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Characterization of the Second Messengers Involved in 1α,25-Dihydroxyvitamin D_3 Stimulated Intestinal Calcium Absorption (Transcaltachia)

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1α,25-Dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] has been shown to generate biological responses via both genomic and nongenomic pathways. In the nongenomic process, 1α,25$(OH)_2D_3$ stimulates the rapid transport of Ca^{2+} across the chick intestinal epithelial cell, a process termed transcaltachia. In previous studies, the involvement of Ca^{2+}-channels, protein kinase C, and cAMP-dependent kinase in the 1α,25$(OH)_2D_3$ stimulated transcaltachic response have been implicated. To further characterize the elements involved in mediating the transcaltachic effect, H7, an inhibitor of protein kinase C, U73122, an inhibitor of phospholipase C, and mastoparan, an activator of G-proteins, were employed. Both H7 and U73122 suppressed 1α,-25$(OH)_2D_3$ stimulated intestinal Ca^{2+} transport. Mastoparan was found to mimic the effect of 1α,25$(OH)_2D_3$ to stimulate transcaltachia. Collectively, these results suggest that 1α,25$(OH)_2D_3$ activations of G-proteins, phospholipase C and protein kinases are essential steps in the rapid stimulation of intestinal Ca^{2+} transport.

Key Words: 1α,25$(OH)_2$-vitamin D_3; intestinal Ca^{2+} absorption; transcaltachia; G-proteins.

Introduction

The vitamin D hormone 1α,25-Dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] is believed to exert many of its effects by interaction with a nuclear receptor to regulate gene expression (Minghetti and Norman, 1988). However, the genomic mechanism does not appear to explain all the actions of the secosteroid. Some of the biological responses of 1α,25-$(OH)_2D_3$ appear to be mediated by a nongenomic or rapid reaction pathway. The nongenomic actions of 1α,25$(OH)_2D_3$ occur very rapidly (seconds to minutes), and are not blocked by inhibitors of transcription and translation (Nemere et al., 1984; Nemere and Norman, 1987; Mezzetti et al., 1988).

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Recently, the rapid actions of 1α,25$(OH)_2D_3$ have been correlated with various cellular processes, such as activation of second messengers (Wali et al., 1990; Barsony and Marx, 1991), stimulation of voltage-gated calcium channels (Caffrey and Farach-Carson, 1989), an increase in intracellular Ca^{2+} concentration (Tien et al., 1993), induction of a change in membrane potentials (Edelman et al., 1986) and modification of enzyme activities (Dick et al., 1990). To date, rapid actions of 1α,25$(OH)_2D_3$ have been observed in many types of tissues, including intestine (Nemere et al., 1987), osteoblasts (Civitelli et al., 1990), hepatocytes (Baran et al., 1988), kidney (Kurnic et al., 1987), parathyroid cells (Bourdeau et al., 1990), leukemic HL-60 cells (Desai et al., 1986), and NB4 cells (Bhatia et al., 1995).

One of the well-defined systems for studying the rapid actions of 1α,25$(OH)_2D_3$ is the isolated vascularly perfused chick duodenum (Nemere et al., 1984). In this system, 1α,25$(OH)_2D_3$ stimulates the rapid transport of Ca^{2+} from the duodenal lumen to the vascular perfusate of the perfused duodenum of vitamin D-replete chicks. This process, transcaltachia, occurs only when 1α,25$(OH)_2D_3$ is applied to the basal lateral membrane, but not the brush-border membrane of the intestinal epithelial cell. This suggests the presence of a 1α,25$(OH)_2D_3$ receptor on the basal lateral membrane surface which may be responsible for the initiation of transcaltachia; this membrane receptor has recently been identified and purified in our laboratory (Nemere et al., 1994). Studies on the mechanism of 1α,25$(OH)_2D_3$-stimulated transcaltachia also indicated that the activation of basal lateral membrane voltage-dependent Ca^{2+} channels is involved in the 1α,25$(OH)_2D_3$ stimulated transcaltachic response (de Boland and Norman, 1990a). Additional evidence from the study of the involvement of the second messengers also suggests that the rapid action of 1α,-25$(OH)_2D_3$ is mediated by the action of protein kinase C (PKC) and cAMP-dependent protein kinase, since the protein kinase C activator, phorbol ester (TPA), and the adenylate cyclase activator, forskolin, were found to stimulate $^{45}Ca^{2+}$ transport in perfused chick intestine (de Boland and Norman, 1990a).

To further elucidate the mechanism of 1α,25(OH)$_2$D$_3$-induced Ca^{2+} transport in the perfused chick duodenum, it is necessary to characterize more of the signal transduction elements in mediating the rapid actions of the secosteroid. In this report we describe the effects on transcaltachia of the PKC inhibitor, H7, the phospholipase inhibitor, U73122, and the G-protein agonist, mastoparan.

Materials and Methods

Chemicals

$^{45}CaCl_2$ (1 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Mastoparan, *vespula lewisii* and H7, 1-(5-isoquinolinesulfony)-2-methylpiperazine.2HCl, were from Sigma Chemical Co. (St. Louis, MO). U73122,1-(6[(17B-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]-hexyl)-1H-pyrrole-2,5-Dione was from BIOMOL research laboratories, Inc. (Plymouth Meeting, PA). 1α,25(OH)$_2$D$_3$ was the kind gift of Milan Uskokovic (Hoffmann-La Roche, Nutley, NJ).

Animals

White Leghorn cockerels (Hyline International, Lakeview, CA) were obtained on the day of hatch and maintained on a vitamin D-supplemented diet (1.2% calcium and 0.4% phosphorus; O. H. Kruse Grain and Milling, Ontario, CA) for 5–6 wk to prepare normal vitamin D$_3$-replete chicks. Protocols of all experiments employing animals were approved by the University of California-Riverside Chancellor's Committee on Laboratory Animal Care.

Intestinal $^{45}Ca^{2+}$ Transport Measurements

Measurements of $^{45}Ca^{2+}$ transport were carried out in perfused chick duodena as previously described (Nemere et al., 1984; Norman et al., 1993). Normal vitamin D-replete chicks weighing approx 500 g were anesthetized with Chloropent (0.3 mL/100 g) through iv injection, and the duodenal loop was surgically exposed. Three pairs of blood vessels branching off from the celiac artery were ligated before cannulation of the celiac artery itself. The duodenal loop was then excised, and after cannulation of the celiac vein, placed between layers of saline-moistened cheesecloth at 24°C.

The arterial perfusion was initiated with modified Gey's balanced salt solution (GBSS) containing 27 m*M* $NaHCO_3$, 5.6 m*M* D-glucose and oxygenated with 95% O_2/5% CO_2 at a flow rate of 2 mL/min. An auxiliary pump was used for introduction of vehicle (ethanol) or test substances plus albumin (0.125% wt/vol final concentration) to the vascular perfusate at a rate of 0.25 mL/min. The intestinal lumen was then flushed and filled with GBSS containing $^{45}Ca^{2+}$ (5 μCi/mL). After the lumen was filled with $^{45}Ca^{2+}$, a basal transport rate was established by perfusion with control medium for 20 min. The tissue was then exposed to 1α,25(OH)$_2$D$_3$ or re-exposed to control medium for an additional 40 min. The vascular perfusate was collected at 2 min intervals during both the basal and treatment periods. Duplicate 100-μL aliquots were taken for determination of the $^{45}Ca^{2+}$ level by liquid scintillation spectrometry. Results are expressed as the ratio of the $^{45}Ca^{2+}$ appearing in the 40 min test period divided by the average initial basal transport period.

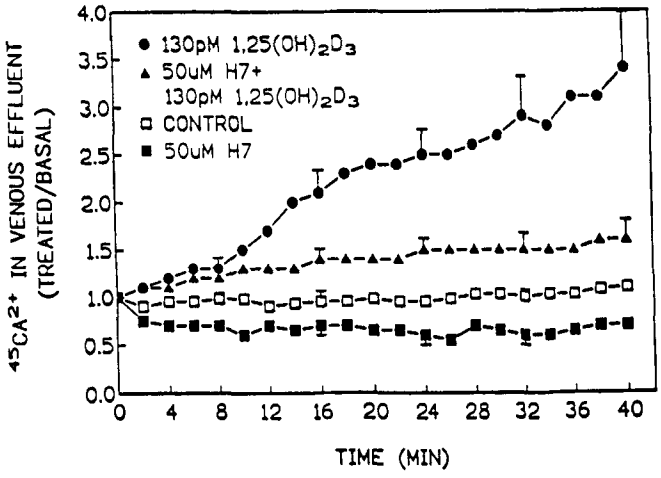

Fig. 1. Suppression of 1a,25(OH)$_2$D$_3$-induced duodenal $^{45}Ca^{2+}$ transport by the protein kinase C inhibitor H7. Each duodenum, filled with $^{45}Ca^{2+}$ (5 μCi/mL) in GBSS, was vascularly perfused (25°C) for the first 20 min with control medium (GBSS containing 0.125% bovine serum albumin [BSA]) and then with 130 p*M* 1α,25(OH)$_2$D$_3$, 50 μ*M* H7 in the presence of 130 p*M*. 1α,25(OH)$_2$D$_3$, 50 μ*M* H7 or control medium. Values are the mean ± SEM for at least three duodena within each experimental group.

Statistics

Statistical analysis was performed using Duncan's multiple comparison test.

Results

The potential involvement of the protein kinases and PI-specific phospholipase C was investigated by utilizing the inhibitors H7 and U73122. H7 is a PKC inhibitor (Thompson et al., 1991; Hermenegildo et al., 1993). Perfusion with 50 μ*M* H7 for 40 min resulted in 75% inhibition of the 1α,25(OH)$_2$D$_3$-enhanced transport of $^{45}Ca^{2+}$ from lumen to the vascular perfusate over the control level (Fig. 1). U73122 has been shown to inhibit agonist-induced phospholipase C activation in human platelets (Smith et al., 1990) and neutrophils (Bleasdale et al., 1990). As shown in Fig. 2, 10 μ*M* U73122 suppressed, the stimulation of $^{45}Ca^{2+}$ transport elicited by a physiological concentration of 1α,25(OH)$_2$D$_3$ after 40 min perfusion. In both cases, perfusion of the isolated duodenum with the inhibitors alone resulted in only a small decrease in the rate of $^{45}Ca^{2+}$ transport over the controls.

G-proteins are central to the transduction of many receptor signals. Many toxins have been reported to modify the function of G-proteins. Mastoparan from wasp venom is a tetradecapeptide that is capable of direct activation of G-

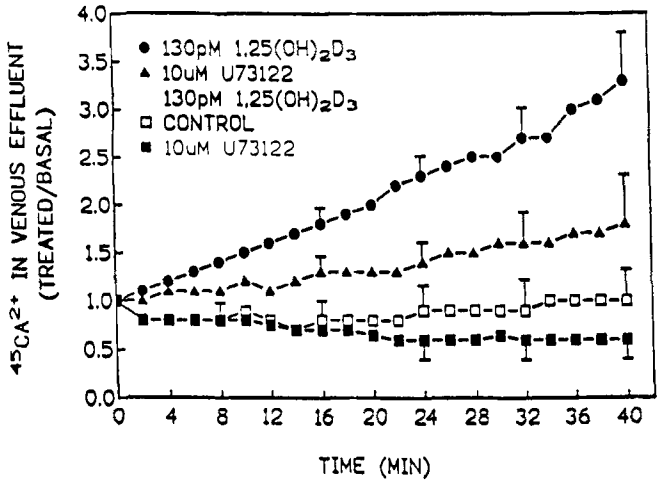

Fig. 2. Suppression of $1\alpha,25(OH)_2D_3$-induced duodenal $^{45}Ca^{2+}$ transport by the phospholipase C inhibitor U73122. Each duodenum, filled with $^{45}Ca^{2+}$ (5 μCi/mL) in GBSS, was vascularly perfused (25°C) for the first 20 min with control medium (GBSS containing 0.125% BSA) and then with 130 p*M* $1a,25(OH)_2D_3$, 10 μ*M* U73122 in the presence of 130 p*M* $1\alpha,25(OH)_2D_3$, 10 μ*M* U73122 or control medium. Values are the mean ±SEM for at least three duodena within each experimental group.

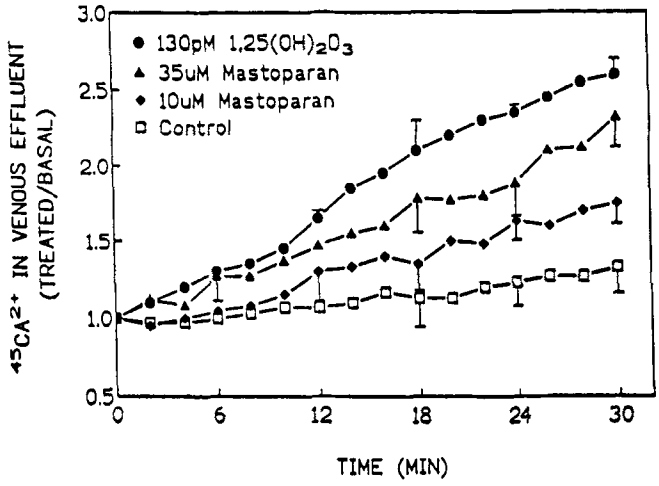

Fig. 3. Effect of mastoparan on the appearance of $^{45}Ca^{2+}$in the venous effluent of perfused duodena from vitamin D-replete chicks. Each duodenum, filled with $^{45}Ca^{2+}$ (5 μCi/mL) in GBSS, was vascularly perfused (25°C) for the first 20 min with control medium (GBSS containing 0.125% BSA) and then with 130 p*M* $1\alpha,25(OH)_2D_3$, 35 μ*M* mastoparan, 10 μ*M* mastoparan or control medium. Values are the mean ±SEM for at least three duodena within each experimental group.

proteins by mimicking the role normally played by agonist-liganded receptors (Gusovsky et al., 1991; Higashijima et al., 1988; Wallace and Carter, 1989). Introduction of mastoparan in the perfused intestinal system, resulted in stimulation of rapid Ca^{2+} transport by 83 and 52% that were obtained after 30 min perfusion with 35 and 10 μ*M* mastoparan, respectively (Fig. 3). Analogous to $1\alpha,25(OH)_2D_3$, the initial stimulatory effect of mastoparan was observed within 4–12 min.

Discussion

A first level analysis of the effects of second messengers on the $1\alpha,25(OH)_2D_3$-stimulated transcaltachia was conducted in our laboratory by de Boland and Norman (1990a,b). The activation of cAMP-dependent protein kinase A (PKA) and PKC was shown to stimulate $^{45}Ca^{2+}$ transport from the lumen to the vascular effluent to the same extent that application of physiological levels (130 p*M*) of $1\alpha,25(OH)_2D_3$ achieved. Also forskolin and TPA-enhanced duodenal $^{45}Ca^{2+}$ transport was abolished by the Ca^{2+}-channel antagonists nifedipine and verapamil (de Boland and Norman, 1990a). It was proposed that the PKA- and PKC-dependent activation of Ca^{2+}-channels may be the primary mechanisms underlying the rapid $1\alpha,25(OH)_2D_3$-mediated increase of duodenal Ca^{2+} transport.

In this paper a second level evaluation of the signal transduction pathway associated with transcaltachia is presented. The suggestion that both PKA- and PKC-dependent events are involved in transcaltachia is supported by the effect that H7, an inhibitor of PKC activity, abolished the rise in $^{45}Ca^{2+}$ transport induced by $1\alpha,25(OH)_2D_3$ in the perfused duodenum. The mechanism of inhibition by H7 involves the competition for the adenosine triphosphate (ATP) site of the kinases. Thus, H7 is not a specific inhibitor of PKC, although it may have this effect under some experimental conditions (Thompson et al., 1991; Raya et al., 1992). H7 was reported to inhibit three different protein kinases with equal activity, having K_i values of 3–6 n*M* for cyclic nucleotide (cAMP, cGMP)-dependent kinases as well as for PKC. Therefore, the inhibitory effect of H7 on transcaltachia is likely a result of its actions on one or other of these protein kinases.

The suppression of $1\alpha,25(OH)_2D_3$-induced stimulation of duodenal Ca^{2+} transport (transcaltachia) by the inhibitor of phospholipase C (U73122) implies that the generation of IP_3 and diacyl glycerol (DAG) are also important events in mediating the secosteroid mode of action. Both IP_3 and DAG may stimulate the Ca^{2+}-channel activity associated with the process of transcaltachia. IP_3 may stimulate Ca^{2+}-channel directly, similar to the report by De Waard et al. (1992) who reported that the opening of Ca^{2+}-channels is dependent on the direct binding of IP_3 to the Ca^{2+} channel. Alternatively, while DAG can act via PKC-mediated phosphorylation events that are known to be linked to Ca^{2+} channel activity.

In the transmembrane signaling pathway, G-proteins often may serve as signal transducers, linking extracellularly oriented receptors to membrane-bound effectors (Birnbaumer et al., 1990). Many types of G-proteins have been identified, including G_s, G_i, and G_o. G-proteins can be specifically stimulated or inhibited by certain toxins, which has proven extremely useful in examining G-protein function. One such toxin is mastoparan, which is obtained from wasp venom. It is a cell-permeable, 14-amino acid peptide. Higashijima et al. (1988) have shown that mastoparan under

in vitro conditions activates the G-proteins (G_o and G_i) by accelerating the dissociation of G-protein-bound guanosine diphosphate (GDP). In addition, they noted that mastoparan, on binding to phospholipid membranes, formed an amphiphilic α-helix that bore similarities to the two basic loops of G-protein-coupled receptors (Dohlman et al., 1987). The structure function similarities of mastoparan to hormone receptors led Higashijima and coworkers (1988) to suggest that mastoparan stimulates G-proteins by mimicking agonist liganded receptors.

Mastoparan has been employed in the present work to study the rapid stimulatory effect of $1\alpha,25(OH)_2D_3$ on intestinal Ca^{2+} transport. Mastoparan reproduced the rapid stimulatory effect of $1\alpha,25(OH)_2D_3$ on intestinal $^{45}Ca^{2+}$ transport when the toxin was presented to basal lateral membrane of the vitamin D-replete chick duodena. The stimulatory effect is rapid and concentration dependent (*see* Fig. 3). The action of mastoparan on intestinal Ca^{2+} transport could be mediated by activation of G_o and G_i, since there is direct evidence for involvement of G_o and G_i in the stimulation of the formation of IP_3 (Kikuchi et al., 1986). Consistent with this result, mastoparan has been found to stimulate phosphoinositide breakdown in HL-60 cells and cell-free preparations (Gusovsky et al., 1991) and activate PI-specific phospholipase C (PLC) purified from rabbit brain membranes (Wallace and Carter, 1989).

The vesicular transport model of intestinal Ca^{2+} absorption has been proposed previously (Nemere et al., 1986). In this model intestinal Ca^{2+} transport is effected via a vesicular pathway that includes intracellular internalization of Ca^{2+} in endocytic vesicles, fusion of the vesicles with lysosomes, and movement of the lysosomes along the microtubule to the basal lateral membrane where exocytosis of the contents completes the transport process. $1\alpha,25(OH)_2D_3$ may initiate this process by binding to its receptor on the basal lateral membrane surface to stimulate the G-protein coupled activation of second messenger systems, such as cAMP-dependent protein kinase, PKC and IP_3. The stimulation of Ca^{2+}-channels by these second messengers directly or indirectly may provide the route for Ca^{2+} entry into the cell; in turn the transient increase in intracellular Ca^{2+} may initiate the vesicular transport and/or exocytosis of Ca^{2+}.

Acknowledgments

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