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Studies on mammalian

25-hydroxyvitamin D_3-24-hydroxylase

by

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May, 1992

A Thesis Submitted to the Faculty of Graduate Studies and Research

in Partial Fulfillment of the Requirements for

the Degree of Doctor of Philosophy

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Abstract

This thesis describes three studies on mammalian 25-hydroxyvitamin D_3-24-hydroxylase (24-hydroxylase), the first enzyme in the C24-oxidation pathway, a major catabolic pathway for vitamin D metabolites in kidney and other target tissues for vitamin D hormone. The first study examines the involvement of protein kinase C in the regulation of 24-hydroxylase activity in mouse kidney. Evidence is presented supporting a stimulatory role for protein kinase C in the regulation of constitutive, but not inducible, renal 24-hydroxylase. The kinase is also implicated in the aberrant expression of renal vitamin D metabolism in the mutant X-linked hypophosphatemic (Hyp) mouse. The second study investigates the mechanism(s) by which forskolin, a classic activator of adenylate cyclase, inhibits 24-hydroxylase activity in mouse kidney. Both the traditional cAMP-dependent mechanism and a novel cAMP-independent mode of action are observed. A direct interaction between forskolin and the substrate binding site of 24-hydroxylase is suggested for the latter based on kinetic analyses and structural similarities between the diterpene and the steroid substrate for the hydroxylase. The third study addresses the structural relationship between renal 1-hydroxylase and renal and target cell 24-hydroxylase(s) by assessing 24-hydroxylase activity in patients with vitamin D dependency rickets type I (VDDR-I), a Mendelian disorder of 1-hydroxylase function. Both constitutive renal 24-hydroxylase, indirectly ascertained through measurement of circulating levels of relevant vitamin D metabolites, and inducible target cell 24-hydroxylase, directly measured in cultured skin fibroblasts, are shown to be intact in VDDR-I patients undergoing calcitriol therapy. These findings suggest that the 1- and 24-hydroxylase activities likely represent or contain distinct gene products.

Sommaire

Cette thèse comprend trois études sur la 25-hydroxyvitamine D_3-24-hydroxylase (24-hydroxylase), la première enzyme dans la voie d'oxydation C24, une importante voie de dégradation des métabolites de la vitamine D dans le rein et dans d'autres tissus-cibles pour l'hormone de la vitamine D. La première étude porte sur le rôle de la protéine kinase C dans la régulation de la 24-hydroxylase rénale chez la souris. Les résultats suggèrent que la protéine kinase C agirait comme stimulateur de l'activité basale de la 24-hydroxylase, mais pas de l'activité enzymatique inductible. La kinase serait aussi impliquée dans le métabolisme anormal de la vitamine D chez la souris mutante hypophosphatémique (Hyp). La deuxième étude examine l'inhibition de la 24-hydroxylase rénale chez la souris par la forskoline, activeur connu de l'adénylate cyclase. Deux mécanismes d'inhibition sont observés: le mécanisme déjà connu, dépendant de l'AMP cyclique ainsi qu'un nouveau mode d'action indépendant de celui-ci. La cinétique de l'inhibition et la similitude des structures de la forskoline et du substrat stéroïde de l'hydroxylase suggèrent une intéraction directe entre la forskoline et le site de liaison du substrat de la 24-hydroxylase. Dans la troisième étude, la relation structurale entre la 1-hydroxylase et la 24-hydroxylase est examinée en évaluant l'état de la 24-hydroxylase chez des patients atteints de rachitisme vitamino-dépendant de type I (VDDR-I), un désordre mendélien de la 1-hydroxylase. La 24-hydroxylase rénale constitutive (déterminée à partir des niveaux plasmatiques des métabolites de la vitamine D pertinents) ainsi que l'activité enzymatique inductible des cellules-cibles (mesurée dans des fibroblastes en culture) semblent intactes chez les patients VDDR-I traités avec l'hormone de la vitamine D. Ces résultats suggèrent que la 1- et la 24-hydroxylase soient ou comprennent des produits génétiques différents.

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To my grandmother,
for beating the odds
and teaching me that nothing is impossible

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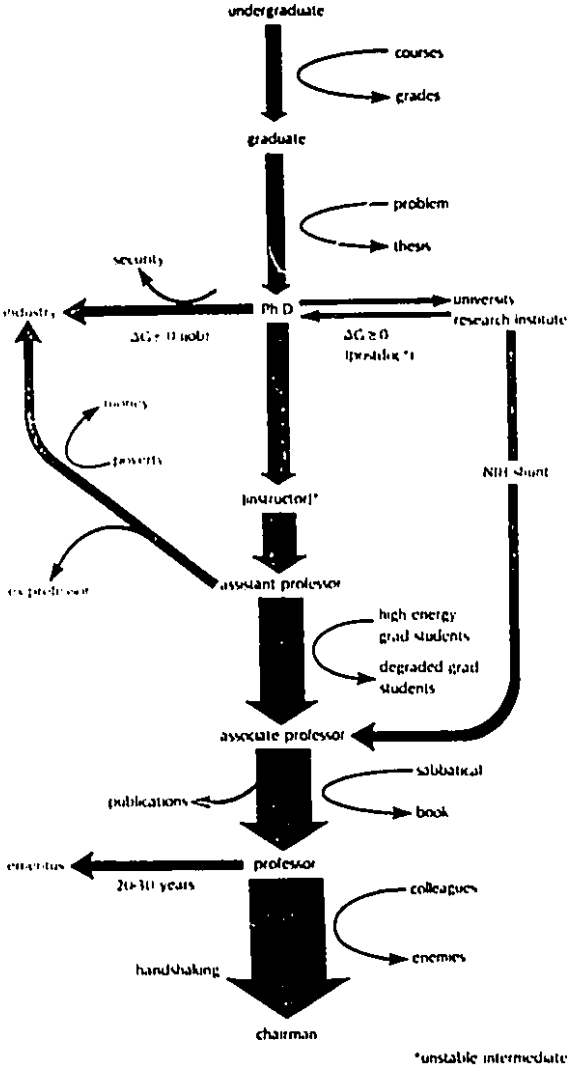
Academic Metabolism



"The pathway of academic metabolism"
(by Elizabeth J. Kovacs)

Acknowledgements

Although the manuscripts included in this thesis offer acknowledgements in appreciation of specific technical contributions, these are typically brief and formal, and fail to recognize those less tangible contributions, which we all know to be of equal (if not greater) importance. Thus, I would like to take this opportunity to express my sincere thanks to the contributors of those "less-tangibles" as well as provide the "extended version" of those acknowledgements so briefly and generically cited in the manuscripts. First, a general word of thanks to all the catalysts and co-factors, past and present, who contributed in various ways to my processing along the Pathway of Academic Metabolism. Specific thanks are offered to:

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And most importantly, special thanks to my parents, for seeing me through yet one more degree. Thank you for your support, understanding, patience and unwavering faith in me. (Would now be a good time to mention medical school? - just kidding!!)

Abbreviations

ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	adenosine 3',5'-monophosphate
cDNA	complementary DNA (deoxyribonucleic acid)
cGMP	cyclic guanosine monophosphate
DAG	diacylglycerol
DBP	vitamin D binding protein
DMSO	dimethylsulfoxide
EC_{50}	half-maximal effective concentration
EDTA	ethylenediamine tetraacetic acid
EGTA	[ethylenebis(oxyethylenenitrilo)]tetraacetic acid
FBS	fetal bovine serum
G_i	inhibitory guanine nucleotide regulatory protein (G protein)
G_p	phospholipase C-specific G protein
G_s	stimulatory G protein
H-7	1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	high performance liquid chromatography
Hyp	X-linked hypophosphatemia, murine mutation
IP_3	inositol-1,4,5-trisphosphate
K	kilodaltons
K_D	dissociation constant
Ki	inhibitory constant
Km	apparent Michaelis-Menten constant
MEM	minimal essential medium
mol wt	molecular weight
mRNA	messenger RNA (ribonucleic acid)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
OAG	1-oleoyl-2-acetyl-sn-glycerol
OK cells	opossum kidney cells
PBS	phosphate-buffered saline
4α-phorbol	4α-phorbol 12,13-didecanoate
PIP_2	phosphatidylinositol-4,5-bisphosphate
PMA (or TPA)	phorbol 12-myristate 13-acetate
Protein kinase A	cAMP-dependent protein kinase
Protein kinase C	calcium-activated, phospholipid-dependent protein kinase
PTH	parathyroid hormone
$P450_{scc}$	side-chain cleavage cytochrome P450
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
Tris	tris-(hydroxymethyl)-aminomethane
VDDR-I	vitamin D dependency rickets type I

VDDR-II	vitamin D dependency rickets type II
Vmax	maximum velocity of reaction
XLH	X-linked hypophosphatemia, human disorder
1-hydroxylase	25-hydroxyvitamin D_3-1α-hydroxylase
24-hydroxylase	25-hydroxyvitamin D_3-24-hydroxylase
25OHD_3	25-hydroxyvitamin D_3
25OHD	25-hydroxyvitamin D_2 and D_3
1,25-$(OH)_2D_3$	1,25-dihydroxyvitamin D_3
1,25-$(OH)_2D$	sum of 1,25-dihydroxyvitamin D_2 and D_3
24,25-$(OH)_2D_3$	24,25-dihydroxyvitamin D_3
24,25-$(OH)_2D$	sum of 24,25-dihydroxyvitamin D_2 and D_3
1,24,25-$(OH)_3D_3$	1,24,25-trihydroxyvitamin D_3

Preface

Thesis format

This thesis is submitted in the form of manuscripts, in accordance with the regulations stated in the *Guidelines Concerning Thesis Preparation*, which read as follows:

> "The candidate has the option, subject to the approval of the Department, of including as part of the thesis the text, or duplicated published text, of an original paper or papers. Manuscript-style theses must still conform to all other requirements explained in the *Guidelines Concerning Thesis Preparation*. Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (e.g. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported. The thesis should be more than a mere collection of manuscripts published or to be published. It must include a general abstract, a full introduction and literature review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interest of cohesion.
>
> It is acceptable for theses to include, as chapters, authentic copies of papers already published, provided these are duplicated clearly and bound as an integral part of the thesis. In such instances, connecting texts are mandatory and supplementary explanatory material is almost always necessary. Photographs or other materials which do not duplicate well must be included in their original form.
>
> While the inclusion of manuscripts co-authored by the candidate and others is acceptable, the candidate is required to make an explicit statement of who contributed to such work and to what extent, and supervisors must attest to the accuracy of the claims at the Ph.D. Oral Defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make the responsibilities of authors perfectly clear."

Accordingly, the present thesis is written in manuscript style with the modifications required to achieve a cohesive discourse. All three manuscripts presented have been published. The first manuscript, which deals with the regulation of mouse renal 25-hydroxyvitamin D_3-24-hydroxylase by protein kinase C, and the second, which examines the mechanism for forskolin-mediated inhibition of 24-hydroxylase activity in mouse kidney, have been

published in *Endocrinology*. The third paper, describing 24-hydroxylase activity in patients with vitamin D-dependency rickets type I (VDDR-I), appears in *The Journal of Clinical Endocrinology and Metabolism*. Supplementary material, such as technical details and complementary data which have not been published elsewhere, has been included in Appendices.

Contributions by Co-authors

All three manuscripts presented in this thesis have been co-authored by myself and my thesis supervisor, Dr. Harriet S. Tenenhouse. Two of the three manuscripts bear the name of a third co-author. According to the regulations stated in the *Guidelines Concerning Thesis Preparation*, the inclusion of co-authored manuscripts is acceptable, provided that the candidate clearly state the contribution of each co-author (excluding the thesis supervisor) to the final work.

The non-supervisory co-author of the manuscript entitled "Evidence for protein kinase C involvement in the regulation of 25-hydroxyvitamin D_3-24-hydroxylase" is Dr. Avihu Boneh. Dr. Boneh was a graduate student of Dr. Tenenhouse, working on the characterization of protein kinase C in mouse kidney, and his contributions to the manuscript comprise the data in Figure 1 and the associated procedural description, "Phosphorylation of endogenous substrates", in Materials and Methods.

Dr. Glenville Jones is the non-supervisory co-author of the manuscript entitled "Normal 24-hydroxylation of vitamin D metabolites in patients with vitamin D dependency rickets type I. Structural implications for the vitamin D hydroxylases". Dr. Jones is an expert in the chromatographic analysis of vitamin D metabolites and has collaborated with Dr. Tenenhouse on several occasions. Dr. Jones' contribution to the present study was three-fold: 1) the

chromatographic separation and quantitation of serum 25-hydroxyvitamin D_3 [25OHD_3] and 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$] using an HPLC system based on cyano-bonded phase packing (Zorbax-CN) not available in our laboratory; 2) the chromatographic analysis of 24-hydroxylation products of 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] in cultured cell extracts, the identification of which required commercially unavailable standards; both Dr. Jones' laboratory and ours shared in the analysis of 24-hydroxylation products of 25OHD_3; 3) the provision of [1β-^{3}H]1α,25-$(OH)_2D_3$, a commercially unavailable substrate, which was used to assay calcitroic acid production. In addition, Dr. Jones provided helpful discussion and constructive criticism of the manuscript.

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Chapter I: Introduction

1. Objectives

The common thread which binds together the three studies presented in this thesis is the mitochondrial enzyme, 25-hydroxyvitamin D_3-24-hydroxylase [24-hydroxylase], which catalyzes the first reaction in the C24-oxidation pathway, a major catabolic pathway for vitamin D metabolites in kidney as well as other target tissues for vitamin D. In all other respects, these studies differ appreciably from one another, as they examine different issues, ranging from regulatory aspects to structural considerations; in different systems, including intact cells and isolated subcellular fractions; in different species, notably, in man and mouse. To help orient the reader to the diverse objectives considered, a brief outline of each study is provided below.

Calcium-activated, phospholipid-dependent protein kinase [protein kinase C] is a ubiquitous, serine/threonine kinase that has been shown to participate in the regulation of a variety of cellular responses ranging from cell growth and proliferation to specialized functions such as secretion, membrane transport and muscle contraction. Recently, it has been implicated in the regulation of steroidogenesis in a number of tissues and cell types. Comparatively little is known of its role in the modulation of renal vitamin D metabolism. The first study presented in this thesis (Chapter II) examines the involvement of protein kinase C in the regulation of renal 24-hydroxylase activity in normal mice, as well as in a mutant mouse strain in which both renal vitamin D metabolism and protein kinase C activity are perturbed.

Parathyroid hormone is a major regulator of renal vitamin D metabolism and has been shown to inhibit 24-hydroxylase activity by a mechanism involving cAMP. Part of the evidence for cAMP involvement in this process

stems from the observation that forskolin, a classical activator of adenylate cyclase, mimics the inhibitory effect of PTH on 24-hydroxylase. Recently, however, a number of reports have described cAMP-independent effects of forskolin on certain proteins of the plasma membrane. The second study (Chapter III) examines whether similar cAMP-unrelated mechanisms participate in the forskolin-mediated inhibition of renal mitochondrial membrane-associated 24-hydroxylase activity.

The third and final study (Chapter IV) assesses the status of 24-hydroxylase activity in patients with vitamin D dependency rickets type I, an inherited disorder of vitamin D metabolism attributable to a defect in 25-hydroxyvitamin D_3-1-hydroxylase [1-hydroxylase] function, with the objective of gaining some insight into the structural relationship between the renal 1- and 24-hydroxylases.

The remainder of this first chapter serves to develop a context for these studies by reviewing our current state of knowledge regarding vitamin D metabolism, regulation and action, in both health and disease. Space limitations prevent the citation of all the relevant primary works that have contributed to our understanding of this rapidly evolving field. Although original articles will be cited wherever possible, frequent references will be made to the numerous specialized and detailed reviews that have been written on these topics.

2. Biological role and mode of action of vitamin D

1,25-Dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], the hormonally active form of the vitamin, is a major regulator of mineral metabolism and, thus, plays a pivotal role in normal growth and development. Vitamin D hormone fulfills its homeostatic role in the maintenance of circulating levels of calcium and

phosphorus by acting on its three classic target tissues, intestine, bone and kidney. It acts on intestine to stimulate transepithelial transport of calcium, as well as phosphate, from lumen to plasma (reviewed by DeLuca and Schnoes, 1983). 1,25-$(OH)_2D_3$ also acts, in concert with parathyroid hormone, to mobilize calcium and phosphorus from bone (DeLuca and Schnoes, 1983) and promotes reabsorption of both these minerals by the kidney (Yamamoto et al., 1984; Kurnik and Hruska, 1984).

It is generally accepted that these established actions of 1,25-$(OH)_2D_3$ are mediated by a classical steroid receptor mechanism which involves binding of the hormone to its intracellular receptor, association of the activated hormone-receptor complex with specific target genes, resulting in the stimulation (or suppression) of target gene expression and elicitation of the appropriate cellular responses (Reichel et al., 1989; Pike, 1990). The occurrence of disease states in which 1,25-$(OH)_2D_3$ receptor function is absent or altered underscores the importance of the receptor in mediating the actions of vitamin D hormone (reviewed by Marx, 1989; see section I.6). The 1,25-$(OH)_2D_3$ receptor is a 50-60 K protein with high affinity (K_D, $5x10^{-11}$ M) and selectivity for the hormonal form of vitamin D (DeLuca, 1988; Pike, 1990), although it can bind other vitamin D metabolites, albeit with lesser affinity (Stern, 1981; DeLuca, 1988). The cDNAs for both the human (Baker et al., 1988) and avian (McDonnell et al., 1987) 1,25-$(OH)_2D_3$ receptors have been cloned and sequenced, and found to belong to the superfamily of genes encoding true steroid, thyroid and retinoid receptors (Evans, 1988). Like other members of this gene family, the 1,25-$(OH)_2D_3$ receptor comprises at least two domains, including a carboxy-terminal steroid-binding domain with 1,25-$(OH)_2D_3$ recognition sequences and an amino-terminal DNA-binding domain consisting of a cysteine-rich region with two putative zinc fingers (Evans, 1988;

Pike, 1990). The recent identification of vitamin D response elements in the genes for osteocalcin (Kerner et al., 1989), osteopontin (Noda et al., 1990) and calbindin D-9K (Darwish and DeLuca, 1992), three known targets of 1,25-$(OH)_2D_3$ action, further supports a classic steroid hormone mechanism for 1,25-$(OH)_2D_3$.

Evidence for a genomic mechanism for 1,25-$(OH)_2D_3$ does not preclude the existence of non-genomic actions of the hormone. Indeed, a number of so-called "early effects", which occur on a scale of seconds to minutes and which are insensitive to inhibitors of protein synthesis, have been described for 1,25-$(OH)_2D_3$. These include the stimulation of intestinal calcium transport (Nemere et al., 1984), intracellular cGMP accumulation (Barsony and Marx, 1988) and membrane phosphoinositide hydrolysis (Lieberherr et al., 1989). Because these rapid responses do not occur in the absence of functional 1,25-$(OH)_2D_3$ receptors, it is believed that they too represent receptor-mediated events (Barsony and Marx, 1988; Lieberherr et al., 1989).

Recent studies have revealed the presence of 1,25-$(OH)_2D_3$ receptors in tissues previously not considered targets of vitamin D action. Endocrine tissues, such as the parathyroid gland, cells of ectodermal origin, such as skin fibroblasts, even pathological tissues, including tumours and established cancer cell lines, are among the more than two dozen "new" target tissues for 1,25-$(OH)_2D_3$ currently listed (Reichel et al., 1989). The relative ubiquity of the 1,25-$(OH)_2D_3$ receptor suggests a much wider biological role for vitamin D hormone. Indeed, 1,25-$(OH)_2D_3$ has been implicated in the control of differentiation and proliferation of hematopoietic cells (Abe et al., 1981) as well as other cell types (Reichel et al., 1989). Clearly, the full range of 1,25-$(OH)_2D_3$ actions remains to be defined.

3. Metabolism of vitamin D

Figure 1 illustrates the remarkable anatomical dispersion of 1,25-$(OH)_2D_3$ biosynthesis (reviewed by Fraser, 1980), which begins in the skin with the photochemical conversion of 7-dehydrocholesterol to vitamin D_3. Alternatively, vitamin D_3, as well as vitamin D_2, the equivalent sterol in plants and fungi, can be absorbed by the intestine from dietary sources; both are metabolized similarly in humans. Vitamin D_3 then enters the circulation where it is transported bound to a specific vitamin D binding protein, known as Gc (group specific component) protein or transcalciferin, which increases the capacity of plasma for the hydrophobic vitamin and its metabolites.

Further metabolism occurs in the liver where vitamin D_3 undergoes hydroxylation at carbon 25 to yield 25-hydroxyvitamin D_3 [25OHD_3], the major circulating form of the vitamin. This reaction is not tightly regulated, determined chiefly by the plasma concentration of vitamin D_3 and is catalyzed by a classical mitochondrial cytochrome P450 steroid hydroxylase, 25-hydroxylase (Björkhem and Holmberg, 1978). Although a hepatic microsomal 25-hydroxylase activity has also been decribed in the rat, it is unlikely to be of importance in 25OHD_3 synthesis, since it is present exclusively in the male of the species (Dahlbäck and Wikvall, 1987) and absent from humans of both sexes (Oftebro et al., 1981).

Final metabolic activation takes place in the kidney, where 25OHD_3 is hydroxylated in the 1α position by 25OHD_3-1-hydroxylase [1-hydroxylase], giving rise to 1,25-$(OH)_2D_3$, the major biologically active metabolite of vitamin D, with ≈1000 times the potency of its immediate precursor (Stern, 1981). Extra-renal 1,25-$(OH)_2D_3$ production has also been reported, although primarily in association with pregnancy (Weisman et al., 1978; DeLuca and Schnoes, 1983) and pathological conditions such as sarcoidosis (Barbour et al., 1981; Reichel et al., 1989). The physiological relevance of ectopic 1,25-$(OH)_2D_3$ synthesis is

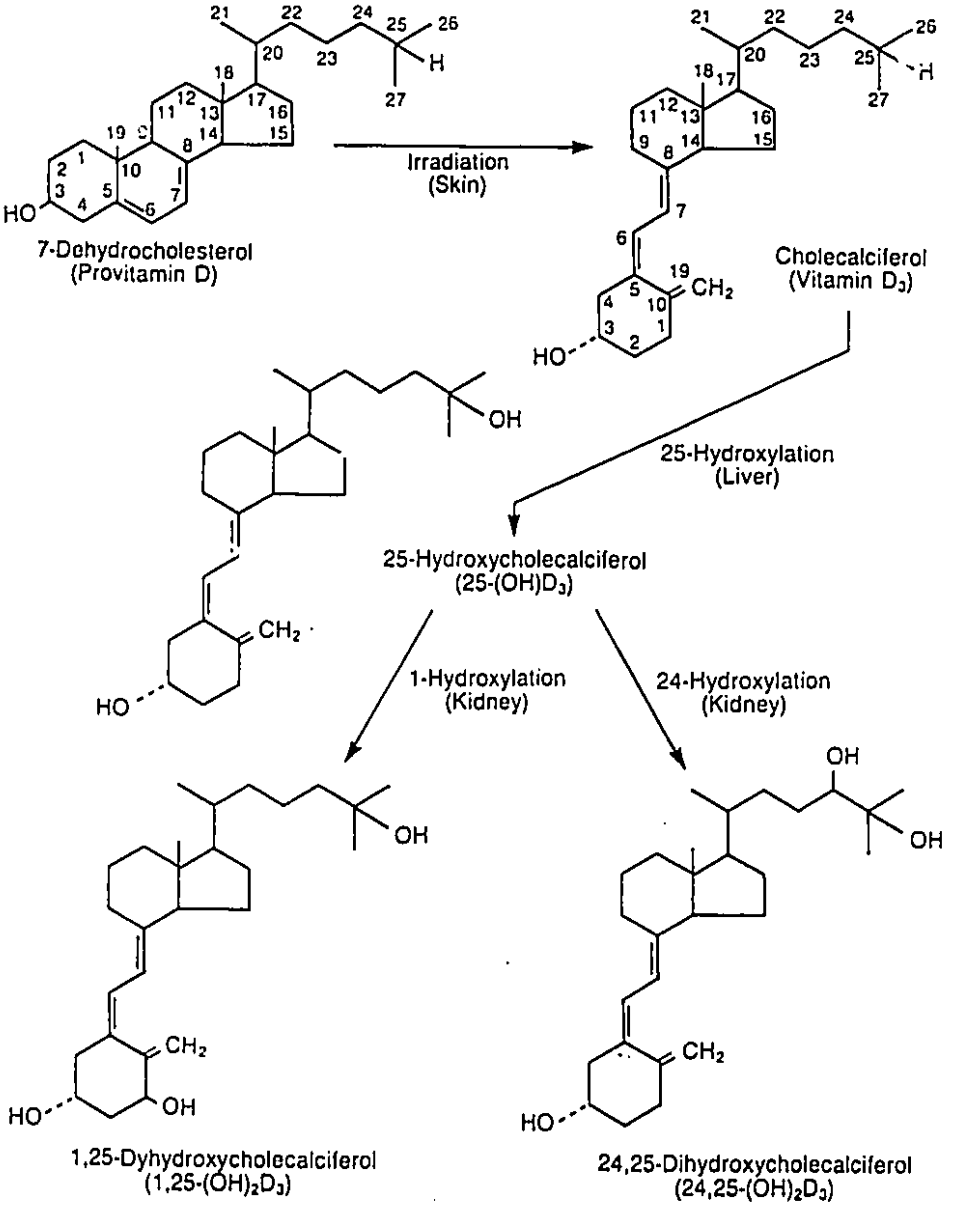

Figure 1. Vitamin D metabolism: biosynthesis of $1,25\text{-}(OH)_2D_3$ and $24,25\text{-}(OH)_2D_3$.

[From Breslau NA: Calcium Homeostasis. In: *Textbook of Endocrine Physiology*, JE Griffin and SR Ojeda, eds., Oxford University Press, Inc., New York, p280, 1988]

unclear and the phenomenon itself remains a controversial issue. There is evidence to suggest that at least some ectopic 1,25-$(OH)_2D_3$ production is non-metabolic, occurring by free-radical chemistry (Hollis, 1989), or that the metabolite produced may not represent true 1,25-$(OH)_2D_3$, but a contaminant which conventional methodologies fail to resolve from vitamin D hormone (Brown and DeLuca, 1985; Horst and Napoli, 1988). It is generally agreed that, under normal physiological conditions, the kidney is the exclusive site of 1,25-$(OH)_2D_3$ synthesis.

The kidney is also the site of an alternative hydroxylation reaction, catalyzed by 25OHD_3-24-hydroxylase [24-hydroxylase], which converts 25OHD_3 to 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$], a major metabolite of as yet undetermined function. Although a role for this metabolite has been suggested in embryonic development (Henry and Norman, 1978) and in bone formation (Ornoy et al., 1978), its physiological importance remains a point of contention. Current theory holds that 24-hydroxylation represents the initial inactivation step in the disposal of 25OHD_3 and its metabolites [see section I.4]. Unlike 1,25-$(OH)_2D_3$ synthesis which is confined to the kidney, 24,25-$(OH)_2D_3$ production occurs in other target tissues for vitamin D such as intestine (Kumar et al., 1978; Mayer et al., 1983a; Tomon et al., 1990ab), bone (Howard et al., 1981; Makin et al., 1989) and skin fibroblasts (Feldman et al., 1982; Gamblin et al., 1985), in addition to kidney. Whereas renal 24,25-$(OH)_2D_3$ synthesis is readily measurable under basal conditions, extra-renal production of this metabolite is detectable only after prior exposure to 1,25-$(OH)_2D_3$ (or its precursors, 25OHD_3 and vitamin D_3) and appears to be receptor-dependent. Indeed, 1,25-$(OH)_2D_3$-inducible 24-hydroxylase activity is considered a sensitive and reliable index of tissue responsiveness to 1,25-$(OH)_2D_3$ (Gamblin et al., 1985).

The renal 1- and 24-hydroxylation reactions share a number of features

in common. Studies using isolated nephron segments microdissected from chick (Brunette et al., 1978) and rat (Kawashima et al., 1981) have localized both catalytic activities to the renal proximal tubule. Both hydroxylation reactions are mediated by mitochondrial cytochrome P450 mixed-function oxidases (Henry and Norman, 1974; Ghazarian et al., 1974; Ohyama et al., 1989; Ohyama and Okuda, 1991) requiring molecular oxygen, NADPH, a non-heme iron sulfur protein or ferredoxin, and a ferredoxin reductase for activity [see Figure 2]. Both activities are tightly and reciprocally regulated by the same physiological factors including phosphorus, calcium, parathyroid hormone and vitamin D itself (Fraser, 1980). Because of these many similarities, it has been suggested that the 1- and 24-hydroxylase activities may represent alternate functions of the same enzyme. Indeed, precedents for this situation have been described for cytochrome P450 hydroxylases in the adrenal cortex (Rapp and Dahl, 1976; Wada et al., 1984; Zuber et al., 1986). However, differences in susceptibility to inhibition by carbon monoxide (Knutson and DeLuca, 1974) and cytochrome P450 inhibitors (Kung et al., 1988), as well as the existence of extra-renal 24-hydroxylase activity in the absence of 1-hydroxylase function, has led to speculation that the two catalytic activities may in fact be mediated by distinct enzyme entities.

Recent advances in the isolation and purification of the renal vitamin D hydroxylases provide further insight into this controversy. Both 1-hydroxylase, partially purified from pig kidney (Gray and Ghazarian, 1989) and 24-hydroxylase, purified to homogeneity from rat kidney (Ohyama et al., 1989; Ohyama and Okuda, 1991) catalyze their respective reactions when reconstituted with the appropriate electron carrying system (NADPH, ferredoxin and ferredoxin reductase); however, neither preparation supports the alternative reaction. These findings provide strong evidence in support of the

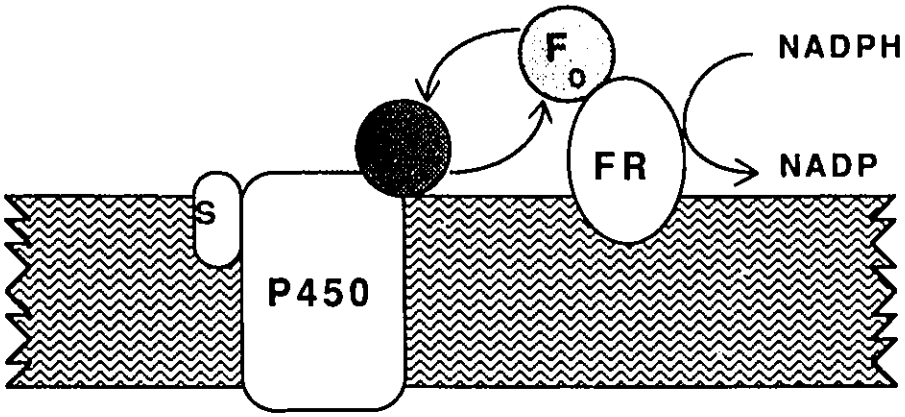

Figure 2. Schematic representation of the steroidogenic electron carrying system of the inner mitochondrial membrane.

Reducing equivalents are transferred sequentially from NADPH to ferredoxin reductase **(FR)**, ferredoxin **(Fo**, oxidized; **Fr**, reduced), cytochrome P450 **(P450)** and substrate **(S)**.

two-enzyme theory. However, whether these two catalytic activities are encoded by separate genes or result from post-translational modification of a single gene product (Ghazarian, 1990) remains to be determined. Expression studies and other molecular biological strategies using the recently cloned cDNA for renal 24-hydroxylase (Ohyama et al., 1991) may provide more conclusive evidence regarding the structural relationship between the renal vitamin D hydroxylases. A non-molecular approach to address this issue was undertaken as part of this thesis and is described in Chapter IV.

4. C24-oxidation and vitamin D catabolism

The steady-state plasma concentration of 1,25-$(OH)_2D$ is determined by the relative rates of its biosynthesis via renal 1-hydroxylase and its metabolism to other compounds via several pathways including hepatic conjugation (Kumar, 1984), C23-oxidation (Napoli and Horst, 1983ab; Ishizuka and Norman, 1987) and C24-oxidation (Jones et al., 1987a; Reddy and Tserng, 1989; Makin et al., 1989). The present section concentrates on the pathways involved in vitamin D catabolism, with particular emphasis on C24-oxidation [see Figure 3], since the first enzyme in this degradative pathway, 24-hydroxylase, forms the main focus of this thesis.

C24-Oxidation. The first suggestion of further metabolism of 1,25-$(OH)_2D_3$ was provided by the work of Frolik and DeLuca (1971,1972), which showed that rats dosed with [^{3}H-26,27]1,25-$(OH)_2D_3$ produced lipid-soluble metabolites more polar than the hormone, as well as substantial amounts of water-soluble radioactive products. Moreover, not all the radioactivity administered as [^{3}H-26,27]1,25-$(OH)_2D_3$ was recovered from these animals, suggesting metabolism of the radiolabel to a volatile product. Confirmation of this hypothesis was obtained in later studies from the same laboratory (Kumar et al., 1976; Harnden

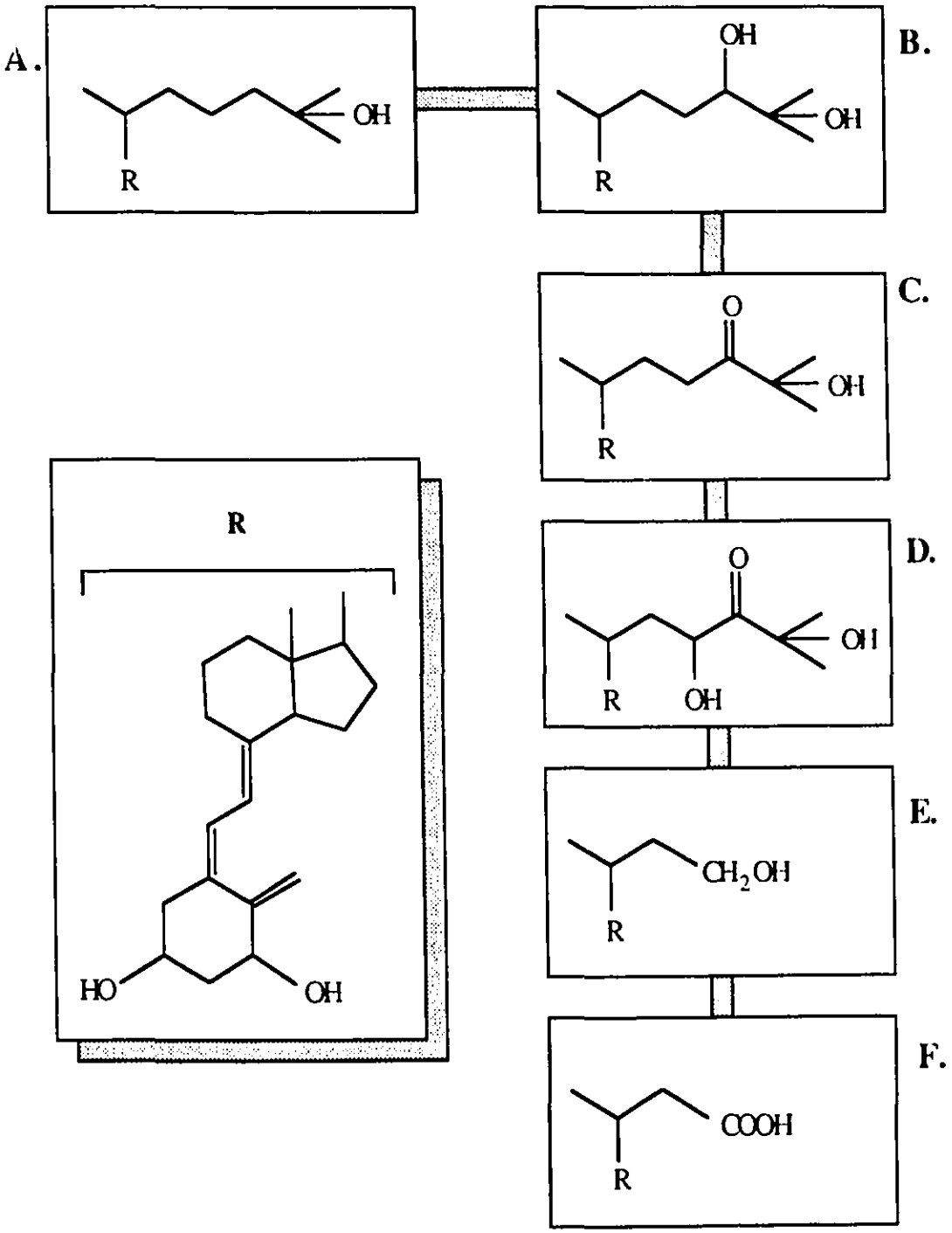

Figure 3. C24-Oxidation pathway for 1,25-$(OH)_2D_3$.

A. 1,25-$(OH)_2D_3$ [vitamin D hormone]; **B.** 1,24,25-$(OH)_3D_3$; **C.** 1,25-$(OH)_2$-24-oxo-D_3; **D.** 1,23,25-$(OH)_3$-24-oxo-D_3; **E.** 1,23-$(OH)_2$-24,25,26,27-tetranor-D_3; **F.** 1-OH-23-COOH-24,25,26,27-tetranor-D_3 [calcitroic acid].

et al., 1976), which showed the release of $^{14}CO_2$ in the air expired from rats administered [^{14}C-26,27]1,25-$(OH)_2D_3$. Because, in both sets of experiments, the radiolabel was located in the side chain of vitamin D hormone [carbons 26 and 27], the findings suggested that oxidative cleavage of the side chain had occurred.

Direct evidence for side chain cleavage was established by Esvelt et al. (1979) who isolated a major side chain-cleaved, water-soluble metabolite of vitamin D hormone from the livers of rats that had been dosed with [^{3}H-3]1,25-$(OH)_2D_3$. The use of 1,25-$(OH)_2D_3$ substrate radiolabelled in the A ring of the steroid backbone permitted detection of the side chain-cleaved product which was identified as 1-hydroxy-23-carboxy-24,25,26,27-tetranor-vitamin D_3 or calcitroic acid. Subsequent investigation of the possible physiological role of this metabolite revealed that it was virtually inactive biologically [in terms of its ability to mobilize calcium from bone and stimulate intestinal calcium absorption] and did not bind the vitamin D receptor or the vitamin D binding protein with great affinity (Esvelt and DeLuca, 1981). Furthermore, calcitroic acid was found to be excreted in bile (Esvelt and DeLuca, 1981). Together, these observations suggested that calcitroic acid was likely a degradative product of 1,25-$(OH)_2D_3$ metabolism. However, the sequence of intermediary metabolites formed during the conversion of 1,25-$(OH)_2D_3$ to its putative inactivation product remained unknown. Similarly, despite its isolation from rat liver (Esvelt et al., 1979) and subsequent discovery in other tissues including intestine, bone and kidney (Esvelt and DeLuca, 1981), the exact site(s) of calcitroic acid production had not been determined.

In 1973, Holick et al. identified 1,24,25-trihydroxyvitamin D_3 [1,24,25-$(OH)_3D_3$] as the initial product of further metabolism of 1,25-$(OH)_2D_3$ in rat kidney, thereby establishing 24-hydroxylation as the first step of what is known

today as the C24-oxidation pathway. Subsequent studies (reviewed by Jones et al., 1987a), primarily in the kidney, revealed the complete sequence of reactions known to date, shown in Figure 3. The final link in the catabolic chain was provided by Reddy and Tserng (1989) who established the C-23 alcohol, 1,23-dihydroxy-24,25,26,27-tetranor-vitamin D_3, as the immediate precursor of calcitroic acid in the kidney. Their study also demonstrated that calcitroic acid was the major water-soluble renal metabolite produced under physiological conditions. Although another minor metabolite (less than 10% of total water-soluble radioactivity) more polar than calcitroic acid was also detected, its identity remains unknown.

Thus, it is well established that the kidney contains the full complement of enzymatic reactions required for the conversion of 1,25-$(OH)_2D_3$ to calcitroic acid. As mentioned in the previous section, 24-hydroxylase, the first enzyme in this sequence, can be induced in a number of vitamin D target tissues such as intestine (Kumar et al., 1978), bone (Howard et al., 1981) and skin (Feldman et al., 1982). One might anticipate that these other target tissues, like the kidney, also possess the complete sequence of reactions which constitute the C24-oxidation pathway. Indeed, the entire C24-oxidation pathway has recently been demonstrated in cultured bone cells (Makin et al., 1989) and most of the intermediates have been identified in intestine (Mayer et al., 1983a; Napoli and Horst, 1983a).

It is generally agreed that calcitroic acid is the major excretory product of 1,25-$(OH)_2D_3$. Accordingly, the C24-oxidation pathway which generates calcitroic acid may be considered primarily a degradative pathway for vitamin D hormone. Further support for a catabolic role derives from the demonstration that biological activity [as determined by intestinal calcium absorption and bone calcium mobilization] declines as 1,25-$(OH)_2D_3$ is sequentially metabolized to

1,24,25-$(OH)_3D_3$, 1,25-$(OH)_2$-24-oxo-D_3 and 1,23,25-$(OH)_3$-24-oxo-D_3 (Mayer et al., 1983b) and that C24-oxidation products, in general, exhibit lower affinities for the vitamin D receptor than does the native hormone (Stern, 1981; Mayer et al., 1983b). In addition, the pathway is accelerated in kidney and induced in other target tissues by 1,25-$(OH)_2D_3$ itself.

The catabolic C24-oxidation pathway would confer a major advantage to the organism by helping to guard it against the toxic effects of 1,25-$(OH)_2D_3$ in conditions of hormone excess. In this respect, it is of interest that the C24-oxidation pathway can also effectively metabolize $25OHD_3$. Although the biological activity of $25OHD_3$ is considerably lower than that of 1,25-$(OH)_2D_3$, owing to its comparatively lower affinity for the vitamin D receptor (Stern, 1981; DeLuca, 1988), at high concentrations, such as those occurring during hypervitaminosis D, $25OHD_3$ can bind the vitamin D receptor in sufficient amounts to elicit the physiological effects of the hormone. Thus, C24-oxidation would provide protection against the potentially harmful effects of excess $25OHD_3$, as well as 1,25-$(OH)_2D_3$. Although the possibility remains that C24-oxidation may represent a pathway for the generation of metabolites with specialized functions distinct from the classical effects associated with vitamin D hormone, until conclusive evidence for such a role is established, this pathway may be regarded as a process for the inactivation and disposal of vitamin D metabolites.

As mentioned above, 1,25-$(OH)_2D_3$ treatment stimulates C24-oxidation in the kidney and induces the catabolic pathway in other vitamin D target tissues. The C24-oxidation cascade comprises a series of sequential reactions which include 24-hydroxylation, 24-oxidation, 23-hydroxylation and side chain cleavage [Figure 3]. It is not clear at present whether the various reactions in this catabolic sequence are catalyzed by several separate enzymes whose

activities are tightly coordinated or a single enzyme with multiple activities. It is well established that the first reaction in the C24-oxidation pathway involves a cytochrome P450, 24-hydroxylase (Ohyama et al., 1989; Ohyama and Okuda, 1991). Steroidogenic cytochrome P450s are well known for their ability to catalyze several sequential and coordinated reactions at a single active site (Hall, 1985; Kühn-Velten et al., 1991).

Similarly, it is not evident whether the renal and extra-renal 24-hydroxylase activities are mediated by the same gene product. Although kinetically similar (Tenenhouse and Jones, 1987), the two catalytic activities can be distinguished by a number of features. Whereas detection of extra-renal 24-hydroxylase activity requires prior exposure to supraphysiological doses of vitamin D hormone (or its precursors, $25OHD_3$ or vitamin D_3), the renal enzyme activity is readily measurable under un-induced or basal conditions. Furthermore, only basal renal 24-hydroxylase activity is perturbed in the mutant X-linked hypophosphatemic (Hyp) mouse [see section I.6]; $1,25\text{-}(OH)_2D_3$-inducible activity is comparable in both normal and mutant animals (Tenenhouse and Jones, 1987; Jones et al., 1987b). A number of studies have shown $1,25\text{-}(OH)_2D_3$-mediated induction of 24-hydroxylase activity to be sensitive to inhibitors of mRNA and protein synthesis (Henry, 1979; Colston and Feldman, 1982; Tomon et al., 1990ab), suggesting that de novo synthesis of 24-hydroxylase may be required for the induction process. This hypothesis was confirmed by the recent demonstration that induction of 24-hydroxylase activity is associated with an increase in mRNA levels for the cytochrome P450 component of 24-hydroxylase (Ohyama et al., 1991; Armbrecht and Boltz, 1991). One possible interpretation of these findings is that the inducible 24-hydroxylase may represent an isozyme of the constitutively-expressed renal enzyme. The studies presented in Chapters II, III and IV of this thesis, as

well as the data included in Appendix A, have some interesting implications in this regard.

C23-Oxidation. Another route by which certain vitamin D target tissues, such as kidney and intestine, can further metabolize 1,25-$(OH)_2D_3$ is the C23-oxidation pathway (Napoli and Horst, 1983ab; Napoli and Martin, 1984; Ishizuka and Norman, 1987), depicted in Figure 4. As with the C24-oxidation pathway, both 1,25-$(OH)_2D_3$ and its immediate precursor, 25OHD_3, can act as substrates for C23-oxidation. The end products of this pathway, 1,25-$(OH)_2D_3$-26,23-lactone (Ishizuka et al., 1984) or 25OHD_3-26,23-lactone (Wichmann et al., 1979), depending on the substrate, have no known physiological function at present. However, the fact that metabolites of C23-oxidation have much lower affinities for the vitamin D receptor than does the native hormone (Napoli and Horst, 1983b) and that the pathway can be induced several-fold by exposure to either vitamin D_3 or 1,25-$(OH)_2D_3$ (Horst and Littledike, 1980; Engstrom et al., 1986) suggests a degradative role for C23-oxidation.

The relative contributions of the C23- and C24-oxidation pathways to the overall disposal of vitamin D metabolites is unclear. Engstrom et al. (1986) compared 23- and 24-hydroxylase activities, in renal homogenates prepared from eight animal species, before and after 1,25-$(OH)_2D_3$ treatment. Although their findings suggest that the physiological importance of the C23-oxidation pathway may well depend on the species examined, their results also indicate that in most species, C24-oxidation is the dominant pathway. This is particularly true of the mouse, in which renal 23-hydroxylation represents less than 10% of 24-hydroxylase activity (Engstrom et al., 1986). Further support for these findings derives from studies of 1,25-$(OH)_2D_3$ metabolism in perfused rat kidney (Reddy and Tserng, 1989; Makin et al., 1989) and both cultured bone cells [UMR-106 (Makin et al., 1989)] and porcine kidney cells [LLC-PK_1 (Napoli and

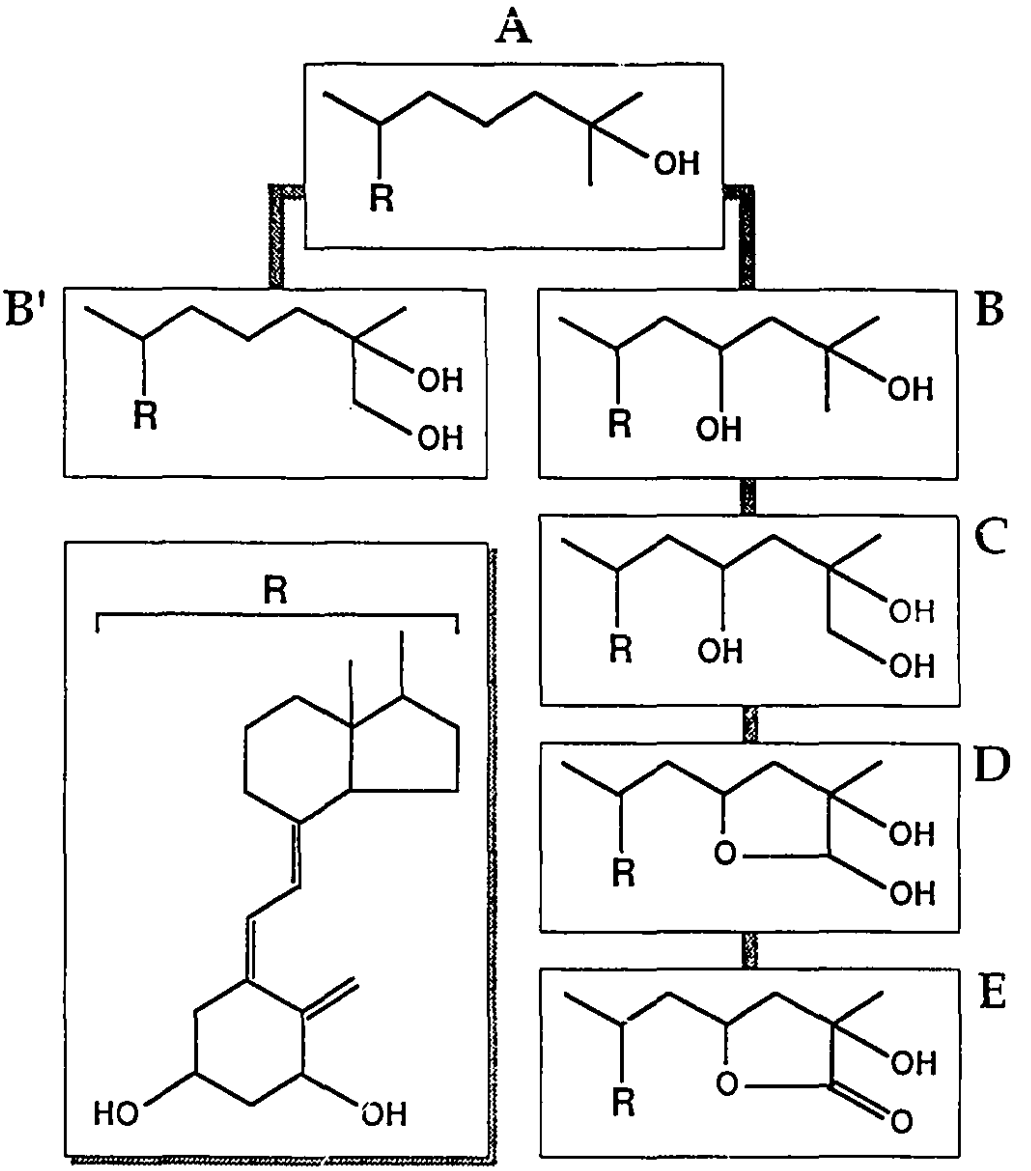

Figure 4. C23-Oxidation pathway for 1,25-$(OH)_2D_3$.

A. 1,25-$(OH)_2D_3$ [vitamin D hormone]; **B.** 1,23(*S*),25-$(OH)_3D_3$; **B'.** 1,25(*R*),26-$(OH)_3D_3$; **C.** 1,23(*S*),25(*R*),26-$(OH)_4D_3$; **D.** 1,25(*R*)-$(OH)_2D_3$-26,23(*S*)-lactol; **E.** 1,25(*R*)-$(OH)_2D_3$-26,23(*S*)-lactone.

Martin, 1984)], which have either described 1,25-$(OH)_2D_3$-26,23-lactone as a minor metabolite relative to C24-oxidation products or have failed to detect the presence of the lactone or any of its precursors. By contrast, in vivo intoxication studies have shown both 1,25-$(OH)_2D_3$-26,23-lactone (Ishizuka et al., 1984) and 25OHD_3-26,23-lactone (Horst, 1979; Wichmann et al., 1979) to be major circulating metabolites, suggesting that C23-oxidation is a prominent vitamin D catabolic pathway. However, higher circulating levels of the lactone forms need not imply a higher rate of conversion via the C23-oxidation pathway, but may instead reflect the higher affinity of these metabolites, relative to C24-oxidation products, for the vitamin D binding protein (Horst, 1979), which would favour their accumulation in plasma. Thus, metabolism of 1,25-$(OH)_2D_3$ to 1,25-$(OH)_2D_3$-26,23-lactone via the C23-oxidation pathway does not appear to represent a major degradative route for the hormone, compared to C24-oxidation. However, the demonstration of preferential 23-hydroxylation of 1,25-$(OH)_2$-24-oxo-D_3, relative to 1,25-$(OH)_2D_3$, suggests that the physiologically relevant role for the C23-oxidation pathway might be the further processing of C24-oxidized metabolites (Napoli and Horst, 1983a; Napoli and Martin, 1984).

Hepatic conjugation. Although calcitroic acid was originally isolated from rat liver (Esvelt et al., 1979), it is unclear whether the liver has the intrinsic capacity to synthesize this metabolite or whether it merely concentrates calcitroic acid produced elsewhere, from plasma, for excretion into bile. Nevertheless, there is evidence supporting hepatic metabolism of 1,25-$(OH)_2D_3$ to compounds other than calcitroate. Analysis of bile samples collected from rats dosed with [^{3}H]1,25-$(OH)_2D_3$ revealed the presence of both glucuronides and sulfates of vitamin D hormone (Kumar. 1984). Whereas the 1,25-$(OH)_2D_3$-sulfates were completely inert with respect to bone calcium mobilization and intestinal calcium absorption, the glucuronides showed marginal biological

activity. However, the latter effect was attributed to free hormone released from 1,25-$(OH)_2D_3$-glucuronides by the action of endogenous glucuronidases.

5. Regulation of renal vitamin D metabolism

As mentioned above [section I.3], the renal 1- and 24-hydroxylase activities are tightly and reciprocally regulated by a number of physiological factors including, calcium, phosphorus, parathyroid hormone (PTH) and vitamin D itself (Fraser, 1980). In conditions associated with hypocalcemia, hypophosphatemia, hyperparathyroidism and vitamin D deficiency, renal 1,25-$(OH)_2D_3$ synthesis is stimulated, whereas in the opposite states, 24,25-$(OH)_2D_3$ production predominates. Although the effects of these and other regulatory signals on renal vitamin D metabolism have been well documented, the precise biochemical mechanism(s) involved have not yet been fully elucidated.

There are a number of mechanisms or pathways whereby extracellular factors, such as peptide hormones, neurotransmitters and growth factors, transmit their regulatory messages across target cell membranes and elicit specific cellular responses. Two of the better known transmembrane signal transduction pathways are the cAMP pathway and the phosphoinositide cascade, both of which are depicted in Figure 5. Both pathways operate on a single principle which integrates conversion of an extracellular signal into an intracellular message, translation of the internal message into a regulatory activity and modulation of specific cellular proteins, resulting ultimately in the appropriate cellular response. Only the specific components which mediate these cellular processes differ between the two pathways.

The cAMP pathway (reviewed by Krebs, 1989), illustrated in Figure 5, begins with a plasma membrane receptor which specifically recognizes and binds the incoming signal or "primary messenger". The agonist-bound receptor

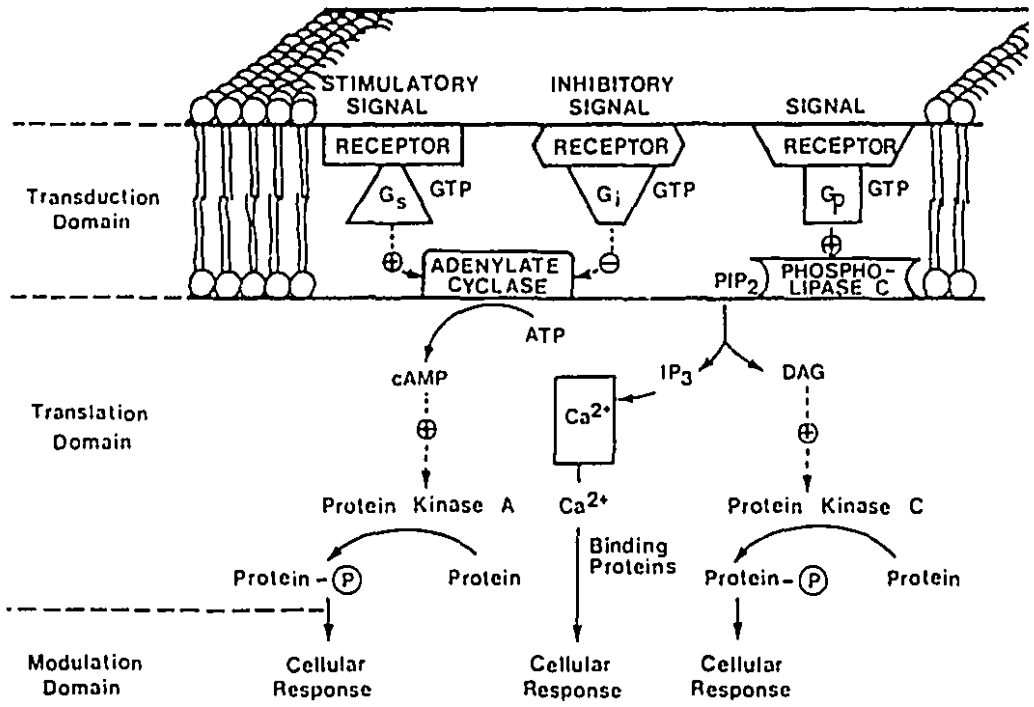

Figure 5. Schematic representation of the cAMP and phosphoinositide signal transduction pathways.

Abbreviations: G_S, stimulatory G protein; G_I, inhibitory G protein; G_p, phospholipase C-specific G protein; PIP_2, phosphatidylinositol-4,5-bisphosphate; IP_3, inositol-1,4,5-trisphosphate; **DAG**, diacylglycerol. [From Mooibroek MJ, Wang JH: Biochem Cell Biol 66:557-566; 1988]

then activates the next component, a guanine nucleotide regulatory protein or G protein, which couples the receptor to a third element, a membrane-associated effector protein, adenylate cyclase. There are two types of G protein that can associate with adenylate cyclase, G_s and G_i; these are involved in the stimulation and inhibition of the enzyme, respectively. If coupling occurs through G_s, the cyclase elaborates cAMP, an intracellular message or "second messenger", which transmits its information to a regulatory protein, cAMP-dependent protein kinase (protein kinase A). The kinase then phosphorylates key cellular proteins, thereby altering their activities, resulting in specific cellular responses. If receptor-effector coupling occurs through G_i, then the opposite effects are observed.

Initiation of signal transduction through the phosphoinositide pathway (reviewed by Pfeilschifter, 1989; Rana and Hokin, 1990), depicted in Figure 5, also requires a cell surface receptor and a G protein (G_p), however, in this case, the G protein is coupled to a membrane-bound phosphodiesterase, phospholipase C. The phospholipase catalyzes the hydrolysis of the membrane phospholipid, phosphatidylinositol-4,5-bisphosphate (PIP_2), to generate two intracellular second messengers, inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 diffuses into the cytoplasm and mobilizes calcium from intracellular stores, primarily the endoplasmic reticulum. Most of the effects of the newly released calcium are mediated by specific calcium-binding proteins, such as calmodulin, which interact with selected enzymes or proteins and modulate their activities. The other second messenger, DAG, remains in the plasma membrane, where it acts in concert with specific membrane phospholipids and the newly released calcium to facilitate the activation of another protein kinase, protein kinase C. Changes in the activities of specific target proteins, as a result of protein kinase C-mediated phosphorylation, elicit

the cellular responses dictated by the original incoming signal.

PTH is a major regulator of renal vitamin D metabolism. It is also one of the most extensively studied. In addition, PTH has been shown to exert its regulatory effects on renal vitamin D metabolism via both major cellular signalling pathways, cAMP/protein kinase A and diacylglycerol/protein kinase C. For these reasons and also because all three of these subjects - PTH, cAMP and protein kinase C - are implicated in the studies described in the chapters that follow (Chapters II and III), the present section will focus on the regulation of renal vitamin D metabolism by PTH.

Parathyroid hormone. PTH synthesis (reviewed by Potts et al., 1982) occurs in the chief cells of the parathyroid glands and is regulated primarily by the plasma calcium concentration, although recent evidence suggests a role for vitamin D hormone as well (Silver et al., 1986). Hypocalcemia stimulates PTH synthesis and secretion whereas hypercalcemia suppresses both processes. PTH is synthesized as a 115 amino acid precursor, preproPTH, which undergoes two sequential enzymatic cleavages to yield the final 84 amino acid secretory product. The biological activity of PTH is confined to amino acid residues 1-34 of the amino terminal portion of the molecule, which interacts with specific PTH receptors located on the plasma membrane of target cells. Both the cDNA and the gene for PTH (reviewed in Kemper, 1986) as well as the cDNA for its receptor (Jüppner et al., 1991) have been cloned, and their full sequences and structures are known.

The physiological role of PTH, like that of vitamin D hormone, involves the maintenance of calcium homeostasis, which it achieves by acting on its two target tissues, bone and kidney (Potts et al., 1982). It acts directly on bone to stimulate resorption and promote the release of calcium (and phosphorus) into the extracellular fluid. In the kidney, PTH increases calcium reabsorption and

phosphate excretion, and also stimulates the synthesis of 1,25-$(OH)_2D_3$ which, in turn, enhances intestinal calcium absorption [see section I.2]. Concomitant with its stimulatory effect on renal 1,25-$(OH)_2D_3$ synthesis, PTH also inhibits 24,25-$(OH)_2D_3$ production.

Some of the earliest evidence suggesting a role for PTH in the regulation of renal vitamin D metabolism derives from the work of Garabedian et al. (1972) which showed that rats maintained on a calcium-deficient diet exhibited elevated plasma levels of 1,25-$(OH)_2D_3$ which decreased dramatically following parathyroidectomy and that the fall in circulating 1,25-$(OH)_2D_3$ could be prevented by injecting the animals with PTH after surgery. Similar experimental findings in other species such as the chick (Fraser and Kodicek, 1973) as well as clinical observations in humans (Haussler et al., 1976) provided further support for PTH involvement in the regulation of renal vitamin D metabolism.

Evidence for a direct effect of PTH on renal vitamin D metabolism was provided by Rasmussen et al. (1972) and Larkins et al. (1974) who showed, independently, that addition of PTH to renal tubules, freshly isolated from vitamin D-deficient chicks, stimulated the conversion of 25OHD_3 to 1,25-$(OH)_2D_3$. Subsequent studies confirmed the direct stimulatory action of PTH on renal 1,25-$(OH)_2D_3$ synthesis in a number of species, using various renal tissue preparations including, slices from chick (Rost et al., 1981) and rat (Armbrecht et al., 1982; 1984ab), tubule segments from guinea pig (Kremer and Goltzman, 1981) and cultured cells isolated from chick (Trechsel et al., 1979; Henry, 1981,1985) and mouse (Fukase et al., 1982; Korkor et al., 1987). A number of these studies also demonstrated a direct inhibitory effect of PTH on renal 24,25-$(OH)_2D_3$ production (Henry, 1981,1985; Armbrecht et al., 1982,1984ab).

PTH and the cAMP pathway. Based on the fact that one of the earliest manifestations of PTH action on the kidney is the stimulation of cAMP

production (Chase and Aurbach, 1967) and that cAMP had already been implicated as an intracellular mediator of the actions of other peptide hormones (Sutherland and Rall, 1960), the involvement of cAMP in the regulation of renal vitamin D metabolism by PTH was examined.

Intravenous infusion of whole animals with either cAMP or its more permeable derivative, dibutyryl cAMP, elicited changes in renal vitamin D metabolism analogous to those observed in response to PTH (Horiuchi et al., 1977; Shigematsu et al., 1986). Addition of exogenous cAMP or dibutyryl cAMP to various renal tissue preparations resulted in PTH-like stimulation of 1,25-$(OH)_2D_3$ synthesis (Rasmussen et al., 1972; Larkins et al., 1974; Rost et al., 1981; Henry, 1981) and inhibition of 24,25-$(OH)_2D_3$ production (Henry, 1981). Similarly, increasing endogenous cAMP accumulation through the use of forskolin, a known activator of adenylate cyclase [see below (Seamon and Daly, 1981)], also mimicked the effects of PTH on renal vitamin D metabolism (Armbrecht et al., 1984a; Henry, 1985). The lack of additivity of forskolin and PTH effects suggested a common mechanism for the two agonists, one involving cAMP.

The subsequent steps of the cAMP pathway [see Figure 5], those extending beyond PTH-stimulated generation of cAMP, have also been documented. Armbrecht et al. (1984b) demonstrated that protein kinase A activity was stimulated following PTH-treatment of rat kidney slices and established a temporal correlation between PTH-induced increases in cAMP accumulation, protein kinase A activity and changes in vitamin D metabolism. Studies in primary cultures of chick kidney cells showed that protein kinase A-mediated phosphorylation of endogenous proteins was also enhanced in response to PTH treatment (Noland and Henry, 1983). Moreover, the PTH-induced phosphorylation pattern could be mimicked by dibutyryl cAMP.

Although the identities of these endogenously phosphorylated substrates were unknown, the possibility remained that one or more of these proteins might be responsible for the PTH-related changes in vitamin D metabolism.

Studies of the adrenal mitochondrial steroid hydroxylating system revealed that 11β-hydroxylase, a cytochrome P450, was phosphorylated by protein kinase A (DeFaye et al., 1982). Although the physiological significance of this event, if any, was not known, it nevertheless prompted investigators to examine whether any of the components of the renal mitochondrial vitamin D hydroxylating system (cytochrome P450, ferredoxin and ferredoxin reductase) might be modified in a similar manner. Preliminary observations suggested that in vitro phosphorylation of the cytochrome P450 component of the 1-hydroxylase complex had no effect on enzyme activity (Ghazarian and Yanda, 1985). However, the same study suggested that ferredoxin phosphorylation may be involved in the modulation of 1-hydroxylase function. Later studies by Armbrecht and associates supplied three lines of evidence in support of this hypothesis. They demonstrated that purified ferredoxin could be directly phosphorylated and reversibly dephosphorylated in vitro; that in vitro phosphorylation of ferredoxin was associated with inhibition of 1-hydroxylase activity in a reconstituted system; and that dephosphorylation of ferredoxin in response to PTH treatment of rat renal cortical slices accompanied the stimulation of 1-hydroxylase activity (Nemani et al., 1989; Siegel et al., 1986). More recently, Mandel et al. (1990) showed that ferredoxin phosphorylation not only inhibited 1-hydroxylase function but also stimulated 24-hydroxylase activity.

Although it may seem somewhat paradoxical that stimulation of 1-hydroxylase activity by PTH is associated with an increase in protein kinase A activity and a decrease in the phosphorylation state of ferredoxin, this apparent

inconsistency may be resolved by implicating a phosphatase as the intermediary in this cascade of events. Thus, PTH binds to its cell surface receptor, thereby activating adenylate cyclase and increasing intracellular cAMP content. The cAMP then activates protein kinase A which phosphorylates the putative phosphatase. The activated phosphatase then dephosphorylates ferredoxin, thereby modulating renal vitamin D metabolism. It is not clear how phosphorylation alters ferredoxin function. It is known that ferredoxin acts as a shuttle, ferrying electrons from the reductase component to the cytochrome P450 moiety of the enzyme complex (Hall, 1985). Phosphorylation of the iron-sulfur protein might alter its ability to interact with one or both of these components. It this regard, it is noteworthy that upon phosphorylation, ferredoxin becomes less effective at supporting 1-hydroxylase activity but more efficient at supporting 24-hydroxylase function (Mandel et al., 1990), suggesting that the association between ferredoxin and the cytochrome P450 component(s) is altered.

The above findings suggest that ferredoxin phosphorylation-dephosphorylation is an important mechanism in the regulation of renal vitamin D metabolism by PTH. It is of interest that a similar regulatory mechanism has recently been proposed for the adrenal mitochondrial steroid hydroxylating system (Monnier et al., 1987). However, in that system, in vitro phosphorylation of adrenal ferredoxin (adrenodoxin) by protein kinase A is associated with stimulation of two enzyme activities, namely 11β-hydroxylase and cholesterol side chain cleavage enzyme, rather than inhibition of enzyme function.

There is evidence to suggest that PTH may regulate renal vitamin D metabolism by an additional cAMP-dependent mechanism, distinct from the rapid phosphorylation-dephosphorylation process described above. A five minute incubation with PTH is sufficient to elicit near-maximal levels of protein

kinase A activity (Armbrecht et al., 1984b) and maximal dephosphorylation of ferredoxin (Siegel et al., 1986) in rat renal cortical slices. Although rapid (30 minutes or less) alterations in renal vitamin D metabolism, consistent with the above time frame, are commonly observed in response to PTH treatment, there are a number of studies in which the vitamin D response does not follow this pattern. Studies involving cultured renal epithelial cells (Henry, 1981, 1985; Korkor et al., 1987) and renal tissue preparations from thyroparathyroidectomized, vitamin D-deprived animals (Armbrecht et al., 1984ab) show that several hours of exposure to PTH (or forskolin) are required for changes in vitamin D metabolism to become manifest, despite a rapid increase in intracellular cAMP levels. The demonstration that this "slow" increase in 1,25-$(OH)_2D_3$ production is sensitive to inhibition by both cycloheximide and actinomycin D (Korkor et al., 1987) suggests a requirement for de novo protein synthesis and implies an additional cAMP-dependent regulatory mechanism for PTH. It is well established that cAMP can alter gene expression, primarily through transcriptional activation, via distinct promoter elements located within specific target genes. These cAMP response elements or CREs are known to bind specific phosphoprotein transcription factors, some of which are phosphorylated by protein kinase A and may be involved in the regulation of cAMP-responsive genes. Such a mechanism has been demonstrated for the better characterized adrenal steroid hydroxylase system (Simpson et al., 1990) and may well be operative in renal vitamin D metabolism.

Forskolin. As mentioned above, forskolin [depicted in Figure 7, Chapter III] is a naturally occurring diterpene well known for its ability to activate adenylate cylcase directly (reviewed by Seamon and Daly, 1981; 1986). Originally isolated from the root of an Indian medicinal plant, forskolin was rapidly recognized for its unique pharmacological properties which included potent

cardiotonic and vasodilatory effects. The basis for these actions was revealed in subsequent studies which showed that forskolin treatment of rabbit heart slices produced a rapid, dose-dependent stimulation of adenylate cyclase and protein kinase A activities, suggesting that forskolin effects were mediated via adenylate cyclase activation and subsequent generation of cAMP. Direct activation of adenylate cyclase by forskolin was established based on the observation that forskolin-induced stimulation of cAMP synthesis was rapid and reversible, and occurred in the absence of the G protein (G_s) required for hormonal stimulation of adenylate cyclase as well as in solubilized preparations of the enzyme.

The ability of forskolin to directly activate adenylate cyclase in both isolated membranes as well as in intact cells and tissues has made it an invaluable tool to probe cAMP involvement in various physiological processes (Seamon and Daly, 1986). Recently, however, forskolin has been shown to exert a number of its effects independently of cAMP production (Laurenza et al., 1989). Virtually all cAMP-independent effects of forskolin reported to date involve the modulation of plasma membrane transporter and channel functions such as glucose transport (Kashiwagi et al., 1983; Joost and Steinfelder, 1986), phosphate transport (Cole et al., 1988) and voltage-dependent potassium channel activity (Hoshi et al., 1988). There is no evidence at present to support adenylate cyclase-independent actions of forskolin on proteins that are not transporters or channels of the plasma membrane.

Part of the evidence supporting cAMP involvement in the regulation of renal vitamin D metabolism by PTH derives from studies demonstrating that PTH-like effects on both 1,25-$(OH)_2D_3$ synthesis and 24,25-$(OH)_2D_3$ production can be elicited by forskolin [see above (Armbrecht et al., 1984a; Henry, 1985)]. It would be of interest to know whether these effects of forskolin might involve a

cAMP-independent mechanism. Such a finding would not, in any way, dispute the involvement of the cAMP pathway in the regulation of renal vitamin D metabolism by PTH. However, the demonstration of a cAMP-independent effect of forskolin on the mitochondrial membrane-associated hydroxylase(s) would indicate that non-classical effects of forskolin are not restricted to plasma membrane transporters and channels, but may in fact represent a more generalized phenomenon. This issue forms the basis for the study described in Chapter III of the present thesis.

PTH and the phosphoinositide pathway. The first suggestion of an association between PTH and the phosphoinositide pathway [Figure 5] in kidney was provided by Lo et al. (1976) who showed that upon exposure to PTH, cat renal cortical slices increased ^{32}P incorporation into inositol phosphate and phosphatidic acid, a phenomenon known as "the phosphoinositide effect", indicating that membrane phosphoinositide turnover had been stimulated.

Phospholipase C, the phosphodiesterase responsible for initiating receptor-stimulated phosphoinositide breakdown, has been identified in both the brush-border (Schwertz et al., 1983) and basolateral (Rogers and Hammerman, 1987) surfaces of the polarized renal epithelial cell. Because the basolateral membrane is in contact with the peritubular fluid and the various circulating regulatory factors therein and because it has traditionally been viewed as the exclusive site for peptide hormone receptors, including the receptor for PTH (Shlatz et al., 1975; Zull et al., 1977), the demonstration of phospholipase C at this surface has been considered supportive of an association between PTH and the phosphoinositide pathway. By contrast, the occurrence of high levels of phospholipase C activity at the renal brush-border membrane has remained somewhat of an enigma. Recent studies, however, suggest that peptide hormone receptors may also be present at the renal brush-

border membrane. Receptors for angiotensin II, a peptide hormone which regulates renal fluid reabsorption, have been found to be uniformly distributed on both the brush-border and basolateral surfaces of the renal epithelial cell (reviewed by Douglas, 1987). More interestingly, a similar, bipolar distribution has recently been proposed for the PTH receptor based on the observation that PTH effectively regulates brush-border membrane phosphate transport in opossum kidney (OK) cells grown on permeable supports when applied to either cell surface (Reshkin et al., 1990). Thus, the presence of phospholipase C at the renal brush-border membrane, like that at the basolateral surface, may indeed be related to the presence of peptide hormone receptors. In addition, other ligand-binding components of the renal epithelial cell membrane, such as ion transport systems and sodium-solute cotransporters, may also be coupled to phospholipase C, as has been proposed for the small intestine (Vaandrager et al., 1990; MacLeod et al., 1992).

Direct evidence linking PTH to the two second messengers of the phosphoinositide pathway, IP_3 and diacylglycerol, was obtained by Hruska et al. (1987) who demonstrated that PTH elicited an immediate, concentration-dependent increase in IP_3 production, diacylglycerol release and PIP_2 hydrolysis in OK cells, as well as in primary cultures of canine proximal tubule cells and basolateral membranes isolated from dog kidney. The increase in PIP_2 hydrolysis was evident within ten seconds of exposure to PTH; levels returned to normal within one minute. A concomitant, transient increase in cytosolic calcium was also observed in PTH-treated OK cells and was found to be dependent upon intracellular calcium stores. None of these effects of PTH was mimicked by cAMP. More recent studies have confirmed the original observations with respect to PTH-stimulated IP_3 production, diacylglycerol release and calcium transient formation (reviewed by Murer et al., 1991).

Together, these findings provide strong evidence supporting PTH activation of the phosphoinositide pathway.

The demonstration that PTH can evoke both the formation of a calcium transient and the release of diacylglycerol in renal epithelial cells suggests that both branches of the phosphoinositide pathway may be involved in mediating the actions of the peptide hormone in kidney. Preliminary findings suggest that PTH stimulates calcium-calmodulin-dependent protein phosphorylation in isolated renal basolateral membranes (Hruska et al., 1987) and there is some evidence supporting a second messenger role for calcium in the stimulation of renal gluconeogenesis by PTH (Hruska et al., 1986). Despite these indications, our understanding of the calcium branch of the phosphoinositide pathway in mediating the biological effects of PTH remains limited and, for this reason, will not be addressed here. Instead, the present discussion will focus on the other, better characterized limb of the phosphoinositide pathway, that involving diacylglycerol and the activation of protein kinase C.

Protein kinase C is a widely-distributed serine/threonine kinase which requires calcium, phospholipid (phosphatidylserine) and diacylglycerol for optimum activity (reviewed by Nishizuka, 1986; Huang, 1989). Molecular analyses indicate that protein kinase C comprises a family of at least eight closely-related yet distinct isozymes which are derived from multiple genes as well as from alternative splicing. These isozymes are characterized by unique structural features and exhibit subtle differences in tissue and subcellular distribution, substrate specificity, co-factor preference and developmental expression (Kikkawa et al., 1989; Huang, 1989).

Protein kinase C is activated endogenously by diacylglycerol which, as mentioned earlier, is formed transiently in the membrane as a result of receptor-stimulated, phospholipase C-mediated PIP_2 hydrolysis. Protein kinase C can

also be activated by phorbol esters, potent tumour-promoting agents, such as phorbol 12-myristate 13-acetate [PMA or TPA (reviewed by Castagna, 1987)]. These agents mimic the effect of diacylglycerol by virtue of their structural similarity to this endogenous activator and offer a useful experimental tool to probe the involvement of protein kinase C in cellular function. Activation of protein kinase C, whether by diacylglycerol or phorbol ester, is associated with translocation of the enzyme from the cytosolic compartment to the membrane compartment. Chronic activation of the kinase leads to eventual cellular depletion of the enzyme or "down-regulation", most likely as a result of proteolytic degradation. The latter phenomenon is frequently observed in response to prolonged exposure to phorbol esters which, in contrast to diacylglycerol, are not rapidly metabolized and persist in the cell.

Protein kinase C mediates the effects of various regulatory factors by phosphorylating specific target proteins, thereby modulating their activities, resulting in specific cellular responses. Although many endogenous substrates have been demonstrated for protein kinase C in various tissues, for the most part, their identities remain unknown. Conversely, many functionally defined proteins have been proposed as substrates for protein kinase C on the basis of in vitro experiments (Nishizuka, 1986), however, the physiological significance of these phosphorylation reactions remains to be defined. Several of these putative protein kinase C substrates have also been shown to be targets for protein kinase A, also a serine/threonine kinase, suggesting that certain proteins may act as regulatory foci for multiple signal transduction pathways.

One of the first studies to suggest an association between protein kinase C activation and PTH action in the kidney was provided by Noland and Henry (1983) who examined the effect of PTH on endogenous protein phosphorylation in primary cultures of chick kidney cells. They found that although most of the

endogenous proteins phosphorylated in response to PTH treatment could be attributed to the action of protein kinase A, a number of phosphoproteins were shown to be targets of protein kinase C. Direct evidence for protein kinase C activation by PTH was recently provided by Tamura et al. (1989) who demonstrated an immediate, dose-dependent translocation of protein kinase C in OK cells in response to the peptide hormone. Translocation reached maximal levels within fifteen seconds of exposure to PTH and returned to normal within one minute, a time course reminiscent of that described for PTH-induced phosphoinositide hydrolysis (Hruska et al., 1987). Together, these findings suggest that protein kinase C may be involved in mediating PTH action in the kidney.

Although protein kinase C has been implicated in the regulation of a wide variety of cellular functions including steroid hormone biosynthesis (reviewed by Nishizuka, 1986), its role in the modulation of renal vitamin D metabolism has been investigated only recently. Henry (1986) demonstrated that treatment of primary cultures of chick kidney cells with the phorbol ester, TPA, resulted in increased 24,25-$(OH)_2D_3$ production and decreased 1,25-$(OH)_2D_3$ synthesis. However, the lack of direct evidence for protein kinase C activation (e.g. translocation) and the lengthy incubation times used (four hours) raised the possibility that the effects of TPA on renal vitamin D metabolism might be the result of phorbol ester-induced down-regulation rather than activation of the kinase.

The observations in avian kidney described above prompted the first investigation of protein kinase C involvement in the regulation of mammalian renal vitamin D metabolism, which was undertaken as part of the present thesis and is described in Chapter II. In addition to providing the first evidence for protein kinase C-dependent modulation of vitamin D metabolism in mammalian

kidney, our study also addresses a number of questions which the previous report left unanswered. During the course of our investigation, a second report, also from the laboratory of Henry, was published (Henry and Luntao, 1989). Their findings are discussed in Chapter II as well as in the General Discussion of the present thesis.

Although the evidence thus far suggests a role for protein kinase C in the regulation of renal vitamin D metabolism, little is known of the mechanism(s) whereby this regulation might occur. Earlier in this section, it was stated that alterations in the phosphorylation state of ferredoxin elicit changes in renal vitamin D metabolism. Although ferredoxin phosphorylation was discussed in the context of the cAMP pathway, this protein could serve as a potential regulatory target for protein kinase C as well. In this regard, it is of interest that phosphorylation modification of ferredoxin occurs on specific serine and threonine residues (Nemani et al., 1989). The fact that protein kinase C is a serine/threonine kinase and that its presence has been demonstrated in renal mitochondria (Boneh and Tenenhouse, 1988) lends support to this hypothesis.

Studies of the adrenal mitochondrial steroid hydroxylase system have shown in vitro protein kinase C-dependent phosphorylation of the cytochrome P450 catalyzing cholesterol side chain cleavage (Vilgrain et al., 1984). Although the physiological significance of this phosphorylation is unknown, it could represent a potential regulatory mechanism. It remains to be determined whether the cytochrome P450 component of the renal mitochondrial vitamin D hydroxylating system undergoes protein kinase C-dependent phosphorylation and whether this modification affects the metabolism of vitamin D.

Another mechanism whereby protein kinase C might regulate renal vitamin D metabolism is through transcriptional activation, not unlike that described above for cAMP. A number of genes have been described whose

expression is either induced or enhanced in response to treatment with the phorbol ester, TPA. These TPA-responsive genes have been shown to contain specific enhancer sequences, called TPA-responsive elements or TREs which, like their cAMP-responsive counterparts (CREs), are known to bind nuclear transcription factors. It is thought that alterations in either activity or abundance of these nuclear factors as a result of phosphorylation by protein kinase C, the primary target of TPA action, are involved in the modulation of TPA-responsive gene transcription (Angel et al., 1987).

Protein kinase C might also alter gene function via direct interaction with DNA. Preliminary studies show that purified preparations of protein kinase C contain a protein, presumably the kinase, that binds specifically to DNA enriched in repetitive sequences (Testori et al., 1988). Although unequivocal proof of the identity of this protein is still lacking, it is of interest that protein kinase C contains a tandem repeat of a cyteine-rich, zinc finger-like motif, similar to that found in many DNA-binding proteins that are active in transcriptional regulation. The demonstration that this region is involved in phorbol ester binding (Ono et al., 1989), does not preclude a potential interaction with DNA. Structural similarities between DNA and phospholipids, such as phosphodiester linkages and hydrophobic regions, may promote interaction of the nucleic acid with protein kinase C.

6. Inherited disorders of vitamin D metabolism and action

The present section describes three inherited disorders in which either vitamin D metabolism or action is perturbed, namely, vitamin D dependency rickets types I and II, and X-linked hypophosphatemia. The purpose of this section is two-fold. First, to illustrate the importance of 1,25-$(OH)_2D_3$ in the maintenance of mineral homeostasis and normal growth and development, and

second, to acquaint the reader with these three disorders, as they are pertinent to the studies described in the chapters that follow. A more complete discussion of these and other inherited disorders of vitamin D is offered in the current edition of *The Metabolic Basis of Inherited Disease* (Marx, 1989; Rasmussen and Tenenhouse, 1989); only the more salient features are summarized below.

Vitamin D dependency rickets type I. First described by Prader et al. in 1961, vitamin D dependency rickets type I [VDDR-I], formerly known as pseudovitamin D deficiency rickets type I, is an autosomal recessive disorder characterized by early onset of hypocalcemia, secondary hyperparathyroidism, ensuing hypophosphatemia and severe rachitic lesions, despite adequate diet. Although the gene responsible for the disease has recently been mapped by linkage analysis to 12q14 (Labuda et al., 1990), the actual gene product remains unknown. The demonstration of low or undetectable serum concentrations of 1,25-$(OH)_2D$ (Scriver et al., 1978; Delvin et al., 1981) despite normal or slightly elevated circulating levels of 25OHD precursor and of complete remission of the disease in response to physiological doses of 1,25-$(OH)_2D_3$ (Fraser et al., 1973) provided indirect evidence for a defect in renal 1-hydroxylase activity. Direct evidence was later obtained from studies of an animal model of VDDR-I, the Hannover rachitic pig (Plonait, 1962), which revealed undetectable levels of 1-hydroxylase activity in renal homogenates prepared from mutant animals (Fox et al., 1985; Winkler et al., 1986). Interestingly, 24-hydroxylase activity was also absent from these preparations. Moreover, the absence of renal 24-hydroxylase activity was not secondary to the hypocalcemia, hyperparathyroidism and hypophosphatemia associated with the disease, since normalization of the above serum parameters following treatment with 1,25-$(OH)_2D_3$ failed to restore enzyme activity (Winkler et al., 1986).

The observation that a single genetic mutation is associated with the impairment of both 1- and 24-hydroxylase activities in the animal model for VDDR-I is consistent with the hypothesis that both activities are mediated by a single gene product. However, one cannot exclude the possibility that the target of the mutation may be a gene encoding a shared component or common regulator of two separate enzymes. Furthermore, it is not yet known whether the disturbance in renal 24-hydroxylase activity observed in the rachitic pig model is also expressed in the human counterpart of VDDR-I. Although circulating levels of 24,25-$(OH)_2D$ appear to be normal in VDDR-I patients (Nguyen et al., 1979; Aarskog et al., 1983), suggesting that 24-hydroxylase activity is intact, direct evidence for normal enzyme function is still lacking. These issues form the basis for the study presented in Chapter IV.

Vitamin D dependency rickets type II. Also known as pseudovitamin D deficiency rickets type II, vitamin D dependency rickets type II [VDDR-II], like VDDR-I, is characterized by an autosomal recessive mode of inheritance, hypocalcemia, secondary hyperparathyroidism, hypophosphatemia and early onset rickets (Brooks et al., 1978; Marx et al., 1978). Unlike VDDR-I, it is frequently associated with alopecia. VDDR-II is further distinguished from the type I disorder by elevated plasma levels of 1,25-$(OH)_2D$ and refractoriness to treatment with physiological doses of vitamin D hormone, suggestive of a generalized target organ resistance to the hormone (Brooks et al., 1978; Marx et al., 1978), analogous to that described previously for syndromes of androgen-insensitivity (Keenan et al., 1974). Studies in cultured human skin fibroblasts which express both the 1,25-$(OH)_2D_3$ receptor (Feldman et al., 1980; Eil et al., 1981) and inducible 24-hydroxylase activity (Feldman et al., 1982), a receptor-mediated process, identified aberrant vitamin D receptor function as the likely cause of VDDR-II. Since then, a number of receptor defects have been

described for VDDR-II, the most common involving either decreased or absent 1,25-$(OH)_2D_3$-binding or decreased DNA-binding to the receptor (reviewed by Marx, 1989). More recently, studies at the molecular level have characterized several mutations in both steroid-binding (Ritchie et al., 1989) and DNA-binding (Hughes et al., 1988; Sone et al., 1990) domains of the vitamin D receptor. As more VDDR-II families are examined, more mutations will no doubt be discovered, providing further insight into the mechanism of 1,25-$(OH)_2D_3$ receptor function and the means by which mutations can give rise to this disease.

X-Linked hypophosphatemia in mouse and man. X-linked hypophosphatemia (XLH) is a dominantly inherited disorder of phosphate homeostasis, characterized by hypophosphatemia, rickets and osteomalacia, reduced renal reabsorption of phosphate without striking hypocalcemia or secondary hyperparathyroidism, and abnormal renal vitamin D metabolism (Rasmussen and Tenenhouse, 1989). Also known as familial vitamin D resistant rickets, XLH is the most commonly occurring form of inherited rickets in man. Although it was first described more than fifty years ago (Albright et al., 1937) and has recently been mapped to Xp22.31➡p21.3 (Thakker et al., 1987), the identity of the mutant gene product involved remains unknown. Much of what is known about the pathophysiology of XLH derives from studies of the animal model for this disorder, the X-linked hypophosphatemic or Hyp mouse (Eicher et al., 1976), with whom it shares many features. The following discussion will be confined to studies of the mutant mouse model and its renal defects.

Phosphate transport. Like its human counterpart, the Hyp mouse is characterized by low plasma phosphate levels, attributable to a defect in renal phosphate reabsorption (Eicher et al., 1976). The defect has been localized by

micropuncture studies to the proximal tubule of Hyp kidney (Giasson et al., 1977). The reabsorptive abnormality in Hyp mice is independent of PTH, since it persists following thyroparathyroidectomy (Cowgill et al., 1979). Transport studies in renal cortical slices, which preferentially expose the basolateral membrane, demonstrate that phosphate uptake is comparable in Hyp and normal mice, suggesting that the defect is not expressed at this membrane surface (Tenenhouse et al., 1978). By contrast, studies in purified renal brush-border membrane vesicles isolated from Hyp mice have demonstrated a specific deficit in Na^+-dependent phosphate co-transport (Tenenhouse et al., 1978; Tenenhouse and Scriver, 1978), associated with a 50% decrease in apparent Vmax of the high affinity/low capacity transport system, with no change in apparent Km (Tenenhouse et al., 1989). A corresponding defect in Hyp intestine has not been found (Tenenhouse et al., 1981).

Vitamin D metabolism. The Hyp mouse also exhibits abnormalities in renal vitamin D metabolism, affecting both synthesis and degradation of vitamin D hormone.

Despite significant hypophosphatemia, a known stimulus for renal 1,25-$(OH)_2D$ production, circulating levels of 1,25-$(OH)_2D$ in the Hyp mouse are not elevated relative to normal (Meyer et al., 1980), a feature it shares with human XLH (Isogna et al., 1983). In addition, renal synthesis of 1,25-$(OH)_2D_3$ is significantly lower in Hyp mice than in normal mice with comparable hypophosphatemia, achieved with dietary phosphate restriction (Lobaugh and Drezner, 1983). Decreased 1,25-$(OH)_2D_3$ synthesis in the (vitamin D/calcium-deprived) Hyp mouse is associated with a decrease in apparent Vmax for 1-hydroxylase (Tenenhouse, 1984). Furthermore, Hyp mice exhibit a blunted 1-hydroxylase response to stimulators of 1,25-$(OH)_2D_3$ synthesis including, phosphate deprivation (Meyer et al., 1980; Lobaugh and Drezner,

1983), vitamin D deficiency (Tenenhouse, 1983; 1984), calcium restriction (Tenenhouse, 1984; Nesbitt et al., 1986) and PTH infusion (Nesbitt et al., 1986).

Decreased renal 1,25-$(OH)_2D_3$ biosynthesis is accompanied by accelerated catabolism of the hormone, as evidenced by increased renal C24-oxidation of vitamin D metabolites in Hyp mice relative to normal littermates (Jones et al., 1987b; Tenenhouse et al., 1988). Increased renal degradation of vitamin D hormone is thought to contribute to the pathophysiology of this disorder. Kinetic analysis shows that the increase in 24-hydroxylase activity is associated with an increase in apparent Vmax whereas the affinity of the enzyme for its substrate is unaltered (Tenenhouse and Jones, 1987). There is also evidence for abnormal regulation of renal 1,25-$(OH)_2D_3$ catabolism in the Hyp mouse in response to dietary phosphate manipulation (Tenenhouse and Jones, 1990). By contrast, Hyp mice exhibit an appropriate increase in renal 24-hydroxylase activity in response to treatment with 1,25-$(OH)_2D_3$ (Tenenhouse and Jones, 1987; Jones et al., 1987b).

Protein kinase C. The Hyp mouse is characterized by elevated protein kinase C activity in renal cytosol, but not in cytosolic fractions prepared from heart, liver or spleen, suggesting that this defect is specific to kidney (Tenenhouse and Henry, 1985). By contrast, Hyp mice do not exhibit any abnormalities in cytosolic protein kinase A or protein kinase inhibitor activities (Tenenhouse and Henry, 1985), nor in cAMP-dependent phosphorylation of renal brush-border membrane proteins (Hammerman and Chase, 1983; Brunette et al., 1984).

Because protein kinase C has been implicated in the regulation of both renal phosphate transport (Cole et al., 1987; Boneh et al., 1989) and vitamin D metabolism (Henry, 1986; Henry and Luntao, 1989), the two renal functions perturbed in the Hyp mouse, it has been suggested that the kinase may be

involved in the expression of the mutant renal phenotype. Consistent with this hypothesis is our demonstration that the defect in renal phosphate transport characteristic of the Hyp mouse can be mimicked in normal mouse kidney through activation of protein kinase C (Boneh et al., 1989). Whether a similar relationship exists between renal protein kinase C and the abnormalities in vitamin D metabolism associated with the Hyp mutation is one of the questions examined in the study described in Chapter II of this thesis.

It is unclear, at present, how the abnormalities in renal phosphate transport, vitamin D metabolism and protein kinase C activity interact to produce XLH in man and mouse. Mendelian inheritance dictates that a single mutation must be responsible for all three defects, although it is not evident which of these, if any, is the primary target. Despite its pivotal regulatory position, protein kinase C is not a likely target, since none of the protein kinase C isozymes known to date maps to the X chromosome (Coussens et al., 1986). Nor is the vitamin D metabolizing enzyme system a likely candidate, since both the catabolic (Tenenhouse and Jones, 1990) and biosynthetic (Yamaoka et al., 1986; Davidai et al., 1991) defects in the Hyp mouse are alternately corrected by dietary phosphate supplementation and exacerbated by phosphate deprivation, suggesting that they are secondary to the disturbance in phosphate homeostasis. Nor is there any evidence at present identifying the phosphate transporter as the mutant gene product.

Because the abnormalities in phosphate transport and vitamin D metabolism characteristic of Hyp kidney are expressed in primary cultures of renal epithelial cells derived from Hyp mice, it has been suggested that the defect is intrinsic to the kidney (Bell et al., 1988). However, the recent demonstration that normal mice surgically joined to Hyp mice develop many of the phenotypic features of XLH including hypophosphatemia, decreased renal

phosphate reabsorption, decreased renal brush-border membrane sodium-dependent phosphate transport and inappropriately low plasma levels of vitamin D hormone, suggests the involvement of a humoral factor (Meyer et al., 1989ab). The factor is not PTH, since the above responses are not altered following parathyroidectomy of both members of the pair (Meyer et al., 1989b). The fact that these abnormalities do not persist in the normal mouse after it is surgically separated from its Hyp partner and are not apparent in parabiosed normal mouse pairs further supports a humoral basis for this disorder. It is of interest that a circulating phosphaturic factor has been implicated in the pathogenesis of oncogenic hypophosphatemic osteomalacia (OHO), a rare form of hypophosphatemia which occurs in association with certain tumours (Agus, 1983). The clinical features of this disorder are similar to those characterizing XLH and are thought to arise from abnormalities in renal phosphate reabsorption and vitamin D metabolism caused by a tumour-derived humoral factor. A similar factor may well be involved in XLH. Although the findings from parabiosis experiments are at variance with the data from studies in cultured renal cells, the two can be reconciled if the putative humoral factor is of renal origin. However, until such a factor is found and mapped to the X chromosome, the primary cause of XLH will remain largely a matter for speculation.

Chapter II:
Evidence for protein kinase C involvement in the regulation of renal 25-hydroxyvitamin D_3-24-hydroxylase

Preliminary findings have implicated protein kinase C in the regulation of vitamin D metabolism in avian kidney. The present study was undertaken to determine whether protein kinase C is involved in the modulation of mammalian renal vitamin D metabolism. Although our investigation focusses primarily on the constitutively-expressed or basal 24-hydroxylase of normal mouse kidney, we also examine potential protein kinase C involvement in the regulation of inducible 24-hydroxylase activity in the 1,25-$(OH)_2D_3$-treated mouse, as well as in the aberrant expression of 24-hydroxylase function in the mutant Hyp mouse.

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Evidence for Protein Kinase C Involvement in the Regulation of Renal 25-Hydroxyvitamin D_3-24-Hydroxylase*

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ABSTRACT. Although calcium-activated, phospholipid-dependent protein kinase (protein kinase C) has been implicated in the regulation of various steroidogenic pathways, comparatively little is known of its role in the metabolism of vitamin D. The present study was undertaken to determine whether protein kinase C is involved in the regulation of renal mitochondrial 25-hydroxyvitamin D_3-24-hydroxylase (24-hydroxylase), the first enzyme in the C-24 oxidation pathway, a major catabolic pathway for vitamin D metabolites in kidney and other target tissues. We examined the effect of phorbol 12-myristate 13-acetate (PMA), a potent activator of protein kinase C, on 24-hydroxylase activity in fresh mouse renal tubules and correlated the changes in 24,25 dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$] production with translocation of protein kinase C and phosphorylation of mitochondrial proteins. PMA stimulated 24,25-$(OH)_2D_3$ synthesis, protein kinase C translocation from the cytosolic to the mitochondrial fraction, and phosphorylation of 30-35 K, 40 K, and 50 K mitochondrial proteins derived from ^{32}P-labeled tubules. 4α-Phorbol 12,13 didecanoate, an inert analog of PMA, did not elicit any of these effects. The synthetic diacylglycerol, oleoylacetyl glycerol, also stimulated 24,25-$(OH)_2D_3$ synthesis, whereas the protein kinase C inhibitors, H-7 and staurosporine, inhibited 24-hydroxylase activity. PMA did not further stimulate 24,25-$(OH)_2D_3$ production in tubules derived from mutant (*Hyp*) mice in which 24-hydroxylase and protein kinase C activities are elevated relative to normal. However, after treatment with H-7, 24-hydroxylase activity was reduced in both strains, and genotype differences were no longer apparent. Finally, H-7 failed to inhibit the induced renal 24-hydroxylase in tubules isolated from 1,25-dihydroxyvitamin D_3-treated mice. These findings suggest a role for protein kinase C in the regulation of constitutive renal 24-hydroxylase and implicate the kinase in the aberrant expression of the hydroxylase in the *Hyp* mouse. (*Endocrinology* 127: 2639-2647, 1990)

1,25-DIHYDROXYVITAMIN D_3 [1,25-$(OH)_2D_3$] is a prominent regulator of bone and mineral metabolism, and thus, plays a pivotal role in normal growth and development. The C-24 oxidation pathway is a major catabolic pathway for 1,25-$(OH)_2D_3$ in many target tissues including kidney and serves to control physiological levels of 1,25-$(OH)_2D_3$ and guard against the toxic effects of excess hormone (1-4). This pathway involves sequential 24-hydroxylation, 24-oxidation, 23-hydroxylation, and eventual side-chain cleavage of 1,25-$(OH)_2D_3$ to yield calcitroic acid, the final inactivation product (5, 6). The first enzyme in this degradative sequence, the 24-hydroxylase, catalyzes the conversion of 1,25-$(OH)_2D_3$ to the biologically less active metabolite 1,24,25-trihydroxyvitamin D_3. It can also metabolize 25-hydroxyvitamin D_3 (25OHD_3), the renal precursor of 1,25-$(OH)_2D_3$, to 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$]. The C-24 oxidation pathway is expressed constitutively only in the kidney. However, after pretreatment with 1,25-$(OH)_2D_3$, the catabolic pathway is inducible in intestine (2, 4), bone (5), and fibroblasts (7) as well as kidney (2-6). The constitutive renal 24-hydroxylase is regulated by a number of physiological factors including PTH, calcium, phosphate, and vitamin D itself (see Ref. 8 for review). Although the effects of these regulatory factors on 24-hydroxylase activity have been well documented, little is known of the biochemical mechanism(s) involved.

Based on observations that cAMP (9) and forskolin (10, 11) could mimic the effects of PTH on both the renal 1- and 24-hydroxylases, it was suggested that the activation of cAMP-dependent protein kinase (protein kinase A) is involved in the regulation of renal vitamin

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D metabolism. However, a direct correlation between protein kinase A-mediated phosphorylation of endogenous substrates and modulation of either 1,25-(OH)$_2$D$_3$ or 24,25-(OH)$_2$D$_3$ production have not been reported.

Calcium-activated, phospholipid-dependent protein kinase (protein kinase C) is a ubiquitous serine and threonine kinase (12) that has been implicated in the regulation of steroidogenesis in various tissues and cell types including adrenal cortical, ovarian, testicular, and adipose cells (see Ref. 12 for review). Recent studies in cultured chick kidney cells (13, 14) demonstrated that phorbol 12-myristate 13-acetate (PMA), a potent activator of protein kinase C (15), inhibits 1,25-(OH)$_2$D$_3$ synthesis, stimulates 24,25-(OH)$_2$D$_3$ production, and attenuates the effects of PTH and forskolin on these two processes. However, neither study presented evidence for protein kinase C activation, nor protein kinase C-mediated phosphorylation of endogenous proteins, potential mediators of the changes in vitamin D metabolism.

In the present study, we examine the effect of PMA on 24-hydroxylase activity in freshly prepared mouse renal tubules and correlate these effects with translocation of protein kinase C and endogenous phosphorylation of mitochondrial proteins. The renal tubule preparation constitutes a well-characterized model of the renal proximal tubule cell (16, 17), the site of vitamin D metabolism in the kidney, and circumvents many of the problems associated with cell culture systems, such as dedifferentiation and transformation. We use two mouse models in our investigation: the mutant X-linked hypophosphatemic (*Hyp*) mouse (18), in which renal 24-hydroxylase activity (19) and cytosolic protein kinase C (20) are elevated relative to normal, and the 1,25-(OH)$_2$D$_3$-treated mouse, in which the renal C-24 oxidation pathway has been induced (21).

Materials and Methods

Animals

Normal C57Bl/6 male mice were obtained from Charles River Canada Inc. (St-Constant, Quebec). Mutant *Hyp* mice and normal littermates were obtained by breeding C57Bl/6J *Hyp*/+ females with C57Bl/6J +/Y males; the original breeding pairs were purchased from Jackson Laboratory (Bar Harbor, ME). All mice were maintained on Wayne Lab Blox diet (Allied Mills, Inc., Chicago, IL) containing 1.2% calcium, 0.99% phosphorus, and 4.41 IU vitamin D$_3$/g. For the 1,25-(OH)$_2$D$_3$ treatment protocol, mice were injected ip with 1,25-(OH)$_2$D$_3$ (1.5 ng/g body wt) or the mineral oil vehicle and killed 16 h after injection. Two- to 4-month-old mice were used in all experiments.

Materials

Modified minimum essential medium [MEM, 10-121-22], phosphate-free minimum essential medium [16-287-49], and nonessential amino acid concentrate [16-810-49] were purchased from Flow Laboratories (Mississauga, Ontario, Canada). Collagenase (from *Clostridium histolyticum*, grade II), was purchased from Boehringer Mannheim (Dorval, Quebec, Canada). 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride [H-7] was obtained from Seikagaku America Inc. (St. Petersburg, FL). PMA, 4α-phorbol 12,13-didecanoate (4α-phorbol), 1-oleoyl-2-acetyl-sn-glycerol (OAG), lysine-rich histone (type III-S), L-phosphatidyl-L-serine, 1,2-dioleoyl-rac-glycerol (diacylglycerol), leupeptin, 3-isobutyl-1-methyl-xanthine, BSA fraction V, sodium dodecyl sulfate (SDS), and mol wt protein standards were purchased from Sigma Chemical Co. (St. Louis, MO). Acrylamide, bis-acrylamide, and other gel reagents and equipment were purchased from Bio-Rad Laboratories (Richmond, CA). γ[^{32}P]ATP and carrier-free ^{32}P were purchased from New England Nuclear (Montreal, Quebec, Canada) and [^{3}H]25OHD$_3$, [^{3}H]-1,25-(OH)$_2$D$_3$, and [^{3}H]-24,25-(OH)$_2$D$_3$, as well as the cAMP assay kit [TRK.432], from Amersham (Oakville, Ontario, Canada). 25OHD$_3$ was a gift from Upjohn Company, (Kalamazoo, MI) and Hoffmann-LaRoche Inc. (Etobicoke, Ontario, Canada). Staurosporine was a gift from R. John MacLeod.

Preparation of renal cortical tubules

Mouse renal cortical tubules were prepared as described previously (22). Briefly, cortical slices were incubated in 15 ml MEM-HEPES, pH 7.5 containing 4.5 U collagenase and 0.75 ml 10% (wt/vol) BSA for 45 min at 37 C in a shaking water bath. Digestion was stopped by addition of 30 ml ice-cold MEM-HEPES buffer. Collagenase-treated slices were dispersed, filtered through a wire mesh, washed three times, and resuspended in MEM-HEPES, pH 7.5. As a viability index, lactic dehydrogenase activity was assayed in the supernatant fraction of a tubule suspension at various times after preparation. When kept on ice, tubules remained viable for at least 3 h after preparation (data not shown). Tubule preparations and subsequent experiments were carried out in plastic containers.

Metabolism of 25OHD$_3$

One milliliter aliquots of tubule suspension [2–4 mg protein/ml] were incubated with 50 nM [^{3}H]25OHD$_3$ (2000 cpm/pmol) in a shaking waterbath at 30 C for the times indicated. Under these conditions, the percentage conversion of substrate to product was less than 5%, indicating that substrate was not rate limiting. Incubations were treated with either OAG, PMA, 4α-phorbol [dissolved in dimethylsulfoxide (DMSO), final concentration, 0.1%], staurosporine (dissolved in DMSO, final concentration, 1%) or H-7 (dissolved in water). Parallel incubations treated with an equivalent volume of the appropriate vehicle served as controls, except for PMA-treated tubules in which 4α-phorbol-treated tubules served as controls. Activities obtained with 4α-phorbol did not differ significantly from those observed with DMSO vehicle alone (data not shown). Reactions were stopped by addition of 3.75 ml methanol-chloroform (2:1), and samples were stored at −20 C under N$_2$ until extraction. Reaction mixtures in which [^{3}H]25OHD$_3$ was incubated with MEM-HEPES buffer either in the absence of tubules or in the

presence of boiled tubules served as background in the quantification of product formation.

Extraction and assay of vitamin D metabolites

Reaction mixtures were extracted twice as described by Bligh and Dyer (23). Both organic phases were pooled, dried, reconstituted in hexane-isopropanol-methanol (93.5:5.5:1) and an aliquot fractionated on a Zorbax Sil column (25 cm × 6.2 mm, DuPont Co., Wilmington, DE) equilibrated with the same solvent mixture, as described by Jones (24). Radioactivity was measured in Formula 963 (New England Nuclear, Montreal, Quebec) by liquid scintillation counting. Elution positions of 25OHD$_3$, 24,25-(OH)$_2$D$_3$, and 1,25-(OH)$_2$D$_3$ were determined with either crystalline or radioactive standards. Recovery of ^{3}H label was greater than 90%, and comparable for both blank (where no significant conversion of substrate is apparent) and experimental samples, indicating that all reaction products could be accounted for under our conditions.

Translocation of protein kinase C

The effect of PMA on the subcellular distribution of protein kinase C was determined as described previously (22). Briefly, tubules were incubated with 1 μg/ml (1.6 μM) either PMA or 4α-phorbol, at 30 C for 10 min, centrifuged at 60 × *g* for 1 min, resuspended in 250 μl homogenizing buffer [0.25 M sucrose, 20 mM Tris-HEPES (pH 7.5), 2 mM EGTA, 10 mM dithiothreitol and 10 μg/ml leupeptin], and disrupted by sonication. Mitochondrial and cytosolic fractions were prepared from tubule lysates as described previously (25). After centrifugation at 120 × *g* for 10 min, the resulting supernatants were centrifuged at 9,000 × *g* for 10 min to yield a mitochondrial pellet and a postmitochondrial supernatant. Postmitochondrial supernatants were centrifuged at 105,000 × *g* for 60 min to yield cytosolic fractions, which were assayed directly for protein kinase C as described below. Mitochondrial pellets were resuspended in homogenizing buffer, solubilized in 0.2% Triton X-100 for 60 min on ice, and centrifuged. Supernatants thus generated were further diluted with the same buffer and assayed for protein kinase C as described below. The mitochondrial fraction was shown to be enriched 8- to 10-fold with respect to the mitochondrial enzyme marker succinate cytochrome *c* reductase (21, 25). Assay of specific subcellular markers (21, 25) demonstrated that 67% of tubular 24-hydroxylase activity, 5% of tubular lactic dehydrogenase activity, 21% of tubular nicotinamide adenine dinucleotide phosphate (reduced form)-cytochrome *c* reductase activity, 11% of tubular DNA, and 29% of tubular trehalase activity were recovered in the mitochondrial fraction.

Protein kinase C assay

Enzyme activity was measured as ^{32}P incorporation from γ[^{32}P]ATP to calf thymus histone (type III-S) as described previously (25). Protein concentration was approximately 20 μg/100 μl assay for both cytosolic and mitochondrial fractions. The reaction was initiated by the addition of γ[^{32}P]ATP (≈200 cpm/pmol, final concentration, 0.3 mM) after 1.5 min preincubation at 30 C, and stopped after 3 min of incubation by spotting 50 μl aliquots of reaction mixture onto 2 × 2 cm squares of phosphocellulose paper as described previously (25). The squares were immediately immersed in 75 mM phosphoric acid, washed three times in the same solution, dried, and counted. All values were corrected for background activity (determined from a reaction mixture containing buffer instead of enzyme). Protein kinase C activity was obtained by subtracting kinase activity measured in the absence of calcium, phosphatidylserine, and diacylglycerol (basal activity) from kinase activity measured in the presence of all three components (total activity). We demonstrated in a previous study that the calcium- and phospholipid-dependent component (total − basal) is attributable to protein kinase C (25).

Phosphorylation of endogenous substrates

Tubules were resuspended in phosphate-free MEM supplemented with nonessential amino acids and 2 mM glutamine (pH 7.5). Tubule cells were labeled with carrier-free ^{32}P [250–400 μCi] for 45 min at 30 C in a shaking waterbath and then incubated with 1 μg/ml either 4α-phorbol (10 min) or PMA (0, 10, 20 min) as described previously (22). The incubation was stopped by the addition of 10 ml ice-cold MEM-HEPES buffer containing phosphate and centrifugation at 60 × *g* for 1 min. Mitochondrial fractions, prepared as described previously (21, 25), were treated with an equal volume of loading buffer (20% glycerol, 4% SDS, 1.66% NaH_2PO_4, 10% 2-β-mercaptoethanol, and 10% bromophenol blue, pH 6.8) and boiled for 1.5 min. Aliquots containing equivalent amounts of protein (30 μg/lane) and radioactivity were applied to 10% SDS-polyacrylamide gels and electrophoresed according to the method of Laemmli (26). Autoradioagraphy was performed using Kodak X-Omat AR-5 films in exposure holders with intensifying screens (3 days exposure). Phosphorylation of endogenous protein substrates was quantified by scanning autoradiograms on a LKB-2202 ultroscan laser densitometer using a KB-2220 recording integrator (LKB Instruments, Inc., Rockville, MD). The scanner was calibrated by exposure to an area of film with no phosphorylation. Intensity in the autoradiogram was proportional to the amount of ^{32}P present. Phosphorylation of protein bands after incubation of tubules with PMA was compared to both phosphorylation at time zero and phosphorylation in the presence of 4α-phorbol (basal phosphorylation). Mitochondrial proteins were designated substrates for protein kinase C when they adhered to the following criteria: 1) PMA-induced phosphorylation exceeded basal phosphorylation; 2) basal phosphorylation did not exceed phosphorylation at time zero; 3) criteria 1 and 2) were consistently observed in at least four separate preparations.

cAMP assay

Tubules were incubated in the presence or absence of PMA, OAG, or H-7 as described above for vitamin D assay. cAMP in reaction mixtures was measured as described previously (27) using a cAMP assay kit.

Other determinations

Protein concentration was determined by the method of Lowry *et al.* (28) using BSA as standard.

Statistics

Results represent mean ± SEM. Statistical significance was determined by Students *t* test, paired or unpaired, as appropriate.

Results

Preliminary studies showed that 24-hydroxylase activity in mouse renal tubules was linear with time for at least 40 min and with protein concentrations up to 5 mg/ml (data not shown). Kinetic analysis revealed an apparent Michaelis-Menten constant (K_m) of 490 nM for 25OHD, and a maximum velocity of reaction (V_{max}) of 173 fmol/mg protein·min. These parameters are consistent with those described previously for 24-hydroxylase activity measured in isolated renal mitochondria (19).

We demonstrated in a previous study (22) that PMA treatment of freshly prepared mouse renal tubules elicits a rapid (maximal after 10 min exposure) translocation of protein kinase C activity from the cytosolic to the particulate fraction. In the present study, we examined whether PMA elicits a translocation of protein kinase C activity to the mitochondrial fraction, the site of 24-hydroxylase activity in the renal epithelial cell. Table I shows that PMA-treated tubules exhibit a significant decrease in cytosolic protein kinase C activity concomitant with a significant increase in the activity of the kinase associated with the mitochondrial fraction, relative to their 4α-phorbol-treated counterparts. The increment in protein kinase C activity in the mitochondrial fraction represents approximately 16% of the activity lost from the cytosol.

Figure 1 shows the effect of PMA treatment on the endogenous phosphorylation of mitochondrial proteins

TABLE I. Protein kinase C activity in cytosolic and mitochondrial fractions prepared from PMA- and 4α-phorbol-treated tubules

	Protein kinase C activity (pmol/min·mg protein)		
	4α-Phorbol	PMA	Difference
Cytosolic fraction	3909 ± 264	1330 ± 92*	−2479
Mitochondrial fraction	401 ± 67	796 ± 121†	+395

Mouse renal tubules were treated with 1 μg/ml either 4α-phorbol or PMA for 10 min at 30°C. Cytosolic and mitochondrial fractions were prepared and protein kinase C activity determined as described in *Materials and Methods*. PMA treatment had no significant effect on basal kinase activity in either cytosolic or mitochondrial fractions (data not shown). Each value represents the mean ± SEM of five experiments assayed in duplicate. Effect of PMA is indicated by footnotes as follows:

* $P < 0.001$.

† $P < 0.01$.

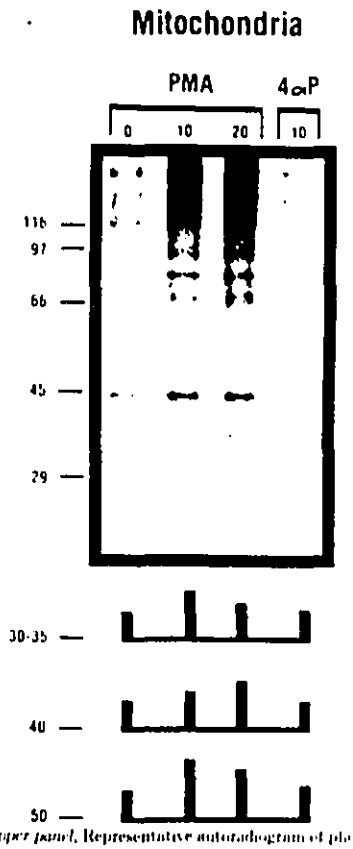

FIG. 1. *Upper panel*, Representative autoradiogram of phosphorylated mitochondrial proteins isolated from PMA- and 4α-phorbol-treated, ^{32}P-labeled tubules. Mitochondria were prepared from ^{32}P-labeled renal tubules treated with 1 μg/ml either PMA or 4α-phorbol, and electrophoresed on SDS polyacrylamide gels as described in *Materials and Methods*. Autoradiography was performed as described in *Materials and Methods*. Migration positions of protein standards (β-galactosidase, 116 K; phosphorylase B, 97.4 K; bovine plasma albumin, 66 K; ovalbumin, 45 K; carbonic anhydrase, 29 K) run simultaneously are indicated. The autoradiogram may not clearly show all phosphorylated bands indicated in the histograms below.

Lower panel, Histograms summarize the results of at least four separate experiments and depict the degree of phosphorylation of three protein bands that were consistently phosphorylated to a greater extent in the presence of PMA than 4α-phorbol. Phosphorylation was quantitated by laser densitometry as described in *Materials and Methods*. Each *bar* in the histogram represents the extent of phosphorylation of a given band under the conditions specified in the lane above.

in ^{32}P-labeled mouse renal tubules. On the basis of densitometric analysis of replicate experiments, three protein bands, 30–35 K, 40 K, and 50 K, were designated substrates for protein kinase C, *i.e.* their phosphorylation in the presence of PMA exceeded that observed in the

[1]Photograph of autoradiogram is available in *Addendum*, on page 52b

presence of 4α-phorbol. Phosphorylation of the 30–35 K and 50 K protein bands was maximal after a 10-min incubation with PMA and diminished over the next 10 min. By contrast, phosphorylation of the 40 K protein band was greatest after 20 min incubation with PMA.

Figure 2 depicts the effect of PMA concentration on 24-hydroxylase activity in mouse renal tubules. Whereas PMA concentrations of 500 ng/ml (0.8 μM) or less had no effect on 24,25-(OH)$_2$D$_3$ synthesis, higher concentrations were observed to stimulate production of this metabolite. Figure 3 shows that this stimulatory response was also dependent on the length of exposure of the tubules to the activator, with maximal stimulation occurring after 10 min of incubation.

Treatment of mouse renal tubules with the synthetic dicylglycerol, OAG (final concentration, 80 μM), a more physiological activator of protein kinase C than PMA, also resulted in stimulation of 24-hydroxylase activity [19.1 ± 3.6 *vs.* 27.4 ± 4.2 (n = 4), fmol/mg protein·min for vehicle- and OAG-treated tubules, respectively; $P < 0.05$].

Figure 4 shows that the protein kinase C inhibitor, H-7 (29), inhibited 24-hydroxylase activity in mouse renal tubules in a dose-dependent manner. Maximal inhibition (≈60%) was reached at 500 μM H-7, the same concentration that achieved 50% inhibition of protein kinase C activity in cytosolic fractions isolated from mouse renal tubules (data not shown).

Staurosporine (final concentration, 10 μM), a more potent and specific inhibitor of protein kinase C than H-7 (30), also attenuated 24-hydroxylase activity in mouse renal tubules [29.8 ± 5.1 *vs.* 16.5 ± 2.2 fmol/mg protein·min, (n = 4), for vehicle- and staurosporine-treated tubules, respectively; $P < 0.05$]. The same concentration of staurosporine inhibited renal cytosolic protein kinase C by ≈80% (data not shown).

The effects of PMA and H-7 on 24-hydroxylase activity were also examined in renal tubules derived from a mutant mouse strain, designated *Hyp* or hypophosphatemic, which exhibits significantly elevated renal mitochondrial 24-hydroxylase (19) and cytosolic protein kinase C (20) activities relative to normal mice. Table 2 illustrates that whereas PMA elicited a small but signif-

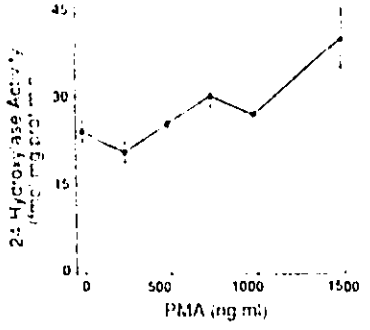

FIG. 2. Effect of PMA concentration on 24-hydroxylase activity in mouse renal tubules. Tubules were incubated with the indicated concentrations of PMA and 50 nM [^{3}H]25OHD$_3$ for 20 min, as described in *Materials and Methods*. Control (i.e. 0 μg/ml PMA) represents incubation with 1 μg/ml 4α-phorbol. 24-Hydroxylase activity measured in the presence of 4α-phorbol did not differ significantly from that observed in the presence of DMSO vehicle alone (data not shown). Metabolites were extracted, fractionated by HPLC, and counted as indicated in *Materials and Methods*. Each value represents the mean and range of duplicate determinations of a representative experiment. Experiments were repeated at least two times with similar results.

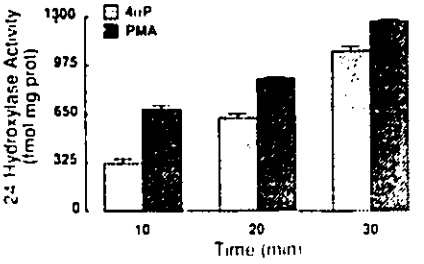

FIG. 3. Time dependence of PMA effect on 24-hydroxylase activity in mouse renal tubules. Tubules were incubated with 1 μg/ml either PMA or 4α-phorbol and 50 nM [^{3}H]25OHD$_3$ for the times indicated, as described in *Materials and Methods*. 24-Hydroxylase activity measured in the presence of 4α-phorbol did not differ significantly from that observed in the presence of DMSO vehicle alone (data not shown). Metabolites were extracted, fractionated by HPLC, and counted, as described in *Materials and Methods*. Each value represents the mean and range of duplicate determinations of a representative experiment. Experiments were repeated at least two times with similar results.

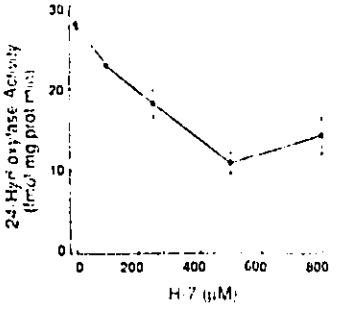

FIG. 4. Effect of H-7 concentration on 24-hydroxylase activity in mouse renal tubules. Tubules were incubated with the indicated concentrations of H-7 and 50 nM [^{3}H]25OHD$_3$ for 20 min, as described in *Materials and Methods*. Metabolites were extracted, fractionated by HPLC, and counted as indicated in *Materials and Methods*. Each value represents the mean and range of duplicate determinations of a representative experiment. Experiments were repeated at least two times with similar results.

TABLE 2. Effect of PMA (A) and H-7 (B) on 24-hydroxylase activity in renal tubules from normal and Hyp mice

	24-Hydroxylase activity (fmol/mg protein/min)	
	Normal	*Hyp*
A. 4α-phorbol	15 ± 2 (24)	43 ± 4 (9)*
PMA	18 ± 2 (24)*	45 ± 6 (9)*
B. Control	22 ± 4 (4)	32 ± 2 (4)*
H-7	12 ± 3 (4)‡	13 ± 2 (4)‡

Tubules were incubated with 50 nM [^{3}H]25OHD$_3$ and (A) 1 μg/ml either PMA or 4α-phorbol or (B) 500 μM H-7 or vehicle for 20 min, as described in *Materials and Methods*. Metabolites were extracted, fractionated by HPLC, and counted, as described in *Materials and Methods*. Values represent mean ± SEM of the number of experiments indicated in parentheses.

* Effect of *Hyp* mutation, $P < 0.01$.

† Effect of PMA, $P < 0.01$.

‡ Effect of H-7, $P < 0.05$.

icant increase in 24,25-(OH)$_2$D$_3$ synthesis in renal tubules prepared from normal mice, it did not significantly stimulate the enzyme activity associated with tubules derived from the mutant strain. By contrast, PMA effectively stimulated protein kinase C activity in renal cytosolic fractions isolated from both normal and mutant mice [PMA-stimulated component, 1116 ± 79 vs. 1428 ± 114 pmol/mg protein·min for normal and *Hyp* mice, respectively (n = 6)]. Table 2 also shows that H-7 inhibited 24-hydroxylase activity in both normal- and *Hyp*-derived renal tubules. While 24-hydroxylase activity measured in the absence of inhibitor was significantly higher in tubules from *Hyp* mice than that from normal mice, treatment with H-7 reduced 24-hydroxylase activity in both genotypes and abolished the interstrain difference.

In order to determine whether H-7 also inhibited 1,25-(OH)$_2$D$_3$-inducible 24-hydroxylase, we examined the effect of the inhibitor on 24,25-(OH)$_2$D$_3$ production in renal tubules prepared from 1,25-(OH)$_2$D$_3$-treated mice. In contrast to our findings with the constitutive enzyme, we found that H-7 had no effect on the 1,25-(OH)$_2$D$_3$-induced 24-hydroxylase (Fig. 5).

In order to ascertain whether the agonists or antagonists of protein kinase C used in the present study exerted their effects on vitamin D metabolism through the adenylate cyclase/protein kinase A pathway, we measured cAMP accumulation in mouse renal tubules treated with each of the three reagents. We found that cAMP levels in mouse renal tubules were not significantly altered by either PMA [178.1 ± 26.6 (n = 6) *vs.* 171.7 ± 20.9 (n = 6) pmol cAMP/mg protein·20 min, for PMA- and 4α-phorbol-treated tubules, respectively; $P > 0.4$], OAG [163.6 ± 24.6 (n = 6) *vs.* 174.4 ± 11.5 (n = 6), for OAG- and vehicle-treated tubules, respectively; $P > 0.6$], or H-7 [153.4 ± 25.1 (n = 9) *vs.* 143.0 ± 21.5 (n = 9), for H-7 and vehicle-treated tubules, respectively, $P > 0.3$].

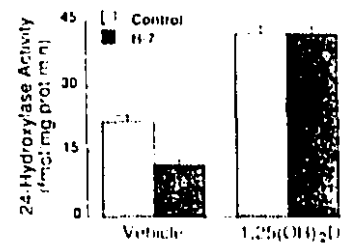

FIG. 5. Effect of H-7 on constitutive and 1,25-(OH)$_2$D$_3$-inducible 24-hydroxylase activities in mouse renal tubules. Mice were injected ip with either 15 ng/g body wt 1,25-(OH)$_2$D$_3$ or equivalent volume of vehicle 16 h before death, as described in *Materials and Methods*. Renal cortical tubules were prepared and incubated with 50 nM [^{3}H]25OHD$_3$ for 20 min, either in the presence or absence of 500 μM H-7, as indicated in *Materials and Methods*. Metabolites were extracted, fractionated by HPLC, and counted, as described in *Materials and Methods*. Each value represents the mean ± SEM of four to six preparations, assayed in duplicate.

Discussion

The present study was undertaken to examine whether protein kinase C is involved in the regulation of renal 24-hydroxylase, the first enzyme in the C-24 oxidation pathway, a major degradative pathway for vitamin D metabolites in kidney as well as other target tissues (1-6). Our results demonstrate a temporal correlation between PMA-induced translocation of protein kinase C from the cytosolic to the mitochondrial fraction, endogenous phosphorylation of mitochondrial proteins, and stimulation of 24-hydroxylase activity in fresh mouse renal cortical tubules. 4α-Phorbol, an inert analog of PMA, did not elicit any of these effects. The synthetic diacylglycerol OAG, like PMA, stimulated 24-hydroxylase activity, whereas H-7 and staurosporine, inhibitors of protein kinase C (29, 30), decreased 24,25-(OH)$_2$D$_3$ synthesis. Although PMA failed to further stimulate the already elevated renal 24-hydroxylase activity of mutant *Hyp* mice, H-7 inhibited the enzyme in both genotypes and abolished the genotype difference. By contrast, H-7 did not inhibit induced 24-hydroxylase activity in tubules prepared from 1,25-(OH)$_2$D$_3$-treated mice. Taken together, our results suggest protein kinase C involvement in the regulation of constitutive but not 1,25-(OH)$_2$D$_3$-inducible renal 24-hydroxylase and implicate the kinase in the aberrant expression of the hydroxylase in the *Hyp* mouse. Our demonstration of PMA-induced stimulation of 24-hydroxylase activity in mouse renal tubules is consistent with two previous studies in cultured chick kidney cells (13, 14). However, neither of the earlier studies provided evidence for either PMA-induced acti-

vation of protein kinase C or phosphorylation of endogenous proteins, nor were the effects of protein kinase C inhibitors or control experiments using an inactive phorbol analog described.

Recently (22) we provided evidence for PMA-induced activation of protein kinase C in mouse renal tubules by demonstrating that PMA stimulated the phosphorylation of 45 K and 87–90 K cytosolic proteins, ubiquitous cellular substrates for protein kinase C and, thus, useful markers for protein kinase C activation in intact cells (31). In that same study (22), we also showed that PMA, but not 4α-phorbol, stimulated the translocation of protein kinase C from the cytosolic to the particulate fraction. In the present study, we provide evidence for PMA-induced translocation of protein kinase C from the cytosolic to the mitochondrial fraction. This latter fraction, although enriched in mitochondria, is not free of brush border membrane and microsomal contamination. On the basis of previously published values for the specific activity of brush border membrane-associated protein kinase C (25) and renal cellular brush border membrane protein content (25), we estimate that less than 6% of protein kinase C associated with the mitochondrial fraction is brush border membrane-derived. Since values for microsomal protein kinase C activity are not available, we are unable to provide a similar estimate for microsomal protein kinase C contribution to the mitochondrial preparation. Thus, although we cannot rule out the possibility that a small portion of the protein kinase C activity associated with the mitochondrial fraction may indeed be derived from extramitochondrial sources, our findings are compatible with translocation of protein kinase C to the mitochondrial fraction. These results are of interest to the present study considering the mitochondrial localization of the 24-hydroxylase in the renal tubular cell.

In the present study, three proteins, 30–35 K, 40 K, and 50 K, were designated substrates for protein kinase C in mitochondria prepared from PMA-treated, ^{32}P-labeled tubules. We have previously reported evidence for protein kinase C-mediated phosphorylation of a 35 K protein in isolated renal mitochondria incubated with calcium, phospholipid, and diacylglycerol (25). The present demonstration of PMA-induced phosphorylation of the same mol wt protein band in intact tubular cells suggests that it is an endogenous mitochondrial substrate for protein kinase C in mammalian kidney. Neither the identity nor the role of the 30–35 K protein in vitamin D metabolism, if any, has been determined. Similarly, we have not yet identified the 40 K or 50 K mitochondrial substrates for protein kinase C. However, it is of interest that the renal 24-hydroxylase is a mitochondrial cytochrome P450 with a mol wt in the 52–53 K range (32) and that Vilgrain *et al.* (33) have demonstrated protein kinase C-mediated phosphorylation of an adrenal mitochondrial cytochrome P450 ($P450_{SCC}$ or side-chain cleavage enzyme) which catalyzes the first reaction in the steroid biosynthetic pathway in that tissue (33). Clearly, further study will be required to establish whether the 50 K mitochondrial substrate observed in the present study is the 24-hydroxylase and whether its phosphorylation is associated with alterations in vitamin D metabolism.

Our data show that two known activators of protein kinase C, PMA and OAG, stimulated 24-hydroxylase activity in mouse renal cortical tubules. The PMA effect was small but significant, and occurred in a dose- and time-dependent manner. We also demonstrate that renal 24-hydroxylase activity was inhibitable by H-7 and staurosporine, two inhibitors of protein kinase C which exert their effects via distinct mechanisms [the former, by competing with ATP (29), the latter, by noncompetitive interaction with the catalytic site of the kinase (30)]. Together these data provide strong evidence supporting protein kinase C-mediated regulation of renal 24-hydroxylase activity.

Previous studies have shown that both PMA (34) and OAG (14) can stimulate intracellular cAMP production which, in turn, has the potential to activate protein kinase A. To determine whether the observed changes in 24-hydroxylase activity could be mediated by protein kinase A, we examined the effects of the various agonists and antagonists of protein kinase C used in the present study on cAMP accumulation in mouse renal tubules. We found that none of these reagents had any effect on cAMP accumulation under our incubation conditions. Although the measurement of intracellular cAMP levels may not always provide the most sensitive assessment of protein kinase A activation (35), our findings are, nevertheless, consistent with the notion that the changes in 24-hydroxylase activity described herein are mediated by protein kinase C and not protein kinase A.

The murine *Hyp* mutation is characterized by accelerated renal catabolism of vitamin D metabolites as a consequence of increased renal mitochondrial 24-hydroxylase activity (19). *Hyp* mice also exhibit elevated renal cytosolic protein kinase C activity (23). In the present study, we compared the effects of PMA and H-7 on 24-hydroxylase activity in renal tubules prepared from normal mice and mutant *Hyp* littermates. In contrast to our findings with tubules from normal mice, we found that PMA could not further stimulate 24-hydroxylase activity in *Hyp*-derived tubules. Our demonstration that PMA can stimulate protein kinase C activity in renal cytosolic fractions isolated from mutant mice indicates that refractoriness of the kinase to PMA stimulation cannot account for our results. We propose that the constitutive renal vitamin D catabolic pathway in the *Hyp* mouse

may be maximally up-regulated by protein kinase C, and therefore insensitive to further stimulation by activators of the kinase. On the other hand, inhibition of protein kinase C by H-7 led to an inhibition of 24-hydroxylase activity in *Hyp*-derived renal tubules. Moreover, the level of 24-hydroxylase activity observed after H-7 treatment was similar in both genotypes. Taken together, these observations suggest that protein kinase C may be involved in the abnormal expression of 24-hydroxylase activity in the *Hyp* mouse.

In addition to abnormalities in renal vitamin D metabolism and protein kinase C activity, the *Hyp* mouse also exhibits impaired renal phosphate reabsorption attributable to a specific defect in Na^+-dependent phosphate transport at the renal brush border membrane (36). We have shown previously that PMA-induced activation of protein kinase C in renal tubules from normal mice elicits a decrease in Na^+-dependent phosphate uptake (22). The fact that stimulation of renal protein kinase C activity in normal mice results in perturbations of renal phosphate transport and vitamin D metabolism, which are characteristic of *Hyp* mice, further supports the hypothesis that protein kinase C plays a role in the expression of the mutant renal phenotype.

The 24-hydroxylase, the first enzyme in the catabolic C-24 oxidation pathway, is expressed constitutively in mouse kidney but can be induced to higher levels of activity by pretreating animals with the vitamin D hormone (21). We asked whether protein kinase C also participates in the regulation of the inducible 24-hydroxylase. We found that whereas H-7 inhibited constitutive 24-hydroxylase activity, it failed to attenuate the induced enzyme. We reported in a previous study (4) that the induction of 24-hydroxylase by 1,25-$(OH)_2D_3$ is actinomycin D-sensitive, suggesting that RNA and protein synthesis are required for this process. Furthermore, 24-hydroxylase activity is expressed constitutively only in the kidney, whereas the 1,25-$(OH)_2D_3$-inducible enzyme has been found in other target tissues such as intestine (2, 4), bone (5), and skin fibroblasts (7). Thus, one possible interpretation of our findings could be that the inducible 24-hydroxylase represents another form or isozyme of the constitutively expressed renal hydroxylase, one that is not subject to regulation by protein kinase C. Further support for this hypothesis derives from our previous demonstration that only constitutive renal 24-hydroxylase activity is perturbed in the *Hyp* mouse whereas the inducible enzyme activity is comparable in mutant and normal animals (21). Clearly, further study will be required to resolve this issue.

In summary, we have demonstrated PMA-induced translocation of protein kinase C from the cytosolic to the mitochondrial fraction, phosphorylation of endogenous mitochondrial proteins, and stimulation of 24-hydroxylase activity in freshly isolated mouse renal tubules. The temporal correlation between these events suggests that protein kinase C may play a role in the regulation of renal 24-hydroxylase activity. The observations that OAG, another known activator of protein kinase C, and H-7 and staurosporine, inhibitors of the kinase, also alter 24-hydroxylase activity in mouse renal tubules, lend further support to our hypothesis. Our findings suggest protein kinase C involvement in the regulation of constitutive but not 1,25-$(OH)_2D_3$-inducible renal 24-hydroxylase activity and implicate the kinase in the aberrant expression of renal vitamin D metabolism in the *Hyp* mouse.

Acknowledgments

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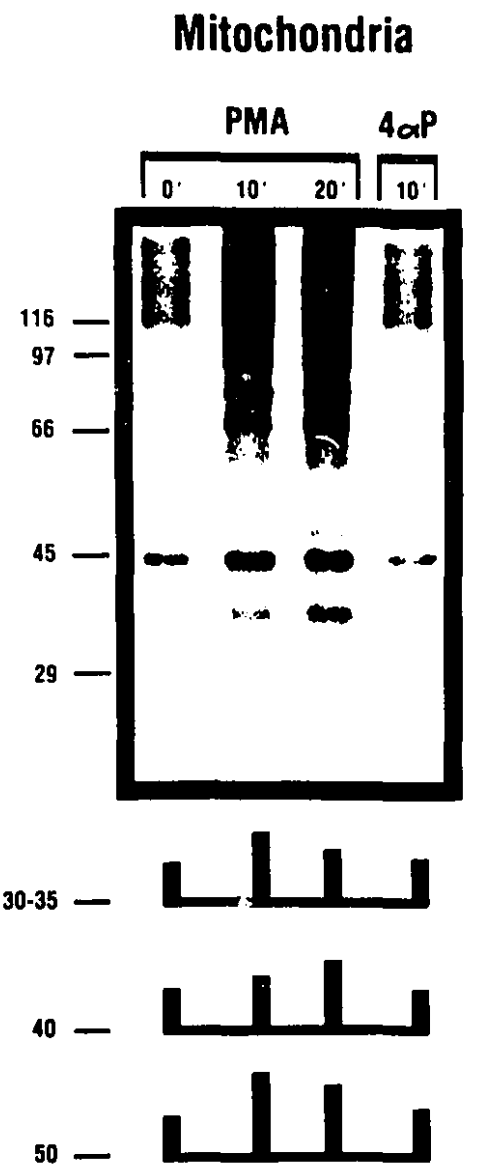
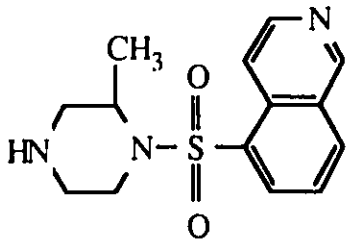
Addendum II-A

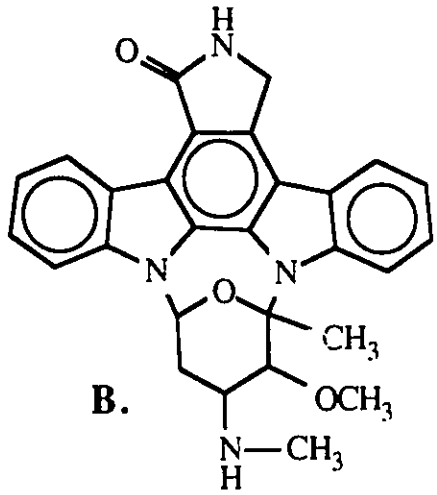


Photograph of autoradiogram shown in upper panel of Figure 1 of manuscript [see page 47 of thesis], i.e. "Representative autoradiogram of phosphorylated mitochondrial proteins isolated from PMA- and 4α-phorbol-treated ^{32}P-labeled tubules. "

Addendum II-B



A.



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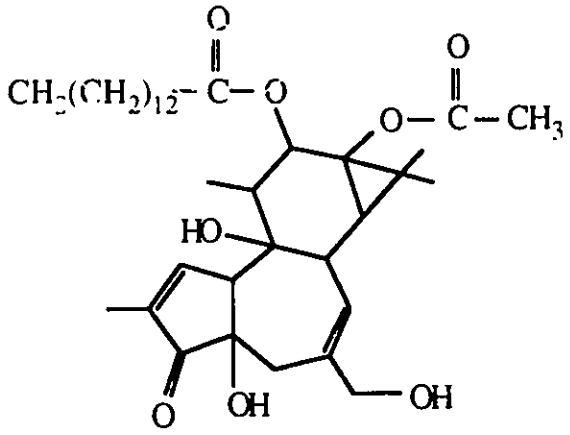
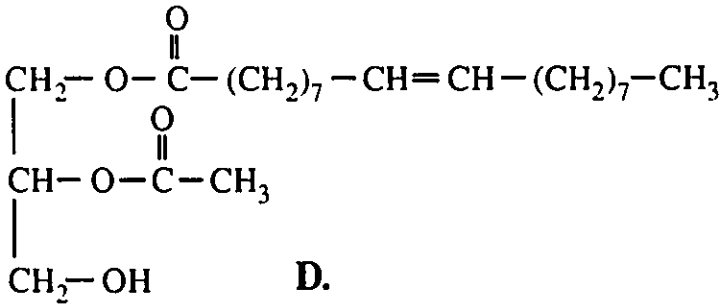


C.



D.

Structures of protein kinase C inhibitors and activators.

A. H-7; **B.** Staurosporine; **C.** Phorbol ester (PMA); **D.** Oleoylacetylglycerol (OAG)

Chapter III:
Inhibition of 25-hydroxyvitamin D_3-24-hydroxylase by forskolin: Evidence for a cAMP-independent mechanism

Whereas the previous chapter focussed on the role of protein kinase C and, thus, the phosphoinositide pathway in the regulation of renal 24-hydroxylase activity, the present study has its origins in an aspect of the cAMP pathway and its involvement in this process. PTH is a major regulator of renal vitamin D metabolism and has been shown to inhibit 24-hydroxylase activity by a mechanism involving cAMP. Part of the evidence for this stems from the observation that forskolin, a well known activator of adenylate cyclase, mimics the inhibitory effect of PTH on 24-hydroxylase. Recently, however, forskolin has been shown to exert some of its effects independently of cAMP production. To date, all reports of forskolin's cAMP-independent actions have been confined to transporters and channel proteins of the plasma membrane. The present study was undertaken to determine whether a cAMP-unrelated mechanism might be involved in the forskolin-induced inhibition of the mitochondrial membrane-associated 24-hydroxylase.

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Inhibition of 25-Hydroxyvitamin D_3-24-Hydroxylase by Forskolin: Evidence for a 3′,5′-Cyclic Adenosine Monophosphate-Independent Mechanism*

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ABSTRACT. Forskolin has long been used to demonstrate the involvement of cAMP in the regulation of cellular function, by virtue of its ability to stimulate adenylate cyclase directly. Recently, however, forskolin has been shown to affect plasma membrane transporter and channel function in a manner unrelated to cAMP. The present study examines whether forskolin-mediated inhibition of a mitochondrial membrane-associated enzyme, 25-hydroxyvitamin D_3-24-hydroxylase (24-hydroxylase), also occurs by a cAMP-independent mechanism.

Both forskolin and PTH stimulated cAMP accumulation and inhibited 24-hydroxylase activity in a dose-dependent manner in fresh mouse renal tubules. However, the level of inhibition of 24-hydroxylase achieved with forskolin was consistently greater than that obtained with PTH, at comparable levels of cAMP. 1′,9′-Dideoxyforskolin, a cyclase-inactive analog of forskolin, also inhibited 24-hydroxylase activity, without stimulating cAMP production. Moreover, both forskolin and 1′,9′-dideoxyforskolin directly inhibited 24-hydroxylase in isolated renal mitochondria. Kinetic analysis revealed a competitive mode of inhibition for both agents; however, 1′,9′-dideoxyforskolin proved to be a more potent inhibitor of 24-hydroxylase than forskolin (inhibitory constant, 0.25 *vs.* 22 μM, respectively). Finally, both forskolin and 1′,9′-dideoxyforskolin also inhibited inducible 24-hydroxylase in renal tubules prepared from 1,25-$(OH)_2D_3$-treated mice. However, inducible 24-hydroxylase activity was less susceptible to inhibition by the diterpenes than the basal enzyme activity.

The present study provides evidence for cAMP-independent inhibition of 24-hydroxylase by forskolin and represents the first demonstration of a cAMP-independent effect of forskolin on a protein that is not a plasma membrane-associated transporter or channel. Our data advocate caution in the interpretation of studies using forskolin to assess the role of cAMP in cellular processes. (*Endocrinology* 130: 2145-2151, 1992)

PARATHYROID hormone is a prominent regulator of renal vitamin D metabolism. PTH has been shown to act on the kidney to stimulate 1,25-dihydroxyvitamin D_3[1,25-$(OH)_2D_3$] synthesis (1-3) and inhibit 24,25-dihydroxyvitamin D_3[24,25-$(OH)_2D_3$] production (4-6), primarily through a mechanism involving cAMP. Part of the evidence supporting cAMP involvement in this process derives from studies demonstrating that PTH-like effects on renal vitamin D metabolism can be elicited by forskolin (5, 6), a cardiotonic diterpene that has been shown to activate adenylate cyclase directly (7). However, recent evidence suggests that forskolin can exert a number of its effects independently of cAMP production (8). Forskolin-induced inhibition of several membrane transport processes has been shown to occur via direct interaction of the diterpene with the transporter or channel, rather than by stimulation of adenylate cyclase and subsequent generation of cAMP (8). There is no evidence at present for the involvement of cAMP-independent effects of forskolin in the modulation of membrane proteins that are not transporters or channels. The present study examines whether forskolin-mediated inhibition of 25-hydroxyvitamin D_3-24-hydroxylase (24-hydroxylase), a mitochondrial membrane-associated cytochrome P-450 which plays a major role in the catabolism of vitamin D (9–13), occurs by a mechanism independent of cAMP production.

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† Recipient of Studentships from Fonds pour la Formation des chercheurs et l'aide à la recherche and the McGill University Medical Faculty.

Materials and Methods

Materials

PTH, bovine 1–34, was purchased from Bachem Inc. (Torrance, CA). Forskolin and 1′,9′-dideoxyforskolin were obtained from Sigma Chemical Co. (St. Louis, MO.). 25OHD$_3$, 1,25-

$(OH)_2D_3$, and 24,25-$(OH)_2D_3$ were gifts from Upjohn Company (Kalamazoo, MI) and Hoffmann-LaRoche Inc. (Etobicoke, Ontario, Canada). [^{3}H]25OHD$_3$ and the cAMP assay kit (TRK432) were purchased from Amersham (Oakville, Ontario, Canada).

Mice

Normal C57Bl/6 male mice, 2–5 months of age, were obtained either from Charles River Canada Inc. (St-Constant, Quebec, Canada) or from our breeding colony at the Montreal Children's Hospital. In the latter case, the original breeding pairs were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were maintained on Wayne Lab Blox diet (Allied Mills, Inc., Chicago, IL) containing 1.2% calcium, 0.99% phosphorus and 4.41 IU vitamin D_3/g. For induction of 24-hydroxylase activity, mice were injected ip with 1,25-$(OH)_2D_3$ (1.5 ng/g body wt) 16 h before the experiment, as described previously (12). Control mice received an equivalent volume of mineral oil vehicle.

Preparation of tubules and mitochondria

Tubules were prepared by collagenase digestion of mouse renal cortical slices, as described previously (14). Mitochondria were prepared by differential centrifugation of mouse renal cortical homogenates, as indicated previously (15). Protein concentration was determined by the method of Lowry *et al.* (16), using BSA as standard.

24-Hydroxylase activity

One-milliliter aliquots of either tubular or mitochondrial suspension were incubated with 50 nM [^{3}H]25OHD$_3$ (2000 cpm/pmol), under initial rate conditions, with either PTH (bovine 1–34), forskolin, or 1′,9′-dideoxyforskolin, at the concentrations indicated. Parallel incubations, treated with an equivalent volume of the appropriate vehicle, served as controls. Reaction mixtures in which substrate was incubated in the absence of tubules or mitochondria served as background in the quantification of product formation. Reactions were stopped by addition of 3.75 ml chloroform-methanol (1:2), and samples were stored under N_2 until extraction.

Extraction and analysis of vitamin D metabolites

Reaction mixtures were extracted twice, according to Bligh and Dyer (17). Aliquots of the reconstituted organic phase were fractionated on a Zorbax Sil column (25 cm × 6.2 mm; Dupont Co., Wilmington, DE) equilibrated with hexane-isopropanol-methanol (93.5:5.5:1), as described by Jones (18). Elution positions of vitamin D metabolites were determined with either crystalline or radioactive standards. Recovery of ^{3}H label was greater than 90% and comparable for both blank (where no significant conversion of substrate is apparent) and experimental samples, indicating that all reaction products could be accounted for under our incubation conditions.

cAMP accumulation

Tubules or mitochondria were incubated as described above for 24-hydroxylase assay. cAMP in reaction mixtures was measured, as described previously (19), using a competitive binding assay kit.

Statistics

Results represent mean ± SEM. Statistical significance was determined by Student's *t* test, paired or unpaired, as appropriate.

Results

We examined the effect of PTH on 24-hydroxylase activity and cAMP accumulation in freshly prepared mouse renal tubules. The renal tubule preparation represents a well characterized model of the renal proximal tubular cell (20, 21), the site of vitamin D metabolism in the kidney, and provides a system in which cellular signaling machinery is intact. Figure 1 shows that, as expected, PTH inhibited 24-hydroxylase activity and increased cAMP production in a dose-dependent fashion, suggesting that the inhibitory effect of PTH on 24-hydroxylase is likely mediated by cAMP. The maximal inhibition of 24-hydroxylase activity achieved by PTH (10^{-7} M) was 25%.

We next examined the effect of forskolin concentration on 24-hydroxylase activity and cAMP accumulation in mouse renal tubules and found that, like PTH, forskolin inhibited 24-hydroxylase function and stimulated cAMP production in a dose-dependent manner (Fig. 2). However, the level of inhibition of 24-hydroxylase achieved by forskolin was consistently greater than that observed with PTH, despite comparable levels of cAMP accumulation. These results suggest that at least part of the effect of the diterpene occurs independently of cAMP.

To further investigate the possibility of a cAMP-

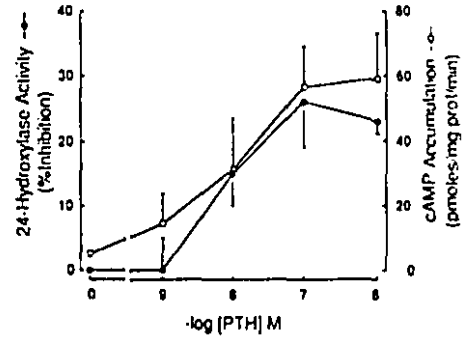


FIG. 1. Effect of PTH concentration on 24-hydroxylase activity and cAMP accumulation in mouse renal tubules. Tubules were prepared and incubated with the indicated concentrations of PTH (bovine 1–34) and 50 nM [^{3}H]25OHD$_3$ as described in *Materials and Methods*. Metabolites were extracted and fractionated by HPLC, as indicated in *Materials and Methods*. 24-Hydroxylase activity (●) is expressed as percent inhibition, where 0% inhibition (or 100% control activity) equals 15 ± 2 fmol/mg protein·min. cAMP accumulation (○) was determined as described in *Materials and Methods*. Each value represents the mean ± SEM of three to eight separate experiments.

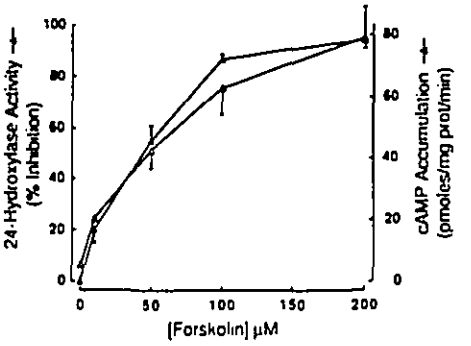


FIG. 2. Effect of forskolin concentration on 24-hydroxylase activity and cAMP accumulation in mouse renal tubules. For experimental details on measurement of 24-hydroxylase activity (▲) and cAMP accumulation (△), see legend to Fig. 1. 24-Hydroxylase activity is expressed as percent inhibition, where 0% inhibition (or 100% control activity) equals 23 ± 2 fmol/mg protein·min. Each value represents the mean ± SEM of four to eight separate experiments.

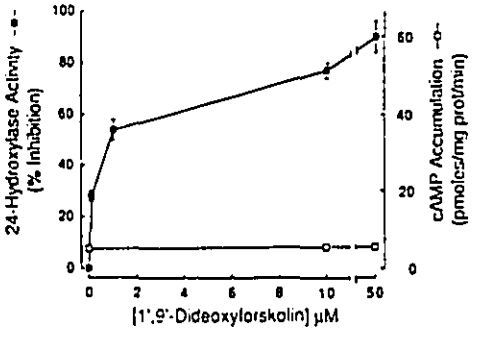


FIG. 3. Effect of 1',9'-dideoxyforskolin concentration on 24-hydroxylase activity and cAMP accumulation in mouse renal tubules. For experimental details on measurement of 24-hydroxylase activity (■) and cAMP accumulation (□), see legend to Fig. 1. 24-Hydroxylase activity is expressed as percent inhibition, where 0% inhibition (or 100% control activity) equals 16 ± 2 fmol/mg protein·min. Each value represents the mean ± SEM of three to four separate experiments.

independent mechanism for forskolin action, we examined the effect of 1',9'-dideoxyforskolin, a cyclase-inactive analog of forskolin (22), on 24-hydroxylase activity in mouse renal tubules. Figure 3 shows that 1',9'-dideoxyforskolin achieved a dose-dependent inhibition of 24-hydroxylase activity in the renal tubule preparation, without stimulating cAMP accumulation. We further demonstrated that both forskolin and 1',9'-dideoxyforskolin directly inhibited 24-hydroxylase activity in isolated renal mitochondria, a preparation virtually free of adenylate cyclase activity (Fig. 4). In both preparations, 1',9'-dideoxyforskolin was a more potent inhibitor of 24-hydroxylase activity than forskolin (EC_{50} in tubules, 1 *vs.* 50 μM, respectively; in mitochondria, 0.1 *vs.* 50 μM,

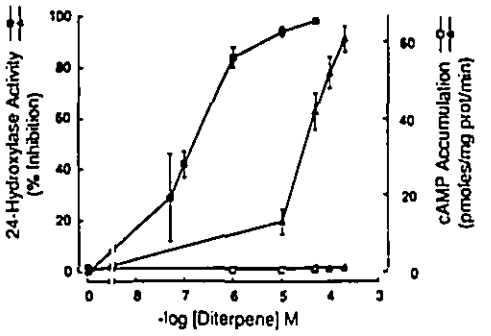


FIG. 4. Effects of forskolin and 1',9'-dideoxyforskolin concentrations on 24-hydroxylase activity and cAMP accumulation in isolated renal mitochondria. Mitochondria were prepared and incubated with the indicated concentrations of forskolin (▲, △) or 1',9'-dideoxyforskolin (■, □), and 50 nM [^{3}H]25OHD$_3$ as described in *Materials and Methods*. Metabolites were extracted and fractionated by HPLC, as indicated in *Materials and Methods*. 24-Hydroxylase activity (▲, ■) is expressed as percent inhibition, where 0% inhibition (100% control activity) equals 76 ± 7 fmol/mg protein·min for forskolin and 111 ± 20 fmol/mg protein·min for 1',9'-dideoxyforskolin. cAMP accumulation (△, □) was determined as described in *Materials and Methods*. Each value represents the mean ± SEM of three to eight separate experiments.

respectively). Moreover, whereas forskolin was equally effective in both preparations (EC_{50}, 50 μM), 1',9'-dideoxyforskolin achieved a greater inhibition of 24-hydroxylase activity in isolated mitochondria than in tubules (EC_{50}, 0.1 *vs.* 1 μM, respectively).

To characterize the nature of the cAMP-independent mechanism of forskolin action, we examined the effect of the diterpene on the kinetics of 24-hydroxylase activity in isolated mouse renal mitochondria. Figure 5A depicts an Eadie-Hofstee transformation of 24-hydroxylase activity measured either in the presence or the absence of 50 μM forskolin. Analysis of the kinetic parameters thus derived (Table 1) reveals a significant increase in the apparent Michaelis-Menten constant (K_m) for the substrate (25OHD$_3$) with no significant alteration in the maximal velocity of reaction (V_{max}), consistent with a competitive mode of inhibition for forskolin. Similar results were obtained with the cyclase-inactive analog, 1',9'-dideoxyforskolin (Fig. 5B and Table 1). However, the analog was a far more potent competitive inhibitor of 24-hydroxylase activity than forskolin (inhibitory constant, 0.25 *vs.* 22 μM, respectively).

Finally, to determine whether forskolin also inhibits 1,25-(OH)$_2$D$_3$-inducible 24-hydroxylase, we examined the effect of the diterpene on 24,25-(OH)$_2$D$_3$ synthesis in renal tubules derived from 1,25-(OH)$_2$D$_3$-treated mice. Figure 6A shows that although forskolin inhibited both basal and 1,25-(OH)$_2$D$_3$-inducible 24-hydroxylase, the inducible enzyme activity was less susceptible to inhibition by the diterpene than the basal activity (maximal

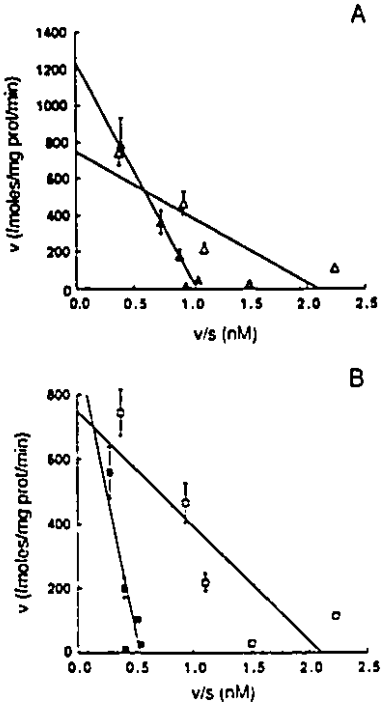


FIG. 5. Effects of forskolin and 1',9'-dideoxyforskolin on kinetics of 24-hydroxylase activity in isolated renal mitochondria. A, Mitochondria were prepared and incubated under initial rate conditions, over substrate concentrations ranging from 20–2000 μM [^{3}H]25OHD$_3$, either in the presence (▲) or absence (△) of 50 μM forskolin, as described in *Materials and Methods*. Metabolites were extracted and fractionated by HPLC, as indicated in *Materials and Methods*. Shown is an Eadie-Hofstee plot of the data. Each point represents the mean ± SEM of three (▲) or six (△) separate experiments. Regression lines were obtained by least-squares analysis [r = 0.98 for (▲) and 0.84 for (△)]. B, Experimental details are as described in A, except incubations were carried out either in the presence (■) or absence (□) of 1 μM 1',9'-dideoxyforskolin. Shown is an Eadie-Hofstee plot of the data. Each point represents the mean ± SEM of three (■) or six (□) separate experiments. Regression lines were obtained by least-squares analysis [r = 0.82 for (■) and 0.84 for (□)].

inhibition, 51% and 94%, respectively). Similar results were obtained with 1',9'-dideoxyforskolin in isolated renal mitochondria (Fig. 6B).

Discussion

24-Hydroxylase is a mitochondrial membrane-associated enzyme which plays a key role in the catabolism of vitamin D metabolites in the kidney and other vitamin D target tissues (9–13). It is well established that PTH inhibits renal 24-hydroxylase activity by a classical mechanism involving cAMP. Support for cAMP involvement derives from the demonstration that PTH effects

TABLE 1. Effect of forskolin and 1',9'-dideoxyforskolin on kinetic parameters of 24-hydroxylase

	Control	Forskolin	ddForskolin
V_{max} (fmol/min·mg protein)	750 ± 111	1185 ± 338	864 ± 171
K_m (μM)	345 ± 44	1063 ± 224*	1556 ± 158*

Mitochondria were prepared from mouse kidney as described in *Materials and Methods*. 24-Hydroxylase activity was measured under initial rate conditions, over substrate concentrations ranging from 20–2000 μM [^{3}H]25OHD$_3$, in the presence or absence of 50 μM forskolin or 1 μM 1',9'-dideoxyforskolin (ddForskolin), as specified in *Materials and Methods*. Metabolites were extracted and fractionated by HPLC, as described in *Materials and Methods*. Each value represents the mean ± SEM of three (inhibitor) or six (control) separate experiments. Difference in V_{max} between inhibitor and control is not statistically significant.

* Statistically significant difference ($P < 0.005$; Student's t test) in K_m between inhibitor and control.

on renal vitamin D metabolism can be mimicked by cAMP (1–4) and forskolin (5, 6), a known activator of adenylate cyclase (7). Recent studies, however, have shown that forskolin can exert a number of its effects on plasma membrane transporter and channel activity independently of adenylate cyclase activation (8). The present study demonstrates that these nontraditional effects of forskolin extend to other membrane-associated proteins, such as mitochondrial 24-hydroxylase. We show that although both PTH and forskolin inhibit 24-hydroxylase and stimulate cAMP accumulation in mouse renal tubules, the level of 24-hydroxylase inhibition achieved by forskolin is consistently greater than that obtained with PTH, despite comparable levels of cAMP production. We also show that 1',9'-dideoxyforskolin, a cyclase-inactive analog of forskolin, not only inhibits 24-hydroxylase activity, but does so more effectively than forskolin, without stimulating cAMP production. Our results further demonstrate that both forskolin and 1',9'-dideoxyforskolin directly inhibit 24-hydroxylase in isolated renal mitochondria which are virtually free of adenylate cyclase activity. Taken together, our findings indicate that at least part of the inhibition of 24-hydroxylase by forskolin in intact cells occurs via a cAMP-independent mechanism. Our findings in no way negate the results of previous studies showing a cAMP-dependent mechanism for PTH regulation of renal 1- and 24-hydroxylase (1–6).

One of the better characterized examples of a cAMP-independent action of forskolin is the forskolin-mediated inhibition of glucose transport. A cAMP-independent mechanism was suggested by the demonstration that 1',9'-dideoxyforskolin, a cyclase-inactivate analog of forskolin, inhibited glucose transport in adipocytes (23) and muscle cells (24) without increasing cAMP production, and that forskolin attenuated glucose transport in isolated adipocyte membrane vesicles, which lack the ATP

*correction: units for Km are nM (Table 1)

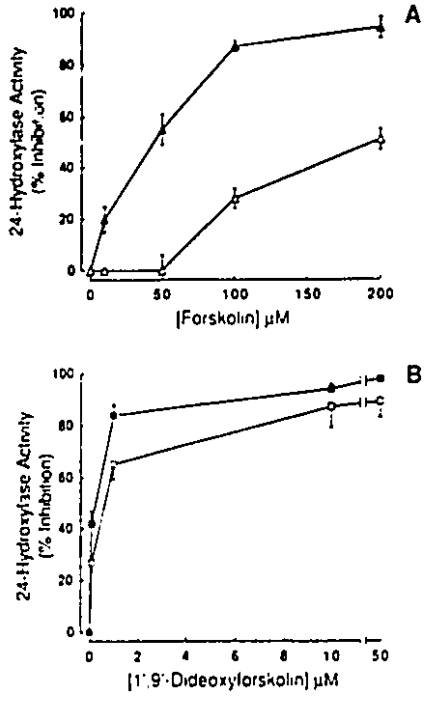


FIG. 6. Effects of forskolin and 1',9'-dideoxyforskolin on basal and 1,25-$(OH)_2D_3$-inducible 24-hydroxylase activities. A, Mice were injected ip with either 1.5 ng/g body wt 1,25-$(OH)_2D_3$ (△) or equivalent volume of mineral oil vehicle (▲) 16 h before the experiment, as described in *Materials and Methods*. Tubules were prepared as described in *Materials and Methods*. For experimental details on measurement of 24-hydroxylase activity, see legend to Fig. 1. 24-Hydroxylase activity is expressed as percent inhibition, where 0% inhibition (or 100% control activity) equals 23 ± 2 fmol/mg protein·min for ▲ and 76 ± 7 fmol/mg protein·min for △. Each value represents the mean ± SEM of three to seven separate experiments. B, Injection protocol is as described in legend to Fig. 6A. Mitochondria were prepared from 1,25-$(OH)_2D_3$ (□) or mineral oil (■)-treated mice as described in *Materials and Methods*. For experiments details on the measurement of 24-hydroxylase activity, see legend to Fig. 4. 24-Hydroxylase activity is expressed as percent inhibition, where 0% inhibition (or 100% control activity) equals 111 ± 20 fmol/mg protein·min for ■ and 303 ± 31 fmol/mg protein·min for □. Each value represents the mean ± SEM of three to five separate experiments.

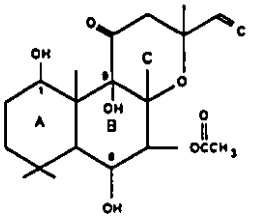
required for cAMP synthesis (25, 26). Direct evidence for an interaction between forskolin and the glucose transporter was provided by photoaffinity labeling experiments, which showed covalent binding of forskolin and its derivatives to the erythrocyte glucose transporter and inhibition of this process by cytochalasin B and D-glucose but not by L-glucose (27, 28). Structural homologies between forskolin and the transported hexoses have been invoked as a basis for forskolin binding to the glucose transporter (23) and have been used to develop forskolin derivatives with different potencies for the inhibition of sugar transport (24, 29).

Some insight into the nature of the cAMP-independent mechanism of 24-hydroxylase inhibition by forskolin may be derived from our kinetic studies in isolated renal mitochondria, which show that forskolin significantly increases the apparent K_m of the 24-hydroxylase for its substrate, $25OHD_3$, without significantly altering the V_{max}. This finding is consistent with a competitive mode of inhibition and suggestive of a direct interaction between forskolin and the substrate binding site of the hydroxylase. A basis for such interaction may be found in the structural similarities which exist between the diterpene and the steroid substrate of 24-hydroxylase. The ring structures of forskolin have their counterparts in $25OHD_3$, and both compounds have axial methyl groups at positions 10 and 13 (Fig. 7). A major structural difference between the two compounds is the existence of hydroxyl groups at positions 1, 6, and 9 of forskolin and their absence from the steroid substrate (Fig. 7). In this regard, the cyclase-inactive analog, 1',9'-dideoxyforskolin, which lacks two of the three hydroxyl groups (Fig. 7), shares greater homology with the steroid substrate. This may account for our observation that 1',9'-dideoxyforskolin has a greater affinity than forskolin for the substrate binding site of 24-hydroxylase.

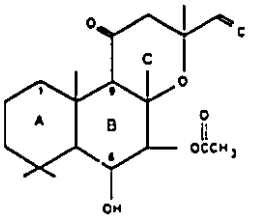
Whereas 1',9'-dideoxyforskolin achieves a greater inhibition of 24-hydroxylase activity in isolated renal mitochondria than in tubules, forskolin is equally effective at inhibiting 24-hydroxylase in both preparations. A possible explanation for this observation may reside in the inhibitory mechanisms employed by the two diterpenes, as well as the differential accessibility of the 24-hydroxylase in the two tissue preparations. Because the inhibition of 24-hydroxylase by 1',9'-dideoxyforskolin depends entirely on direct interaction between the diterpene and the mitochondrial enzyme, it may be less effective in the tubule preparation, where 24-hydroxylase is less accessible to the inhibitor than in isolated mitochondria. By contrast, the inhibition of 24-hydroxylase by forskolin, which can occur by activation of adenylate cyclase at the plasma membrane in addition to direct interaction with the mitochondrial enzyme, should not be compromised in this way.

24-Hydroxylase activity, expressed in mouse kidney, can be increased severalfold by pretreating the animals with 1,25-$(OH)_2D_3$, the vitamin D hormone (30). We show in the present study that forskolin inhibits not only basal 24-hydroxylase, but also attenuates 1,25-$(OH)_2D_3$-inducible enzyme activity. The same holds true for 1',9'-dideoxyforskolin. It is of interest that in both cases, the inducible 24-hydroxylase is less sensitive to inhibition by the diterpenes than the basal activity. These findings are reminiscent of previously reported differences be-

Forskolin



1',9'-Dideoxyforskolin



25-Hydroxyvitamin D_3

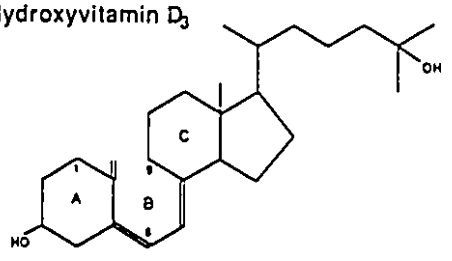


FIG. 7. Structures of forskolin, 1',9'-dideoxyforskolin, and 25-hydroxyvitamin D_3.

tween basal and 1,25-$(OH)_2D_3$-inducible 24-hydroxylase activities. We demonstrated that whereas basal 24-hydroxylase activity is strongly inhibited by the protein kinase C inhibitor, H-7, 1,25-$(OH)_2D_3$-inducible enzyme activity is completely insensitive to this compound (31). Similarly, only the basal 24-hydroxylase activity is perturbed in the mutant X-linked hypophosphatemic (*Hyp*) mouse; the inducible enzyme activity is comparable in both normal and mutant animals (30). The basis for these differences is not fully understood. Induction of 24-hydroxylase is sensitive to inhibitors of RNA and protein synthesis (12) and associated with an increase in 24-hydroxylase messenger RNA abundance (13). Furthermore, whereas basal 24-hydroxylase is expressed only in the kidney, the inducible enzyme activity can be found in other vitamin D target tissues such as intestine (10, 12), bone (32), and skin fibroblasts (33). Thus, one may speculate that the inducible 24-hydroxylase could represent an isozyme of the basally expressed enzyme. Clearly, further study will be required to resolve this issue.

Although cAMP-independent effects of forskolin have been described previously, such reports have been limited to transport proteins and channels of the plasma membrane (8). The present study documents the first example of a cAMP-independent effect of forskolin on a mitochondrial membrane-associated enzyme and suggests that such nontraditional actions of forskolin may represent a more generalized phenomenon. Our study further demonstrates that forskolin concentrations need not be high for cAMP-independent effects to become manifest. In fact, forskolin concentrations as low as 10 μM, well within the range of the EC_{50} for forskolin-mediated elevation of cAMP [1–20 μM (34)] and typically used in physiological studies, are sufficient to elicit cAMP-independent inhibition of 24-hydroxylase. Our findings emphasize the importance of exercising caution in the design and interpretation of studies using forskolin to assess cAMP involvement in cellular function.

In summary, our findings present strong evidence for cAMP-independent inhibition of 24-hydroxylase activity by forskolin. Our study is the first to describe a cAMP-independent effect of forskolin involving the modulation of a protein which is not a plasma membrane-associated transporter or channel, and suggests that this may be a more generalized phenomenon. Our kinetic data suggest a direct interaction of forskolin with the substrate binding site of the 24-hydroxylase. Compelling structural similarities between the diterpene and the steroid substrate of the enzyme support this type of interaction. Finally, our results advocate caution in the design and interpretation of experiments using forskolin to assess cAMP involvement in cellular function.

Acknowledgments

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Chapter IV:
Normal 24-hydroxylation of vitamin D metabolites in patients with vitamin D dependency rickets type I. Structural implications for the vitamin D hydroxylases

Whereas Chapters II and III were concerned with regulatory pathways and related subjects, structural considerations form the basis for the study described in the present chapter. In order to address this issue, we developed a strategy which takes advantage of a Mendelian disorder of vitamin D metabolism, namely, vitamin D dependency rickets type I (VDDR-I), which is attributable to a defect in 1-hydroxylase function. The status of 24-hydroxylase activity in VDDR-I patients is unclear. Studies in the animal model for this disorder, the rachitic pig, reveal a defect not only in renal 1-hydroxylase activity, but in 24-hydroxylase function as well. Because Mendelian inheritance dictates that a single mutation be responsible for both enzyme defects, it has been postulated that both catalytic activities are mediated by a single gene product. The present study was designed to assess 24-hydroxylase function in patients with VDDR-I, to determine whether the defect in 24-hydroxylase activity observed in the porcine disorder is also expressed in the human counterpart, with the aim of gaining some insight into the structural relationship between the vitamin D hydroxylases.

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Normal 24-Hydroxylation of Vitamin D Metabolites in Patients with Vitamin D-Dependency Rickets Type I. Structural Implications for the Vitamin D Hydroxylases*

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ABSTRACT. The steady state serum concentration of 1,25-dihydroxyvitamin D [1,25-$(OH)_2D$] is determined by the relative rates of its biosynthesis via the renal mitochondrial 1-hydroxylase and catabolism via renal and target cell 24-hydroxylases. It is not yet known whether the two catalytic activities are mediated by the product of a single gene or products of distinct genes. To address this question, we undertook to assess 24-hydroxylase function in patients with vitamin D-dependency rickets type I (VDDR-I), a Mendelian disorder of 1,25-$(OH)_2D$ synthesis attributable to a defect in renal 1-hydroxylase activity. To assess renal 24-hydroxylase activity, we measured the serum concentration of 24,25-dihydroxyvitamin D [24,25-$(OH)_2D$] and its 25-hydroxyvitamin D (25OHD) precursor. We also measured target cell, 1,25-$(OH)_2D_3$-inducible 24-hydroxylase activity and calcitroic acid production in skin fibroblasts from VDDR-I patients and age- and sex-matched controls. Serum levels of 24,25-$(OH)_2D$ and 25OHD were similar in VDDR-I patients and controls [ratio of product to substrate, 0.062 ± 0.013 (n = 5) *vs.* 0.067 ± 0.005 (n = 10), mean ± SEM, for patients and controls, respectively]. Circulating levels of 1,25-$(OH)_2D$ were also comparable in both groups [60.6 ± 15.5 (n = 5) *vs.* 86.1 ± 5.2 (n = 10) pmol/L, for patients and controls, respectively], presumably indicative of compliance with calcitriol therapy. Skin fibroblasts from VDDR-I patients exhibited 24-hydroxylase activity which was indistinguishable from that observed in control fibroblasts [108 ± 14 (n = 5) *vs.* 96 ± 25 fmol/10^6 cells·min (n = 6), for patients and controls, respectively]. Similarly, calcitroic acid production was comparable in fibroblast cultures derived from the two groups of subjects [31 ± 6 *vs.* 33 ± 3 fmol/10^6 cells·min (n = 3), for patients and controls, respectively]. Our data demonstrate that renal and target cell 24-hydroxylase activities are normal in patients with VDDR-I and suggest that the renal 1- and 24-hydroxylases likely represent, or contain, distinct polypeptides encoded by different genes. (*J Clin Endocrinol Metab* 74: 814–820, 1992)

THE STEADY state serum concentration of 1,25-dihydroxyvitamin D [1,25-$(OH)_2D$], the vitamin D hormone, is determined by the relative rates of its biosynthesis via the renal mitochondrial 25-hydroxyvitamin-D_3-1α-hydroxylase (1-hydroxylase) and catabolism via pathways including C24- and C23-oxidation, and hepatic conjugation. C24-oxidation constitutes a major degradative pathway for vitamin D metabolites and involves renal and target cell 25-hydroxyvitamin D_3-24-hydroxylases (24-hydroxylase). Despite recent advances in the isolation and purification of the 1- and 24-hydroxylases (1, 2), the precise structural relationship between the two catalytic activities remains controversial. Because both activities have been localized to the inner mitochondrial membrane, are mediated by cytochrome P450s, and are tightly and reciprocally regulated by the same physiological factors, it has been suggested that they represent alternate functions of a single enzyme (3). Precedents for this situation have been described for cytochrome P450 hydroxylases in the adrenal cortex (4–6). Alternatively, differences in susceptibility to inhibition by carbon monoxide (7) and cytochrome P450 inhibitors (8), as well as the existence of extra-renal 24-hydroxylase activity in the absence of 1-hydroxylase activity, have led to speculation that the two catalytic activities may in fact be mediated by distinct gene products. Similar uncertainty surrounds the structural relationship between the renal and extra-renal 24-hydroxylase activities.

Vitamin D-dependency rickets type I (VDDR-I) is an autosomal recessive disorder characterized by early onset

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† Recipient of Studentship Awards from Fonds pour la formation de chercheurs et l'aide à la recherche and the Faculty of Medicine, McGill University.

of hypocalcemia, secondary hyperparathyroidism, hypophosphatemia, and severe rachitic lesions (9). Although the gene responsible for the disease has recently been mapped to 12q14 (10), the actual gene product remains unknown. The demonstration of low or undetectable serum levels of 1,25-$(OH)_2$D despite normal or slightly elevated circulating levels of the 25-hydroxyvitamin D (25OHD) precursor and of complete correction of the clinical phenotype in response to physiological doses of 1,25-$(OH)_2D_3$ provided indirect evidence for a defect in renal 1-hydroxylase activity (11). Direct evidence was later obtained from studies of a rachitic pig model of VDDR-I (12), which revealed undetectable levels of 1-hydroxylase activity in renal homogenates prepared from mutant animals (13, 14). Interestingly, renal 24-hydroxylase activity was also undetectable in these animals. Moreover, the absence of 24-hydroxylase activity could not be attributed to the hypocalcemia, hyperparathyroidism, and hypophosphatemia associated with the disease, since treatment of rachitic pigs with 1,25-$(OH)_2D_3$ did not restore enzyme activity (14).

The observation that a single genetic mutation is associated with the impairment of both 1- and 24-hydroxylase activities in the rachitic pig is consistent with the hypothesis that both activities are mediated by a single gene product. The present study was undertaken to examine the status of 24-hydroxylase activity in patients affected with VDDR-I, with the aim of gaining further insight into the structural relationship between the renal 1-hydroxylase and renal and target cell 24-hydroxylases. To assess renal 24-hydroxylase activity, we measured concentrations of both 24,25-dihydroxyvitamin D [24,25-$(OH)_2$D], and its precursor, 25OHD, in plasma samples from both VDDR-I patients and sex- and age-matched controls. To ascertain target cell 24-hydroxylase activity, we measured 1,25-$(OH)_2D_3$-inducible 24-hydroxylase activity in cultured skin fibroblasts derived from both VDDR-I patients and sex- and age-matched controls. In addition, we measured 1,25-$(OH)_2D_3$-inducible production of calcitroic acid, the final inactivation product of 1,25-$(OH)_2D_3$ via the C24-oxidation pathway (15–18), in both VDDR-I and control cultures.

Materials and Methods

Materials

Cell culture media were obtained from Flow Laboratories Inc. (Mississauga, Ontario, Canada). Fetal bovine serum (FBS) was purchased from GIBCO Canada Inc. (Burlington, Ontario, Canada). BSA, fraction V, was from Sigma Chemical Co. (St. Louis, MO). Plastic ware (Falcon Laboratories) was obtained through Baxter Corporation (Pointe Claire, Quebec, Canada). [26,27-^{3}H]1,25-$(OH)_2D_3$ and [26,27-^{3}H]25OHD$_3$ were purchased from Amersham (Oakville, Ontario, Canada). Crystalline 1,25-$(OH)_2D_3$ and 25OHD$_3$ were gifts from Hoffmann-LaRoche Inc. (Etobicoke, Ontario, Canada). [1β-^{3}H]1α,25-$(OH)_2D_3$ was prepared by the method of Makin *et al.* (18). In brief, 1-oxo-25-hydroxyprevitamin D$_2$ was reduced with [^{3}H] sodium borohydride (13 Ci/mmol) to give a mixture of 1α- and 1β,25-$(OH)_2$ previtamin D$_3$ isomers which were separated and heated to give pure [1β-^{3}H]1α,25-$(OH)_2D_3$ ($\approx$3.5 Ci/mmol). [1β-^{3}H]1α,25-$(OH)_2D_3$ was purified before use and was always greater than 95% pure, where purity is defined as giving a single peak by HPLC on Zorbax-SIL (19). 1,25-$(OH)_2$D assay kit was purchased from Incstar (Stillwater, MN).

Subjects

Five patients with VDDR-I (see Table 2), three male and two female, aged between 8 and 28 yr, were recruited from our Biochemical Genetics Service (Montreal Children's Hospital) for the present study. Two patients (WG1729 and 1736) were sibs. Three patients (WG1728, 1729, and 1736) were French-Canadians from the Saguenay-Charlevoix region of Quebec, a region with high incidence of VDDR-I (20). All had been diagnosed on the basis of clinical and biochemical features associated with VDDR-I, namely early onset of hypocalcemia, hypophosphatemia, elevated serum alkaline phosphatase, and severe rachitic bone lesions despite adequate diet. Clinical details of three patients (WG1717, 1729, and 1736) have been described previously (21). All were receiving treatment (15–20 ng/kg·day Rocaltrol, administered orally in two doses, 12 h apart) at the time of study.

Cell lines

Fibroblasts from the five VDDR-I patients described above, six control subjects and one VDDR-II patient, were studied. VDDR-I fibroblast cultures were established from skin biopsies obtained and processed as described previously (22). Sex- and age-matched control human dermal fibroblasts were grown from frozen stocks maintained by the Repository for Mutant Human Cell Strains (Montreal Children's Hospital). VDDR-II fibroblasts were a gift from Dr. S. J. Marx (NIH, Bethesda, MD) and have been characterized previously (23).

Cell culture

For 24-hydroxylase assay, fibroblasts were grown in 75 cm^2 plastic flasks, at 37 C, under 5% CO_2/95% air, in Eagles minimal essential medium (MEM) with Earle's salts, L-glutamine, and nonessential amino acids, 10% FBS, supplemented with 0.025 mM $Fe(NO_3)_3$ $9H_2O$, 1.4 mM NaCl, 0.03 mM KCl, 0.16 mM NaH_2PO_4, 0.006 mM KH_2PO_4, 8.4 mM glucose, and 1 mM sodium pyruvate, as described previously (22). All experiments were performed with confluent cultures between the 6th and 30th passage. For assay of calcitroic acid production, conditions were identical to those described for 24-hydroxylase assay, except that cells were maintained in plastic multiwell plates (35 cm^2 × 6). For subculture, cells were detached by treatment with 0.59 mM disodium EDTA in 25 mM Tris-buffer (pH 7.4) and 0.25% trypsin in Puck's saline G. Cells were harvested with either the above method, for 24-hydroxylase assay, or 0.05% trypsin and 0.54 mM EDTA in PBS, for measurement of calcitroic acid production.

24-Hydroxylase induction

24-Hydroxylase, the first enzyme in the C24-oxidation pathway, was induced in cultured human fibroblasts as described previously (24). Confluent cultures were washed with PBS, placed in fresh MEM containing 1% FBS, and incubated at 37 C for 16 h with either 10 nM $1,25\text{-}(OH)_2D_3$ or an equivalent volume of the ethanol vehicle (final concentration, 0.2%).

Assay of 24-hydroxylase activity and calcitroic acid production

24-Hydroxylase activity was assessed as described previously (24). After pretreatment with either $1,25\text{-}(OH)_2D_3$ or vehicle, medium was removed and monolayers were washed, harvested (see above), and resuspended in assay medium (serum-free MEM buffered with HEPES, 3.38 g/L, pH 7.5) to yield approximately 1×10^6 cells/mL. One milliliter aliquots of cell suspension were incubated with 50 nM either [26,27-^{3}H]$1,25\text{-}(OH)_2D_3$ or [26,27-^{3}H]$25OHD_3$, under initial rate conditions, in a shaking waterbath at 25 C. Reactions were stopped with addition of 3.75 mL ice-cold chloroform:methanol (1:2) and stored under nitrogen at −20 C until extraction.

Measurement of calcitroic acid production was achieved by the method of Jones, G., C. Haussler, and M. R. Haussler (in preparation). Briefly, after preincubation with either $1,25\text{-}(OH)_2D_3$ or vehicle, medium was removed and monolayers were washed twice with PBS, then twice with assay medium (see above) containing 2% BSA. Assay medium containing 0.2% BSA and substrate (25 nM [1β-^{3}H]1α, $25\text{-}(OH)_2D_3$) was then added and cultures incubated for 1–2 h at 37 C. Preliminary studies showed that the production of aqueous-soluble vitamin D metabolites, greater than 90% of which consisted of calcitroic acid, was linear for at least 2 h and that these aqueous-soluble metabolites represented approximately 75% of the total metabolic products under our experimental conditions (data not shown). Control incubations, performed in the absence of cells, served as background in the quantitation of calcitroic acid production; unconverted [1β-^{3}H]$1\alpha,25\text{-}(OH)_2D_3$ substrate partitioning into the aqueous phase accounted for less than 5% of total radioactivity. Reactions were stopped with addition of 1.25 mL methanol. Microwell contents were transferred to glass test tubes (13 mm × 100 mm) to which 0.625 ml chloroform was added.

Extraction and measurement of vitamin D metabolites

24-Hydroxylase assay. Reaction mixtures were extracted according to Bligh and Dyer (25). Aliquots of the organic phase were fractionated on Zorbax-Sil (25 cm × 6.2 mm) equilibrated with hexane-isopropanol-methanol (93.5:5.5:1) as described previously (19) for separation of $25OHD_3$ from 24-hydroxylation products (24,25-dihydroxyvitamin D_3, 24-oxo-25-hydroxyvitamin D_3 and 24-oxo-23,25-dihydroxyvitamin D_3). Chromatographic separation of $1,25\text{-}(OH)_2D_3$ from 24-hydroxylation products (1,24,25-trihydroxyvitamin D_3, 24-oxo-1,25-dihydroxyvitamin D_3 and 24-oxo-1,23,25-trihydroxyvitamin D_3) was achieved on Zorbax-Sil (25 cm × 6.2 mm) with hexane-isopropanol-methanol (80:10:2) as described previously (26). Fractions were collected and radioactivity measured by liquid scintillation counting. Elution positions of products were confirmed with authentic metabolites. Recovery of ^{3}H label was greater than 85% and comparable for incubations of $1,25\text{-}(OH)_2D_3$-treated cells, vehicle-treated cells and medium alone (in the latter two instances, no significant conversion of substrate was apparent), indicating that all reaction products could be accounted for under our conditions.

Calcitroic acid production

The extraction procedure of Bligh and Dyer (25) was modified by replacing 4% KCl with water. Phases were allowed to separate and stabilize for 1–2 h at room temperature. Replicate aliquots of the aqueous phase were taken and radioactivity measured by liquid scintillation counting.

Measurement of vitamin D metabolites in serum

Venous blood samples were drawn from 5 VDDR-I patients and 10 sex- and age-matched control subjects under nonfasting conditions, usually in late morning. Thus, for VDDR-I patients, sampling time fell within 4–6 h of their morning dose of $1,25\text{-}(OH)_2D_3$. Serum was separated within 1 h of sampling and frozen at −20 C until analysis. Serum concentrations of $1,25\text{-}(OH)_2D$ were determined by a competitive binding assay using a calf thymus radioreceptor assay kit. $24,25\text{-}(OH)_2D$ and 25OHD concentrations were measured by the method of Cunningham *et al.* (27). Briefly, vitamin D metabolites were extracted by methanol-methylene chloride and then separated by Zorbax-CN chromatography (28) into 25OHD and $24,25\text{-}(OH)_2D$ fractions. 25-Hydroxyvitamin D-26,23-lactone (29), a common interfering metabolite in such assays, was separated by this chromatographic step and discarded. $24,25\text{-}(OH)_2D$ and 25OHD were quantitated by a competitive protein binding assay based upon rat DBP (27).

Statistics

All values are expressed as mean ± SEM. Statistical significance was determined by the Student's *t* test, paired or unpaired, as appropriate.

Results

Plasma concentrations of vitamin D metabolites

Because the VDDR-I patients investigated in the present study are being treated with physiological doses of calcitriol, their circulating levels of $1,25\text{-}(OH)_2D$ should be comparable to those observed in sex- and age-matched control individuals. Table 1 shows that plasma concentrations of $1,25\text{-}(OH)_2D$ did not differ significantly between the two groups, indicating that VDDR-I patients are compliant with calcitriol therapy and have circulating levels of $1,25\text{-}(OH)_2D$ within the normal physiological range.

When circulating concentrations of $1,25\text{-}(OH)_2D$ are physiologically normal, such as in our subjects, the plasma concentration of $24,25\text{-}(OH)_2D$ is a reflection of two phenomena: 1) the intrinsic activity of the consti-

TABLE 1. Serum vitamin D metabolites in VDDR-I patients and sex- and age-matched control subjects

	Control (10)	VDDR-I (5)
1,25-$(OH)_2D$ (pmoles/L)	86 ± 5	81 ± 16
24,25-$(OH)_2D$ (nmoles/L)	3.4 ± 0.4	4.2 ± 1
25OHD (nmoles/L)	57 ± 4	68 ± 5

VDDR-I patients were undergoing calcitriol therapy (15-20 ng/kg·day Rocaltrol), as indicated in *Materials and Methods*. Circulating levels of 1,25-$(OH)_2D$ were determined by calf thymus radioreceptor assay, as described in *Materials and Methods*. 24,25-$(OH)_2D$ and 25OHD were measured by competitive binding assay after chromatographic separation, as described in *Materials and Methods*. (n) indicates number of subjects. Data represent mean ± SEM.

tutive renal 24-hydroxylase, since extra-renal target cell enzyme activity does not appear to be expressed under such conditions (24, 30); and 2) 25OHD precursor availability (31). We, therefore, measured circulating levels of both 24,25-$(OH)_2D$ and 25OHD precursor in both VDDR-I patients and sex- and age-matched control subjects. The results depicted in Table 1 clearly indicate that plasma concentrations of both 24,25-$(OH)_2D$ and 25OHD are comparable in VDDR-I patients and controls [ratio of 24,25-$(OH)_2D$: 25OHD, 0.062 ± 0.013 (n = 5) *vs.* 0.067 ± 0.005 (n = 10), mean ± SEM, for patients and controls, respectively], and suggest that constitutive renal 24-hydroxylase activity is intact in VDDR-I and, as in normals, is directly proportional to the concentration of substrate, 25OHD.

Catabolism of vitamin D metabolites in cultured skin fibroblasts

1,25-$(OH)_2D_3$-Inducible 24-hydroxylase activity is linear with time, up to 45 min and with cell number, up to 5×10^6 cells/mL (data not shown). Kinetic analysis of 1,25-$(OH)_2D_3$-inducible 24-hydroxylase revealed a maximal velocity of reaction (V_{max}) of 185 fmol/10^6 cells·min and an apparent Michaelis Menten constant (K_m) of 167 nM for $25OHD_3$ and a V_{max} of 57 fmol/10^6 cells·min and an apparent K_m of 4 nM for 1,25-$(OH)_2D_3$. These parameters are consistent with those described previously for inducible 24-hydroxylase in mouse kidney and intestine (30).

We compared 1,25-$(OH)_2D_3$-inducible 24-hydroxylase activity in skin fibroblasts isolated from five VDDR-I patients and six sex- and age-matched controls. Table 2 demonstrates that fibroblasts derived from patients with VDDR-I exhibit 1,25-$(OH)_2D_3$-inducible 24-hydroxylase activity which is indistinguishable from that measured in control fibroblasts, whether the substrate used is 1,25-$(OH)_2D_3$ or $25OHD_3$. Thus, it would appear that 1,25-$(OH)_2D_3$-inducible 24-hydroxylase activity is intact in patients with VDDR-I.

The production of calcitroic acid, the final inactivation product of 1,25-$(OH)_2D_3$ via the C24-oxidation pathway (15-18), was measured in cultured skin fibroblasts obtained from patients with VDDR-I and sex- and age-matched controls. Table 3 demonstrates that calcitroic acid production is comparable in fibroblasts derived from patients with VDDR-I and control subjects, suggesting that the entire C24-oxidation pathway is unperturbed in VDDR-I. By contrast, skin fibroblasts derived from a patient with VDDR-II show negligible calcitroic acid production (Table 3).

TABLE 2. 1,25-$(OH)_2D_3$-inducible 24-hydroxylase activity in fibroblasts derived from VDDR-I patients and controls

Cell line	Sex, age	24-Hydroxylase activity (fmoles/10^6 cells·min)			
		1,25-$(OH)_2D_3$ substrate [Mean ± SEM (n)]		$25OHD_3$ substrate [Mean ± SEM (n)]	
VDDR I patients					
WG1717	M,22	144 ± 31	(4)	84	(1)
WG1728	M,11	139	(2)	71	(2)
WG1729	M,28	95	(2)	48	(2)
WG1735	F,10	83 ± 4	(3)	44	(2)
WG1736	F,21	81 ± 5	(3)	32	(2)
All lines		108 ± 14		56 ± 10	
Controls					
WG972	M,23	19	(2)	ND	
MCH70	M,19	116	(1)	36	(1)
MCH39	M,11/12	56 ± 3	(4)	51 ± 3	(3)
WG122	M,25	178 ± 57	(3)	45	(1)
WG1052	F,6	152 ± 1	(3)	65	(2)
WG123	F,24	58	(2)	ND	
All lines		96 ± 25		54 ± 11	

24-Hydroxylase activity was induced by 1,25-$(OH)_2D_3$ treatment of confluent skin fibroblast cultures as described in *Materials and Methods*. 1,25-$(OH)_2D_3$-inducible 24-hydroxylase activity was measured under initial rate conditions using 50 nM either [^{3}H]1,25-$(OH)_2D_3$ or [^{3}H] $25OHD_3$, as indicated in *Materials and Methods*. Metabolites were extracted, fractionated by HPLC, and counted, as described in *Materials and Methods*. ND = not determined. (n) represents number of experiments.

Discussion

The present study was undertaken to examine, in a systematic fashion, the status of 24-hydroxylase function in patients with VDDR-I, a hereditary disorder of vitamin D metabolism, attributable to a defect in renal 1-hydroxylase activity (11). We present evidence supporting normal 24-hydroxylation of vitamin D metabolites in VDDR-I. Plasma concentrations of 25OHD and 24,25-$(OH)_2D$ in VDDR-I patients undergoing calcitriol therapy are similar to those observed in sex- and age-matched control subjects, suggesting that constitutive renal 24-hydroxylase activity is intact in VDDR-I. Skin fibroblasts derived from VDDR-I patients exhibit 1,25-$(OH)_2D_3$-inducible 24-hydroxylase activity which is comparable to that measured in control cultures, indicating

TABLE 3. 1,25-$(OH)_2D_3$-inducible calcitroic acid production in fibroblasts derived from VDDR-I and VDDR-II patients and controls. 1β-^{3}H]1,25-$(OH)_2D_3$ as substrate

Cell line	Sex, age	Calcitroic acid production (fmoles/10^6 cells·min)	
		Mean ± SEM	(n)
VDDR I patients			
WG1717	M,22	25 ± 3	(4)
WG1729	M,28	25 ± 4	(4)
WG1735	F,10	43 ± 6	(6)
All lines		31 ± 6	
Controls			
MCH39	M,11/12	38 ± 1	(6)
WG122	M,25	27	(2)
WG1052	F,8	33 ± 1	(4)
All lines		33 ± 3	
VDDR-II patient			
WG1851	F,10	5 ± 1	(4)

The C24-oxidation pathway was induced by 1,25-$(OH)_2D_3$ treatment of confluent skin fibroblast cultures as described in *Materials and Methods*. 1,25-$(OH)_2D_3$-inducible calcitroic acid production was measured using 25 nM[1β-^{3}H]1,25-$(OH)_2D_3$, as indicated in *Materials and Methods*. Metabolites were extracted and radioactivity counted, as described in *Materials and Methods*. (n) represents number of experiments.

that inducible, target cell 24-hydroxylase function is also unperturbed in VDDR-I. Calcitroic acid production is also indistinguishable in VDDR-I patient-derived and control fibroblasts, suggesting that the entire C24-oxidation pathway is intact in VDDR-I. Our demonstration of normal 24-hydroxylase activity in patients with a Mendelian disorder of 1-hydroxylase function suggests that these two catalytic activities likely represent, or contain, distinct polypeptides encoded by separate genes.

The VDDR-I patients examined in the present study are treated with physiological doses of calcitriol and, as expected, exhibit plasma concentrations of 1,25-$(OH)_2D$ which are in the normal physiological range. Under such conditions, the plasma concentration of 24,25-$(OH)_2D$ presumably reflects constitutive renal 24-hydroxylase activity, since the expression of extra-renal 24-hydroxylase is only induced by administration of pharmacological doses of vitamin D hormone (24, 30). Plasma 24,25-$(OH)_2D$ levels are also determined by 25OHD precursor availability (31). Accordingly, plasma concentrations of both metabolites must be measured to ensure a reliable estimate of renal 24-hydroxylase activity. We show that both VDDR-I patients undergoing calcitriol therapy and control subjects have similar plasma concentrations of both 24,25-$(OH)_2D$ and 25OHD, suggesting that constitutive renal 24-hydroxylase activity is intact in VDDR-I. Our findings confirm the results of two earlier studies (32, 33) which reported normal plasma levels of 24,25-$(OH)_2D$ in VDDR-I patients. However, identification of the plasma metabolite as 24,25-$(OH)_2D$ in those studies is somewhat equivocal, since neither used a chromatographic method capable of resolving 24,25-$(OH)_2D$ from 25-hydroxyvitamin D-26,23-lactone, an abundant metabolite found in plasma known to interfere with the quantitation of 24,25-$(OH)_2D$ by competitive protein binding assay (29). Our study employs a chromatographic method (28) which provides unequivocal separation and analysis of vitamin D metabolites.

In the present study, we show that 1,25-$(OH)_2D_3$-inducible 24-hydroxylase activity in human skin fibroblasts is dependent on cell number, duration of incubation, and substrate concentration, and that the relative affinities of 1,25-$(OH)_2D_3$ and 25OHD_3 substrates for the enzyme system are similar to those reported previously for 24-hydroxylase in renal and intestinal tissues (30). Our data also show that the amount of 24-hydroxylase activity observed does not correlate with either sex or age of subject. Finally, when we compare 1,25-$(OH)_2D_3$-inducible 24-hydroxylase activity in skin fibroblasts derived from VDDR-I patients and control subjects, we observe no significant difference between the two, suggesting that inducible target cell 24-hydroxylase function is intact in VDDR-I.

In addition to measuring 1,25-$(OH)_2D_3$-inducible 24-hydroxylase activity in VDDR-I skin fibroblasts, we also measure the production of calcitroic acid, the final inactivation product of 1,25-$(OH)_2D_3$ via the C24-oxidation pathway (16–18). Our demonstration of normal calcitroic acid production in skin fibroblasts derived from VDDR-I patients confirms our findings with respect to 24-hydroxylase activity and indicates that the entire C24-oxidation pathway is intact in VDDR-I. As expected, the 1,25-$(OH)_2D_3$-inducible catabolic pathway shows negligible expression in skin fibroblasts from a VDDR-II patient, deficient in 1,25-$(OH)_2D_3$ receptor function (23).

Previous studies in the rachitic pig (12), a putative animal model for VDDR-I, showed a complete absence of 24-hydroxylase activity, as well as 1-hydroxylase function, in renal homogenates (13) and mitochondria (14) prepared from the mutant animals. Our demonstration of normal 24-hydroxylase function in humans with VDDR-I is in striking contrast with the rachitic pig data and suggests that the two disorders, despite their many shared clinical and biochemical features, may not have the same genetic basis.

Our demonstration of normal 24-hydroxylase activity in patients with VDDR-I, a Mendelian disorder of 1-hydroxylase function, supports the hypothesis that the two hydroxylases either represent or contain distinct gene products. Although our findings do not exclude the possibility that both catalytic activities are mediated by a single polypeptide chain, as proposed by Ghazarian (34), they are in agreement with previous studies which

demonstrated biochemical differences between the 1- and 24-hydroxylases with respect to sensitivity to carbon monoxide (7) and cytochrome P450 inhibitors (8). More recently, purified rat renal 24-hydroxylase was shown to support the synthesis of 24,25-$(OH)_2D_3$, but not 1,25-$(OH)_2D_3$, in a reconstituted system containing 25OHD_3 substrate, NADPH, ferredoxin, and ferredoxin reductase (35). Clearly, unequivocal evidence for the two gene hypothesis must await expression studies in cells transfected with the recently cloned complementary DNA (cDNA) encoding rat renal 24-hydroxylase (36). It should be noted that the 24-hydroxylase clone was isolated from a kidney cDNA library prepared from rats treated with pharmacological doses of vitamin D and, thus, represents the inducible 24-hydroxylase. It is not yet known whether the inducible and constitutive 24-hydroxylase activities are mediated by the same enzyme or different isozymes encoded by separate genes (17). Preliminary data from our laboratory indicate different biochemical and regulatory properties for the inducible and constitutive 24-hydroxylase activities. Whereas the protein kinase C inhibitor, H-7, effectively inhibits constitutive 24-hydroxylase activity in mouse renal tubules, it fails to attenuate the 1,25-$(OH)_2D_3$-inducible enzyme activity (37). In addition, studies in the *Hyp* mouse have shown that only constitutive renal 24-hydroxylase activity is perturbed in the mutant animal; the 1,25-$(OH)_2D_3$-inducible enzyme activity is comparable in both normal and mutant mice (38).

Although our findings in human VDDR-I oppose observations in the rachitic pig (13, 14), the resulting structural implications for the 1- and 24-hydroxylases are not necessarily irreconcilable. Although the findings in the rachitic pig are consistent with the hypothesis that both activities are mediated by a single gene product, they do not rule out the possibility that the target of the mutation may be a gene encoding a shared component or common regulator of two distinct enzymes.

In conclusion, we provide evidence for normal 24-hydroxylase activity in patients with VDDR-I, a Mendelian disorder of vitamin D metabolism attributable to a defect in 1-hydroxylase function. Our findings have interesting implications for the structural relationship between the 1- and 24-hydroxylases and suggest that the two catalytic activities likely represent or contain distinct gene products.

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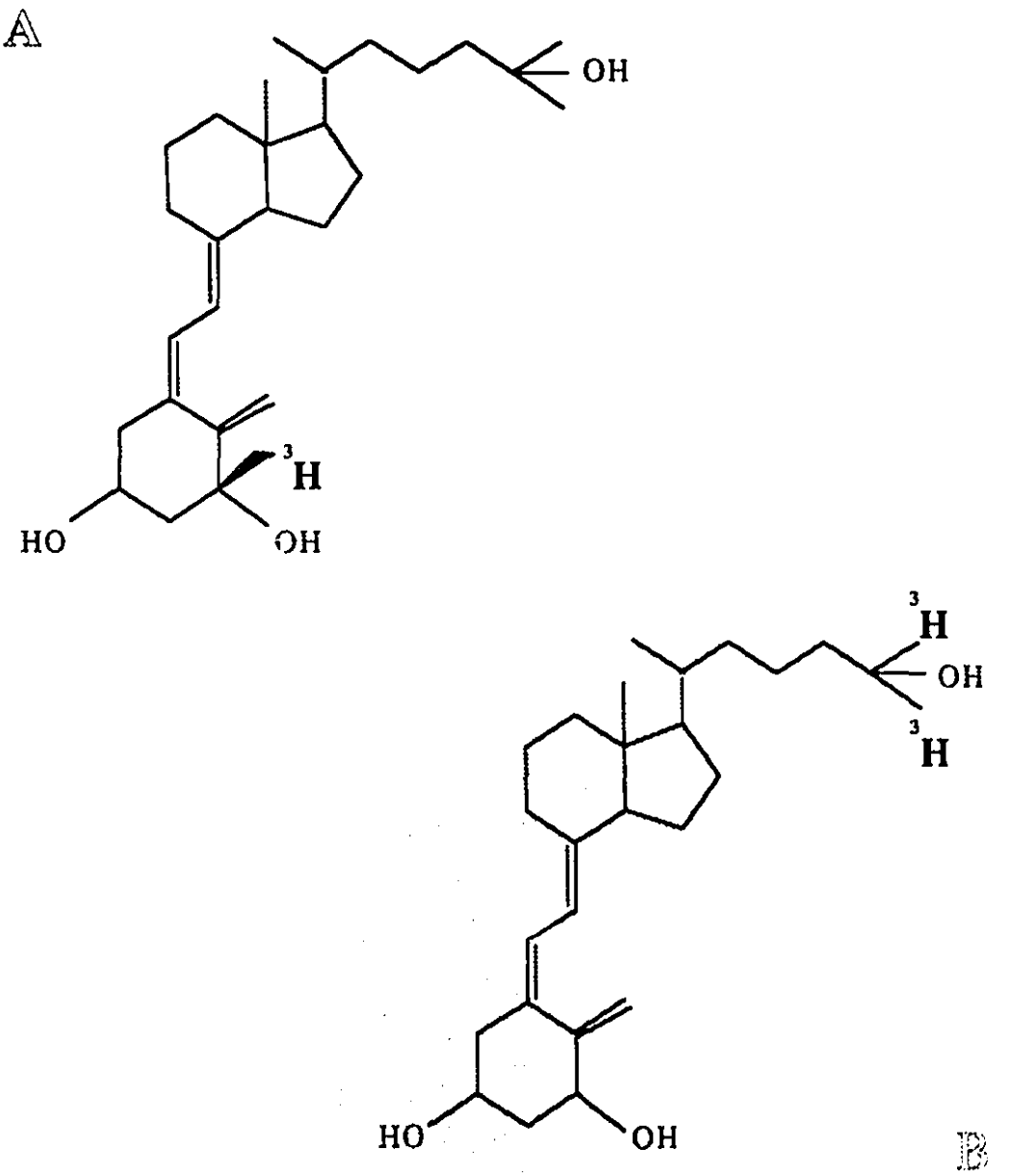
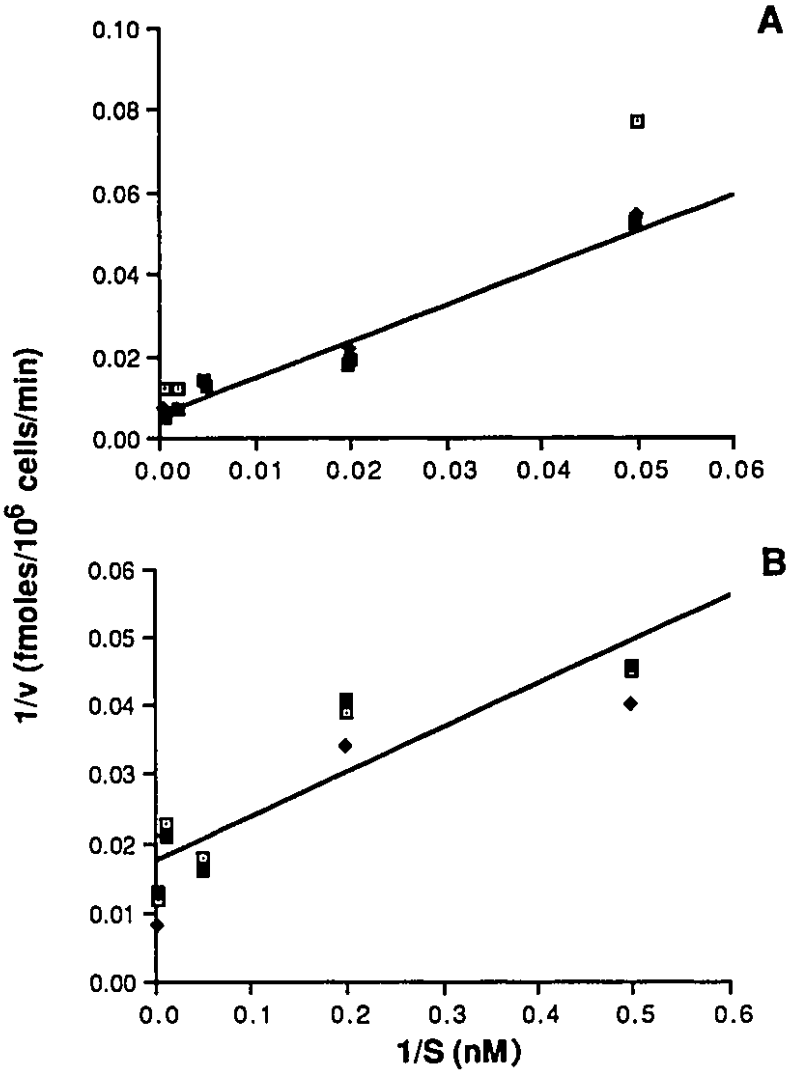
Addendum IV-A



Structures of [^{3}H]1,25-(OH)$_2$D$_3$ substrates used in VDDR-I study.

A. [1β-^{3}H]1,25-(OH)$_2$D$_3$: ^{3}H label in A ring of steroid backbone allows detection following side chain cleavage; used for measurement of calcitroic acid production; **B. [26,27-^{3}H]1,25-(OH)$_2$D$_3$:** ^{3}H label in side chain of molecule; used for standard 24-hydroxylase assay.

Addendum IV-B



Kinetics of 1,25-$(OH)_2D_3$-inducible 24-hydroxylase activity in control skin fibroblasts.

A. [^{3}H]25OHD_3 as substrate; **B.** [^{3}H]1,25-$(OH)_2D_3$ as substrate. 24-Hydroxylase activity was induced by 1,25-$(OH)_2D_3$ treatment of confluent skin fibroblast cultures as described in Materials and Methods (Chapter IV). 1,25-$(OH)_2D_3$-inducible 24-hydroxylase activity was measured under inital rate conditions, over substrate concentrations ranging from 20-2000 nM for 25OHD_3 and 2-500 nM for 1,25-$(OH)_2D_3$ as indicated in Materials and Methods (Chapter IV). Metabolites were extracted, fractionated by HPLC and counted, as described in Materials and Methods (Chapter IV). Shown is a Lineweaver-Burk plot of the data from three separate experiments. Regression lines were obtained by least-squares analysis [r=0.98 for 25OHD_3 and 0.90 for 1,25-$(OH)_2D_3$].

Chapter V: General Discussion

Evidence for protein kinase C involvement in the regulation of renal 25-hydroxyvitamin D_3-24-hydroxylase.

In 1986, Henry reported that treatment of chick kidney cell cultures with the phorbol ester, TPA, elicits an increase in 24,25-$(OH)_2D_3$ production and a decrease 1,25-$(OH)_2D_3$ synthesis, thereby implicating protein kinase C in the regulation of renal vitamin D metabolism (Henry, 1986). However, her study failed to provide direct evidence for phorbol ester-induced protein kinase C activation (i.e. translocation). Moreover, the lengthy incubation times (four hours) used in her study raised the possibility that the observed changes in vitamin D metabolism might be the result of TPA-induced down-regulation rather than activation of the kinase.

The initial observations reported for avian kidney by Henry (1986) prompted our investigation of protein kinase C involvement in the regulation of mammalian renal vitamin D metabolism, which was undertaken as part of the present thesis. While our study was in progress, a second report from Henry's laboratory appeared, essentially confirming the original observations with respect to TPA-induced modulation of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ synthesis and providing some additional support for protein kinase C involvement in this process (Henry and Luntao, 1989). They demonstrated that the effect of TPA on 1,25-$(OH)_2D_3$ synthesis in cultured chick renal cells could be mimicked by another well known activator of protein kinase C, the membrane-permeable, synthetic diacylglycerol analogue, OAG. In addition, they showed that the effects of TPA were not likely the result of protein kinase C down-regulation, since the number of phorbol ester binding sites (which presumably represent protein kinase C) was not decreased in TPA-treated cells

relative to control cultures. However, as in their first study, no direct evidence was provided for phorbol ester-induced activation of protein kinase C, nor were control experiments using an inactive phorbol ester described.

Our study provides strong evidence supporting a role for protein kinase C in the regulation of mammalian renal 24-hydroxylase, the first enzyme in the C24-oxidation pathway, a major catabolic pathway for vitamin D metabolites in kidney as well as in other target tissues for vitamin D hormone. We provide direct evidence for phorbol ester (PMA)-induced activation of protein kinase C in mouse renal cortical tubules by demonstrating translocation of the kinase from the cytosol to the mitochondrial compartment, the site of 24-hydroxylase activity in the renal epithelial cell. We also establish a temporal correlation between activation of protein kinase C, endogenous phosphorylation of mitochondrial proteins - potential mediators of kinase action on vitamin D metabolism - and stimulation of 24-hydroxylase activity in response to PMA treatment. We further show that none of these effects is elicited by the inactive phorbol ester analogue, 4α-phorbol. We also demonstrate that another activator of protein kinase C, OAG, also stimulates 24,25-$(OH)_2D_3$ production whereas H-7 and staurosporine, two inhibitors of the kinase which operate via different mechanisms, decrease 24,25-$(OH)_2D_3$ synthesis. Since none of these agents had any effect on cAMP accumulation under our experimental conditions, it is unlikely that their effects on 24,25-$(OH)_2D_3$ production are the result of fortuitous activation or inhibition of protein kinase A. These findings further support the contention that the observed changes in 24-hydroxylase activity are mediated directly by protein kinase C.

Based on our current understanding of the mechanisms involved in the regulation of renal vitamin D metabolism, it is clear that activation of the cAMP pathway results in increased 1,25-$(OH)_2D_3$ production and decreased 24,25-

$(OH)_2D_3$ synthesis whereas stimulation of the phosphoinositide cascade elicits the opposite responses. It is also evident that both signal transduction cascades are activated by the peptide hormone, PTH [see section I.5]. The ability of PTH to activate two signalling pathways which inversely regulate renal vitamin D metabolism suggests a remarkable economy and versatility in the organization of cellular signal transduction. However, at the same time, it raises a logistic problem: if both pathways are triggered coordinately by PTH, then what prevents them from cancelling out each others effects? Alternatively, if each pathway is activated separately and independently by the peptide hormone, then how is one pathway selected over the other? Another point of interest concerns the mechanism whereby PTH is coupled to both signal transduction cascades. Is each pathway coupled to a different PTH receptor subtype or does a single class of PTH receptor interact with both the cAMP and phosphoinositide cascades?

Until recently, it was generally assumed that a given receptor subtype was coupled to either the cAMP pathway or the phosphoinositide cascade, but not both. Consequently, the ability of certain agonists to trigger both cellular signalling pathways was attributed to receptor heterogeneity. Although the existence of two PTH receptor subtypes has been suggested in a recent study (Reshkin et al., 1990), most of the evidence accumulated so far supports a single receptor class for this peptide hormone (Pfeilschifter, 1989). Thus, an alternative explanation is required to account for the ability of PTH to activate both signal transduction cascades. Expression studies using cell lines transfected with individual muscarinic acetylcholine receptor subtypes have shown that each receptor subclass can couple to both the cAMP pathway and the phosphoinositide cascade although, in each case, one of the pathways is predominant (Peralta et al., 1988). Similar findings have recently been

obtained with cloned polypeptide hormone receptors, such as the receptor for luteinizing hormone [LH (Gudermann et al., 1992)]. These studies suggest that a single receptor class can couple effectively to multiple signal transduction pathways and offer a possible explanation for the observations obtained with PTH. Direct evidence supporting this hypothesis has only just become available. Segre and associates have recently cloned a cDNA encoding the PTH receptor and have shown that PTH receptor-deficient renal cell lines transfected with this cDNA exhibit PTH-stimulated cAMP accumulation, indicating that the receptor is functionally coupled to the cAMP cascade (Jüppner et al., 1991). Although not yet published, results from preliminary transfection studies by the same group suggest that the receptor clone can also stimulate phosphoinositide hydrolysis (Segre et al., 1992).

As mentioned above, it remains unclear whether PTH regulates renal vitamin D metabolism by activating both signal transduction pathways coordinately or by triggering each pathway independently of the other. Some insight into this issue may be derived from studies of another target of PTH action, namely, renal phosphate transport. As indicated earlier [section I.5], one of the functions of PTH in the proximal tubule is to increase phosphate excretion which it achieves by inhibiting sodium-dependent phosphate uptake across the renal brush-border membrane. Like renal vitamin D metabolism, renal phosphate transport is regulated by PTH via a dual mechanism involving both the cAMP and phosphoinositide cascades (reviewed by Murer, 1991). However, unlike vitamin D metabolism, PTH activation of either pathway results in the inhibition of phosphate uptake. In 1987, Cole et al. documented a discrepancy between the dose-response curves for PTH-dependent inhibition of sodium-phosphate cotransport and activation of cAMP production in OK cells. They showed that whereas half-maximal inhibition of phosphate transport was

observed between 10^{-11}-10^{-12} M PTH, a concentration of 10^{-8} M PTH was required to achieve half-maximal stimulation of cAMP production (Cole et al., 1987; 1988). By contrast, PTH-dependent activation of phospholipase C, also measured in OK cells, was evident at PTH concentrations as low as 10^{-12} M, concentrations at which inhibition of renal phosphate transport was observed (Quamme et al., 1989). These findings led to the proposal that low or physiologically normal concentrations of PTH regulate phosphate transport *exclusively* via the phosphoinositide pathway whereas higher concentrations of the hormone *selectively* activate the cAMP cascade. Thus, PTH would appear to activate each pathway separately, in a concentration-dependent fashion.

A more recent study by Martin et al. (1989) offers findings that are in striking contrast with the observations described above. In their study, Martin et al. (1989) compared the dose-response curves for PTH-mediated inhibition of phosphate transport, cAMP accumulation and protein kinase A activation in OK cells. They found that although PTH-induced changes in phosphate uptake were not reflected in intracellular cAMP levels (in agreement with Cole et al., 1987; 1988), alterations in protein kinase A activity were demonstrable at the lowest dose of PTH which inhibited phosphate transport. These findings suggest that low concentrations of PTH elicit minute, perhaps localized, changes in intracellular cAMP content which, although undetectable, are sufficient to activate protein kinase A. Thus, one cannot exclude a role for the cAMP pathway in the regulation of phosphate transport at low or physiologically normal concentrations of PTH.

Although they may appear contradictory, the proposals of Cole et al. (1987) and Martin et al. (1989) are not irreconcilable. The cAMP and phosphoinositide second messenger systems could conceivably coregulate renal vitamin D metabolism (and phosphate transport) over the entire range of

PTH concentrations examined, however, at low or physiological levels of PTH, the balance would be shifted toward the phosphoinositide pathway whereas at higher concentrations, the cAMP cascade would be favoured. In this regard, it is of interest that normal circulating levels of PTH [10^{-12} to 10^{-10} M] are within the range reported to stimulate the phosphoinositide pathway and that the *predominant*, but not exclusive, vitamin D metabolite produced under normal physiological conditions is 24,25-$(OH)_2$D. Under circumstances in which PTH concentrations become elevated, such as in hypocalcemia, the balance would shift toward the cAMP pathway and a preponderance of 1,25-$(OH)_2$D.

A similar mechanism has been proposed for another peptide hormone, LH. The recently cloned LH receptor has been shown to activate both the cAMP pathway and the phosphoinositide cascade with EC_{50} s of <100 pM and >2 nM, respectively (Gudermann et al., 1992). Although normal circulating levels of LH are well below the threshold for triggering phosphoinositide hydrolysis, ovulatory levels of LH and pregnancy levels of human chorionic gonadotropin (hCG), a homologue of LH also known to bind the LH receptor, are sufficient to stimulate the phosphoinositide pathway. Thus, it has been suggested that under normal physiological conditions, cellular responses to LH (and hCG) are mediated by the cAMP pathway whereas during ovulation and pregnancy, *both* signalling systems are likely activated and participate in the response (Gudermann et al., 1992).

The proposal described above must be further tempered with the consideration that, in vivo, the cell is subject to a variety of regulatory factors and constantly changing conditions which may affect the ultimate cellular response to a given agonist. For example, Henry (1981) showed that cultured renal epithelial cells maintained in serum-free growth medium fail to respond to PTH with the expected increase in 1,25-$(OH)_2D_3$ synthesis, but that PTH-

responsiveness can be restored with the addition of insulin to the growth medium. These findings suggest a permissive role for insulin in this process (Henry, 1981). Vitamin D status has also been shown to influence PTH action in the kidney. Armbrecht et al. (1984b) demonstrated that whereas PTH stimulates 1-hydroxylase activity in renal slices prepared from thyroparathyroidectomized rats previously fed a vitamin D-deficient, low calcium diet, slices from normal, vitamin D-replete animals do not respond to the peptide hormone with the appropriate increase in 1,25-$(OH)_2D_3$ production. It is not clear how insulin or vitamin D hormone modulate the effects of PTH on renal vitamin D metabolism. However, these observations do suggest that a cell does not respond to a given agonist solely on the basis of the signal transduction pathway(s) activated by that agonist; rather, it integrates additional regulatory influences, which together determine the ultimate cellular response.

The mutant Hyp mouse is characterized by a number of renal abnormalities including accelerated catabolism of vitamin D metabolites as a result of increased 24-hydroxylase activity (Tenenhouse et al., 1988), elevated cytosolic protein kinase C activity (Tenenhouse and Henry, 1985) and decreased brush-border membrane sodium-dependent phosphate transport (Tenenhouse and Scriver, 1978; Tenenhouse et al., 1978). We have shown in a previous study that the phosphate transport defect characteristic of the Hyp mutation can be elicited in normal mouse kidney through phorbol ester-induced activation of protein kinase C (Boneh et al., 1989). In the present study, we provide evidence implicating the kinase in the aberrant expression of renal 24-hydroxylase activity in this mouse mutant. We show that whereas phorbol ester treatment stimulates 24-hydroxylase activity in normal mouse kidney, it fails to further increase the already elevated 24-hydroxylase activity of Hyp kidney, despite its ability to stimulate protein kinase C in this strain. These findings

suggest that 24-hydroxylase activity in the Hyp mouse is maximally up-regulated by protein kinase C and, therefore, resistant to further stimulation by activators of the kinase. Further support for our hypothesis derives from our demonstration that the protein kinase C inhibitor H-7 not only decreases the level of 24-hydroxylase activity in the Hyp mouse, but abolishes the interstrain difference. Together, these findings support a role for protein kinase C in the aberrant expression of renal vitamin D metabolism and phosphate transport associated with the Hyp mutation.

Our findings are consistent with the hypothesis that X-linked hypophosphatemic rickets in the mouse and, perhaps, in man as well are caused by a humoral factor which acts on both renal phosphate transport and vitamin D metabolism by stimulating the cellular signalling pathway involving protein kinase C. A humoral basis for this inherited disorder was initially suggested by the intriguing parabiosis studies of Meyer et al. [(1989ab), see section I.6] and is further supported by observations of a deviant gene dose effect on both plasma phosphorus levels and renal phosphate transport in Hyp mice (Scriver and Tenenhouse, 1990). Conventional gene dosage requires that the phenotype of the heterozygote - in the case of the X-linked Hyp mutation, the Hyp/+ female - be intermediate between those of the normal female homozygote (+/+) or male hemizygote (+/Y) and the mutant female homozygote (Hyp/Hyp) or male hemizygote (Hyp/Y). However, heterozygous females exhibit the same degree of hypophosphatemia as homozygous mutant females and hemizygous mutant males (Scriver and Tenenhouse, 1990). Similarly, brush-border membrane sodium-dependent phosphate transport is as severely affected in Hyp/+ females as it is in Hyp/Hyp females and Hyp/Y males (Scriver and Tenenhouse, 1990). A transacting humoral factor would account for the occurrence of atypical gene dosage as well as the elevated

levels of protein kinase C activity and the abnormalities in renal phosphate transport and vitamin D metabolism characteristic of the Hyp mouse.

Finally, our study shows that whereas constitutive 24-hydroxylase activity is strongly inhibited by the protein kinase C inhibitor, H-7, 1,25-$(OH)_2D_3$-inducible enzyme activity is completely insensitive to this compound, suggesting that the inducible 24-hydroxylase, unlike its constitutively-expressed counterpart, is not subject to regulation by protein kinase C. This interpretation is consistent with the observation that only constitutive 24-hydroxylase activity is perturbed in the Hyp mouse; inducible enzyme activity is indistinguishable between normal and mutant animals (Jones et al., 1987b). In addition, as discussed below, the observed difference in susceptibility to H-7 inhibition has interesting structural implications for the constitutive and inducible 24-hydroxylase activities.

Inhibition of 25-hydroxyvitamin D_3-24-hydroxylase by forskolin: Evidence for a cAMP-independent mechanism.

By virtue of its unique ability to activate adenylate cyclase directly, forskolin has long been used to demonstrate cAMP involvement in a host of physiological processes, including PTH-dependent regulation of renal vitamin D metabolism [see section I.5]. Recently, however, a number of reports have appeared documenting cAMP-independent effects of forskolin on various transport functions and channel activities of the plasma membrane (Laurenza et al., 1989). Our study presents several lines of evidence supporting the involvement of a cAMP-independent mechanism in the inhibition of renal mitochondrial 24-hydroxylase activity by forskolin. We demonstrate that although both forskolin and PTH stimulate cAMP accumulation and inhibit 24-hydroxylase activity in mouse renal tubules, the level of 24-hydroxylase

inhibition achieved by the diterpene is significantly greater than that obtained with the peptide hormone, at comparable levels of cAMP production. We also show that 1',9'-dideoxyforskolin, a cyclase-inactive analogue of forskolin, is a more potent inhibitor of 24-hydroxylase activity than forskolin itself, despite its inability to stimulate cAMP production. We further demonstrate that both forskolin and 1',9'-dideoxyforskolin directly inhibit 24-hydroxylase activity in isolated renal mitochondria, a preparation virtually free of adenylate cyclase activity. A mechanism involving direct interaction of forskolin (or 1',9'-dideoxyforskolin) with the substrate binding site of 24-hydroxylase is suggested based on the kinetics of 24-hydroxylase inhibition by the diterpenes, and the structural similarities between the steroid substrate of the enzyme and the two inhibitors. Our study is the first to document a cAMP-independent action of forskolin on a protein which is neither a transporter nor a channel of the plasma membrane, but an inner mitochondrial membrane-associated enzyme. Our findings suggest that such non-traditional effects of forskolin may well represent a more generalized phenomenon and advocate caution in the design and interpretation of studies using forskolin to assess cAMP involvement in cellular processes.

Although our study focusses primarily on the effects of forskolin and 1',9'-dideoxyforskolin on constitutively-expressed or basal 24-hydroxylase activity, we also consider the effects of the two diterpenes on 1,25-$(OH)_2D_3$-inducible 24-hydroxylase activity. We show that although inducible renal 24-hydroxylase activity is also inhibited by both forskolin and 1',9'-dideoxyforskolin, it is less susceptible to inhibition than the un-induced activity. This observation recalls previously reported differences between renal constitutive and 1,25-$(OH)_2D_3$-inducible 24-hydroxylase activities. As mentioned above, only basal 24-hydroxylase activity is perturbed by the Hyp mutation (Jones et al., 1987b) and

is inhibitable by H-7; 1,25-$(OH)_2D_3$-inducible enzyme activity is unaffected in both situations. A greater sensitivity to inhibition by cytochrome P450 inhibitors further distinguishes the constitutive 24-hydroxylase from its inducible counterpart [see Appendix A]. The basis for these differences is not fully understood. However, it is of interest that the induction of 24-hydroxylase activity is associated with an increase in mRNA levels for the cytochrome P450 component of the 24-hydroxylase complex (Ohyama et al., 1991; Armbrecht and Boltz, 1991). This finding makes it tempting to speculate that the inducible 24-hydroxylase may represent an isozyme of the constitutively-expressed enzyme. Certainly, the ability of various chemical compounds to discriminate between these two catalytic activities suggests a structural basis for these differences and further implies that the two catalytic activities may be mediated by different proteins. Although it is unclear precisely what advantages might be conferred by these putative structural differences, they are unlikely to be of a kinetic nature, as the two 24-hydroxylase activities are virtually indistinguishable from one another in terms of their substrate affinities (Tenenhouse and Jones, 1987). Nevertheless, the concept of multiple 24-hydroxylase isozymes is intriguing and merits further study.

Normal 24-hydroxylation of vitamin D metabolites in patients with vitamin D-dependency rickets type I: Structural implications for the vitamin D hydroxylases.

Clinical studies in patients with vitamin D dependency rickets type I (VDDR-I), a Mendelian disorder of vitamin D metabolism, suggested a defect in 1-hydroxylase activity as the underlying cause of this phenotype (Fraser et al., 1973). Direct evidence for a 1-hydroxylase defect was provided by studies of the animal model for this disorder, the rachitic pig, which showed undetectable

levels of 1-hydroxylase activity in renal tissue preparations derived from mutant animals (Fox et al., 1985; Winkler et al., 1986). In addition, these same studies demonstrated a complete absence of renal 24-hydroxylase activity in the rachitic pigs. The observation that a single mutation was associated with the impairment of both 1- and 24-hydroxylase activities in the animal model for VDDR-I led to the proposal that both activities were mediated by a single gene product. However, it was not known whether the disturbance in renal 24-hydroxylase activity observed in the rachitic pig was also expressed in the human counterpart of VDDR-I. Renal 24-hydroxylase activity had not been directly ascertained in patients with VDDR-I. Circulating levels of 24,25-$(OH)_2D$, which were measured to provide an indirect estimate of renal 24-hydroxylase activity, appeared to be normal in VDDR-I patients (Nguyen et al., 1979; Aarskog et al., 1983), suggesting that renal 24-hydroxylase function was intact in this disorder. However, the identity of the plasma metabolite as 24,25-$(OH)_2D$ in these studies was somewhat equivocal, since neither used a chromatographic method capable of resolving 24,25-$(OH)_2D$ from 25-hydroxyvitamin D-26,23-lactone (25OHD-26,23-lactone), an abundant plasma metabolite known to co-chromatograph with 24,25-$(OH)_2D$ and interfere with its quantitation by competitive protein binding assay (Horst, 1979).

Our study was undertaken to examine, in a systematic fashion, the status of 24-hydroxylase activity in VDDR-I patients. Our results indicate that, unlike the rachitic pig, the VDDR-I patient does not exhibit a defect in 24-hydroxylase function. We established that our calcitriol-treated VDDR-I patients and control subjects exhibit physiologically normal circulating concentrations of 1,25-$(OH)_2D$; under such conditions, plasma 24,25-$(OH)_2D$ levels are a reflection of renal 24-hydroxylase function (Tomon et al., 1990ab) and 25OHD precursor availability (Marx et al., 1989). Thus, we ascertained renal 24-hydroxylase

function indirectly, by measuring the ratio of the circulating concentrations of 24,25-$(OH)_2$D:25OHD, using a chromatographic method that provides unequivocal separation and analysis of vitamin D metabolites (Jones, 1983). We found the plasma 24,25-$(OH)_2$D:25OHD concentration ratio in VDDR-I patients undergoing calcitriol therapy to be similar to that observed in sex- and age-matched control subjects, indicating that constitutively-expressed or basal renal 24-hydroxylase activity is intact in VDDR-I. We also assessed target cell, inducible 24-hydroxylase function by directly measuring 24-hydroxylase activity in 1,25-$(OH)_2D_3$-treated skin fibroblast cultures established from VDDR-I patients and controls. We found comparable levels of enzyme activity in fibroblasts derived from both groups of subjects, suggesting that 1,25-$(OH)_2D_3$-inducible 24-hydroxylase activity is also unperturbed in human VDDR-I. In addition, we measured the production of calcitroic acid (the final inactivation product of vitamin D hormone via the C24-oxidation pathway) in these same cell cultures, using a substrate labelled in the A ring of the steroid backbone which allows detection of the product after side chain cleavage. Calcitroic acid production was indistinguishable in VDDR-I patient-derived and control fibroblasts, indicating that the entire C24-oxidation pathway is intact. Our demonstration of normal 24-hydroxylase activity in patients with a Mendelian disorder of 1-hydroxylase function suggests that the two catalytic activities are likely mediated by distinct proteins.

Our findings are consistent with previously reported differences in the biochemical properties of the 1- and 24-hydroxylase activities (Knutson and DeLuca, 1974; Kung et al., 1988) and the more recent demonstration that purified renal 24-hydroxylase (Ohyama et al., 1989; Ohyama and Okuda, 1991) and 1-hydroxylase (Mandel et al., 1990a) preparations catalyze their respective reactions in a reconstituted system (containing 25OHD_3 substrate, NADPH,

ferredoxin and ferredoxin reductase) but fail to support the alternative reaction. Together, these findings provide strong evidence in support of the two-enzyme hypothesis. However, it is not clear whether these two catalytic activities are encoded by separate genes or result from differential processing of a single gene product. There is evidence at present to support both hypotheses.

Using HPLC-based methodologies, Ghazarian and associates purified chick renal mitochondrial 1-hydroxylase (Mandel et al., 1990a) and subsequently used this preparation to generate monoclonal antibodies against the enzyme (Mandel et al., 1990b). In addition to recognizing the 1-hydroxylase, the antibodies also detected the 24-hydroxylase, but failed to interact with cytochrome P450s from other sources such as liver microsomes or adrenal mitochondria. The apparent molecular weights of the 1- and 24-hydroxylase enzymes were 57 K and 55 K, respectively. One of the monoclonal antibodies was used to immunopurify the two hydroxylases from chick renal mitochondria (Moorthy et al., 1991). The purified enzymes were subjected to amino-terminal sequencing and amino acid composition analysis. The overall amino acid compositions of the two hydroxylases are almost identical. Moreover, the amino-terminal sequences of the first 10 residues are 100% homologous. These observations have led Ghazarian (1990) to postulate a precursor-product relationship between the two hydroxylases whereby the smaller 24-hydroxylase enzyme is derived from the larger 1-hydroxylase by proteolytic cleavage of a 2 K carboxy-terminal fragment. This modification would presumably alter the active site of the enzyme sufficiently to shift its catalytic specificity (from carbon 1 to carbon 24) without changing its substrate specificity ($25OHD_3$). In the aggregate, these findings suggest that the renal 1- and 24-hydroxylases represent separate but nearly identical enzymes that are derived from a single gene.

Recent findings by DeLuca's group (Burgos-Trinidad et al., 1992) are in striking contrast to the observations of Ghazarian and associates, described above. Burgos-Trinidad et al. (1992) isolated electrophoretically homogeneous preparations of chick renal 1- and 24-hydroxylases using an immunopurification strategy similar to that employed by Moorthy et al. (1991) only, in this case, the monoclonal antibodies used to prepare the affinity column were generated against partially purified 24-hydroxylase rather than 1-hydroxylase. Amino-terminal sequencing analysis of the immunopurified proteins shows that the first 20 residues of the two hydroxylases, although similar, are not identical. Furthermore, the amino acid compositions of the two hydroxylases are different, with eight amino acids differing in percent abundance by 1.8-fold or greater. Yet, the fact that monoclonal antibodies to the 24-hydroxylase can be used to immunoisolate the 1-hydroxylase clearly indicates significant structural similarities between the two enzymes. Collectively, these findings suggest that the renal 1- and 24-hydroxylases represent similar but distinct entities and imply that they are encoded by different genes.

It is of interest that the 1- and 24-hydroxylases isolated by Burgos-Trinidad et al. (1992) differ appreciably in both amino-terminal amino acid sequence and amino acid composition from the proteins purified by Moorthy et al. (1991). In an attempt to reconcile these disparate findings, Burgos-Trinidad et al. (1992) invoke the presence of two different forms for each hydroxylase. Indeed, the existence of two isozymes for 24-hydroxylase - one constitutive, the other inducible - has already been proposed on the basis of various experimental observations [see above]. It is noteworthy that the renal 24-hydroxylase isolated by Burgos-Trinidad et al. (1992) was prepared from chicks maintained on a vitamin D-supplemented diet and pre-treated with both 1,25-$(OH)_2D_3$ and $25OHD_3$ prior to the isolation procedure and, therefore, represents

inducible enzyme activity. By contrast, the 24-hydroxylase purified by Moorthy et al. (1991) was derived from chicks fed a control diet neither enriched nor deficient in vitamin D and, thus, reflects constitutive enzyme activity. It is unfortunate that our study was not able to offer any insight into the structural relationship between the two 24-hydroxylase activities. Had our data shown reduced plasma levels of 24,25-$(OH)_2D$ in VDDR-I patients, reflective of decreased renal 24-hydroxylase activity, concomitant with normal levels of fibroblast 24-hydroxylase activity, they may have suggested the existence of two isozymes of 24-hydroxylase. However, our study clearly demonstrates that both basal and inducible 24-hydroxylase activities are intact in patients with VDDR-I and is, therefore, uninformative in this regard.

Although our finding of normal 24-hydroxylase activity in VDDR-I patients is in striking contrast with observations in the animal model for this disorder (Fox et al., 1985; Winkler et al., 1986), the resulting structural implications for the two renal vitamin D hydroxylases are not necessarily irreconcilable. We propose two possible unifying hypotheses, one based on the single-gene model postulated by Ghazarian (1990) and the other, on the separate-gene model suggested by the findings of Burgos-Trinidad et al. (1992). These are depicted in Figures 1 and 2, respectively, and are explained below.

If we consider Ghazarian's model (see above), a mutation occurring in that part of the gene which is shared by the two hydroxylases, i.e. the amino-terminal region, would affect both enzyme activities and could account for the observations in the rachitic pig [see Figure 1]. By contrast, a mutation in the carboxy-terminal region, which is specific to the 1-hydroxylase and removed by proteolytic cleavage to generate the 24-hydroxylase, would selectively alter 1-hydroxylase function and spare 24-hydroxylase activity. Such would be the

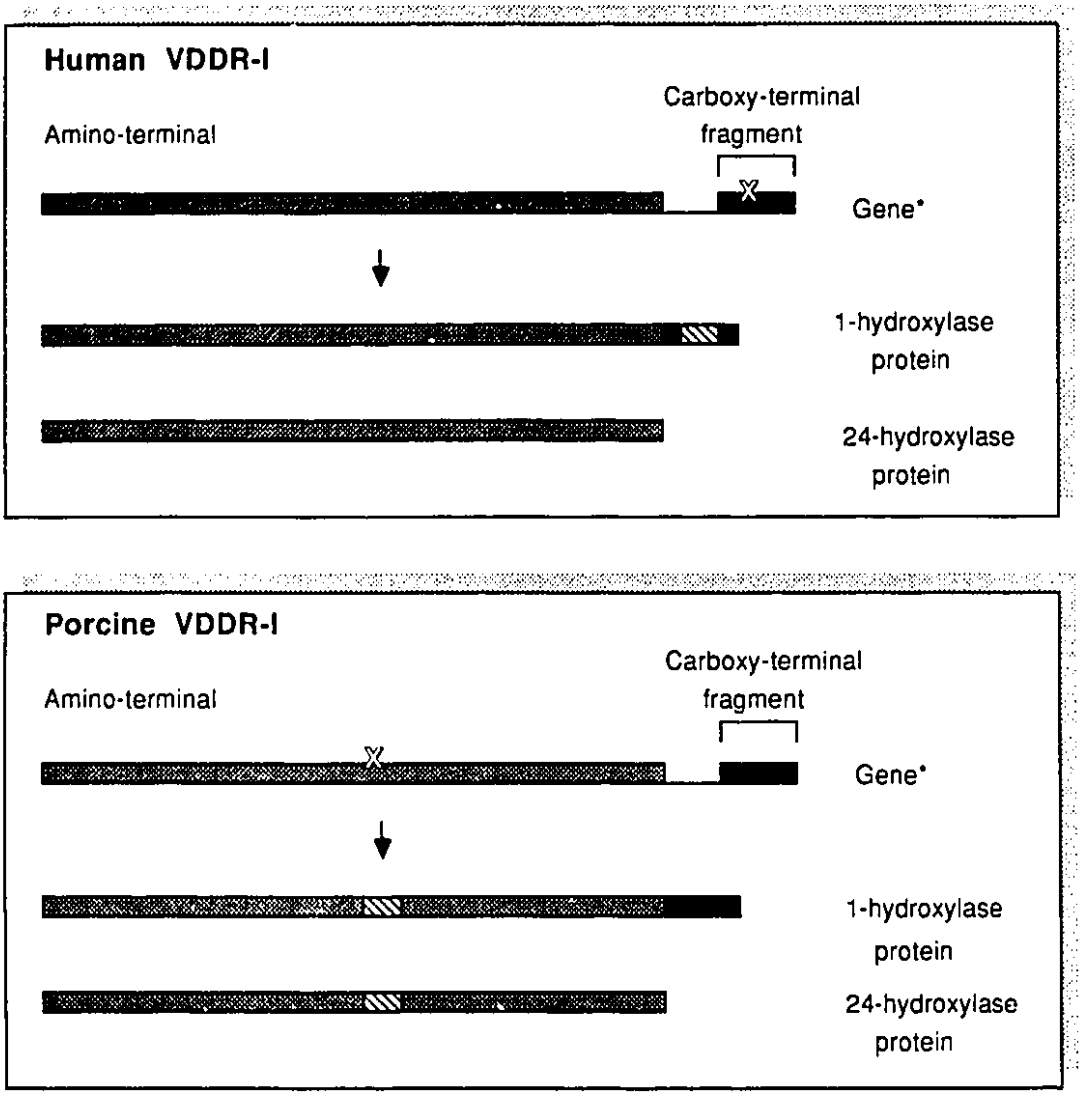


Figure 1. Possible mechanisms for human and porcine VDDR-I: single-gene model.

The observations in human and porcine VDDR-I may be accomodated by a model in which the 1- and 24-hydroxylases represent separate enzymes encoded by a single gene, such as that proposed by Ghazarian (1990). A mutation [denoted by X] in the gene's carboxy-terminal region, which is specific to the 1-hydroxylase, would selectively disrupt 1-hydroxylase function, leaving 24-hydroxylase activity intact, thereby resulting in the human disorder. By contrast, a mutation in any other part of the gene, which is shared by both hydroxylases, would perturb both enzyme activities, thereby accounting for the porcine phenotype. *Gene structure (introns/exons) is hypothetical; actual gene has not been isolated.

situation in human VDDR-I [see Figure 1].

Alternatively, the observations in human and porcine VDDR-I could be accomodated by a model involving two distinct gene products (as suggested by the findings of Burgos-Trinidad et al., 1992). A mutation targetting the gene for the 1-hydroxylase, leaving the 24-hydroxylase gene intact, would explain VDDR-I in man [see Figure 2]. By contrast, a mutation in a gene encoding a shared co-factor or common regulator of the two hydroxylases would account for the disorder in the rachitic pig [see Figure 2]. Although the identity of this putative shared component is unknown, it is not likely to be either the ferredoxin or ferredoxin reductase moieties of the renal vitamin D hydroxylating system. The cDNAs for both ferredoxin (Morel et al., 1988) and ferredoxin reductase (Solish et al., 1988) have recently been cloned from human adrenal cDNA libraries. These studies have shown that both electron transport intermediates are encoded by single-copy genes that are expressed in various steroidogenic tissues and are, therefore, shared with other mitochondrial cytochrome P450s (Morel et al., 1988; Solish et al., 1988). Since VDDR-I patients do not exhibit abnormalities in any other cytochrome P450 system, it is unlikely that either of these components represents a target of the mutation responsible for this disorder.

Both models as well as the underlying observations which they attempt to explain suggest that, despite their many clinical and biochemical similarities, human and porcine VDDR-I may not have the same genetic basis. Preliminary evidence for genetic heterogeneity in VDDR-I may be found in the work of Labuda et al. (unpublished results) which indicates different genomic locations for the VDDR-I phenotype in French Canadian and Polish populations.

More conclusive evidence regarding the structural relationship between the renal 1- and 24-hydroxylases, as well as that between the constitutive and

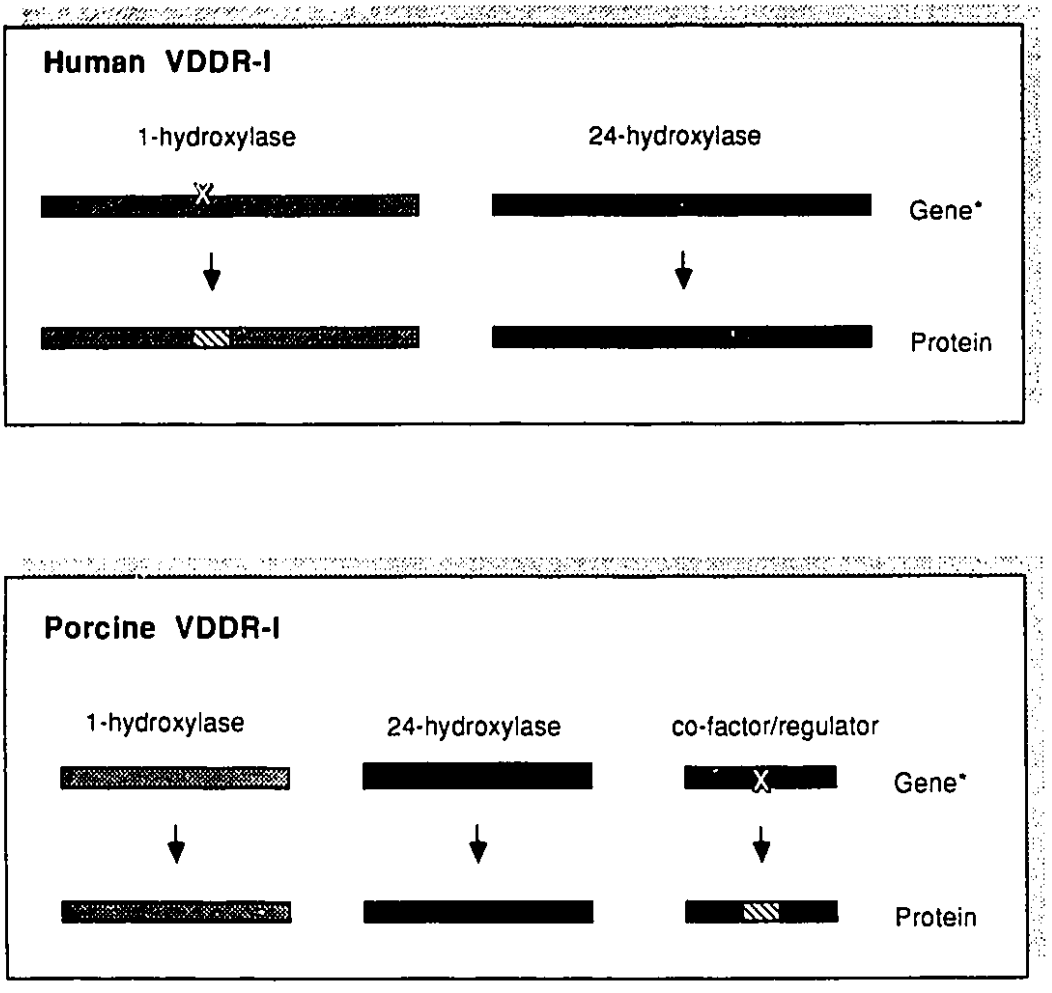


Figure 2. Possible mechanisms for human and porcine VDDR-I: separate-gene model.

The observations in human and porcine VDDR-I may be explained by a model in which the 1- and 24-hydroxylases represent separate enzymes encoded by separate genes, as suggested by the findings of Burgos-Trinidad et al. (1992). A mutation [denoted by X] in the gene encoding the 1-hydroxylase would selectively disrupt 1-hydroxylase function, leaving 24-hydroxylase activity intact, thereby resulting in the human disorder. By contrast, a mutation in a gene encoding a shared co-factor or common regulator of the two hydroxylases would perturb both enzyme activities, thereby accounting for the porcine phenotype. *Gene structures (introns/exons) are not known.

inducible 24-hydroxylase activities must await expression studies and other molecular biological strategies using the recently cloned cDNA encoding rat renal 24-hydroxylase (Ohyama et al., 1991). Studies directed at cloning the 1-hydroxylase from chick kidney are currently in progress (Burgos-Trinidad et al., 1992).

Claims to originality

To the best of my knowledge, the following represent original contributions:

Protein kinase C involvement in the regulation of renal 24-hydroxylase activity:

1. This study provides the first evidence supporting a role for protein kinase C in the regulation of mammalian renal 24-hydroxylase, the first enzyme in the catabolic C24-oxidation pathway.
2. This is the first study to *directly* demonstrate phorbol ester-induced activation and translocation of the kinase from the cytosol to the mitochondrial compartment, the site of 24-hydroxylase activity in the renal epithelial cell.
3. This is the only study to describe control experiments examining the effect of an inert phorbol ester analogue on renal 24-hydroxylase activity.
4. This is the first study to examine the effects of *both* activators *and* inhibitors of protein kinase C on renal 24-hydroxylase activity. The protein kinase C activator OAG, like PMA, stimulates 24-hydroxylase activity in mouse renal tubules (the same observation was reported by another group while this study was in progress). H-7 and staurosporine, two known inhibitors of protein kinase C which act via different mechanisms, inhibit renal 24-hydroxylase activity. These complementary findings strengthen the evidence for protein kinase C involvement in the modulation of renal 24-hydroxylase activity.
5. PMA fails to further increase the already elevated renal 24-hydroxylase activity characteristic of the mutant Hyp mouse, despite its ability to stimulate renal protein kinase C in this strain. By contrast, H-7 not only inhibits 24-hydroxylase activity in Hyp mouse renal tubules as it does in normal mouse

tubules, but also abolishes the interstrain difference. These observations are the first to implicate protein kinase C in the aberrant expression of 24-hydroxylase activity associated with the Hyp mutation.

6. This is also the first study to address protein kinase C involvement in the regulation of inducible 24-hydroxylase activity. The findings suggest that the inducible enzyme activity, unlike the basal activity, is not regulated by protein kinase C.

cAMP-independent inhibition of renal 24-hydroxylase activity by forskolin:

1. This study demonstrates for the first time that forskolin, a direct activator of adenylate cyclase, exerts part of its inhibitory effect on renal 24-hydroxylase activity in intact cells by a mechanism independent of cAMP formation. This conclusion derives from the observation that: 1) forskolin achieves consistently greater inhibition of 24-hydroxylase activity in mouse renal tubules than does PTH, despite comparable levels of cAMP production; 2) 1',9'-dideoxyforskolin, the cyclase inactive analogue of forskolin, not only inhibits 24-hydroxylase activity in renal cortical tubules, but does so more effectively than forskolin, despite its failure to stimulate cAMP production; 3) both forskolin and 1',9'-dideoxyforskolin directly inhibit 24-hydroxylase activity in isolated renal mitochondria which lack adenylate cyclase.
2. Kinetic analysis reveals a competitive mode of inhibition for forskolin (and 1',9'-dideoxyforskolin), suggesting a direct interaction between the diterpene and the substrate binding site of 24-hydroxylase as the cAMP-independent mechanism. Compelling structural similarities between the inhibitors and the steroid substrate of 24-hydroxylase support such an interaction.
3. The cAMP-independent effect does not require excessive concentrations of

forskolin to become manifest, but is evident at concentrations within the range of the EC_{50} for forskolin-mediated elevation of cAMP and typically used in physiological studies. This finding emphasizes caution in the design and interpretation of experiments using forskolin to assess cAMP involvement in cellular function.

4. In addition to inhibiting constitutively-expressed or basal renal 24-hydroxylase activity, both forskolin and 1',9'-dideoxyforskolin also inhibit inducible 24-hydroxylase function. However, the inducible enzyme activity is less susceptible to the effects of these inhibitors than the basal activity.
5. This study provides the first description of a cAMP-independent effect of forskolin on a protein which is not a transporter or channel of the plasma membrane, but a mitochondrial membrane-associated enzyme, and suggests that such non-traditional actions of forskolin may represent a more widespread phenomenon.

24-Hydroxylase activity in patients with vitamin D dependency rickets type I (VDDR-I):

1. This study is the first to systematically evaluate 24-hydroxylase function in patients with VDDR-I, a Mendelian disorder of 1-hydroxylase function.
2. Constitutive renal 24-hydroxylase activity, assessed indirectly by measuring circulating levels of 24,25-$(OH)_2$D and 25OHD precursor, is comparable in both VDDR-I patients undergoing calcitriol therapy and sex- and age-matched control subjects.
3. This is the first time that 24-hydroxylase activity was measured *directly* in cultured skin fibroblasts derived from VDDR-I patients. Skin fibroblast cultures from VDDR-I patients and control subjects exhibit similar levels of 24-hydroxylase activity, indicating that inducible 24-hydroxylase function is

unperturbed in this disorder.

4. This is also the first study in which the production of calcitroic acid, the final inactivation product of 1,25-$(OH)_2D$ via the C24-oxidation pathway, was determined in skin fibroblast cultures established from VDDR-I patients. Normal levels of calcitroic acid production in VDDR-I cultures not only corroborate our findings of normal 24-hydroxylase activity in these patients but also indicate that the entire C24-oxidation pathway is intact in VDDR-I.
5. The present study shows that patients with VDDR-I do not express the abnormality in renal 24-hydroxylase activity associated with the rachitic pig model for this disorder. This finding suggests that human and porcine VDDR-I do not have the same genetic basis.
6. This study presents a novel approach to address the issue of the structural relationship between the renal vitamin D hydroxylases and provides evidence consistent with the hypothesis that the renal 1- and 24-hydroxylase activities are mediated by separate proteins.

Structural relationship between the constitutively-expressed and inducible 24-hydroxylase activities:

The present thesis describes the first attempt to address the nature of the structural relationship between the constitutively-expressed (or basal) and inducible 24-hydroxylase activities. The demonstration that various chemical agents including a panel of cytochrome P450 inhibitors, forskolin and its cyclase-inactive analogue, 1',9'-dideoxyforskolin, as well as the protein kinase C inhibitor, H-7, can discriminate between these two catalytic activities suggests a structural basis for these differences and implies that the two catalytic activities may be mediated by different proteins.

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[1]All the references cited in the *Introduction* and *General Discussion* sections of the present thesis are listed here. The publications cited in the manuscripts are listed in the *References* section of each individual manuscript.

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Appendix A

Differential sensitivity of constitutive and 1,25-$(OH)_2D_3$-inducible 24-hydroxylase activities to cytochrome P450 inhibitors

A recurring theme in each of the three manuscripts presented herein is the relationship between constitutively-expressed (or basal) and 1,25-$(OH)_2D_3$-inducible 24-hydroxylase activities and whether they may represent different cytochrome P450 moieties. Our speculation that 1,25-$(OH)_2D_3$-inducible 24-hydroxylase may represent an isozyme of the constitutive enzyme is based on a growing number of studies documenting a variety of intriguing differences between the two enzyme activities [Tenenhouse and Jones, 1987; Mandla et al., 1990 (see Chapter II); Mandla and Tenenhouse, 1992 (see Chapter III)]. In the present study, we examine whether constitutive and inducible 24-hydroxylase activities can be differentiated on the basis of yet another property, namely, sensitivity to inhibitors of cytochrome P450.

We examined the effects of four known cytochrome P450 inhibitors [SKF-525A, ketoconazole, aminoglutethimide and metyrapone; see Fig. 1] on constitutive and inducible 24-hydroxylase activities, measured in renal mitochondria isolated from control and 1,25-$(OH)_2D_3$-treated mice, respectively. The results of this initial screening, in which previously reported inhibitor concentrations were used, are illustrated in Figure 2. The data show that although both enzyme activities were inhibited by each of the four compounds, constitutive 24-hydroxylase activity appeared to be significantly more sensitive than inducible activity to three of the inhibitors, SKF-525A, ketoconazole and aminoglutethimide [Fig. 2]. Although the same trend was observed in response to metyrapone, the difference between the two activities was not statistically significant [Fig. 2]. The effects of SKF-525A and ketoconazole on constitutive

and inducible 24-hydroxylase activities were further investigated in dose-response experiments. The results depicted in Figure 3 show that although SKF-525A inhibited both activities in a concentration-dependent manner, their relative sensitivities were significantly different, with constitutive activity demonstrating a greater susceptibility to inhibition than inducible activity over most of the concentrations examined [EC_{50}, 0.25 and 0.35 mM, respectively]. Figure 4 shows that similar results were observed with the inhibitor ketoconazole [EC_{50}, 0.3 and 0.7 μM, for constitutive and inducible 24-hydroxylase activities, respectively].

If constitutive and inducible 24-hydroxylase activities were mediated by the same enzyme, one might expect them to be comparably affected by cytochrome P450 inhibitors. The present data show that constitutive 24-hydroxylase activity was consistently and significantly more sensitive than inducible activity to inhibitors of cytochrome P450. Our findings are reminiscent of previous studies in which differences between constitutive and inducible 24-hydroxylase activities have been observed [Tenenhouse and Jones, 1987; Mandla et al., 1990 (see Chapter II); Mandla and Tenenhouse, 1992 (see Chapter III)] and further support, but do not prove, the hypothesis that the two catalytic activities are associated with distinct gene products.

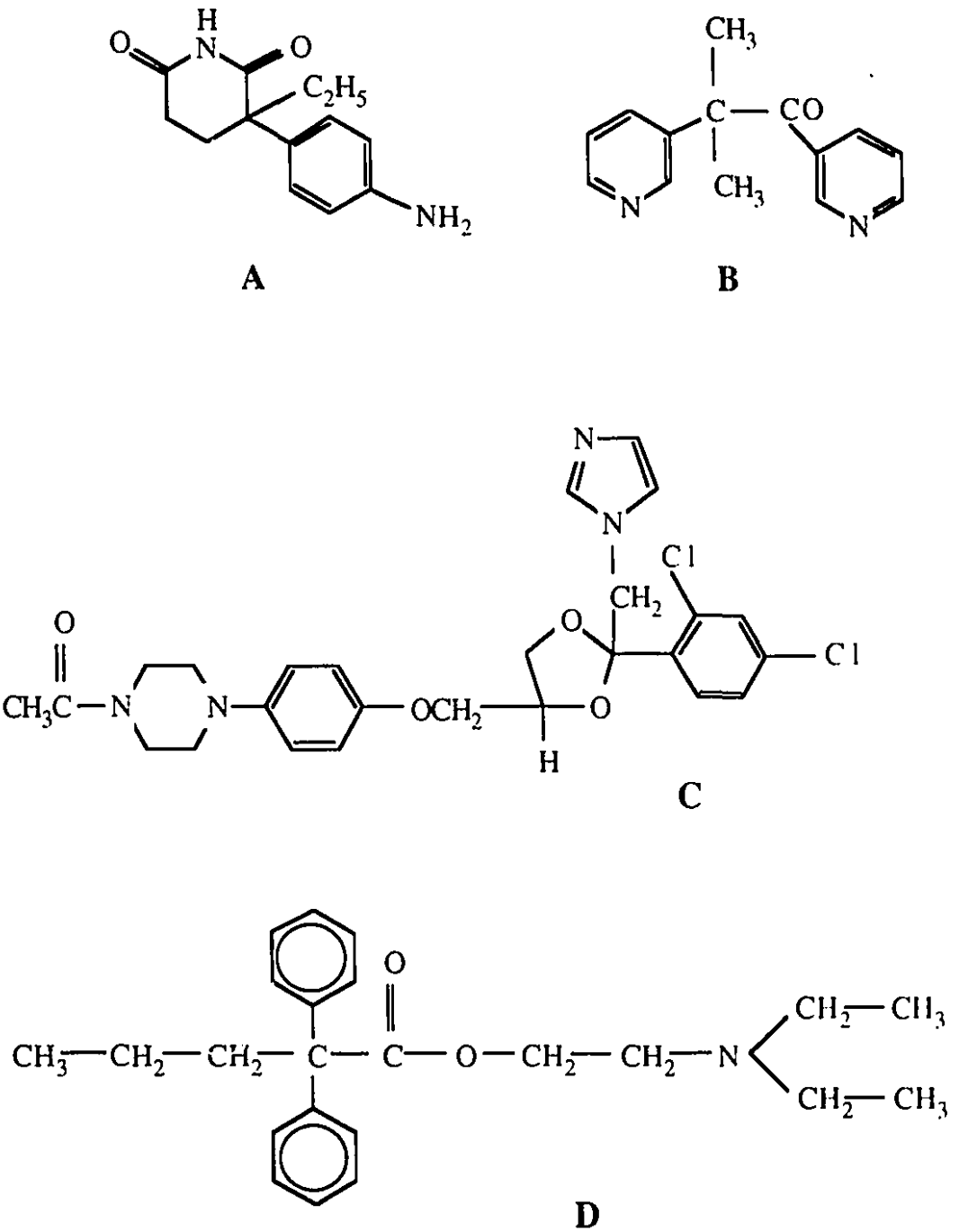

Figure 1. Chemical structures of cytochrome P450 inhibitors.

A. Aminoglutethimide; **B.** Metyrapone; **C.** Ketoconazole; **D.** SKF-525A.

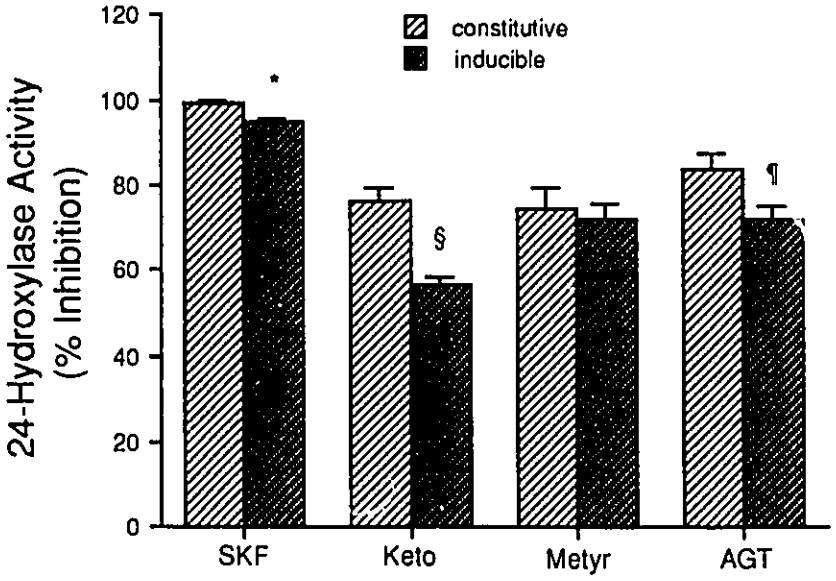

Figure 2. Effect of cytochrome P450 inhibitors on constitutive and 1,25-$(OH)_2D_3$-inducible 24-hydroxylase activities in isolated renal mitochondria.

Mice were injected intraperitoneally with either 1.5 ng/g body wt 1,25-$(OH)_2D_3$ or equivalent volume of mineral oil vehicle 16 h before the experiment, as described in Materials and Methods (see Chapters II and III). Renal mitochondria were prepared and incubated with 50 nM [^{3}H]25OHD$_3$, under initial rate conditions, either in the absence or presence of cytochrome P450 inhibitor [1 mM SKF-525A (SKF), metyrapone (Metyr) or aminoglutethimide (AGT); or 1 μM ketoconazole (Keto)] as indicated in Materials and Methods. Metabolites were extracted, fractionated by HPLC and counted, as described in Materials and Methods. Each value represents mean ± SEM of six to eight preparations. Symbols denote statistically significant difference between constitutive and inducible activities (*, $p<0.005$, §, $p<0.0001$ and ¶, $p<0.05$, by Student's t test).

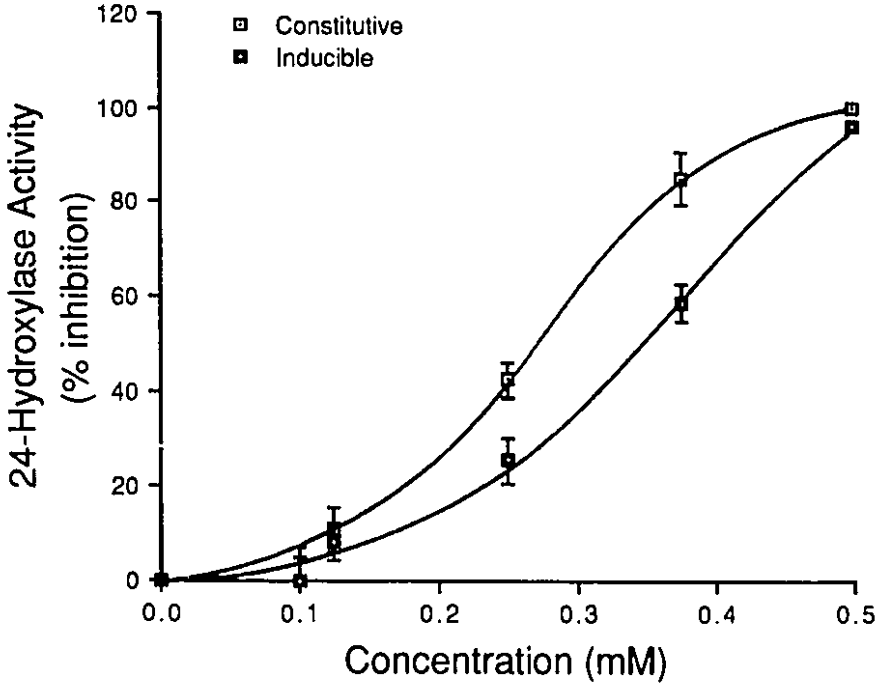

Figure 3. Effect of SKF-525A concentration on constitutive and 1,25-$(OH)_2D_3$-inducible 24-hydroxylase activities in isolated renal mitochondria.

Mice were injected intraperitoneally with either 1.5 ng/g body wt 1,25-$(OH)_2D_3$ or equivalent volume of mineral oil vehicle 16 h before the experiment, as described in Materials and Methods (see Chapters II and III). Renal mitochondria were prepared and incubated with the indicated concentrations of SKF-525A and 50 nM [^{3}H]25OHD_3, under initial rate conditions, as indicated in Materials and Methods. Metabolites were extracted, fractionated by HPLC and counted, as described in Materials and Methods. Each value represents mean ± SEM of three to five preparations.

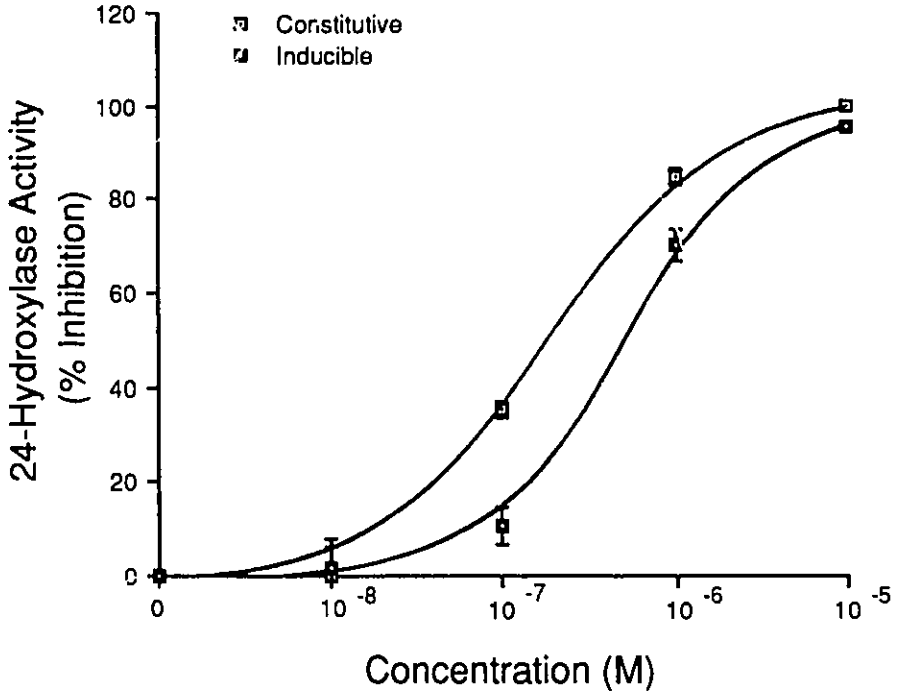

Figure 4. Effect of ketoconazole concentration on constitutive and 1,25-$(OH)_2D_3$-inducible 24-hydroxylase activitites in isolated renal mitochondria.

Mice were injected intraperitoneally with either 1.5 ng/g body wt 1,25-$(OH)_2D_3$ or equivalent volume of mineral oil vehicle 16 h before the experiment, as described in Materials and Methods (see Chapters II and III). Renal mitochondria were prepared and incubated with the indicated concentrations of ketoconazole and 50 nM [^{3}H]25OHD_3, under initial rate conditions, as indicated in Materials and Methods. Metabolites were extracted, fractionated by HPLC and counted, as described in Materials and Methods. Each value represents mean ± SEM of three to four preparations.

Appendix B

The present section contains additional procedural information for the studies described in Chapters II through IV.

Preparation of renal cortical tubules (Chapters II and III). Mice were decapitated and the kidneys removed and immediately placed in ice-cold MEM buffer [containing minimal essential medium (MEM) and 10 mM HEPES, pH 7.5]. Kidneys were decapsulated and hemisectioned. Medullae were removed and discarded. Slices were prepared from cortices, using a Stadie-Riggs microtome, and incubated in 15 ml MEM buffer containing 4.5 units of collagenase and 0.75 ml of 10% (wt/vol) BSA (previously dialyzed against saline for 48 hours) in a 22 C shaking waterbath [Dubnoff metabolic shaking incubator] for 45 minutes. Digestion was stopped with the addition of 30 ml ice-cold MEM buffer. Collagenase-treated slices were dispersed by repeated aspiration through a 10 ml serological pipette, filtered through a wire mesh tea strainer and centrifuged at 60 x g for 45 seconds [IEC HN-sII centrifuge]. The supernatant was discarded and the pellet washed three times in 30 ml ice-cold MEM buffer. After the third wash, the pellet was finally resuspended in ice-cold MEM buffer to yield the desired protein concentration. All manipulations were carried out in plasticware.

Preparation of renal cytosolic and mitochondrial fractions (Chapter II). Following incubation with the desired reagent (e.g. PMA, 4α-phorbol, etc) the tubule suspension (described above) was diluted with 10 ml ice-cold MEM buffer and centrifuged at 60 x g for 1 minute [IEC HN-sII centrifuge]. The tubule pellet was then resuspended in homogenizing buffer [containing 0.25 M

sucrose, 20 mM Tris-HEPES, pH 7.5, 2 mM EGTA, 10 mM dithiothreitol and 10 μg/ml leupeptin] and disrupted by sonication (2 bursts of 30 seconds each) on ice. The tubule lysate was centrifuged at 120 x g for 10 minutes, at 4 C [Beckman J2-21 centrifuge, JA-17 rotor]. The pellet was discarded and the supernatant centrifuged at 9000 x g to yield a mitochondrial pellet and postmitochondrial supernatant. The postmitochondrial supernatant was centrifuged at 105,000 x g for 60 minutes [Beckman L8-55 ultracentrifuge, Ti50 rotor]; the resulting supernatant was used as the *cytosolic fraction.* The mitochondrial pellet was resuspended in homogenizing buffer, solubilized in 0.2% Triton X-100 for 60 minutes on ice and centrifuged at 105,000 x g for 60 minutes; the resulting supernatant was used as the *mitochondrial fraction.*

Preparation of renal mitochondria (Chapter III). Mice were decapitated and the kidneys removed and immediately placed in ice-cold 0.9% (wt/vol) saline. Kidneys were decapsulated and hemisectioned. Medullae were discarded. Cortices were homogenized (10 strokes) in 10 volumes (wt/vol) of ice-cold homogenizing buffer [containing 0.25 mM sucrose, 10 mM HEPES, pH 7.4 and 10 mM KCl] using a motor-driven teflon pestle and glass homogenizer. The homogenate was centrifuged at 120 x g for 10 minutes, at 4 C [Beckman J2-21 centrifuge, JA-17 rotor]. The pellet was discarded and the supernatant centrifuged at 9750 x g for 10 minutes. The resulting pellet was washed in fresh homogenizing buffer and finally resuspended in incubation buffer [containing 0.125 mM KCl, 20 mM HEPES, pH 7.4, 10 mM malate, 2 mM $MgSO_3$, 1 mM dithiothreitol, and 0.25 mM EDTA] to yield the desired protein concentration (2-4 mg/ml).

Protein kinase C assay (Chapter II). Enzyme activity was measured as

^{32}P incorporation from [$\gamma^{32}P$]ATP to calf thymus histone. Reaction mixtures (100 µl) consisted of 25 mM Pipes (pH 6.8), 10 mM $MgCl_2$, 1mM 2-β-mercapto-ethanol, 40 µg histone (type III-S), 0.2 mM EGTA and 0.1 mM EDTA. When present, $CaCl_2$ concentration was 500 µM (buffered with EGTA according to Barfait [(1979); Adv Cyclic Nucleotide Res 10:219-242] to give a final calcium concentration of 300 µM); phosphatidylserine was used at 3 µg/100 µl assay; diacylglycerol was used at 1.25 µg/100 µl assay. A five-fold concentrated stock of phosphatidylserine and diacylglycerol was prepared in water by sonication under nitrogen. Protein concentration was approximately 20 µg/100 µl assay for both cytosolic and mitochondrial fractions. The assay was carried out as described in the manuscript.

Extraction and analysis of vitamin D metabolites (Chapters II through IV). Reaction mixtures were transferred from incubation vials to conical centrifuge tubes and 1.25 ml of chloroform were added. Tubes were capped and shaken, and 1.25 ml of 4% (wt/vol) KCl were added. Tubes were again capped, shaken and then centrifuged at 3000 x g for 10 minutes [IEC HN-sII centrifuge]. The lower organic layer was transferred to a conical glass vial. The upper aqueous layer was extracted a second time, after addition of 3.75 ml chloroform:methanol (1:2). Both organic phases were pooled and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 1 ml of hexane:isopropanol:methanol (93.5:5.5:1) and stored at -20 C for at least one hour. Samples were then centrifuged at 3000 x g for 10 minutes and the supernatants transferred to glass vials, dried under nitrogen and reconstituted in 200 µl hexane:isopropanol:methanol (93.5:5.5:1). Chromatographic analysis was performed as described in the manuscripts, using a Waters solvent delivery system (model 6000A) and injector (U6K).

Measurement of cAMP accumulation (Chapters II and III). Tubules or mitochondria (described above) were incubated under the same conditions used to assay 24-hydroxylase activity. Reactions were stopped with the addition of 10 μl of concentrated acetic acid and boiling for five minutes. After cooling on ice, the samples were centrifuged at 16,000 x g for 3-5 minutes [Eppendorf microfuge] and the resulting supernatants were transferred to fresh tubes and frozen immediately. The cAMP content of the supernatants was determined by competitive binding assay using a commercially available kit.

All other procedures were carried out as described in the manuscripts.

Appendix C

The following is a list of the works which I have either authored or co-authored.

Publications:

Lyon MF, Scriver CR, Baker LRI, Tenenhouse HS, Kronick J and **Mandla S** (1986) The Gy mutation: another cause of X-linked hypophosphatemia in mouse. Proc Natl Acad Sci 83:4899-4903

Mandla S, Scriver CR and Tenenhouse HS (1988) Decreased taurine transport in renal basolateral membrane vesicles from hypertaurinuric mice. Am J Physiol 255 (Renal Fluid Electrolyte Physiol 24):F88-F95

Mandla S (1986) Master's thesis: Transport studies in mouse renal basolateral membrane vesicles.

Boneh A, **Mandla S** and Tenenhouse HS (1989) Phorbol myristate acetate activates protein kinase C, stimulates the phosphorylation of endogenous proteins and inhibits phosphate transport in mouse renal tubules. Biochim Biophys Acta 1012:308-316

Mandla S, Boneh A and Tenenhouse HS (1990) Evidence for protein kinase C involvement in the regulation of renal 25-hydroxyvitamin D_3-24-hydroxylase. Endocrinology 127 (6):2639-2647

Mandla S, Jones G and Tenenhouse HS (1992) Normal 24-hydroxylation of vitamin D metabolites in patients with vitamin D dependency rickets type I. Structural implications for the vitamin D hydroxylases. J Clin Endocrinol Metab 74:814-820

Mandla S and Tenenhouse HS (1992) Inhibition of 25-hydroxyvitamin D_3-24-hydroxylase by forskolin: Evidence for a 3',5'-cyclic adenosine monophosphate-independent mechanism. Endocrinology 130:2145-2151

Tenenhouse HS, Meyer Jr RA, **Mandla S**, Meyer MH, Gray RW (1992) Renal phosphate transport and vitamin D metabolism in X-linked hypophosphatemic Gy mice: Responses to phosphate deprivation. Endocrinology 131 (in press)

Abstracts/Communications:

Lyon MF, Scriver CR, Baker LRI, Deol M, Tenenhouse HS, Kronick J and **Mandla S** (1985) The Gy mutation: another cause of X-linked hypophosphatemia in mice; implications for man. Am J Hum Gen 37(4): A12

Mandla S, Scriver CR and Tenenhouse HS (1986) Decreased taurine (tau) transport in renal cortical basolateral membrane vesicles (BLMVs) from a hypertaurinuric mouse strain. Am J Hum Gen 39(3): A15; Amer Soc Hum Gen, Philadelphia (PA), Nov 1986.

Mandla S, Boneh A and Tenenhouse HS (1989) A role for protein kinase C involvement in the regulation of renal vitamin D metabolism. Can Fed Biol Soc, Calgary (AL), June 1989

Mandla S, Tomon M, Byford V, Jones G and Tenenhouse HS (1991) Normal 24-hydroxylation of vitamin D metabolites in vitamin D dependency rickets type I: Structural implications for the vitamin D hydroxylases. Proceedings of the Eighth Workshop on Vitamin D, p 273; Paris (France), July 1991

Mandla S, Martel J and Tenenhouse HS (1991) Inhibition of renal 25-hydroxyvitamin D-24-hydroxylase activity by forskolin: evidence for cAMP-dependent and -independent mechanisms. Amer Soc Bone & Min Res, San Diego (CA), Aug 1991

Meyer Jr RA, **Mandla S**, Tenenhouse HS, Gray RW and Meyer MH (1991) Increased renal 25-hydroxyvitamin-D-24-hydroxylase may explain the failure of Gyro (Gy) mice to respond to low phosphate diet with increased plasma 1,25-dihydroxyvitamin D. Amer Soc Bone & Min Res, San Diego (CA), Aug 1991