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Short communication

Investigation of the effects of vitamin D and calcium on intestinal motility: *In vitro* tests and implications for clinical treatment

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The presence of vitamin D receptors in small intestine muscle cells may lead one to think that vitamin D may act locally, influencing intracellular calcium concentration and contributing to the contraction-relaxation regulation of the intestinal smooth muscle cells. This study investigates the potential effects of vitamin D and calcium on intestinal motility using an *in vitro* test.
Different calcium concentrations added to the tissue not pre-treated with 1,25-dihydroxycholecalciferol [1α,25$(OH)_2D_3$] produced no response at low doses (1.25×10^{-3} and 2.0×10^{-3} mol L^{-1}) and only a very weak response at higher concentration (3.0×10^{-3} mol L^{-1}). The addition of 1α,25$(OH)_2D_3$ (1.44×10^{-10} mol L^{-1}) had no effect on isolated ileum motility. When calcium (3.0×10^{-3} mol L^{-1}) was added after at least 3 hours, it evoked evident and persistent contractions for 60–90 minutes. The contractions were at about 40 % of the peak produced by acetylcholine. Thus, simultaneous intake of vitamin D and calcium might be a useful co-adjuvant in intestinal atony therapy aimed to stimulate normal gut motility in humans. These findings imply that supplemental vitamin D may be important in all cases where calcium has to be prescribed.

Keywords: vitamin D, calcium, intestinal motility, *in vitro* tests

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Vitamin D is a group of fat-soluble compounds that include ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3). The most abundant form of vitamin D is its hydroxylation product in liver, 25-hydroxy-vitamin D_3 [25$(OH)D_3$], whose serum concentration is used to indicate the vitamin D status of a human individual. The most active metabolite is obtained from further hydroxylation of 25$(OH)D_3$ in the kidney to 1α,25-dihydroxy-vitamin D_3 [1α,25$(OH)_2D_3$] (1, 2).

Health consequences of vitamin D deficiency are important; recent studies confirm that vitamin D deficiency is widespread in children and elderly people (3, 4). Vitamin D

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deficiency has also been implicated in the pathogenesis of certain cancers, including breast, ovarian, prostate, and colon, and of other diseases, for example multiple sclerosis, diabetes, *Lupus erythematosus*, cardiovascular diseases, and hypertension (5–7).

Two major signal transduction pathways activated by $1\alpha,25(OH)_2D_3$ in target cells have been identified: the so-called genomic pathway, where the nuclear receptors play a major role, and the non-genomic signal transduction pathway. In order to ensure full biological activity of $1\alpha,25(OH)_2D_3$ both pathways need to be activated. The non-genomic pathway is faster than that induced following changes in gene expression. It induces rapid changes in intracellular calcium concentrations, alterations in membrane phospholipid metabolism and it activates several signalling transduction pathways (8).

Biological effects induced by the active form of $1\alpha,25(OH)_2D_3$ are mediated by the vitamin D receptor (VDR), also known as the NR1I1 receptor. This receptor belongs to the superfamily of the nuclear receptor (NR), which includes receptors for steroid hormones, retinoids and thyroid hormones (9).

The presence of the receptor outside the classical target tissues, even in the small intestine muscle cells, may imply that vitamin D acts locally, influencing the intracellular calcium concentration and participating in contraction-relaxation regulation of the intestinal smooth muscle cell.

The aim of this study is to investigate the potential effects of vitamin D and calcium on intestinal motility using an *in vitro* test.

EXPERIMENTAL

Chemicals and reagents

The following reagents were used: NaCl, USP grade (Research Organic, Inc., USA), KCl (Carlo Erba Reagenti s.r.l., Italy), $CaCl_2 \times 2H_2O$ (Merck, Germany), $NaHCO_3$, ACS reagent (Research Organic), $MgCl_2 \times 6H_2O$ (Merck), $NaH_2PO_4 \times H_2O$ (Merck), D(+) glucose (Merck), acetylcholine chloride (TCI Europe, Belgium) and $1\alpha,25(OH)_2D_3$, 99 % TLC (Sigma-Aldrich Inc., USA).

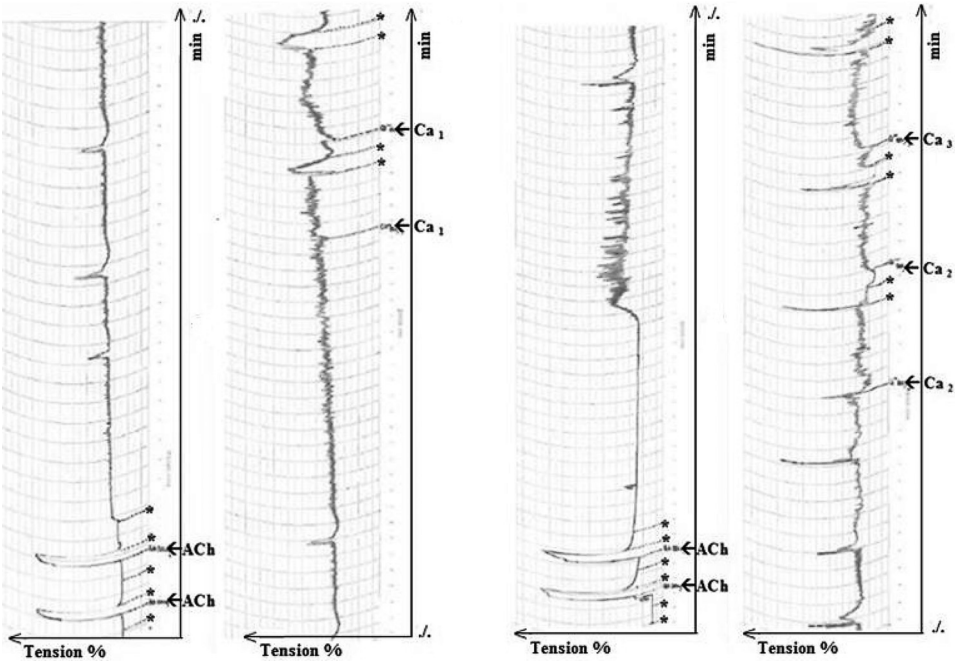
Tyrode's solution. – It was composed of NaCl (137 mmol L^{-1}), KCl (2.7 mmol L^{-1}), $CaCl_2 \times 2H_2O$ (1.8 mmol L^{-1}), $NaHCO_3$ (11.9 mmol L^{-1}), $MgCl_2 \times 6H_2O$ (1.05 mmol L^{-1}), NaH_2PO_4 (0.4 mmol L^{-1}), D(+) glucose (5.55 mmol L^{-1}).

Instruments

The following instruments were used: Isotonic Transducer no 7006 calibrated to tension of 1 g (Ugo Basile S.p.A, Italy), recording device 'UNIRECORD' for transducer: model 7050 Serial. n. 86503 (Ugo Basile), isolated organ bath, model 4050. Serial No. 00387 – 2 Chamber (Ugo Basile S.p.A.), O_2 (95 %) and CO_2 (5 %) tanks (Linde – Arluno, Italy).

Animals, tissue preparation and in vitro *tools*

The experimental procedure has been described previously (10). Six male guinea-pigs weighing 300–400 g (aged 2–3 months, provided by Morini, Italy) were housed in



*washing; c(ACh) = 1.1×10^{-7} mol L^{-1}.

Fig. 1. Response to different concentrations of calcium ($Ca_1 = 1.25 \times 10^{-3}$ mol L^{-1}; $Ca_2 = 2.0 \times 10^{-3}$ mol L^{-1}; $Ca_3 = 3.0 \times 10^{-3}$ mol L^{-1}) of the isolated guinea pig ileum strip without pre-treatment with $1\alpha,25(OH)_2D_3$.

groups with food and water available *ad libitum*, in a room with controlled temperature (22 ± 1 °C) and under artificial 12-h light/12-h dark cycle for at least 4 days before use. The ileum was excised and kept in Tyrode's solution. Each segment, 2–3 cm long, was cleaned and set up under 1 g tension in a 10-mL organ bath containing Tyrode's solution, maintained at 37 °C and gassed with 95 % O_2 and 5 % CO_2. Changes in tension were recorded under isotonic conditions by a transducer connected to a recorder, which was calibrated before each experiment. The preparations were allowed to equilibrate for 30–40 min and were then stimulated two or three times with ACh (10^{-7} mol L^{-1}) to ascertain their responses. Contractile responses recorded by the transducer were expressed as percentage of the ACh maximum response. The same tissue preparations were generally used for several consecutive tests. After each test, the preparations were allowed to rest for 25 min and were washed three times between tests with Tyrode's solution. Each experimental test was performed on tissue preparations coming from at least four animals. A series of *in vitro* tests was designed to investigate the potential effects of $1\alpha,25(OH)_2D_3$ and calcium ($CaCl_2 \times 2H_2O$) on intestinal motility. Acetylcholine chloride served as a reference substance.

The experimental procedure was approved by the University Ethics Committee "La Sapienza" concerning the care and use of mammals in experimental practice.

RESULTS AND DISCUSSION

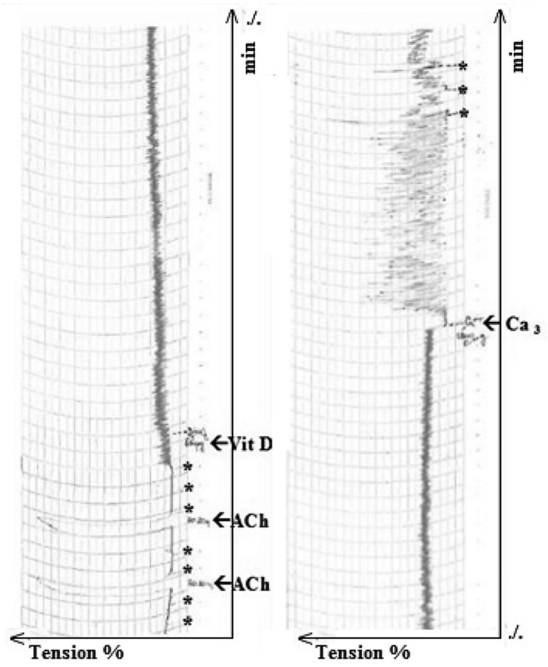
Effect of calcium concentration on isolated guinea pig ileum not pre-treated with 1α,25(OH)$_2$D$_3$

Different concentrations of calcium added to the tissues not pre-treated with 1a,25(OH)$_2$D$_3$ gave no response at low doses (Ca_1: 1.25×10^{-3} mol L^{-1} and Ca_2: 2.0×10^{-3} mol L^{-1}) and only a very weak response at higher concentration (Ca_3: 3.0×10^{-3} mol L^{-1}) (Fig. 1). The lack of response to calcium could be due to the inability of this ion to enter the cell, bind the contracting proteins and trigger contraction mechanisms (Fig. 1).

Effect of calcium on the motility of isolated guinea pig ileum pre-treated with 1α,25(OH)$_2$D$_3$

Addition of 1α,25(OH)$_2$D$_3$ (1.44×10^{-10} mol L^{-1}) had no effect on isolated ileum motility. As shown in Fig. 2, the tissue kept its stable basal contraction for hours.

However, after at least 3 hours, when calcium (Ca_3: 3.0×10^{-3} mol L^{-1}) was added, evident contractions were evoked and persisted for 60–90 minutes, until the tissue was



*washing; *c*(ACh) = 1.1×10^{-7} mol L^{-1}.

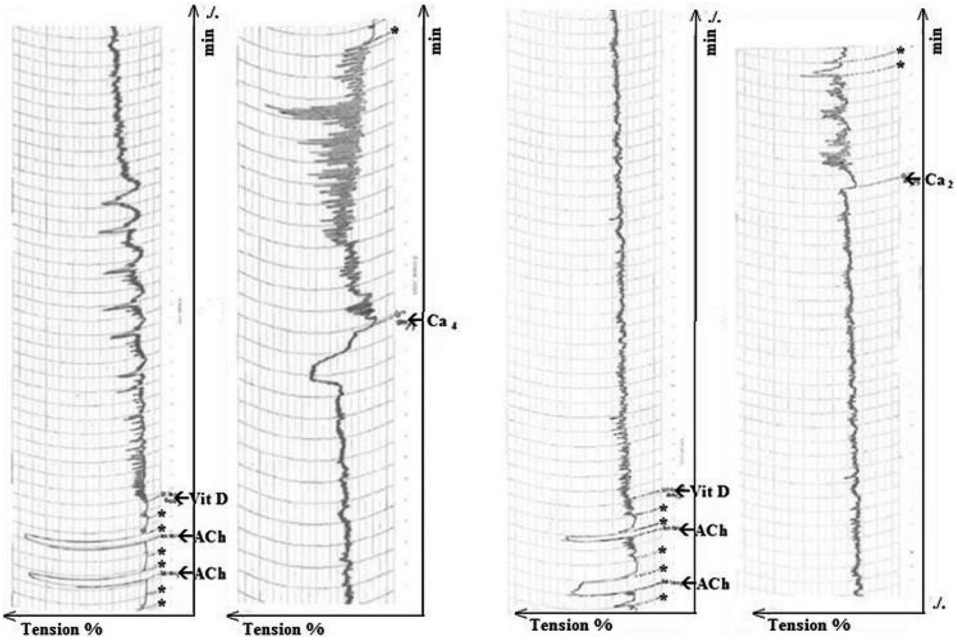
Fig. 2. Response of the isolated guinea pig ileum strip to 1α,25(OH)$_2$D$_3$ (1.44×10^{-10} mol L^{-1}) followed by calcium (Ca_3 = 3.0×10^{-3} mol L^{-1}).

washed with Tyrode's solution to remove the tested substances. The mean percent contraction was 39.1 ± 4.1 (mean ± SD, six experiments) of peak of acetylcholine contraction (Fig. 2).

Experiments were also performed using different calcium concentrations (Ca_2: 2.0×10^{-3} mol L^{-1} and Ca_4: 2.5×10^{-3} mol L^{-1}), in different tissues (six guinea pig ileums for each concentration), maintaining unchanged the time and modality of $1\alpha,25(OH)_2D_3$ pre-addition (1.44×10^{-10} mol L^{-1}, contact time more than 3 hours). Calcium-evoked contractions (mean ± SD, six experiments) were 31.1 ± 3.4 for Ca_2 and 37.0 ± 3.4 % for Ca_4 of the recorded acetylcholine contraction peak (Fig. 3).

The results confirm the role of vitamin D in guinea pig ileum muscle cell contractility. Calcium influx in the smooth muscle cell seems to be favoured in tissues pre-treated with $1\alpha,25(OH)_2D_3$ and it is followed by contractions evoked by its interaction with proteins specifically involved in the contraction (11). Calcium triggers the contraction process; in fact, this happens after it is in the cell and its intracellular concentration is enhanced, activating the muscle contraction.

This late effect seems to be due to genomic response rather than non-genomic activity on intestinal smooth muscle. Reported data confirm that vitamin D promotes calcium transfer into the cell (11). The contracting activity is a function of intracellular calcium



*washing; c(ACh) = 1.1×10^{-7} mol L^{-1}.

Fig. 3. Response to different concentrations of calcium (Ca_4 = 2.5×10^{-3} mol L^{-1}; Ca_2 = 2.0×10^{-3} mol L^{-1}) of the isolated guinea pig ileum strip pre-treated with $1\alpha,25(OH)_2D_3$ (1.44×10^{-10} mol L^{-1}).

concentration. This, in turn, is determined by homeostatic mechanisms operating within the cells, which act on membrane channels, transporters and ATP dependent pumps. We found that $1\alpha,25(OH)_2D_3$ may be included among these mechanisms and the indirect role of vitamin D on isolated guinea pig ileum contractility was confirmed in our research because it promoted the response to calcium.

$1\alpha,25(OH)_2D_3$ stimulates calcium absorption, increasing permeability at the intestinal level and active transport. In addition, $1\alpha,25(OH)_2D_3$ modifies the binding between calmodulin and myosin I, a protein found in the intestinal villous (12). The binding of calmodulin to myosin promotes calcium entrance into the intestinal cells, while transport within the cytoplasm is ensured by calbindin D, whose synthesis is stimulated by calcitriol itself. Calbindin D is a low molecular mass protein; increased synthesis of calbindin D results in extended absorption of calcium. The calbindin calcium binding domain is similar to that of calmodulin (12).

The relationship between the intracellular calcium level and contraction evoked is due to the action of ACh on muscarinic receptors located in intestinal smooth muscle cells (muscarinic acetylcholine receptor M3) (13). The binding of ACh to its receptor triggers a G protein with the production of inositol 1,4,5-triphosphate (IP3). The opening of calcium channels in the endoplasmic reticulum increases calcium levels in the cytoplasm. Calcium binds the troponin belonging to the troponin-tropomyosin system. This complex causes the actin to bind to the myosin filament which produces the contraction. All these mechanisms could be applied to our results to emphasize the role of the vitamin in the contraction process.

CONCLUSIONS

Calcium added to isolated guinea pig ileum not pre-treated with $1\alpha,25(OH)_2D_3$ showed no response, probably because the calcium membrane transporters were scarcely expressed or the calcium channels were inactivated and the ion was not able to enter the cells. However, when $1\alpha,25(OH)_2D_3$ was added, followed after at least 3 hours by calcium, the response to this ion was evident. $1\alpha,25(OH)_2D_3$ can increase the synthesis of the calcium channel in the cell. In addition, other preliminary results, which are not shown, indicated a potential cholinergic modulation. The main strength of our study was to show that vitamin D has an indirect role in the mechanism of contraction. However, further studies are needed to clarify the role of the receptors involved in the mechanism of action of vitamin D. The current results suggest that supplemental vitamin D and calcium may be useful co-adjuvants in intestinal atony therapy, stimulating normal intestinal motility.

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