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Review

Age-related changes in the response of intestinal cells to 1α,25(OH)$_2$-vitamin D$_3$

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ABSTRACT

The hormonally active form of vitamin D$_3$, 1α,25(OH)$_2$-vitamin D$_3$, acts in intestine, its major target tissue, where its actions are of regulatory and developmental importance: regulation of intracellular calcium through modulation of second messengers and activation of mitogenic cascades leading to cell proliferation. Several causes have been postulated to modify the hormone response in intestinal cells with ageing, among them, alterations of vitamin D receptor (VDR) levels and binding sites, reduced expression of G-proteins and hormone signal transduction changes. The current review summarizes the actual knowledge regarding the molecular and biochemical basis of age-impaired 1α,25(OH)$_2$-vitamin D$_3$ receptor-mediated signaling in intestinal cells. A fundamental understanding why the hormone functions are impaired with age will enhance our knowledge of its importance in intestinal cell physiology.

1. Introduction

Vitamin D$_3$ is a prohormone produced by the action of ultraviolet sunlight from 7-dehydrocholesterol in the skin. Vitamin D$_3$ is then metabolized, first into 25OHD$_3$ in the liver by D$_3$-25-hydroxylase, and then into the hormonally active form, 1α,25(OH)$_2$D$_3$, in the kidney by 25OHD$_3$-1α-hydroxylase; this is followed by systemic transport of 1α,25(OH)$_2$D$_3$ to distal target organs bound to plasma vitamin D binding protein (DBP) (Ikekawa and Ishizuka, 1993). Regulation of the vitamin D endocrine system occurs at the level of the renal 1α-hydroxylase by 1α,25(OH)$_2$D$_3$ itself, parathyroid hormone, and calcium and phosphate serum concentrations. 1α,25(OH)$_2$D$_3$ is an important regulator of extracellular calcium and phosphorus homeostasis and also modulates cell proliferation and differentiation, muscle growth and contractility, and the immune system, being the intestine the major target tissue of the hormone.

As in other target cells (Haussler, 1986; Minghetti and Norman, 1988; Boland and Boland, 1994), 1α,25(OH)$_2$D$_3$ elicits responses in intestinal cells both through nuclear receptor-mediated gene transcription and a fast mechanism independent of new RNA and protein synthesis (Norman et al., 1992) 1α,25(OH)$_2$D$_3$ rapid responses include the activation of the adenylyl cyclase/cAMP/PKA, PLC/DAG/IP$_3$/PKC signal transduction pathways and the Ca^{2+} messenger system (Boland et al., 1990a,b,c; Boland and Nemere, 1992; Massheimer et al., 1994; Picotto, 2001).

Many changes occur during cellular ageing, such as decreased membrane fluidity, increased protein oxidation, decreased DNA methylation and defects in mitogenic signaling (Kirkland, 1992; Lin and Beal, 2003). Aging is associated with increased circulating PTH levels (Chan et al., 1992) and decreased serum vitamin D metabolites (Armbrecht et al., 1984; Liang et al., 1989), intestinal calcium absorption (Bullamore et al., 1970; Armbrecht, 1990), and bone density (Hui et al., 1988). Moreover, ageing develops intestinal resistance to 1α,25(OH)$_2$D$_3$ (Wood et al., 1998; Pattanaungkul et al., 2000; Song and Fleet, 2007). Vitamin D$_3$ deficiency is prevalent in the elderly population (Eriksen and Glerup, 2002). Low serum concentrations of the hormone precursor 25OHD$_3$ are associated with an increased risk of several chronic diseases including osteoporosis, cancer, diabetes, autoimmune disorders, hypertension, atherosclerosis and muscle weakness all of which can be considered aging-related diseases. There is a study suggesting that Vitamin D receptor (VDR) genetic ablation promotes premature aging in mice, and that vitamin D$_3$ homeostasis regulates physiological ageing (Keisala et al., 2009). VDR expression in human muscle tissue decreases with age (Bischoff-Ferrari et al., 2004) and muscle contractility and growth, processes modulated by the steroid hormone, are also affected by ageing (Boland, 1986). Age-related alterations of 1α,25(OH)$_2$D$_3$-signal transduction have been demonstrated. In skeletal muscle isolated from aged rats, the transient production of IP$_3$ and DAG generated by the hormone decreased significantly (Facchinetti et al., 1998a,b). In addition, the hormone-dependent increase of total and membrane PKC activity were completely blunted in muscle from aged rats. Furthermore, ageing impairs PKC membrane translocation and calcium-dependent PKC (alpha and beta) and calcium-independent PKC (delta, epsilon, theta and zeta) signal transduction pathways under selective regulation by

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1α,25$(OH)_2D_3$ (Facchinetti and Boland, 1999). In addition, the stimulation of cAMP/protein kinase A-dependent calcium uptake by the steroid hormone in these cells is severely impaired with senescence (Massheimer et al., 1995). However, alterations of the direct effects of 1α,25$(OH)_2$-Vitamin D_3 on duodenal cell signaling with ageing remains incompletely understood. The purpose of this review is to analyze age-related changes in the activation of signal transduction pathways by 1α,25$(OH)_2$-Vitamin D_3 in intestinal epithelial cells.

2. Vitamin D receptor in intestinal cells

Two types of receptors has been reported, the classic nuclear vitamin D receptor (VDR) and the putative plasma membrane vitamin D receptor (VDRm), which with their shape-sensitive and stereoselective ligand binding domains, determine the signal transduction pathways which become activated and result in biologic responses (Norman et al., 2001). The classic VDR is a DNA-binding protein and transcription factor with a molecular weight of about 50 kDa. VDR was discovered in the intestine of vitamin D-deficient chickens (Haussler and Norman, 1969), but so far the presence of VDR was described in more than 37 cell types and tissues (Mizwicki and Norman, 2009).

The genomic responses to 1α,25$(OH)_2D_3$ result from its stereospecific interaction with its nuclear receptor. 1α,25$(OH)_2D_3$-VDR-dependent transcriptional activity is modulated through synergistic ligand-binding and dimerization with retinoic X receptor (RXR). The activated 1α,25$(OH)_2D_3$-VDR–RXR complex specifically binds to vitamin D response elements within the promoter region of vitamin D_3 responsive genes. The VDR not only directly up-regulates gene transcription but also directly down-regulates the transcription of several genes (Bouillon et al., 1995). Co-factor proteins also have the ability to modulate VDR-mediated gene expression as well as 1α,25(OH)2D3 binding induces phosphorylation and conformational changes in VDR, which causes the release of co-repressors allowing the gene expression (Haussler et al., 1998, 2011). The genes that have been identified to be responsive to 1α,25$(OH)_2D_3$ are extensive, and many of them are regulated by the hormone in a tissue-specific fashion. Most of these genes play a direct role in Ca^{2+} endocrinology or bone formation and also in cell proliferation and differentiation (Bouillon et al., 1995). Despite the fact that nuclear localization of VDR-binding to the hormone is well established, (Haussler and Norman, 1969; Norman, 2006; Haussler et al., 2011) a mechanism for targeting the VDR to subnuclear compartments remains undefined and little is known about the receptor half life in the nuclear compartment and the signal on/off mechanism. It has been reported that a significant proportion of VDR in living cells resides in the cytoplasm, colocalized with the endoplasmic reticulum, the Golgi complex and microtubules and discrete regions of the nucleus and along the nuclear envelope (Barsony et al., 1997). Moreover, in rat enterocytes the VDR localize in membrane, cytosol and, to a lesser extent, in the nucleus and mitochondria. In competition binding assays, specific binding in all subcellular fractions, with maximum binding in mitochondrial and nuclear fractions has been detected (Gonzalez Pardo et al., 2008). In addition, it is necessary to establish whether specific signaling pathways, including those activated by 1α,25$(OH)_2D_3$ through non-genomic actions at the cell membrane (Norman et al., 2004) are also contributing to VDR subnuclear localization.

First studies have suggested the existence of a 1α,25$(OH)_2D_3$ binding protein different from the nuclear VDR in the plasma membrane of chick enterocytes which mediates rapid hormone stimulation of intestinal Ca^{2+} transport and PKC activity (Nemere et al., 1994, 1998). Alternatively, it has been reported that annexin II may be the membrane receptor that mediates 1α,25$(OH)_2D_3$-induced rapid increases in cytosolic Ca^{2+} in rat osteoblast-like cells ROS 24/1 which do not express the VDR (Baran et al., 2000). However, subsequent studies have provided evidence that annexin II does not bind 1α,25$(OH)_2D_3$ in a physiologically relevant manner casting doubts on the possibility that it is a putative membrane receptor for the hormone (Mizwicki et al., 2004a,b). It has also been reported that another cell surface receptor for 1α,25$(OH)_2D_3$ termed MARRS (Membrane Associated, Rapid Response Steroid binding), identical to the thiol-protein disulphide oxidoreductase PDIA3/Erp57/GRp58/ERp60 may mediate several 1α,25$(OH)_2D_3$-induced effects in various cell types, including certain cancer cells, chondrocytes and muscle (Boyan et al., 2006; Khanal and Nemere, 2007; Chen et al., 2010; Richard et al., 2010). Furthermore, the targeted disruption of the 1,25D_3-MARRS receptor gene in intestinal epithelial cells eliminates the rapid response to 1,25$(OH)_2D_3$ with respect to rapidly enhanced calcium uptake and PKA signaling (Nemere et al., 2010). However, it is not yet clear whether MARRS deletion has an impact on intestinal Ca^{2+} absorption, whole body Ca^{2+} metabolism, or bone. Studies in different cell types have also demonstrated the presence of VDR associated with plasma membrane caveolae (Norman et al., 2002; Huhtakangas et al., 2004), although no evidence was presented on a functional role of the membrane VDR in non-genomic modulation of signaling pathways. Interestingly, the identification of an alternative ligand-binding pocket in the nuclear VDR has allowed to generate by computer docking a receptor conformational ensemble model providing an explanation for how VDR can have genomic and non-genomic functions (Mizwicki et al., 2004a,b, 2005).

Although the nongenomic, membrane initiated actions of 1α,25$(OH)_2D_3$ have been investigated mostly in the duodenum, recent data shows that all intestinal segments strongly expressed both types of receptors with the VDRm expression in the jejunum to the colon being higher than VDR expression, especially in the ileum, indicating that distal small intestine and large intestine could respond to the nongenomic actions of 1α,25$(OH)_2D_3$ (Tudpor et al., 2008). The nongenomic actions mediated by 1α,25$(OH)_2D_3$ are rapid and not dependent on transcription. However, nongenomic signaling may indirectly affect transcription through cross-talk with other signaling pathways. As a membrane receptor the VDRm acts as a primary regulator of lipid and cytosolic second messengers that modulate the activity of kinases and phosphatases that in-turn can control the opened-state of ionic channels (Mizwicki and Norman, 2009; Norman, 2006). Alternatively, other lines of evidence point to a role of the nuclear vitamin D receptor itself in mediating some of the rapid, membrane-initiated effects of the hormone in other cell types (Barsony et al., 1994; Buitrago et al., 2001; Capiati et al., 2002).

VDR genetic ablation promotes premature aging in mice, suggesting that vitamin D_3 homeostasis regulates physiological aging (Keisala et al., 2009). Furthermore, transgenic expression of the human Vitamin D receptor in the duodenum of VDR-null mice attenuates the age-dependent decline in calcium absorption (Marks et al., 2007). The VDR control the expression of Alpha-klotho, an anti-ageing gene expressed predominately in kidney and brain (Forster et al., 2011), and at lower levels in several endocrine organs, but not found in intestine (Kuro-o, 2010). While the VDR is not considered a longevity factor *per se*, the 1α,25$(OH)_2D_3$-dependent regulation of FoxO proteins, a well-known transcription factors involved in stress resistance and longevity, also links VDR function to ageing (An et al., 2010). Whether there is a decrease in intestinal VDR with age has been a matter of debate. No change in intestinal VDR with age has been reported, suggesting that there is intestinal resistance to 1α,25$(OH)_2D_3$ with age which results in decreased intestinal calcium absorption (Wood et al., 1998). However, with ageing, in rat duodenal cells, it has been shown that both, VDR protein levels and 1α,25$(OH)_2D_3$ binding, are diminished (Gonzalez Pardo et al., 2008; Takamoto et al., 1990) and in humans,

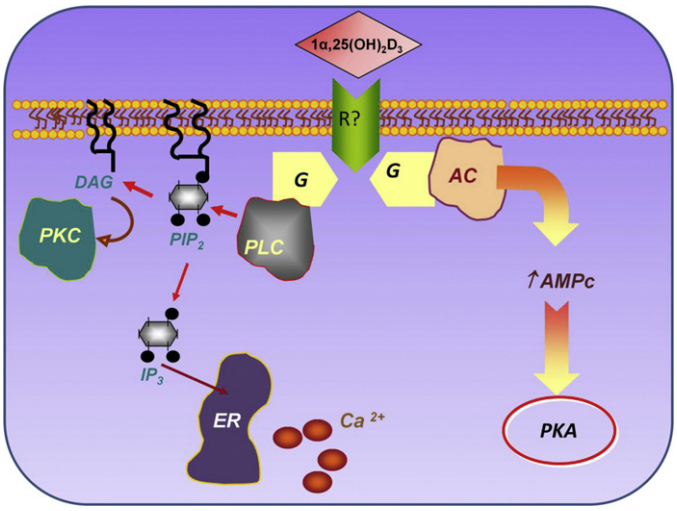

Fig. 1. $1\alpha,25(OH)_2D_3$ regulates AC/cAMP/PKA and PLC/IP_3/DAG pathways in intestinal cells. $1\alpha,25(OH)_2D_3$ stimulates adenylyl cyclase (AC) activity increasing cAMP and resulting in PKA activation. The hormone also induces PLC hydrolysis with generation of the second messengers IP_3 and DAG, and ultimately PKC activation and release of Ca^{2+} from intracellular stores.

similarly to rats, an age related decrease in intestinal VDR occurs (Ebeling et al., 1992). In addition, decreased expression of VDRm (MARRS) and its mRNA in aged basal lateral chicken membranes has been reported (Larsson and Nemere, 2003a,b). Alterations in mRNA expression of duodenal VDR in aged rats also were observed (Liang et al., 1994). Age-related declines in VDR/VDRm may have important consequences for correct receptor/effector coupling in the duodenal tissues and may explain age-related declines in the hormonal regulation of signal transduction pathways and intestinal cell proliferation.

3. Regulation of enterocyte AC/cAMP/PKA and PLC/IP_3/DAG pathways by $1\alpha,25(OH)_2$-vitamin D_3

Adenylyl cyclase (AC) plays a key role in signal transduction generating the second messenger cAMP, which in turn, activates protein kinase A (PKA) by binding to its regulatory subunit (Soderling, 1990). Stimulation of AC in the intestinal epithelial cell by $1\alpha,25(OH)_2D_3$ and the participation of its product cAMP in Ca^{2+} transport across the intestine has been reported (Walling et al., 1967; Neville and Hodsworth, 1969; Corradino, 1977; Boland et al., 1990a,b). The AC/cAMP/PKA pathway is rapidly activated by $1\alpha,25(OH)_2D_3$ in rat enterocytes (Fig. 1), the hormone induces early significant elevations in cAMP levels and increases PKA activity independently of changes in Ca^{2+} levels. As a consequence, the cAMP cascade mediates the non-genomic activation of rat duodenal calcium fluxes by the hormone (Massheimer et al., 1994). In enterocytes from senescent animals, the effects of $1\alpha,25(OH)_2D_3$ on the AC/cAMP/PKA pathway is abolished (Massheimer et al., 1995). A conspicuous feature of duodenal cells from aged rats is their high basal AC, cAMP levels and PKA activity (Massheimer et al., 2000). Increased basal cAMP content during aging and related diseases has also been observed in rat osteoblasts (Donahue et al., 1988) and brain tissue (Sugawa and May, 1993). A conceivable interpretation for these changes is an age-related increase in AC and/or a decline of cAMP-phosphodiesterase activities or a different PKA activity in the older rat. It is possible that alterations in G proteins involved in the coupling of $1\alpha,25(OH)_2D_3$ membrane receptor with AC may take place during senescence. Age-induced changes in subunit concentration and functional properties of G proteins have been reported (Hanai et al., 1989). Interestingly, there is evidence which involves G proteins in $1\alpha,25(OH)_2D_3$ fast actions in other cell types (Boland et al., 1991; Vazquez et al., 1997; Santillán et al., 1999;). Reduced expression of duodenal inhibitory $G\alpha_i$ with ageing and unchanged levels of $G\alpha_s$, may explain the enhanced adenylyl cyclase and cAMP levels detected in old enterocytes (Facchinetti and Russo de Boland, 2001).

$1\alpha,25(OH)_2D_3$ also activates the PLC/DAG/PKC messenger pathway in several cell types (Oshima et al., 1987; Civitelli et al., 1990; Morelli et al., 1993). Mammalian phosphoinositide-specific phospholipase C (PI-PLC) catalyzes the hydrolysis of PIP_2 to yield the two second messengers IP_3 and DAG. IP_3 interacts specifically with a family of IP_3 receptor-operated Ca^{2+} channels to mobilize non-mitochondrial intracellular Ca^{2+} stores, whereas DAG activates plasma membrane-associated protein kinase C enzymes, which can, in turn, phosphorylate a number of target proteins (Berridge, 1993). In enterocytes, $1\alpha,25(OH)_2D_3$ induces a rapid and transient release of IP_3 and DAG (Lieberherr et al., 1989; Wali et al., 1990) (Fig. 1).

In enterocytes isolated from aged rats, the transient and biphasic production of IP_3 and DAG generated by the hormone decreased significantly. The first phase of DAG production which temporally coincided with IP_3 production is abolished by neomycin. The second DAG phase occurred in the absence of IP_3 production and is not affected by the PLC inhibitor, implying that phospholipids other than phosphoinositides are the source of DAG (Boland et al., 1996). In other cell types (Lipschitz et al., 1991), diminished IP_3 production with ageing has also been related to a decrease of metabolically active phosphoinositides (PI, PIP, PIP_2) in the plasma membrane, which are precursors of IP_3 and DAG. Moreover, composition changes in the plasma membrane and cytoskeleton accompany the ageing process in human and experimental animals (Allalouf et al., 1988; Viani et al., 1991). The hormone also increases PKC activity in the mammalian duodenum by a non-genomic mechanism which involves the rapid influx of extracellular Ca^{2+},and activation of PKC, and in turn, mediates $1\alpha,25(OH)_2D_3$ stimulation of intestinal Ca^{2+} uptake (Boland et al., 1996). Similar fast effects on the enzyme activity were observed in both rat colonic epithelium (Wali et al., 1990) and in Caco-2 cells, a human epithelial colorectal adenocarcinoma cell line (Bissonnette et al., 1994; Wali et al., 1992).

PKC is distributed between the cytosolic and particulate fractions depending on cell type and growth characteristics. Upon cell activation by agonists the soluble form of PKC usually translocate to the membrane (Nishizuka, 1992). Both long and short-term treatments with $1\alpha,25(OH)_2D_3$ have been shown to increase membrane-associated PKC activity and/or immunoreactivity in different kinds of cells (Wali et al., 1990, 1992; Simboli-Campbell et al., 1994). In rat duodenum the hormone is able to translocate the isoforms α, β and δ of PKC from cytosol to membranes (Balogh and Russo de Boland, 2000). With ageing, higher levels of basal PKC activity are found in rat duodenum and the hormone failed to activate PKC (Boland et al., 1996) and its ability to translocate PKC isoforms α, β and δ to the membrane is severely impaired (Balogh and Russo de Boland, 2000). Age-related abnormalities in signal transduction pathways linking receptor to effector functions are seen in various tissues. Changes in PKC expression, activity and translocation have been evidenced in brain during the ageing process (Martinez-Serrano et al., 1992; Battaini et al., 1995). A direct stimulation of PKC by physiological concentrations of $1\alpha,25(OH)_2D_3$, in an *in vitro* assay system, (Slater et al., 1995) suggests that alterations in hormone-PKC interaction may also contribute to the absence of $1,25(OH)_2D_3$ dependent changes in PKC activity in the aged duodenum. The impairment in the $1\alpha,25(OH)_2D_3$-activated phosphoinositide cascade in aged mammalian duodenum may be the result of a decrease in the number or affinity of receptors linked to PLC, or of an inefficient coupling of receptor to GTP-binding proteins. However, the mechanisms by

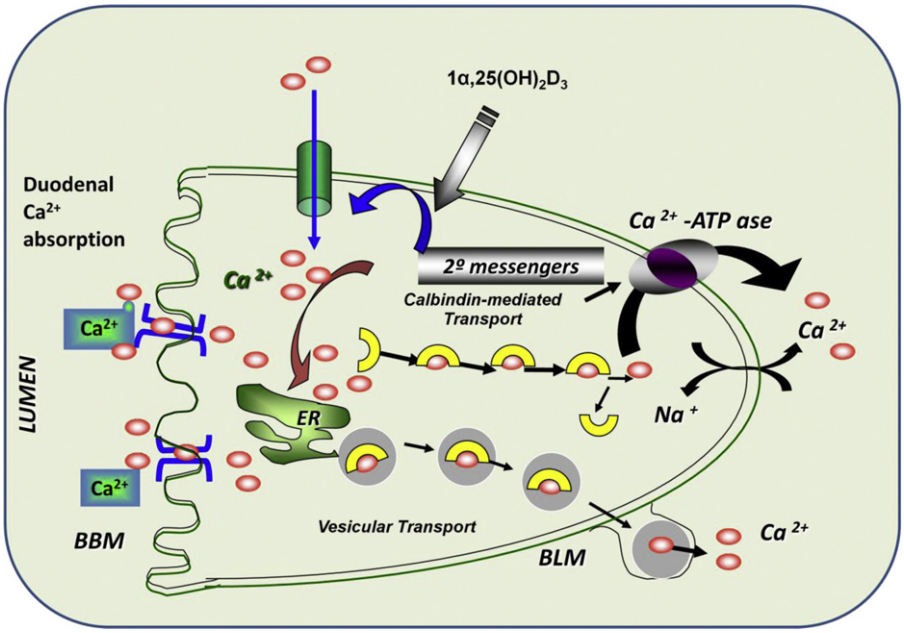

Fig. 2. $1\alpha,25(OH)_2D_3$ non-genomic effects on intestinal Ca^{2+} transport $1\alpha,25(OH)_2D_3$ mediates intestinal calcium absorption by increasing the transcellular flux of calcium through enterocytes. Transepithelial transport of calcium involves uptake at the apical membrane (BBM), movement across the cell, and extrusion at the basolateral membrane (BLM). The hormone regulates the latter two processes by induction of calbindin D and the plasma membrane Ca^{2+} ATPase, respectively. The fast $1\alpha,25(OH)_2D_3$-induced increase in intestinal cell Ca^{2+} influx in mammals and the Ca^{2+} transport in avian is mediated by a dihydropyridine-sensitive (L-type) calcium channels. This rapid stimulation of calcium channels may provide the signal which triggers exocytosis of Ca^{2+} by two possible mechanisms: vesicular and a calbindin mediated transport to the vasculature.

which the signals provided by hormone binding to its membrane receptor are transduced remain to be elucidated.

$1\alpha,25(OH)_2D_3$ through PKC and PKA also modulates duodenal membrane protein phosphorylation and this mechanism is severely altered with ageing (Balogh and Russo de Boland, 1999). Basal phosphorylation of membrane proteins were shown to be similar in young and aged rats in spite of the fact that PKA (Massheimer et al., 1994) and PKC (Balogh et al., 1997) basal activities are higher in the duodenum from aged rats than that seen in young animals. Because phosphorylation results from the combined action of a variety of protein kinases and phosphatases, it is then possible that defective activation of enzymes different from PKC and PKA or higher phosphatase activity occurs with ageing, resulting in similar basal phosphorylation of target proteins. As a result of alterations of the PKC pathway and the cAMP signal transduction pathway and of impaired $1\alpha,25(OH)_2D_3$-protein phosphorylation with ageing, the regulation of many biological process such as cell growth, differentiation, and Ca^{2+}-channel activity may be altered and may explain the impairment of the fast Ca^{2+} uptake response of rat intestinal cells to $1\alpha,25(OH)_2D_3$ during ageing.

4. $1\alpha,25(OH)_2$-vitamin D_3 and enterocyte Ca^{2+} signaling

Calcium availability is reduced in the elderly on account of decreased synthesis in the skin and dietary intake of vitamin D and diminished calcium absorption by the small intestine. $1\alpha,25(OH)_2D_3$ regulates intestinal Ca^{2+} transport through genomic actions involving the classical vitamin D receptor (VDR) (Walters et al., 1999; Jones et al., 1998; Li et al., 2001; Bouillon et al., 2003; Norman, 2006; Pike et al., 2007) and non-genomic actions through a separate membrane receptor (Nemere et al., 2004; Rohe et al., 2005; Khanal and Nemere, 2007).

$1\alpha,25(OH)_2D_3$ mediates intestinal calcium absorption by increasing the transcellular flux of calcium through enterocytes (Nellans, 1990).Transepithelial transport of calcium involves uptake at the apical membrane, movement across the cell, and extrusion at the basolateral membrane (Christakos et al., 2011). The hormone regulates the latter two processes by induction of calbindin D and the plasma membrane ATPase (calcium pump), respectively (Khanal and Nemere, 2008) (Fig. 2).

In addition to transcellular transport of calcium, calcium is also absorbed by the paracellular pathway that occurs through tight junctions and structures present within the intercellular spaces. The paracellular pathway functions throughout the length of the intestine but predominates in the more distal regions when dietary calcium is adequate or high (Wasserman, 2004). However, its regulation by vitamin D remains undefined.

It has been reported that only 2–4 h after $1\alpha,25(OH)_2D_3$ treatment to deficient or normal animals is intestinal calcium absorption significantly increased (Wasserman and Fullmer, 1995). Also, induction of TRPV6 and calbindin occurs hours after $1\alpha,25(OH)_2D_3$ treatment and precedes intestinal calcium absorption (Fleet and Schoch, 2010; Xue and Fleet, 2009). Duodenal expression of the calcium-channel TRPV6 is vitamin D dependent in men, but not in older women, where expression of TRPV6 and VDR are both reduced. These findings can explain, at least in part, the lower fractional calcium absorption seen in older postmenopausal women (Walters et al., 2006).

In the rat duodenum age-induced decrease in transepithelial Ca^{2+} transport is accompanied by a reduction in the levels of $1\alpha,25(OH)_2D_3$ receptor occupancy (Takamoto et al., 1990) immunoreactive-calbindin D9k and the calcium pump (Armbrecht et al., 1999, 2003), which have been related to reduced serum levels of $1\alpha,25(OH)_2D_3$ (Armbrecht et al., 1989; Liang et al., 1989; Takamoto et al., 1990). *In vivo* administration of $1\alpha,25(OH)_2D_3$ partially abolishes the difference in duodenal Ca^{2+} uptake between young and old rats (Liang et al., 1989). $1\alpha,25(OH)_2D_3$ is capable of provoking direct effects on intestinal cells (Nemere and Norman, 1986; Boland et al., 1990a,b). Although mediation of the $1\alpha,25(OH)_2D_3$ effect on intestinal calcium transport by voltage dependent calcium channels is controversial, there is evidence that in avian and mammalian intestinal cells, the hormone enhances duodenal cell Ca^{2+} influx through a mechanism which involves

the activation of voltage-dependent calcium channels, PKC and the cAMP second messenger system (Boland et al., 1990a,b; Massheimer et al., 1994). The fast 1α,25(OH)$_2$D$_3$-induced increase in intestinal cell Ca^{2+} influx in mammals (Massheimer et al., 1994) and the Ca^{2+} transport in avian (Boland et al., 1990a,b,c) is mediated by a dihydropyridine-sensitive (L-type) calcium channels. It has been proposed that this rapid stimulation of calcium channels provides the signal which triggers exocytosis of Ca^{2+} from the intestinal cell to the vasculature (Boland et al., 1990a,b,c). However, additional work is needed to link the non-genomic actions of the hormone to whole body calcium metabolism and active transcellular calcium transport. In enterocytes isolated from aged rats, 1α,25(OH)$_2$D$_3$ stimulation of Ca^{2+} channels through the cAMP/PKA pathway is blunted (Massheimer et al., 1995), suggesting that intestinal resistance to non-genomic hormone stimulation of duodenal cell Ca^{2+} uptake develops in rats upon ageing. *In vivo* administration of 1α,25(OH)$_2$D$_3$ or its precursor (25OHD$_3$) to senescent rats restores the ability of the hormone to stimulate duodenal cell calcium influx through the cAMP messenger system (Massheimer et al., 1999), probably by increasing membrane receptor affinity for 1α,25(OH)$_2$D$_3$ as have been demonstrated for peptide hormones (Supiano et al., 1987) or restoring the functional coupling of the receptor complex with G proteins.

Due to the age-related decline in intestinal calcium transport, serum parathyroid hormone (PTH) levels increase with age (Riggs and Khosla, 1994). Resistance to 1α,25(OH)$_2$D$_3$ action at the parathyroid gland is also associated with ageing, which results in a diminished ability of the hormone to inhibit PTH secretion and therefore contributes to the increase in PTH. The secondary hyperparathyroidism increases bone turnover, and, as a consequence, more bone is resorbed than is formed in the elderly (Riggs and Khosla, 1994). This allows for maintenance of plasma Ca^{2+} concentration within the normal range in spite of intestinal resistance to the hormone (Wemeau, 1995).

5. 1α,25(OH)$_2$-vitamin D$_3$ modulation of enterocyte c-Src kinase and protein tyrosine phosphorylation

In addition to modulation of PKA and PKC activity (Massheimer et al., 1994; Balogh et al., 1997), in isolated rat enterocytes, 1α,25(OH)$_2$D$_3$ also stimulates the tyrosine phosphorylation of several duodenal proteins (Boland and Norman, 1998; Balogh and Russo de Boland, 1999), including PLCγ (Gonzalez Pardo and Russo de Boland, 2004). Because the nuclear and membrane vitamin D receptor have no intrinsic tyrosine kinase activity, hormone stimulation must activate one or more cytosolic tyrosine kinases. In the basolateral membrane of rat colonic cells, 1α,25(OH)$_2$D$_3$ activates the non-receptor tyrosine kinase c-Src (Khare et al., 1997). Changes in Src tyrosine phosphorylation and activity induced by the hormone have been also reported in embryonic skeletal muscle cells (Buitrago et al., 2000) and human keratinocytes (Gniadecki, 1998). In rat enterocytes (Gonzalez Pardo and Russo de Boland, 2004) and in rat colonocytes (Khare et al., 1997), activation of the non-receptor tyrosine kinase c-Src mediates 1α,25(OH)$_2$D$_3$ stimulation of PLCγ phosphorylation. Two structurally related isozymes PLCγ1 and PLCγ2 have been found in association with several signaling molecules, including kinases of the Src and Syk families (Khare et al., 1997; Law et al., 1996).

Src proteins play a critical role in the proliferation and differentiation of normal intestinal epithelial cells (Cartwright et al., 1993). In the normal intestinal mucosa, proliferative, undifferentiated cells at the base of the crypts express high c-Src activity; in contrast, differentiated cells at the tips of intestinal villi show decreased c-Src activity (Cartwright et al., 1993).

The mechanisms by which the signals provided by 1α,25(OH)$_2$D$_3$ to the membrane receptor are transduced to non receptor Src tyrosine kinase is not known at present. However, the involvement of G protein in 1α,25(OH)$_2$D$_3$ signal transduction has been evidenced (Boland et al., 1991, 1995). Studies in COS-7 cells suggest that the βγ subunits of G-protein-coupled receptors mediate activation of the Src family of non-receptor tyrosine kinases (Luttrell and Luttrell, 2004). Moreover, Src-family tyrosine kinases were shown to be direct effectors of $G\alpha_s$ and $G\alpha_i$ proteins (Ma and Huang, 2002). In skeletal muscle cells the participation of PKC and PTPα in 1α,25(OH)$_2$D$_3$-dependent Src activation was recently reported (Buitrago et al., 2011). Further studies are necessary to evaluate whether the hormone employs a similar pathway in rat enterocytes.

Age-related alterations in signaling events related to protein tyrosine kinase activity have been less studied. 1α,25(OH)$_2$D$_3$-induced PLCγ phosphorylation in duodenal cells is greatly decreased in old animals (Gonzalez Pardo and Russo de Boland, 2004). These alterations, coupled with G-protein changes found with ageing, may combine to produce an impairment of PLC activity with advanced age which may result in abnormal 1α,25(OH)$_2$D$_3$ regulation of cell Ca^{2+} and proliferation in the duodenum.

6. Effects of 1α,25(OH)$_2$-vitamin D$_3$ on map kinases activation

Mitogen-activated protein kinases are a family of Ser/Thr protein kinases, that are activated by phosphorylation in response to a wide array of extracellular stimuli and that play an important role in signal transduction mechanisms linked to the regulation of cell proliferation and differentiation (Seger and Krebs, 1995). The MAPK superfamily consists of three main protein kinase families: the extracellular signal-regulated protein kinases (ERKs), the c-Jun N-terminal kinases (JNKs) and the p38 family of kinases. These enzymes are regulated by a characteristic phosphorelay system in which a series of three protein kinases phosphorylate and activate one another. Each is proving to have major roles in the regulation of intracellular metabolism and gene expression and integral actions in many areas including growth and development, disease, apoptosis and cellular responses to external stresses (Chang and Karin, 2001).

In avian and mammalian enterocytes (Fig. 3), 1α,25(OH)$_2$D$_3$ rapidly and transiently stimulates the tyrosine phosphorylation and the activity of the of the MAP kinase isoforms ERK1 and ERK2 (Boland and Norman, 1998; Gonzalez Pardo and Russo de Boland, 2004). In chick enterocytes, hormone analogs which can achieve the 6-s-cis- (steroid-like conformation) shape increase the tyrosine phosphorylation and rapid activation of ERK1/2 while, 1β,25(OH)$_2$D$_3$, a known antagonist of 1α,25(OH)$_2$D$_3$ mediated rapid responses (Norman et al., 1993), blocked the hormone effects on ERK1/2 (Boland and Norman, 1998).In these cells, it has been shown that PKC is necessary, but not sufficient, for ERK1/2 response to 1α,25(OH)$_2$D$_3$ and in rat intestinal cells, the tyrosine kinase cSrc play a role upstream in the signaling pathway leading to ERK1/2 activation by the hormone (Gonzalez Pardo and Russo de Boland, 2004).

There is concordant information on the pathway by which the hormone activates ERK kinase in other cell types. It has been reported that the steroid triggers tyrosine phosphorylation of Shc and complex formation between Shc, Grb2 and Sos in keratinocytes (Gniadecki, 1996) and stimulates Raf kinase in hepatic Ito cells (Lissoos et al., 1993). Furthermore, in HL-60 promyelocytic leukemia cells it has been shown that 1α,25(OH)$_2$D$_3$ activation of ERK1/2 kinase is mediated by upstream PKC regulation

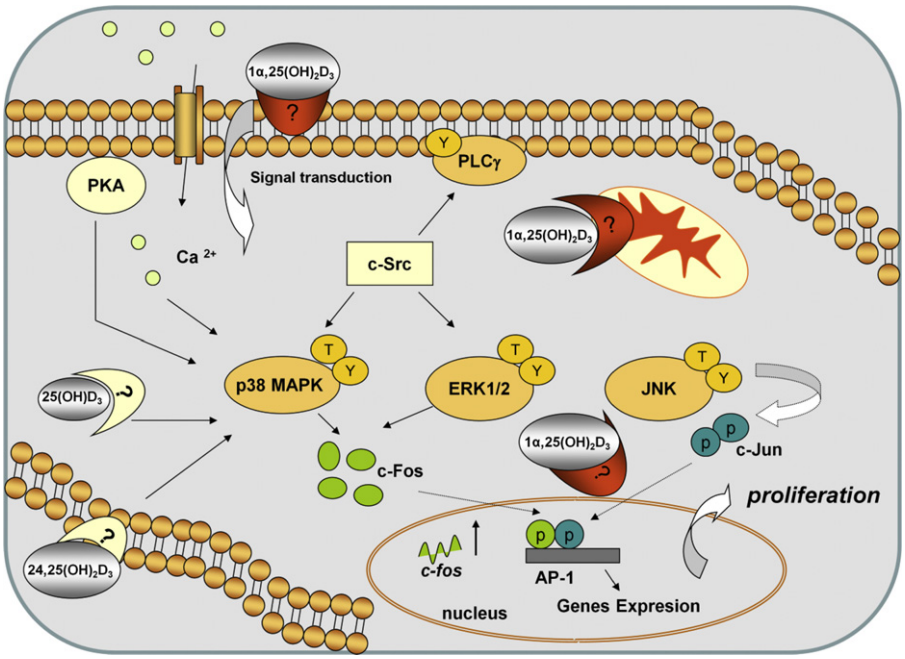

Fig. 3. Src, tyrosine phosphorylation and MAPKs activation induced by $1\alpha,25(OH)_2D_3$ in enterocytes. The cytosolic tyrosine kinase c-Src mediates $1\alpha,25(OH)_2D_3$-dependent PLCγ, ERK1/2 and p38 MAPK phosphorylation. PKA and Ca^{2+} are also part of the mechanism of activation of p38 MAPK. $24.25(OH)_2D_3$ and $25OHD_3$, similar to the hormone, induced phosphorylation of p38 MAPK. JNK and its substrate c-jun are already activated in basal conditions and their phosphorylation is not affected by $1\alpha,25(OH)_2D_3$. The hormone also increases c-fos protein and mRNA levels. c-fos may form heterodimers with c-jun to regulate gene expression.

(Marcinkowska et al., 1997) and in skeletal muscle cells is mediated by PKCα and Ca^{2+} (Morelli et al., 2001; Buitrago et al., 2003).

Although the relative levels of ERK1 and ERK2 did not substantially change with age, the magnitude of $1\alpha,25(OH)_2D_3$-dependent ERK1/2 phosphorylation and activity are significantly lower in enterocytes of aged rats compared with those of young animals (Gonzalez Pardo and Russo de Boland, 2004). Age-related decline in MAP Kinase activation upon mitogenic stimulation has been demonstrated in other cell types (Gorgas et al., 1997; Palmer et al., 1999; Zhen et al., 1999).

In addition, $1\alpha,25(OH)_2D_3$,through Ca^{2+} and the non-receptor tyrosine kinase c-Src as upstream regulators, activates in rat duodenal cells the p38 MAPK cascade and this pathway participates in the regulation of $1\alpha,25(OH)_2D_3$-induced c-fos expression (Gonzalez Pardo et al., 2006) (Fig. 3). How $1\alpha,25(OH)_2D_3$-induced increases in cytosolic Ca^{2+} stimulate tyrosine phosphorylation of p38 MAPK is not known, but they might involve activation of a Ca^{2+}-dependent isoform of adenylyl clyclase (Mons et al., 1998), c-Src kinase activation (Simonson et al., 1996), or Ca^{2+}-dependent assembly of some component(s) upstream involved in the activation of the MAPK pathway. Basal levels of p38 MAPK phosphorylation decreases in enterocytes from old rats and the hormone response is greatly diminished (González Pardo et al., 2007). Understanding the age-related differences in $1\alpha,25(OH)_2D_3$ signaling will require more information about the subtle molecular mechanisms that modulate membrane receptor-p38MAPK signaling pathway. Although a role for $25(OH)D_3$ or $24,25(OH)_2D_3$ by binding to the basolateral membrane in the intestine is speculative and their membrane effects are controversial, in intestinal cells, $25OHD_3$ and $24,25(OH)_2D_3$, also enhanced p38 phosphorylation, and to a similar extent than $1\alpha,25(OH)_2D_3$, an ability that is lost with ageing. $25OHD_3$, may act through one of the identified basolateral membrane binding protein (Phadnis and Nemere, 2003). Moreover, specific binding for the other vitamin D_3 metabolite $24,25(OH)_2D_3$ has been observed in the basal lateral membrane of intestinal cells (Nemere et al., 2002).

There is, however, contradicting data about changes in MAPK activity during ageing (Chung et al., 2000). For instance, ERK and p38 MAPK activities were reported to be impaired in aged rat brain (Zhen et al., 1999). Also, a reduced activation of the MAPK pathway has been detected in isolated T cell and splenic lymphocyte (Pahlavani and Vargas, 2000; Li et al., 2000) and decreased expression of p38 MAPK was reported in skeletal muscle (Mylabathula et al., 2006). Furthermore, the kinase activity of p38 MAPK, and the levels of phosphorylation of downstream regulators of translation were found to be decreased in dwarf mouse livers (Hsieh and Papaconstantinou, 2002). Also, p38 MAPK activity decreased in the gastric mucosa of 24-month-old Fischer 344 rats (Xiao and Majumdar, 2000). Other investigators found that the basal kinase activities of p38α, its upstream activator, MKK3, and its downstream substrate, ATF-2, are elevated in livers of aged C57BL/6 male mice (Hsieh and Papaconstantinou, 2002). The ageing process also enhanced p38 MAPK basal activity in both the liver (Kim et al., 2002) and brain of rats (Suh, 2001). However, an increase in the basal expression of p38 MAPK was detected in the brain but not in the liver of old rats. Thus, it appears that the ageing effect on MAPK activity and expression is likely cell- and tissue specific.

Intestine is lined by low-resistance epithelium that experiences transient mucosal osmotic load during the process of normal digestion (Madara, 1983). Gastrointestinal epithelium faces osmotic stress, both at physiological and pathophysiological conditions. Activation of JNK is well known as one of the early signals activated by various types of stress in various cells, including the intestinal epithelial cells (Samak et al., 2010). Moreover, In Caco-2 cell, osmotic stress induced activation of JNK, which was suggested to mediate the hyperosmotic stress-induced IL-8 production (Hubert et al., 2004). Also, JNK pathway and one of its most important substrates, the AP-1 transcription factor c-Jun, modulates Wnt signaling strength in the intestine (Sancho et al., 2009).

$1\alpha,25(OH)_2D_3$ have been shown to induced a rapid activation of JNK1 and increased AP-1 gene-transactivating activity in Caco-2 colonic cells (Chen et al., 1999). As aging progresses, the basal levels of activity of the stress-activated pathways increase, thereby becoming a basic factor in the development of a state of chronic stress in aged tissues and the ability of these

stress-activated signaling pathways to respond is altered in aged tissues (Papaconstantinou, 1994). In line with these observations, basal JNK phosphorylation and activity have been shown to increase with age in the brain (Suh, 2001) and in rat enterocytes (Buzzi et al., 2007). In addition, rac1, a JNK activator involved in stress response, is up-regulated at old age (Lee et al., 1999). Moreover, age-related increases in JNK basal activities *in vivo* have been reported (Xiao and Majumdar, 2000). The increase in basal activities of JNKs could be due to the well-documented increase in genotoxic stress during aging (Vijg, 2000).

Knowledge on the molecular mechanisms involved in the age-related differences in $1\alpha,25(OH)_2D_3$ signaling through MAP Kinase cascades could contribute to the understanding of alterations in cell proliferation and differentiation that occur during senescence.

7. $1\alpha,25(OH)_2D_3$ modulation of intestinal cell proliferation and differentiation

$1\alpha,25(OH)_2D_3$ regulates the proliferation and differentiation of several cell types (Abe et al., 1981; Farquahrson et al., 1993; Suda, 1989; Bikle and Pillai, 1993). The hormone also plays a role in the regulation of intestinal cells growth, reduced colonic (Thomas et al., 1992) and human duodenal (Thomas et al., 1997) mucosal cell proliferation have been seen in response to $1\alpha,25(OH)_2D_3$. The hormone bound to VDR regulates the expression of more than 60 genes that exert pro-differentiation, antiproliferative and anti-metastatic effects. In some cases the hormone indirectly influences the cell cycle, apoptosis and cell differentiation or interacts with other transcriptional regulators or signaling pathways. $1\alpha,25(OH)_2D_3$ upregulates proteins that control the cell cycle and decreases cell proliferation of both normal and aberrant cells (Holick, 2007). In most cell types that express a functional VDR, incubation with $1\alpha,25(OH)_2D_3$ results in an accumulation of cells in the G0/G1 phase of the cell cycle (Jensen et al., 2001). Activation of the cyclin-dependent kinase inhibitors such as p18, p19, p21, or p27, repression of cyclin D1 expression, as well as down-regulation of the activity of complexes between cyclins and cyclin-dependent kinases, have been suggested to be early events, whether or not directly mediated by $1\alpha,25(OH)_2D_3$ and may be responsible for the growth-inhibitory effect of the hormone (Jensen et al., 2001; Gedlicka et al., 2006; Verlinden et al., 1998). In the colonic Caco-2 cells, the hormone may also inhibit cell growth by interfering with signaling pathways initiated by Wnt-ligands (Aguilera et al., 2007; Palmer et al., 2001).

Of physiological significance, and in agreement with the well-known participation of MAP Kinases in pathways leading to mitogenic effects, ERK2 and JNK1 modulation also mediates $1\alpha,25(OH)_2D_3$ stimulation of Caco-2 cell differentiation (Chen et al., 1999). Moreover, p38 MAPK plays a crucial role in intestinal epithelial cell differentiation by enhancing the transactivation capacity of CDX2 (Houde et al., 2001), an intestine-specific homeobox gene product well known for its broad effect on enterocyte differentiation (Traber and Silberg, 1996). *In vitro* experiments have shown that the establishment of cell-cell contacts in intestinal cell cultures could be a critical step in initiating p38 MAPK action and induction of the differentiation process (Aliaga et al., 1999, Houde et al., 2001). $1\alpha,25(OH)_2D_3$-induced gene expression increases as Caco-2 cells differentiate. The blunted transcriptional responsiveness of proliferating Caco-2 cells is due to higher Alien corepressor levels and to impaired hormone-induced recruitment of VDR to chromatin and vitamin D response elements (VDRE) (Cui et al., 2009). VDR knockout mice exhibit enhanced colonic proliferation (Kallay et al., 2001), indicating that the fundamental actions of VDR is to promote cell differentiation and apoptosis and that $1\alpha,25(OH)_2D_3$ is an important regulator of normal cell proliferation (Egan et al., 2009) playing an important role in reducing the risk of age-related epithelial cell cancers, such as those of the colon and breast.

The proto-oncogene c-fos, as an immediate early gene, is known to provide a link between short-term signals elicited at the membrane and long-term cellular responses. The best-characterized aspect of c-fos is its regulation of cell proliferation and transformation (Angel and Karin, 1991). There is increasing evidence that altered gene activity occurs during the normal ageing of all mammalian cells. Some genes show increased expression with age while others diminish their expression (Barron et al., 1999; Linskens et al., 1995). In enterocytes from old rats, $1\alpha,25(OH)_2D_3$-dependent increase of c-fos protein level diminished, whereas c-fos mRNA expression is not different from young animals (Gonzalez Pardo et al., 2006). However, in aged gastric mucosa a rise in c-jun and c-fos levels has been reported (Xiao and Majumdar, 2000). Furthermore, it has been proposed that proteins that control cell cycle progression and that are inactivated in tumor cells, such as the restriction point proteins Rb and p53 and various cyclin-dependent kinase inhibitors, play an important role in the establishment of senescence (Campisi, 2005).

8. Regulation of intestinal cells PI3K pathway by $1\alpha,25(OH)_2D_3$

The double-enzymatic activity of PI3K (lipid kinase and protein kinase) as well as the ability of this enzyme to activate a number of signal proteins including some oncoproteins determines its fundamental significance in regulation of cell functions such as growth and survival, ageing, and malignant transformation (Fruman et al., 1998). As lipid kinase, PI3K catalyzes the addition of a phosphate moiety specifically to the 3′-OH position of the inositol ring of phosphatidylinositols (Fruman et al., 1998). The resulting 3′-phosphorylated phosphatidylinositols serve as secondary messengers to activate many downstream signaling targets, initiating the physiological effects of PI3K, they bind to the PH domain of both protein kinase B (Akt) and phosphoinositide dependent kinase-1 (PDK-1) and induce their translocation to the plasma membrane where PDK-1 phosphorylates and activates Akt kinase (Chan et al., 1999).

The PI3K/Akt pathway transduces mitogenic signals from growth factor receptors to the cell cycle machinery and plays a critical role in regulation of intestinal epithelial proliferation (Sheng et al., 2003). A study in rats showed that $1\alpha,25(OH)_2D_3$ rapidly induced vascular smooth muscle migration *via* the PI3K pathway independently of gene transcription (Rebsamen et al., 2002). Furthermore, the PI3K pathway was also involved in the regulation of the charge-selective property as well as the enhanced paracellular calcium transport in the duodenum of rats (Jantarajit et al., 2007).

PI3K is necessary for the functional and morphological differentiation of intestinal epithelial cells. The enzyme is an important modulator of extracellular signals, including those elicited by E-cadherin-mediated cell-cell adhesion, which plays an important role in maintenance of the structural and functional integrity of epithelia. PI3K promotes assembly of adherens junctions, which, in turn, control p38 MAPK activation and enterocyte differentiation (Laprise et al., 2002). Moreover, it has been shown that, in a PI3K/Akt-dependent pathway, E-cadherin leads to MEK/ERK inhibition, and this mechanism may account for the role of E-cadherin in proliferation/differentiation transition along the crypt-villus axis of the human intestinal epithelium (Laprise et al., 2004).

The PI3K/Akt pathway is one of the most critical signaling pathways involved in the regulation of cell survival. In osteoblasts, the anti-apoptotic effects of $1\alpha,25(OH)_2D_3$ occur through nongenomic activation of a VDR/PI3K/Akt survival pathway that includes

phosphorylation of multiple p-Akt substrates and reduction of caspase activities (Zhang and Zanello, 2008; Vertino et al., 2005).

PI3K activity changes during cell ageing have been observed in other cell types. A decrease in PI3K activity was reported to cause an increase in ageing rate in normal fibroblasts (Tresini et al., 1998) but the mechanism of PI3K-dependent control of cell ageing and the role of individual effectors of PI3K are still unknown. PI3K activity after insulin stimulation was dramatically reduced in liver and muscle of aged rats (Carvalho et al., 1996) and insulin signaling defects due to a reduced activation of PI3K and Akt with no changes in the protein levels of the p85 subunit of PI3K, have been reported in fat cells from old rats (Carvalho et al., 2000). Moreover, the p85α subunit of PI3K in cardiac muscle declined in old mouse whereas the skeletal muscle content of this protein was unaffected by ageing (Martineau et al., 1999).

In rats, aging is associated with activation of PI3K/Akt signaling, as evidenced by the higher levels of phosphorylated forms of p85, the regulatory subunit of PI3K and of Akt in the proximal and distal colonic mucosa, of aged than in young animals. The constitutive activation of the PI3K/Akt-signaling pathway is partly responsible for the age-related increase in colonic mucosal cell survival (Majumdar and Du, 2006).

Induction of PI3K, which leads to subsequent activation of Akt, promotes cell survival directly by means of its ability to phosphorylate and inactivate several proapoptotic targets, including Bad and the forkhead transcription factors (Datta et al., 1999; Downward, 2004). Akt also promotes cell survival through its indirect effect on NF-κB and p53 (Datta et al., 1999; Downward, 2004).

It is becoming increasingly clear that the PI3K pathway is activated in response to the activation of a number of growth factor receptors, including EGFR (King et al., 1997; Moghal and Sternberg, 1999). Because aging has been shown to be associated with increased expression and activation of EGFR in the gastric and colonic mucosa (Xiao and Majumdar, 2001; Schmelz et al., 2004), age-related increases in PI3K/Akt signaling could partly be the consequence of constitutive activation of EGFR in the colonic mucosa.

9. $1\alpha,25(OH)_2D_3$ modulation of PLD and PLA_2 activity

Hydrolysis of phosphatidylcholine (PC) by phospholipase D (PLD) leads to the generation of the versatile lipid second messenger, phosphatidic acid (PA) and choline. PA is involved in fundamental cellular processes, including membrane trafficking, actin cytoskeleton remodeling, cell proliferation and cell survival. In mammals two types of PLDs (PLD1 and PLD2) are well-known. They share about 50% sequence identity and differ in their basal activity and regulatory interactions (Pettitt et al., 2001). PLD activity can be dramatically stimulated by a large number of cell surface receptors and is elaborately regulated by intracellular factors, including PKC isoforms, small GTPases of the ARF, Rho and Ras families and, particularly, by the phosphoinositide, phosphatidylinositol 4,5-bisphosphate (PIP2) (Mansfeld and Ulbrich-Hofmann, 2009). Most cellular responses following PLD activation are probably mediated by the immediate reaction product PA. PA is a multifunctional lipid that can be further metabolized to the bioactive lipids, lysophosphatidic acid (LPA) and diacylglycerol (DAG), can by itself alter membrane curvature, and can serve as a protein attachment site and affect both cellular localization and activity of various proteins, including Raf-1 kinase, protein phosphatase 1, sphingosine kinase 1, and mTOR (mammalian target of rapamycin), a key regulator of cell growth and proliferation (Jenkins and Frohman, 2005; Cockcroft, 2001). Phosphatidylinositides which are generally known as potent specific activators of many membrane binding proteins (Lemmon, 2008) can influence PLD activity directly.

$1\alpha,25(OH)_2D_3$ have been shown to stimulate PC cleavage by PLD in rat skeletal muscle through a mechanism that is dependent on extracellular Ca^{2+}, partially dependent on PKC activation and involves a G protein (Facchinetti et al., 1998b), and in keratinocytes, the hormone increases the expression and activity of PLD-1 (Zheng et al., 2003). The hormone also activates PLD in the colonic Caco-2 cells, generating phosphatidic acid and contributing to the sustained rise in DAG. In these cells, PLD stimulation occurred by both PKC-dependent and -independent mechanisms. Inhibitors of G-proteins, c-Src, and PKC blunted the seco-steroid-mediated activation of PLD (Sitrin et al., 1999). Cells stably transfected with sense PKCα showed increased $1\alpha,25(OH)_2D_3$-stimulated PLD activation, whereas transfectants with antisense PKCα had an attenuated response. In addition, $1\alpha,25(OH)_2D_3$ also regulates PLD in this colonic cell line by activating the monomeric G-protein Rho A by a mechanism independent of the G-protein/c-Src/PKC pathway (Sitrin et al., 1999). The exact identity of this G protein is, however, currently unknown. Moreover, it is not clear whether the α- or βγ subunits of this protein(s) are involved in the activation of c-Src by $1\alpha,25(OH)_2D_3$. Also, the possibility that the hormone response to PLD activation is due to arachidonic acid metabolites with their potential contribution to cellular regulation in these cells remains to be investigated.

Because several human isoforms of PLD have been described (Mansfeld and Ulbrich-Hofmann, 2009), it will be of interest to identify the specific isoforms activated by $1\alpha,25(OH)_2D_3$ in intestinal cells. The differential regulation of PLD observed in Caco-2 cells may reflect PLD isoform-specific activation by the steroid hormone. The products of PLD have been implicated in the regulation of a number of important physiological processes, including proliferation and differentiation (Cook and Wakelam, 1992; Jenkins and Frohman, 2005; Cockcroft, 2001). Moreover, $1\alpha,25(OH)_2D3$ have been shown to stimulate PLD activity by increasing the expression of a specific PLD isoform (PLD-1), and that this expression correlates with late keratinocyte differentiation (Griner et al., 1999). Therefore it is possible that activation of PLD by the hormone may be part of the mechanism by which the hormone regulates intestinal cell proliferation and differentiation.

In senescence, PLD activity is greatly diminished (Venable et al., 1994). It has been suggested that the defect in PLD/PKC in cellular senescence is a result of elevated cellular ceramide levels which inhibit PLD activation. Ceramide acts to inhibit the activation of PLD by possibly three mechanisms, inhibiting activation by Rho, translocation to the membrane and gene expression. Addition of ceramide to young cells not only inhibits PLD but also recapitulates all the standard measures of cellular senescence (Venable and Obeid, 1999).

There is no information about the effects of ageing on PLD activation in intestinal cells, further understanding of the mechanism of PLD regulation by $1\alpha,25(OH)_2D_3$ and of the action of ceramide may allow important insight into the molecular mechanisms involved in cellular senescence.

Phospholipase A_2 (PLA_2) comprises a set of extracellular and intracellular enzymes that catalyze the hydrolysis of the sn-2 fatty acyl bond of phospholipids to yield free fatty acids and lysophospholipids (Kudo and Murakami, 2002). PLA_2 enzymes play an important role in regulatory processes in as much as they interact with the cell membrane by altering both its chemical composition and consequently its physical state, thereby controlling its function. PLA_2s are subdivided into three categories: secreted PLA_2 ($sPLA_2$s; implicated in a number of biological processes, such as modification of eicosanoid generation, inflammation, and host defense), cytosolic PLA_2 ($cPLA_2$s) and Ca^{2+} independent PLA_2 ($iPLA_2$s) and these three categories are further subdivided into 13 different groups (Canaan et al., 2004; Six and Dennis, 2000). Group IIA PLA_2 is one of the key enzymes in the process of inflammation that regulates the

synthesis of arachidonic acid and lysophospholipids (Nevalainen, 1993). High concentrations of group IIA PLA_2 are expressed in Paneth cells of the small intestine (Nevalainen et al., 1995), and it has been suggested that the main physiological role of PLA_2-IIA, could be the defence of the intestine against bacterial invasions (Karray et al., 2011). In a rat small intestinal cell line (IEC-18) and in a mouse colon cancer cell line (WB-2054-M4), inhibitors of $cPLA_2$ and the $sPLA_2$ promoted apoptosis, and decreased mitogenesis, suggesting that PLA_2 and its metabolites may be involved in intestinal epithelial cell replication and colorectal carcinogenesis (Longo et al., 1999). With the growing list of knockout and transgenic mouse strains for individual PLA_2s, much progress has been made in delineating the physiological functions of each mammalian PLA_2, even though knockout strains for several PLA_2s are still unavailable (Murakami et al., 2011) and there is not information on the physiological functions of individual PLA_2s in intestinal cells.

It is now becoming obvious that $cPLA_2\alpha$ is a central regulator of AA metabolism. However, $sPLA_2$s and $iPLA_2$s can also participate in the generation of lipid mediators according to pathophysiologic context. The $iPLA_2$ family is an integral regulator of cellular membrane homeostasis and energy metabolism, many of the enzymes in the $cPLA_2$ and $iPLA_2$ families also possess lysophospholipase activities. The $iPLA_2$ shows a unique tissue distribution, yet in general terms this enzyme is ubiquitously expressed in a wide variety of cells and tissues, including the rat small intestine (Fukushima and Serrero, 1994).

Activation of PLA_2 by $1\alpha,25(OH)_2D_3$ was demonstrated in different cell types, such as skeletal muscle cells (Boland et al., 1995), chondrocytes (Schwartz and Boyan, 1988) and hepatocytes (Baran and Kelly, 1988), being dihydropyridine-sensitive extracellular Ca^{2+} influx required for the modulatory effects of $1\alpha,25(OH)_2D_3$ on PLA_2 (Boland and Boland, 1993). In rat duodenal cells, $1\alpha,25(OH)_2D_3$ activates a Ca^{2+}-dependent cytosolic PLA_2 and attendant arachidonic acid release and this activation requires prior stimulation of intracellular ERK1/2 (Gentili et al., 2004a).

Phospholipases, particularly PLA_2, are key factors in the membrane hypothesis of ageing (Zs-Nagy et al., 1988). In this regard, PLA_2 plays a major role in phospholipids membrane destabilization, the synthesis of inflammatory mediators, and the generation of and/or response to free radicals (Rosenthal and Franson, 1989). Free fatty acids released by PLA_2 are converted to inflammatory mediators such as leukotrienes and prostaglandins, and in this process, free radicals are formed which in turn can damage more membrane phospholipids. In addition the lyso derivatives released by PLA_2 action have detergent effects which can also damage cell membranes (Phillips et al., 1965).

Basal levels of $cPLA_2$ serine-phosphorylation are higher in enterocytes from old rats (Gentili et al., 2004b), but Information on hormone PLA_2s activation in intestinal cells with ageing is lacking. Clearly, more work is required to dissect the effects of $1\alpha,25(OH)_2D_3$ on the mechanisms of the regulatory actions of individual PLA_2s, as well as the roles of the pathways in specific cell responses during ageing in intestinal cells.

10. Concluding remarks

The intestine is one of the main target tissues for $1\alpha,25(OH)_2D_3$ where its actions are of regulatory and developmental importance. It is clear that in enterocytes and colonocytes, the hormone has two important roles: stimulation of active intestinal calcium absorption through modulation of second messengers and, regulation of mitogenic cascades leading to intestinal cell proliferation and differentiation. The ability of the hormone to stimulate Ca^{2+} influx markedly decreases with ageing and thus provoking regulation of intestinal calcium absorption and calcium homeostasis imbalance in the elderly. In addition, $1\alpha,25(OH)_2D_3$ as regulator of normal cell proliferation plays an important role in reducing the risk of age-related epithelial cell cancers such as those of the colon. The controversies in this field are whether the rapid, non-genomic effects of the hormone are mediated by the nuclear VDR. There has been much written on whether the non-genomic effects of $1\alpha,25(OH)_2D_3$ are mediated by membrane-bound hormone receptors (VDRm) and whether or not these receptors are identical to the nuclear VDR. Thus, depending on the cell type, some of the rapid actions of $1\alpha,25(OH)_2D_3$ may be dependent upon nuclear VDR, whereas others are not. Elucidation of the effects and mechanisms of $1\alpha,25(OH)_2D_3$ in intestinal cell can provide clues to essential pathways in cell control of this important hormone with ageing. The information presented here, may also provide useful insights into the mode of action of this hormone in target cells, where it appears to play an important role in both the regulation of cellular calcium and development. The facts summarized in this review should facilitate future analysis of $1\alpha,25(OH)_2D_3$ action in the physiology and pathophysiology of ageing and will open new approaches to therapeutical support of clinical cases manifesting an imbalance of calcium homeostasis and growth.

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