

**Regulation of calbindin D-28K by $1,25(\text{OH})_2\text{D}_3$
in MDBK cells**

by
AnneMarie Gagnon

Thesis submitted to the Department of Biochemistry
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

University of Ottawa
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DEDICATION

Cette thèse est dédiée

*à la mémoire de ma mère, Renée Saint-Germain Gagnon,
pour m'avoir donné curiosité et persévérance*

à ma famille pour leur support

*and to Paul
whose love and understanding made it all possible.*

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ABSTRACT

1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), the hormonally active form of vitamin D, mediates its effects, at least partially, via binding to its nuclear receptor, the vitamin D receptor (VDR), which then interacts with vitamin D-response elements in the promoter region of vitamin D-regulated genes. Our studies have focused on the regulation of calbindin D-28K, a vitamin D-dependent calcium-binding protein, in Madin-Darby bovine kidney (MDBK) cells. MDBK cells are renal epithelial cells which display distal tubular characteristics including the expression of calbindin D-28K and VDR. Consistent with data derived in primary cultures and *in vivo* models, we have characterized the dose- and time-dependence of calbindin D-28K regulation by 1,25(OH)₂D₃, offering the first established *in vitro* model system for studying the molecular regulation of calbindin D-28K.

Our data also emphasize the role of post-transcriptional mechanisms of regulation in calbindin D-28K induction by 1,25(OH)₂D₃. The observation that protein kinase C (PKC) activators and inhibitors can modulate the expression of vitamin D-dependent proteins implicates this signalling pathway in 1,25(OH)₂D₃ regulation. We have investigated the potential role of PKC in the regulation of calbindin D-28K in MDBK cells. Time course analysis with 1,25(OH)₂D₃ and TPA, a well-characterized PKC modulator, suggests a temporal correlation between PKC activity and calbindin D-28K expression in MDBK cells. More precisely, both long term treatment with 1,25(OH)₂D₃ and short term treatment with TPA induce PKC activity, PKC α immunoreactivity and calbindin D-28K expression. Long term treatment with TPA which down-regulates PKC activity and expression also causes a decrease in calbindin D-28K levels. The observation that phosphatase inhibitors blunt the down-regulating effect of TPA on calbindin D-28K expression further suggests a role for phosphorylation in this regulation.

Amino acid sequence analysis of calbindin D-28K reveals the presence of five casein kinase II (CKII) and two PKC phosphorylation sites. *In vitro* phosphorylation assays demonstrate that PKC, and more precisely PKC α , phosphorylates calbindin D-28K in a calcium- and phospholipid-dependent manner. In agreement with the amino acid sequence, the phosphorylated form of calbindin D-28K is detected with an anti-phosphothreonine antibody. CKII does not phosphorylate calbindin D-28K under our experimental conditions. Immunoprecipitation studies of radiolabeled MDBK cells further support the phosphorylation of calbindin D-28K. Both TPA and 1,25(OH)₂D₃ treatments enhance the phosphorylation state of a 28 kDa protein specifically immunoprecipitated in the presence of calbindin D-28K antibodies.

These data have been compiled into a hypothetical model involving the phosphorylation of calbindin D-28K as a 1,25(OH)₂D₃ regulatory step. Our results strongly implicate the PKC signalling pathway in calbindin D-28K regulation.

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LIST OF ABBREVIATIONS

ATCC	american type culture collection
ATP	adenosine 5'-triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
cAMP	cyclic adenosine monophosphate
Ca ²⁺	calcium (ion)
cDNA	complementary DNA
CKII	casein kinase II
CO ₂	carbon dioxide
Cyclohex	cycloheximide
DAG	diacylglycerol
DBP	vitamin D-binding protein
dCTP	2'-deoxycytidine 5'-triphosphate
DNA	deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DR3	direct repeats separated by three nucleotides
DR4	direct repeats separated by four nucleotides
DTT	dithiothreitol
EDAC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol-bis(β-aminoethyl ether)
ELISA	enzyme-linked immunoabsorbent assay
EtOH	ethanol
FITC	fluorescein
GTP	guanosine 5'-triphosphate
³ H	tritium
HCl	hydrochloric acid
H ₂ O ₂	hydrogen peroxide
IgG	immunoglobulin
IP ₃	inositol tri-phosphate
ITS	insulin, transferrin, selenous acid (serum-substitute)
KCl	potassium chloride
K _d	dissociation constant
kDa	kilodalton
LLC-PK ₁ cells	pig kidney cells
MARCKS	myristolated alanine-rich C kinase substrate
MDBK cells	Madin-Darby Bovine Kidney cells
MDCK cells	Madin-Darby Canine Kidney cells
MgCl ₂	magnesium chloride

mRNA	messenger ribonucleic acid
Na ⁺	sodium
NaCl	sodium chloride
NaF	sodium fluoride
NaHCO ₃	sodium bicarbonate
NaOH	sodium hydroxide
NBT	nitro blue tetrazolium
OK cells	opossum kidney cells
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCA	perchloric acid
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PMSF	phenylmethyl sulfonyl fluoride
RNA	ribonucleic acid
RD α	regulatory domain of PKC α
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
TBS	Tris-buffered saline
TCA	trichloroacetic acid
TPA	12-O-tetradecanoyl phorbol-13-acetate
TRE	TPA-response element
Tris	tris[hydroxymethyl]aminomethane
VDR	vitamin D receptor
VDRE	vitamin D response element
1,25(OH) ₂ D ₃	1,25-dihydroxyvitamin D ₃
25(OH)D ₃	25-hydroxyvitamin D ₃
4 α -PDD	4 α -phorbol-12,13-didecanoate

LITERATURE REVIEW

In the early 1900s, the childhood bone disease rickets was shown to be cured by administration of cod liver oil, from which an anti-rachitic activity was isolated (McCollum, Simmonds, Becker and Shipley, 1922). This activity was termed vitamin D₃. Concomitant studies revealed that rickets could also be cured by exposure to sunlight (Huldshinsky, 1919; Chick, Palzell and Hume, 1922). These observations led to the discovery that vitamin D₃ is produced in the skin by the action of ultra-violet light on 7-dehydrocholesterol (Windaus, Schenck and von Weder, 1936; Windaus and Bock, 1937; Esvelt, Schnoes and DeLuca, 1978). The seco-steroid previtamin D₃ is the initial product of photolysis and results from the rupture of the 9-10 carbon bond of 7-dehydrocholesterol; previtamin D₃ then isomerizes to vitamin D₃ (Velluz, Amiard and Petit, 1949; Holick and Clark, 1978). 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) has since been identified as the biologically active hormonal form of vitamin D₃. Activation occurs through sequential hydroxylations catalyzed by specific mixed function oxygenases (DeLuca, 1974). The first hydroxylation takes place in the liver, where the 25-hydroxylase catalyzes the formation of 25-hydroxyvitamin D₃, the predominant vitamin D₃ metabolite in the circulation (Ponchon, DeLuca and Suda, 1970). The second hydroxylation occurs in the proximal tubules of the kidney, where the 1 α -hydroxylase is localized (Fraser and Kodicek, 1970). This final activation step is tightly regulated by serum calcium levels (Wong, Norman, Reddy and Coburn, 1972; Omdahl, Gray, Boyle, Knutson and DeLuca, 1972), parathyroid hormone (Garabedian, Holick, DeLuca, and Boyle, 1972), and

1,25(OH)₂D₃ itself (Tanaka, Lorenc and DeLuca, 1975). 1,25(OH)₂D₃ can be further hydroxylated at the 24-position, and this reaction is thought to be the first step in the catabolism of the hormone (DeLuca, Krisinger, and Darwish, 1990).

1,25(OH)₂D₃ is now recognized as a member of the steroid/thyroid hormone family and has been further assigned to a subgroup of hormones including thyroid hormone and retinoic acid (Forman and Samuels, 1990). The classical function of 1,25(OH)₂D₃ is to promote bone formation by maintaining adequate levels of calcium and phosphorus in plasma. The hormone acts on three main target tissues: the bone, the intestine, and the kidney. 1,25(OH)₂D₃ makes bone calcium available to support plasma calcium concentration by promoting the formation of bone resorbing osteoclasts (Suda, Shinki, and Takahashi, 1990). 1,25(OH)₂D₃ also stimulates intestinal absorption of calcium from the diet and renal reabsorption of calcium and phosphorus. These effects of the hormone are thought to be mediated, at least partially, through the activation or induction of calcium transport proteins and induction of specific calcium-binding proteins, including the calbindins (Schachter, Kowarski, Finkelstein and Wang, 1966; Wasserman and Taylor, 1966).

1,25(OH)₂D₃ mediates most of its effects via a mechanism similar to that of other steroid hormones. In the plasma, vitamin D metabolites circulate bound to a vitamin D-binding protein (DBP), a member of the albumin multigene family (McLeod and Cooke, 1989). 1,25(OH)₂D₃ is taken up by target tissues by a mechanism that is still poorly understood. In the cell, 1,25(OH)₂D₃ binds to its nuclear receptor, the vitamin D receptor (VDR) which then interacts with specific vitamin D-response elements (VDREs) located in the promoter region of vitamin D-regulated genes. The binding of the hormone-receptor

complex to the VDRE is thought to modulate the transcription rate of the target gene (Norman, Roth, and Orci, 1982; Minghetti and Norman, 1988).

The VDR, at 48-60 kDa, is one of the smallest members of the steroid/nuclear receptor superfamily. The unoccupied VDR is thought to be mainly nuclear, although controversial reports of cytosolic localization exist (Barsony, Pike, DeLuca and Marx, 1990). The VDR consists of three characteristic functional domains. The DNA-binding domain is the most conserved region amongst the steroid/nuclear receptor family (Evans, 1988). It consists of 68 highly conserved amino acids forming a common DNA binding structure, the two zinc-fingers (Freedman, Luisi, Korszun, Basavappa, Sigler, and Yamamoto, 1988). Discrimination between specific nucleotide binding sites is determined by the three amino acids located at the base of the first zinc finger (Umesono and Evans, 1989). Although more centrally located in other members of this family of receptors, the DNA-binding domain lies close to the N-terminus of the VDR, resulting in a very short N-terminal domain. The N-terminal domain of the glucocorticoid, estrogen and progesterone receptors have been involved in the transcriptional activity of the receptors, but deletion of the VDR N-terminal region does not affect transcriptional induction by $1,25(\text{OH})_2\text{D}_3$ (Sone, Kerner and Pike, 1991). The third domain is located at the C-terminal and has been assigned two functions: ligand-binding and dimerization. The VDR is characterized for its low capacity, high affinity binding site for $1,25(\text{OH})_2\text{D}_3$ ($K_D = 10^{-10}$ - 10^{-11}M) and must accommodate the distinct mobile A-ring of the hormone (Minghetti and Norman, 1988). Receptor dimerization is required for the high affinity binding of the VDR to the VDRE and for transcriptional activation. Both homodimers and heterodimers with retinoic X receptor and other members of the

steroid/nuclear receptor family have been shown to confer vitamin D-responsiveness through VDREs (Greer, 1993; Rosen, Beninghof, and Koenig, 1993; Yu, Delsert, Andersen, Holloway, Devary, Näär, Kim, Boutin, Glass, and Ronsenfeld, 1991). Most of the VDREs identified to date consist of two direct repeats of the characteristic hexanucleotide steroid response element half-site separated by three nucleotides (DR3) (Lian and Stein, 1992), but other structurally distinct VDREs may also confer $1,25(\text{OH})_2\text{D}_3$ -responsiveness (Carlberg, Bendik, Wyss, Mcir, Sturzenbecker, Grippo and Hunziker, 1993).

Recently, the localization of the VDR in non-classical target tissues, such as the brain, skin, and pancreas, has prompted researchers to expand the role of $1,25(\text{OH})_2\text{D}_3$ in biological systems (Eisman, Martin, MacIntyre, Frampton, Moscley and Whitehead, 1980). The demonstration that $1,25(\text{OH})_2\text{D}_3$ could induce the differentiation of promyelocytes to monocytes (Tanaka, Abe, Miyaura, Kuribayashi, Konno, Nishii, and Suda, 1982) first suggested the potential role of this hormone in the regulation of cell differentiation and development. Since these initial studies, other groups have shown the cellular growth regulating ability of $1,25(\text{OH})_2\text{D}_3$ in various systems including cancer cell lines (Frampton, Omond, and Eisman, 1983), intestinal cells (Suda et al, 1990), and keratinocytes (Hosomi, Hosoi, Abe, Suda, and Kuroki, 1983). It has been suggested that the growth regulating function of $1,25(\text{OH})_2\text{D}_3$ is mediated, at least partially, through down-regulation of the steady-state levels of *c-myc* mRNA (Reitsma, Rothberg, Astrin, Trial, Bar-Shavit, Hall, Teitelbaum and Kahn, 1983). The expression of *c-myc*, a proto-oncogene, has been linked to cellular proliferation in various cell lines (Kelly, Cochran, Stiles and Leder, 1983; Armelin, Armelin, Kelly, Stewart, Leder, Cochran and Stiles, 1984). The regulation of *c-myc* by

1,25(OH)₂D₃ has been correlated to activation and induction of protein kinase C (PKC), an enzyme associated with cell growth and differentiation (Martell, Simpson and Taylor, 1987; Simpson, Hsu, Wendt and Taylor, 1989).

One important contribution to the vitamin D₃ research field was the discovery of calbindin D-28K by Wasserman and Taylor in 1966. Calbindin D-28K was first described as a calcium-binding activity induced in the avian intestine after 1,25(OH)₂D₃ injection. Since its discovery, calbindin D-28K has been localized in various tissues, including intestine, kidney, pancreas and skin (Christakos, Gabriellides and Rhoten, 1989). Calbindin D-28K can also be found in amphibian, reptilian, avian and mammalian brain, suggesting that it evolved primarily as a neuronal protein. Calbindin D-28K is highly conserved through evolution. At the protein sequence level, mammalian calbindin D-28K proteins are 98% homologous, and share 79% homology with the avian protein (Goodman, 1980; Parmentier, Lawson, and Vassart, 1987a). The high degree of conservation through evolution observed in the primary structure of calbindin D-28K indicates a restriction in the freedom for mutation, and suggests a primordial role for this calcium-binding protein.

Structurally, calbindin D-28K consists of a single polypeptide chain of 261 amino acids, including six consensus helix-loop-helix domains (Fullmer and Wasserman, 1987). These domains, termed EF-hands, are responsible for the calcium-binding ability of calbindin D-28K (Tufty and Kretsinger, 1975). EF-hands are the characteristic feature of a family of calcium-binding proteins which include calmodulin, parvalbumin, troponin C, calretinin, and calbindin D-9K, another vitamin D-dependent calcium-binding protein (van Eldik, Zendegui, Marshak, and Watterson, 1982). Only four of the six potential EF-hands found in calbindin

D-28K contain the number and positioning of oxygen containing amino acids required for high affinity calcium binding (Hunziker, 1986; Hunziker and Schriekel, 1988). Consistent with this observation, calbindin D-28K has been shown to bind up to four calcium ions with an affinity of 10^{-6} - 10^{-7} M (Pansini and Christakos, 1984).

The calcium-binding affinity of calbindin D-28K suggests a role in calcium-buffering or calcium transport. Unfortunately, only scarce functional studies have been performed to determine the function of calbindin D-28K. Studies using neuronal tissues have demonstrated that calbindin D-28K is protective against high calcium currents (Sonnenberg, Frantz, Lee, Heick, Chu, Tobin and Christakos, 1991). Other data suggest a role for calbindin D-28K in enzyme activation (Morgan, Welton, Heick and Christakos, 1986; Reisner, Christakos and Vanaman, 1992). In the mammalian kidney, calbindin D-28K expression is restricted to distal tubules and convoluted tubules of the nephron, the sites of hormone-dependent renal reabsorption of calcium and phosphorus, suggesting a role for calbindin D-28K in this process. However, the current data are insufficient to clearly define the function of this vitamin D-dependent calcium-binding protein.

The predominant regulator of calbindin D-28K in the avian intestine and avian and mammalian kidney is $1,25(\text{OH})_2\text{D}_3$. Evidence also supports a role for serum calcium and phosphorus levels in the modulation of calbindin D-28K expression, but the mechanism underlying this regulation is unclear (Meyer, Fullmer, Wasserman, Komm and Haussler, 1992; Theofan, King, Hall and Norman, 1987). $1,25(\text{OH})_2\text{D}_3$ regulates mammalian calbindin D-28K at both the transcriptional (Varghese, Lee, Huang and Christakos, 1988; Mayel-Afshar, Lane and Lawson, 1988) and post-transcriptional levels (Norman, Nemere, Zhou, Bishop, Lowe,

Maiyar, Collins, Taoka, Sergeev, and Farach-Carson, 1992; Craviso, Garrett and Clemens, 1987; Theofan and Norman, 1986). Transcriptionally, $1,25(\text{OH})_2\text{D}_3$ is thought to modulate calbindin D-28K mRNA synthesis via VDR binding to a VDRE in the promoter region of the calbindin D-28K gene. Three putative VDREs have been identified in the mouse calbindin D-28K gene: two of which are consistent with other defined VDREs (DR3) (Takeda, Arakawa and Kuwano, 1994), while the other differs slightly from the consensus with four nucleotides separating the two direct repeats of the steroid response element half-site (DR4) (Christakos, Gill, Lee and Li, 1992; Lian and Stein, 1992; Gill and Christakos, 1993). The post-transcriptional mechanism regulating calbindin D-28K expression is poorly understood, although $1,25(\text{OH})_2\text{D}_3$ reportedly exerts post-transcriptional effects on other vitamin D-dependent proteins as well. Recently, activators and inhibitors of PKC have been shown to modulate the effects of $1,25(\text{OH})_2\text{D}_3$ on osteocalcin and osteopontin, two bone matrix proteins (van Leeuwen, Birkenhager, van den Bermd, Buurman, Staal, Bos and Pols, 1992a), VDR (van Leeuwen, Birkenhager, Buurman, van den Bermd, Bos and Pols, 1992b; Krishnan and Feldman, 1991a), and 1α -hydroxylase (Henry, Dutta, Cunningham, Blanchard, Penny, Tang, Marchetto and Chou, 1992; Welsh, Weaver and Simboli-Campbell, 1991).

PKC is described as a serine/threonine kinase implicated in the regulation of several cellular functions including growth and proliferation. PKC consists of a family of isozymes subdivided into calcium-dependent and calcium-independent isoforms. Activation of PKC occurs in the presence of second messengers such as diacylglycerol (DAG), arachidonic acid, and/or calcium, generated from the hydrolysis of membrane phosphoinositides. Various growth factors and peptide hormones have also been shown to rapidly increase PKC activity

in a variety of cell lines. The tumor-promoting phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA) can replace DAG and directly activate PKC (Castagna, Takai, Kaibuchi, Sano, Kikkawa and Nishizuka, 1982). $1,25(\text{OH})_2\text{D}_3$ has also been shown to regulate PKC activity and gene expression in HL-60 cells. Although no VDR has yet been identified in the promoter region of PKC genes, induction of PKC by $1,25(\text{OH})_2\text{D}_3$ is thought to occur through VDR-mediated transcriptional activation (Obeid, Okazaki, Karolak and Hannun, 1990). The potential involvement of the PKC signal transduction pathway in the mechanism of post-transcriptional regulation of calbindin D-28K by $1,25(\text{OH})_2\text{D}_3$ has yet to be investigated, primarily due to a lack of model system in which to study calbindin D-28K expression.

The first objective of my research project was to identify a suitable renal model in which to investigate calbindin D-28K regulation by $1,25(\text{OH})_2\text{D}_3$. Evidence reported in Chapter 1 indicates that MDBK cells, a non-transformed established renal cell line, represent a novel model to investigate calbindin D-28K expression, and Chapter 2 characterizes the vitamin D-dependence of this system. The following chapters investigate the potential involvement of signal transduction pathways, including the PKC pathway, in the regulation of calbindin D-28K by $1,25(\text{OH})_2\text{D}_3$. A hypothetical model of calbindin D-28K regulation by $1,25(\text{OH})_2\text{D}_3$, which integrates our findings, is also presented. Investigation into the regulation of calbindin D-28K expression hopefully will bring insight into the function of this evolutionary important protein.

OBJECTIVES

1. To establish an *in vitro* model system in which to investigate renal calbindin D-28K regulation by $1,25(\text{OH})_2\text{D}_3$.
2. To characterize the regulation of calbindin D-28K by $1,25(\text{OH})_2\text{D}_3$ in MDBK cells.
3. To investigate the post-transcriptional regulation of calbindin D-28K expression in MDBK cells.

CHAPTER 1: ESTABLISHMENT OF AN *IN VITRO* MODEL SYSTEM TO INVESTIGATE CALBINDIN D-28K REGULATION BY 1,25(OH)₂D₃

1.1 INTRODUCTION

The kidney is not only the site of production of the biologically active form of vitamin D, 1,25(OH)₂D₃, it is also a major target organ. In keeping with its classical function of maintaining calcium homeostasis, 1,25(OH)₂D₃ increases the reabsorption of calcium and phosphates in the kidney. The detailed mechanism involved in this regulation is still unknown, but is thought to include the induction of the two vitamin D-dependent calcium-binding proteins, calbindin D-9K and calbindin D-28K (Thomasset, Parkes and Cuisinier-Greyis, 1982; Wasserman and Taylor, 1966). The lack of a suitable *in vitro* model system has hindered research on the regulation and role of the vitamin D-dependent calbindins. My first objective was to establish an *in vitro* model system in which to study calbindin D-28K regulation by 1,25(OH)₂D₃.

1.1.1 Renal physiology

The kidneys play a vital role in homeostasis by regulating the concentration of waste products of metabolism and maintaining the ionic composition of the extracellular fluid. The nephron is the basic functional unit of the kidney; it is divided into several segments which differ in cellular structure and function. The glomerulus serves to produce an ultrafiltrate of

the plasma. This filtrate then passes through the proximal tubules, the loop of Henle, the distal tubules and finally, the collecting ducts, where the urine is collected. These renal segments respond specifically and differentially to various signals, such as hormones and cytokines, to ensure plasma homeostasis.

1.1.2 Effects of $1,25(\text{OH})_2\text{D}_3$ in the kidney

The kidney has long been known to be a major target for $1,25(\text{OH})_2\text{D}_3$. Although the VDR has been shown to be present throughout the nephron (Kawashima and Kurokawa, 1982), most effects of $1,25(\text{OH})_2\text{D}_3$ occur in the distal portion corresponding to the distal tubules and the collecting ducts, the sites of vitamin D-dependent calcium transport (Costanzo, Sheeche and Weiner, 1974; Bouhciauy, Lajeunesse and Brunette, 1993). Other well-described renal effects of $1,25(\text{OH})_2\text{D}_3$ include the regulation of its own metabolism. It has been demonstrated by several groups that the expression and activity of the 24-hydroxylase, the first enzyme required for $1,25(\text{OH})_2\text{D}_3$ catabolism, is induced in the presence of $1,25(\text{OH})_2\text{D}_3$ (Tanaka et al, 1975). In proximal tubules $1,25(\text{OH})_2\text{D}_3$, in conjunction with the parathyroid hormone, has been shown to modulate its own production by regulating the expression and activity of the 1α -hydroxylase, the enzyme required for its production (Welsh et al, 1991; Henry et al, 1992). Both renal vitamin D-dependent calcium-binding proteins (calbindin D-9K and calbindin D-28K) are localized exclusively in the distal tubules and collecting ducts. In these cells, calbindin D-28K has been subcellularly localized in the cytoplasm, being more concentrated at the apical pole of the cell, and less frequently in the nucleus (Roth, Thorens, Hunziker, Norman and Orci, 1981; Roth, Brown, Norman and Orci,

1982). The correlation between the localization of these proteins and the role of $1,25(\text{OH})_2\text{D}_3$ in increasing calcium and phosphorus reabsorption implicate the calbindins in these processes, perhaps acting as calcium buffers or calcium transport proteins. The heterogeneity in kidney structure and the differential effects of vitamin D on the various segments of the nephron have contributed to the difficulties in investigating specific renal effects of $1,25(\text{OH})_2\text{D}_3$.

1.1.3 Renal model systems

Most studies of $1,25(\text{OH})_2\text{D}_3$ effects in the kidney, including the regulation of calbindin D-28K, have been done using *in vivo* model systems or primary cultures of renal cells. *In vivo* studies are limited by their use of whole kidney homogenates which include various cells types or involve tedious isolation procedures of specific tubules. To date, the only successful *in vitro* models exhibiting $1,25(\text{OH})_2\text{D}_3$ -dependent expression of renal calbindin D-28K are primary cultures. Besides the experimental difficulties in working with primary cultures, these systems are heterogeneous, and the epithelial cells display both distal and proximal tubule characteristics. The best described models include primary cultures of chick kidney cells (Craviso et al, 1987), rabbit collecting duct cells (Bindels, Hartog, Timmermans and van Os, 1991), and vitamin D_3 -replete rat kidney cells (Chen, Boltz, Christakos and Armbrecht, 1992), all of which have been reported to express calbindin D-28K in a vitamin D-dependent manner. In most of these systems, fibroblast contamination is low, but only 5-10% of the cells express calbindin D-28K (Craviso et al, 1987). It has also been reported that the avian model retains the ability to produce $1,25(\text{OH})_2\text{D}_3$ from $25-(\text{OH})\text{D}_3$, indicating contamination with proximal tubular cells (Brunette, Chan, Ferriere and Roberts,

1978). In the rat primary cultures, the observation of Na-dependent phosphate transport likewise indicates the presence of proximal tubular cells (Chen, King and Armbrrecht, 1990). These limitations warranted the search for a new *in vitro* renal model system in which to study calbindin D-28K regulation by $1,25(\text{OH})_2\text{D}_3$.

1.1.4 Established renal cell lines

The lack of an established renal cell line known to express calbindin D-28K in a vitamin D-dependent manner has hindered investigation into the function and regulation of this calcium-binding protein. A number of commercially available renal cell lines express the VDR, but none has been reported to express calbindin D-28K. Our first objective was to identify a renal cell model expressing calbindin D-28K in a vitamin D-dependent manner. We therefore assessed the presence of calbindin D-28K in four different renal epithelial cell lines: Madin Darby Bovine Kidney (MDBK) cells, Madin Darby Canine Kidney (MDCK) cells, LLC-PK₁ (pig kidney) cells, and opossum kidney (OK) cells. MDBK and MDCK cells originated from normal bovine and canine kidneys respectively, and represent well-differentiated renal epithelial cells with distal tubular characteristics (Madin and Darby, 1958). Although the characteristics of the MDBK cell line have not been extensively studied, MDCK cells retain enzyme markers, morphological features, and antigenic determinants characteristic of distal tubules (Rindler, Chuman, Shaffer and Saier, 1979; Valentich, 1981; Herzlinger, Easton and Ojakian, 1982). On the other hand, LLC-PK₁ and OK cells exhibit Na-dependent transport of glucose, phosphate and amino acids, suggesting their proximal tubule origin (Gstraunthaler, Pfaller and Kotanko, 1985; Malmstrom and Murer, 1986; Quamme,

Pfeilschifter and Murer, 1989).

In these studies, the levels of calbindin D-28K and the VDR were assessed in four distinct renal cell lines. As anticipated, VDR expression was detected in all cell lines, with the highest levels found in MDBK cells. Calbindin D-28K expression was detected only in MDBK cells. Further, the expression of calbindin D-28K in these cells was enhanced by $1,25(\text{OH})_2\text{D}_3$, offering the first report of an established renal cell line expressing calbindin D-28K in a vitamin D-dependent manner.

1.2 METHODS

1.2.1 Cell culture

Stock cultures of MDBK, MDCK, and LLC-PK₁ cells were obtained from American Type Culture Collection (ATCC) (Rockville, Maryland, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Burlington, Ontario, Canada) supplemented with 10% newborn calf serum (Life Technologies). Cells were plated at a density of 1.5×10^4 cells/ml and grown to confluence at 37°C in an atmosphere of 5% CO₂. OK cells were obtained from ATCC, plated at a density of 10^5 cells/ml, grown in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal calf serum (Woodlyn Laboratories, Guelph, Ontario, Canada) and allowed to reach confluence.

In some experiments, confluent MDBK cells were changed to serum-free DMEM for 24 hours prior to processing or treatment with 1,25(OH)₂D₃ (Biomol Research Laboratories, Plymouth Meeting, Pennsylvania, USA), vehicle (absolute ethanol), or serum for up to 24 hours.

Cells were harvested by scraping, washed with ice-cold PBS and lysed by vortexing for two minutes in hypotonic buffer (1 mM NaHCO₃, 5 mM MgCl₂, 5 mM dithiothreitol (DTT), 100 µM phenylmethyl sulfonyl fluoride (PMSF), and 20 µg/ml leupeptin, pH 7.5). Ice-cold Tris-EGTA buffer was added to the cell lysate to give a final concentration of 50 mM Tris-Base and 500 µM EGTA. Crude nuclear fractions were obtained after centrifugation at 500 x g for five minutes at 4°C, further centrifugation through a 45% sucrose gradient yielded purified nuclei. Cytosolic and membrane fractions were obtained by centrifugation of the

post-nuclear fraction at 100,000 x g for one hour at 4°C. Glucose-6-phosphate dehydrogenase activity (cytosol marker), forskolin-stimulatable adenylate cyclase activity (membrane marker) and lamin B presence (nuclear marker) were assessed in all fractions to confirm purity. Cytosolic fractions were used for calbindin D-28K analysis.

Whole bovine kidney, obtained from a slaughterhouse and kept frozen at -70°C, was homogenized in hypotonic buffer for preparation of cytosolic fractions as described above.

Protein concentrations were determined by the method of Bradford (1976).

1.2.2 Immunofluorescence

MDBK, MDCK, LLC-PK₁, and OK cells were plated on glass coverslips in 35 mm dishes at a density of 4×10^4 cells/ml and incubated in the appropriate growth medium for 72 hours. In some experiments, MDBK cells were changed to serum-free medium 24 hours prior to treatment with 10^{-7} M $1,25(\text{OH})_2\text{D}_3$, or ethanol vehicle (0.05%). Cells were washed with ice-cold PBS and fixed in ethanol for 20 minutes at -20°C. Cells were then washed and non-specific binding sites were blocked overnight in PBS containing 0.5% skim milk and 0.2% sodium azide. Cells were washed again and incubated at room temperature for one hour with a distal tubule marker antibody (MAB 474, Chemicon International, Temecula, California, USA) or with a monoclonal anti-calbindin D-28K antibody (Sigma Immunochemicals, Saint-Louis, Missouri, USA), diluted 1:10 in blocking solution. Following washing, cells were incubated for one hour at room temperature with a fluorescein (FITC)-conjugated goat anti-rabbit IgG (for MAB 474) or FITC-conjugated donkey anti-mouse IgG (for calbindin D-28K) (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) diluted 1:50 in

blocking solution. Cells were washed, mounted on slides, and photographed using a Zeiss microscope equipped with epi-fluorescence. Non-specific binding was assessed by incubation of the coverslips in the absence of primary antibodies.

1.2.3 Immunoblotting

Proteins from cytosolic, membrane, nuclear fractions or total homogenates of MDBK, MDCK, LLC-PK₁, OK cells, or bovine kidney were separated on 12 or 15% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose. The effectiveness of the transfer was monitored by reversibly staining the nitrocellulose with 0.2% Ponceau S in 3% trichloroacetic acid (TCA). Non-specific binding sites were saturated by incubation of nitrocellulose with PBS containing 0.5% skim milk for 2 hours. The membranes were then washed in PBS containing 0.1% Tween-20 and incubated for one hour at room temperature with a mouse monoclonal anti-calbindin D-28K (Sigma Immunochemicals) diluted 1:200 in blocking solution. Following washing, filters were incubated for one hour at room temperature with biotinylated donkey anti-mouse IgG (1:1000) (Jackson ImmunoResearch Laboratories). Nitrocelluloses were washed again and incubated for 20 minutes at room temperature with streptavidin peroxidase (Jackson ImmunoResearch Laboratories). In some experiments, parallel samples were immunoblotted with polyclonal rabbit anti-rat renal calbindin D-28K antiserum (provided by S. Christakos, University of Medicine and Dentistry of New Jersey) diluted 1:200 in blocking solution, followed with biotinylated-goat anti-rabbit IgG (1:2000). In all experiments, the peroxidase product was detected with 4-chloro-1-naphthol (625 µg/ml) in PBS with 20% methanol and 0.12% H₂O₂. Non-specific binding,

assessed in the absence of primary antibodies, was negligible.

1.2.4 VDR analysis

VDR numbers were assayed by [^3H]-1,25(OH) $_2\text{D}_3$ competition binding. Cells were homogenized in high salt buffer (10 mM Tris-HCl, 1 mM EDTA, 5 mM DTT, 10 mM sodium molybdate, 0.02% soybean trypsin inhibitor, 300 mM KCl, pH 7.6) and centrifuged at 100,000 x g, for one hour at 4°C to yield nuclear extracts. Extracts (1.0 mg protein/ml) were incubated with 0.5 nM [^3H]-1,25(OH) $_2\text{D}_3$ (Amersham, Oakville, Ontario, Canada) overnight at 4°C. In some experiments, the nuclear extracts were incubated at 37°C for one hour. Bound and free hormones were separated by addition of dextran-coated charcoal, incubation for 15 minutes, and centrifugation at 3,500 x g for 15 minutes. Bound [^3H]-1,25(OH) $_2\text{D}_3$ in the supernatants was counted in a Beckman scintillation counter. Data for specific [^3H]-1,25(OH) $_2\text{D}_3$ binding were generated by subtraction of non-specific binding, assessed in parallel tubes incubated with 200-fold excess of unlabelled 1,25(OH) $_2\text{D}_3$, from total [^3H]-1,25(OH) $_2\text{D}_3$ binding.

1.2.5 Enzyme-Linked Immuno-Absorbent Assay (ELISA) for calbindin D-28K

Calbindin D-28K was quantitated by ELISA techniques, based on the method of Miller and Norman (1983). Purified rat recombinant calbindin D-28K (SWANT, Bellinzona, Switzerland) and cytosolic fractions of MDBK cells (25 µg protein/well) were incubated overnight with monoclonal anti-calbindin D-28K (1:80,000) in wells coated with 10 ng calbindin D-28K. Wells were washed and incubated with an alkaline phosphatase-conjugated

donkey anti-mouse IgG antibody (1:10,000; Jackson ImmunoResearch Laboratories). Bound antibodies were detected using p-nitrophenyl phosphate (1 mg/ml in diethanolamine buffer). The colorimetric reaction was stopped after 30 minutes with 1 N NaOH and absorbance at 405 nm was determined using a Bio-Tek microplate reader. Calbindin D-28K in the samples was calculated from a standard curve obtained by plotting the absorbance against the log of recombinant rat calbindin D-28K. Absorbance decreased linearly up to 200 ng calbindin D-28K standard.

1.2.6 [^3H]-Thymidine incorporation into DNA

MDBK cells were grown to confluence in 35 mm dishes and changed to serum-free media 24 hours prior to treatment with 10^{-7} M $1,25(\text{OH})_2\text{D}_3$, ethanol (0.05%), or serum (10%). [^3H]-Thymidine (0.5 $\mu\text{Ci/ml}$) (Amersham) was also added to the media, and cells were incubated for 24 hours. Cells were washed with ice-cold PBS and DNA was solubilized with 0.5 N perchloric acid (PCA) at 90°C for 20 minutes. [^3H]-Thymidine incorporated into DNA was counted using a Beckman scintillation counter.

1.2.7 Analysis of cell cycle kinetics

Confluent MDBK cells were changed to serum-free media 24 hours prior to treatment with 10^{-7} M $1,25(\text{OH})_2\text{D}_3$, vehicle, or serum (10%). After 24 hours of treatment, cells were washed with ice-cold PBS, trypsinized, pelleted (500 x g, 5 minutes, 4°C), and fixed with 95% ethanol at -20°C for at least 30 minutes. Fixed cells were stained with propidium iodide (Coulter Corporation, Burlington, Ontario, Canada) for 15 minutes at room temperature, and

analyzed within two hours on an EPICS XL v35195 (1.5/1.22) flow cytometer. Cell cycle kinetics were analyzed using XL and Multicycle analysis softwares (Phoenix Flow Systems, San Diego, California, USA).

1.2.8 Statistical analysis

Unless otherwise stated, experiments were done at least three times. Data are expressed as mean $\pm$ standard error (SEM). Statistical analysis was evaluated using either Student's *t*-test or ANOVA, as appropriate. Differences between means were considered significant if a *P* value less than 0.05 was obtained.

1.2.9 Materials

All chemicals were obtained from Sigma Chemical Corp., Saint-Louis, Missouri, USA, unless otherwise stated.

1.3 RESULTS

1.3.1 Expression of distal tubule marker, VDR and calbindin D-28K in four renal cell lines.

To determine which of the four cell lines exhibit distal tubule characteristics, a commercially available monoclonal antibody raised against a human marker protein characteristic of distal tubules (MAB 474), was examined by immunofluorescence techniques. The majority of MDBK cells exhibit strong immunoreactivity (Fig. 1.1a) indicating that this cell line retains this distal tubule marker protein in culture. The closely related cell line MDCK, also expresses the distal tubule marker, but immunoreactivity is faint relative to the MDBK cells analyzed in parallel (Fig. 1.1b). As expected because of their documented proximal tubular characteristics, the distal tubule marker is not expressed in OK and LLC-PK₁ cells (Fig. 1.1c, d).

VDR expression was assessed by specific ³H-1,25(OH)₂D₃ binding. As expected, all four cell lines express the VDR but at markedly different levels. As shown in Figure 1.2, 1,25(OH)₂D₃ binding is significantly higher in MDBK cells (260 fmol/mg protein) than in either MDCK (13 fmol/mg protein), LLC-PK₁ (47 fmol/mg protein), or OK (43 fmol/mg protein) cells. Previous work in our laboratory has determined that the approximate molecular weight of the VDR and its dissociation constant ($K_D = 10^{-10}$ M) for 1,25(OH)₂D₃ in MDBK cells (Simboli-Campbell, Franks and Welsh, 1992a) are comparable to the values previously reported in various tissues and cell lines (Colston and Feldman, 1982; Stone, Weaver, Bruns and Welsh, 1991).

Figure 1.1 Immunofluorescence of a distal tubule marker protein in MDBK, MDCK, OK and LLC-PK₁ cells.

Cells were grown on coverslips in media containing 10% serum, washed and fixed in ice-cold ethanol. Fixed cells were incubated with MAB 474 antibody directed against a characteristic distal tubule protein, followed by incubation with FITC-labelled secondary antibody as described in **Methods**. Cells were mounted and viewed under phase contrast (left panels) and fluorescence (right panels) microscopy. A. MDBK cells; B. MDCK cells; C. OK cells; D. LLC-PK₁ cells. In all cases, non-specific immunoreactivity was negligible (not shown).

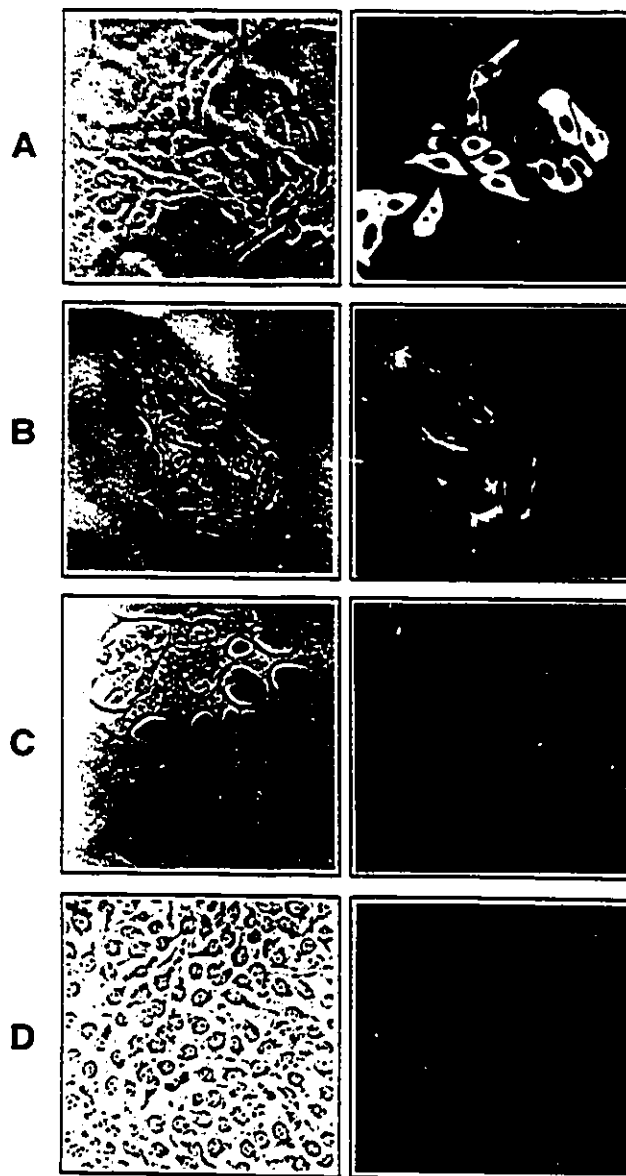
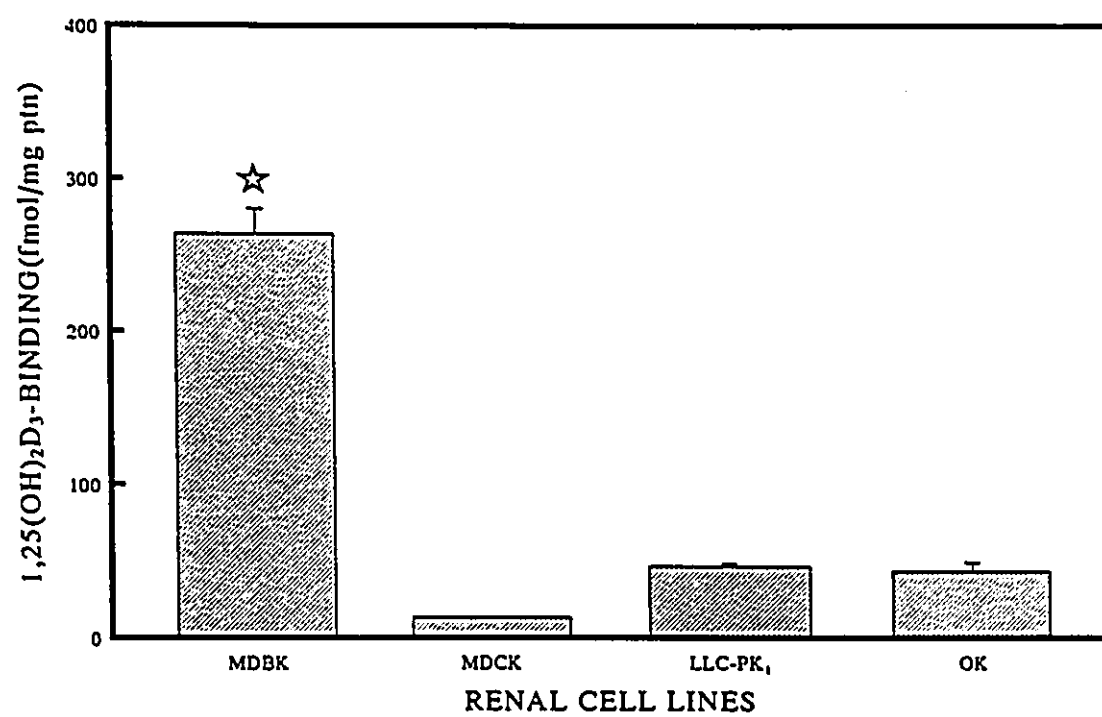


Figure 1.2 *Specific ^3H -1,25(OH) $_2\text{D}_3$ -binding in MDBK, MDCK, LLC-PK, and OK cells.*

Cells were grown to confluence in media containing 10% serum, harvested by scraping, and high salt extracts were incubated with 0.5 nM ^3H -1,25(OH) $_2\text{D}_3$ overnight at 4°C as described in Methods. Data are expressed as mean $\pm$ SEM ($N = 4$ to 8) of specific ^3H -1,25(OH) $_2\text{D}_3$ -binding, obtained by subtraction of non-specific binding from total binding. Statistical differences between groups were assessed by ANOVA with significance level at $P < 0.05$ (indicated by * on the graph).



Calbindin D-28K expression was assessed in all four renal cell lines by immunoblotting. Bovine kidney cytosol was used as positive control. A single immunoreactive band of approximately 28 kDa is detected in bovine kidney and MDBK cells using a monoclonal antibody directed against chick intestinal calbindin D-28K (Fig. 1.3a). Although MDCK cells have distinct distal tubules determinants (Rindler et al, 1979; Valentich 1981), these cells do not express calbindin D-28K. As expected due to the exclusive localization of calbindin D-28K in the distal tubules, this protein is not expressed in the proximal tubule-like LLC-PK₁ and OK cells (Fig. 1.3a). Similar results for all cell lines were obtained using rabbit polyclonal antibodies raised against rat renal calbindin D-28K (Fig. 1.3b). Subcellular fractionation of MDBK cells indicated that calbindin D-28K is predominantly localized in the cytosol (Fig. 1.4). In agreement with previous reports, some expression of calbindin D-28K is evident in the nucleus (Roth et al, 1981). In light of the expression of the VDR and calbindin D-28K in MDBK cells, further studies were conducted to determine the vitamin D-dependence of these proteins in this distal tubular-like cell line.

1.3.2 Effects of serum and 1,25(OH)₂D₃ on calbindin D-28K and VDR expression in MDBK cells.

Previous reports have suggested that both VDR and calbindin D-28K expression are increased in rapidly proliferating cells (Chen and Feldman, 1981; Chen et al, 1992). 1,25(OH)₂D₃-binding and calbindin D-28K levels in quiescent and growing MDBK cells were therefore assessed. As shown in Figure 1.5 (top), calbindin D-28K expression, determined by immunoblotting, is significantly increased in cells grown in the presence of 10% serum.

Figure 1.3 *Calbindin D-28K expression in MDBK, MDCK, LLC-PK, and OK cells.*
Cells were grown in serum-containing media and harvested by scraping. Bovine kidney was obtained from a slaughterhouse. Cytosolic proteins (50 µg/lane) were separated on 15% SDS-PAGE, transferred to nitrocellulose and blotted with either A. a monoclonal antibody raised against chick intestinal calbindin D-28K or B. polyclonal antibodies raised against rat renal calbindin D-28K as described in Methods. In each panel, samples were derived from bovine kidney (*lane 1*), MDBK cells (*lane 2*), MDCK cells (*lane 3*), LLC-PK₁ cells (*lane 4*) and OK cells (*lane 5*).

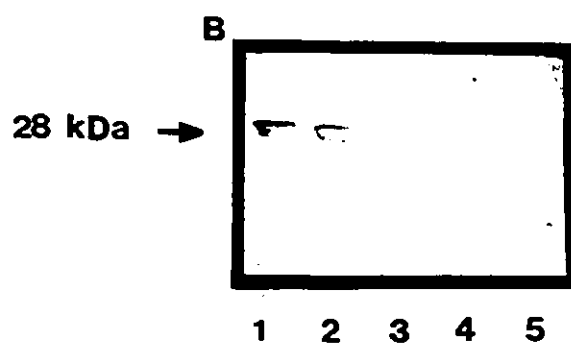
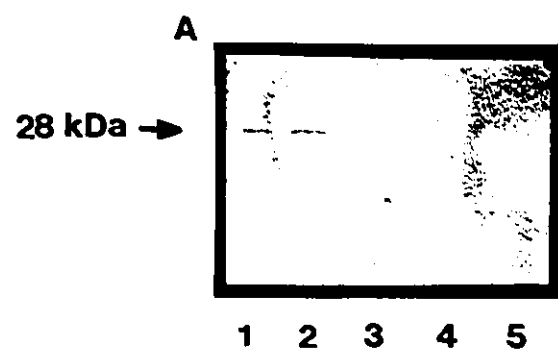


Figure 1.4 *Subcellular localization of calbindin D-28K in MDBK cells.*

Cells were grown to confluence and harvested by scraping. Membrane, cytosolic and nuclear fractions were prepared as described in Methods. Proteins from each fraction (50 µg/lane) were separated on 12% SDS-PAGE, electrophoretically transferred to nitrocellulose and blotted with monoclonal anti-calbindin D-28K antibody (S). Non-specific immunoreactivity was assessed in the absence of primary antibodies (NS), as previously described. *Lane N*, nuclear fraction; *lane C*, cytosolic fraction; *Lane M*, membrane fraction.

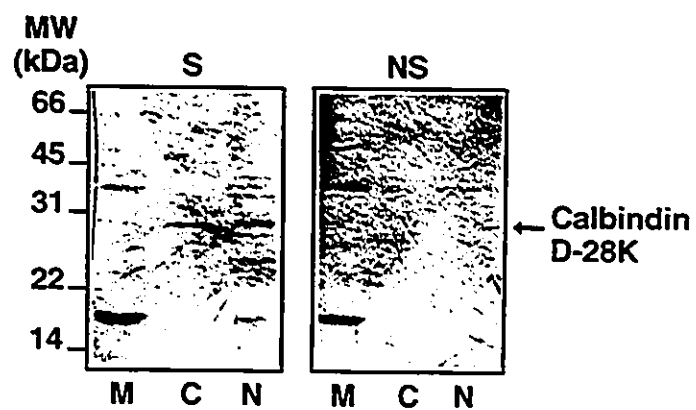
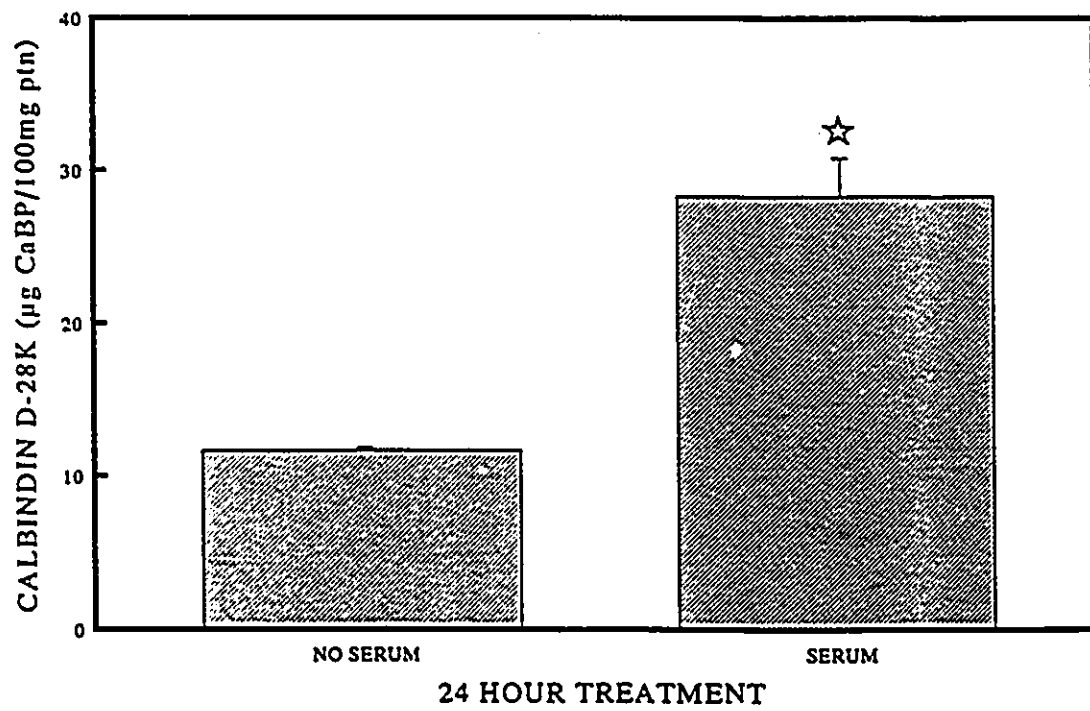
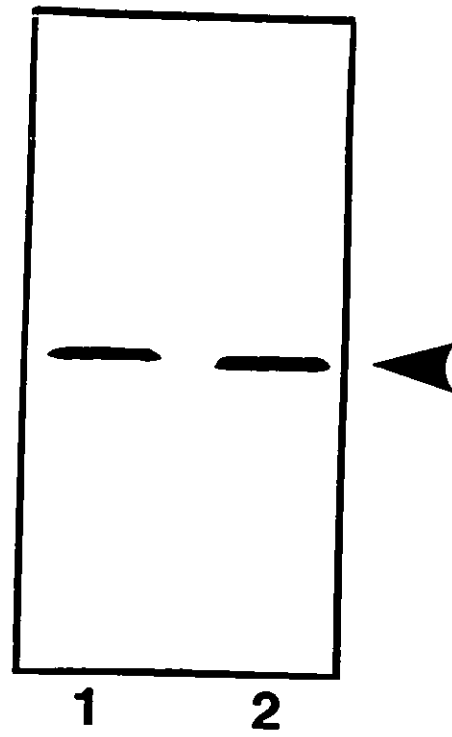


Figure 1.5 *Effect of 24 hour serum stimulation on calbindin D-28K expression in MDBK cells.*

Cells were grown to confluence and changed to serum-free media 24 hours prior to treatment. Serum, to 10%, was added to some cells for 24 hours. Cells were harvested by scraping and cytosolic fractions were prepared. **Top:** Cytosolic proteins (100 µg/lane) were separated on 15% SDS-PAGE, transferred to nitrocellulose and blotted with anti-calbindin D-28K monoclonal antibody as described in Methods. *Lane 1*, quiescent cells; *lane 2*, serum-stimulated cells. **Bottom:** Cytosolic levels of calbindin D-28K were measured by ELISA as described in Methods. Data are expressed as mean $\pm$ SEM ($N = 4$) of calbindin D-28K (µg/100 mg of protein). Calbindin D-28K was significantly ($P < 0.05$) increased in serum-stimulated cells compared to serum-starved cells, as assessed by Student's *t*-test for unpaired data.



Quantitation of calbindin D-28K levels by ELISA also indicate significantly elevated calbindin D-28K protein in serum-stimulated ($27.8 \pm 1.3 \mu\text{g}/100 \text{ mg protein}$) compared to serum-starved cells ($11.3 \pm 0.2 \mu\text{g}/100 \text{ mg protein}$) (Fig. 1.5, bottom). The basal level of calbindin D-28K in MDBK cells is comparable to that reported in primary cultures of chick (5-24 $\mu\text{g}/100 \text{ mg protein}$) and rat renal cells (37-85 $\mu\text{g}/100 \text{ mg protein}$). Serum-stimulated cells also exhibit a 2.5-fold increase in VDR numbers as compared to serum-starved cells (Fig. 1.6). These results confirm previous studies reporting elevated expression of these two vitamin D-dependent proteins in proliferating cells compared to quiescent cells.

To study the effects of $1,25(\text{OH})_2\text{D}_3$ on calbindin D-28K and VDR expression in MDBK cells, cells were grown in serum-free media for 24 hours prior to treatment with 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ or ethanol (vehicle). Under these conditions, induced expression of calbindin D-28K is evident by both immunoblotting and immunofluorescence (Fig. 1.7). A 1.6-fold induction in calbindin D-28K levels in response to $1,25(\text{OH})_2\text{D}_3$ treatment was calculated from ELISA results (Fig. 1.8). This increase is similar to that previously reported in primary renal cultures (Chen et al, 1992; Craviso et al, 1987), and constitutes the first report of calbindin D-28K up-regulation by $1,25(\text{OH})_2\text{D}_3$ in an established renal cell line. The induction of calbindin D-28K expression by $1,25(\text{OH})_2\text{D}_3$ in MDBK cells was accompanied by an increase in VDR numbers, as compared to vehicle control (Fig. 1.9). The observed 2.5-fold increase in $1,25(\text{OH})_2\text{D}_3$ -binding in response to 24 hour treatment with $1,25(\text{OH})_2\text{D}_3$ is consistent with previous reports of autologous up-regulation of the VDR in other systems (Costa, Hirst and Feldman, 1985; Wiese, Uhland-Smith, Ross, Prahl and DeLuca, 1992). Taken together, these results suggest that the MDBK cell line constitutes a relevant model

Figure 1.6 *Effect of 24 hour serum stimulation on specific 1,25(OH)₂D₃-binding in MDBK cells.*

Cells were grown to confluence and changed to serum-free media 24 hours prior to stimulation with 10% serum. Cells were harvested 24 hours after treatment and high salt extracts were incubated with 0.5 nM ³H-1,25(OH)₂D₃ overnight at 4 °C as described in Methods. Data are expressed as mean ± SEM (*N* = 3) of specific ³H-1,25(OH)₂D₃-binding obtained by subtraction of non-specific binding from total binding. VDR number was significantly (*P* < 0.05) increased in serum-stimulated cells compared to serum-starved cells, as assessed by Student's *t*-test for unpaired data.

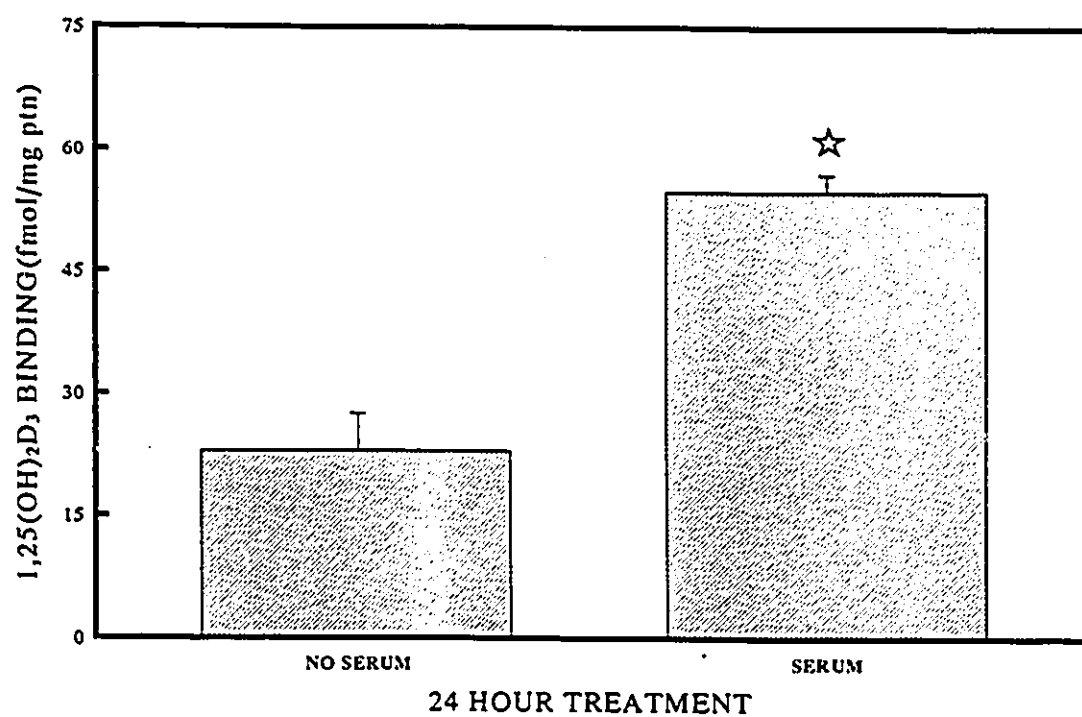


Figure 1.7 *Effect of 24 hour treatment with 1,25(OH)₂D₃ on calbindin D-28K expression in MDBK cells.*

Scrum-free MDBK cells were treated for 24 hours with 10^{-7} M 1,25(OH)₂D₃ or ethanol (0.05%). A. Cells were harvested by scraping and cytosolic fractions were prepared as described in Methods. Cytosolic proteins (100 µg/lane) were separated on 15% SDS-PAGE, transferred to nitrocellulose and blotted with monoclonal anti-calbindin D-28K antibodies, as described in text. *Lane 1*, vehicle; *lane 2*, 1,25(OH)₂D₃. B, C. Fixed cells were incubated with monoclonal anti-mouse calbindin D-28K antibody, followed by incubation with FITC-labelled secondary antibody, as described in Methods. B. vehicle; C. 1,25(OH)₂D₃.

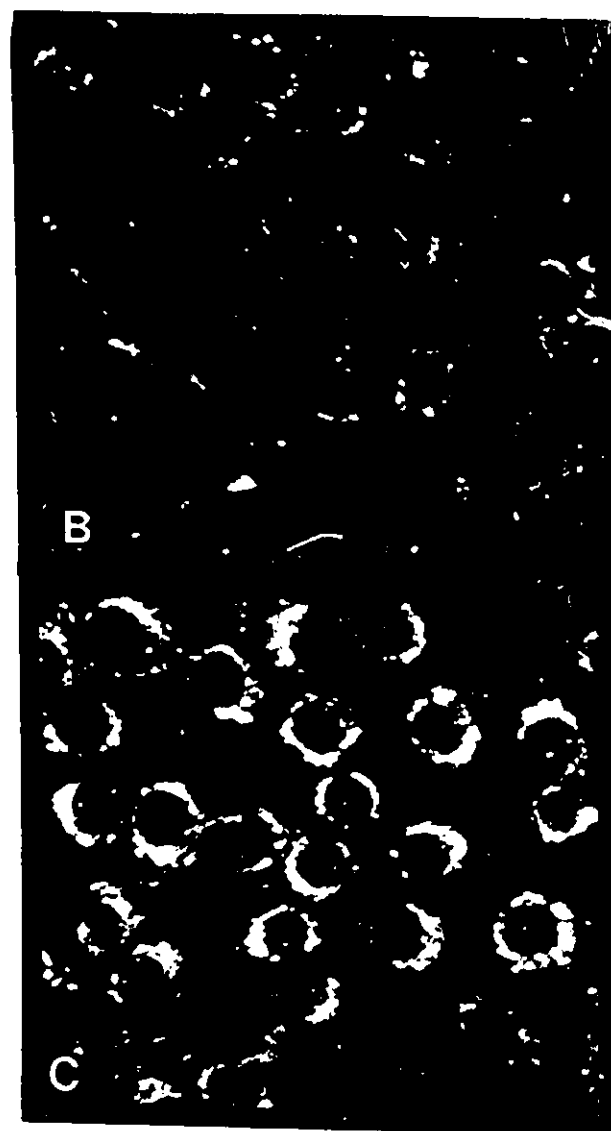
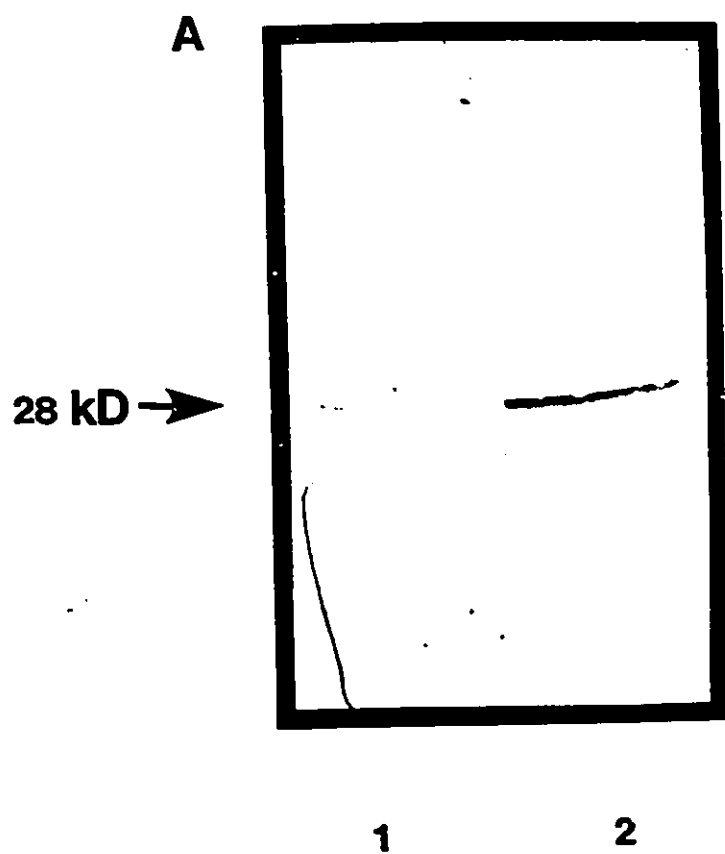


Figure 1.8 *Effect of 24 hour treatment with 1,25(OH)₂D₃ on calbindin D-28K expression in MDBK cells, assessed by ELISA.*

Serum-free MDBK cells were treated for 24 hours with 10^{-7} M 1,25(OH)₂D₃ or ethanol (0.05%). Cells were harvested by scraping and cytosolic fractions were prepared. Cytosolic levels of calbindin D-28K were quantitated by ELISA, as described in Methods. Data are expressed as mean $\pm$ SEM ($N = 4$) of calbindin D-28K ($\mu\text{g}/100$ mg of protein). Calbindin D-28K was significantly ($P < 0.05$) increased in 1,25(OH)₂D₃-treated cells compared to vehicle-treated cells, as assessed by Student's *t*-test for unpaired data.

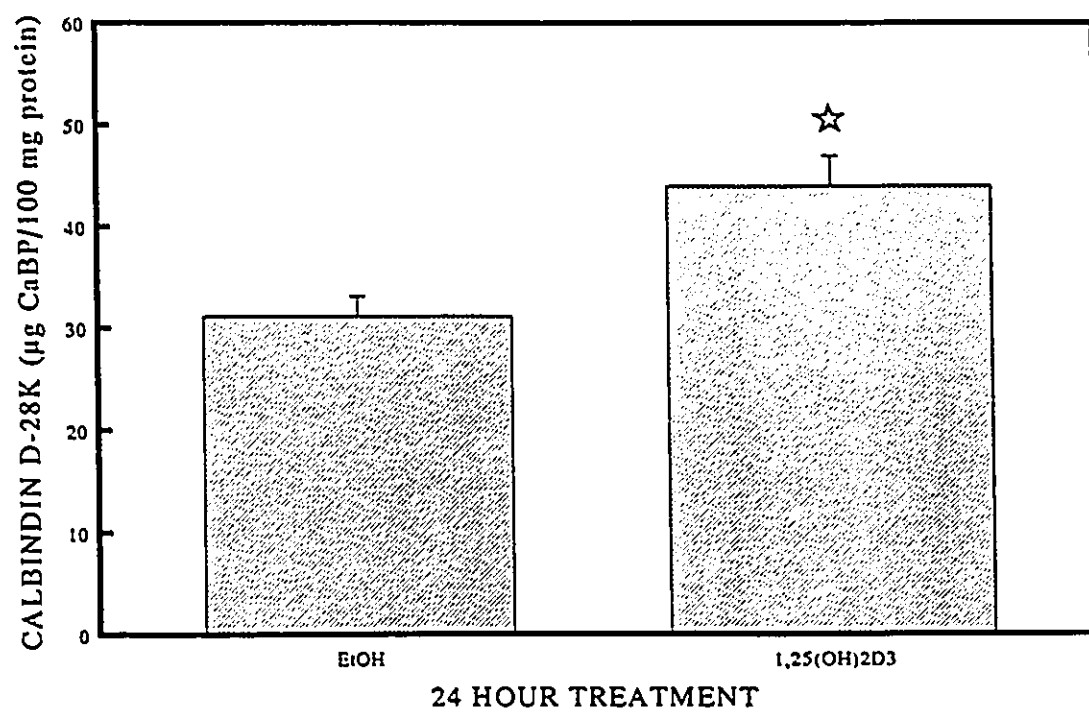
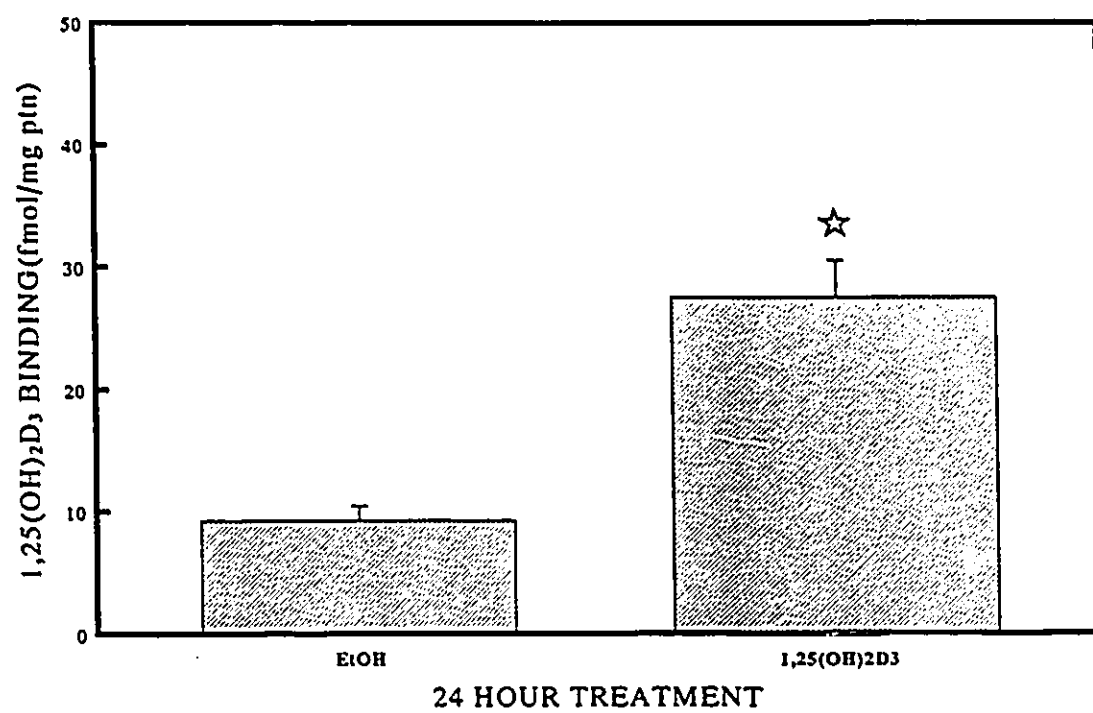


Figure 1.9 *Effect of 24 hour treatment with $1,25(\text{OH})_2\text{D}_3$ on specific $1,25(\text{OH})_2\text{D}_3$ -binding in MDBK cells.*

Serum-free MDBK cells were treated with 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ or ethanol (0.05%) for 24 hours prior to harvest. High salt extracts were incubated for 1 hour at 37°C with 0.5 nM ^3H - $1,25(\text{OH})_2\text{D}_3$ as described in Methods. Data are expressed as mean $\pm$ SEM ($N = 4$) of specific ^3H - $1,25(\text{OH})_2\text{D}_3$ -binding, obtained by subtraction of non-specific binding from total binding. VDR number was significantly ($P < 0.05$) increased in $1,25(\text{OH})_2\text{D}_3$ -treated cells compared to vehicle-treated cells, as assessed by Student's t -test for unpaired data.



to further investigate the mechanism underlying calbindin D-28K regulation by $1,25(\text{OH})_2\text{D}_3$.

1.3.3 Effects of serum and $1,25(\text{OH})_2\text{D}_3$ on MDBK cell proliferation.

Our data indicate that both VDR and calbindin D-28K levels are elevated in serum-stimulated MDBK cells, consistent with earlier work demonstrating induction of both proteins in rapidly proliferating cells (Chen et al, 1992). Recent evidence emphasizes the involvement of $1,25(\text{OH})_2\text{D}_3$ in the regulation of cell proliferation and differentiation (Minghetti and Norman, 1988; Reitsma et al, 1983). To determine whether the observed changes in calbindin D-28K and VDR expression in response to $1,25(\text{OH})_2\text{D}_3$ treatment or serum-stimulation are related to effects on cellular proliferation and/or differentiation, we next assessed cell cycle parameters.

Cell proliferation was first determined by ^3H -thymidine incorporation into DNA. As shown in Figure 1.10, serum-stimulation for 24 hours causes a significant increase in ^3H -thymidine incorporation (3900 ± 400 cpm) as compared to serum-starved cells (1200 ± 100 cpm), indicating an elevated cell proliferation rate in the presence of serum. In contrast, MDBK cells treated for 24 hours with either 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ or ethanol vehicle exhibit similar rates of ^3H -thymidine incorporation into DNA, suggesting that $1,25(\text{OH})_2\text{D}_3$ does not alter MDBK cell proliferation rate under these conditions.

More precise characterization of cell cycle kinetics was obtained using flow cytometric analysis. The results of these experiments are summarized in Table 1.1. As expected, serum-stimulated cultures exhibit a higher percentage of cells in S and G_2/M phases (22.8 ± 0.7 % and 13.0 ± 2.3 % respectively) than do serum-starved cultures (2.6 ± 0.1 and 4.2 ± 0.2 %).

Figure 1.10 Effects of serum and 1,25(OH)₂D₃ on ³H-thymidine incorporation into DNA in MDBK cells.

Serum-free MDBK cells were incubated for 24 hours with either 10% serum, 10⁻⁷ M 1,25(OH)₂D₃ or ethanol vehicle (0.05%). ³H-thymidine (0.5 μCi/ml) was added simultaneously with the various treatments. ³H-thymidine incorporation into DNA was assessed as described in Methods. Data are expressed as mean ± SEM (N = 3). ³H-thymidine incorporation was significantly increased (*P* < 0.05) in serum-stimulated cells compared to serum-starved cells, as assessed by Student's *t*-test for unpaired data.

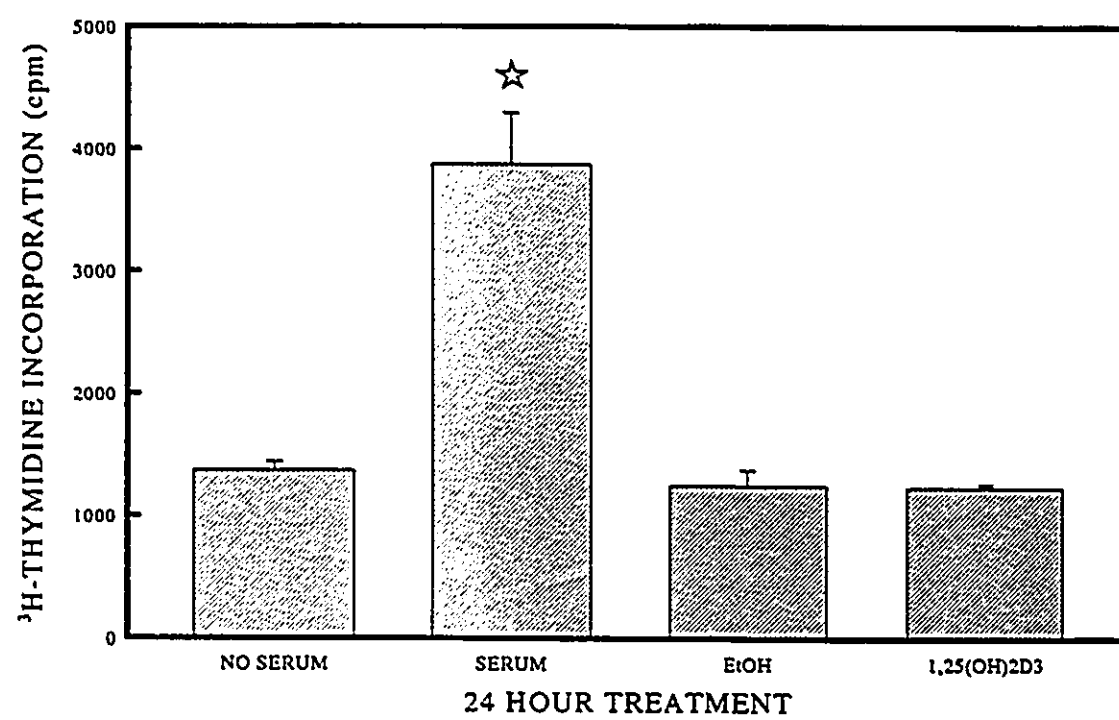


Table 1.1

*Flow cytometric analysis of MDBK cells.
Effects of serum and 1,25(OH)₂D₃.*

TREATMENT	% of Cells in G ₀ /G ₁	% of Cells in S	% of Cells in G ₂ /M
No Serum	93.3±0.3*	2.6±0.1*	4.2±0.2*
Serum	65.0±1.8*	21.8±0.7*	13.0±2.3*
EtOH	89.1±0.6	2.7±0.2	3.0±0.2
1,25(OH) ₂ D ₃	88.1±0.8	2.6±0.3	3.2±0.4

Serum-free MDBK cells were treated for 24 hours with either 10% serum, 10⁻⁷ M 1,25(OH)₂D₃, or ethanol vehicle. Cells were lysed, stained with propidium iodide and analyzed by flow cytometry as described in Methods. Statistical differences between groups were determined by Student's *t*-test for unpaired data. * Denotes statistically significant (*P* < 0.05) differences between serum-stimulated and serum-starved cells.

There is also a concomitant decrease in the number of cells in G_0/G_1 in serum-stimulated cells. As was observed with the 3H -thymidine incorporation experiments, $1,25(OH)_2D_3$ does not alter the cell cycle characteristics of MDBK cells when compared to ethanol treated cells. This is evident from the results displayed in Table 1.1. There are no significant differences in the percentage of cells in any phase of the cell cycle between the two treatments. These results suggest that $1,25(OH)_2D_3$ induces calbindin D-28K and VDR levels in MDBK cells in a specific and cell cycle-independent manner, further validating the use of this cell model in investigating the vitamin D-dependence of calbindin D-28K.

1.4 DISCUSSION

Investigation into the molecular regulation of calbindin D-28K expression in the kidney has been hindered by the lack of an established cell line expressing calbindin D-28K in a vitamin D-dependent manner. The results described here examine the expression of calbindin D-28K in four renal cell lines, OK (opossum kidney), LLC-PK₁ (pig kidney), MDCK (Madin-Darby Canine Kidney) and MDBK (Madin-Darby Bovine Kidney) cells, to identify a model cell line in which to research calbindin D-28K regulation by 1,25(OH)₂D₃. The primary culture systems currently used to study calbindin D-28K regulation are derived from collagenase digestion of fresh kidney (Craviso et al, 1987; Chen et al, 1992). Both avian and rat renal primary cultures exhibit characteristics of proximal tubules such as Na-dependent phosphate transport and 25-(OH)D₃ 1 α -hydroxylase activity (Brunette et al, 1978; Chen et al, 1990). Considering that renal calbindin D-28K is localized exclusively to distal tubules and collecting ducts *in vivo* (Rhoten and Christakos, 1981; Roth et al, 1981; Roth et al, 1982), the heterogeneous cellular mixture in primary cultures complicates investigations into the regulation of calbindin D-28K. Our first objective was to find an established renal epithelial cell line which expresses calbindin D-28K and is responsive to 1,25(OH)₂D₃. We first analyzed four renal cell lines for distal tubular characteristics and the presence of VDR and calbindin D-28K.

1.4.1 Establishment of distal tubule character of four renal cell lines

Both OK and LLC-PK₁ cells display Na⁺-dependent transport of glucose, amino acids and phosphate, characteristics of the proximal nephron *in vivo* (Gstraunthaler et al, 1985; Malmstrom and Murer, 1986; Quamme et al, 1989). Thus it was not unexpected that these cell lines expressed neither the distal tubular marker protein nor calbindin D-28K (Figs. 1.1c,d and 1.3). Although the lack of immunoreactivity for calbindin D-28K could reflect species differences in recognition by the monoclonal antibody, the known amino acid sequence homology between calbindin D-28K proteins from divergent species argues against this interpretation. Moreover, no immunoreactive calbindin D-28K was detected in OK and LLC-PK₁ cells using distinct polyclonal antibodies raised against rat renal calbindin D-28K (Fig. 1.3). This antibody has been shown to cross-react with calbindin D-28K protein from various species including birds and reptiles (Wasserman, Fullmer and Taylor, 1977; Rhoten, Lubit and Christakos, 1984; Parmentier, Ghysens, Rypen, Lawson, Pasteeels, and Pochet, 1987). Consistent with previous reports documenting the presence of VDR throughout the nephron (Kawashima and Kurokawa, 1982), both OK and LLC-PK₁ cells exhibit ³H-1,25(OH)₂D₃ binding, although the level observed is significantly lower than that measured in MDBK cells (Fig. 1.2). However, the VDR number determined in LLC-PK₁ cells in our experiments is comparable to other reports in the literature (Colston and Feldman, 1982). These results suggests that OK and LLC-PK₁ cells represent proximal tubular cells, which lack calbindin D-28K despite the presence of VDR.

MDCK and MDBK cells were originally established from normal canine and normal bovine kidney respectively. MDCK cells have been described as a well-differentiated distal

tubular-like cell line retaining several characteristics of the distal nephron (Rindler et al, 1979; Valentich et al, 1981; Herzlinger et al, 1982), although other studies have suggested heterogeneity within and between MDCK cell cultures (Richardson, Scalera and Simmons, 1981; Valentich, 1981; Meier, Snavely, Brown, Brown and Insel, 1983), including the characteristics of at least two separate nephron tubule segments. Both MDCK and MDBK cell lines exhibit immunoreactivity towards the distal tubule marker protein, although the relative intensity is markedly greater in MDBK cells (Fig. 1.1a,b). This difference could reflect species differences in antibody recognition or depend on the sub-clone of MDCK cells used. In parallel with the expression of the distal tubule marker, calbindin D-28K immunoreactivity is observed as a single 28 kDa band in MDBK cells but is undetectable in MDCK cells (Fig. 1.3). Further, VDR expression in MDCK cells is the lowest of all four cell lines examined (Fig. 1.2). These findings are consistent with previous studies reporting the lack of specific $1,25(\text{OH})_2\text{D}_3$ -binding sites in MDCK cells (Colston and Feldman, 1982). Taken together these results suggest that although retaining some distal tubule characteristics, MDCK cells apparently lack the vitamin D-dependent pathway resulting in the expression of calbindin D-28K. In contrast, MDBK cells express both the VDR and calbindin D-28K, consistent with its distal tubule origin. MDBK cells also express the smaller renal vitamin D-dependent calcium-binding protein, calbindin D-9K (data not shown), which is also exclusively localized in the mammalian distal nephron *in vivo* (Taylor, Gleason and Lankford, 1984; Rhoten, Bruns and Christakos, 1985). These observations suggest that MDBK cells represent a distal tubular cell line expressing proteins known to be involved in vitamin D actions.

1.4.2 Calbindin D-28K and VDR regulation by serum and $1,25(\text{OH})_2\text{D}_3$ in MDBK cells.

Twenty-four hour serum-stimulation of MDBK cells results in induction of both VDR and calbindin D-28K expression (Figs. 1.5 and 1.6), corroborating earlier reports suggesting increased levels of these two vitamin D-dependent calcium-binding proteins in rapidly proliferating cells (Chen and Feldman, 1981; Chen et al, 1992). Although the increase in VDR numbers resulting from serum-stimulation has previously been demonstrated in other cell systems (Krishnan and Feldman, 1991a), this is the first report of calbindin D-28K induction by serum-stimulation. The mechanism by which serum induces calbindin D-28K expression is unknown but may reflect an increased sensitivity to $1,25(\text{OH})_2\text{D}_3$ due to the elevated numbers of VDRs.

Both calbindin D-28K and VDR were up-regulated within 24 hours after a single dose of 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ (Figs. 1.7, 1.8 and 1.9). This dose of $1,25(\text{OH})_2\text{D}_3$ was previously shown to induce these vitamin D-dependent proteins in several systems (Chen et al, 1992; Craviso et al, 1987; Mayel-Afshar et al, 1988; Wiese et al, 1992; Krishnan and Feldman, 1992; van Leeuwen et al, 1992a). Quantitatively, the calbindin D-28K induction observed in MDBK cells (1.6 fold) is comparable to that reported *in vivo* (less than 3.0 fold) (Sonnenberg, Pansini and Christakos, 1984) and in primary cultures of rat and chick kidney cells (1.3-2.0 fold) (Craviso et al, 1987; Chen et al, 1992). In contrast to primary culture models, calbindin D-28K-specific immunoreactivity is detectable and induced by $1,25(\text{OH})_2\text{D}_3$ in every MDBK cell. Although calbindin D-28K is generally defined as a cytosolic calcium-binding protein, the lack of cell culture systems which express calbindin D-28K has precluded

extensive investigation into its subcellular localization. In MDBK cells, immunoreactive calbindin D-28K is predominantly detected in the cytosol with a clear peri-nuclear distribution. However, consistent with previous reports, calbindin D-28K is also found in the nucleus, more prominently in $1,25(\text{OH})_2\text{D}_3$ -treated cells (Fig. 1.7). Purification of subcellular fractions confirms the presence of calbindin D-28K in the cytosol of MDBK cells with some expression also evident in the nucleus (Fig. 1.4). The significance of nuclear calbindin D-28K is unclear, and may be a consequence of passive diffusion through the nuclear pore. These findings identify the MDBK cell line as a novel renal cell model which expresses calbindin D-28K in a vitamin D-dependent manner.

1.4.3 Effects of serum and $1,25(\text{OH})_2\text{D}_3$ on MDBK cell proliferation

As previously mentioned, rapidly proliferating cells display elevated levels of calbindin D-28K and VDR, suggesting the possibility that the rise in calbindin D-28K and VDR expression in serum-stimulated cells may reflect an increase in cellular proliferation. Serum, an established proliferative agent, is thought to regulate cell proliferation by inducing the levels or activity of mitogenic proteins such as proto-oncogenes (Treisman, 1992). Consistent with these observations, stimulation of MDBK cells with serum increased the proliferation rate, as compared to serum-starved cells. However, $1,25(\text{OH})_2\text{D}_3$, also a known regulator of cellular proliferation and differentiation (Suda et al, 1990), did not alter the cell cycle kinetics of MDBK cells, compared to vehicle-treated cells (Fig. 1.10 and Table 1.1). Using epidermal growth factor (EGF) and serum-substitute (ITS) treatments we have reported the dissociation of calbindin D-28K and VDR induction from increased cellular proliferation

(Gagnon, Donepudi and Welsh, in preparation), suggesting that the increased expression of calbindin D-28K and VDR in response to serum stimulation is not related to enhanced cellular proliferation. Instead, serum may act by sensitizing the cells to $1,25(\text{OH})_2\text{D}_3$, increasing VDR levels or altering an unidentified factor involved in calbindin D-28K regulation. Serum is composed of a variety of hormones, growth factors and other agents which may independently or synergistically influence the levels of VDR and calbindin D-28K. Further investigation of the promoter region of both the calbindin D-28K and VDR genes may reveal specific response elements involved in the regulation of these proteins by serum. On the other hand, the effects of $1,25(\text{OH})_2\text{D}_3$ on calbindin D-28K and VDR levels in MDBK cells, which are also mediated independently of cell cycle kinetics, are thought to involve a specific direct effect of the hormone, as demonstrated in various systems (Chen et al, 1992; Costa et al, 1985).

1.5 CONCLUSIONS

The results described in this chapter identify a novel *in vitro* renal cell model suitable for the study of calbindin D-28K regulation by $1,25(\text{OH})_2\text{D}_3$. MDBK cells are an established renal epithelial cell line which display distal tubular characteristics, including the expression of the VDR and calbindin D-28K in a vitamin D-responsive manner. Other conclusions derived from these findings include:

- Serum stimulation of cell proliferation is accompanied by increased expression of calbindin D-28K and VDR in MDBK cells.

- $1,25(\text{OH})_2\text{D}_3$ induction of calbindin D-28K and VDR is not related to alterations in cell cycle kinetics, suggesting a direct regulation by the hormone.

CHAPTER 2: CHARACTERIZATION OF CALBINDIN D-28K

REGULATION BY $1,25(\text{OH})_2\text{D}_3$ IN MDBK CELLS

2.1 INTRODUCTION

Calbindin D-28K was first described as an intestinal calcium-binding activity induced in the presence of $1,25(\text{OH})_2\text{D}_3$ (Wasserman and Taylor, 1966). Since its discovery, calbindin D-28K has been localized in various tissues, both classical and non-classical targets of vitamin D. The vitamin D-dependence of calbindin D-28K expression has also been demonstrated in kidneys (Christakos and Norman, 1980; Thomasset et al, 1982) and bone (Christakos, Friedlander, Frandsen and Norman, 1979; Mouc, Holt, Brehier, Lomri and Thomasset, 1988). However, uterine and neuronal calbindin D-28K which are induced by estrogen and nerve growth factor respectively, appear unresponsive to $1,25(\text{OH})_2\text{D}_3$ (Bar, Rosenberg and Hurwitz, 1984; Pasteels, Pochet, Surardt, Hubeau, Chimoaga, Parmentier and Lawson, 1986), suggesting tissue-specific regulation of calbindin D-28K.

In the kidney, calbindin D-28K levels generally reflect the vitamin D status of the organism albeit, unlike intestinal cells, renal distal tubules constitutively express calbindin D-28K under conditions of vitamin D-deficiency (Christakos and Norman, 1980; Craviso et al, 1987). The induction of renal calbindin D-28K by $1,25(\text{OH})_2\text{D}_3$ has been characterized to be dose- and time-dependent both *in vivo* (Pansini and Christakos, 1984) and in primary cultures (Enomoto, Hendy, Andrews and Clemens, 1992). However, investigations into the mechanism underlying this regulation have been limited due to the lack of an established renal

cell line expressing calbindin D-28K in a vitamin D-dependent manner. Having demonstrated the vitamin D-dependence of calbindin D-28K expression in MDBK cells, we next focused on the characterization of the mechanism of action of $1,25(\text{OH})_2\text{D}_3$.

2.1.1 Calbindin D-28K gene

The calbindin D-28K gene is approximately 18.5 kilobases long and is divided into 11 coding exons. Analysis of the coding sequences of mammalian (rat, mouse and human) calbindin D-28K revealed 79% identity with that of the chicken (Parmentier et al, 1987a; Hunziker and Schriecke!, 1988; Wood, Kobayashi, Franz, Varghese, Christakos and Tobin, 1988; Boland, Minghetti, Lowe and Norman, 1991); however, little homology was found between the extensive mammalian 5' and 3' untranslated regions and their chick counterparts (Parmentier, 1989). Common regulatory sequences have been found in the promoter region of mammalian and avian calbindin D-28K genes, including TATA box and CAT box sequences (Ferrari, Drusiani, Battini and Fregni, 1988).

Putative VDREs have also been described in chicken and mouse calbindin D-28K genes. The putative mammalian VDREs consist of two direct repeats of the steroid hormone element separated by three (DR3) (Takeda et al, 1994) or four (DR4) nucleotides (Christakos et al, 1992). These sequences are fairly consistent with previously reported VDREs which usually have three or four nucleotides separating the two direct repeats (Lian and Stein, 1992; Rhodes, Chen, DiMattia, Scully, Kalla, Lin, Yu, Rosenfeld, 1993). The vitamin D-responsiveness of the mouse calbindin D-28K VDRE (DR3) has been demonstrated in MDBK cells transfected with discrete promoter regions (Takeda et al, 1994). The lack of

1,25(OH)₂D₃-dependence of these promoters in other cell lines (Ferrari et al, 1988) suggest a requirement for other tissue-specific factors in renal calbindin D-28K gene transcription. The 3' untranslated region of the calbindin D-28K gene is quite extensive and contains several simple repeated sequences which may represent regulatory signals (Parmentier, 1989). In addition, other hormone response-elements, specific for estrogen and glucocorticoid, have been identified in the calbindin D-28K promoter and may be responsible for the modulation of calbindin D-28K expression by these hormones in specific tissues (Christakos et al, 1989). Thus, additional signals may act either independently or in conjunction with 1,25(OH)₂D₃ to regulate calbindin D-28K mRNA stability and/or translation processes, as well as transcription.

2.1.2 Transcriptional regulation of renal calbindin D-28K by 1,25(OH)₂D₃

Administration of 1,25(OH)₂D₃ *in vivo* or to primary cultures induces renal calbindin D-28K in a time- and dose-dependent manner (Thomasset et al, 1982; Christakos, Rhoten and Feldman, 1987; Craviso et al, 1987; Chen et al, 1992). The long lag time between maximal induction of calbindin D-28K mRNA and maximal calbindin D-28K expression in response to 1,25(OH)₂D₃ implicates both transcriptional and post-transcriptional regulatory components. Transcriptionally, 1,25(OH)₂D₃ mediates its effects by binding to the VDR which then interacts with specific VDREs located in the promoter region of vitamin D-dependent genes. As previously mentioned, putative VDREs have been identified in the promoter region of the chicken (Ferrari et al, 1988; Boland et al, 1991; MacDonald, Whitfield, Haussler, Hocker, Haussler and Komm, 1992) and mouse (Christakos et al, 1992;

Takeda et al, 1994) calbindin D-28K genes.

In rat kidney, steady-state levels of calbindin D-28K mRNA are induced within two hours of treatment with $1,25(\text{OH})_2\text{D}_3$, reaching maximum levels within 12 hours, after which calbindin D-28K mRNA levels start to decline (Varghese et al, 1988; Enomoto et al, 1992). Significant increases in calbindin D-28K protein are observed after four hours of treatment with $1,25(\text{OH})_2\text{D}_3$, but maximal induction occurs within 24 to 48 hours (Craviso et al, 1987; Varghese et al, 1988). In contrast with calbindin D-28K mRNA regulation, the induced expression of calbindin D-28K is sustained for up to 72 hours after exposure to a single dose of $1,25(\text{OH})_2\text{D}_3$ (Craviso et al, 1987; Varghese et al, 1988; Chen et al, 1992). This temporal inconsistency between calbindin D-28K mRNA levels and expression in response to $1,25(\text{OH})_2\text{D}_3$ suggests the involvement of post-transcriptional mechanisms in the regulation of renal calbindin D-28K.

2.1.3 Post-transcriptional regulation of renal calbindin D-28K by $1,25(\text{OH})_2\text{D}_3$

Several reports demonstrate increased mRNA stability of target genes in response to steroid hormones (Nielsen and Shapiro, 1990; Vercaeren, Winderickx, Devos, Peeters and Heyns, 1992). Vitamin D-induced increases in calbindin D-28K mRNA stability have also been suggested as a regulatory mechanism, but studies have demonstrated that $1,25(\text{OH})_2\text{D}_3$ does not alter the half-life of calbindin D-28K transcripts in chick primary renal cultures (Enomoto et al, 1992). Altered translation rates of calbindin D-28K mRNA could explain the lag time between peak induction of calbindin D-28K mRNA and expression, but this possibility remains to be investigated. The sustained induction of calbindin D-28K expression,

in contrast to its mRNA levels, may reflect an increase in calbindin D-28K stability in response to $1,25(\text{OH})_2\text{D}_3$. This may reflect direct post-translational modifications to calbindin D-28K itself or association with additional proteins, such as microtubules (Nemere, Opperman, Ross and Norman, 1992).

Studies in vitamin D-replete chick intestine (Theofan and Norman, 1986) and renal primary cultures (Enomoto et al, 1992) have demonstrated that the induction of calbindin D-28K expression by $1,25(\text{OH})_2\text{D}_3$ is blocked by cycloheximide pre-treatment, implying the importance of *de novo* protein synthesis in the induction of calbindin D-28K by $1,25(\text{OH})_2\text{D}_3$. These results, however, fail to reveal the precise mechanism by which $1,25(\text{OH})_2\text{D}_3$ might post-transcriptionally regulate calbindin D-28K.

To further validate the use of the MDBK cell line as a model to investigate vitamin D-dependent calbindin D-28K regulation, we characterized the induction of calbindin D-28K by $1,25(\text{OH})_2\text{D}_3$ in these cells. Our results demonstrate a time- and dose-dependent effect of $1,25(\text{OH})_2\text{D}_3$ on calbindin D-28K in MDBK cells. As previously shown in renal primary cultures (Craviso et al, 1987), cycloheximide pre-treatment blocks the vitamin D-dependent induction of calbindin D-28K in MDBK cells. We also report that EB 1089, a non-calcemic vitamin D analog, induces calbindin D-28K in MDBK cells in a time- and dose-dependent manner.

2.2 METHODS

2.2.1 Cell culture

MDBK cells obtained from ATCC were grown in DMEM supplemented with 10% newborn calf serum. Confluent cells were changed to serum free media for 24 hours prior to treatment with up to 10^{-5} M $1,25(\text{OH})_2\text{D}_3$, up to 10^{-7} M EB 1089 (kindly provided by Lise Binderup, Leo Pharmaceuticals, Ballerup, Denmark) or ethanol vehicle (0.05%) for up to 72 hours. In some cases, cells were pre-treated for 2 hours with cycloheximide (2 μM) prior to treatment with 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ or ethanol for up to 24 hours. This concentration of cycloheximide blocked > 60% of protein synthesis without affecting RNA synthesis. Cells were harvested and fractions prepared as described in section 1.2.1.

2.2.2 Immunoblotting

Proteins from cytosolic fractions (for calbindin D-28K) or total homogenates (for VDR) were separated on 12% SDS-PAGE and transferred electrophoretically to nitrocellulose. Immunoblotting was performed as previously described in 1.2.3 using monoclonal mouse anti-calbindin D-28K (Sigma) or monoclonal rat anti-VDR antibodies (Chemicon, Temecula, California), diluted 1:200 and 1:100 respectively, in blocking solution. Membranes were washed and incubated with alkaline phosphatase-conjugated goat anti-mouse (for calbindin D-28K) or goat anti-rat (for VDR) antibodies (diluted 1:7500) (Promega, Madison, Wisconsin, USA) for one hour at room temperature. The colorimetric product was obtained by incubation of the membranes with the substrates nitro blue

tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Promega). Alternatively, biotinylated secondary antibodies (1:1000) (Jackson ImmunoResearch Laboratories) were used, followed by incubation with streptavidin peroxidase. In these cases, immunoreactivity was detected with 4-chloro-1-naphthol (625 µg/ml) in PBS containing 20% methanol and 0.12% H₂O₂. Non-specific binding, assessed in the absence of primary antibodies, was negligible.

2.2.3 ELISA for calbindin D-28K

Serum-free MDBK cells were treated for up to 24 hours with 10⁻⁷ M 1,25(OH)₂D₃ or ethanol vehicle. In some case, cells were pre-incubated with 2 µM cycloheximide prior to the indicated treatments. Calbindin D-28K levels in cytosolic fractions of MDBK cells were quantitated by ELISA, based on the method of Miller and Norman (1983), as described in section 1.2.4.

2.2.4 1,25(OH)₂D₃-binding assay

High salt fractions of MDBK cells treated for 24 hours with 10⁻⁷ M 1,25(OH)₂D₃, 10⁻⁹ M EB 1089 or ethanol vehicle (0.05%) were prepared and incubated with 0.5 nM [³H]-1,25(OH)₂D₃ for 1 hour at 37°C. Bound and free hormone were separated and specific [³H]-1,25(OH)₂D₃-binding was determined as described in 1.2.6.

2.2.5 Northern analysis

Serum-free MDBK cells were treated for up to 48 hours with 10⁻⁷ M 1,25(OH)₂D₃ and

RNA was extracted using TRIZOL, following the manufacturer's instructions (Life Technologies). RNA (up to 25 µg/lane) was electrophoresed on 1% agarose gels under denaturing conditions (6.7% formaldehyde). Gels were stained with ethidium bromide and photographed as a control for loading. RNA was then electrophoretically transferred to Photogene nylon membranes (Life technologies) and hybridized with ³²P-labeled rat calbindin D-28K cDNA (kindly provided by Dr. Monique Thomasset, Institut National de la Santé et de la Recherche Médicale, Le Vésinet, France) or human tubulin cDNA (kindly provided by Dr. Martin Tenniswood, W. Alton Jones Cell Science Center, Lake Placid, New York). After a final wash (0.2 x SSC/0.1% SDS, at 50°C), membranes were exposed for up to 5 days to Kodak X-OMAT autoradiography film.

Calbindin D-28K and tubulin cDNAs were labeled to high specific activity with ³²P-dCTP using random primer reactions (Rediprime kit, Amersham) based on the method of Feinberg and Vogelstein (1983 and 1984).

2.2.6 Miscellaneous

Protein concentrations were determined by the method of Bradford (1976). Experiments were performed at least three times unless otherwise stated. Data are expressed as mean ± SEM unless otherwise stated. Statistical differences were assessed using Student's *t*-test for unpaired data or the Bonferroni procedure with a significance level of $P < 0.05$, as appropriate.

2.3 RESULTS

2.3.1 Dose- and time-dependence of calbindin D-28K induction by $1,25(\text{OH})_2\text{D}_3$ in MDBK cells

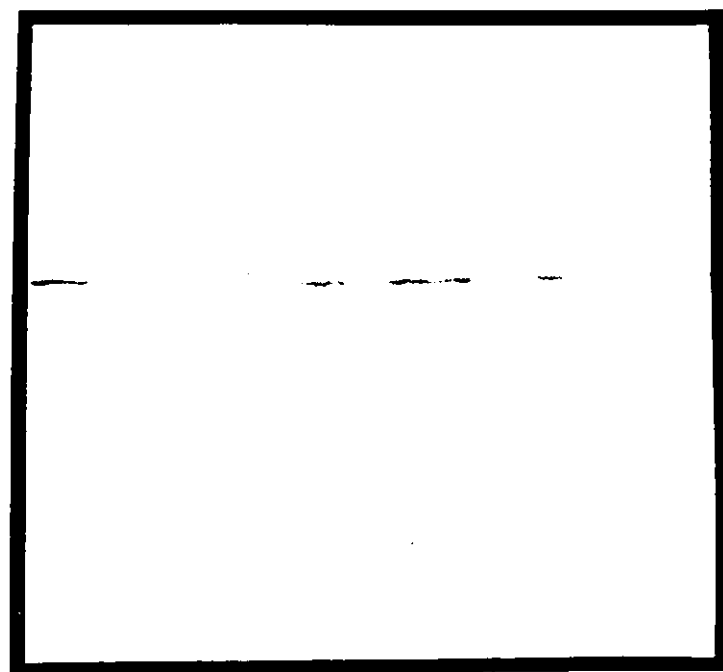
Concentrations as low as 10^{-9} M $1,25(\text{OH})_2\text{D}_3$ induce calbindin D-28K levels within 24 hours of treatment, and maximal induction is observed in the presence of 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ (Fig. 2.1). As previously demonstrated in other systems, $1,25(\text{OH})_2\text{D}_3$ -induction of calbindin D-28K is not only dose-dependent but also time-dependent (Chen et al, 1992). In MDBK cells, western blotting analysis demonstrates elevated levels of calbindin D-28K within 6 hours of treatment with 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ (Fig. 2.2). ELISA studies were performed to quantitate the effect of $1,25(\text{OH})_2\text{D}_3$ on calbindin D-28K expression. These results reveal significant increases in calbindin D-28K levels after 8 and 24 hours of treatment with $1,25(\text{OH})_2\text{D}_3$, compared to ethanol-treated cells (Fig. 2.3). The levels of calbindin D-28K in vehicle-treated cells do not significantly vary over the 24 hour experiment. The vitamin D-dependent induction of calbindin D-28K in MDBK cells appears to be sustained for up to 72 hours in MDBK cells (Fig. 2.4), although quantitative analysis (ELISA) would be necessary to assess the statistical significance of the induction at each time point. The temporal effect of $1,25(\text{OH})_2\text{D}_3$ on calbindin D-28K expression in MDBK cells is consistent with vitamin D-dependent induction of calbindin D-28K in other systems.

Consistent with results reported in other systems, MDBK cells exhibit very low to undetectable levels of calbindin D-28K mRNA in the absence of $1,25(\text{OH})_2\text{D}_3$ (Fig. 2.5, zero time point). In MDBK cells, only one calbindin D-28K transcript is detected at 2.8 kb.

Figure 2.1 *Calbindin D-28K expression in response to increasing concentrations of 1,25(OH)₂D₃ in MDBK cells.*

Serum-free MDBK cells were treated for 24 hours with up to 10^{-5} M 1,25(OH)₂D₃ or ethanol vehicle (0.05%). Cytosolic proteins (100 µg/lane) were separated on 12% SDS-PAGE, transferred to nitrocellulose, and antigenic sites were saturated. Membranes were incubated with monoclonal calbindin D-28K antibodies, followed with biotinylated secondary antibodies and streptavidin peroxidase as described in Methods. *Lane 1*, ethanol; *lane 2*, 10^{-9} M 1,25(OH)₂D₃; *lane 3*, 10^{-8} M 1,25(OH)₂D₃; *lane 4*, 10^{-7} M 1,25(OH)₂D₃; *lane 5*, 10^{-6} M 1,25(OH)₂D₃; *lane 6*, 10^{-5} M 1,25(OH)₂D₃.

28 Kd →



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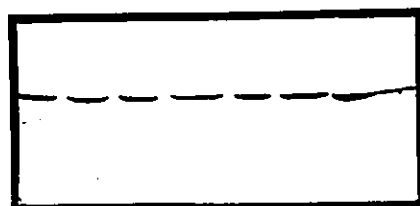
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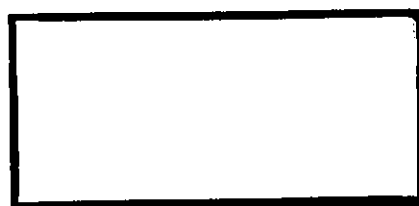
Figure 2.2 *Temporal effect of 1,25(OH)₂D₃ on calbindin D-28K immunoreactivity in MDBK cells.*

Scrum-free MDBK cells were treated for up to 24 hours with 10^{-7} M 1,25(OH)₂D₃ (top) or ethanol vehicle (bottom). Cytosolic proteins (100 µg/lane) were separated on 12% SDS-PAGE, transferred to nitrocellulose and antigenic sites were saturated. Membranes were incubated with monoclonal calbindin D-28K antibodies, followed by biotinylated secondary antibodies and streptavidin peroxidase as described in Methods. *Lane 1*, 0 hour; *lane 2*, 1 hour; *lane 3*, 2 hours; *lane 4*, 4 hours; *lane 5*, 6 hours; *lane 6*, 8 hours; *lane 7*, 12 hours; and *lane 8*, 24 hours.



1 2 3 4 5 6 7 8

← CALBINDIN D-28K



1 2 3 4 5 6 7 8

← CALBINDIN D-28K

Figure 2.3 *Temporal effect of 1,25(OH)₂D₃ on cytosolic calbindin D-28K levels in MDBK cells, as assessed by ELISA.*

Scrum-free MDBK cells were treated for up to 24 hours with 10^{-7} M 1,25(OH)₂D₃ in ethanol or vehicle. Cytosolic levels of calbindin D-28K were measured by ELISA as described in **Methods**. Data represent the mean $\pm$ SEM ($N = 3$ to 5) of calbindin D-28K ($\mu\text{g}/100$ mg protein). Calbindin D-28K was significantly ($P < 0.05$) higher in 1,25(OH)₂D₃-treated cells compared to vehicle-treated cells at 8 and 24 hours, as determined by Student's *t*-test for unpaired data at each time point.

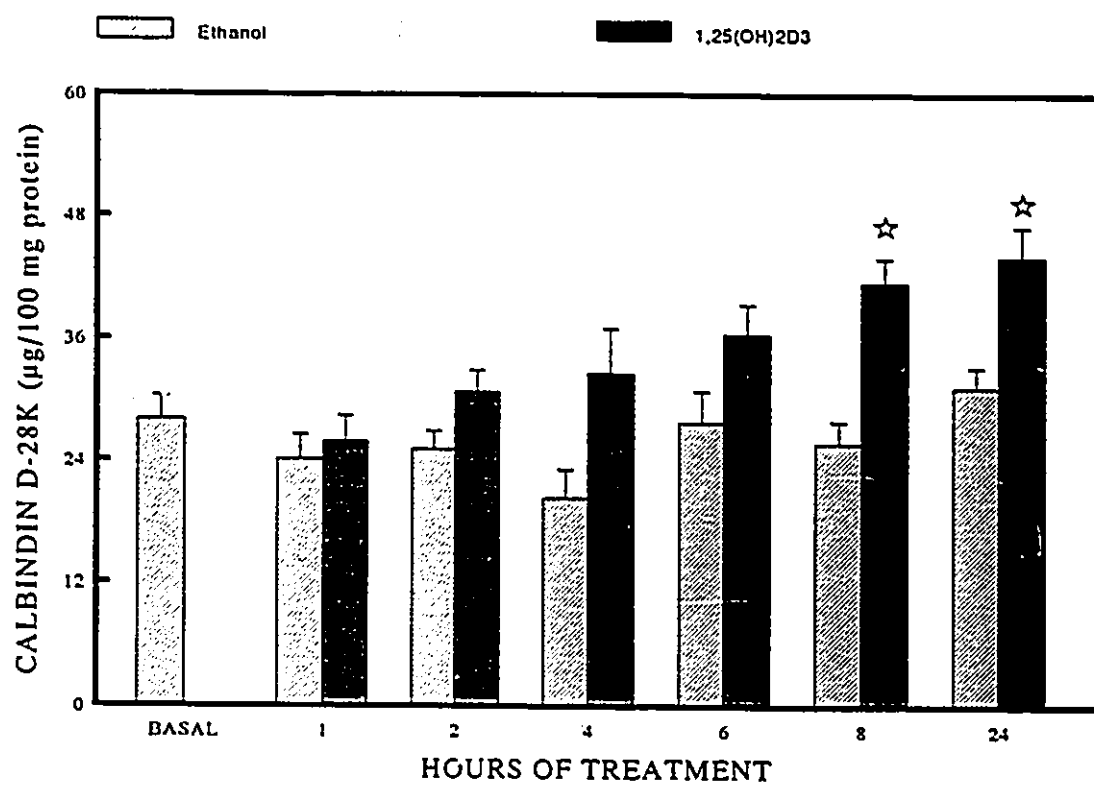


Figure 2.4 *Long term induction of calbindin D-28K immunoreactivity in response to 1,25(OH)₂D₃ in MDBK cells.*

Scrum-free MDBK cells were treated for up to 72 hours with 10^{-7} M 1,25(OH)₂D₃ or ethanol vehicle. Cytosolic proteins (50 µg/lane) were separated on 12% SDS-PAGE, transferred to nitrocellulose and antigenic sites were saturated. Membranes were incubated with monoclonal calbindin D-28K antibodies, followed with alkaline phosphatase-conjugated secondary antibodies as described in Methods. *Lane 1*, ethanol; *lane 2*, 24 hours with 1,25(OH)₂D₃; *lane 3*, 48 hours with 1,25(OH)₂D₃; *lane 4*, 72 hours with 1,25(OH)₂D₃.

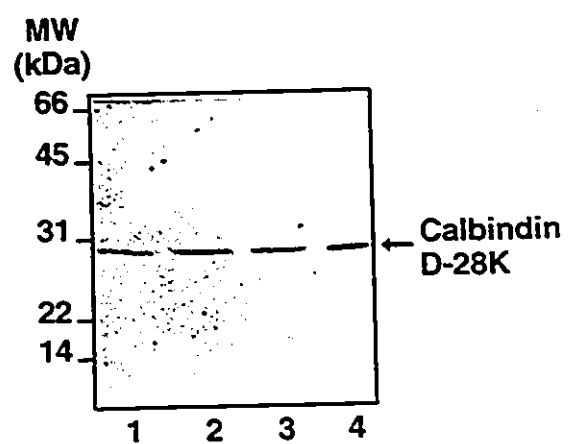
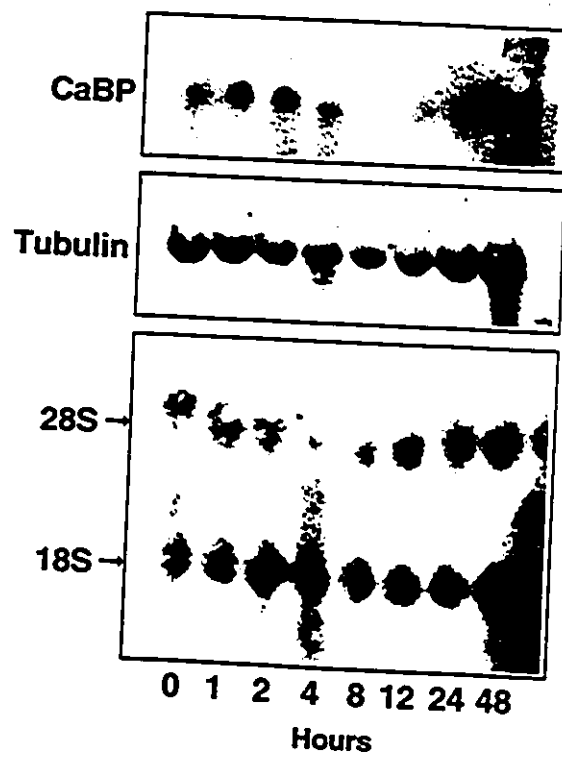


Figure 2.5 *Temporal effect of 1,25(OH)₂D₃ on calbindin D-28K and tubulin mRNAs in MDBK cells.*

Serum-free MDBK cells were treated for the indicated times with 10^{-7} M 1,25(OH)₂D₃ in ethanol. RNA was extracted as described, electrophoresed on 1% denaturing agarose gel and stained with ethidium bromide (bottom). RNA was electrophoretically transferred to nylon membranes and probed with a ³²P-labeled rat calbindin D-28K or human tubulin cDNAs as described in Methods. Bands were visualized by autoradiography after a five day exposure. *N* = 1.



Treatment with $1,25(\text{OH})_2\text{D}_3$ increases calbindin D-28K mRNA levels in a biphasic manner, with a rapid induction within 1 to 2 hours of treatment followed by a decrease to control levels and a second induction after 24 to 48 hours of treatment with $1,25(\text{OH})_2\text{D}_3$ (Fig. 2.5). Steady-state levels of tubulin mRNA are also increased in the presence of $1,25(\text{OH})_2\text{D}_3$.

2.3.2 Dose- and time-dependence of calbindin D-28K induction by EB 1089 in MDBK cells

EB 1089 is a biologically active analogue of $1,25(\text{OH})_2\text{D}_3$ which has been extensively tested for its growth regulating properties. EB 1089 has been shown to be ten to sixty times more potent than $1,25(\text{OH})_2\text{D}_3$ in inhibiting the growth of cancer cells, while having little effect on calcium metabolism (Mathiasen et al, 1993). Since studies on the effect of this analogue on the classical functions of vitamin D are scarce, we examined the effect of EB 1089 on calbindin D-28K, a classical target of $1,25(\text{OH})_2\text{D}_3$. As observed with $1,25(\text{OH})_2\text{D}_3$, EB 1089 stimulates calbindin D-28K expression in a dose-dependent manner, maximally inducing calbindin D-28K at a concentration of 10^{-9} M, which is 100-fold less than the dose required for maximal effect with $1,25(\text{OH})_2\text{D}_3$ (Fig. 2.6). The time course of induction of calbindin D-28K by EB 1089 is comparable to that previously demonstrated with $1,25(\text{OH})_2\text{D}_3$, increasing within 4-8 hours and lasting for up to 24 hours after exposure to a single 10^{-9} M dose of EB 1089 (Fig. 2.7). The increase in calbindin D-28K expression in response to either EB 1089 or $1,25(\text{OH})_2\text{D}_3$, is accompanied by increases in VDR numbers and expression, which are evident after 24 hours of treatment (Fig. 2.8). Previous time course studies in MDBK cells have demonstrated a significant, but transient up-regulation of

Figure 2.6 *Calbindin D-28K expression in response to increasing concentrations of EB 1089 in MDBK cells.*

Scrum-free MDBK cells were treated for 24 hours with up to 10^{-8} M EB 1089 or ethanol. Cytosolic proteins (100 μ g/lane) were separated on 12% SDS-PAGE, transferred to nitrocellulose and antigenic sites were saturated. Membranes were incubated with monoclonal calbindin D-28K antibodies, followed by alkaline phosphatase-conjugated secondary antibodies as described in Methods. *Lane 1*, ethanol; *lanes 2-5*, increasing concentrations of EB 1089, as indicated.

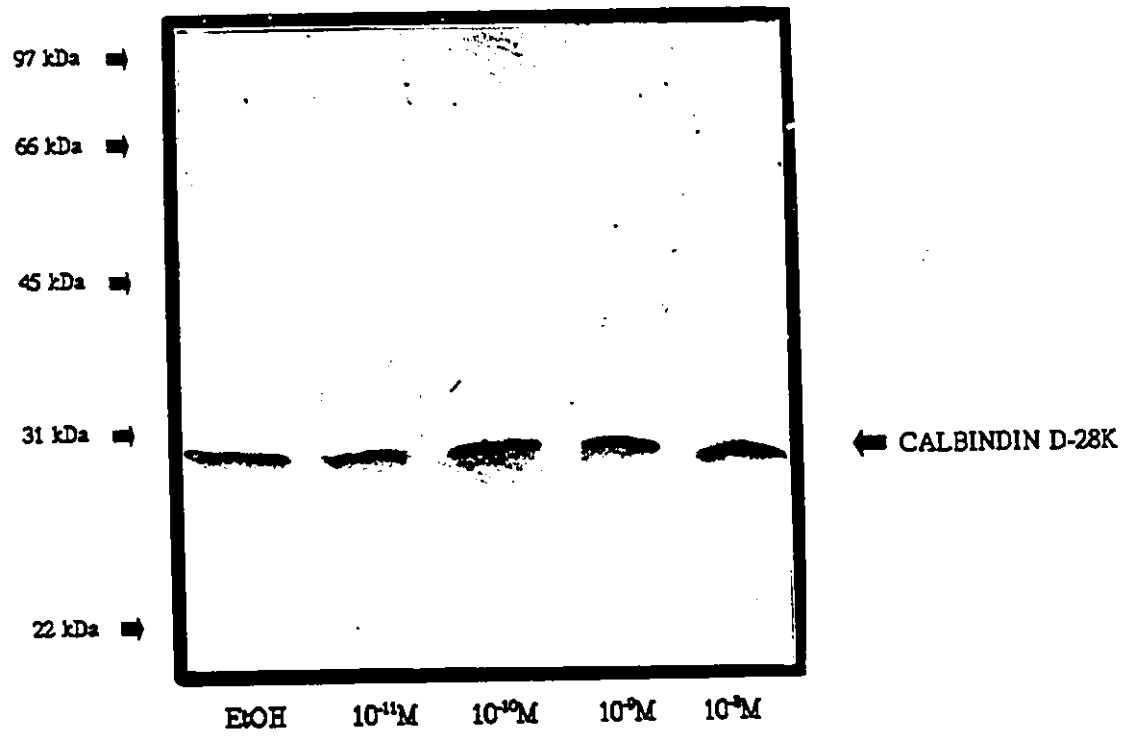


Figure 2.7 Temporal effect of EB 1089 on calbindin D-28K immunoreactivity in MDBK cells.

Serum-free MDBK cells were treated for the indicated times with 10^{-9} M EB 1089. Cytosolic proteins (100 μ g/lane) were separated on 12% SDS-PAGE, transferred to nitrocellulose and antigenic sites were saturated. Membranes were incubated with monoclonal calbindin D-28K antibodies, followed by alkaline phosphatase-conjugated secondary antibodies as described in Methods.

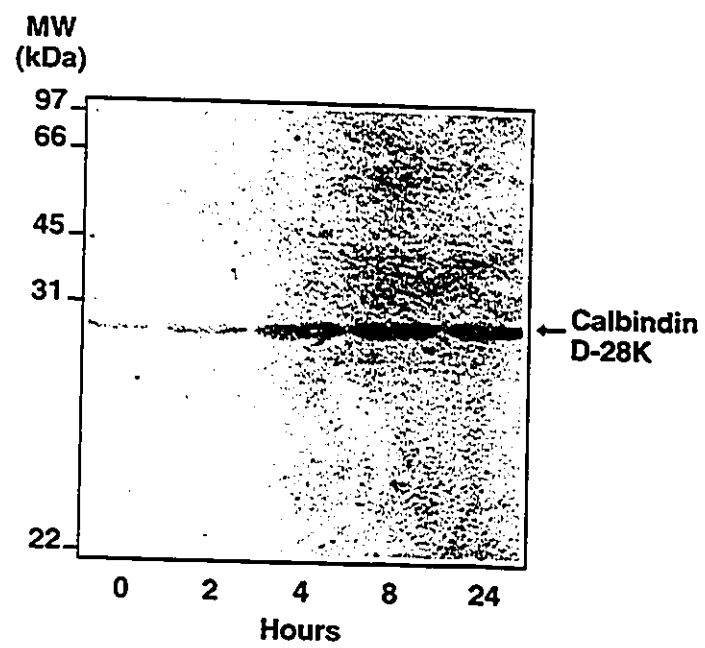
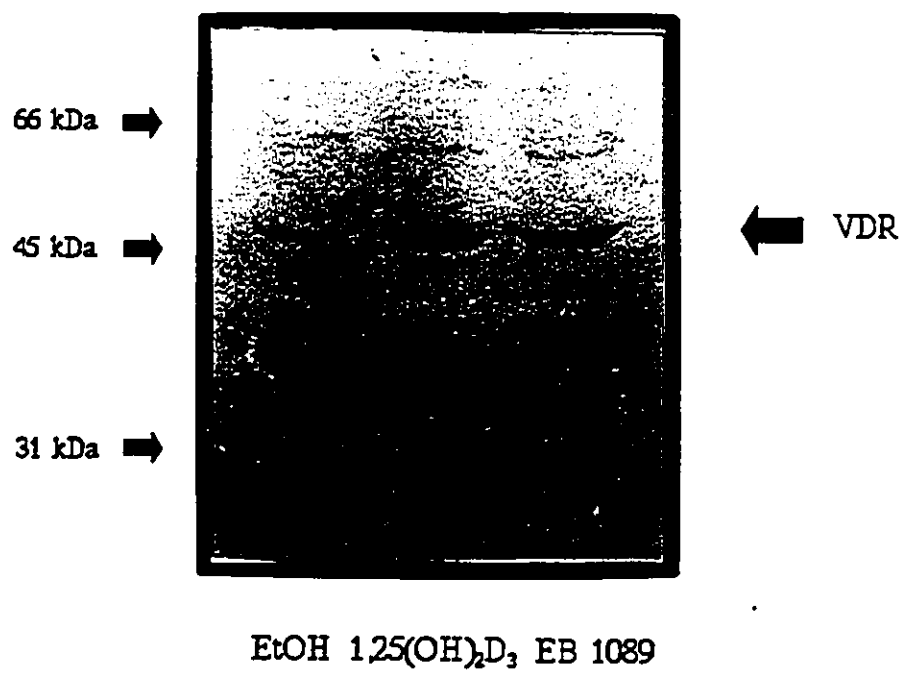
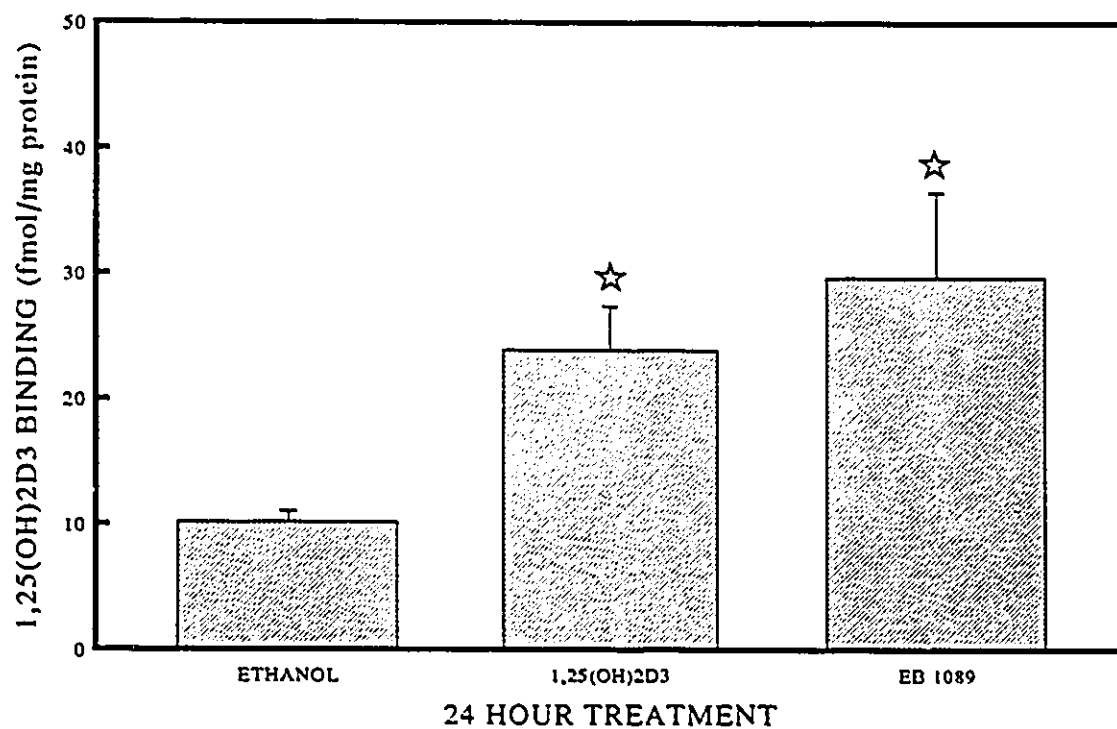


Figure 2.8 *Effects of 1,25(OH)₂D₃ and EB 1089 on VDR expression in MDBK cells.* Serum-free MDBK cells were treated for 24 hours with either ethanol vehicle, 10⁻⁷ M 1,25(OH)₂D₃ or 10⁻⁹ M EB 1089. **Top:** Cells were harvested by scraping and high salt extracts were incubated with 0.5 nM ³H-1,25(OH)₂D₃ overnight at 4°C as described in **Methods**. Data are expressed as mean ± SEM (*N* = 4) of specific ³H-1,25(OH)₂D₃ binding, obtained by subtraction of nonspecific binding from total binding. 1,25(OH)₂D₃-binding was significantly (*P* < 0.05) increased in cells treated with 1,25(OH)₂D₃ or EB 1089 as compared to ethanol-treated cells, as assessed by the Bonferroni procedure. **Bottom:** Total homogenates (200 µg protein/lane) were separated on 10% SDS-PAGE, transferred to nitrocellulose and antigenic sites were saturated. Membranes were incubated with monoclonal VDR antibodies, followed with alkaline phosphatase-conjugated antibodies as described in **Methods**. Non-specific immunoreactivity, assessed in the absence of primary antibodies, was negligible.



VDR numbers within 24 hours of treatment with $1,25(\text{OH})_2\text{D}_3$, which return to control levels within 48 hours of treatment (Simboli-Campbell, 1993).

2.3.3 $1,25(\text{OH})_2\text{D}_3$ -dependent post-transcriptional regulation of calbindin D-28K in MDBK cells

Calbindin D-28K regulation by $1,25(\text{OH})_2\text{D}_3$ involves both transcriptional and post-transcriptional mechanisms. Attenuation of $1,25(\text{OH})_2\text{D}_3$ -stimulated calbindin D-28K expression by protein synthesis inhibitors has been demonstrated both *in vivo* and in primary cultures of renal cells. The role of *de novo* protein synthesis in calbindin D-28K regulation by $1,25(\text{OH})_2\text{D}_3$ was investigated using pre-treatment of MDBK cells with 2 μM cycloheximide followed by exposure to $1,25(\text{OH})_2\text{D}_3$ or vehicle for up to 24 hours. In both vehicle- and $1,25(\text{OH})_2\text{D}_3$ -treated cells, cycloheximide slightly reduced calbindin D-28K expression below that of ethanol-treated cells (Fig. 2.9). These results were confirmed by ELISA, revealing significant decreases in calbindin D-28K levels in MDBK cells treated with cycloheximide, regardless of the vitamin D status (Fig 2.9). Our data, collectively with that of other groups, strongly suggest that calbindin D-28K expression is at least partially regulated post-transcriptionally, possibly through the induction by $1,25(\text{OH})_2\text{D}_3$ of additional proteins.

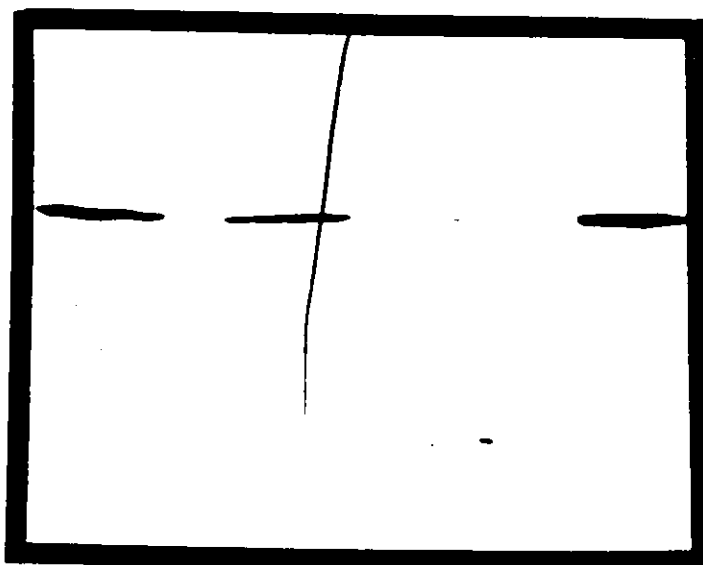
Figure 2.9 *Effect of cycloheximide pre-treatment on calbindin D-28K induction by 1,25(OH)₂D₃ in MDBK cells.*

Scrum-free MDBK cells were pre-treated for 2 hours with 2 μ M cycloheximide in PBS or PBS alone, and treated with 10^{-7} M 1,25(OH)₂D₃ for 24 hours. Top: Cytosolic proteins (100 μ g/lane) were separated on 12% SDS-PAGE, transferred to nitrocellulose and antigenic sites were saturated. Membranes were incubated with monoclonal calbindin D-28K antibodies, followed with biotinylated secondary antibodies and streptavidin peroxidase as described in Methods. Lane 1, vehicle; lane 2, cycloheximide; lane 3, 1,25(OH)₂D₃ and cycloheximide; and lane 4, 1,25(OH)₂D₃. Bottom: Cytosolic levels of calbindin D-28K were measured by ELISA as described in Methods. Data represent mean $\pm$ SEM ($N = 3$) of calbindin D-28K (μ g/100 mg protein) and statistical significance ($P < 0.05$) between two groups were determined by the Bonferroni procedure.

^a $P < 0.05$ compared to control values

^b $P < 0.05$ compared to 1,25(OH)₂D₃ values

28 kD →

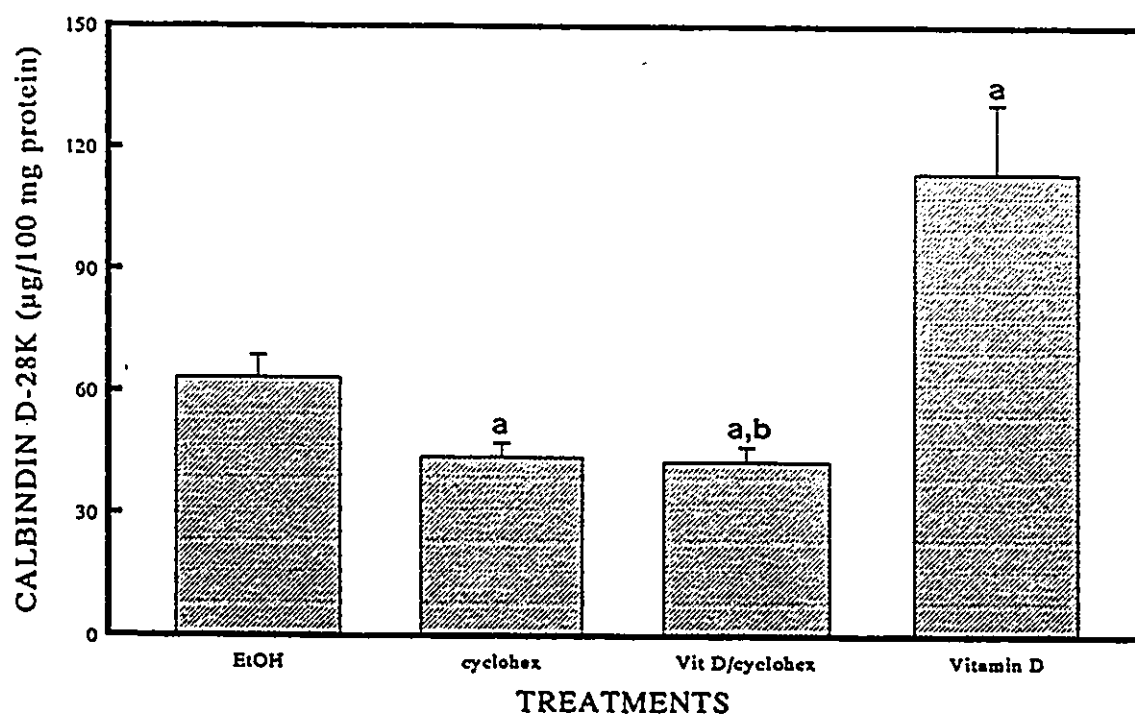


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2.4 DISCUSSION

Few studies have examined the regulation of renal calbindin D-28K by $1,25(\text{OH})_2\text{D}_3$ due to the lack of a suitable *in vitro* system. We have described that MDBK cells express calbindin D-28K and VDR in a vitamin D-responsive manner. We now further characterize this model as a valuable tool to investigate the molecular regulation of calbindin D-28K by $1,25(\text{OH})_2\text{D}_3$.

2.4.1 Induction of calbindin D-28K by $1,25(\text{OH})_2\text{D}_3$ in MDBK cells

Calbindin D-28K induction by $1,25(\text{OH})_2\text{D}_3$ is evident upon exposure to concentrations as low as 10^{-9} M for 24 hours, with a maximal effect observed with 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ (Fig. 2.1). A similar dose response was described in avian primary cultures (Craviso et al, 1987). Although this dose of $1,25(\text{OH})_2\text{D}_3$ does not reflect physiological levels, many effects of this hormone *in vitro*, including osteocalcin and osteopontin production and inhibition of *c-myc* expression (Noda, Vogel, Craig, Prah, DeLuca and Denhardt, 1990; Evans, Russell, Brown and Dobson, 1989; Simpson et al, 1989), are maximally observed with 10^{-7} M $1,25(\text{OH})_2\text{D}_3$. The requirement for higher doses of $1,25(\text{OH})_2\text{D}_3$ in these studies is unclear, but may represent decreased solubility or efficacy in transport and nuclear uptake. The observation that VDR binding to VDREs *in vitro* also requires elevated (10^{-7} M) concentrations of $1,25(\text{OH})_2\text{D}_3$ (Liao, Ozono, Sone, McDonnell and Pike, 1990) suggests a lack of solubility of the hormone in aqueous-based systems. *In vivo*, $1,25(\text{OH})_2\text{D}_3$ circulates bound to the vitamin D-binding protein (DBP) which may be

necessary for cellular entry or trafficking.

Consistent with results derived from both *in vivo* and in renal primary cultures (Craviso et al, 1987), calbindin D-28K induction by $1,25(\text{OH})_2\text{D}_3$ in MDBK cells is time-dependent. Increases in calbindin D-28K expression are evident within 6 hours and persist for up to 72 hours after a single dose of $1,25(\text{OH})_2\text{D}_3$ (Figs. 2.2, 2.3 and 2.4). We have also observed induction of calbindin D-28K mRNA steady-state levels in response to $1,25(\text{OH})_2\text{D}_3$ in MDBK cells (Fig. 2.5). However, the time course of induction observed in our studies differs from that reported by Takeda et al (1994) who demonstrated the induction of calbindin D-28K mRNA within 6 to 24 hours of treatment with $1,25(\text{OH})_2\text{D}_3$ using our model system. These discrepancies may be resolved by repeating the Northern analysis and quantitating the results by densitometric scanning.

The long lag time between the peak of induction of calbindin D-28K mRNA levels (1-2 hours) and calbindin D-28K expression (24-72 hours) implicates post-transcriptional mechanisms of regulation. In these studies, we have detected only one calbindin D-28K transcript in MDBK cells. This observation is consistent with that of Takeda et al (1994) but is in contrast to data suggesting two or three calbindin D-28K transcripts *in vivo* (Varghese et al, 1988) and in primary cultures (Clemens, McGlade, Garrett, Craviso and Hendy, 1989). This discrepancy may reflect the inability of the heterogeneous calbindin D-28K cDNA probe used in these experiments to recognize the smaller (1.8 and 2.2 kb) calbindin D-28K transcripts. Consistent with previous results, tubulin mRNA levels are increased for up to 48 hours in response to $1,25(\text{OH})_2\text{D}_3$ (Nemere, Theofan and Norman, 1987). The induction of tubulin may play a role in calbindin D-28K regulation since an interaction between these two

proteins has been reported in intestinal cells (Nemere et al, 1992). Our results indicate that $1,25(\text{OH})_2\text{D}_3$ up-regulates calbindin D-28K expression in a dose- and time-dependent manner in MDBK cells, consistent with the regulation of this renal calcium-binding protein *in vivo*.

2.4.2 Effect of EB 1089 on calbindin D-28K and VDR expression in MDBK cells

EB 1089, a biologically active analogue of vitamin D, mimics the growth regulatory effects of $1,25(\text{OH})_2\text{D}_3$, but its effect on classical vitamin D targets, such as calbindin D-28K and VDR, has yet to be investigated (Binderup, Latini and Kissmeyer, 1991; Mathiasen, Colston and Binderup, 1993). We assessed the effects of EB 1089 in MDBK cells and observed that, like $1,25(\text{OH})_2\text{D}_3$, EB 1089 up-regulates calbindin D-28K expression in a dose- and time-dependent manner. Maximal induction of calbindin D-28K is obtained with 10^{-9} M EB 1089, 100-fold less than that observed with $1,25(\text{OH})_2\text{D}_3$ (Fig. 2.6). This difference in potency in inducing calbindin D-28K between EB 1089 and $1,25(\text{OH})_2\text{D}_3$ is comparable with their potency in growth regulation (Mathiasen et al, 1993). Although the metabolic clearance of these two ligands is similar *in vivo* (Binderup, Latini and Kissmeyer, 1991), the difference in potency may reflect different rates of catabolism or nuclear uptake in MDBK cells. The time-dependence of calbindin D-28K induction by EB 1089 is comparable to that of $1,25(\text{OH})_2\text{D}_3$, with calbindin D-28K levels increasing within 4-8 hours of treatment (Fig. 2.7). In MDBK cells, the induction of calbindin D-28K observed after 24 hour treatment with EB 1089 is accompanied by increased VDR numbers (Fig. 2.8). This induction in $1,25(\text{OH})_2\text{D}_3$ -binding in response to EB 1089 is similar to that obtained in MDBK cells exposed to 10^{-7} M $1,25(\text{OH})_2\text{D}_3$. These results demonstrate that EB 1089 regulates the expression of calbindin

D-28K and VDR in MDBK cells and are interesting in view of the lower hypercalcemic activity of this analogue *in vivo* (Mathiasen, et al, 1993). The observation that EB 1089 can induce calbindin D-28K and VDR to the same extent as $1,25(\text{OH})_2\text{D}_3$, while exerting lesser effects on calcium homeostasis suggests the flexibility of the vitamin D transduction pathway. Most actions of $1,25(\text{OH})_2\text{D}_3$ and EB 1089, including the regulation of calcium metabolism and cellular proliferation and differentiation, are mediated through an interaction with the VDR. The differential regulation of some, but not all, targets of $1,25(\text{OH})_2\text{D}_3$ by EB 1089 suggests that small variations in the VDR-hormone complex, due to the distinct structure of the analogue, may alter the transactivation potential of the VDR complex on a specific subset of genes. $1,25(\text{OH})_2\text{D}_3$ has been shown to bind the VDR with higher affinity than EB 1089 (Mathiasen et al, 1993), this difference could affect the conformation of the VDR and the regulation of vitamin D-dependent proteins. The increasing diversity of VDREs and the number of proteins that potentially dimerize with the VDR (Carlberg et al, 1993) emphasize the complexity of $1,25(\text{OH})_2\text{D}_3$ gene regulation. Consistent with this proposal, EB 1089 was reported to increase the affinity of the VDR for the retinoid X receptor, a well-described dimerization partner (Peleg, Sastry, Collins, Bishop and Norman, 1995). Further research to define which VDREs are responsive to the various vitamin D analogues would clarify the role of these agents and the mechanism of action of $1,25(\text{OH})_2\text{D}_3$.

The observation that EB 1089 up-regulates calbindin D-28K in MDBK cells without altering plasma calcium concentrations (Mathiasen et al, 1993) is in contradiction with the calcium transporter function associated with calbindin D-28K. However, calcium homeostasis is attained by complex mechanisms involving various tissues, including kidney.

Hormone-dependent renal calcium reabsorption, which occurs primarily in distal tubules, accounts for only 10% of total renal calcium reabsorption (Friedman and Gesek, 1993); and as such only contributes a small fraction to calcium regulation. Further studies measuring renal calcium currents in response to EB 1089 and the effect of this analogue on other calcium-regulating tissues may prove helpful in determining the contribution of calbindin D-28K to renal calcium reabsorption and overall calcium homeostasis.

2.4.3 Role of *de novo* protein synthesis in the regulation of calbindin D-28K by $1,25(\text{OH})_2\text{D}_3$

The $1,25(\text{OH})_2\text{D}_3$ transcriptional regulation of calbindin D-28K is fairly well understood (Chen et al, 1992; Varghese et al, 1988), while the post-transcriptional features of this regulation are unclear. As previously reported in avian primary cultures (Craviso et al, 1987), inhibition of new protein synthesis using cycloheximide abolishes the $1,25(\text{OH})_2\text{D}_3$ -induction of calbindin D-28K in MDBK cells (Fig. 2.9). This observation is consistent with an effect of the hormone that requires *de novo* protein synthesis. However, our results describe an effect of cycloheximide which reduces calbindin D-28K expression below basal levels, in the presence or absence of $1,25(\text{OH})_2\text{D}_3$. Considering that the half-life for calbindin D-28K has been assessed at approximately 36 hours (Corradino and Fullmer, 1991), it is unlikely that the reduction of calbindin D-28K expression by cycloheximide results solely from calbindin D-28K turnover. Taken together, these observations suggest the involvement of specific, yet unidentified proteins in both the constitutive and vitamin D-induced expression of calbindin D-28K.

Recently, some effects of vitamin D have been proposed to be mediated, at least partially, via activation of various signal transduction pathways, such as the cyclic 3', 5' adenosine monophosphate (cAMP) and PKC pathways. The mechanism by which $1,25(\text{OH})_2\text{D}_3$ regulates these signal transduction pathways is unknown, but may involve a direct transcriptional effect or activation of a putative membrane receptor, generating second messengers. In the kidney, cAMP-dependent protein kinase (PKA) and PKC activation have been shown to modulate VDR expression and vitamin D-dependent hydroxylase activities (Henry and Luntao, 1989; Simboli-Campbell et al, 1992b; Welsh et al, 1991). The observation that extracellular calcium concentrations modulate renal calbindin D-28K and the tremendous literature on $1,25(\text{OH})_2\text{D}_3$ regulation of PKC prompted us to focus on the role of this particular signalling pathway in the post-transcriptional regulation of calbindin D-28K.

2.5 CONCLUSIONS

In summary, the results reported in this chapter describe the MDBK cell line as the first renal *in vitro* model mimicking calbindin D-28K regulation by $1,25(\text{OH})_2\text{D}_3$ *in vivo* and provide for a suitable renal system to investigate the detailed molecular regulation of calbindin D-28K. Other conclusions drawn from these results are:

- In MDBK cells, $1,25(\text{OH})_2\text{D}_3$ up-regulates calbindin D-28K expression in a dose- and time-dependent manner and this induction is sensitive to protein synthesis inhibitors.

- EB 1089, a non-calcemic analogue of $1,25(\text{OH})_2\text{D}_3$, also up-regulates calbindin D-28K and VDR expression in MDBK cells.

- Cycloheximide pre-treatment inhibits both the constitutive and vitamin D-inducible expression of calbindin D-28K in MDBK cells.

CHAPTER 3: EFFECT OF TPA ON CALBINDIN D-28K AND VDR EXPRESSION IN MDBK CELLS

3.1 INTRODUCTION

External signals stimulate cells by triggering signal transduction pathways which amplify the stimuli by modulating the expression and activity of specific proteins, ultimately resulting in altered cellular functions. Recently, some effects of $1,25(\text{OH})_2\text{D}_3$ have been shown to be modulated by signal transduction pathways. In cardiac muscle, $1,25(\text{OH})_2\text{D}_3$ affects cell calcium uptake by regulating calcium channel activity via a cAMP-dependent pathway (Selles and Boland, 1991; Kaune, Munson and Bikle, 1994). This signalling pathway is also implicated in the $1,25(\text{OH})_2\text{D}_3$ -induced differentiation of HL-60 cells which is characterized by altered phosphorylation state of nuclear proteins (Ueda, Sawada, Okuda, Yumoto, Kato, Ogawa, Tashima, Yoshida and Okuma, 1991; Martell, Strahler and Simpson, 1992). Phosphorylation of the VDR which occurs upon hormone binding, may represent a primary event in $1,25(\text{OH})_2\text{D}_3$ action (Hilliard, Cook, Weigel and Pike, 1994). The mechanisms by which these signalling pathways interact with the vitamin D endocrine system are unclear.

3.1.1 Involvement of PKC in $1,25(\text{OH})_2\text{D}_3$ actions

The PKC signalling pathway plays a role in mediating some effects of $1,25(\text{OH})_2\text{D}_3$. In HL-60 cells, $1,25(\text{OH})_2\text{D}_3$ induces monocytic differentiation, at least partially, through a

PKC-dependent down-regulation of c-myc expression (Taoka, Itano, Tokuda, Tanaka, Hatase and Irino, 1989; Obeid et al, 1990; Biskobing and Rubin, 1993; Taoka, Collins, Irino and Norman, 1993). Phorbol ester-induced differentiation of U937 cells is enhanced in the presence of $1,25(\text{OH})_2\text{D}_3$ (Dodd, Cohen, Newman and Gray, 1983). This effect is correlated with increased PKC-dependent phosphorylation of endogenous proteins observed in vitamin D-treated cells (Ways, Dodd, Bennett, Gray and Earp, 1987). In bone, inhibitors of PKC blunt the $1,25(\text{OH})_2\text{D}_3$ -dependent induction of osteocalcin secretion and bone resorption (van Leeuwen et al, 1992b). The PKC signalling pathway has also been implicated in the rapid stimulation of intestinal calcium transport (transcaltachia) by $1,25(\text{OH})_2\text{D}_3$ (de Boland and Norman, 1990), possibly by regulating calcium channels and/or calcium transporters activity (Lagast, Pozzan, Waldvogel and Lew, 1984).

Although the precise function of PKC in the processes described above is unknown, these observations strongly suggest a role for this signal transduction pathway in mediating some actions of $1,25(\text{OH})_2\text{D}_3$.

3.1.2 Regulation by phorbol esters

Tumor-promoting phorbol esters, such as TPA, bind to PKC and rapidly activate the enzyme at physiological concentrations of Ca^{2+} (Nishizuka, 1984). In contrast, long term treatment with TPA is usually associated with a sustained down-regulation of PKC activity (Rodriguez-Pena and Rozengurt, 1984). We used TPA as a tool to investigate the potential involvement of the PKC signalling pathway in calbindin D-28K regulation in MDBK cells.

Phorbol esters regulate the expression and/or activity of proteins, many of which are

substrates of PKC, by modulating their phosphorylation state (Fletcher and Gallin, 1980; Schlessinger, 1986). TPA also modulates protein levels, notably proto-oncogenes, by inducing an altered program of gene transcription (Angel and Karin, 1991). Although activation of PKC has been implicated in this effect (Imbra and Karin, 1987; Brenner, O'Hara, Angel, Chojker and Karin, 1989), the detailed pathway transducing the genomic effects of TPA are unclear. Analysis of the promoter region of several TPA-regulated genes reveals a conserved region, referred to as the TPA response element (TRE) or AP-1 site (Angel, Imagawa, Chiu, Stein, Imbra, Rahmsdorf, Jonat, Herrlich and Karin, 1987). The Fos and Jun family of nuclear phosphoproteins interact with AP-1 response elements and modulate gene transcription (Angel and Karin, 1991). However, the mechanism of TPA-regulated gene expression is complicated by the observation that proteins, including steroid hormone receptors, can interfere with this pathway by interacting either directly or indirectly with Fos and Jun (Miner, Diamond and Yamamoto, 1991).

3.1.3 Phorbol ester regulation of vitamin D-dependent proteins

Activation of PKC by TPA has been associated with changes in the expression and activity of various vitamin D-regulated proteins. In fibroblasts, TPA causes a decrease in steady-state levels of VDR mRNA, concurrent with a decrease in VDR numbers (Krishnan and Feldman, 1991b; Simboli-Campbell et al, 1992b). These results infer the presence of an AP-1 site in the promoter region of the VDR gene. However, the effect of TPA on VDR numbers differs in different cell lines and these contradictory results may be related to the specific PKC isozymes expressed in each cell line. In any case, TPA regulation of VDR does

not appear to simply involve a transcriptional effect. In addition, the recent observation that VDR serves as an *in vitro* substrate for PKC β (Hsieh, Jurutka, Galligan, Terpening, Haussler, Samuels, Shimizu, Shimizu and Haussler, 1991) indicate that phorbol esters may have direct post-transcriptional effects on the VDR. The significance of VDR phosphorylation is unclear but appears important for receptor turnover and transactivation (Hsieh, Jurutka, Nakajima, Galligan, Haussler, Shimizu, Shimizu, Whitfield and Haussler, 1993).

As observed in other steroidogenic pathways, phorbol esters modulate the activity of the 1 α -hydroxylase and 24-hydroxylase, two enzymes involved in vitamin D metabolism. A temporal correlation between PKC activity, phosphorylation of mitochondrial proteins and stimulation of 24-hydroxylase activity in response to TPA has been demonstrated in mouse renal tubules (Mandla, Bonch and Tenenhouse, 1990). In addition, a role for phorbol ester-activated PKC activity has been postulated in the mRNA accumulation of the 24-hydroxylase cytochrome P₄₅₀, a specific component of this enzyme (Chen, Boltz and Armbrecht, 1993). TPA has also been reported to modulate the activity of the 1 α -hydroxylase, the enzyme required for 1,25(OH)₂D₃ production (Welsh et al, 1991).

In bone, TPA modulates the production of osteocalcin, a non-collagenous matrix protein. PKC-dependent transcriptional regulation is mediated through an AP-1 site located within the VDRE of the human and mouse osteocalcin genes (Schüle, Umesono, Mangelsdorf, Bolado, Pike and Evans, 1990; Owen, Bortell, Yocum, Smock, Zhang, Abate, Shalhoub, Ardnin, Wright, van Wijnen, Stein, Curran, Lian and Stein, 1990). Further investigations indicate a complex interaction between 1,25(OH)₂D₃- and phorbol ester-regulation of osteocalcin which is physiologically related to osteoclast differentiation and bone

resorption (Ozono, Liao, Kerner, Scott and Pike, 1990; Lian, Stein, Bortell and Owen, 1991).

Taken together, these results implicate the PKC signalling pathway in mediating some actions of $1,25(\text{OH})_2\text{D}_3$. Although the regulation of calbindin D-28K by $1,25(\text{OH})_2\text{D}_3$ likely involves post-transcriptional mechanisms, the role of PKC in calbindin D-28K expression has yet to be investigated. In this chapter, we characterize the regulation of both calbindin D-28K and VDR by phorbol esters in MDBK cells. We report that TPA differentially regulates calbindin D-28K and VDR in these cells and propose a role for PKC-dependent phosphorylation events in the regulation of these vitamin D-dependent proteins.

3.2 METHODS

3.2.1 Cell culture

MDBK cells obtained from ATCC were grown to confluence in DMEM supplemented with 10% newborn calf serum as described in 1.2.1. Cells were changed to serum-free media 24 hours prior to treatment with up to 1.5×10^{-7} M TPA, 10^{-7} M 4 α -phorbol-12,13-didecanoate (4 α -PDD) or 125 nM okadaic acid in PBS:DMSO, 10^{-7} M 1,25(OH) $_2$ D $_3$ in ethanol or vehicles for up to 24 hours. In some cases, cells were pre-treated with 2 μ M cycloheximide prior to treatment with the indicated agents. Cells were harvested by scraping and purified cellular fractions were prepared as previously described (1.2.1).

3.2.2 3 H-Thymidine incorporation into DNA

Serum-free MDBK cells were treated for 24 hours with 10^{-7} M TPA or PBS:DMSO (0.05%). [3 H]-thymidine incorporation into DNA was quantitated as described in 1.2.6.

3.2.3 Analysis of cell cycle kinetics

Serum-free MDBK cells were treated for 24 hours with 10^{-7} M TPA or PBS:DMSO (0.05%). Cells were washed, trypsinized, fixed with 95% ethanol for 30 minutes at -20°C and stained with propidium iodide. Cell cycle kinetics were analyzed by flow cytometry as described in 1.2.7.

3.2.4 Immunoblotting

Cytosolic proteins (100 μ g/lane) were separated on 12% SDS-PAGE and processed

for immunoblotting with mouse monoclonal calbindin D-28K antibodies, diluted 1:200. Following washing, membranes were incubated with alkaline phosphatase-conjugated antibodies and immunoreactivity was detected as previously described (2.2.2).

3.2.5 ELISA for calbindin D-28K

Scrum-free MDBK cells were treated for up to 24 hours with up to 1.5×10^{-7} M TPA, 125 nM okadaic acid or PBS:DMSO (0.05%). In some cases, cells were pre-incubated with 2 μ M cycloheximide prior to the indicated treatments. Calbindin D-28K levels in cytosolic fractions were quantitated by ELISA as described in 1.2.4.

3.2.6 1,25(OH)₂D₃-binding assay

High salt extracts of MDBK cells treated for up to 24 hours with 10^{-7} M TPA or PBS:DMSO (0.05%) were prepared as stated in 1.2.6. Extracts (0.8-1.0 mg/ml) were incubated overnight at 4°C with 0.5 nM [³H]-1,25(OH)₂D₃. Bound and free hormone were separated and specific [³H]-1,25(OH)₂D₃-binding was determined as previously described (1.2.4).

3.2.7 Miscellaneous

Protein concentrations were determined by the method of Bradford (1976). Each experiment was performed at least three times. Unless otherwise stated, data are expressed as mean $\pm$ SEM. Statistical differences were assessed using Student's *t*-test for unpaired data or the Bonferroni procedure with a significance level of $P < 0.05$, as appropriate.

3.3 RESULTS

3.3.1 Effect of TPA on MDBK cell morphology and cell cycle kinetics

Dramatic morphological changes are observed in MDBK cells treated with 10^{-7} M TPA (Fig. 3.1). While vehicle-treated cells display a typical tight epitheloid-like morphology, TPA treatment induces cell elongation and formation of fibroblast-like morphology. These changes are evident within hours and become more prevalent after 24 hour exposure. The TPA-dependent morphological changes described in MDBK cells are consistent with observations reported in other cell systems and may result from cytoskeletal reorganization (Driedger and Blumberg, 1977; Rifkin, Crowe and Pollack, 1979; Fey and Penman, 1984).

To ascertain whether the effects of TPA on calbindin D-28K and VDR expression are independent of alterations in cell cycle parameters, we assessed the growth kinetics of MDBK cells exposed to TPA. ^3H -thymidine incorporation into DNA is similar between vehicle-treated and TPA-treated cells (Fig. 3.2). Consistent with these data, flow cytometric measurements of cell cycle parameters reveal no significant differences between the percentage of TPA-treated and vehicle-treated cells in each phase of the cell cycle (Table 3.1). Taken together, these results clearly indicate that, although inducing profound morphological changes, TPA does not regulate cell growth or cell cycle kinetics in MDBK cells.

3.3.2 Effect of TPA on calbindin D-28K and VDR expression in MDBK cells

Our results demonstrate a dose-dependent decrease in calbindin D-28K expression in response to 24 hour treatment with TPA (Fig. 3.3). Maximal reduction of calbindin D-28K

Figure 3.1 *Effects of TPA and 1,25(OH)₂D₃ on the morphology of MDBK cells.*
Serum-free MDBK cells were treated for 24 hours with A. Ethanol; B. 10^{-7} M 1,25(OH)₂D₃;
C. PBS:DMSO or D. 10^{-7} M TPA Cells were washed with PBS, viewed by phase contrast
light microscopy and photographed on Ilford XP-1 black and white film.

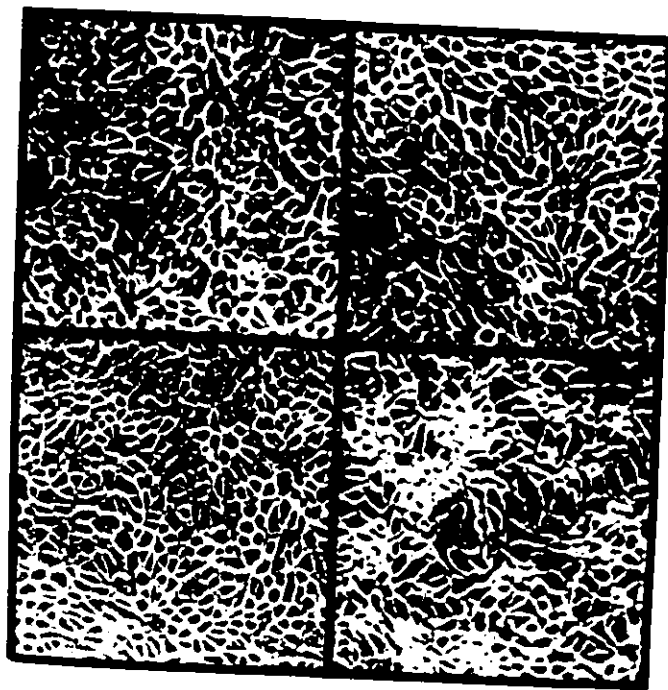


Figure 3.2 *Effect of TPA on ^3H -thymidine incorporation in MDBK cells.*

Serum-free MDBK cells were incubated for 24 hours with 10^{-7} M TPA or PBS:DMSO (0.05%). ^3H -thymidine (0.5 $\mu\text{Ci/ml}$) was added simultaneously with the treatments. ^3H -thymidine incorporation into DNA was assessed as described in Methods. Data are expressed as mean $\pm$ SEM ($N = 3$).

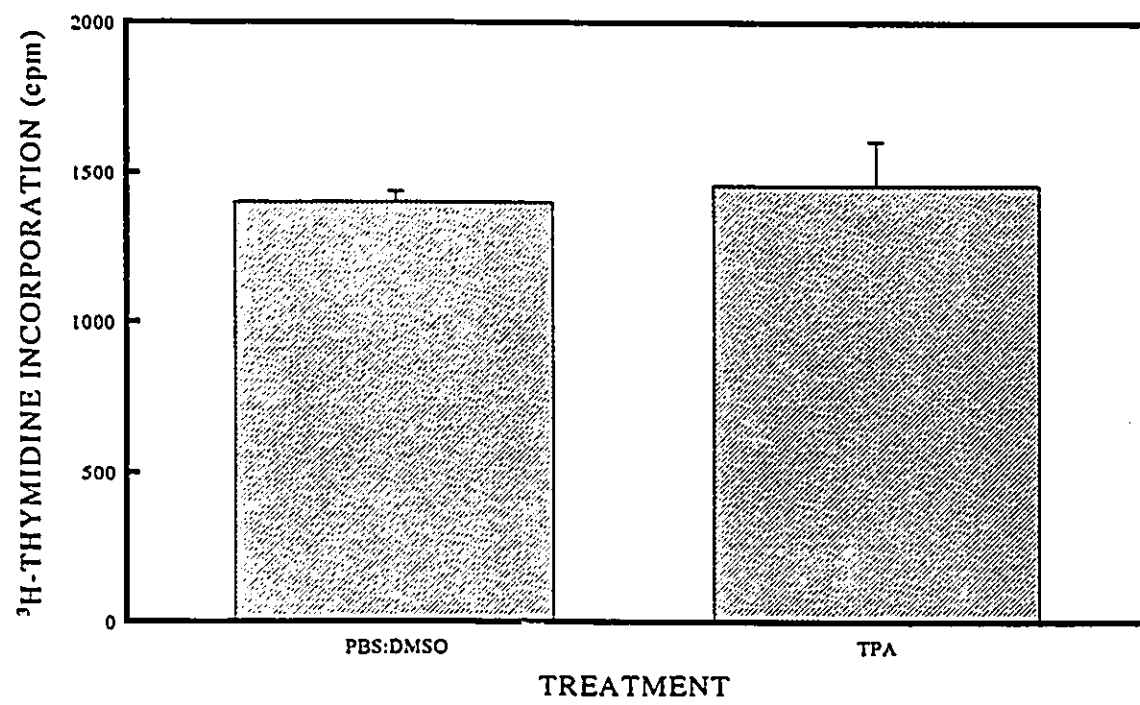


Table 3.1

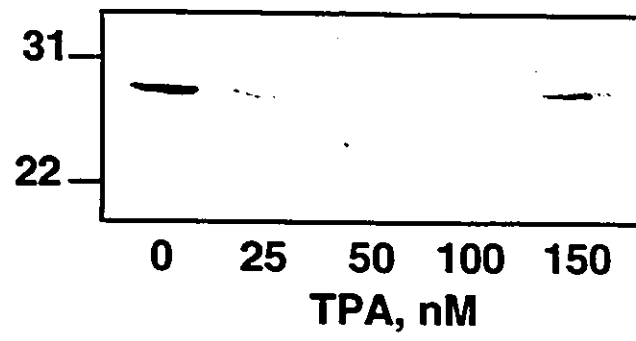
*Flow cytometric analysis of MDBK cells.
Effect of TPA treatment.*

TREATMENT	% of Cells in G ₀ /G ₁	% of Cells in S	% of Cells in G ₂ /M
PBS:DMSO	88.1 ± 0.7	4.2 ± 0.7	3.8 ± 1.1
TPA	87.6 ± 2.3	5.2 ± 1.4	4.4 ± 0.9

Serum-free MDBK cells were treated for 24 hours with 100 nM TPA or vehicle (PBS:DMSO, 0.05%). Cells were lysed, stained with propidium iodine and analyzed by flow cytometry as described in Methods. Values represent mean ± SEM of 3 independent experiments. Statistical differences between groups were determined by Student's *t*-test for unpaired data.

Figure 3.3 Calbindin D-28K expression in response to increasing concentrations of TPA in MDBK cells.

Serum-free MDBK cells were treated for 24 hours with up to 1.5×10^{-7} M TPA or PBS:DMSO (0.05%). Cytosolic proteins (100 μ g/lane) were separated on 12% SDS-PAGE, transferred to nitrocellulose, and antigenic sites were saturated. Membranes were incubated with monoclonal calbindin D-28K antibodies, followed with biotinylated secondary antibodies and streptavidin peroxidase as described in Methods.



levels was obtained with 10^{-7} M TPA, a concentration previously shown to maximally down-regulate PKC activity in MDBK cells (Simboli-Campbell, 1993).

We next characterized the acute temporal effect of 10^{-7} M TPA on both calbindin D-28K and VDR expression in MDBK cells. Immunoblotting of cytosolic fractions of MDBK cells reveals that TPA rapidly induces calbindin D-28K expression within 6 hours, reaching maximal induction after 8 hours of treatment (Fig. 3.4a). The down-regulative effect of TPA observed after 24 hours is specific for tumor-promoting phorbol esters, since 4α -PDD does not decrease calbindin D-28K expression (Fig. 3.4b). 4α -PDD is biologically inert and has previously been reported not to bind PKC (Slaga, Fischer, Nelson and Gleason, 1980; Nidel, Kuhn and Vandenbark, 1983). Quantitation by ELISA indicates a significant increase in calbindin D-28K within 4 hours of treatment with TPA. Maximal induction occurs after 8 hours of treatment, with calbindin D-28K levels increasing approximately 2.5-fold compared to time-matched vehicle controls. Calbindin D-28K expression then starts to decline and falls below control levels within 24 hours of treatment with TPA (Fig. 3.5). The down-regulative effect of TPA on calbindin D-28K is sustained for up to 48 hours (data not shown).

In contrast to our observations with $1,25(\text{OH})_2\text{D}_3$, the early rise in calbindin D-28K levels in response to TPA is not accompanied by a similar increase in VDR levels, suggesting different regulatory pathways which may involve distinct PKC isozymes. A significant (50%) reduction in VDR numbers is evident within 8 hours and persists for up to 24 hours of treatment with TPA (Fig. 3.6). The down-regulation of VDR in response to TPA is also dose-dependent with maximal effect observed with 10^{-7} M TPA (Simboli-Campbell et al, 1992b). These results demonstrate the differential regulation of these two vitamin D-

Figure 3.4 *Temporal effect of TPA on calbindin D-28K expression in MDBK cells, assessed by immunoblotting.*

Cells were grown to confluence and changed to serum-free media 24 hours prior to treatment with 10^{-7} M TPA, 10^{-7} M 4α -PDD or vehicle for up to 24 hours. Cells were harvested by scraping and cytosolic fractions were prepared. Cytosolic proteins (100 μ g/lane) were separated on 12% SDS-PAGE, transferred to nitrocellulose and blotted with anti-calbindin D-28K monoclonal antibody as described in **Methods**. **A.** Cells were treated for up to 8 hours with TPA. *Lane 1*, 0 hour; *lane 2*, 1 hour; *lane 3*, 2 hours; *lane 4*, 4 hours; *lane 5*, 6 hours; *lane 6*, 8 hours. **B.** Cells were treated for 24 hours with the following agents: *lane 1*, PBS:DMSO (vehicle); *lane 2*, TPA; *lane 3*, 4α -PDD.

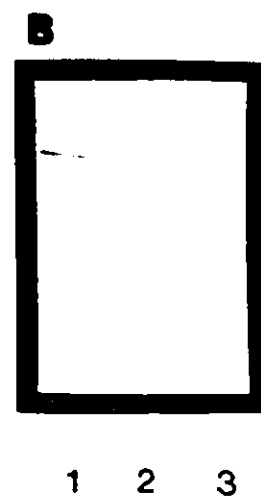
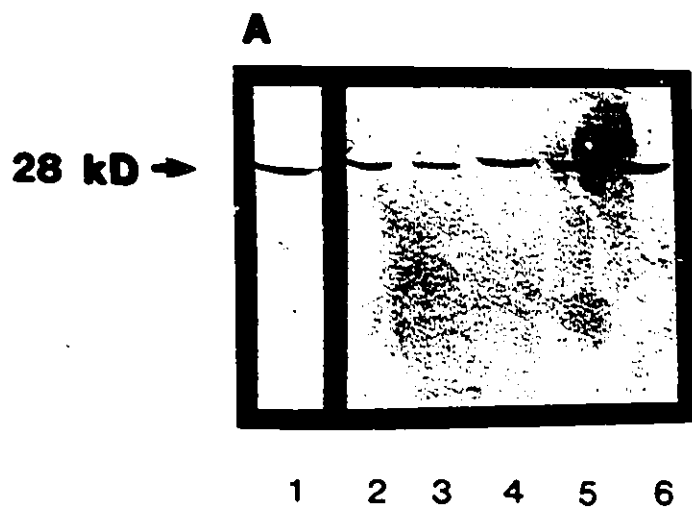


Figure 3.5 *Temporal effect of TPA on calbindin D-28K expression in MDBK cells, assessed by ELISA.*

Cells were grown to confluence and changed to serum-free media 24 hours prior to treatment with 10^{-7} M TPA or vehicle for up to 24 hours. Cells were harvested by scraping and cytosolic fractions were prepared. Cytosolic levels of calbindin D-28K were measured by ELISA as described in Methods. Data are expressed as mean $\pm$ SEM ($N=3$) of calbindin D-28K ($\mu\text{g}/100$ mg of protein). * Indicates significantly ($P < 0.05$) different calbindin D-28K levels in TPA-treated cells compared to time-matched controls.

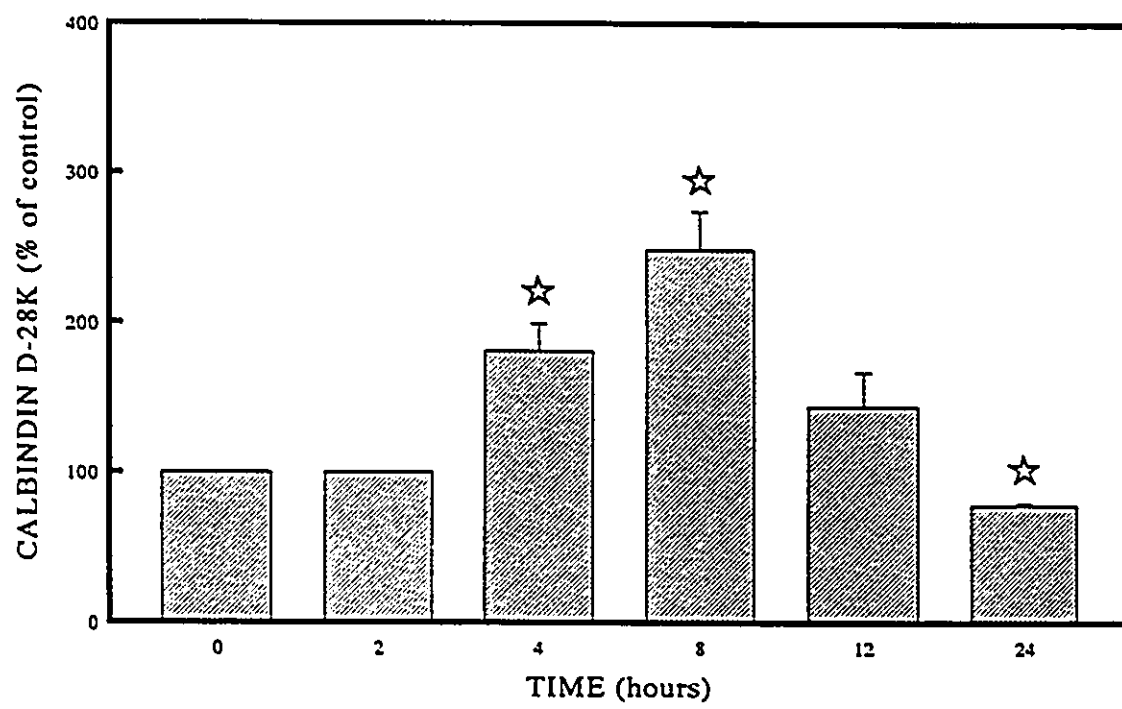
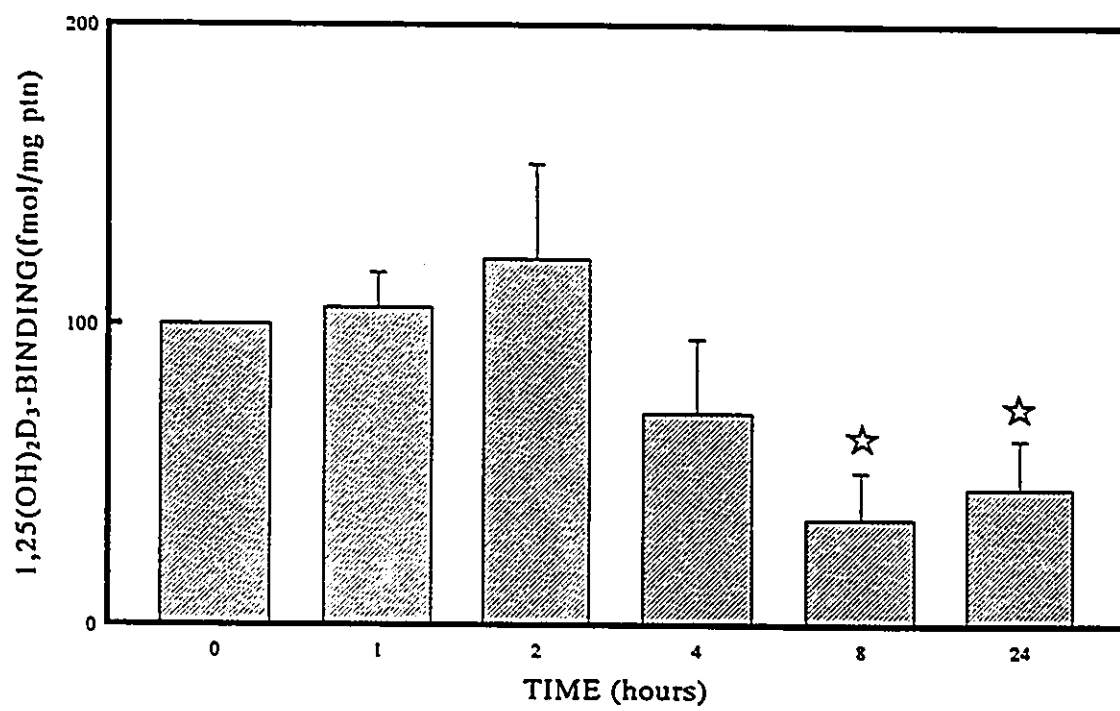


Figure 3.6 *Temporal effect of TPA on specific ^3H -1,25(OH) $_2\text{D}_3$ -binding in MDBK cells.*

Cells were grown to confluence and changed to serum-free media 24 hours prior to treatment with 10^{-7} M TPA or vehicle for up to 24 hours. Cells were harvested by scraping and high salt extracts were incubated with 0.5 nM ^3H -1,25(OH) $_2\text{D}_3$ overnight at 4°C as described in Methods. Data are expressed as mean $\pm$ SEM ($N = 3$) of specific ^3H -1,25(OH) $_2\text{D}_3$ -binding obtained by subtraction of non-specific binding from total binding. * Indicates significantly ($P < 0.05$) different 1,25(OH) $_2\text{D}_3$ -binding in TPA treated cells compared to time-matched controls.



dependent proteins by TPA in MDBK cells.

3.3.3 Effect of protein synthesis and phosphatase inhibitors on calbindin D-28K regulation by TPA in MDBK cells

The role of *de novo* protein synthesis in calbindin D-28K regulation by TPA was investigated by pre-treating MDBK cells with cycloheximide. The rapid induction of calbindin D-28K expression is partially but not significantly blunted by cycloheximide pre-treatment (Fig. 3.7). Similarly, inhibition of protein synthesis does not alter the decrease in calbindin D-28K expression associated with long term TPA treatment (Fig. 3.8). However, as we have previously observed, long term cycloheximide exposure results in a reduction in the constitutive expression of calbindin D-28K compared to controls. This decrease in basal calbindin D-28K levels is comparable to that shown with TPA treatment alone. No further decrease is observed when both agents are present, suggesting a common regulatory pathway.

Since many effects of TPA are mediated through modulation of PKC activity, we investigated whether the phosphatase inhibitor okadaic acid (Bialojan and Takai, 1988) could modulate the effect of TPA on calbindin D-28K. As previously shown, long term TPA treatment decreases the expression of calbindin D-28K. This down-regulation is significantly prevented by simultaneous exposure to okadaic acid. Okadaic acid alone consistently increases calbindin D-28K levels in MDBK cells (Fig. 3.9), although the induction is not statistically significant at this time point.

Figure 3.7 Effect of cycloheximide pre-treatment on calbindin D-28K induction by TPA in MDBK cells.

Scrum-free MDBK cells were pre-treated for 2 hours with 2 μ M cycloheximide in PBS or PBS alone, and treated with 10^{-7} M TPA or vehicle for 4 hours. Cells were harvested by scraping and cytosolic fractions were prepared. Cytosolic levels of calbindin D-28K were measured by ELISA as described in Methods. Statistical significance ($P < 0.05$) between two groups were determined by the Bonferroni procedure. * Indicates significantly different levels of calbindin D-28K levels compared to vehicle-treated cells.

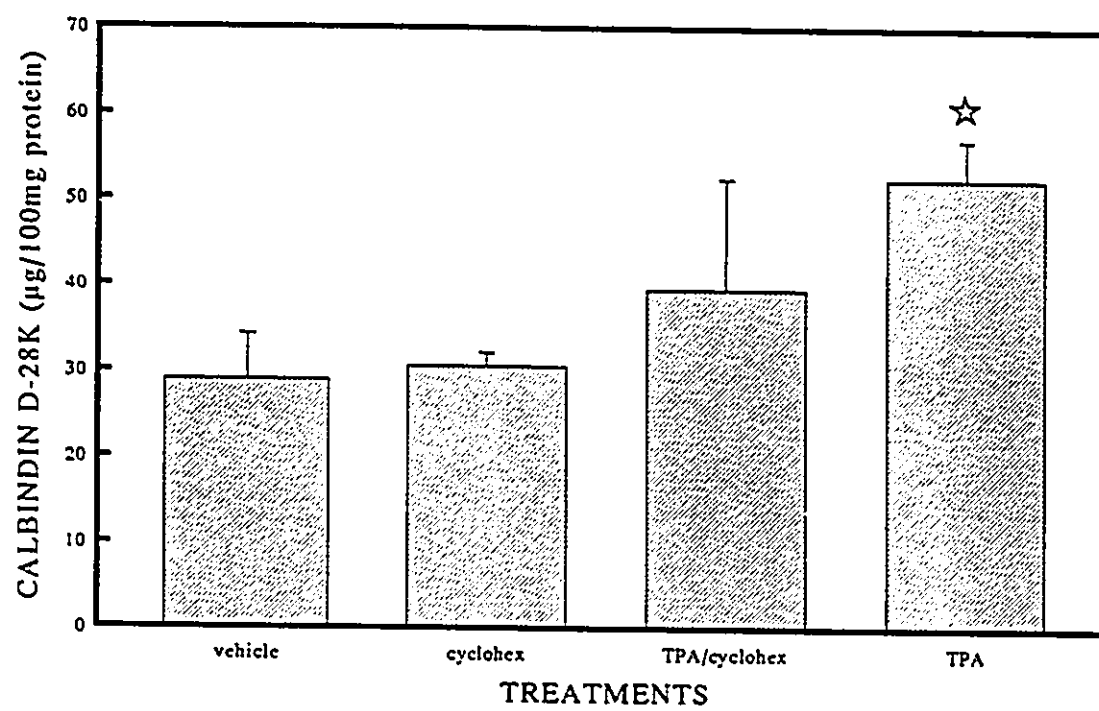


Figure 3.8 *Effect of cycloheximide pre-treatment on calbindin D-28K down-regulation by TPA in MDBK cells.*

Scrum-free MDBK cells were pre-treated for 2 hours with 2 μ M cycloheximide in PBS or PBS alone, and treated with 10^{-7} M TPA or vehicle for 24 hours. Cells were harvested by scraping and cytosolic fractions were prepared. Cytosolic levels of calbindin D-28K were measured by ELISA as described in Methods. Statistical significance ($P < 0.05$) between two groups were determined by the Bonferroni procedure. * Indicates significantly different levels of calbindin D-28K levels compared to vehicle-treated cells.

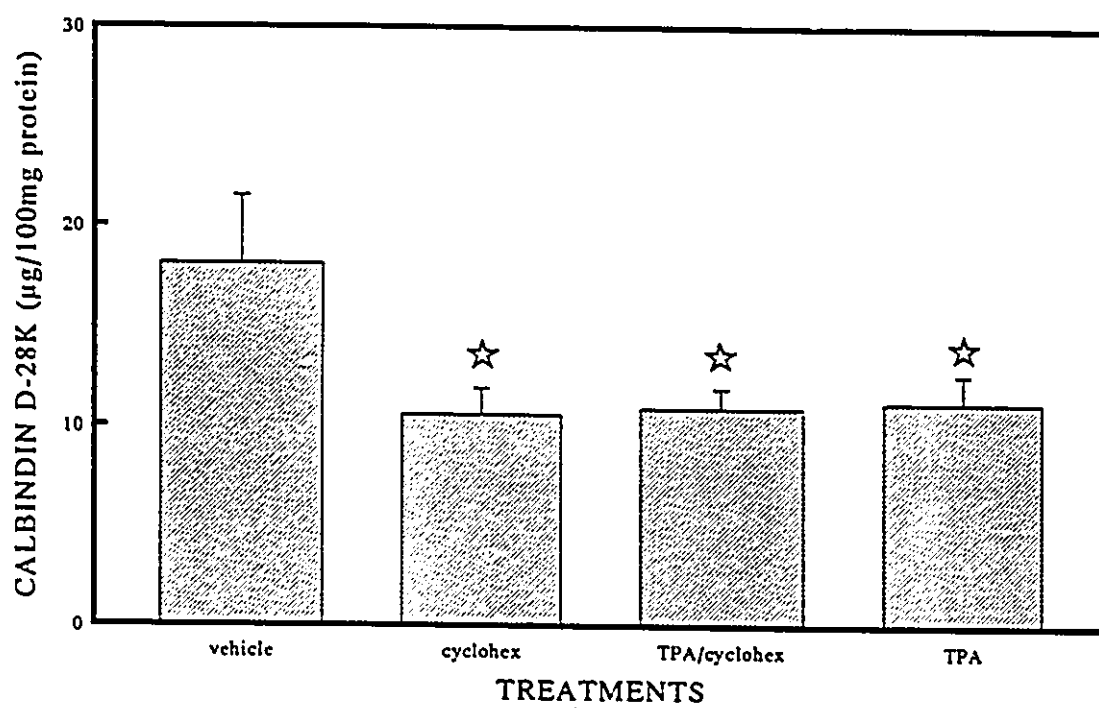
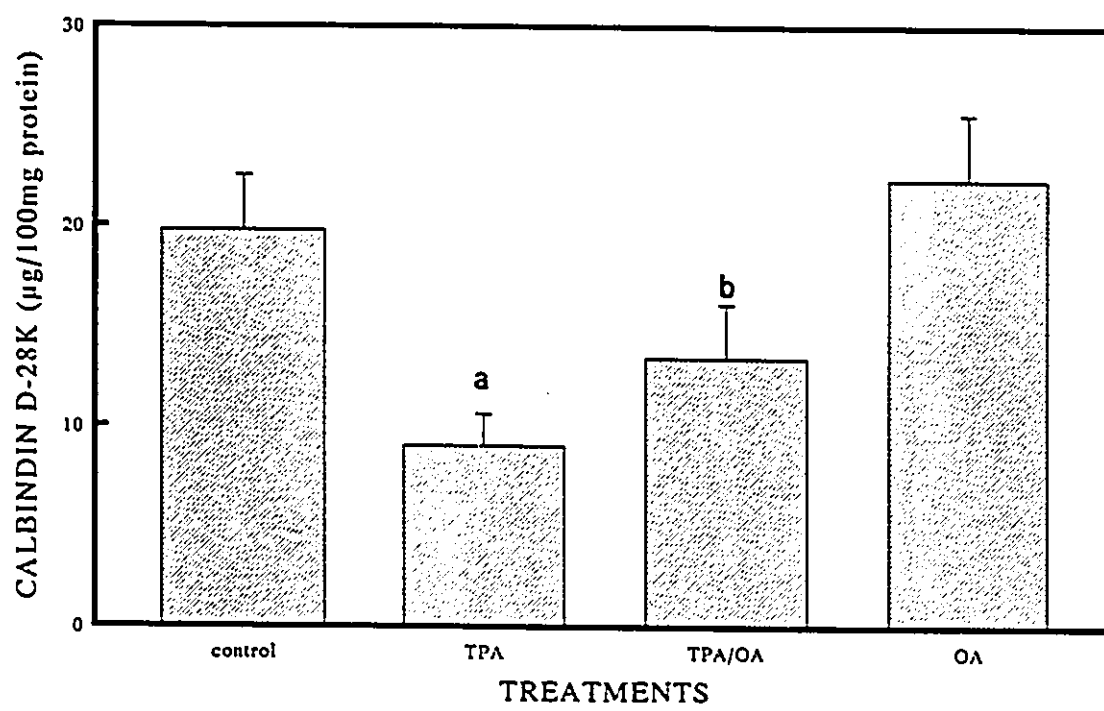


Figure 3.9 *Effect of okadaic acid on the TPA-dependent down-regulation of calbindin D-28K in MDBK cells.*

Scrum-free MDBK cells were treated for 24 hours with 10^{-7} M TPA or vehicle in the presence or absence of 125 nM okadaic acid. Cells were harvested by scraping and cytosolic fractions were prepared. Cytosolic levels of calbindin D-28K were measured by ELISA as described in Methods. Statistical significance ($P < 0.05$) between two groups were determined by the Bonferroni procedure.

^a $P < 0.05$ compared to control values

^b $P < 0.05$ compared to TPA-treated values



3.4 DISCUSSION

The details of the post-transcriptional regulation of calbindin D-28K by $1,25(\text{OH})_2\text{D}_3$ are unclear. The growing evidence implicating the PKC signalling pathway in mediating some actions of $1,25(\text{OH})_2\text{D}_3$ prompted us to investigate the potential role of PKC in calbindin D-28K regulation. We characterized the effects of TPA, a well-described PKC modulator, on the expression of both calbindin D-28K and VDR in MDBK cells and speculate a critical role for this kinase in the regulation of these vitamin D-dependent proteins.

3.4.1 Differential regulation of calbindin D-28K and VDR by TPA in MDBK cells

Our results demonstrate that TPA rapidly up-regulates calbindin D-28K expression whereas, long term (24 hours) TPA treatment results in decreased expression of calbindin D-28K in MDBK cells (Figs. 3.4 and 3.5). The down-regulative effect of TPA on calbindin D-28K is dose-dependent with maximal reduction obtained at 10^{-7} M TPA (Fig. 3.3). This concentration is consistent with that resulting in maximal down-regulation of PKC activity and VDR levels after 24 hours of treatment of MDBK cells with TPA (Simboli-Campbell et al, 1992b), and these observations suggest an association between PKC activity and VDR/calbindin D-28K expression. The apparent lesser effect of higher concentrations of TPA is unclear but may reflect stress-induced conditions.

The rapid up-regulation of calbindin D-28K expression by TPA could be consistent with a genomic effect of this phorbol ester. TPA-dependent gene regulation is thought to be mediated by induction of the Fos and Jun family of proteins which interact with AP-1 sites

located in the promoter region of specific genes (Angel and Karin, 1991). However, in contrast to the mammalian osteocalcin genes which were reported to contain such response elements (Schüle et al, 1990; Owen et al, 1990), no AP-1 sites have been identified in the promoter region of mammalian calbindin D-28K genes (Christakos et al, 1992), arguing against a direct genomic regulation by TPA. Alternatively, TPA could induce a protein which in turn binds to the calbindin D-28K promoter and activates transcription. Further studies assessing the steady-state levels of calbindin D-28K mRNA transcripts in response to TPA are required to confirm this hypothesis.

The long term down-regulation of calbindin D-28K expression observed in TPA-treated MDBK cells may result from either inhibition of calbindin D-28K gene transcription or increased turnover rate of calbindin D-28K mRNA transcripts or calbindin D-28K itself. Unfortunately, the very low abundance of calbindin D-28K mRNA has precluded definitive conclusions on the effect of TPA on these transcripts. In any case, further studies are required to distinguish between these potential mechanisms of action.

The regulation of VDR by phorbol esters has previously been investigated in other systems (Haussler, Mangelsdorf, Donaldson, Marion, Sleator and Pike, 1985; Merke, Milde, Lewicka, Hugel, Klaus, Mangelsdorf, Haussler, Rauterberg and Ritz, 1989). The progressive decrease in $1,25(\text{OH})_2\text{D}_3$ -binding observed in TPA-treated MDBK cells (Fig. 3.6) is similar to that reported in NIH 3T3 cells (Krishnan and Feldman, 1991b; Krishnan and Feldman, 1992). In contrast, TPA causes a rapid but transient decrease followed by up-regulation of VDR numbers in osteoblast-like cells (van Leeuwen et al, 1992a). In MCF-7 cells, $1,25(\text{OH})_2\text{D}_3$ -binding is also up-regulated by TPA (Simboli-Campbell and Welsh, unpublished

observations). These apparent discrepancies may reflect distinct patterns of PKC isozyme expression and activity in various cell lines.

Since most, if not all effects of TPA are mediated through activation of PKC, our results implicate this signalling pathway in the regulation of renal calbindin D-28K and VDR. In contrast to the tight correlation commonly observed between calbindin D-28K and VDR (Theofan, Nguyen and Norman, 1986; Gagnon et al, in preparation), TPA differentially regulates these vitamin D-dependent proteins in MDBK cells. This observation argues against a secondary influence of VDR regulation on calbindin D-28K expression and suggest distinct regulatory pathways.

3.4.2 Role of *de novo* protein synthesis and phosphatases in calbindin D-28K regulation by TPA in MDBK cells

Although no AP-1 site has been identified in the promoter of the mouse calbindin D-28K gene, regulation via an as yet unidentified AP-1 site is a possibility. To further define the regulation of calbindin D-28K by TPA, MDBK cells were pre-treated with a protein synthesis inhibitor. Cycloheximide pre-treatment partially prevented the rapid up-regulation, but had no effect of the long term down-regulation of calbindin D-28K by TPA, implying that TPA regulation of this calcium-binding protein is, at least partially, independent of *de novo* protein synthesis. However, as previously described, long term treatment with cycloheximide alone decreases calbindin D-28K levels, suggesting a role for a short-lived protein in calbindin D-28K regulation. The similarity between the responses to individual treatments (cycloheximide alone or TPA alone) and the lack of synergism in the presence of both TPA and

cycloheximide implicate a common regulatory pathway. The reported super-induction of Fos observed after cycloheximide treatment (Edwards and Mahadevan, 1992) may complicate these results if an AP-1 site is indeed present in the calbindin D-28K gene; albeit an enhancement of the TPA effect would be expected under these conditions.

Okadaic acid is a potent inhibitor of phosphatases 1 and 2a, two serine/threonine protein phosphatases (Bialojan and Takai, 1988). Treatment with okadaic acid often mimics PKC activation, enhancing the phosphorylation of various proteins (Nagamine and Ziegler, 1991). In MDBK cells, okadaic acid significantly blunts the TPA-dependent down-regulation of calbindin D-28K. Okadaic acid may prevent the long term dephosphorylation of a PKC substrate involved in calbindin D-28K regulation, preventing the long term (24 hours) down-regulation of calbindin D-28K expression in response to TPA. Okadaic acid alone slightly increased calbindin D-28K expression, implicating phosphorylation events in calbindin D-28K regulation.

3.4.3 Regulation of cell morphology and cell growth by TPA in MDBK cells

Various cell systems have been reported to undergo extensive morphological changes after exposure to tumor promoters, including TPA (Sivak and van Duuren, 1967; Croop, Toyama, Dlugosz and Holtzer, 1980; Ojakian, 1981). Epithelial cells such as keratinocytes and MDCK cells treated with TPA display a distinct fibroblast-like morphology which has been attributed, at least partially, to the reorganization of the intermediate filament network and the disruption of junctional complexes (Parkinson and Emmerson, 1982; Fey and Penman, 1984). Consistent with these reports, we observed rapid and profound morphological

alterations in MDBK cells exposed to TPA, including loss of cell-cell contact, elongation and appearance of fibroblast-like features (Fig. 3.1).

However, these changes in MDBK cell morphology were not associated with dysregulation of cell growth. In contrast to the proliferative effects of TPA reported in various systems (Dotto, Parada and Weinberg, 1985; Nishikawa, Komada, Uemura, Hiidaka and Shirakawa, 1990; Weinstein, Berner, Krauss, O'Driscoll, Choi, Moritomi, Hsieh, Tchou-Wong, Nichols-Guadagno, Ueffing and Guillem, 1990), cell division, as assessed by ^3H -thymidine incorporation into DNA, and cell cycle parameters were unchanged in TPA-treated MDBK cells (Fig. 3.2 and Table 3.1). These results indicate that the effects of TPA on calbindin D-28K and VDR in MDBK cells are independent of cell cycle regulation. However, our data does not rule out the possibility that the cytoskeletal reorganization induced by long term TPA treatment is linked to the down-regulation of calbindin D-28K and VDR, since interactions with tubulin have been reported for both proteins (Nemere et al, 1992; Barsony and McCoy, 1992).

Taken together, our results demonstrate that TPA modulates the expression of both calbindin D-28K and VDR in MDBK cells, implicating PKC in the regulation of these vitamin D-dependent proteins. The temporal effect of TPA on calbindin D-28K and VDR is quite distinct from the regulation by $1,25(\text{OH})_2\text{D}_3$. Further investigations into the effects of these agents on PKC activity and isozyme expression may help distinguish between the regulation of constitutive and $1,25(\text{OH})_2\text{D}_3$ -induced expression of renal calbindin D-28K.

3.5 CONCLUSIONS

The results described in this chapter implicate the PKC signalling pathway in the regulation of the renal vitamin D-dependent proteins, calbindin D-28K and VDR. The characteristics of this regulation are:

- Short term TPA treatment up-regulates calbindin D-28K expression while significantly decreasing VDR levels in the absence of $1,25(\text{OH})_2\text{D}_3$.
- Long term TPA treatment down-regulates both calbindin D-28K and VDR levels in MDBK cells.
- The regulation of calbindin D-28K by TPA is independent of new protein synthesis but is influenced by a phosphatase inhibitor.

CHAPTER 4: EFFECTS OF 1,25(OH)₂D₃ AND TPA ON PKC ACTIVITY, EXPRESSION AND SUBCELLULAR LOCALIZATION IN MDBK CELLS

4.1 INTRODUCTION

The PKC signalling pathway is now recognized as a major regulator of various cellular processes such as proliferation (Guy, Gordon, Michell and Brown, 1985; Kaibuchi, Takai and Nishizuka, 1985), membrane ion conductance (Rosoff, Stein and Cantley, 1984; Johnson, Ware, Cliveden, Smith, Dvorak and Salzman, 1985; Greene and Lattimer, 1986) and cytoskeletal organization (Werth, Niedel and Pastan, 1983; Kawamoto, and Hidaka, 1984). This phospholipid-dependent serine/threonine protein kinase is generally thought to be activated by DAG and inositol phosphates which are generated by the hydrolysis of membrane phospholipids (Takai, Kishimoto, Kikkawa, Mori and Nishizuka, 1979; Sano, Takai, Kamanishi and Nishizuka, 1983). Identified as a cytoplasmic protein kinase in 1977 (Inoue, Kishimoto, Takai and Nishizuka, 1977; Takai, Kishimoto, Inoue and Nishizuka, 1977), PKC has since been recognized as a family of structurally related isozymes, which differ in their localization, regulation and substrate specificity (Nishizuka, 1988). The PKC isozymes identified to date can be grouped into two categories: calcium-dependent (conventional) PKC and calcium-independent (novel) PKC (Osada, Mizuno, Saido, Akita, Suzuki, Kuroki and Ohno, 1990). The specific role of each isozyme in cellular regulation has yet to be defined. The differential regulation of calbindin D-28K by 1,25(OH)₂D₃ and TPA prompted us to investigate the effects of these agents on PKC isozyme expression and subcellular localization

in MDBK cells. Our results suggest a role for calcium-dependent PKCs (PKC α and PKC β) in the regulation of calbindin D-28K.

4.1.1 Structure of PKC

Structurally, PKC isozymes are single chain polypeptides containing an amino-terminal regulatory domain and a carboxy-terminal catalytic domain. The various isozymes share 3-4 regions of homology (conserved regions: C1-C4) which are separated by variable regions (V1-V5) (Coussens, Parker, Rhce, Yang-Feng, Chen, Waterfield, Francke and Ullrich, 1986). Region C1 is characterized by tandem repeats of cysteine-rich sequences, reminiscent of zinc fingers, which have been implicated in DAG and phorbol ester binding (Parker, Coussens, Totty, Rhce, Young, Chen, Stabel, Waterfield and Ullrich, 1986). Although lacking a characteristic calcium-binding structure, the C2 region has been suggested as the calcium-binding site since this sequence is missing in novel (calcium-independent) PKC isozymes (Parker et al, 1986; Ono, Fujii, Igarashi, Kuno, Tanaka, Kikkawa and Nishizuka, 1989). The regulatory domain of PKC isozymes also contains a pseudo-substrate domain which can interact with the catalytic domain, inhibiting enzyme activity (House and Kemp, 1990). Such autoinhibitory regions are a common feature of protein kinases and provide for another level of regulation of enzyme activity (Soderling, 1990). The catalytic region of PKC reveals extensive sequence similarities with other families of protein kinases (Parker et al, 1986). Both the C3 and C4 sections have ATP binding sites, although their significance remains unknown. However, deletion studies suggest that the C3 region solely constitutes the catalytic domain of the kinase (Kaibuchi, Fukumoto, Oku, Takai, Arai and Muramatsu, 1989).

The variable sections (V1-V5) are thought to be implicated in the substrate specificity of each isozyme (Pears, Schaap and Parker, 1991).

4.1.2 Activation of PKC

Growth factors, hormones and cytokines interact with surface receptors, often activating phospholipases which catalyse the hydrolysis of membrane phosphoinositides, yielding elevated levels of DAG and inositol tri-phosphate (IP_3) (Berridge, 1987). IP_3 acts on calcium channels to release calcium from intracellular stores (Streb, Irvine, Berridge and Schulz, 1983). Elevated DAG and calcium levels are thought to act synergistically to activate PKC (Azzi, Boscoboinik and Henscy, 1992). Activation of PKC by calcium and DAG or phorbol ester involves the redistribution of the enzyme, most commonly from the cytosol to a membrane-associated location (Kraft and Anderson, 1983). The association of PKC with cellular membranes also appears dependent on PKC autophosphorylation, which increases its affinity for calcium, and therefore may play a role in enzyme activation (Wolf, Cuatrecasa and Sahyoun, 1985).

4.1.3 $1,25(OH)_2D_3$ regulation of PKC activity and expression

The ability of $1,25(OH)_2D_3$ to induce PKC activity and gene expression has been described in several systems. In isolated colonocytes, $1,25(OH)_2D_3$ stimulates the breakdown of phosphoinositides and activates PKC within minutes (Wali, Baum, Sitrin and Brasitus, 1990). These effects may mediate the regulation of calcium transport and proliferation by $1,25(OH)_2D_3$ in these cells. More recently, *in vitro* studies have shown a direct and specific

activation of PKC by $1,25(\text{OH})_2\text{D}_3$ and suggest that PKC may act as a 'membrane-receptor' for $1,25(\text{OH})_2\text{D}_3$ (Slater, Kelly, Taddeo, Larkin, Yeager, McLane, Ho and Stubbs, 1995). These rapid (within minutes) effects of $1,25(\text{OH})_2\text{D}_3$ on phosphoinositide metabolism and PKC activity may be implicated in the non-genomic actions of this hormone, including transcalcachia and intracellular calcium regulation. However, $1,25(\text{OH})_2\text{D}_3$ also exerts long term effects on PKC isozyme activity, expression and subcellular localization (Obcid et al, 1990; Solomon, O'Driscoll, Sosne, Weinstein and Cayre, 1991; Simboli-Campbell, Gagnon, Franks and Welsh, 1994). In HL-60 cells, $1,25(\text{OH})_2\text{D}_3$ increases calcium- and phospholipid-dependent phosphorylation (Zylber-Katz and Glazer, 1985), ^3H -phorbol ester binding (Martell et al, 1987) and PKC β gene transcription (Obcid et al, 1990). The $1,25(\text{OH})_2\text{D}_3$ -mediated induction of PKC gene expression and activity appears to be required for the inhibition of *c-myc* expression, a well-described long term effect of $1,25(\text{OH})_2\text{D}_3$ in HL-60 cells (Simpson et al, 1989).

In kidneys, it has been suggested that the modulation of 1α -hydroxylase and 24-hydroxylase activities by $1,25(\text{OH})_2\text{D}_3$ may occur at the level of reversible phosphorylation (Siegcl, Wongsurawat and Ambrecht, 1986). *In vitro* activation of renal PKC also increases $25(\text{OH})\text{D}_3$ - 1α -hydroxylation and decreases $25(\text{OH})\text{D}_3$ -24-hydroxylation (Henry and Luntao, 1989; Mandla et al, 1990; Weaver, Franks and Welsh, 1992), mimicking the effects of $1,25(\text{OH})_2\text{D}_3$ on these enzymes. However, the specific PKC isozymes involved in these effects have not been determined.

4.1.4 Regulation of PKC by phorbol esters

Tumour-promoting phorbol esters are non-carcinogenic agents characterized by their ability to induce tumour formation when administered with subthreshold doses of carcinogens. Biologically active phorbol esters cause a complex array of biochemical changes, including morphological transformation (Driedger and Blumberg, 1977), loss of fibronectin (Blumberg, Driedger and Rossow, 1976), altered rates of DNA synthesis (Huberman and Callahan, 1979; Hamilton and Dientsman, 1981) and alteration of surface receptors (Fletcher and Gallin, 1980).

In recent years, the PKC signalling pathway has been implicated in mediating most of the biological effects of tumour-promoting phorbol esters. More specifically, phorbol esters have been reported to bind PKC with high affinity ($K_D = 7$ nM), suggesting PKC as a functional 'phorbol ester receptor' (Niedel et al, 1983). Biologically active phorbol esters, such as TPA, are structurally similar to DAG and can directly activate PKC both *in vivo* and *in vitro*. Like DAG, phorbol esters increase the affinity of PKC for Ca^{2+} , resulting in full activation of the enzyme at physiological concentrations of Ca^{2+} (Castagna et al, 1982). However, in contrast to DAG which is transiently produced in membranes, phorbol ester exposure results in sustained activation of PKC. This persistent stimulation of PKC in response to phorbol esters also influences its degradation, most commonly catalyzed by the proteolytic enzyme calpain, ultimately resulting in the disappearance of PKC from cells (Young, Parker, Ullrich and Stabel, 1987; Ase, Berry, Kikkawa, Kishimoto and Nishizuka, 1988). The long term down-regulation of PKC has been implicated in the tumour-promoting ability of phorbol esters, by preventing the negative feedback control of cellular proliferation

ability of phorbol esters, by preventing the negative feedback control of cellular proliferation exerted by PKC (Schlessinger, 1986). Although phorbol esters represent non-physiological agents, they are often used to investigate the role of the PKC signalling pathway in specific biological events.

Previous results from this lab implicate the PKC signalling pathway in mediating some of the renal actions of $1,25(\text{OH})_2\text{D}_3$ (Welsh et al, 1991). The differential regulation of calbindin D-28K and VDR expression in response to $1,25(\text{OH})_2\text{D}_3$ and TPA prompted us to investigate the specific effects of these two agents on PKC activity, expression and subcellular localization and to identify the isozymes responsible for these responses. Our results suggest the involvement of the PKC signalling pathway in the induction of calbindin D-28K by $1,25(\text{OH})_2\text{D}_3$ and, by analogy to other vitamin D-regulated proteins, may offer an alternate mechanism for the regulation of this calcium-binding protein.

4.2 METHODS

4.2.1 Cell culture

MDBK cells obtained from ATCC were grown in DMEM supplemented with 10% newborn calf serum. Confluent cells were changed to serum-free media for 24 hours prior to treatment with 10^{-7} M $1,25(\text{OH})_2\text{D}_3$, ethanol vehicle (0.05%), 10^{-7} M TPA or PBS:DMSO (0.05%). Cells were harvested by scraping and cellular fractions were prepared as described in 1.2.1.

Murine cycS49T lymphoma cells, used in the preparation of the PKC substrate (Chakravarthy, Franks, Whitfield and Durkin, 1989) were grown as suspension cultures in RPMI 1640 media (Life Technologies) supplemented with 10% horse serum (Life Technologies). Cultures were seeded at $2-4 \times 10^5$ cells/ml and maintained for 3 days at 37°C in a humidified atmosphere of 95% air and 5% CO_2 .

4.2.2 Immunoblotting of PKC isozymes

Cytosolic and membrane proteins (25 $\mu\text{g}/\text{lane}$) were processed for immunoblotting as previously described in 1.2.3, using rabbit polyclonal isozyme-specific PKC antibodies (2 $\mu\text{g}/\text{ml}$, Life Technologies). In some experiments, antibodies were pre-incubated with their specific antigenic peptides to assess non-specific binding. Following washing, membranes were incubated for one hour at room temperature with alkaline phosphatase-conjugated anti-rabbit IgG antibodies (Promega) and immunoreactivity detected with NBT and BCIP as previously described. Alternatively, membranes were incubated with streptavidin peroxidase-

conjugated anti-rabbit IgG antibodies. Immunoreactivity was detected using enhanced chemiluminescence techniques (Amersham).

4.2.3 PKC activity

MDBK cell monolayers were washed twice with PBS and frozen at -20°C in hypotonic lysis buffer (1 mM NaHCO_3 , 5 mM MgCl_2 and 100 μM PMSF, pH 7.5). Cells were thawed, scraped and vortexed. Intact nuclei were removed by centrifugation (500 x g, 5 min, 4°C) and supernatants (25 μg protein) were centrifuged to obtain a membrane pellet (14,000 x g, 15 min, 4°C).

PKC activity was determined in membrane pellets resuspended in assay buffer (1 mM NaHCO_3 , 5 mM MgCl_2 , 100 μM PMSF, 50 mM Tris-HCl, 10 μM EGTA, 1 μM CaCl_2 , 100 μM sodium vanadate, 100 μM sodium pyrophosphate and 1 mM sodium fluoride, pH 7.5), based on the method of Chakravarthy et al (1989). Reactions were initiated by addition of a 85 kDa PKC-specific substrate protein, isolated from cyc⁻S49T cells, and $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ (10 μM , 4400 cpm/pmol; NEN Research Products, Markham, Ont., 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 (1 mM). Membranes were removed by centrifugation (14,000 x g, 15 min, 4°C) and supernatant proteins were separated on 8% SDS-PAGE. The phosphorylated 85 kDa substrate protein was visualized by autoradiography. Phosphorylation of the substrate was quantitated by densitometry (LKB 2222-020 Ultrosan XL).

4.2.4 Immunocytochemistry of PKC isozymes

Serum-free MDBK cells grown on coverslips were treated for up to 24 hours with 100 nM 1,25(OH)₂D₃, 100 nM TPA or vehicle. Cells were washed in PBS and fixed for 5 min at -20°C with ethanol:acetone (1:1). Cells were washed with PBS and non-specific binding sites were blocked overnight at 4°C with PBS containing 0.5% skim milk and 0.2% sodium azide. After washing, coverslips were incubated overnight at 4°C with PKC α , PKC β , PKC ϵ or PKC ζ specific antibodies (Life Technologies) diluted 10 μ g/ml in blocking solution. Coverslips were washed again and incubated for 2 hours at room temperature with FITC-labelled secondary antibodies (Jackson ImmunoResearch Laboratories) diluted 1:50 in blocking solution. Coverslips were washed, mounted on slides and viewed with a Zeiss fluorescent microscope. Non-specific fluorescence, assessed in the absence of primary antibodies or in the presence of excess antigenic peptide, was negligible.

All results were confirmed by immunoblotting of purified fractions of MDBK cells (not shown).

4.2.5 Miscellaneous

Protein concentrations were determined by the method of Bradford (1972). Each experiment was performed at least three times. Data are expressed as mean $\pm$ SEM unless otherwise stated. Statistical differences were assessed using Student's *t*-test for unpaired data, with a significance level of $P < 0.05$.

4.3 RESULTS

4.3.1 Expression of PKC isozymes in MDBK cells

Using isozyme-specific PKC antibodies, we detected four PKC isozymes in total lysates of MDBK cells: two calcium-dependent (PKC α and PKC β) and two calcium-independent (PKC ϵ and PKC ζ) isozymes (Fig. 4.1). These isozymes have previously been detected in homogenates of whole kidney. No specific bands were identified with antibodies directed against PKC δ or PKC γ . The specificity of the individual PKC isozyme antibodies was demonstrated using antigenic peptides; the immunoreactive bands are competed out by the respective PKC isozyme antigenic peptide (Fig. 4.1), while no competition for antibody binding is observed with the antigenic peptides specific for other PKC isozymes (Simboli-Campbell et al, 1993; Simboli-Campbell et al, 1994).

4.3.2 Effect of 1,25(OH) $_2$ D $_3$ on PKC activity, expression and subcellular localization in MDBK cells

In light of reports suggesting interactions between vitamin D and PKC signalling pathways, we investigated the effect of 1,25(OH) $_2$ D $_3$ on PKC activity in MDBK cells. Our results demonstrate that 1,25(OH) $_2$ D $_3$ induces calcium- and phospholipid-dependent PKC activity, measured in its native membrane-associated state, compared to ethanol-treated MDBK cells. The induction in PKC activity is evident within 24 hours and is sustained for up to 72 hours after a single dose of 10^{-7} M 1,25(OH) $_2$ D $_3$ (Fig. 4.2), temporally correlating with the increase in calbindin D-28K expression in response to 1,25(OH) $_2$ D $_3$ (Fig. 2.4). This

Figure 4.1 Expression of PKC isozymes in MDBK cells.

Proteins from total homogenates of MDBK cells (200 µg/lane) were separated on 10% SDS-PAGE, transferred to nitrocellulose and blotted with polyclonal isozyme-specific PKC antibodies (PKC α , PKC β , PKC γ , PKC δ , PKC ϵ and PKC ζ) in the presence (+) or absence (-) of the appropriate antigenic peptide, followed with alkaline phosphatase-conjugated secondary antibodies as described in Methods.

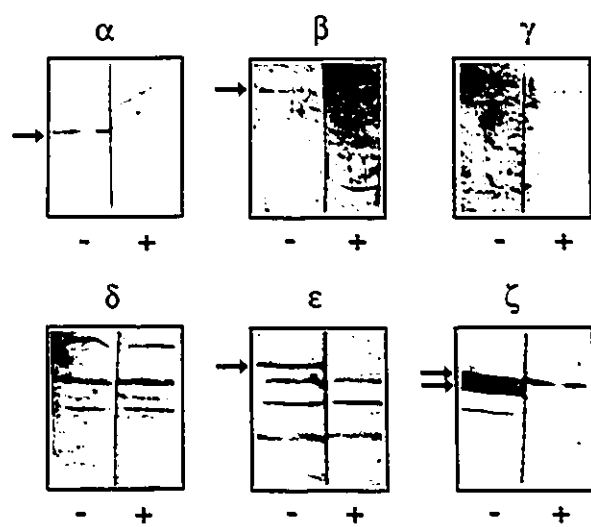
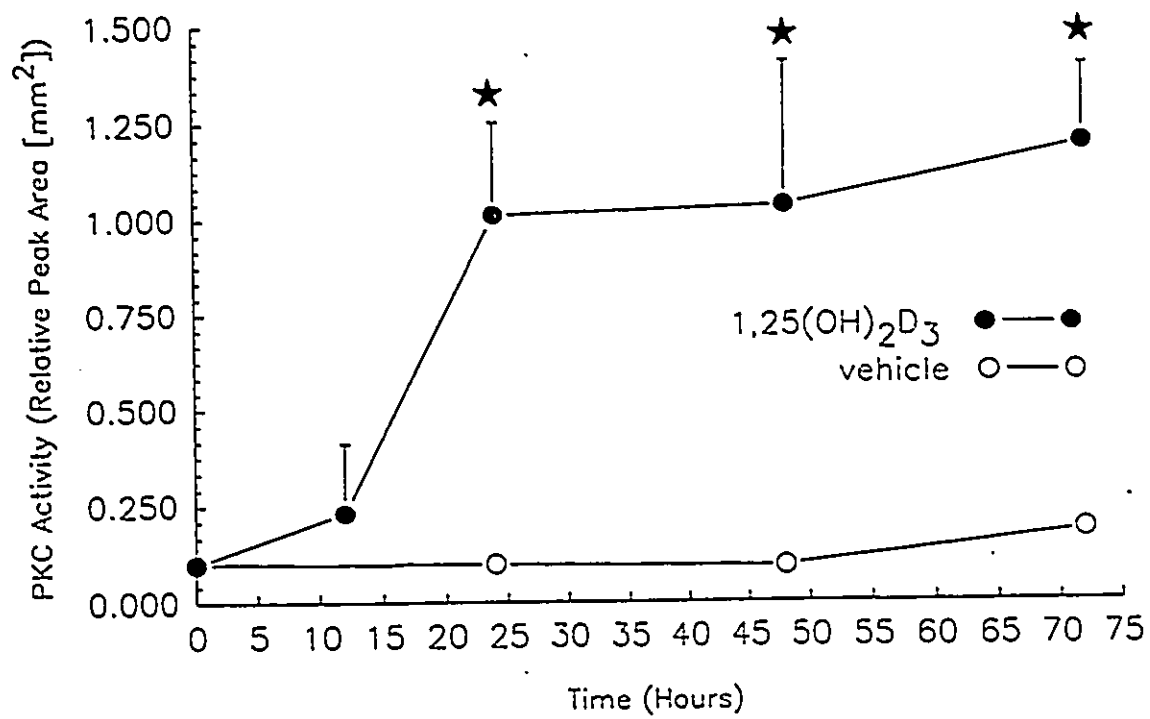


Figure 4.2 *Effect of 1,25(OH)₂D₃ on calcium- and phospholipid-dependent PKC activity in particulate fractions of MDBK cells.*

Scrum-free MDBK cells were treated for up to 72 hours with 10^{-7} M 1,25(OH)₂D₃ or ethanol (0.05%). Membrane fractions were prepared as previously described. PKC activity was measured by incubating membrane fractions with [γ -³²P]-ATP and a 85 kDa PKC-specific substrate as described in Methods. Data was obtained by densitometric scanning of autoradiographs. Values represent mean $\pm$ SEM ($N=4$) and statistical significances ($P < 0.05$) were assessed by Student's *t*-test for unpaired data (indicated by * on graph).



dose of $1,25(\text{OH})_2\text{D}_3$ has previously been shown to maximally increase PKC activity in MDBK cells, and is comparable with doses inducing calbindin D-28K and VDR levels (Simboli-Campbell et al, 1992a; Fig. 2.1).

To identify the PKC isozyme(s) potentially involved in $1,25(\text{OH})_2\text{D}_3$ -mediated actions, we next examined the effects of this hormone on isozyme-specific expression in MDBK cells, using immunofluorescence techniques. Within 24 hour of treatment, consistent with the activity data, $1,25(\text{OH})_2\text{D}_3$ -treated cells exhibit a pronounced increase in PKC α immunoreactivity associated with plasma membranes (Fig. 4.3), however no increase in PKC α expression is observed in whole extracts of vitamin D-treated MDBK cells compared to ethanol-treated cells (Simboli-Campbell et al, 1994). As observed with PKC α , $1,25(\text{OH})_2\text{D}_3$ does not detectably alter the total cellular levels of PKC β (Simboli-Campbell et al, 1994), although the hormone causes the translocation of this isozyme to the peri-nuclear region (Fig. 4.4). These results are consistent with data from our lab showing translocation of PKC β to the nucleus and increased PKC α immunoreactivity in membrane fractions of MDBK cells treated with $1,25(\text{OH})_2\text{D}_3$ (Simboli-Campbell et al, 1994). The expression and subcellular localization of PKC ϵ and PKC ζ , two calcium-independent PKC isozymes, were unaffected by $1,25(\text{OH})_2\text{D}_3$ treatment (Figs. 4.5 and 4.6). This preferential regulation of the calcium-dependent PKC isozymes may be related to the calciotropic nature of this hormone.

4.3.3 Effect of TPA on PKC activity, expression and subcellular localization in MDBK cells.

As previously reported in several other systems, TPA induces membrane PKC activity

Figure 4.3 Effect of 1,25(OH)₂D₃ on PKC α expression and subcellular localization in MDBK cells.

Scrum-free MDBK cells grown on coverslips were treated for 24 hours with 10^{-7} M 1,25(OH)₂D₃ or ethanol (0.05%). Cells were fixed in ethanol:acetone (1:1), blocked and incubated with isozyme-specific PKC α antibodies, followed by incubation with FITC-labelled secondary antibodies as described in Methods. Cells were mounted and viewed under phase contrast (left panels) and fluorescence (right panels) microscopy. In all cases, non-specific immunoreactivity was negligible.

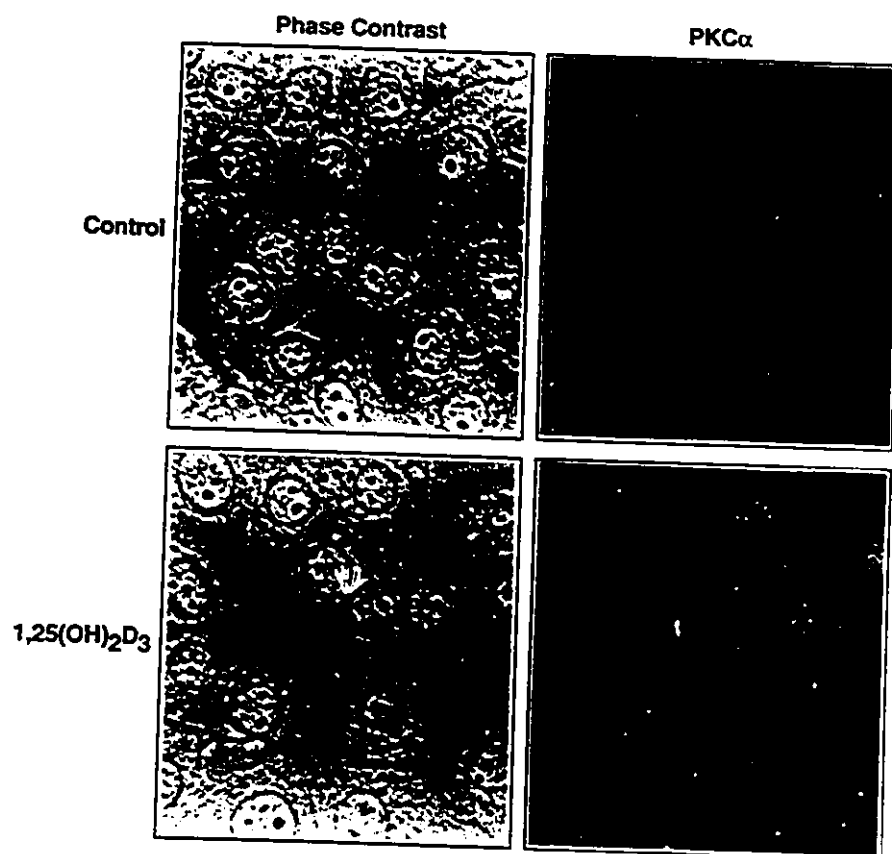


Figure 4.4 Effect of 1,25(OH)₂D₃ on PKC β expression and subcellular localization in MDBK cells.

Serum-free MDBK cells grown on coverslips were treated for 24 hours with 10^{-7} M 1,25(OH)₂D₃ or ethanol (0.05%). Cells were fixed in ethanol:acetone (1:1), blocked and incubated with isozyme-specific PKC β antibodies, followed by incubation with FITC-labelled secondary antibodies as described in Methods. Cells were mounted and viewed under phase contrast (left panels) and fluorescence (right panels) microscopy. In all cases, non-specific immunoreactivity was negligible.

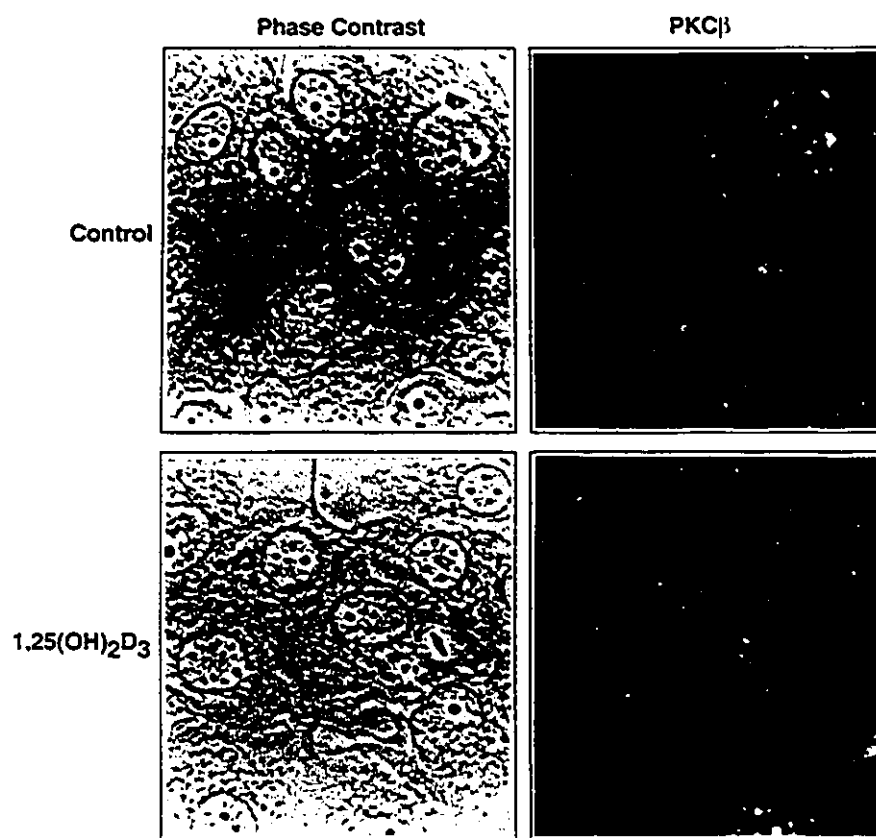


Figure 4.5 *Effect of 1,25(OH)₂D₃ on PKC ϵ expression and subcellular localization in MDBK cells.*

Serum-free MDBK cells grown on coverslips were treated for 24 hours with 10^{-7} M 1,25(OH)₂D₃ or ethanol (0.05%). Cells were fixed in ethanol:acetone (1:1), blocked and incubated with isozyme-specific PKC ϵ antibodies, followed by incubation with FITC-labelled secondary antibodies as described in Methods. Cells were mounted and viewed under phase contrast (left panels) and fluorescence (right panels) microscopy. In all cases, non-specific immunoreactivity was negligible.

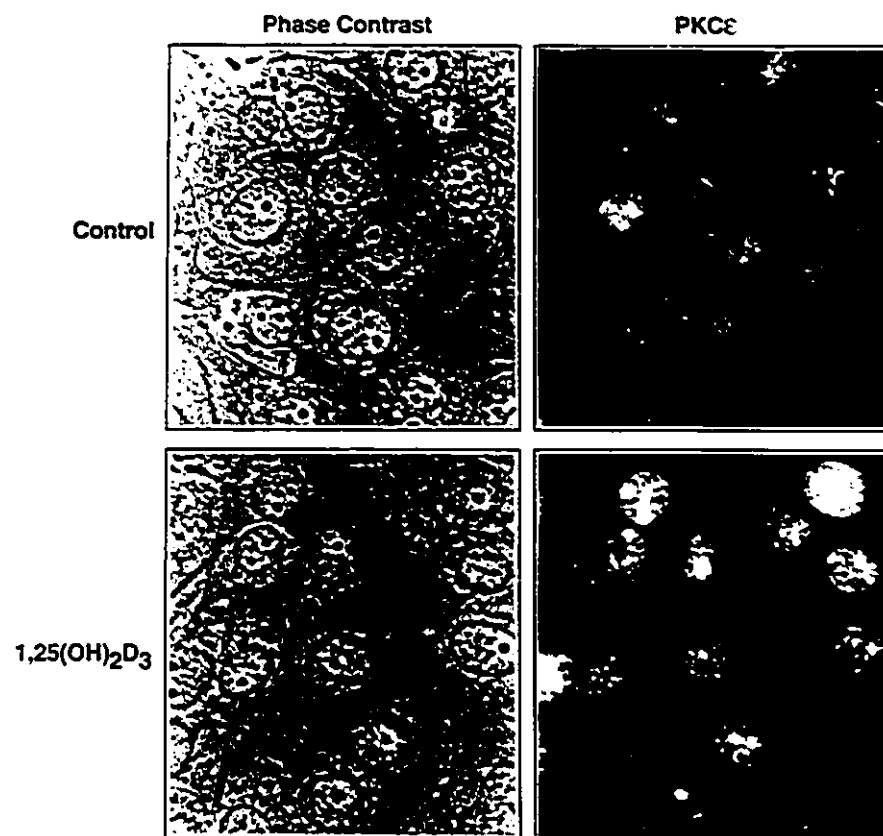
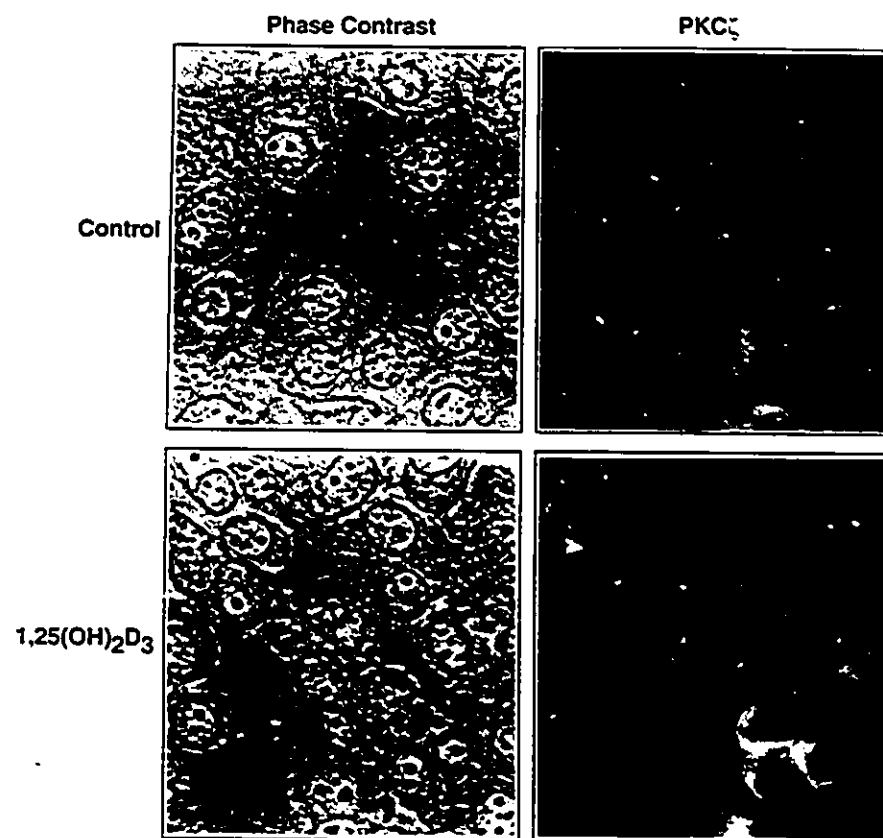


Figure 4.6 *Effect of 1,25(OH)₂D₃ on PKC ζ expression and subcellular localization in MDBK cells.*

Serum-free MDBK cells grown on coverslips were treated for 24 hours with 10^{-7} M 1,25(OH)₂D₃ or ethanol (0.05%). Cells were fixed in ethanol:acetone (1:1), blocked and incubated with isozyme-specific PKC ζ antibodies, followed by incubation with FITC-labelled secondary antibodies as described in Methods. Cells were mounted and viewed under phase contrast (left panels) and fluorescence (right panels) microscopy. In all cases, non-specific immunoreactivity was negligible.



rapidly (within 1 hour) but the effect is transient (Fig. 4.7). PKC activity in TPA treated cells falls below control levels within 24 hours of treatment in MDBK cells. The temporal biphasic effect of TPA on PKC activity resembles its effect on calbindin D-28K, which increases rapidly before falling below control levels after 24 hours (Figs. 3.4 and 3.5). The early rise in membrane PKC activity in response to TPA is associated with increased PKC α immunoreactivity in particulate fractions (Fig. 4.8) and plasma membrane staining (Fig. 4.9) of MDBK cells, with a concomitant decrease in cytosolic levels. The translocation of PKC α from cytosol to membrane occurs within 45 minutes, a time course which is consistent with the induction of PKC activity. The enhanced membrane association of PKC α is sustained for up to 4 hours after TPA treatment, although PKC activity has returned to control levels by 2 hours (Fig. 4.7). Short term TPA treatment also results in a rapid translocation of PKC β to the nucleus (Fig. 4.11), which persists for up to 24 hours and is accompanied by a similar decrease in cytosolic PKC β , detected by immunofluorescence (Fig. 4.13). As previously mentioned, long term treatment of various cells with TPA is usually associated with the down-regulation of PKC activity and expression. This characteristic of phorbol esters is also evident in MDBK cells, which display decreased PKC α , PKC ϵ and PKC ζ immunoreactivity in TPA-treated MDBK cells as compared to vehicle controls (Figs. 4.12, 4.14 and 4.15). In contrast, the total cellular levels of PKC β appears unaffected by 24 hour treatment with TPA (Fig 4.13; Simboli-Campbell et al, 1994), suggesting the presence of a mechanism protecting PKC β from proteolytic degradation.

Figure 4.7 *Effect of TPA on calcium- and phospholipid-dependent PKC activity in particulate fractions of MDBK cells.*

Serum-free MDBK cells were treated for up to 24 hours with 10^{-7} M TPA or vehicle (PBS:DMSO, 0.05%). Membrane fractions were prepared as previously described. PKC activity was measured by incubating membrane fractions with [γ - 32 P]-ATP and a 85 kDa PKC-specific substrate as described in Methods. Data was obtained by densitometric scanning of autoradiographs. Values represent mean $\pm$ SEM ($N = 4$) and statistical significances between treatments ($P < 0.05$) were assessed by Student's t -test for unpaired data (indicated by * on graph).

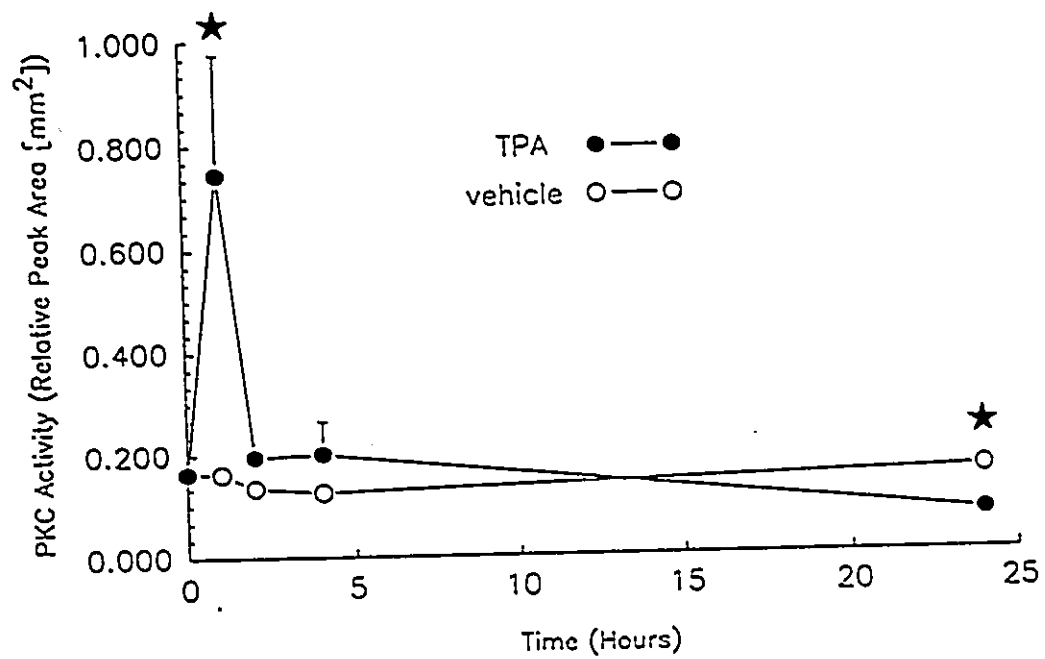


Figure 4.8 *Temporal effect of TPA on PKC α expression and subcellular localization in MDBK cells, assessed by immunoblotting.*

Scrum-free MDBK cells were treated for 0, 0.75, 1, 2 and 4 hours with 10^{-7} M TPA or vehicle (PBS;DMSO, 0.05%). Cytosolic and membrane proteins (25 μ g/lane) were separated on 10% SDS-PAGE, transferred to nitrocellulose and blotted for PKC α as described in **Methods**. The numbers on the right of the figure indicate the migration and molecular weight (kDa) of protein markers.

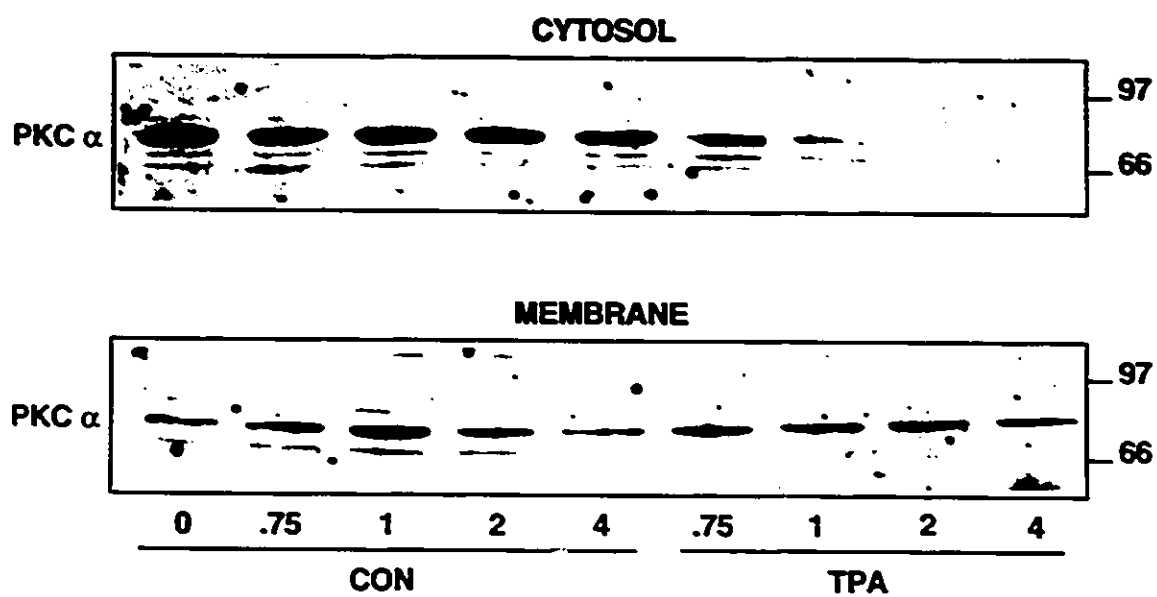


Figure 4.9 Short term effect of TPA on PKC α expression and subcellular localization in MDBK cells, assessed by immunofluorescence.

Serum-free MDBK cells grown on coverslips were treated for 1 hour with 10^{-7} M TPA or vehicle (PBS:DMSO, 0.05%). Cells were fixed with ethanol:acetone (1:1), blocked and incubated with PKC α antibodies, followed by incubation with FITC-labelled secondary antibodies as described in Methods. Cells were mounted and viewed under phase contrast (left panels) and fluorescence (right panels) microscopy. Non-specific immunoreactivity, assessed in the absence of primary antibodies, was negligible.

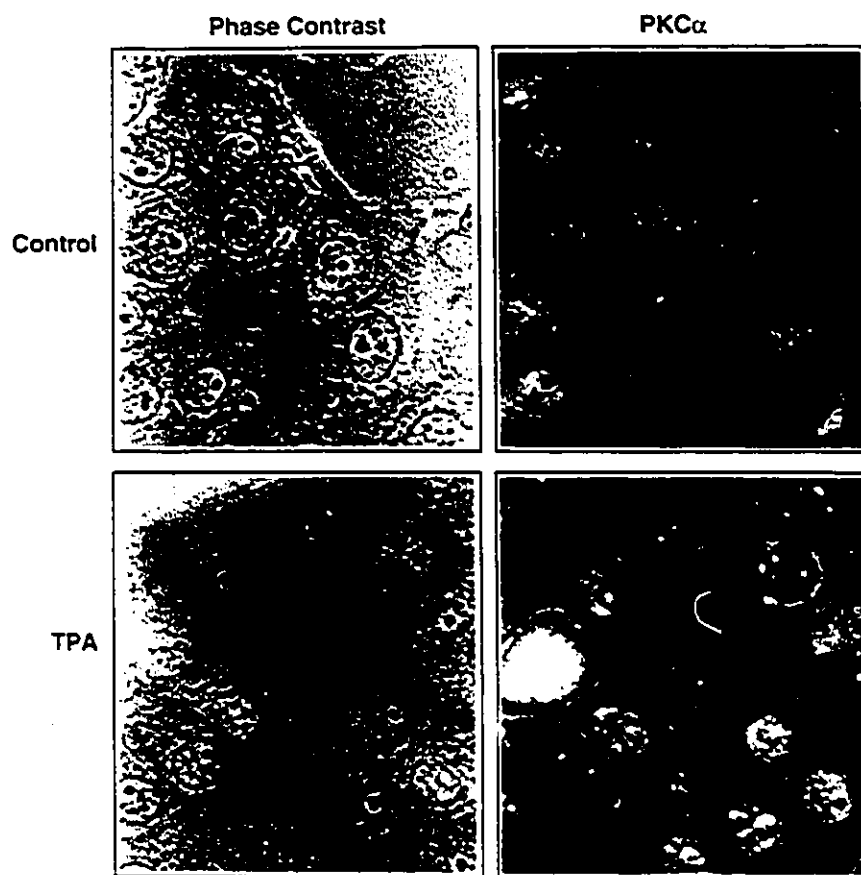


Figure 4.10 Temporal effect of TPA on PKC β expression and subcellular localization in MDBK cells, assessed by immunoblotting.

Serum-free MDBK cells were treated for up to 2 hours with 10^{-7} M TPA or vehicle (PBS;DMSO, 0.05%). Cytosolic and membrane proteins (25 μ g/lane) were separated on 10% SDS-PAGE, transferred to nitrocellulose and blotted for PKC β as described in Methods.

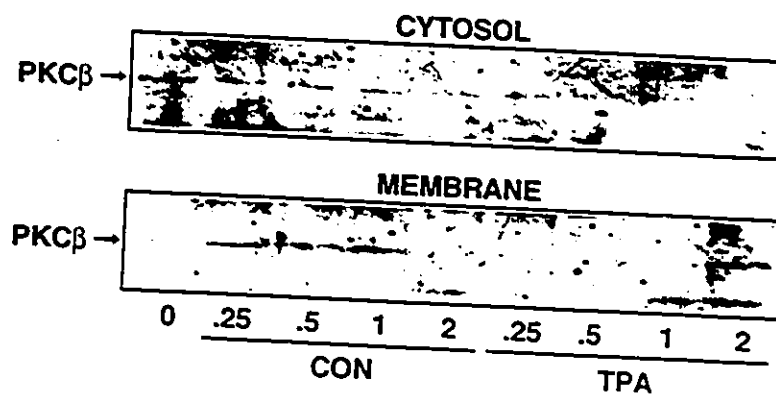


Figure 4.11 Short term effect of TPA on PKC β expression and subcellular localization in MDBK cells, assessed by immunofluorescence.

Serum-free MDBK cells grown on coverslips were treated for 1 hour with 10^{-7} M TPA or vehicle (PBS:DMSO, 0.05%). Cells were fixed with ethanol:acetone (1:1), blocked and incubated with PKC β antibodies, followed by incubation with FITC-labelled secondary antibodies as described in Methods. Cells were mounted and viewed under phase contrast (left panels) and fluorescence (right panels) microscopy. Non-specific immunoreactivity, assessed in the absence of primary antibodies, was negligible.

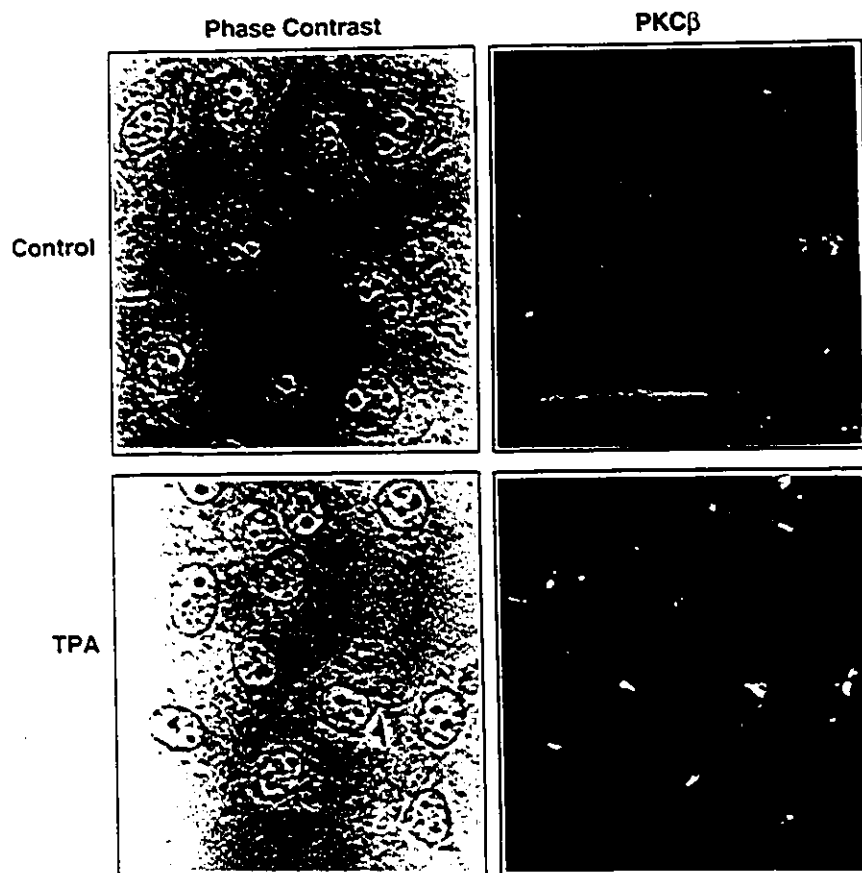


Figure 4.12 Long term effect of TPA on PKC α expression and subcellular localization in MDBK cells.

Serum-free MDBK cells grown on coverslips were treated for 24 hours with 10^{-7} M TPA or vehicle (PBS:DMSO, 0.05%). Cells were fixed in ethanol:acetone (1:1), blocked and incubated with isozyme-specific PKC α antibodies, followed by incubation with FITC-labelled secondary antibodies as described in **Methods**. Cells were mounted and viewed under phase contrast (left panels) and fluorescence (right panels) microscopy. In all cases, non-specific immunoreactivity was negligible.

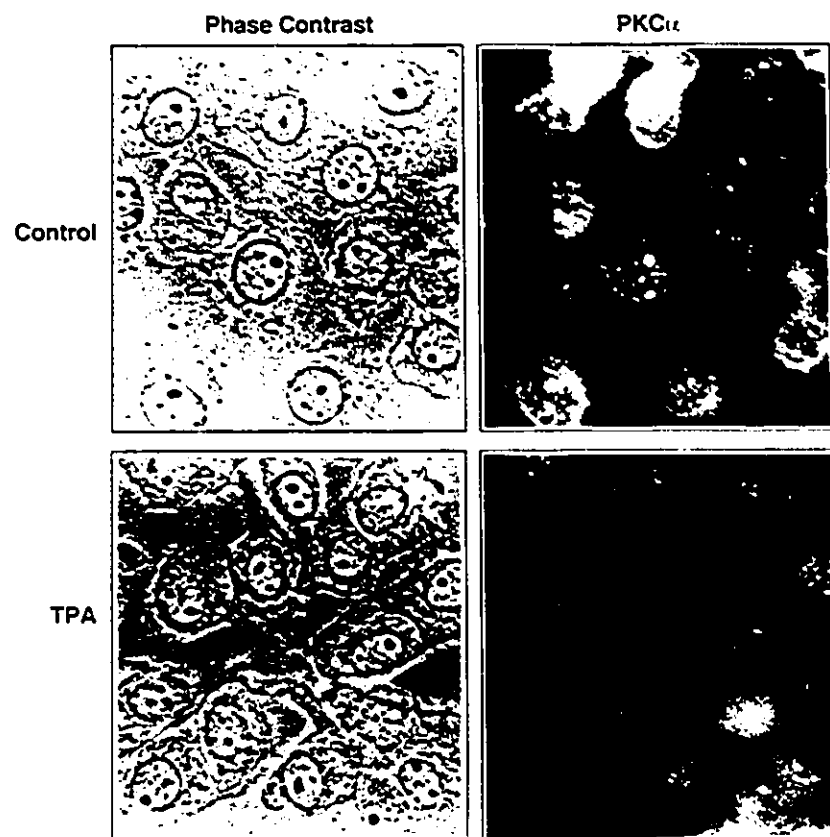


Figure 4.13 Long term effect of TPA on PKC β expression and subcellular localization in MDBK cells.

Serum-free MDBK cells grown on coverslips were treated for 24 hours with 10^{-7} M TPA or vehicle (PBS:DMSO, 0.05%). Cells were fixed in ethanol:acetone (1:1), blocked and incubated with isozyme-specific PKC β antibodies, followed by incubation with FITC-labelled secondary antibodies as described in Methods. Cells were mounted and viewed under phase contrast (left panels) and fluorescence (right panels) microscopy. In all cases, non-specific immunoreactivity was negligible.

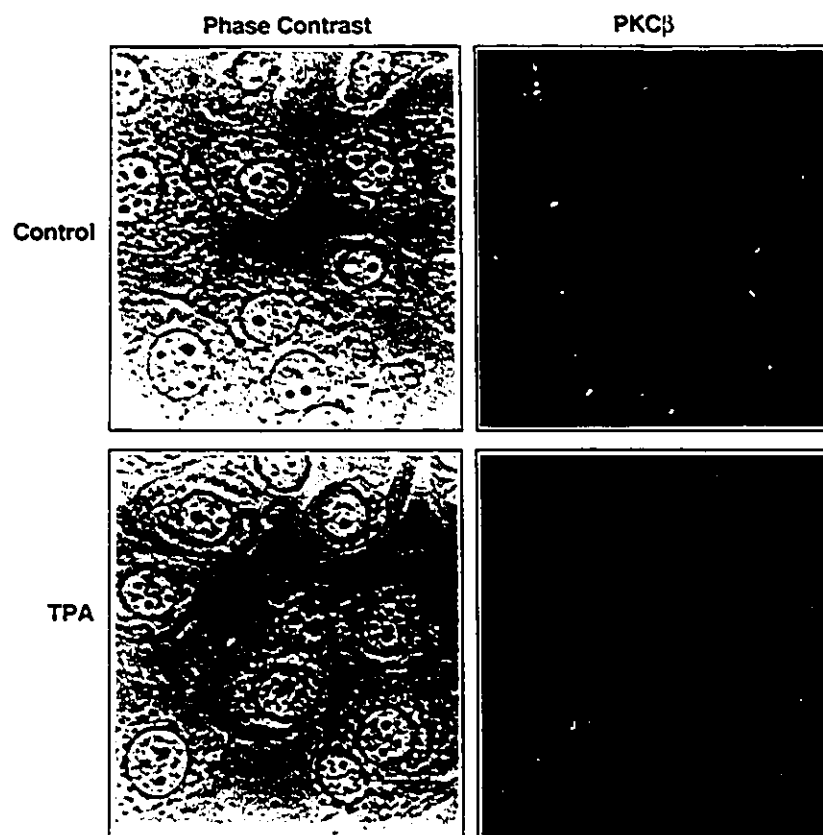


Figure 4.14 Long term effect of TPA on PKC ϵ expression and subcellular localization in MDBK cells.

Scrum-free MDBK cells grown on coverslips were treated for 24 hours with 10^{-7} M TPA or vehicle (PBS:DMSO, 0.05%). Cells were fixed in ethanol:acetone (1:1), blocked and incubated with isozyme-specific PKC ϵ antibodies, followed by incubation with FITC-labelled secondary antibodies as described in Methods. Cells were mounted and viewed under phase contrast (left panels) and fluorescence (right panels) microscopy. In all cases, non-specific immunoreactivity was negligible.

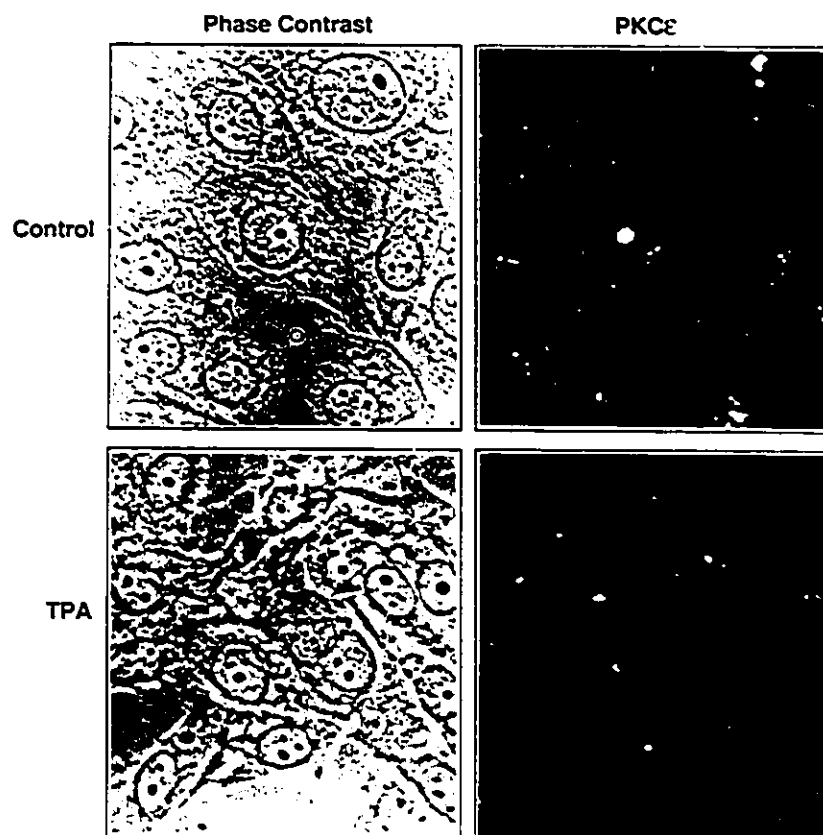
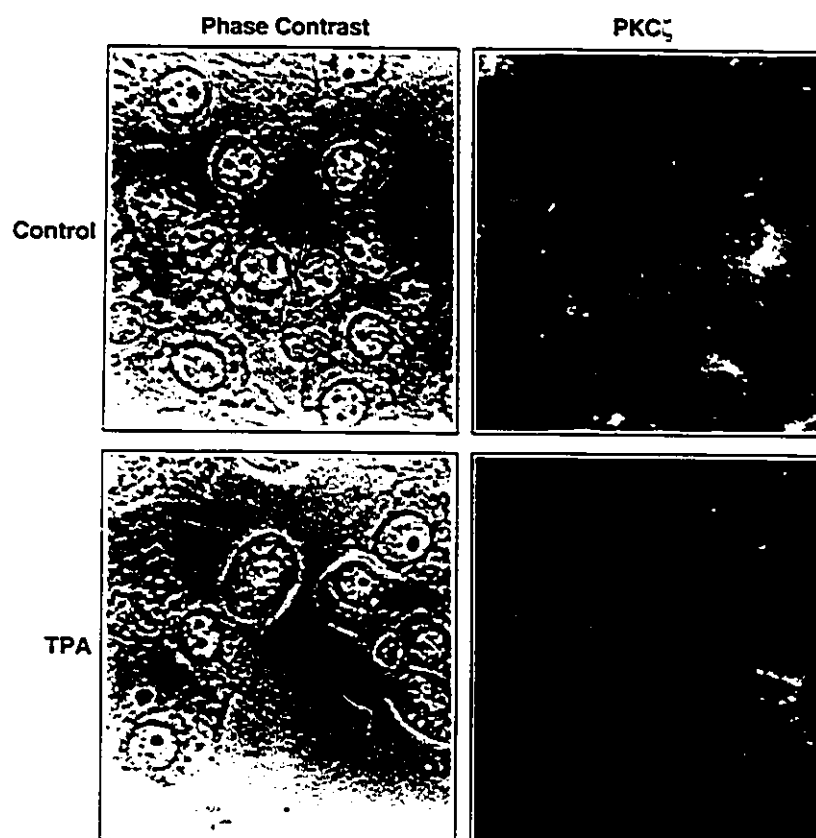


Figure 4.15 Long term effect of TPA on PKC ζ expression and subcellular localization in MDBK cells.

Scrum-free MDBK cells grown on coverslips were treated for 24 hours with 10^{-7} M TPA or vehicle (PBS:DMSO, 0.05%). Cells were fixed in ethanol:acetone (1:1), blocked and incubated with isozyme-specific PKC ζ antibodies, followed by incubation with FITC-labelled secondary antibodies as described in Methods. Cells were mounted and viewed under phase contrast (left panels) and fluorescence (right panels) microscopy. In all cases, non-specific immunoreactivity was negligible.



4.4 DISCUSSION

Calbindin D-28K regulation has been shown to involve post-transcriptional mechanisms. The demonstration that $1,25(\text{OH})_2\text{D}_3$ and phorbol esters regulate both calbindin D-28K and VDR in MDBK cells implicate the PKC signal transduction pathway in this regulation. Differential regulation of these proteins by TPA and $1,25(\text{OH})_2\text{D}_3$ prompted us to characterize the specific effects of these two agents on PKC activity and expression in MDBK cells.

4.4.1 PKC isozyme expression in MDBK cells

Previous groups have reported the expression of four PKC isozymes in whole kidney extracts (α , β , ϵ , and ζ) (Dong, Stevens and Jaken, 1991; Nakanishi and Exton, 1992; Balboa, Firestein, Godson, Bell and Insel, 1994; Mühl and Pfeilschifter, 1994). Consistent with this data, MDBK cells specifically express two calcium-dependent ($\text{PKC}\alpha$ and $\text{PKC}\beta$) and two calcium-independent ($\text{PKC}\epsilon$ and $\text{PKC}\zeta$) PKC isozymes (Fig. 4.1). The role of each isozyme in kidney function is unknown, although PKC has been implicated in various processes including renal compensatory growth and the regulation of Na-K-ATPase (Caramelo, Tsai, Okada and Schrier, 1988; Satoh, Cohen and Katz, 1993) and vitamin D hydroxylases (Welsh et al, 1991).

4.4.2 Regulation of PKC activity, expression and subcellular localization by 1,25(OH)₂D₃ in MDBK cells.

Results presented in this chapter demonstrate that 1,25(OH)₂D₃ increases calcium-dependent PKC activity in membrane fractions of MDBK cells (Fig. 4.2). In contrast to the recently reported direct activation of PKC by 1,25(OH)₂D₃ (Slater et al, 1995), the long term regulation described here is consistent with a VDR-mediated genomic effect of the hormone on PKC gene expression, as demonstrated in HL-60 cells (Solomon et al, 1991). The rapid actions of 1,25(OH)₂D₃ on PKC activity and expression may be involved in the non-genomic effects of this hormone which occur within minutes of treatment, while persistent induction of PKC activity may regulate phenotypic changes in cell growth and differentiation. Sustained induction of PKC activity in response to 1,25(OH)₂D₃ may result from a direct effect of the hormone on PKC gene transcription or from the activation/induction of phospholipases C or A₂, maintaining elevated levels of PKC activators. In kidney, the stimulation of calcium pumps and calcium channels involved in the 1,25(OH)₂D₃-stimulated renal reabsorption of calcium results in elevations of intracellular calcium levels (Kumar, 1995). Consequently, the sustained induction in PKC activity observed in 1,25(OH)₂D₃-treated MDBK cells may be related to a rise in intracellular calcium levels. Further studies are required to determine the mechanism by which 1,25(OH)₂D₃ regulates PKC activity.

Consistent with the PKC activity data, 1,25(OH)₂D₃ increases the expression of PKC α in membrane fractions of MDBK cells (Fig. 4.3). The lack of induction of PKC α expression in total cell extracts of MDBK cells treated with 1,25(OH)₂D₃ compared to vehicle-treated cells (Simboli-Campbell et al, 1994) argues against a genomic effect of the hormone, which

would result in increased gene transcription. $1,25(\text{OH})_2\text{D}_3$ may regulate PKC α activity and subcellular localization through sustained elevation of DAG and Ca^{2+} concentrations or by modulating proteins involved in the stability/membrane-localization of PKC α . Further studies are required to define the mechanism underlying the regulation of PKC α by $1,25(\text{OH})_2\text{D}_3$.

$1,25(\text{OH})_2\text{D}_3$ treatment also results in the translocation of cytosolic and membrane PKC β to a peri-nuclear localization (Figs. 4.3 and 4.4), which is accompanied by increased calcium- and phospholipid-dependent phosphorylation of endogenous nuclear proteins (Simboli-Campbell et al, 1994). A bipartite nuclear localization signal has been identified in PKC β ; it has been suggested to be unmasked upon activation/calcium binding, resulting in the nuclear translocation of this isozyme (Goss et al, 1994; Dekker and Parker, 1994). Such sites have yet to be identified in other PKC isozymes, although nuclear localization has been observed in various cell lines. The $1,25(\text{OH})_2\text{D}_3$ -dependent nuclear translocation of PKC β , which was confirmed by immunoblotting of purified cellular fractions of MDBK cells (not shown, Simboli-Campbell et al, 1994), was not accompanied by an increase in total cellular expression of this isozyme (Simboli-Campbell et al, 1994), inferring that the effect of $1,25(\text{OH})_2\text{D}_3$ may not simply involve activation of PKC β gene transcription.

The physiological relevance of vitamin D regulation of calcium-dependent PKC isozyme activity, expression and subcellular localization is unknown. In HL-60 cells, increased expression of PKC β and phosphorylation of nuclear proteins in response to $1,25(\text{OH})_2\text{D}_3$ has been associated with decreased expression of *c-myc*. Inhibition of *c-myc* is thought to be implicated in the differentiation process of HL-60 cells. In kidneys, a classical target of vitamin D, $1,25(\text{OH})_2\text{D}_3$ -mediated increases in PKC may be related to

calbindin D-28K expression, VDR numbers/or and vitamin D metabolism. Our present data further suggest a temporal correlation between membrane PKC α activation and calbindin D-28K levels in MDBK cells treated with 1,25(OH) $_2$ D $_3$. On the other hand, the report that VDR serves as an *in vitro* substrate for PKC β suggests that 1,25(OH) $_2$ D $_3$ -dependent nuclear translocation of this PKC isozyme may be important for VDR regulation or activity (Fig. 4.3b). The functional relevance of VDR phosphorylation is unclear, but may play a role in receptor stability or transactivation potential.

In contrast, 1,25(OH) $_2$ D $_3$ did not have any detectable effect on the expression or subcellular localization of the calcium-independent isozymes, PKC ϵ and PKC ζ (Figs. 4.5 and 4.6). Restriction of the effect of 1,25(OH) $_2$ D $_3$ to the calcium-dependent PKC isozymes (PKC α and PKC β) may indicate that intracellular calcium fluxes, which are generated in various systems in response to 1,25(OH) $_2$ D $_3$ (Tornquist and Tashjian, 1989; Caffrey and Farach-Carson, 1989), are involved in the mechanism of translocation and activation of PKC α and PKC β .

4.4.3 Regulation of PKC activity, expression and subcellular localization by TPA in MDBK cells.

As previously described in various systems, PKC activity is acutely and transiently activated (within 1 hour) in membrane fractions of MDBK cells, followed by a long term down-regulation of the enzyme (Fig. 4.7). This regulation by TPA is in contrast to the more persistent effects of 1,25(OH) $_2$ D $_3$ on PKC activity, clearly indicating different pathways for PKC activation. The expression of PKC α in membranes of MDBK cells follows a similar

trend: increasing rapidly (within 45 min) but transiently, decreasing to below control levels within 24 hours of treatment with TPA (Figs. 4.8, 4.9 and 4.12). The apparent lesser effect of TPA on the membrane translocation of PKC α observed in immunoblots of purified cellular fractions of MDBK cells (Fig. 4.8) compared to immunofluorescence data (Fig. 4.9) may reflect a relocalization of this PKC isozyme during cellular fractionation. This time course of PKC activity and expression is comparable to the effect of TPA on calbindin D-28K expression, again suggesting a temporal correlation between PKC α expression and activity and calbindin D-28K levels in MDBK cells.

Interestingly, as observed with 1,25(OH) $_2$ D $_3$, 24 hour treatment with TPA also induces PKC β translocation to the nucleus (Fig. 4.13). However, this translocation is not associated with increased calcium- and phospholipid-dependent phosphorylation of endogenous nuclear proteins (Simboli-Campbell et al, 1994). It is presently unclear why nuclear fractions from 1,25(OH) $_2$ D $_3$ -treated MDBK cells, but not TPA-treated cells, exhibit increased phosphorylation of nuclear proteins, since both treatments apparently induce nuclear translocation of PKC β . It is possible that TPA translocates PKC β to the nucleus without inducing the activity of this isozyme, at the time point tested (24 hours) (Simboli-Campbell et al, 1994). The translocation of PKC β to the nucleus in response to TPA is a rapid event, occurring within one hour, suggesting that increased activity may occur prior to 24 hours (Fig. 4.11). Nuclear staining of PKC β may persist longer than the increase in activity, as was observed for PKC α (Figs. 4.7 and 4.8). It is also possible that 1,25(OH) $_2$ D $_3$ and TPA differentially regulate subtypes of PKC β (β_1 and β_2), which were not distinguished in our experiments. Further studies, including kinetics of nuclear PKC activity in response to both

treatments and investigating the phosphorylation state of specific nuclear PKC substrates, are required to distinguish between these various possibilities.

Long term treatment with TPA characteristically down-regulates PKC expression, depleting the cell of this enzyme. This effect is evident with PKC α , PKC ϵ and even PKC ζ (Figs. 4.12, 4.14 and 4.15), which has been reported not to bind phorbol esters *in vitro*. The regulatory domain of PKC ζ contains only one of the cysteine-rich, zinc finger-like regions and is thus not directly regulated by phorbol esters. The down-regulation of this isozyme observed in MDBK cells treated with TPA for 24 hours may reflect a secondary effect of TPA on calpain activation. In contrast, 24 hour treatment with TPA does not cause a detectable decrease in PKC β expression in total extracts of MDBK cells (Simboli-Campbell et al, 1994). The nuclear localization of PKC β may be protective against proteolytic cleavage, usually catalyzed by calpain; however, PKC ϵ , whose subcellular localization is predominantly nuclear in MDBK cells, was down-regulated in response to long term TPA treatment. The fact that PKC β expression is not reduced in MDBK cells treated with TPA for 24 hours, a treatment that significantly decreases calbindin D-28K levels, suggest that this isozyme is not implicated in calbindin D-28K regulation. Our results emphasize the distinct regulation of the various isozymes and suggest their involvement in distinct regulatory pathways.

The results described here suggest a role for the PKC signalling pathway in mediating the renal effects of 1,25(OH) $_2$ D $_3$, including calbindin D-28K and VDR regulation. The mechanism of regulation of these vitamin D-dependent proteins by PKC and modulation of

PKC isozyme activity and subcellular localization are unclear but probably involve reversible phosphorylation steps.

4.5 CONCLUSIONS

The results presented in this chapter implicate PKC α and PKC β in the renal actions of 1,25(OH) $_2$ D $_3$, including the regulation of calbindin D-28K and VDR expression. More precisely, we demonstrate:

- a temporal relationship between membrane PKC α activity and expression and calbindin D-28K levels.
- that 1,25(OH) $_2$ D $_3$ preferentially regulates calcium-dependent PKC isozymes.
- that both 1,25(OH) $_2$ D $_3$ and TPA treatments result in the translocation of PKC β to a perinuclear localization.

CHAPTER 5: PHOSPHORYLATION OF CALBINDIN D-28K

5.1 INTRODUCTION

Reversible phosphorylation events have been implicated in several regulatory pathways. The phosphorylation of transcription factors ultimately controls the rate of transcription of specific genes (Angel and Karin, 1991). Translation rates are also modulated by phosphorylation of various regulatory factors (Mendez and deHaro, 1994; Duncan, Cavener and Qu, 1995). Direct phosphorylation of proteins may affect their function, localization and turnover rate (Kawamoto and Hidaka, 1984; Ainsztein and Purich, 1994). Our previous data suggest that calbindin D-28K expression may be altered by phosphorylation event(s), which may occur at any level described.

5.1.1 Vitamin D regulation of phosphorylated proteins

1,25(OH)₂D₃-dependent phosphorylation events have been described in several systems. The differentiative effect of 1,25(OH)₂D₃ on HL-60 cells is associated with phosphorylation of nuclear proteins such as lamin B and histone H1 (Martell et al, 1992) and an unidentified cytosolic 75 kDa protein (pp75) (Bushkin, Roth, Heffetz and Zick, 1991). In cardiac muscle and rat colonocytes, 1,25(OH)₂D₃ induces the phosphorylation of two membrane proteins in correlation with increased calcium transport (Wali et al, 1990; Selles and Boland, 1991). Similar phosphorylation patterns are observed in myocytes treated with

forskolin, a known regulator of adenylyl cyclase, implicating the cAMP-dependent signalling pathway in these events (Selles and Boland, 1991).

Osteopontin, a vitamin D-regulated secreted protein expressed predominantly in bone and kidney, is phosphorylated on tyrosine, serine and threonine residues. The induction of osteopontin expression by $1,25(\text{OH})_2\text{D}_3$ is well documented (Terpening, Haussler, Jurutka, Galligan, Komm and Haussler, 1991; Crivello and Delvin, 1992). In contrast, controversial reports exist on the role of this hormone in modulating osteopontin phosphorylation. In JB6 epidermal cells, $1,25(\text{OH})_2\text{D}_3$ increases the production of a non-phosphorylated form of osteopontin (Chang and Prince, 1991); while in osteosarcoma and RCA1 osteoblastic cells, the hormone induces both osteopontin levels and phosphorylation (Kasugai, Zhang, Overall, Wrana, Butler and Sodek, 1991). The kinase responsible for this phosphorylation is currently unknown, but both CK II and PKA have been shown to phosphorylate osteopontin *in vitro* (Ashkar, Teplow, Glimcher and Saavedra, 1993).

The VDR can be phosphorylated by CK II and PKC both *in vitro* and *in vivo* (Hsieh et al, 1991; Jurutka, Terpening and Haussler, 1993; Hilliard et al, 1994; Hsieh et al, 1993). The functional relevance of these phosphorylation events are unknown. CK II phosphorylation of the VDR occurs on Ser²⁰⁵, a residue located between the DNA-binding and ligand-binding domains, but may not be required for transcriptional activation (Jurutka, Hsieh, MacDonald, Terpening, Haussler, Haussler and Whitfield, 1993). In contrast, PKC phosphorylation of the VDR is hormone-independent and may play a role in receptor stability/turnover (Hsieh et al, 1993).

5.1.2 Sequence analysis of calbindin D-28K

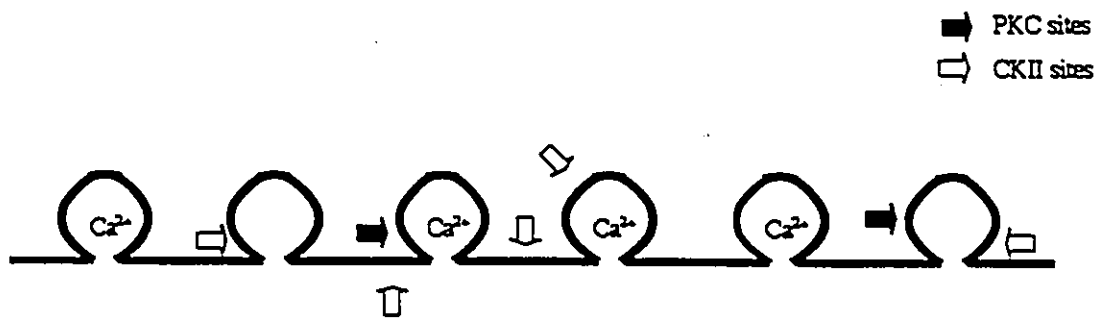
Sequence analysis of calbindin D-28K reveals a total of seven consensus phosphorylation sites, five of which are specific for casein kinase II (CK II) and two for PKC (Fig. 5.1). All five CK II sites in calbindin D-28K are characterized by the presence of an acidic amino acid located three residues C-terminal to the phosphorylatable serine/threonine (Glu/Asp-Xaa-Xaa-Ser/Thr), consistent with the minimal requirement for a CK II phosphorylation site (Mcggio, Perich, Meycr, Hoffmann-Posorske, Lennon, Johns and Pinna, 1989). The presence of other acidic amino acids in the vicinity of the phosphorylatable residue also greatly increases substrate suitability (Kuenzel, Mulligan, Sommercorn and Krebs, 1987). However, in spite of the high degree of homology displayed by calbindin D-28K from different species, these CK II phosphorylation sites are not all conserved in avian and human calbindin D-28K (Fullmer and Wasserman, 1987; Parmentier, 1989).

In contrast, the two PKC consensus phosphorylation sites are conserved in rat, human and avian calbindin D-28K (Wood et al, 1988; Parmentier, 1989; Fullmer and Wasserman, 1987), suggesting their functional relevance. PKC consensus sequences are not as well defined as CK II phosphorylation sites, but are characterized by the presence of two or more basic residues located near the phosphorylatable serine/threonine (Pearson and Kemp, 1991). These sites are similar to those recognized by other serine/threonine kinases, including PKA and myosin light chain kinase; however, each kinase phosphorylates distinct substrates, suggesting that other structural components are important in enzyme recognition (Zetterqvist, Ragnarsson, Humble, Berglund and Engstrom, 1976; Kemp, Pearson and House, 1983).

The large spectrum of phosphorylation sites associated with PKC may be related to

Figure 5.1 Potential phosphorylation sites in calbindin D-28K.

Schematic of rat calbindin D-28K describing 6 helix-loop-helix (EF-hand) domains and potential phosphorylation sites. The sequence was derived from a rat calbindin D-28K cDNA and translated using the PC\Gene program. Potential phosphorylation sites were also identified using this computer program and meets with the minimum requirement phosphorylation sites of the specified kinases.



CKII phosphorylation sites:

⁶⁰Ser: *Ser*-Phe-Val-Asp
⁹⁸Ser: *Ser*-Cys-Glu-Glu
¹³⁷Thr: *Thr*-Val-Asp-Asp
¹⁵⁶Ser: *Ser*-Asn-Asn-Asp
²³⁷Ser: *Ser*-Ala-Gly-Asp

PKC phosphorylation sites:

¹⁰⁶Thr: Glu-Glu-Phe-Met-Lys-*Thr*-Trp-Arg-Lys-Tyr
²³⁹Thr: Ile-Asn-Asn-Ile-Thr-*Thr*-Lys-Lys-Asn-Ile

the high number of isozymes composing this family of serine/threonine kinases. Mounting evidence suggests the substrate specificity of each isozyme (Turner, Kemp, Su and Kuo, 1985; Marais, Nguyen, Woodgett and Parker, 1990; Kochs, Hummel, Meyer, Hug, Marme and Sarre, 1993). For example, cEF-1 α is phosphorylated by PKC δ , while little phosphorylation is detected in the presence of calcium-dependent PKC isozymes (Kielbassa, Muller, Meyer, Marks and Gschwendt, 1995). The specific sequences recognized by each PKC isozyme have yet to be characterized.

5.1.3 Involvement of CK II in 1,25(OH) $_2$ D $_3$ actions

CK II is described as an ubiquitous, multifunctional, second messenger-independent heterotetrameric serine/threonine kinase (Pinna, 1990). A distinguishing feature of CK II is its ability to utilize as phosphate donor both GTP and ATP, with almost the same efficiency (Cochet, Feige, Pirollet, Keramidas and Chambaz, 1982). Although structurally reminiscent of PKA, with two catalytic (α) and two non-catalytic (β) subunits (Kemp, Froscio, Rogers and Murray, 1975), CK II recognizes acidic phosphorylation sites rather than the basic sequences associated with most other serine/threonine kinases (Tuazon, Bingham and Traugh, 1979).

Due to its constitutive activity, CK II does not appear to be regulated by incoming signals. Nevertheless, increases in CK II activity have been observed in response to mitogenic factors, suggesting its important role in cell growth regulation (Geahlen and Harrison, 1984; Sommercorn and Krebs, 1987). The mechanism underlying this regulation is unclear but may involve phosphorylation of the enzyme (Ackerman and Osheroff, 1989). CK II has also been

involved in mediating some actions of $1,25(\text{OH})_2\text{D}_3$, including osteopontin and VDR phosphorylations (Ashkar et al, 1993; Jurutka et al, 1993). The mechanism via which $1,25(\text{OH})_2\text{D}_3$ regulates CK II activity towards these specific substrates is currently unknown.

5.1.4 Involvement of PKC in $1,25(\text{OH})_2\text{D}_3$ actions

As previously discussed, PKC plays an important role in mediating some effects of $1,25(\text{OH})_2\text{D}_3$. In bone, the vitamin D-dependent increase in osteocalcin production is enhanced in response to PKC activation by phorbol esters (van Leeuwen et al, 1992b). Similarly, transcalcitachia, the rapid induction of intestinal calcium transport by $1,25(\text{OH})_2\text{D}_3$, is further induced in the presence of TPA (de Boland and Norman, 1990). The activities of both the 24- and 1α -hydroxylases are modulated by PKC activators and inhibitors, mimicking the effects of $1,25(\text{OH})_2\text{D}_3$ on these enzymes (Henry and Luntao, 1989; Weaver et al, 1992; Chen et al, 1993). Although containing various consensus phosphorylation sites, calbindin D-28K has never been investigated as a phosphoprotein. Only one report detecting various forms of calbindin D-28K in rat kidney using 2-dimensional gel electrophoresis suggests possible post-translational modifications of this calcium-binding protein *in vivo* (Kendrick, Bishop and DeLuca, 1984).

Our studies demonstrated the role of a short-lived protein and a phosphorylation event in the regulation of calbindin D-28K (Chapters 2 and 3). Further, we have observed a temporal correlation between calbindin D-28K expression and membrane PKC α expression and activity (Chapter 4). However, a direct role of signal transduction pathways in calbindin

D-28K regulation has yet to be determined. We examined the possibility that calbindin D-28K serves as a substrate for CK II and/or PKC, and assessed the phosphorylation state of this calcium-binding protein in MDBK cells treated with TPA and $1,25(\text{OH})_2\text{D}_3$.

5.2 METHODS

5.2.1 Cell culture and transfections

MDBK cells obtained from ATCC were grown in DMEM supplemented with 10% newborn calf serum. Confluent cells were changed to serum-free media 24 hours prior to treatment with 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ in ethanol, 10^{-7} M TPA in PBS:DMSO or vehicles. Cells were harvested by scraping and cellular fractions were prepared as described in 1.2.1. In some cases, cells were lysed in denaturing buffer (containing 9 M urea) and processed for immunoblotting.

A construct of the regulatory domain of PKC α (RD α) was prepared from an EcoRI/ApaI fragment (1154 bp) of PKC α (Ono, Fujii, Igarashi, Kikkawa, Ogita and Nishizuka, 1988), ligated in pSVK3 and subcloned in DH5 α strand of *E. coli* (kindly provided by Dr. Sue Jaken, W. Alton Jones Cell Science Center, Lake Placid, New York). MDBK cells were cotransfected with the purified pSVK3 plasmids with or without the RD α construct in a 50:1 ratio with pSV2 neo, which carries the G418 resistance gene, using lipofectin following the manufacturer's protocol (Life Technologies). Transfectants were selected in G418-containing medium (800 ng/ml, Life Technologies).

5.2.2 CKII *in vitro* phosphorylation assay

Purified rat recombinant calbindin D-28K or casein (1 $\mu\text{g}/\text{assay}$) were diluted in assay buffer (10 mM MgCl_2 , 200 mM NaCl and 0.1 $\mu\text{Ci}/\mu\text{l}$ [γ - ^{32}P]-ATP). Reactions were initiated by the addition of 10 units of purified CK II (Promega) and incubated for 15 minutes at 37°C.

Reactions were terminated by boiling the samples in Laemmli buffer. Proteins were separated on 12% SDS-PAGE and phosphorylated proteins visualized by autoradiography on Kodak X-OMAT film.

5.2.3 PKC/PKC α *in vitro* phosphorylation assay

Purified rat recombinant calbindin D-28K (0.5 μ g/assay) or PKC-specific peptide substrate (300 μ M, Amersham) was diluted in assay buffer (50 mM Tris-HCl, 3 mM DTT, 15 mM MgCl₂, 50 μ M ATP and 0.2 μ Ci [γ -³²P]-ATP/assay) in the presence or absence of calcium and phospholipids (final concentrations: 1.25 mM CaCl₂, 2 μ g/ml phosphatidylserine and 24 μ g/ml TPA; or 1.25 mM EGTA). Reactions were initiated by the addition of 3 μ g partially purified PKC mixture from rabbit brain (provided by Dr. Sue Jaken, W. Alton Jones Cell Science Center, Lake Placid, New York) or 25 ng recombinant PKC α (UBI, Lake Placid, New York) and incubated at room temperature for the indicated times. Reactions were terminated by boiling the samples in Laemmli buffer. Proteins were separated on 12% SDS-PAGE and phosphorylated proteins visualized by autoradiography. Alternatively, reactions were terminated by the addition of 30% TCA and samples were spotted onto acid-treated filter papers. Papers were thoroughly washed and radioactivity determined by liquid scintillation counting.

5.2.4 Immunoblotting

Proteins from cytosolic fractions (50 μ g/lane) or total homogenates (150 μ g/lane) of MDBK cells, transfected MDBK cells or samples from non-radioactive *in vitro* PKC

phosphorylation assay were separated on 12% SDS-PAGE and processed for immunoblotting as described in 1.2.3, using monoclonal calbindin D-28K antibodies (Sigma), diluted 1:200, monoclonal phosphothreonine or phosphoserine antibodies (Sigma), diluted 1:25 or monoclonal VDR antibodies (Chemicon), diluted 1:50 in blocking solution. Membranes were washed and incubated with appropriate alkaline phosphatase-conjugated secondary antibodies (Promega), diluted 1:7500 in blocking solution. Immunoreactivity was detected with NBT and BCIP as described in 2.2.2.

5.2.5 Calbindin D-28K immunoprecipitation

Scrum-free MDBK cells were treated for 24 hours with 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ or ethanol. Cells were washed and incubated in phosphate-free media containing 100 $\mu\text{Ci/ml}$ [^{32}P]-orthophosphate (Amersham) for 4 hours prior to harvest. In some cases, cells were treated with 10^{-7} M TPA or PBS:DMSO within 1 hour of harvest. Cells were harvested by scraping in RIPA buffer (50 mM Tris-HCl, 0.15 M NaCl, 1 mM EDTA, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 50 mM NaF, 1 mM Na vanadate, 10 $\mu\text{g/ml}$ aprotinin, 10 $\mu\text{g/ml}$ leupeptin, 1 mM PMSF and 50 nM okadaic acid, pH 7.4). Samples were heat-treated (65°C, 10 minutes), centrifuged and calbindin D-28K-containing supernatants were pre-cleared with protein A-agarose (final dilution, 1:50, Life Technologies). After centrifugation, samples were incubated for 1 hour at room temperature with polyclonal rabbit calbindin D-28K or calretinin antibodies (final dilution 1:1000, Swant) and protein A agarose (final dilution 1:40) in the presence or absence of 2 μg purified recombinant rat calbindin D-28K. Pellets were washed and proteins were solubilized by boiling in Laemmli buffer.

Immunoprecipitated proteins were separated on 12% SDS-PAGE and phosphorylated proteins were visualized by autoradiography. Alternatively, to determine the amount of calbindin D-28K immunoprecipitated, proteins were electrophoretically transferred to nitrocellulose and blotted for total calbindin D-28K as described above.

5.2.6 Overlay assay

Purified rat recombinant calbindin D-28K (1 µg/lane) was run on 12% SDS-PAGE and electrophoretically transferred to nitrocellulose. Blotted calbindin D-28K was renatured overnight in renaturing buffer (150 mM NaCl, 15 mM Tris-HCl, 2 mM MgCl₂, 1 mM DTT and 0.1 mM PMSF, pH 7.3). Membranes were incubated for 3 hours with or without 40 µg/ml partially purified PKC mixture in Tris-buffered saline (TBS) containing 1.2 mM CaCl₂ in the presence or absence of 1 mM EGTA. Following washing, bound proteins were cross linked with 10 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC, Sigma) for 20 min. After quenching the reaction (50 mM Tris-HCl, pH 7.6), membranes were washed for 1 hour in the appropriate buffer. Membranes were then processed for immunoblotting. Membranes were incubated in PBS containing 0.5% skim milk to block antigenic sites. Washed membranes were incubated for 1 hour at room temperature with monoclonal calbindin D-28K (diluted 1:200, Sigma) or polyclonal PKCα (diluted 1:100, Life Technologies) antibodies, followed with alkaline phosphatase-conjugated anti-mouse (for calbindin D-28K) or anti-rabbit (for PKCα) IgGs (Promega), diluted 1:7500 in blocking solution. Immunoreactivity was detected using NBT and BCIP as described in 2.2.2.

5.2.7 Miscellaneous

Protein concentrations were determined by the method of Bradford (1972). Each experiment was performed at least three times. Unless otherwise stated, data are expressed as mean $\pm$ SEM. Statistical differences were assessed using Student's *t*-test for unpaired data, with a significance level of $P < 0.05$.

5.3 RESULTS

5.3.1 Phosphorylation of calbindin D-28K *in vitro*

We first investigated the ability of CK II and PKC to phosphorylate calbindin D-28K *in vitro* in the presence of the appropriate activators. Although casein phosphorylation by CK II was readily detected in our assay, no phosphorylation of calbindin D-28K by this enzyme was observed after up to fifteen minutes of incubation (Fig. 5.2). The recombinant enzyme used in these assays also phosphorylated a positive control CK II-specific peptide substrate under the same conditions (data not shown).

In contrast, calbindin D-28K was phosphorylated within minutes of exposure to a partially purified PKC mixture from rabbit brain (Fig. 5.3). This PKC mixture contains all isozymes tested (α , β , γ , δ , ϵ , and ζ isozymes; data not shown). PKC phosphorylation of calbindin D-28K was maximal in the presence of calcium and phospholipids, albeit some phosphorylation was detected in the absence of these activators. The identity of the various phosphorylated bands detected in addition to calbindin D-28K are unknown. However, PKC is known to autophosphorylate once activated which may account for the highly phosphorylated band observed at approximately 80 kDa; other bands may be PKC substrates in the partially purified PKC mixture. Consistent with the sequence analysis, PKC-phosphorylated calbindin D-28K is recognized by anti-phosphothreonine antibodies (Fig. 5.4). Interestingly, purified PKA, which also recognizes basic sequences, did not phosphorylate calbindin D-28K under similar conditions (data not shown).

In light of our results correlating PKC α activity and expression with calbindin D-28K

Figure 5.2 *In vitro phosphorylation of calbindin D-28K and casein by casein kinase II (CKII).*

Purified recombinant CKII (10 units) and purified rat recombinant calbindin D-28K or casein (1 µg) were incubated in assay buffer and [γ - 32 P]-ATP for 15 minutes. Reactions were terminated by boiling the samples in Laemmli buffer, and proteins were separated on 12% SDS-PAGE, as described in Methods. Phosphorylated proteins were visualized by autoradiography. *Lane 1*, assay buffer; *lane 2*, casein; *lane 3*, casein + CKII; *lane 4*, calbindin D-28K; *lane 5*, calbindin D-28K + CKII; *lane 6*, CKII.

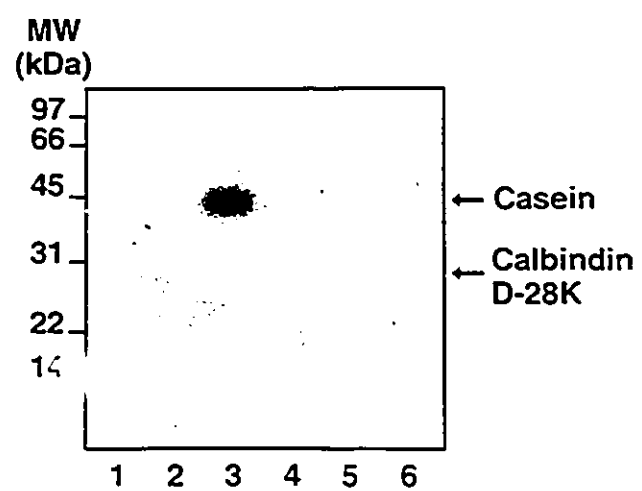


Figure 5.3 In vitro phosphorylation of calbindin D-28K by PKC.

Partially purified PKC mixture from rabbit brain and purified rat recombinant calbindin D-28K were incubated with [γ - 32 P]-ATP for the indicated times, in the presence or absence of PKC activators. Reactions were terminated by boiling the samples in Laemmli buffer, and proteins were separated on 12% SDS-PAGE, as described in Methods. Phosphorylated proteins were visualized by autoradiography. *Lane 1 of each panel*, control incubation in which no substrate calbindin D-28K was added.

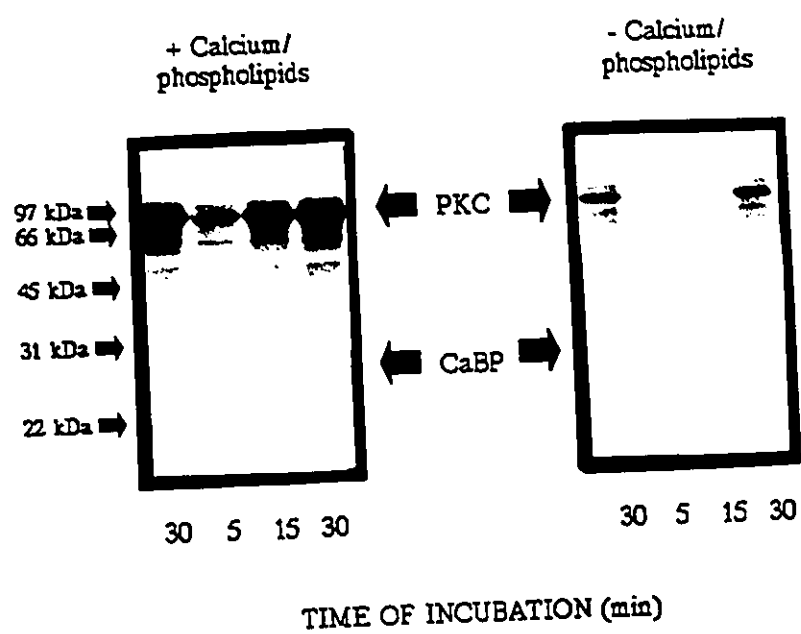
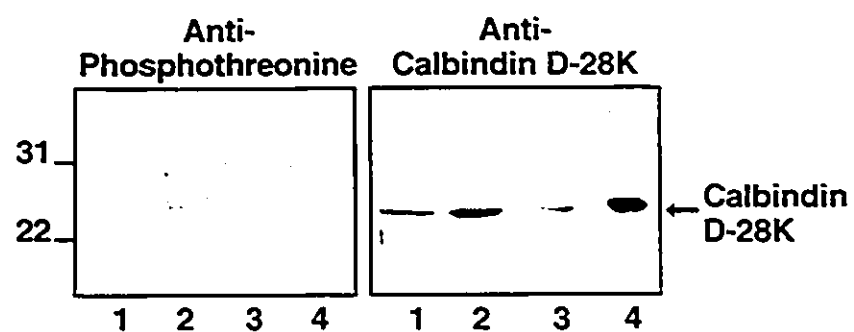


Figure 5.4 Immunoblotting of PKC-phosphorylated calbindin D-28K.

Calbindin D-28K was phosphorylated for up to 30 minutes with partially purified PKC mixture from rabbit brain, as described in Methods. Proteins were separated on 12% SDS-PAGE, transferred to nitrocellulose and blotted for calbindin D-28K (right panel) or phosphothreonine (left panel) as previously described. *Lane 1*, 0; *lane 2*, 5 minutes; *lane 3*, 15 minutes; *lane 4*, 30 minutes.



levels, we examined the ability of purified recombinant PKC α to phosphorylate calbindin D-28K. Again, calbindin D-28K phosphorylation was detected within thirty minutes of incubation and was greatly enhanced in the presence of calcium and phospholipids (Fig. 5.5 top). Interestingly, autophosphorylation of PKC α was slightly but consistently accentuated in the presence of calbindin D-28K. Other PKC substrates have been shown to promote autophosphorylation of the enzyme, however the regulatory mechanism and functional significance of this observation is currently unknown (Newton, 1995). The phosphorylation rate of calbindin D-28K by PKC α was similar in magnitude to that displayed against a PKC-specific peptide substrate (Fig. 5.5 bottom). Taken together, these results demonstrate that calbindin D-28K serves as an *in vitro* substrate for PKC (specifically PKC α), but not for CK II or PKA.

5.3.2 Phosphorylation of calbindin D-28K in MDBK cells

In vitro phosphorylation of a protein does not always correlate with *in vivo* observations. Therefore, we next assessed the phosphorylation state of calbindin D-28K in MDBK cells treated with TPA or 1,25(OH) $_2$ D $_3$. Immunoprecipitation studies of radiolabelled MDBK cells reveals the presence of a 28 kDa phosphoprotein (Fig. 5.6, lane 1). The intensity of this phosphorylation band is increased after one hour exposure to TPA (Fig. 5.6, lane 2) without a corresponding increase in calbindin D-28K expression as assessed by immunoblotting. This band appears specific for calbindin D-28K since it is not present in extracts precipitated with calretinin antibodies (Fig. 5.6, lane 4). Non-specific immunoprecipitations were performed using calretinin antibodies, because of the high degree

Figure 5.5 *In vitro phosphorylation of calbindin D-28K by PKC α*

Recombinant PKC α and purified rat recombinant calbindin D-28K or a PKC-specific peptide substrate were incubated for 30 minutes with [γ - 32 P]-ATP in the presence or absence of PKC activators. Top: Reactions were terminated by boiling the samples in Laemmli buffer, and proteins were separated on 12% SDS-PAGE, as described in Methods. Phosphorylated proteins were visualized by autoradiography. *lanes 1 and 3*, PKC α alone; *lanes 2 and 4*, PKC α and calbindin D-28K. Bottom: Reactions were terminated by the addition of 30% TCA, and precipitated proteins were spotted onto acid-treated filter papers, washed and radioactivity was determined by liquid scintillation counting. Results represent mean $\pm$ SEM of calcium/phospholipid-specific phosphorylation determined in four separate experiments ($N = 4$).

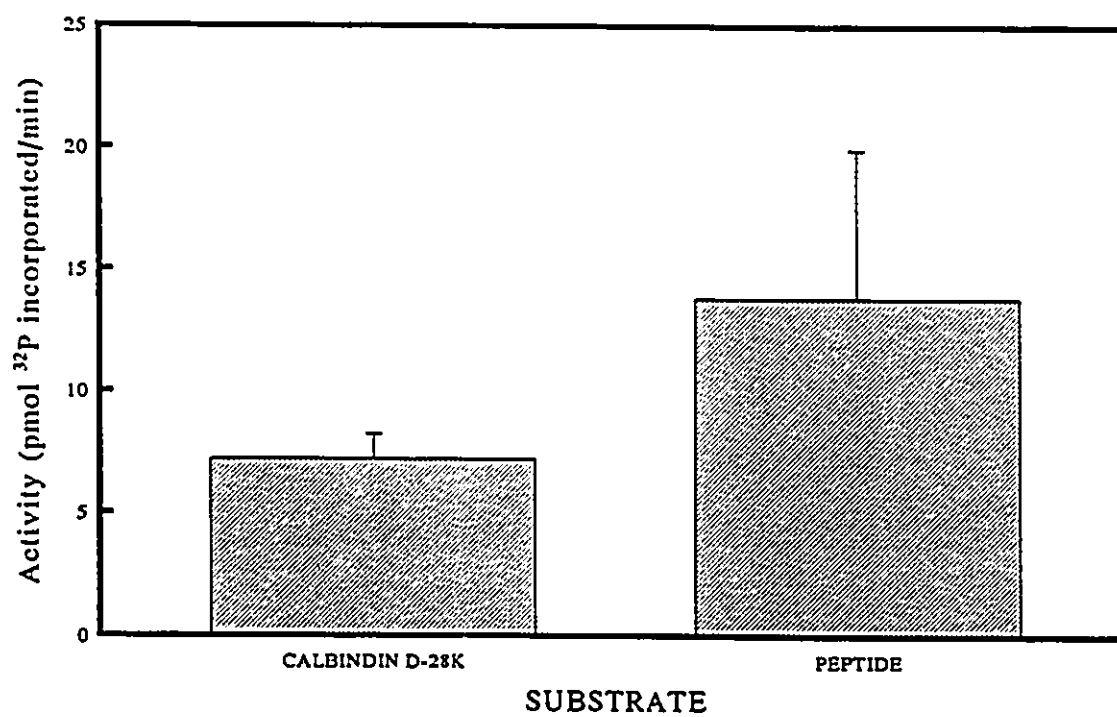
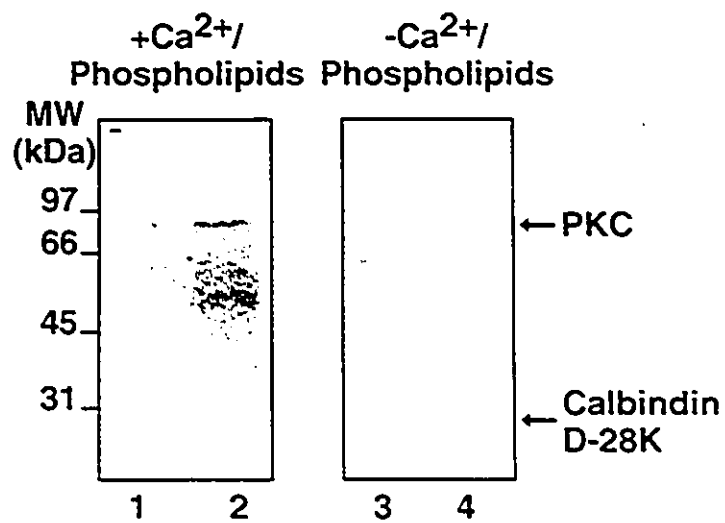
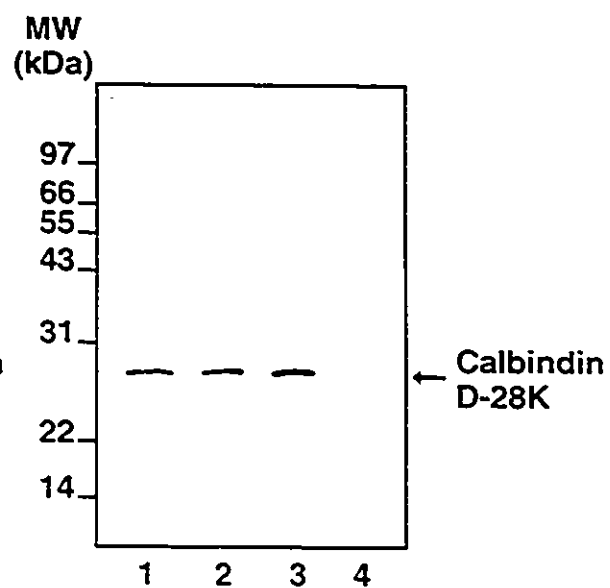
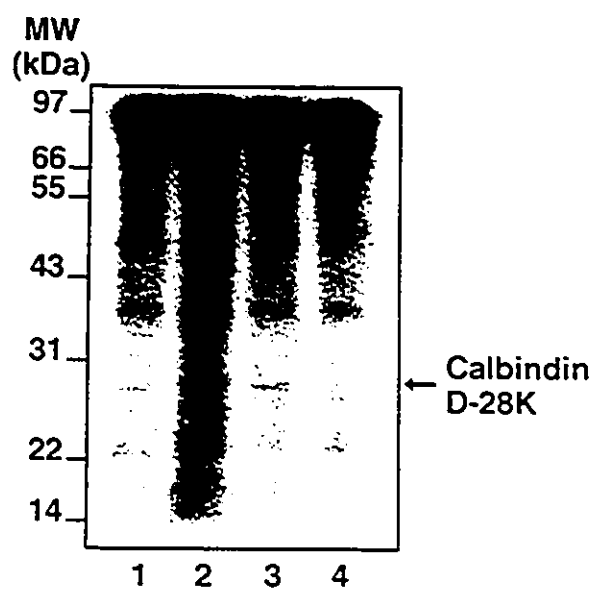


Figure 5.6 *Immunoprecipitation of calbindin D-28K from radiolabelled MDBK cells.*
Effect of TPA treatment.

MDBK cells were grown to confluence, changed to serum-free media 24 hours prior to treatment. Cells were washed in phosphate free media and labelled for 4 hours with [32 P]-orthophosphate (100 μ Ci/ml) in phosphate free media. TPA (10^{-7} M) or vehicle was added to dishes one hour prior to harvest. Cells were scraped in RIPA buffer, heat treated, and calbindin D-28K was immunoprecipitated as described in Methods. Immunoprecipitated proteins were separated on 12% SDS-PAGE. Left panel: Phosphorylated proteins were visualized by autoradiography. Right panel: Proteins were electrophoretically transferred to nitrocellulose and blotted for calbindin D-28K. *Lane 1*, vehicle; *lanes 2, 3 and 4*, TPA; *lane 3*, immunoprecipitation in the presence of 2 μ g excess purified rat recombinant calbindin D-28K; *lane 4*, non-specific immunoprecipitation.



of homology (88%) displayed between calbindin D-28K and calretinin (Parmentier, 1989), and the very low cross-reactivity of either antibody (Celio, Baier, Schärer, Gregersen, de Viragh and Norman, 1990). Further, excess unlabelled purified calbindin D-28K partially competes out this 28 kDa phosphorylated band (Fig. 5.6, lane 3). Partial sequence determination is required to definitively identify the 28 kDa phosphorylated protein as calbindin D-28K. Most other phosphorylated proteins detected in the immunoprecipitates appear non-specific, since they are observed in all conditions assayed. Again consistent with the sequence analysis, a 28 kDa band, co-migrating with calbindin D-28K, is detected by anti-phosphothreonine, but not anti-phosphoserine antibodies, in MDBK cell extracts (Fig. 5.7). Although calbindin D-28K levels remain the same after one hour treatment with TPA, the intensity of the phosphothreonine band is enhanced, suggesting increased phosphorylation.

Similar experiments in $1,25(\text{OH})_2\text{D}_3$ - or vehicle-treated MDBK cells also detected a 28 kDa phosphorylated band, which is specifically immunoprecipitated by anti-calbindin D-28K antibodies. The intensity of this phosphorylated band is increased in $1,25(\text{OH})_2\text{D}_3$ -treated compared to vehicle-treated cells (Fig. 5.8). Taken together, these results implicate regulatory phosphorylation events in both TPA and $1,25(\text{OH})_2\text{D}_3$ modulation of calbindin D-28K expression in MDBK cells.

5.3.3 Involvement of PKC α in calbindin D-28K regulation

Our previous data demonstrating a temporal correlation between calbindin D-28K expression and membrane PKC α activity and expression prompted us to further investigate the role of this isozyme in calbindin D-28K regulation. Using crosslinking techniques, we first

Figure 5.7 Immunoblotting of calbindin D-28K immunoprecipitated from MDBK cells. Effect of TPA.

MDBK cells were grown to confluence and changed to serum-free media 24 hours prior to treatment with 10^{-7} M TPA (T) or vehicle (C) for one hour. Cells were scraped in denaturing buffer and proteins were separated on 12% SDS-PAGE, electrophoretically transferred to nitrocellulose and blotted for calbindin D-28K, phosphothreonine and phosphoserine as described in Methods.

C T C T C T

28 kDa ▶

Ab ▶ CaBP P-Thr P-Ser

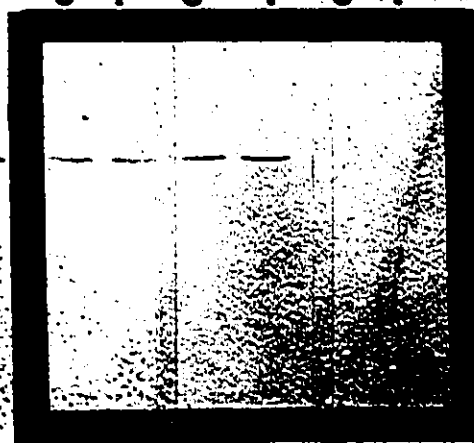
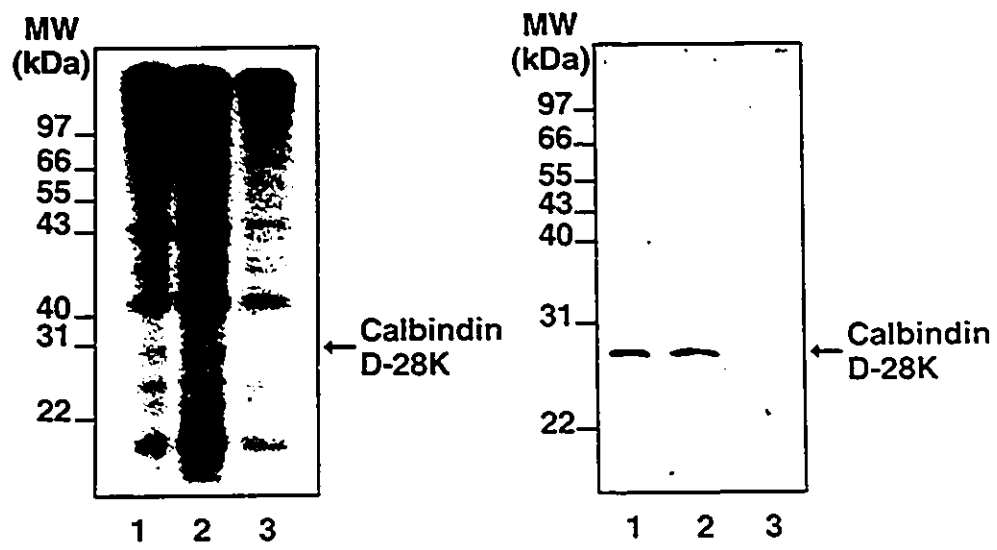


Figure 5.8 *Immunoprecipitation of calbindin D-28K from radiolabelled MDBK cells.
Effect of 1,25(OH)₂D₃ treatment.*

MDBK cells were grown to confluence, changed to serum-free media 24 hours prior to treatment with 10^{-7} M 1,25(OH)₂D₃ or vehicle for 24 hours. Cells were washed in phosphate free media and labelled for 4 hours with [³²P]-orthophosphate (100 µCi/ml) in phosphate free media. Cells were scraped in RIPA buffer, heat treated, and calbindin D-28K was immunoprecipitated as described in Methods. Immunoprecipitated proteins were separated on 12% SDS-PAGE. Left panel: Phosphorylated proteins were visualized by autoradiography. Right panel: Proteins were electrophoretically transferred to nitrocellulose and blotted for calbindin D-28K. Lane 1, vehicle; lanes 2 and 3, 1,25(OH)₂D₃; lane 3, non-specific immunoprecipitation.



examined a potential physical interaction between calbindin D-28K and PKC α . Purified calbindin D-28K, immobilized on nitrocellulose filters, was incubated with partially purified PKC from rabbit brain in the presence or absence of calcium. Interacting proteins were crosslinked to calbindin D-28K and filters subjected to immunoblotting. PKC α antibodies detected the 28 kDa calbindin D-28K band, suggesting a physical interaction between these two proteins (Fig. 5.9). This interaction was calcium-dependent since no bands were observed when the incubation was done in the presence of EGTA. PKC α antibodies did not recognize calbindin D-28K alone, demonstrating the specificity of this antibody. However, the *in vitro* interaction between these two proteins does not necessarily represent *in vivo* conditions.

The role of PKC α in calbindin D-28K regulation in MDBK cells was further examined using transfection models. The regulatory domain (RD) of PKC isozymes has been shown to inhibit enzyme activity by 'folding over' the catalytic domain. Overexpression of the cDNA coding for the specific RD for each isozyme (prepared by restriction digest of the full clone) has been reported to inhibit PKC in an isozyme-specific manner (Kahn, O'Driscoll, Jiang, Borner, Xu, Blackwood, Zhang, Nomoto and Weinstein, 1994). We transfected MDBK cells with an expressing vector containing the RD α construct. MDBK cells expressing RD α display a lower level of calbindin D-28K compared to cells transfected with control vector, implicating PKC α in the constitutive regulation of calbindin D-28K in MDBK cells (Fig. 5.10). In contrast, VDR levels in transfected MDBK cells are unchanged. Unfortunately, transfections with RD α are unstable, limiting experiments and hindering strong conclusions. Other studies using other isozyme-specific RD expression vector should help distinguish

Figure 5.9 Cross-linking overlay of calbindin D-28K and PKC α

Purified rat recombinant calbindin D-28K was run on 12% SDS-PAGE and electrophoretically transferred to nitrocellulose. After renaturation, calbindin D-28K was incubated with partially purified PKC mixture from rabbit brain in the presence or absence of calcium. Membranes were washed and bound proteins cross-linked with EDAC. Proteins were visualized by immunoblotting duplicate lanes with calbindin D-28K- and PKC α -specific antibodies, as described in Methods.

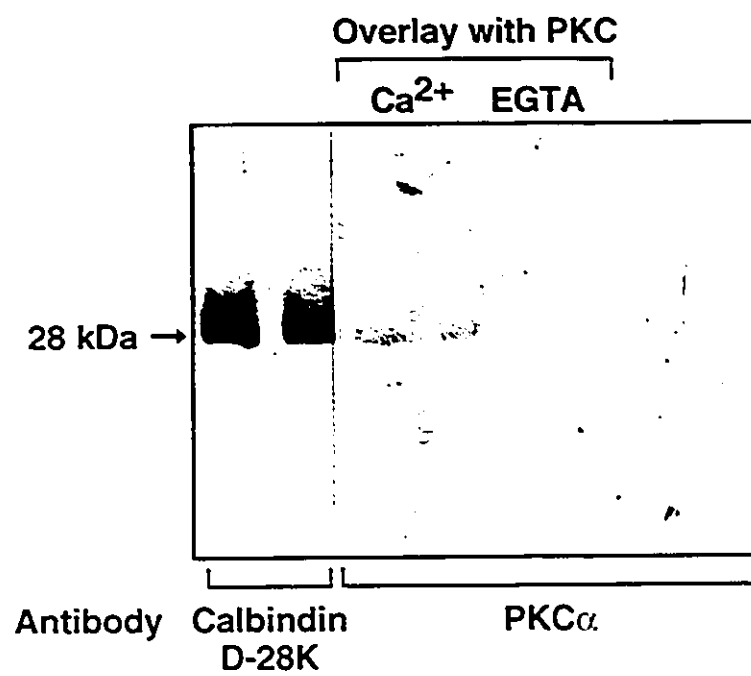
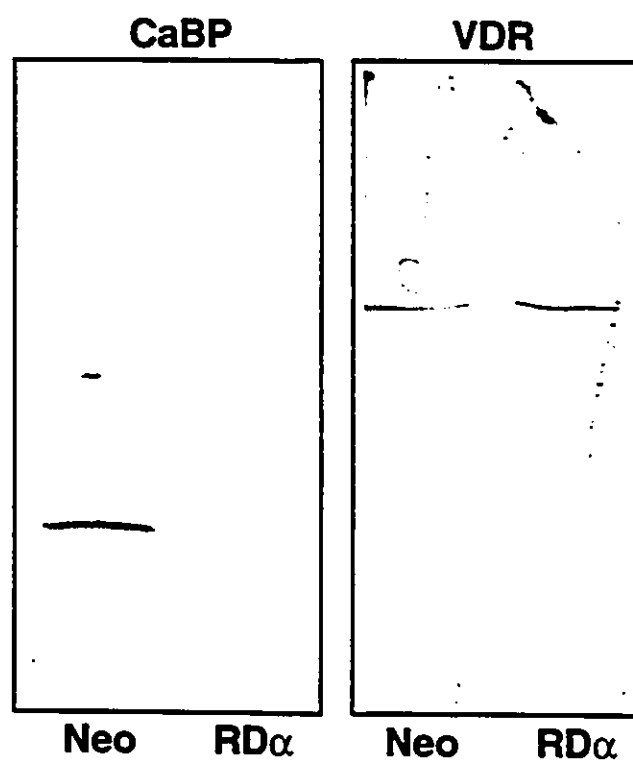


Figure 5.10 *Basal levels of calbindin D-28K and VDR in MDBK cells transfected with the regulatory domain of PKC α (RD α).*

Proteins from total homogenates of MDBK cells transfected with the regulatory domain of PKC α (RD α) or control vector (Neo) were separated on 12% SDS-PAGE, electrophoretically transferred to nitrocellulose and blotted for calbindin D-28K and VDR as described in Methods.



between a specific role of PKC α or a non-specific secondary effect of PKC deregulation on calbindin D-28K expression.

5.4 DISCUSSION

We and others have demonstrated that $1,25(\text{OH})_2\text{D}_3$ regulates renal calbindin D-28K, at least partially, via a post-transcriptional mechanism. We have further observed that this post-transcriptional regulation may involve a phosphorylation event. This chapter investigates the direct phosphorylation of calbindin D-28K by various kinases and emphasizes the role of the PKC signalling pathway in calbindin D-28K regulation in MDBK cells.

5.4.1 *In vitro* phosphorylation of calbindin D-28K

The two PKC sites in calbindin D-28K are conserved in all species examined, implying their functional relevance (Fig. 5.1). Consistent with this observation, calbindin D-28K was phosphorylated within five minutes of exposure to PKC in the presence of enzyme activators (Fig. 5.3). Some autophosphorylation of PKC, indicative of endogenous enzyme activity (Wolf, Sahyoun, LeVine and Cuatrecasas, 1984), was evident even in the absence of calcium and phospholipids and may explain the low but detectable level of calbindin D-28K phosphorylation observed under these conditions. The rabbit brain PKC mixture used in these *in vitro* assays is only partially purified and contains many other proteins, some of which could be kinases. However, the calcium- and phospholipid-dependence of calbindin D-28K phosphorylation argues against the involvement of other kinases in this case. Further, the observation that purified recombinant PKC α phosphorylates calbindin D-28K under identical conditions directly implicates this enzyme in calbindin D-28K phosphorylation (Fig. 5.5).

Consistent with the sequence analysis, phosphorylated calbindin D-28K is recognized

by anti-phosphothreonine antibodies (Fig. 5.4). Both threonine residues identified as potential PKC phosphorylation sites are located within or near EF-hands, the calcium-binding domains of calbindin D-28K. The appearance of a phosphate group within an EF-hand could alter the calcium-binding affinity of the protein by providing another ionic group coordinating the divalent cation. A conformational change resulting from calbindin D-28K phosphorylation may also alter the function of calbindin D-28K without affecting its calcium-binding affinity. However, phosphoamino acid analysis and phosphopeptide mapping are required to appropriately identify the PKC-dependent calbindin D-28K phosphorylation sites.

Calbindin D-28K is not phosphorylated by CK II under the conditions utilized. In contrast, CK II rapidly phosphorylates casein, a well-defined substrate for CK II, under these conditions (Fig. 5.2). As is often observed, the presence of a consensus phosphorylation site in the primary sequence of a protein does not always correlate with this post-translational modification *in vivo*. On the other hand, the conditions used in *in vitro* assays may not completely represent the cellular environment, precluding the demonstration of the phosphorylation of specific substrates. This is especially true for CK II, whose substrate specificity also depends on the secondary and tertiary structure of both the substrate and the enzyme itself and the presence of modulators such as polylysine or spermine (Meggio, Boldyreff, Marin, Marchiori, Perich, Issinger and Pinna, 1992). However, the observation that the CK II phosphorylation sequences in calbindin D-28K are not well conserved despite the high degree of homology displayed by this protein in general suggests that these sites may not be physiologically important. Further studies, including the identification of the calbindin D-28K sites phosphorylated *in vivo*, will help determine the role, if any, of CK II in calbindin

D-28K regulation in MDBK cells.

5.4.2 Phosphorylation of calbindin D-28K in MDBK cells

The phosphorylation of purified proteins by purified enzyme preparations under *in vitro* conditions does not represent *in vivo* observations. We have identified a 28 kDa phosphorylated protein specifically immunoprecipitated with calbindin D-28K antibodies from MDBK cell extracts. Our data strongly suggests that this phosphoprotein is indeed calbindin D-28K, however, partial sequence analysis is required for accurate identification. The intensity of this 28 kDa band increased within one hour of TPA treatment (Fig. 5.6), in correlation with the induction of membrane PKC activity previously described (Fig. 4.5). This phosphorylation event precedes the up-regulation of calbindin D-28K expression observed in TPA-treated MDBK cells (Fig. 3.3 and 3.4) and suggests that this post-translational modification is involved in calbindin D-28K regulation. As previously observed in *in vitro* assays and consistent with the sequence analysis, phosphorylated calbindin D-28K is recognized by phosphothreonine but not phosphoserine antibodies (Fig. 5.7). These antibodies, although specific for the indicated phosphorylated residue do not always recognize such a site within a protein. Therefore, we cannot rule out the possibility that calbindin D-28K is also phosphorylated on serine residues. Phosphopeptide mapping and phosphoamino acid analysis should answer some of these questions and identify the calbindin D-28K residues phosphorylated in MDBK cells.

Enhancement of calbindin D-28K phosphorylation was also detected after 24 hour treatment with 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ (Fig. 5.8), again correlating with induction of PKC

activity and calbindin D-28K expression in MDBK cells (Figs. 4.1, 2.3 and 2.4). These results constitute the first report of calbindin D-28K phosphorylation both *in vitro* and *in vivo* and are consistent with the post-transcriptional regulation of this protein demonstrated in various systems (Theofan and Norman, 1986; Enomoto et al, 1992; Gagnon, Simboli-Campbell, and Welsh, 1994). However, the role of this phosphorylation step in calbindin D-28K regulation still need to be addressed. As previously mentioned, calbindin D-28K induction by $1,25(\text{OH})_2\text{D}_3$ involves a receptor-mediated increase in calbindin D-28K gene transcription; however, phosphorylation of calbindin D-28K may be involved in the long term, sustained (24 to 72 hours) induction of calbindin D-28K expression, after calbindin D-28K mRNA levels start to decline. Calbindin D-28K phosphorylation may increase calbindin D-28K stability, resulting in increased expression of the protein. This hypothesis is supported by our observation that induction of PKC activity by $1,25(\text{OH})_2\text{D}_3$ occurs after 24 hours of treatment, while induction of calbindin D-28K expression is evident within 6-8 hours. A more detailed time course of calbindin D-28K phosphorylation in response to $1,25(\text{OH})_2\text{D}_3$ and TPA may help strenghten the correlation between PKC activity and calbindin D-28K expression and define the mechanism underlying this regulation.

5.4.3 Involvement of PKC α in calbindin D-28K regulation in MDBK cells

The correlation between PKC activity and calbindin D-28K phosphorylation observed with both TPA and $1,25(\text{OH})_2\text{D}_3$ treatments suggests a direct role for PKC in calbindin D-28K regulation. Our previous data correlating the activity of membrane PKC α and calbindin D-28K expression prompted us to investigate the role of this isozyme in calbindin D-28K

regulation. Using overlay assays, a calcium-dependent interaction between calbindin D-28K and PKC α was demonstrated (Fig. 5.9), indicative of possible enzyme-substrate interaction. Transfection of MDBK cells with the RD α construct, which specifically inhibits PKC α activity, further emphasizes the role of this kinase in calbindin D-28K expression. MDBK cells expressing the RD α show decreased basal levels of calbindin D-28K, supporting a role for this isozyme in the constitutive regulation of calbindin D-28K (Fig. 5.10). Unfortunately, the unstable nature of the transfection hindered additional studies on the effects of both TPA and 1,25(OH) $_2$ D $_3$ on calbindin D-28K expression and phosphorylation in these cells.

Our data do not rule out the possibility that another kinase, activated by PKC, is responsible for calbindin D-28K phosphorylation *in vivo*. A secondary effect of PKC α activation may account for the regulation of calbindin D-28K expression. Further studies are required to distinguish between these various possibilities.

5.4.4 Potential mechanism of action

As previously shown, calbindin D-28K is predominantly localized in the cytosol of MDBK cells. However, the correlation that we have demonstrated implicates membrane-bound PKC α . This discrepancy in the localization of the enzyme and its potential substrate argues against a direct involvement of PKC in calbindin D-28K phosphorylation. However, some groups have reported the localization of vesicular and even membrane-bound calbindin D-28K in various systems. Membrane localization of calbindin D-28K was detected in avian intestinal cells but only after treatment with Triton X-100 (Shimura and Wasserman, 1984), which may explain the lack of calbindin D-28K immunoreactivity observed in intact MDBK

cells. Non-cytoplasmic localization of calbindin D-28K has also been reported in kidney and brain, where calbindin D-28K appears partially localized in tubulin-associated vesicles (Nemere et al, 1992; Leathers and Norman, 1993). Vesicular distribution of calbindin D-28K is evident in MDBK cells under high resolution microscopy (data not shown). One can speculate that vesicular or membrane-associated calbindin D-28K is in close enough proximity to activated PKC to be effectively phosphorylated by this kinase. Phosphorylation of calbindin D-28K may promote the association within vesicles and increase the stability of the protein, resulting in increased expression. Pulse-chase analysis of ^{35}S -methionine-labelled MDBK cells could be used to assess calbindin D-28K turnover in relation to its phosphorylation state.

The regulation of calbindin D-28K by PKC suggests that other PKC modulators may control the expression of this calcium-binding protein. Consistent with this premise, we have reported that serum, EGF and insulin, agents known to regulate PKC activity (Iwashita and Kobayashi, 1992), modulate calbindin D-28K expression in a cell growth-independent manner in MDBK cells (Gagnon, et al, in preparation). In contrast to growth factors and tumor promoters which acutely regulate PKC activity (Castagna et al, 1982; Breyer, Jacobson and Breyer, 1988; Iwashita and Kobayashi, 1992), the persistent long term increase in PKC activity in response to $1,25(\text{OH})_2\text{D}_3$ is consistent with a genomic control by the hormone. Our results complement that of Solomon et al (1991), who demonstrated a genomic regulation of $\text{PKC}\beta$ by $1,25(\text{OH})_2\text{D}_3$ in HL-60 cells. However, the regulation of $\text{PKC}\alpha$ by $1,25(\text{OH})_2\text{D}_3$ is not likely to simply involve transcriptional effects of the hormone.

Our results describe a strong correlation between calbindin D-28K phosphorylation

and levels with PKC α activity and expression in MDBK cells, implying a direct role of the kinase in this regulation. However, an indirect effect of PKC activation or the induction of other PKC isozymes may also be involved in this regulation. As previously mentioned, VDR phosphorylation by PKC β , an isozyme regulated by both TPA and 1,25(OH) $_2$ D $_3$, has been demonstrated and may affect transcriptional activity (Hsieh et al, 1993) of specific genes such as calbindin D-28K. The distinct time courses of expression of VDR and calbindin D-28K in TPA and 1,25(OH) $_2$ D $_3$ -treated MDBK cells argue against this hypothesis (Chapters 2 and 3). However, PKC has also been implicated in cytoskeletal reorganization (Omary, Baxter, Chou, Riopel, Lin and Strulovici, 1992; Murti, Kaur and Goorha, 1992; Ainsztein and Purich, 1994), which in turn may affect calbindin D-28K expression whose association with tubulin has been previously discussed. The regulation of transcription and translation by PKC-dependent phosphorylations could also be responsible for calbindin D-28K regulation, albeit this possibility is inconsistent with the observed increase in calbindin D-28K phosphorylation. The activation of an unidentified kinase may in turn phosphorylate calbindin D-28K, independently of PKC activation.

In summary, our results describe calbindin D-28K as a phosphoprotein whose phosphorylation state is modulated by regulators of PKC. The direct involvement of PKC in this phosphorylation event is suggested by the observations of a temporal correlation between PKC activity and calbindin D-28K phosphorylation and direct phosphorylation under *in vitro* conditions. PKC α is further implicated as the isozyme responsible for calbindin D-28K phosphorylation. However, the detailed mechanism regulating calbindin D-28K still has

to be investigated. The results presented here indicate that other agents, modulators of PKC, can also modulate calbindin D-28K expression and therefore broadens the physiological role of this evolutionary important calcium-binding protein.

5.5 CONCLUSIONS

The results presented in this chapter describe the phosphorylation of calbindin D-28K, providing for the first report of calbindin D-28K as a phosphoprotein. More precisely, the conclusions attained from these data are:

- Calbindin D-28K can be phosphorylated *in vitro* by PKC but not CK II.
- A calbindin D-28K antibody-specific 28 kDa phosphoprotein is observed in MDBK cells.
- Correlating with the regulation of calbindin D-28K expression and membrane PKC activity, phosphorylation of this 28 kDa protein is enhanced in TPA and $1,25(\text{OH})_2\text{D}_3$ -treated MDBK cells.
- Calbindin D-28K and PKC α interact *in vitro* in a calcium-dependent manner.
- MDBK cells expressing the RD α construct display a lower basal level of calbindin D-28K, implicating this isozyme in calbindin D-28K regulation.

GENERAL CONCLUSION

Calbindin D-28K is a vitamin D-dependent calcium-binding protein of the EF-hand family. While the induction of renal calbindin D-28K by $1,25(\text{OH})_2\text{D}_3$, the hormonally active form of vitamin D, has been well-documented, the lack of an established *in vitro* model system for renal calbindin D-28K has hindered investigations of the detailed mechanism of this regulation. The studies presented here describe a novel model system, the MDBK cell line, to investigate calbindin D-28K regulation and function. The role of transcriptional and post-transcriptional mechanisms in calbindin D-28K regulation by $1,25(\text{OH})_2\text{D}_3$ were also investigated. In view of the increasing literature implicating the PKC signalling pathway in mediating some actions of $1,25(\text{OH})_2\text{D}_3$, we further examined the role of these serine/threonine kinases in calbindin D-28K regulation.

Due to the inherent problems associated with primary cultures and *in vivo* studies, our first objective was to establish an *in vitro* renal system in which to study calbindin D-28K. In view of the exclusive localization of renal calbindin D-28K in the distal nephron (Roth et al, 1981), we first determined the distal tubule origin of various renal cell lines. We demonstrate that while both MDBK and MDCK cell lines display distal tubular characteristics, only MDBK cells significantly express calbindin D-28K and VDR. Although characterized as a well-differentiated distal tubular-like cell line (Ojakian, 1981), MDCK cells do not appear to express critical renal components of the vitamin D signalling pathway. We

further investigated the regulation of calbindin D-28K and VDR in MDBK cells and show that, as previously described in primary cultures and *in vivo* systems, calbindin D-28K and VDR levels are highest in rapidly proliferating cells (Chen et al, 1992; Chen and Feldman, 1981). In addition, both calbindin D-28K and VDR are up-regulated in the presence of $1,25(\text{OH})_2\text{D}_3$ in MDBK cells, providing the first report of vitamin D-dependence of calbindin D-28K in an established cell line. Although $1,25(\text{OH})_2\text{D}_3$ was shown to modulate proliferation in various cell lines (Minghetti and Norman, 1988), the $1,25(\text{OH})_2\text{D}_3$ induction of calbindin D-28K and VDR in MDBK cells is not accompanied by increased proliferation, suggesting an effect of the hormone which is independent of cell cycle regulation.

We further characterized the vitamin D-dependence of calbindin D-28K and VDR expression in MDBK cells and compared our data with that previously described in primary cultures and *in vivo* studies. These results are presented in Chapter 2. Consistent with previous reports, calbindin D-28K induction by $1,25(\text{OH})_2\text{D}_3$ is dose-dependent, reaching maximal induction with 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ (Craviso et al, 1987). Similar doses were used to demonstrate well-characterized effects of the hormone, including induction of vitamin D-dependent gene transcription (Liao et al, 1990). We also describe the time-dependence of calbindin D-28K regulation by $1,25(\text{OH})_2\text{D}_3$ in MDBK cells. Again consistent with previous reports on renal calbindin D-28K regulation, calbindin D-28K levels increase within 4 hours of treatment of MDBK cells with the hormone and are sustained for up to 72 hours after a single dose of $1,25(\text{OH})_2\text{D}_3$ (Thecofan et al, 1986; Varghese et al, 1988). VDR levels also increased reaching a maximum after 24 hours of exposure to the hormone, followed by a slow return to basal levels. The early rise in calbindin D-28K levels in response to $1,25(\text{OH})_2\text{D}_3$

was accompanied with increased steady-state mRNA levels. Consistent with the data of Craviso et al (1987), the long term induction of calbindin D-28K expression by $1,25(\text{OH})_2\text{D}_3$ was blocked by cycloheximide pre-treatment, involving post-transcriptional levels of regulation. Taken together, these results demonstrate the MDBK cell model as an *in vitro* system suitable to investigate calbindin D-28K regulation, since calbindin D-28K induction by $1,25(\text{OH})_2\text{D}_3$ is dose- and time-dependent and can be blocked by treatment with protein synthesis inhibitors.

Recent evidence implicate the PKC signalling pathway in the actions of $1,25(\text{OH})_2\text{D}_3$. In the kidney, PKC activation has been shown to mimic the effects of $1,25(\text{OH})_2\text{D}_3$ on 24- and 1α -hydroxylase activities and expression (Henry and Luntao, 1989; Mandla et al, 1990; Welsh et al, 1992). The regulation of PKC activity and expression by $1,25(\text{OH})_2\text{D}_3$ has been documented in various systems including MDBK cells (Martell et al, 1987; Simboli-Campbell et al, 1992; Wali et al, 1992; Simboli-Campbell et al, 1994; Slater et al, 1995). We investigated the involvement of this serine/threonine kinase in the regulation of calbindin D-28K in MDBK cells. In the absence of $1,25(\text{OH})_2\text{D}_3$, the phorbol ester TPA, a well-characterized PKC modulator, causes a rapid, but transient increase in calbindin D-28K levels, followed by down-regulation which reaches below control levels within 24 hours of treatment. The early increase in calbindin D-28K expression is not accompanied by a similar increase in VDR numbers, suggesting two distinct regulatory pathways. The regulation of calbindin D-28K by TPA is unaffected by protein synthesis inhibitors, although okadaic acid, a potent phosphatase inhibitor, blunts the down-regulative effect observed after 24 hours. Taken together, these results describe a direct regulation of calbindin D-28K expression by

TPA, an effect which apparently involves a phosphorylation event and is independent of *de novo* protein synthesis.

The regulation of PKC activity, expression and subcellular localization by $1,25(\text{OH})_2\text{D}_3$ and TPA was next examined. $1,25(\text{OH})_2\text{D}_3$ induces membrane PKC activity within 24 hours of treatment and this induction was sustained for up to 72 hours after a single dose of the hormone, temporally correlating with the increase in calbindin D-28K levels. Interestingly, $1,25(\text{OH})_2\text{D}_3$ modulates the expression and subcellular localization of calcium-dependent PKC isozymes ($\text{PKC}\alpha$ and $\text{PKC}\beta$), having no effect on $\text{PKC}\epsilon$ and $\text{PKC}\zeta$. Correlating with the activity data, membrane $\text{PKC}\alpha$ expression is enhanced in the presence of $1,25(\text{OH})_2\text{D}_3$. Nuclear translocation of $\text{PKC}\beta$ in response to $1,25(\text{OH})_2\text{D}_3$ is also evident and may be involved in the regulation of the VDR, a known substrate for this isozyme. The long term regulation of PKC activity, expression and subcellular localization by $1,25(\text{OH})_2\text{D}_3$ is consistent with a genomic effect of the hormone; however, further studies are required to examine this issue. In contrast, TPA rapidly induces membrane PKC activity in MDBK cells, in agreement with the reported direct interaction of phorbol esters with the kinase. Membrane PKC activity peaks within one hour of TPA-treatment then starts to decline, reaching below control levels within 24 hours. This time course again correlates with the observed regulation of calbindin D-28K by TPA. Short term treatment with TPA also enhances membrane $\text{PKC}\alpha$ immunoreactivity and results in $\text{PKC}\beta$ translocation to the nucleus. As previously reported, down-regulation of PKC expression is evident in MDBK cells after 24 hour treatment with TPA. Our data describe a temporal correlation between membrane PKC activity, membrane $\text{PKC}\alpha$ immunoreactivity and calbindin D-28K expression

in response to both $1,25(\text{OH})_2\text{D}_3$ and TPA, implicating this signalling pathway in calbindin D-28K regulation.

$1,25(\text{OH})_2\text{D}_3$ has been reported to modulate the phosphorylation state of various proteins, including osteopontin and VDR. We examined the possibility that calbindin D-28K is also a phosphoprotein. Amino acid sequence analysis of calbindin D-28K reveals five potential consensus phosphorylation sites for CKII and two for PKC. We assessed the ability of either kinase to phosphorylate calbindin D-28K *in vitro*. Although CKII does not phosphorylate calbindin D-28K under our experimental conditions, PKC phosphorylation of calbindin D-28K is evident within five minutes of incubation. This phosphorylation is calcium- and phospholipid-dependent and, consistent with the sequence analysis, appears to occur on threonine residues. These results constitute the first report of calbindin D-28K phosphorylation. We further demonstrate that $\text{PKC}\alpha$ directly interacts with and phosphorylates calbindin D-28K *in vitro*, again implicating this isozyme in calbindin D-28K regulation. However, the phosphorylation of calbindin D-28K under *in vitro* conditions may not represent *in vivo* observations. Studies in MDBK cells further describe calbindin D-28K as a phosphoprotein whose phosphorylation state is modulated by both TPA and $1,25(\text{OH})_2\text{D}_3$. These results suggest a direct phosphorylation step in calbindin D-28K regulation. The functional relevance of this phosphorylation is unknown but may affect the calcium-binding affinity of calbindin D-28K or its interaction with other cellular components.

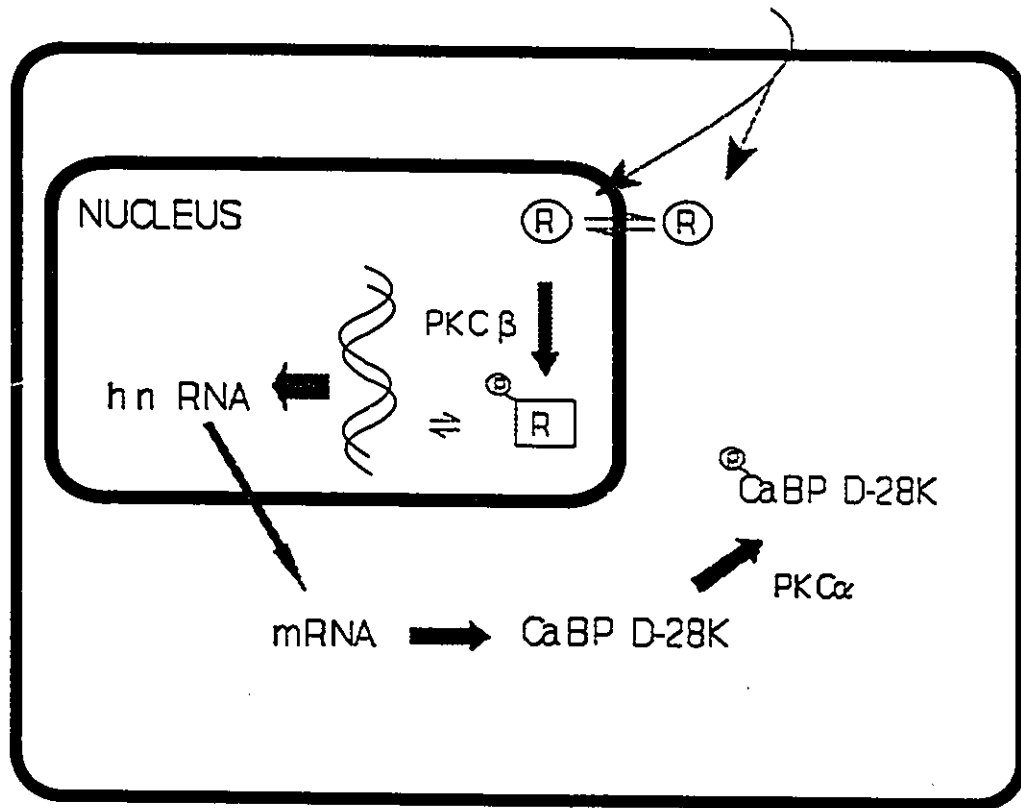
The role of $\text{PKC}\alpha$ in calbindin D-28K phosphorylation in MDBK cells was assessed using MDBK cells transfected with a cDNA coding for the regulatory domain of $\text{PKC}\alpha$ ($\text{RD}\alpha$). This construct was previously shown to specifically inhibit $\text{PKC}\alpha$ activity. The lower

basal levels of calbindin D-28K expression observed in MDBK cells expressing RD α implicate PKC α in the constitutive regulation of calbindin D-28K. VDR levels are unaffected by this transfection, suggesting the specificity of the effect. Further studies are required to determine the role of this PKC isozyme in the regulation of calbindin D-28K by TPA and 1,25(OH) $_2$ D $_3$.

A hypothetical model of calbindin D-28K regulation has been compiled from our results. In this model, 1,25(OH) $_2$ D $_3$ regulates calbindin D-28K expression both at the transcriptional and post-transcriptional levels. As previously described, 1,25(OH) $_2$ D $_3$ transcriptionally regulates calbindin D-28K via a VDR-mediated induction of gene transcription. This is supported by the identification of VDREs in the promoter region of mammalian calbindin D-28K genes (Gill and Christakos, 1993; Takeda et al, 1994). The post-transcriptional regulation of calbindin D-28K by 1,25(OH) $_2$ D $_3$ would involve direct phosphorylation of calbindin, mediated by PKC α . The mechanism underlying activation of PKC α by 1,25(OH) $_2$ D $_3$ is unclear, but may involve genomic actions of the hormone. We hypothesize that phosphorylation increases calbindin D-28K stability, possibly through interaction with other cellular components such as tubulin. This model is also consistent with the results observed with TPA. Rapid activation of PKC α by TPA results in increased phosphorylation and expression of calbindin D-28K. Since TPA directly activates PKC, protein synthesis inhibitors would have no effect on this induction. In addition, the down-regulation of PKC observed after long term treatment with TPA would reduce calbindin D-28K expression. The presence of phosphatase inhibitors would partially prevent the dephosphorylation of calbindin D-28K, blunting the effect of TPA down-regulation.

Other important effects of 1,25(OH) $_2$ D $_3$ observed in MDBK cells, include VDR up-

Hypothetical model of calbindin D-28K regulation in MDBK cells.



regulation and nuclear translocation of PKC β . Although the VDR has been shown to be phosphorylated by PKC β , this phosphorylation step appears to be hormone-independent. Further studies involving nuclear translocation of PKC β and VDR phosphorylation and expression are required to determine the function of this isozyme in 1,25(OH) $_2$ D $_3$ actions.

In summary, we have described a new *in vitro* model system to investigate renal calbindin D-28K regulation by 1,25(OH) $_2$ D $_3$. We also demonstrated the interaction between 1,25(OH) $_2$ D $_3$ and PKC signalling pathways in modulating calbindin D-28K and VDR expression in MDBK cells. The characterization of calbindin D-28K as a phosphoprotein modulated by PKC activation may prove important in defining the biological function of this evolutionary important calcium-binding protein.

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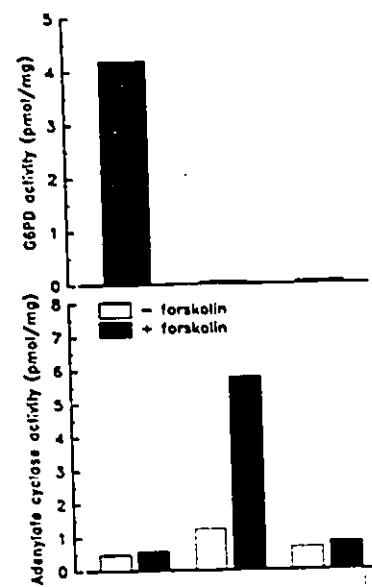
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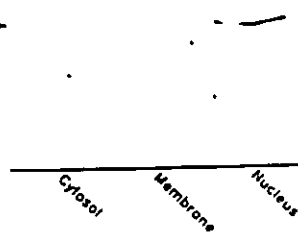
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Appendix 1 Characteristics of subcellular fractions.

MDBK cells were homogenized and fractionated as described in 1.2.1. Subcellular fractions were analyzed for glucose-6-phosphate dehydrogenase (G6PD) activity (*top panel*), a cytosolic marker, forskolin-stimulated adenylate cyclase activity (*middle panel*), a plasma membrane marker, and lamin B immunoreactivity (*bottom panel*), a nuclear marker. In each case, *left lane* represents cytosol, *center lane* represents membrane and *right lane* represents nuclear fractions. Data presented in the *top* and *middle panels* represent means of triplicate determinations.

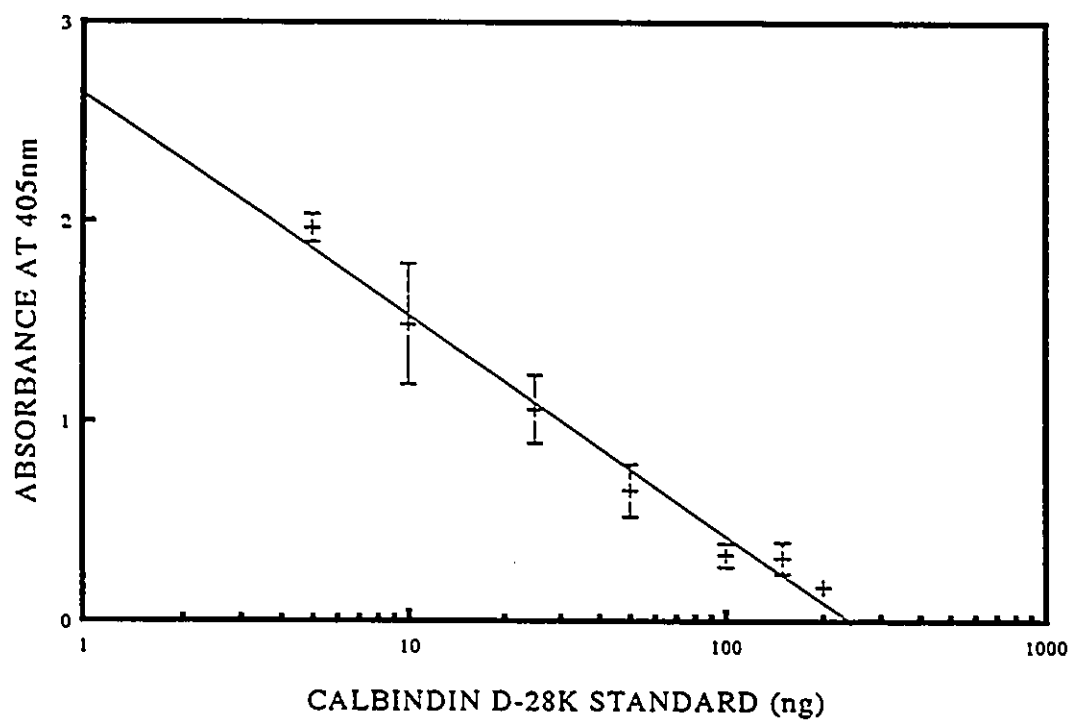


Lamin B
70kDa



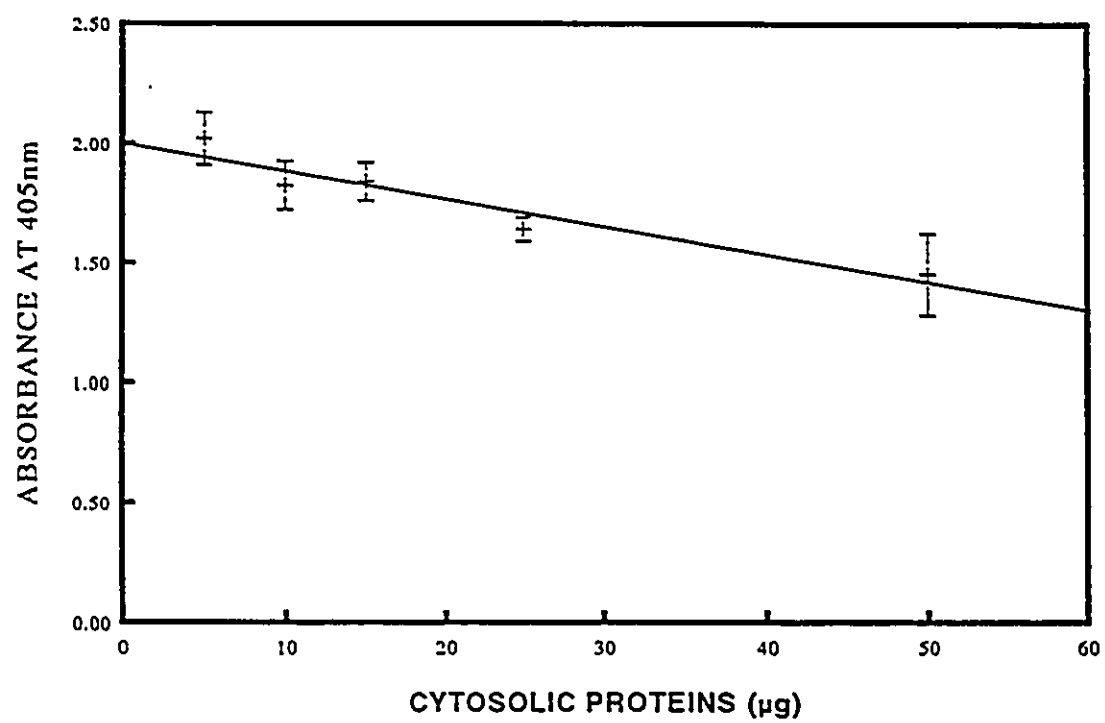
Appendix 2 Representative standard curve used in calbindin D-28K ELISA.

Rat recombinant calbindin D-28K (up to 200 ng) was incubated with monoclonal mouse calbindin D-28K antibodies in wells coated with 10 ng calbindin D-28K. After washing, secondary alkaline phosphatase-conjugated antibodies were added to the wells. Wells were washed again and p-nitrophenyl phosphate, used as a substrate, was added, as described in 1.2.5. Absorbances were determined at 405 nm. The standard curve was generated by plotting the absorbance at 405 nm against the log of calbindin D-28K standard (ng), and is representative of at least 50 determinations.



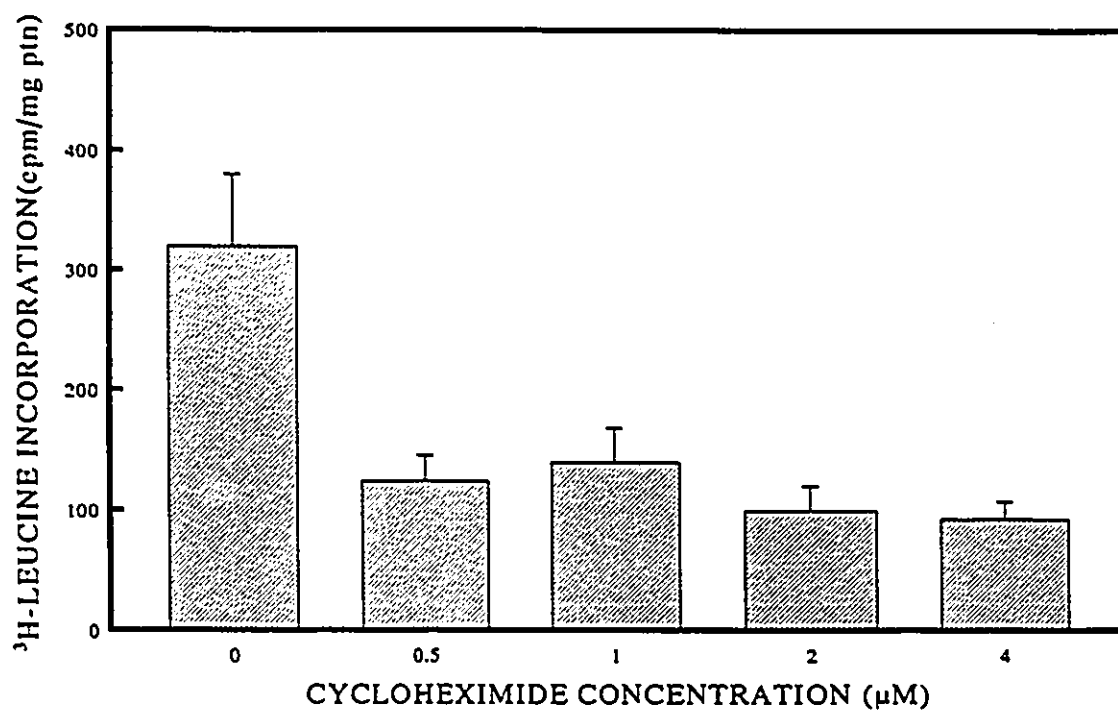
Appendix 3 Protein concentration curve of cytosolic calbindin D-28K levels as assessed by ELISA.

Cytosolic fractions of MDBK cells were prepared as described in 1.2.1. Cytosolic proteins (up to 50 µg) were incubated with monoclonal mouse calbindin D-28K antibodies in wells coated with 10 ng purified rat recombinant calbindin D-28K. After washing, wells were incubated with secondary alkaline phosphatase-conjugated antibodies. Wells were washed again and p-nitrophenyl phosphate, used as a substrate, was added as described in 1.2.5. Absorbance was determined at 405 nm. The protein curve was generated by plotting the absorbance at 405 nm against protein concentration. Data represent the mean $\pm$ SEM of three separate triplicate determinations ($N = 3$).



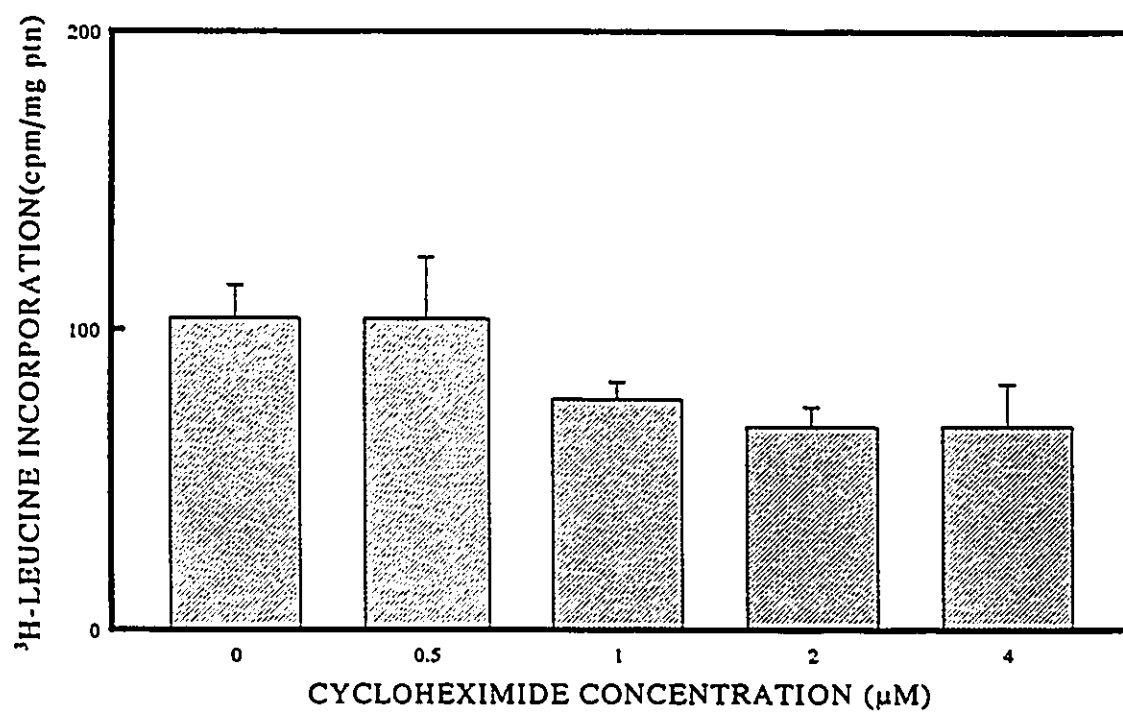
Appendix 4 [³H]-leucine incorporation into protein after two hour treatment with cycloheximide.

Serum-free MDBK cells were treated for 2 hours with up to 2 μ M cycloheximide or vehicle (PBS). After one hour, [³H]-leucine (0.1 μ Ci/ml) was added to each dish. Cells were harvested by scraping and proteins purified by TCA precipitation. [³H]-leucine incorporation into proteins were determined by liquid scintillation counting. Data represent the mean $\pm$ SEM of three separate duplicate determinations ($N = 3$).



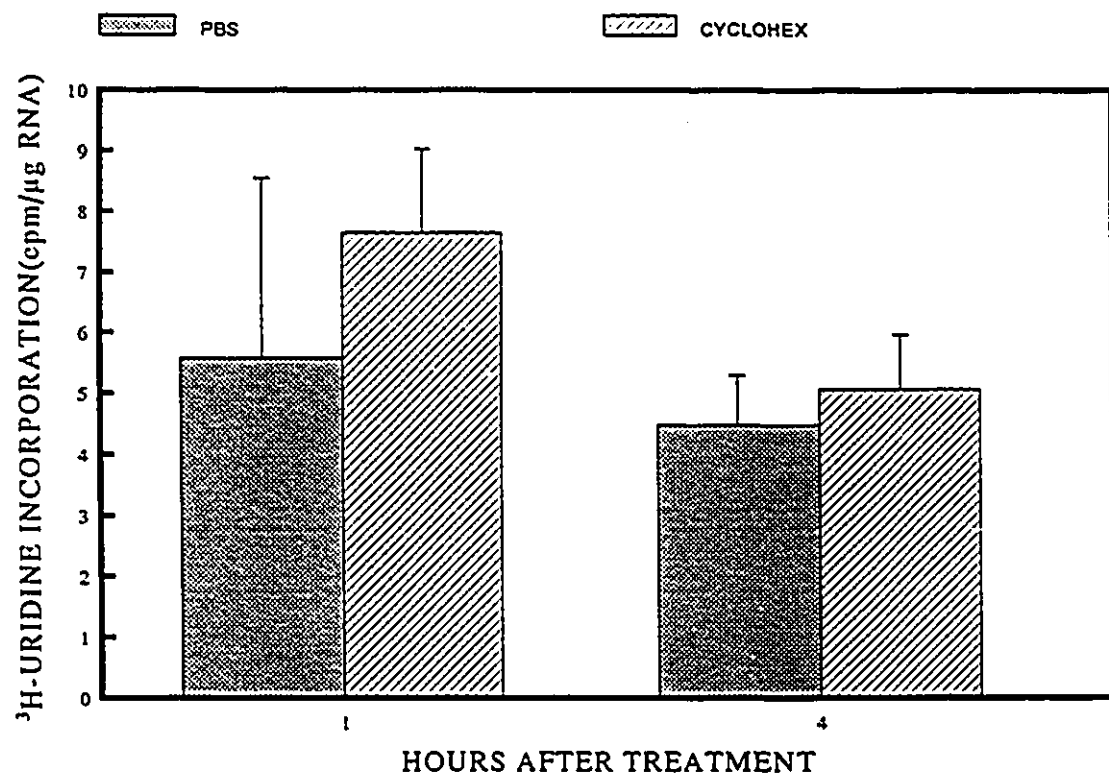
Appendix 5 $[^3\text{H}]$ -leucine incorporation into protein after 24 hour treatment with cycloheximide.

Serum-free MDBK cells were pretreated with up to 2 μM cycloheximide or vehicle (PBS) followed by treatment with 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ for 24 hours. In the last hour of treatment, MDBK cells were labelled with $[^3\text{H}]$ -leucine (0.1 $\mu\text{Ci/ml}$). Cells were harvested by scraping and proteins were purified by TCA precipitation. $[^3\text{H}]$ -leucine incorporation was determined by liquid scintillation counting. Data represent the mean $\pm$ SEM of three separate duplicate determinations ($N = 3$).



Appendix 6 [3H]-uridine incorporation into RNA following treatment with cycloheximide.

Serum-free MDBK cells were treated for up to 4 hours with 2 μ M cycloheximide or vehicle (PBS). [3H]-uridine (0.1 μ Ci/ml) was added to each dish within the final hour of treatment. RNA was extracted as previously described and [3H]-uridine incorporation into RNA was determined by liquid scintillation counting. Data represent the mean $\pm$ SEM of three separate duplicate determinations ($N = 3$).



AnneMarie Gagnon*Curriculum vitae***Personal data**

Date of birth: May 26th, 1968
Place of birth: Quebec City, Quebec
Citizenship: Canadian

Degrees

D.E.C. Sciences, CEGEP Garneau, Quebec City, Quebec, 1988.
B.Sc. Biochemistry, Honours Nutrition, University of Ottawa, Ottawa Ontario, 1991.
Ph.D. Biochemistry, University of Ottawa, Ottawa, Ontario, expected fall 1995.

Employment history

Student research assistant, Department of Biotechnology, Laval University, Quebec City, Quebec (summers 1987-1988).

Demonstrator, Undergraduate Student Labs, Department of Biochemistry, University of Ottawa, Ottawa, Ontario (1991-1995).

Awards

Osteoporosis Society Travel Award, 1995
Medical Research Council of Canada Graduate Studentship, 1991-present
University of Ottawa Excellence Scholarship, 1991-present
Young Investigator Award, Ninth International Workshop on Vitamin D, 1994.
Department of Biochemistry Travel Awards, 1992, 1994, 1995
University of Ottawa Travel Awards, 1992, 1994
Society of Chemical Industry Merit Award, 1991
Dairy Bureau Award, 1991
University of Ottawa Entrance Scholarship, 1988

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