Enhancement of calcium transport in Caco-2 monolayer through PKCε-dependent Ca\textsubscript{v}1.3-mediated transcellular and rectifying paracellular pathways by prolactin

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PROLACTIN (PRL) is a crucial calcium-regulating hormone in pregnant and lactating animals since it increases intestinal calcium absorption, renal calcium reabsorption, and bone turnover ultimately to supply more calcium for fetal growth and milk production (4, 5). In vivo studies in rats suggested that PRL could stimulate transcellular and paracellular calcium absorption in several intestinal segments, particularly in the duodenum and proximal jejunum in which PRL receptors (PRLR) are strongly expressed (20, 25, 26). Although direct stimulatory actions of PRL on intestinal calcium transport has been demonstrated, little is currently known regarding the detailed mechanisms of PRL in intestinal epithelial cells.

Under normal conditions, calcium predominantly traverses the intestinal epithelium via the paracellular pathway, especially after a calcium-enriched meal that favors the calcium gradient-dependent paracellular calcium transport, whereas the transcellular calcium transport becomes more significant during high calcium demand, such as pregnancy and lactation (5, 23). A number of calcium transport proteins are essential for the transcellular calcium transfer, namely, transient receptor potential vanilloid family calcium channel (TRPV)-6 and voltage-dependent L-type calcium channel Ca\textsubscript{v}1.3 for the apical calcium entry, calcium-binding protein (CaBP) calbindin-D\textsubscript{9k} for cytoplasmic translocation, and plasma membrane Ca\textsuperscript{2+}/Na\textsuperscript{+} exchanger (NCX)-1 for basolateral extrusion (18, 34, 35). It was apparent that, under the influence of various hormones and physiological conditions, different transport proteins may be recruited to augment calcium absorption. For example, L-type calcium channels were responsible for the enhanced transcellular calcium transport in response to parathyroid hormone-related peptide and luminal glucose (34, 51), while TRPV6 was required for the 1,25(OH)\textsubscript{2}D\textsubscript{3} and 17β-estradiol-stimulated calcium absorption (23, 46). Regarding the PRL-stimulated calcium transport, it was not known which transporter proteins were involved.

The paracellular calcium transport driven by an electrochemical gradient (i.e., calcium gradient and voltage difference), on the other hand, is regulated by several tight junctions proteins, e.g., claudin-2 and -12, which polymerize to form an array of channel-like paracellular pores (15, 43, 47). Thus, the paracellular channels appeared to exhibit charge and size selectivity, voltage response, and rectification similar to those found in typical ion channels (12, 30, 41, 43). Physiological significance of the voltage-dependent calcium transport in the small intestine is controversial, but it may be of greater importance when the transepithelial potential difference (PD) is increased by some nutrients, such as glucose, short-chain fatty acid, phenylalanine, cysteine, and proline (14, 40, 48). Recently, PRL has been shown to modify some properties of the paracellular channel, i.e., charge selectivity and calcium permeability, which led to an enhancement of the calcium gradient-dependent paracellular calcium transport (45). The effects of PRL on other paracellular properties, such as response to calcium uptake; protein kinase C; rectification; small interfering RNA; voltage-dependent calcium transport

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voltage change and rectification, or the voltage-dependent calcium transport remained unknown. However, they could not be demonstrated in the presence of calcium gradient because high calcium concentration occluded the paracellular pores (known as the paracellular conductance block), which in turn altered the transepithelial resistance (TER) (43, 45). Such paracellular properties should be validated by a voltage-clamp study in an absence of calcium gradient.

In addition to the calcium transport mechanism, the signaling pathways of PRL were also elusive. In the rat duodenum and intestinal epithelium-like Caco-2 monolayer, phosphoinositide 3-kinase (PI3K) was required for PRL signaling (20, 45). Further investigations in Caco-2 cells suggested that the PRL-stimulated transcellular and gradient-dependent paracellular calcium transport relied on protein kinase C (PKC) and RhoA-associated coiled-coil forming kinase (ROCK) (45), but which PKC isoform was involved was not known.

Therefore, the objectives of the present study were 1) to identify the transcellular transporters and PKC isoform that were essential for the PRL-stimulated transcellular calcium transport, 2) to investigate the paracellular properties altered by PRL, and 3) to demonstrate the effects of PRL on the voltage-dependent calcium transport as well as the responsible signaling pathways. The human colorectal adenocarcinoma Caco-2 cells were used in this study because they have functional similarities to the small intestinal cells, including the presence of brush border, expression of sucrase-isomaltase, and expression of the transcellular calcium transporters and charge-selective paracellular proteins, e.g., TRPV6, PMCA, claudin-1, -2, -3, and -5 (32, 37, 38, 50, 52). Caco-2 monolayer was also a standard model for calcium absorption study and was responsive to PRL (20, 45).

MATERIALS AND METHODS

Cell culture. Caco-2 intestinal cells [no. HTB-37, American Type Culture Collection (ATCC)] were grown in Dulbecco’s modified Eagle’s medium (Sigma, St. Louis, MO) supplemented with 15% fetal bovine serum (Gibco, Grand Island, NY), 1% L-glutamine (Gibco), Eagle’s medium (Sigma, St. Louis, MO) supplemented with 15% fetal bovine serum (Gibco, Grand Island, NY), and 1% L-glutamine (Gibco, Grand Island, NY). Cells were propagated in 75-cm² T flask (Corning, NY) under a humidified 5% CO₂ in air (Sigma), and 0.25% nonessential amino acid (Sigma), 100 U/ml penicillin/streptomycin (Sigma), and 50 µg/ml amphotericin B (Sigma). Cells were seeded at 5.0 ×10⁶ cells/cm² on polyester Snapwell at day 12 after seeding, in vitro transfection was performed with 10 µg/ml polyethylenimine and 1 µmol/ml siRNA molecules. At day 14 (i.e., 48 h after transfection), transepithelial calcium flux was determined. Efficiency of siRNA that had no homology to any other genes was used as a negative control. As previously described (45), Caco-2 cells were first plated on Snapwell at 5.0 × 10⁶ cells/cm². At day 12 after seeding, in vitro transfection was performed with 10 µg/ml polyethylenimine and 1 µmol/ml siRNA molecules. At day 14 (i.e., 48 h after transfection), transepithelial calcium flux was determined. Efficiency of siRNA was evaluated by quantitative real-time PCR (qRT-PCR). The knockdown protocol was approved by the Institutional Biosafety Committee (IBC) of Mahidol University.

mRNA isolation, quantitative real-time PCR, and sequencing. By using TRizol reagent (Invitrogen, Carlsbad, CA), total RNA was prepared from Caco-2 cells, as previously described (45). One microgram of total RNA was reverse-transcribed with iScript kit (Bio-Rad, Hercules, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, served as a control gene to check the consistency of the reverse transcription. Sense and antisense primers used for qRT-PCR are presented in Table 1. The amplification reaction using real-time PCR (model MiniOpticon; Bio-Rad) was performed with iQ SYBR Green SuperMix (Bio-Rad). Real-time expression of studied genes over GAPDH was calculated from the threshold cycle (Ct) values by using 2⁻ΔΔCt method. After qRT-PCR, the PCR products were also visualized on a 1.5% agarose gel stained with 1.0 µg/ml ethidium bromide. Thereafter, all PCR products were extracted by the HiYield Gel/PCR DNA Extraction kit (Real Biotech, Taipei, Taiwan) and were sequenced by the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Bathing solution. The bathing solution for Ussing chamber experiments contained (in mmol/l) 118 NaCl, 4.7 KCl, 1.1 MgCl₂, 1.25 CaCl₂, 23 NaHCO₃, 12 Na-glucose, and 2 mannitol (all purchased from Sigma). The solution, continuously gassed with humidified 5% CO₂ in 95% O₂, was maintained at 37°C, pH 7.4, and had an osmolality of 290–293 mmol/kg water as measured by a freezing point-based osmometer (3302; Advanced Instruments, Norwood, MA).

Table 1. Homo sapiens oligonucleotide sequences used in the quantitative real-time PCR experiments

<table>
<thead>
<tr>
<th>Name</th>
<th>Accession No.</th>
<th>Primer (forward/reverse) Product Length, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPV6</td>
<td>AF365928</td>
<td>5’-CTCGAGATCTGAGGATGATCCAGC-3’ 144</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’-AGATCTTGGGCAAGTTGATC-3’</td>
</tr>
<tr>
<td>Ca1.3</td>
<td>EU363339</td>
<td>5’-TCACCTGCTGAGGATGATCCAGC-3’ 113</td>
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<tr>
<td></td>
<td></td>
<td>5’-AGATCTTGGGCAAGTTGATC-3’</td>
</tr>
<tr>
<td>Calbindin-D₉k</td>
<td>L13220</td>
<td>5’-CAGCTGAGGATGATCCAGC-3’ 106</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’-GGGAGCTTGGGCAAGTTGATC-3’</td>
</tr>
<tr>
<td>PKCᵦ</td>
<td>NM_002744</td>
<td>5’-CAATGACGACTTGAGGATGATCCAGC-3’ 138</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’-CGATGAGGATGATCCAGC-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_002046</td>
<td>5’-GGATGAGGATGATCCAGC-3’ 359</td>
</tr>
</tbody>
</table>

TRPV6, transient receptor potential vanilloid family Ca²⁺ channel 6; Ca1.3, voltage-dependent L-type Ca²⁺ channel 1.3; PKCᵦ, ε-isozyme of protein kinase C; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Distilled water used in the present work had a resistance >18.3 MΩ-cm and free-ionized calcium <2.5 mmol/l.

**Measurement of electrical parameters.** Electrical parameters, i.e., potential (voltage) difference (PD), short-circuit current (∆Isc), and TER, were determined as described previously (7, 20). In brief, a pair of Ag/AgCl electrodes connected to a preamplifier (model EVC-4000; World Precision Instruments, Sarasota, FL) were connected to another pair of Ag/AgCl electrodes placed at the end of each hemichamber to supply solution on one side of the Snapwell. TER, were determined as described previously (7, 20). In brief, a pair of Ag/AgCl electrodes connected to a preamplifier (model EVC-4000; World Precision Instruments, Sarasota, FL) were connected to another pair of Ag/AgCl electrodes placed at the end of each hemichamber to supply solution on one side of the Snapwell. TER, were determined as described previously (7, 20).

**Calcium flux measurement.** Calcium fluxes were determined by modified method of Chaoenphandhu et al. (7). After a 20-min incubation in Ussing chamber, the bathing solution was changed to a fresh one. The solution on one side contained 45Ca (initial specific activity of 5 mCi/ml, final specific activity of ~450–500 mCi/mol; Amersham, Buckinghamshire, UK). Samples were collected from the chamber to calculate the unidirectional flux (JH–C) from the hot side (H) to the cold side (C), as follows:

\[
J_{H\rightarrow C} = R_{H\rightarrow C}(S_h \times A)
\]

where \(R_{H\rightarrow C}\) was the rate of tracer appearance in the cold side (cpm/h), \(S_h\) was the specific activity in the hot side (cpm/mmol), \(A\) was the surface area of Snapwell (cm²), \(C_{H\rightarrow C}\) was the mean radioactivity in the hot side (cpm), and \(C_t\) was the total calcium in the cold side (nmol).

Radioactivity of 45Ca in counts per minute (cpm) was analyzed by liquid scintillation spectrophotometer (model Tri-Carb 3100; Packard, Meriden, CT). Total calcium concentration in the hot side was determined by atomic absorption spectrophotometry (model SpectrAA-300; Varian Techtron, Springvale, Victoria, Australia). In the absence of transepithelial calcium gradient, i.e., bathing solution in both hemichambers contained equal calcium concentration of 1.25 mmol/l, the measured calcium fluxes represented the transcellular active calcium transport (45).

As for the experimental protocol, Caco-2 monolayer was directly incubated on the basolateral side for 60 min with 600 ng/ml recombinant human prolactin (rhPRL) (purity >97%; catalog no. 682-Pl; R&D Systems, Minneapolis, MN), which is the maximal effective concentration reported by Jantarajit et al. (20). In some experiments, the monolayers were also exposed to PKC activator [100 nmol/l phorbol-12-myristate-13-acetate (PMA), Calbiochem, San Diego, CA], or inhibitors for RNA polymerase II [50 nmol/l 5,6-dichloro-1-b-ribobenzimidazole (DRB), Calbiochem], protein biosynthesis (70 nmol/l cycloheximide, Sigma), L-type calcium channel (1, 5, or 10 nmol/l nifedipine, or 100 nmol/l verapamil; Sigma), calmodulin-dependent PMCA (100 nmol/l trifluoperazine, Sigma), PKCα (5 nmol/l Go-6976, Calbiochem), PKCz (40 nmol/l myristoylated PKCζ pseudosubstrate peptide; Calbiochem), PKCα (30 nmol/l myristoylated PKCα pseudosubstrate peptide; Calbiochem), PKCζ (4 μmol/l PKCζ translocation inhibitor peptide; Calbiochem), or PKA (10 μmol/l myristoylated PKA inhibitor 14–22 amide; Calbiochem).

**Voltage clamping.** Calcium fluxes in clamped preparations represented the voltage-dependent calcium transport through the paracellular route (22). Transepithelial potential differences across the control group.

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**Table 2. Epithelial electrical parameters of Caco-2 monolayers**

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>PD, mV</th>
<th>∆Isc, μA/cm²</th>
<th>TER, Ω·cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>1.01±0.12</td>
<td>2.73±0.70</td>
<td>376.67±37.56</td>
</tr>
<tr>
<td>Vehicle</td>
<td>10</td>
<td>0.94±0.32</td>
<td>2.60±0.84</td>
<td>386.67±35.83</td>
</tr>
<tr>
<td>600 nmol rhPRL</td>
<td>15</td>
<td>0.88±0.19</td>
<td>3.47±0.92</td>
<td>253.22±28.01*</td>
</tr>
</tbody>
</table>

**Inhibitors**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Control</th>
<th>Vehicle</th>
<th>600 nmol rhPRL</th>
<th>PMA, 100 μmol/l</th>
<th>PKA, 10 μmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 nmol/l DRB</td>
<td>12</td>
<td>1.03±0.28</td>
<td>2.50±0.67</td>
<td>418.75±82.28</td>
<td></td>
</tr>
<tr>
<td>70 nmol/l CCH</td>
<td>13</td>
<td>1.03±0.23</td>
<td>2.62±0.51</td>
<td>396.15±55.76</td>
<td></td>
</tr>
<tr>
<td>10 nmol/l Nifedipine</td>
<td>14</td>
<td>1.17±0.33</td>
<td>3.00±0.88</td>
<td>395.48±48.35</td>
<td></td>
</tr>
<tr>
<td>100 nmol/l Verapamil</td>
<td>10</td>
<td>0.95±0.22</td>
<td>2.50±0.53</td>
<td>381.67±46.78</td>
<td></td>
</tr>
<tr>
<td>100 nmol/l TFP</td>
<td>10</td>
<td>0.96±0.28</td>
<td>2.40±0.70</td>
<td>405.00±66.23</td>
<td></td>
</tr>
<tr>
<td>5 μmol/l Go-6976</td>
<td>6</td>
<td>1.27±0.19</td>
<td>3.17±0.40</td>
<td>394.44±15.47</td>
<td></td>
</tr>
<tr>
<td>40 μmol/l myr-PKCζ</td>
<td>6</td>
<td>1.28±0.14</td>
<td>3.50±0.43</td>
<td>371.39±15.09</td>
<td></td>
</tr>
<tr>
<td>30 μmol/l myr-PKCζ</td>
<td>6</td>
<td>1.08±0.12</td>
<td>2.67±0.33</td>
<td>430.56±23.34</td>
<td></td>
</tr>
<tr>
<td>4 μmol/l trans-PKCζ</td>
<td>6</td>
<td>1.03±0.11</td>
<td>2.25±0.31</td>
<td>413.89±23.37</td>
<td></td>
</tr>
<tr>
<td>100 nmol/l PMA</td>
<td>4</td>
<td>1.08±0.13</td>
<td>2.25±0.25</td>
<td>408.33±49.30</td>
<td></td>
</tr>
<tr>
<td>10 μmol/l PKAI 14-22</td>
<td>4</td>
<td>1.00±0.12</td>
<td>2.50±0.58</td>
<td>385.48±41.11</td>
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</tr>
<tr>
<td>600 nmol rhPRL+</td>
<td>12</td>
<td>0.83±0.31</td>
<td>3.33±1.03</td>
<td>241.39±40.14*</td>
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<tr>
<td>50 nmol/l DRB</td>
<td>12</td>
<td>0.83±0.31</td>
<td>3.33±1.03</td>
<td>241.39±40.14*</td>
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</tr>
<tr>
<td>70 nmol/l CCH</td>
<td>13</td>
<td>0.89±0.27</td>
<td>3.46±0.88</td>
<td>256.28±36.57*</td>
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<tr>
<td>10 nmol/l Nifedipine</td>
<td>14</td>
<td>0.77±0.22</td>
<td>3.14±0.77</td>
<td>243.45±29.63*</td>
<td></td>
</tr>
<tr>
<td>100 nmol/l Verapamil</td>
<td>10</td>
<td>0.92±0.40</td>
<td>3.67±1.37</td>
<td>254.83±34.86*</td>
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<tr>
<td>100 nmol/l TFP</td>
<td>10</td>
<td>0.87±0.16</td>
<td>3.50±0.55</td>
<td>246.61±35.13*</td>
<td></td>
</tr>
<tr>
<td>5 μmol/l Go-6976</td>
<td>6</td>
<td>1.02±0.21</td>
<td>3.83±0.98</td>
<td>268.06±19.62*</td>
<td></td>
</tr>
<tr>
<td>40 μmol/l myr-PKCζ</td>
<td>6</td>
<td>1.00±0.19</td>
<td>4.50±0.89</td>
<td>225.00±6.80*</td>
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</tr>
<tr>
<td>30 μmol/l myr-PKCζ</td>
<td>6</td>
<td>1.13±0.20</td>
<td>4.33±0.71</td>
<td>259.80±88.52*</td>
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<tr>
<td>4 μmol/l trans-PKCζ</td>
<td>6</td>
<td>0.93±0.19</td>
<td>3.50±1.05</td>
<td>276.94±49.99*</td>
<td></td>
</tr>
<tr>
<td>100 nmol/l PMA</td>
<td>4</td>
<td>1.00±0.21</td>
<td>4.00±0.82</td>
<td>250.00±10.21*</td>
<td></td>
</tr>
<tr>
<td>10 μmol/l PKAI 14-22</td>
<td>4</td>
<td>0.95±0.13</td>
<td>3.75±0.50</td>
<td>254.17±22.05*</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. Caco-2 monolayer was exposed to recombinant human prolactin (rhPRL), RNA polymerase II inhibitor [5,6-dichloro-1-b-ribobenzimidazole (DRB), protein synthesis inhibitor [cycloheximide (CH)], L-type calcium channel blockers (nifedipine and verapamil), plasma membrane Ca2+-ATPase (PMCA) inhibitor [trifluoperazine (TFP)], PKC inhibitors [Go-6976 for PKCζ, myristoylated (myr) pseudosubstrates for PKCζ, and translocation (trans) inhibitor peptide for PKCζ, PKC activator (PMA), myristoylated PKA inhibitor 14–22 amide (PKA 14-22), or rhPRL plus inhibitors.** P < 0.01 compared with the control group.**
trol and 600 ng/ml rhPRL-treated monolayers were clamped to −25, −10, −5, 0, +5, +10, or +25 mV (the serosal side was considered zero) during 60-min experiment by using EVC-4000 system. Transepithelial calcium fluxes in apical-to-basolateral (A-to-B) and basolateral-to-apical (B-to-A) directions were determined. Net calcium flux was a subtraction of B-to-A flux from A-to-B flux. In some experiments, Caco-2 monolayers (n = 5 per each voltage; total 280 Snapwells used) were exposed to 600 ng/ml rhPRL plus PI3K inhibitor (75 μmol/l LY-294002; Tocris Bioscience, Bristol, UK), pan-specific PKC inhibitor (1 μmol/l GF-109203X; AG Scientific, San Diego, CA), or ROCK inhibitor (1 μmol/l Y-27632; Calbiochem).

Calcium uptake study. The calcium uptake protocol was modified from the method of Charoenphandhu et al. (6). In brief, Caco-2 cells (1.0 × 10⁶ cells/cm²) were propagated in 6-well plates for 3 days. Confluent Caco-2 cells were then incubated with 600 ng/ml rhPRL for 60 min until ⁴⁵Ca was added to obtain a final specific activity of ~500 mCi/mol. After exactly 1-, 3-, 6-, 9-, 12-, 20-, 30-, or 40-min exposure to ⁴⁵Ca (n = 5 per each time interval; total 225 setups used; cells for 0 min were not incubated with ⁴⁵Ca), cells were immediately washed in ice-cold ⁴⁵Ca-free solution to stop the physiological processes, placed in 5 mmol/l EGTA-containing solution to eliminate the adsorbed extracellular calcium, centrifuged at 550 g for 5 min, and finally lysed by 30% vol/vol Triton X-100. Radioactivity of ⁴⁵Ca in the homogenate was analyzed by a liquid scintillation spectrophotometer (Packard). Cellular protein was determined by bicinchoninic acid method using a commercial kit (Sigma). In some experiments, 600 ng/ml rhPRL-treated cells were also incubated with 75 μmol/l LY-294002, 1 μmol/l GF-109203X, or 1 μmol/l Y-27632.

Statistical analysis. Results are expressed as means ± SE. Two sets of data were compared using the unpaired Student’s t-test. One-way analysis of variance with Dunnett’s multiple-comparison test was used for multiple sets of data. The level of significance for all statistical tests was P < 0.05. The curves of calcium uptake vs. time and of calcium flux vs. voltage were obtained using one-phase association and second-order polynomial equations, respectively (36). Data were analyzed by GraphPad Prism 4.0 for Mac OS X (GraphPad Software, San Diego, CA).

RESULTS

PRL signaling in Caco-2 monolayer was nongenomic and protein synthesis independent. Calcium flux studies using Ussing chamber technique demonstrated that 600 ng/ml rhPRL significantly increased the transcellular calcium transport by ~2.13-fold (Fig. 1), while decreasing TER in Caco-2 monolayer (Table 2). PRL effects occurred within 60 min after treatment. Exposure to PRL plus inhibitors of de novo gene transcription (50 μmol/l DRB) or protein biosynthesis (70 μmol/l cycloheximide) had no effect on the transcellular calcium transport (Fig. 1). DRB or cycloheximide alone were without effect on the basal calcium transport (Fig. 1) and

![Fig. 2. A: expression of transient receptor potential vanilloid family calcium channel 6 (TRPV6), voltage-dependent L-type Ca²⁺ channel 1.3 (Ca₁.3) and calbindin-D₉k (CaBP₉k) in Caco-2 cells transfected with scramble small interfering RNA (siRNA; negative control) or siRNA targeting (si) TRPV6, Ca₁.3, or CaBP₉k mRNAs. Quantitative real-time PCR (qRT-PCR) results are expressed as log means ± SE. Representative electrophoretic bands and fold differences between the expression in control and knockdown cells (conventional PCR, 36 cycles) are also presented along with qRT-PCR results. GAPDH was used for normalization. **P < 0.01, ***P < 0.001 compared with scramble. B: transcellular calcium transport in TRPV6, Ca₁.3, and CaBP₉k knockdown Caco-2 monolayers with (+rhPRL) or without (−rhPRL) 600 ng/ml rhPRL in the basolateral solution. In some experiments, TRPV6 knockdown monolayers were exposed to 600 ng/ml rhPRL plus 100 nmol/l verapamil (Ca₁.3 blocker; on the apical side). Control group denotes normal Caco-2 monolayers (without siRNA transfection). **P < 0.01 compared with the control group. Numbers in parentheses represent the number of independent Snapwells.](image-url)
bated with 100 nmol/l verapamil, which inhibited Cav1.3 activity. *eral side. In some experiments, TRPV6-knockdown monolayers were incu-
containing solution. The apical side was negative with respect to the basolat-
Mounted monolayer was bathed on both sides with 1.25 mmol/l calcium-

calcium channel blockers (dihydropyridine group), on the apical side, but not the basolateral side, diminished the PRL-stimulated transcellular calcium transport in a concentration-dependent manner (Fig. 3). Similar inhibition was observed after incubating Caco-2 monolayer on the apical side with another L-type calcium channel blocker, verapamil (phenylalkylamine group) (Fig. 3). The involvement of Ca,1.3 in the calcium transport response to PRL was supported by the finding that 100 nmol/l verapamil could prevent the PRL-stimulated calcium transport in TRPV6 knockdown monolayer (Fig. 2B). The results, therefore, indicated that Ca,1.3 at the apical membrane was required for calcium entry during the PRL-stimulated transcellular calcium transport. Basolateral calcium extrusion under PRL stimulation was likely to be mediated by PMCA rather than NCX1, since calcium flux was totally abolished by a calmodulin-dependent PMCA inhibitor trifluoperazine (Fig. 3).

PRL stimulated apical calcium uptake via the PKC signaling pathway. Although Ca,1.3, a channel for calcium entry, was required for PRL actions, the effects of PRL on apical calcium uptake in Caco-2 cells as well as responsible signaling pathways were not known. Herein, the calcium uptake study showed that, under normal conditions, the relationship between calcium uptake and time complied with the one-phase association equation \(r^2 = 0.98\), and calcium accumulation in Caco-2 cells ascended to the maximal value \(Y_{\text{max}}\) of \(\sim 15\) mmol/kg protein with a rate constant of \(-0.15\) min\(^{-1}\) (Fig. 4). Exposure to 600 ng/ml rhPRL did not change \(Y_{\text{max}}\) value. However, the rate constant that represented the rate of calcium uptake was increased in PRL-treated cells by approximately twofold (Fig. 4D).

To demonstrate the PRL signaling pathways for stimulation of apical calcium uptake, Caco-2 cells were incubated with inhibitors for PI3K, PKC, and ROCK, all of which have been reported to mediate PRL actions in intestinal epithelial cells (20, 45). The results showed that PI3K inhibitor (LY-294002) and panspecific PKC inhibitor (GF-109203X), but not ROCK electrical parameters (Table 2). The effect of DRB were consistent with that reported previously (45). The data indicated that PRL exerted stimulatory effects on the transcellular calcium transport via nongenomic, protein synthesis-independent signaling pathways.

**PRL increased transcellular calcium transport via L-type calcium channel Ca,1.3 and PMCA.** In the transcellular calcium transport, calcium has been reported to traverse the apical membrane through TRPV6 and Ca,1.3 before binding to calbindin-D\(_{9k}\) for cytoplasmic translocation to the basolateral side (18, 31, 34). We therefore generated siRNA-transfected Caco-2 monolayers to verify the significance of each putative protein in the PRL-stimulated calcium transport. We show in Fig. 2 that knockdown of TRPV6, Ca,1.3, and calbindin-D\(_{9k}\) markedly reduced mRNA expressions by 26.52-, 61.22-, and 49.34-fold, respectively, with no significant effect on the basal calcium transport or electrical parameters (Table 3). Interestingly, the PRL-enhanced calcium transport was completely abolished in Ca,1.3 knockdown monolayer, and not in the TRPV6 and calbindin-D\(_{9k}\) knockdown monolayers (Fig. 2B). Exposure to 1, 5, and 10 \(\mu\)mol/l nifedipine, a classical L-type calcium channel blocker (phenylalkylamine group), on the apical side to various concentrations of nifedipine or plasma membrane Ca\(^{2+}\)-ATPase (PMCA) inhibitor (100 \(\mu\)mol/l trifluoperazine) in the presence (+ rhPRL) and absence (− rhPRL) of 600 ng/ml rhPRL. DMSO was used as vehicle for inhibitor preparation. Numbers in parentheses represent the number of independent Snapwells. **P < 0.01 compared with the control group.
inhibitor (Y-27632), could abolish the effect of PRL on calcium uptake (Fig. 4).

The effect of PRL on the transcellular calcium transport was mediated by PKC \(_{\zeta}\). Since the calcium uptake study showed that PKC was required for the PRL-stimulated transcellular calcium transport, we proceeded to identify the responsible PKC isozyme by exposing Caco-2 monolayer to various PKC isozyme inhibitors in the presence or absence of 600 ng/ml rhPRL. We demonstrated that PKC inhibitors alone had no effect on the basal calcium transport (Fig. 5). PKC \(_{\zeta}\) inhibitor, but not inhibitors of PKC\(_{\alpha/\beta}\) (Gö-6976), PKC\(_{\alpha}\), or PKC\(_{\varepsilon}\), abolished the PRL-enhanced transcellular calcium transport in Caco-2 monolayer (Fig. 5). Since a high concentration of Gö-6976 (5 μmol/l) used in the present study also inhibited PKC\(_{\mu}\) (16), it was likely that PRL action did not involve PKC\(_{\mu}\). A classical PKC activator, phorbol-12-myristate-13-acetate (PMA), which could not activate PKC\(_{\zeta}\), also itself stimulated the transcellular calcium transport but with a smaller magnitude than PRL (Fig. 5). Exposure to 600 ng/ml rhPRL further increased calcium transport in PMA-treated monolayer (Fig. 5). Neither PKC inhibitors nor PMA had effect on the PRL-induced decrease in TER (Table 2).

Since a number of hormones, such as parathyroid hormone and 1,25(OH)\(_2\)D\(_3\), enhanced calcium transport through the

**Fig. 4.** Calcium uptake in Caco-2 cells (\(n = 5\) per each time interval; total 225 Snapwells used) treated with 600 ng/ml rhPRL, or rhPRL plus phosphoinositide 3-kinase (PI3K) inhibitor (75 μmol/l LY-294002) (A), panspecific PKC inhibitor (1 μmol/l GF-109203X) (B), or RhoA-associated coiled-coil forming kinase (ROCK) inhibitor (1 μmol/l Y-27632) (C). Cells were incubated in \(^{45}\)Ca-containing solution for 0, 1, 3, 6, 9, 12, 20, 30, or 40 min. Control and rhPRL data in A were reused in B and C for better comparison. \(*P < 0.01\) rhPRL vs. control. \(D\): rate constant, which is indicative of the rate of calcium uptake, in Caco-2 cells exposed to rhPRL or rhPRL plus inhibitors. Data were obtained from the one-phase association curves in A–C. **\(P < 0.01\) compared with the control group.

**Fig. 5.** Transcellular calcium transport in Caco-2 monolayers exposed to selective PKC\(_{\alpha/\beta}\) inhibitor (5 μmol/l Gö-6976), 40 μmol/l myristoylated PKC\(_{\zeta}\) pseudosubstrate peptide, 30 μmol/l myristoylated PKC\(_{\alpha}\) pseudosubstrate peptide, 4 μmol/l PKC\(_{\varepsilon}\) translocation inhibitor peptide, PKC activator (100 nmol/l phorbol-12-myristate-13-acetate; PMA), or 10 μmol/l myristoylated PKA inhibitor 14-22 amide (PKAI 14-22), in the presence (+ rhPRL) or absence (−rhPRL) of 600 ng/ml rhPRL. All cell-permeable inhibitor peptides were dissolved in water. Gö-6976 and PMA were dissolved in DMSO (vehicle). Numbers in parentheses represent the number of independent Snapwells. **\(P < 0.01\)** compared with the control group.
transcellular route in a protein kinase A (PKA)-dependent manner (10, 11, 23), we examined a possibility of PKA-mediated PRL action. However, exposure to a cell-permeable PKA inhibitor, myristoylated PKA inhibitor 14-22 amide, did not prevent the PRL effect on the transcellular calcium transport (Fig. 5).

PKC\textsubscript{\textgamma} knockdown abolished the PRL-stimulated transcellular calcium transport. To confirm that PRL exerted its actions through PKC\textsubscript{\textgamma}, calcium fluxes were determined in PKC\textsubscript{\textgamma} knockout Caco-2 monolayer, which manifested a decrease in PKC\textsubscript{\textgamma} mRNA expression by 68.86-fold (Fig. 6A). The results showed that 600 ng/ml rhPRL did not increase the transcellular calcium flux in PKC\textsubscript{\textgamma} knockout monolayer (Fig. 6B), while neither scramble siRNA nor PKC\textsubscript{\textgamma} siRNA altered the basal calcium flux (Fig. 6B) or electrical parameters (Table 3). However, PKC\textsubscript{\textgamma} knockdown had no effect on the PRL-induced decrease in TER (Table 3).

PRL enhanced the voltage-dependent paracellular calcium via ROCK pathway. In addition to the transcellular pathway, PRL has been reported to augment calcium movement through the paracellular space. However, effects of PRL on the voltage-dependent paracellular calcium transport were not known. Under normal conditions, the voltage-clamp study revealed that the apical-to-basolateral (A-to-B; Fig. 7A) calcium fluxes were dramatically increased with positive apical voltage (basolateral voltage was zero), whereas the increase in basolateral-to-apical (B-to-A; Fig. 7B) calcium fluxes with negative apical voltage was comparatively much smaller. Given an equal driving force (i.e., transepithelial voltage) of opposite signs, the presence of unidirectional calcium fluxes being larger in one direction than the other suggested that the paracellular space of Caco-2 monolayer possessed a property known as rectification. This phenomenon, therefore, resulted in a large net calcium flux (equivalent to electrical current) at apical voltage positive and a relatively small net calcium flux at voltage negative (Fig. 7, C and F).

PRL-treated Caco-2 monolayer exhibited significant increases in A-to-B and B-to-A calcium fluxes at the apical voltage ranges of 0 to +25 mV and −25 to −5 mV, respectively, thereby increasing the net calcium transport (Fig. 7C). Interestingly, after PRL exposure, the B-to-A calcium flux vs. voltage curve nearly became a mirror image of the A-to-B curve. In other words, paracellular space of PRL-treated Caco-2 monolayer no longer exhibited rectification. Such PRL effects on the voltage-dependent paracellular calcium transport were completely abolished by 75 μmol/l LY-294002 (Fig. 7, A–C) and 1 μmol/l Y-27632 (Fig. 7, D–F), which were PI3K and ROCK inhibitors, respectively.

**DISCUSSION**

Although the cellular mechanism of PRL action was still not fully understood, a twofold increase in the intestinal calcium absorption both in vivo and in vitro induced by PRL suggested a significant role of this hormone as a regulator of calcium homeostasis in conditions of high circulating PRL levels, such as in pregnancy and lactation (5). In the present study, we provided further information on the cellular mechanism of PRL as well as the possible signaling pathways. Acting via PI3K and PKC\textsubscript{\textgamma}, PRL enhanced the transcellular active calcium transport by increasing calcium entry through Ca\textsubscript{1.3} and the basolateral calcium extrusion by PMCA. PRL also stimulated the voltage-dependent paracellular calcium transport through PI3K and ROCK.

The theoretical concept of the transcellular calcium transport in the intestine was recently challenged by the findings that calbindin-D\textsubscript{9k} knockout and TRPV6/calbindin-D\textsubscript{9k} double-knockout mice were normocalcemic and still exhibited active calcium absorption (1, 2, 27). We also found in this study that the rapid nongenomic effects of PRL on the transcellular active calcium transport in Caco-2 monolayer did not require the
The presence of TRPV6 or calbindin-D9k, although both transport proteins may contribute to the responses to other hormones, such as genomic 1,25(OH)2D3, 17β-estradiol, and glucocorticoids (19, 46). It appeared that redundancy in the mechanisms underlying calcium absorption may involve other calcium channel(s), such as L-type and T-type calcium channels, which could provide alternative route for apical calcium entry (9).

Supporting evidence was provided by recent reports that dihydropyridine (nifedipine)- and phenylalkylamine (verapamil)-sensitive L-type calcium channel Cav1.3 could mediate calcium entry for the transcellular calcium absorption, especially during ingestion of high-calcium diet (31, 34). Nifedipine and verapamil also inhibited the stimulatory effects of parathyroid hormone-related peptide, nongenomic 1,25(OH)2D3, and luminal glucose on intestinal calcium absorption (10, 34, 51). Our previous microarray study has identified several L-type calcium channels upregulated in the duodenal epithelial cells of the pituitary-grafted hyperprolactinemic rats (8). Here, the absence of PRL effect on calcium transport after Cav1.3 knockdown or nifedipine/verapamil exposure strongly suggested a physiological role of Cav1.3 during PRL stimulation.

Similar to the apical calcium entry, the cytoplasmic calcium translocation across the intestinal epithelial cells may be mediated by other calcium-binding proteins (CaBP) besides calbindin-D9k, e.g., calmodulin and parvalbumin, as well as vesicular calcium transport (3, 13). Interestingly, the duodenal epithelial cells of hyperprolactinemic rats were found to manifest a 17-fold increase in parvalbumin mRNA expression as demonstrated by microarray (8), but its physiological significance in calcium transport remains to be investigated. Regarding the basolateral calcium extrusion, 80–85% of calcium efflux normally occurs through PMCA, whereas the remaining is performed by NCX1 (18, 49). PRL was previously found to increase PMCA activity in purified basolateral membrane vesicles from the duodenum (6); therefore, PMCA was likely to mediate the PRL-enhanced calcium transport.

As for the paracellular pathway, the present results together with that reported by Thongon et al. (45) suggest that PRL augmented the paracellular calcium absorption in a concentration- and voltage-dependent manner. In the study of passive calcium absorption, PRL was found to alter the charge-selective property of tight junction without changing the pore size, thereby increasing calcium permeability and calcium flux through the paracellular route (44, 45). Our present investigation (Fig. 7) further suggests that the paracellular pores for calcium transport are rectifiers. In other words, the paracellular pores can conduct much larger A-to-B calcium flux at apical voltages positive than B-to-A calcium flux at apical voltage

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Fig. 7. Effects of PI3K inhibitor (75 μmol/l LY-294002) (A–C) and ROCK inhibitor (1 μmol/l Y-27632) (D–F) on the PRL-enhanced voltage-dependent paracellular calcium transport across Caco-2 monolayer (n = 5 per each voltage point; total 280 Snapwells used). The apical voltage was clamped to −25, −10, −5, 0, +5, +10, or +25 mV, with the basolateral side being a reference. Calcium fluxes in the apical-to-basolateral (A-to-B) (A and D) and basolateral-to-apical (B-to-A) (B and E) directions as well as net calcium fluxes (C and F) are presented. By using paired Snapwells, the net calcium flux was a subtraction of B-to-A flux from A-to-B flux. The mounted Snapwell was bathed on both sides with 1.25 mmol/l Ca2+ containing solution. *P < 0.01 rhPRL vs. control. †P < 0.01, value of the rhPRL-treated group vs. value of the rhPRL-treated group at −25 mV (for A-to-B and net flux) or +25 mV (for B-to-A). ‡P < 0.01 value of the control group vs. value of the control group at −25 mV (for A-to-B and net flux) or +25 mV (for B-to-A). Values of the control and rhPRL-treated groups in D, E, and F are from those in A, B, and C, respectively.
negative even in an absence of the transepithelial calcium gradient, rendering the degree of rectification \( J_{\text{B}} / J_{\text{A}} = 46.23/6.95 \approx 7 \) (where \( J \) represents calcium flux in mmol·h\(^{-1}\)·cm\(^{-2}\) at +25 mV or -25 mV; \( i \) and \( o \) denote A-to-B and B-to-A, respectively). The paracellular rectification could be explained by a model of a channel (or pore) with an energy barrier that impedes calcium movement in a B-to-A direction (for review see Ref. 41). Although the exact paracellular structures acting as the energy barrier have never been identified, we speculated that they might be tight junction proteins, claudins, which had their charge-selective extracellular loops protruding into the paracellular space (47). Several claudins, such as claudin-2, -3, and -12, are known to be involved in the intestinal calcium absorption (7, 15).

After exposure to PRL, the voltage-dependent calcium transport in both A-to-B and B-to-A directions were markedly increased, and the degree of rectification was reduced to ~1, suggesting that an energy barrier or cation-selective barriers of the paracellular pores were removed by PRL. Changes in the paracellular barrier properties could have resulted from the PRL-induced claudin phosphorylation (L. Nakkrasae and N. Charoenphandhu, unpublished observation, 2009). PRL-enhanced voltage-dependent paracellular calcium transport may be of physiological importance, presumably when the intestinal absorptive cells were exposed to nutrients that could alter the transepithelial potential difference, e.g., glucose, short-chain fatty acid, and neutral amino acids (14, 40, 48). Although the modulation of potential difference could also facilitate calcium transport via the transcellular pathway (11), the transcellular calcium transport was much smaller in magnitude when compared with the paracellular calcium transport, particularly in Caco-2 monolayer (7, 45). However, there was a disparity regarding the magnitude of paracellular calcium flux between different models. For example, in perfused intestinal loop, nifedipine inhibited calcium absorption in pregnant and lactating animals.

The action of PRL on calcium absorption involved the signaling molecules PI3K, PKC, and ROCK (20, 45). PI3K was likely to be the most upstream mediator, which transmitted PRL signals to PKC and ROCK for the transcellular and paracellular calcium transport, respectively (17, 21, 29, 45). In some cells, e.g., L6 muscle cells, PKC\(\varepsilon\) is a key molecule within the PI3K signal cascade (39). PKC is a large family of serine/threonine kinases, consisting of ~12 members (33). Some PKC isozymes, e.g., PKC\(\alpha\), PKC\(\beta\), and PKC\(\varepsilon\), but not PKC\(\varepsilon\), are activated by diacylglycerol (DAG), rendering them sensitive to DAG analog phorbol ester (i.e., PMA) (33). Several investigators provided evidence that activities of the transcellular calcium transport proteins TRPV6, Ca\(_{1.3}\), and PMCA could be modulated by PKA and/or PKC, especially PKC\(\beta\) (23, 24, 34). However, PKA and phorbol-sensitive PKCs (e.g., PKC\(\alpha\), PKC\(\beta\), and PKC\(\varepsilon\)) were not involved in PRL signaling, since PRL actions were blocked only by an inhibitor or siRNA of PKC\(\varepsilon\), which is a phorbol-insensitive isozyme (33). The presence of the additive effect of PRL in PMA-treated monolayer also suggested that PRL signaled through another signaling pathway that was not phorbol-sensitive PKCs. The absence of PRL-PMA synergism or truly additive effect, on the other hand, probably indicated that PKC\(\varepsilon\) and phorbol-sensitive PKCs shared some target proteins.

In addition to the calcium gradient-dependent calcium transport, PRL exerted its stimulatory effects on the voltage-dependent calcium transport via ROCK pathway. PRL was previously reported to use the Rho-associated pathway in endothelial cells (28), in which a GTP-bound RhoA activated the serine/threonine kinase ROCK. In T84 colonic adenocarcinoma cell monolayer, the presence of Rho/ROCK activities was essential for a decrease in TER and disassembly of apical junctional complex triggered by extracellular calcium depletion (42). However, it is currently unknown as to why the PRL-induced decrease in TER, which was closely related to the increased paracellular permeability, was not abolished by LY-294002 and Y-27632. It was possible that PRL may have other signaling molecules for modulation of paracellular properties. PKC\(\varepsilon\) might be not involved in the PRL-enhanced calcium transport via the paracellular pathway because PKC\(\varepsilon\) inhibitor was without effect on the PRL-induced decrease in TER.

In conclusion, we demonstrated that PRL stimulated the transcellular active calcium transport via Ca\(_{1.3}\) and PMCA, but not TRPV6, calbindin-D\(_{9k}\), or NCX1. Such PRL action was mediated by PI3K and phorbol-insensitive PKC\(\varepsilon\) in a non-genomic manner. Moreover, PRL removed the paracellular rectification and enhanced the voltage-dependent paracellular calcium transport via the PI3K and ROCK pathways. The present findings provide more detailed information on the cellular mechanisms of PRL in the intestinal epithelial cells, which could be used to explain how PRL enhances calcium absorption in pregnant and lactating animals.

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