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Prolactin stimulates the L-type calcium channel-mediated transepithelial calcium transport in the duodenum of male rats

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ABSTRACT

Elevated plasma levels of prolactin (PRL) have been reported in several physiological and pathological conditions, such as lactation, prolactinoma, and dopaminergic antipsychotic drug uses. Although PRL is a calcium-regulating hormone that stimulates intestinal calcium absorption in lactating rats, whether PRL is capable of stimulating calcium absorption in male rats has been elusive. Herein, the transepithelial calcium transport and electrical characteristics were determined in ex vivo duodenal tissues of male rats by Ussing chamber technique. We found that PRL receptors were abundantly present in the basolateral membrane of the duodenal epithelial cells. PRL (200–800 ng/mL) markedly increased the active duodenal calcium transport in a dose-dependent fashion without effect on the transepithelial resistance. The PRL-enhanced active duodenal calcium transport was completely abolished by L-type calcium channel blocker (nifedipine) as well as inhibitors of the major basolateral calcium transporters, namely plasma membrane Ca^{2+}-ATPase and Na^{+}/Ca^{2+} exchanger. Several intracellular mediators, such as JAK2, MEK, PI3K and Src kinase, were involved in the PRL-enhanced transcellular calcium transport. Moreover, PRL also stimulated the paracellular calcium transport in the duodenum of male rats in a PI3K-dependent manner. In conclusion, PRL appeared to be a calcium-regulating hormone in male rats by enhancing the L-type calcium channel-mediated transcellular and the paracellular passive duodenal calcium transport. This phenomenon could help restrict or alleviate negative calcium balance and osteoporosis that often accompany hyperprolactinemia in male patients.

1. Introduction

Hyperprolactinemia—a condition with elevated plasma prolactin (PRL) levels—occurs in male patients with PRL-producing tumors or chronic uses of dopaminergic antagonists (e.g., antipsychotic drugs) [1,2]. It has been reported that prolonged exposure to high PRL levels profoundly affects bone calcium metabolism, leading to massive bone resorption and osteoporosis [3,4]. Since high plasma levels of PRL during pregnancy and lactation have been found to markedly stimulate the duodenal calcium absorption [5], PRL itself might help restrict this bone loss by enhancing intestinal calcium uptake to supply extra calcium for deposition in bone. Although PRL has been reported to stimulate intestinal calcium absorption in vitamin D-deficient male rats [6], little is known whether PRL can act directly on the intestinal cells and then induces calcium absorption in male rats. Since PRL is known to enhance the conversion of inactive vitamin D_3 to 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$]—a potent genomic stimulator of intestinal calcium absorption [7], whether the intestine is a direct target of PRL action remains inconclusive. Thus, the ex vivo PRL exposure was used in the present study to exclude possible indirect effects of PRL.

Cellular mechanism of the PRL-stimulated calcium absorption in male rats remains elusive. Generally, calcium traverses the intestinal epithelium via both transcellular and paracellular pathways, the latter of which contributes up to 80% of the total calcium absorption especially when luminal calcium concentration is greater than 5 mM [8,9]. The capacity of $1,25(OH)_2D_3$-induced calcium absorption is gender-dependent with the rate in females being greater than in males [10]. The transcellular calcium transport begins with apical calcium uptake via the transient receptor potential vanilloid calcium channel (TRPV)-5, TRPV6 or voltage-dependent L-type calcium channel (Ca_v)-1.3, followed by cytoplasmic translocation in a calbindin-D_{9k}-bound form, and the basolateral extrusion via the plasma membrane Ca^{2+}-ATPase (PMCA)-1b and Na^{+}/Ca^{2+} exchanger (NCX)-1 [3,11].

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On the other hand, the paracellular calcium transport is regulated by the tight junction permselectivity, which is roughly represented by the transepithelial electrical resistance (TER) [12]. In lactating rats and intestinal epithelium-like Caco-2 monolayer, PRL has been shown to stimulate both transcellular and paracellular calcium transport by increasing the transport activity of Ca_v and tight junction permeability to calcium, respectively [5,13,14]. Although the intracellular signaling pathways of PRL in the intestinal epithelial cells of male rats had not been studied, they possibly involved the Janus kinase (JAK)-2, mitogen-activated protein kinase/extracellular signal regulated kinase (MEK), phosphoinositide 3-kinase (PI3K) and Src kinase, as previously reported in Caco-2 cells, endometrial cells, hepatocytes, and mammary cells [15–18].

Therefore, the objectives of the present study were to determine the stimulatory effects of PRL on the transepithelial calcium transport in the duodenum of male rats, and to elaborate its cellular mechanisms and signaling pathways. The duodenum was used in this study because, compared to other small intestinal segments, it is regarded as the efficient site of transcellular and paracellular calcium transport [11].

2. Materials and methods

2.1. Animals

Eight-week-old male and female Sprague–Dawley rats (weighing 200–300 g) were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. Animals were housed in hanging stainless steel cages at 25 ± 2 °C and 55 ± 5% humidity under 12:12-h light–dark cycle. All rats were fed standard chow and reverse osmosis water ad libitum. This study has been approved by the Institutional Animal Care and Use Committee of the Faculty of Science, Mahidol University.

2.2. Experimental design

The expression and localization of PRL receptor proteins were first determined in the duodenum of male rats by immunohistochemistry. Duodenal calcium fluxes and epithelial electrical parameters were measured by the Ussing chamber technique. In Ussing chamber, the mounted duodenal tissue was directly exposed on the basolateral side to 50–1000 ng/mL PRL (catalog No. L6520; Sigma) during a 70-min experiment. Transepithelial calcium fluxes determined in the present study were the transcellular active calcium transport and paracellular passive transport. Since the dose–response study showed that 200 ng/mL PRL was the most efficient dose for stimulating duodenal active calcium transport in male rats, this concentration was used in the subsequent experiments. To identify the transporters responsible for the transcellular calcium transport, duodenal tissues of male rats were incubated in Ussing chamber with 200 ng/mL PRL plus L-type calcium channel blocker (1–10 μM nifedipine; catalog No. 1075; Tocris Bioscience), PMCA inhibitors [10–1000 μM vanadate (catalog No. P0758; New England Biolabs) and 50–200 μM trifluoperazine (TFP; Sigma)], specific NCX1 inhibitor (30–70 μM KB-R7943; catalog No. 420336; Calbiochem), or KB-R7943 (30 and 70 μM) plus 100 μM vanadate. Nifedipine was added in the apical solution, whereas vanadate, TFP and/or KB-R7943 were added in the basolateral solution.

To identify possible PRL signaling pathways, 200 ng/mL PRL plus JAK2 inhibitor (50 μM AG490; catalog No. 658401; Calbiochem), MEK inhibitor (30 μM U0126; A.G. Scientific), PI3K inhibitor (75 μM LY294002; catalog No. 1130; Tocris Bioscience) or Src kinase inhibitor (10 μM SU6656; catalog No. S9692; Sigma) were added in the basolateral solution. In the last experiment, the paracellular calcium fluxes in the duodenum of male and female rats were determined after direct exposure to PRL or PRL plus 75 μM LY294002. The PRL concentration of 600 ng/mL, the most effective dose in female rats [19], was used to stimulate the duodenum of female rats in the present study.

2.3. Surgery and tissue preparation

Rats were anesthetized by 40 mg/kg sodium pentobarbitone i.p. (Ceva Santé Animale). Thereafter, 1.5-cm median laparotomy was performed. A duodenal segment (0–5 cm from the pylorus) was dissected, and was then subjected to histological examination and calcium flux measurement. For the calcium flux study, the duodenal segments were cut longitudinally to expose the mucosal surface and were mounted between the two halves of Ussing chamber.

2.4. Measurement of transepithelial electrical resistance (TER)

As described previously [5,19], duodenal tissue was mounted in Ussing chamber filled on both sides with solution containing (in mM) 118 NaCl, 4.7 KCl, 1.1 $MgCl_2$, 1.25 $CaCl_2$, 23 $NaHCO_3$, 12 D-glucose, 2.5 L-glutamine, and 2 mannitol at 37 °C. The solution was continuously aerated with humidified 5% CO_2 in 95% O_2. In Ussing chamber setup, a pair of salt bridges (2 M KCl and 2% wt/vol agar) was placed near each surface of the mounted duodenal tissue for measurement of transepithelial potential difference (PD), while another pair of salt bridges was placed at both ends of the hemi-chambers to provide the short-circuit current (Isc). Each Ag/AgCl half-cell connected a salt bridge to a preamplifier or direct current-generating unit (model ECV-4000; World Precision Instrument). PowerLab/4SP operated with Chart version 5.4.1 (ADInstruments) was used for digital recording. TER was calculated form PD and Isc by Ohm's equation.

2.5. Measurement of transepithelial calcium flux

As described previously [5,19], the tissue was first equilibrated in the chamber with physiological bathing solution for 30 min. Thereafter, the apical chamber was filled with bathing solution containing radioactive ^{45}Ca (final specific activity of 360 mCi/mol; catalog No. NEZ013; PerkinElmer). Unidirectional calcium flux ($J_{H\rightarrow C}$; nmol h^{-1} cm^{-2}) from the hot side (H; apical side) to cold side (C; basolateral side) was calculated by Eqs. (1) and (2).

$$J_{H\rightarrow C} = R_{H\rightarrow C}/(S_H \times A) \quad (1)$$

$$S_H = C_H/C_{To} \quad (2)$$

where $R_{H\rightarrow C}$ is the rate of tracer appearance in the cold side (cpm h^{-1}); S_H is the specific activity in the hot side (cpm $nmol^{-1}$); A is the surface area of the tissue (cm^2); C_H is the mean radioactivity in the hot side (cpm); and C_{To} is the total calcium content in the hot side (nmol). Radioactivity of ^{45}Ca was analyzed by liquid scintillation spectrophotometer (model Tri-Carb 3100; Packard). Total calcium concentration in the bathing solution was analyzed by an atomic absorption spectrophotometer (model SpectrAA-300; Varian Techtron). In the absence of transepithelial calcium gradient (i.e., bathing solution in both hemi-chambers contained the same calcium concentration of 1.25 mM), the measured calcium flux represented active calcium transport. The calcium gradient-dependent paracellular passive fluxes were measured by determining transepithelial calcium fluxes in the presence of various apical calcium concentrations, i.e., 1.25, 2.5, 5, 10, and 20 mM.

2.6. Immunohistochemistry

Tissues were preserved overnight at 4 °C in 0.1 M phosphate-buffered saline containing 4% paraformaldehyde prior to paraffin embedding. The sections were incubated at 4 °C overnight with 1:50 rabbit polyclonal primary antibody against PRL receptors (catalog No. sc-30225; Santa Cruz Biotechnology). After washing, the sections were incubated for 1 h with 1:500 biotinylated goat anti-rabbit IgG (Santa Cruz Biotechnology) followed by streptavidin-conjugated horseradish peroxidase solution (Zymed) and 3,3′-diaminobenzidine chromogen (Pierce). As for the negative controls, the sections were incubated with blocking solution in the absence of PRL receptor primary antibody. Finally, all sections were counterstained with hematoxylin and examined under a light microscope.

2.7. Statistical analyses

The results are expressed as means ± SE. Comparisons between the two sets of data were performed by unpaired Student's *t*-test. One-way analysis of variance with Dunnett's post-test was used for multiple sets of data. The level of significance was $p < 0.05$. All statistical tests were analyzed by GraphPad Prism 5.

3. Results

3.1. PRL receptors were abundantly expressed in the duodenal epithelial cells of male rats

As shown in Figs. 1A–B, the absorptive cells in the duodenal villi—the principal sites of calcium absorption [20]—abundantly expressed PRL receptor proteins. Most PRL receptor signals were localized in the basolateral regions of the epithelial cells (arrows). Some positive signals were also observed in the villous core, presumably in the vascular endothelial cells. The negative control sections that were not incubated with primary antibody against PRL receptor showed no positive signals (Fig. 1C). These immunohistochemical data indicated that the duodenal absorptive cells of male rats were direct targets of PRL action.

3.2. PRL markedly stimulated the transcellular and paracellular calcium transport

A direct exposure to 100–800 ng/mL PRL, but not 50 or 1000 ng/mL PRL, significantly increased the active calcium flux across the duodenal epithelium of male rats (Fig. 1D). Since 200 ng/mL PRL produced the maximal response, this concentration was used in subsequent experiments. Although 50–1000 ng/mL PRL did not alter the duodenal TER (Fig. 2A), an indicator of tight junction permeability, 200 ng/mL PRL markedly enhanced the paracellular calcium transport (Fig. 2B).

3.3. The PRL-enhanced active calcium flux was a Ca_v-mediated transcellular process dependent on PMCA and NCX

As shown in Fig. 3, the 200 ng/mL PRL-enhanced active calcium flux was totally abolished by L-type calcium channel blocker (10 μM nifedipine), suggesting that the apical calcium uptake under PRL stimulation occurred solely through Ca_v. Both PMCA inhibitor (200 μM TFP) and specific NCX inhibitor (70 μM KB-R7943) completely diminished the PRL-enhanced active calcium flux (Fig. 3C). Vanadate (100 and 1000 μM), which could inhibit PMCA and Na^+/K^+-ATPase [21–23], as well as vanadate plus KB-R7943 also blocked the duodenal response to PRL (Fig. 3C). The results thus indicated that PRL directly stimulated the Ca_v-mediated transcellular calcium transport in the duodenum of male rats.

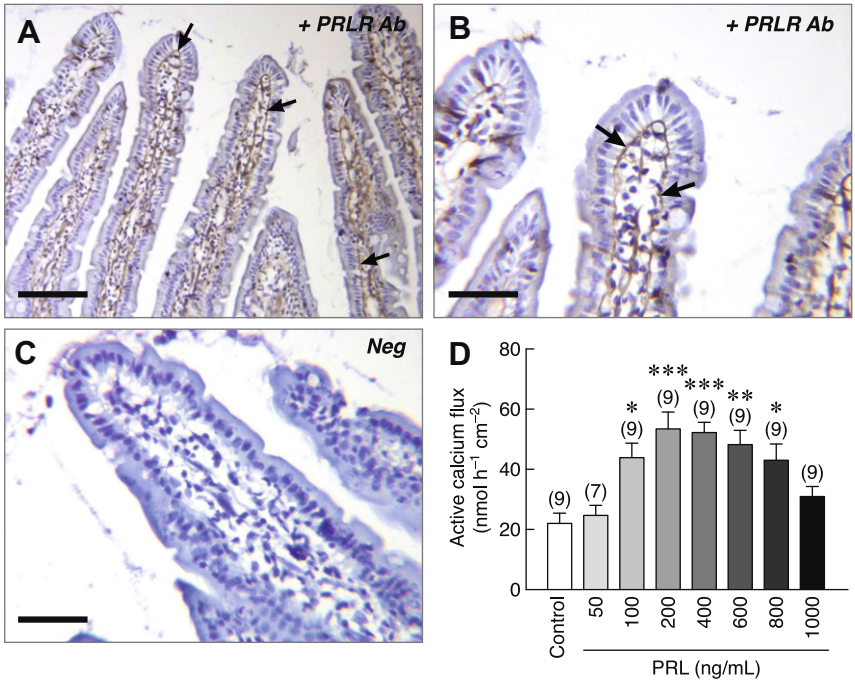

Fig. 1. Representative immunohistochemical photomicrographs of PRL receptor protein expression (+PRLR Ab) in the duodenal villi of male rats at 200× (scale bar, 200 μm; A) and 400× magnifications (scale bar, 100 μm; B). The positive brownish signals of PRL receptor proteins (arrows) are localized predominantly in the basolateral membrane of villous absorptive cells. The negative control (Neg) shows no brownish signal (400× magnification; scale bar, 100 μm; C). (D) The duodenal active calcium flux in male rats after direct exposure to 50, 100, 200, 400, 600, 800 or 1000 ng/mL PRL. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the control group. Numbers in parentheses represent the numbers of animals.

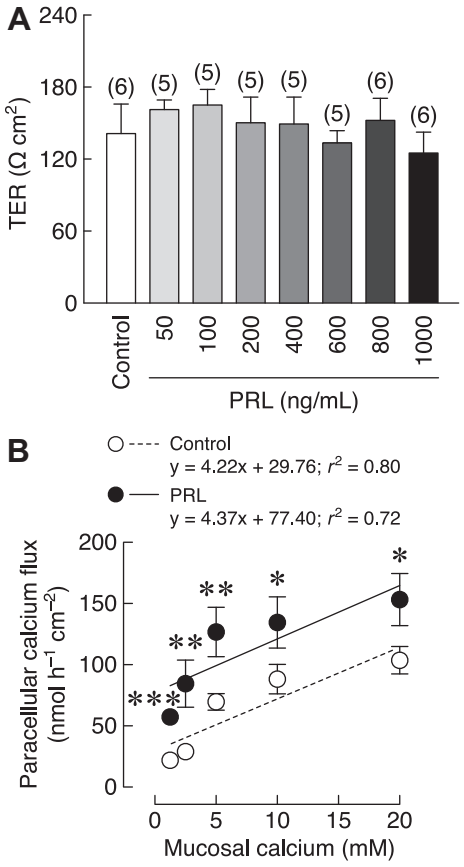

Fig. 2. (A) TER of the duodenal epithelia of male rats after direct exposure to 50–1000 ng/mL PRL. Numbers in parentheses represent the numbers of animals. (B) Paracellular calcium flux in the duodenum of male rats in the absence (control) or presence of 200 ng/mL PRL. The duodenal tissue was bathed on the apical side with various calcium concentrations (1.25, 2.5, 5, 10 or 20 μM; n = 6–14 per each calcium concentration). Linear regression lines, equations, and coefficients of determination (r^2) are also presented. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the corresponding control group.

3.4. The PRL-enhanced calcium transport was mediated by JAK2, MEK, PI3K and Src kinase

To identify the signaling pathways of PRL in the duodenal epithelial cells, various inhibitors of kinases were used in this series of experiments. The results showed that the PRL-stimulated transcellular calcium transport was completely abolished by inhibitors of JAK2 (50 μM AG490), MEK (30 μM U0126), PI3K (75 μM LY294002), and Src kinase (10 μM SU6656) (Fig. 4A). Moreover, as depicted in Fig. 4B, 75 μM LY294002 also inhibited the PRL-enhanced paracellular calcium transport across the duodenal epithelia of male and female rats (apical calcium concentration of 10 μM). However, in both male and female rats, the duodenal epithelia of control and PRL-treated groups showed comparable electrical parameters, either PD (male 6.50 ± 1.02 vs. 6.72 ± 1.86 mV; female 6.68 ± 2.15 vs. 6.56 ± 1.44 mV) or Isc (male 51.05 ± 8.03 vs. 48.41 ± 10.72 μA/cm^2; female 55.39 ± 21.68 vs. 66.15 ± 14.48 μA/cm^2).

4. Discussion

Although PRL has been established as an important regulator of the intestinal calcium transport in pregnant and lactating rats [3], whether PRL could directly stimulate the intestinal calcium absorption in male rats has long been enigmatic. Herein, we have provided corroborative evidence that the duodenal absorptive cells of male rats directly responded to PRL in a dose-dependent manner. After an acute exposure to PRL in the Ussing chamber, duodenum increased the rate of both transcellular and paracellular calcium transport. Interestingly, these findings suggested that the hyperprolactinemia-induced increase in the intestinal calcium absorption actually provided extra calcium, which could help lessen bone loss due to the PRL-induced bone resorption in hyperprolactinemic individuals [2].

Since the L-type calcium channel blocker nifedipine completely abolished the PRL-stimulated calcium transport, the present nongenomic transcellular mechanism was dependent on the apical Ca_v, which was distinct from the actions of $1,25(OH)_2D_3$ and estrogen that stimulated the TRPV5/6-dependent calcium absorption [9]. In a previous study using the intestinal epithelium-like Caco-2 monolayer, it was found that double knockdown of TRPV5 and TRPV6 did not abolish the PRL-stimulated calcium transport, whereas $Ca_v1.3$ knockdown and TRPV5-TRPV6-$Ca_v1.3$ triple knockdown did [13]. Since the opening of the apical Ca_v is generally triggered by the presence of luminal glucose that enters the intestinal cells with Na^+ through sodium-dependent glucose transporter (SGLT)-1, and in doing so depolarizes the apical plasma membrane [11], the PRL-stimulated calcium transport should be robust during the postprandial period. Therefore, calcium supplementation with diet, rather than between meals, might best benefit for hyperprolactinemic male patients with bone loss. Nevertheless, TRPV6 may be important for the genomic action of PRL since it was reported that long-term exposure to PRL could upregulate TRPV6 expression in the intestinal cells of female rats [5].

Regarding the basolateral calcium extrusion, both PMCA and NCX contributed to the PRL-stimulated transcellular calcium transport. Since an inhibition of one of the two basolateral transporters by either 200 μM TFP or 70 μM KB-R7943 totally diminished the PRL-enhanced calcium flux, PMCA and NCX may function in an interdependent manner. Specifically, it was possible that inhibition of either PMCA or NCX raised the intracellular calcium concentration, which in turn decreased the apical calcium uptake by a mechanism known as calcium-dependent inactivation [24], thereby leading to a decrease in the basolateral calcium efflux even though the other transporter was still functional. This phenomenon would provide protection against calcium toxicity and apoptosis secondary to elevated intracellular calcium concentration [25].

To activate the transcellular calcium transport, PRL first bound to the basolateral PRL receptors, which in turn signaled through several intracellular mediators. We herein found that the PRL-stimulated transcellular calcium transport was mediated by JAK, MEK, PI3K and Src kinase. Involvement of the multiple signaling cascades was not uncommon since many calcium-transporting proteins with a variety of domains for modification and phosphorylation are required for the transcellular calcium transport process. Indeed, these kinases have been reported to mediate PRL signaling in other cell types, such as hepatocytes, endometrial cells, mammary cells, Caco-2 cells [15–18]. Acosta et al. [18] provided evidence that, in the PRL-exposed T47D and MCF7 breast cancer cells, Src kinase was an upstream activator of both MEK and PI3K. Nevertheless, further investigation is required to demonstrate in depth the signal transduction of PRL in the duodenal epithelial cells.

Besides the transcellular calcium transport, PRL also enhanced the paracellular calcium movement across the duodenal epithelium of male rats in a PI3K-dependent manner. No change in the duodenal TER suggested that PRL did not simply widen the tight junctions to enhance the paracellular ion movement. Rather, overexpression or phosphorylation of some proteins in the claudin family (e.g., claudin-2, -12 and -15) can selectively increase tight junction permeability to calcium without tight junction widening

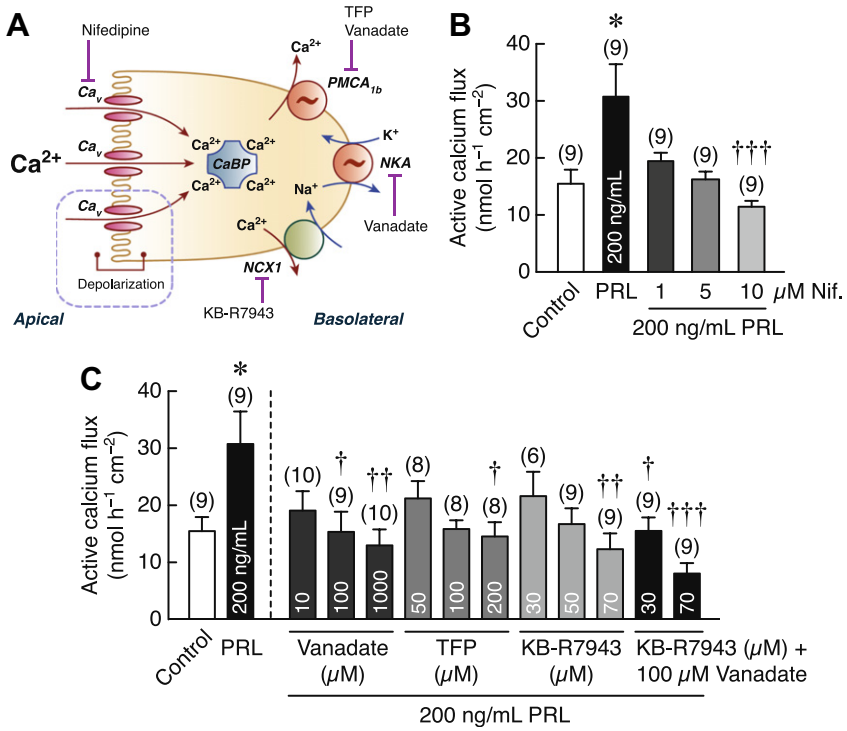

Fig. 3. (A) A diagram shows the voltage-dependent L-type calcium channel (Ca_v)-mediated transcellular calcium transport, which requires the apical Ca_v, calbindin-D_{9k} (CaBP), Na^+/Ca^{2+} exchanger (NCX)-1, and plasma membrane Ca^{2+}-ATPase (PMCA)-1b. Depolarization of the apical membrane is necessary for the activation of Ca_v. The NCX activity is dependent on Na^+/K^+-ATPase (NKA) that maintains sodium gradient across the basolateral membrane. Therefore, vanadate that can inhibit NKA also diminishes NCX activity. Inhibitors used in this study are also presented in the figure. (B) Active calcium flux in the duodenum of male rats after direct exposure to L-type calcium channel blocker [1, 5 and 10 μM nifedipine (Nif.)] or (C) various concentrations of PMCA inhibitors (vanadate and TFP), NCX inhibitor (KB-R7943), or KB-R7943 + 100 μM vanadate in the presence of 200 ng/mL PRL. $^*p < 0.05$ compared to the control group. The control and 200 ng/mL PRL data in panel *B* were reused in panel *C*. $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ compared to the PRL-treated group. Numbers in parentheses represent the numbers of animals.

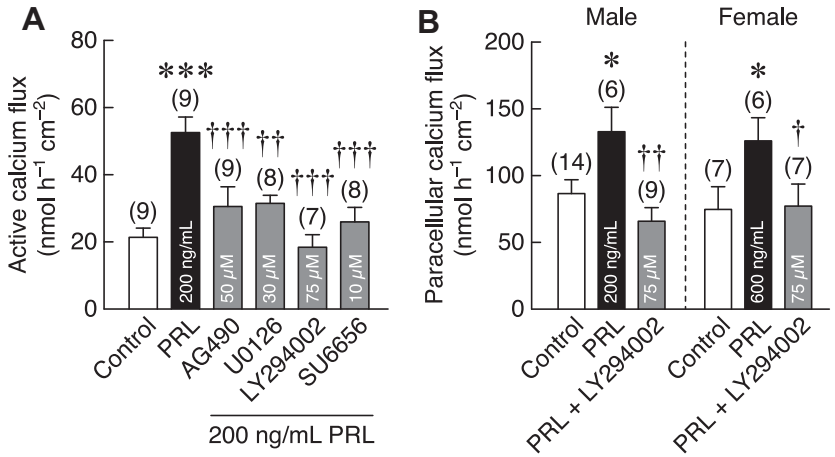

Fig. 4. (A) Active calcium flux in the duodenum of male rats after direct exposure to JAK2 inhibitor (50 μM AG490), MEK inhibitor (30 μM U0126), PI3K inhibitor (75 μM LY294002), or Src kinase inhibitor (10 μM SU6656) in the presence of 200 ng/mL PRL. (B) Paracellular calcium flux in the duodenum of male and female rats after a direct exposure to 200 ng/mL PRL plus 75 μM LY294002. $^*p < 0.05$, $^{***}p < 0.001$ compared to the control group. $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ compared to the PRL-treated group. Numbers in parentheses represent the numbers of animals.

[5]. We recently showed that serine phosphorylation of the tight junction protein claudin-15 was essential for the PRL-enhanced paracellular calcium transport in Caco-2 monolayer [5].

Interestingly, since an approximately 1.5-fold increase in the paracellular calcium flux (apical calcium concentration of 10 mM) was observed when the duodenal tissues of male and female rats were exposed to 200 and 600 ng/mL PRL, respectively, the duodenal cells of male rats appeared to be more sensitive to the acute action of PRL than those of female rats. Similarly, the duodenal cells of male rats responded to 200 ng/mL PRL by increasing the transcellular calcium flux, whereas the duodenal cells of females required 600–800 ng/mL PRL [19]. In addition, the typical biphasic response to PRL was also observed in male rats as 100–800 ng/mL PRL induced a positive effect on the duodenal calcium transport, whereas extremely low or high PRL concentrations of 50 and 1000 ng/mL, respectively, were without effect (Fig. 1D), consistent to that reported in chondrocytes [26]. This biphasic phenomenon could be explained by the fact that low-to-moderate PRL

concentrations allow formation of functional PRL receptor dimer with 1:2 ligand/receptor stoichiometry, whereas excess PRL ligands occupy most PRL receptors leading to non-functional 1:1 ligand/receptor complexes, which markedly reduce downstream PRL signal transduction [27].

In conclusion, the duodenal absorptive cells of male rats strongly expressed PRL receptor proteins in the basolateral membrane, and responded to the rapid non-genomic action of PRL by increasing the transcellular and paracellular calcium transport, the former of which appeared to depend on the nifedipine-sensitive Ca_v as well as the basolateral PMCA and NCX. Several intracellular mediators, namely JAK2, MEK, PI3K and Src kinase, signaled the stimulatory effect of PRL on the Ca_v-mediated transcellular calcium transport. Moreover, PRL also used PI3K to enhance the paracellular duodenal calcium transport in both male and female rats. Because PRL stimulated the intestinal calcium absorption, oral calcium supplementation to hyperprolactinemic male patients could counterbalance the negative effects of PRL on bone calcium metabolism.

Acknowledgments

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