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THE ROLE OF PROTEIN KINASE C IN VITAMIN D-MEDIATED EFFECTS IN KIDNEY

by
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ABSTRACT

The role of the calcium/phospholipid-dependent serine/threonine kinase Protein Kinase C (PKC), in the effects of vitamin D on kidney was studied. Madin Darby Bovine Kidney (MDBK) cells, a normal epithelial-like cell line, were found to express the Vitamin D Receptor (VDR) and the vitamin D-dependent calcium binding protein Calbindin D-28K (CaBP D-28K). In MDBK cells, $1,25(OH)_2D_3$ increased PKC activity in a time- and dose-dependent manner as measured by two different phosphorylation assays. This activation appeared to result from translocation of PKC from the cytosol to the membrane and was accompanied by an increase in immunoreactive PKC in the membrane. PKC was also activated by short term exposure of MDBK cells to TPA, whereas PKC activity was completely down-regulated by long term exposure to TPA. Down-regulation of PKC activity was accompanied by a loss of immunoreactive PKC. The phorbol ester analogue 4αPDD had no effect on PKC activity or amount. Concurrent with activation of PKC $1,25(OH)_2D_3$ homologously up-regulated the VDR and increased total immunoreactive CaBP D-28K in MDBK cells. In contrast, down-regulation of PKC activity in TPA treated cells was associated with decreased expression of the VDR and CaBP D-28K. The phorbol ester analogue 4αPDD, which had no effect on PKC, did not affect the expression of the VDR or CaBP D-28K. Short term TPA treatment, which activated PKC, increased CaBP D-28K without altering VDR levels. The divergent effects of $1,25(OH)_2D_3$ and TPA were associated with differential regulation of PKC isozymes. Treatment of MDBK cells with $1,25(OH)_2D_3$ increased membrane association of PKC α, induced nuclear translocation of PKC β and had no effect on PKC ζ. In contrast, long term treatment of MDBK cells with TPA induced down-regulation of PKC α, nuclear translocation of PKC β, and decreased PKC ζ. Nuclear translocation of PKC β by $1,25(OH)_2D_3$ treatment was accompanied by an increase in phosphorylation of endogenous nuclear proteins. However nuclear translocation of PKC β by TPA treatment did not affect phosphorylation of endogenous nuclear proteins. The data have been incorporated into a model based on the hypothesis that the VDR could be a nuclear substrate for PKC β and CaBP D-28K could be a cytosolic substrate for PKC α. This model predicts a novel role for PKC-dependent phosphorylations in the renal actions of $1,25(OH)_2D_3$.

DEDICATION

To my Husband, my Son and my Parents

who through their support, encouragement and love have made this work possible.

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I would like express my sincere gratitude to my two supervisors Dr. JoEllen Welsh and Dr. Doug Franks for their direction and support during this project.

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1-(5-isoquinolinesulfonyl)-2-methylpeperazine (H-7)

4-α-phorbol-12,13-didecanoate (4αPDD)

12-O-Tetradecanoyl phorbol-13-acetate (TPA)

1,24,25-trihydroxyvitamin D_3 ($1,24,25(OH)_3D_3$)

1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$)

25-hydroxyvitamin D_3 (25-OH-D_3).

adenosine triphosphate (ATP)

bovine serum albumin (BSA)

Calbindin D-28K (CaBP D-28K)

calcium chloride ($CaCl_2$)

counts per minute (cpm)

deoxyribonucleic acid (DNA)

diacylglycerol (DAG)

diethylaminoethyl (DEAE)

dimethyl sulfoxide (DMSO)

dissociation constant (K_d)

Dulbecco's modified Eagle medium (DMEM)

estrogen receptor (ER)

ethylene diaminetetra-acetic acid (EDTA)

ethyleneglycol-bis-(β-aminoethyl ether)
N,N,N,N'-tetracetic acid (EGTA)

fast protein liquid chromatography (FPLC)

human promyelocytic Leukemia cells (HL-60 cells)

hydrochloric acid (HCl)

kilodalton (kD)

Madin Darby Canine Kidney cells (MDCK cells)

Madin Darby Bovine Kidney cells (MDBK cells)

magnesium chloride ($MgCl_2$)

maximum number binding sites (N_{max})

messenger ribonucleic acid (mRNA)

opossum kidney cells (OK cells)

perchloric acid (PCA)

phenylmethylsulfonyl fluoride (PMSF)

phosphate-buffered saline (PBS)

Phosphatidylserine (PS)

polyacrylamide gel (PAGE)

potassium chloride (KCl)

Protein Kinase C (PKC)

sodium hydroxide (NaOH)

sodium bicarbonate ($NaHCO_3$)

sodium dodecyl sulfate (SDS)

standard error of mean (SEM)

trichloroacetic acid (TCA)

vitamin D receptor (VDR)

GENERAL INTRODUCTION

Vitamin D_3 is classified as a vitamin, because it can be supplied by the diet, however it is primarily present in the skin as a precursor which upon ultraviolet irradiation via sunlight is converted to Vitamin D_3. Vitamin D_3 from both of these sources is transported to the liver where it is metabolized to 25-hydroxyvitamin D_3 (25-OH-D_3). 25-OH-D_3 is subsequently transported to the proximal tubular cells of the kidney where it is further metabolized by 1α-Hydroxylase to form 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), the biologically active form of vitamin D_3. 1,25$(OH)_2D_3$ can in turn be metabolized by 24-Hydroxylase to form 1,24,25-trihydroxyvitamin D_3 (1,24,25$(OH)_3D_3$); this is believed to be the first step in a catabolic pathway present in most target cells (DeLuca, Krisinger and Darwish, 1990).

The effects of 1,25$(OH)_2D_3$ are primarily, if not exclusively, mediated via the intracellular vitamin D receptor (VDR) (Haussler, 1986; Haussler et al., 1988a); a member of the steroid, thyroid and retinoic acid receptor gene family (Beato, 1988). The VDR was initially discovered in 1969, when Haussler and Norman provided evidence for the existence of a chromosomal protein capable of binding 1,25$(OH)_2D_3$ with low capacity and high affinity. Since then, the VDR has been extensively studied in crude extracts from a variety of target tissues including intestine, bone, kidney and promyelocytes

(Brumbaugh and Haussler, 1975). Generally, the VDR has a molecular weight of approximately 50-60 kD and an equilibrium dissociation constant (K_D) of approximately 10^{-10}-10^{-11} M. The receptor is stabilized by sulfhydryl reagents, is susceptible to proteolytic activity and is found predominantly in the nuclear fraction upon cell fractionation in low salt medium. Under high salt conditions (300 mM) the VDR is extracted from the nucleus. Immunocytochemical data has confirmed that the VDR is predominantly localized to the nucleus (Pike et al., 1982; Dame et al., 1986) and has provided insight into its functional domains. The VDR binds $1,25(OH)_2D_3$ in the C-terminal portion of the molecule. Similar to other steroid receptors (Petkovich et al., 1987), DNA-binding loops or fingers have been identified in the VDR (McDonell et al., 1987), in the N-terminal portion of the molecule, that are similar to the zinc-fingers proposed for the 5S transcription factor TFIIIA (Miller, McLachlan and Klug, 1985). As a DNA-binding protein, the VDR is capable of up-regulating or down-regulating the transcription of RNA polymerase II genes (Beato, 1991) and thus modulating the synthesis of proteins which in turn mediate the many diverse biological effects of $1,25(OH)_2D_3$.

The biological effects of $1,25(OH)_2D_3$ include: (1) mineral homeostasis via the induction of calcium binding proteins (Kumar, 1991); (2) vitamin D catabolism via the induction of the 24 hydroxylase (Henry and Norman, 1984); (3) bone

remodelling via recruitment of osteoclast precursors (Mangelsdorf et al., 1984) and induction of osteocalcin in osteoblasts (Price and Baukol, 1980); (4) immunomodulation via regulation of interleukins such as interleukin 2 (Rigby, Denone and Fanger, 1987); and most recently (5) regulation of cell proliferation and differentiation (Minghetti and Norman, 1988).

$1,25(OH)_2D_3$ induces differentiation of human promyelocytic Leukemia (HL-60) cells into monocyte-like cells. This differentiation is preceded by a decrease in the steady-state mRNA levels of the protooncogene c-*myc* (Reitsma et al., 1983; Simpson et al., 1989); expression of the c-*myc* gene is closely linked to proliferation (Kelly et al., 1983; Armelin et al., 1984). The regulation of c-*myc* transcription by $1,25(OH)_2D_3$ is related to an increase in Protein Kinase C (PKC) levels (Martell, Simpson and Taylor, 1987; Simpson et al., 1989). PKC is a signal transducing calcium/phospholipid-dependent kinase that phosphorylates target protein substrates on serine/threonine residues. It is also the cellular receptor for phorbol esters (Nishizuka, 1984; 1986). Classical activation of PKC results from signal-induced hydrolysis of membrane phosphoinositides which produces diacylglycerol (DAG) and calcium. This activation generally results in a rapid, transient translocation of PKC from the cytosol, where it is inactive, to the membrane, where in the presence of phospholipid, DAG and calcium the enzyme becomes fully active.

12-O-Tetradecanoyl phorbol-13-acetate (TPA), a tumor-promoting phorbol ester, can substitute for DAG and directly activate PKC without signal-induced hydrolysis of membrane phosphoinositides (Castagna, 1982). In HL-60 cells, activation of PKC by $1,25(OH)_2D_3$ results from VDR-mediated induction of PKC gene expression (Obeid et al., 1990) rather than signal-induced hydrolysis of membrane phosphoinositides.

The objective of my Ph.D studies was to investigate whether PKC is involved in mediating other effects of $1,25(OH)_2D_3$. One of the best characterized effects of $1,25(OH)_2D_3$ is the regulation of Calbindin D-28K (CaBP D-28K). This 28 kD soluble calcium binding protein is primarily found in renal distal convoluted tubular cells and in intestinal cells. In the intestine, correlations between the rate and time course of calcium absorption and the induction and concentration of CaBP D-28K provide strong evidence for a role of CaBP D-28K in vitamin D-dependent calcium transport (Wasserman and Fullmer, 1989). It has been speculated that renal CaBP D-28K plays a similar role in renal calcium absorption. Renal CaBP D-28K is exclusively localized to the distal nephron (Christakos, Brunette and Norman, 1981; Taylor et al., 1982), where vitamin D-dependent calcium reabsorption occurs (Winaver et al., 1980; Puschett et al., 1972 a,b). *In vivo* (Christakos and Norman, 1980) and *in vitro* (Craviso, Garrett and Clemens, 1987; Chen et al., 1992) renal CaBP D-28K expression is stimulated by $1,25(OH)_2D_3$. Recently, a

correlation between induction of $1,25(OH)_2D_3$, CaBP D-28K and active calcium transport has been shown in primary culture of rabbit renal cells (Bindels et al., 1991). There is mounting evidence that $1,25(OH)_2D_3$ stimulates renal and intestinal CaBP D-28K via a classic steroid hormone mechanism. CaBP D-28K mRNA transcription is increased in response to $1,25(OH)_2D_3$ treatment of cultured chicken intestinal cells (Ferrari et al., 1992). In intestine, $1,25(OH)_2D_3$ induction of CaBP D-28K protein synthesis (Hunziker et al., 1982) and mRNA transcription (Theofan, Nguyen and Norman, 1986) is correlated with VDR occupancy. In kidney an autoradiographic study demonstrated nuclear localization of the $1,25(OH)_2D_3$-VDR complex in distal convoluted tubules (Stumpf et al., 1980), which coincides with immunocytochemical localization of CaBP D-28K. Additionally, a maximum stimulatory dose of $1,25(OH)_2D_3$ results in induction of CaBP D-28K mRNA in chick kidney (Clemens et al., 1988) and in primary culture of vitamin D-deficient chick kidney cells (Clemens et al., 1989; Enomoto et al., 1992).

When I started these studies, primary culture systems were the only model in which regulation of CaBP D-28K expression by $1,25(OH)_2D_3$ had been reported. However primary culture systems are heterogenous mixtures of proximal and distal tubular cells, of which only a small percentage actually express CaBP D-28K (Craviso, Garrett and Clemens, 1987). In order to perform my studies I initially had to

establish an *in vitro* model of the renal distal tubule, which I describe in Chapter One. In studies described in Chapter Two I used this model to determine the effects of $1,25(OH)_2D_3$ and TPA on PKC activity and immunoreactivity. In studies described in Chapter Three I correlated the effects of these two agents on PKC with expression of the VDR and CaBP D-28K. During the course of the studies comprising the initial three chapters, isozyme-specific antibodies for PKC became available. As it is now evident that PKC exists as a family of kinases that differ in activation requirements, subcellular distribution and substrate specificity (Nishizuka, 1988; 1989), my final Chapter describes studies of the differential effects of $1,25(OH)_2D_3$ and TPA on renal PKC isozymes.

THESIS OBJECTIVES

The overall objective of this thesis was to study the role of PKC in renal effects of $1,25(OH)_2D_3$; specifically, the regulation of CaBP D-28K expression. The specific objectives were as follows;

-To establish an *in vitro* model for vitamin D-mediated effects in the renal distal tubule (Chapter One).

-To determine the effects of $1,25(OH)_2D_3$ and TPA on PKC activity and immunoreactivity in this model (Chapter Two).

-To determine the effects of $1,25(OH)_2D_3$ and TPA on VDR and CaBP D-28K expression in this model and in relation to their effects on PKC (Chapter Three).

-To determine the effects of $1,25(OH)_2D_3$ and TPA on subcellular localization of specific PKC isozymes in this model (Chapter Four).

CHAPTER 1: ESTABLISHMENT OF AN *IN VITRO* MODEL TO STUDY THE ROLE OF PROTEIN KINASE C IN EFFECTS OF $1,25(OH)_2D_3$ ON RENAL CALBINDIN D-28K.

1.1 Introduction

The basic functional unit of the kidney, the nephron, possesses a structural and functional segmentation. The main segments are the proximal tubule, the diluting segment, the distal tubule and the collecting tubule. The structural organization implies that the nephron displays a great deal of biochemical heterogeneity; distinct sites of hormone binding and action exist in each of these segments (Morel, 1981). Because of this heterogeneity, studies of hormone action *in vivo* or in whole kidney homogenates are difficult to interpret. This is especially true for $1,25(OH)_2D_3$, whose synthesis is limited to the proximal tubule, whereas the effects of $1,25(OH)_2D_3$ are predominantly confined to distal sites of the nephron (Kawashima and Kurokawa, 1986; DeLuca, Krisinger and Darwish, 1990). The VDR is present in both proximal and distal tubules; in the proximal tubule $1,25(OH)_2D_3$ presumably mediates effects on the vitamin D hydroxylases while in the distal tubule, $1,25(OH)_2D_3$ is involved in mediating effects on calcium transport, CaBP D-28K and catabolism of $1,25(OH)_2D_3$. Thus for studies focusing on actions of $1,25(OH)_2D_3$, renal epithelial cell culture could be

a better alternative than studies *in vivo* or in whole kidney. Two possible *in vitro* models could be pursued: Primary culture of renal tubules or a renal cell line.

1.1.1 Primary Culture of Renal Tubules

The best characterized *in vitro* system available for the study of $1,25(OH)_2D_3$ regulation of renal CaBP D-28K is primary culture of vitamin D-deficient chick kidney cells (Craviso, Garrett and Clemens, 1987; Clemens et al., 1989; Enomoto et al., 1992). These cells, which are obtained by collagenase digestion of whole chick kidney, display an epithelial morphology with little fibroblast contamination. However, immunocytochemistry indicates that only a minority of cells, likely from distal tubules and collecting ducts (Taylor, McIntosh and Bourdeau, 1982), retain the ability to synthesize CaBP D-28K in culture. As well, these cultures can synthesize $1,25(OH)_2D_3$ from $25(OH)D_3$, indicating the presence of proximal tubular cells (Brunette et al., 1978). Thus, these cultures are apparently a heterogenous mixture of distal and proximal tubular cells. When these cells are administered a dose of 100 nM $1,25(OH)_2D_3$, the cellular CaBP D-28K content increases linearly with time up to 48 hours; this two-fold increase is blocked by cycloheximide. Since CaBP D-28K expression appears to be regulated in a vitamin D-dependent manner in these cells, it has been inferred, but not reported, that these

cells express the VDR in culture. A similar primary culture system derived from vitamin D-replete rats (Chen et al., 1992) also expresses CaBP D-28K in a vitamin D-responsive manner. As determined by enzyme markers, these cultures consist of both renal proximal and distal tubular cells (Chen, King and Armbrecht, 1990). A primary culture system consisting of microdissected rabbit connecting tubules and cortical collecting ducts also expresses CaBP D-28K in a vitamin D-dependent manner (Bindels et al., 1991). Thus primary culture systems, in addition to the obvious disadvantages of frequent contamination and labor intensive isolation, are often heterogenous mixtures of renal tubular cells.

1.1.2 Established Renal Cell Lines

Although a number of established renal cell lines express the VDR, none have been reported to express CaBP D-28K. LLC PK_1 (pig kidney) cells have VDR and can metabolize $25(OH)D_3$ to $1,25(OH)_2D_3$, which suggests that they are predominantly proximal tubular cells (Colston and Feldman, 1981). OK (opossum kidney) cells are a well established model of proximal tubule cells, (Biber, Forgo and Murer, 1988), but it is not known whether they have VDR. Since CaBP D-28K is localized to distal tubules *in vivo*, these cell lines of proximal origin would not be expected to express CaBP D-28K. Madin Darby Canine Kidney (MDCK) cells closely resemble

differentiated kidney distal tubule cells (Taub et al., 1979); although no VDR can be detected by ^{3}H-1,25(OH)$_2$D$_3$ binding techniques (Colston and Feldman, 1982). A more recent report indicates that the MDCK cell line is a heterogenous mixture of tubular cells (Gstraunthaler, Pfaller and Kotanko, 1985). A related cell line, Madin Darby Bovine Kidney (MDBK) cells, was therefore chosen for further characterization. At the time there were no reports concerning the suitability of this cell line as a model of the renal distal tubule. As well, it had not been reported whether this cell line contained VDR and CaBP D-28K. However MDBK cells had been used to study the effects of tumor promoters on the cytoskeleton (Ben-Ze'ev, 1986). Twenty-four hour treatment with TPA induced gross morphological changes in MDBK cells, reportedly due to alterations in the cytoskeleton. Phorbol esters such as TPA can exert generalized effects on cell proliferation and differentiation (Nishizuka, 1989). However, it is unclear whether these effects are involved in the induction of morphological changes reported for MDBK cells. As mentioned previously, similar effects on cell proliferation and differentiation are induced by 1,25(OH)$_2$D$_3$ (Minghetti and Norman, 1988). *In vitro*, the expression of the VDR (Chen and Feldman, 1981), PKC (Halsey et al., 1987) and CaBP D-28K (Chen et al., 1992) are related to the rate of cell proliferation. Thus, I initially had to determine whether MDBK cells contain VDR, CaBP D-28K, PKC and other distal tubular marker proteins

and to ensure that any effects of TPA and $1,25(OH)_2D_3$ observed in MDBK cells are not due to generalized effects on cell proliferation.

1.2 Objectives

The overall objective of the studies described in this chapter was to establish an *in vitro* system of distal tubular epithelial cells in which to study the role of PKC in the $1,25(OH)_2D_3$ regulation of renal CaBP D-28K expression. The specific objectives were:

1.2.1 To establish that MDBK cells are a renal distal tubular epithelial cell line.

1.2.2 To establish that MDBK cells express CaBP D-28K, VDR and PKC.

1.2.3 To establish the effect of serum on MDBK cell proliferation and on levels of CaBP D-28K, VDR and PKC.

1.2.4 To establish the effect of TPA and $1,25(OH)_2D_3$ on MDBK cell proliferation.

1.2.5 To establish the effect of TPA and $1,25(OH)_2D3$ on MDBK cell morphology.

1.3 Methods

Cell Culture

Stock cultures of MDBK cells were kindly donated by Dr. K. Dimock (Department of Microbiology, University of Ottawa, Ottawa, ON., Canada). The MDBK cell line was originallly derived from a kidney of an adult steer by Madin and Darby (1958). These cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco IBRL, Burlington, ON., Canada) supplemented with 10% newborn calf serum (Gibco IBRL). Cells were plated in 60 mm dishes at a density of 1.5 x 10^4 cells/ml and grown to confluence. Alternatively, for immunofluorescent studies, cells were plated on glass coverslips in 35 mm dishes. For experiments on cell proliferation and morphology, confluent cells were fluid changed to serum-free DMEM for 24 hours followed by treatment with 100 nM $1,25(OH)_2D_3$ (Biomol Research Laboratories, Plymouth Meeting, PA., USA)(in ethanol), 100 nM TPA (in PBS/DMSO (50/50;v/v)) or the vehicles for 24 hours.

OK cells were generously provided by Dr. L. E. Limbird (Department of Pharmacology, Vanderbilt University, Nashville, TN., USA). The OK cell line was originally established by Koyama et al., (1978) from the whole kidneys of an adult female opossum. Cells were grown in RPMI 1640 medium (Gibco IBRL) supplemented with 10% fetal calf serum (Woodlyn

Laboratories, Guelph, ON., Canada), plated at a density of 10^5 cells/ml and allowed to reach confluence.

Murine *cyc*⁻S49T lymphoma cells, which were used as the source of substrate for PKC assay (see 1.3.4), were grown in suspension culture in RPMI 1640 medium (Gibco IBRL) supplemented with 10% horse serum (Gibco IBRL). Cultures were seeded at 2-4 x 10^5 cells/ml and maintained for three days before harvesting. All cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

1.3.1 Identification of Distal Tubule Origin

Cells on coverslips were washed twice with PBS, fixed for 20 min at -20°C in ethanol:acetone (50/50;v/v) rinsed with PBS and then blocked overnight at 4°C in PBS/skim milk with sodium azide included as an antimicrobial agent. After washing with PBS, coverslips were incubated with a mouse anti-human kidney distal tubule monoclonal antibody (MAB 474, Chemicon International Inc., Temecula, CA., USA) diluted 1:10 with blocking solution for one hour in a humid box at room temperature. This antibody is believed to be directed against an epithelial cytokeratin. Non-specific binding was determined by incubating with blocking solution alone. Coverslips were rinsed several times with PBS and then incubated with fluorescein-conjugated donkey anti-mouse secondary antibody (Jackson Immunoresearch Laboratories) for

60 min at a dilution of 1:50 in blocking solution. Coverslips were then rinsed extensively with PBS and mounted with glycerol-containing 0.1% (w/v) p-phenylenediamine, viewed with a Zeiss fluorescent microscope and photographed using Ilford XP1 black and white film.

1.3.2 Characterization of Vitamin D Receptor

For FPLC analysis to determine the approximate molecular weight/size of the $1,25(OH)_2D_3$ binding component, MDBK cells were homogenized in high salt KTED buffer (10 mM Tris-HCl, 1 mM EDTA, 5 mM dithiothreitol, 10 mM molybdate, 0.02% (w/v) soybean trypsin inhibitor, 300 mM KCl) and centrifuged (105,000g, 4°C, 60min) to yield a chromatin extract. Cell extracts (4.5 mg protein/ml) were incubated for 24 hours at 4°C, with 2.5 nM ^3H-$1,25(OH)_2D_3$ (Amersham Canada LTD, Oakville, ON., Canada). The time course of specific ^3H-$1,25(OH)_2D_3$ binding indicated that binding was maximal by 24 hours of incubation with ^3H-$1,25(OH)_2D_3$ (Appendix 1). Non-specific binding was assessed in companion tubes containing 250-fold excess of unlabelled $1,25(OH)_2D_3$ (Biomol Research Laboratories). Bound and free hormone were separated by addition of dextran-coated charcoal, incubation for 15 min and centrifugation at 3,500g for 15 min (Manolagas et al, 1980); this charcoal solution was found to adsorb 97% of ^3H-$1,25(OH)_2D_3$ in KTED buffer (data not shown). The supernatants

were then applied to a Superose 12 FPLC column (Pharmacia, Dorval, PQ., Canada) and eluted at 0.3 ml/min with KTED buffer. ^{14}C-albumin (molecular weight = 66 kD) was used as an internal standard (Stone et al., 1991). One ml fractions were counted for ^{14}C and ^{3}H radioactivity.

For determination of N_{max} and K_d, chromatin extracts from MDBK and OK cells (1.0 mg protein/ml) were incubated overnight with increasing concentrations of ^{3}H-1,25(OH)$_2$D$_3$ (0.05-1.0 nM) alone or in the presence of a 250-fold molar excess of unlabelled 1,25(OH)$_2$D$_3$ and then charcoal treated as described above. The equilibrium binding data was analyzed by the method of Scatchard and best fit lines were generated by computerized linear regression of data points.

For some experiments measurement of VDR number was achieved by incubation of chromatin extracts (1 mg protein/ml) with 0.5 nM ^{3}H-1,25(OH)$_2$D$_3$ (Amersham Canada LTD) for 24 hours at 4°C. Non-specific binding was assessed in companion tubes containing 250-fold excess of unlabelled 1,25(OH)$_2$D$_3$ (Biomol Research Laboratories). Bound and free hormone were separated as described above and bound ^{3}H-1,25(OH)$_2$D$_3$ in the supernatants was then counted in a Beckman liquid scintillation counter.

1.3.3 Analysis of Calbindin D-28K

The initial analyses of CaBP D-28K in MDBK cells were performed by Dr. S. Christakos (Department of Biochemistry,

University of Medicine and Dentistry of New Jersey, Newark, NJ., USA); samples were sent and immunoblotted with a rabbit polyclonal antibody to rat renal CaBP D-28K (Sonnenberg, Pansini and Christakos, 1984). Subsequently, techniques for immunoblotting were set up in our laboratory, using a commercially available CaBP D-28K monoclonal antibody. For these studies, cytosolic supernatants of MDBK and OK cells were diluted in 1 mM $NaHCO_3$, 5 mM $MgCl_2$, 100 μM PMSF, 50 mM Tris-HCl (pH 7.5), 0.01% (w/v) bromophenol blue, 0.05% (v/v) glycerol, 1% (w/v) sodium dodecyl sulfate (SDS) and 1.25% (v/v) ß-mercaptoethanol. 100 μg of protein was electrophoresed on 15% SDS-polyacrylamide gels (SDS-PAGE) (Laemmli, 1970), equilibrated for 30 min in transfer solution containing 192 mM glycine, 25 mM Tris and 20% (v/v) methanol, pH 8.3 and then transferred electrophoretically to nitrocellulose. The effectiveness of transfer was monitored by staining the nitrocellulose with 0.2% (w/v) Ponceau S in 3% (w/v) TCA. Non-specific binding sites were blocked by incubation of nitrocellulose in blocking solution (PBS with 0.5% (w/v) skim milk (Gibco)) for two hours at room temperature. The papers were then washed in PBS/Tween (PBS with 0.1% (v/v) Tween 20) and incubated for one hour at 37°C with a mouse anti-avian intestinal CaBP D-28K monoclonal antibody (Sigma Immunochemicals, St. Louis, MO., USA) diluted 1:200 with blocking solution. The characteristics of this antibody have been previously described by Celio et al (1990).

The paper was then washed again with PBS/Tween and incubated for one hour at room temperature with a biotin-conjugated goat anti-mouse secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA., USA) diluted 1:1000 with blocking solution. The paper was washed once more with PBS/Tween, incubated for 20 min at room temperature with streptavidin peroxidase (Jackson Immunoresearch Laboratories) diluted to 2 μg/ml with blocking solution, washed a final time with PBS/Tween and then with PBS alone. The peroxidase-product was detected with 4-chloro-1-napthol (4 mg/ml in PBS with 20% (v/v) methanol and 0.12% (v/v) H_2O_2) (Aldrich Chemical Company Inc., Milwaukee, WI., USA) (Cadrin et al., 1990).

1.3.4. Assay of Protein Kinase C Activity

PKC activity was measured as phosphorylation of an 85 kD protein substrate. As reported by Chakravarthy et al., (1989) this substrate is specific for PKC and can be readily isolated from murine *cyc*⁻S49T lymphoma cells. Due to its electrophoretic mobility and heat stability, this protein substrate is believed to be analogous to the myristoylated alanine-rich C kinase protein substrate (MARCKS) isolated from rat brain (Blackshear et al., 1986). The advantage of this novel assay is that it allows the measurement of PKC activity *in vitro* while it is still in the native membrane associated state and does not require detergent extraction, partial

purification and/or reconstitution with phospholipid as do more conventional PKC activity assays.

To obtain membrane associated PKC, MDBK cell monolayers were washed with PBS, frozen at -20°C in 1.0 ml of hypotonic lysis buffer (1 mM $NaHCO_3$, 5 mM $MgCl_2$, 100 μM PMSF), thawed, scraped from the dish and vortexed for 2 min. Intact nuclei were removed by centrifugation (500g, 4°C, 5 min) and 25 μg of post nuclear fraction protein was centrifuged (14,000g, 4°C, 15 min) to obtain a membrane pellet. This amount of post nuclear fraction protein was determined to be within the linear range for phosphorylation of the PKC-specific 85 kD protein substrate by membrane associated PKC from MDBK cells (Appendix 2).

For this novel phosphorylation assay, the membrane pellet was resuspended in an assay buffer consisting of 1 mM $NaHCO_3$, 5mM $MgCl_2$, 100 μM PMSF, 50 mM Tris-HCl, 10 μM EGTA, 1μM $CaCl_2$, 100 μM sodium vanadate, 100 μM sodium pyrophosphate and 1 mM sodium fluoride, pH 7.5. The reaction was initiated by addition of the PKC-specific 85 kD protein substrate (previously isolated from murine *cyc*$^-$S49T lymphoma cells essentially as described in Chakravarthy et al., 1989) and [γ-^{32}P]-ATP (10 μM, 4400 cpm/pmol; NEN Research Products, Markham, ON., Canada). To some tubes, 100 nM TPA was added to activate PKC. Reactions were terminated after 10 min at 37°C by addition of EGTA for a final concentration of 1mM. This assay can be used to measure calcium dependent PKC isozyme activity.

Membranes were removed by centrifugation (14,000g, 4°C, 15 min) and the supernatant proteins were separated on 8% SDS-PAGE (Laemmli, 1970). The phosphorylated 85 kD protein was visualized by autoradiography on Kodak X-OMAT AR film (Eastman Kodak Company, Rochester, NY, USA) for 48 hours.

1.3.5. Assessment of Cell Proliferation and Morphology

Cell proliferation was assessed as ^{3}H-thymidine incorporation into DNA; MDBK cells were treated with ^{3}H-thymidine (NEN) at a final concentration of 0.5 uCi/ml for 24 hours. After removal of media and washing with PBS, cells were fixed with PBS/formalin. They were then washed once with H_2O, twice with ice cold 0.5N perchloric acid (PCA) and heated for 20 min at 90°C in 0.5N PCA to solubilize DNA. The radioactivity of the PCA-extracted DNA was determined in a Beckman liquid scintillation counter and DNA synthesis was expressed as cpm ^{3}H-thymidine incorporated per dish (Franks, Plamondon and Hamet, 1984).

Assessment of cell cycle parameters was performed by flow cytometry of MDBK cell suspensions. MDBK monolayers were detached by addition of trypsin/EDTA (0.25%/1 mM), cells were counted and centrifuged (500g, 5 min, 4°C) and then a single cell suspension was prepared. 100 µl of a lysing reagent (Coulter Corporation, Hialeah, FL, USA) was added to 100 µl of cell suspension followed by 2 ml of propidium iodide/RNAse

solution (Coulter Corporation). This mixture was incubated for 15 min at room temperature. DNA histograms were obtained by analysing 1 x 10^6 cells in an Epics-Profile II Cytofluorometer (Coulter Corporation). The percentage of cells in G_0/G_1, S and G_2/M of the cell cycle were determined by planimetry using the "Elite" software (Coulter Corporation).

The effect of TPA and $1,25(OH)_2D_3$ on MDBK cell morphology was assessed by phase contrast light microscopy and photographed with Ilford XP1 black and white film.

Miscellaneous

Protein concentration was determined by the method of Bradford (1976). All materials were obtained from Sigma Chemical Corp, St. Louis, MO unless otherwise stated. Data are expressed as mean +/- standard error (SEM); when indicated in the figure legend, statistical significance was evaluated with the True Epistat computer program.

1.4 Results

1.4.1 Identification of Distal Tubule Origin

Immunofluorescent techniques were used to evaluate the presence of a distal tubule epithelial marker in MDBK cells (Figure 1.1). The homogenous immunoreactivity clearly indicates that MDBK cells retain this distal tubule characteristic.

1.4.2 Characterization of Vitamin D Receptor

FPLC analysis indicated that the ^{3}H-1,25$(OH)_2D_3$ binding component in MDBK cells eluted in one major peak corresponding to a molecular weight of approximately 66-70 kD. This binding peak was effectively competed out by the inclusion of unlabelled 1,25$(OH)_2D_3$ (Figure 1.2).

Scatchard analysis of binding data indicated the presence of one high affinity binding site for 1,25$(OH)_2D_3$ in MDBK cells, while similar analysis of an established proximal tubule (OK) cell line indicated little detectable specific binding (Figure 1.3). In MDBK cells, the number of binding sites were 17.3 fmol/mg protein (N_{max}) with a K_d of 0.07 nM.

Figure 1.1 Immunofluorescence of Distal Tubule Marker in MDBK cells.

Cells were fixed and incubated with a distal tubule marker monoclonal antibody and antigen-antibody complexes were detected with a fluorescein-conjugated secondary antibody as described in 1.3.1. Specific immunofluorescence is shown in (A) while the nonspecific is shown in (C). A representative field of each is shown on the left with the corresponding phase contrast photograph shown to the right of each fluorescent image.

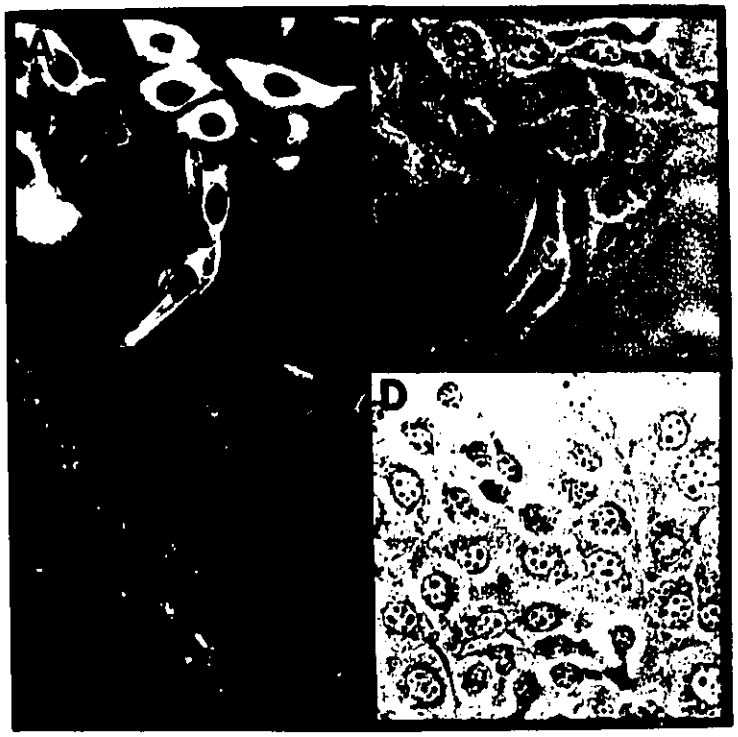

A
D

Figure 1.2 FPLC Analysis of Specific and Non-Specific ^{3}H-1,25(OH)$_2$D$_3$ Binding in MDBK Cells.

Cell extracts (4.5 mg protein/ml) prepared from MDBK cells were incubated with 2.5 nM ^{3}H-1,25(OH)$_2$D$_3$ alone or in the presence of a 250-fold molar excess of 1,25(OH)$_2$D$_3$ and incubated as described in 1.3.2. Bound sterol was applied to a Superose 12 FPLC column and eluted with KTED buffer. Total (●) and non-specific (O) binding were used to generate the radioactivity profile. Arrow indicates the elution position of ^{14}C-albumin (molecular weight = 66 kD).

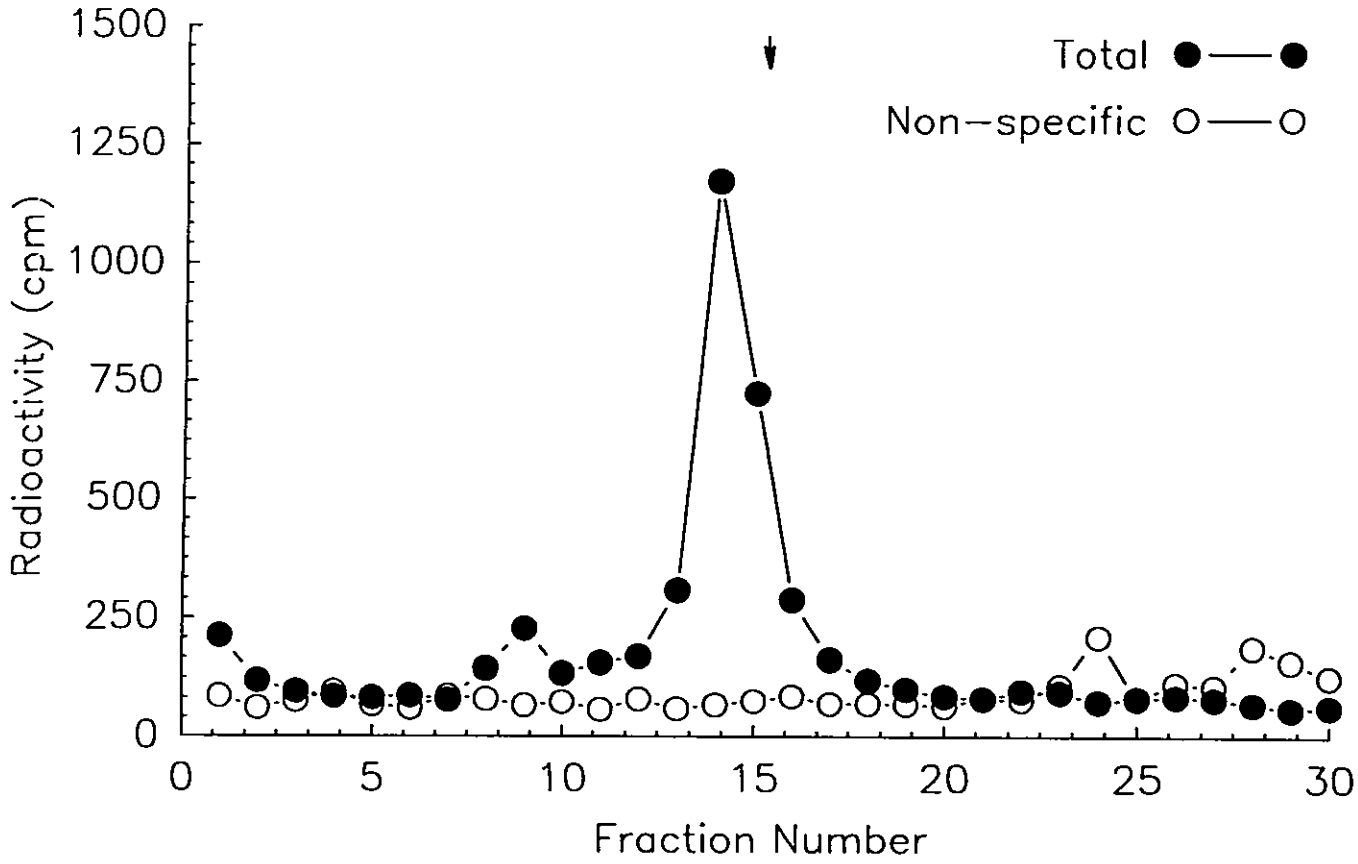
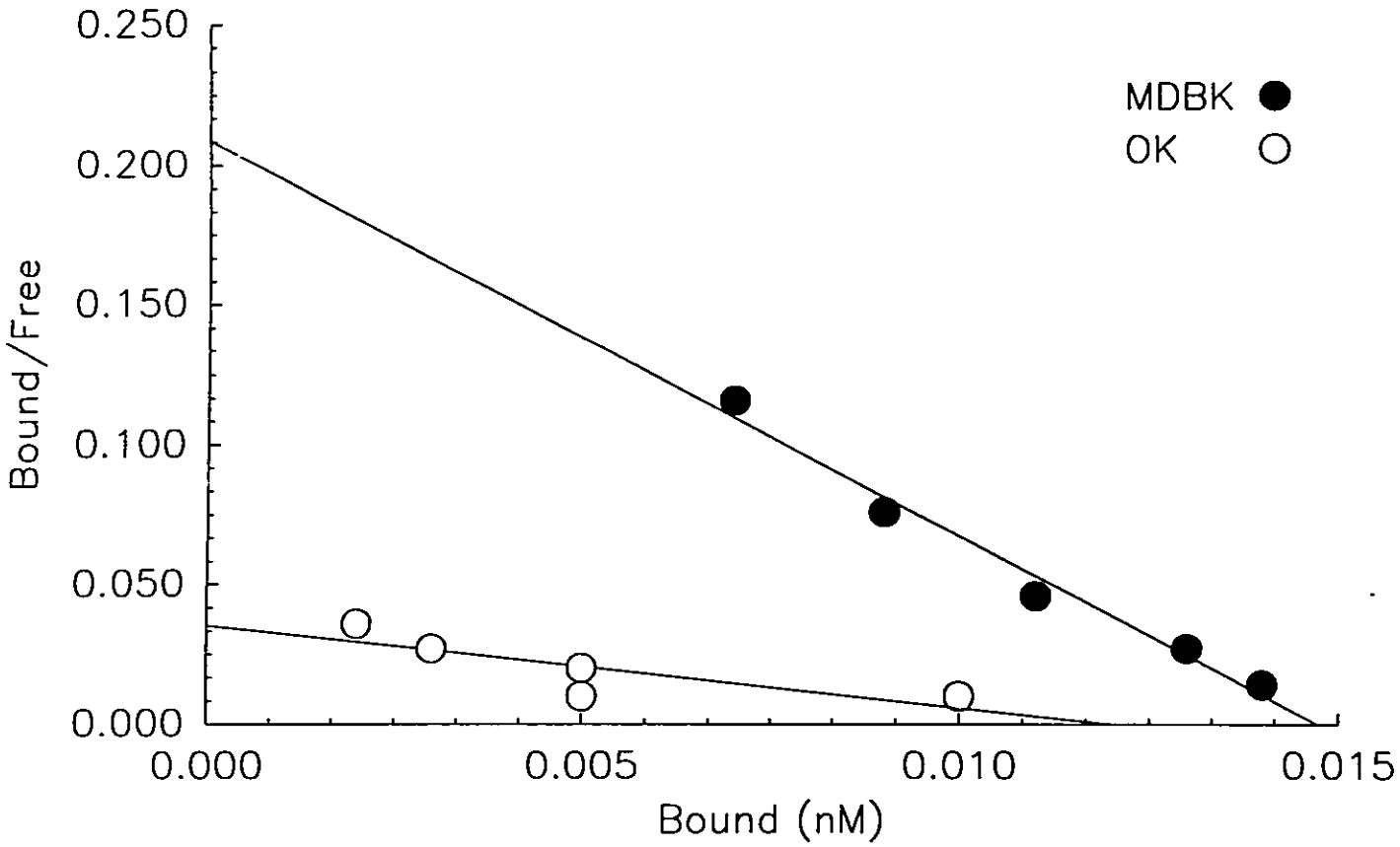

1500
1250
1000
750
500
250
0
Radioactivity (cpm)
0
5
10
15
20
25
30
Fraction Number
Total
Non-specific

Figure 1.3 Scatchard Analysis of Specific ^3H-1,25$(OH)_2D_3$ Binding in MDBK and OK Cells

For Scatchard analysis, chromatin extracts (1.0 mg protein/ml) prepared from MDBK (●) and OK (O) cells were incubated with 0.05-1.0 nM ^3H-1,25$(OH)_2D_3$ as described in 1.3.2. Specific binding, derived by subtracting non-specific from total binding was used to generate the Scatchard plots. Each point is the average of duplicates. Best fit lines were generated by computerized linear regression of data points.


0.250
0.200
0.150
0.100
0.050
0.000
Bound/Free
MDBK
OK
0.000
0.005
0.010
0.015
Bound (nM)

1.4.3 Analysis of Calbindin D-28K

In preliminary studies performed by Dr. S. Christakos, CaBP D-28K was detected in MDBK cytosolic extracts using a polyclonal antibody raised in rabbits against rat renal CaBP D-28K (Sonnenberg, Pansini and Christakos, 1984). The immunoreactive CaBP D-28K in MDBK cells displayed a similar molecular weight and electrophoretic behavior as pure rat renal CaBP D-28K (Figure 1.4).

In further studies, we used a distinct monoclonal antibody to CaBP D-28K (Celio et al, 1990), which also recognized a 28 kD protein in MDBK cytosolic extracts; no immunoreactivity was detected in cytosolic extracts from OK cells (Figure 1.5).

1.4.4 Assay of Protein Kinase C Activity

The PKC-specific 85 kD protein substrate purified from murine *cyc*-S49T lymphoma cells was phosphorylated *in vitro* by membranes isolated from MDBK cells (Figure 1.6). As reported in other cell lines (Chakravarthy et al, 1989), addition of the phorbol ester TPA *in vitro* stimulated phosphorylation of the 85 kD PKC-specific substrate. These observations indicate the presence of PKC-specific activity in MDBK cellular membranes.

Figure 1.4 Analysis of Calbindin D-28K in MDBK Cells

Cytosolic supernatants prepared from MDBK cells were immunoblotted with a rabbit anti-rat renal CaBP D-28K polyclonal antibody as described in 1.3.3. Lane 1: MDBK cytosol, Lane 2: rat renal CaBP D-28K standard.

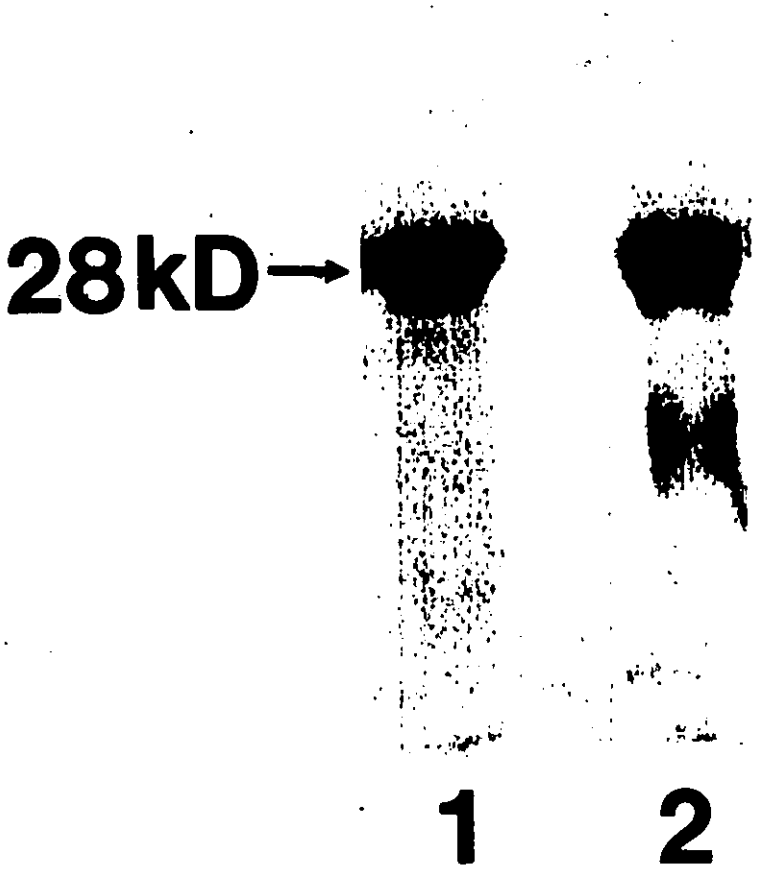

28kD
1
2

Figure 1.5 Calbindin D-28K Immunoreactivity in MDBK and OK Cells.

Cytosolic extracts prepared from MDBK (lane 1) and OK (lane 2) cells were separated on 15% SDS-PAGE, transferred to nitrocellulose and immunoblotted with a CaBP D-28K monoclonal antibody. Antigen-antibody complexes were detected with a biotinylated secondary antibody and streptavidin peroxidase as described in 1.3.3.

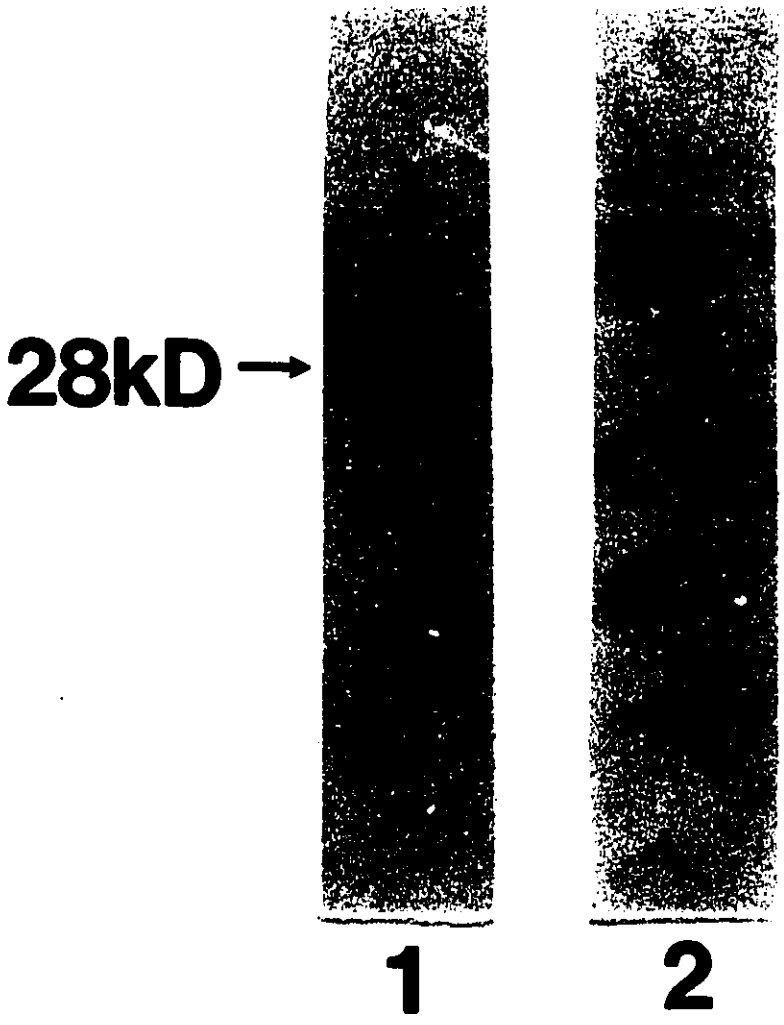
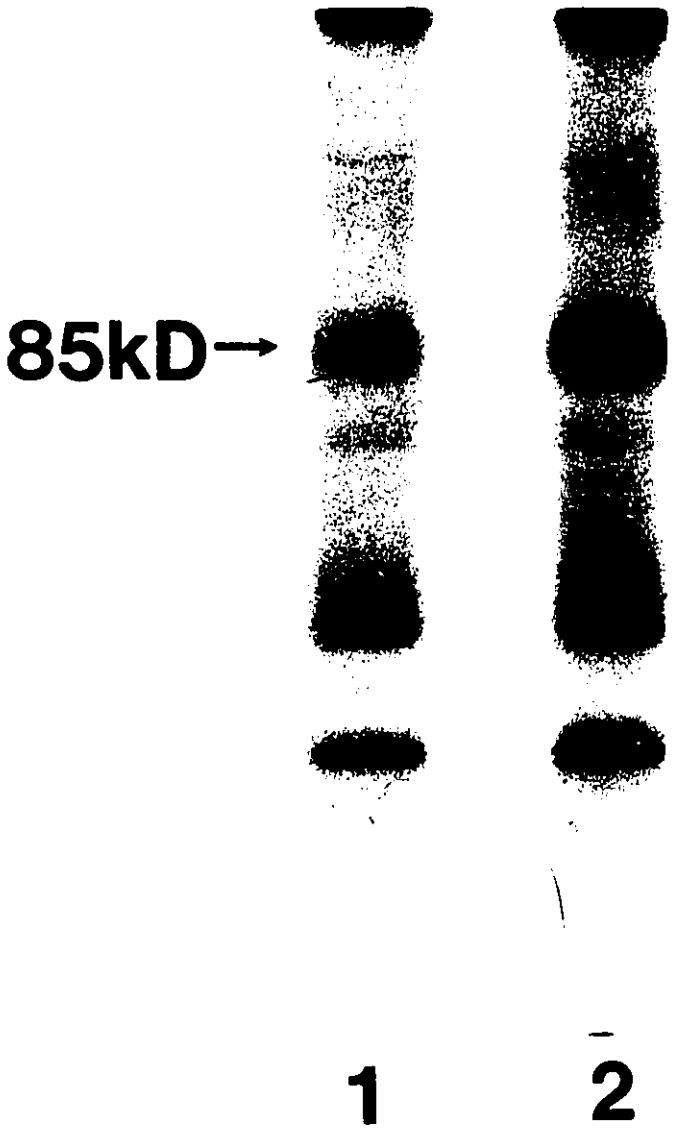

28kD
1
2

Figure 1.6 Membrane-Associated Protein Kinase C Activity in MDBK Cells

PKC activity was measured in the membrane-associated state by incubating crude membrane fractions from MDBK cells with [γ-^{32}P]-ATP and the 85kD PKC-specific substrate in the absence (lane 1) or presence (lane 2) of 100 nM TPA. Phosphorylated proteins were separated on 8% SDS-PAGE and visualized by autoradiography as described in 1.3.4.


85kD
1
2

1.4.5 Assessment of Cell Proliferation and Morphology

MDBK cells cultured in 10% serum exhibited proliferative growth, as evidenced by incorporation of ^{3}H-thymidine into DNA (Figure 1.7) and detection of cells in S phase on flow cytometry (Figure 1.8). In contrast, cells switched to serum-free media for 24 hours were rendered essentially quiescent. In comparison to cells in serum, serum-free cells showed a three-fold reduction of incorporation of ^{3}H-thymidine into DNA (Figure 1.7). Similar results were obtained with flow cytometry; after 24 hours in serum-free conditions there was a three-fold reduction in the percentage of cells found in S phase (Figure 1.8). Treatment of serum-free MDBK cells with either 100 nM TPA or 100 nM $1,25(OH)_2D_3$ for 24 hours did not affect cell proliferation, whether measured as ^{3}H-thymidine incorporation into DNA (Figure 1.7) or by flow cytometry (Figure 1.8). In both cases, proliferative indices were not different in serum-free cells compared to serum-free cells treated with either TPA, $1,25(OH)_2D_3$ or the respective vehicles.

Although TPA did not affect the proliferation of quiescent MDBK cells, gross morphological changes were induced by this phorbol ester (Figure 1.9). Untreated control MDBK cells displayed a tight cuboidal morphology typical of epithelial cells, whereas cells treated with 100 nM TPA became

Figure 1.7 DNA Synthesis in MDBK Cells; Effect of Serum, TPA and $1,25(OH)_2D_3$.

Serum-free MDBK cells were treated with 100 nM $1,25(OH)_2D_3$, 100 nM TPA or vehicles for 24 hours; cells in serum were not treated. ^{3}H-thymidine (0.5 μCi/ml media) was added 24 hours prior to assay. ^{3}H-thymidine incorporation was assessed as described in 1.3.5. Data are expressed as mean +/- SEM (n = 4), statistical significance was evaluated by Students' paired t test. DNA synthesis in cells with serum was approximately three times higher than synthesis in serum-free cells ($p < .001$). There was no significant effect of $1,25(OH)_2D_3$, TPA, DMSO/PBS or ethanol on DNA synthesis in serum-free cells.

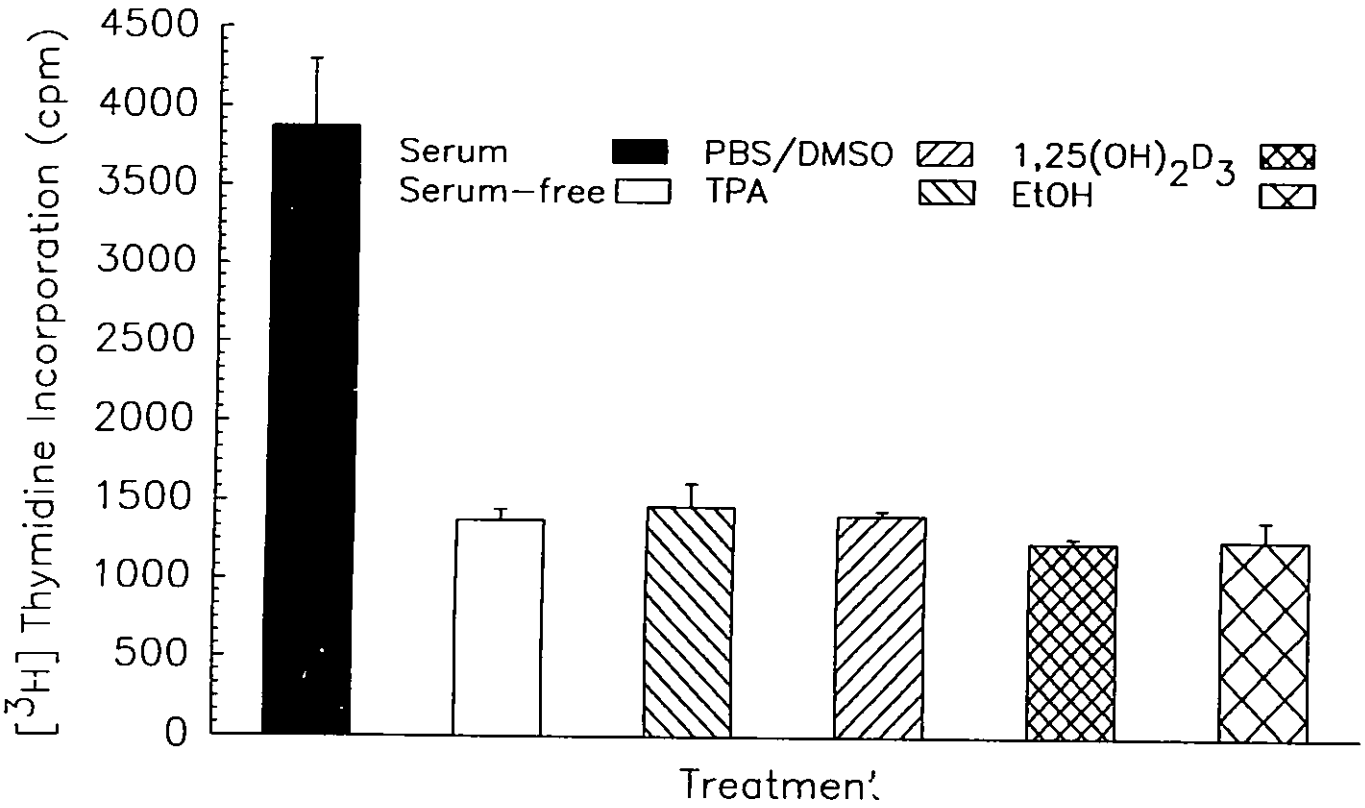
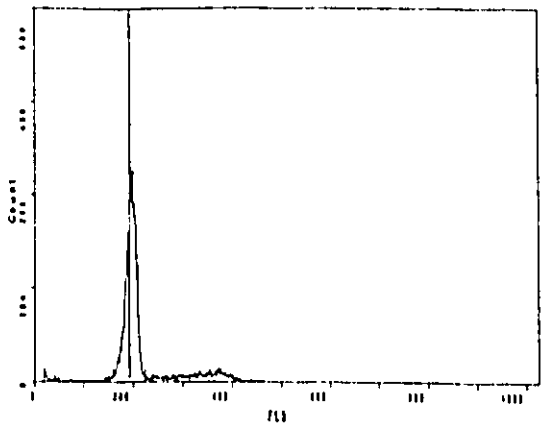
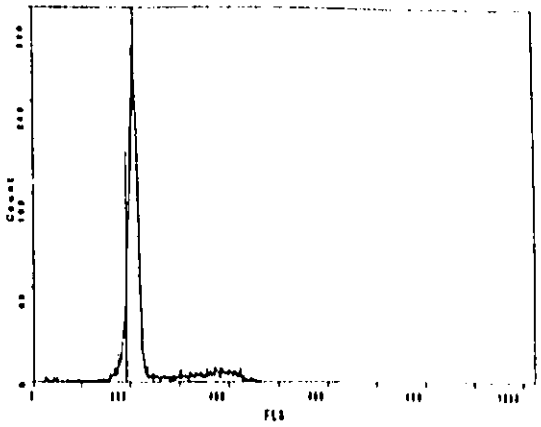

4500
4000
3500
3000
2500
2000
1500
1000
500
0
[3H] Thymidine Incorporation (cpm)
Serum
Serum–free
PBS/DMSO
TPA
1,25(OH)2D3
EtOH
Treatment

Figure 1.8 Flow Cytometry of MDBK Cells; Effect of Serum, TPA and $1,25(OH)_2D_3$.

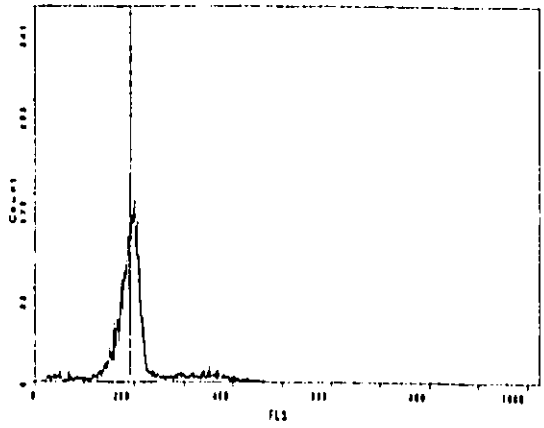
MDBK cells in serum (Serum) or serum-free MDBK cells (NS) were untreated or treated for 24 hours with ethanol (EtOH), 100 nM $1,25(OH)_2D_3$ in ethanol (1.25), PBS/DMSO; 50:50 (DMSO) or 100 nM TPA in PBS/DMSO 50:50 (TPA) were lysed, stained with propidium iodide and analysed by flow cytometry as described in 1.3.5. The percentage of cells in G_o/G_1 (FL3 range 162-247), S (FL3 range 242-359) and G_2/M (FL3 range 364-459) of the cell cycle were determined by planimetry using the "Elite" software as described in 1.3.5.



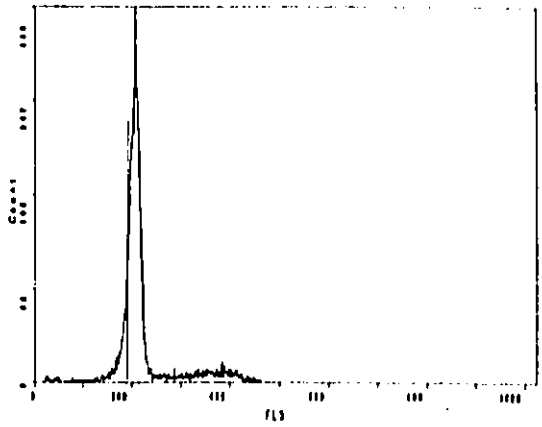
TPA



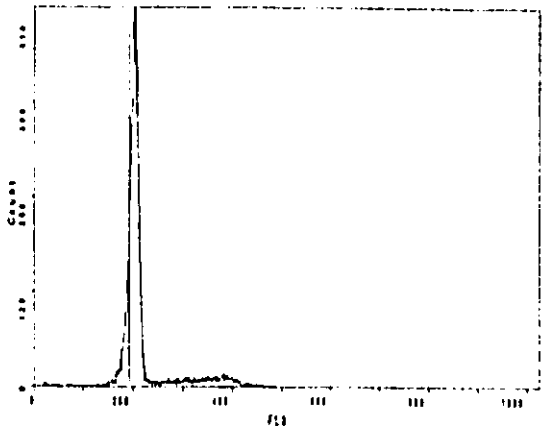
DMSO



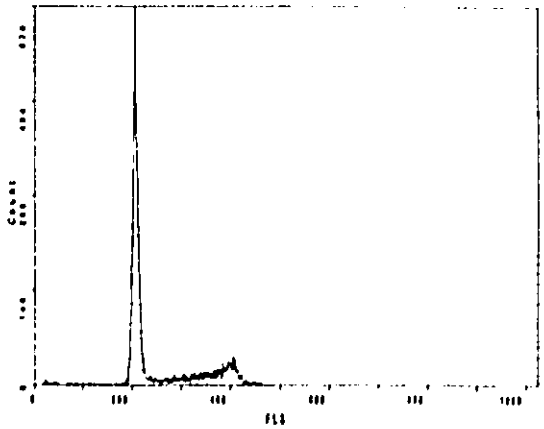
1.25



EtOH



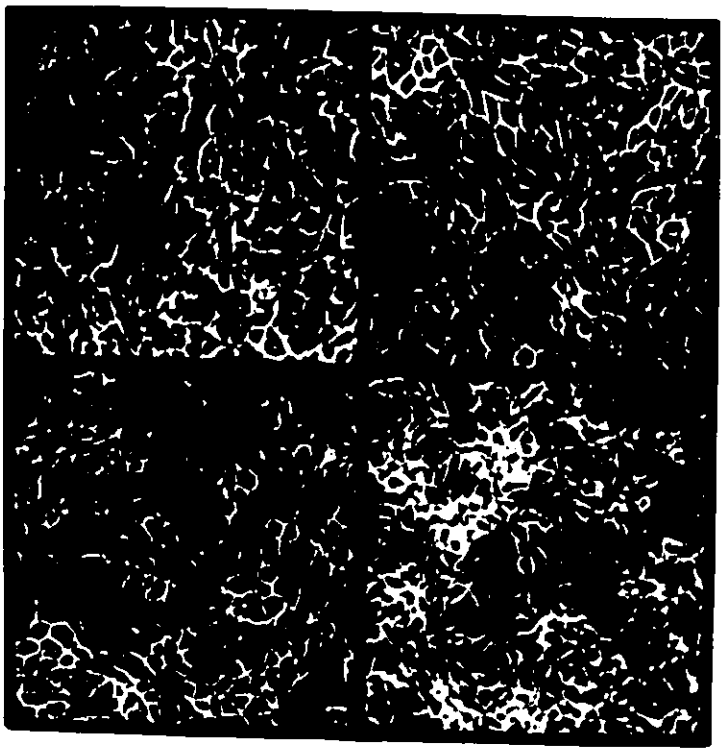
NS



Serum

Figure 1.9 Morphology of MDBK Cells; Effect of TPA and $1,25(OH)_2D_3$.

Serum-free MDBK cells were treated for 24 hours with (A) ethanol, (B) 100 nM $1,25(OH)_2D_3$ in ethanol, (C) PBS/DMSO or (D) 100 nM TPA in PBS/DMSO and then viewed and photographed under light microscopy as described in 1.3.5.

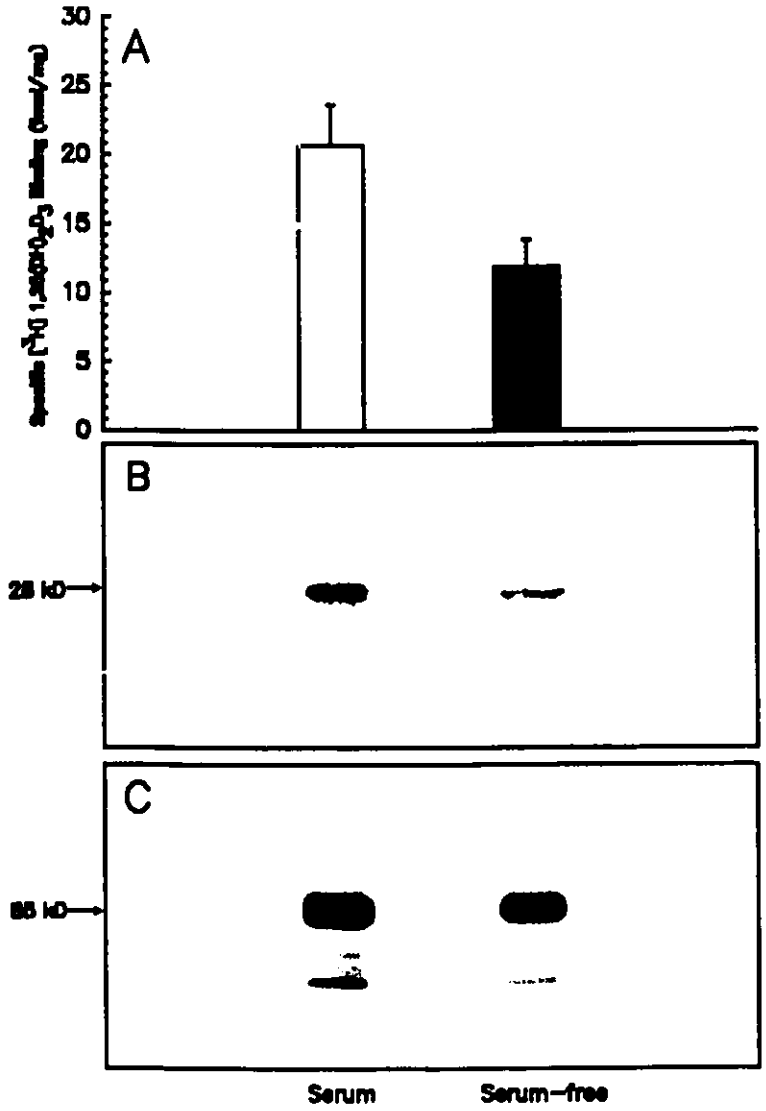


elongated, lost their cuboidal shape and displayed a fibroblastic-like morphology; these morphological changes were evident within 4-6 hours of treatment with TPA (data not shown), were most apparent at 24 hours (Figure 1.9) and persisted to a lesser extent for up to 48 hours. In contrast, $1,25(OH)_2D_3$ did not alter the morphology of quiescent MDBK cells when viewed at the level of the light microscope (Figure 1.9).

In MDBK cells, cell proliferation appeared to have a pronounced effect on expression of VDR, CaBP D-28K and PKC. Compared to serum-free cells, cells in serum displayed higher numbers of VDR, as measured by ligand binding, more intense immunoreactivity for CaBP D-28K and enhanced PKC membrane activity (Figure 1.10).

Figure 1.10 Effect of Serum on Specific ^3H-1,25(OH)$_2$D$_3$ Binding, Calbindin D-28K Immunoreactivity and Membrane-Associated Protein Kinase C Activity in MDBK Cells.

MDBK cells in serum or MDBK cells in serum-free media for 24 hours were analysed for specific ^3H-1,25(OH)$_2$D$_3$ binding (A) as described in 1.3.2, CaBP D-28K immunoreactivity (B) as described in 1.2.3 and membrane associated PKC activity (C) as described in 1.2.4.


A
30
25
20
15
10
5
0
B
28 kD
C
85 kD
Serum
Serum-free

1.5 Discussion

1.5.1 Identification of Distal Tubule Origin

In this chapter we establish that MDBK cells (Madin and Darby, 1958) represent an *in vitro* model for the renal distal tubule. These cells express a distal tubule marker protein (Figure 1.1), VDR (Figure 1.2) and CaBP D-28K (Figure 1.4). CaBP D-28K has been exclusively localized to the distal nephron (Taylor, McIntosh and Bourdeau, 1982). Although the VDR is detectable in both the distal (Stumpf et al., 1979) and proximal (Colston and Feldman, 1982) tubules under normal conditions VDR expression is higher in the distal nephron. As a negative control we examined OK cells (Koyama et al., 1978), an established *in vitro* model for the proximal tubule (Biber, Forgo and Murer, 1988). In comparison to MDBK cells, OK cells contained little detectable VDR (Figure 1.3) and did not express CaBP D-28K (Figure 1.5).

1.5.2 Characterization of Vitamin D Receptor

As determined by FPLC analysis (Figure 1.2), the ^{3}H-$1,25(OH)_2D_3$ binding component in MDBK cells had a molecular weight similar to that of the well characterized intestinal VDR (Minghetti and Norman, 1988) and identical to that reported for whole rat kidney (Stone et al., 1991). As well,

this binding component was specific, since it was competed out by the inclusion of unlabelled $1,25(OH)_2D_3$. The K_d (0.07 nM) and N_{max} (17.3 fmol/mg protein) of the VDR in MDBK cells (Figure 1.3) is consistent with values obtained for LLC-PK1 cells (Colston and Feldman, 1982), renal carcinoma cells (Nagakura et al., 1986) and mammalian kidney (Chandler, Pike and Haussler, 1979).

Thus we conclude that MDBK cells, like distal tubular cells *in vivo* (Stumpf et al., 1979), express the VDR.

1.5.3 Analysis of Calbindin D-28K

Preliminary studies were conducted to determine if MDBK cells expressed CaBP D-28K, the cytosolic vitamin D-dependent calcium binding protein localized to the distal tubule *in vivo* (Taylor, McIntosh and Bourdeau, 1982). Immunoblotting MDBK cell cytosol with a polyclonal rabbit antibody raised against rat renal CaBP D-28K (Sonnenberg, Pansini and Christakos, 1984) detected a protein at approximately 28 kD, that displayed electrophoretic properties indistinguishable from standard rat renal CaBP D-28K on SDS-PAGE (Figure 1.4).

In subsequent studies, a well characterized murine monoclonal antibody to avian intestinal CaBP D-28K (Celio et al.,1990) was used for immunoblotting. It is important to emphasize that this monoclonal antibody does not recognize calretinin, a novel 27 kD calcium binding protein with

sequence similarities to CaBP D-28K (Rogers, 1987). Since it is possible that the available polyclonal antibodies, directed against CaBP D-28K, cross react with calretinin, all subsequent studies utilized the monoclonal CaBP D-28K. The monoclonal antibody to CaBP D-28K detected a 28 kD protein in MDBK cells but not in the OK proximal tubule cell line (Figure 1.5).

1.5.4 Assay of Protein Kinase C Activity

Classical activation of PKC in intact cells is thought to result from the combined action of DAG and calcium. Recent models indicate that activated PKC exists as a complex consisting of the kinase, calcium, phospholipid and DAG, presumably associated with a cellular membrane structure (Ganong et al., 1986). Active tumor-promoting phorbol esters such as TPA are thought to bind to PKC at the DAG binding site, leading to direct activation of PKC (Castagna et al., 1982). TPA can activate PKC both *in vivo* (ie. addition to intact cells) and *in vitro* (ie. addition to membrane fractions) (Chakravarthy et al., 1989). In MDBK cells, constitutively active PKC was detected as phosphorylation of the PKC-specific 85 kD substrate by untreated MDBK membrane fractions. As expected, *in vitro* addition of TPA to MDBK membrane fractions stimulated phosphorylation of the 85 kD substrate (Figure 1.6). These results are consistent with

data obtained in several other cell lines, including normal rat kidney cells (Chakravarthy et al., 1989) and MDCK cells (Portilla et al., 1988) and demonstrate the presence of activatable PKC in MDBK cells.

1.5.5 Assessment of Cell Proliferation and Morphology

As indicated in the introduction, TPA (Nishizuka, 1989) and $1,25(OH)_2D_3$ (Minghetti and Norman, 1988) can exert generalized effects on cell proliferation and differentiation. In cultured cells, the abundance of CaBP D-28K (Chen et al., 1992), VDR (Chen and Feldman, 1981) and PKC (Halsey et al., 1987) are related to the rate of cell proliferation. In MDBK cells, CaBP D-28K expression, VDR number and membrane PKC activity were higher in proliferating cells (cells in serum) compared to quiescent cells (serum-free cells) (Figure 1.10). In order to ensure that any effect of TPA or $1,25(OH)_2D_3$ on these three proteins was not secondary to a general effect of either agent on cell proliferation, their effects on DNA synthesis (by ^{3}H-thymidine incorporation) and DNA content (by flow cytometry) were measured in MDBK cells. It is readily apparent (Figure 1.7, Figure 1.8) that MDBK cells in serum exhibited high rates of proliferation, while cells depleted of serum were essentially quiescent. Furthermore, neither TPA nor $1,25(OH)_2D_3$ were mitogenic in quiescent MDBK cells. This data indicates that any effects of TPA and $1,25(OH)_2D_3$ on VDR,

CaBP D-28K and PKC in MDBK cells would necessarily be independent of effects on cell proliferation.

Despite having no effect on cell proliferation in quiescent MDBK cells, TPA induced dramatic morphological changes similar to those reported by Ben-Ze'ev (1986); including elongation of cells with a change from cuboidal epithelial morphology to a fibroblastic-like one (Figure 1.9). TPA has also been shown to induce these morphological changes in epidermal keratinocytes (Parkinson and Emmerson, 1982) and MDCK cells (Ojakian, 1981). All of these cell types display a breakdown of epithelial organization and development of a fibroblastic-like morphology in response to treatment with TPA. Intermediate filament reorganization is believed to be a major component of the morphological response to tumor-promoters (Fey and Penman, 1984). The characteristic morphological response or "signature" induced in well-differentiated epithelia by tumor-promoting agents like TPA are distinct from that observed in response to mitogens, metabolic inhibitors or agents that disrupt the cytoskeleton. Although $1,25(OH)_2D_3$ induces morphological changes consistent with terminal monocytic differentiation in HL-60 cells (Reitzma et al., 1983), it had no effect on morphology of MDBK cells. This result was not unexpected since MDBK cells are non-transformed distal tubule cells which display a highly differentiated phenotype.

1.6 Conclusions

The overall conclusion of this chapter is that MDBK cells represent a suitable model system for studying the role of PKC in the regulation of renal CaBP D-28K by $1,25(OH)_2D_3$. Other conclusions include:

1.6.1 MDBK cells express the renal distal tubular marker protein.

1.6.2 MDBK cells contain CaBP D-28K, VDR and PKC.

1.6.3 Expression of CaBP D-28K, VDR and PKC in MDBK cells appears to be related to the rate of cell proliferation.

1.6.4 Treatment of quiescent MDBK cells with TPA and $1,25(OH)_2D_3$ does not alter cell proliferation.

1.6.5 TPA treatment induces morphological changes in MDBK cells.

CHAPTER TWO: EFFECTS OF 1,25(OH)$_2$D$_3$ AND PHORBOL ESTERS ON PROTEIN KINASE C ACTIVITY AND IMMUNOREACTIVITY.

2.1 Introduction

PKC is recognized as a major regulatory enzyme and is implicated in the control of a wide variety of physiological processes (Nishizuka, 1984). PKC mediates signal transduction in response to many hormones, growth factors, neurotransmitters and drugs. Specifically PKC phosphorylates serine and threonine residues on target protein substrates, such as the epidermal growth factor receptor and myelin basic protein (Nishizuka, 1986). This kinase was originally shown to require calcium and phospholipid for activity (Takai et al., 1979a). As well, DAG stimulates PKC by decreasing its calcium requirement into a physiological range (<1 μM) (Takai et al., 1979b). Activation of PKC in intact cells is believed to result from the synergistic action of DAG and calcium, both generated secondary to signal-induced hydrolysis of membrane inositol phospholipids. Fully active PKC exists as a quaternary complex composed of phospholipid, calcium, DAG and the enzyme; the ternary complex without DAG exhibits kinase activity only at a 100-fold higher concentration of calcium. Phosphatidylserine (PS) appears to be the most effective phospholipid for reconstitution of enzyme activity *in vitro*. Recent studies on the mechanisms involved in lipid activation

of PKC have established the specificity of the enzyme for the sn-1,2-enantiomer of DAG and have also shown that DAG possessing long-chain, saturated acyl groups is the most effective activator (Boni and Rando, 1985).

Although a number of chemical compounds reportedly inhibit PKC activity, including 1-(5-isoquinolinesulfonyl)-2-methylpeperazine (H-7) (Kawamoto and Hidaka, 1984), sphingosine (Hannun et al., 1986) and staurosporine (Tamaoki et al., 1986), these agents all show substantial inhibition of other kinases. More promising specific inhibitors of PKC include monoclonal antibodies and pseudosubstrate peptides; however, at present, the inhibition of PKC activity by these compounds *in vitro* is not always complete (Leach et al., 1988; Henrich, 1991; Buchner et al., 1992).

Bovine brain PKC has been purified and partially sequenced; its apparent molecular weight is 79-80 kD (Parker and Ullrich, 1987). The amino acid sequence of bovine PKC suggests a two-domain structure: a regulatory domain and a catalytic domain between which lies a hinge region that is susceptible to proteolytic attack. There is direct evidence that the DAG and phospholipid binding sites are located within the regulatory domain. Limited proteolysis of PKC *in vitro* leads to generation of a fragment of PKC, designated PKM, that is constitutively active in the absence of calcium, phospholipid and DAG (Huang and Huang, 1986). Membrane bound PKC is reportedly more susceptible than cytosolic PKC to this

proteolysis (Kishimoto et al., 1983), however, controversy exists concerning the physiological significance of PKM generation as a mode of kinase activation (Nishizuka, 1986).

Molecular cloning and biochemical analysis has revealed the existence of multiple PKC isozymes (Nishizuka, 1988). During the course of the work described in this thesis, isozyme-specific probes became commercially available, which will be described in Chapter Four of this thesis.

2.1.1 Regulation of Protein Kinase C by Phorbol Esters

Phorbol esters can substitute for DAG and directly activate PKC (Castagna, 1982), bypassing the regulated signal-induced hydrolysis of membrane phosphoinositides. TPA, a potent tumor promoter, with a structure similar to DAG (Nishizuka, 1989) has been used extensively to study PKC. The dose response curve for TPA activation of PKC strongly resembles the saturation curve for TPA receptor binding, suggesting that PKC is the cellular receptor for TPA. Data from structure-activity studies of enzyme activation and receptor binding support this suggestion. In this context, 4-α-phorbol-12,13-didecanoate (4αPDD), a phorbol ester analogue of TPA, does not promote tumor formation, bind to the receptor for TPA nor modulate PKC activity (Castagna et al., 1982).

The term translocation refers to a change in intracellular localization of PKC upon treatment with TPA or

natural agonists. Most often translocation appears as a decrease in cytosolic PKC and an increase in membrane bound PKC. Translocation of PKC was first observed by Kraft et al., (1982, 1983) in studies of the subcellular distribution of PKC activity in EL4 mouse thymoma cells after treatment with TPA and has since been noted in a variety of cell types (Neidel and Blackshear, 1986).

Prolonged exposure of intact cells to TPA results in a gradual decline of PKC activity, a phenomenon which has been termed down-regulation of PKC. Although the kinetics vary, TPA induced down-regulation of PKC activity is common to many cells (Stabel et al., 1986). Down-regulation is a useful tool for investigating the importance of PKC-dependent pathways in the actions of hormones, neurotransmitters and growth factors. The mechanism of down-regulation seems to be directly due to a reduction in the PKC protein, as indicated by decreased phorbol ester binding sites and PKC immunoreactivity. It has been suggested that this reduction may result from degradation by a calcium-dependent protease present in plasma membrane (Young et al., 1987). A likely candidate is calpain, a protease which cleaves PKC in the presence of PS and DAG or TPA and is active at the micromolar range of calcium (Kishimoto et al., 1989). It is suggested that exposure of PKC to TPA, which is associated with activation or translocation to membrane, may expose one or more protease sensitive site(s). These sites are presumably localized to the hinge

region between the regulatory and catalytic domains therefore proteolysis could concievably produce the constitutively active fragment PKM. However, PKM is rarely recovered in intact cells and is probably degraded further by the action of other proteases (Nishizuka, 1989).

In summary, TPA elicits a biphasic effect, producing a short term activation (translocation) of PKC, followed by long term inactivation (down-regulation) of the kinase.

2.1.2 Regulation of Protein Kinase C by $1,25(OH)_2D_3$

$1,25(OH)_2D_3$ modulates PKC in HL-60 cells by a mechanism that involves the VDR. HL-60 cells can be induced to differentiate into morphologically, immunologically and biochemically mature monocytes in response to a variety of compounds including $1,25(OH)_2D_3$ (Bar-Shavit et al., 1983). The observation that TPA (Huberman and Callahan, 1979) also induces monocytic differentiation of these cells suggests that PKC may mediate the effects of both $1,25(OH)_2D_3$ and TPA. In HL-60 cells, long term treatment with $1,25(OH)_2D_3$ increases endogenous calcium and PS-dependent protein phosphorylation (Zylber-Katz and Glazer, 1985), phorbol ester binding (Martell, Simpson and Taylor, 1987) and transcription of the gene(s) for PKC (Obeid et al., 1990).

Recent data suggests that in addition to a genomic mechanism of action, $1,25(OH)_2D_3$ may act acutely via poorly

defined mechanisms. In keratinocytes (Tang et al., 1987) and enterocytes (Lieberherr et al., 1989), $1,25(OH)_2D_3$ rapidly (within 15 sec) induces hydrolysis of membrane phosphoinositides; which in turn produces increases in intracellular calcium and DAG and activation of PKC. However, since no membrane receptor has yet been identified for $1,25(OH)_2D_3$ this mechanism apparently precludes involvement of the well characterized intracellular VDR. Until further data defining this potential effect of $1,25(OH)_2D_3$ becomes available, it is difficult to incorporate these findings with existing theory concerning the mediation of $1,25(OH)_2D_3$ actions.

2.2 Objectives

The overall objective of the studies described in this chapter was to characterize the effect of TPA and $1,25(OH)_2D_3$ on PKC in MDBK cells. The specific objectives were:

2.2.1 To characterize the effect of long term treatment with $1,25(OH)_2D_3$ on PKC activity in MDBK cells.

2.2.2 To establish the dose response and time course of $1,25(OH)_2D_3$ effects.

2.2.3 To determine the effect of long term treatment with $1,25(OH)_2D_3$ on PKC immunoreactivity in MDBK cells.

2.2.4 To establish whether TPA causes short term activation and long term down-regulation of PKC activity in MDBK cells.

2.2.5 To characterize the long term effect of TPA on PKC immunoreactivity.

2.2.6 To compare the effects of the phorbol ester analogue 4αPDD on PKC activity and immunoreactivity.

2.3 Methods

Cell Culture

Stock cultures of MDBK cells were maintained in Dulbecco's modified Eagle medium (Gibco IBRL) supplemented with 10% newborn calf serum (Gibco IBRL). For activity assays, cells were plated in 60 mm dishes at a density of 1.5 x 10^4 cells/ml and grown to confluence. For immunoblotting, cells were grown in 150 mm dishes. Confluent cells were rendered quiescent by fluid changing to serum-free DMEM for 24 hours followed by treatment with 100 nM $1,25(OH)_2D_3$ (Biomol Research Laboratories) (in ethanol), 100 nM TPA (in PBS/DMSO (50/50; v/v)), 100 nM 4αPDD (in PBS/DMSO (50/50; v/v)) or the vehicles for up to 24 hours. The time points and concentrations used for time courses and dose response curve are outlined in the figure legends.

Murine *cyc*⁻S49T lymphoma cells, which were used as the source of substrate for PKC assay (see 2.3.1), were grown in suspension culture in RPMI 1640 medium (Gibco IBRL) supplemented with 10% horse serum (Gibco IBRL). Cultures were seeded at 2-4 x 10^5 cells/ml and maintained for three days at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

2.3.1 Assay of Protein Kinase C Activity

2.3.1.1 Preparation of Cytosolic and Membrane Fractions:

MDBK cell monolayers were washed twice with PBS and frozen at -20°C in 1.0 ml of hypotonic lysis medium (1mM $NaHCO_3$, 5 mM $MgCl_2$ and 100 μM PMSF). The cells were thawed, scraped from the dish and vortexed for 2 min. Tris-HCl buffer (500 mM, pH 7.5) was added to the cell lysate to 50 mM and nuclei and debris were removed by centrifugation (500g, 5 min). The post nuclear fraction was then centrifuged (14,000g, 15 min) to obtain a cytosolic supernatant and a membrane pellet.

2.3.1.2 Measurement of Protein Kinase C Activity in Detergent Extracted Membranes and Cytosolic Supernatants:

For this conventional assay PKC was extracted from membrane pellets of MDBK cells by homogenization in 20 mM Tris (pH 7.5) 2mM EDTA, 0.5 mM EGTA, 1mM PMSF, 1% Triton x-100 (w/v) and centrifugation (100,000g, 60 min). PKC was partially purified from the detergent solubilized extracts and cytosolic supernatants on DEAE-Sephacel anion exchange columns. For histone phosphorylation assay, 40 μl of the column effluent was added to an assay mixture (270 μl final volume) containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM

$CaCl_2$, 50 μg of histone IIIs and [γ-^{32}P]-ATP (10 μM, 200 cpm/pmol; NEN Research Products). Assays were conducted at 30°C in the presence or absence of 10 μg PS and 1 μg of diolein C18:1, Cis-9 (DAG) and were terminated after 8 min by addition of 2 ml of 20% trichloroacetic acid (TCA) and 50 μg bovine serum albumin (BSA). TCA precipitable material obtained by centrifugation (500g, 15 min) was dissolved in 1N NaOH, re-precipitated with 20% TCA and re-centrifuged. Final pellets were dissolved in 1N NaOH, added to a mixture of 1 ml of 0.1N HCl and 10 ml liquid scintillation cocktail and counted. PKC activity was calculated as the difference between [^{32}P] incorporation into histone in the presence and absence of PS and DAG and expressed as pmol/mg protein/min (Kleine, Whitfield and Boynton, 1986). The incubation times and protein concentrations chosen for use with cytosolic (Appendices 3 and 4 respectively) and membrane (Appendices 5 and 6 respectively) extracts in all subsequent histone phosphorylation assays were within the linear range for these two variables.

2.3.1.3 Measurement of Protein Kinase C Activity Directly in Intact Cellular Membranes:

Alternately PKC activity was measured directly in the native membrane-associated state by phosphorylation of a PKC-specific 85 kD protein substrate as described in Chapter One

(1.3.4) with the following exceptions. In order to quantitate phosphorylation of the PKC-specific 85 kD protein substrate, the resultant band on the autoradiogram was subjected to laser densitometry (LKB 2222-020 Ultroscan XL). Other phosphorylated bands on the autoradiograms were believed to be proteolytic degradation products of the PKC-specific 85 kD protein substrate. Therefore, in some experiments all phosphorylated proteins and peptides were collected on Whatman GF/A glass micro fibre filters as described (Tanaka et al., 1986), washed, dried and counted in 10 ml of liquid scintillation cocktail.

2.3.2 Immunoblotting of Protein Kinase C

For immunoblotting, the membrane pellet of MDBK cells was solubilized in 1 mM $NaHCO_3$, 5 mM $MgCl_2$, 100 μM PMSF, 50 mM Tris-HCl (pH 7.5), 0.01% (w/w) Bromophenol Blue, 0.05% (v/v) glycerol, 1% (w/v) SDS and 1.25% (v/v) ß-mercaptoethanol. The extracted membrane proteins (150 μg/lane) were electrophoresed on 10% SDS-PAGE (Laemmli, 1970) and transferred and immunoblotted as described in Chapter One (1.3.3) with one exception; the primary antibody used was a mouse anti-bovine PKC α monoclonal antibody (MC5, Amersham Canada Ltd., Oakville, Ontario, Canada) diluted 1:50 with blocking solution. This antibody has been shown to recognize α and β forms of bovine PKC but not the γ form.

Miscellaneous

Protein concentration was determined by the method of Bradford (1976). All materials were obtained from Sigma Chemical Corp., unless otherwise stated. Data are expressed as mean +/- standard error (SEM); when indicated in the figure legend, stastical significance was evaluated with the True Epistat computer program.

2.4 Results

2.4.1 Effect of $1,25(OH)_2D_3$ on Protein Kinase C activity.

PKC activity, measured in detergent extracts of MDBK cellular membranes as calcium and phospholipid dependent phosphorylation of histone IIIs, increased significantly in cells treated for 24 hours with 100 nM $1,25(OH)_2D_3$ ($p<0.01$), compared to vehicle treated cells (Table 2.1). $1,25(OH)_2D_3$ did not alter total PKC activity, measured in whole cell extracts by histone phosphorylation assay (data not shown). Since activation of membrane PKC can occur as a result of translocation of the enzyme from cytosol, the effect of $1,25(OH)_2D_3$ on subcellular distribution of PKC-dependent phosphorylation of histone IIIs was examined. As shown in Figure 2.1, the $1,25(OH)_2D_3$ induced increase in membrane PKC activity was paralleled by a concurrent decrease of similar magnitude in cytosolic PKC activity.

The histone phosphorylation assay method requires extraction of PKC from membrane and *in vitro* reconstitution and activation with phospholipid, this assay thus measures the amount of PKC which is potentially activatable. To determine whether the *in situ* activity of PKC was affected by $1,25(OH)_2D_3$ treatment, PKC activity in the native membrane-associated

TABLE 2.1

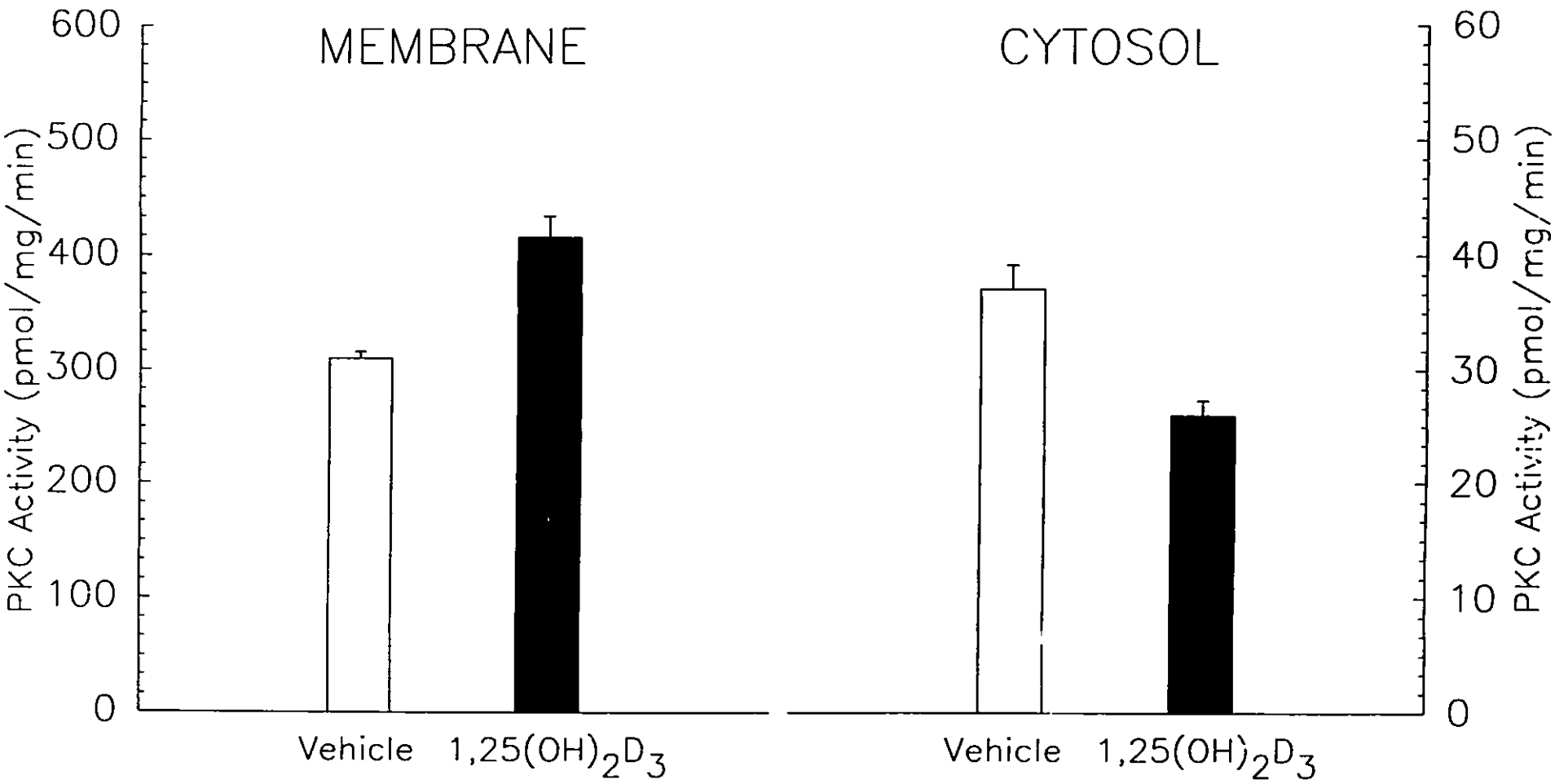
Effect of $1,25(OH)_2D_3$ on Protein Kinase C Activity in MDBK Cellular Membranes

PKC ACTIVITY	VEHICLE	$1,25(OH)_2D_3$
(pmol/mg/min)	363.8 ± 77.7	459.8 ± 76.7
(% of Control)	100%	126%

Quiescent MDBK cells were treated with 100 nM $1,25(OH)_2D_3$ or vehicle for 24 hours. PKC was detergent extracted and partially purified from solubilized membrane fractions. PKC activity was assayed by phosphorylation of histone substrate as described in 2.3.1.2. Data are expressed as both specific activity (pmol ^{32}P incorporated into histone per membrane protein per min) and as a percent of control PKC Activity. PKC specific activity represents calcium/phospholipid-dependent phosphorylation. Values are mean +/- SEM for triplicate determinations in five experiments. The difference between control and treated cells was significant at $p<0.01$ by Student's paired t test.

Figure 2.1 Effect of $1,25(OH)_2D_3$ on Subcellular Distribution of Protein Kinase C Activity in MDBK Cells

Quiescent MDBK cells were treated with 100 nM $1,25(OH)_2D_3$ or vehicle for 24 hours. PKC was detergent extracted and partially purified from cytosolic and solubilized membrane fractions. PKC activity was assayed by phosphorylation of histone substrate as described in 2.3.1.2. Data are expressed as pmol ^{32}P incorporated into histone per mg membrane/cytosol protein per min. PKC-specific activity represents calcium/phospholipid-dependent phosphorylation. Values are mean +/- SEM for triplicate determination in one experiment.


MEMBRANE
CYTOSOL
PKC Activity (pmol/mg/min)
600
500
400
300
200
100
0
60
50
40
30
20
10
0
Vehicle
1,25(OH)2D3
Vehicle
1,25(OH)2D3

state was measured using a murine lymphoma cell extract containing a PKC-specific 85 kD protein substrate. As shown in Figure 2.2, 24 hour treatment with 100 nM $1,25(OH)_2D_3$ increased membrane associated PKC activity in MDBK cells, detected as increased phosphorylation of the 85 kD PKC substrate in $1,25(OH)_2D_3$ treated cells versus vehicle treated cells. Densitometric scanning (Table 2.2) indicated that phosphorylation of the PKC-specific 85 kD substrate by membrane fractions from $1,25(OH)_2D_3$ treated cells was increased approximately 37 % in comparison to vehicle treated cells. The dose response of $1,25(OH)_2D_3$ on membrane-associated PKC activity in MDBK cells is shown in Figure 2.3; in this experiment, the phosphorylated 85 kD protein and any degradation products were collected on glass fibre filters for quantitation. PKC-dependent phosphorylation was significantly enhanced at 10 nM $1,25(OH)_2D_3$ ($p<0.05$) and at 100 nM $1,25(OH)_2D_3$ ($p<0.001$). The time course of the increase in membrane PKC activity in response to 100 nM $1,25(OH)_2D_3$ (Figure 2.4), also measured as phosphorylation of the 85 kD substrate, indicated that within 24 hours of incubation with $1,25(OH)_2D_3$ there was a significant effect ($p<0.01$) and that this effect was sustained for at least 72 hours.

Figure 2.2 Effect of $1,25(OH)_2D_3$ on Phosphorylation of Protein Kinase C-Specific 85 kD Substrate by MDBK Cellular Membranes

Quiescent MDBK cells were treated with 100 nM $1,25(OH)_2D_3$ (lanes 4,5,6) or vehicle (lanes 1,2,3) for 24 hours. PKC activity was measured directly in its native membrane-associated state by incubating crude membrane fractions with [γ-^{32}P]-ATP and the PKC-specific 85 kD substrate as described in 2.3.1.3. The protein source and amount in each lane was as follows: (1) 12.5 μg, (2) 25 μg, (3) 50 μg derived from control cells; (4) 12.5 μg, (5) 25 μg, (6) 50 μg derived from $1,25(OH)_2D_3$ treated cells. Phosphorylated proteins were separated on 8% SDS-PAGE and visualized by autoradiography.

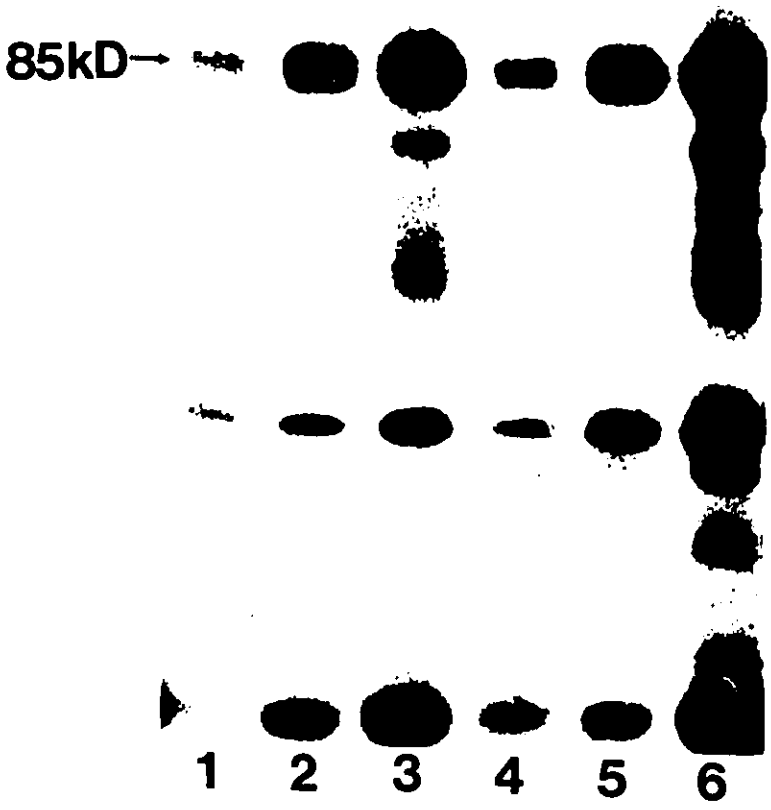

85kD
1
2
3
4
5
6

TABLE 2.2

Densitometric Scanning of Autoradiograph

PNF PROTEIN CONTENT (μg)	RELATIVE PEAK AREA (mm^2)	
	VEHICLE	$1,25(OH)_2D_3$
12.5	0.283	0.487
25	0.842	1.341
50	2.145	3.179

Data is derived from densitometric scanning of the autoradiograph presented in Figure 2.2.

Figure 2.3 Dose Response of Effect of $1,25(OH)_2D_3$ on Phosphorylation of Protein Kinase C-Specific 85 kD Substrate by MDBK Cellular Membranes

Quiescent MDBK cells were treated for 24 hours with the indicated concentrations of $1,25(OH)_2D_3$ or vehicle. PKC activity was measured directly in its native membrane-associated state by incubating crude membrane fractions with [γ-^{32}P]-ATP and the PKC-specific 85 kD substrate as described in 2.3.1.3. The phosphorylated 85 kD protein was collected on glass fibre filters for quantitation. Data are expressed as pmoles ^{32}P incorporated into the 85 kD substrate per mg membrane protein per min. Values are mean +/- SEM for duplicate determinations in three experiments. The difference between vehicle and $1,25(OH)_2D_3$ treated cell membranes was significant at 10 nM $1,25(OH)_2D_3$ ($p<0.05$) and at 100 nM $1,25(OH)_2D_3$ ($p<0.001$) by Student's paired t test.

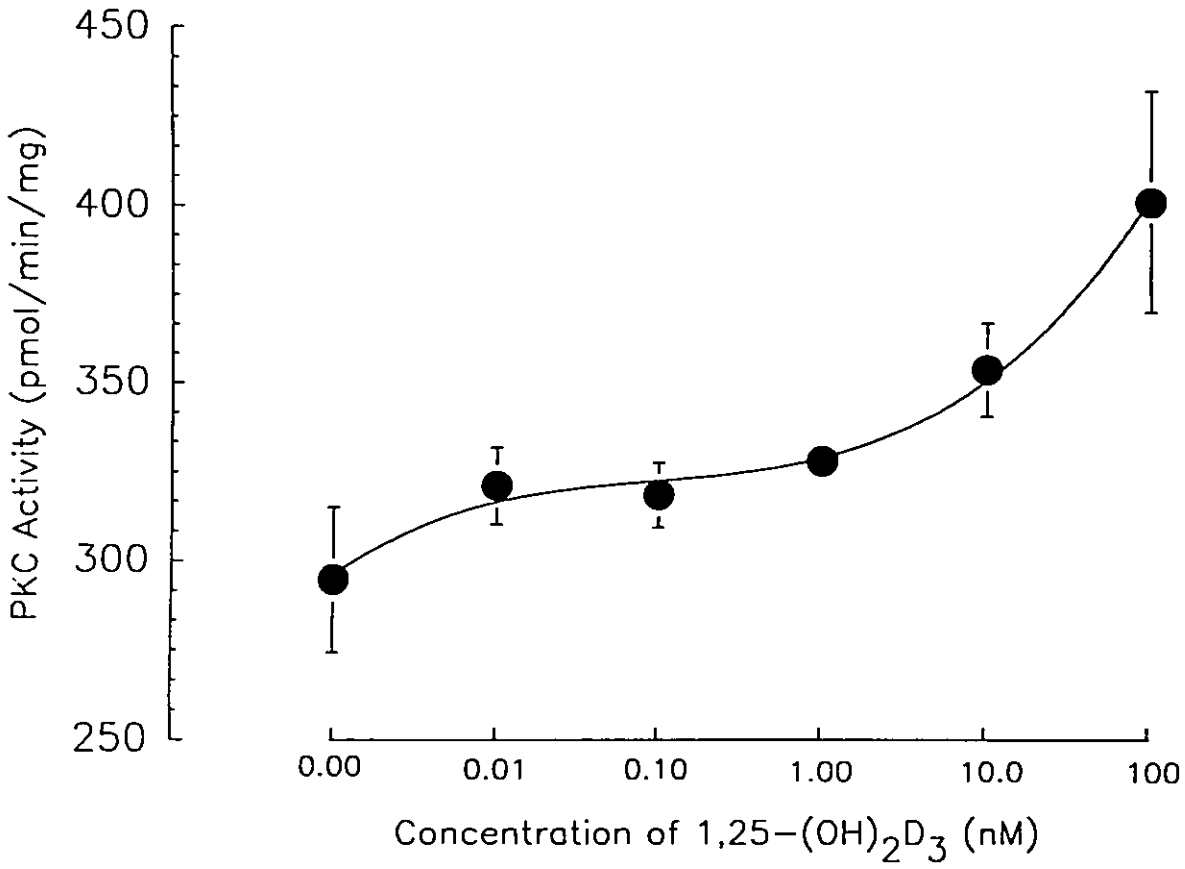
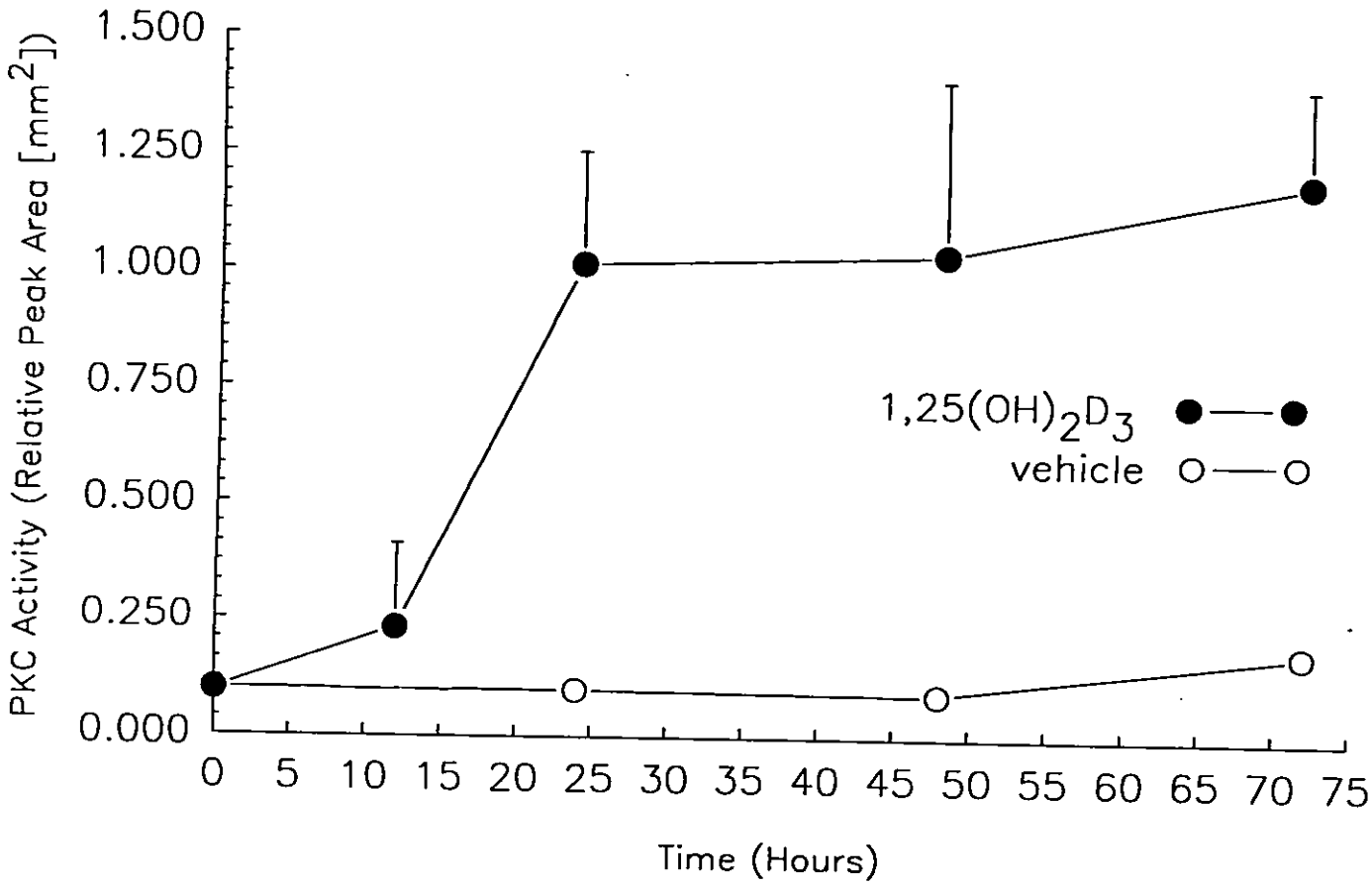

450
400
350
300
250
PKC Activity (pmol/min/mg)
0.00
0.01
0.10
1.00
10.0
100
Concentration of 1,25−(OH)2D3 (nM)

Figure 2.4 Time Course of Effect of $1,25(OH)_2D_3$ on Phosphorylation of Protein Kinase C-Specific 85 kD Substrate by MDBK Cellular Membranes

Quiescent MDBK cells were treated with 100 nM $1,25-(OH)_2D_3$ or vehicle for the times indicated. PKC activity was measured directly in its native membrane-associated state by incubating crude membrane fractions with [γ-^{32}P]-ATP and the PKC-specific 85 kD substrate as described in 2.3.1.3. Data was obtained by densitometric scanning of the autoradiograph. Values are mean +/- SEM of 4 separate determinations. The difference between vehicle and $1,25(OH)_2D_3$ treated became significant at 24 hours ($p<0.01$) by Student's paired t test.


PKC Activity (Relative Peak Area [mm2])
1.500
1.250
1.000
0.750
0.500
0.250
0.000
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75
Time (Hours)
1,25(OH)2D3
vehicle

2.4.2 Effect of $1,25(OH)_2D_3$ on Immunoreactive Protein Kinase C

Immunoblotting was used to detemine whether the increase in membrane PKC activity in response to $1,25(OH)_2D_3$ was paralleled by an increase in the amount of PKC protein. As shown in Figure 2.5, a 79 kD protein was identified as PKC by immunoblotting of membranes from MDBK cells with a mouse monoclonal antibody known to recognize α and ß forms of bovine PKC. Also evident in Figure 2.5, the membranes of MDBK cells treated with 100 nM $1,25(OH)_2D_3$ for 24 hours contained more immunoreactive PKC than did vehicle treated cells.

2.4.3 Effect of Phorbol Esters on Protein Kinase C Activity

PKC activity was measured as phosphorylation of a PKC-specific 85 kD substrate protein by intact membranes derived from MDBK cells treated with vehicle or 100 nM TPA for the times indicated in Figure 2.6. Densitometric scanning of the autoradiographs indicated that TPA significantly, but transiently, increased membrane PKC activity ($p<0.003$) at one hour. Within 24 hours TPA treatment significantly decreased membrane PKC activity in comparison to vehicle treated cells ($p<.01$).

The specificity of the TPA mediated down-regulation of PKC activity was determined by examining the effect of a

Figure 2.5 Effect of $1,25(OH)_2D_3$ on Immunoreactive Protein Kinase C in MDBK Cellular Membranes

Quiescent MDBK cells were treated with 100 nM $1,25(OH)_2D_3$ (lanes 3,4) or vehicle (lanes 1,2) for 24 hours. Solubilized membrane fractions (150 μg/lane) were separated by 10% SDS-PAGE and immunoblotted with monoclonal antibody to PKC as described in 2.3.2. Similar results were obtained in two other experiments.

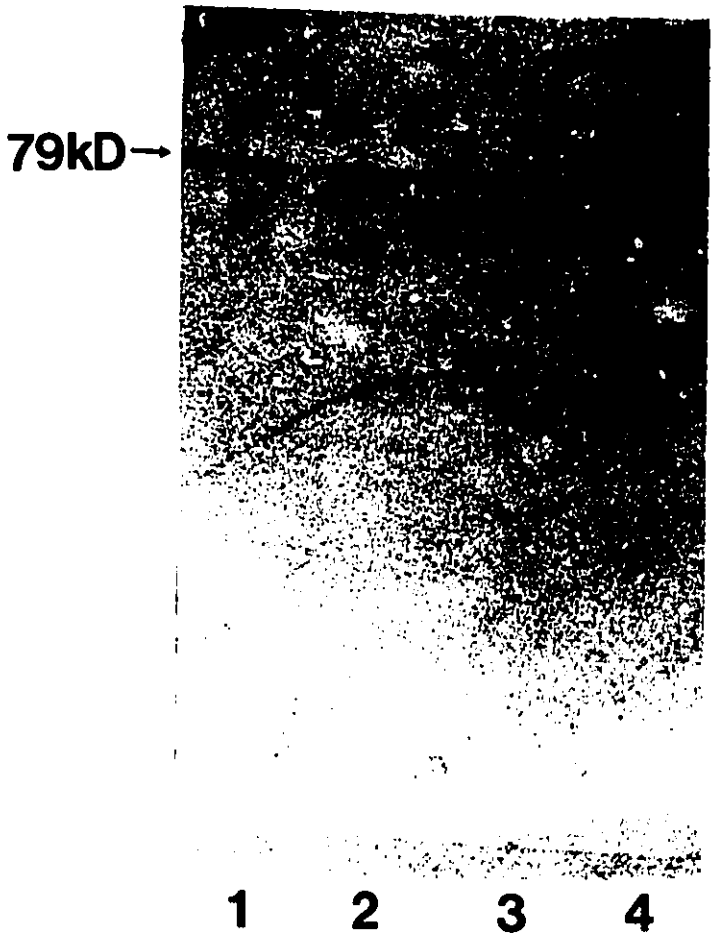
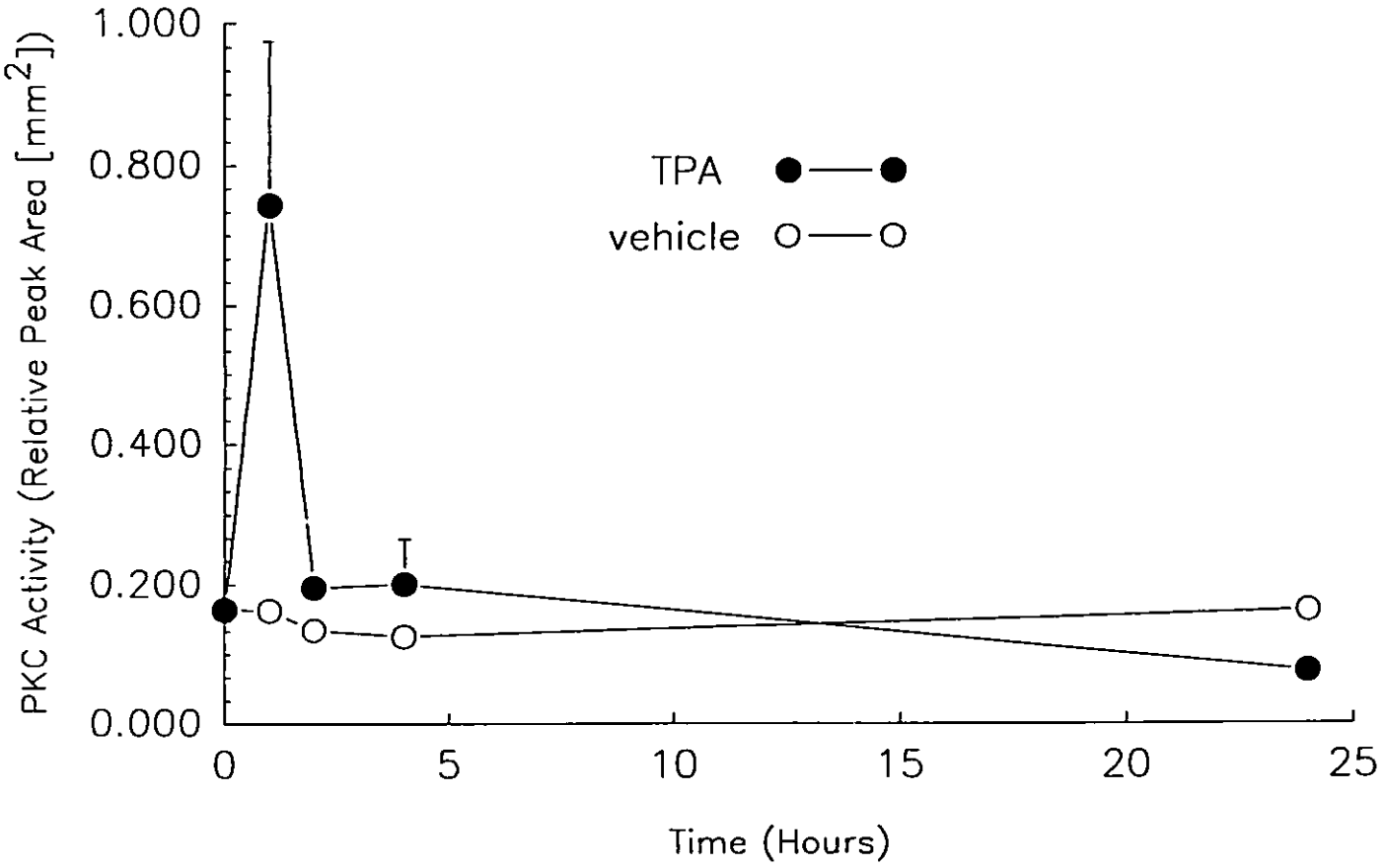

79kD
1
2
3
4

Figure 2.6 Time Course of Effect of TPA on Phosphorylation of Protein Kinase C-Specific 85 kDa Substrate by MDBK Cellular Membranes

Quiescent MDBK cells were treated with 100 nM TPA or vehicle for the times indicated. PKC activity was measured directly in its native membrane-associated state by incubating crude membrane fractions with [γ-^{32}P]-ATP and the PKC-specific 85 kD substrate as described in 2.3.1.3. Data was obtained by densitometric scanning of the autoradiograph. Values are mean +/- SEM of 4 separate determinations. The difference between vehicle and TPA treated was significant at 1 hour ($p<0.003$) and at 24 hours ($p<0.02$) by Student's paired t test.


1.000
0.800
0.600
0.400
0.200
0.000
PKC Activity (Relative Peak Area [mm2])
TPA
vehicle
0
5
10
15
20
25
Time (Hours)

phorbol ester analogue, 4αPDD. In contrast to TPA, long term treatment with 4αPDD did not down-regulate membrane PKC activity. There were no significant differences in PKC activity between untreated control cells and cells treated with 4αPDD or the vehicle (Figure 2.7).

2.4.4 Effect of Phorbol Esters on Immunoreactive Protein Kinase C.

The down-regulation in PKC activity in response to 24 hour treatment with TPA was associated with a dramatic decrease in immunoreactive PKC in the membrane of TPA treated cells compared to vehicle treated cells (Figure 2.8). Treatment of MDBK cells with 4αPDD, which did not down-regulate PKC activity, also did not decrease immunoreactive PKC present in the membrane; there was no visible difference in the amount of PKC present in the membrane fractions from 4αPDD treated cells compared to vehicle treated cells (Figure 2.8).

Figure 2.7 Effect of TPA and a Phorbol Ester Analogue on Phosphorylation of Protein Kinase C-Specific 85 kD Substrate by MDBK Cellular Membranes

Quiescent MDBK cells were treated with 100 nM TPA, the phorbol ester analogue, 4αPDD, or vehicle for 24 hours. Control cells were left untreated. PKC activity was measured directly in its native membrane-associated state by incubating crude membrane fractions with [γ-^{32}P]-ATP and the PKC-specific 85 kD substrate as described in 2.3.1.3. Data was obtained by densitometric scanning of the autoradiograph. Values are an average of duplicate determination of a single experiment.

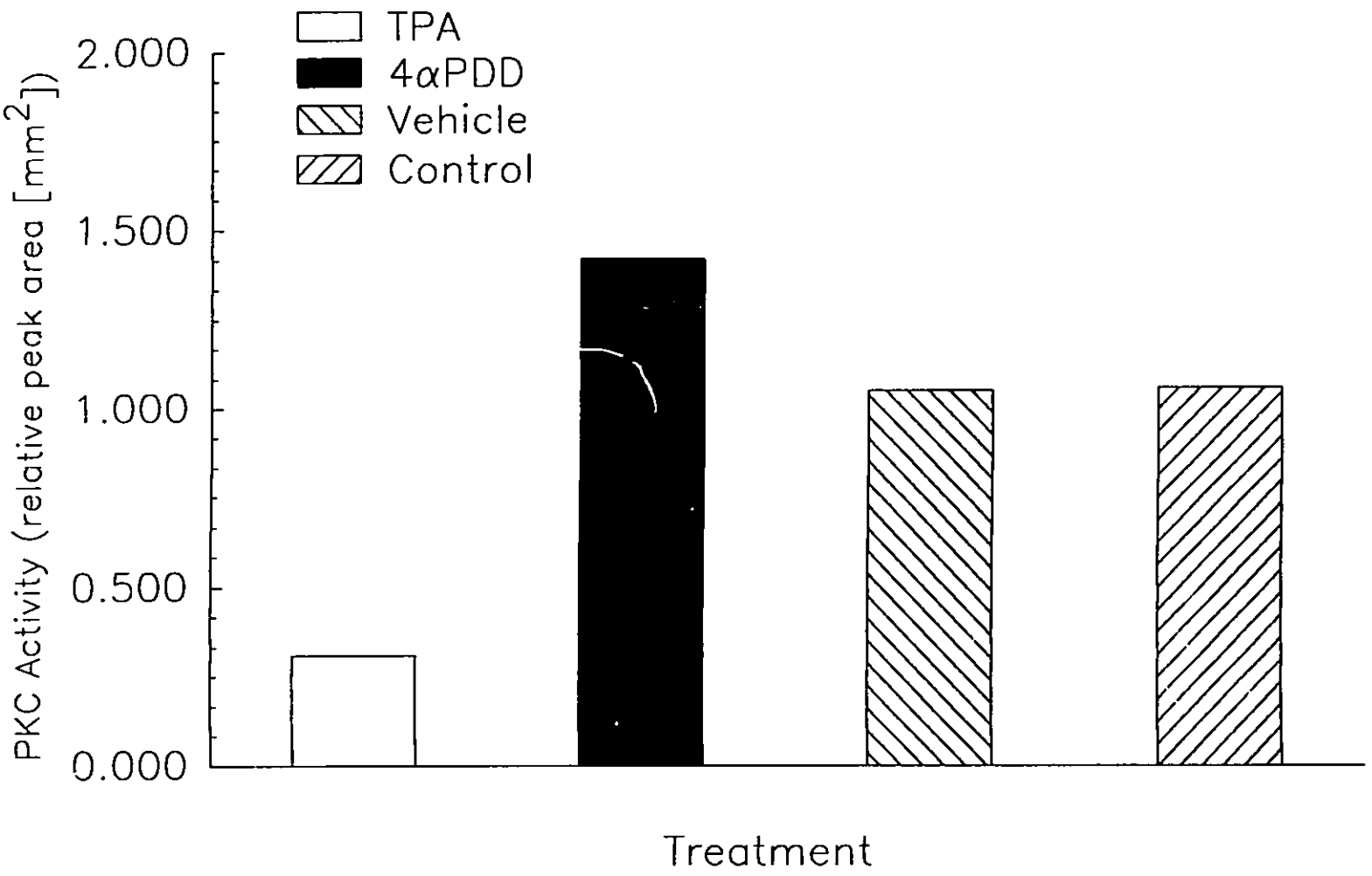
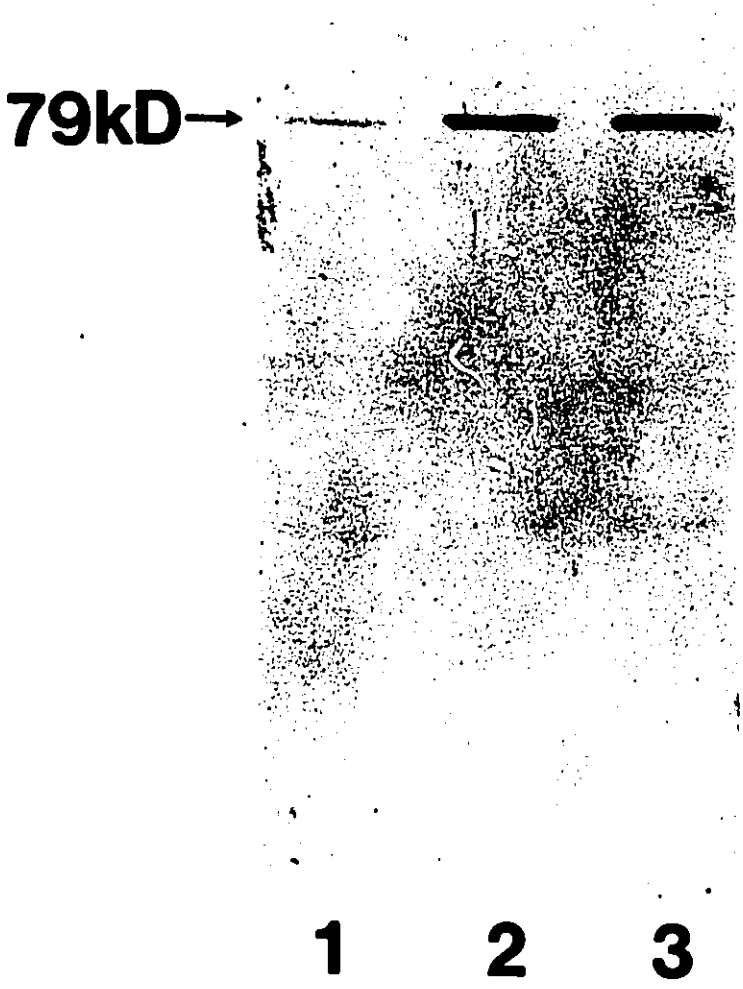

TPA
4αPDD
Vehicle
Control
2.000
1.500
1.000
0.500
0.000
PKC Activity (relative peak area [mm2])
Treatment

Figure 2.8 Effect of TPA and a Phorbol Ester Analogue on Immunoreactive Protein Kinase C in MDBK Cellular Membranes

Quiescent MDBK cells were treated with 100 nM TPA (lane 1), the phorbol ester analogue 4αPDD (lane 2) or vehicle (lane 3) for 24 hours. Solubilized membrane fractions (150 μg/lane) were separated by 10% SDS-PAGE and immunoblotted with a monoclonal antibody to PKC as described in 2.3.2. Similar results were obtained in two other experiments.


79kD
1
2
3

2.5 Discussion

2.5.1 Regulation of Protein Kinase C by $1,25(OH)_2D_3$

Our data clearly demonstrate that $1,25(OH)_2D_3$ treatment increases membrane PKC activity in MDBK cells, whether measured in detergent solubilized membrane fractions (as phosphorylation of histone IIIs in Table 2.1) or in the native, membrane associated state (as phosphorylation of the PKC-specific 85 kD protein derived from *cyc*⁻ cells in Figure 2.2). The magnitude of the response to 100 nM $1,25(OH)_2D_3$ is comparable with both assays (compare Table 2.1 (26%) to Table 2.2 (37%)). The increase in membrane-associated PKC activity appears to result from translocation of cytosolic enzyme to the membrane (Figure 2.1). However, as kidney has been previously shown to contain soluble inhibitors of PKC that can interfere with the measurement of kinase activity (Dong, Stevens and Jaken, 1991), data suggesting translocation in renal systems must be interpreted with caution. The effect of $1,25(OH)_2D_3$ on PKC activity is dose-dependent (Figure 2.3), with significant effects occurring between concentrations of 10 nM and 100 nM. These concentrations of $1,25(OH)_2D_3$ are within the physiological range, with respect to the K_d of specific $1,25(OH)_2D_3$ binding determined in Chapter one. Time course data (Figure 2.4) indicates that 24 hours of incubation with $1,25(OH)_2D_3$ is sufficient for this effect. Immunoblotting

indicated that $1,25(OH)_2D_3$ increases not only PKC activity, but also the amount of PKC, in membranes of MDBK cells (Figure 2.5).

These results are the first demonstration of an increase in PKC activity in response to $1,25(OH)_2D_3$ treatment. This data complements and extends previous reports that $1,25(OH)_2D_3$ increases PKC-dependent phosphorylation (Zylber-Katz and Glazer, 1985) and phorbol ester binding sites (Martell, Simpson and Taylor, 1987) in HL-60 cells. Similar to HL-60 cells, activation of PKC by $1,25(OH)_2D_3$ in MDBK cells could be mediated by the VDR since it requires long incubation times. In contrast to HL-60 cells, where transcription of PKC gene(s) has been implicated in the activation of PKC by $1,25(OH)_2D_3$ (Obeid et al., 1990), in MDBK cells activation of PKC by $1,25(OH)_2D_3$ appears to result from translocation of the kinase from cytosol to membrane. Also in contrast to HL-60 cells, MDBK cells are a non-transformed epithelial cell line derived from kidney, a known target tissue for $1,25(OH)_2D_3$. The relevance of modulation of PKC by $1,25(OH)_2D_3$ in MDBK cells is not readily apparent. In HL-60 cells, the $1,25(OH)_2D_3$ mediated increase in PKC is thought to be necessary for inhibition of c-*myc* expression and subsequent induction of monocytic differentiation (Simpson et al., 1989). In Kidney, PKC may be involved in regulation of CaBP D-28K expression, or other known functional effects of $1,25(OH)_2D_3$ such as regulation of vitamin D hydroxylases (Mandla, Boneh and Tenenhouse, 1990;

Henry and Luntao, 1989; Welsh, Weaver and Simboli-Campbell, 1991).

2.5.2 Regulation of Protein Kinase C by Phorbol Esters

In MDBK cells, treatment with the phorbol ester TPA transiently increases membrane-associated PKC activity at 1 hour and subsequently decreases PKC activity below control levels after 24 hours, indicating that down-regulation of PKC occurs (Figure 2.6). The time course of changes in immunoreactive PKC are similar (data not shown), with down-regulation of PKC activity at 24 hours associated with decreased immunoreactive PKC in membrane (Figure 2.7). Long term treatment with the phorbol ester analogue, 4αPDD, does not cause down-regulation of either PKC activity (Figure 2.7) or immunoreactivity (Figure 2.8).

Among TPA's many potent effects on cell function, its modulation of PKC activity has been best characterized. In most cells, TPA causes a rapid translocation of PKC from cytosol to membrane (Neidel and Blackshear, 1986), therefore short term exposure of cells to TPA usually enhances PKC activity in cellular membranes. Subsequent to translocation, however, membrane-associated PKC becomes susceptible to proteolysis; thus long term exposure of most cells to TPA eventually results in down-regulation of PKC activity (Stabel et al., 1986). The regulation of PKC by phorbol esters in MDBK

cells is similar to that observed in many other cells, activation of PKC by TPA induces a short term translocation to the plasma membrane which sets up the enzyme for long term down-regulation, while the phorbol ester analogue, 4αPDD, does not cause activation (translocation) of PKC and therefore does not induce long term down-regulation of the enzyme either (Castanga, 1982). Thus in MDBK cells TPA is active and 4αPDD is inactive with respect to effects on PKC activity, consistent with their effects on PKC activity and tumor-promoting ability in other cells. These two phorbol esters should be useful for investigating whether PKC is involved in the regulation of CaBP D-28K by $1,25(OH)_2D_3$.

2.6 Conclusions

The overall conclusion of this chapter is that long term treatment with $1,25(OH)_2D_3$ or short term treatment with TPA results in activation of PKC in MDBK cells. In contrast, long term treatment with TPA down-regulates PKC, whereas there is no evidence that long term treatment with $1,25(OH)_2D_3$ (up to 72 hours) down-regulates PKC. Other conclusions include:

2.6.1 Long term (24-72 hours) treatment with $1,25(OH)_2D_3$ increases both detergent extractable and *in situ* membrane PKC activity in MDBK cells.

2.6.2 The $1,25(OH)_2D_3$ mediated increase in PKC activity is dose- and time-dependent.

2.6.3 Long term treatment with $1,25(OH)_2D_3$ increases membrane PKC immunoreactivity.

2.6.4 Short term treatment with TPA increases membrane PKC activity in MDBK cells, while long term treatment down-regulates PKC activity.

2.6.5 Long term treatment with TPA decreases membrane PKC immunoreactivity.

2.6.6 Long term treatment with the phorbol ester analogue 4αPDD does not down-regulate membrane PKC activity or immunoreactivity.

CHAPTER THREE: EFFECTS OF 1,25(OH)$_2$D$_3$ AND PHORBOL ESTERS ON VITAMIN D RECEPTOR AND CALBINDIN D-28K EXPRESSION

3.1 Introduction

The abundance of VDR in target cells is modulated by a variety of factors, including 1,25(OH)$_2$D$_3$, which homologously up-regulates its receptor (Haussler et al., 1988a). Heterologous regulation can also occur, in response to steroid hormones such as glucocorticoids (Hirst and Feldman, 1982; Neilson et al., 1988) and estrogens (Walters, 1981; Duncan, Glass and Wray, 1991) as well as other agents, like retinoic acid (Chen and Feldman, 1985; Petkovich et al., 1984). Restriction of dietary calcium, which increases circulating 1,25(OH)$_2$D$_3$, is also associated with up-regulation of VDR (Favus et al., 1988; Sandgren and DeLuca, 1990). Other modulators, like epidermal growth factor (Bruns et al., 1989), may be important in regulation of VDR number during neonatal development. Recent studies have indicated that VDR levels vary in relation to the rate of cell proliferation in cultured cells (Chen and Feldman, 1981; Krishnan and Feldman, 1991a; Merke et al., 1989). Consistent with these findings, VDR levels are increased in proliferating MDBK cells compared to quiescent cells (Chapter One).

Since the biological effects of 1,25(OH)$_2$D$_3$ are mediated via interaction with the VDR, regulation of receptor

concentration is a possible mechanism for modulating the responsiveness of the target cell to hormone. Co-localization of VDR (Stumpf et al., 1979) and CaBP D-28K (Taylor, McIntosh and Bourdeau, 1982) in the renal distal tubule implies that the $1,25(OH)_2D_3$-VDR complex acts upon specific regulatory elements capable of controlling the rate of transcription of the CaBP D-28K gene. Recently it has been shown that $1,25(OH)_2D_3$ induces CaBP D-28K expression in kidney (Clemens et al., 1989) and that receptor regulation is an important mechanism for modulating renal cell responsiveness to $1,25(OH)_2D_3$ (Huang et al., 1989).

Similar to other steroid hormone receptors including the estrogen (Migliaccio, Rotondi and Auricchio, 1984; Auricchio, 1989), glucocorticoid (Singh and Moudgil, 1985; Mendel et al., 1986; Housley and Pratt, 1983), progesterone (Dougherty, Puri and Toft, 1982; Logeat et al., 1985), androgen (Goueli, Holtzman and Ahmed, 1984) and thyroid hormone (Goldberg et al., 1988) receptors, the VDR has been shown to be phosphorylated in a number of species (Pike and Sleator, 1985; Haussler et al., 1988b; Brown and DeLuca, 1990). In embryonic chick duodenal organ culture (Brown and DeLuca, 1990) VDR phosphorylation is increased by $1,25(OH)_2D_3$ prior to the induction of CaBP D-28K suggesting phosphorylation may play a role in the $1,25(OH)_2D_3$ regulation of transcription in the intestine. Phosphoamino acid analysis performed on the mouse VDR has shown that in this species the VDR is phosphorylated

predominantly on serine residues (Haussler et al., 1988b). The accumulated evidence implies that $1,25(OH)_2D_3$ may activate a serine kinase which participates in the regulation of gene transcription by $1,25(OH)_2D_3$.

3.1.1 Regulation of Vitamin D Receptor by $1,25(OH)_2D_3$

Modulation of target cell responsiveness to hormones can occur by the phenomenon of ligand dependent autoregulation, which has been reported for both peptide and steroid receptors (Catt et al., 1979; Clark et al., 1980). Examples of homologous up-regulation of the VDR *in vivo* include rat intestine (Favus et al., 1988) and kidney (Costa and Feldman, 1986). Examples of homologous up-regulation *in vitro* include mouse fibroblasts (McDonnell et al., 1987), human and rat osteosarcoma cells (Mahonen et al., 1990; Pan and Price, 1987), human promyelocytic leukemia cells (Lee et al.,1989), LLC-PK_1 (pig kidney) cells and human skin fibroblasts (Costa, Hirst and Feldman, 1985). In contrast, mutant skin fibroblasts from patients with vitamin D-dependent rickets type II, containing nonresponsive VDR, fail to exhibit the characteristic homologous up-regulation observed in normal cells. This data indicates that $1,25(OH)_2D_3$ regulation of VDR involves a receptor-mediated induction mechanism. Subsequently, these authors reported that the homologous up-regulation of VDR *in vitro* results primarily from a

prolongation of VDR half-life, with a relatively small contribution from an increase in the rate of receptor synthesis (Costa and Feldman, 1987). These findings are consistent with those of Huang and coworkers (1989), who observed that administration of $1,25(OH)_2D_3$ to vitamin D-deficient rats does not result in an increase in VDR mRNA. More recent *in vivo* studies have indeed indicated that elevation of VDR in response to $1,25(OH)_2D_3$ administration results from ligand-induced stabilization of the VDR and not from increased transcription (Wiese et al., 1992).

3.1.2 Regulation of Vitamin D Receptor by Phorbol Esters

TPA down-regulates the VDR in NIH 3T3 mouse fibroblasts (Krishnan and Feldman, 1991b) and HL-60 cells (Haussler et al, 1987) and estrogen receptors (ER) in MCF-7 cells (Guilbaud et al, 1988). The TPA induced down-regulation of specific $1,25(OH)_2D_3$ binding in mouse fibroblasts is associated with decreased VDR protein levels and decreased steady-state levels of VDR mRNA (Krishnan and Feldman, 1991b). In MCF-7 cells, the TPA induced decrease in specific estradiol binding is also associated with decreased ER protein levels and decreased steady-state levels of ER mRNA (Saceda et al., 1991). Using transcription run-off assays, these investigators demonstrated no effect of TPA on ER gene transcription. However, this study indicated that TPA reduces ER mRNA half-life from 4

hours in control cells to 40 minutes in TPA treated cells; suggesting that the decline in ER expression is mediated by post-transcriptional destabilization of ER mRNA. In contrast, up-regulation of the VDR in response to TPA has been reported in bovine aortic endothelial cells (Merke et al, 1989) and human lymphocytes (Yu et al, 1991). This discrepancy may reflect differences in the time course for activation/down-regulation of PKC in response to TPA in different cell types. Since previous studies on TPA modulation of VDR number have not measured PKC activity after TPA treatment, the relationship between PKC activity and steroid hormone receptor regulation in these studies remains unknown.

3.1.3 Regulation of Calbindin D-28K

$1,25(OH)_2D_3$ up-regulates renal CaBP D-28K expression *in vivo* (Christakos and Norman, 1980) and *in vitro* (Craviso, Garrett and Clemens, 1987). In primary cultures of avian renal epithelial cells, 24 hour treatment with 100 nM $1,25(OH)_2D_3$ increased cellular CaBP D-28K approximately two-fold, in a dose-dependent manner. In this system cycloheximide inhibits CaBP D-28K induction by $1,25(OH)_2D_3$, indicating a requirement for new protein synthesis. Induction of CaBP D-28K mRNA is observed within 4 hours of $1,25(OH)_2D_3$ treatment and peaks at 8-12 hours, preceding the peak increase in CaBP D-28K protein levels at 24-48 hours (Clemens et al.,

1989). Although this data indicates that regulation of CaBP D-28K by $1,25(OH)_2D_3$ occurs at the level of transcription, the long lag time between increases in transcription and the peak of mRNA and protein accumulation suggests the involvement of post-transcriptional mechanisms as well. In a more recent study (Enomoto et al., 1992) the relative contributions of transcriptional and post-transcriptional mechanisms to $1,25(OH)_2D_3$ modulation of CaBP D-28K expression was examined. In this study, nuclear run-on assays, which were sufficiently sensitive to show induction of zinc-mediated metallothionein gene transcription in kidney cells, revealed only a modest induction of CaBP D-28K transcription by $1,25(OH)_2D_3$. These results suggested that a relatively small increase in renal CaBP D-28K gene transcription must be accompanied by a pronounced effect of $1,25(OH)_2D_3$ on post-transcriptional mechanisms. As well, *in vivo* studies have provided evidence that $1,25(OH)_2D_3$ is not an exclusive regulator of CaBP D-28K expression; CaBP D-28K mRNA present in rat brain is unresponsive to $1,25(OH)_2D_3$ (Christakos, Gabrielides and Rhoten, 1989). Apparently, CaBP D-28K is regulated by different agents in different tissues. Even in kidney, $1,25(OH)_2D_3$ is not the only factor modulating CaBP D-28K expression; extracellular calcium concentration modulates the regulation of renal CaBP D-28K expression by $1,25(OH)_2D_3$ (Clemens et al., 1989; Enomoto et al., 1992). Studies to characterize the molecular regulation of CaBP D-28K by

$1,25(OH)_2D_3$ and other independent factors have been limited due to the lack of an *in vitro* system.

In Chapter Two I established that long term treatment of MDBK cells with $1,25(OH)_2D_3$ activates PKC (a serine/threonine kinase), as does short term treatment with TPA, whereas long term treatment with TPA down-regulates PKC. In Chapter Three, I examine the regulation of VDR number by $1,25(OH)_2D_3$ and phorbol esters in MDBK cells and assess the relevance of VDR modulation by measuring CaBP D-28K. Finally $1,25(OH)_2D_3$ and TPA induced changes in VDR and CaBP D-28K expression are correlated with the effects of these two agents on PKC described in Chapter Two.

3.2 Objectives

The overall objective of the studies described in this chapter was to characterize the effects of TPA and $1,25(OH)_2D_3$ on VDR and CaBP D-28K expression in MDBK cells. The specific objectives were:

3.2.1 To establish whether $1,25(OH)_2D_3$ homologously up-regulates the VDR and induces CaBP D-28K in MDBK cells.

3.2.2 To determine the effect of TPA on the VDR and CaBP D-28K in MDBK cells.

3.2.3 To determine the time course of the effect of TPA on the VDR and CaBP D-28K in MDBK cells.

3.2.4 To determine the effect of the inactive phorbol ester analogue, 4αPDD, on the VDR and CaBP D-28K in MDBK cells.

3.3 Methods

Cell Culture

Stock cultures of MDBK cells were maintained in Dulbecco's modified Eagle medium (Gibco IBRL) supplemented with 10% newborn calf serum (Gibco IBRL). Cells were plated at a density of 1.5 x 10^4 cells/ml in 75 mm^2 flasks and grown to confluence. Confluent cells were rendered quiescent by fluid changing to serum-free DMEM for 24 hours followed by treatment with 100 nM $1,25(OH)_2D_3$ (Biomol Research Laboratories) (in ethanol), 100 nM TPA (in PBS/DMSO (50/50; v/v)), 100 nM 4αPDD (in PBS/DMSO (50/50; v/v)) or vehicle for up to 24 hours. The times and concentrations used to generate data for time courses and dose response curve are outlined in the figure legends.

3.3.1 Characterization of Vitamin D Receptor

FPLC analysis, saturation analysis and measurement of VDR number by equilibrium binding were performed in chromatin extracts derived from MDBK cells following the protocols described in Chapter One (1.3.2).

3.3.2 Calbindin D-28K immunoblotting

Immunoblotting of total protein extracts and cytosolic supernatants derived from MDBK cells was performed as described in Chapter One (1.3.3).

Miscellaneous

Protein concentration was determined by the method of Bradford (1976). All materials were obtained from Sigma Chemical Corp, St. Louis, MO unless otherwise stated. Data are expressed as mean +/- standard error (SEM); when indicated in the figure legend, statistical significance was evaluated with the True Epistat computer program.

3.4 Results

3.4.1 Effect of $1,25(OH)_2D_3$ on Specific ^3H-$1,25(OH)_2D_3$ Binding

Homologous up-regulation of ^3H-$1,25(OH)_2D_3$ binding was observed in $1,25(OH)_2D_3$ treated MDBK cells 24 hours after a single dose of 100 nM $1,25(OH)_2D_3$; $1,25(OH)_2D_3$ binding was increased approximately 1.5 fold (Figure 3.1). This effect was delayed (not observed at 12 hours) and transient (back to baseline at 48 hours). Scatchard analysis of equilibrium binding data indicated that homologous up-regulation of $1,25(OH)_2D_3$ binding was due to an increase in VDR number (N_{max}) and not to a change in the affinity (K_d) of the receptor for the hormone (Figure 3.2). In control cells N_{max} was approximately 21 fmol/mg protein with K_d approximately 7.7×10^{-11} M; in $1,25(OH)_2D_3$ treated cells N_{max} was 26 fmol/mg protein and K_d was 7.1×10^{-11} M. In essence N_{max} was increased approximately 1.2 fold with no apparent change in K_d.

3.4.2 Effect of Phorbol Esters on Specific ^3H-$1,25(OH)_2D_3$ Binding

The time course of the effect of 100 nM TPA on specific ^3H-$1,25(OH)_2D_3$ binding in MDBK cells is shown in Figure 3.3. Although VDR number began to decline within 4-6 hours of TPA treatment, 24 hour treatment was necessary for a significant

Figure 3.1 Time Course of Effect of $1,25(OH)_2D_3$ on Specific ^3H-$1,25(OH)_2D_3$ Binding in MDBK Cells

Quiescent MDBK cells were treated with 100 nM $1,25(OH)_2D_3$ for the indicated times or vehicle for 24 hours. Chromatin extracts (1 mg protein/ml) were incubated with 0.5 nM ^3H-$1,25(OH)_2D_3$ in the presence or absence of a 250-fold molar excess of unlabelled $1,25(OH)_2D_3$ as described in 3.3.1. Values are mean +/- SEM for triplicate determinations in one experiment.

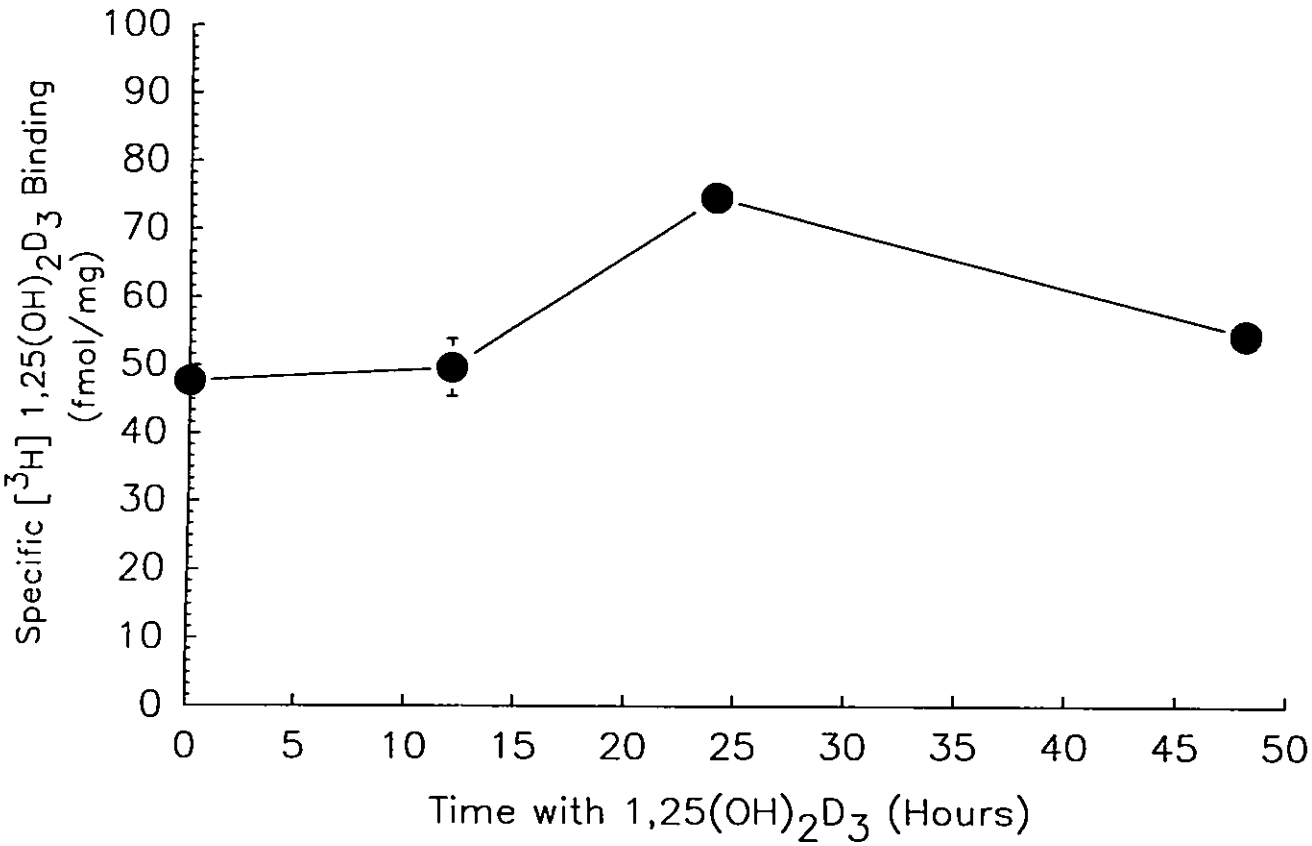

Specific [3H] 1,25(OH)2D3 Binding
(fmol/mg)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
45
50
Time with 1,25(OH)2D3 (Hours)

Figure 3.2 Scatchard Analysis of Specific ^{3}H-1,25(OH)$_2$D$_3$ Binding in 1,25(OH)$_2$D$_3$ Treated MDBK Cells

Quiescent MDBK cells were treated with 100 nM 1,25(OH)$_2$D$_3$ (●) or vehicle (O) for 24 hours. Chromatin extracts (1.0 mg protein/ml) were incubated with 0.05-1.0 nM of ^{3}H-1,25(OH)$_2$D$_3$ in the presence or absence of a 250-fold molar excess of unlabelled 1,25(OH)$_2$D$_3$ as described in 3.3.1. Specific binding, derived by subtracting non-specific from total binding, was used to generate the Scatchard plots. Each point is the average of duplicate determinations in one experiment. Similar results were obtained in two other experiments.

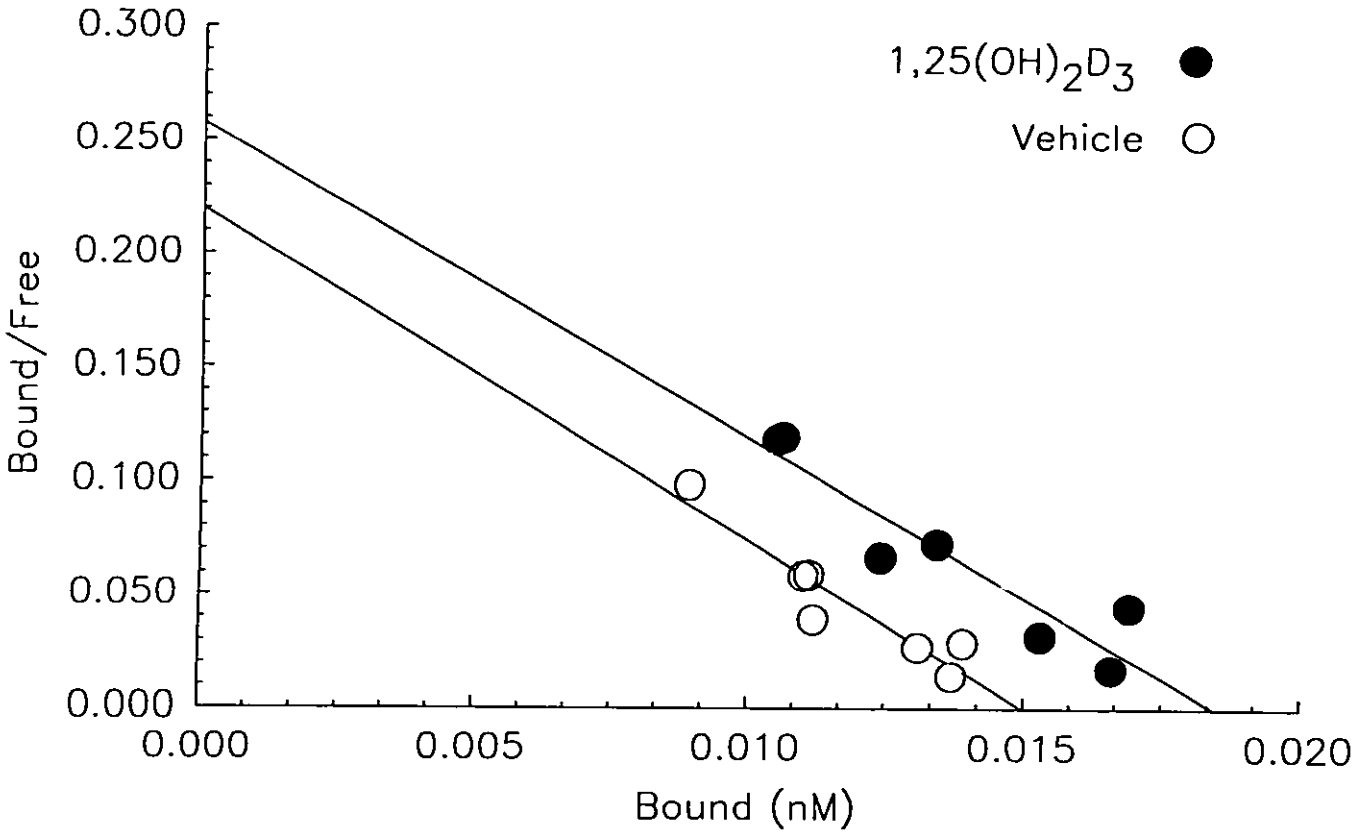
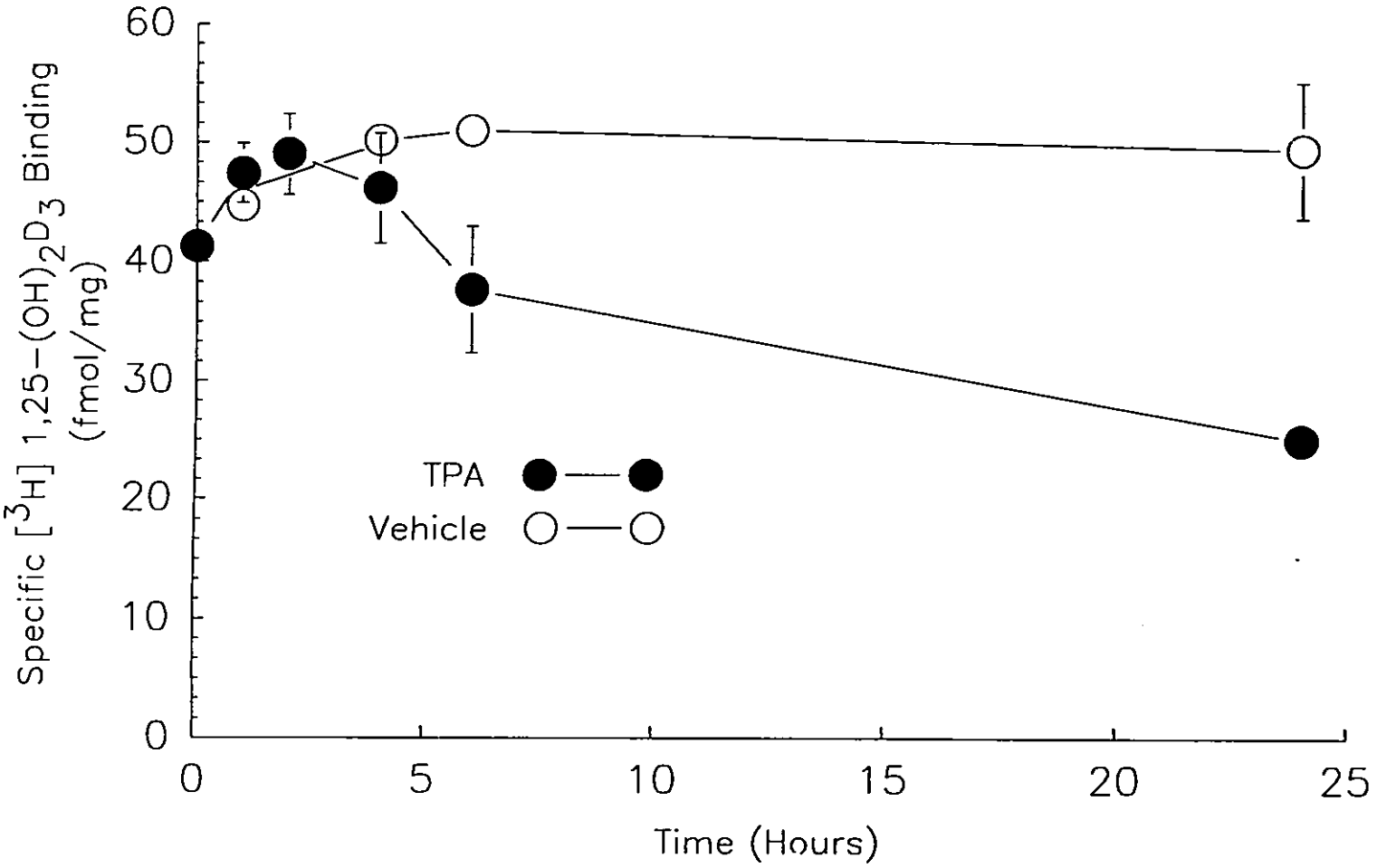

0.300
0.250
0.200
0.150
0.100
0.050
0.000
Bound/Free
0.000
0.005
0.010
0.015
0.020
Bound (nM)
1,25(OH)2D3
Vehicle

Figure 3.3 Time Course of Effect of TPA on Specific ^{3}H-1,25(OH)$_2$D$_3$ Binding in MDBK Cells

Quiescent MDBK cells were treated with 100 nM TPA (●) or vehicle (O) for the indicated times. Chromatin extracts (1 mg protein/ml) were prepared and incubated with 0.5 nM ^{3}H-1,25(OH)$_2$D$_3$ in the presence or absence of a 250-fold molar excess of unlabelled 1,25(OH)$_2$D$_3$ as described in 3.3.1. Values are mean +/- SEM for triplicate determinations in five experiments. The difference between vehicle and TPA treated cells was significant at 24 hours ($p<0.0001$) by Student's paired t test.


Specific [3H] 1,25-(OH)2D3 Binding
(fmol/mg)
60
50
40
30
20
10
0
0
5
10
15
20
25
Time (Hours)
TPA
Vehicle

($p < 0.0001$) decrease in specific ^3H-1,25$(OH)_2D_3$ binding. The magnitude of the decrease in N_{max} of MDBK cells treated with TPA for 24 hours was approximately 50%. VDR numbers remained low in cells treated for up to 48 hours with TPA (data not shown). There was no significant effect of vehicle (PBS/DMSO (50/50; v/v) or incubation time on VDR content in control cells.

The effect of 24 hour treatment with TPA on VDR number was dose-dependent (Figure 3.4), with significant ($p<0.05$) down-regulation of specific ^3H-1,25$(OH)_2D_3$ binding observed at doses as low as 5 nM. No significant effect on VDR number was observed with TPA concentrations less than 5 nM. The maximal decreases in 1,25$(OH)_2D_3$ binding were observed at 10 and 100 nM TPA; which reduced N_{max} by 61% and 47%, respectively ($p < 0.001$) in TPA treated versus vehicle treated cells.

Scatchard analysis of equilibrium binding data yielded an N_{max} of 22.4 fmol/mg protein and a K_d of 1.0×10^{-10} for vehicle treated cells versus an N_{max} of 13.0 fmol/mg protein and a K_d of 2.0×10^{-10} for cells treated for 24 hours with 100 nM TPA (Figure 3.5). Thus, by this analysis, N_{max} was also reduced approximately 50% by TPA treatment, but TPA had little effect on the affinity of the VDR for 1,25$(OH)_2D_3$.

FPLC analysis (Figure 3.6) indicated that the 1,25$(OH)_2D_3$ binding component eluted as one major peak corresponding to a molecular weight of approximately 66 - 70 kD in both control and TPA treated cells. Thus, although specific ^3H-1,25$(OH)_2D_3$

Figure 3.4 Dose Response of Effect of TPA on Specific ^{3}H-1,25(OH)$_2$D$_3$ Binding in MDBK Cells

Quiescent MDBK cells were treated with the indicated concentrations of TPA or vehicle for 24 hours. Chromatin extracts (1 mg protein/ml) were prepared and incubated with 0.5 nM ^{3}H-1,25(OH)$_2$D$_3$ in the presence or absence of a 250-fold molar excess of unlabelled 1,25(OH)$_2$D$_3$ as described in 3.3.1. Values are mean +/- SEM for triplicate determinations in six experiments. The difference between vehicle and TPA treated cells was significant at 5 nM ($p<0.05$), 10 nM ($p<0.001$) and 100 nM ($p<0.001$) by Student's paired t test.

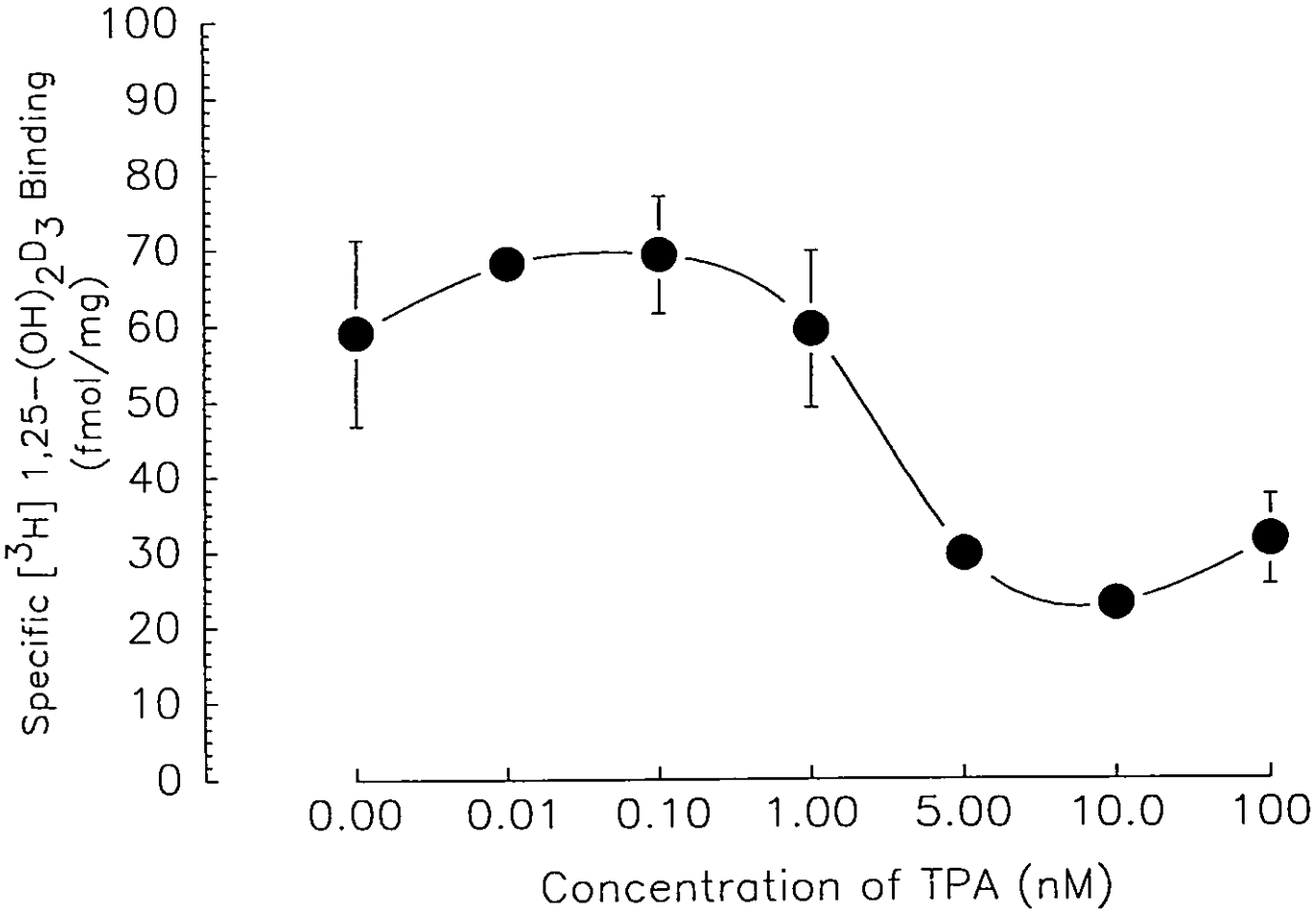
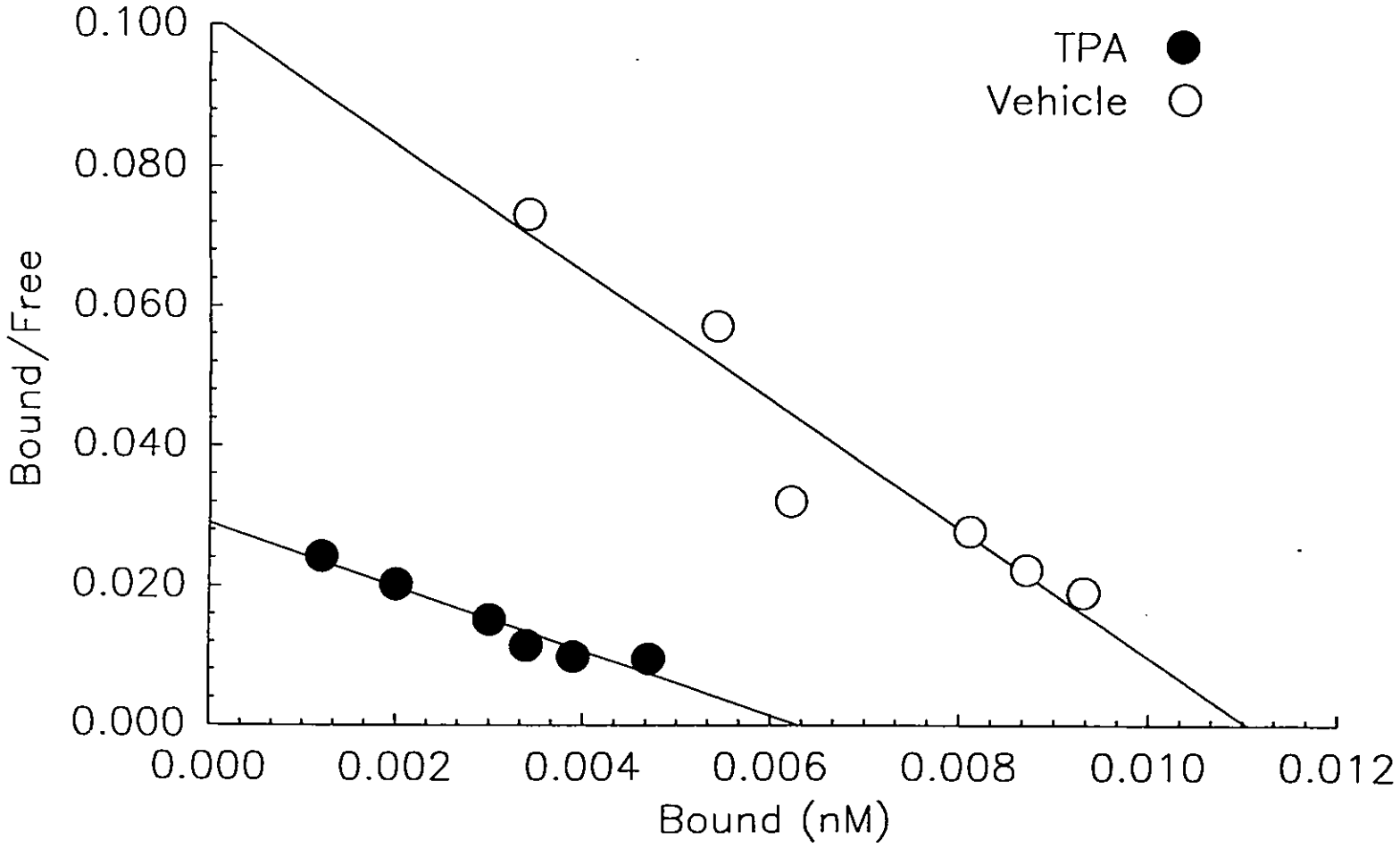

Specific [3H] 1,25-(OH)2D3 Binding
(fmol/mg)
100
90
80
70
60
50
40
30
20
10
0
0.00
0.01
0.10
1.00
5.00
10.0
100
Concentration of TPA (nM)

Figure 3.5 Scatchard Analysis of Specific ^3H-1,25(OH)$_2D_3$ Binding in TPA Treated MDBK Cells

Quiescent MDBK cells were treated with 100 nM TPA (●) or vehicle (O) for 24 hours. Chromatin extracts (1.0 mg protein/ml) were incubated with 0.05-1.0 nM of ^3H-1,25(OH)$_2D_3$ in the presence or absence of a 250-fold molar excess of unlabelled 1,25(OH)$_2D_3$ as described in 3.3.1. Specific binding, derived by subtracting non-specific from total binding, was used to generate the Scatchard plots. Each point is the average of duplicate determinations in one experiment. Similar results were obtained in two other experiments.


0.100
0.080
0.060
0.040
0.020
0.000
Bound/Free
0.000
0.002
0.004
0.006
0.008
0.010
0.012
Bound (nM)
TPA
Vehicle

binding was again reduced by approximately 50% in cells treated with 100 nM TPA for 24 hours, TPA did not alter the apparent molecular weight/size of the ^{3}H-1,25(OH)$_2$D$_3$ binding component.

The specificity of the effect of TPA was demonstrated by the lack of effect of 24 hours incubation with 100 nM 4αPDD on specific ^{3}H-1,25(OH)$_2$D$_3$ binding in MDBK cells (Figure 3.7). 4αPDD is a phorbol ester analogue, structurally similar to TPA, which is considered inactive as it does not modulate PKC activity or promote tumor formation (Castagna et al, 1982). As well treatment of MDBK cells with vehicle had no effect on ^{3}H-1,25(OH)$_2$D$_3$ binding in comparison to control untreated MDBK cells.

3.4.3 Effect of 1,25(OH)$_2$D$_3$ on calbindin D-28K Expression

24 hour treatment with 100 nM 1,25(OH)$_2$D$_3$ increased immunoreactive CaBP D-28K as detected by immunoblotting MDBK cell extracts with a monoclonal antibody to CaBP D-28K (Figure 3.8). Other studies in this laboratory have since demonstrated that this increase is time- and dose- dependent as well as sensitive to cycloheximide, an inhibitor of protein synthesis (personal communication, AM. Gagnon, 1992).

Figure 3.6 FPLC Analysis of Specific ^{3}H-1,25(OH)$_2$D$_3$ Binding in TPA treated MDBK Cells

Quiescent MDBK cells were treated for with 100 nM TPA (●) or vehicle (O) for 24 hours. Chromatin extracts (4.5 mg protein/ml) were incubated with 2.5 nM ^{3}H-1,25(OH)$_2$D$_3$ in the presence or absence of a 250-fold molar excess of unlabelled 1,25(OH)$_2$D$_3$ as described in 3.3.1. Bound sterol was applied to a Superose 12 FPLC column and eluted with KTED buffer. Specific binding, derived by subtracting non-specific from total binding, was used to generate the FPLC plots. Arrow indicates the elution position of ^{14}C-albumin (molecular weight = 66 kD).

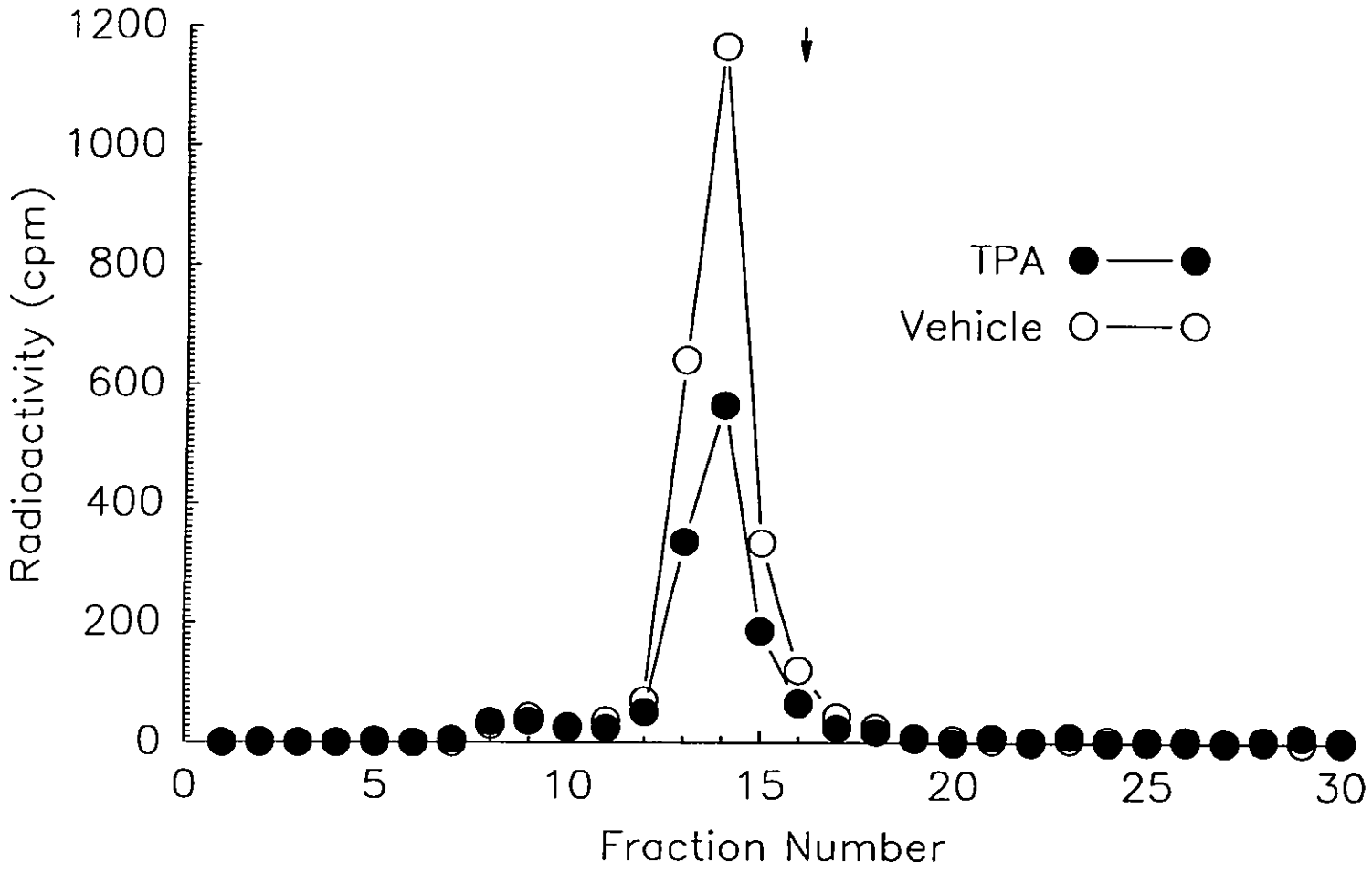

1200
1000
800
600
400
200
0
Radioactivity (cpm)
0
5
10
15
20
25
30
Fraction Number
TPA
Vehicle

Figure 3.7 Effect of TPA and 4αPDD on Specific ^{3}H-1,25(OH)$_2$D$_3$ Binding in MDBK Cells

Quiescent MDBK cells were treated with 100 nM TPA, 100 nM 4αPDD, or vehicle for 24 hours. Control cells were untreated. Chromatin extracts (1.0 mg protein/ml) were incubated with 0.5 nM ^{3}H-1,25(OH)$_2$D$_3$ in the presence or absence of a 250-fold molar excess of unlabelled 1,25(OH)$_2$D$_3$ as described in 3.3.1. Values are mean +/- SEM for triplicate determinations in four experiments. The difference between TPA treated and control untreated, vehicle treated or 4αPDD treated cells was significant ($p<0.05$) by Student's paired t test. There were no significant differences between untreated, vehicle treated and 4αPDD treated cells.

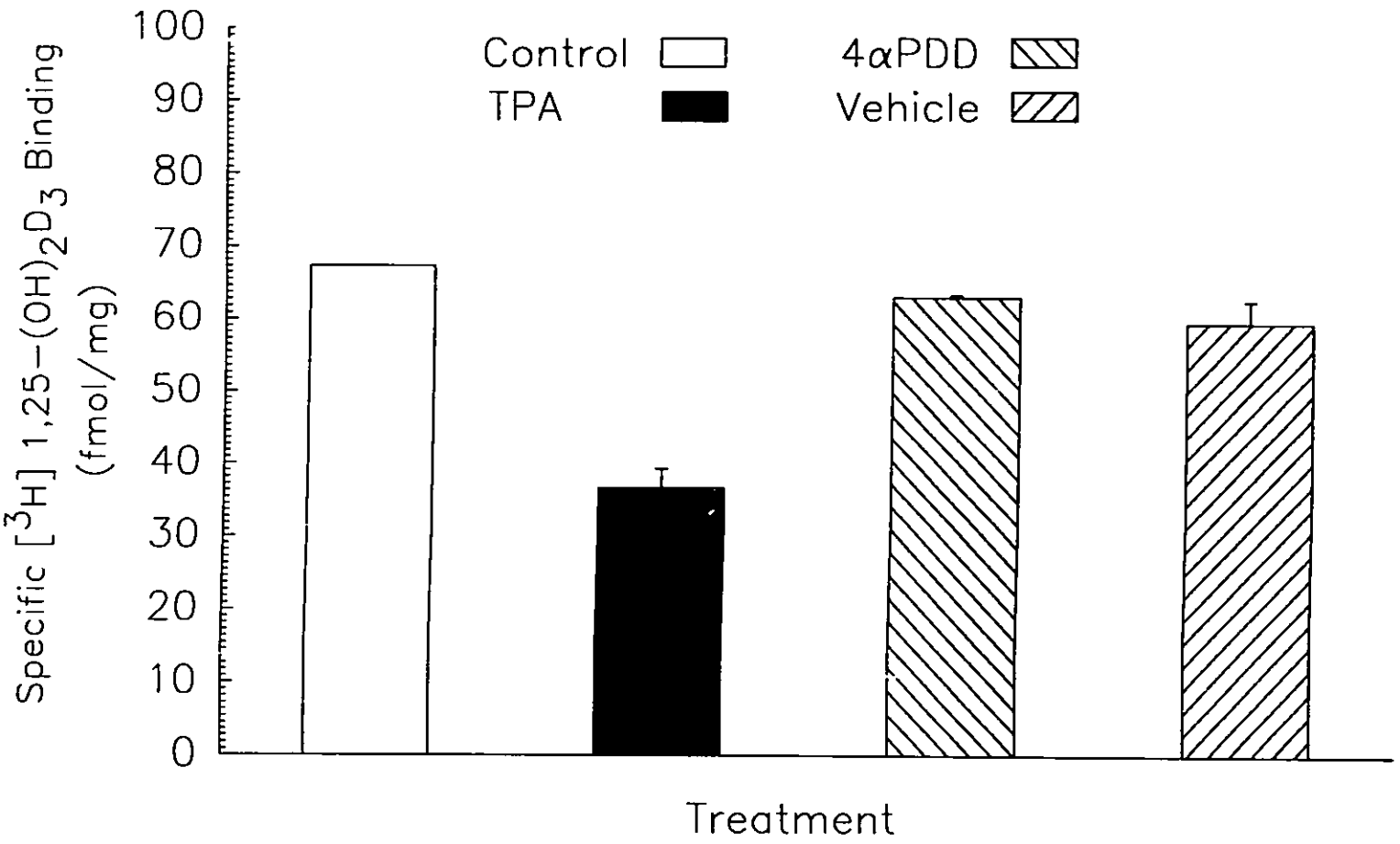
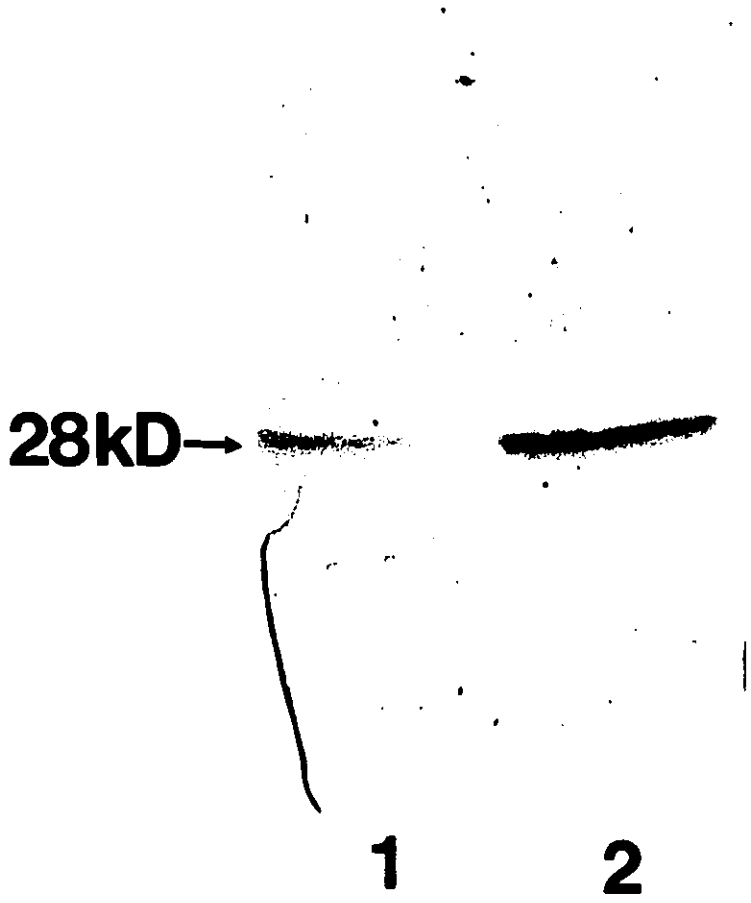

Control
TPA
4αPDD
Vehicle
Specific [3H] 1,25-(OH)2D3 Binding
(fmol/mg)
100
90
80
70
60
50
40
30
20
10
0
Treatment

Figure 3.8 Effect of $1,25(OH)_2D_3$ on Calbindin D-28K Immunoreactivity in MDBK Cells

Quiescent MDBK cells were treated with 100 nM $1,25(OH)_2D_3$ (lane 2) or vehicle (lane 1) for 24 hours. Total cellular protein extracts (100 μg protein) were electrophoresed on 15% SDS-PAGE, transferred to nitrocellulose and immunoblotted with a CaBP D-28K monoclonal antibody. Antigen-antibody complexes were detected with a biotinylated secondary antibody and streptavidin peroxidase as described in 3.3.2.


28kD
1
2

3.4.4 Effect of Phorbol Esters on calbindin D-28K Expression

As shown in Figure 3.9, cytosolic CaBP D-28K was increased within 2 hours of exposure of MDBK cells to 100 nM TPA, this increase was maximal within 4-6 hours of treatment. 24 hour treatment with 100 nM TPA markedly decreased immunoreactive CaBP D-28K. After 48 hours of TPA treatment, CaBP D-28K was barely detectable on immunoblots (data not shown). 24 hour treatment with the inactive phorbol ester, 4αPDD (100 nM), had no effect on CaBP D-28K immunoreactivity in MDBK cells (Figure 3.10).

Figure 3.9 Time Course of Effect of TPA on Calbindin D-28K Immunoreactivity in MDBK Cells

Quiescent MDBK cells were treated with 100 nM TPA (lane 1: 1 hour TPA, lane 2: 2 hour TPA, lane 3: 4 hour TPA, lane 4: 6 hour TPA, lane 5: 24 hour TPA) or vehicle (lane 6) for up to 24 hours. Cytosolic extracts (100 μg protein) were electrophoresed on 15% SDS-PAGE, transferrred to nitrocellulose and immunoblotted with a CaBP D-28K monoclonal antibody. Antigen-antibody complexes were detected with a biotinylated secondary antibody and streptavidin peroxidase as described in 3.3.2.

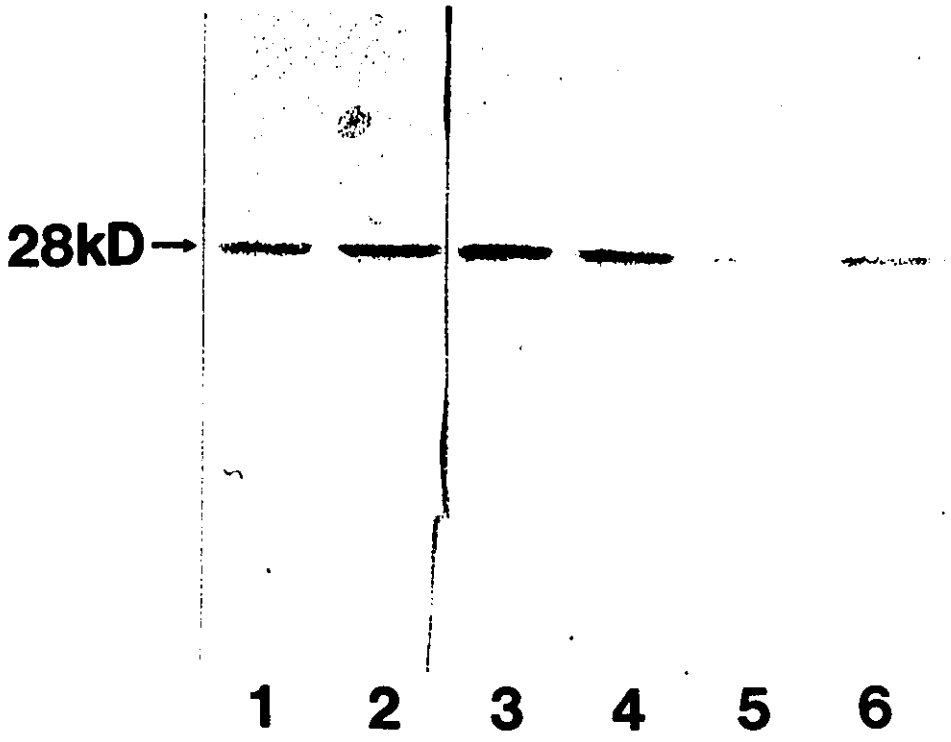
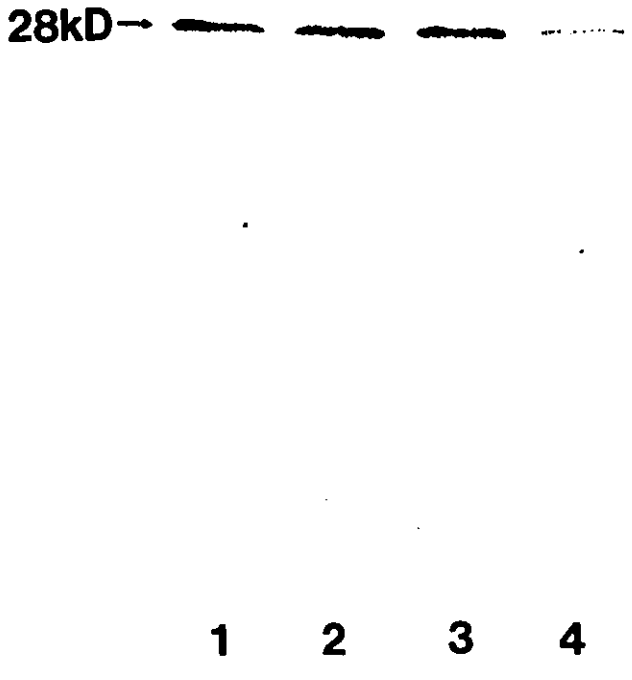

28kD
1
2
3
4
5
6

Figure 3.10 Effect of TPA and 4αPDD on Calbindin D-28K Immunoreactivity in MDBK Cells

Quiescent MDBK cells were treated with 100 nM TPA (lanes 4, 5), 100 nM 4αPDD (lanes 2, 3) or vehicle (lane 1) for 24 hours. Cytosolic extracts (100 μg protein) were electrophoresed on 15% SDS-PAGE, transferrred to nitrocellulose and immunoblotted with a CaBP D-28K monoclonal antibody. Antigen-antibody complexes were detected with a biotinylated secondary antibody and streptavidin peroxidase as described in 3.3.2.


28kD
1
2
3
4
5

3.5 Discussion

3.5.1 Effect of $1,25(OH)_2D_3$ on Specific ^3H-$1,25(OH)_2D_3$ Binding

In MDBK cells, 24 hour treatment with 100 nM $1,25(OH)_2D_3$ homologously up-regulated specific ^3H-$1,25(OH)_2D_3$ binding (Figure 3.1). The increase in VDR levels appeared to be due to an increase in N_{max} and not due to changes in the affinity of the receptor for the hormone (Figure 3.2). These results are consistent with those obtained in LLC-PK_1 cells, a proximal tubular cell line (Costa, Hirst and Feldman, 1985). Treatment of LLC-PK_1 cells with 5 nM $1,25(OH)_2D_3$ for 20 hours results in a 1.7-fold increase in specific ^3H-$1,25(OH)_2D_3$ binding with no apparent change in K_d, 4.0×10^{-11} M. These results also compare favorably to reports for whole rat kidney; VDR protein levels, measured by immunoradiometric assay, are 1.8-fold higher in vitamin D-replete animals in comparison to vitamin D-deficient animals (Sandgren and DeLuca, 1990). Generally, homologous up-regulation of the VDR is modest in kidney in comparison to intestine; the tissue with the highest VDR concentration (DeLuca, Krisinger and Darwish, 1990).

During homologous up-regulation, the degradation of the VDR is slowed (Norman et al., 1983) and receptor half-life is increased (Costa and Feldman, 1987) whereas there is no apparent change in VDR mRNA levels (Huang et al., 1989; Weise

et al., 1992). Thus it is currently believed that homologous up-regulation of the VDR does not result from an increase in receptor synthesis but rather that it results from an increase in receptor protein levels due to enhanced stability.

One mechanism that could potentially alter protein stability may involve post-translational modifications, such as phosphorylation. As mentioned previously, the VDR has been shown to be phosphorylated (Pike and Sleator, 1985; Brown and DeLuca, 1990) and, in the mouse, phosphorylation occurs predominantly on serine residues (Haussler et al., 1988b). The functional significance of VDR phosphorylation is unclear but it has been suggested that phosphorylation may be related to transactivation or receptor turnover (Hseih et al., 1991; Pike, 1991). This finding is intriguing in light of my findings that $1,25(OH)_2D_3$ activates PKC (Chapter Two) and up-regulates the VDR in MDBK cells. I hypothesize that activation of PKC by $1,25(OH)_2D_3$ in MDBK cells might increase the phosphorylation state of the VDR, this in turn might enhance its stability which could result in up-regulation of specific $1,25(OH)_2D_3$ binding. This potential role of PKC in VDR regulation was next probed by examining the effects of two phorbol esters, TPA and 4αPDD, on specific $1,25(OH)_2D_3$ binding.

3.5.2 Effect of Phorbol Esters on Specific ^{3}H-1,25(OH)$_2$D$_3$ Binding

Long term treatment of MDBK cells with the phorbol ester TPA decreased the number of VDRs, as measured by specific 1,25(OH)$_2$D$_3$ binding (Figure 3.3). Scatchard analysis revealed that TPA treatment did not alter the binding affinity of the VDR for 1,25(OH)$_2$D$_3$ as the K_d values obtained in vehicle and TPA treated MDBK cells were similar (Figure 3.5).

Our results complement earlier reports of TPA induced down-regulation of the VDR and estrogen receptor in HL-60 (Haussler et al, 1987) and in MCF-7 (Guilbaud et al, 1988) cells respectively. In those studies, specific hormone binding was decreased after 24 hours of treatment with TPA and was not the result of changes in receptor affinity for hormone. However, the effect of TPA on steroid receptor numbers in both HL-60 and MCF-7 cells was co-incident with inhibition of cell proliferation. In contrast TPA did not affect cell proliferation in MDBK cells (Chapter One), thus the effect of TPA on the VDR in MDBK cells appears to be independent of modulation of cell proliferation. These data are consistent with that of Yu et al (1991) in which a dissociation between VDR expression and cell proliferation was observed in human lymphocytes. It should be noted, however, that in lymphocytes, TPA triggers activation and induces expression of the VDR. Up-regulation of the VDR in response

to long term treatment with TPA has also been reported in osteoblast-like cell lines, UMR 106 and ROS 17/2.8 (Van Leeuwen et al., 1992) and bovine aortic endothelial cells (Merke et al, 1989). These data suggest that TPA affects VDR levels in a cell specific manner.

The best characterized effect of TPA in all cells is modulation of PKC activity (Nishizuka, 1986). The effect of TPA on the VDR in MDBK cells is likely related to effects on PKC activity, since an inactive but structurally similar phorbol ester 4αPDD (Castagna et al., 1982), which does not affect PKC activity or immunoreactivity in MDBK cells (Chapter Two), also does not affect VDR levels (Figure 3.7). TPA modulation of PKC activity is biphasic in most cells: transient short term activation of PKC is followed by long term down-regulation (Nishizuka, 1989). As a result of the biphasic modulation of PKC by TPA, different interpretations can be made as to whether the effects of long term treatment with TPA are due to activation or down-regulation of PKC. In bone cells, up-regulation of VDR by TPA is interpreted to result from down-regulation of PKC (Van Leeuwen et al., 1992); whereas in bovine aortic endothelial cells up-regulation of VDR by TPA is interpreted to result from activation of PKC (Merke et al., 1989). Finally Krishnan and Feldman (1991b) concluded that activation of PKC is responsible for the down-regulation of VDR by TPA in NIH-3T3 cells. However, none of the studies cited above actually measured changes in PKC

activity or amount in response to TPA treatment. Clearly my data indicates that in MDBK cells, short term exposure to TPA results in transient activation of PKC, while long term exposure significantly down-regulates PKC activity and amount (Chapter Two). The decrease in VDR levels in MDBK cells, treated for 24 hours with TPA, is co-incident with the down-regulation of PKC. To concur with my hypothesis in 3.5.1 that homologous up-regulation of VDR could be due to increased PKC-dependent phosphorylation, I further hypothesize that VDR down-regulation is related to decreased PKC-dependent phosphorylation. If this is the case, it is unclear why activation of PKC by short term TPA treatment did not increase VDR levels. However this data is consistent with those of Haussler et al., (1987), who reported that 1 hour treatment with TPA does not affect $1,25(OH)_2D_3$ binding in HL-60 cells; whereas long term TPA exposure results in VDR down-regulation.

Although the normal process by which the VDR is turned over is poorly understood, decreased PKC-dependent phosphorylation of the VDR, due to long term TPA treatment, could initiate or accelerate VDR degradation. Proteolytic cleavage (either by trypsin or endogenous proteases) of the VDR has been shown to generate polypeptide fragments (approximately 40-45 kD) which retain hormone binding capability (Allegretto, Pike and Haussler, 1987). In MDBK cells, the FPLC profile of $1,25(OH)_2D_3$ binding was not altered by TPA, indicating that smaller hormone binding fragments are

not generated after TPA treatment (Figure 3.6). This data, therefore, suggests that the down-regulation of the VDR induced by TPA is not secondary to proteolytic cleavage at this known site; however, an effect of TPA on VDR half life can not be completely discounted.

Alternatively, decreased PKC-dependent phosphorylation might affect transcription of the VDR as Krishnan and Feldman (1991b) have reported decreased VDR mRNA in TPA treated fibroblasts. TPA induced down-regulation of the ER in MCF-7 cells is also reported to be accompanied by a decrease in ER mRNA levels, however TPA does not affect transcription of the ER gene but rather induces post-transcriptional destabilization of ER mRNA (Saceda et al., 1991). This destabilization of ER mRNA by TPA is thought to be due to its effects on PKC activity.

3.5.3 Effect of $1,25(OH)_2D_3$ and Phorbol Esters on Calbindin D-28K Expression

Analogous to other steroid hormones, $1,25(OH)_2D_3$, through the VDR, regulates the synthesis of specific proteins. As mentioned previously, CaBP D-28K, the best characterized vitamin D modulated protein in kidney, is regulated both transcriptionally and post-transcriptionally by $1,25(OH)_2D_3$ (Clemens et al, 1988; 1989). In MDBK cells, homologous up-regulation of the VDR by $1,25(OH)_2D_3$ was associated with

increased CaBP D-28K (Figure 3.8). Exposure of MDBK cells for 24 hours to one dose of 100 nM $1,25(OH)_2D_3$ increased total immunoreactive CaBP D-28K, consistent with effects of $1,25(OH)_2D_3$ on CaBP D-28K in primary cultures (Craviso, Garrett and Clemens, 1987; Chen et al., 1992). Thus MDBK cells are the first cell line found to express CaBP D-28K in a vitamin D-dependent manner. Although the induction of CaBP D-28K by $1,25(OH)_2D_3$ in these cells is modest, the apparent magnitude of this induction is consistent with work in primary cultures, where 24 hour treatment with one dose of 100 nM $1,25(OH)_2D_3$ increased CaBP D-28K 1.3- to 1.9-fold in rat preparations (Chen et al., 1992) and two-fold in chick preparations (Craviso, Garrett and Clemens, 1987), compared to vehicle treated cells. *In vivo*, there is less than a three-fold difference in renal CaBP D-28K levels between vitamin D-replete and vitamin D-deficient rats (Sonnenberg, Pansini and Christakos, 1984). Despite this vitamin D-dependence, constitutive expression of renal CaBP D-28K is detectable in vitamin D-deficient chicks and rats (Christakos and Norman, 1980; Sonnenberg, Pansini and Christakos, 1984). Similarly, in MDBK cells CaBP D-28K expression persists even in the absence of exogenous $1,25(OH)_2D_3$ (serum-free media) for at least 48 hours (data not shown). These data documenting the persistence of CaBP D-28K expression in the absence of $1,25(OH)_2D_3$ and the relatively modest increases in response to $1,25(OH)_2D_3$ treatment imply that MDBK cells mimic the

expression of renal CaBP D-28K *in vivo*.

Down-regulation of the VDR in response to long term TPA treatment was associated with reduced CaBP D-28K (Figure 3.9). No change in CaBP D-28K was observed after treatment with the phorbol ester analogue, 4αPDD (Figure 3.10) which had no effect on VDR levels (Figure 3.7) or PKC activity (Chapter Two). Although my results suggest that TPA might affect CaBP D-28K levels via its effects on the VDR, I cannot exclude an independent effect of TPA on CaBP D-28K. In fact, short term exposure of MDBK cells to TPA was associated with an increase in cytosolic levels of CaBP D-28K in the absence of any change in VDR levels. The increase in CaBP D-28K was preceded by an increase in PKC activity, suggesting that PKC-dependent phosphorylation might directly affect CaBP D-28K expression. In addition to modulating PKC activity, TPA rapidly induces the nuclear phosphoproteins c-*fos* and c-*jun*, which together form the AP-1 transcription factor (Ransone and Verma, 1990). An intrinsic AP-1 binding site within the vitamin D response element (VDRE) has been identified in the promoter region of the gene for human osteocalcin, another $1,25(OH)_2D_3$ regulated protein (Ozono et al, 1990) and the *Fos-Jun* complex can bind to the promoter region of the chicken CaBP D-28K gene (Lowe et al., 1991).

To gain insight into the divergent regulation of VDR and CaBP D-28K expression by TPA and $1,25(OH)_2D_3$, the effect of these two agents on specific PKC isozymes in MDBK cells was

examined in the next series of studies.

3.6 Conclusions

The overall conclusion of this chapter is that $1,25(OH)_2D_3$ and TPA have divergent effects on VDR and CaBP D-28K expression in MDBK cells. The specific conclusions are:

3.6.1 Long term treatment with $1,25(OH)_2D_3$ homologously up-regulates the VDR and increases total immunoreactive CaBP D-28K in MDBK cells.

3.6.2 Long term treatment with TPA down-regulates the VDR and decreases cytosolic levels of CaBP D-28K in MDBK cells.

3.6.3 Short term treatment with TPA increases CaBP D-28K without affecting VDR levels in MDBK cells.

3.6.4 Long term treatment with the phorbol ester analogue, 4αPDD, does not alter VDR or CaBP D-28K expression in MDBK cells.

CHAPTER FOUR: EFFECTS OF 1,25(OH)$_2$D$_3$ AND TPA ON SUBCELLULAR LOCALIZATION OF PROTEIN KINASE C ISOZYMES

4.1 Introduction

Until recently, all biological responses involving PKC were attributed to one molecular species. Now it is well known that PKC exists as a family of related genes which code for a number of highly homologous isozymes (Parker et al., 1989). The reasons for PKC heterogeneity are unknown, although it is clear that diverse stimuli regulate the activity and subcellular distribution of specific PKC isozymes. Emerging data on individual PKC family members have suggested that different isozymes regulate distinct cellular functions (Nishizuka, 1988). The observation that more than one isozyme is usually expressed within a particular cell type (Strulovici, 1991; Kiley et al., 1990a) further supports this notion and could explain the diversity of cellular responses mediated by PKC.

4.1.1 Structure of Protein Kinase C Isozymes

Molecular cloning analysis has revealed the existence of at least eight different PKC isozymes (Nishizuka, 1988; 1989), which can be divided into two major groups. Initially, four isozymes were identified. This group includes PKC α, β_I, β_{II},

and γ, with β_I and β_{II} being derived from a single RNA transcript by alternative splicing. All four of these isozymes have a similar structural organization with an amino-terminal regulatory region and a carboxy-terminal catalytic domain. The regulatory domain consists of two highly conserved constant regions, C_1 and C_2 (Nishizuka, 1988). C_1 possesses two "zinc finger" cysteine-rich regions and is believed to be the phospholipid, DAG/phorbol ester binding region (Ono et al., 1989a), while C_2 is the putative calcium binding region (Nishizuka, 1989). The activity of these four isozymes is strongly dependent on calcium, phospholipid and DAG (Nishizuka, 1988) and as such they have since been designated as conventional PKCs (cPKCs).

Subsequently, a second group of isozymes, specifically PKC δ, ϵ, ζ, and L (murine homologue η), was identified (Ono et al., 1988; Osada et al., 1990). These latter isozymes are designated novel PKCs (nPKCs) since they lack the conserved C_2 region. As well PKC ζ contains only one "zinc finger" cysteine-rich region in its C_1 domain (Bell and Burns, 1991).

4.1.2 Activity of Protein Kinase C Isozymes

Although structurally similar the members of the cPKC group exhibit distinctly different modes of activation, kinetic properties and, most likely, substrate specificity. PKC γ and PKC α show considerably less activation by DAG than

PKC β (Sekiguchi et al., 1988). PKC β_I and β_{II} show virtually identical kinetic and catalytic properties (Ono et al., 1987) and can be distinguished only by immunochemical procedures (Shearman et al., 1987). PKC β shows substantial activity even without added calcium, in the presence of DAG and phospholipid (Sekiguchi et al., 1988). All of the cPKCs are down-regulated by long term exposure to TPA (Ase et al., 1988).

The nPKC group exhibit calcium-independent activity, presumably reflecting the absence of the putative calcium binding domain C_2. As well, PKC δ and PKC ϵ do not exhibit an absolute requirement for phospholipid and DAG, whereas the cPKC group becomes fully active only when these lipids are added (Ono et al., 1988). PKC ϵ, PKC ζ and PKC L(η) (Strulovici et al., 1991; Ways et al., 1992; Greif et al., 1992) appear to be resistant to down-regulation by long term treatment with TPA. In the case of PKC ζ, resistance to down-regulation is somewhat expected, since this isozyme contains only one set of cysteine-rich zinc-finger-like motifs in the C_1 domain (Bell and Burns, 1991) and these motifs are believed to be important for phorbol ester binding (Ono et al., 1989a).

4.1.3 Distribution of Protein Kinase C Isozymes

The tissue and cellular distribution of the cPKC group has been well studied. PKC γ is expressed solely in the brain

and spinal cord (Saito et al., 1988). PKC β_I, β_{II} are expressed in the brain as well as in many other tissues; however, immunological studies indicate a clearly distinct cellular expression for β_I and β_{II} subspecies. For example, in rat cerebellar cortex, β_I is primarily localized in the granular layer while β_{II} is mostly in the molecular layer. In neuronal cells β_I is sometimes associated with the plasma membrane while β_{II} is often localized in the Golgi complex (Hosoda et al., 1989; Saito et al., 1989). PKC α is universally distributed in most tissues and cell types (Kosaka et al., 1988).

Relatively little is known about the tissue and cellular distribution of the nPKC group. By northern blot analysis, PKC δ is present predominantly in the brain and lung; PKC ϵ is present in brain; and PKC ζ is detected mainly in brain, kidney and lung (Ono et al., 1988). By western blot analysis, PKC δ is present in brain, heart, spleen, lung, liver, ovary, pancreas, skin and adrenal tissues; PKC ζ is in most tissues but particularly brain, lung and liver (Wetsel et al., 1992); and PKC L(η) is expressed predominantly in lung, heart and skin (Bacher et al., 1991).

4.1.4 Regulation of Protein Kinase C Isozymes

Regulation of PKC isozymes has been studied in a variety of tissues and cell types. PKC α, β_{II} and γ are differentially regulated during neuronal differentiation of PC12 cells

(Wooten, 1992). PKC α, γ and ϵ exhibit age-dependent expression in rat pancreatic islets (Fletcher and Ways, 1991). The ratio of PKC γ to PKC β is dramatically higher in human placental tissue during the first trimester than during the second and third trimesters (Nomura et al., 1991). Finally, in HL-60 cells, two PKC activators (bryostatin and TPA) elicit disparate effects on the translocation and activation of PKC α and PKC β_{II}; this differential regulation may explain the antagonistic actions of these two PKC activators on HL-60 cell differentiation (Hocevar and Fields, 1991).

In kidney, hormonal responses that involve the phosphoinositide signalling pathway and PKC are distributed throughout the nephron in the proximal and distal tubules and the glomerulus (Pfelischifter, 1989). The known heterogeneity of PKC activity predicts that specific PKC isozymes modulate diverse renal functions within specific cell types of the nephron. Molecular analyses indicate that at least three PKC isozymes, PKC α, PKC β and PKC ζ, are expressed in whole rat kidney (Kosaka et al., 1988); however, little is known about the regulation of these PKC isozymes in renal cells.

In the present studies, I examine whether the divergent effects of TPA and $1,25(OH)_2D_3$ on VDR and CaBP D-28K in MDBK cells, reported in Chapter Three, could result from differential translocation and/or activation of PKC α, PKC β and PKC ζ, three PKC isozymes expressed in kidney.

4.2 Objectives

The overall objective of the studies described in this chapter was to characterize the effect of TPA and $1,25(OH)_2D_3$ on the expression, translocation and activation of PKC isozymes in MDBK cells. The specific objectives were:

4.2.1 To establish whether MDBK cells, like whole kidney, express PKC α, β and ζ.

4.2.2 To develop and validate a protocol for subfractionation of MDBK cells into cytosol, membrane and nuclear components.

4.2.3 To determine the effect of 24 hour treatment with TPA and $1,25(OH)_2D_3$ on the translocation/activation of PKC α, β and ζ in MDBK cells, by immunoblot analysis of cytosol, membrane and nuclear fractions.

4.2.4 To determine the effect of 24 hour treatment with TPA and $1,25(OH)_2D_3$ on the distribution of PKC α, β and ζ by immunofluorescence of intact MDBK cells.

4.3 Methods

Cell Culture

Stock cultures of MDBK cells were maintained, at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2, in Dulbecco's modified Eagle medium (GIBCO IBRL) supplemented with 10% newborn calf serum (GIBCO IBRL). Cells were plated, in 175 mm^2 culture flasks (for immunoblotting) or on glass coverslips in 35 mm culture dishes (for immunofluorescence), at a density or 1.5-4.0 x 10^4 cells/ml. The cells were grown to confluence, changed to serum-free media for 24 hours and then treated for 24 hours with 100 nM $1,25(OH)_2D_3$ (Biomol Inc.) (in ethanol), 100 nM TPA (in PBS/DMSO (50/50;v/v)) or vehicle alone.

4.3.1 Subcellular Fractionation

Cells were washed twice with PBS, scraped, pelleted and lysed in hypotonic buffer (1 mM $NaHCO_3$, 5 mM $MgCl_2$, 100 μM PMSF and 20 μg/ml leupeptin) and made to 50 mM Tris-HCl and 0.5 mM EGTA, pH 7.5. Total cell homogenates were normalised for protein concentration and centrifuged (500g, 5 min, 4°C) to obtain a crude nuclear pellet and a post nuclear fraction. The post nuclear fraction was centrifuged (100,000g, 60 min, 4°C) to give a cytosolic supernatant and a plasma

membrane/microsomal (cellular membranes) pellet which was solubilized in Buffer A (50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5mM EGTA, 1.0% (v/v) β-mercaptoethanol, 1mM PMSF and 20 μg/ml leupeptin) containing 1% (v/v) Triton X-100. The nuclear pellet was washed three times with hypotonic lysis buffer, layered over 45% (w/v) sucrose, centrifuged (1660g, 30 min, 4°C), resuspended and sonicated in Buffer A. The purity of those subcellular fractions was characterized by the following procedures. Glucose 6-phosphate dehydrogenase activity was measured, as a cytosolic marker, essentially as described in Cohen and Rosemeyer (1977). Forskolin-stimulatable adenylate cyclase activity was used as a plasma membrane marker; subcellular fractions were treated with 10 μM forskolin in DMSO or vehicle alone for 10 min and adenylate cyclase activity was measured essentially as described in Franks, Plamondon and Hamet (1984). Finally lamin B immunoreactivity was used as a nuclear marker (Franke, 1987); immunoblotting of lamin B in subcellular fractions was performed as described in Chapter one (1.3.3) except the primary antibody used was a mouse anti-human lamin B monoclonal antibody (MAB 101-B7, Matritek Inc., Cambridge, MA., USA) diluted 1:1000 with blocking solution.

4.3.2 Isolation of Protein Kinase C Isozymes

Total cell homogenates or cytosol, membrane and nuclear

fractions were loaded onto 1ml DEAE-Sephacel columns pre-equilibrated with Buffer A. The columns were washed with 10 bed volumes of Buffer A and bound PKC was eluted with Buffer A containing 200 mM NaCl (Hocevar and Fields, 1991). Eluates were desalted on individual G-25 Sephadex mini-prep columns (Muler, Traish and Wotiz, 1983) and lyophilized overnight.

4.3.3 Immunoblotting of Protein Kinase C Isozymes

For immunoblotting lyophilized samples were redissolved in H_2O containing 0.01% (w/v) bromophenol blue, 0.05% (v/v) glycerol, 1% (w/v) SDS and 1.25% (v/v) β-mercaptoethanol and electrophoresed on 10% SDS-PAGE (Laemmli, 1970) and transferred and immunoblotted as described in Chapter One (1.3.3) with the following exceptions: the primary antibodies used were PKC α, PKC β or PKC ζ isozyme-specific antibodies (GIBCO IBRL) diluted to 2 μg/ml with blocking solution, non-specific binding was assessed in parallel samples by incubating without primary antibody or in the presence of the specific peptide antigen (1 μg/ml) and the secondary antibody used was a Biotin-conjugated goat anti-rabbit antibody (Jackson Immunoresearch Laboratories) diluted 1:2000 with blocking solution.

4.3.4 Immunofluorescence of Protein Kinase C Isozymes

Cells on coverslips were washed twice with PBS, fixed for 20 min at -20°C in ethanol:acetone (50:50;v:v) rinsed with PBS and then blocked overnight at 4°C in PBS/skim milk with sodium azide included as an antimicrobial agent. After washing extensively with PBS, coverslips were incubated with either PKC α, PKC β or PKC ζ isozyme-specific antibody (GIBCO IBRL) diluted to 10 μg/ml with blocking solution overnight at 4°C in a humid box. Coverslips were rinsed several times with PBS and then incubated with fluorescein-conjugated goat anti-rabbit secondary antibody (Jackson Immunoresearch Laboratories) for 60 min at a dilution of 1:50 in blocking solution. Coverslips were then rinsed extensively with PBS and mounted with glycerol-containing 0.1% (w/v) p-phenylenediamine, viewed with a Zeiss fluorescent microscope and photographed with Ilford XP1 black and white film. Non-specific fluorescence, determined by incubation without primary antibody or in the presence of antigenic peptide (1 μg/ml), was neglible.

4.3.5 Assay of Nuclear Protein Kinase C Activity

To measure nuclear PKC activity, the resuspended and sonicated nuclear pellet was used in a modification of a mixed micelle assay (Hannun, Loomis and Bell, 1985) produced

commercially (Amersham Canada Ltd, Oakville, Ontario, Canada). Briefly, 25 µl of nuclear fraction (0.2 µg protein/ml) was incubated with 25 µl of assay buffer (3 mM calcium, 225 µM peptide substrate, 2 mole % PS and 7.5 mM dithiothreitol in 50 mM Tris-HCl, containing 0.05 volume sodium azide) and 25 µl of [^{32}P] ATP buffer at 25°C. Reactions were terminated after 5 min and aliquots pipetted onto phosphocellulose peptide binding papers, washed twice with 5% (v/v) acetic acid and counted in a Beckman liquid scintillation counter. Phosphorylation of the synthetic peptide was calculated as pmol phosphate transferred/mg nuclear protein/min after correction for blanks containing no peptide substrate, 1mM EGTA and no phospholipid, which were run with each sample. PKC-dependent phosphorylation of endogenous nuclear proteins was calculated as calcium/phospholipid-dependent activity in parallel assays from which the synthetic peptide substrate was omitted. The incubation time and nuclear protein concentration were chosen from within the linear ranges of these two variables for the mixed micelle assay (Appendices 7 and 8 respectively).

The contribution of PKC β to phosphorylation was estimated by incubation of nuclear fractions with 1 µg of the anti-PKC β antibody (GIBCO IBRL) for 30 min prior to assay (Leach et al., 1988). Although this antibody has not been reported to have a neutralizing effect on PKC β activity, it is directed against a sequence from the V3 region of PKC β

(Heinrich, 1991). This region is adjacent to the ATP binding site and as a result antibody binding may provide steric hindrance to ATP binding which in turn could blunt catalytic activity.

Miscellaneous

Protein concentration was determined by the method of Bradford (1976). All materials were obtained from Sigma Chemical Corp., St. Louis, MO, USA unless otherwise stated. Data are expressed as mean +/- standard error (SEM).

4.4 Results

4.4.1 Expression of Protein Kinase C Isozymes in MDBK Cells and Antibody Specificity

To determine whether PKC α, PKC β and PKC ζ were expressed in MDBK cells, total cell extracts were immunoblotted with isozyme specific antibodies. As indicated in Figure 4.1, antibodies directed against PKC α and PKC β recognized a single band of approximately 80 kD, which was competed out by inclusion of the appropriate antigenic peptide. The PKC ζ antibody recognized two bands of approximately 70 and 80 kD, both of which were competed out by the PKC ζ antigenic peptide. The characteristics of these three antibodies are consistent with previous reports (Heinrich, 1991).

4.4.2 Characterization of Subcellular Fractions

Marker proteins used to characterize the subcellular fractions included glucose 6-phosphate dehydrogenase (G6PD; a soluble cytosolic enzyme), adenylate cyclase (a plasma membrane bound enzyme) and lamin B (a nuclear envelope protein). As indicated in Figure 4.2 cytosolic fractions contained G6PD activity, but were negative for adenylate cyclase and lamin B; plasma membrane fractions exhibited

Figure 4.1 Expression of PKC Isozymes in MDBK Cells and Antibody Specificity

MDBK cells were homogenized, PKC was isolated as described in 4.3.2, separated on 10% SDS-PAGE, transferred to nitrocellulose and immunoblotted with PKC α, PKC β and PKC ζ isozyme-specific antibodies, in the presence (+) or absence (-) of the appropriate antigenic peptide. Antigen-antibody complexes were detected with a biotinylated secondary antibody and streptavidin peroxidase as described in 4.3.3. Data is representative of two experiments which gave identical results.

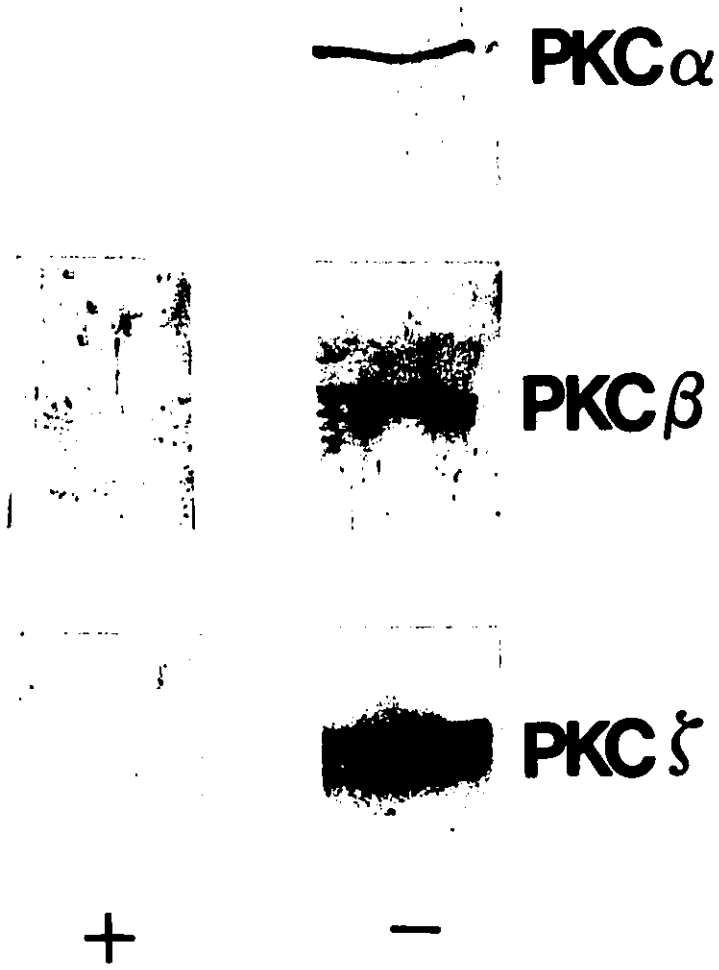
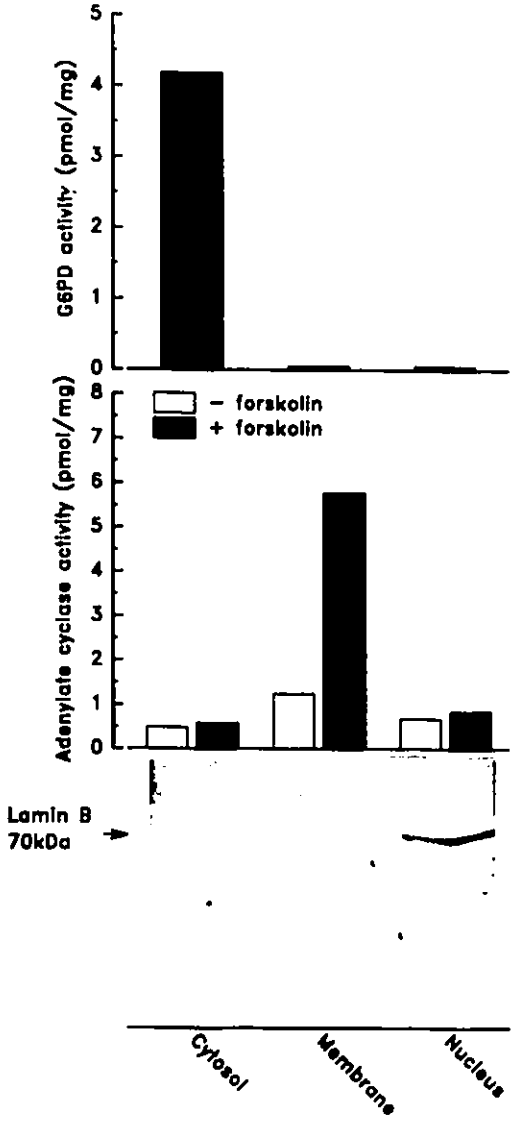

PKCα
PKCβ
PKCζ
+
−

Figure 4.2 Characterisation of Subcellular Fractions

MDBK cells were homogenised and fractionated as described in 4.3.1. Subcellular fractions were analysed for glucose 6-phosphate dehydrogenase activity (top), a cytosol marker; forskolin-stimulatable adenylate cyclase activity (center), a plasma membrane marker; and lamin B immunoreactivity (bottom), a nuclear marker. In each case, left lane represents cytosol, middle lane represents membrane and right lane represents nuclear fraction. Data given in top and center panels represent means of triplicate determinations. Data given in bottom panel is representative of three experiments which gave identical results.


G6PD activity (pmol/mg)
5
4
3
2
1
0
Adenylate cyclase activity (pmol/mg)
8
7
6
5
4
3
2
1
0
– forskolin
+ forskolin
Lamin B
70kDa
Cytosol
Membrane
Nucleus

adenylate cyclase activity but no G6PD activity or lamin B immunoreactivity; nuclear fractions were positive for lamin B, but negative for G6PD and adenylate cyclase.

4.4.3 Effect of $1,25(OH)_2D_3$ and TPA on Subcellular Distribution of Protein Kinase C α

Immunoblots of subcellular fractions derived from vehicle treated cells indicated PKC α was present in both membrane and cytosol (Figure 4.3a, ethanol vehicle; Figure 4.3c, PBS/DMSO vehicle). MDBK cells treated for 24 hours with 100 nM $1,25(OH)_2D_3$ (Figure 4.3b) consistently exhibited a slight increase in PKC α in membrane, compared to vehicle controls, but there was no detectable effect of $1,25(OH)_2D_3$ on PKC α immunoreactivity in the cytosolic fraction. In contrast, 24 hour treatment with 100 nM TPA (Figure 4.3d) resulted in almost complete reduction in immunoreactive PKC α in both membrane and cytosol compared to vehicle controls. No PKC α was apparent in the nuclear fraction of MDBK cells under any incubation conditions.

Immunofluorescence localization indicated that $1,25(OH)_2D_3$ treated cells exhibited increased PKC α immunofluorescence associated with the plasma membrane (Figure 4.4b) compared to vehicle controls (Figure 4.4a), whereas TPA treated cells (Figure 4.4d) exhibited reduced PKC α immunofluorescence in all areas compared to vehicle controls (Figure 4.4c).

Figure 4.3 Effect of 1,25(OH)$_2$D$_3$ and TPA on Subcellular Fractionation of PKC α

Quiescent MDBK cells were treated with ethanol vehicle (A), 100 nM 1,25(OH)$_2$D$_3$ (B), PBS/DMSO vehicle (C) or 100 nM TPA (D) for 24 hours. Cells were homogenised and fractionated into cytosol (1), membrane (2) and nucleus (3) as described in 4.3.1. From these fractions PKC was isolated as described in 4.3.2 and separated on 10% SDS-PAGE, transferred to nitrocellulose and immunoblotted with PKC α antibody, antigen-antibody complexes were detected with a biotynylated secondary antibody and streptavidin peroxidase as described in 4.3.3. Immunoblots shown are representative of five experiments which gave similar results.

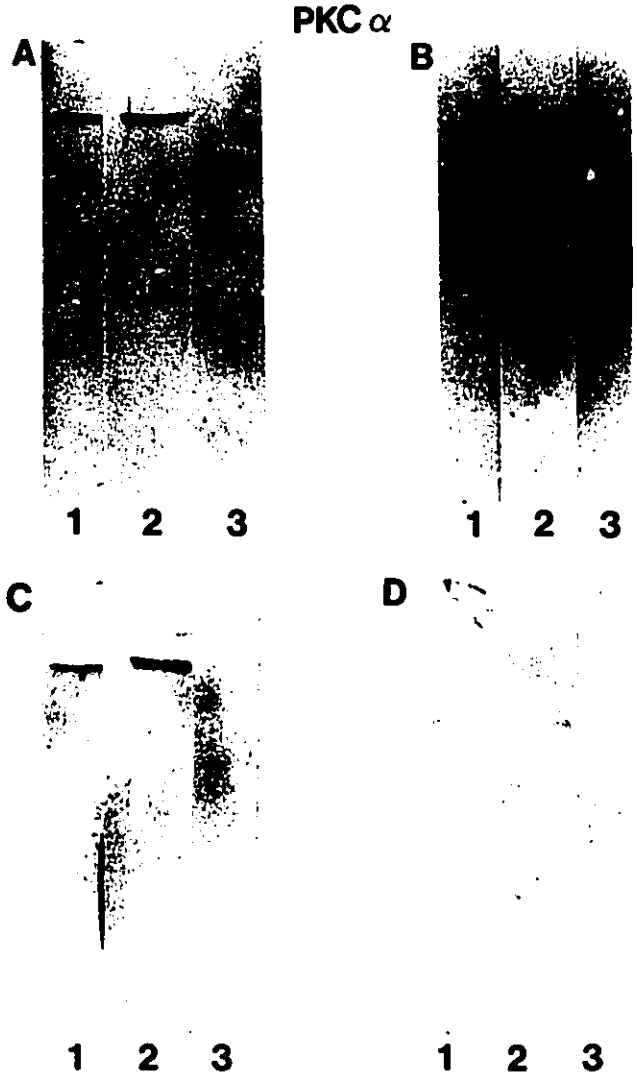
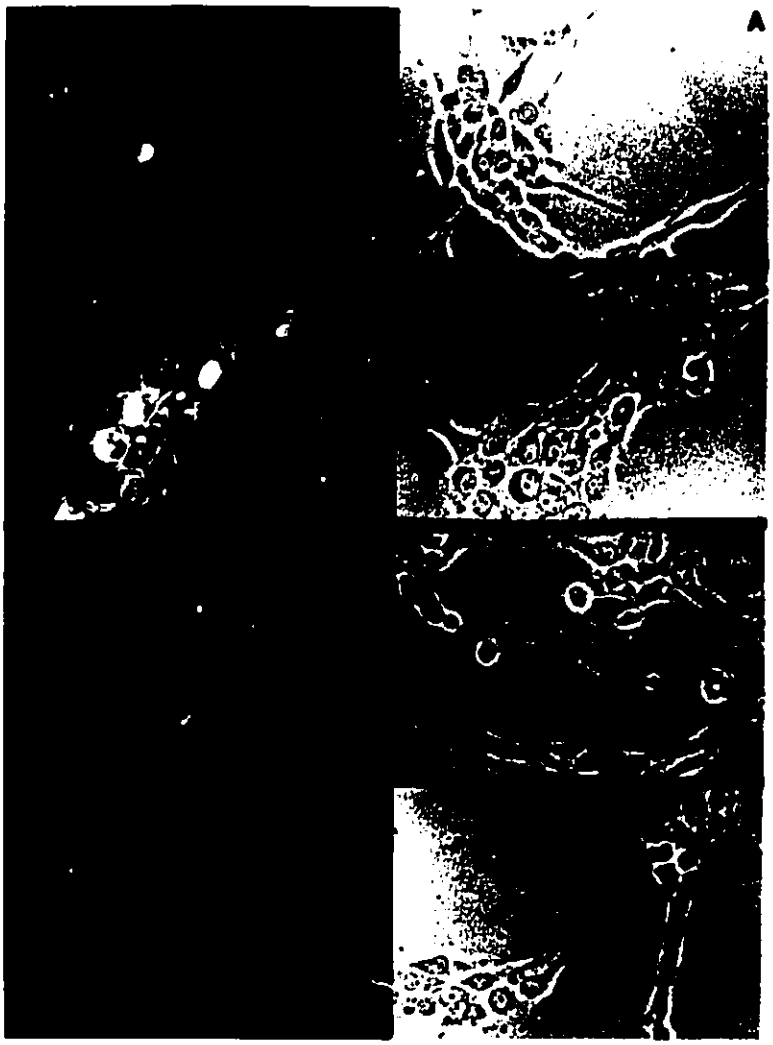

PKC α
A
B
1 2 3
1 2 3
C
D
1 2 3
1 2 3

Figure 4.4 Effect of $1,25(OH)_2D_3$ and TPA on PKC α Immunofluorescence

Quiescent MDBK cells were treated with ethanol vehicle (A), 100 nM $1,25(OH)_2D_3$ (B), PBS/DMSO vehicle (C) or 100 nM TPA (D) for 24 hours. Cells were fixed and incubated with PKC α antibody, antigen-antibody complexes were detected with a fluorescein-conjugated secondary antibody as described in 4.3.4. Left: Immunofluorescence, right: corresponding phase contrast photograph; immunostaining was performed four times with similar results.


A

4.4.4 Effect of $1,25(OH)_2D_3$ and TPA on Subcellular Distribution of Protein Kinase C β

In vehicle treated MDBK cells (Figure 4.5a and 4.5c), PKC β was expressed at low levels in membrane and cytosol fractions; no immunoreactivity was detected in the nuclear fraction. In cells treated with 100 nM $1,25(OH)_2D_3$ for 24 hours, PKC β immunoreactivity was detected in the nuclear fraction and was abolished in membrane and cytosol (Figure 4.5b). TPA treatment for 24 hours also resulted in the appearance of PKC β immunoreactivity in the nuclear fraction and loss of immunoreactivity in membrane and cytosol (Figure 4.5d).

Consistent with immunoblotting results, MDBK cells treated with either $1,25(OH)_2D_3$ or TPA exhibited increased PKC β immunofluorescence in the perinuclear region compared to vehicle controls. In $1,25(OH)_2D_3$ treated cells (Figure 4.6b), PKC β was concentrated in the peri-nuclear area, whereas in TPA treated cells (Figure 4.6d), PKC β immunofluorescence rimmed the nuclear envelope and appeared in punctated areas within the nucleus.

4.4.5 Effect of $1,25(OH)_2D_3$ and TPA on Subcellular Distribution of Protein Kinase C ζ

Immunoblotting of subcellular fractions with anti PKC ζ

Figure 4.5 Effect of $1,25(OH)_2D_3$ and TPA on Subcellular Fractionation of PKC β

Quiescent MDBK cells were treated with ethanol vehicle (A), 100 nM $1,25(OH)_2D_3$ (B), PBS/DMSO vehicle (C) or 100 nM TPA (D) for 24 hours. Cells were homogenised and fractionated into cytosol (1), membrane (2) and nucleus (3) as described in 4.3.1. From these fractions PKC was isolated as described in 4.3.2 and separated by 10% SDS-PAGE, transferred to nitrocellulose and immunoblotted with PKC β antibody, antigen-antibody complexes were detected with a biotynylated secondary antibody and streptavidin peroxidase as described in 4.3.3. Immunoblots shown are representative of three experiments which gave similar results.

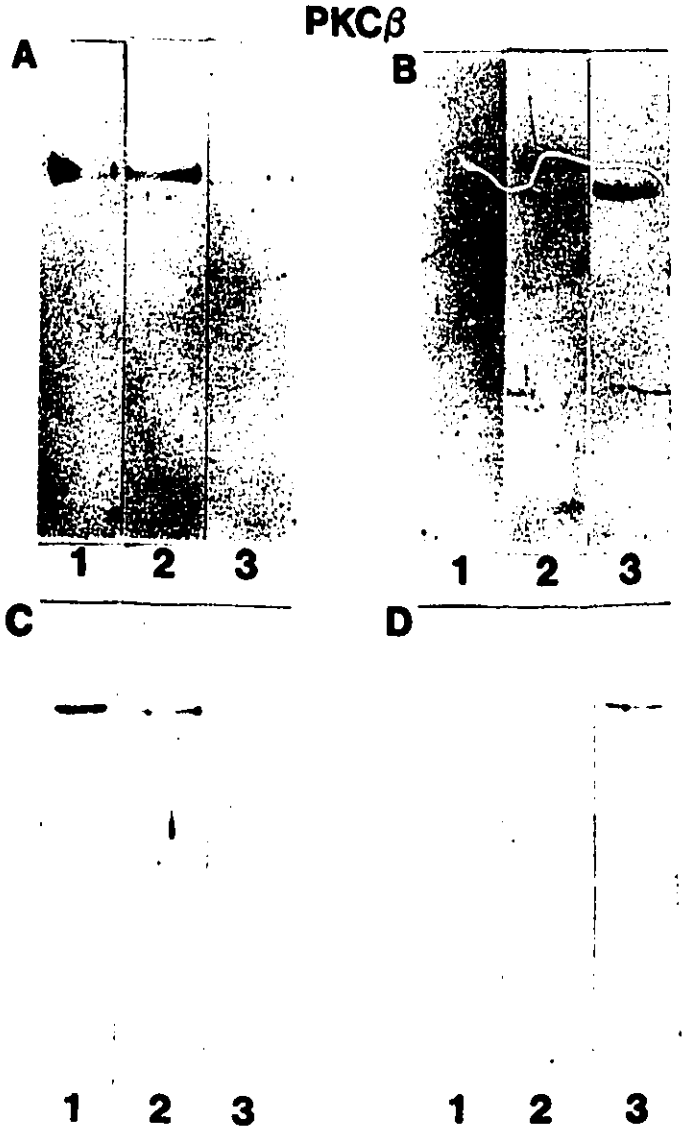
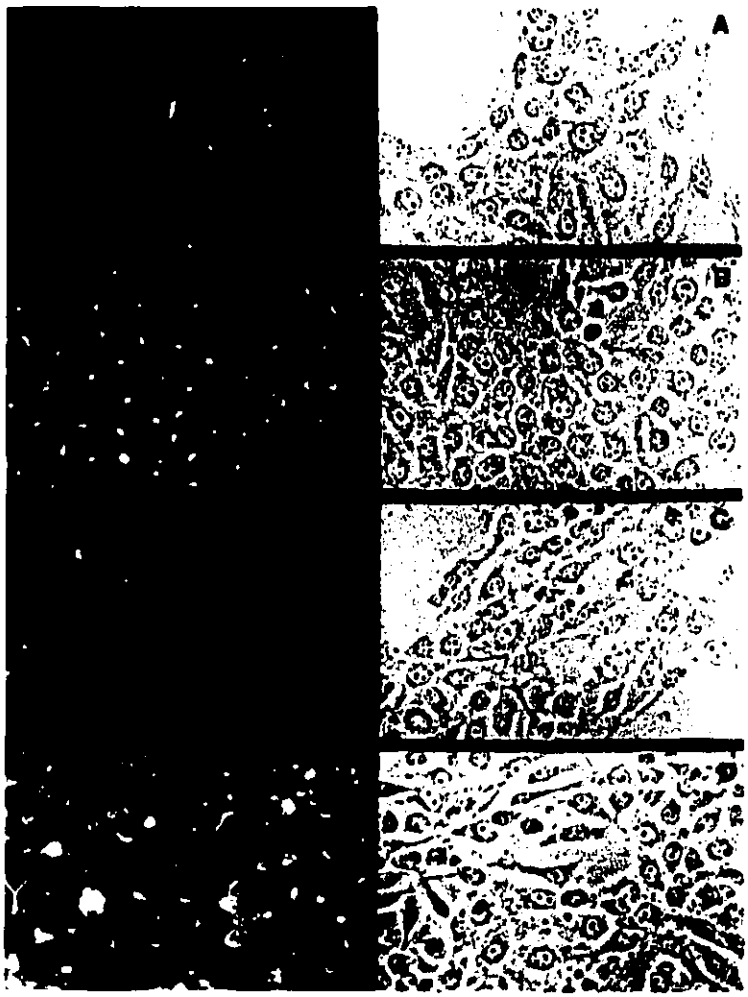

PKCβ
A
B
1 2 3
1 2 3
C
D
1 2 3
1 2 3

Figure 4.6 Effect of 1,25(OH)$_2$D$_3$ and TPA on PKC β Immunofluorescence

Quiescent ML3K cells were treated with ethanol vehicle (A), 100 nM 1,25(OH)$_2$D$_3$ (B), PBS/DMSO vehicle (C) or 100 nM TPA (D) for 24 hours. Cells were fixed and incubated with PKC β antibody, antigen-antibody complexes were detected with a fluorescein-conjugated secondary antibody as described in 4.3.4. Left: Immunofluorescence, right: corresponding phase contrast photograph;immunostaining was performed four times with similar results.


A

antibody identified two proteins (80 kD and 70 kD), highly expressed in cytosol and membrane. The 80 kD protein was predominant in cytosol, whereas the two bands appeared to be equally expressed in membrane. When MDBK cells were treated for 24 hours with 100 nM $1,25(OH)_2D_3$ (Figure 4.7b) there was no detectable difference in the amount or subcellular distribution of PKC ζ compared to vehicle controls (Figure 4.7a). TPA treatment decreased PKC ζ in all fractions (Figure 4.7d) compared to vehicle controls (Figure 4.7c). The reduction in cytosolic PKC ζ immunoreactivity in TPA treated cells was associated with the appearance of a low molecular weight band (approximately 30 kD), suggesting degradation of the 70 and/or 80 kD proteins.

In immunofluorescence, no differences in PKC ζ localization were observed in $1,25(OH)_2D_3$ treated cells (Figure 4.8b), compared to vehicle controls (Figure 4.8a). Down-regulation of PKC ζ in response to TPA treatment (Figure 4.8d) was readily apparent when compared to vehicle controls (Figure 4.8c).

4.4.6 Effect of $1,25(OH)_2D_3$ and TPA on Nuclear Protein Kinase C Activity

To determine whether translocation of PKC β to the nuclear fraction in response to $1,25(OH)_2D_3$ or TPA was associated with changes in nuclear PKC activity,

Figure 4.7 Effect of $1,25(OH)_2D_3$ and TPA on Subcellular Fractionation of PKC ζ

Quiescent MDBK cells were treated with ethanol vehicle (A), 100 nM $1,25(OH)_2D_3$ (B), PBS/DMSO vehicle (C) or 100 nM TPA (D) for 24 hours. Cells were homogenised and fractionated into cytosol (1), membrane (2) and nucleus (3) as described in 4.3.1. From these fractions PKC was isolated as described in 4.3.2 and separated by 10% SDS-PAGE, transferred to nitrocellulose and immunoblotted with PKC ζ antibody, antigen-antibody complexes were detected with a biotynylated secondary antibody and streptavidin peroxidase as described in 4.3.3. Immunoblots shown are representative of three experiments which gave similar results.

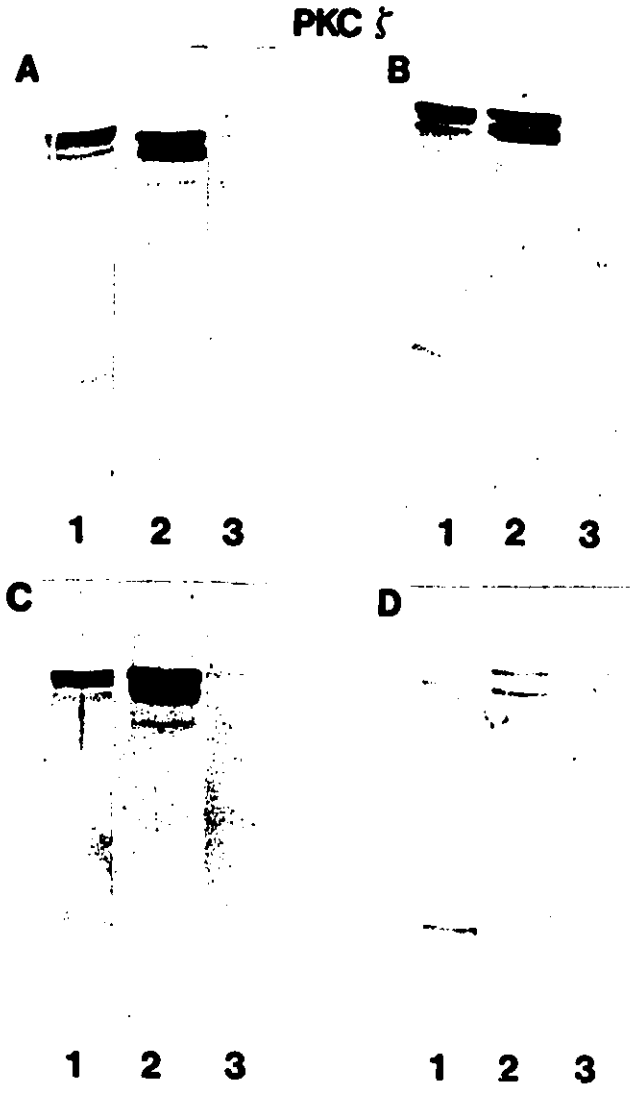
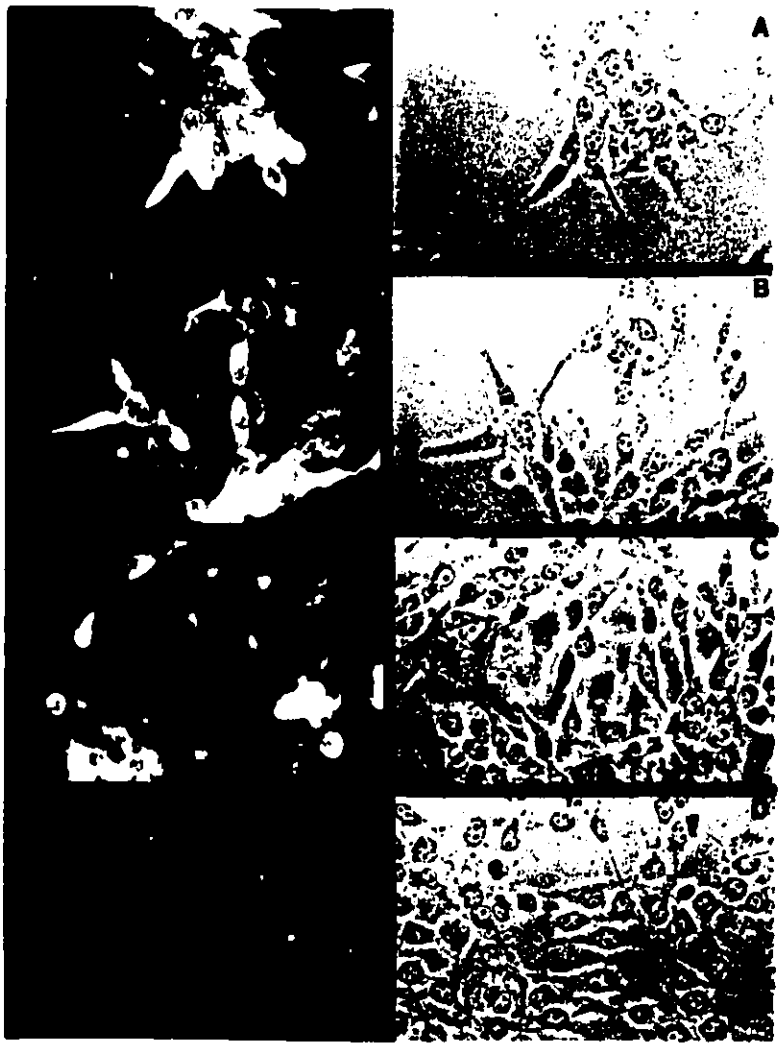

PKC ζ
A
B
1 2 3
1 2 3
C
D
1 2 3
1 2 3

Figure 4.8 Effect of 1,25(OH)$_2$D$_3$ and TPA on PKC ζ Immunofluorescence

Quiescent MDBK cells were treated with ethanol vehicle (A), 100 nM 1,25(OH)$_2$D$_3$ (B), PBS/DMSO vehicle (C) or 100 nM TPA (D) for 24 hours. Cells were fixed and incubated with PKC ζ antibody, antigen-antibody complexes were detected with a fluorescein-conjugated secondary antibody as described in 4.3.4. Left: Immunofluorescence, right: corresponding phase contrast photograph; immunostaining was performed four times with similar results.


A
B
C
D

calcium/phospholipid dependent phosphorylation was measured in nuclear fractions derived from cells treated for 24 hours with 100 nM $1,25(OH)_2D_3$, 100 nM TPA or vehicle. Phosphorylation of both a synthetic peptide substrate and endogenous nuclear proteins was assessed. Calcium/phospholipid dependent phosphorylation of the synthetic peptide substrate was detected in nuclear fractions of MDBK cells (Figure 4.9), but phosphorylation of this substrate was not altered in cells treated with either $1,25(OH)_2D_3$ or TPA. In contrast, calcium/phospholipid dependent phosphorylation of endogenous nuclear proteins was increased in cells treated with 100 nM $1,25(OH)_2D_3$ for 24 hours, compared to vehicle controls (Figure 4.10). The increase in calcium/phospholipid dependent phosphorylation of endogenous nuclear proteins in $1,25(OH)_2D3$ treated cells was partially inhibited by pre-incubation with the PKC β antibody (0.22 pmol/mg/min with PKC β antibody vs. 0.60 pmol/mg/min without PKC β antibody). There was no difference in calcium/phospholipid dependent phosphorylation of endogenous nuclear proteins in cells treated with 100 nM TPA for 24 hours compared to controls.

Figure 4.9 Effect of $1,25(OH)_2D_3$ and TPA on Nuclear Phosphorylation of a PKC-Specific Peptide Substrate

Quiescent MDBK cells were treated with ethanol vehicle, 100 nM $1,25(OH)_2D_3$, PBS/DMSO vehicle or 100 nM TPA for 24 hours. Cells were homogenised and a nuclear fraction, obtained as described in 4.3.1, was assayed for calcium/phospholipid dependent phosphorylation of a synthetic peptide substrate as described in 4.3.5. Results are expressed as pmol phosphate incorporated/mg nuclear protein/min and are mean +/- SEM of triplicate determinations.

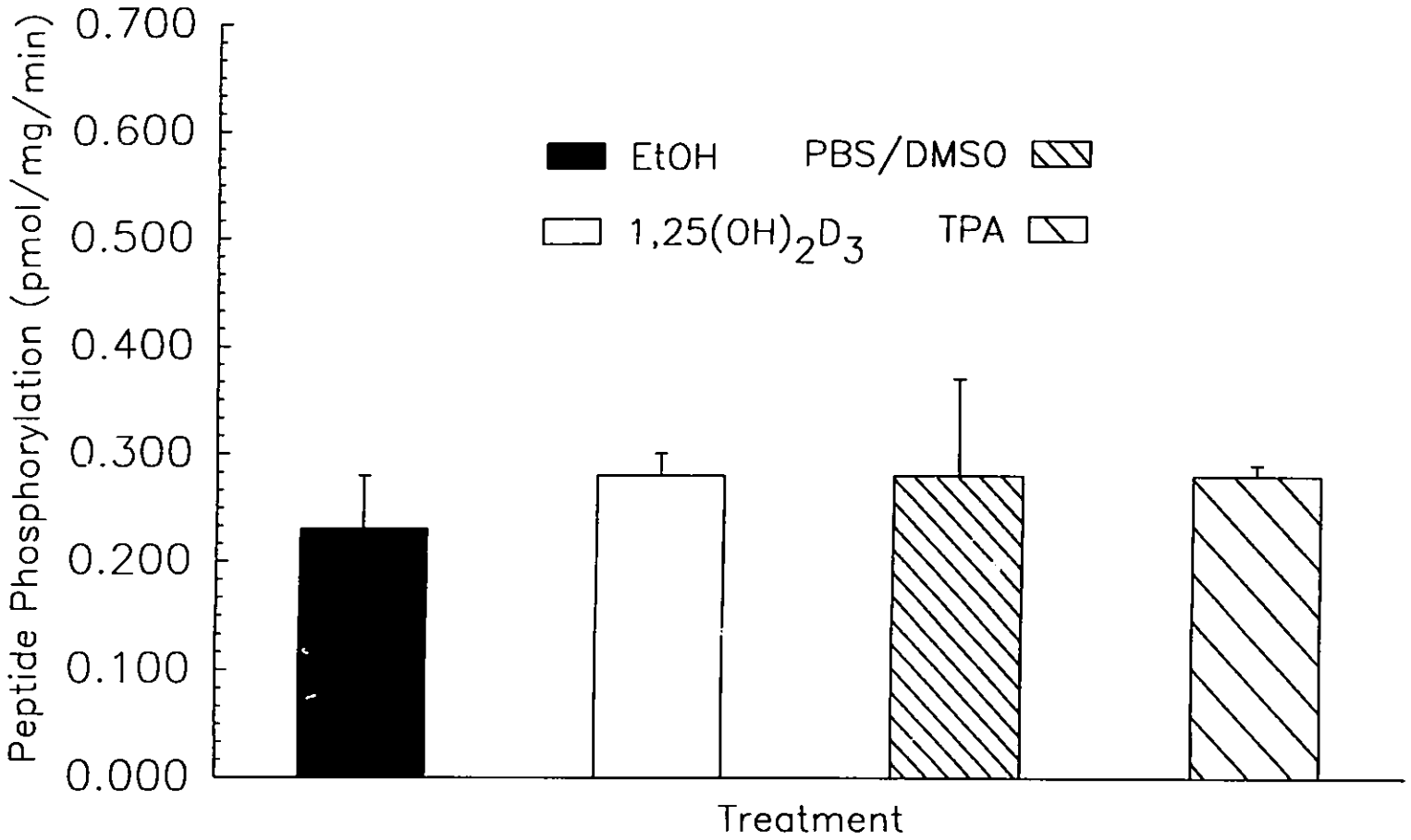
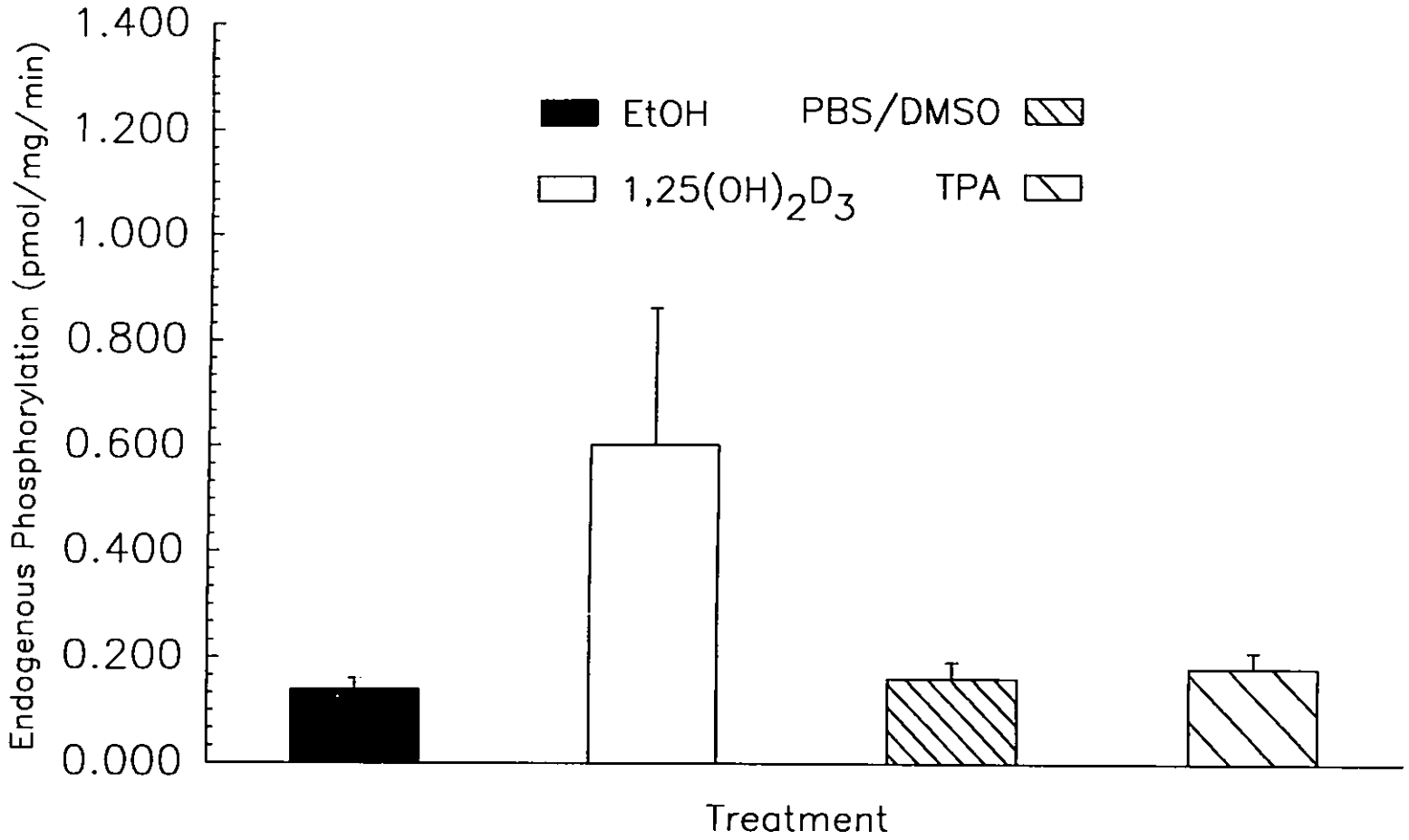

Peptide Phosphorylation (pmol/mg/min)
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
EtOH
PBS/DMSO
1,25(OH)2D3
TPA
Treatment

Figure 4.10 Effect of $1,25(OH)_2D_3$ and TPA on Calcium/Phospholipid-dependent Phosphorylation of Endogenous Nuclear Proteins

Quiescent MDBK cells were treated with ethanol vehicle, 100 nM $1,25(OH)_2D_3$, PBS/DMSO vehicle or 100 nM TPA for 24 hours. Cells were homogenised and a nuclear fraction, obtained as described in 4.3.1, was assayed for calcium/phospholipid dependent phosphorylation of endogenous nuclear proteins as described in 4.3.5. Results are expressed as pmol phosphate incorporated/mg nuclear protein/min and are mean +/- SEM of triplicate determinations.


1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Endogenous Phosphorylation (pmol/mg/min)
EtOH
PBS/DMSO
1,25(OH)2D3
TPA
Treatment

4.5 Discussion

In this chapter I extend my previous observation that $1,25(OH)_2D_3$ increases PKC in MDBK cells (Chapter Two), by characterizing the subcellular localization and isozyme specificity of this response. As well, I compare the effects of $1,25(OH)_2D_3$ to those of TPA, since these agents exert divergent effects on the expression of the VDR and CaBP D-28K in MDBK cells (Chapter Three). The data in this chapter clearly indicate that the divergent effects of $1,25(OH)_2D_3$ and TPA on these vitamin D-related functions in kidney cells may result from differential activation and translocation of PKC α, PKC β and/or PKC ζ.

4.5.1 Expression of Protein Kinase C Isozymes in MDBK Cells and Antibody Specificity

To identify subcellular localization of specific PKC isozymes and their selective modulation by $1,25(OH)_2D_3$ and TPA in MDBK cells, I used isozyme-specific antibodies for immunofluorescence and immunoblotting. Both of these techniques indicate that MDBK cells express PKC α, PKC β and PKC ζ, in agreement with molecular analyses showing that these three isozymes are expressed in whole rat kidney (Kosaka et al., 1988). The specificity of these antibodies in whole cell extracts was confirmed for all three antibodies, since

immunoreactivity was completely abolished by inclusion of the appropriate antigenic peptide (Figure 4.1).

4.5.2 Characterization of Subcellular Fractions

The purity of subcellular fractions was assessed by measurement of G6PD activity as a cytosolic marker, forskolin stimulated adenylate cyclase activity as a plasma membrane marker and lamin B immunoreactivity as a nuclear marker (Figure 4.2). Using these three criteria, I conclude that each of the three fractions is devoid of detectable contamination from the other two.

4.5.3 Effect of $1,25(OH)_2D_3$ and TPA on Subcellular Distribution of Protein Kinase C β

Treatment of MDBK cells with 100 nM $1,25(OH)_2D_3$ for 24 hours results in complete translocation of PKC β from cytosol and membrane fractions to nucleus. On immunoblots, PKC β immunoreactivity is detected in nuclear fractions derived from $1,25(OH)_2D_3$ treated cells, but not in nuclear fractions derived from vehicle control cells (Figure 4.5a,b). In immunofluorescence studies, $1,25(OH)_2D_3$ treatment enhances the perinuclear localization of PKC β (Figure 4.6a,b). Consistent with a translocation event, $1,25(OH)_2D_3$ abolishes PKC β immunoreactivity in cytosolic and membrane fractions.

$1,25(OH)_2D_3$ treatment also enhances calcium/phospholipid-dependent phosphorylation of endogenous nuclear proteins in MDBK cells (Figure 4.10) but not calcium/phospholipid-dependent phosphorylation of a synthetic peptide substrate (Figure 4.9). This data is consistent with that of Hocevar and Fields (1991) who showed that nuclear PKC activator specificity is substrate dependent. In MDBK cells, enhanced phosphorylation of endogenous nuclear proteins is partially blunted by pre-incubation of nuclear preparations with PKC β antibody. This data is the first demonstration that $1,25(OH)_2D_3$ induces nuclear translocation of any PKC isozyme, which I believe is of critical importance in view of the nuclear mechanism of action of this steroid hormone.

Although the significance of nuclear translocation of PKC β in response to $1,25(OH)_2D_3$ is unknown at present, my work complements that of Martell et al (1992), who reported that $1,25(OH)_2D_3$ modulates the phosphorylation state of nuclear proteins in HL-60 cells. The identification of PKC β as the isozyme which is translocated to the nuclear fraction in response to $1,25(OH)_2D_3$ is intriguing in view of reports (referred to in Chapter Three) that the VDR, a nuclear receptor, is phosphorylated upon $1,25(OH)_2D_3$ binding (Pike and Sleator, 1985; Brown and DeLuca, 1990) predominantly on serine residues (Haussler et al., 1988b) and that this phosphorylation can be catalyzed by PKC β *in vitro* (Hseih et al., 1991). As discussed in Chapter Three the functional

significance of VDR phosphorylation is unclear, but it has been suggested that phosphorylation may be related tc transactivation or receptor turnover (Haussler et al., 1988c; Hseih et al., 1991; Pike, 1991). I have previously observed that $1,25(OH)_2D_3$ treatment increases, whereas TPA treatment decreases, VDR number in MDBK cells (Chapter Three). In this respect, although both $1,25(OH)_2D_3$ and TPA induce nuclear translocation of PKC β (Figure 4.5, 4.6), only $1,25(OH)_2D_3$ treated cells exhibit increased calcium/phospholipid-dependent phosphorylation of endogenous nuclear proteins (Figure 4.10). This apparent discrepancy may result from differential effects of TPA and $1,25(OH)_2D_3$ on β_I and β_{II} subtypes, which were not discriminated in this study, since Hocevar and Fields (1991) reported that TPA induces translocation of PKC β_{II} but not PKC β_I to the nuclear membrane of HL-60 cells. My studies also do not distinguish to which nuclear site PKC β is translocated by $1,25(OH)_2D_3$ and TPA treatment. Alternatively, $1,25(OH)_2D_3$ and TPA might differentially modulate the nuclear substrate(s) for PKC β. For example, if the VDR is a substrate for nuclear PKC β, phosphorylation patterns would predictably be different in $1,25(OH)_2D_3$ and TPA treated cells due to differential effects of these agents on VDR number (Chapter Three). Comparison of the kinetics of $1,25(OH)_2D_3$ induced changes in PKC β subcellular localization and activity with VDR expression and phosphorylation will be necessary to distinguish between these possibilities.

4.5.4 Effect of $1,25(OH)_2D_3$ and TPA on Subcellular Distribution of Protein Kinase C α

The effects of $1,25(OH)_2D_3$ and TPA on subcellular distribution of PKC α in MDBK cells differs substantially from their effects on PKC β. $1,25(OH)_2D_3$ increases membrane associated PKC α, which was most evident by immunofluorescence, whereas TPA down-regulates PKC α throughout the cell (Figure 4.3 and 4.4). PKC α is not detectable in nuclear fractions of either control cells or in cells treated with $1,25(OH)_2D_3$ or TPA. The differential regulation of PKC α by $1,25(OH)_2D_3$ and TPA offers an explanation for the previous observation that $1,25(OH)_2D_3$ increases, whereas TPA decreases, membrane associated PKC activity (Chapter Two) and CaBP D-28K expression in MDBK cells (Chapter Three). CaBP D-28K may be a substrate for PKC α, since inspection of its primary sequence indicates two consensus sites for PKC. Although CaBP D-28K is predominantly a cytosolic protein its localization to tubulin-associated vesicular organelles has been reported in chick brain, intestine and kidney (Nemere et al., 1992). Thus CaBP D-28K may be in close enough proximity to membrane associated PKC α to be an effective substrate. If so, phosphorylation might affect CaBP D-28K turnover, such that down-regulation of PKC α in TPA treated cells might enhance CaBP D-28K degradation whereas enhanced PKC α in $1,25(OH)_2D_3$ treated cells might

stabilize the protein. Alternatively one can not rule out the possibility that modulation of CaBP D-28K by $1,25(OH)_2D_3$ and TPA is secondary to effects on PKC β or the VDR discussed previously. Further studies are necessary to probe the relative importance of PKC α and PKC β in the regulation of CaBP D-28K and to discern possible mechanisms.

The effect of TPA on PKC α may also explain the morphological changes induced by TPA in MDBK cells (Chapter One). It has been reported that PKC α is associated with Triton X-100 insoluble material (Kiley and Jaken, 1990b). If PKC α directly interacts with cytoskeletal proteins, TPA mediated down-regulation of PKC α could alter the phosphorylation state of cytoskeletal proteins, affecting their stability and/or conformation and resulting in gross morphological changes (Kiley et al., 1992). Further studies to probe the association of PKC α with cytoskeletal proteins in MDBK cells and effects of TPA will be necessary to test this possibility.

4.5.5 Effect of $1,25(OH)_2D_3$ and TPA on Subcellular Distribution of Protein Kinase C ζ

PKC ζ, a calcium independent isozyme (Ono et al., 1989b; Ways et al., 1992), has been purified from bovine kidney (Nakanishi and Exton, 1992) and is highly expressed in MDBK cells (Figure 4.7). This cell line is the first to be used

successfully in immunofluorescent studies with the PKC ζ antibody (Figure 4.8). In MDBK cells, $1,25(OH)_2D_3$ has no effect on PKC ζ, whereas TPA down-regulates PKC ζ, although not as completely as PKC α (Figure 4.7 and 4.8). These data are in contrast to reports that TPA neither activates nor down-regulates PKC ζ and that purified PKC ζ does not bind phorbol esters (Ono et al., 1988; Ono et al., 1989b). The partial down-regulation of PKC ζ by TPA in MDBK cells, thus, may be an indirect effect of TPA. For example in murine lung cells, PKC ζ is decreased by long term treatment with TPA, reportedly due to an indirect effect of TPA to increase calpain levels (Dwyer, Miller and Malkinson, 1992). If so this might explain the apparent increase in a small molecular weight band that reacts with PKC ζ antibody in immunoblots of cytosolic fractions derived from TPA treated MDBK cells in comparison to vehicle treated control cells (Figure 4.7).

4.5.6 Possible Mechanism of Action

In contrast to rapid, transient PKC translocation seen in response to agents which interact with membrane receptors to induce phospholipid hydrolysis, modulation of PKC isozymes is observed after 24 hours treatment with $1,25(OH)_2D_3$. The persistent activation of PKC by $1,25(OH)_2D_3$ is likely due to the fact that this steroid hormone interacts with an intracellular receptor and modulates gene expression (Pike,

1991). Our findings imply that PKC activation by $1,25(OH)_2D_3$ in MDBK cells results from translocation events in contrast to the transcriptional regulation of PKC gene expression by $1,25(OH)_2D_3$ reported for HL-60 cells (Obeid et al., 1990; Solomon et al., 1991). However in MDBK cells $1,25(OH)_2D_3$ could regulate expression of proteins which in turn modulate membrane association or activity of PKC, for example, by modulating DAG or intracellular calcium concentration. In this respect, $1,25(OH)_2D_3$ increases intracellular calcium, an effect dependent on protein synthesis, in several cell types (Tornquist and Tashjian, 1989; Caffrey and Farach-Carson, 1989). An effect of $1,25(OH)_2D_3$ mediated via intracellular calcium might explain why PKC ζ, which lacks the calcium binding domain and therefore represents a calcium-independent isozyme (Ways et al., 1992; Nakanishi and Exton, 1992), is unaffected by $1,25(OH)_2D_3$ treatment. The underlying basis for the selective effects of $1,25(OH)_2D_3$ on PKC α and PKC β is unclear, since both isozymes are reportedly translocated to the nucleus under different conditions (Hocevar and Fields, 1991; Berry et al., 1989; Leach et al., 1989; Buchner et al., 1992). Similar to HL-60 cells (Hocevar and Fields, 1991) TPA induces nuclear translocation of PKC β, but not PKC α, in MDBK cells. The presence of a bipartite nuclear targeting motif in PKC β (Malviya and Block, 1992), offers some explanation for these observations. It has recently been suggested that truncation of PKC α may also expose similar nuclear targeting

sequences (Eldar, Ben-Chaim and Livneh, 1992), however such an effect was not evident in TPA treated HL-60 or MDBK cells.

In summary, these studies are the first to characterize the effect of $1,25(OH)_2D_3$ on subcellular localization of PKC isozymes. The data indicate that $1,25(OH)_2D_3$ enhances membrane association of PKC α, whereas TPA down-regulates PKC α. Both $1,25(OH)_2D_3$ and TPA translocate PKC β to the nucleus, but only $1,25(OH)_2D_3$ treated cells exhibit enhanced phosphorylation of nuclear proteins. This data offers insight into the disparate effects of $1,25(OH)_2D_3$ and TPA on VDR and CaBP D-28K expression in MDBK cells and provides strong evidence for a role of PKC β in $1,25(OH)_2D_3$-mediated nuclear events.

4.6 Conclusions

The overall conclusion of this chapter is that $1,25(OH)_2D_3$ and TPA have differential effects on the expression, translocation and activation of PKC isozymes in MDBK cells. The specific conclusions are:

4.2.1 MDBK cells express PKC α, β and ζ.

4.2.2 24 hour treatment with $1,25(OH)_2D_3$ increases plasma membrane-association of PKC α, nuclear translocation and activation of PKC β and yet does not affect PKC ζ in MDBK cells.

4.2.3 24 hour treatment with TPA down-regulates PKC α, increases nuclear translocation of PKC β, but does not increase nuclear activity of PKC β and decreases PKC ζ in MDBK cells.

GENERAL CONCLUSION

1,25($(OH)_2D_3$, the active form of vitamin D, is a steroid hormone synthesized in kidney, which primarily modulates extracellular calcium homeostasis (DeLuca, Krisinger and Darwish, 1990). Cumulative studies, including my work (Simboli-Campbell, Franks and Welsh, 1992a; Simboli-Campbell et al., 1992b; Welsh, Weaver and Simboli-Campbell, 1991) and the work of others (Henry and Luntao, 1989; Mandla, Boneh and Tenenhouse, 1990; Weaver, Franks and Welsh, 1992), have implicated the phosphoinositide signalling pathway, specifically PKC, in both synthesis and renal actions of 1,25$(OH)_2D_3$. In this thesis I have studied the interactions between 1,25$(OH)_2D_3$ and PKC in MDBK cells, a distal tubular-like epithelial cell line, since the distal nephron is a major site of calcium reabsorption (DeLuca, Krisinger and Darwish) and because the VDR and CaBP D-28K are localized to distal tubules *in vivo* (Taylor, McIntosh and Bourdeau, 1982; Stumpf et al., 1979).

In Chapter one I establish MDBK cells as a relevant model for the renal distal tubule. I demonstrate that MDBK cells express the VDR, CaBP D-28K and PKC and that all three proteins are up-regulated in proliferating compared to quiescent cells. Although both 1,25$(OH)_2D_3$ and the phorbol ester TPA are potent modulators of proliferation in a variety of cell types (Minghetti and Norman, 1988; Nishizuka, 1989),

quiescent MDBK cells are refractory to the effects of $1,25(OH)_2D_3$ and TPA on cell proliferation. Thus the effects of these agents observed in this thesis are not indirectly related to effects on MDBK cell proliferation. Although TPA does not affect proliferation of quiescent MDBK cells, gross morphological changes similar to those shown in other epithelial cell lines are observed (Parkinson and Emmerson, 1982; Ojakian, 1981). These changes have been reported to be due to TPA-mediated reorganization of the cytoskeleton (Fey and Penman, 1984).

In Chapter two I show that, in MDBK cells, PKC can be activated by long term treatment with $1,25(OH)_2D_3$. This activation is observed both when PKC is extracted from membrane and assayed as phosphorylation of histone and when assayed in its natural membrane-associated state as phosphorylation of PKC-specific substrate. These results are in agreement with previous reports of increases in phorbol ester receptor binding sites and PKC gene expression following long term treatment of HL-60 cells with $1,25(OH)_2D_3$ (Martell, Simpson and Taylor, 1987; Obeid et al., 1990). In MDBK cells, the activation of PKC by $1,25(OH)_2D_3$ appears to result from translocation of kinase activity from the cytosol to the membrane and is accompanied by an increase in PKC immunoreactivity in the membrane. As well the activation of PKC by $1,25(OH)_2D_3$ in MDBK cells, is both time- and dose-dependent, with the maximum effect occuring 24 hours after

treatment with 100 nM $1,25(OH)_2D_3$. In MDBK cells, PKC activity is also increased by short term exposure to TPA and is completely down-regulated by long term treatment with this phorbol ester. This biphasic effect of TPA on PKC is well established (Nishizuka, 1989). The down-regulation of PKC activity in MDBK cells is accompanied by a complete loss of PKC immunoreactivity throughout the cell. This effect is specific for TPA, since long term treatment of MDBK cells with a phorbol ester analogue, 4αPDD (Castagna, 1982), does not affect either PKC activity or immunoreactivity.

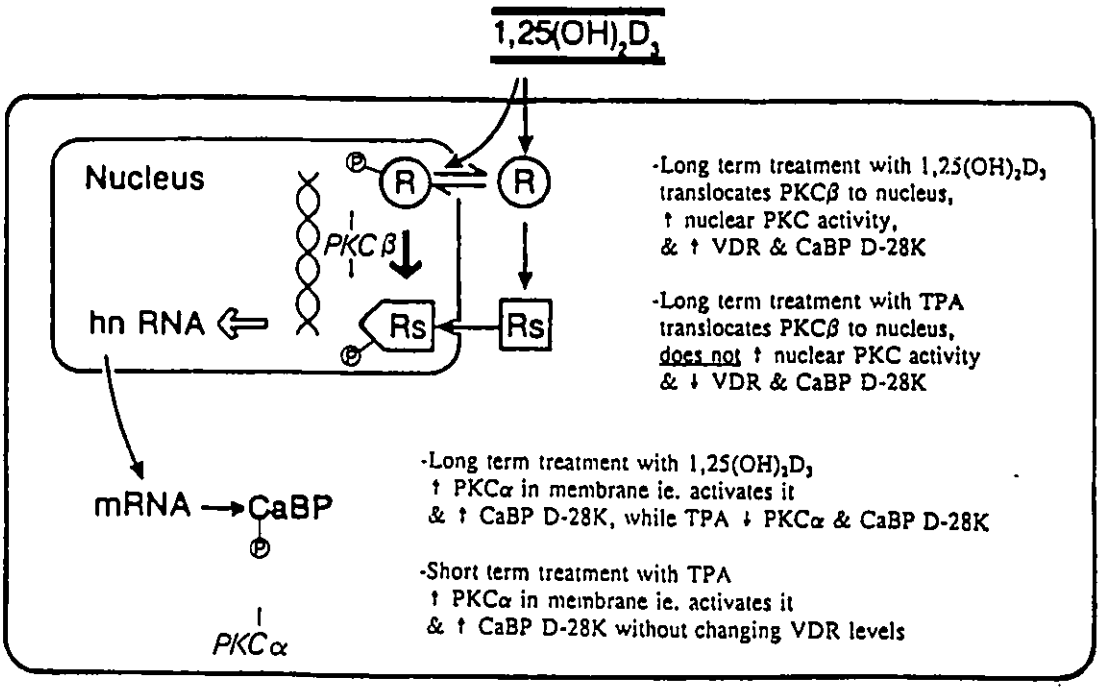
In Chapter Three I report that long term treatment of MDBK cells with $1,25(OH)_2D_3$ and TPA has divergent effects on the VDR and CaBP D-28K. Long term treatment with $1,25(OH)_2D_3$ homologously up-regulates the VDR without affecting its affinity for the ligand, in agreement with reports in LLC-PK_1 renal cells (Costa, Hirst and Feldman, 1985). This homologous up-regulation of the VDR is accompanied by an increase in total immunoreactive CaBP D-28K. Conversely, long term treatment with TPA results in down-regulation of the VDR and CaBP D-28K. These results are in agreement with studies linking VDR occupancy to the regulation of CaBP D-28K expression (Theofan, Nguyen and Norman, 1986). In contrast to TPA, long term treatment with the inactive phorbol ester analogue 4αPDD, does not affect either the VDR or CaBP D-28K. This data, in conjunction with the effects on PKC described previously, suggests a role for PKC in vitamin D-mediated

effects in the kidney, specifically regulation of renal CaBP D-28K by $1,25(OH)_2D_3$. Such a role could involve phosphorylation of the VDR, which has been reported to occur in the intestine prior to the induction of CaBP D-28K (Brown and DeLuca, 1990).

Other results of Chapter Three indicated that the down-regulation of the VDR by TPA is maximal at 24 hours treatment with 100 nM TPA. This decrease does not involve changes in either the affinity of the receptor for the ligand or proteolytic degradation of the receptor. Surprisingly although activation of PKC by short term TPA treatment is followed by an increase in cytosolic CaBP D-28K levels it is not accompanied by an increase in VDR levels. Thus the divergent effects of $1,25(OH)_2D_3$ and TPA on expression of the VDR and CaBP D-28K are not simply general effects on PKC activation/down-regulation.

It is now well established that PKC exists as a family of related kinases which differ in activation requirements, substrate specificity, subcellular localization and sensitivity to TPA induced down-regulation (Nishizuka, 1988; Parker et al., 1989). In whole rat kidney three PKC isozymes have been identified, these include PKC α, PKC β and PKC ζ (Kosaka et al., 1988; Wetsel et al., 1991). In Chapter Four I report that MDBK cells express all three of these known renal PKC isozymes and that these isozymes are differentially regulated by long term treatment with $1,25(OH)_2D_3$ and TPA. To

summarize, $1,25(OH)_2D_3$ translocates PKC β to the nucleus, increases membrane-associated PKC α and has no effect on PKC ζ. In contrast, TPA down-regulates PKC α, decreases PKC ζ and also translocates PKC β to the nucleus. $1,25(OH)_2D_3$, but not TPA, treatment enhances calcium/phospholipid-dependent phosphorylation of endogenous nuclear proteins; I hypothesize this phosphorylation represents an increase in nuclear PKC β activity since the increase in nuclear phosphorylation was blunted by inclusion of PKC β antibody. The differential effects on PKC isozymes, presented in Chapter Four, offer some explanation for the observations made in Chapters One, Two and Three. In the following illustration I propose a model of what I believe is occuring in MDBK cells. PKC β has previously been shown to phosphorylate the VDR *in vitro* (Hseih et al., 1991). The functional significance of this phosphorylation is unknown, but it has been suggested that it could affect induction of transactivation or stabilize the receptor. Either way, the outcome would involve an increase in transcription of the mRNA for vitamin D-regulated genes (ie. CaBP D-28K and possibly VDR as well). This portion of the model would explain our observations that $1,25(OH)_2D_3$ induces nuclear translocation of PKC β as well as increases phosphorylation of endogenous nuclear proteins, homologous up-regulation of the VDR and an increase in CaBP D-28K expression. In contrast although TPA induces a similar translocation of PKC β to the nucleus, this event is not


1,25(OH)2D3
Nucleus
R
R
PKCβ
hn RNA
Rs
Rs
mRNA
CaBP
PKCα
-Long term treatment with 1,25(OH)2D3 translocates PKCβ to nucleus, ↑ nuclear PKC activity, & ↑ VDR & CaBP D-28K
-Long term treatment with TPA translocates PKCβ to nucleus, does not ↑ nuclear PKC activity & ↓ VDR & CaBP D-28K
-Long term treatment with 1,25(OH)2D3 ↑ PKCα in membrane ie. activates it & ↑ CaBP D-28K, while TPA ↓ PKCα & CaBP D-28K
-Short term treatment with TPA ↑ PKCα in membrane ie. activates it & ↑ CaBP D-28K without changing VDR levels

accompanied by an increase in phosphorylation of endogenous nuclear proteins. This effect of TPA on PKC β must be distinct from the effect of $1,25(OH)_2D_3$ on PKC β, since it does not induce activation of PKC β and is accompanied by down-regulation of the VDR and CaBP D-28K. In the other portion of this model, I envision PKC α as phosphorylating CaBP D-28K leading to enhanced stability of the protein and resulting in elevation of cytosolic levels of CaBP D-28K. This portion of the model would explain observations that long term treatment with $1,25(OH)_2D_3$ enhances membrane association of PKC α and increases CaBP D-28K, while long term treatment with TPA down-regulates PKC α and reduces CaBP D-28K. Direct phosphorylation of CaBP D-28K by PKC α might also explain why short term TPA treatment increases cytosolic CaBP D-28K without increasing the VDR. Since TPA treatment eventually down-regulates PKC α, short term treatment must activate this isozyme; the process of activation sets PKC up for subsequent down-regulation. This model could also explain the morphological changes induced in MDBK cells by long term treatment with TPA. Cytoskeletal proteins have been suggested to be targets for the action of PKC α (Kiley et al., 1992), constitutive phosphorylation of these proteins by PKC α may stabilize a specific organization and down-regulation of PKC α by TPA could result in gross morphological changes due to cytoskeleton reorganization.

To summarize, during the course of these studies, I have

observed that $1,25(OH)_2D_3$ and TPA elicit divergent effects on expression of the VDR and CaBP D-28K in MDBK cells. These effects appear to be related to differential regulation of subcellular localization and activity of specific PKC isozymes. A model incorporating these findings implicates PKC α and PKC β as important modulators of $1,25(OH)_2D_3$ effects in renal distal tubules.

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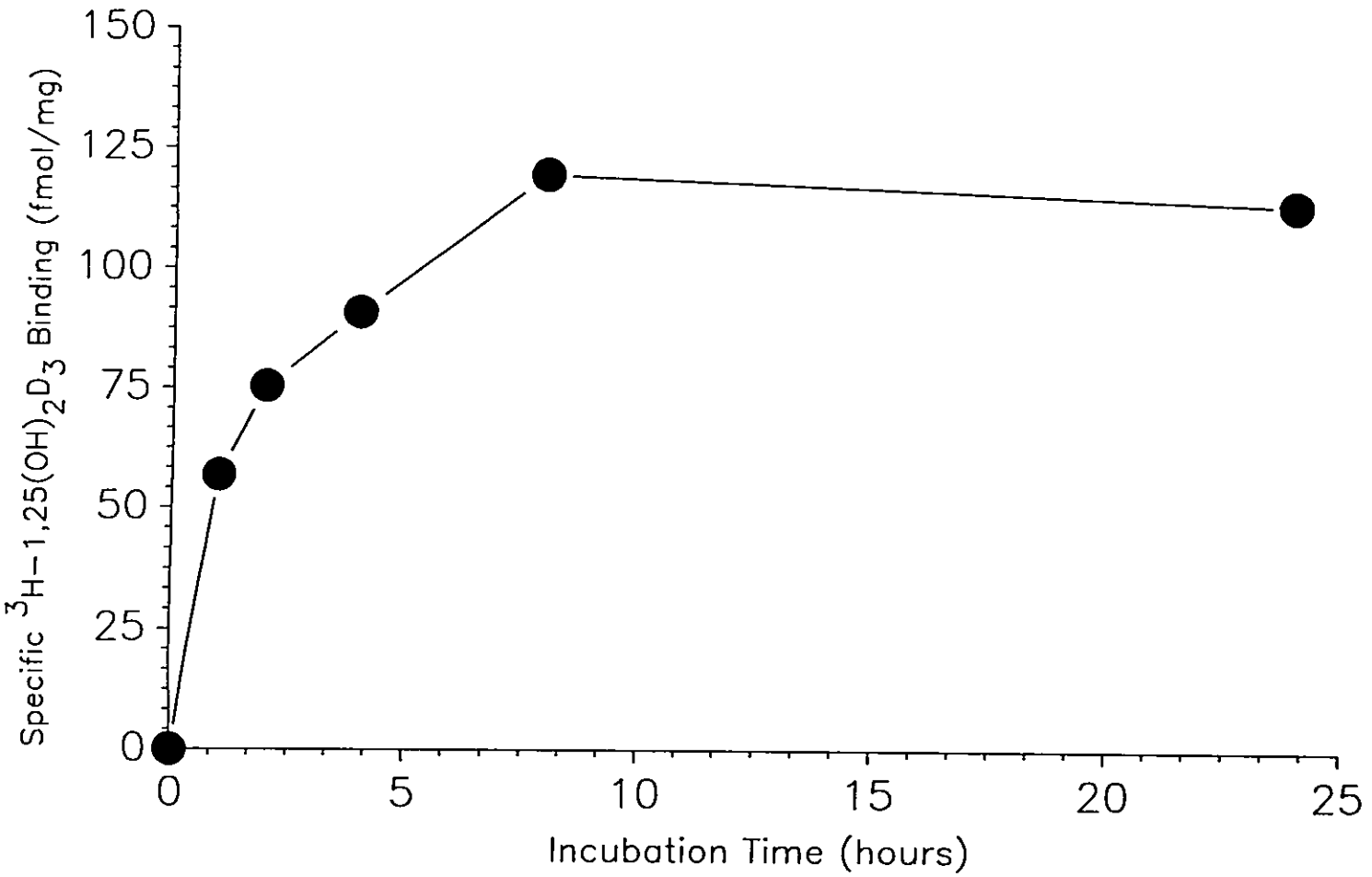
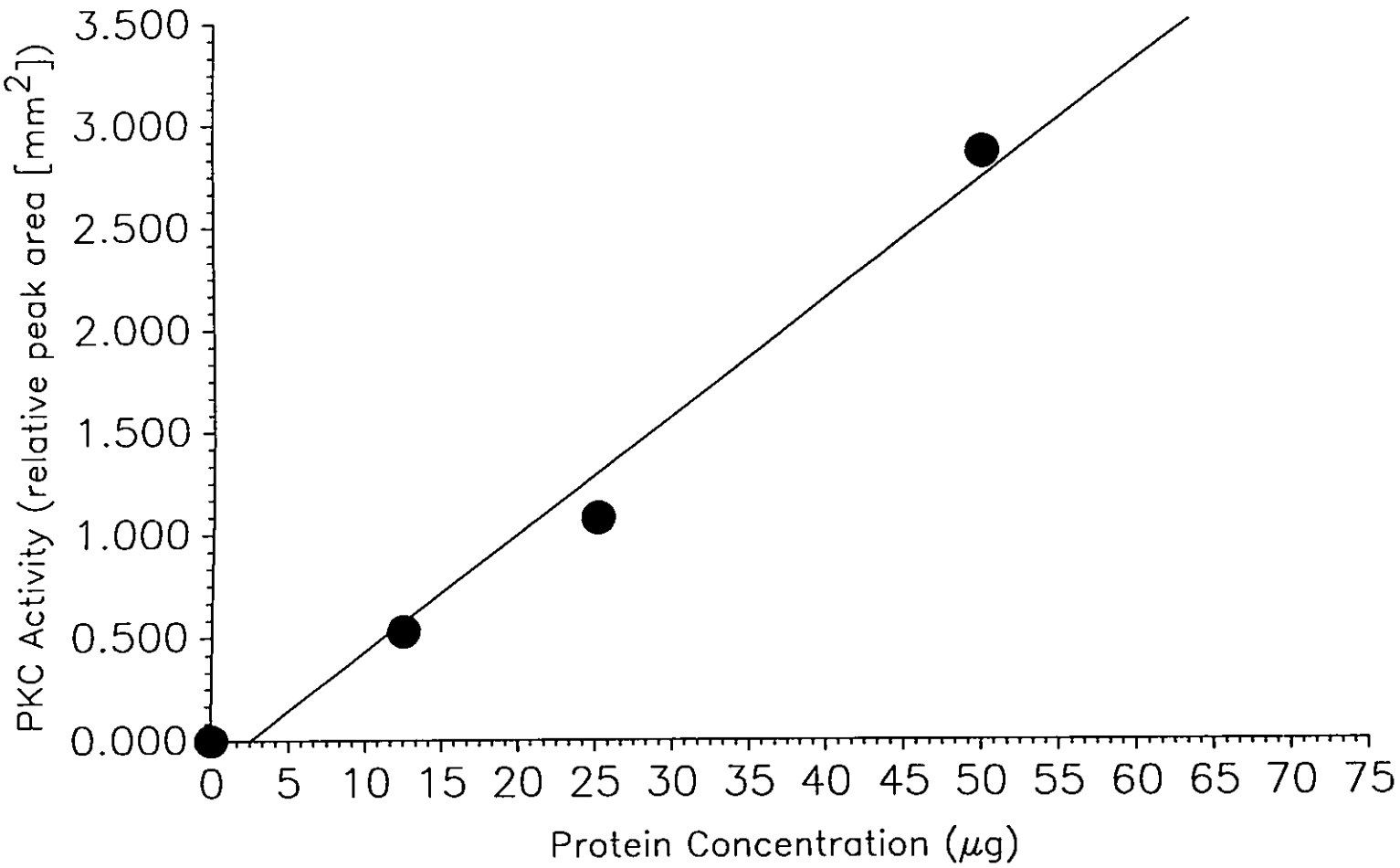
Appendix 1 Time Course Curve of Specific ^{3}H-1,25(OH)$_2$D$_3$ Binding in MDBK Cells

Chromatin extracts (1 mg protein/ml) were prepared and incubated with 0.5 nM ^{3}H-1,25(OH)$_2$D$_3$ in the presence or absence of a 250-fold molar excess of unlabelled 1,25(OH)$_2$D$_3$ as described in 1.3.2. This was performed to find an optimal incubation time for measuring specific ^{3}H-1,25(OH)$_2$D$_3$ binding in MDBK cells.


Specific $^{3}H-1,25(OH)_2D_3$ Binding (fmol/mg)
150
125
100
75
50
25
0
0
5
10
15
20
25
Incubation Time (hours)

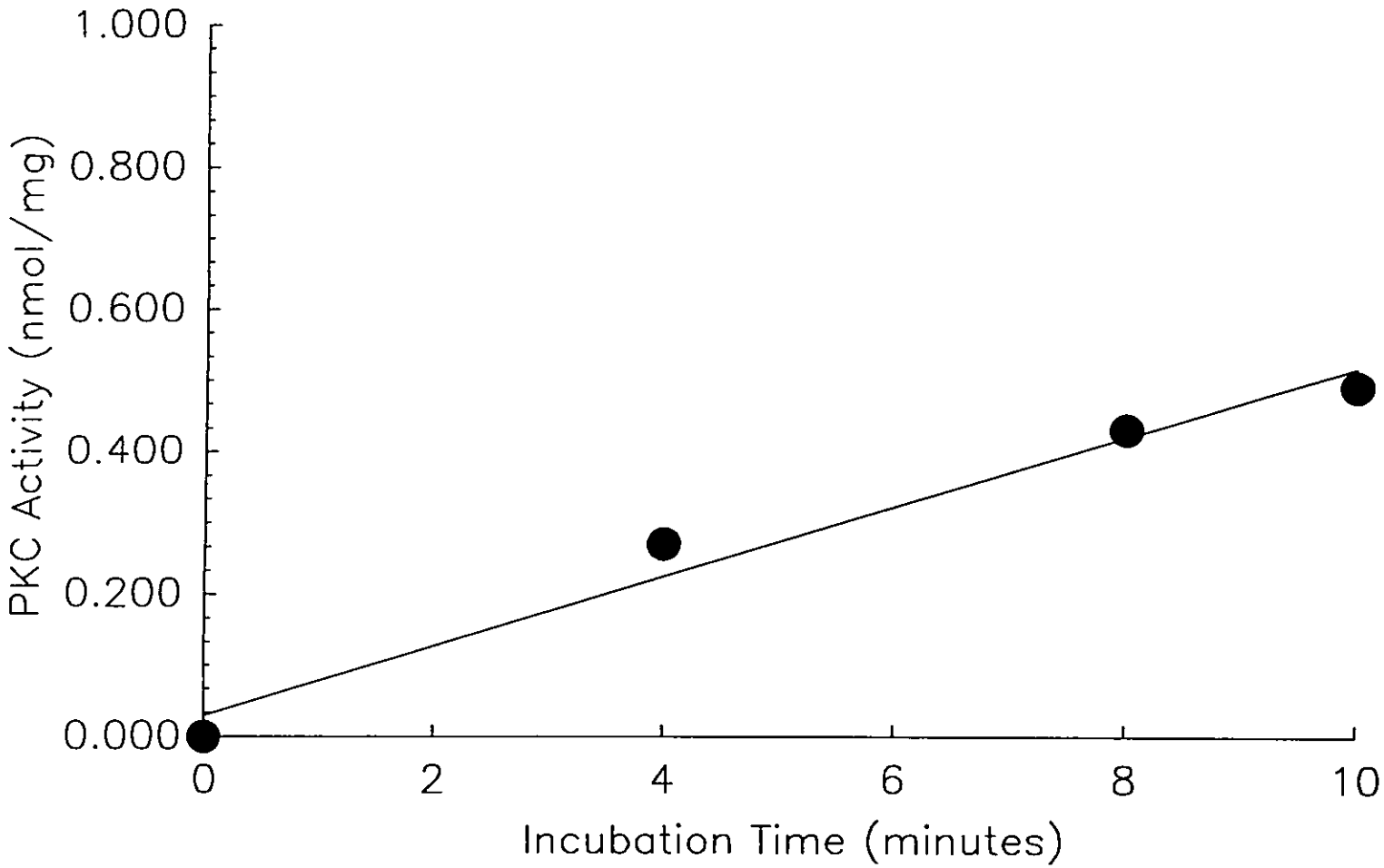
Appendix 2 Protein Concentration Curve of Phosphorylation of Protein Kinase C-Specific 85 kD Substrate by MDBK Cellular Membranes

PKC Activity was measured directly in its native membrane-associated state by incubating crude membrane fractions with [γ-^{32}P]-ATP and the PKC-specific 85 kD substrate as described in 1.3.4. Data was obtained by densitometric scanning of the autoradiograph. This was performed to determine the protein concentrations of the post nuclear fraction which would give a linear rate of phosphorylation of the PKC-specific 85 kD substrate by membrane-associated PKC derived from MDBK cells.


PKC Activity (relative peak area [mm^2])
3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75
Protein Concentration (μg)

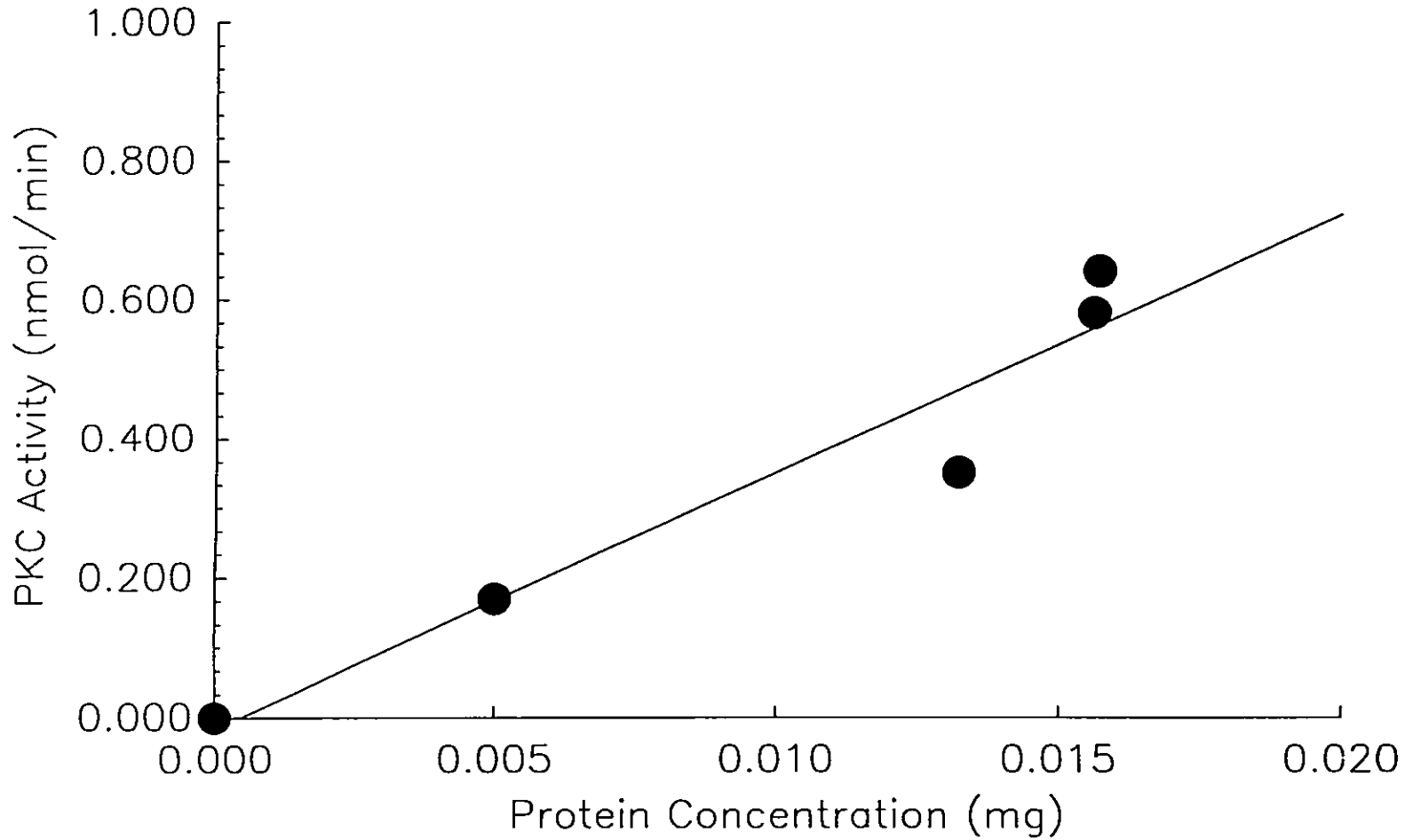
Appendix 3 Time Course Curve of Protein Kinase C Activity in MDBK Cytosolic Fractions

PKC was partially purified from cytosolic fractions. PKC activity was assayed by phosphorylation of histone substrate as described in 2.3.1.2. Data are expressed as nmol ^{32}P incorporated into histone per mg protein. This was performed to determine incubation times which would give a linear rate of phosphorylation of histone substrate by cytosolic PKC isolated from MDBK cells.


1.000
0.800
0.600
0.400
0.200
0.000
PKC Activity (nmol/mg)
0
2
4
6
8
10
Incubation Time (minutes)

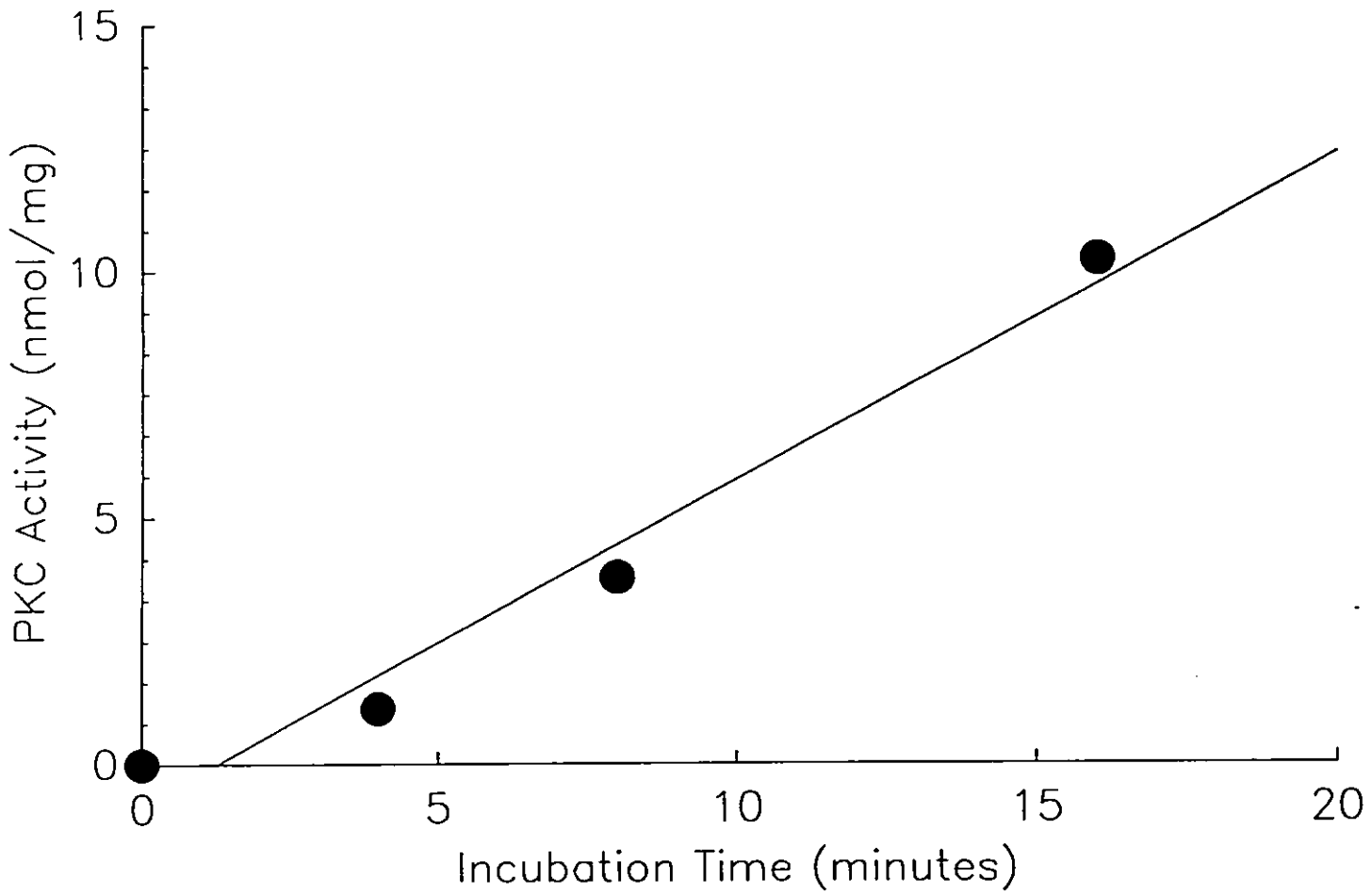
Appendix 4 Protein Concentration Curve of Protein Kinase C Activity in MDBK Cytosolic Fractions

PKC was partially purified from cytosolic fractions. PKC activity was assayed by phosphorylation of histone substrate as described in 2.3.1.2. Data are expressed as nmol ^{32}P incorporated into histone per min. This was performed to determine the protein concentrations which would give a linear rate of phosphorylation of histone substrate by cytosolic PKC isolated from MDBK cells.


1.000
0.800
0.600
0.400
0.200
0.000
PKC Activity (nmol/min)
0.000
0.005
0.010
0.015
0.020
Protein Concentration (mg)

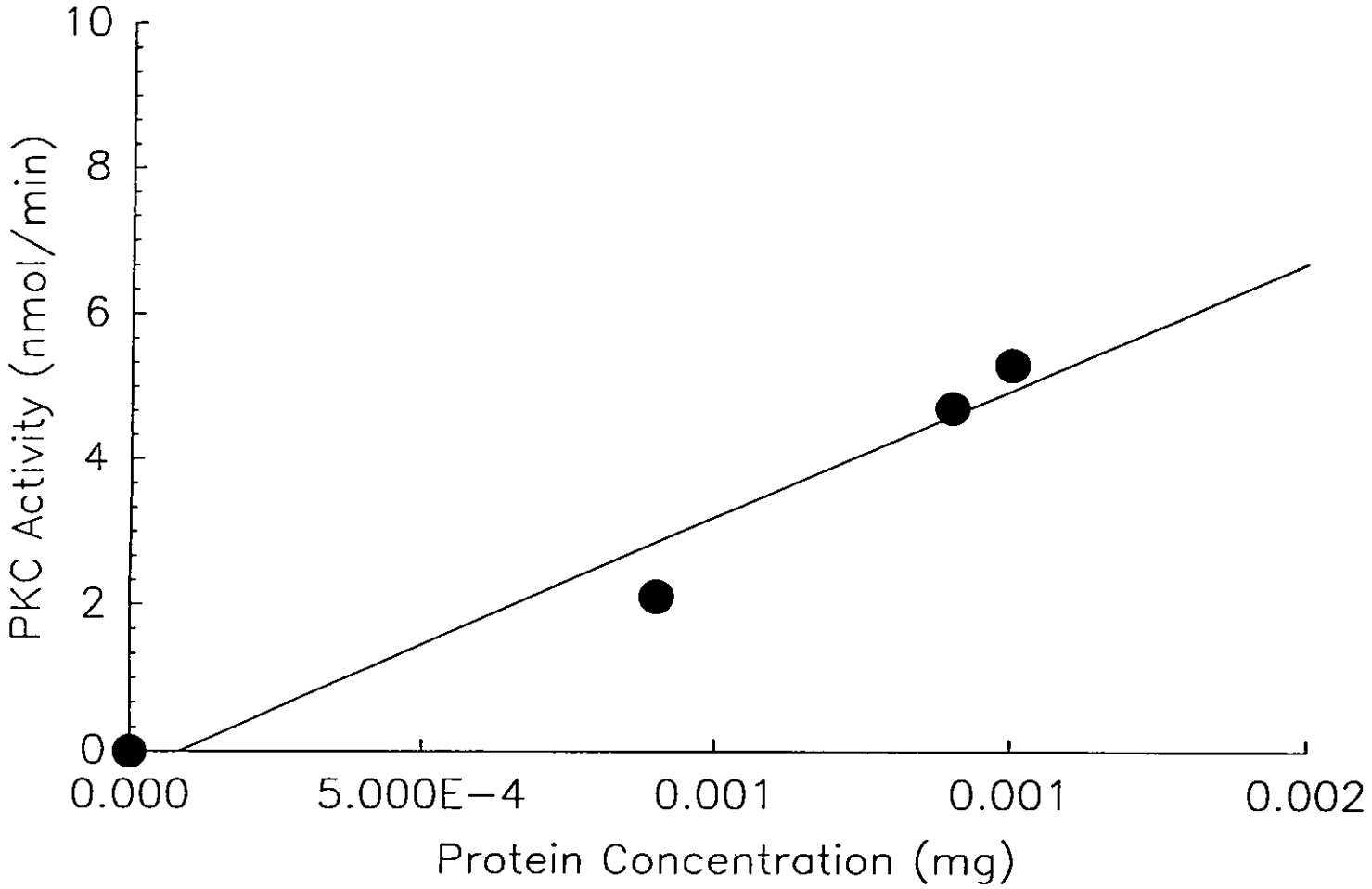
Appendix 5 Time Course Curve of Protein Kinase C Activity in MDBK Solubilized Membrane Fractions

PKC was detergent extracted and partially purified from solubilized membrane fractions. PKC activity was assayed by phosphorylation of histone substrate as described in 2.3.1.2. Data are expressed as nmol ^{32}P incorporated into histone per mg protein. This was performed to determine incubation times which would give a linear rate of phosphorylation of histone substrate by membrane PKC isolated from MDBK cells.


15
10
5
0
PKC Activity (nmol/mg)
0
5
10
15
20
Incubation Time (minutes)

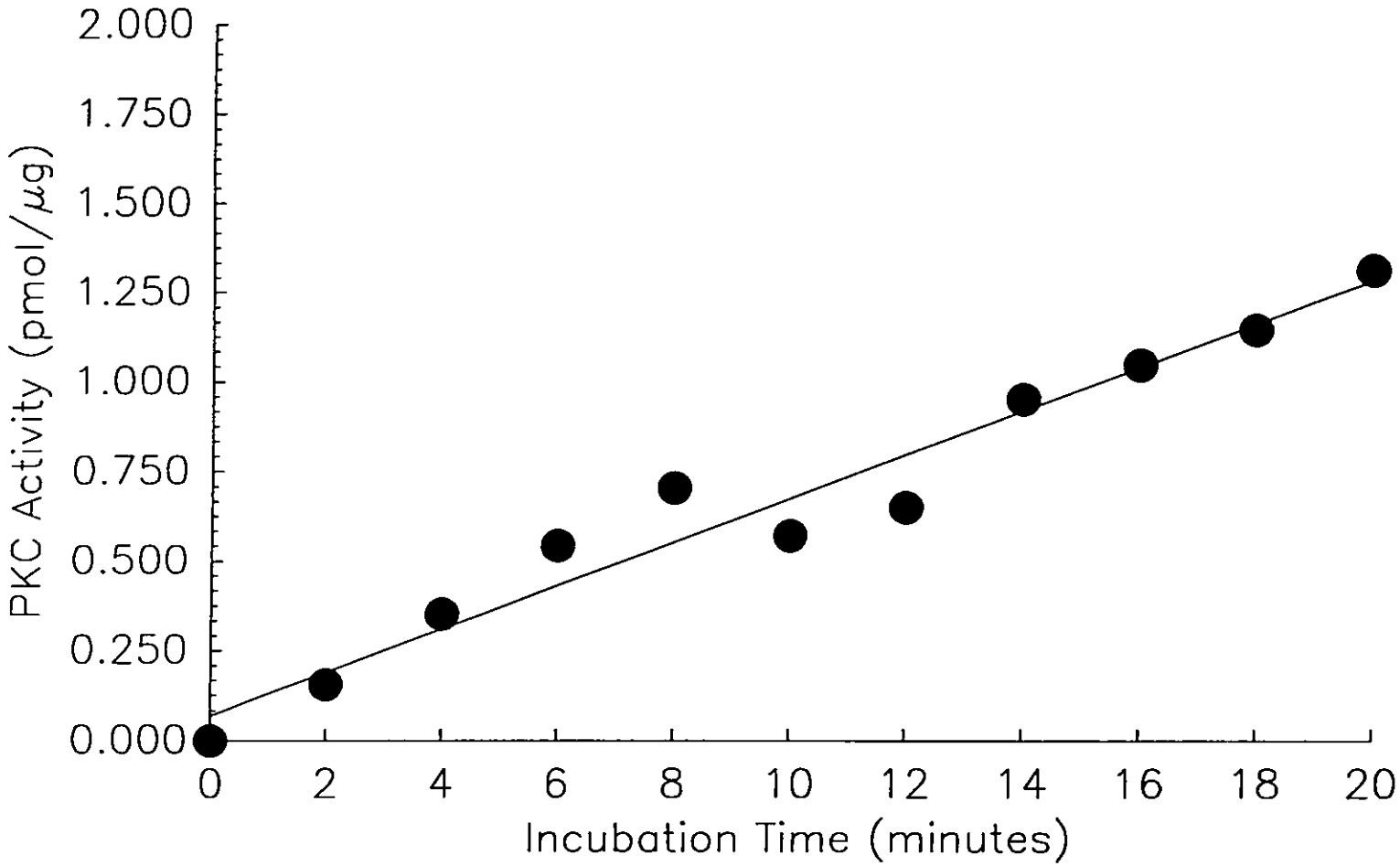
Appendix 6 Protein Concentration Curve of Protein Kinase C Activity in MDBK Solubilized Membrane Fractions

PKC was detergent extracted and partially purified from solubilized membrane fractions. PKC activity was assayed by phosphorylation of histone substrate as described in 2.3.1.2. Data are expressed as nmol ^{32}P incorporated into histone per min. This was performed to determine the protein concentrations which would give a linear rate of phosphorylation of histone substrate by membrane PKC isolated from MDBK cells.


10
8
6
4
2
0
PKC Activity (nmol/min)
0.000
5.000E−4
0.001
0.001
0.002
Protein Concentration (mg)

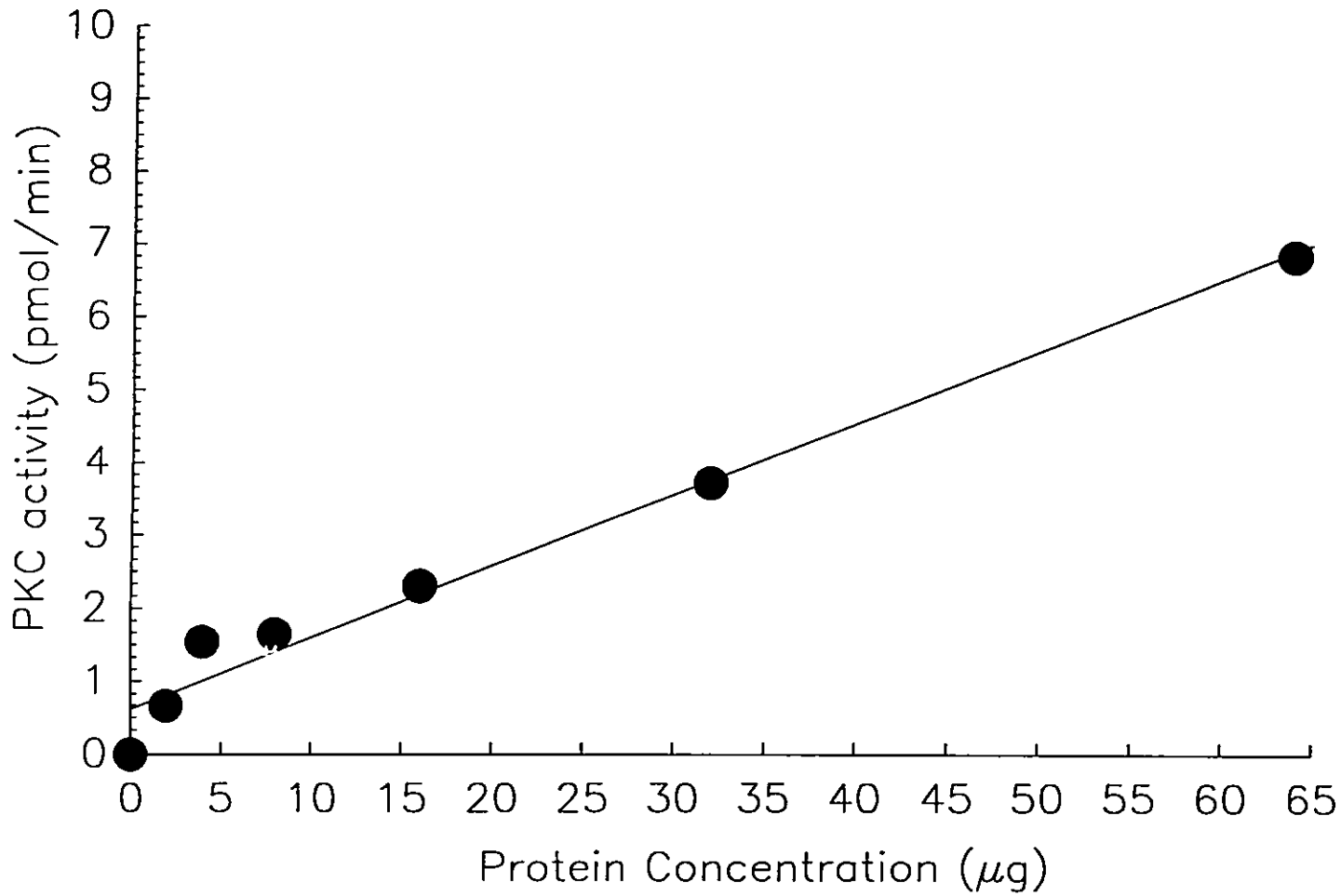
Appendix 7 Time Course Curve of Nuclear Phosphorylation of a PKC-Specific Peptide Substrate

Cells were homogenized and a nuclear fraction, obtained as described in 4.3.1, was assayed for calcium/phospholipid-dependent phosphorylation of a synthetic peptide as described in 4.3.5. Results are expressed as pmol phosphate incorporated/ μg nuclear protein. This was performed to determine incubation times which would give a linear rate of phosphorylation of PKC-specific peptide substrate by nuclear PKC isolated from MDBK cells.


2.000
1.750
1.500
1.250
1.000
0.750
0.500
0.250
0.000
PKC Activity (pmol/μg)
0
2
4
6
8
10
12
14
16
18
20
Incubation Time (minutes)

Appendix 8 Protein Concentration Curve of Nuclear Phosphorylation of a PKC-Specific Peptide Substrate

Cells were homogenized and a nuclear fraction, obtained as described in 4.3.1, was assayed for calcium/phospholipid-dependent phosphorylation of a synthetic peptide as described in 4.3.5. Results are expressed as pmol phosphate incorporated/ min. This was performed to determine the protein concentrations which would give a linear rate of phosphorylation of PKC-specific peptide substrate by nuclear PKC isolated from MDBK cells.


PKC activity (pmol/min)
0
1
2
3
4
5
6
7
8
9
10
0
5
10
15
20
25
30
35
40
45
50
55
60
65
Protein Concentration (μg)

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THESES AND PUBLICATIONS

Simboli, M. **Factors Affecting the Aromatic Hydroxylation of Estradiol.** BSc Thesis, Queens University (1984)

Simboli, M. **The Effect of Dietary Phosphate Deprivation on Vitamin D Metabolism.** MSc Thesis, Queens University (1988)

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Simboli-Campbell, M., Franks, D.J. and Welsh, JE. **$1,25(OH)_2D_3$ Increases Protein Kinase C Activity in the Membrane of Madin Darby Bovine Kidney Cells.** Presented at the Sixth Int. Symposium on Cellular Endocrinology, Lake Placid, NY, USA, August, 1990

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Simboli-Campbell, M. and Franks, D.J. **Subcellular Redistribution of Protein Kinase C Isozymes in Response to Phorbol Esters and Vitamin D.** Presented at the thirty-second Annual Meeting of the American Society for Cell Biology, Denver, CO, USA, November, 1992

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