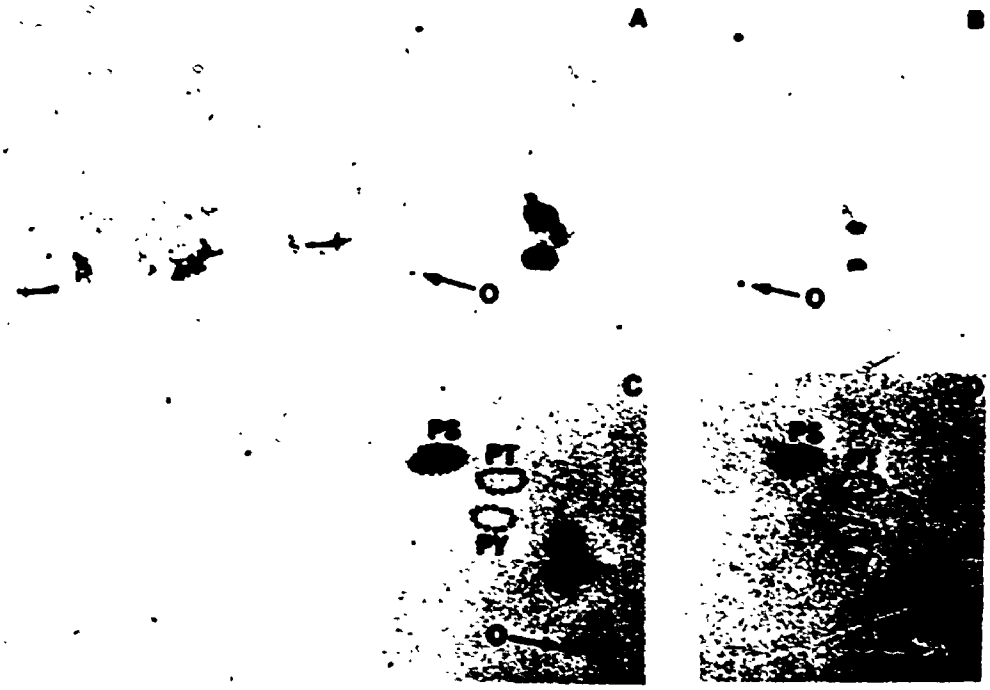
TWO-DIMENSIONAL TRYPTIC PHOSPHOPEPTIDE MAPS AND PHOSPHOAMINO ACID ANALYSIS OF PHOSPHORYLATED BOVINE LIVER CALDESMON FORMS.

Following SDS-polyacrylamide gel electrophoresis, phosphorylated caldesmon$_{77}$ (panels A and C) or caldesmon$_{150}$ (panels B and D) was digested with trypsin. The tryptic phosphopeptides were subjected to peptide mapping (panels A and B) or were partially hydrolysed for phosphoamino acid analysis (panels C and D). Standard phosphoamino acids - phosphoserine (PS), phosphothreonine (PT), phosphotyrosine (PY) - were visualized with ninhydrin.

FIGURE 3.20

TWO-DIMENSIONAL PHOSPHOPEPTIDE MAPPING OF AUTOPHOSPHORYLATED RAT BRAIN PROTEIN KINASE C.

Autophosphorylated protein kinase C was run on 8% SDS-polyacrylamide gel electrophoresis, digested with trypsin and separated in two dimensions on thin layer plates as previously described. The phosphopeptides were visualized by autoradiography.


A
B
C
FOR

yield, purity and stability of the purified protein kinase C, we attempted to purify the enzyme from rat brain using the novel approach of Wolf et al. (1985a). This method utilizes the Ca^{2+}-dependent associations of protein kinase C with brain and erythrocyte membranes to effect a rapid purification. Since the step using erythrocyte membranes was found to be unreliable, a more consistent purification scheme using DEAE-cellulose chromatography was devised. Protein kinase C purified by this procedure from rat brain was significantly superior to that purified from bovine brain on the basis of specific activity, purity, yield, stability, and ease and speed of purification. For these reasons, rat brain replaced bovine brain as the tissue source for the enzyme purification.

As a first step in establishing a potential role for protein kinase C in the regulation of contractile events, we have demonstrated its in vitro activity towards myosin light chain kinase and different forms of the calmodulin-binding protein caldesmon. In the case of myosin light chain kinase, its identification as a substrate for protein kinase C introduces another potential level of control for this important regulatory enzyme. Of particular interest are the two sites of phosphorylation that can be selectively blocked in the presence of calmodulin, since phosphorylation at these sites might have an effect on the ability of calmodulin to activate myosin light chain kinase. Indeed, phosphorylation of myosin light chain kinase with protein kinase C does result in a substantial increase in the amount of calmodulin required to activate myosin light chain kinase (Nishikawa et al., 1985; Ikebe et al., 1985). This result is

similar to that obtained following phosphorylation of myosin light chain kinase with cAMP-dependent protein kinase (Conti and Adelstein, 1981), although only one of the two protein kinase C-dependent phosphorylation sites that are blocked by calmodulin appears to be common to the two kinases.

The particular sites of phosphorylation by the two different kinases which are not blocked by calmodulin are clearly distinct. Although the physiological significance of these additional sites is unknown, it is possible that phosphorylation by the two kinases may actually regulate different properties of the myosin light chain kinase molecule. The effect that phosphorylation of myosin light chain kinase by one protein kinase has on phosphorylation by the other protein kinase also remains to be investigated.

It should also be mentioned that the substrate for myosin light chain kinase (the regulatory light chain of myosin) can be phosphorylated directly by protein kinase C in vitro (Endo et al., 1982; Nishikawa et al., 1983; Nishikawa et al., 1984b) and in intact cells (Naka et al., 1983; Inagaki et al., 1984). The result of this reaction is an apparent decrease in the actin-activated ATPase of myosin which has been previously phosphorylated by myosin light chain kinase (Nishikawa et al., 1983). Thus, although many questions remain unanswered, a number of lines of evidence implicate protein kinase C in the modulation of contractile events through the phosphorylation of several components of the contractile apparatus.

Further support for this suggestion comes form our demonstration that protein kinase C can also phosphorylate various forms of the

calmodulin-binding protein caldesmon. Protein kinase C catalyzes the multisite phosphorylation of caldesmon from chicken gizzard. The presence of calmodulin in the phosphorylation reaction mixture results in a significant decrease in caldesmon phosphorylation. These results suggest that phosphorylation may regulate interactions between caldesmon and calmodulin, as is the case for myosin light chain kinase. However, this possibility has not been experimentally tested. The finding that calmodulin does not block any particular phosphorylation sites (unlike myosin light chain kinase) raises questions concerning the mechanism of inhibition. Several possibilities that would result in a general inhibition of caldesmon phosphorylation could be envisioned. For example, the phosphorylation sites may all be located within the calmodulin-binding domain of the molecule. Alternatively, calmodulin may inhibit caldesmon phosphorylation by inducing conformational changes in caldesmon, or by binding to more than one site on the caldesmon molecule. The possibility that the enzyme is directly inhibited by calmodulin as seen by Albert et al., (1984) must also be considered. Using histones as substrate however, no inhibitory effect of calmodulin was observed in this report or by other investigators (Schatzman et al., 1983; Kikkawa et al., 1982).

Another property of caldesmon which potentially makes this protein an important component of the contractile apparatus is its phosphorylation by a calmodulin-dependent protein kinase. This protein kinase contaminates several preparations of caldesmon from chicken gizzard (Ngai and Walsh, 1984; Ngai and Walsh, 1987; Lash et

al., 1986). The results of the phosphopeptide maps presented in this chapter clearly demonstrate that the calmodulin-dependent kinase and protein kinase C have different sites of phosphorylation. These observations suggest that the functional properties of caldesmon may be controlled via different phosphorylation pathways. The effects of phosphorylation on the known properties of caldesmon are certainly of interest and will be discussed in a succeeding chapter.

Although the precise physiological role of caldesmon in the contractile apparatus has not been completely elucidated, this protein, or a closely related polypeptide, has been identified in a wide variety of tissue and cell types (Bretscher and Lynch, 1985; Owada et al., 1984; Sobue et al., 1985). As discussed earlier, there are two forms of caldesmon which are similar with respect to immunological cross-reactivity, heat stability, actin-binding and calmodulin-binding characteristics (reviewed by Bretscher, 1986). Despite their shared functional properties, the two species of caldesmon differ greatly in molecular weight. The results in this chapter identify both forms of caldesmon from either avian or bovine sources as substrates for protein kinase C, suggesting that the two classes of protein may also be subject to similar regulatory mechanisms. A phosphopeptide map of caldesmon$_{72}$ from chicken liver is a subset of that obtained with the larger chicken gizzard caldesmon$_{150}$ molecule. This result cannot establish the molecular basis of the similarities or differences between the two species of caldesmon, but does demonstrate that the two proteins have regions of homology at their sites of phosphorylation. These results are

confirmed by phosphopeptide maps obtained with phosphopeptides from caldesmon$_{77}$ and caldesmon$_{150}$ from bovine liver. One explanation for the similarity between phosphopeptide maps of the two caldesmon forms is that the smaller caldesmon species is simply generated by proteolytic degradation of the larger species. However, the results of others (Dingus et al., 1986) and Dr. Ball in this laboratory (personal communication) clearly demonstrate this not to be the case.

As was observed with vinculin and talin in the previous chapter, less than stoichiometric phosphorylation of the caldesmon species was achieved. Chicken gizzard caldesmon was phosphorylated on three major sites and a number of minor sites to a level of 2.3 mol phosphate/mol protein. However, significantly higher phosphate incorporation was achieved by Umekawa and Hidaka (1985). It seems likely that higher phosphorylation of the minor sites may have occurred in their study. Measurable quantities of phosphate can be detected on caldesmon as isolated from chicken gizzard, but the quantities (0.21-0.26 mol phosphate/mol protein) are too low to explain the variation between our results and those of Umekawa and Hidaka (1985). In the case of bovine liver caldesmon$_{77}$, phosphate incorporation slightly exceeded one mol phosphate/mol protein while peptide maps clearly showed two major sites of phosphorylation. Again, this observation may result from the isolation of a partially phosphorylated form of the protein. Alternatively, phosphopeptide maps may be complicated by incomplete digestion of certain phosphorylation sites as discussed in the preceding chapter. The reasons for the apparent low stoichiometry of phosphorylation that we

observe are unclear, but it can be noted that the level of *in vitro* phosphorylation observed with protein kinase C is frequently less than one mol phosphate/mol substrate protein (Werth *et al.*, 1983; Kawamoto and Hidaka, 1984).

The results described in this chapter support the hypothesis that protein kinase C is involved in modulation of the contractile response. Myosin light chain kinase and various forms of the calmodulin-binding protein caldesmon can be added to the list of important components of the contractile apparatus that are targets for protein kinase C (reviewed by Nishizuka, 1986; Sellers and Adelstein, 1987). In establishing the physiological significance of the action of protein kinase C, it will be of interest to examine whether or not the reactions that have been described in this chapter happen in intact living cells. In addition, studies on the effects of phosphorylation on substrate proteins will be central to elucidation of the molecular mechanisms involved in regulation by protein kinase C.

CHAPTER 4

PHOSPHORYLATION OF CYTOSKELETAL PROTEINS IN INTACT HUMAN PLATELETS

4.1 INTRODUCTION

Protein phosphorylation plays an extremely important role in the regulation of many cellular processes (reviewed by Krebs, 1986; Krebs and Beavo, 1979). In developing a complete understanding of a particular phosphorylation reaction and its involvement in cellular regulation, *in vitro* experiments conducted using purified protein kinase and purified protein substrate have proven very useful. However, many reactions that occur under *in vitro* conditions do not occur in living cells. Thus, to demonstrate that a particular phosphorylation event is of physiological significance, the reaction must also be shown to occur in living cells.

The results presented in the preceeding chapters have demonstrated that the focal contact proteins vinculin and talin are *in vitro* substrates for protein kinase C, as are the calmodulin-binding proteins myosin light chain kinase and caldesmon. Of these proteins, vinculin is the only one that had been identified in published reports as a probable substrate for protein kinase C in living cells (Werth and Pastan, 1984). Thus, the objective of the work described in this chapter was to extend our *in vitro* observations by examining the role of protein kinase C in phosphorylating the three other cytoskeletal proteins in intact cells. In living cells protein kinase C is the receptor for, and can be directly activated by, tumour-promoting phorbol esters such as TPA

(Ashendel, 1985; Castagna et al., 1982; Niedel et al., 1983). For this reason, this compound has been extensively utilized to study the involvement of protein kinase C in the phosphorylation of a number of different proteins in a variety of intact cell systems.

Platelets were chosen as the experimental cell system for a number of reasons. They contain high levels of protein kinase C (Kikkawa et al., 1983a) and can be rapidly isolated from fresh whole blood (Baenziger and Majerus, 1974; Lyons et al., 1975). The response of platelets to TPA has been well characterized (Chiang et al., 1981; White et al., 1974; Carroll et al., 1982), and results in the phosphorylation of two major substrate proteins, the regulatory light chain of myosin (P20) (Naka et al., 1983) and the 5'-phosphomonoesterase specific for inositol triphosphate (P47) (Connolly et al., 1986; Imaoka et al., 1983; Kaibuchi et al., 1983). In addition, platelet forms of the cytoskeletal proteins talin (Collier and Wang, 1982a; Collier and Wang, 1982b; O'Halloran et al., 1985), myosin light chain kinase (Hathaway and Adelstein, 1979) and caldesmon (Dingus et al., 1986) have all been identified.

As a prerequisite for examining the phosphorylation of cytoskeletal proteins in TPA-treated platelets, a method must be available for the identification and isolation of the protein of interest. Talin was recently identified as the major platelet protein P235 (O'Halloran et al., 1985). This highly abundant protein could be readily seen on, and excised from, one-dimensional SDS-polyacrylamide gels for further analysis. Caldesmon, which is considerably less abundant than P235 in platelets, could not be

identified solely on one-dimensional SDS-polyacrylamide gels. Immunoprecipitation with affinity-purified rabbit antibodies prepared against chicken gizzard caldesmon was used to isolate caldesmon$_{77}$. The phosphorylation of myosin light chain kinase in intact platelets was not examined since this protein could not be unambiguously identified on SDS-polyacrylamide gels, nor were useful antibodies available. The results that are presented in this chapter describe the TPA-induced phosphorylation of the cytoskeletal proteins talin and caldesmon$_{77}$ in intact human platelets.

4.2 MATERIALS AND METHODS

4.2.1 Materials

TPA was purchased from Consolidated Midland Corporation, and 4α-PDD and thrombin were from Sigma. A23187 (Ca^{2+} ionophore) was obtained from Calbiochem and ^{32}Pi (carrier free) from New England Nuclear. Other reagents and chemicals were obtained as previously described or were of analytical grade.

4.2.2 Platelet Preparation and Phosphorylation

Washed human platelets were prepared from fresh whole blood (40 ml) according to the method of Baenziger and Majerus (1974) using EDTA as anticoagulant. To prevent platelet activation, all manipulations were done with plastic labware at room temperature unless specified. Briefly, whole blood was spun for 3 minutes at 1400 X g to remove erythrocytes. The supernatant (platelet rich plasma) was centrifuged at 2250 X g for 15 minutes to pellet the platelets. The platelets were then resuspended with platelet wash-

buffer (0.113 M NaCl, 4.3 mM K_2HPO_4, 4.3 mM Na_2HPO_4, 24.4 mM NaH_2PO_4, 5.5 mM glucose, pH 6.5) and centrifuged at 120 X g for 7 minutes to remove contaminating leukocytes and erythrocytes. To recover platelets which may have been pelleted by this procedure, the 120 X g pellet was resuspended in platelet wash buffer and the spin repeated. The supernatants from the two 120 X g spins were combined and centrifuged at 2000 X g for 15 minutes. The platelet pellet was washed once with platelet wash buffer and resuspended in phosphate-free resuspension buffer (15 mM Tris-Cl, pH 7.5, 0.14 M NaCl, 5.5 mM glucose, 0.3% bovine serum albumin) and incubated for 15 minutes at room temperature. The platelets were pelleted, resuspended once again in phosphate-free resuspension buffer and incubated for another 15 minutes at room temperature. In preparation for phosphate labeling, the platelets were then pelleted and resuspended in phosphate-free resuspension buffer at a concentration of 2 X 10^9 platelets/ml. The platelets were then incubated with ^{32}Pi (1 mCi/ml) for 1 hour at 37°C as outlined by Lyons et al. (1975). Following phosphate labeling, the platelets were washed and treated as indicated with TPA (1 μM in DMSO), 4α-PDD (1 μM in DMSO), A23187 (0.4 μM in DMSO), DMSO alone, or thrombin (1 unit/ml) for the appropriate length of time at room temperature. In those experiments in which platelet phosphorylation was done in the presence of leupeptin, platelets were pre-incubated with leupeptin (0.05 mg/ml) for 10 minutes at room temperature prior to addition of TPA or 4α-PDD.

4.2.3 Determination of Phosphorylation of P20, P47 and P235.

Following treatment of platelets as described above for the

appropriate length of time, an aliquot of the platelet suspension was immediately boiled in sample buffer for SDS-polyacrylamide gel electrophoresis. Aliquots of the extract were subjected to electrophoresis and autoradiography. Levels of phosphorylation were determined by densitometric scanning (LKB Ultroscan XL Laser Densitometer) of the autoradiograms (Garrison, 1983). The phosphorylation of P20 and P47 was monitored using 12% or 6-15% linear gradient polyacrylamide gels. P235 phosphorylation was monitored following electrophoresis on 6% polyacrylamide gels.

4.2.4 Immunoprecipitation

Platelets labeled as described above were lysed by the addition of SDS-polyacrylamide gel sample buffer to give 1% SDS and 1.67% 2-mercaptoethanol (final concentrations) followed by immediate boiling for 2 minutes. The extract was then diluted with 9 volumes of SDS-free immunoprecipitation buffer (1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 20 mM sodium phosphate, pH 7.4) containing protease and phosphatase inhibitors (2 mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4, 1 mM PMSF and 5 µg/ml leupeptin) in preparation for immunoprecipitation which was conducted essentially by the method of Sefton et al. (1978). Caldesmon$_{77}$ was immunoprecipitated using affinity-purified rabbit antibodies which had been raised against chicken gizzard caldesmon.

4.2.5 Determination of Platelet Caldesmon$_{77}$ Phosphorylation

Immunoprecipitates, obtained as described above, were electrophoresed on 6-15% SDS-polyacrylamide gels. The caldesmon$_{77}$ band was visualized by staining with Coomassie blue, excised, and

digested overnight with 30% H_2O_2 at 70°C so that ^{32}Pi content could be determined by scintillation counting.

4.2.6 Other Procedures

All other procedures have been described in previous chapters.

4.3 RESULTS

4.3.1 Analysis of Platelet Proteins by SDS-Polyacrylamide Gel Electrophoresis

Platelets have a characteristic protein profile when subjected to SDS-polyacrylamide gel electrophoresis (figure 4.1, lane 1). Among the proteins that can be identified on this gel are a number of contractile and cytoskeletal proteins including actin (Mr 43,000), vinculin (Mr 130,000), myosin (Mr 200,000), P235 (Mr 235,000) and actin-binding protein (Mr 250,000). Of particular interest to our studies is P235 (marked with arrow) which has recently been identified as the platelet form of the focal contact protein talin (O'Halloran et al., 1985). It is not clear why platelets should contain such an abundance of talin, but it makes platelets an excellent system in which to examine talin phosphorylation.

4.3.2 Phosphorylation of P235, P47 and P20 in Human Platelets

To examine the phosphorylation of P235 in living cells, washed human platelets were loaded with ^{32}Pi and treated with TPA (1 μM), a tumour-promoting phorbol ester known to directly activate protein kinase C in living cells (Castagna et al., 1982). Phosphorylated proteins were visualized by SDS-polyacrylamide gel electrophoresis (figure 4.2, panel A) and autoradiography (figure 4.2, panel B). As

FIGURE 4.1

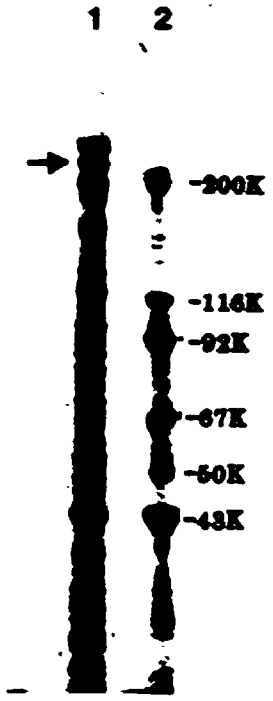
SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF HUMAN PLATELETS.

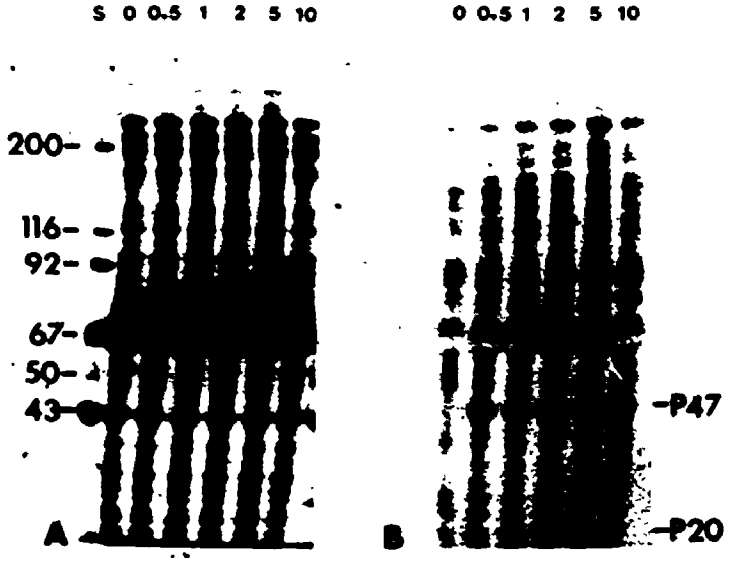
Intact human platelets were extracted with SDS-gel sample buffer and electrophoresed on a 6-15% SDS-polyacrylamide gel which was stained with Coomassie blue. Lane 1, platelets; lane 2 molecular weight markers. The position of P235 is indicated with an arrow.

FIGURE 4.2

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS AND AUTORADIOGRAPHY OF PHOSPHATE-LABELLED PLATELETS TREATED WITH TPA.

Intact human platelets were incubated for 1 hour at 37°C with ^{32}Pi at 1.0 mCi/ml as described in Materials and Methods. Unincorporated ^{32}Pi was then washed away and the platelets treated with TPA (1 μM). At each time point (in minutes following addition of TPA) indicated at the top of each lane, aliquots of the platelets were immediately boiled in SDS-gel sample buffer. These samples were electrophoresed on a 6-15% SDS-polyacrylamide gel which was stained with Coomassie blue (panel A) and then autoradiographed (panel B). Molecular weight markers were run in the lane marked S. The positions of platelet proteins P47 and P20 are indicated.


1
2
-200K
-116K
-92K
-67K
-50K
-43K


S 0 0.5 1 2 5 10
0 0.5 1 2 5 10
200-
116-
92-
67-
50-
43-
-P47
-P20
A
B

seen by a number of other investigators (Naka et al., 1983; Castagna et al., 1982), the platelet proteins P47 and P20 were significantly phosphorylated following TPA-treatment. On the autordiogram of the gel, visualization of P235 phosphorylation was complicated by the observation that myosin (Mr 200,000) and actin binding protein (Mr 250,000) are both phosphorylated following TPA treatment. Therefore, P235 phosphorylation was analyzed on a 6% SDS-polyacrylamide gel (not shown) which enhanced the separation between P235 and the neighbouring myosin and actin-binding protein bands.

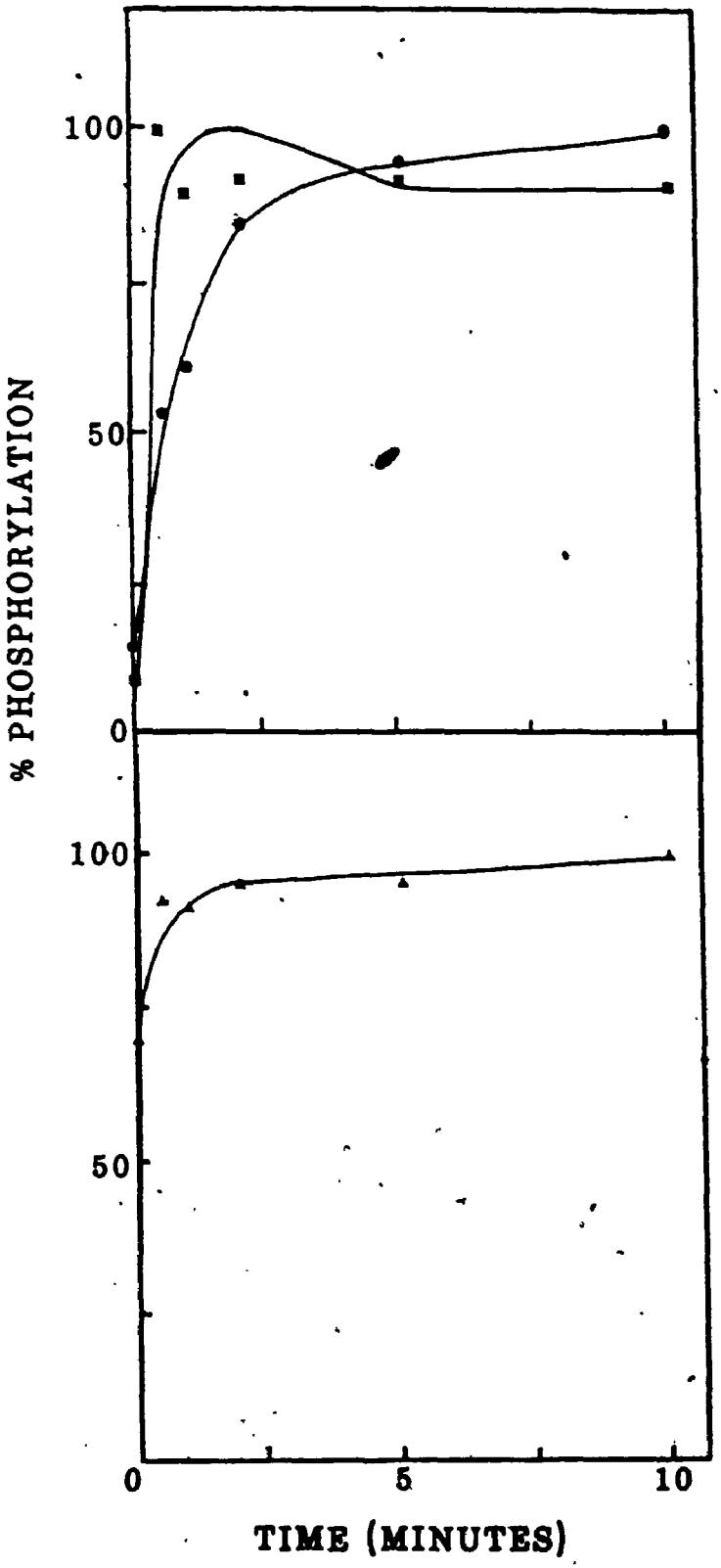
By densitometric scanning of autoradiograms, the time course of phosphorylation of P235, P20 and P47 was measured (figure 4.3). As is evident from figure 4.3 (upper panel), P47 reached maximal levels of phosphorylation after only ~~30~~ seconds of TPA treatment. These results are similar to the findings of Castagna et al., (1983). By comparison, P20 phosphorylation occurs more slowly, approaching maximal levels of phosphorylation after 5 minutes of TPA treatment in agreement with the results of Naka et al. (1983). The time course of P235 phosphorylation (lower panel) most closely resembles that of P47 since the most significant increase in the ^{32}P content of P235 occurred within the first 30 seconds of TPA treatment. Unlike P47 the phosphorylation of P235 continued to increase very slightly for the duration of the experiment.

By SDS-polyacrylamide gel electrophoresis it is obvious that on the basis of protein levels, P235 is a more abundant constituent of platelets than is P47 or P20. However, by autoradiography, the increase in phosphorylation of P235 is barely detected under

FIGURE 4.3

TIME DEPENDENCE OF PHOSPHORYLATION OF P235, P20 AND P47 IN INTACT PLATELETS.

Platelets were labeled with ^{32}Pi and treated with TPA as described in Materials and Methods. At the indicated times, an aliquot of the TPA-treated platelets was immediately boiled in gel sample buffer. Aliquots of this extract were applied to a 12% SDS-polyacrylamide gel and to a 6% SDS-polyacrylamide gel which were subsequently autoradiographed. P235 phosphorylation (lower panel) (▲) was determined by densitometric scanning of the autoradiogram of the 6% gel. Phosphorylation of P20 (●) and P47 (■) (upper panel) was determined by densitometric scanning of the autoradiogram of the 12% gel. The levels of phosphorylation are expressed as a percentage of the maximal level observed for each protein and represent the average of duplicate determinations.


100
50
0
% PHOSPHORYLATION
100
50
0
5
10
TIME (MINUTES)

conditions where the phosphorylation of P47 or P20 is highly noticeable. In fact, when platelets are treated with TPA the increase in phosphorylation of P47 and P20 is several-fold, whereas the phosphate content of P235 increases by only 44%. These results imply that although the phosphate content of P235 increases in TPA-treated platelets, it is relatively poorly phosphorylated in comparison to P47 or P20.

4.3.3 Peptide Mapping of P235 Phosphorylated in Intact Platelets

The examination of P235 phosphorylation in intact human platelets was extended by two-dimensional phosphopeptide mapping (figure 4.4). When phosphorylated P235 is obtained from TPA-treated platelets (panel B), or from untreated (panel A) platelets, the protein contains the same two major sites of phosphorylation; In addition to the two major phosphopeptides, the P235 maps contain a number of minor phosphopeptides. Comparison of the two phosphopeptide maps reveals that there is a cluster of minor spots which is selectively enhanced in P235 from TPA-treated platelets. This observation confirms that P235 is indeed selectively phosphorylated in platelets in response to TPA treatment. However, since those spots that are selectively enhanced in response to TPA are relatively minor, it appears as if only a small fraction of the total cellular P235 is newly phosphorylated. Thus, as was previously suggested (section 4.3.2), it does not appear that P235 is a very good substrate for protein kinase C.

It was not possible to make meaningful comparisons between tryptic phosphopeptide maps of P235 phosphorylated in TPA-treated

FIGURE 4.4

TWO-DIMENSIONAL PHOSPHOPEPTIDE MAPPING OF P235 PHOSPHORYLATED IN INTACT PLATELETS.

Following electrophoresis of phosphate-labelled platelets on 6% SDS-polyacrylamide gels, the P235 band was extensively digested with trypsin. The phosphopeptides were then loaded on thin layer plates at the origin (O) and subjected to electrophoresis at pH 1.9 prior to ascending chromatography as previously described. Panel A, P235 from platelets not treated with TPA; panel B, P235 from TPA-treated platelets.

platelets and talin phosphorylated by protein kinase C *in vitro* (chapter 2) since P235 was isolated from mammalian cells and talin from avian tissue.

4.3.4 Immunoprecipitation of Caldesmon$_{77}$ from Intact Human Platelets

Although a number of cytoskeletal proteins can be identified by one-dimensional SDS-polyacrylamide gel electrophoresis of platelets, the calmodulin-binding protein caldesmon cannot. Thus, as a preliminary step in examining the phosphorylation of platelet caldesmon, the protein was isolated from intact human platelets by immunoprecipitation with affinity-purified rabbit antibodies against chicken gizzard caldesmon. Following immunoprecipitation (figure 4.5, panel A, lane 3), a number of bands can be visualized by staining with Coomassie blue. To establish the identity of caldesmon, the Western blotting technique was utilized (figure 4.5, panel B). In extracts of whole platelets (lane 1) and in immunoprecipitates obtained with affinity-purified anti-caldesmon (lane 3), a single band with Mr 77,000 is detected. This result confirms that platelets contain a single form of caldesmon (Dingus *et al.*, 1986) and demonstrate that immunoprecipitation brings down this protein.

4.3.5 Phosphorylation of Caldesmon$_{77}$, P47 and P20 in Human Platelets

Examination of the phosphorylation of caldesmon$_{77}$ by protein kinase C in living cells was conducted using washed human platelets which were loaded with ^{32}Pi and treated with TPA (1 μM). Immunoprecipitates of ^{32}P-labeled platelet extracts were subjected to

FIGURE 4.5

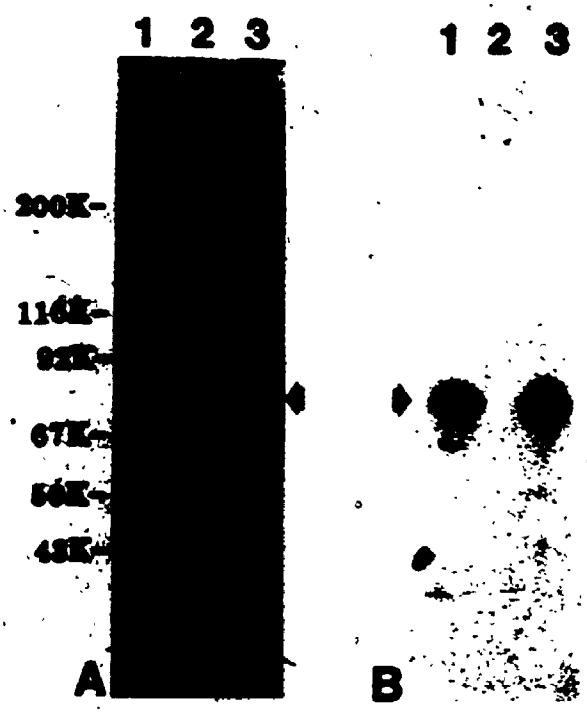
WESTERN BLOT OF PLATELET PROTEINS IMMUNOPRECIPITATED WITH AFFINITY-PURIFIED ANTI-CALDESMON ANTIBODIES.

Platelets were extracted with immunoprecipitation buffer and the resultant lysate was immunoprecipitated with affinity-purified rabbit anti-caldesmon antibodies as previously described. Aliquots of the platelet lysate and the immunoprecipitate were run on a 6-15% polyacrylamide gel (panel A) and transferred to nitrocellulose. The transferred proteins were probed with affinity-purified anti-caldesmon antibodies (panel B). Lane 1, platelets extracted with SDS-gel sample buffer; lane 2, molecular weight markers; lane 3, immunoprecipitated proteins. The position of caldesmon$_{77}$ is marked with an arrow.

FIGURE 4.6

IMMUNOPRECIPITATION OF CALDESMON$_{77}$ FROM INTACT HUMAN PLATELETS.

Platelets labeled with ^{32}Pi for 1 hour at 37°C as described under Materials and Methods were incubated in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of TRA for 10 minutes. The platelet lysates were subjected to immunoprecipitation using either affinity-purified rabbit anti-caldesmon antibodies (lanes 1 and 2) or nonimmune rabbit antibodies (lanes 3 and 4). The immunoprecipitates were run on a 6-15% SDS-polyacrylamide gel which was stained with Coomassie blue (panel A) and autoradiographed (panel B). The position of caldesmon$_{77}$ is marked with an arrowhead. Molecular weight markers (lane 5) are as follows: myosin heavy chain, 200,000; β-galactosidase, 116,000; phosphorylase b, 92,000; bovine serum albumin, 67,000; immunoglobulin heavy chain, 50,000; actin, 43,000.


1 2 3
1 2 3
200K-
116K-
92K-
67K-
50K-
43K-
A
B


1 2 3 4 5
1 2 3 4 5

SDS-polyacrylamide gel electrophoresis (figure 4.6, panel A) and phosphoproteins identified by autoradiography (figure 4.6, panel B). Four protein bands were specifically immunoprecipitated using affinity-purified anti-caldesmon antibodies (panel A, lanes 1,2) in addition to those bands that were non-specifically immunoprecipitated with antibodies from pre-immune serum (panel A, lanes 3,4). Approximately equal amounts of protein were immunoprecipitated with affinity-purified anti-caldesmon antibodies from TPA-treated platelets (panel A, lane 1) and from DMSO-treated platelets (panel A, lane 2). Analysis of the accompanying autoradiogram reveals that the four proteins that are immunoprecipitated with anti-caldesmon antibodies are all phosphoproteins in TPA-treated (panel B, lane 1) and DMSO-treated (panel B, lane 2) platelets. No phosphoproteins were immunoprecipitated from TPA-treated (panel B, lane 3) or DMSO-treated platelets (panel B, lane 4) using antibodies from pre-immune serum.

Although four phosphoproteins were immunoprecipitated with affinity-purified anti-caldesmon antibodies, only the protein with Mr 77,000 (marked by arrow) reacted with anti-caldesmon on Western blots (section 4.3.4). In addition, by phosphopeptide mapping (not shown), no relationship between the four proteins was detected. These results suggest that caldesmon$_{77}$ is immunoprecipitated from platelets in a complex containing three other unrelated polypeptides. Examination of the 77,000 dalton protein on the autoradiogram (figure 4.6, panel B) reveals that incorporation of ^{32}P into caldesmon$_{77}$ is enhanced nearly 4-fold in TPA-treated platelets (lane 1) relative to

DMSO-treated platelets (lane 2). Lesser increases in ^{32}P content are observed for the three other phosphoproteins.

The time course of caldesmon$_{77}$ phosphorylation was determined in TPA-treated platelets (figure 4.7, lower panel) as were the time courses of P47 and P20 phosphorylation (figure 4.7, upper panel). For P47 and P20, the time courses of phosphorylation were similar to those previously observed (section 4.3.2). Rapid phosphorylation of P47 resulted in maximal phosphorylation within 1 minute of TPA treatment, while P20 phosphorylation occurred more slowly reaching maximal levels after 5 minutes of TPA treatment. Caldesmon phosphorylation occurred even more slowly than did P20 phosphorylation and did not reach its highest measured level until the platelets had been treated with TPA for 10 minutes.

4.3.6 Peptide Mapping and Phosphoamino Acid Analysis of Caldesmon$_{77}$ Phosphorylated in Intact Platelets

Phosphopeptides were prepared from caldesmon$_{77}$ isolated by immunoprecipitation from TPA-treated (figure 4.8, panel B) or untreated (figure 4.8, panel A) human platelets and subjected to two-dimensional mapping. To compare the sites of caldesmon$_{77}$ that are phosphorylated in TPA-treated mammalian platelets with those that are phosphorylated by protein kinase C *in vitro*, a phosphopeptide map of mammalian caldesmon$_{77}$ (figure 4.8, panel D) which was phosphorylated *in vitro* by protein kinase C (section 3.3.1i) is also shown.

The phosphopeptide map of caldesmon$_{77}$ from untreated platelets (panel A) shows two sites of phosphorylation which are not the same as either of the two major sites phosphorylated on bovine liver

FIGURE 4.7

TIME DEPENDENCE OF PHOSPHORYLATION OF CALDESMON$_{77}$, P20 AND P47 IN INTACT PLATELETS.

Platelets were labeled with ^{32}Pi and treated with TPA as previously described. At the indicated times, an aliquot of the TPA-treated platelets was immediately boiled in gel sample buffer. An aliquot of this extract was applied to a 6-15% SDS-polyacrylamide gel for the determination of P20 and P47 phosphorylation. The remainder of the extract was immunoprecipitated using affinity-purified rabbit anti-caldesmon antibodies prior to SDS-polyacrylamide gel electrophoresis and autoradiography. Caldesmon$_{77}$ phosphorylation (▲) was measured by scintillation counting of the excised band from SDS-polyacrylamide gel electrophoresis (lower panel). The phosphorylation of the P20 (○) and P47 (●) was determined by densitometric scanning of autoradiograms (upper panel). The levels of phosphorylation are expressed as a percentage of the maximal level observed for each protein and represent the average of duplicate determinations.

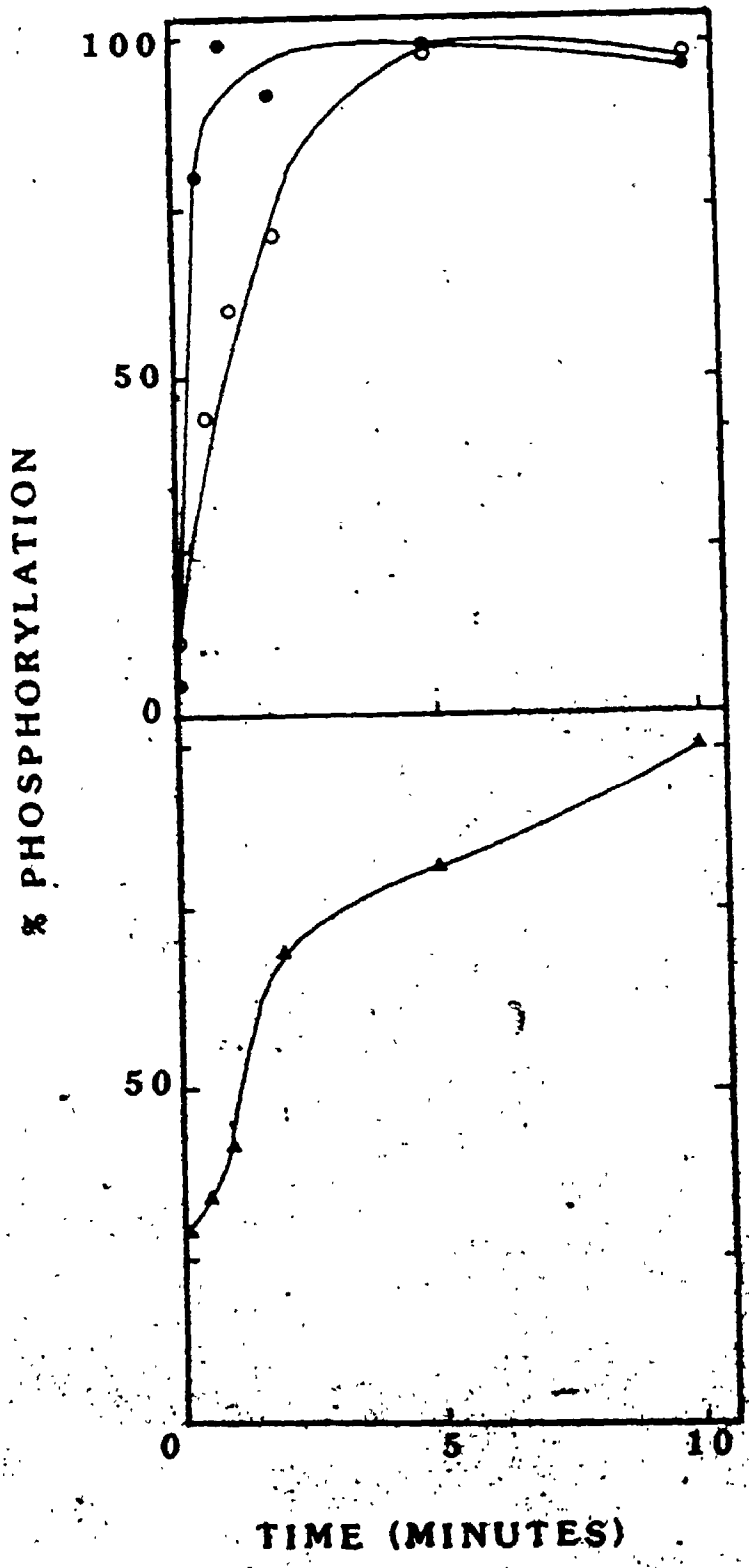
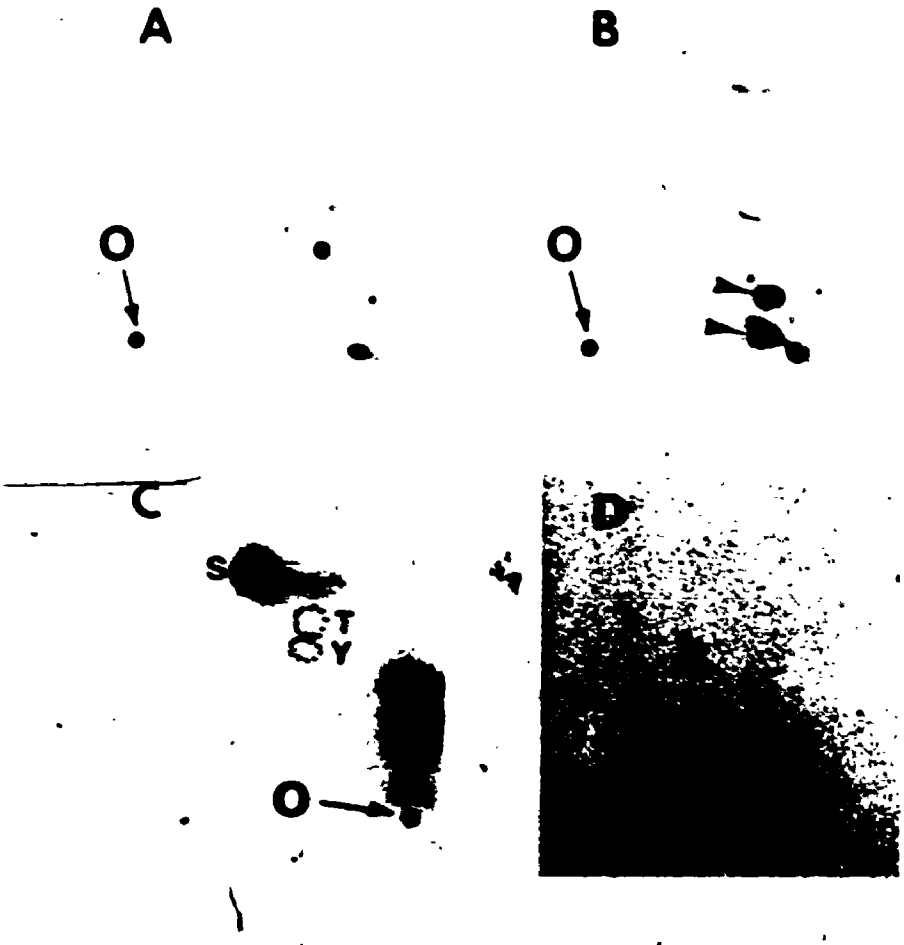

100
50
0
50
0
5
10
% PHOSPHORYLATION
TIME (MINUTES)

FIGURE 4.8

TWO-DIMENSIONAL PHOSPHOPEPTIDE MAPS AND PHOSPHOAMINO ACID ANALYSIS OF CALDESMON$_{77}$ PHOSPHORYLATED IN INTACT PLATELETS.

Following immunoprecipitation of caldesmon$_{77}$ from platelets which were incubated with (panel B) or without (panel A) TPA, the caldesmon$_{77}$ band was extensively digested with trypsin. The phosphopeptides were applied to thin layer plates at the origin (O) and subjected to electrophoresis and ascending chromatography as previously described. Caldesmon$_{77}$ phosphopeptides from TPA-treated platelets were also subjected to partial acid hydroylsis in preparation for phosphoamino acid analysis (panel C). For comparison purposes, a phosphopeptide map of bovine liver caldesmon$_{77}$ phosphorylated by protein kinase C <u>in vitro</u> is also shown (panel D).


A
B
O
O
C
S
CT
CY
O
D

caldesmon$_{77}$ by protein kinase C *in vitro* (panel D). Following TPA-treatment (panel B), a phosphopeptide map of platelet caldesmon$_{77}$ shows two new major phosphopeptides (marked with arrowheads). These two phosphopeptides have the same migration pattern as the two major phosphopeptides of bovine liver caldesmon$_{77}$ which was phosphorylated *in vitro* by protein kinase C. In addition, caldesmon$_{77}$ in TPA-treated platelets was phosphorylated exclusively on serine residues (panel C), as was bovine liver caldesmon$_{77}$ which was phosphorylated *in vitro* (figure 3.19, panel C). On the basis of these results, it is apparent that caldesmon$_{77}$ is phosphorylated on the same two peptides by protein kinase C *in vitro* and in TPA-treated platelets.

4.3.7 *Effects of Various Agents on the Phosphorylation of Caldesmon, P47 and P20 in Intact Platelets*

As a first step in establishing the role of caldesmon$_{77}$ phosphorylation in different aspects of platelet activation, the effects of various agents on the phosphorylation of this protein in intact human platelets was investigated (table 4.1). As expected, when platelets were physiologically activated by treatment with thrombin, P47 and P20 were rapidly phosphorylated (Haslam and Davidson, 1984; Sano *et al.*, 1983; Lyons *et al.*, 1975). In addition, after 5 minutes of thrombin treatment caldesmon$_{77}$ phosphorylation was enhanced by 18%. Although this enhancement of caldesmon$_{77}$ phosphorylation is only approximately 5% of that seen with TPA treatment, phosphopeptide maps of caldesmon$_{77}$ (not shown) from thrombin-treated platelets suggest that protein kinase C is responsible for the elevation in phosphorylation.

TABLE 4.1

PHOSPHORYLATION OF PLATELET PROTEINS IN RESPONSE TO VARIOUS AGENTS.

Addition	Time	Caldesmon$_{77}$	P47	P20
	(minutes)	(Relative Phosphorylation)		
none	5.0	1.00	1.00	1.00
4α-PDD	5.0	1.00	1.00	1.00
TPA	5.0	2.96	24.6	3.15
A23187	0.5	0.88	13.9	3.55
A23187	5.0	1.04	13.6	3.19
TPA + A23187	0.5	1.55	19.5	4.30
TPA + A23187	5.0	3.53	20.0	5.03
Thrombin	0.5	0.97	24.7	3.80
Thrombin	5.0	1.18	22.1	3.37

Platelets were loaded with ^{32}Pi and treated with the indicated agent for the indicated length of time. Phosphorylation of caldesmon$_{77}$, P47 and P20 were determined as indicated in Materials and Methods. The levels of phosphorylation are expressed in relation to the phosphate content of the protein measured without treatment of the platelets. The values represent the average of duplicate determinations.

The action of thrombin on platelets is mediated via at least two distinct molecular pathways (Sano et al., 1983; Kaibuchi et al., 1983; Kaibuchi et al., 1982). One pathway involves activation of protein kinase C and the second pathway is triggered by the mobilization of calcium. As was previously observed (section 4.3.5), activation of the protein kinase C pathway by TPA results in enhanced phosphorylation of caldesmon$_{77}$, P47 and P20. In contrast, treatment with the inactive phorbol ester 4α-PDD does not result in elevated phosphorylation of any of the three proteins. To examine the role of the calcium mobilization pathway in the phosphorylation of caldesmon$_{77}$, platelets were treated with the calcium ionophore A23187 (0.4 μM). Treatment with this agent did not stimulate caldesmon$_{77}$ phosphorylation, but as expected did trigger near complete phosphorylation of P20 and partial phosphorylation of P47 (Haslam et al., 1979; Kaibuchi et al., 1983). This result suggests that in platelets, caldesmon$_{77}$ may not be a substrate for a calcium/calmodulin dependent protein kinase. However, since P47 is partially phosphorylated in ionophore-treated platelets it is possible that it is a substrate for a calcium/calmodulin-dependent kinase. When platelets were treated with a combination of TPA and A23187, caldesmon$_{77}$ phosphorylation was enhanced to a level that was similar to that seen in platelets treated with TPA alone. Furthermore, phosphopeptide maps of caldesmon$_{77}$ from TPA- and A23187-treated platelets (not shown) were indistinguishable from those treated with TPA alone.

When cells, including platelets, are treated with TPA, protein

kinase C translocates to the plasma membrane where it is initially activated by membrane phospholipids and subsequently degraded by proteolysis (Kraft and Anderson, 1983; Tapley and Murray, 1984; Tapley and Murray, 1985; Melloni et al., 1986). Although the actual mechanism of protein kinase C degradation is not fully understood, there is some evidence to suggest that proteolysis initially produces a catalytically active fragment of protein kinase C that is fully active in the absence of phosphatidylserine and calcium (Kishimoto et al., 1983; Tapley and Murray, 1985; Melloni et al., 1986). To investigate the role of this fragment in the phosphorylation of caldesmon$_{77}$, platelets were preloaded with the protease inhibitor leupeptin which was shown to block the conversion of protein kinase C to its catalytically active fragment in platelets (Tapley and Murray, 1985) and in neutrophils (Melloni et al., 1986). In those platelets that were preloaded with leupeptin, the majority of caldesmon$_{77}$ phosphorylation is not prevented since the level of phosphorylation observed in response to TPA was 89% of that observed in platelets that had not been treated with leupeptin (results not shown). Similarly, the phosphorylation of P20 in leupeptin-treated platelets was nearly as high as that seen in platelets not treated with leupeptin, with the level of phosphorylation in the former reaching 83% of that observed in the latter. The level of P47 phosphorylation was altered by less than 2% in TPA-treated platelets that had been pre-incubated with leupeptin. These preliminary results imply that the proteolytic conversion of protein kinase C to its catalytically active fragment is not the major event responsible for the

phosphorylation of caldesmon$_{77}$ in TPA-treated platelets. However, more thorough experimentation is clearly required to permit the definitive elucidation of the role of the proteolytic activation of protein kinase C in the phosphorylation of its substrate proteins.

4.4 DISCUSSION

One requirement to demonstrate that a particular phosphorylation event is of physiological significance is to show that the reaction occurs in living cells (Krebs and Beavo, 1979). By using the tumour-promoting phorbol ester TPA to activate protein kinase C in intact human platelets (Castagna et al., 1982), we have studied the phosphorylation in living cells of talin and caldesmon, two of the cytoskeletal proteins that we identified as in vitro substrates for protein kinase C (chapters 2 and 3).

Although the functional characteristics of talin and its platelet counterpart P235 are not well understood, the similar structural and physical properties of the two proteins suggest that the two proteins may be subject to the same means of control (Collier and Wang, 1982a; Burridge and Connell, 1983b; O'Halloran et al., 1985). Since we had previously identified chicken gizzard talin as an in vitro substrate for protein kinase C (chapter 2), it was of interest to demonstrate that the phosphorylation of the platelet counterpart of this protein increases when cells are treated with TPA.

On the basis of a number of criteria the phosphorylation of P235 in platelets appears to be at very low levels. The protein is a

major component of platelets representing up to 8% of total platelet protein (Collier and Wang, 1982a) and can be clearly visualized when whole platelet protein is electrophoresed on one-dimensional SDS-polyacrylamide gels. The ^{32}Pi content of the protein is however much lower than that of proteins such as P47 and P20 that are much less abundant in platelets. In addition, phosphopeptide maps of P235 from TPA-treated platelets do not contain any major phosphopeptides that are not present prior to TPA treatment.

The results certainly demonstrate that the phosphorylation of P235 in TPA-treated platelets has low overall stoichiometry and suggest that the event may be of limited physiological importance. However, it must be taken into consideration that as a result of subcellular compartmentalization only a specific population of P235 may be accessible to protein kinase C. Thus, phosphorylation could be an important regulatory signal even though the overall level of phosphorylation is low. To more fully evaluate this possibility, a better understanding of the localization of P235 inside platelets and its role in physiological events is required. A low stoichiometry of phosphorylation is also observed when talin is phosphorylated on tyrosine residues in RSV-transformed chick embryo fibroblasts (Pasquale et al., 1986).

The observation that the TPA-induced phosphorylation of P235 slowed down significantly after the first 30 seconds of treatment is somewhat puzzling since it was obvious that the protein was not fully phosphorylated. Again, this result may suggest that only a small fraction of total P235 was available to protein kinase C.

Alternatively, since P235 is known to undergo proteolysis during platelet activation, it may be postulated that P235 is also proteolysed in TPA-treated platelets and that phosphorylation of P235 is a prerequisite for its degradation (O'Halloran et al., 1985; Fox et al., 1985). Thus, the decrease in the apparent rate of phosphorylation would actually result from the disappearance of a significant proportion of the phosphorylated protein. Although this proposed scheme has not been experimentally tested in any way, it is not without precedent. In the case of P20, the regulatory light chain of myosin, phosphorylation by protein kinase C has been shown to increase its susceptibility to proteolysis (Pontremoli et al., 1987).

The demonstration that P235 is phosphorylated in response to TPA in platelets, albeit at a low level, implies that the closely related protein talin may by an in vivo substrate for protein kinase C in other cell systems. Although its physiological functions have not been fully characterized, talin is at least partially localized in focal contacts of cultured cells, where it is presumably involved in the attachment of actin filaments to the plasma membrane (Burridge and Connell, 1983a; Burridge and Connell, 1983b). Talin, which has been shown to interact with vinculin and the transmembrane fibronectin receptor in vitro, must be considered to be an important potential regulator of cytoskeletal organization, adhesion and cell morphology (Burridge and Mangeat, 1984; Horwitz et al., 1986). For this reason, further analysis of the in vivo phosphorylation of this protein and an examination of the role of phosphorylation on its

known properties is in order.

In contrast to the low level of TPA-induced phosphorylation that was observed for P235, caldesmon$_{77}$ is significantly phosphorylated in TPA-treated platelets. Although the stoichiometry of caldesmon$_{77}$ phosphorylation in TPA-treated platelets was not determined, the increase in ^{32}P incorporation after 10 minutes of treatment was nearly 4-fold. In addition, the two phosphopeptides that were enhanced by TPA-treatment increased from nearly undetectable levels to become the two major sites of phosphorylation. Our observation that the protein kinase C-specific phosphopeptides of caldesmon$_{77}$ are enhanced slightly following treatment of platelets with thrombin suggests that this phosphorylation event may play a role in physiological processes. Caldesmon phosphorylation is also stimulated in smooth muscle strips that have been treated with carbachol, which induces rapid contraction, or phorbol esters, which activate protein kinase C and induce a slow sustained contraction (Park and Rasmussen, 1986). These observations imply that the phosphorylation of caldesmon may have a role in the regulation of contractile events in a variety of intact cell systems.

One puzzling feature of our analysis of caldesmon$_{77}$ phosphorylation is the observation that affinity-purified anti-caldesmon antibodies specifically immunoprecipitated four bands from platelet lysates. Since tryptic phosphopeptide mapping failed to demonstrate any structural homology and Western blotting failed to reveal immunological cross-reactivity, it is possible that the proteins exist as a complex in the platelet extracts. Alternatively,

the proteins may share a common conformational antigenic determinant that is lost during SDS-polyacrylamide gel electrophoresis and Western blotting. Further analysis is obviously required to resolve these issues.

The phosphorylation of caldesmon$_{77}$ proceeds at a slower rate than does the phosphorylation of P47 or even P20 in TPA-treated platelets. This observation may reflect the intracellular localization of the activated form of protein kinase C with respect to its different substrates. When protein kinase C is activated by tumour-promoting phorbol esters, the enzyme translocates to, and becomes tightly associated with, the plasma membrane (Kraft and Anderson, 1983; Wolf et al., 1985a; Wolf et al., 1985b). Indeed, many of the substrates for protein kinase C are integral or peripheral membrane proteins (reviewed by Nishizuka, 1986). In contrast, caldesmon$_{77}$ and caldesmon$_{150}$ are cytoskeletal proteins that are localized along actin filaments within the cytoplasm (Bretscher and Lynch, 1985; Owada et al., 1984; Sobue et al., 1985).

A key question raised by the phosphorylation of caldesmon$_{77}$ in intact platelets relates to the issue of how a membrane-activated enzyme such as protein kinase C can act on a cytoplasmic substrate. Perhaps the simplest explanation for this observation is that there may be a portion of the total caldesmon$_{77}$ population that is proximal to or associated with the plasma membrane. Indeed, in adrenal chromaffin cells, caldesmon has been identified as a 70,000 dalton protein that undergoes reversible membrane association (Burgoyne et al., 1986). Moreover, by immunofluorescence microscopy, caldesmon

has been detected in ruffling membranes of cultured cells (Bretscher and Lynch, 1985). Alternatively, the slow phosphorylation of caldesmon$_{77}$ and the regulatory light chain in TPA-treated platelets may reflect a necessity for cytoskeletal reorganization prior to phosphorylation by protein kinase C. A third explanation for the phosphorylation of caldesmon$_{77}$ by protein kinase C involves the possible role of a catalytically active fragment of protein kinase C (Kishimoto et al., 1983; Tapley and Murray, 1985; Melloni et al., 1986). In addition to activating protein kinase C, tumour-promoting phorbol esters such as TPA stimulate the degradation of protein kinase C (Tapley and Murray, 1984; Tapley and Murray, 1985; Melloni et al., 1986). A proposed step in this degradation is the generation of a 51,000 dalton fragment of protein kinase C that is fully active in the absence of phospholipid and calcium (Kishimoto et al., 1983; Tapley and Murray, 1985). This fragment, which is no longer membrane-associated, would have the potential to phosphorylate a new set of soluble substrate proteins such as caldesmon$_{77}$. The physiological significance of this proteolytic activation of protein kinase C is somewhat controversial since there has not yet been any demonstration that the fragment exists in vivo (Woodgett and Hunter, 1987a).

The generation of the catalytically fragment of protein kinase C was blocked in platelets (Tapley and Murray, 1985), and in neutrophils (Melloni et al., 1986) by preloading the cells with leupeptin, an inhibitor of the calcium-dependent protease. In a preliminary experiment (section 4.3.7) however, the effect of pre-incubating

platelets with leupeptin prior to TPA treatment was very limited. Although it was presumed from the studies of Melloni et al., (1986) that leupeptin did traverse the plasma membrane, no direct evidence to confirm this presumption was obtained. Therefore, further analysis of this problem is clearly required.

A role for protein kinase C has been proposed for different aspects of platelet activation (Sano et al., 1983; Kaibuchi et al., 1983; Connolly et al., 1986). The phosphorylation of caldesmon$_{77}$ by protein kinase C in intact platelets suggests that this reaction may be involved in the regulation of the contractile response and cytoskeletal rearrangements that accompany platelet activation (Carroll et al., 1982). To more fully understand the role of caldesmon phosphorylation in these processes however, an investigation of the effects of phosphorylation on the known properties of the prptein must be undertaken.

The observation that the phosphorylation of the platelet forms of talin (P235) and caldesmon (caldesmon$_{77}$) are elevated in TPA-treated platelets extends the results that have been previously presepted (chapters 2 and 3). The demonstration that these proteins can be phosphorylated by protein kinase C in living cells lends support to the postulate that their phosphorylation may have regulatory significance in mediating the effects of tumour-promoting phorbol esters on cell morphology and cytoskeletal organization.

CHAPTER 5

THE EFFECTS OF PHOSPHORYLATION OF CHICKEN GIZZARD CALDESMON BY PROTEIN KINASE C

5.1 INTRODUCTION

There are a number of observations that have implicated the calmodulin- and F-actin-binding protein caldesmon as a potential regulator of contractile events or actin filament organization. From *in vitro* studies, it has been established that the interactions of caldesmon with F-actin and calmodulin are regulated by free calcium concentrations (Kakiuchi and Sobue, 1983; Sobue *et al.*, 1982). In the absence of calcium, caldesmon forms a complex with F-actin that can be at least partially disrupted by the addition of calcium/calmodulin (Sobue *et al.*, 1981; Bretscher, 1984). Several investigators have now shown that caldesmon can inhibit the actin-activated ATPase of myosins from smooth or skeletal muscle (Ngai and Walsh, 1984; Dabrowska *et al.*, 1985; Marston and Lehman, 1985; Clark *et al.*, 1986; Lim and Walsh, 1986; Lash *et al.*, 1986; Smith *et al.*, 1987). In addition, the association between actin and heavy meromyosin was markedly enhanced by caldesmon (Lash *et al.*, 1986).

On the basis of *in vitro* findings, Ngai and Walsh (1984) introduced the idea that the phosphorylation of caldesmon by a calmodulin-dependent kinase regulated its inhibitory activity towards the actin-activated ATPase of smooth muscle myosin. In contrast, phosphorylation of caldesmon did not alter its activity towards the actin-activated ATPase of skeletal muscle myosin (Lim and Walsh,

1986). Although their original findings were not substantiated by other investigators (Lash et al., 1986; Smith et al., 1987), Ngai and Walsh (1987) have very recently confirmed and extended their observations. In addition to the effects on the smooth muscle ATPase, phosphorylation of caldesmon by the calmodulin-dependent kinase led to a decrease in associations between caldesmon and F-actin.

Caldesmon has also been identified as a substrate for protein kinase C in vitro (chapter 3; Umekawa and Hidaka, 1985) and in intact human platelets (chapter 4). Furthermore, the tumour-promoting phorbol esters such as TPA which activate protein kinase C in living cells (Castagna et al., 1982) are known to have effects on different aspects of the contractile response (Rasmussen et al., 1984; Park and Rasmussen, 1986). To determine whether or not there is a relationship between these two observations, it is of interest to examine the effects that the phosphorylation of caldesmon by protein kinase C has on the known properties of caldesmon.

The only demonstration that the phosphorylation of caldesmon could alter its functional properties was the observation that phosphorylated caldesmon, but not non-phosphorylated caldesmon, inhibited myosin light chain kinase in vitro (Umekawa and Hidaka, 1985). The objective of our studies was to further characterize the effects of phosphorylation by protein kinase C on the more widely established activities of caldesmon. By examining the role of phosphorylation by protein kinase C on the ability of caldesmon to inhibit the actin-activated ATPase of skeletal muscle myosin and to

associate with F-actin, we sought to determine whether or not this phosphorylation event was of functional significance.

The demonstration that the phosphorylation of caldesmon by protein kinase C does have functional consequences clearly extends those results presented previously (chapters 3,4). Indeed, the phosphorylation of caldesmon by protein kinase C, which occurs *in vitro* and in living cells may mediate some of the cytoskeletal, contractile, and/or morphological alterations that are seen when cells are acted upon by appropriate stimuli.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Rabbit skeletal muscle myosin was obtained from Sigma and [γ-^{32}P] ATP was purchased from ICN Radiochemicals. All other reagents and chemicals were of analytical grade or were obtained as described in previous chapters.

5.2.2 Protein Purifications

Caldesmon was purified from chicken gizzard by denaturing methods (Bretscher, 1984) as described in Chapter 3. Rat brain protein kinase C, and bovine brain calmodulin were also purified as described in Chapter 3. Chicken gizzard tropomyosin which had been purified by the denaturing methods described by Bretscher (1984) was obtained from Dr. E.H. Ball. Rabbit skeletal muscle actin was purified from rabbit skeletal muscle acetone powder according to the method of Pardee and Spudich (1982).

5.2.3 Measurement of Skeletal Muscle Actin-Activated Myosin ATPase

Actin-activated myosin ATPase measurements were conducted in a manner similar to that used by a number of previous investigators (Ngai and Walsh, 1984; Lash et al., 1986; Smith et al. 1987). Assays were conducted at 25°C in reaction mixtures of 0.1 ml containing myosin (0.125 mg/ml), actin (0.25 mg/ml), tropomyosin (0.125 mg/ml) and caldesmon (as indicated) in a solution comprised of Tris-Cl, pH 7.5, 50 mM KCl, 3.5 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 1 mM [γ-^{32}P]ATP (specific activity 1000 cpm/nmol). To ensure complete equilibration of protein components of the assay, myosin, actin, tropomyosin and caldesmon were pre-incubated at 25°C for a minimun of 30 minutes prior to commencement of the ATPase reaction by the addition of ATP. The ATPase reaction was allowed to proceed for 4 minutes at 25°C so that ATP hydrolysis was linear with respect to time and did not exceed 20% of the total ATP. Release of ^{32}P from ATP was measured essentially by the method of Pollard (1982). Briefly, the reaction was terminated by addition of an aliquot (0.05 ml) of acid solution (2.9N sulphuric acid, 4.3% silicotungstic acid, 0.5 mM K_2HPO_4) and vortexing. An aliquot (0.027 ml) of ammonium molybdate (7.5%) was then added and the solution vortexed vigourously for 10 seconds. Finally, the phosphomolybdate complex was extracted by addition of 0.200 ml of isobutanol-benzene (1:1). Following vortexing and centrifugation of the solution for 2 minutes in a Beckman microfuge, an aliquot (0.1 ml) of the upper organic layer was removed for scintillation counting.

5.2.4 Phosphorylation of Caldesmon

Chicken gizzard caldesmon (3 mg/ml) was phosphorylated by protein kinase C as described in Chapter 3 in a total reaction of 0.05-0.1 ml containing 1 mM ATP for 2 hours at 30°C to ensure maximal phosphorylation. Phosphorylated caldesmon was diluted to the desired concentration directly into the actin-activated myosin ATPase reaction mixture. Non-phosphorylated caldesmon was prepared for addition to the ATPase reaction mixture in an identical phosphorylation mixture that contained heat-killed protein kinase C instead of active protein kinase C. For control purposes, phosphorylation mixtures containing all components except caldesmon were also prepared so that the total volume of phosphorylation reaction mixture added to each assay tube was the same, even though the amount of caldesmon added was varied.

To measure the extent of caldesmon phosphorylation, samples containing caldesmon were subjected to SDS-polyacrylamide gel electrophoresis. The caldesmon bands were excised from the gels and digested as previously described with H_2O_2 prior to scintillation counting.

5.2.5 Sedimentation Assay

Interactions between caldesmon and F-actin were monitored in 0.1 ml reaction mixtures containing 25 mM Mes, pH 6.5, 50 mM KCl, 5 mM $MgCl_2$, F-actin (0.5 mg/ml), caldesmon (0.05 mg/ml), calmodulin (0.04 mg/ml) and either $CaCl_2$ (0.2 mM) or EGTA (0.2 mM). Tropomyosin (0.12 mg/ml) was added as indicated. Following incubation for 30 minutes at room temperature, F-actin was pelleted by centrifugation for 20

minutes at 386,000 X g. Samples of the supernatant and pelleted fractions were then subjected to electophoresis on 6-15% SDS-polyacrylamide gels. The relative quantities of actin and caldesmon in the supernatant and pelleted fractions were determined by densitometric scanning of Coomassie blue stained gels with an LKB Ultroscan XL Laser Densitometer. Phosphorylated or non-phosphorylated caldesmon was prepared as described above by incubation with active or heat-killed protein kinase C.

5.2.6 Other Procedures

Protein determinations were done by the Hartree modification (1972) of the method of Lowry et al. (1951) using bovine serum albumin as standard. Other procedures were done as described in previous chapters.

5.3 RESULTS

5.3.1 Reconstitution of Actin-Activated ATPase of Skeletal Muscle Myosin

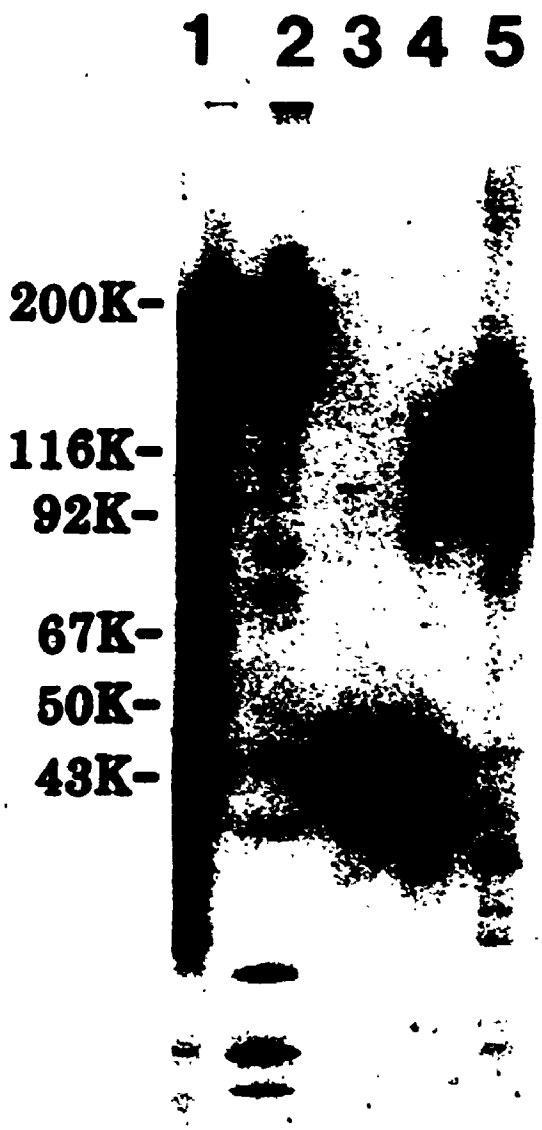
5.3.1.1 Analysis of the Protein Components by SDS-Polyacrylamide Gel Electrophoresis

As a preliminary step in the reconstitution of an actin-activated skeletal muscle myosin ATPase assay system, the protein components of the assay mixture were analysed by subjecting 10 µg of each protein preparation to SDS-polyacrylamide gel electrophoresis (figure 5.1). The preparation of skeletal muscle myosin (Lane 1) contains primarily the myosin heavy chain (200,000 daltons) and the three myosin light chains (all less than 25,000 daltons)(Wagner,

FIGURE 5.1

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF PROTEIN COMPONENTS OF ACTIN-ACTIVATED MYOSIN ATPase ASSAY.

Samples of each protein (10 µg) used in the actin-activated myosin ATPase assay were electrophoresed on a 6-15% polyacrylamide gel. Lane 1, molecular weight markers; lane 2, rabbit skeletal muscle myosin; lane 3, rabbit skeletal muscle actin; lane 4, chicken gizzard tropomyosin; lane 5, chicken gizzard caldesmon.


1 2 3 4 5
200K-
116K-
92K-
67K-
50K-
43K-

1982). A number of other minor bands are also present revealing that the preparation is not completely homogeneous. The preparations of rabbit skeletal muscle actin (lane 2, 43,000 daltons) and chicken gizzard tropomyosin (lane 3, subunits of 43,000 and 35,000 daltons) are very near homogeneity. Chicken gizzard caldesmon (lane 4, 150,000 daltons) which was purified by the denaturing methods of Bretscher (1984) contains a number of minor bands with molecular weights less than that of intact caldesmon. The majority of these bands are likely fragments of caldesmon, since they increase in concentration with repeated freeze/thaws of the protein.

5.3.1.2 Actin-activation of Skeletal Muscle Myosin ATPase

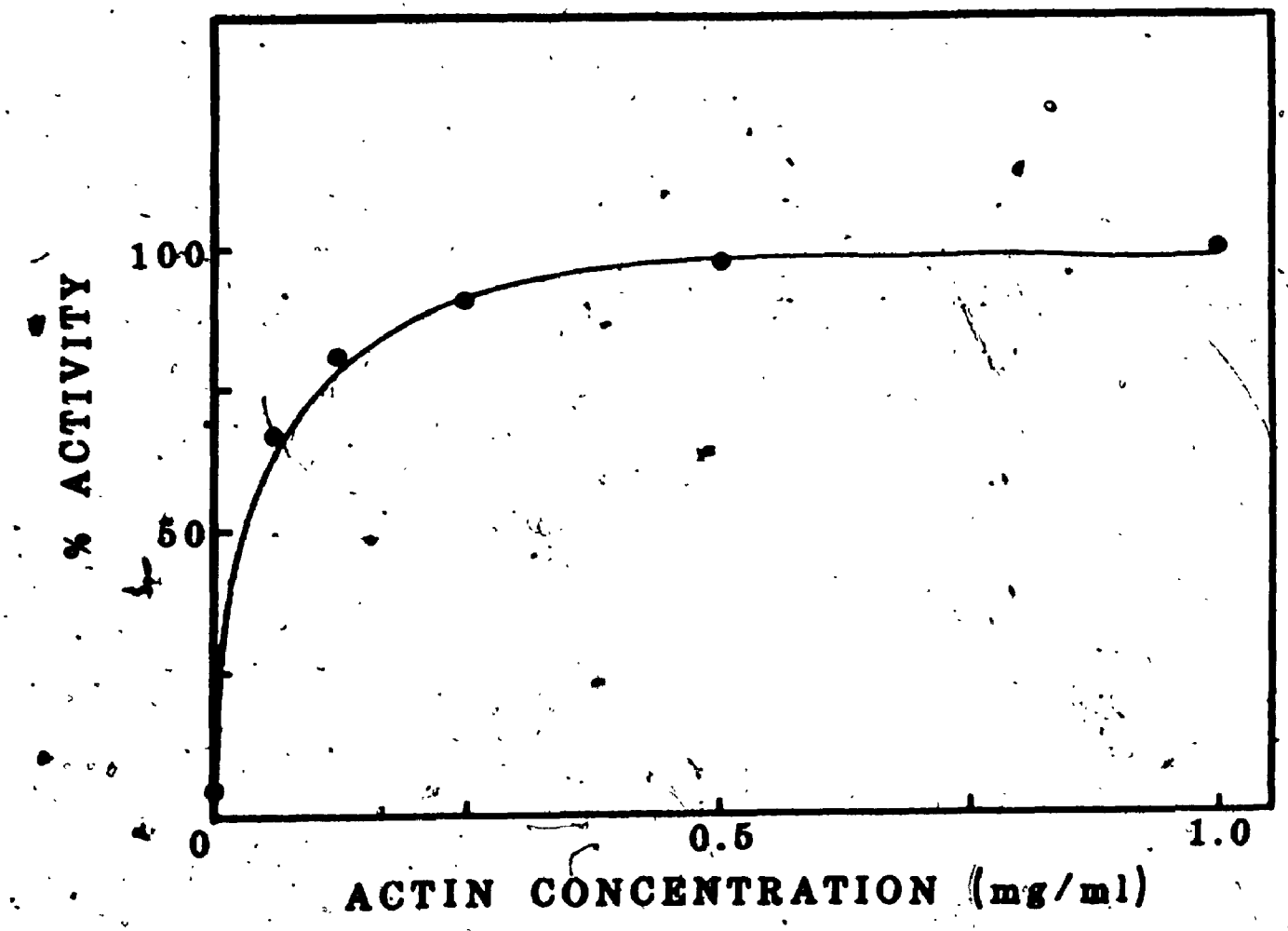
The ATPase activity of skeletal muscle myosin (0.125 mg/ml) was measured as a function of actin concentration (figure 5.2). In the absence of actin, the myosin ATPase activity (17.8 nmol Pi released/min/mg myosin) was nearly 20-fold less than was measured in the presence of actin at a concentration of 0.25 mg/ml (325.5 nmol Pi released/min/mg myosin). At higher concentrations of actin, the activity of the myosin ATPase was further elevated by less than 10 percent. An actin concentration of 0.25 mg/ml was therefore used for all assays.

As mentioned in Materials and Methods, it was determined that it was necessary to mix together and pre-incubate the protein components of the assay mixture for a minimum of 30 minutes to ensure their complete equilibration. When incubated for shorter periods of time, the results of duplicate assay mixtures were somewhat erratic indicating that complete equilibration had not yet been achieved.

FIGURE 5.2

ACTIN-ACTIVATION OF SKELETAL MUSCLE MYOSIN ATPase.

The ATPase activity of rabbit muscle myosin was measured as described in Materials and Methods in the presence of the indicated amounts of rabbit muscle actin. Activities are expressed as a percentage of the maximal ATPase activity measured. The values represent the average of duplicate samples.


% ACTIVITY
100
50
0
0.5
1.0
ACTIN CONCENTRATION (mg/ml)

3 of/de 3

1.0
1.1
1.25
1.4
1.6
2.8
3.2
3.6
2.5
2.2
2.0
1.8

MICROCOPY

5.3.2 Effects of Caldesmon on the Actin-Activated ATPase of Skeletal Muscle Myosin

When chicken gizzard tropomyosin at an approximate molar ratio of 2 tropomyosin: 7 actin monomers was added to the assay, an increase of approximately 16% in the activity of the actin-activated ATPase was observed in the absence of caldesmon (Table 5.1). This modest increase is very similar to that observed by Lim and Walsh (1986), but is significantly less than the 1.6 fold increase observed by Dabrowska et al.,(1985).

Chicken gizzard caldesmon significantly inhibited the activity of the actin-activated ATPase of skeletal muscle myosin (Table 5.1). A similar effect of caldesmon has been previously observed using the ATPase of smooth muscle myosin (Ngai and Walsh, 1984; Clark et al., 1986) smooth muscle heavy meromyosin (Lash et al., 1986), or skeletal muscle myosin (Dabrowska et al., 1985; Marston and Lehman, 1985; Lim and Walsh, 1986; Smith et al., 1987). The inhibitory effect of caldesmon on the ATPase was markedly enhanced by the addition of tropomyosin, confirming the results of a number of studies (Dabrowska et al., 1985; Lim and Walsh, 1986; Smith et al., 1987) . At all the caldesmon concentrations used, the inhibition of the ATPase was more than 5-fold greater in the presence of tropomyosin than in its absence. The inhibition of the ATPase that we have observed in the presence of tropomyosin is similar to that reported previously. However, in the absence of tropomyosin, the maximal 13% inhibition by caldesmon that we have observed is significantly less than the nearly 50% inhibition that was observed by Lim and Walsh (1986) or the 40%

TABLE 5.1

EFFECTS OF TROPOMYOSIN ON CALDESMON INHIBITION OF SKELETAL MUSCLE ACTIN-ACTIVATED MYOSIN ATPase.

Caldesmon (μM)	ATPase (nmol Pi/min/mg myosin)	
	+ Tropomyosin	-Tropomyosin
0	387.4 (100%)	338.9 (100%)
0.25	280.1 (72%)	326.6 (96%)
0.50	204.4 (53%)	311.2 (92%)
1.00	146.6 (38%)	311.9 (92%)
2.00	130.9 (34%)	295.6 (87%)

The actin-activated ATPase of skeletal muscle myosin was measured as described in Materials and Methods with the indicated amount of chicken gizzard caldesmon in the presence or absence of chicken gizzard tropomyosin (0.125 mg/ml). Skeletal muscle myosin (0.125 mg/ml) and skeletal muscle actin (0.25 mg/ml) were utilized. Values in brackets represent the percentage of the ATPase activity that was measured in the absence of added caldesmon. The values represent the average of duplicate determinations.

inhibition seen by Dabrowska et al., (1985).

5.3.3 Effects of Caldesmon Phosphorylation on Its Inhibition of Actin-Activated ATPase of Skeletal Muscle Myosin

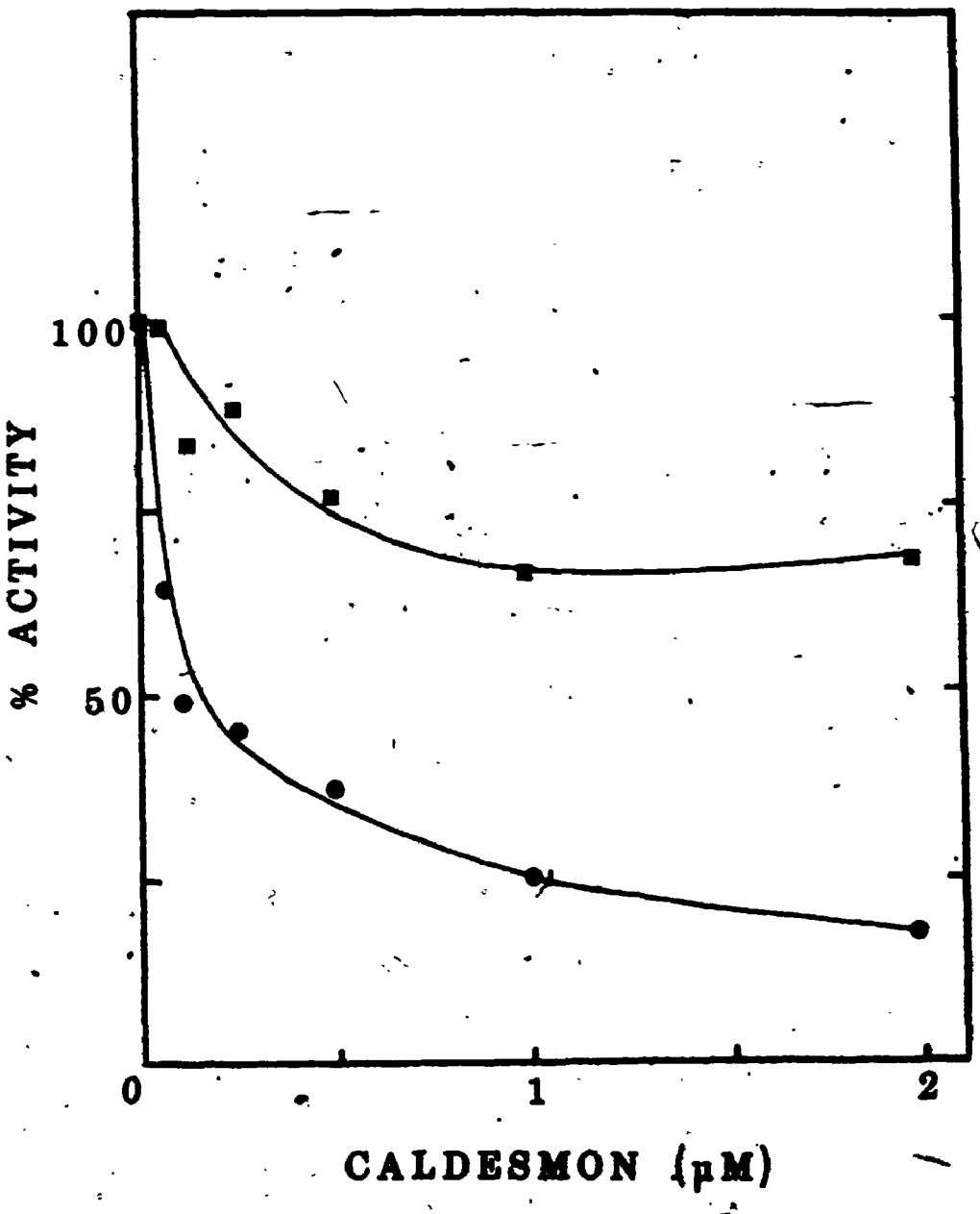
To examine the effects of phosphorylation on the inhibitory activity of caldesmon towards the actin-activated ATPase of skeletal muscle, chicken gizzard caldesmon was phosphorylated with purified rat brain protein kinase (see chapter 3). Caldesmon was phosphorylated to a maximal level of 2.6 mol phosphate/mol protein; approximately 13% higher than we had previously observed (chapter 3). This phosphate was stable during the assay; less than a 2% decrease in the phosphate content of caldesmon was observed.

On the basis of the results described in the previous sections, tropomyosin was included in all reactions used for the measurement of the actin-activated ATPase of skeletal muscle myosin in the presence of varying concentrations of either phosphorylated or non-phosphorylated caldesmon (figure 5.3). Figure 5.3 clearly demonstrates that phosphorylation of caldesmon partially abolishes its inhibitory activity towards the actin-activated ATPase of skeletal muscle myosin. Non-phosphorylated caldesmon inhibited the ATPase to a maximal level exceeding 80%, whereas phosphorylated caldesmon inhibited the ATPase to a maximal level of only 35%. In addition to this change, the caldesmon concentration at which half-maximal inhibition of the ATPase occurred was different for the two forms of the protein. Half-maximal inhibitions were observed at caldesmon concentrations of approximately 0.1 μM and 0.3 μM for non-phosphorylated and phosphorylated caldesmons respectively. These

FIGURE 5.3

INHIBITION OF ACTIN-ACTIVATED MYOSIN ATPase BY CALDESMON AND PHOSPHORYLATED CALDESMON.

Actin-activated myosin ATPase was measured as described in Materials and Methods in the presence of the indicated amounts of caldesmon (●) or phosphorylated caldesmon (■). All measurements were conducted in the presence of Tropomyosin. Activities are expressed as a percentage of ATPase measured in the absence of caldesmon. The values are typical of those obtained in three separate experiments.


100
50
% ACTIVITY
0
1
2
CALDESMON (μM)

observations suggest that the phosphorylation of caldesmon reduces its effectiveness as an inhibitor of the actin-activated ATPase of skeletal muscle. In similar studies of Ngai and Walsh (1984, 1987), the inhibition by caldesmon of the actin-activated ATPase of smooth muscle myosin is alleviated by phosphorylation of caldesmon with the calmodulin-dependent endogenous kinase. However, other investigators have failed to demonstrate any effect of this calmodulin-dependent phosphorylation on the inhibitory activities of caldesmon (Lash et al., 1986). Further, caldesmon that has been phosphorylated with the calmodulin-dependent kinase has the same activity towards the actin-activated ATPase of skeletal muscle myosin as non-phosphorylated caldesmon (Lim and Walsh, 1986). Thus, our results suggest that the phosphorylation of caldesmon by protein kinase C may play a unique role in regulating the inhibitory activity of caldesmon towards the actin-activated ATPase of skeletal muscle myosin.

5.3.4 Effects of Phosphorylation on the Binding of Caldesmon to Skeletal Muscle Actin

Caldesmon is known to be an actin-binding protein (Sobue et al., 1981; Bretscher, 1984; Ngai and Walsh, 1985). To examine the role of caldesmon phosphorylation on this property of the protein, caldesmon was incubated with F-actin and then subjected to ultracentrifugation. Under the assay conditions used, greater than 95% of the F-actin was routinely pelleted. In all cases (see Table 5.2), the percentage of caldesmon bound to F-actin was less for phosphorylated caldesmon than for non-phosphorylated caldesmon. For example, in the presence of tropomyosin without added calcium, more

TABLE 5.2

EFFECTS OF PHOSPHORYLATION ON ACTIN-BINDING OF CALDESMON.

Caldesmon	Tropomyosin	Calcium	% Pelleted with Actin
non-phosphorylated	+	-	91.0
phosphorylated	+	-	53.9
non-phosphorylated	-	-	76.9
phosphorylated	-	-	50.8
non-phosphorylated	+	+	52.3
phosphorylated	+	+	23.7
non-phosphorylated	-	+	38.6
phosphorylated	-	+	18.2

Phosphorylated caldesmon (0.05 mg/ml) or non-phosphorylated caldesmon (0.05 mg/ml) was incubated with F-actin (0.5 mg/ml) and calmodulin (0.04 mg/ml) in the presence (+) or absence (-) of tropomyosin (0.12 mg/ml) and then sedimented as detailed in Materials and Methods. Calcium (+) or EGTA (-) was added as indicated. The values represent the average of four determinations.

than 90% of the non-phosphorylated caldesmon was pelleted with F-actin, whereas in the case of phosphorylated caldesmon just over 50% was pelleted. In the presence of calcium, with or without tropomyosin, the amount of F-actin-associated caldesmon was more than 2-fold higher with the non-phosphorylated form of the protein.

Further examination of Table 5.2 reveals that tropomyosin enhanced the binding of both phosphorylated and non-phosphorylated caldesmons with F-actin. However, the effect of tropomyosin was clearly more significant for non-phosphorylated caldesmon than it was for the phosphorylated protein. One additional trend that is demonstrated by Table 5.2, is that of the two caldesmon forms, the phosphorylated one is more effectively displaced from F-actin by calcium/calmodulin. Collectively, the results of table 5.2 suggest that the phosphorylation of caldesmon by protein kinase C diminishes the interactions of the protein with F-actin. These results parallel the recent findings of Ngai and Walsh (1987) who demonstrated that the phosphorylation of caldesmon by the calmodulin-dependent endogenous kinase decreased its binding to chicken gizzard F-actin.

5.4 DISCUSSION

Although the precise physiological role of caldesmon is not yet fully understood, a number of observations have implicated this protein as a potential regulator of contractile events and/or actin filament organization. Therefore, it is of interest to identify those factors that can regulate the known activities of caldesmon. In

particular, since caldesmon had been identified as a substrate for protein kinase C in vitro (chapter 3; Umekawa and Hidaka, 1985) and in intact human platelets (chapter 4), it was our intention to examine the role of this phosphorylation reaction on key activities of the protein.

In this study, a reconstituted actin-activated myosin ATPase system was established utilizing components from skeletal muscle (actin and myosin) and smooth muscle (tropomyosin and caldesmon). Although an assay system using smooth muscle myosin instead of skeletal muscle myosin may have been more physiologically correct, the hybrid system was selected on the basis of its simplicity. Actin-activation of smooth muscle myosin requires prior phosphorylation of its regulatory light chain by the calmodulin-dependent enzyme myosin light chain kinase, whereas skeletal muscle myosin has no such prerequisite for light chain phosphorylation (Adelstein, 1982; Kamm and Stull, 1985; Sellers and Adelstein, 1987). Previous studies have demonstrated that the inhibitory effect of smooth muscle caldesmon on the actin-activated ATPase of skeletal muscle myosin is similar to that observed for smooth muscle myosin (Marston and Lehman, 1985; Dabrowska et al., 1985; Lim and Walsh, 1986; Smith et al., 1987).

Non-phosphorylated caldesmon consistently inhibited the actin-activated ATPase of skeletal muscle myosin, with maximal inhibition of 82% observed in the presence of tropomyosin. The magnitude of this inhibition was very similar to that reported by a number of investigators (Dabrowska et al., 1985; Lim and Walsh, 1986; Smith et

al, 1987). In the absence of tropomyosin however, the inhibitory effects of caldesmon were much less significant achieving a maximal level of only 13%. Although this inhibition is similar to that seen by Smith et al. (1987), the effect is considerably less than was observed by Lim and Walsh (1986) or Dabrowska et al., (1985) who demonstrated caldesmon inhibitions of approximately 50% and 40% respectively. The variations seen for the effects of caldesmon in the absence of tropomyosin may result from differences in $MgCl_2$ and KCl concentrations in the assay mixtures, since the inhibitory effects of caldesmon are very sensitive to changes in the concentrations of these compounds (Dabrowska et al., 1985). Similarly, the modest elevation by tropomyosin of actin-activated myosin ATPase activity seen in this study, or by Lim and Walsh (1986), was considerably lower than was observed by Dabrowska et al. (1985). Again, differences in the $MgCl_2$ or KCl concentrations may have been responsible for the variations (Nowak and Dabrowska, 1985).

We have observed effects of the phosphorylation of caldesmon by protein kinase C on two properties of this actin- and calmodulin-binding protein. Phosphorylation significantly diminishes the inhibitory activity of caldesmon on the actin-activated ATPase of skeletal muscle myosin and weakens its associations with F-actin. This weakened interaction with F-actin was observed in the presence or absence of tropomyosin. Furthermore, phosphorylated caldesmon was more effectively displaced from actin filaments by calcium/calmodulin. The latter result implies that phosphorylation of caldesmon does not impair its interactions with calmodulin,

contrary to what may have been expected since caldesmon phosphorylation is inhibited by calmodulin (see chapter 3). Attempts to directly examine the effects of phosphorylation on the interactions of caldesmon with calmodulin were all unsuccessful, perhaps due to the low affinity of the calmodulin/caldesmon interactions (Ngai and Walsh, 1985; Smith et al., 1987). It is not yet known whether the effects of phosphorylation on the inhibition of the actin-activated ATPase and the associations of caldesmon with F-actin are related, since the mechanism of the caldesmon inhibition of the actin-activated ATPase is not completely understood. Further experimentation will also be required to determine whether or not the same effects of phosphorylation are observed in a system that has been reconstituted using smooth muscle instead of skeletal muscle actomyosin.

Although caldesmon was originally identified as a calmodulin- and actin-binding protein (Sobue et al., 1981), there have been suggestions that it may also have interactions with myosin as well, perhaps forming cross-bridges between actin and myosin filaments (Bretscher, 1986; Lim and Walsh, 1986; Lash et al., 1986). In this vein, it has been proposed that caldesmon may be involved in the calcium-dependent maintenance of smooth muscle tension, a phenomenon that is independent of the phosphorylation state of the regulatory light chain (Chatterjee and Murphy, 1983). The demonstration by Lim and Walsh (1986) that caldesmon inhibited certain ATPases of skeletal muscle myosin in the absence of F-actin suggested that caldesmon may indeed interact directly with myosin filaments. Furthermore,

caldesmon was capable on enhancing interactions between F-actin and heavy meromyosin (Lash et al., 1986). Clearly, a better understanding of caldesmon and its physiological activities is required.

In addition to its phosphorylation by protein kinase C, chicken gizzard caldesmon has been identified as an in vitro substrate for a calmodulin-dependent protein kinase that copurifies with caldesmon as purified by non-denaturing methods (Ngai and Walsh, 1984; Lash et al., 1986; Ngai and Walsh, 1987). Although it has been reported that this phosphorylation event has no effect on the properties of caldesmon (Lash et al., 1986), Ngai and Walsh (1984, 1987) have consistently demonstrated that phosphorylation partially abolishes the inhibitory activity of caldesmon towards the actin-activated ATPase of smooth muscle myosin. Very recently, Ngai and Walsh (1987) have also observed a decrease in the interactions of caldesmon with F-actin following phosphorylation. Thus, it appears that the phosphorylation of caldesmon with protein kinase C or with the calmodulin-dependent kinase may have similar consequences. However, inhibition of the actin-activated ATPase of skeletal muscle myosin was unaffected by the phosphorylation of caldesmon with the calmodulin-dependent kinase (Lim and Walsh, 1986). Therefore, despite some similarities, the effects induced by the phosphorylation of caldesmon with the two protein kinases have distinct features.

The tumour-promoting phorbol esters have been shown to elicit a slow, sustained contraction of smooth muscle (Rasmussen et al., 1984; Park and Rasmussen, 1985; Park and Rasmussen, 1986). Thus, a role

for protein kinase C, which is directly activated by the tumour-promoting phorbol esters (Castagna et al., 1982), in the regulation of contractile events is implicated. Consistent with this idea is the identification of contractile proteins such as myosin light chain kinase (chapter 3, Ikebe et al., 1985; Nishikawa et al., 1985), myosin regulatory light chain (Endo et al., 1982; Naka et al., 1983; Nishikawa et al., 1983; Inagaki et al., 1987) and caldesmon as substrates for protein kinase C. However, on the basis of in vitro results it appears as if these phosphorylation events may have opposing consequences. The phosphorylations of myosin light chain kinase and the regulatory light chain of myosin both serve to diminish the activation of the actin-activated myosin ATPase, and thus the contractile response. On the other hand, phosphorylation of caldesmon partially relieves its inhibition of the actin-activated ATPase which would be expected to enhance the contractile response. In all likelihood, these observations demonstrate that the control mechanisms for contraction or actin filament organization are complex and involve more than one regulatory system. Clearly, there is a need for further experimentation to more fully elucidate the roles of these proteins, and their phosphorylations, in the regulation of contraction and cytoskeletal organization.

In any event, the results that have been presented in this chapter illustrate that the phosphorylation of caldesmon by protein kinase C can have significant functional consequences. Thus, since caldesmon is phosphorylated by protein kinase C in vitro and in living cells and since an effect of phosphorylation on the properties

of caldesmon has been demonstrated, we can conclude that this phosphorylation event is of physiological significance. Furthermore, studies with phosphorylated caldesmon may prove useful in developing a more complete understanding of the role of caldesmon in the control of contractile or cytoskeletal events.

CHAPTER 6

SUMMARY AND FUTURE PROSPECTS

6.1 SUMMARY OF RESULTS

The overall objective of the work presented in this thesis has been to elucidate the molecular events that are responsible for the alterations in the organization and function of the actin-containing microfilaments that are induced by the tumour-promoting phorbol esters. When cultured cells are treated with these biologically-active compounds, the microfilaments are reorganized and the cells' morphology is altered (Rifkin et al., 1979; Parkinson et al., 1982; Schliwa et al., 1984). Also, contractile events are stimulated in smooth muscle following treatment with tumour-promoting phorbol esters (Rasmussen et al., 1984; Park and Rasmussen, 1985; Park and Rasmussen, 1986). It is now generally accepted that many, if not all, of the effects of the tumour-promoting phorbol esters are mediated by their receptor, a serine/threonine specific protein kinase known as protein kinase C (reviewed by Ashendel, 1985; Nishizuka et al., 1986; Kikkawa et al., 1984; Pasti et al., 1986). Therefore, the functional and structural alterations of the actin-containing filaments that are induced by these compounds may result from the phosphorylation of regulatory components of the cytoskeleton by protein kinase C.

On the basis of their regulatory potential, our investigations focused upon two groups of microfilament-associated proteins, those located within the focal contacts of cultured cells (Burridge et al.,

1982; Mangeat and Burridge, 1984; Geiger, 1985) and those involved in the regulation of contractile events in smooth and non-muscle cells (Kakiuchi and Sobue, 1983; Marston and Smith, 1985; Sellers and Adelstein, 1987). In the former group, we studied the phosphorylation by protein kinase C of two proteins, vinculin and talin, that are thought to be involved in the anchorage of the termini of the actin filaments in stress fibres to the plasma membrane. In the latter group, two calmodulin-binding proteins, myosin light chain kinase and caldesmon, were the subject of investigation. A summary of the phosphorylation events that we examined is schematically presented in figure 6.1.

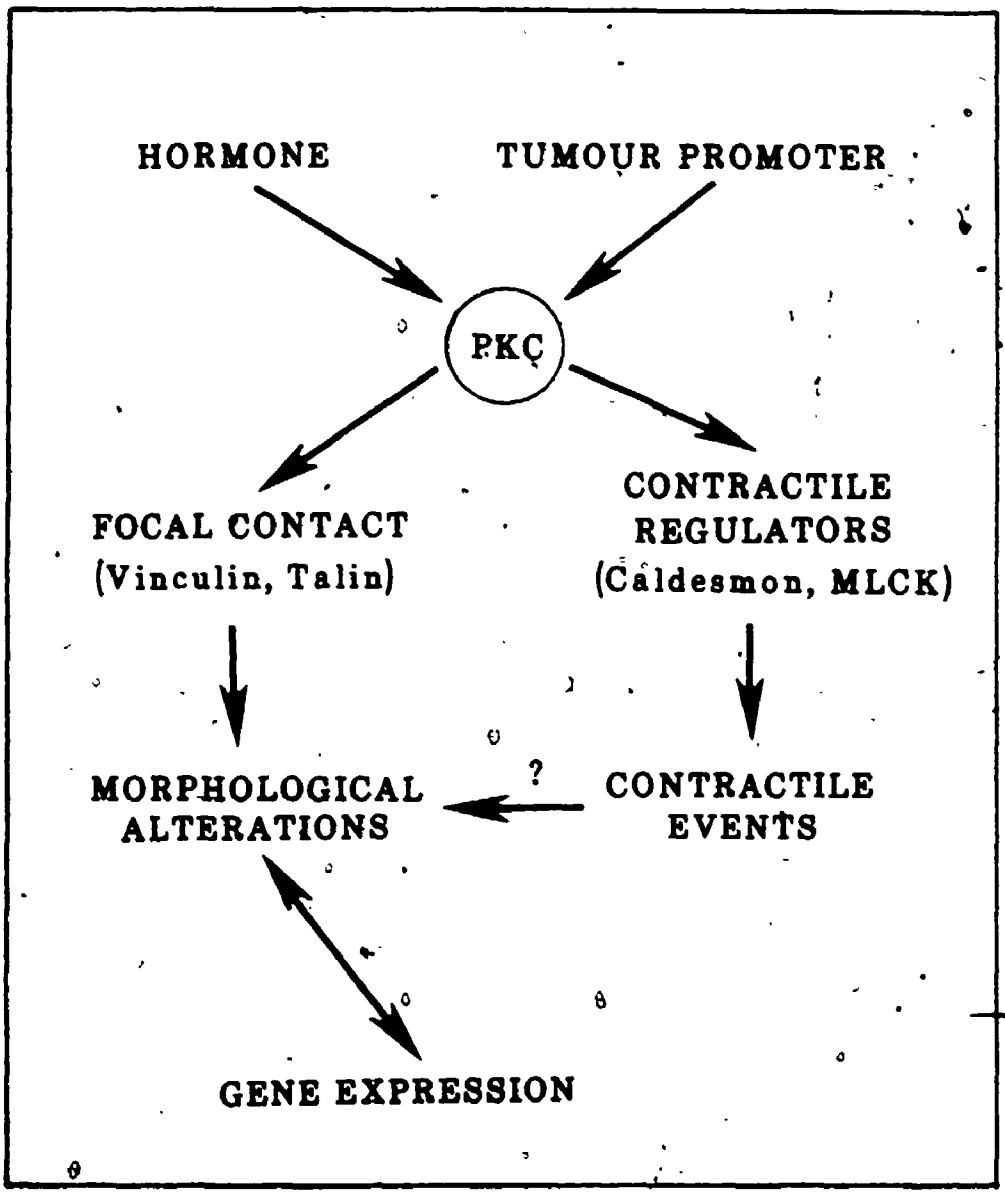
6.1.1 Phosphorylation of Focal Contact Proteins by Protein Kinase C

Since they are localized near the plasma membrane, vinculin and talin are prime candidates for phosphorylation by protein kinase C. Indeed, chicken gizzard vinculin is phosphorylated *in vitro* by purified protein kinase C (chapter 2; Werth *et al.*, 1983; Kawamoto and Hidaka, 1984) as is chicken gizzard talin (chapter 2; Litchfield and Ball, 1986). Vinculin is also phosphorylated in cultured cells in response to phorbol esters, although there was no apparent correlation between the phosphorylation of vinculin and morphological alterations (Werth and Pastan, 1984). Similarly, the phosphorylation of the platelet form of talin, P235, is enhanced following treatment of intact human platelets with TPA (chapter 4). In this instance however, phosphorylation was increased by only 44% and the apparent stoichiometry was low. Nevertheless, the demonstration that vinculin and talin are phosphorylated by protein kinase C *in vitro*, and in

FIGURE 6.1

PROPOSED SCHEME FOR THE ROLE OF PROTEIN KINASE C IN CYTOSKELETAL REGULATION.

Following treatment of cells with tumour promoters or with stimuli that elicit diacylglycerol production, protein kinase C (PKC) is activated. Phosphorylation of focal contact proteins (vinculin, talin) may lead to alterations in the shape and adhesive properties of cells with the result that changes in patterns of gene expression occur. By phosphorylation of proteins involved in contractile regulation (myosin light chain kinase (MLCK), caldesmon), protein kinase C may have a role in mediating contractile events.


HORMONE
TUMOUR PROMOTER
PKC
FOCAL CONTACT
(Vinculin, Talin)
CONTRACTILE
REGULATORS
(Caldesmon, MLCK)
MORPHOLOGICAL
ALTERATIONS
?
CONTRACTILE
EVENTS
GENE EXPRESSION

intact cells, implies that these phosphorylation events may mediate the effects of phorbol esters on microfilament organization and the integrity of focal contacts. Moreover, since patterns of gene expression have been shown to be dependent on cell shape and cell contacts (Ben Ze'ev, 1986; Ungar et al., 1986), these phosphorylation reactions may have significance in the regulation of other biochemical events that are stimulated by the phorbol esters.

In this study, it was observed that the phosphorylation of vinculin is diminished in the presence of talin. This result provides a preliminary indication that phosphorylation may be involved in the regulation of the properties of these proteins. However, at present, the precise functions of vinculin and talin and their roles in physiological processes remain poorly understood. As a result, the effects of phosphorylation on the properties of these proteins have been difficult to assess.

6.1.2 Phosphorylation of Contractile Regulators by Protein Kinase C

The demonstration that the biologically active phorbol esters stimulate contractile events in smooth muscle is indicative of a role for protein kinase C in the regulation of components of the contractile apparatus (Rasmussen et al, 1984; Park and Rasmussen, 1985; Park and Rasmussen, 1986). Consistent with this suggestion is the observation that myosin light chain kinase (chapter 3; Ikebe et al., 1985; Nishikawa et al., 1985) and caldesmon (chapter 3; Umekawa and Hidaka, 1985; Litchfield and Ball, 1987a; Litchfield and Ball, 1987b) can be phosphorylated in vitro by protein kinase C. Although phosphorylation by protein kinase C has been shown to decrease the

activity of myosin light chain kinase by diminishing its affinity for calmodulin, the phosphorylation reaction has not yet been demonstrated in living cells. Clearly, the physiological significance of the reaction cannot be established until such a demonstration is achieved. In contrast, we have demonstrated in intact platelets that TPA enhances by nearly 4-fold the phosphorylation of caldesmon$_{77}$ (chapter 4; Litchfield and Ball, 1987a). Moreover, the two major phosphopeptides from TPA-treated platelets have identical migration patterns to the two major phosphopeptides obtained from caldesmon$_{77}$ that was phosphorylated by purified protein kinase C.

To extend the observations that caldesmon is phosphorylated by protein kinase C *in vitro* and *in* intact platelets, we examined the effects of phosphorylation on the properties of this calmodulin-binding protein (chapter 5). Phosphorylation of chicken gizzard caldesmon by protein kinase C partially diminishes its inhibitory activity towards the actin-activated ATPase of skeletal muscle myosin. Furthermore, phosphorylation apparently weakened the associations of caldesmon with F-actin and enhanced the ability of calmodulin to disrupt these associations. Although these effects of phosphorylation are similar to those observed by Ngai and Walsh (1987) following phosphorylation of caldesmon by a calmodulin-dependent protein kinase that copurifies with caldesmon, we have reason to believe that the effects of the two protein kinases are distinct. First of all, phosphopeptide maps of caldesmon phosphorylated by the two kinases demonstrate that the major sites of

phosphorylations by each kinase are different. Secondly, phosphorylation of caldesmon by the calmodulin-dependent kinase did not alter its inhibition of an actin-activated ATPase of skeletal muscle myosin (Lim and Walsh, 1986), whereas phosphorylation by protein kinase C did. As yet, there has been no demonstration that the calmodulin-dependent phosphorylation of caldesmon occurs in living cells. In fact, since the calcium ionophore A23187 did not stimulate caldesmon phosphorylation in intact platelets, it appears as if the calmodulin-dependant reaction does not occur in these cells.

The results of this study demonstrate that protein kinase C phosphorylates representatives of both classes of the calmodulin-binding protein caldesmon (Litchfield and Ball, 1987b). Thus, in addition to the similarities in the functional properties exhibited by the two forms of the protein, they may be subject to the same mechanisms of regulation. On the basis of phosphopeptide mapping it is obvious that the caldesmon species have regions of structural identity.

6.2 FUTURE PROSPECTS

In consideration of the results of this study, the focal contact proteins vinculin and talin, and the contractile regulators myosin light chain kinase and caldesmon can be added to the growing list of cytoskeletal or contractile proteins that have been identified as substrates for protein kinase C. In addition to these proteins, actin-binding proteins such as microtubule-associated protein 2 (Akiyama et al., 1986), p36 (Gould et al., 1986; Isacke et al., 1986)

and filamin (Kawamoto and Hidaka, 1984), and contractile proteins such as myosin light chain (Endo et al., 1982; Nishikawa et al., 1983; Naka et al., 1983), cardiac C-protein (Lim et al., 1985), troponin-T and troponin-I (Mazzei and Kuo, 1984) have been identified as substrates for protein kinase C. These observations obviously stress the potential importance of protein kinase C in the phosphorylation and regulation of cytoskeletal components. However, in elucidating the role of protein kinase C in various aspects of cytoskeletal regulation, determining its substrates is merely the first step. The effects of phosphorylation on each of the substrate proteins must be characterized before the physiological significance of a particular phosphorylation event can be fully understood. In living cells the occurrence of a physiological event and the extent of the response must also be shown to be correlated with the level of phosphorylation (Krebs and Beavo, 1979).

6.2.1 Phosphorylation of Focal Contact Proteins: Future Prospects

Having identified vinculin and talin as substrates for protein kinase C, it is certainly of interest to examine the effects of phosphorylation on their known properties. In the absence of known enzymatic activities, further analysis will have to concentrate on the binding interactions that have been characterized for vinculin and talin. For example, vinculin interacts with permeabilized cells (Schlessinger and Geiger, 1983; Ball et al., 1986) and with talin in vitro (Burridge and Mangeat, 1984). In addition to its interactions with vinculin, talin has been shown to interact with the transmembrane fibronectin receptor (Horwitz et al., 1986). Further

study directed towards establishing correlations between the phosphorylation of talin and/or vinculin with the alterations in focal contacts or actin filament organization in living cells that have been treated with tumour-promoting phorbol esters is also clearly required.

6.2.2 Phosphorylation of Contractile Regulators: Future Prospects

On the basis of in vitro experiments, a number of effects of phosphorylation have been identified for myosin light chain kinase (Ikebe et al., 1985; Nishikawa et al., 1985) and for caldesmon (chapter 5; Umekawa and Hidaka, 1985). In the case of myosin light chain kinase however, these results are not physiologically significant unless evidence that the reaction occurs in living cells is obtained. For caldesmon, the effects of phosphorylation on a number of its properties remain to be studied. Among these latter properties are its calmodulin-binding capabilities (Sobue et al., 1981; Smith et al., 1987), and its ability to enhance interactions between F-actin and heavy meromyosin (Lash et al., 1986). Furthermore, the effects of phosphorylation were analysed only for chicken gizzard caldesmon. Thus, an examination of the effects of phosphorylation on the lower molecular weight species of caldesmon may prove enlightening in assessing the similarites and differences of the two forms of the protein. At present, an analysis of the extent of caldesmon or myosin light chain kinase phosphorylation in relation to the contractile response is also lacking.

6.2.3 Additional Considerations

It is not surprising that vinculin and talin can serve as

substrates for protein kinase C, since they are at least partially localized near the plasma membrane. Caldesmon though, is not generally believed to be a membrane-associated protein (Sobue et al., 1985). Thus, one extremely interesting aspect of this research relates to the possible mechanisms of how a membrane-activated enzyme can phosphorylate a soluble protein such as caldesmon. As previously discussed (chapter 4), it is possible that there is a sub-population of caldesmon that is associated with the plasma membrane (Burgoyne et al., 1986; Bretscher and Lynch, 1985), or that rearrangements of the cytoskeleton that occur following treatment of cells with TPA permit the association of caldesmon with the plasma membrane. Alternatively, the phosphorylation of caldesmon may reflect the generation of the calcium/phospholipid-independent catalytically active fragment of protein kinase C in TPA-treated cells (Kishimoto et al., 1983; Tapley and Murray, 1985; Melloni et al., 1986). Certainly, further investigation of the mechanism of caldesmon phosphorylation may provide a number of answers relating to the physiological significance of the proteolytic activation of protein kinase C.

Recent investigations have introduced a new, and somewhat unexpected, level of complexity involving protein kinase C that may necessitate a re-evaluation of some aspects of the regulation and involvement of the enzyme in physiological events. Using a molecular biological approach, a number of investigators have recently demonstrated that protein kinase C is not a single entity (Coussens et al., 1986; Housey et al., 1986; Knopf et al., 1986; Makowske et

al., 1986; Ohno et al., 1987; Ono et al., 1986; Ono et al., 1987; Parker et al., 1986). Instead, there is actually a family of closely related protein kinase C species that exhibits a degree of tissue specificity. Multiple forms of protein kinase C have also been characterized using protein chemical and immunological methods (Woodgett and Hunter, 1987a; Woodgett and Hunter, 1987b; Kikkawa et al., 1987; Huang et al., 1986b). At present, differences in substrate specificity or subcellular localization for the different forms of the enzyme have not been demonstrated. However, it is not unreasonable to imagine that the different protein kinase C species may have distinct roles in physiological processes. As a result, it may be of interest, or necessity, to examine the phosphorylation of cytoskeletal proteins by each of the kinase species.

The results of this thesis are significant in that a number of potential regulators of the cytoskeleton have been identified as substrates for protein kinase C. Although it is obvious that much work remains to be done, the results suggest that protein kinase C is an important mediator of the alterations in the organization and function of the actin-containing microfilaments that are induced by the tumour-promoting phorbol esters. Future work will undoubtedly lead to a better understanding of the role of protein kinase C in cytoskeletal regulation and perhaps in other aspects of signal transduction that are stimulated by the tumour-promoting phorbol esters. Obviously, it will be of interest to extend the results of this study by examining the role of protein kinase C in the regulation of cytoskeletal events that occur in response to treatment

with stimuli that trigger generation of diacylglycerol, the natural activator of protein kinase C. Furthermore, as the relationship between the activation of protein kinase C by the phorbol esters or by the receptor-mediated hydrolysis of phosphatidylinositols becomes more fully understood, our findings may gain new significance with respect to the involvement of protein kinase C in the transmission of molecular signals that have been stimulated by receptor-mediated events.

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