# THE REGULATION OF CALBINDIN D-28K BY EPIDERMAL GROWTH FACTOR IN MDBK CELLS AND THE INVOLVEMENT OF SIGNAL TRANSDUCTION PATHWAYS

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## ABSTRACT

$1,25(OH)_2D_3$ and EGF both modulate cell proliferation and differentiation in several cell systems and tissues (Franceschi et al, 1985, Brackman et al, 1992, Chen et al, 1995). The renal distal tubule is one of the key target sites for $1,25(OH)_2D_3$ actions. $1,25(OH)_2D_3$ increases the expression of renal distal tubular proteins involved in calcium reabsorption including the VDR, and the calcium binding proteins calbindin D-28K and calbindin D-9K (Johnson et al, 1994, Bouthiauy et al, 1994, Pansini et al, 1994). Mitogenic agents such as serum and EGF have been shown to modulate VDR in various normal and transformed cell lines (Krishnan et al, 1991a, Krishnan et al, 1991b, Gagnon et al, 1994). We investigated the ability of $1,25(OH)_2D_3$ and EGF to modulate cell proliferation and VDR levels in renal distal tubular cells. As well, we characterized the effects of EGF on the vitamin D-dependent calcium binding protein calbindin D-28K in order to determine if changes in its expression were linked to cell proliferation. The MDBK cells represent an ideal in vitro system for studying the regulation of calbindin D-28K and VDR since both proteins are expressed in a $1,25(OH)_2D_3$-dependent manner in these cells. Assessment of DNA synthesis, cell cycle kinetics and cell number indicated that EGF significantly increased DNA synthesis (40%), cell number and the population of cells in S phase of the cell cycle compared to vehicle treated MDBK cells. On the other hand, $1,25(OH)_2D_3$ did not affect proliferation of MDBK cells nor did it blunt the mitogenic actions of EGF in these cells.

Previous studies have shown that serum and growth factors such as EGF and insulin stimulate VDR gene expression in 3T3 fibroblasts and in MCF-7 cells (Krishnan et al, 1991a).

We observed that EGF down-regulated $1,25(OH)_2D_3$-binding levels (33%) after 4 hour and 24 hour treatment compared to PBS treatment for the same time. Pre-treatment with $1,25(OH)_2D_3$, prior to the addition of EGF, restored VDR binding to levels observed when cells were exposed to $1,25(OH)_2D_3$ alone.

The changes in $1,25(OH)_2D_3$ binding in response to EGF prompted us to examine EGF effects on calbindin D-28K expression. MDBK cells were stimulated with EGF for up to 24 hours and calbindin D-28K levels were assessed by ELISA and immunoblotting. After 24 hours with EGF calbindin D-28K was down-regulated compared to control treated cells. Calbindin D-28K down-regulation was prevented by $1,25(OH)_2D_3$ and therefore correlated with the changes in VDR binding observed under the same conditions. However, acute (4 hour) EGF treatment up-regulated calbindin D-28K protein levels as assessed by ELISA and immunoblotting. This increase in calbindin D-28K was observed despite the decrease in VDR levels under the same conditions and therefore may represent post-transcriptional regulation of calbindin D-28K by EGF. We have observed similar effects of the PKC activator TPA on calbindin D-28K expression in MDBK cells, suggesting that growth factor signalling pathways involving CKII or PKC may modulate calbindin D-28K independently of the $1,25(OH)_2D_3$ signalling pathway.

EGF interacts with its tyrosine kinase receptor and activates several signal transduction pathways including the serine/threonine kinases PKC and casein kinase II (CKII). Previous data in this lab demonstrated that TPA modulates calbindin D-28K expression in MDBK cells, but the mechanism is unknown (Gagnon et al, 1994). Calbindin D-28K contains both PKC and CK II consensus phosphorylation sites. Our lab has recently

demonstrated that PKC phosphorylates calbindin D-28K *in vitro*. We hypothesized that EGF might regulate calbindin D-28K in MDBK via its effect on CK II and/or PKC signalling pathways. To date there have been no studies on the effect of EGF on the regulation of calbindin D-28K, or the effect of CK II on calbindin D-28K phosphorylation status. Our results suggest that CKII does not directly phosphorylate calbindin D-28K and is not likely involved in the regulation of this protein *in vivo*. On the other hand, MDBK cells stimulated with EGF for up to 4 hours exhibited a 2-fold increase in PKC activity compared to control cells. These early changes in PKC activity, after 2 hour EGF treatment, did not directly correspond to changes in PKCα expression monitored in soluble and particulate fractions by immunoblotting. Thus, these acute effects of EGF on PKC activity may be due to effects on PKCβ or other isozymes present in MDBK cells. However, we observed an increase in PKCα expression in particulate fractions of MDBK cells following 4 hour EGF treatment. Contrastingly, soluble PKCα expression did not change significantly in response to EGF treatment. Long-term (24 hour) EGF treatment down-regulated particulate PKC activity and PKCα expression, while soluble PKC activity returned to basal levels. The increase in membrane-associated PKC activity following 4 hour EGF treatment coincided with the increase in calbindin D-28K expression observed under the same conditions, suggesting that PKC may be involved in mediating the effects of EGF on calbindin D-28K at the post-transcriptional level in MDBK cells.

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Figure 4.11B: Changes in PKCα expression in membrane fractions of MDBK cells treated with EGF for 4 hours and 24 hours.

## LIST OF ABBREVIATIONS

ATP - adenosine triphosphate

BSA - bovine serum albumin

cAMP- cyclic adenosine monophosphate

cdc - cell cycle dependent kinase

CKI - casein kinase I

CKII- casein kinase II

CNS - central nervous system

cpm - counts per minute

CSN - casein

DAG- diacylglycerol

DBD - DNA binding domain

DCT - distal convoluted tubule

DMEM- Dulbecco's Modified Eagle Medium

DNA - deoxyribonucleic acid

DR3 - direct repeat three

DTT - dithiotriethol

EDTA - ethylene diaminetetracetic acid

EGTA - ethylene glycoltetracetic acid

EGF- Epidermal growth factor

## LIST OF ABBREVIATIONS

EGFR - Epidermal growth factor receptor

ELISA - Enzyme Linked Immunosorbent Assay

EtOH - ethanol

FGF - fibroblast growth factor

fmol - femtomole

GAP - GTP activating protein

hVDR - human vitamin D receptor

IGF-1 - insulin-like growth factor-1

IgG- Immunoglobulin

$IP_3$ - inositol 3-phosphate

$K_d$ - dissociation constant

kDa - kilodalton

LBD - ligand binding domain

MAPK - mitogen activated protein kinase

mg - microgram

mL - microliter

mg - milligram

mL - milliliter

MDBK - Madin Darby Bovine Kidney

## LIST OF ABBREVIATIONS

mRNA - messenger ribonucleic acid

NaOH - sodium hydroxide

ng - nanogram

NGF - nerve growth factor

nm - nanometer

nM - nanomolar

$N_{max}$ - maximum number

PAGE - polyacrylamide gel electrophoresis

PBS - phosphate buffered saline

PDGF - platelet derived growth factor

PL - phospholipid

PLC - phospholipase C

PKC - protein kinase C

PNP - phenyl nitrophosphate

PTH - parathyroid hormone

RAR - retinoic acid receptor

RNA - ribonucleic acid

RXR - retinoid X receptor

SDS - sodium dodecyl sulfate

## LIST OF ABBREVIATIONS

SEM - standard error of the mean

TALH - thick ascending loop of Henle

TCA - trichloroacetic acid

TFII - transcription factor II

TGF - transforming growth factor

TNF - tumor necrosis factor

TPA - 12-O-tetradecanoyl phorbol 13-acetate

TR - thyroid hormone receptor

VDR - vitamin D receptor

VDRE - vitamin D response element

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# 1.INTRODUCTION

## 1.1 $1,25(OH)_2D_3$

$1,25(OH)_2D_3$, the biologically active form of vitamin $D_3$, was identified by its ability to eliminate the bone disease rickets. $1,25(OH)_2D_3$ increases bone resorption, intestinal calcium absorption, and tubular calcium reabsorption in kidney; these effects are partially mediated by the interaction of the hormone with the vitamin D receptor (DeLuca, 1984). The role of this steroid hormone in calcium homeostasis is well established; however, recent evidence indicates that $1,25(OH)_2D_3$ also modulates proliferation and differentiation of normal and malignant cells (Bikle, 1995 and Norman, 1995)

## 1.2 $1,25(OH)_2D_3$ SYNTHESIS

Vitamin $D_3$ is synthesized in the skin following UV irradiation of the precursor 7-dehydrocholesterol. Alternatively, vitamin $D_3$ can be obtained from the diet. In either case, vitamin $D_3$ is converted to $1,25(OH)_2D_3$ via two sequential hydroxylations. The first occurs in the liver and generates $25(OH)D_3$. The second hydroxylation step, which produces the biologically active form of vitamin $D_3$, occurs in the proximal tubules of the kidney (Kumar, 1995) and is regulated primarily by parathyroid hormone (PTH). In response to low plasma calcium levels PTH activates the renal cytochrome P450 1α-hydroxylase which hydroxylates $25(OH)D_3$ at C-1 to produce $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ circulates bound to D-binding protein (DBP) and is taken up by the cells in target tissues by a mechanism which is still not clear.

## 1.3 $1,25(OH)_2D_3$ AND RENAL CALCIUM TRANSPORT

The kidney reabsorbs 98% of filtered calcium. Approximately 60% of this occurs at the proximal tubule, 10-15% at the loop of Henle, and the remaining 15-25% at the distal and collecting tubules. Proximal tubular calcium reabsorption is dependent on sodium transport, however at the distal tubule calcium reabsorption is independent of sodium transport. The distal tubule is the site of hormonal regulation including $1,25(OH)_2D_3$-mediated regulation of calcium reabsorption. This region of the kidney has been shown to contain the proteins involved in calcium reabsorption such as the basolateral plasma membrane pump, the $Na^+/Ca^{2+}$ exchanger, VDR, and vitamin D dependent calcium binding proteins such as the calbindins (Thomasset et al, 1982, Kumar, 1995). *In vivo* studies using rabbit proximal and distal tubules showed that vitamin D-depletion decreases calcium uptake by distal tubules while proximal tubule calcium uptake is unaffected. The decrease in calcium uptake appeared to be due to a decrease in ATP-dependent calcium uptake rather than any changes in $Na^+/Ca^{2+}$ exchange (Bouhtiauy et al, 1993).

## 1.4 CALBINDIN D-28K

The vitamin D-dependent calbindins have been implicated in calcium transport in kidney and intestine. Calbindin D-9K is found predominantly in mammalian intestine although low levels are expressed in kidney (Christakos et al, 1989). Calbindin D-28K is present in avian intestine and kidney, and mammalian intestine, brain, kidney and other tissues (Thomasset, 1982). Both proteins belong to a family of intracellular proteins with high affinity for binding calcium ($K_d=10^{-8}$-$10^{-6}$). Phylogenetic studies have shown that calbindins

are evolutionarily conserved proteins which are present in a variety of species and tissues indicating that these proteins potentially play a key role in maintaining calcium balance (Christakos, 1989).

Calbindin D-28K is a vitamin D-dependent protein found in high concentrations in the kidney (Pansini, et al, 1984) and is immunolocalized in the cytosol of distal and collecting tubule cells (Kumar et al, 1994). Calbindin D-28K protein and mRNA is detected in kidney as early as 12 days of gestation in mice and before birth to one week postnatal in rats, suggesting a role in embryological and neonatal development (Thomasset et al, 1982, Lanting et al, 1993, Varghese et al, 1988). The function of calbindin D-28K with respect to renal tubular calcium handling is unclear, although some evidence indicates that calbindin D-28K binding to transported calcium may increase the concentration gradient for calcium, thus facilitating calcium uptake (Bouthiauy et al, 1994). In addition, overexpression of calbindin D-28K in rat sensory neurons, NIH 3T3 cells, and $GH_3$ cells indicates that this protein buffers intracellular calcium and may protect against calcium overload (Chard et al, 1993, Lledo P.-M et al, 1992, Muir et al, 1993).

## i. CALBINDIN D-28K GENE STRUCTURE AND REGULATION

$1,25(OH)_2D_3$ binds to the VDR and acts in the nucleus of target cells to either up or down-regulate RNA polymerase II genes such as PTH, collagen type II, bone mineral-binding proteins containing gamma-carboxyglutamic acid (gla) domains, c-myc and the calbindins. The human calbindin D-28K gene is present in single copy on chromosome 8. The 5'-untranslated region (UTR) of the avian calbindin D-28K gene contains G+C rich sequences,

TATA and CAT boxes, and sequences homologous to vitamin $D_3$-responsive elements identified in other vitamin D-responsive genes (Minghetti et al, 1988). Studies in rat osteosarcoma (ROS) cells using promoter constructs of the mouse calbindin D-28K gene identified sequences which confer basal activation and a $D_3$-inducible response (Christakos et al, 1992). Further studies identified a D-responsive region between -1265 and -1110 bp in the 5'flanking region of mouse calbindin D-28K which is similar to the consensus DR3 identified in other D-responsive genes (Takeda et al, 1994). The promoter also contains a high density of CpG islands (important for binding nuclear factors), potential nuclease recognition signals, enhancer-like core elements, a glucocorticoid-like response element and a metal-responsive element (Minghetti et al, 1988). The 3'end of the gene contains a large uninterrupted exon with an A+T rich region (common to calcium-binding proteins), and a pair of polyadenylation signals.

The multiple polyadenylation sites generate three mRNA species in rats and chickens. Northern analysis reveals the major calbindin D-28K mRNA species found in chicken intestine, kidney, and brain are approximately 2.0, 2.6, and 3.1 kb (Wood et al, 1988, Varghese et al, 1988). While it is known that all three mRNA species are transcribed from a single gene the functional and regulatory significance of these three mRNA species is not clear, although in rat kidney all three mRNA species are induced by $1,25(OH)_2D_3$.

Calbindin D-28K gene expression is regulated in a tissue specific manner by a variety of factors in addition to $1,25(OH)_2D_3$, including development (Davies, 1994), dietary calcium (Bogden et al, 1992, Meyer et al, 1992) and phosphorus, glucocorticoids, serum, growth factors and phorbol esters (Gagnon et al, 1994, Simboli Campbell et al, 1992). Studies in

primary cultures of chick renal cells show that $1,25(OH)_2D_3$ dose-dependently increases calbindin D-28K linearly up to 48 hours following treatment. $1,25(OH)_2D_3$ exerts transcriptional effects on renal calbindin D-28K gene expression, as evidenced by the rapid appearance (2h) and accumulation (12h) of calbindin D-28K mRNA following $1,25(OH)_2D_3$ administration to D-deficient rats. Induction of gene expression is correlated with nuclear localization of the VDR and VDR occupancy (Theofan et al, 1986). *In vitro* studies in avian kidney cells have also suggested the involvement of post-transcriptional mechanisms to stabilize calbindin mRNA levels (Clemens et al, 1992 ).

In contrast to renal calbindin, $1,25(OH)_2D_3$ treatment does not induce calbindin mRNA in brain (Wood et al, 1988, Varghese et al, 1988). Consistent with these observations, dietary changes in calcium and phosphorus affect renal but not brain calbindin D-28K mRNA levels. Expression of calbindinD-28K mRNA is induced by dexamethasone in brain and retinoic acid, TNFα and TNFβ in medulloblastoma and hippocampal cells (Christakos et al, 1994). These differences suggest significant differences in gene expression and regulation, which may involve cell and tissue-specific factors.

## ii. CALBINDIN D-28K PROTEIN STRUCTURE

Calbindin D-28K belongs to the family of EF-hand calcium binding proteins including calbindin D-9K, calmodulin, troponin C, paravalbumin (Heizmann and Hunziker, 1991), and the S-100 proteins (Van Eldik et al, 1995, Chazin et al, 1995). EF-hand proteins contain multiple helix-loop-helix domains which bind calcium with high affinity depending on the amino acid content of the loop. EF hands with higher affinity ($K_d=10^{-9}$) for calcium contain

amino acids with at least 3-5 oxygen atoms in the loop which facilitate calcium binding; EF-hands with less than 3 oxygen atoms have lower affinity for calcium ($K_d=10^{-5}$). Other structural features important for high affinity calcium binding are the appropriate folding of the EF-hand domains, helix-helix interactions, the location of amino acid side chain oxygens, and N-terminal and C-terminal helices (Gross et al, 1993, Heizman et al, 1991).

Proteins containing multiple EF-hand domains are believed to have originated from a single EF-hand domain through duplications, triplications and deletions. These changes gave rise to four sub-groups of calcium binding proteins containing two (calbindin D-9K), three (paravalbumin), four (calmodulin), or six (calretinin, calbindin D-28K) EF-hand domains. Although calbindin D-28K contains 6 putative EF-hands, only 4 of these bind calcium ions since EF-hands 2 and 6 contain mutations in the loop sequence which prevent high affinity calcium binding (Parmentier et al, 1987).

Amino acid sequence analysis of calbindin D-28K indicates that this protein is highly conserved among avian, murine, rodent, human and bovine species. For example, human and chick calbindin D-28K show 78.5% homology. Sequence analysis of bovine cerebellar calbindin D-28K reveals a high content of acidic amino acids (36.8, 42.2 aspartate and glutamate residues respectively) (Takagi et al, 1986). Mutations in EF hands 2 and 6 described above result in substitutions of alanine for aspartate, resulting in the loss of calcium binding in these motifs.

Further protein sequence analysis reveals consensus sequences for CKII and PKC dependent phosphorylations (Figure A1). Five CKII phosphorylation sites are present at Thr 60, Ser 99, Thr 137, Ser 156, and Ser 257; however only Ser 99 is conserved among rodent,

murine, chicken, human and bovine species. There are conserved PKC consensus phosphorylation sites at Thr 106 (EEFMKTWRKYD) and Thr 233 (IINITTYKKNI) which are located in the α-helical segments before EF-hands 3 and 6 respectively. The functional and regulatory significance of either CKII or PKC consensus phosphorylation sites is currently not known, although their presence suggests that reversible phosphorylation may play a role in regulation of calbindin D-28K by growth factors, phorbol esters and other agents (Simboli-Campbell et al, 1994).

## 1.5 VITAMIN D RECEPTOR

### i. Subcellular Localization

The genomic effects of $1,25(OH)_2D_3$ are mediated by the vitamin D receptor (VDR). The human VDR is a 60 kDa soluble protein expressed in many tissues including the intestine, bone, kidney, parathyroid glands, placenta, brain and various normal and malignant cell lines. Occupied VDR is found exclusively in the nucleus (MacDonald, 1991, Chandler et al, 1979, Colston et al, 1979, Pike et al, 1983, Pike, 1991). Although most studies indicate that unoccupied VDR is cytosolic, the subcellular localization of bound or unbound VDR remains controversial since immunoreactive VDR has been detected in chick epithelial nuclei in the absence of $1,25(OH)_2D_3$ (Clemens et al, 1991).

### ii. VDR Protein

The VDR belongs to the steroid/thyroid hormone receptor superfamily. These receptors have the same general structure consisting of a well conserved N-terminal DNA-

binding domain (DBD) containing a cysteine-rich zinc-finger domain and a C-terminal ligand binding domain (LBD) that is unique to each receptor. These domains are separated by a highly variable and immunogenic hinge region. In addition, the C-terminus of these receptors contains a dimerization domain, nuclear localization signal and other regions important for protein-protein interaction (Beato, 1989). It is believed that the presence of hormone increases the receptor's affinity for DNA by inducing conformational changes which enhance the receptor's affinity for nuclear accessory factors, or promote dimerization (Liao et al, 1990, Perdew et al, 1991). Nuclear factors which interact with VDR include RXR, TR and TFIIb. The LBD in the C-terminus of the VDR binds $1,25(OH)_2D_3$ in a saturable manner and has a $K_d$ of $10^{-10}$ to $10^{-11}$. A specific region in the C-terminus between amino acids 200 and 300 is responsible for hormone binding. Sequence comparison of different steroid hormone receptors show this region to be highly variable and unique to each receptor. In addition to ligand binding, this region is also involved in protein-protein interactions. Several heptad repeats throughout the LBD of the VDR contain hydrophobic amino acids which form a coiled-coil α-helix, exposing the hydrophobic surface for dimerization and protein-protein interactions (MacDonald et al, 1994). Finally, several mutations in the hormone binding domain of the VDR have been identified in patients with vitamin D-resistant rickets type II, who have non-functional VDR (Kristjansson et al, 1993, Ritchie et al, 1989).

The DBD in the VDR is the smallest of all the receptors in the superfamily and is highly basic as it contains nine conserved cysteine residues which coordinate two zinc atoms to form zinc-finger domains. Mutations in any of the first eight cysteine residues disrupt VDR structure and prevent it from binding DNA sequence elements resulting in vitamin D-resistant

rickets type II (Sone et al, 1990). VDR-DNA interaction is specified by three amino acids (glutamine, glycine, glycine) at the base of the first zinc finger motif. The first zinc finger is responsible for recognition of hormone response elements and direct interaction with the major groove of DNA via specific base contacts; the second zinc finger lies perpendicular across the first and stabilizes the interaction. The second zinc finger contains many positively charged amino acids (lysine, arginine) which interact with the negatively charged phosphate backbone of the DNA helix. Studies have demonstrated the presence of a nuclear accessory factor in mammalian (rat liver, kidney) nuclear extracts required for VDR-DNA binding in the presence of hormone (Ross et al, 1992, Sone et al, 1991).

### iii. Receptor Gene

The human VDR gene is located in the 12q13-14 region of chromosome 12. This region of genomic DNA consists of nine coding exons interrupted by intronic sequences. The two zinc finger motifs are encoded by separate exons (MacDonald et al, 1994). A comparison of DNA sequences from diverse receptors such as VDR, thyroid hormone receptor (TR) and retinoic acid receptor (RAR) revealed regions of homology in the DBD and LBD regions while the hinge regions showed more variability. The coding regions from human, rat, and avian VDRs are 95% identical in the DBD and 93% in the LBD (Pike, 1991). The structural similarity of these domains between species indicates that this protein has been well conserved throughout evolution and emphasizes the importance of these structures in genomic function of the VDR.

iv. General Mechanism of Action

Once bound by hormone the VDR binds specific VDREs in target genes. Two types of VDREs have been described, one that is bound by VDR homodimers and one that is bound by VDR heterodimers, such as the osteopontin gene promoter (Carlberg et al, 1993, Green, 1993, Schräder et al, 1993). Retinoid X receptor (RXR) is a major dimerization partner for several nuclear steroid hormone receptors including VDR. Dimerization with RXR enhances the DNA binding affinity and transcriptional activity of VDR for DR3 and inverted palindromes (Kliewer et al, 1992, Yu et al, 1991). In addition, VDR has been shown to dimerize with the TR and RAR.

**v. VDR Regulation**

The VDR is up-regulated by its ligand in several systems including pig kidney cells (LLC-PK1) (Costa et al, 1985), mouse fibroblasts (Mangelsdorf et al, 1987, McDonnell et al, 1987), human promyelocytic leukemia cells (HL-60) (Lee et al, 1989), rat osteosarcoma cells (Pan et al, 1987) and in vivo in rat intestine (Wiese et al, 1992). $1,25(OH)_2D_3$ increases VDR mRNA and protein levels in the intestine 10-fold above deficiency levels and maintains constant total receptor levels (Strom et al, 1989). Studies by Wiese et al (1992) have shown that elevation of VDR following $1,25(OH)_2D_3$ treatment *in vivo* is due to increased receptor protein half-life rather than enhanced transcription. Ligand induced stabilization of the VDR has also been reported in COS-1 cells and S.Cerevisiae over-expressing VDR (Santiso-Mere et al, 1993).

VDR expression is altered in response to mitogens, phorbol esters, or physiological

needs such as growth, development, and pregnancy (Colston et al, 1988). Some studies have shown VDR levels to be closely associated with the rate of cell proliferation in vitro (Chen et al, 1981, Manolagas et al, 1984, Krishnan et al, 1991). Mitogens such as serum, EGF, IGF-1, and high concentrations of insulin up-regulate VDR mRNA and protein (Krishnan et al, 1991). In distal tubular cells serum stimulation increases VDR binding (Gagnon et al, 1994) while TPA (12-O-tetradecanoylphorbol 13-acetate) decreases VDR binding by decreasing receptor number (Simboli-Campbell et al, 1992). Krishnan et al (1991) showed that serum and EGF increase VDR mRNA levels in 3T3 mouse fibroblasts and MCF-7 human breast cancer cells. However, later studies by this group showed that FGF, an activator of PKC and calcium mobilization, down-regulates VDR mRNA levels. Similarly, decreased VDR levels are observed in NIH 3T3 fibroblasts (Krishnan et al, 1992) treated with forskolin and TPA, and in osteoblast-like cells in response to cAMP and TPA (vanLeeuwen et al, 1992). VDR mRNA and protein levels are down-regulated in response to cAMP, forskolin and TPA in both cell types. The inconsistency in VDR regulation may reflect cell type specific regulation of VDR mRNA and protein. Therefore, growth factors whose effects are mediated by distinct signalling pathways may differentially modulate VDR abundance and ultimately affect cell responsiveness to $1,25(OH)_2D_3$. PKC and CKII can both mediate phosphorylation of serine residues on VDR and thus reversible phosphorylation may modulate transactivation ability of the VDR (Brown et al, 1990, Hilliard et al, 1994, Darwish et al, 1993, Hsieh et al, 1991, Hsieh et al, 1993, Jurutka Peter W., 1993).

## 1.6 EPIDERMAL GROWTH FACTOR

Epidermal growth factor (EGF) is a 6 kDa protein that belongs to a diverse group of polypeptides which modulate cell growth and differentiation (Carpenter et al, 1990b, Harris et al, 1990). EGF was initially identified during purification of nerve growth factor (NGF) from mouse submaxillary glands as a fraction that did not have nerve growth factor activity but that induces precocious opening of eyelids in neonates. Members of this growth factor family include transforming growth factorα (TGFα) and amphiregulin (Carpenter et al, 1990a, Segal et al, 1990). EGF binds to the EGF receptor (EGFR) in target cells and activates the intrinsic receptor tyrosine kinase, initiating a signalling cascade to promote cell proliferation. EGF has been immunodetected in a variety of tissues including salivary glands, intestine, pancreas, bone marrow, pituitary and kidney. The physiological effects of EGF include increased lipogenesis in adipocytes, eyelid opening in neonates, inhibition of acid secretion from gastrointestinal mucosa and decreased renal glomerular filtration rate. EGF increases cell proliferation in many tissues including intestine, heart, liver, adipocytes, and kidney (Carpenter et al, 1990b, Fisher et al, 1989, Rall et al, 1985). In vitro studies using fetal mouse metanephri identified EGF as one of the heterologous growth factors involved in regulating tubulogenesis and renal maturation (Chailler et al, 1991). Radioimmunoassays have identified mature human EGF in various body fluids including milk, plasma, serum, saliva and urine (Carpenter et al, 1990a, Fisher et al, 1989, Mroczkowski et al, 1993); however, circulating levels are low indicating that EGF does not function as a classical hormone.

## 1.7 EGF SYNTHESIS AND KIDNEY

EGF is synthesized as a membrane attached precursor (preproEGF) glycoprotein and is present in almost all tissues. Mature EGF is secreted into the lumen of kidney tubules. PreproEGF and EGF are both found in high concentrations in the thick ascending loop of Henle (TALH) and distal convoluted tubules (DCT) of the kidney (Hamm et al, 1993, Rall et al, 1985, Salido et al, 1986). In fact, the precursor accumulates in mammary glands as well as in kidney, suggesting that it may have physiological effects as well (Mroczkowski et al, 1993).

Hydropathy analysis shows that preproEGF contains two hydrophobic sequences - an N-terminal sequence which potentially acts as a signal related to biosynthesis, and a C-terminal hydrophobic sequence followed by a short sequence of basic amino acids which is characteristic of transmembrane proteins (Carpenter et al, 1990a, Fisher et al, 1989). Although the mechanism is unclear, mature EGF is believed to be cleaved from the membrane-attached precursor by an arginine specific endopeptidase (Carpenter et al, 1990a).

### i. EFFECT OF EGF ON KIDNEY FUNCTION

The physiological effects of EGF on renal function include increased cell proliferation and decreased cell differentiation, decreased glomerular filtration rate and increased urinary volume. Cellular changes in response to EGF involve stimulation of $Na^+/H^+$ exchange, increase in eicosanoid and prostaglandin synthesis, stimulation of inositol phosphate formation and increase in intracellular calcium (Carpenter et al, 1990a, Rall et al, 1985, Hamm et al, 1993). The location of preproEGF in the TALH and DCT of kidney and the high urinary

EGF concentration suggest that in addition to its function as a mitogen (Gansler et al, 1990) it may also maintain tubular and uro-epithelial integrity and distal tubular transport processes. Furthermore, EGF may be involved in compensatory renal growth following kidney damage, such as ischemia or nephrotoxicity (Hamm et al, 1993, Harris et al, 1991), since renal production of EGF increases following a decrease in renal mass.

## 1.8 EGF RECEPTOR

EGF binds to the EGF receptor (EGFR) in target tissues to exert its biological effects. The human EGFR gene has been mapped to p14-p12 and is expressed in diverse adult and fetal tissues (Lee and Han, 1990). Sequence analysis of the primary structure of the EGFR shows 78% identity between avian and human EGFR (Carpenter et al, 1990a, Carpenter et al,1990b). In the developing bovine kidney, immunocytochemical staining shows the EGFR localized in ductal and tubular epithelium of metanephric and mesonephric cells and reproductive organs (Winters et al, 1993). Other studies show that EGFR tyrosine kinase activity is detected in rat kidney membranes (Cybulsky et al, 1994) as well as in mouse skin, developing skeletal muscle, and various internal organs except liver and brain (Lee and Han, 1990).

### i. EGF RECEPTOR STRUCTURE

The organization of the EGFR is similar to other growth factor receptors such as the PDGF receptor, insulin receptor, and IGF-1 receptor. The mature EGFR, originally isolated from A431 carcinoma cells, is a 170 kDa transmembrane glycoprotein with intrinsic tyrosine

kinase activity (Schlessinger, 1986). It is organized into three regions: the extracellular ligand-binding domain, the hydrophobic membrane-spanning sequence, and the cytosolic tyrosine kinase domain.

The extracellular domain binds EGF and EGF-like molecules such as TGFα and has 10 or 11 N-linked glycosyl chains which are important in receptor degradation. The EGFR contains two clusters of cysteine residues which are most likely involved in the formation of disulfide bridges and receptor dimerization following ligand-binding The region between the two cysteine clusters is the putative ligand-binding domain (Carpenter et al, 1990a, Schlessinger 1986, Carpenter et al, 1990b).

The C-terminal cytoplasmic domain of human EGFR encodes the tyrosine kinase domain which is 90% homologous with the avian v-erbB oncogene. The cytoplasmic domain contains an ATP binding site, required for receptor activation, at lysine 721. In addition to tyrosine kinase activity, this portion of the receptor is phosphorylated at multiple serine, threonine, and tyrosine residues following ligand binding. The non-tyrosine phosphorylations (Thr 654) are the result of phosphorylation by cellular serine/threonine kinases including PKC, which are thought to be involved in the heterologous regulation of the receptor (Carpenter et al, 1990b, Schlessinger, 1986).

## ii. EGFR REGULATION

The currently accepted model for receptor regulation involves EGF binding to the receptor, dimerization of the receptor via formation of disulfide bonds, and subsequent tyrosine kinase activation. Following receptor activation, the receptor-ligand complex is

internalized and sorted through cellular compartments until both are degraded by lysosomes. Several studies have demonstrated heterologous regulation of the EGFR by a variety of factors including cell cycle (Newberry et al, 1995), $1,25(OH)_2D_3$ (Petkovich et al, 1987, Desprez et al, 1991), calcium status (Éthier et al, 1993), and PKC (Mantzouris et al, 1993, Sharma et al, 1994).

### iii. EGFR SIGNALLING

Upon ligand binding, the EGFR tyrosine kinase activity is induced and triggers the EGF signal transduction pathway activating a series of serine/threonine kinases. There are five known enzymes which are activated as a consequence of EGFR tyrosine kinase activity: GAP, MAPK (Boudewijn et al, 1993, Howe et al, 1992, Wu et al, 1993), PI3 kinase, raf kinase, and PLCγ-1 (Carpenter et al, 1990b, Carpenter et al, 1990a, Teitelbaum et al, 1990). Activation of the raf kinase pathway leads to the activation of CKII (Ackerman et al, 1989, Ackerman et al, 1990) and the phosphorylation of several transcription factors including c-myc. Activation of PLCγ-1 stimulates inositol 3-phosphate ($IP_3$) and diacylglycerol (DAG) formation, increases intracellular calcium, and eventually leads to PKC activation (Iwashita et al, 1992, Carpenter et al, 1990b, Carpenter et al, 1990a).

### iv. EGF AND CALCIUM

Several studies have shown the involvement of EGF in modulating calcium levels in different cell types including osteoblasts, hepatocytes, and renal cells (Tashjian et al, 1986). EGF decreases intracellular calcium levels in $GH_3$ cells by blunting L-type voltage gated

channels (Hinkle et al, 1993). In contrast, hepatocytes (Mine et al, 1991, Tanaka et al, 1992) and osteoblasts (Loza et al, 1995) stimulated with EGF show a calcium influx. Bruns et al (1989) demonstrated that EGF increases circulating $1,25(OH)_2D_3$ and VDR abundance which enhances intestinal calbindin D-9K induction and calcium absorption. Thus it appears that EGF may have physiological effects on calcium homeostasis through actions on the gut, suggesting potential interactions between the EGF signalling system and the vitamin $D_3$ endocrine system.

## 1.9 CASEIN KINASES

Casein kinases were first identified as enzymes catalyzing the transfer of phosphates from ATP to casein or phosvitin. Casein kinases are now defined as ubiquitous serine/threonine kinases present in both the cytosol and nucleus of most eukaryotic cells (Pinna, 1990). There are two members of the casein kinase family, casein kinase I (CKI) and CKII, which differ in structure, substrate specificity, and in responsiveness to activators and inhibitors. Both CKI and CKII are evolutionarily conserved proteins among distant organisms such as Drosophila, yeast, and human emphasizing their phsysiological importance in cells. CKI is a monomeric 40 kDa enzyme which uses only ATP as a phosphate donor, phosphorylates serine residues only, and is almost unaffected by compounds that affect CKII activity.

### i. CASEIN KINASE II

CKII is a heterotetrameric serine/threonine kinase composed of two $\alpha$ ($\alpha$ and/or $\alpha_2$)

catalytic subunits and two smaller β regulatory subunits (Mitev et al, 1994). It is found most commonly as $\alpha_2\beta_2$ or $\alpha\alpha_2\ \beta_2$ complexes. Purification of the yeast_α and β subunits showed that the α subunit of CKII is required for catalysis, is autophosphorylated, and is directly inhibited by heparin (Lin et al, 1991). The β subunit stabilizes the holoenzyme, protects against thermal inactivation, mediates the effects of polybasic (polylysine, polyarginine, protamine, spermine) stimulatory compounds (Meggio et al, 1992), increases enzyme activity, recognizes and binds to physiological substrates (Boldyreff et al, 1994, Meggio et al, 1992, Meggio et al, 1993). There has been some controversy surrounding the importance of phosphorylation of the β subunit and in relation to holoenzyme activity. Recently, human β subunit deletional mutants that have lost their ability to undergo autophosphorylation were shown to display basal catalytic activity and thermostability, suggesting that phosphorylation of this subunit is not essential for activation of the holoenzyme (Meggio et al, 1993).

## ii. CKII FUNCTION, REGULATION, AND SUBSTRATES

Disruption of the regulatory subunits of CKII in S.Cerevisiae has indicated that depletion of CKII activity results in cell death (Reed et al, 1994). Other studies have implicated CKII in cell cycle progression (Padmanabha et al, 1990). CKII displays distinctly high activity in transformed and proliferating cells and tissues, and in response to mitogenic stimuli such as growth factors (Ackerman et al, 1989, Ackerman et al, 1990) indicating that it may be a marker of proliferation (Pinna, 1990). Furthermore, the ability of CKII to phosphorylate non-histone proteins such as transcription factors, RNA polII, and initiation factors indicates that CKII plays a role in regulating protein synthesis as well as cell

proliferation (Dahmus, 1981, Reed et al, 1994).

As mentioned above, CKII catalyzes the phosphorylation of a diverse group of physiological substrates including the calcium binding protein calmodulin (Bidwai et al, 1993, Quadroni et al, 1994) and casein (Dahmus, 1981). Synthetic peptides have been used in in vitro phosphorylation assays to identify consensus sequences recognized and phosphorylated by CKII (Glass et al, 1978, Kuenzel et al, 1985, Kuenzel et al, 1987, Perich et al, 1992, Marin et al, 1994). A short sequence of amino acids surrounding the phosphate acceptor site seem to determine the specificity of CKII for the substrate (Perich et al, 1992). The best substrates are those which contain a cluster of acidic amino acids on either side of phosphorylatable serine or threonine residues (Kuenzel et al, 1987, Pinna, 1990), with the crucial acidic cluster located on the C-terminal side of serine or threonine.

Studies indicate that CKII activity is stimulated by growth factors such as IGF-1, transferrin (Wang et al, 1995), bombesin (Agostinis et al, 1992) and EGF (Ackerman et al, 1989, Ackerman et al, 1990), however the role of second messengers has not been clarified. CKII activity increases significantly in cytosolic extracts of A-431 carcinoma cells treated with EGF without any change in CKII protein concentrations or increases in protein synthesis (Ackerman et al,1989). However, CKII activity is correlated with the status of β subunit phosphorylation (Ackerman et al, 1990). Other known inducers of CKII activity include polyamines and basic polypeptides which enhance via different mechanisms (Linnala-Kankkunen et al, 1984, Ahmed et al, 1985, Meggio et al, 1983). On the other hand, heparin, a polycation, potently inhibits CKII activity by directly altering the activity of the α-subunit towards many substrates (Pinna et al, 1990).

## 1.10 PROTEIN KINASE C

Protein kinase C (PKC) represents a family of ubiquitous, structurally conserved serine/threonine kinases first identified on the basis of their activation in vitro by calcium, phospholipids (PL), and diacylglycerol (DAG) (Dekker et al, 1994, Azzi et al, 1992). Presently, the mammalian PKC family is comprised of 12 known isozymes classified into three established groups distinguished by structure, cofactor requirements, in vitro phospholipid responsiveness, and substrate specificity (Kiley et al, 1991).

PKC-α, βI, βII, and γ are known as classical or conventional PKCs (cPKCs) and are ATP, calcium, phospholipid, and DAG-dependent (Akita et al, 1990, Nishizuka et al, 1992, Huang et al, 1989, Walker et al, 1989, Kochs et al, 1993). On the other hand, members of the novel PKC (nPKC) group of isozymes which includes PKC-δ,_ϵ,_η(Λ), and θ do not require calcium (Akita et al, 1990, Nishizuka et al, 1992, Huang et al, 1989, Kochs et al, 1993). The members of the third group known as atypical PKC (aPKC) are ζ and λ, of which the former exhibits calcium, phospholipid, DAG independent activity (Kochs et al, 1993, Azzi et al, 1992).

Structurally, PKC consists of an amino terminal regulatory domain which mediates calcium, PL, DAG, tumor promoter binding and membrane association. Members of the nPKC family lack the calcium-binding region of the regulatory domain, but are still activated by PL and DAG (Azzi et al, 1992). The atypical (aPKC) isozymes lack the membrane binding domain and calcium binding domains. The carboxy-terminus contains the catalytic site and also the ATP-binding site (Nishizuka et al, 1992, Housey et al, 1989). In the absence of activators a short sequence of conserved amino acids, known as the pseudosubstrate site,

binds to the catalytic domain to inhibit kinase activity. Activators like calcium, PL and tumor promoters reduce the affinity of the pseudosubstrate site for the catalytic site (Dekker et al, 1994).

The PKC family is present throughout a wide variety of tissues although some isoforms show tissue-specific distribution (Nishizuka et al, 1992). Immunofluorescence studies of various rat tissues shows that PKCα and PKC-γ are present in high concentrations in various cells in the CNS and that they are closely associated with the perinuclear region. Cells from peripheral tissues such as thymoma, promyelocytic leukemia, myeloblastic leukemia and pheochromocytoma cells, express both PKC-α and -β. Fibroblast cell lines also express a variety of isozymes in the cytosol, membrane, and cytoskeleton (Huang et al, 1989, Azzi et al, 1992) each with differentially regulated subcellular distribution, activation, and down-regulation (Borner et al, 1992). The heterogeneous tissue and subcellular distribution of the four calcium dependent PKC isozymes is indicative of their unique biochemical characteristics.

## 1.11 PKC ACTIVATION

External signals like hormones or growth factors such as EGF (Reynolds et al, 1993, Teitelbaum et al, 1990, Tsuda et al, 1985) induce the hydrolysis of membrane phospholipids to generate $IP_3$ and DAG, and mediate cellular calcium rises which in turn activate PKC (Slater et al, 1995, Kaibuchi et al, 1986). Sustained DAG levels generated from phosphatidylcholine hydrolysis maintain PKC activity for prolonged periods of time (Nishizuka et al, 1992, Kiley et al, 1991). Upon activation, PKC is autophosphorylated on

both the regulatory and catalytic domains (Huang et al, 1986, Mochly-Rosen et al, 1987). Autophosphorylation modifies the affinity of PKC for phorbol esters, increases its sensitivity to calcium and translocates it to the plasma membrane (Ohno et al, 1990, Huang et al, 1986). Prolonged PKC activation and membrane-association leads to down-regulation via proteolytic degradation by a calcium-dependent cysteine protease known as calpain (Takai et al, 1977).

Membrane association is a characteristic feature of PKC activation. Several studies have shown that TPA, and $1,25(OH)_2D_3$, translocate PKC to the membrane (Simboli-Campbell et al, 1994, Azzi et al, 1993, Kraft et al, 1983, Gopalakrishna et al, 1986, Huang et al, 1989). Increases in cell calcium have also been shown to induce PKC translocation in various cultured cell lines (Wolf et al, 1985).

Evidence has been generated which demonstrates the presence of a pool of membrane-associated endogenously active PKC. This form of PKC is either reversibly bound to the plasma membrane, or nuclear membrane (Buchner et al, 1992), or is membrane-inserted. Its activity is not augmented in the presence of calcium or other activators (Bazzi and Nelsestuen, 1988 a,b,c). Studies with bovine brain nuclei have described the presence of nuclear membrane-associated PKC (Buchner et al, 1992). This form of PKC appears to be membrane-inserted, constitutively active, and binds phorbol ester but is not further activated by phorbol esters or calcium.

## 1.12 PKC SUBSTRATES AND CELLULAR EFFECTS

Activation of PKC triggers a variety of cellular responses secondary to the phosphorylation of serine and threonine residues on target proteins PKC substrates involved

in cell regulation include the EGFR (Schlessinger, 1986, Iwashita et al, 1992), the VDR (Hseih et al, 1991, Hseih et al, 1993), MAPK, glycogen synthase kinase, renal $Na^+/H^+$ transporter (Weinman et al, 1986) and $Na^+/K^+$-ATPase (Middleton et al, 1993). Additional substrates include nuclear proteins such as topoisomerase II, polymerase II and lamin B (Kuo et al, 1989) which are involved in DNA and protein synthesis and cell cycle regulation. Activation of PKC in fibroblasts dephosphorylates the nuclear protein JunB and increases its DNA-binding affinity

(Boyle et al, 1992). The function of these target proteins and the importance of their phosphorylation will help to elucidate the role of PKC in the regulation of cellular events.

Cultured cells overexpressing various PKC isozymes exhibit enhanced responsiveness to TPA (Borner et al, 1992, Hata et al, 1993), display altered morphology and growth rates, and form tumors due to the loss of anchorage-dependent growth (Mischak et al, 1993). PKC activation has been shown to modulate the EGF signalling pathway (Sharma et al, 1994) most likely by phosphorylation of the EGFR tyrosine kinase (Schlessinger, 1986). .....

## 1.13 THE CELL SYSTEM

As stated in Sections 1.1 and 1.6, $1,25(OH)_2D_3$ and EGF both modulate cell proliferation and differentiation in several cell systems and tissues (Franceschi et al, 1985, Brackman et al, 1992, Chen et al, 1995). The renal distal tubule is a major target for the $1,25(OH)_2D_3$-mediated regulation of calcium reabsorption (Stumpf et al, 1979, Bouthiauy et al, 1993). The VDR is localized to the nuclei of glomerular, proximal, and distal tubular epithelial cells (Kumar et al, 1994, Clemens et al, 1988). In addition, $1,25(OH)_2D_3$ increases

the expression of renal distal tubular proteins involved in calcium reabsorption, including the VDR, and the calcium binding proteins calbindin D-28K and calbindin D-9K (Johnson et al, 1994, Bouthiauy et al, 1994, Pansini et al, 1984). Both EGF and the EGFR are present in the distal collecting tubules of the kidney, as well EGF increases proliferation in renal cells.

The studies described in this thesis were designed to investigate the ability of $1,25(OH)_2D_3$ and EGF to modulate cell proliferation and VDR levels in renal distal tubular cells. In addition, we wanted to characterize the effects of mitogens such as EGF on the regulation of renal calbindin D-28K. The detailed mechanism of the cellular regulation of calbindin D-28K has been limited due to the absence of a system which expresses the VDR and calbidnin D-28K in a hormone-dependent manner. We used the Madin-Darby bovine kidney cell line for our studies. MDBK cells are non-transformed, distal tubular epithelial cells derived from normal bovine kidney (Madin et al, 1958) which express calbindin D-28K, VDR and the EGFR. The VDR is expressed in relatively high levels in these cells compared to other renal cell lines (Gagnon et al, 1994). Calbindin D-28K is expressed at low levels in MDBK cells and is induced by $1,25(OH)_2D_3$, similar to its induction in mammalian kidney *in vivo* (Gagnon et al, 1994, Bouhtiauy et al, 1993). Calbindin D-28K can also be up-regulated by serum and increases in the proliferative phases of the cell cycle (Gagnon et al, 1994). Our primary goal was was to investigate the concordance between cell proliferation, VDR and calbindin D-28K expression and the possible interaction between the EGF and $1,25(OH)_2D_3$ signalling pathways to determine whether EGF regulates calbindin D-28K in MDBK cells. Our secondary goal was to investigate the concordance between cell proliferation, VDR and calbindin D-28K expression and the possible interaction between the EGF and $1,25(OH)_2D_3$

signalling pathways.

## 2. OBJECTIVES

1. To characterize the effects of $1,25(OH)_2D_3$ on EGF stimulation of MDBK cell proliferation.
2. To characterize the effects of $1,25(OH)_2D_3$ and EGF, alone and in combination, on VDR levels and calbindin D-28K expression.
3. To investigate the effects of EGF on calbindin D-28K protein concentrations and expression.
4. To examine whether EGF activates CKII and/or PKC signalling pathways in MDBK cells and the relevance of these signalling pathways in the regulation of calbindin D-28K.

# 3. METHODS

## 3.1 Cell Culture

Stock cultures of Madin Darby Bovine Kidney (MDBK) cells were obtained from ATCC (Rockville, MD, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Burlington, ON, Canada) supplemented with 10% newborn calf serum (Life Technologies). Cells were plated at a density of $1.5 \times 10^5$ cells/ml and grown to confluence at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$. For all experiments, sub-confluent cells were fluid changed to serum-free DMEM prior to treatment with 100 nM $1,25(OH)_2D_3$ (Biomol Research Laboratories, Inc., Plymouth Meeting, PA),(EtOH vehicle control for $1,25(OH)_2D_3$), 100 ng/ml EGF (Sigma Cell Culture reagents, St-Louis, MO, USA) or PBS(vehicle control for EGF). In some experiments, cells were pretreated with $1,25(OH)_2D_3$ for 4 hours prior to EGF treatment for 20 hours, resulting in 24 hour exposure to $1,25(OH)_2D_3$.

Cells were harvested by scraping, washed with ice-cold PBS, pelleted, and frozen at -70°C. Cells were lysed by vortexing for two minutes with hypotonic buffer (1 mM $NaHCO_3$, 5 nM $MgCl_2$, 5 mM dithiothreitol (DTT), 100 mM phenylmethylsulfonyl fluoride, 20 µg/mL leupeptin, 50mM NaF, 10mM sodium orthovanadate for PKC assays, pH 7.5). After vortexing, ice-cold Tris-EGTA buffer was added to cell lysates for a final concentration of 50 mM Tris-Base and 500 mM EGTA. Nuclear pellets were obtained by centrifugation at 1500 rpm for 5 minutes at 4°C and discarded, resulting in crude cytosolic fractions which were used for calbindin D-28K experiments. Cytosolic and membrane fractions were obtained by centrifugation of the post-nuclear fraction at 100,000 x g for one hour at 4°C

(Simboli-Campbell et.al, 1994). Membrane fractions were used for EGFR westerns and both cytosolic and membrane fractions were used for all PKC experiments. For VDR immunoblotting, proteins were precipitated from high-salt extracts with 1:2 (v/v) of a methanol:acetone (1:1) mixture and centrifuged (5 minutes/4°C/1500 rpm).

Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Mississauga, ON).

### 3.2 Cell Proliferation

Cell proliferation was assessed by cell number, $^{3}$H-thymidine (Amersham, Oakville, ON, Canada) incorporation into DNA, and flow cytometry.

Cells were grown to sub-confluence and changed to serum-free media. After cells were treated with test agents, cell number was determined by detaching cells with trypsin/EDTA (0.25%/1mM), resuspending in 2mL media, and counting on a hemacytometer. Results are expressed as the mean ± SEM of the cells/ml from four separate experiments.

For thymidine incorporation, cells were grown to sub-confluence in 35 mm dishes, fluid changed to serum-free media, treated, and incubated with $^{3}$H-thymidine (0.5 µCi/ml) for 24 hours prior to harvesting. DNA was solubilized with 0.5N PCA at 90°C for 20 minutes and radioactivity was counted in Beckman scintillation cocktail. Total DNA was quantitated fluorometrically using Hoescht dye 33258 (Sigma) as described in Downs and Wilfinger (1983). Results are expressed as cpm $^{3}$H-thymidine incorporated/mg DNA.

For flow cytometry, sub-confluent cells were changed to serum-free DMEM 24 hours prior to treatment. After the appropriate treatments, cells were washed with PBS,

trypsinized, pelleted and fixed with 95% (v/v) EtOH at 20°C for 30 minutes. Fixed cells were resuspended and stained with propidium iodide (Coulter Corporation, Hialeah, Florida), for 15 minutes at room temperature, and analyzed within 2 hours on a Coulter Epics XLv35195 (1.5/1.22) (Coulter Corporation, Miami Florida) flow cytometer. Cell cycle kinetics were analyzed using XL and Multicycle analysis software (Phoenix Flow Systems, San Diego, CA, USA).

### 3.3 Calbindin D-28K ELISA

Calbindin D-28K was quantitated by enzyme-linked immunosorbent assay based on the method of Miller and Norman (1983). Standard rat recombinant calbindin D-28K (Swant, Bellizona, Switzerland) and cytosolic fractions (50 µg) from MDBK cells were pipetted into wells pre-coated with 10 ng calbindin D-28K. After addition of monoclonal anti-calbindin D-28K (Sigma) diluted 1:80,000 in PBS/0.05% BSA (w/v)/0.05% Tween 20 (w/v), samples were incubated overnight at 4°C. Wells were then washed three times with PBS/BSA/Tween 20 and incubated with a 1:10,000 dilution of alkaline phosphatase donkey anti-mouse IgG antibody (Jackson Immunoresearch Laboratories, West Grove, PA) for 2 hours. Non-specific binding was determined by incubating cytosolic samples in parallel with PBS/skim milk/Tween 20 in the absence of primary antibody. After washing three times with PBS/BSA/Tween 20, p-nitrophenyl phosphate in diethanolamine buffer (1mg/mL) was added. Reactions were terminated after 30-40 minutes with 1N NaOH and absorbance of PNP was determined at 405 nm using a Bio-Tek EL-310 EIA microplate reader. Calbindin D-28K content was determined by comparison to a standard curve obtained with up to 100 ng of recombinant rat

calbindin D-28K. Results are expressed as μg cytosolic calbindin D-28K/mg protein (mean ± SEM) of four separate experiments.

### 3.4 Immunoblotting

Proteins (150mg per lane) from total homogenates, cytosolic, membrane and nuclear fractions were separated on 15%, 12%, or 7.5% SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. Membranes were stained with 0.5% Ponceau S in 3% TCA to monitor the effectiveness of transfer.

The non-specific binding sites were blocked in 0.5% PBS-skim milk for up to 2 hours. For detecting calbindin D-28K, nitrocellulose membranes were incubated for 1.5 h with monoclonal anti-calbindin D-28K antibody (Sigma) diluted 1:200 in blocking solution. Then the membranes were washed with 0.1% PBS-Tween 20 and incubated for 1 hour at room temperature with an alkaline phosphatase conjugated donkey anti-mouse secondary antibody (1:5000). Immunoreactive calbindin-D28K was detected using the Problot detection system (Promega, Madison, WI, USA).

The VDR was detected by incubating membranes for 1 hour with rat anti-VDR monoclonal antibody diluted 1:100 (Chemicon, Temeluca, CA) followed by a 45 minute incubation with a biotinylated donkey anti-rat secondary antibody (Jackson) diluted 1:5000, and a 15 minute incubation with streptavidin peroxidase diluted 1:1000.

PKC α was detected by incubating membranes for 1 hour with anti-PKCα rabbit anti-peptide polyclonal antibody (Life Technologies) diluted 1:200 in blocking solution for 1 hour, shaking at room temperature. This was followed by a 1 hour incubation with peroxidase

conjugated goat anti-rabbit secondary antibody diluted 1:1000 in blocking solution.

The EGF receptor (EGFR) was detected by incubating membranes with a mouse anti-human EGFR monoclonal antibody (Sigma) diluted 1:5000 in blocking solution, followed by a 1 hour incubation with peroxidase conjugated goat anti-mouse secondary antibody diluted 1:1000.

Immunoreactive VDR, PKCα, and EGFR were visualized using the enhanced chemiluminescence detection system (Amersham, Oakville, ON, Canada).

### 3.5 Assessment of Vitamin D Receptor by Ligand Binding

VDR levels were assayed in high-salt extracts of MDBK cells by $^3$H-1,25(OH)$_2$D$_3$ binding assay (Simboli-Campbell et al, 1992). Cell pellets were thawed and resuspended in TKED buffer (10 mM TRIS-HCl, 1mM EDTA, 5mM DTT, 10 mm sodium molybdate, 100 mM KCl and 0.2 mg/mL soybean trypsin inhibitor), sonicated, and then centrifuged at 4°C (105,000xg) for 1 hour to yield a high-salt extract containing the VDR and a nuclear-membrane pellet which was discarded. For experiments including cells treated with 1,25(OH)$_2$D$_3$, 180mg of high-salt extract was incubated with (0.53nM) $^3$H-1,25(OH)$_2$D$_3$ for 1 hour at 37°C to facilitate the exchange of $^3$H-1,25(OH)$_2$D$_3$ for 1,25(OH)$_2$D$_3$ from occupied VDR. For experiments assessing the effect of EGF, extracts were equalized for protein as described above and incubated with (0.53 nM) $^3$H-1,25(OH)$_2$D$_3$ for 24 hours at 4°C. In both cases, parallel tubes measuring non-specific binding contained both radioactive ligand and a 240-fold excess of radioinert ligand. In all experiments, bound and free hormone were separated by incubating samples with dextran coated charcoal for 30 minutes at 4°C and

centrifugation (15 minutes/3500xg/4°C). Bound radioactivity in the supernatant was counted with Beckman scintillation cocktail in a Beckman LS8000 scintillation counter. Data for specific binding were generated by subtracting non-specific binding from total $^3$H-1,25$(OH)_2D_3$ binding. For determination of the effect of EGF on the $N_{max}$ and $K_d$ of the VDR, nuclear extracts were incubated for 24 hours at 4°C with increasing concentrations of $^3$H-1,25$(OH)_2D_3$ (0.05-0.6 nM) alone or in the presence of a 240-fold molar excess of unlabelled 1,25$(OH)_2D_3$ and then charcoal treated as described above. Data are expressed as the mean ± SEM of three separate trials. The equilibrium binding data was analyzed by Scatchard plot as described in Simboli-Campbell et al (1992) and receptor number ($N_{max}$) and affinity ($K_d$) were determined by the method of least squares regression analysis.

### 3.6 Casein Kinase II *in vitro* Phosphorylation Assay

The ability of casein kinase II (CKII) to phosphorylate casein and calbindin D-28K was examined in vitro using a modified paper binding method (Glass et al, 1978, Goueli et al,1980). Assays were carried out using CKII (Promega) purified from rat liver, purified bovine casein (Sigma) and rat recombinant calbindin D-28K (Swant). All reactions were carried out in 25 mL of assay buffer ( 0.1 µCi/mL of [$\gamma$-$^{32}$] ATP (Amersham), 10 mM $MgCl_2$, 200 mM NaCl, pH 7.4), with either casein or calbindin D-28k as substrate. Reactions were inititated with 1 µL of kinase (10 units of enzyme activity) and allowed to proceed for 15 minutes at 37°C (Kuenzel et al, 1987). To determine the appropriate amount of substrate to be used, a dilution series was determined using increasing amounts of casein and calbindin D-28K (0-5 µg). In all cases, phosphorylation was stopped with 125 mL of cold 5% (v/v) TCA

and 25 mL of each reaction mixture was spotted onto 0.22 mm cellulose ester/acetate filters (Millipore, Mississauga, ON, Canada). Filters were air-dried and washed once with 30% (v/v) TCA for 30 minutes at 65°C, twice with 15% (v/v) TCA for 5 minutes each (65°C), and finally with 70% (v/v) EtOH. Filters were again air-dried and radioactivity was counted in the Beckman LS8000 liquid scintillation counter. Results are reported as the mean ± SEM of three separate experiments.

Phosphorylation of casein or calbindin D-28K by CKII was also assessed by autoradiography. Reaction conditions were as described above except phosphorylation was stopped with 5 mL of 10X SDS reducing buffer (Laemmli, 1970) and mixtures were separated on 15% SDS-PAGE. The gel was dried overnight, exposed to KODAK Scientific Imaging Film for 8 hours, and phosphorylated proteins were detected by autoradiography.

### 3.7 Assay of Protein Kinase C Activity

Protein kinase C (PKC) activity was measured in cytosolic and membrane fractions of MDBK cells using a commercially available enzyme assay system (Amersham). The system measures the PKC-catalyzed transfer of the γ-phosphate group of ATP to a PKC-specific peptide. In all cases, cytosolic and membrane fractions were equalized for protein (1μg/μL) and 25μL of protein was added to all reaction mixtures which contained 25μL each of calcium buffer (12mM calcium acetate in 50mM Tris/HCl), lipid buffer (8 mole% LαPhosphatidyl-L-serine and 24 mg/mL phorbol 12-myristate 13-acetate in 50mM Tris/HCl), peptide buffer (900mM peptide in 50mM Tris/HCl), and DTT buffer (30mM DTT in 50mM Tris/HCl). Appropriate control tubes substituting 50mM Tris/HCl (pH 7.5) for the peptide

substrate and lipid buffer, or 50mM Tris/HCl with 12mM EGTA (pH 7.5) for the calcium buffer were assayed in parallel to account for phosphorylation of endogenous substrates. All reactions were initiated with the addition of 25μL of Mg[$^{32}$P]ATP solution (0.1μCi of radioactivity per tube) and mixtures were incubated for 15 minutes at room temperature. Phosphorylation was stopped with the addition of 50mL of 5% Ponceau S solution and 25μL of each reaction mixture was spotted onto peptide binding paper. After washing the papers twice with 5% (v/v) acetic acid (65°C), bound radioactivity on the filters was counted in a Beckman LS8000 liquid scintillation counter. Results are reported as mean ± SEM of three independent trials.

### 3.8 Statistical Analysis

Data are expressed as mean ± SEM. Comparison between two treatment groups was by the unpaired t-test and differences between means were considered statistically significant if $p<0.05$ was obtained.

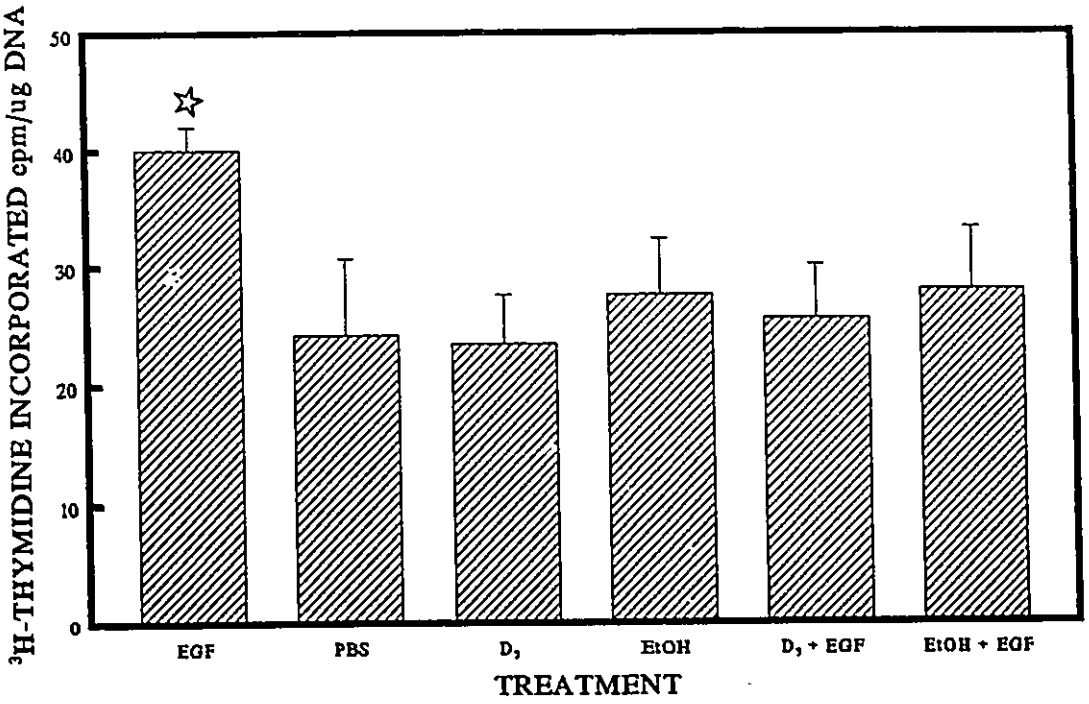
# 4. RESULTS

## 4.1 Cell Proliferation Assessment

To determine the effects of $1,25(OH)_2D_3$ and EGF on MDBK cell proliferation $^3$H-thymidine incorporation into DNA, flow cytometry, and cell number were measured. Thymidine incorporation (Figure 4.1 A) was 2-fold higher in cells treated with EGF (100 ng/mL) for 24 hours compared to PBS and $1,25(OH)_2D_3$ treated cultures. However, when cells were treated with both EGF and $1,25(OH)_2D_3$ thymidine incorporation was not significantly lower than cells treated with EGF alone and was not significantly different from that of ethanol, EGF treated cultures. Since DNA synthesis only measures cells actively undergoing cell division, cell cycle kinetics (Figure 4.1 B) were also analyzed. When serum-free sub-confluent cultures were treated with EGF for 24 hours there was a 1.1-fold decrease in the population of cells in $G_o/G_1$ and a 1.5-fold increase in the $G_2/M$ phase compared to PBS treated cells. Similarly, EGF treatment increased the population of cells in S phase (15.5 ± 4.5%) 3.5-fold compared to PBS (4.5 ± 1.0%). On the other hand, $1,25(OH)_2D_3$ (8.6 ± 3.1% in S phase) treatment did not significantly change the distribution of cell populations in cell cycle phases compared to ethanol (7.8 ± 3.1% in S phase). Similarly, exposing cells to EGF and $1,25(OH)_2D_3$ resulted in 12.3 ± 2.6% in S phase, 80.3 ± 2.7% in $G_o/G_1$, which was comparable to cells treated with ethanol and EGF (12.5±1.6% S phase, 78.7±1.4% $G_o/G_1$).

**Figure 4.1 Changes in MDBK cell proliferation measured by $^3$H-thymidine incorporation into DNA and cell cycle kinetics analysis.**

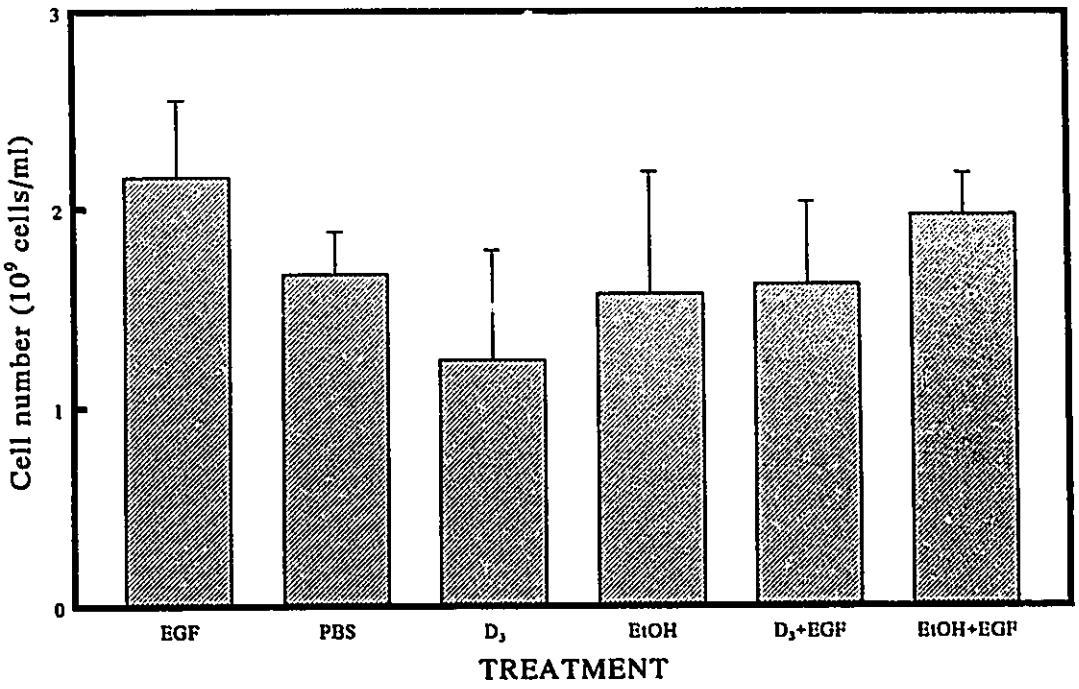
Cell proliferation was assessed by $^3$H-thymidine incorporation and flow cytometry as described in Methods 3.2. Sub-confluent cells were fluid changed to serum-free media for 24 hours prior to treatment with EGF (100 ng/ml), 1,25$(OH)_2D_3$ (100 nM), or appropriate controls for 24 hours. Some cells were pretreated with 1,25$(OH)_2D_3$ for 4 hours and were then given EGF for the remainder of 24 hours. (A) After 24 hours, cells were harvested and $^3$H-thymidine incorporated was measured, and in separate experiments (B) cells were harvested, trypsinized, stained with propidium iodide and cell cycle kinetics were assessed by flow cytometry. Results are expressed as the mean ± SEM of four separate trials done in duplicate, statistically significant results are indicated by * ($p<0.05$).




| Treatment | % of Cells in $G_0/G_1$ | % of Cells in S | % of Cells in $G_2/M$ |
|---|---|---|---|
| EGF | 77.5±7.0 | ☆ 15.5±4.5 | 7.3±0.5 |
| PBS | 90.5±1.9 | 4.5±1.0 | 4.8±0.9 |
| $1,25(OH)_2D_3$ | 85.1±3.9 | 8.6±3.1 | 6.3±0.7 |
| EtOH | 86.3±3.6 | 7.8±3.1 | 5.7±0.3 |
| $1,25(OH)_2D_3$+EGF | 80.3±2.7 | 12.3±2 6 | 7.3±0.2 |
| EtOH+EGF | 78.7±1.4 | 12.5±1.6 | 8.7±0.8 |

**Figure 4.2 Changes in cell number in response to EGF, and $1,25(OH)_2D_3$**

Sub-confluent cells were serum-deprived for 24 hours prior to treatment for 24 hours with EGF (100 ng/ml), and $1,25(OH)_2D_3$ (100nM), or appropriate controls as described in Methods 3.2. Following treatment, cells were harvested, trypsinized and counted as described in Methods 3.2. Results are reported as the mean ± SEM of four separate experiments in which each treatment was done in duplicate.


3
2
1
0
Cell number ($10^9$ cells/ml)
EGF
PBS
$D_3$
EtOH
$D_3$+EGF
EtOH+EGF
TREATMENT

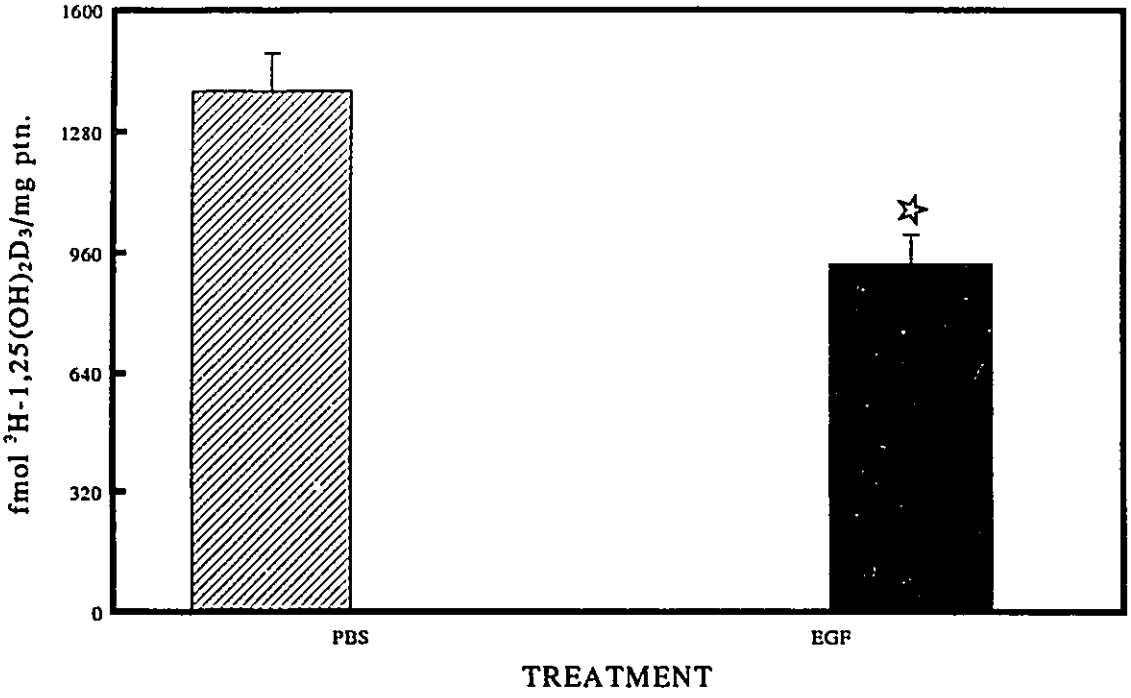
Cell number was measured to determine if the changes observed with DNA synthesis resulted in changes in total cell number. The data (Figure 4.2) show a 1.3-fold increase in cell number in dishes treated with EGF ($2.16x10^9 \pm 3.9x10^8$ cells/mL) as compared to PBS ($1.67x10^9 \pm 2.1x10^8$cells/mL) for 24 hours, or dishes treated with $1,25(OH)_2D_3$ ($1.24x10^9 \pm 5.5x10^8$ cells/mL). Treatment with both EGF and $1,25(OH)_2D_3$ ($1.62x10^9 \pm 4.1x10^8$ cells/mL) resulted in 1.3-fold lower cell number compared to EGF treated dishes; these numbers were comparable to results obtained when MDBK cells were treated with EtOH ($1.57x10^9 \pm 6.2x10^8$ cells/mL). Taken together these data demonstrate that EGF stimulates MDBK cell proliferation and DNA synthesis, however these mitogenic effects are not blunted by $1,25(OH)_2D_3$.

**4.2 Vitamin D Receptor Binding and Protein Levels**

Previous studies have shown that serum and growth factors such as EGF (200 ng/mL), and insulin stimulate VDR gene expression in 3T3 fibroblasts and in MCF-7 cells (Krishnan et al, 1991a). Initial studies indicated that 4 hour treatment with EGF resulted in a 33% down-regulation in $1,25(OH)_2D_3$ binding in MDBK cells (Figure 4.3A). These data were confirmed by western blotting with a rat anti-VDR monoclonal antibody (Figure 4.3 B) which showed a decrease in VDR in cells treated with EGF for 4 hours compared to PBS treated cells. Scatchard analysis of equilibrium binding data indicated that EGF treatment did not significantly alter receptor affinity ( $K_d$: PBS treatment = 56.2 pmoles, EGF treatment = 58.7 pmoles).

**Figure 4.3: Changes in VDR levels and protein expression following 4 hour EGF treatment in MDBK cells.**

Panel A (top) Near-confluent, serum-free MDBK cells were treated with EGF (100 ng/mL) or PBS for 4 hours. High-salt extracts, prepared from cell lysates as described in Methods 3.5, were incubated with 0.53 nM $^3$H-1,25$(OH)_2D_3$ in the presence or absence of 240-fold molar excess of non-radioactive 1,25$(OH)_2D_3$ overnight at 4°C. Bound and free hormone were separated and radioactivity was measured as described in Methods. Results are expressed as the mean ± SEM of data pooled from three independent experiments in which binding for each treatment was assessed in triplicate. Data were statistically significant at $p<0.05$. In panel B, (bottom) 100 µg of cytosolic proteins from cells treated with EGF or PBS (as indicated) were assessed for VDR protein. Immunoreactive VDR was detected by immunoblotting at approximately 60 kDa, and non-specific binding was not present in parallel blots.


1600
1280
960
640
320
0
fmol $^3$H-1,25(OH)$_2$D$_3$/mg ptn.
PBS
EGF
TREATMENT

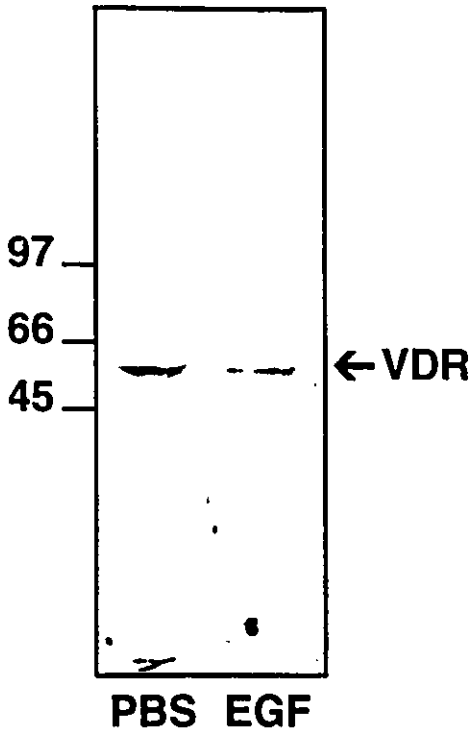
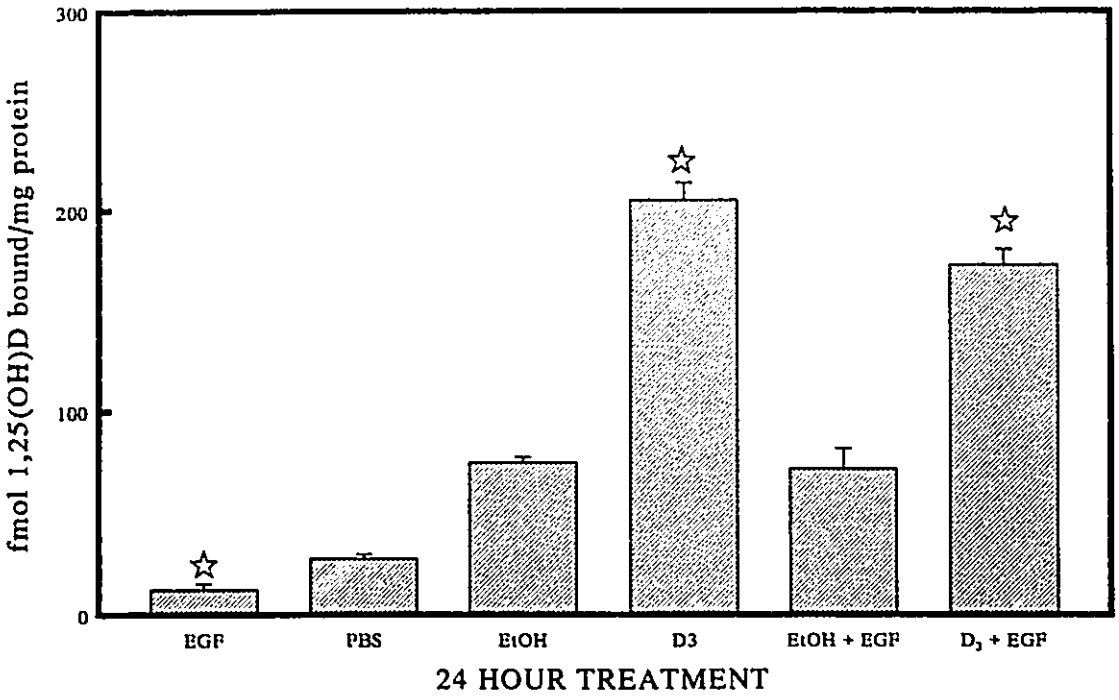
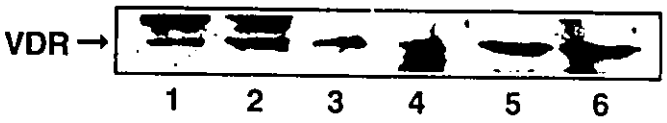

97
66
45
←VDR
PBS EGF

**Figure 4.4: Changes in VDR binding and expression in response to 24 hour treatment with $1,25(OH)_2D_3$ or EGF in MDBK cells.**

In A (top), sub-confluent serum-deprived MDBK cells were treated for 24 hours with EGF (100 ng/mL), PBS (vehicle control), $1,25(OH)_2D_3$ (100 nM), or EtOH. Some dishes were pretreated with $1,25(OH)_2D_3$ or EtOH for 4 hours prior to EGF treatment (described in Methods). High-salt extracts prepared from cells (Methods 3.5) were incubated with 0.53 nM $1,25(OH)_2D_3$ in the presence or absence of 240-fold molar excess of unlabelled hormone and bound hormone was counted. The results reported are the mean ± SEM of data pooled from three independent experiments in which binding for each treatment was measured in triplicate. Data were statistically significant at $p<0.05$. In panel B (bottom), 150 µg of protein precipitated from high-salt extracts (Methods 3.1) were separated by 12% SDS-PAGE. Immunoreactive VDR was detected in cells treated with EGF (lane 1), PBS (lane 2), EtOH lane 3), $1,25(OH)_2D_3$ (lane 4), EtOH and EGF (lane 5), and $1,25(OH)_2D_3$ and EGF (lane 6) at approximately 60 kDa. No non-specific bands were detected in parallel blots.


300
200
100
0
fmol 1,25(OH)D bound/mg protein
EGF
PBS
EtOH
D3
EtOH + EGF
D3 + EGF
24 HOUR TREATMENT


VDR
1
2
3
4
5
6

The effects of long-term exposure to EGF or $1,25(OH)_2D_3$ on VDR binding were also assessed (Figure 4.4A). After 24 hours, VDR levels significantly decreased with EGF (12.15 ± 3.1 fmol/mg protein) compared to PBS (27.73 ± 2.25 fmol/mg protein) treatment. Pretreatment with $1,25(OH)_2D_3$ for 4 hours prior to the addition of EGF restored $^3$H-$1,25(OH)_2D_3$ binding to levels observed with $1,25(OH)_2D_3$ treatment alone. Interestingly, binding levels in cells pretreated with EtOH for 4 hours prior to the addition of EGF are not different from levels in cells exposed to EtOH alone for 24 hours. Changes in immunoreactive VDR were also assessed by immunoblotting (Figure 4.4B). EGF treatment (lane 1) for 24 hours down-regulated VDR compared to PBS (lane 2), whereas $1,25(OH)_2D_3$ treatment (lane 4) moderately up-regulated VDR compared to EtOH (lane 3) treatment. But, EGF in the presence of $1,25(OH)_2D_3$ VDR (lane 6) is not significantly different compared to EtOH and EGF treatment (lane 5) or EGF treatment alone (lane 1). Thus, EtOH appears to have an effect on VDR binding while pretreatment with $1,25(OH)_2D_3$ blunts the effect of EGF on VDR down-regulation, even though the proliferative action of EGF is unaffected by $1,25(OH)_2D_3$.

### 4.3 EGF effects on calbindin D-28K protein expression and concentration

The changes in $1,25(OH)_2D_3$ binding following acute and long-term EGF exposure led to the examination of EGF effects on the expression of calbindin D-28K. Cells were stimulated with EGF (100 ng/mL) for up to 24 hours and calbindin D-28K was assessed by immunoblotting (Figure 4.5).

**Figure 4.5: Changes in calbindin D-28K protein expression following treatment of MDBK cells with EGF or $1,25(OH)_2D_3$ for 24 hours.**

Sub-confluent, serum-free MDBK cells were treated with $1,25(OH)_2D_3$ (lane 1), $1,25(OH)_2D_3$ and EGF (lane 2), EGF (lane 3) or PBS (lane 4) for 24 hours as described in Methods 3.1. Cytosolic proteins (150 mg) obtained from cell lysates (Methods 3.1) were separated by 15% SDS-PAGE. Immunoreactive calbindin D-28K was detected, as described in Methods 3.4, as a 28kDa band by immunoblotting. Data represent three separate trials.

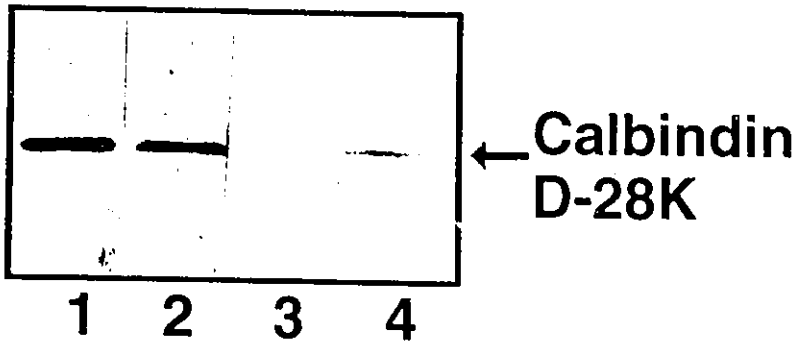
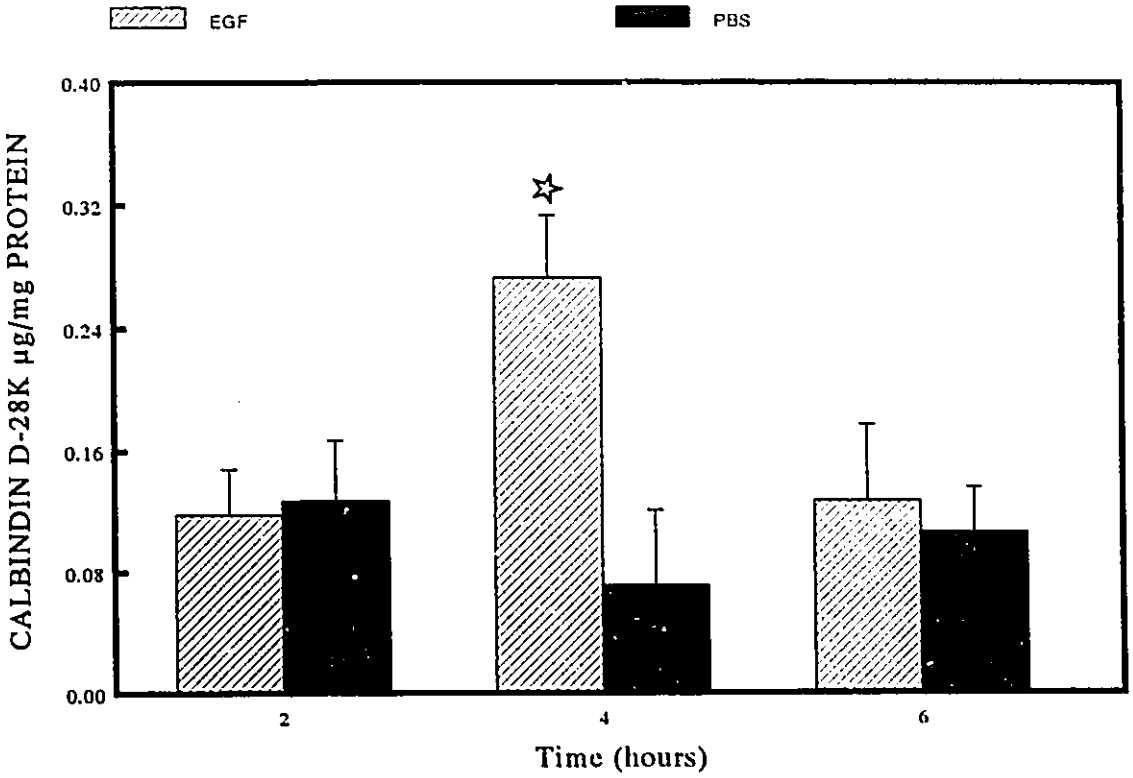
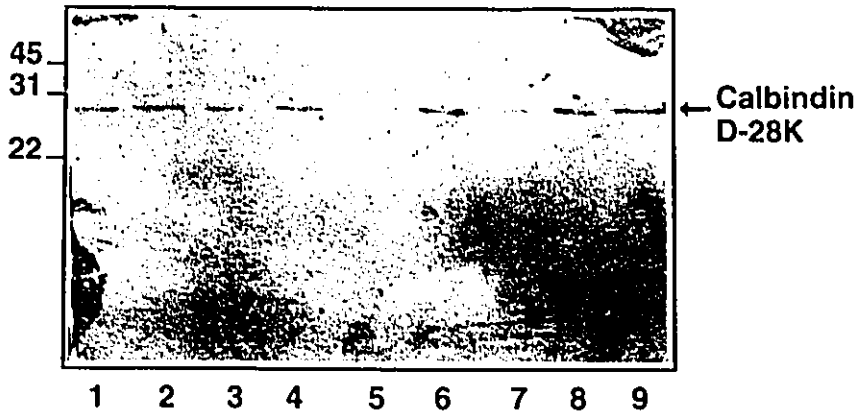

Calbindin
D-28K
1
2
3
4

**Figure 4.6: Time course of the effect of EGF on calbindin D-28K protein expression and protein concentrations in MDBK cells.**

Sub-confluent, serum-free MDBK cells were treated with EGF (100 ng/ml) or PBS for up to 6 hours. (A, top) 50 µg of cytosolic proteins were assayed for calbindin D-28K levels by ELISA as described in Methods 3.3. The blot represents three independent experiments. In panel B (bottom), Following treatment, 150 µg of cytosolic proteins were separated by 15% SDS-PAGE. Lanes 1,3,5,7,9 represent control treated cells for 0,1,2,4,and 6 hours, while lanes 2,4,6,8 represent cells exposed to EGF for 1,2,4 and 6 hours. Calbindin D-28K was detected as a 28kDa immunoreactive band by immunoblotting as described in Methods 3.4. Results are expressed as the mean ± SEM of data pooled from four separate experiments, in which calbindin D-28K levels were measured in triplicate for each treatment. Statistically significant results are indicated by * if $p<0.05$.


EGF
PBS
CALBINDIN D-28K µg/mg PROTEIN
0.40
0.32
0.24
0.16
0.08
0.00
2
4
6
Time (hours)


45
31
22
Calbindin
D-28K
1 2 3 4 5 6 7 8 9

After 24 hours with EGF (lane 3), calbindin D-28K was down-regulated compared to PBS (lane 4) treated cells. This down-regulation was prevented by pretreatment with $1,25(OH)_2D_3$ (lane 2), comparable to treatment with $1,25(OH)_2D_3$ alone (lane 1), and therefore correlated with the increase in VDR observed under the same conditions.

The temporal changes in calbindin D-28K expression in response to EGF treatment for up to 6 hours showed no increases in calbindin D-28K expression after 1 and 2 hour treatment (Figure 4.6A lanes 2 and 4), compared to PBS treatment (lanes 3 and 5). After 4 hours however, calbindin D-28K expression increased in EGF treated cells (Figure 4.6 A, lane 6), whereas calbindin D-28K decreased with time in PBS treated cells (Figure 4.6A, lane 7). These effects were transient and no differences were observed between cells treated for 6 hours with PBS or EGF. These data were confirmed when calbindin D-28K concentrations in cytosolic fraction of MDBK cells were quantitated by ELISA (Figure 4.6B). Calbindin D-28K concentration was 3-fold higher in cells exposed to EGF for 4 hours ($0.273 \pm 0.04$ µg/mg protein) compared to PBS ($0.071 \pm 0.05$ µg/mg protein). The acute up-regulation in calbindin D-28K observed in both experiments occurred despite the down-regulation of VDR observed under the same conditions, and therefore may represent $1,25(OH)_2D_3$-independent effects of EGF on calbindin D-28K. We have observed similar effects of TPA, a PKC activator, on calbindin D-28K expression in MDBK cells (Gagnon et al, 1994). This similarity suggests effects of growth factor signalling pathways on calbindin D-28K which are independent of $1,25(OH)_2D_3$.

## 4.4 Involvement of Casein kinase II in regulating calbindin D-28K

Based on the observations summarized in Section 4.2 of the Results and Section 1.4ii of the Introduction, we hypothesized that the temporal increase in calbindin D-28K in response to EGF treatment may be due to post-transcriptional phosphorylation of the protein via EGF-induced signalling pathways. The involvement of CKII in this regulation was investigated since calbindin D-28K contains five CKII consensus phosphorylation sites in its amino acid sequence. *In vitro* CKII phosphorylation assays using purified CKII (Figure 4.7) demonstrated that calbindin D-28K is not an effective substrate for CKII (33.7±16.1 nmol $^{32}$P/µg protein/minute), when compared to casein (1168 ± 90.7 nmol $^{32}$P/µg protein/minute). The level of calbindin D-28K phosphorylation was not significantly higher than that obtained with CKII assay buffer alone (18.9 ± 2.02 nmol $^{32}$P/µg protein/minute) or with CKII and assay buffer in the absence of any substrate (20.0 ± 1.75 nmol $^{32}$P/µg protein/minute). In other experiments, increasing calbindin D-28K concentration in the reaction mixture did not enhance phosphorylation of calbindin D-28K by CKII. These results suggest that CKII does not directly phosphorylate calbindin D-28K *in vitro*, and may not be involved in the regulation of this protein *in vivo*. We therefore decided not to continue with studies to evaluate whether EGF activates CKII in MDBK cells.

**Figure 4.7: *In vitro* phosphorylation of casein and calbindin D-28K by CKII.**

Phosphorylation of casein (1 µg) or calbindin D-28K (1 µg) was assessed in vitro with purified CKII as described in Methods. Duplicate assay tubes containing mixtures of buffer, CKII, casein (CSN), or calbindin D-28K (CBP) as indicated were incubated with $^{32}P\gamma$-ATP for 15 minutes. Phosphorylation incorporated into casein or calbindin D-28K was measured by a modified cellulose paper binding method. Results are reported as $^{32}P\gamma$-ATP incorporated/µg protein/minute and are the mean ± SEM of data pooled from three separate trials in which activity was measured in duplicate for each assay condition. Statistical significance, compared to buffer and substrate alone, is indicated by * if $p<0.05$.

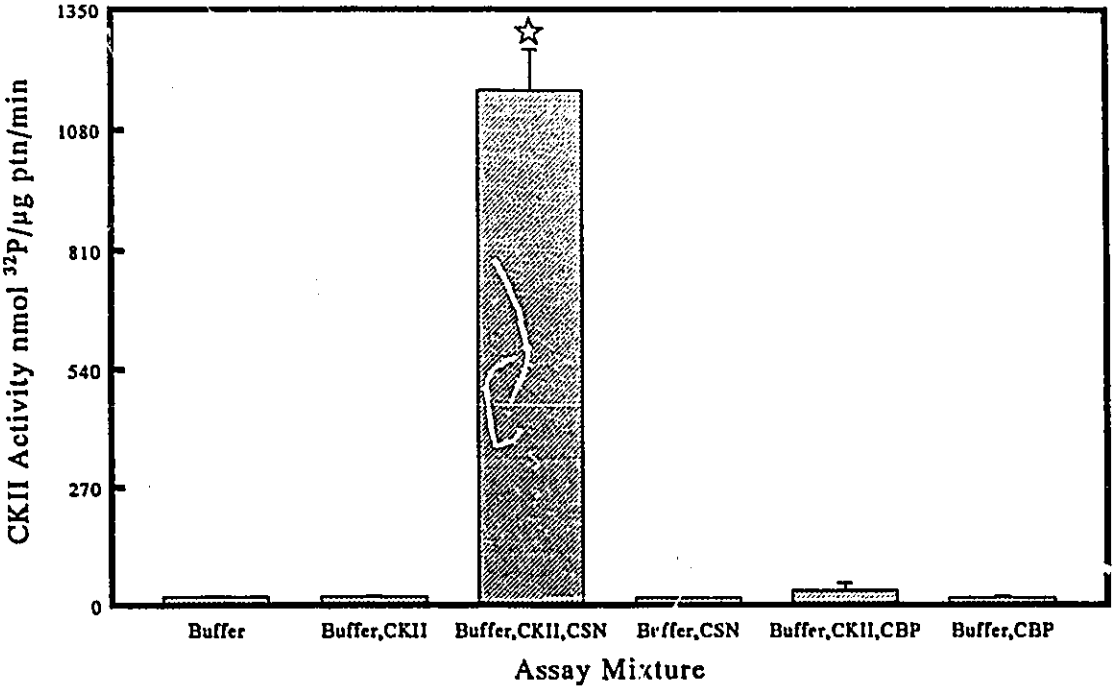
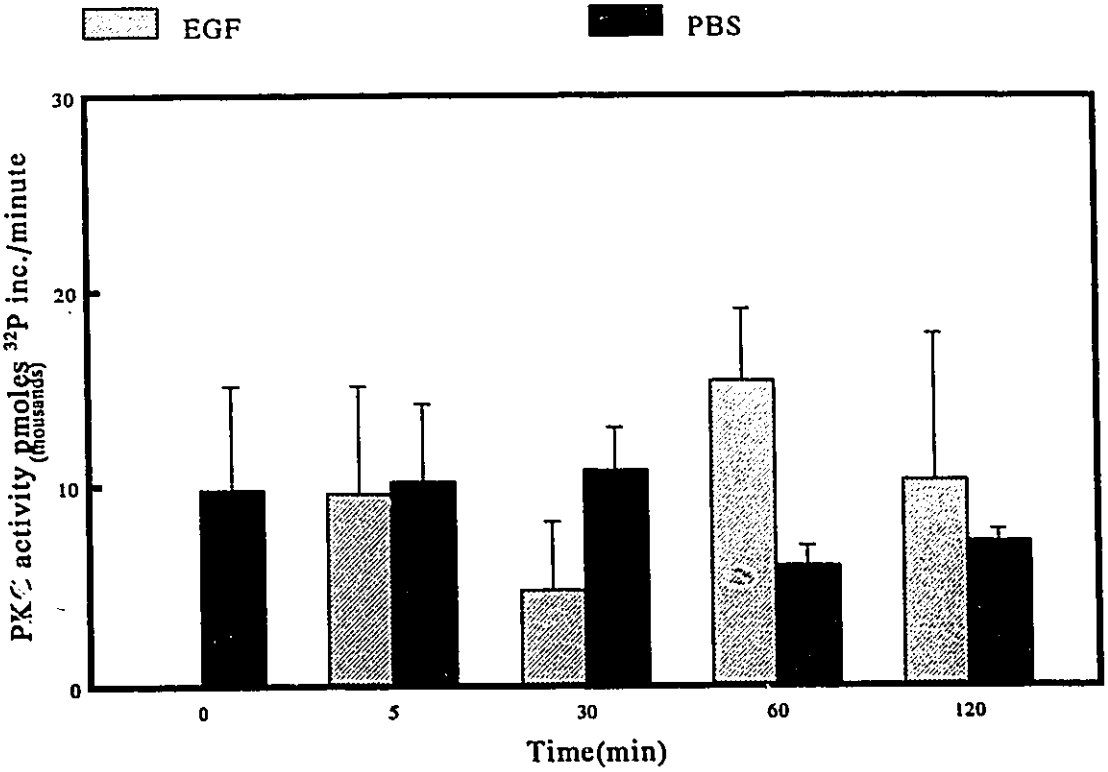

1350
1080
810
540
270
0
CKII Activity nmol $^{32}$P/µg ptn/min
Buffer
Buffer,CKII
Buffer,CKII,CSN
Buffer,CSN
Buffer,CKII,CBP
Buffer,CBP
Assay Mixture

**Figure 4.8: Time course of changes in PKC activity and protein levels in cytosolic fractions from MDBK cells following acute EGF exposure.**

Changes in PKC activity and protein expression was assessed in cytosolic fractions prepared, as described in Methods 3.1, from confluent MDBK cells treated with EGF (100 ng/mL) or PBS (vehicle control), for up to 2 hours. Panel A (top) shows changes in cytosolic PKC activity against a PKC-specific phosphorylatable peptide as discussed in Methods 3.7. Phosphorylated peptide was separated from the mixture by phosphocellulose paper binding and radioactivity was counted. The results are reported as pmoles $^{32}P$ incorporated into the peptide/minute and are the mean ± SEM of data pooled from three independent trials. In panel B (bottom) 150 µg of proteins from cytosolic fractions were separated by 15% SDS-PAGE. Immunoreactive PKCα was detected in cells treated as indicated with EGF or PBS for 5, 30, 60, and 120 minutes. Non-specific binding was not detected in parallel blots and the blot is representative of three independent trials.


EGF
PBS
30
20
10
0
PKC activity pmoles 32P inc./minute (thousands)
0
5
30
60
120
Time(min)

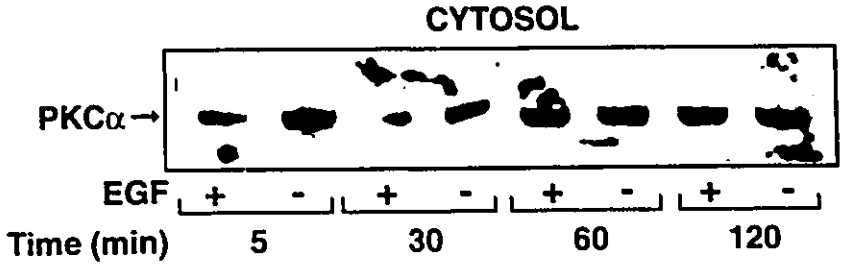
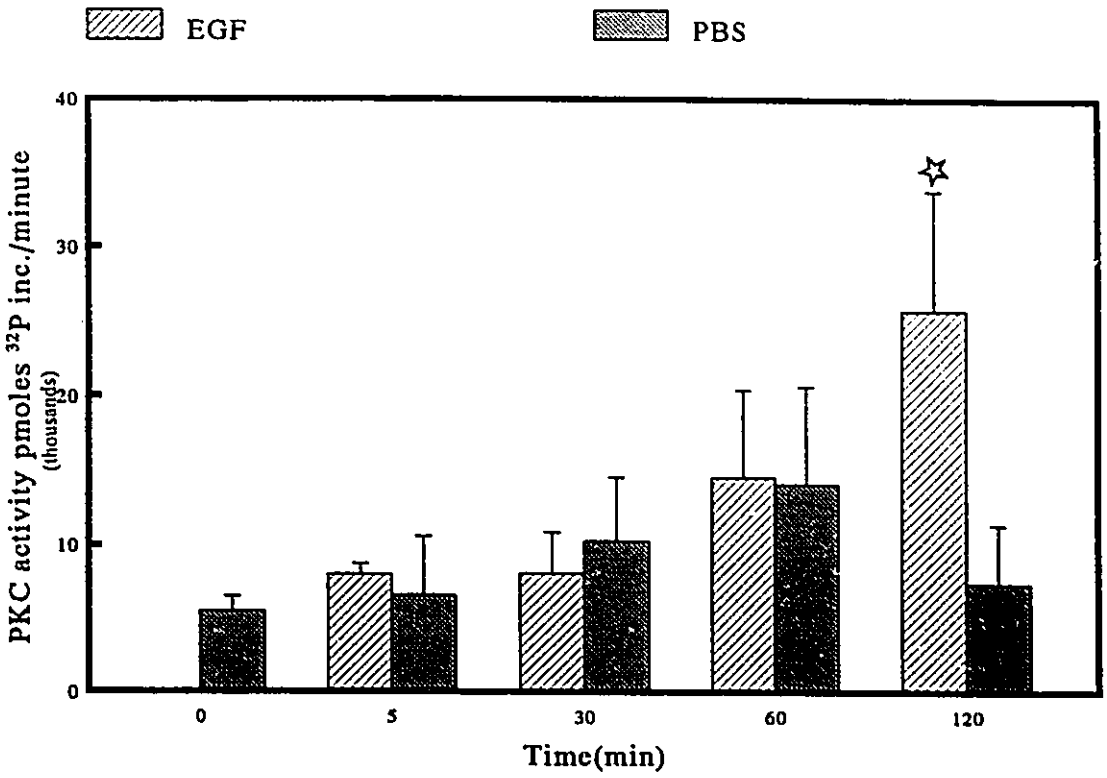

CYTOSOL
PKCα
EGF
+
-
+
-
+
-
+
-
Time (min)
5
30
60
120

**Figure 4.9: Time course of the changes in PKC activity and protein levels in membrane fractions of MDBK cells treated with EGF for up to 2 hours.**

Changes in PKC activity and protein expression was assessed in membrane fractions, prepared as described in Methods 3.1, from confluent MDBK cells treated with EGF (100 ng/mL) or PBS (vehicle control), for up to 2 hours. Panel A (top) shows changes in membrane PKC activity against a PKC-specific phosphorylatable peptide as discussed in Methods 3.7. Phosphorylation of a PKC phosphorylatable peptide bound to phosphocellulose paper was counted. The results are reported as pmoles $^{32}P$ incorporated into the peptide/minute and are the mean ± SEM of data pooled from three independent trials. Statistically significant results are indicated by *, if $p<0.05$. In panel B (bottom) 150 µg of proteins from membrane fractions were separated by 15% SDS-PAGE. Immunoreactive PKCα was detected in cells treated as indicated with EGF for 5, 30, 60, and 120 minutes. Non-specific binding was not detected in parallel blots. Data are representative of three independent tirals.


EGF
PBS
PKC activity pmoles $^{32}$P inc./minute
(thousands)
40
30
20
10
0
0
5
30
60
120
Time(min)

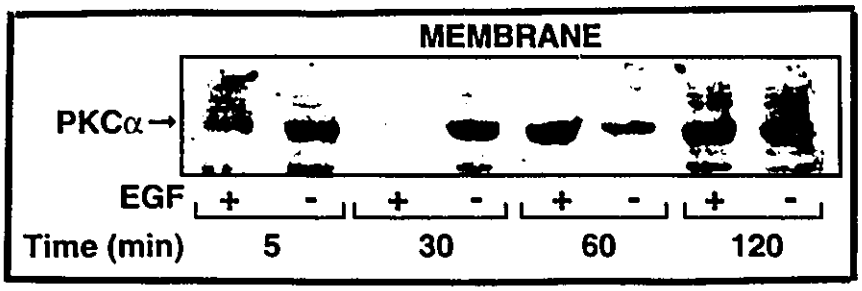
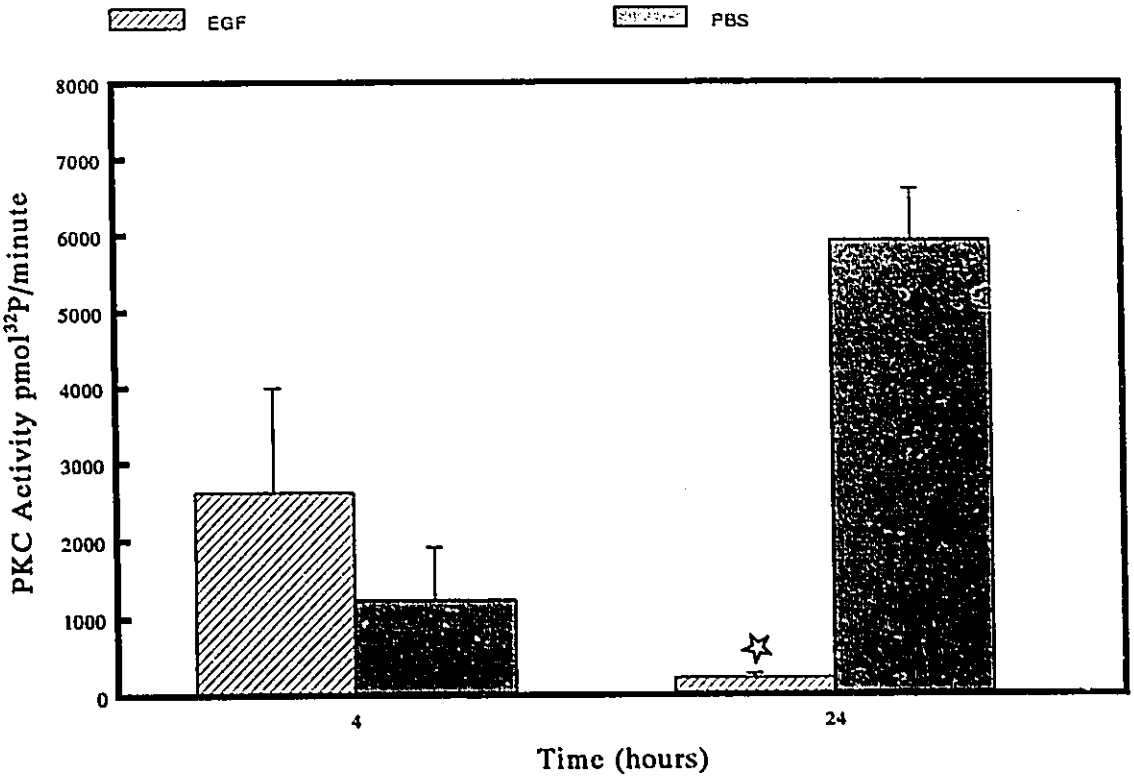

MEMBRANE
PKCα→
EGF
+
-
+
-
+
-
+
-
Time (min)
5
30
60
120

**Figure 4.10: Changes in PKC activity and protein expression in cytosolic fractions of MDBK cells treated with EGF for 4 hours and 24 hours.**

Cytosolic fractions prepared from near-confluent MDBK cells (Methods 3.1) treated with EGF (100 ng/mL) or PBS (vehicle control), for up to 24 hours were assayed for PKC activity and PKC protein levels. Panel A (top) shows changes in cytosolic PKC activity against a PKC-specific phosphorylatable peptide as discussed in Methods. The results are reported as pmoles $^{32}P$ incorporated into the peptide/minute and are the mean ± SEM of data collected from three independent trials. Statistically significant results are indicated by *, if $p<0.05$. In panel B bottom 150 µg of proteins from cytosolic fractions were separated by 15% SDS-PAGE. Immunoreactive PKCα was detected in cells treated with EGF for 4 and 24 hours (lanes 4, and 2 respectively) or PBS (lanes 3, and 1 respectively). No non-specific binding was detected in parallel blots. Data represent three separate experiments.


EGF
PBS
8000
7000
6000
5000
4000
3000
2000
1000
0
PKC Activity pmol $^{32}$P/minute
4
24
Time (hours)

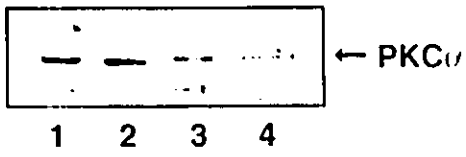
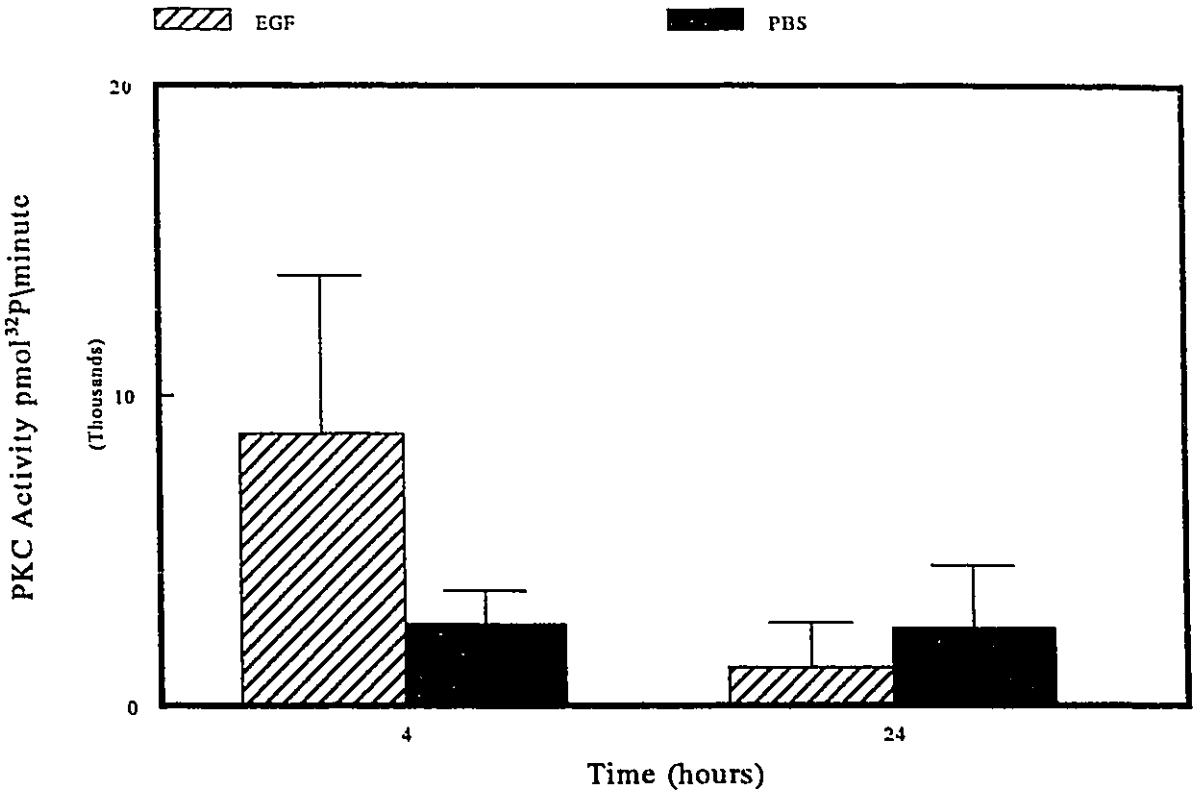
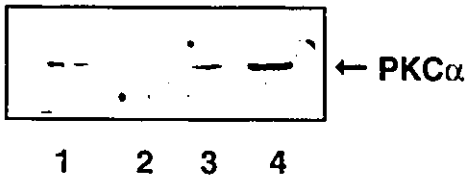

← PKCι
1 2 3 4

**Figure 4.11: Changes in PKC activity and protein levels in membrane fractions of MDBK cells treated with EGF for 4 hours and 24 hours.**

Membrane fractions, prepared according to Methods 3.1, from near-confluent MDBK cells treated with EGF (100 ng/mL) or PBS (vehicle control), for up to 24 hours were assayed for PKC activity and PKC protein levels. Panel A (top) shows changes in membrane PKC activity against a PKC-specific peptide. The results are reported as pmoles $^{32}P$ incorporated into the peptide/minute and are the mean ± SEM of data pooled from three independent trials. Statistically significant results are indicated by *, if $p<0.05$. In panel B (bottom) 150 µg of proteins from membrane fractions were separated by 15% SDS-PAGE. Immunoreactive PKCα was detected in cells treated with EGF for 4 and 24 hours (lanes 4, and 2 respectively) or PBS (lanes 3, and 1 respectively). Non-specific binding was not detected in parallel blots. Data are representative of three separate experiments.


EGF
PBS
20
10
0
(Thousands)
PKC Activity pmol $^{32}$P\minute
4
24
Time (hours)


← PKCα
1
2
3
4

**4.5 Involvement of PKC in the EGF-modulation of calbindin D-28K**

Since calbindin D-28K contains PKC consensus sites at Thr106 and Thr 133 and is phosphorylated by PKC *in vitro* the next step was to determine if PKC may be involved in the effects of EGF on calbindin D-28K in MDBK cells. We measured the time course of changes in PKC activity, in the presence or absence of substrate, and with or without calcium or phospholipids, in soluble and particulate fractions of MDBK cells treated with EGF or PBS for up to 24 hours.

When cells were stimulated with EGF, soluble PKC activity increased transiently after 1 hour and was subsequently down-regulated by 2 hours (Figure 4.8A). Membrane PKC activity (Figure 4.9A) was up-regulated almost 2.5-fold after two hours of EGF treatment (Figure 4.11A). Following 4 hour EGF treatment, membrane PKC activity (Figure 4.11A,) remained 2.5-fold higher than that of control. Long-term (24 hour) EGF treatment down-regulated cytosolic activity 25-fold (Figure 4.10A) and membrane activity 2-fold (Figure 4.11A) compared to PBS treatment. We assessed the expression of the calcium dependent PKC isozyme, PKCα, in cytosolic and membrane fractions of MDBK cell lysates following acute (2 hours) and long-term (4 hours and 24 hours) EGF treatment. Immunoblotting with an anti-PKCα antibody showed no significant changes in the expression of soluble PKCα with EGF treatment, compared to PBS treatment, for up to 2 hours (Figure 4.8B). On the other hand, 4 hour exposure to EGF translocated PKCα from the cytosol (Figure 4.10B, lane 4)) to the membrane (Figure 4.11, lane 4) in comparison to PBS treatment (Figure 4.10B, cytosol=lane 3; Figure 4.11B, membrane=lane 3) which showed little effect on the subcellular location of PKCα in MDBK cells. By 24 hours, cells treated with EGF showed translocation

of PKCα from the particulate fraction (Figure 4.11B, lane 2) back to the soluble fraction (Figure 4.10, lane 2).

## 5. DISCUSSION

$1,25(OH)_2D_3$ regulates calcium homeostasis and gene transcription in target tissues including the kidney (Whitfield et al, 1995, Stumpf et al, 1979). Further to its role as a calcium regulator, studies have demonstrated a role for $1,25(OH)_2D_3$ in the inhibition of cell proliferation and the induction of differentiation in fibroblasts, normal human keratinocytes, human osteosarcoma cells and other cell types (Brackman et al, 1992, Sebag et al, 1994, Chen et al, 1995, Bikle, 1995, and Franceschi et al, 1985). EGF is a known mitogen and is essential in culture medium for the progression of many cell types from $G_1$ to the S phase of the cell cycle (Reddy, 1994, Cross et al, 1991). In these studies, we investigated the interaction between the steroid hormone and growth factor activated signalling pathways.

In MDBK cells, treatment with EGF for 24 hours induced a significant increase in the percentage of cells in the S phase and a corresponding decrease in the $G_o/G_1$ population. These results are consistent with parallel experiments indicating a marked increase in $^3$H-thymidine incorporation and cell number following EGF treatment for the same time. $1,25(OH)_2D_3$ treatment alone did not change MDBK cell cycle kinetics, $^3$H-thymidine incorporation or cell number. Further, pretreatment with $1,25(OH)_2D_3$ prior to the addition of EGF did not blunt the mitogenic effects of this growth factor. These data are the first to suggest that kidney distal tubule cells may be resistant to the antiproliferative effects of $1,25(OH)_2D_3$, despite the presence of functional VDR. However, further studies using higher concentrations of $1,25(OH)_2D_3$ are necessary to address this issue. In addition, these observations are in contrast to data derived from a variety of normal and transformed cells,

which demonstrate that $1,25(OH)_2D_3$ inhibits proliferation and induces differentiation (Brackman et al, 1992, Bikle et al, 1995, Franceschi et al, 1985). As well, our data are contradictory to studies in which exposing keratinocytes to $1,25(OH)_2D_3$ and EGF simultaneously inhibited cell proliferation compared to EGF treatment alone (Sebag et al, 1994).

It is not clear why $1,25(OH)_2D_3$ elicits antiproliferative actions in some cells but not in others. MDBK cells express the vitamin D receptor and display induction of VDR proteins following $1,25(OH)_2D_3$ treatment under the conditions used in the present studies (Gagnon et al, 1994). Other studies in our lab demonstrated that IEC-6 (rat intestine) cell proliferation was inhibited by concentrations of $1,25(OH)_2D_3$ higher than 100nM. It may be that higher doses of $1,25(OH)_2D_3$ are required to blunt the proliferative effects of EGF in MDBK cells. If so, this would suggest different profiles for mediating calcemic effects, such as increasing calbindin D-28K expression, as opposed to antiproliferative effects. In breast cancer cells, it has been demonstrated that while the presence of a functional VDR may be necessary for the antiproliferative effects of $1,25(OH)_2D_3$, its activation is not sufficient for triggering these effects (Buras et al, 1994). Since different mitogens elicit their effects through distinct signalling pathways, $1,25(OH)_2D_3$ may not "cross-talk" to inhibit proliferation depending on which signals initiate proliferation in distinct cell types. Evidence suggests that antiproliferative effects of $1,25(OH)_2D_3$ in sensitive cells results from alteration in the expression of the components of the AP-1 transcription factors which are involved in the control of cell growth and differentiation. These genes, known as early response genes, are also regulated by growth factors such as EGF. The changes in early response genes in

response to EGF and $1,25(OH)_2D_3$ need to be examined further to elucidate the interaction of these two agents in the control of cell proliferation and differentiation in MDBK cells and other cell types. Furthermore, since EGF has also been shown to increase intracellular calcium levels, the role of calcium in mediating the effects of EGF and $1,25(OH)_2D_3$ in controlling cell cycle progression needs to be elucidated.

Since the genomic actions of $1,25(OH)_2D_3$ are mediated through the VDR, we assessed the effect of EGF on the VDR in MDBK cells. Acute (4h) EGF treatment decreased VDR binding by 50%. Scatchard analysis indicated that the binding affinity of the VDR was not decreased by EGF. The observed decrease may be due to the instability of the unoccupied receptor, or due to active down-regulation of VDR mRNA by EGF signalling. After 24 hours of EGF exposure, when cells are rapidly proliferating, no further decrease in VDR binding was observed. Although several groups have reported correlation between VDR and the rate of cellular proliferation (Chen et al, 1981, Krishnan et al, 1991), our data are consistent with other studies reporting reduced VDR in cells in the logarithmic phase of proliferation (Shabahang et al, 1993). These contradictory results may reflect the different stimuli used to induce proliferation or differentiation in different cell (Krishnan et al, 1991, Chen et al, 1981).

Pretreating cultures with $1,25(OH)_2D_3$ prior to EGF treatment prevented a decrease in VDR binding. VDR levels were restored to the levels previously observed with $1,25(OH)_2D_3$ treatment alone, supporting the theory that VDR is up-regulated by its own ligand. Following treatment with $1,25(OH)_2D_3$ most of the receptors are in the occupied state. $1,25(OH)_2D_3$ elevated receptor mRNA and increased total receptor levels in vivo (Pan

et al, 1987, Strom et al, 1989) and in vitro (Santiso-Mere et al, 1993, Wiese et al, 1992) to maintain constant levels of the unoccupied receptor. These observations are consistent with Wiese et al (1992) who also observed that VDR half-life was 4 hours in the absence of ligand and 8 hours in the presence of ligand. Our data demonstrate that the VDR is regulated by agents other than $1,25(OH)_2D_3$. These data support studies by several other groups demonstrating the regulation of VDR by glucocorticoids, estrogen, serum, growth factors, non-calcemic vitamin D analogues, and physiological stress (Duncan et al, 1984, Duncan et al, 1991, Krishnan et al, 1991, van Leeuwen et al, 1992, Costa et al, 1985, Colston et al, 1988).

Several groups have investigated the involvement of specific signalling pathways in the modulation of VDR. In NIH 3T3 fibroblasts and in osteoblast-like cells, PKC activation by tumor promoting phorbol esters decreased VDR levels by reducing steady state VDR mRNA levels (Krishnan and Feldman, 1991, van Leeuwen et al, 1992). In addition, down-regulation of PKC prevented the decrease in VDR protein and mRNA expression, further implicating PKC in the regulation of the VDR. Phosphorylation of the steroid hormone receptors has been well documented (Beato et al, 1988). Phosphorylation of the hVDR on serine 51, between the two zinc-fingers, by PKCβ has been demonstrated in vitro and in vivo (Beato et al, 1988, Hsieh et al, 1991). This phosphorylation is believed to decrease the transactivating VDRE- binding capacity of the receptor (Hsieh et al, 1991). Additional phosphorylation events have been shown at serine 205 and 208 of the hVDR. In vitro phosphorylation of serine 208 on hVDR has been demonstrated using purified bovine CKII in the absence of $1,25(OH)_2D_3$ (Jurutka et al, 1993). Jurutka et al (1993) also discuss the

phosphorylation of the hVDR by cell cycle-dependent kinases such as $P34^{cdc2}$ or mitogen activated protein kinases, implying cell cycle-dependent regulation of the hVDR. Phosphorylation of the VDR by various kinases implies that it is post-transcriptionally regulated by various stimuli. Since these kinases are activated primarily by growth factors, our results suggest a link between mitogenic signalling pathways and the functional consequences of steroid hormone receptor phosphorylation. The regulation of VDR by mitogens appears to be complex and dependent on several factors such as the rate of cell proliferation, and the activation of various kinases. Further investigations to assess the role of PKC in the regulation of VDR phosphorylation, ligand-induced stabilization, and growth factor stimulated mitogenesis are required.

Despite reports of the effects of mitogens on VDR expression, our studies are the first to assess the effects of these agents on the vitamin D-dependent protein calbindin D-28K. Since VDR was modulated by serum and growth factors in MDBK cells, we hypothesized that $1,25(OH)_2D_3$-dependent genes downstream of the VDR would also be regulated by these agents. We therefore assessed the concordance between the expression of VDR and calbindin D-28K in MDBK cells. Exposing MDBK cells to EGF for 24 hours significantly down-regulated calbindin D-28K levels as expected due to the decrease in VDR. This down-regulation was prevented by pretreatment with $1,25(OH)_2D_3$. The attenuation of calbindin D-28K down-regulation was again not surprising since it correlated to VDR binding ability under these conditions. Theofan et al (1986) have shown that the expression of calbindin D-28K mRNA is tightly related to the occupancy of the VDR by its ligand. These data, thus, support the thesis that $1,25(OH)_2D_3$ regulates target gene expression through the direct

interaction of the hormone-ligand complex with a vitamin D response element (VDRE). Cloning and characterization of bovine calbindin D-28K is essential to determine whether the promoter contains a hormone-responsive VDRE.

Surprisingly, we observed an up-regulation of calbindin D-28K expression levels after short-term (4h) treatment with EGF in the absence of $1,25(OH)_2D_3$. The increase in calbindin D-28K occurred despite a significant decrease in VDR binding observed under the same conditions, suggesting an alternate growth factor-mediated up-regulation of the protein. Several groups have reported the post-transcriptional regulation of calbindin D-28K (Varghese et al, 1988, Enomoto et al, 1992, Christakos et al, 1993). In MDBK cells, activation of PKC also rapidly increased calbindin D-28K without affecting VDR binding (Gagnon and Welsh, 1994). Recent studies in our lab have demonstrated the phosphorylation of calbindin D-28K *in vitro* by PKC (Gagnon et al, 1994). Since there are five CKII and two PKC consensus phosphorylation sites on calbindin D-28K, we hypothesized that EGF may be regulating calbindin D-28K post-transcriptionally through phosphorylation by either PKC or CKII. Other studies have demonstrated that changes in the phosphorylation state of calmodulin, another calcium binding protein (Pinna, 1990, Bidwai et al, 1993, Quadroni et al, 1994) alters its biological activity. Although the effects of calbindin D-28K phosphorylation are unknown, we hypothesize that changes in phosphorylation status may stabilize the protein, alter its conformation, or increase its association with other proteins such as tubulin (Nemere et al, 1992).

The interaction of EGF with the EGFR activates the EGF receptor tyrosine kinase and CKII (Ackerman et al, 1989, Ackerman et al, 1990). CKII is a serine/threonine kinase which

phosphorylates a variety of cellular substrates in response to mitogenic stimuli. CKII preferentially phosphorylates acidic proteins like phosvitin, nonhistone proteins *in vivo*, calmodulin *in vitro*, and casein (Quadroni et al, 1994, Kuenzel et al, 1985, Kuenzel et al, 1987, Pinna et al, 1990). Since calbindin D-28K is also an acidic protein and contains CKII consensus phosphorylation sites, we investigated the possibility that calbindin D-28K is a substrate for CKII. *In vitro* phosphorylation assays with purified CKII indicated that CKII was unable to phosphorylate calbindin D-28K although CKII-dependent phosphorylation of casein was demonstrated under the same conditions. CKII activity in the presence of calbindin D-28K was comparable to background levels, in the absence of substrate, and was not due to endogenous autophosphorylation of CKII. These data were confirmed by autoradiography (data not shown). Our studies demonstrate that CKII does not phosphorylate calbindin D-28K under optimized conditions *in vitro*, suggesting that CKII is unlikely to directly regulate this protein *in vivo*.

Having determined that CKII was not likely to be involved in the regulation of calbindin D-28K, we investigated the possibility that EGF-mediated effects on PKC may be linked to calbindin D-28K regulation. In other cell systems, the EGF receptor is a PKC substrate and this has been shown to affect the EGF-mediated signalling pathway (Sharma et al, 1994). In the present studies EGF increased membrane-associated PKC activity prior to the up-regulation of calbindin D-28K suggesting that EGF signalling through PKC may be involved in calbindin D-28K regulation. In unstimulated cells, cytosolic and membrane PKC activity was detected under basal conditions, consistent with studies reporting a pool of constitutively active membrane-attached PKC (Bazzi et al, 1988a, Bazzi et al, 1988b). In

MDBK cells, EGF induced a transient increase in cytosolic PKC activity above control levels. The transient nature of this increase may reflect either PKC translocation or degradation by proteases (Inoue et al, 1977, Takai et al, 1977). Indeed, we observed enhanced membrane PKC activity following acute EGF treatment, consistent with reports that EGF increases intracellular calcium and translocates PKC from the cytosol to the membrane. The observed membrane translocation is similar to the reported effect of phorbol esters on PKC subcellular localization and activity. Consistent with this suggestion, our lab has demonstrated that PKC can directly phosphorylate calbindin D-28K in a calcium and phospholipid dependent manner. We have detected two threonine residues, Thr106 and Thr233, in calbindin D-28K which are consensus PKC phosphorylation sites. These residues are completely conserved in avian, mouse, rodent, and bovine calbindin D-28K. Studies in our lab have shown that short term (4 hour) TPA treatment of MDBK cells activates PKCα and up-regulates calbindin D-28K expression (Gagnon and Welsh, submitted).

We next investigated the subcellular location of PKC following EGF stimulation. Studies of rat kidney showed that PKC is abundant in whole rat kidney (Caramelo et al, 1988, Weinman et al, 1986, Hise et al, 1989) and studies from this lab have demonstrated that PKCα, PKCβ, and PKCζ are expressed in MDBK cells (Simboli-Campbell et al, 1994). Our studies focussed on PKCα since this calcium-dependent isozyme is abundant in MDBK cells and has been linked to TPA-mediated calbindin D-28K regulation in MDBK cells (Gagnon et al, 1994). We examined the temporal changes in subcellular distribution of PKCα in MDBK cells following EGF treatment. No significant changes in either cytosolic or membrane PKCα expression were detected in response to short term (2 hours) EGF

treatment, as assessed by immunoblotting. These data do not directly match the changes in total calcium-dependent PKC activity. The differences between measured activity and PKCα expression may result from contributions from PKCβ, another calcium-dependent PKC isozyme present in MDBK cells. Activation of PKCα already present in the membrane may confer an increase in activity without translocation. The expression of PKCα, in response to early EGF treatment (5,30 minutes) does not correlate to the changes in total calcium-dependent activity since this assay measures total PKC activity and is not isozyme specific. Subcellular distribution of PKC β needs to be examined to further determine the correlation between changes in PKC activity and subcellular distribution of PKC isozymes. However, we detected PKCα translocation, by immunoblotting, to the particulate fraction following four hour EGF treatment. This result correlated with a significant increase in membrane-associated PKC activity observed under the same conditions. Furthermore, the subsequent translocation of PKCα back to the cytosol, after 24 hour EGF treatment, corresponded to the decrease in membrane-associated PKC activity. We therefore hypothesize that the EGF-mediated temporal changes in PKC activity may be similar to the TPA-mediated temporal changes in PKC activity.

Stimulation with EGF did not induce time-dependent changes in calcium/phospholipid-independent PKC activity, possibly because this subgroup of isozymes is not modulated by changes in intracellular calcium, which is enhanced as a consequence of EGFR activation. Although total calcium/phospholipid-independent PKC activity was unaffected by EGF treatment, we cannot rule out the possibility that EGF may increase one or more calcium-independent PKC isozymes. Assessing the changes in subcellular

distribution of additional PKC isozymes, including newly discovered forms, would provide more information about the effects of EGF on these isozymes and the potential role they may play in the EGF-mediated regulation of calbindin D-28K and VDR.

Previous studies from our lab (Simboli-Campbell et al, 1994) have shown that $1,25(OH)_2D_3$-induced membrane translocation of PKCα is linked to the up-regulation of calbindin D-28K, and may explain the ability of $1,25(OH)_2D_3$ to prevent the EGF-induced decrease in calbindin D-28K expression. Further studies to characterize the interactions of $1,25(OH)_2D_3$ and EGF in the regulation of PKCα will be necessary to test this possibility.

In summary, our studies are the first to report that EGF regulates renal calbindin D-28K expression. Since EGF up-regulates calbindin D-28K, in the absence of $1,25(OH)_2D_3$, these studies suggest post-transcriptional effects of growth factor signalling on calbindin D-28K. These studies were conducted in MDBK cells in which calbindin D-28K regulation closely resembles *in vivo* conditions. Collectively, our data support studies demonstrating the regulation of calbindin D-28K by factors other than $1,25(OH)_2D_3$ such as PTH, estrogen, retinoic acid, NGF and TPA in various cell systems (Mutema et al, 1994, Christakos et al, 1994, Simboli-Campbell et al, 1992). The data indicate that EGF stimulates cell proliferation but that the mitogenic effects of EGF can be dissociated from its regulation of VDR and calbindin D-28K. Discordance of VDR levels and calbindin D-28K expression following acute EGF treatment further suggests a post-transcriptional mechanism regulating calbindin D-28K. We have demonstrated that CKII does not phosphorylate calbindin D-28K *in vitro* and therefore does not likely play a role in the regulation of this protein in response to EGF *in vivo*. In contrast, EGF-induced changes in PKC activity correlate with altered calbindin

D-28K expression, consistent with data indicating *in vitro* phosphorylation of calbindin D-28K by PKC. Taken together, our data implicate the PKC signalling pathway in the regulation of calbindin D-28K in MDBK cells in response to EGF.

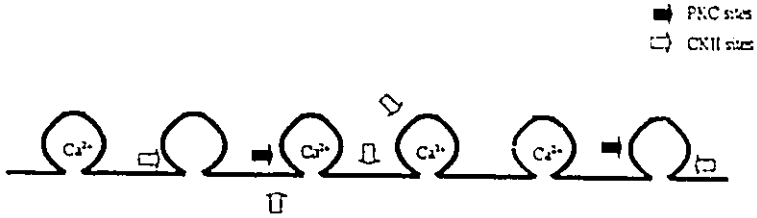
# 6. CONCLUSIONS

The data presented in this thesis demonstrate that EGF is a potent mitogen for MDBK cells. In contrast to other vitamin D responsive epithelial cell lines, $1,25(OH)_2D_3$ does not inhibit MDBK cell proliferation or block the mitogenic effect of EGF. However, $1,25(OH)_2D_3$ up-regulates VDR and prevents the EGF-mediated decrease in VDR number under these conditions. Assessment of calbindin D-28K in relation to VDR indicated that acute EGF treatment up-regulates calbindin D-28K expression in the absence of $1,25(OH)_2D_3$ and independently of changes in VDR binding. Follow-up studies to identify potential signalling cascades induced by EGF, which may impinge on calbindin D-28K involved the assessment of CKII and PKC activity. Our data indicate that CKII does not directly phosphorylate calbindin D-28K, arguing against its involvement in EGF-mediated regulation. Initial studies with PKC suggest that its activation is correlated with EGF-mediated short-term up-regulation of calbindin D-28K. Further understanding of PKC regulation by EGF will be necessary to link specific isoforms of PKC to the regulation of this calcium binding protein and the possible relationship with proliferation. Elucidating the interactions between EGF, $1,25(OH)_2D_3$ and calbindin D-28K in normal cells may provide insight into the role these agents play during renal hypertrophy in response to renal injury.

# APPENDIX

**Figure A1: Consensus CKII and PKC phosphorylation sites for mouse calbindin D-28K**

CKII phosphorylation sites are indicated by open arrows and PKC consensus phosphorylation sites are indicated by shaded arrows.




| | | |
|---|---|---|
| CKII phosphorylation sites: | $^{60}$Ser: | *Ser*-Phe-Val-Asp |
| | $^{99}$Ser: | *Ser*-Cys-Glu-Glu |
| | $^{137}$Thr: | *Thr*-Val-Asp-Asp |
| | $^{156}$Ser: | *Ser*-Asn-Asn-Asp |
| | $^{237}$Ser: | *Ser*-Ala-Gly-Asp |
| PKC phosphorylation sites: | $^{106}$Thr: | Glu-Glu-Phe-Met-Lys-*Thr*-Trp-Arg-Lys-Tyr |
| | $^{233}$Thr: | Ile-Asn-Asn-Ile-Thr-*Thr*-Lys-Lys-Asn-Ile |

**Figure A2: Changes in calbindin D-28K expression with increasing EGF concentration.**

Near-confluent serum-deprived MDBK cells were treated with EGF in increasing concentrations for 24 hours. Cell lysates were separated by 15% SDS-PAGE and immunoblotted for calbindin D-28K as described in Methods 3.4. Immunoreactive calbindin D-28K was detected in cells treated with EGF (0, 25, 50, 100, 200 ng/mL) in lanes 1-5 respectively and is down-regulated in response to 100 ng/mL of EGF.

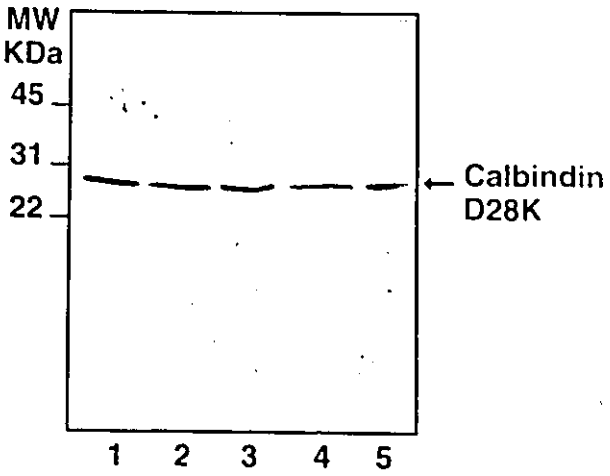

MW
KDa
45
31
22
Calbindin
D28K
1
2
3
4
5

**Figure A3: Scatchard analysis of the changes in VDR Binding following treatment of MDBK cells with EGF.**

Near-confluent, serum-starved MDBK cells were exposed to EGF (100 ng/mL) or PBS for 4 hours. Cells were harvested and high salt extracts, prepared as described in Methods 3.5, were incubated with increasing amounts (0.05-0.6 nM) of $^3$H-1,25(OH)$_2$D$_3$ alone or in the presence of 240-fold molar excess of non-radiolabelled hormone. Bound hormone was measured as described in Methods 3.5. The results are reported as the mean ratio of bound/free hormone versus bound of three separate trials. Analysis of equilibrium binding data results shows $N_{max}$=50.2 and 46.8 in PBS and EGF treated cells respectively with no significant changes in VDR affinity.

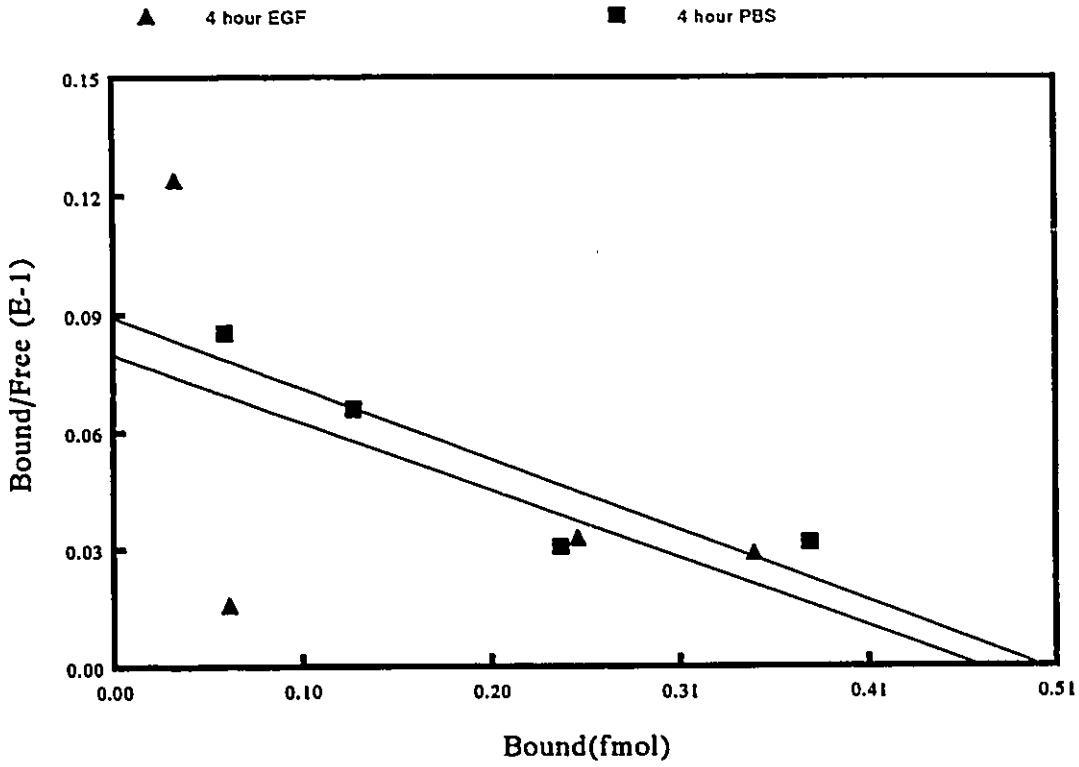
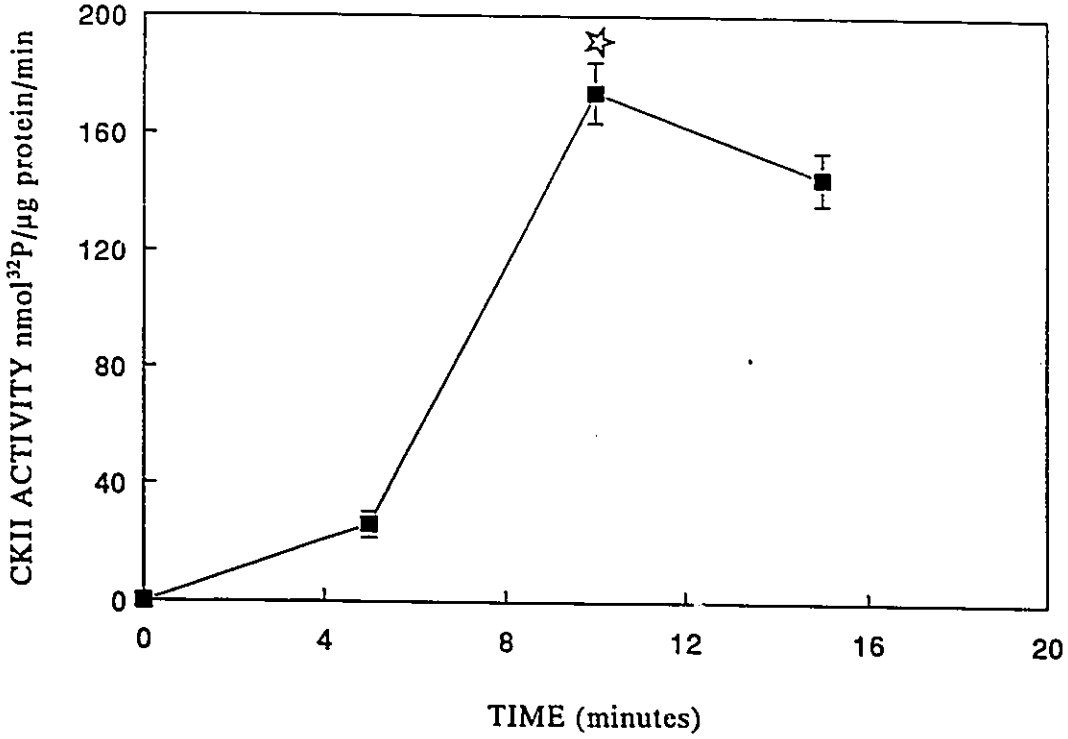

4 hour EGF
4 hour PBS
0.15
0.12
0.09
0.06
0.03
0.00
Bound/Free (E-1)
0.00
0.10
0.20
0.31
0.41
0.51
Bound(fmol)

**Figure A4: Time-dependent and concentration-dependent changes in CKII activity.** In the top panel (I), purified casein kinase II, and 1 µg of casein were incubated with $^{32}P\gamma$-ATP for up to 15 minutes at 37°C. The bottom figure (II) shows casein kinase II activity with increasing amounts (0-10 µg) of casein. In both cases, reactions were terminated, phosphorylated casein was precipitated onto cellulose filters and radioactivity incorporated was counted. Data are reported as nmoles $^{32}P$ incorporated per µg protein per minute. Results are the mean ± SEM of data pooled from three independent trials and statistical significance is indicated by *, if $p<0.05$.


CKII ACTIVITY nmol32P/µg protein/min
200
160
120
80
40
0
0
4
8
12
16
20
TIME (minutes)

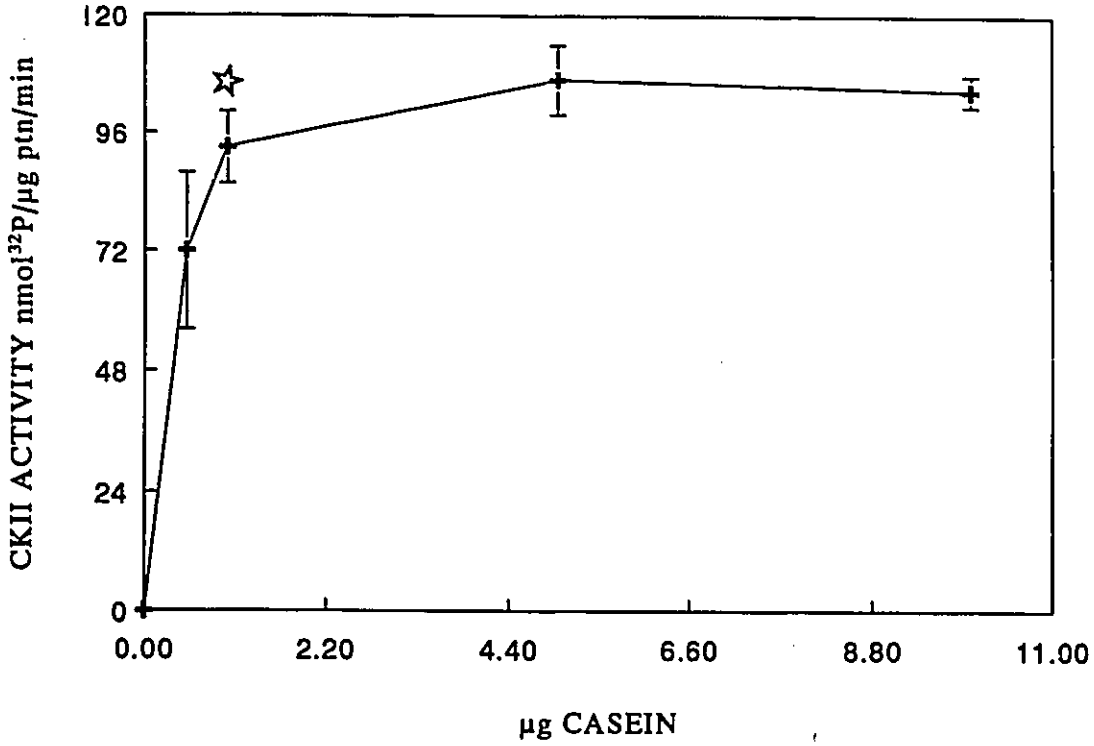

CKII ACTIVITY nmol32P/µg ptn/min
120
96
72
48
24
0
0.00
2.20
4.40
6.60
8.80
11.00
µg CASEIN

**Figure A5: Calbindin D-28K concentration-dependent changes in CKII activity.**

To determine the appropriate amount of calbindin D-28K for the assay, purified casein kinase II, and increasing amounts (0-10 µg) of calbindin D-28K were incubated with $^{32}P\gamma$-ATP for up to 15 minutes at 37°C. Reactions were terminated, phosphorylated calbindin D-28K was precipitated onto cellulose filters and radioactivity incorporated was counted. Data are reported as nmoles $^{32}P$ incorporated per µg protein per minute and are the mean ± SEM of data pooled from three independent trials. Statistical significance, compared to 0 µg calbindin D-28K, is indicated by * if $p<0.05$.

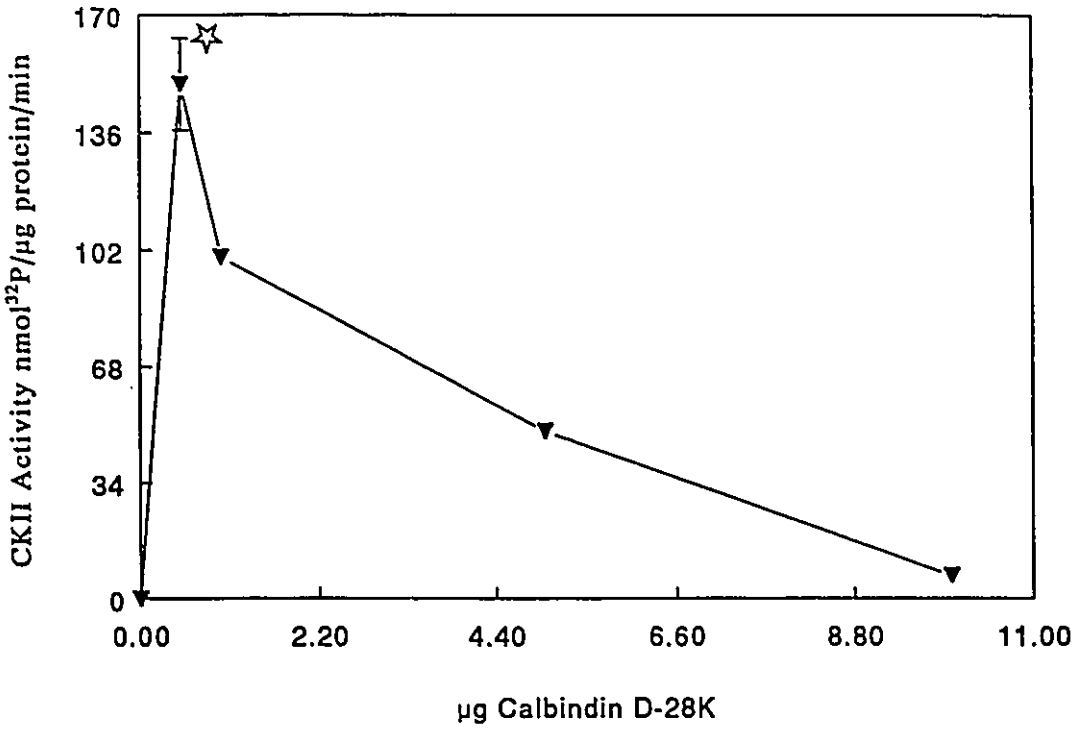
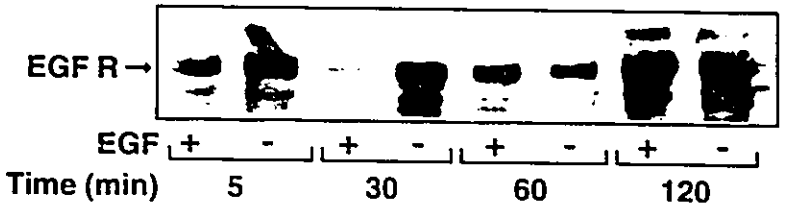

170
136
102
68
34
0
CKII Activity nmol32P/µg protein/min
0.00
2.20
4.40
6.60
8.80
11.00
µg Calbindin D-28K

**Figure A6: Time course of the changes in EGFR protein levels in MDBK cells treated with EGF for up to 24 hours.**

Sub-confluent serum-deprived MDBK cells were treated with EGF (100 ng/mL) or PBS for up to 24 hours. Membrane fractions were obtained from cell lysates and were separated by 12% SDS PAGE. The top figure (I) shows immunoreactive EGFR detected as a 170 kDa band in cells treated with EGF for 5, 30, 60, and 120 minutes and PBS. In the bottom panel (II), immunoreactive EGFR was detected as a 170 kDa band in cells treated with EGF for 4 hours and 24 hours (lanes 1, and 3 respectively) and PBS (lanes 2, 4 respectively).


EGF R →
EGF + - + - + - + -
Time (min) 5 30 60 120

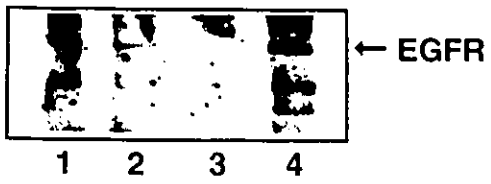
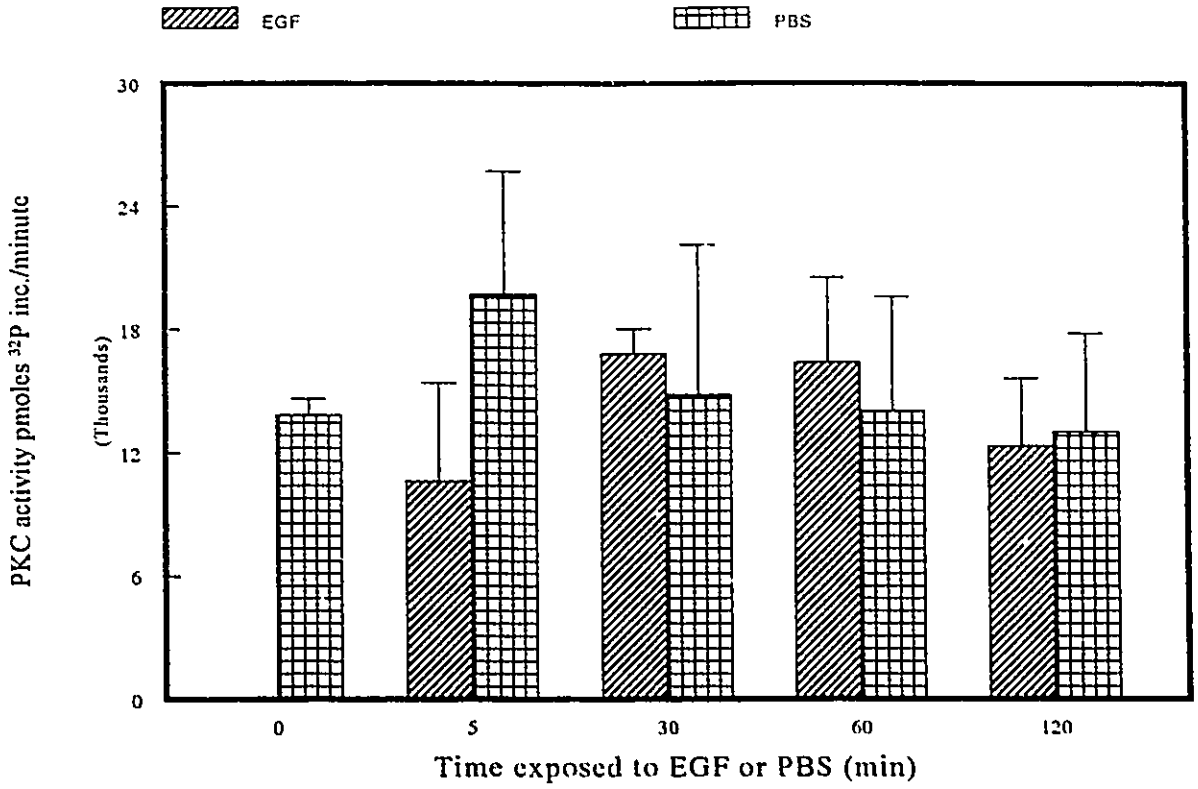

← EGFR
1 2 3 4

**Figure A7: Time course of the changes in PKC activity in response to EGF treatment in soluble and particulate fractions of MDBK cells in the absence of calcium and phospholipids.**

Sub-confluent serum-starved MDBK cells were treated with EGF (100 ng/mL) or PBS for up to 2 hours. Cytosolic extracts (I, top) from cell lysates, prepared as described in Methods 3.1, were assayed for PKC activity as described in Methods 3.7 in the absence of calcium and phospholipids. The bottom figure (II) Changes in calcium and phospholipid independent PKC activity in membrane fractions of MDBK cells. In both cases, results are expressed as picomoles $^{32}P$ incorporated per minute and are the mean ± SEM of data pooled from three independent trials. Statistically significance between treatment groups is indicated by * when $p<0.05$.


EGF
PBS
PKC activity pmoles $^{32}$P inc./minute
(Thousands)
30
24
18
12
6
0
0
5
30
60
120
Time exposed to EGF or PBS (min)

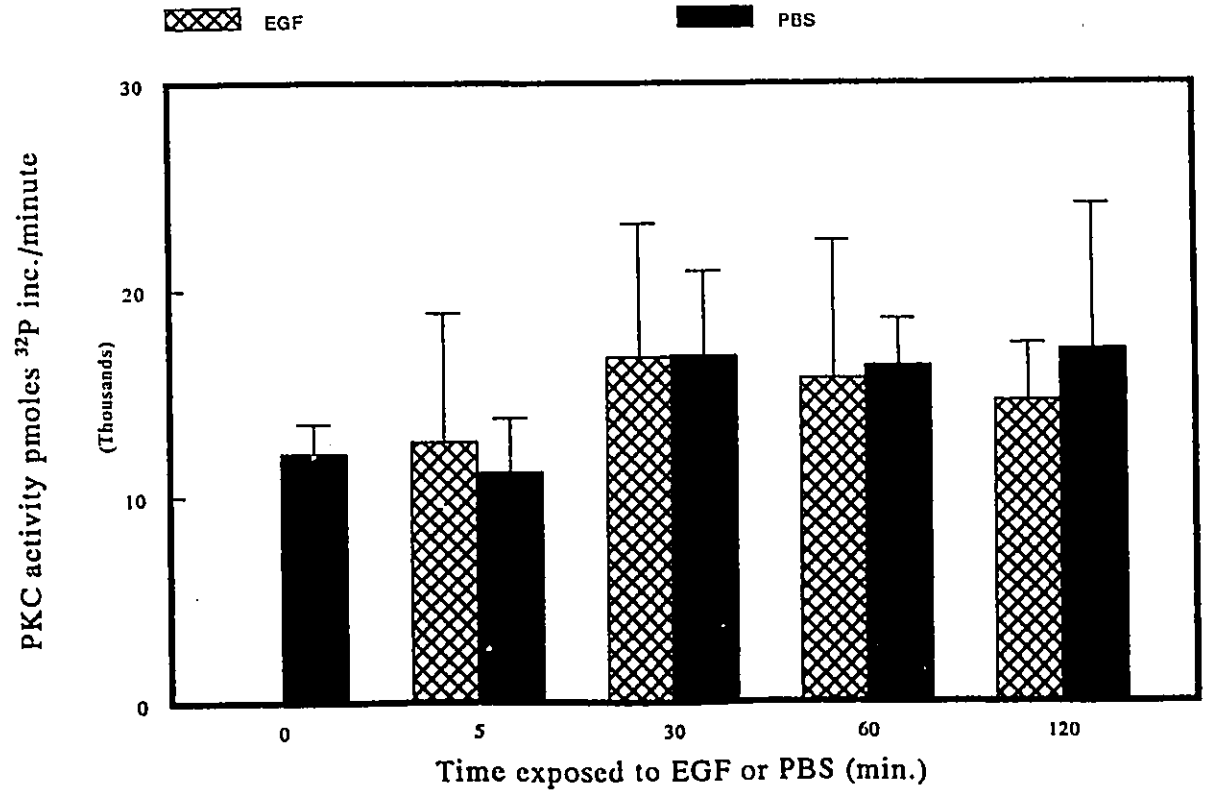
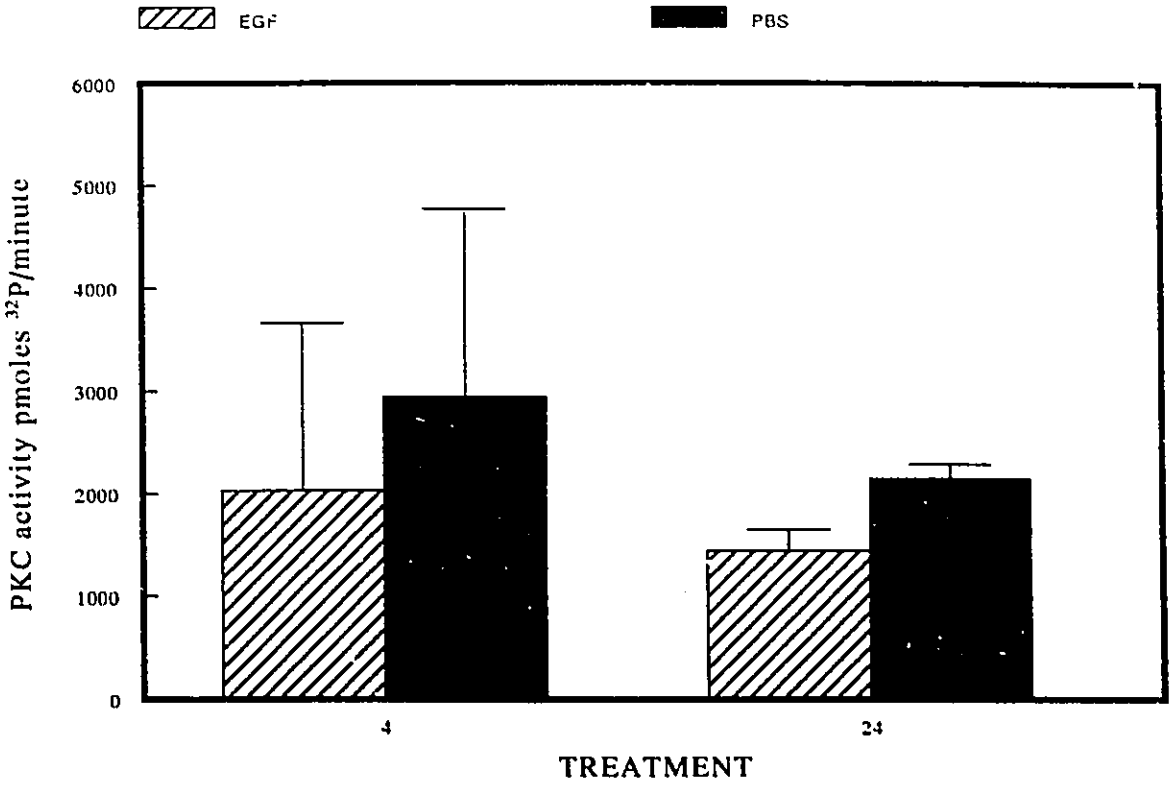
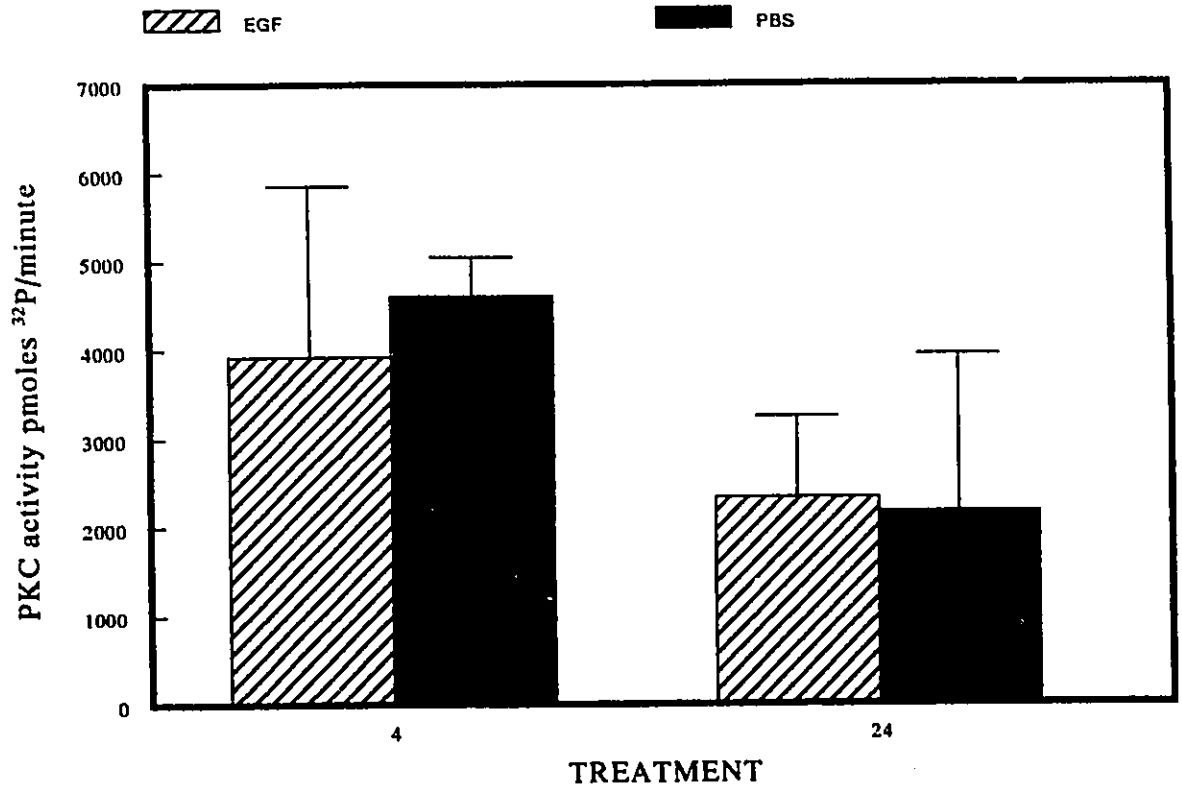

EGF
PBS
PKC activity pmoles $^{32}$P inc./minute
(Thousands)
30
20
10
0
0
5
30
60
120
Time exposed to EGF or PBS (min.)

**Figure A8: Time course of the changes in PKC activity in response 4 hour and 24 hour EGF treatment in cytosolic and membrane fractions of MDBK cells in the absence of calcium and phospholipids.**

Sub-confluent serum-starved MDBK cells were treated with EGF (100 ng/mL) or PBS for up to 24 hours. (I, top) Cytosolic extracts from cell lysates, prepared as described in Methods 3.1, were assayed for PKC activity as described in Methods 3.7 in the absence of calcium and phospholipids. (II, bottom) Changes in calcium and phospholipid independent PKC activity in membrane fractions of MDBK cells. In both cases, results are expressed as picomoles $^{32}P$ incorporated per minute and are the mean ± SEM of three independent trials. Statistically significance between treatment groups is indicated by * when $p<0.05$.


EGF
PBS
6000
5000
4000
3000
2000
1000
0
PKC activity pmoles $^{32}$P/minute
4
24
TREATMENT


EGF
PBS
7000
6000
5000
4000
3000
2000
1000
0
PKC activity pmoles $^{32}$P/minute
4
24
TREATMENT

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