Extra- and Intracellular Calcium and Phosphate Regulation:

From Basic Research to Clinical Medicine

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PREFACE

In recent years, biological research, including research in calcium and phosphate metabolism, has greatly accelerated in pace and provided answers to what until recently were only questions or speculations. The second Chemofux prize, awarded to a distinguished research contribution in the general area of bone and mineral metabolism published between 1988 and 1990, was shared by two investigators whose studies were characterized by the application of contemporary approaches to two important questions: The first of these — what is the nature of the gene that specifies the calcium-binding protein, a major component of transcellular calcium transport? — was answered by Monique Thomasset of Paris. The second — what are the details of the cellular events that take place in the course of bone resorption? — was analyzed by Anna Teti of Bari, Italy. As in the past, the two prize winners presented their papers at a scientific meeting in which members of the jury participated. As a result, it has been possible to structure an overview of current research and concepts, with the present book representing the outcome of this effort.

The current volume deals with four general topics: transport processes involving calcium and phosphate; genetic and functional analysis of the calbindins, molecules involved in calcium transport; hormonal regulation of the two minerals; and bone and bone diseases.

Chapter 1 analyzes the process by which calcium enters the cell, referring among other aspects to calcium channels, now recognized to be ubiquitous and of major importance in regulating calcium traffic. Chapter 2 describes two potential model cell systems for the study of cellular calcium transport. The human colon carcinoma cell line, CaCo-2, responds to stimulation by vitamin D, yet exhibits no increase in net calcium flux. It also does not express calbindin-9K. In contrast, cultured cells from the rabbit nephron, specifically the collecting duct, were found to transport calcium, to increase transport when stimulated by 1,25-dihydroxyvitamin D_3, the active form of vitamin D, and to contain calbindin 28K, the concentration of which increased upon stimulation by vitamin D. In Chapter 3, Caverzasio and colleagues discuss phosphate transport in osteoblastic cells, responsible for bone growth and modeling. They report that the sodium-dependent transport of phosphate by bone cells is enhanced by fluoride addition to the cell culture and raise the question whether this effect plays a role in the beneficial effect that has been attributed to fluoride treatment of osteoporosis.

Section II deals with the calbindins, relatively low molecular weight intracellular calcium-binding proteins thought to enhance the rate of intracellular diffusion of calcium, an enhancement necessary to account for the measured rate of transcellular calcium transport in the face of a very limited rate of self-diffusion of the calcium ion. Chapter 4 describes the prize winning work of Monique Thomasset, summarizing the structure of the gene for calbindin-9K (CaBP, Mr-9kD), its tissue-specific expression and its regulation by vitamin D. Chapter 5, by R. H. Wasserman, the discoverer of the calbindins, provides an overview of the role of the intestinal calbindins and of diseases that affect function or distribution of these molecules.

Section III, Hormonal Aspects, deals with a variety of regulatory phenomena. In Chapter 6, Martin elaborates on the functional role of the parathyroid hormone-related protein, for the purification, cloning and sequencing of which he had been awarded the first Chemofux prize. The protein is of significance because it appears to play a major role in the humoral hypercalcemia of malignancy and because many of its functional and biochemical characteristics are similar to those of the parathyroid hormone. Of particular interest is the possibility that this protein may play a role in the maintenance of the positive calcium gradient that exists between the fetal and maternal blood. Moreover, this control appears to be exerted by regions of the molecule that are not homologous with the parathyroid hormone molecule.

Chapter 7 deals with another new regulatory protein, chromogranin A, part of a family of molecules that seem to play a role in endocrine and neuroendocrine secretion. Chromo-

granin A in particular may function in the sorting and packaging of secretory proteins in endocrine cells, according to a scheme proposed by Cohn and Gorr.

Chapter 8 deals with a question that has puzzled endocrinologists, namely, why diseases of the thyroid gland also involve disturbances of calcium and mineral metabolism. Using findings from experiments with a variety of organ and cell cultures, the authors propose that thyroid hormones act in three ways, modulating the biosynthesis of 1,25-dihydroxyvitamin D_3 on the one hand, having a direct, vitamin D-like action on the cells of the principal organs involved in calcium and phosphate metabolism (gut, kidney and bone), on the other, and, finally, potentiating the effects of vitamin D on the cells of these three organs.

Section IV addresses a variety of questions related to bone and bone diseases. In Chapter 9 the other winner of the second Chemofux prize, Anna Teti, together with Alberta Zambonin Zallone, offers a detailed description of how the bone salt-dissolving activity of the osteoclast, the multinucleated cell responsible for bone destruction, may serve a feedback function that causes the osteoclast ultimately to stop its osteolytic activity on one site and move on to another. In Chapter 10, Stern deals with a topic of importance to the physician, namely, how drugs used for one purpose may deleteriously act on the skeletal system. For example, cyclosporin, a widely used immunosuppressant, has now been found to induce undesirable alterations in bone mass and turnover. Other drugs discussed are gonadotropin-releasing hormone, anti-estrogens, glucocorticoids, anticonvulsants, and thyroid hormones. Throughout *in vitro* and *in vivo* effects are compared and evaluated.

The discussion on secondary osteoporosis, Chapter 11, follows and expands on the description of the side effects of drugs on bone. Raisz emphasizes that osteoporosis that is secondary to such drug treatment may not result in the fractures typical of primary osteoporosis (postmenopausal or senile) and that the mechanisms by which secondary osteoporosis is brought about may vary widely, depending on which cells or metabolic pathways are targeted by the drugs. The final chapter provides a wide-ranging survey of the various drugs and therapeutic regimens used to treat metabolic bone disorders. Benefits and risks involved in treatment are discussed for virtually all drugs used, with emphasis on the fact that no effective agent is without side effects, and that side effects can differ even with the same agent and in different patients.

The title of this book may at first sight seem unduly ambitious, but we hope that our brief descriptions and the chapters themselves demonstrate how short the distance has become between experiment and therapy. We therefore hope the book will evoke interest from experimentalists and clinicians alike. At the same time, we wish to thank our coauthors for their contributions and the publishers for their help.

November, 1991

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THE EDITORS

Felix Bronner, Ph.D., is Professor Emeritus of BioStructure and Function and of Nutritional Sciences at The University of Connecticut. A graduate of the University of California at Berkeley and Davis and of the Massachusetts Institute of Technology, Dr. Bronner has worked in the general area of calcium metabolism for the past 40 years. He was among the first to study the kinetics of ^{45}Ca in humans, did detailed kinetic and balance studies in rats, and then proceeded to an analysis of calcium transport in the intestine and kidney. In recent years, he has returned to the problem of calcium homeostasis and its regulation. Throughout his career he has attempted to describe in quantitative fashion the events by which calcium moves in the body at the subcellular, cellular and organ levels, aiming for a conceptual synthesis.

Dr. Bronner has published over 80 research articles, has contributed some 50 chapters to texts and symposia volumes, and has edited more than 40 volumes of texts and symposia. He was founding editor of *Current Topics in Membranes and Transport* and first chair of the Gordon Research Conference on Bones and Teeth. He is currently on the editorial boards of the *Journal of Nutrition* and *The American Journal of Physiology*.

A member of numerous scientific societies, including fellowship in The American Association for the Advancement of Science, he was the 1974 recipient of the André Lichtwitz prize, awarded by the French National Institutes of Medical Research (INSERM) for outstanding work in calcium and phosphorus metabolism and the honoree of the 4th International Workshop on Calcium and Phosphate Transport Across BioMembranes, held in Lyon in 1989 under NATO sponsorship. He has organized and chaired national and international symposia and workshops and trained graduate students and many postdoctoral fellows.

Meinrad Peterlik, Ph.D., M.D., is Head of the Department of General and Experimental Pathology at the University of Vienna Medical School. In 1963 he received his Ph.D. in Chemistry and in 1972 his M.D. from the University of Vienna, Austria. In 1974–1975 he was granted a Max Kade Fellowship to pursue postdoctoral studies in Robert H. Wasserman's laboratory at Cornell University, Ithaca, NY. In 1978 he was promoted to Associate Professor (with tenure), and in 1983 he was appointed Full Professor of General and Experimental Pathology (Pathophysiology) at the University of Vienna.

Dr. Peterlik is a member of the American Institute of Nutrition, American Society for Bone and Mineral Research, and is currently President of the Austrian Society for Bone and Mineral Research. He serves as member of the Scientific Board of the European Calcified Tissue Society and of the Editorial Board of *Bone*. He is also the head of the International Jury for the Chemofux Prize for Bone and Mineral Research.

Dr. Peterlik (together with Dr. Felix Bronner) has organized a series of International Workshops on Calcium and Phosphate Transport Across Biomembranes in Vienna (1981, 1984 and 1987) and was Head of the Organizing Committee of the XXII European Symposium on Calcified Tissues (Vienna 1991).

Dr. Peterlik has published more than 70 original papers and has edited a textbook for medical students on pathophysiology (*Funktionelle Pathologie*). His current research interests include cellular actions and interactions of vitamin D and thyroid hormones, calcium and phosphate homeostasis, cytokines and bone turnover, and hormonal regulation of intestinal cell differentiation.

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Section I. Calcium and Phosphate Transport

CALCIUM ENTRY AND CALCIUM CHANNELS

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The resting free calcium ion concentration in mammalian cells tends to be less than 100 nM. Three systems are involved in regulating intracellular calcium: calcium entry, calcium extrusion and intracellular calcium binding sites of the organelles (mitochondria, endoplasmic reticulum, lysosomes, etc.). The binding capacity of the various organelles is so large that entry and extrusion may be considered the principal vectors of acute regulation. For example, when the calcium concentration of the buffer in which intestinal cells were bathed was increased from 1 to 3 mM, total calcium content of the cells increased in direct proportion.[1] Since the cells remained viable, their free calcium concentration may have increased, but most of the entering calcium must have been taken up by the many intracellular fixed binding sites.[1] Moreover, the calcium extrusion capacity of the Ca-ATPase is far in excess (perhaps 2-3 orders of magnitude) of the measured influx rate.[2] Inasmuch as the capacity to bind calcium that enters the cell is very large and its extrusion capacity is so much greater than the rate of entry, it is logical to consider entry as the rate-limiting step. As it turns out, however, the situation is not so simple, at least as far as the calcium-transporting cells are concerned.

The major regulator for transcellular calcium transport is vitamin D. Indeed, in the total absence of this compound - more specifically of the active metabolite, 1,25-dihydroxy-vitamin D_3, 1,25-$(OH)_2D_3$ - transcellular calcium transport grinds to a standstill. Yet calcium entry is only partly (30-40%) reduced in vitamin D deficiency.[3,4] Moreover, administration of 1,25-$(OH)_2D_3$ to vitamin D-deficient chicks [3] or rats [4] also enhances brush border membrane vesicle calcium uptake by only about 30-50 percent, whereas total transport increases in direct proportion to the vitamin D administered.[5]

The nature of vitamin D regulation of calcium entry may also differ from its effect on transcellular calcium transport. In the case of total transport it is becoming increasingly clear that 1,25-$(OH)_2D_3$ acts genomically, i.e. induces the biosynthesis of the cytosolic calcium-binding protein (calbindin D-9K, M_r=8.8 kD) via transcriptional, translational and possibly posttranslational mechanisms.[6] As far as the entry mechanism is concerned, Kowarski and Schachter[7] have reported the existence of a vitamin D-dependent component of a brush-border-membrane-related particulate complex and have attributed to it a transport role. Miller and colleagues [8] have identified a membrane-bound, vitamin D-dependent calcium-binding protein from the brush-border that differs in several characteristics from the cytosolic calbindin. The brush-border calcium-binding protein identified by Miller et al[8] has an estimated molecular weight of 19kD and could therefore constitute the monomer of the protein identified by Kowarski and Schachter.[7] However, no further studies have appeared characterizing these molecules.

It is of course possible that 1,25-$(OH)_2D_3$ induces both the cytosolic and the brush-border calcium-binding proteins, but in that case the time required to induce the proteins should not differ significantly. In the case of calbindin D-9K, transcription takes some

4h[9], yet 1,25-$(OH)_2D_3$ has been shown[10] to act on brush-border membrane uptake of calcium in a substantially shorter period, provided the vitamin D metabolite was administered to the animal (and not in vitro). In osteosarcoma-derived osteoblast-like cells the addition of 1,25-$(OH)_2D_3$ to a cell culture produced an increase in calcium uptake in 1-2 min.[11] In similar cells the existence of calcium channels has been demonstrated[12] and a very rapid response leading to increased calcium influx was documented.[13] Although no reports on the existence of calcium channels in the brush border membranes of enterocytes have as yet appeared, these cells, like most others, are likely to have calcium channels. If so, the rapid response of such channels could account for the increase in calcium uptake observed in brush-border membrane preparations.[3,4] Furthermore, on a quantitative basis, the increase in calcium uptake by brush border membrane vesicles from treated animals matches the increase found in treated isolated osteosarcoma cells. However, this increase cannot, as stated above, account for most of the increase in transcellular calcium transport.

Calcium entry can be studied with the aid of brush-border membrane vesicles prepared from either the intestine or kidney. These vesicles are right-side out[14] and take up calcium in a time-dependent and concentration-dependent fashion, with an apparent K_m of 1.1 mM. Calcium that enters the vesicle becomes bound to the inner membrane. The membrane has two calcium-binding sites, a high-affinity site with modest binding capacity and a low affinity site with great binding capacity.[4]

Total calcium uptake by duodenal brush-border membrane vesicles is nearly two orders of magnitude too low as compared with the V_{max} of transcellular transport.[15] This may be the result of channel inactivation, as well as of difficulty in measuring an appropriate initial rate. Recent approaches[16] have improved measuring techniques, but current experimental values do not yet come close to the experimental transport value. If entry were to be a rate-limiting process, its basal rate would have to be either near the actual transport rate or be subject to modulation so as to increase it to the V_{max}. The intracellular self-diffusion rate of Ca^{2+} is two orders of magnitude smaller than the V_{max} for transcellular transport and can be increased in direct proportion to the tissue content of calbindin.[5] Calbindin D-9K content in turn varies directly with the quantity of 1,25-$(OH)_2D_3$ administered to vitamin D-deficient or vitamin D-replete animals.[5]

Since the available values on the V_{max} of calcium uptake by brush border membrane vesicles are too low for transcellular transport to take place at its measured rate and since modulation of the brush border calcium uptake is modest and insufficient to raise its V_{max} to that of transport, one cannot assert that calcium entry is the rate limiting step. One can, however, assert that the entry rate is significantly lower than is the exit or extrusion rate.[2] Moreover, if entry were to be blocked or diminished, this would necessarily limit the amount that is transported transcellularly.

The calcium channel blocker verapamil has been shown to decrease calcium uptake by about 55% in isolated brush border membrane vesicles.[4] Verapamil, administered to fasting rats intraluminally, diminished duodenal calcium absorption, evaluated by in situ intestinal sacs, when the calcium concentration of the instilled buffer was relatively low (2 mM),[17] i.e. when transcellular calcium transport predominated.[18] When however the instilled calcium concentration was high (50 mM), most of the calcium therefore being absorbed by the paracellular route,[15,18] verapamil was without effect.[17] These findings suggest that at least some calcium enters the transporting enterocyte via a dihydropyridine-

sensitive channel. Calcium channels that are similar in characteristics to L-type channels[19] have also been found in renal cells.[20] In the latter, verapamil addition to the bath "reduced the fractional open time at negative but not at positive clamp potentials."[20] Nifedipine (100 mM) completely abolished channel activity which was restored to near maximal by addition of the agonist Bay K-644.[20] Saunders and Isaacson[20] therefore conclude that the channels they studied are specific for calcium. Hess and colleagues,[12] discussing the interaction of calcium with dihydropyridine-sensitive calcium channels, have concluded that the best documented mechanism for channel selectivity is one that involved differential affinities of permeant ions to intra-channel binding sites. In their view, at least one high-affinity calcium-binding site can be reached from either side of the channel, with access from the cytoplasmic side voltage-dependent. In such a situation, "externally located high-affinity Ca-binding sites with possible allosteric effects on L-type Ca channels..." would not be necessary.[21]

Petersen[22] has called attention to at least two other routes of calcium uptake by electrically non-excitable cells such as enterocytes. One is a non-specific or non-selective cation channel, the existence of which has been widely documented (see Ref. 20 for a listing of some). The other is a calcium-selective, voltage insensitive channel.[23] It is quite apparent (cf 19) that an increasing number of cation channels are being described. It will therefore be of interest to catalog the various calcium channels in cells that transport calcium and to quantitate how much of the entering calcium is handled by each.

The addition of 1,25-$(OH)_2$-D_3 to isolated clonal osteosarcoma cells produces a nearly instantaneous two-fold increase in calcium uptake that appears mediated by an inward calcium current, with maximal effects at negative test potentials.[13] The shifts in activation voltage dependence and the lengthening of the open times of single channels that may be attributed to the effect of low (<3nM) concentrations of 1,25-$(OH)_2D_3$ in the osteosarcoma cells could reflect, in the words of Caffrey and Farach-Carsen,[13] stabilization of channel open states. However, by what mechanism 1,25-$(OH)_2D_3$ acts on this high threshold L-type channel is not known. Thus whether intestinal cells respond like the osteosarcoma cells, whether they also possess these L-type channels and how the latter might respond to the vitamin D metabolite remain open questions. The 1,25-$(OH)_2D_3$ receptors that mediate the genomic response in intestinal and renal cells are cytosolic receptors[24] that, when charged with the hormone, travel to the nucleus. The nature of the receptor that permits the channel to alter its open time is as yet unknown.

Before concluding, attention should be called to the possibility that calcium entry into transporting cells may be via a carrier. This possibility was advanced by Holdworth[25] many years ago and has found indirect support in brush-border membrane studies by Schedl and collaborators.[26,27] The latter, like others[4,28], have analyzed brush-border membrane uptake as the sum of two processes, a linear and a saturating one, and have proposed that the saturating process is a mediated one, representing perhaps a carrier process. Others[4] have instead proposed that the saturating process represents binding to the inner aspect of the vesicle membrane. Indeed one can visualize three processes - rapid binding to the outer aspect of the vesicle membrane, transmembrane movement and binding to the inner aspect. There is general agreement that binding to the outer aspect is fastest, but no detailed kinetic analysis of calcium uptake by vesicles is available. As pointed out above, two classes of binding sites have been identified on brush-border membranes,[4] with high affinity and modest capacity and with relatively low affinity and high capacity, but their relative distribution on the inner and outer aspects of the brush border membranes is not known. Moreover, how these binding sites affect the kinetics of vesicular uptake is

also not known. The possibility of a carrier cannot therefore be excluded. One can, however, argue that a cell equipped with two pump-leak systems - one involving facilitated diffusional uptake, the other an active extrusion pump - seems more complicated than necessary, particularly since the existence of a modulated calcium channel alone could account for other known features of cellular calcium uptake.

Finally one might ask questions concerning possible defects of calcium entry. In this connection it is worth recalling the words of Fleckenstein, the pioneer discoverer of calcium antagonists, that "...Ca channel blockade, which is producible in vitro only with huge overdoses,... can never occur in a living animal."[29] In other words, transcellular calcium flux is essential in a variety of cells, notably muscle, heart and calcium transporting cells, even if one were to ignore the intracellular calcium-signaling mechanisms that have evolved in most cells and that alone require some calcium entry. Calcium channels are therefore likely to be ancient, from an evolutionary viewpoint, and mutations that drastically affect channel function are probably lethal. Moreover, since calcium channels seem to alter calcium entry only about twofold, a defect that alters this ability may have effects that are relatively subtle and hard to detect. To be sure, in situations such as myocardial calcium overload, leading to necrosis, calcium antagonists are very useful,[29] but it is unclear whether the defect lies in excessive entry or defective extrusion capacity or some combination of both. Cloning of the genes of calcium channels, directed mutagenesis and the use of transgenic animals may shed light on the possible existence and nature of calcium channel diseases or defects.

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TRANSCELLULAR CALCIUM TRANSPORT ACROSS CULTURED INTESTINAL AND RENAL CELLS

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SUMMARY

The human colon carcinoma cell line CaCo-2 and a primary culture of rabbit kidney collecting duct system were tested whether they provide good model systems for studying active transcellular Ca^{2+} transport. Confluent monolayers of CaCo-2 cells grown on permeable supports were placed in modified Ussing chambers. J_{MS} and J_{SM} fluxes of Ca^{2+} increased significantly after 48 h preincubation with 10^{-7}M $1,25(OH)_2D_3$, without a significant effect on the net Ca^{2+} flux. $^{45}Ca^{2+}$ uptake across the luminal membrane of CaCo-2 cells grown on plastic dishes was also responsive to $1,25(OH)_2D_3$. Calbindin-D_{9K} could not be detected in CaCo-2 cells with a Mab against human Calbindin-D_{9K}.

Rabbit collecting duct system cells were isolated by immunodissection and subsequently cultured on permeable supports. In this way 80% of Calbindin-D_{28K} containing cells were isolated. After 6 days in culture, confluent monolayers transported 83±6 nmol Ca^{2+}/h.cm^2 from the apical to the basal solution. This Ca^{2+} transport was largely dependent on serosal Na^+. Exposure to 0.1 μM $1,25(OH)_2D_3$ for 48 h increased transcellular Ca^{2+} transport by 53% and PTH stimulated net Ca^{2+} transport by 25%. The cells in primary culture contain Calbindin-D_{28K}, which content is increased after 48 h exposure to $1,25(OH)_2D_3$.

In conclusion, CaCo-2 cells are responsive to $1,25(OH)_2D_3$, but do not express net Ca^{2+} transport. The primary culture of renal collecting duct system proved to be a promising model system to study regulation of active transcellular Ca^{2+} transport.

INTRODUCTION

In intestinal and renal epithelia there are two routes available for Ca^{2+} transport. Passive Ca^{2+} movements in both directions occur across the paracellular route, while active Ca^{2+} transport is exclusively transcellular from lumen to blood side. The transcellular route consists of a passive influx, diffusion through the cytosol and active extrusion mediated by the plasma membrane type of Ca^{2+}-ATPase and/or a Na^+/Ca^{2+} exchanger[1]. In addition, a