1,25-Dihydroxyvitamin D3 Rapidly Stimulates the Solvent Drag–Induced Paracellular Calcium Transport in the Duodenum of Female Rats

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Abstract: A calcium-regulating hormone 1α,25-dihydroxyvitamin D3 (1,25-[OH]2D3) has been known to rapidly stimulate the transcellular active calcium transport in the chick duodenum. However, its effects on the solvent drag–induced paracellular calcium transport, which normally contributes ~70% of the total active calcium transport, and the underlying mechanism were unknown. The present study aimed to investigate the rapid non-genomic actions of physiological concentrations of 1,25(OH)2D3, i.e., 1, 10, and 100 nmol/l, on the duodenal calcium absorption in female rats. Quantitative real-time PCR revealed strong expressions of the classical vitamin D receptor (VDR) and the membrane-associated rapid response steroid binding receptors (MARRS) in both small and large intestines. By using the Ussing chamber technique, we found that duodenal epithelia acutely exposed to 10 and 100 nmol/l 1,25(OH)2D3 rapidly increased the solvent drag–induced calcium transport, but not the transcellular calcium transport, in a dose-response manner. On the other hand, 3-day daily injections of 1,25(OH)2D3 enhanced the transcellular active duodenal calcium transport. The 1,25(OH)2D3–stimulated solvent drag–induced transport was abolished by the phosphatidylinositol 3-kinase (PI3K) inhibitors, 200 nmoll wortmannin and 75 μmol/l LY294002, as well as PKC (1 μmol/l GF109203X) and MEK inhibitors (10 μmol/l U0126). Although 100 nmol/l 1,25(OH)2D3 did not alter the transepithelial mannitol flux, indicating no widening of the tight junction, it decreased the transepithelial resistance and increased both sodium and chloride permeability through the paracellular channel. We conclude that 1,25(OH)2D3 uses the nongenomic signaling pathways involving PI3K, PKC, and MEK to rapidly enhance the solvent drag–induced calcium transport, partly by altering the charge-selective property of the duodenal epithelium at least for the pathways involving PI3K and MEK.

Key words: dilution potential, MARRS, nongenomic action, transcellular transport, vitamin D.

1α,25-Dihydroxyvitamin D3 (1,25-[OH]2D3) has been known to stimulate the transcellular calcium transport in the duodenum of rats and humans [1]. This mode of calcium transport consists of apical calcium entry via the transient receptor potential vanilloid family Ca2+ channel 6 (TRPV6), cytoplasmic calcium translocation in a calbindin-D9K–bound form, and basolateral calcium extrusion via the plasma membrane Ca2+-ATPase (PMCA) [1]. A small amount of calcium can be transported transcellularly by vesicular trafficking [2]. The binding of 1,25(OH)2D3 to its classical vitamin D receptors (VDR) in the nuclei of enterocytes activates the genomic actions to enhance the transcription of TRPV6, calbindin-D9K, and PMCA, thereby increasing the transcellular calcium absorption [3, 4].

Besides the putative genomic actions, nongenomic or rapid actions of 1,25(OH)2D3 have been demonstrated in many types of cells, such as intestinal absorptive cells, osteoblasts, and myoblasts [5–7]. These rapid actions require the binding of 1,25(OH)2D3 to its plasma membrane receptors known as the membrane-associated rapid response steroid-binding protein (MARRS) [7–10]. Nemere and co-workers showed that in isolated chick enterocytes, interaction between 1,25(OH)2D3 and MARRS rapidly stimulated the uptake and vesicular transport of phosphate across the cytoplasm [9]. The rapidly enhanced vesicular transport, but not the PMCA-mediated transcellular active transport, has long been implicated in the nongenomic effect of 1,25(OH)2D3 on the duodenal calcium transport in chickens [11]. However, it is possible that 1,25(OH)2D3 also rapidly stimulates other modes of calcium absorption, such as the solvent drag–induced transport, in the rat duodenum.

The components of the metabolically energized active calcium transport that contribute significantly to the duodenal calcium absorption are the aforementioned tran-
scellular active and solvent drag–induced paracellular calcium transport [12, 13]. Although the transcellular active calcium transport is important under the conditions of low dietary calcium intake and high calcium demand, such as pregnancy and lactation [14], we found that in the duodenum and proximal jejunal segment of nonmated female rats fed a normal calcium diet (0.7–1.0% wt/wt Ca), the transcellular active calcium flux was relatively low (less than 20 nmol h⁻¹ cm⁻²) compared to the paracellular flux [13, 15]. Therefore in an absence of calcium gradient across the epithelial sheet, the solvent drag–induced paracellular calcium transport accounted for ~70% of the total active calcium transport [13]. Solvent drag is normally generated by the paracellular sodium gradient created by Na⁺/K⁺-ATPase on the lateral membrane, and it can be abolished by reducing the glucose-dependent transepithelial sodium uptake [13, 16–18]. Since the movement of ions by the solvent drag mechanism occurs via the paracellular channel, it is regulated by the size- and charge-selective properties of the tight junction [19]. Until now, the nongenomic effects of 1,25-(OH)₂D₃ on the solvent drag–induced calcium transport and the epithelial size/charge selectivity had not been examined.

Little was known regarding the nongenomic signaling pathways of 1,25-(OH)₂D₃ in the mammalian intestine. A study in rats showed that 1,25-(OH)₂D₃ rapidly induced vascular smooth muscle migration via the phosphatidylinositol 3-kinase (PI3K) pathway independently of gene transcription [20]. It is interesting that 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 [21]. Besides PI3K, protein kinase C (PKC) and mitogen-activated protein kinase (MAPK)/extracellular signal–regulated kinase (MEK) are also essential for the nongenomic action of 1,25-(OH)₂D₃ [22–24], especially in the chick and rat enterocytes, chondrocytes, and myoblasts [5, 25, 26].

Therefore the objectives of this study were (i) to demonstrate the rapid nongenomic effect of 1,25-(OH)₂D₃ on the solvent drag–induced duodenal calcium transport, (ii) to investigate the 1,25-(OH)₂D₃–induced alterations in the size- and charge-selective properties of the duodenal epithelia, and (iii) to demonstrate the possible nongenomic signaling pathways of 1,25-(OH)₂D₃ in the rat duodenum. Since the expressions of VDR and MARRS are age-dependent and have not been completely identified in the rat intestine [7], we first investigated their expressions in all intestinal segments by using the quantitative real-time PCR technique.

**MATERIALS AND METHODS**

**Animals.** Female Sprague-Dawley rats (8 weeks old, weighing 180–200 g) were obtained from the National Laboratory Animal Centre, Thailand. They were placed in hanging stainless steel cages, fed standard pellets containing 1% wt/wt calcium and 4,000 IU/kg vitamin D (Perfect Companion, Bangkok, Thailand), and distilled water ad libitum under a 12 h/12 h light/dark cycle. Room temperature and humidity were controlled at 23°C–25°C and 50%–60%, respectively. Body weight and food intake were recorded daily. The animals were cared for in accordance with the “Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences.” This study has been approved by the Institutional Animal Care and Use Committee of the Faculty of Science, Mahidol University, Thailand.

**Bathing solution.** The bathing solution, continuously gassed with humidified 5% CO₂ in 95% O₂, contained (in mmol/l) 118 NaCl, 4.7 KCl, 1.1 MgCl₂, 1.25 CaCl₂, 23 NaHCO₃, 12 D-glucose, and 2 mannitol (all purchased from Sigma, St. Louis, MO, USA). The solution was maintained at 37°C, pH 7.4, and had an osmolality of 290–293 mmol kg⁻¹ water as measured by a freezing-point–based osmometer (model 3320; Advanced Instruments, Norwood, MA, USA). Water used in the present work had a resistance higher than 18.3 MΩ cm and a free-ionized calcium concentration less than 2.5 mmol/l.

**Tissue preparation.** After 7-day acclimatization, median laparotomy was performed under 50 mg/kg sodium pentobarbitone (Abbott, North Chicago, IL, USA) i.p. anesthesia. Intestinal segments, including duodenum (10 cm), proximal and distal jejunal segment (10 cm), ileum (8 cm), cecum (4 cm), and proximal and distal colon (8 cm) were removed, rinsed in an ice-cold bathing solution, and dissected longitudinally along the radix mesenterii to expose the mucosa. In the calcium transport experiments, the duodenal segment was mounted in a modified Ussing chamber as described previously [21]. The tissue was incubated for 20 min in the chamber before the 60 min experiment was carried out. As for the mRNA expression studies, intestinal epithelial cells were collected by scraping the mucosal surface of intestinal segments with an ice-cold glass slide [27]. Duodenum was used in the present study because it is the most efficient site for calcium absorption and has been shown to have significant solvent drag–induced calcium flux [13, 14].

**Total RNA preparation.** Total RNA was extracted from mucosal scrapings by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and purified with RNeasy Mini kit (Qiagen, Valencia, CA, USA). Purity of the total RNA was determined by the ratio of absorbance readings at 260 and 280 nm. The integrity of RNA was analyzed by denaturing agarose gel electrophoresis. One microgram of the total RNA was reverse-transcribed with the oligo(dT)₁₅ primer and the iScript kit (Bio-Rad, Hercules, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, served as a control gene to examine the consistency of the reverse transcription (percent coefficient of variation <1%, n = 20).
Quantitative real-time PCR (qRT-PCR) and sequencing. The primers used in this study were designed by OLIGO 6 (Molecular Biology Insights, Cascade, CO, USA) and Primer Validator 1.4 (Naratt Software, Bangkok, Thailand), as shown in Table 1. We performed qRT-PCR and melting curve analysis by the Bio-Rad MiniOpticon with the iQ SYBR Green SuperMix (Bio-Rad) as previously described [21]. Relative expressions of VDR and MARRS over GAPDH were calculated from the threshold cycle (Ct) values by using the $2^{ΔΔCt}$ method. The PCR products were also visualized on a 1.5% agarose gel stained with 1.0 μg/ml ethidium bromide under a UV transilluminator (Alpha Innotech, San Leandro, CA, USA). After electrophoresis, all PCR products were purified from a gel by the HiYield Gel/PCR DNA Extraction kit (Real Biotech Corporation, Taipei, Taiwan) and sequenced by the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Measurement of electrical parameters. Three electrical parameters, i.e., potential difference (PD), short-circuit current ($I_{sc}$), and transepithelial resistance (TER), were determined as previously described [21]. In brief, a pair of Ag/AgCl electrodes connected to agar bridges was placed near each surface of the mounted duodenal tissue for the measurement of PD. The other ends of the PD-sensing electrodes were connected to a preamplifier (model EVC-4000; World Precision Instruments, Sarasota, FL, USA), and lastly to a PowerLab 4/30 operated with the software Chart 5.2.2 for Mac OS X (ADInstruments, Colorado Springs, CO, USA). Another pair of Ag/AgCl electrodes was placed at the end of each hemichamber to supply Isc, which was also measured by a PowerLab 4/30 connected in series to the EVC-4000 current-generating unit. Fluid resistance was automatically subtracted by the EVC-4000 system. TER and conductance ($G; G = 1/TER$) were calculated by Ohm’s equation.

Measurement of calcium and mannitol flux. Duodenal calcium fluxes were determined by the modified methods of Charoenphandhu et al. [12] and Jantarajit et al. [21]. After 20 min incubation, the Ussing chamber was filled with new fresh bathing solution. One side was $^{45}$CaCl$_2$-containing bathing solution with or without $^3$H-mannitol (specific activity of 500 mCi/mol) (Amersham, Buckinghamshire, UK). Unidirectional flux ($J_{H→C}$) from the hot side (H) to the cold side (C) was calculated with Eqs. 1 and 2.

$$J_{H→C} = R_{H→C} \times (S_H \times A)$$  \hspace{1cm} (1)

$$S_H = C_H / C_T$$  \hspace{1cm} (2)

where $R_{H→C}$ was the rate of tracer appearance in the cold side (cpm h$^{-1}$); $S_H$ was the specific activity in the hot side (cpm nmol$^{-1}$); A was the surface area of the tissue (i.e., 0.69 cm$^2$); $C_H$ was a mean of radioactivity in the hot side (cpm); and $C_T$ was the total calcium or mannitol in the hot side (nmol). Radioactivities of $^{45}$Ca and $^3$H-mannitol were analyzed by liquid scintillation spectrophotometry (model Tri-Carb 3100; Packard Instruments, Meriden, CT, USA). Total calcium concentration of the bathing solution was analyzed by atomic absorption spectrophotometry (model SpectrAA-300; Varian Techtron, Springvale, Australia).

The total calcium transport in the absence of calcium concentration gradient (i.e., bathing solution in both hemichambers contained equal calcium concentration) consisted of the solvent drag–induced and transcellular active calcium fluxes [13]. To measure the solvent drag–induced calcium flux, we added 0.1 mmol/l trifluoperazine (TFP, Sigma) to the serosal solution to inhibit calmodulin-dependent plasma membrane Ca$^{2+}$-ATPase activity, thereby diminishing the transcellular active calcium flux [12, 28]. To measure the transcellular active calcium flux, 12 mmol/l mucosal glucose was replaced with the same concentration of mannitol to minimize sodium entry into the cells, thus disrupting the paracellular sodium gradient and abolishing the solvent drag–induced calcium flux [12]. At the end of each experiment, D-glucose was added to the mucosal solution to obtain a final concentration of 30 mmol/l to check tissue viability [29].

Determination of the epithelial charge selectivity. Permeability of sodium ($P_{Na}$) and chloride ($P_{Cl}$), which indicated the charge-selective property of the duodenal epithelium, were measured by the dilution potential technique, modified from the methods of Charoenphandhu et al. [15] and Hou et al. [30]. Briefly, the mounted duodenum was equilibrated for 20 min in normal bathing solu-

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**Table 1. Rattus norvegicus oligonucleotide sequences used in the qRT-PCR experiments (Protocol 1).** VDR, nuclear receptor of vitamin D; MARRS, membrane-associated rapid response steroid binding receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

| Name       | Accession No. | Primer (forward/reverse)          | Product length (bp) |
|------------|---------------|-----------------------------------|---------------------|
| VDR        | NM_017058     | 5´-GACCTTGGTACATCATC-3´ 5´-AGACTGGTTGGACGCATAA-3´ | 141                 |
| MARRS      | NM_017319     | 5´-ATTGCTGACTTGGCCTTAG-3´ 5´-CAGCATATCATGTCTATCA-3´ | 248                 |
| GAPDH      | NM_017008     | 5´-AGCTACTGGGTCTTCAAC-3´ 5´-TCATATTTCTGTGGTTCAC-3´ | 133                 |
tion containing 145 mmol/l NaCl before the apical solution was substituted with 72.5 mmol/l NaCl-containing solution. Osmolality of the solution was maintained by an equivalent amount of mannitol. Changes in the electrical parameters before and after fluid substitution were recorded every 10 s until stable. The ion permeability ratio ($P_{Na}/P_{Cl}$) was calculated from the dilution potential ($V_{d}$) using the Goldman-Hodgkin-Katz equation (Eq. 3) [31].

$$V_{d} = \frac{RT}{F} \ln \frac{P_{Na} C_{a} + P_{Cl} C_{b}}{P_{Na} C_{b} + P_{Cl} C_{a}}$$

where $P_{Na}$ was the absolute permeability of sodium; $P_{Cl}$ was the absolute permeability of chloride; $C_{a}$ was the mucosal NaCl concentration; $C_{b}$ was the serosal NaCl concentration; $R$, $T$, and $F$ had their conventional meanings. When given $r = P_{Na}/P_{Cl}$, $\phi = C_{b}/C_{a}$, and $\nu = F V_{d}/RT$, Eq. 3 could be rewritten as

$$r = \frac{(\phi - e^\nu) / \phi (e^\nu - 1)}$$

$P_{Na}$ and $P_{Cl}$ were calculated from the conductance ($G$) and $r$, using the simplified Kimizuka-Koketsu equations [30], as follows.

$$P_{Na} = \frac{G R T}{C_{a} F^{2} \times 1 + r}$$

$$P_{Cl} = \frac{P_{Na}}{r}$$

**Experimental protocols**

**Protocol 1:** The objective of this protocol was to demonstrate the rapid action of 1,25-(OH)$_{2}$D$_{3}$ on the duodenal calcium transport. Expressions of VDR and MARRS in 7 intestinal segments of rats, i.e., duodenum, proximal and distal jejunum, ileum, cecum, proximal and distal colon, were first examined by using the qRT-PCR technique. As for the calcium transport studies, the duodenum was directly exposed to 1,25-(OH)$_{2}$D$_{3}$ (Cayman Chemical, Ann Arbor, MI, USA) at physiological concentrations of 0.1, 1, 10, or 100 mmol/l, or a pharmacological concentration of 1,000 mmol/l during the 60 min experiment. Measured parameters included the solvent drag–induced transport, transcellular active transport, and electrical parameters. Since 100 mmol/l 1,25-(OH)$_{2}$D$_{3}$ led to the greatest increase in the solvent drag–induced calcium flux, this concentration was used in the following protocols.

**Protocol 2:** Since the results in Protocol 1 showed that 1,25-(OH)$_{2}$D$_{3}$ rapidly enhanced the active calcium transport by increasing the solvent drag component, but not the transcellular active component, and since the latter is associated with the genomic actions of 1,25-(OH)$_{2}$D$_{3}$, the underlying mechanism of the rapid response of duodenum may be different from the classical genomic responses to 1,25-(OH)$_{2}$D$_{3}$. The objective of this protocol was therefore to demonstrate that the stimulatory actions of 1,25-(OH)$_{2}$D$_{3}$ on the transcellular active calcium transport was produced by its genomic action. In this protocol, the rats were administered once daily with 3 μg/kg 1,25-(OH)$_{2}$D$_{3}$ or an equivalent amount of 100% ethanol (control) i.p. for 3 days before the total active and transcellular calcium fluxes were determined.

**Protocol 3:** This protocol was (i) to confirm that the effect of 1,25-(OH)$_{2}$D$_{3}$ on the solvent drag–induced calcium transport was nongenomic, and (ii) to elucidate the signaling pathways of 1,25-(OH)$_{2}$D$_{3}$. The mounted duodenum was directly exposed to 0.3% v/v DMSO (vehicle), 100 nmol/l 1,25-(OH)$_{2}$D$_{3}$, 50 μmol/l 5,6-dichloro-1-β-D-ribofuranosylimidazole (DRB, RNA polymerase II inhibitor for inhibition of transcription; Calbiochem, La Jolla, CA, USA), 200 nmol/l or 50 μmol/l wortmannin (PI3K inhibitor; Tocris Bioscience, Bristol, UK), 75 μmol/l LY294002 (PI3K inhibitor; Tocris Bioscience), 1 μmol/l GF109203X (PKC inhibitor; A.G. Scientific, San Diego, CA, USA), or 10 μmol/l U0126 (MEK inhibitor; A.G. Scientific). Wortmannin was more potent but less specific than LY294002 [32, 33]. Concentrations of inhibitors used in this protocol were the maximal effective concentrations reported previously [21]. Each inhibitor was dissolved in DMSO (Sigma). The concentration of DMSO in bathing solution was 0.3% v/v, which did not affect the viability of cells [21].

**Protocol 4:** The objectives of this protocol were to show whether 1,25-(OH)$_{2}$D$_{3}$ rapidly changed the transepithelial flux of a neutral paracellular marker, i.e., mannitol, and/or the charge-selective property of the duodenal epithelium. The mounted duodenum was directly exposed to 100 nmol/l 1,25-(OH)$_{2}$D$_{3}$ during the 60 min experiment. Both $^{45}$Ca and $^{3}$H-mannitol were simultaneously added to the Ussing chamber to demonstrate the concurrent transport of calcium (solvent drag component) and mannitol, as previously described [13]. Dilution potential technique was used to determine $P_{Na}/P_{Cl}$, which indicated the charge-selective property of the duodenal epithelium.

**Statistical analyses.** Results are expressed as means ± SE. Two sets of calcium or mannitol flux data were compared by using the unpaired Student’s t-test. Multiple comparisons were performed by using one-way analysis of variance (ANOVA) with Dunn’s multiple comparison test. The Mann-Whitney U-test was used to analyze the difference between the expressions of VDR and MARRS in each intestinal segment, and the Kruskal-Wallis nonparametric test with Dunn’s multiple comparison method was used to compare the expression of each gene in various intestinal segments. The level of significance for all statistical tests was $P < 0.05$. The dose-response curve complied with the four-parameter logistic equation,

$$y = \Psi_{min} + \frac{\Psi_{max} - \Psi_{min}}{1 + 10^{(log EC50 - x)/S}}$$

where $x$ was the logarithm of concentration (log mol/l); $y$ was the solvent drag–induced calcium flux (nmol h$^{-1}$ cm$^{-2}$), i.e., the response; EC50 was the half maximal effective
concentration (mol/l); \( S \) was the Hill slope; \( y \) started at the minimal response plateau (\( \psi_{\text{min}} \)) and went to the maximal response plateau (\( \psi_{\text{max}} \)) with a sigmoid shape. All data were analyzed by GraphPad Prism 4.0 for Mac OS X (GraphPad Software, San Diego, CA, USA).

**RESULTS**

**All segments of the small and large intestines of rats expressed VDR and MARRS**

As demonstrated by qRT-PCR, all intestinal segments intensely expressed mRNAs of VDR and MARRS (Fig. 1). The expression levels of VDR in all segments were comparable, whereas those of MARRS were highest in the ileum (\( P < 0.001 \)), followed by the cecum (\( P < 0.01 \)) and proximal colon (\( P < 0.05 \) compared with the duodenum). With an exception of the duodenum, expressions of MARRS in more distal segments, i.e., proximal and distal jejunum, ileum, cecum, proximal and distal colon, were higher than those of VDR by the factors of 5.3 (\( P < 0.01 \)), 30 (\( P < 0.001 \)), 21.5 (\( P < 0.001 \)), 128 (\( P < 0.001 \)), and 16.5 (\( P < 0.001 \)), respectively. The amplicon sequencing confirmed the results of qRT-PCR. The results indicated that the small and large intestine of rats could be targets of the genomic and nongenomic actions of 1,25-(OH)\(_2\)D\(_3\).

**1,25-(OH)\(_2\)D\(_3\) directly and rapidly stimulated the solvent drag-induced calcium transport**

During the 60 min Ussing chamber experiments, 10, 100, and 1,000 nmol/l 1,25-(OH)\(_2\)D\(_3\) significantly increased the solvent drag-induced calcium flux by \(-1.5\text{--}1.8\) fold, from the control value of 33.79 ± 1.74 (\( n = 10 \)), to 50.83 ± 3.65 (\( n = 10, P < 0.01 \)), 62.10 ± 3.32 (\( n = 10, P < 0.01 \)), and 58.30 ± 5.25 nmol h\(^{-1}\) cm\(^{-2}\) (\( n = 9, P < 0.01 \)), respectively (Fig. 2A). The dose-response curve plotted from the data in Fig. 2A, i.e., \( y = 33.99 + 26.37/(1 + 10^{-(x + 8.14) \times 1.89}) \), exhibited the EC50 of 7.32 nmol/l (95% confidence intervals of 2.37--22.63 nmol/l). The same concentrations of 1,25-(OH)\(_2\)D\(_3\) had no rapid effect on the transcellular active calcium transport (Fig. 2B). However, the genomic action of 1,25-(OH)\(_2\)D\(_3\) induced by 3 days of daily 1,25-(OH)\(_2\)D\(_3\) administration enhanced the transcel-
**Fig. 3.** (A) The total calcium flux consisting of the solvent drag–induced and transcellular active fluxes in the duodenum. The rats were administered once daily with 3 μg/kg 1,25-(OH)₂D₃ i.p. for 3 days prior to the calcium flux measurement. Mucosal and serosal solutions had equal calcium concentrations of 1.25 mmol/l. (B) Transcellular active calcium flux in the duodenum of 3 μg/kg 1,25-(OH)₂D₃–treated rats. The mucosal glucose was replaced with an equivalent amount of mannitol to abolish the solvent drag–induced transport. ***P < 0.001 compared with the control group. Numbers in parentheses represent the number of animals.

Luminal calcium transport from 9.69 ± 0.48 (n = 7) to 18.29 ± 0.67 nmol h⁻¹ cm⁻² (n = 6, P < 0.001), which partly accounted for the increase in the total calcium absorption from 46.32 ± 1.15 (n = 9) to 65.64 ± 1.13 nmol h⁻¹ cm⁻² (n = 9, P < 0.001) (Fig. 3). Regarding the electrical parameters, 100 nmol/l 1,25-(OH)₂D₃ reduced the PD (P < 0.01) and TER (P < 0.01) of the epithelia, but they had no effect on the Isc (Table 2). Lower concentrations of 1,25-(OH)₂D₃ did not alter the electrical parameters in the duodenum. Because the most effective concentration of 1,25-(OH)₂D₃ was 100 nmol/l, this dose was used in the subsequent experiments.

**Fig. 4.** Solvent drag–induced calcium flux in the duodenum directly exposed to 0.3% v/v DMSO (vehicle); 100 nmol/l 1,25-(OH)₂D₃; RNA polymerase inhibitor (50 μmol/l DRB); PI3K inhibitors (200 nmol/l wortmannin, or 75 μmol/l LY294002); PKC inhibitor (1 μmol/l GF109203X); MEK inhibitor (10 μmol/l U0126); 1,25-(OH)₂D₃+50 μmol/l DRB; 1,25-(OH)₂D₃+200 nmol/l, or 50 μmol/l wortmannin; 1,25-(OH)₂D₃+75 μmol/l LY294002; 1,25-(OH)₂D₃+1 μmol/l GF109203X; or 1,25-(OH)₂D₃+10 μmol/l U0126. All inhibitors in the absence of 1,25-(OH)₂D₃ (~1,25-(OH)₂D₃) had no effect on the solvent drag–induced calcium flux. **P < 0.01 compared with the control group. Numbers in parentheses represent the number of animals.

**Table 2.** Epithelial electrical parameters of the duodenal epithelium. Electrical parameters consisting of transepithelial potential difference (PD), short-circuit current (Isc), and transepithelial resistance (TER) in the duodenum directly exposed to various concentrations of 1,25-(OH)₂D₃, or 100 nmol/l 1,25-(OH)₂D₃+PK3 inhibitors (200 nmol/l wortmannin or 75 μmol/l LY294002), PKC inhibitor (1 μmol/l GF109203X), or MEK inhibitor (10 μmol/l U0126). The apical side was negative with respect to the basolateral side. The TER value of each tissue was an average of several TER values periodically measured throughout the 60 min experimental period. Values are means ± SE. **P < 0.01 compared with the control group.

| Condition                  | n  | PD (mV) | Isc (μA cm⁻²) | TER (Ω cm²) |
|----------------------------|----|---------|---------------|-------------|
| Control                    | 6  | 6.28 ± 0.28 | 78.27 ± 10.16 | 93.17 ± 9.76 |
| 1,25-(OH)₂D₃              |    |          |               |             |
| 1 nmol/l                   | 6  | 5.73 ± 0.13 | 61.93 ± 2.94  | 93.41 ± 4.40 |
| 10 nmol/l                  | 6  | 6.06 ± 0.35 | 82.40 ± 4.02  | 74.62 ± 6.26 |
| 100 nmol/l                 | 6  | 4.54 ± 0.32** | 70.82 ± 2.15  | 63.97 ± 4.09** |
| 100 nmol/l 1,25-(OH)₂D₃+   |    |          |               |             |
| Wortmannin                 | 6  | 5.34 ± 0.11 | 64.70 ± 3.83  | 83.53 ± 3.71 |
| LY294002                   | 6  | 5.99 ± 0.34 | 80.91 ± 3.73  | 85.85 ± 1.95 |
| GF109203X                  | 6  | 5.43 ± 0.12 | 67.77 ± 0.94  | 80.12 ± 1.84 |
| U0126                      | 6  | 5.30 ± 0.16 | 64.40 ± 3.25  | 83.19 ± 2.33 |
hormones, including 1,25-(OH)2D3 [20]. Figure 4 shows that 50 μmol/l DRB alone did not alter the basal rate of calcium absorption, and exposure to 100 nmol/l 1,25-(OH)2D3 plus 50 μmol/l DRB did not suppress the solvent drag–induced transport. These results suggested that 1,25-(OH)2D3 directly and rapidly stimulated the solvent drag–induced calcium transport via the nongenomic signaling pathways.

Rapid effects of 1,25-(OH)2D3 on calcium transport were abolished by PI3K, PKC, and MEK inhibitors

Since the nongenomic signaling pathways of 1,25-(OH)2D3 have been reported in chick enterocytes, osteoblasts, and myoblasts to involve the PI3K, PKC, and/or MEK [5, 20, 22, 24], these pathways were investigated in the present study by using the panspecific inhibitors of the key enzymes. The results demonstrated that 200 nmol/l and 50 μmol/l wortmannin, as well as 75 μmol/l LY294002, both of which were inhibitors of PI3K, completely abolished the 1,25-(OH)2D3–enhanced solvent drag transport (Fig. 4). We found that 1 μmol/l GF109203X, a PKC inhibitor, and 10 μmol/l U0126, a MEK inhibitor, also diminished the actions of 1,25-(OH)2D3. Moreover, all four inhibitors restored the decreased PD and TER in 100 nmol/l 1,25-(OH)2D3–treated epithelia to the control values (Table 2).

Inhibitor alone or DMSO, the vehicle for the preparation of inhibitors (final concentration of 0.3% v/v) had no effect on either the solvent drag–induced fluxes (Fig. 4) or the electrical parameters (data not shown). These findings indicated that 1,25-(OH)2D3 exerted its nongenomic action on the duodenal calcium transport through the signaling pathway(s) involving PI3K, PKC, and MEK.

1,25-(OH)2D3 rapidly altered the charge-selective property of the duodenal epithelium

Because the solvent drag–induced calcium transport occurs via the paracellular channel, it may be regulated by alterations of the size- and charge-selective properties of the epithelium (or tight junction) [19]. Herein, a neutral paracellular marker, 3H-mannitol, concurrently added with a tracer of calcium transport, 45Ca, was used to demonstrate the widening of the tight junction. However, as shown in Fig. 5, the transepithelial mannitol flux of 100 nmol/l 1,25-(OH)2D3–treated tissue was comparable to that of the control, even though the solvent drag–induced calcium flux was significantly increased. Therefore the enhanced solvent drag–induced calcium transport may not be due to alteration of the size selectivity of the epithelium.

By using the dilution potential technique, we evaluated the effect of 1,25-(OH)2D3 on the epithelial charge-selective property (Fig. 6). There were significant increases in both PD and TER in 100 nmol/l 1,25-(OH)2D3, i.e., from the control values of 14.12 ± 1.64 (n = 7) to 29.53 ± 2.55 × 10–6 cm s–1 (n = 8, P < 0.01), and from 7.13 ± 0.93 (n = 7) to 14.50 ± 1.65 × 10–6 cm s–1 (n = 8, P < 0.01), respectively. Since both PNa and PCl were proportionally increased, the PNa/PCl ratios of the 1,25-(OH)2D3–treated epithelia did not change from those of the control group. Nevertheless, the apparent PNa/PCl ratios being greater than 1.0 in both the control (i.e., 1.90 ± 0.10, n = 7) and 1,25-(OH)2D3–exposed duodenum (i.e., 2.01 ± 0.07, n = 8) confirmed that the rat duodenum possessed cation-selective property. The PI3K and MEK inhibitors (i.e., 75 μmol/l LY294002 and 10 μmol/l U0126, respectively), but not PKC inhibitor (1 μmol/l GF109203X), abolished the effects of 1,25-(OH)2D3 on both PNa and PCl. DMSO (vehicle) had no effect on PNa and PCl (data not shown). Taken together, 1,25-(OH)2D3 increased the paracellular permeability for both cations and anions by 2-fold; however, the 1,25-(OH)2D3–exposed duodenal epithelia still favored the permeation of positively charged ions.

DISCUSSION

In the present study, we provided evidence, for the first time, that 1,25-(OH)2D3 stimulated the solvent drag–induced calcium transport in the duodenum of rats via the nongenomic signaling pathways. Normally, the nongenomic actions of 1,25-(OH)2D3 are very rapid with the activation of its signaling enzymes, e.g., mitogen-activated protein kinases (MAPK) in the MEK pathway, occurring within 2 min [24]. Its stimulatory effect on the solvent drag–induced calcium transport was considerably fast, being observed within 60 min, and was too short to be mediated by the complete genomic processes of transcribe-
tion, translation, and protein localization. Besides, the absence of interference by RNA polymerase II inhibitor (50 μmol/DRB) with the actions of 1,25-(OH)₂D₃ confirmed that de novo gene transcription was not involved in these rapid responses.

It has been accepted that MARRS is required for the rapid 1,25-(OH)₂D₃–enhanced calcium uptake in the intestinal absorptive cells isolated from chickens and rats [25]. In chickens, a highly specific antibody for the N-terminus of MARRS (known as Ab099), but not for VDR, completely abolished the 1,25-(OH)₂D₃–stimulated enterocytic calcium uptake [25]. However, in rats the antibodies against both MARRS and VDR were able to inhibit the enterocytic calcium uptake, suggesting that the classical VDR may be required for the full activation of the nongenomic 1,25-(OH)₂D₃ responses [25]. The classical VDR for nongenomic signaling was localized in the caveolae-enriched plasma membranes of rat intestinal cells and ROS 17/2.8 osteoblast-like cells [34]. How MARRS interacted with the membrane-localized VDR remained to be investigated.

Since the expressions of VDR and MARRS are age-dependent and may be regulated by humoral factors, such as transforming growth factor–β and 1,25-(OH)₂D₃ itself [10], we first examined their expressions in the rat intestine. Although the nongenomic actions of 1,25-(OH)₂D₃ have been investigated mostly in the duodenum, we found that all intestinal segments strongly expressed both types of receptors with MARRS expression in the jejunum to the colon being higher than VDR expression, especially in the ileum. Thus the distal small intestine and large intestine could respond to the nongenomic actions of 1,25-(OH)₂D₃.

Transepithelial calcium transport in the duodenum occurs via two mechanisms, passive and active transport. Passive calcium transport down the concentration gradient via the paracellular channel contributes significantly when the luminal-free calcium exceeds 5 mmol/l [25, 35]. However, in an absence of a substantial calcium gradient or during increased calcium demand, the metabolically energized active calcium absorption, which is composed of the solvent drag–induced transport and transcellular calcium transport, becomes important. The solvent drag–induced active calcium transport, like the passive transport, occurs via the paracellular channel [13]. But unlike the passive transport, it is dependent on the activity of Na⁺/K⁺-ATPase that creates paracellular hyperosmotic gradient resulting from a sodium concentration of ~15 mmol/l above the surrounding milieu [17, 36], thus resembling a secondary active transport. The solvent drag–induced calcium transport is considered physiologically significant, since it contributes about 70% of the total active calcium transport [13]. Its contributions markedly increase in conditions such as high-glucose absorption, chronic metabolic acidosis, and high physiological prolactin exposure [13, 15]. On the other hand, the physiological importance of the transcellular calcium transport is
still debatable, especially when a recent investigation showed that the calbindin-D_{9K}-null mice manifested normal calcium absorption [37].

Via its genomic actions 1,25-(OH)_{2}D_{3} has long been known to stimulate the transepithelial active calcium transport by various mechanisms, e.g., enhancing the expressions and activities of TRPV6 and PMCA [1]. van Abel and co-workers also reported that 1,25-(OH)_{2}D_{3} supplementation to the 1α-hydroxylase–knockout mice restored the expression of TRPV6, calbindin-D_{9K}, and PMCA [4].

Regarding the nongenomic actions, in vitro and in vivo experiments in chick duodenum demonstrated a rapid stimulatory effect of 1,25-(OH)_{2}D_{3} on calcium and phosphate absorption, which involved the transepithelial calcium-enriched vesicles and endoplasmic reticulum-mediated calcium transport [9, 11, 25]. However, nothing is known pertaining to the amount of calcium transported by the membrane-bound organelles in the mammalian absorptive cells, but it probably contributes very little to the 1,25-(OH)_{2}D_{3}-enhanced duodenal calcium transport in adult female rats, since we saw no change in the transepithelial fluxes. Moreover, using a mathematical model-based technique, we previously showed that in the absence of the transepithelial calcium gradient, calcium traversed the duodenal epithelium through the paracellular channel via the solvent drag mechanism, and through the transepithelial pathway via PMCA [13].

Herein, it seemed very likely that the nongenomic effect of 1,25-(OH)_{2}D_{3} did not involve the transepithelial calcium flux (either via vesicular or PMCA-mediated transport), or if it did, such effect was insignificant. However, when 1,25-(OH)_{2}D_{3} was administered for 3 days to induce the genomic response, the transepithelial active flux was significantly increased, consistent with the previous report [38]. Therefore in the rat duodenum, various transport pathways respond differently to the genomic and nongenomic actions of 1,25-(OH)_{2}D_{3}.

Our results, which showed the nongenomic effect of 1,25-(OH)_{2}D_{3} on the solvent drag–induced calcium transport, were consistent with the findings of the 1,25-(OH)_{2}D_{3}-enhanced paracellular flux in Caco-2 monolayer by Chirayath et al. [39], and in rat intestine by Karbach [40]. The enhanced paracellular calcium transport was found together with decreases in PD and TER, especially when exposed to 100 nmol/l 1,25-(OH)_{2}D_{3}. Since TER reflects the permeability of the paracellular channel to small ions, and decreases in PD and TER indicate a leakier tight junction, 1,25-(OH)_{2}D_{3} appeared to increase the ionic permeability of the paracellular channel, thus facilitating the movement of cations and anions through this space in a dose-response manner. This hypothesis was confirmed by the findings that 1,25-(OH)_{2}D_{3} altered both $P_{Na}$ and $P_{Cl}$. Furthermore, the Isc, which reflects net ion current across the duodenal epithelium, remained unchanged, suggesting that 1,25-(OH)_{2}D_{3} did not interfere with the electrogenic transepithelial ion transport, particularly the sodium transport via Na^+/K^+-ATPases, which contribute up to 95% of the Isc and empower the solvent drag [41].

Generally, transport of ions through the paracellular space is controlled by the size- and charge-selective properties of the tight junction, which restrict paracellular transport of solutes with molecular radii larger than ~350 pm. The size selectivity could be altered by the contraction of the perijunctional actomyosin ring complex and cytoskeletal arrangement [42, 43]. In the duodenum, cytochalasin E, which disrupted actin polymerization and resulted in the widening of a tight junction, did not increase the solvent drag–induced paracellular calcium flux [13]. Conversely, as shown in this study, the transepithelial transport of mannitol, a neutral paracellular marker with a molecular radius of ~350 pm, was not accompanied by a parallel increase in calcium flux. Therefore it appeared that the epithelial charge selectivity, but not the size selectivity, regulated the duodenal paracellular calcium transport. In other words, 1,25-(OH)_{2}D_{3} augmented the paracellular transport of charged molecules or ions, including calcium, by altering the charge-selective property of the paracellular channel without the widening of the tight junction.

Experimentally, $P_{Na}/P_{Cl}$ and $P_{Na}/P_{Cl}$ could be used as indicators of the epithelial charge-selective property [30]. It could be implicated that calcium permeability was also increased with an increase in $P_{Na}$ because Na^+ and Ca^{2+} have comparable ionic radii of ~115 pm. Herein, 1,25-(OH)_{2}D_{3} increased both $P_{Na}$ and $P_{Cl}$ by approximately 2-fold; however, the duodenal epithelium was still basically cation-selective, i.e., $P_{Na}/P_{Cl}$ being greater than 1.0. The prominent net change in $P_{Na}$ in the present data explained an increase in the paracellular calcium transport. Generally, the charge-selective property of epithelia is regulated by proteins of the claudin family, which have charged amino acids on their extracellular loops [19]. Different types of epithelial cells express different fingerprints of claudin patterns, and a distribution of claudins along the paracellular membrane can facilitate ion movement without the widening of the tight junction [44]. We recently reported the expressions of 20 claudins in the duodenum of female rats [27]. The molecular mechanisms of 1,25-(OH)_{2}D_{3}–modulated expression and function of claudins in the duodenum are, however, unknown. Claudin-16 is well recognized as an important protein for the paracellular calcium and magnesium reabsorption in the thick ascending limb of the loop of Henle [45]. It has been reported that 1,25-(OH)_{2}D_{3} modulates the activity of claudin-16 promoter in opossum kidney cells [46]. In the duodenum, claudin-3 expression is under the regulation of 1,25-(OH)_{2}D_{3} [47]. Several investigations showed that changes in claudin expression in the intestinal absorptive cells were closely related to the enhanced paracellular calcium transport [48], as well as conditions that led to increased calcium absorption, e.g., chronic metabolic acidosis [15]. Moreover, PKC-mediated claudin-4 phosphorylation rap-
indly resulted in a decrease in TER in ovarian cancer monolayer [49]. Thus besides the genomic action of 1,25-(OH)_2D_3 on claudin expression, nongenomic 1,25-(OH)_2D_3 may also enhance the solvent drag–induced paracellular calcium transport in the duodenum by modulating the functions of claudins.

The nongenomic signaling pathways of 1,25-(OH)_2D_3 in the rat duodenum are still unresolved. The 1,25-(OH)_2D_3–activated PI3K signaling has been reported in vascular smooth muscle cells (VSMC), squamous cell carcinoma cells, osteoblasts, and osteocytes [6, 20, 50]. Direct interaction between membrane-associated VDR and PI3K in VSMC was suggested. Recently, we demonstrated that the PI3K pathway was essential for the enhanced solvent drag–induced calcium transport and changes in charge selectivity in the rat duodenum [21]. In isolated chick intestinal epithelial cells, PKC was activated by 1,25-(OH)_2D_3–MARRS interaction [9], but the final downstream targets of PKC remain inconclusive. Furthermore, 1,25-(OH)_2D_3 could rapidly activate Src kinase in myoblasts and osteoblasts [5, 6], and this kinase is known to be upstream of PI3K and MEK (or MAPK) activation [23]. A study in human acute promyelocytic leukemia NB4 cells showed that 10 nmol/l 1,25-(OH)_2D_3 rapidly induced phosphorylation of MAPK within 30 s [51]. Cross talk between PI3K, PKC, and MEK pathways were possible [52]. In our study, all three pathways, i.e., PI3K, PKC, and MEK, were involved in the 1,25-(OH)_2D_3–stimulated solvent drag–induced calcium transport, suggesting that these kinases were either in the same signaling pathway or were able to interfere with cross talk to each other. Although 1,25-(OH)_2D_3 also used PI3K and MEK pathways to increase the paracellular P_{Na} and P_{Cl}, the PKC pathway was not involved in this effect. The PKC pathway may be utilized by 1,25-(OH)_2D_3 to increase the solvent drag through mechanisms other than alteration of the charge selectivity, for instance, by increasing paracellular water flow or preventing a dissipation of paracellular osmotic gradient. Further investigation is required to demonstrate the detailed signaling cascade and to identify the final target downstream from MARRS and membrane-associated VDR, e.g., phosphorylation or redistribution of claudins.

In conclusion, we demonstrated that all intestinal segments expressed both VDR and MARRS, suggesting that 1,25-(OH)_2D_3 could exert nongenomic actions on the entire intestinal tract. Direct exposure to 10 and 100 nmol/l 1,25-(OH)_2D_3 rapidly stimulated the solvent drag–induced calcium transport, which is the major component of the transepithelial calcium absorption. This nongenomic action was mediated by the PI3K, PKC, and MEK pathways. Our findings therefore provided novel evidence on the 1,25-(OH)_2D_3–enhanced paracellular calcium movement, as well as the nongenomic action of 1,25-(OH)_2D_3 in the mammalian duodenum.

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