Non-genomic mechanisms of vitamin D-regulated bone formation in osteoblasts

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Summary

Hormone 1α,25(OH)$_2$-vitamin D$_3$ (1,25D) is considered a bone anabolic hormone. It increases bone matrix production and mineralization via the nuclear vitamin D receptor (VDR), which is abundantly expressed in osteoblasts. In addition, 1,25D exerts rapid, non-genomic actions that do not involve gene transactivation (1). These include: 1) activation of signal transduction pathways, 2) cytoplasmic Ca^{2+} increase, and 3) ion channel potentiation. While genomic mechanisms have been extensively studied, the signaling involved in non-genomic, membrane-initiated 1,25D actions is less well understood. Moreover, the physiological significance of 1,25D non-genomic effects in osteoblasts and its contribution to bone formation is a field relatively unexplored. Recently we demonstrated, for the first time in single live osteoblasts, a rapid (1-5 min) 1,25D-induced increase of chloride currents at depolarizing potentials (2, 3). This voltage-gated Cl^- channel is selectively potentiated by 1,25D (0.5-50 nM) in osteoblasts expressing a functional VDR (4). We showed that this 1,25D-induced Cl^- current develops simultaneously with activation of exocytosis in single osteoblasts (5). In addition, nanomolar concentrations of 1,25D activate L-type calcium channels at low depolarizing potentials. These contribute to a rapid increase in cytoplasmic Ca^{2+} concentration promoted by the hormone. The purpose of this paper is to overview the current understanding on molecular mechanisms of non-genomic 1,25D-mediated processes of bone formation in osteoblasts. We found that 1,25D acting at a membrane-associated VDR leads to a rapid, regulated exocytotic response that is coupled to Cl^- and Ca^{2+} channel activation in osteoblasts and explains in part the anabolic effects of the hormone in bone.

KEY WORDS: osteoblasts, vitamin D$_3$, rapid responses, ion channels, exocytosis.

Introduction

Vitamin D was discovered originally as an antirachitic agent (6). The development of new therapies for treatment and prevention of bone mass loss depends on a full understanding of the molecular mechanisms by which osteoblasts produce and secrete bone, and how these processes are regulated by hormones such as vitamin D. Here I review studies on non-genomic, membrane-initiated molecular mechanisms of secretion of bone materials in single, live osteoblasts. We utilize techniques of high spatial-temporal resolution applied to individual osteoblasts in culture such as patch-clamp electrophysiology and confocal microscopy. The biologically active 1α,25(OH)$_2$-vitamin D$_3$ (1,25D) potentiates chloride and calcium channel activities within minutes through mechanisms that involve signal transduction pathways and develop only in the presence of a functional vitamin D receptor (4). Secretory activities are coupled to 1,25D-activation of ion channels and elevation of cytoplasmic calcium, and lead to bone matrix production (7). Although the primary focus of this review is on basic research, the long-term objective of our work is to identify molecular targets in the treatment of bone pathologies characterized by decreased bone mass and mineralization. This typifies skeletal diseases such as osteoporosis and osteomalacia, respectively. Osteoporosis in particular affects a large sector of the aging population and constitutes a significant financial burden for the society.

Vitamin D and bone

Bone anabolic effects of 1,25D (8, 9) comprise matrix production and mineralization (10). In osteoblasts, the bone-forming cells, activation of bone matrix protein synthesis by 1,25D occurs via the nuclear vitamin D receptor (VDR), which modulates gene transcription (11, 12). In addition, 1,25D exerts non-genomic effects in osteoblasts. Known as *rapid actions*, they have been described for different steroids (13). Rapid actions are characterized by: 1) developing within seconds to minutes, which excludes the possibility of new mRNA and protein synthesis; 2) insensitivity to inhibitors of gene transcription and protein synthesis, and 3) appear in response to steroids bound to large molecules such as BSA, which prevents them from entering the cell. In osteoblasts, rapid non-genomic 1,25D actions are characterized by: a) activation of intracellular signal transduction pathways (14, 15), b) elevation of cytoplasmic Ca^{2+} concentration (16, 17), and c) potentiation of ion channel activities (2, 3, 18, 19). However, the physiological significance of these rapid, membrane-initiated effects of 1,25D in bone cells remains a field relatively unexplored.

It has been known for over a century that vitamin D deficiency leads to rickets and osteomalacia, characterized by bone lesions and deformities from inadequate mineralization (20). In addition, dietary calcium and vitamin D supplementation have proven to be effective in the treatment of osteoporosis, a disease characterized by bone mass loss (21). A number of effects have been described when primary osteoblasts or osteoblastic cell lines are treated with 1,25D *in vitro*. By acting on the VDR in the cell nucleus, 1,25D modulates the expression of genes associated with osteoblast differentiation and bone production, such as alkaline phosphatase (22), type I collagen, and non-collagenous proteins (23-26). In addition, 1,25D stimulates mineralization in cultures of osteoblast-like cell lines (27). Some research groups postulate indirect effects of vitamin D metabolites on bone formation, mainly centered around the idea that 1,25D acts primarily on calcium intestinal absorp-

tion and delivery of calcium to the bone (28, 29). In fact, the effect of 1,25D on bone *in vivo* appears to be a combination of direct actions on bone cells (30) and indirect actions to regulate calcium absorption and delivery to the bone (31).

Non-genomic, rapid effects of 1,25D in osteoblasts

The study of membrane-initiated, non-genomic actions of steroids in different cell systems has become a rapidly expanding field of research in recent years. The idea of steroids exerting a rapid effect at the cell membrane level was first formulated by Szego and collaborators several decades ago (32, 33). However, the majority of our current knowledge on steroid mechanisms of action resides mainly at the genomic level, with relatively less research addressing rapid or non-genomic studies. Among different functions attributed to non-genomic actions of steroids are those that involve rapid ion movements across the cell membrane and rapid membrane fusion as part of secretory processes in different tissues involving cytoplasmic signaling pathways (34, 35). In osteoblasts, however, there is no current consensus on how the non-genomic 1,25D signaling cascade is initiated. Some groups propose that the VDR or a slightly modified protein is responsible for initiating rapid responses (36); others propose the existence of a membrane-associated receptor of different molecular structure (31, 37).
It has been shown that hormone 1,25D rapidly increases intracellular Ca^{2+} in confluent mouse osteoblasts (16); 70% of this response comes from external Ca^{2+} entering through Ca^{2+} channels in the plasma membrane, and 30% from Ca^{2+} released from the ER. In osteosarcoma ROS 17/2.8 cells, low doses (0.1-1 nM) of 1,25D promote an acute, transient (1 min long) rise in intracellular Ca^{2+} concentration in 40% of the cells that is entirely dependent on extracellular Ca^{2+} (it is blocked by Ca^{2+} channel blockers such as dihydropyridines). At higher doses (over 1 nM), 1,25D-promotes an increase in intracellular Ca^{2+} due to both the influx of extracellular Ca^{2+} and its release from intracellular stores (38). Figure 1 shows the increase in cytoplasmic Ca^{2+} concentration in a single live ROS 17/2.8 cell 1 min after the addition of 5 nM 1,25D to the bath. The Ca^{2+} signal lasted approximately 1 sec, and progressed inside the cytoplasm in a wave fashion.
In addition, hormone 1,25D rapidly activates (sec-min) the phosphatidylinositol (PI) cycle leading to formation of the second messenger IP_3 in mouse osteoblasts (14) and ROS 17/2.8 cells (38). Local intracellular Ca^{2+} elevation is crucial for the fusion of secretory vesicles to the plasma membrane and exocytosis (39). This occurs via activation of phospholipase C (PLC)-β_1 linked to a pertussis toxin (PTX)-insensitive G-protein (15, 40). IP_3 promotes the release of Ca^{2+} from intracellular stores. PLCs are effectors of different subunits of various G-proteins. Several G-protein subunits ($G\alpha_q$, $G\alpha_s$, $G\alpha_i$, $G\beta$ and $G\gamma$) have been identified in female rat osteoblasts. It has been known that the effect of 1,25D on IP_3 formation and Ca^{2+} mobilization in osteoblasts involves $G\alpha_q$ (40).

1,25D effects on ion channels in osteoblasts

Steroids affect in different ways the activity of ion channels present in the plasma membrane of target cells. Our studies showed for the first time that physiological nanomolar concentrations of 1,25D increase outward Cl^- currents in primary osteoblasts (4) and osteosarcoma ROS 17/2.8 cells (2, 3, 19) within the first 5 minutes of treatment (see Figure 2A, B). This 1,25D-sensitive Cl^- channel is a voltage-gated, volume-sensitive anionic channel that activates upon depolarization (2). The presence of different Cl^- channel types in osteoblasts has been demonstrated by means of electrophysiological studies. The 1,25D-sensitive Cl^- channel that we characterized in ROS 17/2.8 cells and mouse calvarial osteoblasts shares some electrical and pharmacological characteristics with a volume-sensitive Cl^- channel described by Gosling et al. (41, 42) and a cAMP-activated Cl^- channel found in primary osteoblasts (43). It opens upon depolarization and is blocked by the specific Cl^- channel blocker DIDS in a time and voltage-dependent manner.
Chloride channels regulate a variety of physiological and cellular functions, including cell volume, electrical excitability, transport of salts, and exocytosis (39). Most mammalian cells express members of the ClC gene family of voltage-gated chloride channels (44). The ClC gene family is composed of at least nine members (ClC-1 through 7, ClC-Ka and Kb) present in the plasma membrane or in the membranes of intracellular compartments. The ClC protein structure has recently been revealed by X-ray crystallography (45). To our knowledge, however, the molecular identity of Cl^- channels expressed in osteoblasts remains unknown. Among these is the 1,25D-sensitive Cl^- channel. One possible candidate is the ClC-2 gene (46). The ClC-2 is a broadly expressed channel that typically activates by cell swelling and hyperpolarization (44). It has been described in epithelial cells, and is involved in transport of ions across epithelia, and cell volume regulation. The Cl^- channel described by Gosling et al. (41) in ROS 17/2.4 cells activates in hyposmotic conditions. The 1,25D-sensitive Cl^- channel that we described in the same osteoblastic cells is also a volume-sensitive channel. However, 1,25D-potentiation of the currents occurs also under isotonic conditions.
Another possible candidate for the 1,25D-sensitive Cl^- channel protein is the one encoded by the ClC-3 channel gene (47).

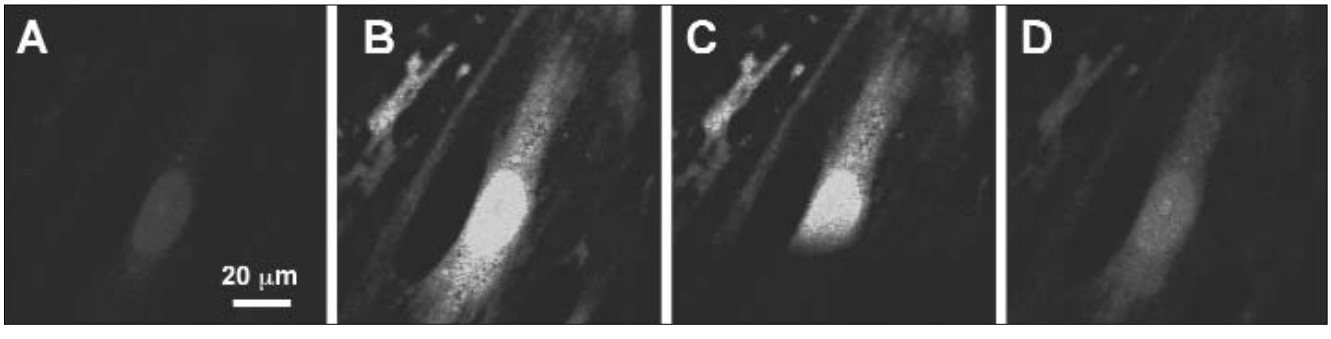

Figure 1 - 1,25D stimulated cytoplasmic calcium wave in a ROS 17/2.8 cell. Series of confocal microscope Ca^{2+} images obtained from a single cell after the addition of 5 nM 1,25D to the bath. Sequential images were obtained with a confocal fluorescence microscope every 0.33 sec. Cells were

This channel is also expressed broadly, and seems to be mostly associated with endosomal and synaptic membranes (44). It is inhibited by DIDS and shows outward rectification, and in these respects it resembles the osteoblast 1,25D-sensitive Cl^- channel. On the other hand, there is a ClC-7 channel described in osteoclasts. Mutation of this gene has been associated with severe osteopetrosis in mice and humans (48). ClC-7 is present in vesicle membranes in the osteoclast ruffled border, and thus participates in acidic dissolution of the inorganic components of the bone. There are no reports on ClC-7 in osteoblasts so far. With these facts in mind, we searched for the expression of ClC-2, ClC-3 and ClC-7 channel genes in the osteoblastic ROS 17/2.8 cell line by means of PCR amplification. As shown in Figure 2C, we found that the ClC-3 gene is expressed in the osteoblasts, which agrees with our electrical and pharmacological characterization of the 1,25D-sensitive Cl^- channel.

Some cation channels activate within seconds-minutes with physiological concentrations of 1,25D in osteoblasts. The best known 1,25D-sensitive cation channel in osteoblasts is a high voltage activated calcium channel shown in Figure 3. When added to the bath, 1,25D causes a shift in the activation of Ca^{2+} currents to more negative membrane potentials within the first 5 sec (2, 3, 18). This is a dihydropyridine-sensitive, or L-type Ca^{2+} (L-Ca) channel. The precise molecular mechanisms of 1,25D effects on the osteoblast L-Ca channel remain unknown. There is evidence that voltage-dependent L-Ca channels need to be phosphorylated to respond to membrane depolarization. Protein kinases A and C, cAMP, and GTP-binding proteins are involved in the phosphorylation of Ca^{2+} channel subtypes (see review, 49). In osteoblasts, we have shown that cAMP mimicks 1,25D-promoted changes in voltage-activation of the L-Ca channel (3). In addition, in muscle cells, 1,25D rapidly activates L-Ca channels via a non-genomic mechanism that involves a G protein-mediated stimulation of the adenylate cyclase/cAMP/PKA messenger system (50).

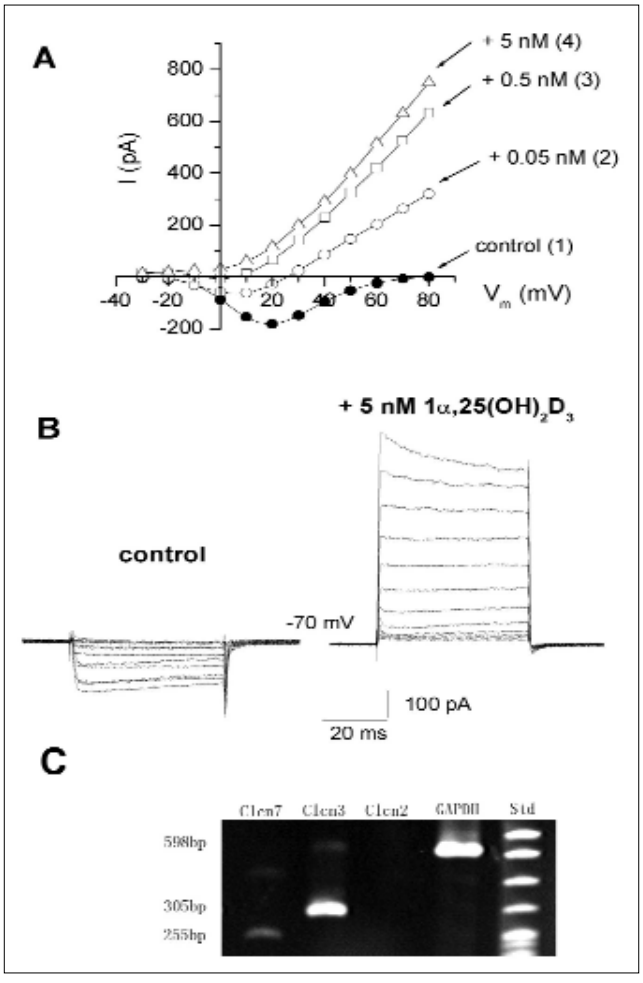

Figure 2 - Effect of 1,25D on outward Cl^- currents in ROS 17/2.8 cells. **A**, Current to voltage (I/V) relations. Inward Ca^{2+} currents were recorded at the beginning of the experiment (closed circles). Subsequent addition of 0.05-5 nM 1,25D to the bath activated an outward Cl^- current (open symbols) within 2.5 min at depolarizing potentials. Increasing concentrations of 1,25D (0.5 nM and 5 nM, open squares and triangles, respectively) promoted increasingly larger outward Cl^- currents over the entire range of depolarizing potentials. Test voltages for I/V curves were 50 ms-long and ranged from 30 to 80 mV. Holding potential –70 mV. **B**, Current traces corresponding to amplitude values depicted in A, for control inward (Ca^{2+}) currents before the addition of 1,25D, and 1,25D-potentiated outward (Cl^-) currents. **C**, Expression at the mRNA level of the voltage-gated ClC-3 channel in ROS 17/2.8 cells cultured for 4 days. For RT-PCR experiments, we used templates designed from previously cloned rat Cl^- channels (cDNA sequences were obtained from GenBank). ROS 17/2.8 cells expressed the outward rectifier, DIDS-sensitive ClC-3, but not the ubiquitous swelling-activated ClC-2. Transcripts of the intracellular ClC-7 channel were also found, although in significantly lower amounts.

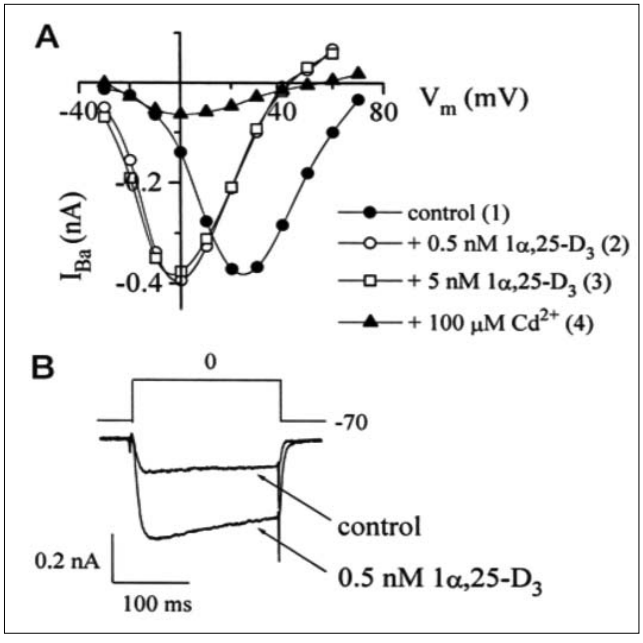

Figure 3 - Effect of 1,25D on inward Ca^{2+} currents in ROS 17/2.8 cells. I/V relations obtained for L-Ca channels (**A**) before (closed circles) and after (open symbols) the addition of 0.5 and 5 nM 1,25D to the bath. Calcium currents were blocked by 100 μM $CdCl_2$ added to the bath at the end of the experiment. **B**, Current traces corresponding to amplitude values depicted in **A**, for control Ca^{2+} currents before the addition of 1,25D, and for 1,25D-potentiated L-Ca currents. Pulse protocols are as in Figure 2 for 200 ms-long voltage pulses. [Duplicated with permission from (2)].

Modulation of ion currents by 1,25D in ROS 17/2.8 cells occurs via intracellular signaling pathways

Stimulation of phosphorylation pathways in ROS 17/2.8 cells mimics the effect of 1,25D on ion channel activities. There is pre-

Table I - Summary of 1,25D effects on voltage-gated Ca^{2+} and Cl^- channels in osteoblasts. V: voltage; PTX: Pertussis toxin.

CHANNEL TYPE	L-Ca	Cl^-
1,25D EFFECTS	Shifts V-sensitivity (seconds)	Increases conductance (minutes)
1,25D MODE OF ACTION	Direct interaction and signal transduction from a membrane-associated receptor	Signal transduction from a membrane-associated receptor coupled to a PTX-insensitive Gq protein
	cAMP	cAMP (40% of cells)
	Phosphorylation	Phosphorylation (PK)
	VDR is necessary	VDR is necessary

vious evidence for modulation of L-Ca channels in osteoblasts by cAMP associated with actions of the natural vitamin D metabolite 24,25$(OH)_2$-vitamin D_3 and parathyroid hormone (51). We found that the cell permeant cAMP analog diBucAMP potentiates (about 2.5-fold at 0 mV) inward Ca^{2+} currents after 5 min in a fashion clearly similar to hormone 1,25D, and causes a significant shift in current to voltage relations to negative values (3). This shift facilitates the opening of L-Ca channels at membrane potentials close to the resting value, and promotes the influx of Ca^{2+} ions from the extracellular medium.

In addition, we found that forskolin (20 mM), an adenylate cyclase activator, causes a significant Cl^- current increase in 40% of ROS 17/2.8 cells after 2 min. Pre-incubation of cells with 0.05 mM staurosporine, a broad spectrum PK inactivator, inhibited 1,25D-potentiation of Cl^- currents (3). Taken together, these results indicate that 1,25D appears to potentiate Cl^- channel functions in ROS 17/2.8 cells through phosphorylation of the channel molecule via a cAMP/PKA pathway.

It has been observed that, in general terms, steroids affect ion channel activities through different mechanisms. Most studies have shown steroid blocking effects at micromolar concentrations (52-56). Our work in osteoblasts, however, uses nanomolar, physiological concentrations of 1,25D. The hormone modulates the voltage-sensitivity of L-Ca channels by shifting current to voltage relations to more negative potentials (18), on one hand, and increases the conductance of voltage-gated Cl^- channels, on the other (2, 3, 18). Our observations therefore reveal multiple mechanisms of action of 1,25D on ion channel activities that take place simultaneously in the osteoblast plasma membrane. Table I summarizes 1,25D effects on ion channel types in ROS 17/2.8 cells, and the mechanisms of action that we propose [extracted from (57)].

We recently reported that the VDR is necessary for rapid 1,25D-potentiation of Cl^- and Ca^{2+} currents in osteoblasts. Treatment with 5 nM 1,25D remarkably increased Cl^- currents within 1-5 min in cells expressing a functional VDR, but not in osteoblasts isolated from a VDR KO mouse (see Figure 4) (4, 7). In addition, the hormone did not alter the voltage-sensitivity of Ca^{2+} channels in VDR KO osteoblasts. To carry out these studies, we used primary calvarial osteoblasts isolated from neonatal VDR KO mice. We used the Tokyo VDR KO mice generated by targeted ablation of exon 2 (58). VDR wild type (WT) and knockout (KO) mice had been used previously to characterize the physiological status caused by an abolished vitamin D metabolism (59, 60). We detected the presence of the VDR molecule by immunocytochemistry in ROS 17/2.8 cells with the use of a primary antibody against VDR and a FITC-conjugated anti-mouse secondary antibody. Figure 5 shows that the VDR localizes abundantly in the cytoplasm and in the proximity to the plasma membrane (61).

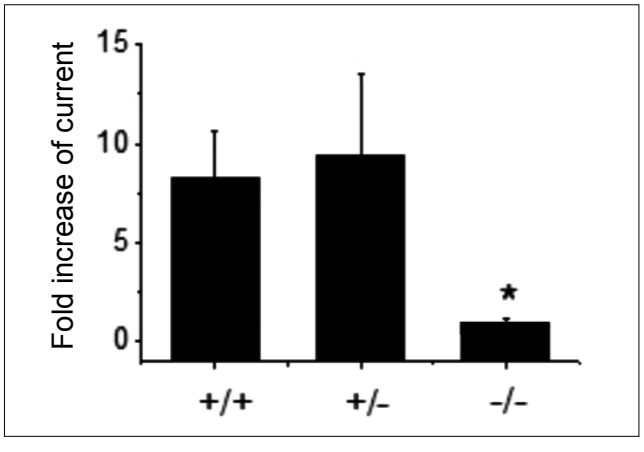

Figure 4 - 1,25D potentiation of Cl^- currents in VDR WT but not in KO osteoblasts. Fold increase (average ± S.D.) of whole-cell outward Cl^- currents promoted by the addition of 5 nM 1,25D. Current amplitudes were measured at -80 mV from genotyped VDR +/+ (n = 6), +/- (n = 6), and -/- (n = 11) mouse calvarial osteoblasts. [Duplicated with permission from (7)].

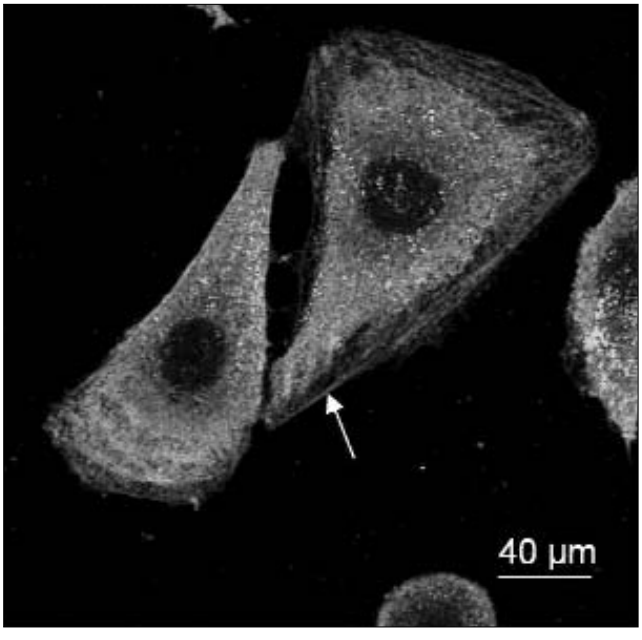

Figure 5 - Subcellular localization of the VDR in ROS 17/2.8 cells. This confocal immunofluorescence micrograph shows a strong signal in the cell cytoplasm and portions of the plasma membrane region (arrow). Bar = 40 µM.

Ion channels and VDR play a role in exocytosis in osteoblasts

In excitable cells, Ca^{2+} channels function as transducers of an electrical signal (action potential) into a chemical signal (exocytosis of neurotransmitters). Osteoblasts, however, are non-excitable cells, and 1,25D appears to act *in lieu* of action potentials to facilitate the opening of Ca^{2+} channels, Ca^{2+} influx, and an immediate exocytotic response (7). Recently, Cl^- channels have been shown to play a crucial role in exocytosis. Located in the secretory vesicle membrane and the plasma membrane, Cl^- channels provide the electrical shunt for acidification of the vesicle content by a H^+-ATPase and loading of secretory proteins. In β-pancreatic cells, for example, insulin secretion is strongly inhibited by the stilbene derivative DIDS, and the ClC-3 channel is localized in the secretory vesicle membrane (62). Thus, 1,25D appears to modulate osteoblast exocytosis by acting on the ion channels directly involved in the secretory process. The 1,25D-sensitive L-Ca and Cl^- channels are therefore likely to be the transducers of a hormonal signal into an electric signal (local depolarization-repolarization) coupled to secretion.

Osteoblasts produce new bone by secreting a complex extracellular matrix that has the capacity to mineralize when adequate amounts of calcium and phosphate are supplied. Bone matrix proteins include type I collagen as the major component, and proteoglycans, glycoproteins and gamma-carboxylated proteins. Osteoblasts express the protein components of the SNARE complex involved in the docking of secretory vesicles to the plasma membrane during regulated secretion (63). Regulated secretion of proteins, which is typical of neurosecretory, endocrine and exocrine cells, occurs as a rapid fusion of vesicles docked to the plasma membrane in response to a specific electrical or chemical stimulus. This is associated with changes in the electrical state of the plasma membrane and elevation of cytoplasmic Ca^{2+} (39). On the other hand, constitutive secretion of proteins had been traditionally associated with osteoblast physiology until recently. This is a continuous process controlled at the genomic level that comprises the production, packing, shipping, and continuous release of secretory products such as matrix proteins (64). Our recent finding of a regulated type of secretion stimulated by a steroid in osteoblasts is a novel concept. The molecular mechanisms underlying this secretion remain to be elucidated.

Figure 6A shows the presence of secretory granules docked to the plasma membrane of a single ROS 17/2.8 ostoeblast. SEM images in Figures 6B, C show collagen fiber production by secretory mechanisms in 8 day-old cultures. We found that steroid-regulated secretion occurs in osteoblasts expressing a functional VDR, but not in cells obtained from a KO VDR mouse (58). Hormone 1,25D, by acting rapidly at the osteoblast plasma membrane level via interaction with a membrane-associated VDR, promotes the release within seconds-minutes of the secretory content -typically collagen- of membrane-docked vesicles. The anabolic effects of the hormone in bone can therefore be explained at both the genomic (synthesis of proteins) and non-genomic (exocytosis of proteins) levels.

We demonstrated in osteoblasts that a rapid physiological response to 1,25D is the activation of secretion (7). This happens simultaneously with Cl^- channel activation. We performed continuous recordings of whole-cell capacitance as a measure of exocytosis in single cells (65). The addition of 1,25D to the external solution increased the frequency of individual exocytotic events within the first 1-5 min only in osteoblasts isolated from VDR wild type mice. Figure 7 shows typical capacitance responses to 1,25D obtained from a single live VDR WT osteoblast.

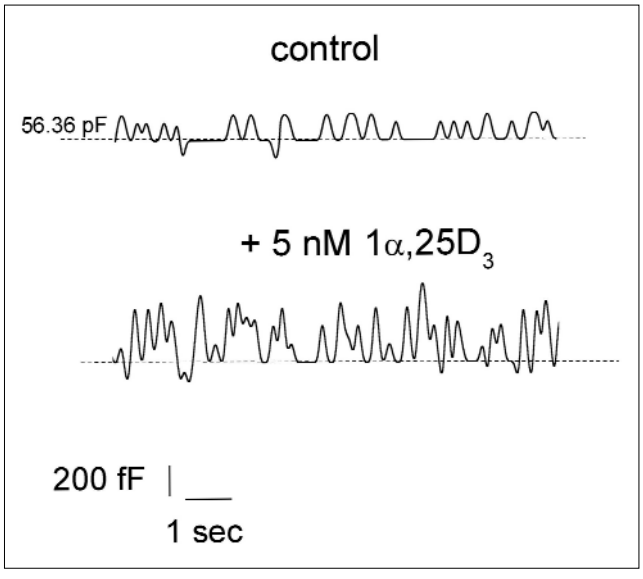

Figure 7 - 1,25D stimulation of exocytotic activities in osteoblasts. Whole-cell capacitance recordings obtained from a single VDR WT osteoblast in the absence (top trace) and presence (bottom trace) of 5 nM $1,25D_3$ added to the bath. Upward deflections from an initial capacitance value of 53.4 pF (dotted line) represent the fusion of individual secretory granules to the osteoblast plasma membrane (exocytotic events). Notice increased frequency of events obtained after hormone addition (bottom trace). [Duplicated with permission from (7)].

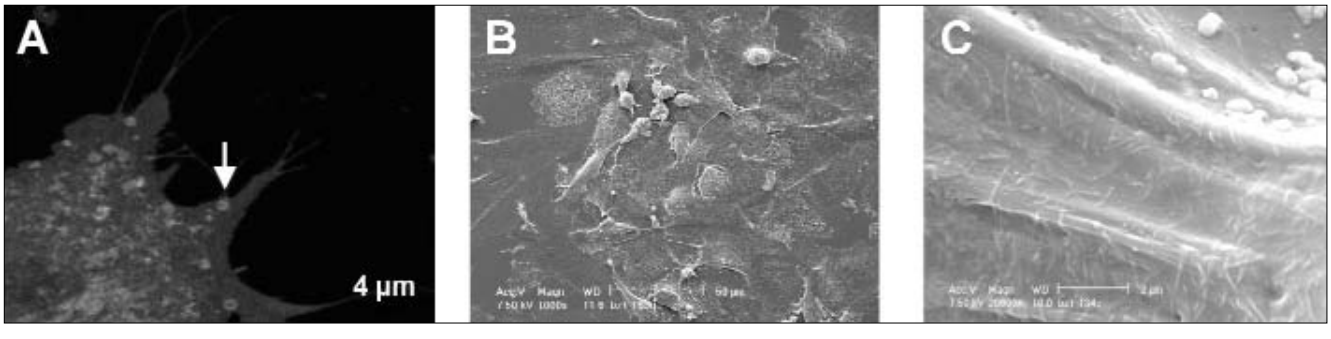

Figure 6 - Images of secretory vesicles (**A**) and associated secretory activities (**B, C**) in ROS 17/2.8 cells at early stages of bone formation. **A**, Secretory vesicles (arrow in **A**) can be observed in close proximity to the plasma membrane. Cells were stained with 3 µM octadecyl rhodamine and observed with confocal fluorescence microscopy. **B, C**: SEM images obtained from a 4-day old ROS 17/2.8 cell culture. Cells were treated with 5 nM 1,25D for 5 min before fixation with 2.5% glutaraldehyde in Na cacodylate buffer. In **B**, ROS 17/2.8 cells colony. In **C**, detail of the plasma membrane surface of a ROS 17/2.8 cell at high magnification. Notice the presence of collagen fibers on the cell surface.

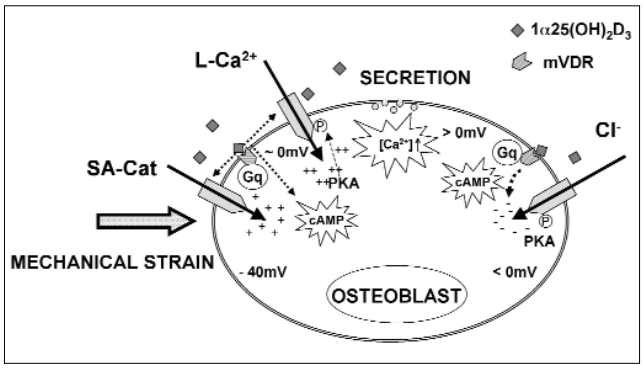

Figure 8 - 1,25D non-genomic signaling pathways in osteoblasts. The scheme summarizes 1,25D membrane-initiated pathways overviewed in this paper. The explanation is given in the text.

Summary model for non-genomic mechanisms of 1,25D electrical effects in osteoblasts

Figure 8 summarizes our current hypothesis of the sequence on molecular events that take place at the osteoblast cell membrane and lead to rapid 1,25D electrical effects coupled to exocytosis (3). Mechanical strain exerted on the cell surface is the initial natural primary stimulus that activates stretch-activated cation channels (SA-Cat) expressed in osteoblasts (66). Bones are subjected to mechanical load due to gravity force and muscle activity. This translates into movement of fluids within bone trabeculae and a shear force applied to the osteoblast surface. The influx of positive charges into the cytoplasm due to SA-Cat channel activation and potentiation by 1,25D causes an initial, local depolarization of the osteoblast membrane from -40 mV at rest, to around 0 mV. This depolarization opens L-Ca channels at low positive membrane potentials. As we showed in our research, calcium channel opening is potentiated at low depolarizing potentials by physiological concentrations of 1,25D (2). Hormone binding to a membrane-associated receptor (possibly VDR) appears to stimulate the production of cAMP via a Gq / adenylate cyclase pathway. This in turn may activate PKA, and ultimately the phosphorylation of L-Ca channels necessary for channel opening. A local elevation of cytoplasmic Ca^{2+} concentration near L-Ca channels promotes the fusion of secretory vesicles to the plasma membrane, and release of secretory contents into the extracellular medium. This local elevation of cytoplasmic Ca^{2+} causes a further depolarization of the plasma membrane to slightly positive values, which in turn promotes the opening of Cl^- channels. These Cl^- channels are also potentiated by 1,25D through similar VDR / Gq / PKA pathways. The influx of Cl^- ions into the cytoplasm contributes to the repolarization of the cell membrane to regenerate negative initial values.

Conclusions

Our work addresses molecular mechanisms of bone formation by investigating non-genomic effects of the steroid 1,25D in osteoblasts. The study of bone formation has traditionally focused on understanding the control of bone cell proliferation, differentiation and apoptosis by different metabolic agents with effects on bone. From this viewpoint, bone formation during the process of bone remodelling in the adult skeleton has been evaluated as the result of an increased osteoblast relative to osteoclast activity (67, 68). Similar perspective has been applied to the study of the pathophysiology of bone mass loss, in which a disordered control of relative bone cell numbers – in osteoporosis, for example, too many osteoclasts relative to fewer osteoblasts – has been described (69). Therefore, the emphasis of these studies has typically been at the bone tissue level.
By focusing at the single-cell level, our studies contribute to the understanding of the molecular basis of bone diseases characterized by decreased bone formation and mineralization, and therefore to their treatment. These include osteoporosis, rickets and osteomalacia. In particular, osteoporosis constitutes a major financial burden for the American society, which will increase in the near future owing to the extended life expectancy of the population. Every possible effort including a full understanding of the molecular basis of these diseases should be made to diminish the financial impact on society and to improve the quality of life of those who suffer from these diseases.

Experimental procedures

Cell culture. Osteosarcoma ROS 17/2.8 cells were cultured according to previously published protocols (2). Briefly, ROS 17/2.8 cells (kindly provided by M.C. Farach-Carson, University of Delaware) were grown in DMEM/Ham's F-12 (50:50, v/v) supplemented with 10% fetal bovine serum and antibiotics. Cell cultures were kept in a humidified atmosphere containing 5% CO_2 at 37°C. For patch-clamp experiments, cells were used within the first 4 days after each passage.
Primary osteoblast cultures were established from 3-5 day old mouse calvaria as published before (4). Stripped parietal bones were fragmented (~1 mm^2), washed several times in sterile HEPES buffer, and transferred to 35 mm Falcon culture dishes. Typically, osteoblasts migrated from the bone fragments and settled on the bottom of culture dishes after 1-2 days. Mature osteoblasts showing a typical polyhedral shape were used for patch-clamp recordings within the first 2-4 weeks in culture.

Vertebrate animals. We used the Tokyo VDR KO mice generated by targeted ablation of exon 2 at the University of Tokyo, and kindly provided by S. Kato (58). Heterozygous (+/-) mice were bred to generate (-/-) homozygous VDR KO mice, which were raised on a normal diet. Wild type heterozygous (+/-) and homozygous (+/+) siblings were used as controls. Animals were genotyped using a 1-cm tail sample at 21-30 days of age. Newborn and adult mice were euthanized by decapitation and cervical dislocation, respectively, to isolate calvaria. Primary cells from bone samples were kept in culture for up to 2 months.

Electrophysiology. Whole-cell patch-clamp recordings (70) were performed with a HEKA EPC-9 amplifier (ALA Scientific Instruments Inc., Westbury, NY) on individual osteoblasts. Patch pipettes of about 2 MΩ were fabricated with a DMZ Universal micropipette puller from Drummond capillaries (Drummond Scientific Co., Broomall, PA), coated with Sylgard elastomer (Dow Corning Corp., Midland, MI) to reduce capacitative transients, and fire-polished. Ion channel activities were recorded in a bath (external) solution composed of (in mM): 150 TEA-Cl, 3.1 KCl, 20 $BaCl_2$, 1 $MgCl_2$, 4 $NaHCO_3$, 10 Hepes, 20 sucrose, pH 7.4 (adjusted with TEA-OH). The pipette (internal) solution consisted of (in mM): 150 CsCl, 15 NaCl, 4 $MgCl_2$, 5 EGTA, 10 HEPES, pH 7.4 (adjusted with NaOH).
Whole-cell capacitance values were measured using the software-based lock-in implementation of Pulse (v.8, HEKA EPC-9). We applied a sine wave with a frequency of 500 Hz and peak amplitude of 20 mV, which was superimposed on a hold-

ing potential of 0 mV. Whole-cell capacitance was continuously monitored for 10-20 min, sampling every 0.1-1 sec.

Microscopy. Rat osteosarcoma ROS 17/2.8 cells were cultured on cover slips in 35 mm Petri-dishes for 48 hours. Cytoplasmic calcium signals were visualized in live, single osteoblasts with a laser scanning confocal Leica TCS SP2 Microscope (Leica Microsystems, Inc., Exton, PA). Cells were loaded with the calcium sensitive dye Fluo 3-AM (5 μM, Molecular Probes), for 30 min at 30°C. The raise in cytoplasmic Ca^{2+} concentration was detected as an increase in fluorescence intensity 1-3 min after the addition of 5 nM 1,25D to the bath.

For immunocytochemistry observations, ROS 17/2.8 cells were fixed with 3.7% (v/v) formaldehyde at room temperature for 20 min, and permeabilized with ice-cold ethanol for 5 min. We used a primary antibody against VDR (D-6, Santa Cruz Biotechnology CA) and a FITC-conjugated anti-mouse secondary antibody (Sigma Immunochemicals St. Louis, MO). Immunostaining was visualized with an Olympus IX50 inverted fluorescence microscope. For SEM observations, cells were grown on glass cover slips for 5 days and fixed with 4% formalin (Sigma) in 0.1 M Na cacodylate buffer, for 1 hour at room temperature. Coverslips were then washed 3 times with 0.1 M sodium cacodylate. Samples were incubated with 1% osmium tetroxide in the same buffer, for 1 hour at room temperature. Dehydration was performed in an ethanol series (30%, 50%, 70%, 80%, 95%, and 100%), and samples were critical point dried. Samples were then covered by a thin gold/palladium layer, and observed on a Philips SEM XL-30 microscope.

Acknowledgements

I wish to acknowledge Dr. Anthony W. Norman for his important contributions to this manuscript, and Dr. David Carter from the Center for Plant Cell Biology at U C Riverside for facilitating the use of the confocal microscope. This work was supported in part by an USPHS grant DK-071115-01 to LPZ.

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