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Prolactin directly stimulated the solvent drag-induced calcium transport in the duodenum of female rats

Chaiyot Tanrattana, Narattaphol Charoenphandhu, Liangchai Limlomwongse, Nateetip Krishnamra*

Department of Physiology, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand

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Abstract

Prolactin has been reported to stimulate the calcium absorption of the duodenum where three components of the active calcium transport, namely transcellular active, voltage-dependent and solvent drag-induced calcium transport, were identified. It was known that the transcellular active, but not the voltage-dependent, duodenal calcium transport was directly stimulated by prolactin. The present study thus aimed to evaluate the direct action of prolactin on the solvent drag-induced duodenal calcium transport by using the Ussing chamber technique. The jejunum was used as a reference for the existence of solvent drag and the widening of tight junction induced by cytochalasin E. Results showed that the solvent drag-induced calcium transport existed in both intestinal segments, but the magnitude was significantly greater in the duodenum (29.27±2.27 vs. 17.31±1.65 nmol h^{-1} cm^{-2}, $P<0.001$). We further demonstrated that 200, 600 and 800, but not 1000 ng/ml, prolactin significantly promoted the solvent drag-induced duodenal calcium transport in a dose–response manner, i.e. from the control value of (nmol h^{-1} cm^{-2}) 24.31±2.36 to 45.42±3.47 ($P<0.01$), 63.82±5.28 ($P<0.001$) and 53.93±5.41 ($P<0.01$), respectively. However, prolactin did not manifest any effect on the jejunum. Because the paracellular transport was suggested to be size-selective as well as charge-selective, further experiments were designed to evaluate the mechanism by which prolactin stimulated the solvent drag-induced calcium transport. The duodenum was exposed to 20 μM cytochalasin E, 600 ng/ml prolactin or the combination of both in the presence of a paracellular marker ^{3}H-mannitol, while the jejunum was a positive reference. The results showed that, in the jejunum, cytochalasin E alone and cytochalasin E plus prolactin significantly increased the mannitol fluxes from (μmol h^{-1} cm^{-2}) 0.29±0.04 to 0.49±0.03 ($P<0.05$) and 0.48±0.05 ($P<0.05$), respectively, while having no effect on the calcium fluxes. Prolactin alone had no effect on the jejunal calcium flux. In the duodenum, neither mannitol nor calcium fluxes were enhanced by cytochalasin E, however, prolactin still increased the solvent drag-induced calcium flux from 27.74±2.41 to 51.03±4.35 nmol h^{-1} cm^{-2} ($P<0.001$). It was concluded that prolactin directly stimulated the solvent drag-induced duodenal calcium transport in a dose–response and biphasic manner without the widening of tight junction.

Keywords: Calcium transport; Duodenum; Prolactin; Solvent drag; Tight junction

1. Introduction

Prolactin has been demonstrated to be a novel calcium-regulating hormone during pregnancy and lactation [1–5]. Both conditions place a stress on maternal calcium metabolism as a result of a high calcium loss for the intrauterine fetal development and lactogenesis. Because 1,25 $(OH)_2D_3$, a putative calcium-regulating hormone, does not contribute to an increase in the intestinal calcium absorption during these periods [6,7], the enhanced calcium absorption should be under the influence of other calcium-regulating hormones, particularly prolactin [8–11]. The physiological significance of prolactin has recently been demonstrated by our finding which showed reductions in the intestinal calcium absorption and vertebral mineral density in growing rats during bromocriptine-induced suppression of endogenous prolactin [2].

* Corresponding author. Tel./fax: +66 2 246 1375.
E-mail addresses: naratt@naratt.com (N. Charoenphandhu), scnks@mahidol.ac.th (N. Krishnamra).

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The acute effect of prolactin on the intestinal calcium absorption was shown in pregnant and lactating rats by Krishnamra et al. [12]. Moreover, the elevated duodenal calcium absorption of pregnant and lactating rats was found to correlate with changes in the plasma level of prolactin [13]. Generally, the small intestine has two mechanisms of calcium transport, known as gradient-dependent passive transport and metabolically energised active calcium transport [14,15]. It is apparent that the duodenum possesses both types of transport, whereas the remaining parts possess mostly the passive transport [16–18]. Duodenum is a site where calcium absorption is tightly controlled, especially during increased calcium requirement [13,17,18]. To gain further insight into the effects of prolactin on each mechanism, our pioneer investigations provided evidence that 0.2 mg/kg prolactin injected intraperitoneally (i.p.) enhanced the passive calcium transport by in situ perfused small intestine [11] and the active calcium transport by in vitro everted intestinal sacs [19] within an hour. Its effect was confined to the duodenum and the proximal jejunum [11] where highly expressed prolactin receptors were reported [20,21]. We also showed that both exogenous and endogenous prolactin significantly enhanced the gradient-dependent passive calcium transport in the small intestine of female rats [2,11].

In the absence of a calcium gradient, the metabolically energised active calcium transport could be measured [16,22], and its three fractions, namely transcellular active, solvent drag-induced- and voltage-dependent calcium transport, could be studied separately [3]. Transcellular active calcium transport is a primarily active process composed of apical facilitated calcium entry, cytoplasmic calcium translocation and basolateral Ca^{2+}-ATPase-dependent calcium extrusion [15,23]. In 2001, Charoenphandhu et al. [3] elucidated that prolactin directly and acutely stimulated the transcellular active calcium transport in a dose–response manner.

The solvent drag-induced and voltage-dependent calcium transports are secondary to the transcellular active sodium transport which produces a convective water flow [17,24–26] and an electrodiffusive force from transepithelial potential difference (PD) [27–29], respectively, for paracellular calcium movement. In the duodenum, the voltage-dependent mechanism is presumptive but has been reported to be negligible and could be considered absent under both control and prolactin-exposed conditions [3]. However, the presence and importance of the solvent drag-induced duodenal calcium transport has not been studied, and the direct action of prolactin on this component of calcium transport has not been investigated yet.

The perijunctional actomyosin-regulated widening of the tight junction has been postulated to increase the tight junction permeability as well as the solvent drag [30,31]. Some inert chemicals such as mannitol, inulin and polyethyleneglycol were utilised to determine the widening of the tight junction [30–33]. Nevertheless, a growing number of tight junction-associated proteins have been reported, and the tight junction was evinced to share physiological properties with conventional charge-selective ion channels [34–36]. The regulation of solvent drag through tight junction may, therefore, not solely be regulated by the widening of the paracellular pores. We hypothesised that, under the influence of prolactin, the widening of tight junction was not sufficient to enhance the solvent drag-induced duodenal calcium transport. To prove the hypothesis, we administered cytochalasin E to induce the widening of duodenal tight junction. The jejunum was used as a reference because changes in the permeability of tight junction have been intensively studied in the rat jejunum [11,31,37].

The principal objectives of the present study were to elucidate that (1) the solvent drag-induced calcium transport in the duodenum was present and significant; (2) prolactin, as a calcium-regulating hormone, directly stimulated the solvent drag-induced duodenal calcium transport; and (3) widening of the tight junction was not required for the stimulatory effects of prolactin.

2. Materials and methods

2.1. Animals

Sexually mature female Wistar rats weighing 200–250 g were obtained from the Animal Centre of Thailand, Salaya Campus, Mahidol University. They were placed in hanging stainless steel cages, fed with standard laboratory pellets (C.P., Bangkok, Thailand) and tap water ad libitum under a 12:12 h light/dark cycle for at least 14 days before the start of the experiments. The room temperature was controlled at 23–25 °C, and relative humidity was about 50–60%. Food pellets contained 1.4% calcium and 0.9% phosphorus. All animals were cared for in accordance with the principles and guidelines of the Laboratory Animal Ethical Committee of Mahidol University, Bangkok, Thailand.

2.2. Tissue preparation

The technique was based on the methods of Charoenphandhu et al. [3]. Some parts were modified from the methods of Carroll et al. [38] and Karbach [17]. Under 60 mg/kg i.p. sodium pentobarbitone (Nembutal®, Abbott Laboratory, North Chicago, IL) anesthesia, a median laparotomy was performed. Either a duodenal or a jejunal segment was obtained 2–10 and 15–25 cm distal to the pyloric sphincter, respectively. After being rinsed in an ice-cold bathing solution pregassed with 5% CO_2 in 95% O_2 (TIG Ltd., Bangkok, Thailand), a longitudinal incision was made along the radix mesenterii. The dissected segment was rinsed again in the bathing solution to remove the luminal contents. An intestinal segment from a rat was then mounted in a modified Ussing chamber with an exposed surface area

of 0.69 cm^2 and bathed on both sides with 3 ml of buffer maintained at 37 °C and continuously aerated with 5% CO_2 in 95% O_2. The tissue was firmly affixed to the chamber by using waterproof adhesive silicone (Bayer, Germany). Gas bubbles were fine but vigorous to minimize the unstirred water layer and to provide good mixing [39]. The tissue was incubated for 20 min before the 40–60 min experiment was carried out.

2.3. Bathing solution

The bathing solution contained (in mM): 118 NaCl, 4.7 KCl, 1.1 $MgCl_2$, 1.25 $CaCl_2$, 23 $NaHCO_3$, 12 D-glucose, and 2 mannitol. The solution was continuously gassed with humidified 5% CO_2 in 95% O_2, and had a pH of 7.4 and osmolality of 298 mosM. At the end of each experiment, the osmolality of the bathing solution was verified to ascertain that its physiological optimum was maintained [3].

2.4. Electrical measurements

Electrical properties of the intestinal segments were for monitoring the viability and integrity of the tissue. The transepithelial PD was continuously measured whereas the values of the short-circuit current (Isc), needed to nullify the PD, were measured every 10 min. Epithelial resistance (R) was calculated by Ohm's equation [27]. The proximal ends of agar bridges made of 4 M KCl per 4 g% agar were located near each surface of the tissue to measure the PD. The distal ends of the bridges were placed in two separate flasks, containing 4 M KCl and calomel electrodes (No. 476350, Corning, USA) which were in turn connected to a Grass Polygraph (Model 79, Grass Instrument, Quincy, MA). At the distant end of each hemichamber, a platinum electrode (BDH Laboratory Supplies, UK) was placed to supply the Isc. Each platinum electrode was connected to a constant direct current (DC) generator (Thaiphan Electrics, Bangkok, Thailand). The values of Isc were displayed by a computer-assisted micromultimeter (Yokogawa model 7551, Japan). The normal values of PD, R and Isc were 5.52±0.21 mV, 70.11±4.02 Ω cm^2 and 80.10±5.90 μA cm^{-2} (n=10), respectively. At the end of each experiment, D-glucose was added to the mucosal solution to give a final concentration of 30 mM to confirm tissue viability [40]. Setups which had abnormal electrical parameters were discarded.

2.5. Unidirectional flux measurement

After 20 min incubation, the bathing solution was replaced with a fresh bathing solution. One side was ^{45}Ca-containing solution (initial specific activity of 2 mCi/ml, Radiochemical Centre, Amersham International, UK) while the other side contained ^{45}Ca-free bathing solution. A 100 μl sample was collected from each side into separate micro-centrifuge tubes to determine the calcium flux. Seven samples were collected per setup. Radioactivity of ^{45}Ca was analysed by liquid scintillation spectrophotometry (LKB-Wallac model 1219, LKB Wallac, Finland). Total calcium concentration of the bathing solution was analysed by an atomic absorption spectrophotometry (SpectrAA-300, Varian Techtron Ltd., Springvale, Australia). Both unidirectional calcium fluxes (nmol h^{-1} cm^{-2}), i.e. mucosa-to-serosa and serosa-to-mucosa fluxes, were calculated by using the following equations [41]:

$$\text{Flux}(\text{nmol h}^{-1})\ \text{cm}^{-2} = \frac{\text{rate of tracer appearance in cold side}(\text{cpm h}^{-1})}{\text{specific activity of hot side}\left(\text{cpm nmol}^{-1}\right)\text{area}\times(\text{cm}^2)} \quad (1)$$

$$\text{specific activity}(\text{cpm nmol}^{-1}) = \frac{\text{mean of radioactivity in hot side}(\text{cpm})}{\text{total calcium in hot side}\times 10^{-6}} \quad (2)$$

In some experiments, the ^3H-mannitol was used for measuring unidirectional fluxes (μmol h^{-1} cm^{-2}) of mannitol, which was a paracellular marker for the widening of tight junction [31,32,42]. In this case, transepithelial movements of calcium and mannitol were measured simultaneously. Unidirectional fluxes of mannitol were also calculated from Eq. (1).

2.6. Experimental protocols

2.6.1. Protocol 1

This study was performed to validate the presence of the solvent drag-induced calcium transport in the duodenum of rats. Both sides of a duodenal segment were exposed to the aforementioned bathing solution for 20 min prior to the experimental period. Electrical parameters were tightly monitored as previously described. Samples were withdrawn from both sides of the chamber to analyse for radioactivity of ^{45}Ca. To manifest the solvent drag-induced calcium transport, 0.1 mM trifluoperazine or TFP (ICN Biomedicals Inc., Ohio, USA) was constantly present in the serosal side to inhibit the transcellular active calcium transport [3,16,43,44]. TFP was first dissolved in 50% dimethylsulfoxide or DMSO (ICN Biomedicals) prior to dilution with the bathing solution to give a final concentration of 0.1 mM. Final concentration of DMSO of 0.05% had effect on neither tissue viability nor paracellular transport [45]. The unidirectional calcium flux (nmol h^{-1} cm^{-2}) was calculated and plotted against the sampling intervals (min) to obtain a flux (Eq. (3)) and flux ratio equation (Eq. (4)) which was originally derived by Ussing in 1949 [46].

$$J = J_{\max}\left(1-\exp^{(xt)}\right) \quad (3)$$

$$\frac{J_{ms}(t)}{J_{sm}(t)} = \frac{C_m}{C_s}\exp\left(\frac{N_A W}{RT}\right) \quad (4)$$

where J denoted the unidirectional calcium fluxes in either mucosa-to-serosa (ms) or serosa-to-mucosa (sm) direction at time t. J_{max} was the maximal calcium flux in respective direction. C was the concentration of calcium in mucosal (m) or serosal (s) compartment. N_A was the Avogadro's number. W was the work done on calcium ions moving through the studied pathway. R and T had their conventional meaning. x was a constant.

According to Ussing's theory, the pre-steady state and steady state flux ratio of a particular ion via a single pathway, either paracellular or transcellular, must be independent of time or time-invariant, as shown in Eq. (4) [29]. In other words, if each value of flux ratio at any time was not a single constant, the analysed system must have at least two pathways for ion transport [47,48].

2.6.2. Protocol 2

This study was to demonstrate the significance of the solvent drag-induced calcium transport in the rat duodenum. Calcium transport of the duodenum and jejunum were compared since the significance of solvent drag has been shown in the jejunum [11]. The duodenal experiments were performed as described in Protocol 1. In the jejunal experiments, the jejunal segment was prepared and mounted between two halves of the hemichambers as usual. Both sides of the tissue were exposed to the aforementioned bathing solution. TFP (0.1 mM) was added to the serosal solution to eliminate the transcellular active calcium transport, and was present during both incubating and experimental periods. After 20 min of incubation, the unidirectional calcium fluxes were measured. Electrical parameters were tightly monitored to access the tissue viability.

2.6.3. Protocol 3

This study was to investigate changes in the solvent drag-induced calcium transport in the duodenum in response to direct exposure to prolactin. Prolactin (Sigma, St. Louis, MO) was first dissolved in normal saline adjusted to pH 9 before adding to the bathing solution to give the final concentrations of 200, 600, 800 or 1000 ng/ml. The vehicle, i.e. 0.9% NaCl at pH 9, was used as a control. Throughout the incubating and experimental periods, the serosal side of the duodenal segments was directly exposed to various doses of prolactin. TFP (0.1 mM) was also added to the serosal solution to abolish the transcellular active calcium transport. After 20 min of incubation, unidirectional calcium fluxes, i.e. solvent drag-induced calcium transport, were measured for 60 min.

2.6.4. Protocol 4

This study was to investigate the direct action of prolactin on the solvent drag-induced calcium transport in the jejunum. Prolactin in a dose of 600 ng/ml was used. The serosal side of jejunal segments was directly exposed to both prolactin and 0.1 mM TFP throughout the incubating and experimental periods. After 20 min of incubation, unidirectional calcium fluxes were measured for 60 min.

2.6.5. Protocol 5

The objective of this protocol was to evaluate the relationship between the solvent drag-induced calcium transport and the widening of tight junction induced by administration of a cytoskeleton inhibitor cytochalasin E in the duodenum and jejunum. The rats were randomly divided into eight groups for two experiments. Four groups were for the duodenal experiments and the other four were for the jejunal experiments. In each experiment, the serosal side of the intestinal segment was directly exposed to 0.9% NaCl (control), 600 ng/ml prolactin, 20 μM *Aspergillus clavatus* cytochalasin E (Sigma) or 600 ng/ml prolactin plus 20 μM cytochalasin E. Cytochalasin E was first dissolved in 50% DMSO to make a stock solution which was diluted with the bathing solution to give a final concentration of 20 μM. Final concentration of DMSO was 0.05%. The tissues were continuously exposed to respective chemicals during both incubating and experimental periods. After 20 min incubation, the mounted tissues were gently washed and then further incubated in a new solution. In addition to the respective chemicals, the new solution contained ^{45}Ca plus ^{3}H-mannitol, an indicator of the changes in permeability or the widening of tight junction. Unidirectional fluxes of ^{45}Ca and ^{3}H-mannitol were measured simultaneously during the 60 min experiment. Viability of mounted tissues was closely monitored.

2.7. Statistical analyses

Results were expressed as mean±S.E. Two sets of data were compared using the unpaired Student's t-test. Multiple comparisons were made by one-way analysis of variance (one-way ANOVA). The level of significance for statistical tests was $P<0.05$. Data were analysed by GraphPad Prism 4.0 for Microsoft Windows (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. Normal duodenum exhibited the solvent drag-induced calcium transport

The results of Protocol 1 are shown in Figs. 1 and 2. Unidirectional calcium fluxes of the total transepithelial active transport, i.e. solvent drag-induced plus transcellular active calcium transport, in both mucosa-to-serosa and serosa-to-mucosa directions are depicted in panel A of Fig. 1. The steady state fluxes (n=8) are 26.12 nmol h^{-1} cm^{-2} for the mucosa-to-serosa direction

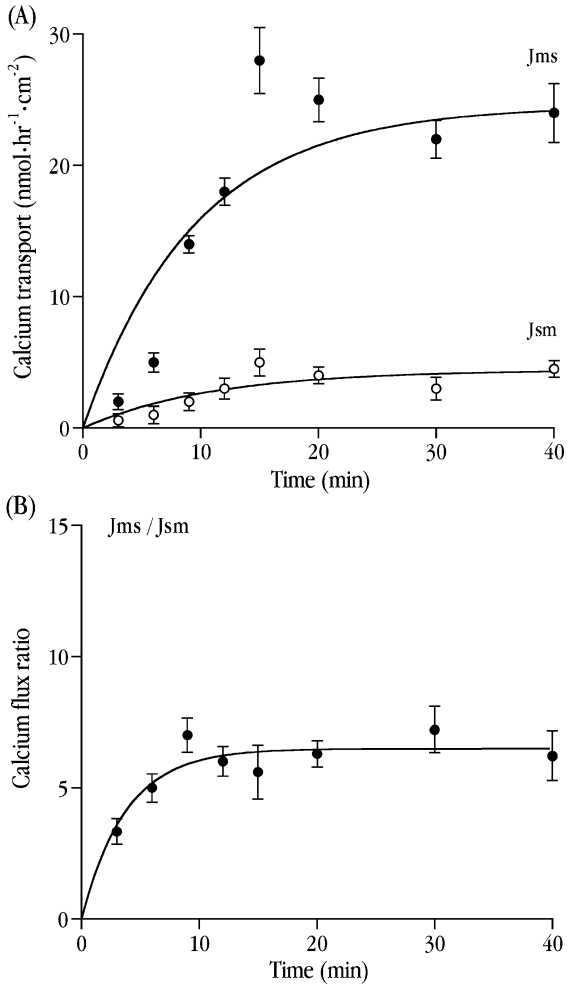

Fig. 1. Total active calcium fluxes in the duodenum of female rats (n=8). Panel A: Unidirectional fluxes in the mucosa-to-serosa (J_{ms}) and serosa-to-mucosa (J_{sm}) direction. Panel B: The flux ratio (J_{ms}/J_{sm}) was derived from the results in panel A. The flux ratio is time-variant indicating that the transepithelial calcium transport was composed of more than one pathway, i.e. transcellular and paracellular pathways.

and 2.92 nmol h^{-1} cm^{-2} for the serosa-to-mucosa direction. Panel B shows that the flux ratio derived from panel A is time-variant, indicating that more than one pathway existed for the transepithelial active calcium transport.

In the presence of 0.1 mM TFP, which blocked the transcellular active calcium transport, panel A of Fig. 2 shows the existence of the solvent drag-induced calcium transport in the duodenum. The fluxes in both directions obey a one phase exponential association of the flux equation (Eq. (3)). The steady state fluxes (n=8) are 7.32 nmol h^{-1} cm^{-2} for the mucosa-to-serosa direction and 2.43 nmol h^{-1} cm^{-2} for the serosa-to-mucosa direction while the half-times of the curves are 7.24 and 5.28 min, respectively. In panel B, the flux ratio derived from panel A of Fig. 2 is time-invariant, i.e. 2.37±0.14 (n=8), indicating that the current system exhibited only one pathway for calcium transport.

3.2. Solvent drag-induced calcium transport in duodenum is greater than that in jejunum

In this series of experiments (Protocol 2), the jejunal segments of the rats were directly exposed to 0.1 mM TFP to abolish the transcellular active calcium transport. Fig. 3 shows that the jejunum (n=7) also exhibited the solvent drag-induced calcium transport with a magnitude of 17.31±1.65 nmol h^{-1} cm^{-2}. However, the solvent drag-induced calcium transport in the duodenum (n=7) was significantly higher, i.e. 29.27±2.27 nmol h^{-1} cm^{-2} (P<0.001).

3.3. Prolactin directly stimulated the solvent drag-induced calcium transport in duodenum

In this series of experiments (Protocol 3), to demonstrate a direct action of prolactin, the duodenal segments were

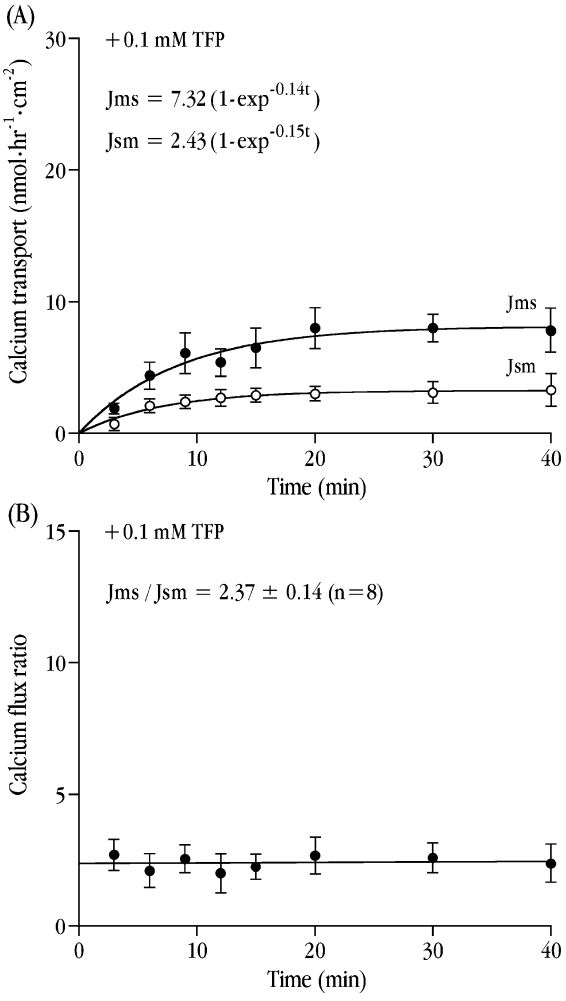

Fig. 2. Active calcium transport in the duodenum (n=8) in the presence of 0.1 mM TFP, which was used to abolish the transcellular active calcium transport. Panel A: Unidirectional fluxes in the mucosa-to-serosa (J_{ms}) and serosa-to-mucosa (J_{sm}) direction. Panel B: The flux ratio (J_{ms}/J_{sm}) was derived from the results in panel A. The flux ratio is constant in time or time-invariant indicating that only a single pathway, i.e. the solvent drag-induced calcium transport, was present.

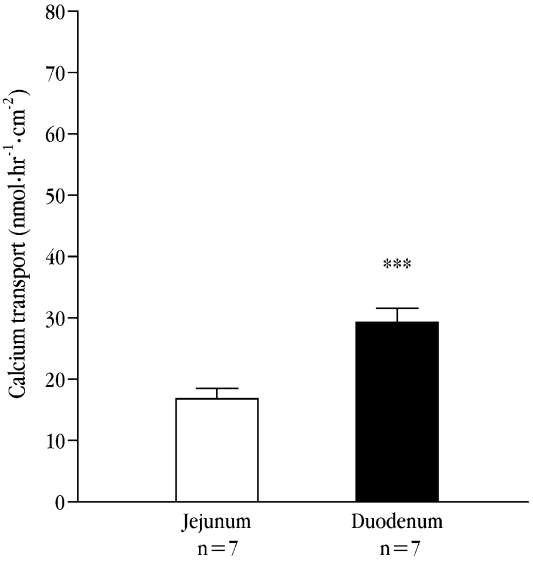

Fig. 3. The solvent drag-induced calcium transport in the control duodenum (n=7) and jejunum (n=7) were compared. The experiment was performed in the presence of 0.1 mM TFP, which abolished the transcellular active calcium transport. ***P<0.001 compared with control.

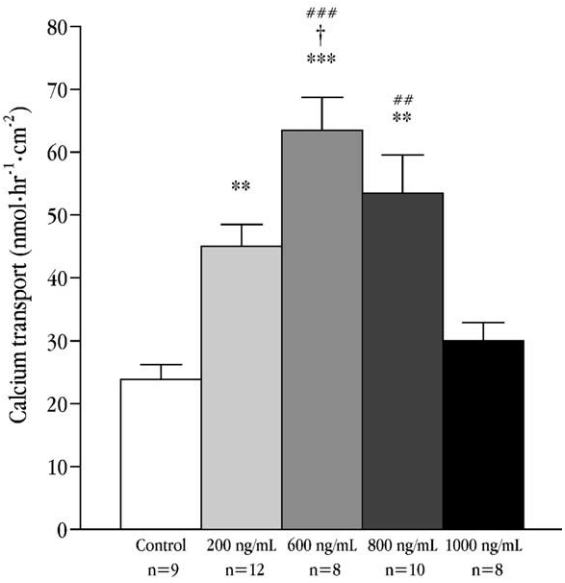

Fig. 4. The dose–response study of 200, 600, 800, and 1000 ng/ml prolactin on the solvent drag-induced calcium transport in the duodenum. The serosal surface of tissues was directly exposed to prolactin throughout the 60-min experimental period. **P<0.01, ***P<0.001 compared with the control group. †P<0.05 compared with the corresponding 200 ng/ml prolactin-treated group. ##P<0.01, ###P<0.001 compared with the corresponding 1000 ng/ml prolactin-treated group.

exposed to 0.9% NaCl (control), 200, 600, 800 or 1000 ng/ml prolactin plus 0.1 mM TFP in the serosal solution. The results of the solvent drag-induced duodenal calcium transport are illustrated in Fig. 4. Prolactin at the concentrations of 200, 600 and 800 ng/ml significantly increased the calcium transport in a dose–response manner, i.e. from the control value of (nmol h^{-1} cm^{-2}) 24.31±2.36 (n=9) to 45.42±3.47 (P<0.01, n=12), 63.82±5.28 (P<0.001, n=8) and 53.93±5.41 (P<0.01, n=10), respectively. Interestingly, the pathological dose of prolactin, i.e. 1000 ng/ml, did not have any effect on the solvent drag-induced calcium transport (29.05±2.61 nmol h^{-1} cm^{-2}, n=8). The acute effect of prolactin could be observed within 20 min after the serosal exposure.

3.4. Prolactin did not have an effect on the solvent drag-induced calcium transport in jejunum

The direct action of prolactin on the solvent drag-induced calcium transport of the jejunum was studied in Protocol 4. The results are graphically displayed in Fig. 5. It could be seen that the stimulatory effect of prolactin on the solvent drag-induced calcium transport seen in the duodenum was absent in the jejunum. The rates of calcium transport in the control group and the 600 ng/ml prolactin-treated group were 18.46±2.18 nmol h^{-1} cm^{-2} (n=7) and 19.57±3.54 nmol h^{-1} cm^{-2} (n=8), respectively.

3.5. Actions of prolactin on the duodenum were not related to the widening of the tight junction

In Protocol 5, ^{3}H-mannitol was used as a marker for the widening of the tight junction caused by serosal administration of 20 μM cytochalasin E. The solvent drag-induced calcium transport in the duodenum and jejunum was measured concurrently. The results are illustrated in Figs. 6 and 7. In the jejunum (Fig. 6), as expected, cytochalasin E

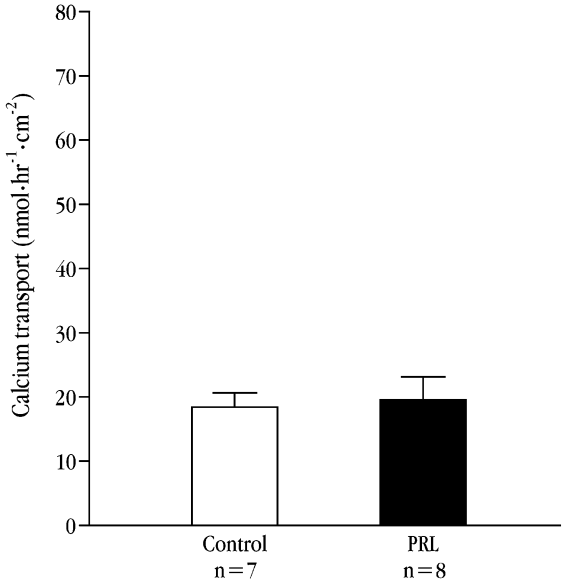

Fig. 5. The solvent drag-induced calcium transport in the control jejunum. The serosal surface of the tissues was directly exposed to 600 ng/ml prolactin (PRL). The experiment was performed in the presence of 0.1 mM TFP to abolish the transcellular active calcium transport. The illustration shows no statistical significance.

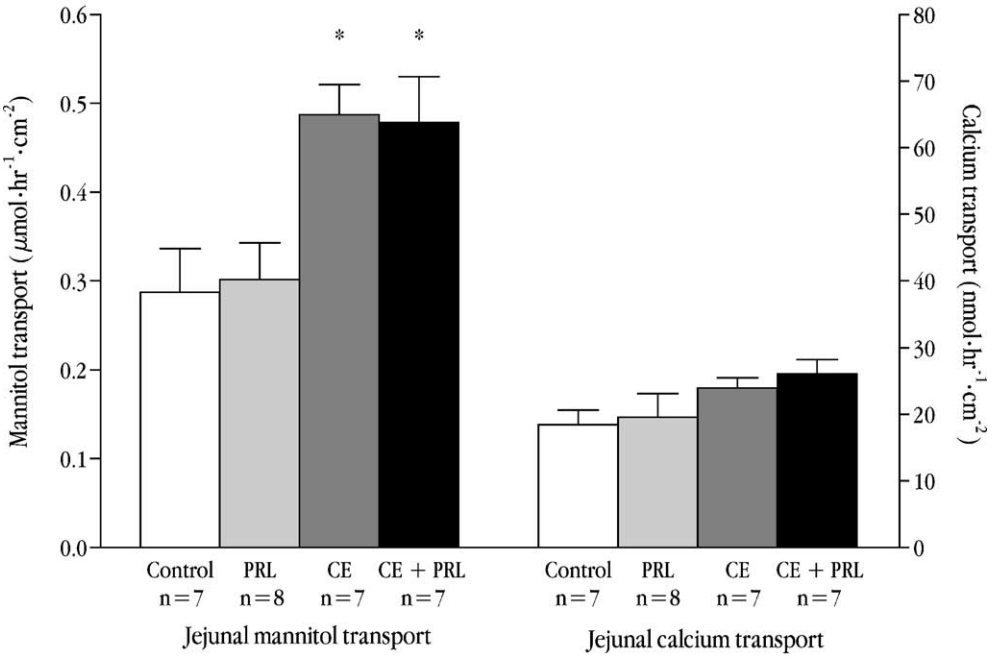

Fig. 6. The mannitol transport and the solvent drag-induced calcium transport in the jejunum. The serosal surface of the jejunal segments was directly exposed to 600 ng/ml prolactin (PRL), 20 μM cytochalasin E (CE), or 20 μM cytochalasin E plus 600 ng/ml prolactin (CE+PRL). Mannitol transport and calcium transport were measured simultaneously. *P<0.05 compared with the respective control group.

significantly increased the mannitol flux (P<0.05) from the control value of 0.29±0.04 μmol h^{-1} cm^{-2} (n=7) to 0.49±0.03 μmol h^{-1} cm^{-2} (n=7). Similarly, 20 μM cytochalasin E plus 600 ng/ml prolactin manifested a 66% increase in the flux of mannitol to 0.48±0.05 μmol h^{-1} cm^{-2} (P<0.05, n=7). Prolactin alone (600 ng/ml), on the other hand, had no effect on the jejunal mannitol flux, i.e. 0.30±0.04 μmol h^{-1} cm^{-2} (n=8). In contrast to the mannitol flux, neither 20 μM cytochalasin E and 600 ng/ml prolactin alone nor the combination of both altered the solvent drag-induced jejunal calcium transport, i.e. in nmol h^{-1} cm^{-2}; 18.46±2.18 (n=7) for the control group, 19.57±3.54 (n=8) for the 600 ng/ml prolactin-treated group, 23.99±1.49 (n=7) for the 20 μM cytochalasin E-treated group, and 26.10±2.13 (n=7) for the 600 ng/ml prolactin plus 20 μM cytochalasin E-treated group.

Unlike the results found in the jejunum, neither 600 ng/ml prolactin and 20 μM cytochalasin E alone nor the combination of both significantly increased the duodenal mannitol flux from the control value of 0.27±0.03 μmol h^{-1} cm^{-2} (n=6). However, 600 ng/ml prolactin and 600 ng/ml prolactin plus 20 μM cytochalasin E significantly enhanced the solvent drag-induced duodenal calcium transport by more than 80% (P<0.001), i.e. from the control value of (nmol h^{-1} cm^{-2}) 27.74±2.41 (n=6) to 51.03±4.35 (n=6) and 50.08±2.99 (n=9), respectively. Cytochalasin E alone (20 μM) had no effect on the solvent drag-induced duodenal calcium transport. The mannitol fluxes in the duodenum and jejunum were not statistically different under the control condition.

4. Discussion

Our work showed, for the first time, that the duodenum of sexually mature female rats acutely responded to the direct action of prolactin by increasing the solvent drag-induced calcium transport. The solvent drag-induced calcium transport in the duodenum was substantial, and was greater than that in the jejunum. Interestingly, the present results also demonstrated that the widening of tight junction was not required for this increased calcium transport, and that the convective force alone was sufficient to enhance calcium movement.

The present study first investigated the existence of the solvent drag-induced calcium transport in the duodenum. Since the serosal and mucosal solutions were of the same composition, calcium fluxes measured in the absence of the external electrochemical gradient must be active [46]. As depicted in Fig. 1, because the flux ratio was time-variant, i.e. being dependent on time, it indicated that more than one pathway existed for the transepithelial active calcium transport [29,47]. These pathways could only be the transcellular active and paracellular solvent drag-induced calcium transport as the third component, the voltage-dependent active calcium transport, had been shown to be negligible in the duodenum [3]. When a calmodulin/calbindin-dependent Ca^{2+}-ATPase inhibitor trifluoperazine [16,43,44,49] was added to the serosal solution to abolish the transcellular active calcium transport, the calcium flux became time-invariant indicating that only the solvent drag-induced calcium transport was present (Fig. 2). These findings confirmed that the current system could be used

for demonstrating the action of prolactin on the solvent drag-induced calcium transport.

The present study clearly showed that the duodenum, similar to the jejunum and ileum [17,18,50], exhibited the solvent drag-induced calcium transport. A novel theory of the solvent drag was recently elaborated on the basis of the Na^+ recirculation theory [47,51]. In general, the solvent drag-induced calcium transport was defined as the paracellular movement of calcium ions carried in the moving stream of water traversing the tight junction, lateral intercellular space, and the infraepithelial basement membrane into the serosal compartment. The driving energy for the solvent drag-induced transport was from the convective force generated by an intercellular standing hyperosmotic environment which was maintained by the Na^+/K^+-ATPase lining the lateral border of the two neighbouring cells [52,53]. Chatton and Spring [25] demonstrated that the concentration of intracellular sodium was about 15 mM above that of the bathing solution. Because the hyperosmotic milieu in the intercellular space was created by sodium ions entering the cells with glucose [54,55], replacement of mucosal glucose with an equivalent amount of mannitol could abolish the solvent drag-induced calcium transport in the duodenum [3].

Significance of the solvent drag-induced duodenal calcium transport was also evaluated by comparing it with substantial solvent drag-induced calcium transport in the jejunum [11,17,18]. Our results testified that the solvent drag-induced calcium transport in the duodenum was higher than that in the jejunum (Fig. 3), thus, expressing the importance of the solvent drag in the duodenum. The results were consistent with the findings of Karbach [17,18] that the solvent drag-induced portion accounted for approximately two-thirds of the total active calcium absorption. The solvent drag could be a mechanism of the glucose-enhanced calcium absorption previously reported in the duodenum [38,50].

Regarding the effect of prolactin, we were able to demonstrate that physiological doses of prolactin, i.e. 200 and 600 ng/ml [13,56], as well as the pharmacological dose of 800 ng/ml directly stimulated the solvent drag-induced calcium transport in a dose–response manner. Moreover, Fig. 4 clearly confirmed the biphasic action of prolactin, reported earlier [57–59]. The mechanism has been related to the dimerization concept of prolactin receptors. At a very high concentration of prolactin, the receptors are occupied to greater extents as non-functional 1:1 complexes, therefore, fewer empty receptors are available for functional 1:2 complexes [57,60].

Since the solvent drag-induced calcium transport of the duodenum was regulated by prolactin, we further investigated the same action of prolactin in the jejunum (Fig. 5). In contrast to the duodenum, prolactin had no effect on this component of calcium transport in the jejunum. The results agreed with findings of Boass and Toverud [50] in that there was no correlation between jejunal calcium absorption and water absorption in lactating rats. Because the prolactin-stimulated solvent drag-induced jejunal calcium transport was absent, we confirmed that the stimulatory effects of prolactin on the calcium transport previously observed in the jejunum were succeeded by the gradient-dependent passive calcium transport [10,11].

The present study also demonstrated the relationship between the widening of tight junction, using mannitol as a marker, and the solvent drag-induced calcium transport (Figs. 6 and 7). Up till now, dilatation of the tight junction has been shown to augment the solvent drag as well as the solvent drag-coupled ion transport [37,42,45,61]. Thus, the perijunctional actomyosin ring was possibly involved in this phenomenon. Several groups elaborated that chemical agents, such as cytochalasin D and E, which perturbed the organisation of the actins could increase the transepithelial flux of mannitol [30,31]. This reflected the widening of tight junction after cytochalasin-mediated contraction of the circumferential actomyosin ring [62]. However, because prolactin was capable of enhancing the solvent drag-induced duodenal calcium transport in the absence of a change in mannitol flux, i.e. no widening of tight junction, we concluded that an increase in convective force or solvent drag alone was sufficient to enhance the calcium movement. The enhanced convective force has been speculated to result from the stimulatory effect of prolactin on the Na^+/K^+-ATPase lining the lateral intercellular space [11,63].

Although the widening of paracellular pores and size-selective properties of the tight junction had been proposed in several epithelia, Madara et al. [45] also introduced the charge-selective properties of the tight junction. Recently, Simon et al. [34] reported an existence of a tight junction protein, called paracellin-1, in the thick ascending limb of Henle. This protein had a negatively charged extracellular portion which possibly contributed to the selective paracellular transport of calcium and magnesium [34,64,65]. Mutation of the paracellin gene led to a genetic disease known as familial hypomagnesemia with hypercalciuria [65]. Although paracellin-1 was renal-specific and absent in the small intestine [35], it provided the supportive evidence that paracellular transport of small ions, like calcium, might be charge-selective rather then size-selective. As seen in Fig. 6, an increase in the jejunal mannitol flux was not accompanied by an increase in the solvent drag-induced calcium transport, indicating that the widening of tight junction was not a prerequisite for the solvent drag-induced calcium movement and indirectly supported the charge-selective properties of the tight junction. Vice versa, the results in Fig. 7 showed that the prolactin-stimulated solvent drag-induced calcium transport in the duodenum occurred independently of changes in tight junction permeability, and possibly involved the charge-selective property of the paracellular transport mechanism. It was likely that, in the duodenum, the widening of tight junction was not required

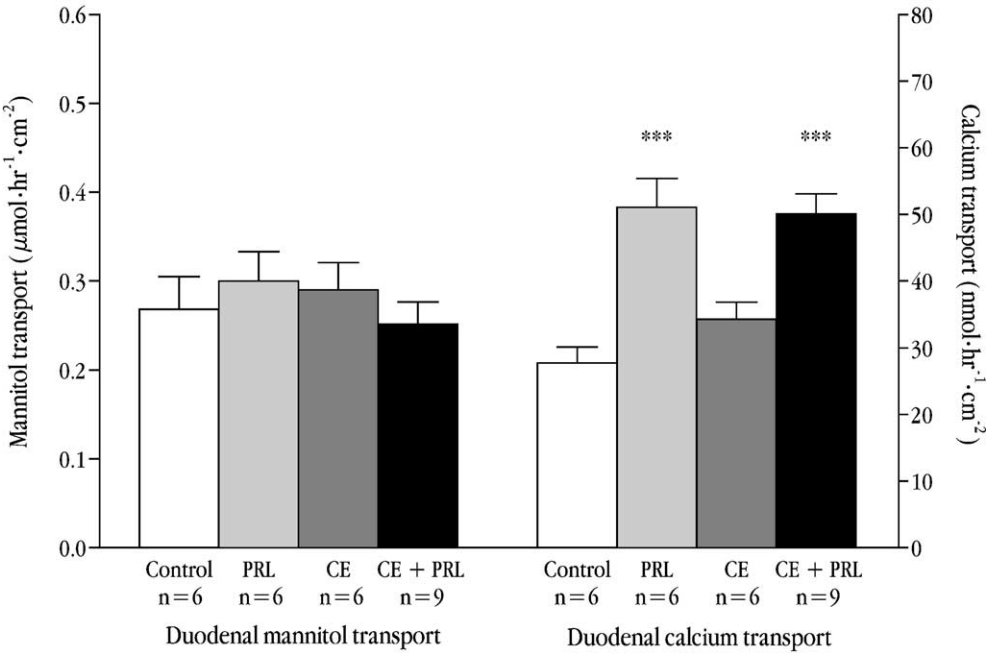

Fig. 7. The mannitol transport and the solvent drag-induced calcium transport in the duodenum. The serosal surface of the duodenal segments was directly exposed to 600 ng/ml prolactin (PRL), 20 μM cytochalasin E (CE), or 20 μM cytochalasin E plus 600 ng/ml prolactin (CE+PRL). Mannitol transport and calcium transport were measured simultaneously. ***$P<0.001$ compared with the respective control group.

for the direct action of prolactin on the solvent drag-induced calcium transport.

Therefore, it was concluded that, first, the duodenum of sexually mature female rats exhibited the solvent drag-induced calcium transport. Second, the solvent drag-induced calcium transport in the duodenum was significant, and was of a higher magnitude than that in the jejunum. Third, prolactin, as a calcium-regulating hormone, directly and acutely stimulated the solvent drag-induced calcium transport in the duodenum, but not in the jejunum. And fourth, the prolactin-stimulated solvent drag-induced duodenal calcium transport did not require the widening of tight junction. An increase in the activity of Na^+/K^+-ATPase which later potentiated the paracellular convective force accounted for the stimulatory effect of prolactin [11]. We also proposed that the properties of the tight junction which limited the paracellular calcium traversing the epithelia were charge-selective rather than size-selective since there was no association between the widening of tight junction and the solvent drag-induced calcium transport.

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References

[1] S. Lotinun, L. Limlomwongse, N. Krishnamra, The study of a physiological significance of prolactin in the regulation of calcium metabolism during pregnancy and lactation in rats, Can. J. Physiol. Pharm. 76 (1998) 218–228.
[2] P. Piyabhan, N. Krishnamra, L. Limlomwongse, Changes in the regulation of calcium metabolism and bone calcium content during growth in the absence of endogenous prolactin and during hyperprolactinemia: a longitudinal study in male and female Wistar rats, Can. J. Physiol. Pharm. 78 (2000) 757–765.
[3] N. Charoenphandhu, L. Limlomwongse, N. Krishnamra, Prolactin directly stimulates transcellular active calcium transport in the duodenum of female rats, Can. J. Physiol. Pharm. 79 (2001) 430–438.
[4] N. Krishnamra, J. Ousingsawat, L. Limlomwongse, Study of acute pharmacologic effects of prolactin on calcium and water transport in the rat colon by an in vivo perfusion technique, Can. J. Physiol. Pharm. 79 (2001) 415–421.
[5] S. Lotinun, L. Limlomwongse, V. Sirikulchayanonta, N. Krishnamra, Bone calcium turnover, formation, and resorption in bromocriptine- and prolactin-treated lactating rats, Endocrine 20 (2003) 163–170.
[6] B.P. Halloran, H.F. DeLuca, Calcium transport in small intestine during pregnancy and lactation, Am. J. Physiol. 239 (1980) E64–E68.
[7] A. Boass, S.U. Toverud, J.W. Pike, M.R. Haussler, Calcium metabolism during lactation: enhanced intestinal calcium absorption in vitamin D-deprived hypocalcemic rats, Endocrinology 109 (1981) 900–907.
[8] J.R. Mainoya, Effects of bovine growth hormone, human placental lactogen and ovine prolactin on intestinal fluid and ion transport in the rat, Endocrinology 96 (1975) 1165–1170.
[9] D.N. Pahuja, H.F. DeLuca, Stimulation of intestinal calcium transport and bone calcium mobilization by prolactin in vitamin D-deficient rats, Science 214 (1981) 1038–1039.
[10] N. Krishnamra, S. Lotinun, L. Limlomwongse, Acute effect and mechanism of action of prolactin on the in situ passive calcium absorption in rat, Bone Miner. 23 (1993) 253–266.

[11] N. Krishnamra, Y. Wirunrattanakij, L. Limlomwongse, Acute effects of prolactin on passive calcium absorption in the small intestine by in vivo perfusion technique, Can. J. Physiol. Pharm. 76 (1998) 161–168.
[12] N. Krishnamra, R. Thumchai, L. Limlomwongse, Acute effect of prolactin on the intestinal calcium absorption in normal, pregnant and lactating rats, Bone Miner. 11 (1990) 31–41.
[13] A. Boass, J.A. Lovdal, S.U. Toverud, Pregnancy- and lactation-induced changes in active intestinal calcium transport in rat, Am. J. Physiol. 263 (1992) G127–G134.
[14] H.N. Nellans, D.V. Kimberg, Cellular and paracellular calcium transport in rat ileum: effect of dietary calcium, Am. J. Physiol. 235 (1978) E726–E727.
[15] F. Bronner, Mechanisms and functional aspects of intestinal calcium absorption, J. Exp. Zool. 300 (2003) 47–52.
[16] F. Bronner, D. Pansu, W.D. Stein, An analysis of calcium transport across the rat intestine, Am. J. Physiol. 250 (1986) G556–G561.
[17] U. Karbach, Segmental heterogeneity of cellular and paracellular calcium transport across the rat duodenum and jejunum, Gastroenterology 100 (1991) 47–58.
[18] U. Karbach, Paracellular calcium transport across the small intestine, J. Nutr. 122 (1992) 672–677.
[19] N. Krishnamra, P. Taweerathitam, Acute effect of prolactin on active calcium absorption in rats, Can. J. Physiol. Pharm. 73 (1995) 1185–1189.
[20] I. Dusanter-Fourt, L. Belair, C. Gespach, J. Djiane, Expression of prolactin (PRL) receptor gene and PRL-binding sites in rabbit intestinal epithelial cells, Endocrinology 130 (1992) 2877–2882.
[21] A. Ouhtit, P.A. Kelly, G. Morel, Visualization of gene expression of short and long forms of prolactin receptor in rat digestive tissues, Am. J. Physiol. 266 (1994) G807–G815.
[22] F. Bronner, Intestinal calcium transport: the cellular pathway, Miner. Electrolyte Metab. 16 (1990) 94–100.
[23] B.M. Slepchenko, F. Bronner, Modeling of transcellular Ca transport in rat duodenum points to coexistence of two mechanisms of apical entry, Am. J. Physiol., Cell Physiol. 281 (2001) C270–C281.
[24] J.M. Diamond, W.H. Bossert, Standing-gradient osmotic flow: a mechanism for coupling of water and solute transport in epithelia, J. Gen. Physiol. 50 (1967) 2061–2083.
[25] J.Y. Chatton, K.R. Spring, The sodium concentration of the lateral intercellular spaces of MDCK cells: a microspectrofluorimetric study, J. Membr. Biol. 144 (1995) 11–19.
[26] K.R. Spring, Routes and mechanism of fluid transport by epithelia, Annu. Rev. Physiol. 60 (1998) 105–119.
[27] T.W. Clarkson, S.R. Toole, Measurement of short-circuit current and ion transport across the ileum, Am. J. Physiol. 206 (1964) 658–668.
[28] R.C. Rose, S.G. Schultz, Studies on the electrical potential profile across rabbit ileum. Effects of sugars and amino acids on transmural and transmucosal electrical potential differences, J. Gen. Physiol. 57 (1971) 639–663.
[29] O. Sten-Knudsen, H.H. Ussing, The flux ratio equation under nonstationary condition, J. Membr. Biol. 63 (1981) 233–242.
[30] J.L. Madara, R. Moore, S. Carlson, Alteration of intestinal tight junction structure and permeability by cytoskeletal contraction, Am. J. Physiol. 253 (1987) C854–C861.
[31] M. Pérez, A. Barber, F. Ponz, Modulation of intestinal paracellular permeability by intracellular mediators and cytoskeleton, Can. J. Physiol. Pharm. 75 (1997) 287–292.
[32] P. Krugliak, D. Hollander, C.C. Schlaepfer, H. Nguyen, T.Y. Ma, Mechanisms and sites of mannitol permeability of small and large intestine in the rat, Dig. Dis. Sci. 39 (1994) 796–801.
[33] J.B. Lloyd, Intestinal permeability to polyethyleneglycol and sugars: a re-evaluation, Clin. Sci. 95 (1998) 107–110.
[34] D.B. Simon, Y. Lu, K.A. Choate, H. Velazquez, E. Al-Sabban, M. Praga, G. Casari, A. Bettinelli, G. Colussi, J. Rodriguez-Soriano, D. McCredie, D. Milford, S. Sanjad, R.P. Lifton, Paracellin-1, a renal tight junction protein required for paracellular Mg^{2+} resorption, Science 285 (1999) 103–106.
[35] S. Weber, K.P. Schlingmann, M. Peters, L.N. Nejsum, S. Nielsen, H. Engel, K.H. Grzeschik, H.W. Seyberth, H.J. Grone, R. Nusing, M. Konrad, Primary gene structure and expression studies of rodent paracellin-1, J. Am. Soc. Nephrol. 12 (2001) 2664–2672.
[36] V.W. Tang, D.A. Goodenough, Paracellular ion channel at the tight junction, Biophys. J. 84 (2003) 1660–1673.
[37] M. O'Rourke, X. Shi, C. Gisolfi, H. Schedl, Effect of absorption of D-glucose and water on paracellular transport in rat duodenum–jejunum, Am. J. Med. Sci. 309 (1995) 146–151.
[38] K.M. Carroll, R.J. Wood, E.B. Chang, I.H. Rosenberg, Glucose enhancement of transcellular calcium transport in the intestine, Am. J. Physiol. 255 (1988) G339–G345.
[39] M.D. Levitt, J.K. Furne, D.G. Levitt, Shaking of the intact rat and intestinal angulation diminish the jejunal unstirred layer, Gastroenterology 103 (1992) 1460–1466.
[40] D.B. Sachar, J.O. Taylor, J.R. Saha, R.A. Phillips, Intestinal transmural electric potential and its response to glucose in acute and convalescent cholera, Gastroenterology 56 (1969) 512–521.
[41] S.G. Schultz, R. Zalusky, Ion transport in isolated rabbit ileum: I. Short-circuit current and Na fluxes, J. Gen. Physiol. 47 (1964) 567–584.
[42] J.L. Madara, Loosening of tight junctions, J. Clin. Invest. 83 (1989) 1089–1094.
[43] M.J. Favus, E. Angeid-Backman, M.D. Breyer, F.L. Coe, Effects of trifluoperazine, ouabain, and ethacrynic acid on intestinal calcium transport, Am. J. Physiol. 244 (1983) G111–G115.
[44] C. Roche, C. Bellaton, D. Pansu, A. Miller III, F. Bronner, Localization of vitamin D-dependent active Ca^{2+} transport in rat duodenum and relation to CaBP, Am. J. Physiol. 251 (1986) G314–G320.
[45] J.L. Madara, D. Barenberg, S. Carlson, Effects of cytochalasin D on occluding junctions of intestinal absorptive cells: further evidence that the cytoskeleton may influence paracellular permeability and junctional charge selectivity, J. Cell Biol. 97 (1986) 125–136.
[46] H.H. Ussing, The distinction by means of tracers between active transport and diffusion, Acta Physiol. Scand. 19 (1949) 43–56.
[47] S. Nedergaard, E.H. Larsen, H.H. Ussing, Sodium recirculation and isotonic transport in toad small intestine, J. Membr. Biol. 168 (1999) 241–251.
[48] E.H. Larsen, S. Nedergaard, H.H. Ussing, Role of lateral intercellular space and sodium recirculation for isotonic transport in leaky epithelia, Rev. Physiol. Biochem. Pharmacol. 141 (2000) 153–212.
[49] O. Scharff, B. Foder, Effect of trifluoperazine, compound 48/80, TMB-8 and verapamil on the rate of calmodulin binding to erythrocyte Ca^{2+} ATPase, Biochim. Biophys. Acta 772 (1984) 29–36.
[50] A. Boass, S.U. Toverud, Enhanced nonsaturable calcium transport in the jejunum of rats during lactation, but not during pregnancy, J. Bone Miner. Res. 12 (1997) 1577–1583.
[51] E.H. Larsen, J.B. Sørensen, J.N. Sørensen, Analysis of the sodium recirculation theory of solute-coupled water transport in small intestine, J. Physiol. 542 (2002) 33–50.
[52] M. Cereijido, J. Ehrenfeld, I. Meza, A. Martínez-Palomo, Structural and functional membrane polarity in cultured monolayers of MDCK cells, J. Membr. Biol. 52 (1980) 147–159.
[53] R.G. Contreras, G. Avila, C. Gutiérrez, J.J. Bolívar, L. González-Mariscal, A. Darszon, G. Beaty, E. Rodríguez-Boulan, M. Cereijido, Repolarization of Na^{+}–K^{+} pumps during establishment of epithelial monolayers, Am. J. Physiol. 257 (1989) C896–C905.
[54] J.R. Pappenheimer, K.Z. Reiss, Contribution of solvent drag through intercellular junctions to absorption of nutrients by the small intestine of the rat, J. Membr. Biol. 100 (1987) 123–136.
[55] J.R. Pappenheimer, On the coupling of membrane digestion with intestinal absorption of sugars and amino acids, Am. J. Physiol. 265 (1993) G409–G417.

[56] L.S. Jacob, L.K. Mariz, W.H. Daughaday, A mixed heterogenous radioimmunoassay for human prolactin, J. Clin. Endocrinol. Metab. 34 (1972) 484–490.
[57] G. Fuh, P. Colosi, W.I. Wood, J.A. Wells, Mechanism-based design of prolactin receptor antagonists, J. Biol. Chem. 268 (1993) 5376–5381.
[58] P.M. Reber, Prolactin and immunomodulation, Am. J. Med. 95 (1993) 637–644.
[59] P.R. Manna, T. El-Hefnawy, J. Kero, I.T. Huhtaniemi, Biphasic action of prolactin in the regulation of murine Leydig tumor cell functions, Endocrinology 142 (2001) 308–318.
[60] M.M. Ilondo, A.B. Damholt, B.A. Cunningham, J.A. Wells, P.D. Meyts, R.M. Shymko, Receptor dimerization determines the effects of growth hormone in primary rat adipocytes and cultured human IM-9 lymphocytes, Endocrinology 134 (1994) 2397–2403.
[61] J.L. Madara, Regulation of the movement of solutes across tight junctions, Annu. Rev. Physiol. 60 (1998) 143–159.
[62] J.L. Madara, J.R. Pappenheimer, Structural basis for physiological regulation of paracellular pathways in intestinal epithelia, J. Membr. Biol. 100 (1987) 149–164.
[63] G.E. Pickford, R.W. Griffith, J. Torretti, E. Hendlez, F.H. Epstein, Branchial reduction and renal stimulation of Na^+–K^+ ATPase by prolactin in hypophysectomized killifish in fresh water, Nature 228 (1970) 378–379.
[64] A. Blanchard, X. Jeunemaitre, P. Coudol, M. Dechaux, M. Froissart, A. May, R. Demontis, A. Fournier, M. Paillard, P. Houillier, Paracellin-1 is critical for magnesium and calcium reabsorption in the human thick ascending limb of Henle, Kidney Int. 59 (2001) 2206–2215.
[65] S.J. Knohl, S.J. Scheinman, Inherited hypercalciuric syndromes: Dent's disease (CLC-5) and familial hypomagnesemia with hypercalciuria (paracellin-1), Semin. Nephrol. 24 (2004) 55–60.