Long-Term Prolactin Exposure Differentially Stimulated the Transcellular and Solvent Drag-Induced Calcium Transport in the Duodenum of Ovariectomized Rats

KUKIAT TUDPOR, NARATTAPHOL CHAROENPHANDHU, WASANA SAENGAMNART, AND NATEETIP KRISHNAMRA[1]

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

Prolactin, having been shown to stimulate transcellular active and solvent drag-induced calcium transport in the duodenum of female rats, was postulated to improve duodenal calcium transport in estrogen-deficient rats. The aim of the present study was, therefore, to demonstrate the effects of long-term prolactin exposure produced by anterior pituitary (AP) transplantation on the duodenal calcium transport in young (9-week-old) and adult (22-week-old) ovariectomized rats. We found that ovariectomy did not alter the transcellular active duodenal calcium transport in young and adult rats fed normal calcium diet (1.0% w/w Ca) but decreased the solvent drag-induced duodenal calcium transport from 75.50 ± 10.12 to 55.75 ± 4.77 nmol·hr^{-1}·cm^{-2} ($P < 0.05$) only in adult rats. Long-term prolactin exposure stimulated the transcellular active calcium transport in young and adult AP-grafted ovariectomized rats fed with normal calcium diet by more than 2-fold from 7.56 ± 0.79 to 16.54 ± 2.05 ($P < 0.001$) and 9.78 ± 0.72 to 15.99 ± 1.75 ($P < 0.001$) nmol·hr^{-1}·cm^{-2}, respectively. However, only the solvent drag-induced duodenal calcium transport in young rats was enhanced by prolactin from 95.51 ± 10.64 to 163.20 ± 18.03 nmol·hr^{-1}·cm^{-2} ($P < 0.001$) whereas that in adult rats still showed a decreased flux from 75.50 ± 10.12 to 47.77 ± 5.42 nmol·hr^{-1}·cm^{-2} ($P < 0.05$). Because oral calcium supplement has been widely used to improve calcium balance in estrogen-deficient animals, the effect of a high-calcium diet (2.0% w/w Ca) was also investigated. The results showed that stimulatory action of long-term prolactin on the transcellular active duodenal calcium transport in both young and adult rats was diminished after being fed a high-calcium diet. The same diet also abolished prolactin-enhanced solvent drag-induced duodenal calcium transport in young and further decreased that in adult AP-grafted ovariectomized rats. We concluded that the solvent drag-induced duodenal calcium transport in adult rats was decreased after ovariectomy. Long-term prolactin exposure stimulated the transcellular active duodenal calcium transport in both young and adult rats whereas enhancing the solvent drag-induced duodenal calcium transport only in young rats. Effects of prolactin were abolished by a high-calcium diet. Exp Biol Med 230:836–844, 2005.

Key words: calcium transport; duodenum; ovariectomy; prolactin; solvent drag

Introduction

Prolactin, as a novel calcium-regulating hormone, has been demonstrated to stimulate active calcium transport in the duodenum of rats (1–3). Three components of active duodenal calcium transport were identified, that is, transcellular active, solvent drag-induced, and voltage-dependent calcium transport (4). However, the latter portion was considered negligible (4). By using the Ussing chamber technique, we reported that 200 to 800 ng/ml prolactin directly stimulated both transcellular active (4) and solvent drag-induced (5) calcium transport in the duodenum of sexually mature female rats in a dose-response manner. Stimulatory action of prolactin was not dependent on the prolactin-induced production of 1α,25-dihydroxycholecalciferol [1,25$(OH)_2D_3$] because such effect was observed within 20 mins after direct exposure to prolactin (3, 6).

Long-term estrogen deficiency caused by either bilateral oophorectomy in the young or menopause in adult places a stress on human calcium metabolism, thus resulting in osteopenia and osteoporosis (7, 8). Besides putative estrogen depletion-induced osteoporosis, a decrease in intestinal calcium absorption was also reported by Kalu *et al.* in 1989 (9). However, quantitative study of the active duodenal calcium transport after estrogen depletion has

This research was supported by the Thailand Research Fund (TRF) and the National Center for Genetic Engineering and Biotechnology (BIOTEC).

[1] To whom correspondence should be addressed at Department of Physiology, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand. E-mail: scnks@mahidol.ac.th

Received May 13, 2005.
Accepted July 29, 2005.

1535-3702/05/23011-0836$15.00

never been performed. On the other hand, endogenous estrogen has been shown to have a role in the intestinal responsiveness to $1,25(OH)_2D_3$ (10, 11) and to up-regulate 1α-hydroxylase, the key enzyme for $1,25(OH)_2D_3$ synthesis (12). We, therefore, hypothesized that active calcium transport in the duodenum, which is the most efficient site for calcium absorption (13), was compromised by long-term estrogen deficiency caused by ovariectomy.

Although hormone replacement therapy by either estrogen alone or in combination with progesterone is widely known to improve calcium balance in humans (14, 15), detrimental adverse effects of both hormones have been reported (16). Because prolactin has a stimulatory effect on the duodenal calcium transport, administration of a high physiological dose of prolactin alone or with a calcium supplement might provide an alternative therapy to benefit estrogen-deficient subjects. Because several of the actions of prolactin on calcium metabolism, including stimulation of intestinal calcium transport (17), reduction in urinary calcium secretion (18), and enhancement of bone calcium deposition (18, 19), are age-dependent, it would also be interesting to investigate the effect of prolactin in different age groups. To study a long-term effect of high physiological level of prolactin, we used an anterior pituitary transplant technique to induce sustained hyperprolactinemia (18, 20–22). After transplantation of pituitary glands under the renal capsule, pituitary tissues lacking hypothalamic peptides would stop producing hormones except for prolactin, which is normally under dopaminergic inhibitory regulation (23). Sustained circulating prolactin level of about 91 ng/ml after transplantation (18) is comparable to the prolactin levels during pregnancy in rats (2).

Another effective method to improve the calcium balance during estrogen deficiency is high-calcium supplementation (24). Beneficial effects of a high-calcium diet are the results of an increase in the calcium concentration gradient across the intestinal epithelium, thus enhancing calcium absorption throughout the entire length of the small intestine via the passive paracellular mechanism (25, 26). Although passive paracellular calcium transport went up with an increase in dietary calcium content, Pansu *et al.* (27) reported a 50% decrease in the maximal rate of the active duodenal calcium transport in male rats fed with high-calcium diet (3.0% w/w Ca). Nevertheless, there has been no report pertaining a down-regulation of active duodenal calcium transport in estrogen-deficient female rats fed a high-calcium diet. Combined effects of prolactin plus fortified calcium diet were also not known.

Therefore, the objectives of the present study were (i) to elucidate the effects of bilateral ovariectomy on the two components of the active duodenal calcium transport, that is, the transcellular active and solvent drag-induced calcium transport, in 9-week-old young and 22-week-old adult female rats; (ii) to investigate the stimulatory effects of long-term prolactin exposure induced by anterior pituitary transplantation on the active duodenal calcium transport in ovariectomized rats of both age groups; and (iii) to demonstrate whether a high-calcium diet together with long-term prolactin exposure would alter the two components of the active duodenal calcium transport.

Materials and Methods

Animals. Young (5-week-old) and adult (18-week-old) female Sprague-Dawley rats weighing 135–150 g and 200–220 g, respectively, were obtained from the Animal Center of Thailand, Salaya Campus, Mahidol University. They were placed in hanging stainless steel cages, fed with either normal calcium (1.0% w/w Ca) or high-calcium (2.0% w/w Ca) laboratory pellets (C.P. Co., Ltd., Bangkok, Thailand) and tap water *ad libitum* under 12 hr:12 hr light to dark cycle. Room temperature was controlled at 23–25°C, and relative humidity was about 50–60%. All animals were cared for in accordance with the principles and guidelines of the Laboratory Animal Ethical Committee of Mahidol University, Bangkok, Thailand.

Surgery. Anterior pituitary (AP) transplantation was performed in AP-grafted experimental groups (Protocols 2 and 3) by implanting two pituitary glands taken from two sexually mature female 18-week-old donors. All rats (i.e., both transplanted and nontransplanted rats) were housed for 2 weeks before ovariectomy was performed at 7-week-old for young and 20-week-old for adult rats. The sham group was subjected to a sham operation for both AP transplantation and ovariectomy. The ovariectomized rats also underwent sham AP transplantation whereas the AP-grafted rats underwent sham ovariectomy.

Anterior Pituitary Transplantation. The surgical procedure was modified from the methods of Adler *et al.* (20) and Piyabhan *et al.* (18). During diethyl ether anesthesia, the recipient rat was laid prone on the operating board. A 1-cm paracostal incision was made on the left side of the body to expose the left kidney, which was then covered with a warm 0.9% NaCl-soaked gauze ready for transplantation. Thereafter, two anesthetized donors were decapitated, and the interparietal bone and bregma were cut along the sagittal plane with a pair of bone-cutting scissors to remove the pituitary gland. On the recipient, the soft renal fascia was separated by forceps. The pituitary gland from the first donor was inserted into the prepared renal capsule of the recipient rat. Immediately, the second pituitary gland was implanted next to the first one and finally covered with the renal fascia. Muscle and skin were sutured, and cleaned with 70% ethanol. Hypophysectomy and AP-transplantation processes were completed within 2 mins. Sham operations consisted of exposure of the left kidney and touching the renal fascia with forceps. Visual examination of well-vascularized hypophyseal graft was performed at the end of each experiment to assure successful AP transplantation (18).

Bilateral Ovariectomy. Bilateral ovariectomy has been a widely accepted surgical procedure to diminish the serum 17β-estradiol to about 20% of normal level (28). The

rat was anesthetized with diethyl ether before two 1.5-cm paralumbar incisions were made. Adipose tissue was then retracted away to expose the ovaries. The distal part of the fallopian tubes was ligated before both ovaries were removed. The adipose tissue was then placed back into the original position. The skin was finally sutured and cleaned with 70% ethanol. Vital signs were carefully observed until the rat recovered from anesthesia. Atrophy of the uterus confirmed the success of the surgery. The sham operation was similar to the bilateral ovariectomy except that both ovaries were gently touched with forceps and left in place. Calcium transport experiments were performed 2 weeks after ovariectomy.

Duodenal Preparation. Under 50 mg/kg sodium pentobarbitone ip (Nembutal, Abbott Laboratory, North Chicago, IL) anesthesia, a median laparotomy was performed. A duodenal segment was obtained 2–10 cm distal to the pyloric sphincter. 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 to expose the mucosa. The tissue 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 bathing solution. The tissue was firmly affixed to the chamber with waterproof adhesive silicone (Bayer, Leverkusen, Germany). The tissue was incubated for 20 mins before the 50-min experiment was carried out.

Bathing Solution. The bathing solution contained (in m*M*): 118 NaCl, 4.7 KCl, 1.1 $MgCl_2$, 1.25 $CaCl_2$, 23 $NaHCO_3$, 12 D-glucose, and 2 mannitol. The solution continuously gassed with humidified 5% CO_2 in 95% O_2 was maintained temperature at 37°C, a pH of 7.4, and an osmolality of 280–290 mOsm (4, 5).

Electrical Measurements. Electrical parameters were measured after 20-mins incubation in the Ussing chamber as described by Charoenphandhu *et al.* (4) and Karbach (13). Transepithelial potential difference (PD) was measured using a Grass recorder (Polygraph Model 79, Grass Instrument, Quincy, MA). Platinum electrodes (BHD Laboratory Supplies, Poole, UK) were placed at the end of each hemichamber to supply short-circuit currents (Isc), which were monitored by a computed micromultimeter (Model 7551, Yokogawa Electrical, Tokyo, Japan) connected in series to a constant current generator (Thaiphan Electrics Inc., Bangkok, Thailand). The epithelial resistance (R) was calculated from PD and Isc by using Ohm's equation.

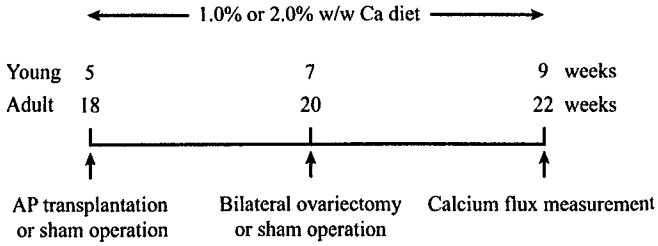
Unidirectional Flux Measurement. After 20-mins incubation in the Ussing chamber, bathing solution was replaced with a new fresh solution. One side was ^{45}Ca-containing solution (initial specific activity of 2 mCi/ml, Radiochemical Center, Amersham International, Buckinghamshire, UK) whereas the other side contained ^{45}Ca-free bathing solution. A 100-µl sample was collected from each side into separate microcentrifuge tubes to determine the calcium flux. Five samples at 10-min intervals were collected per setup. Radioactivity of ^{45}Ca was analyzed by liquid scintillation spectrophotometry (LKB-Wallac model 1219, LKB Wallac, Turku, Finland). Total calcium concentration on the hot side was analyzed by atomic absorption spectrophotometry (SpectrAA-300, Varian Techtron Ltd., Springvale, Australia). Unidirectional calcium fluxes ($nmol \cdot hr^{-1} \cdot cm^{-2}$) were calculated by using the following equations (29):

$$\text{Flux } (nmol \cdot hr^{-1} \cdot cm^{-2}) = \frac{\text{Rate of tracer appearance in cold side } (cpm \cdot hr^{-1})}{\text{Specific activity of hot side } (cpm \cdot nmol^{-1}) \times \text{area } (cm^2)} \quad (1)$$

$$\text{Specific activity } (cpm \cdot nmol^{-1}) = \frac{\text{Mean of radioactivity in hot side (cpm)}}{\text{Total calcium in hot side (mmol)} \times 10^{-6}} \quad (2)$$

To measure the transcellular active calcium flux, 12 m*M* mucosal glucose was replaced with the same concentration of mannitol to minimize the sodium entry into the cells, thus abolishing the solvent drag-induced calcium flux (4, 5). To measure the solvent drag induced-calcium flux, 0.1 m*M* trifluoperazine (ICN Biomedicals Inc., Aurora, OH) was added to the serosal solution to inhibit calmodulin-dependent plasma membrane Ca^{2+}-ATPase activity, thereby diminishing the transcellular active calcium flux (4, 30).

Experimental Protocols.



Protocol 1. This study was to demonstrate the significance of bilateral ovariectomy, which produced 2-week estrogen depletion on the transcellular active and solvent drag-induced calcium transport in the duodenum of young (9-week-old) and adult (22-week-old) female rats. The rats fed with normal calcium diet (1.0% w/w Ca) of each age group were randomly divided into two groups: sham-operated (Sham) and ovariectomized (OVX) rats. Calcium fluxes were determined as previously described. Three electrical parameters were recorded.

Protocol 2. This study was to investigate the significance of long-term exposure to endogenous prolactin on the transcellular active and solvent drag-induced calcium transport in the duodenum of estrogen-depleted young and adult female rats. The rats fed with normal calcium diet (1.0% w/w Ca) of each age group were divided into two groups: sham-operated (Sham) and AP-grafted ovariectomized (OVX+AP) rats. Calcium fluxes and three electrical parameters were determined.

Protocol 3. This series of experiments was to investigate the significance of calcium supplement on the transcellular active and solvent drag-induced calcium transport in the duodenum of estrogen-deficient young and adult female rats with and without hyperprolactinemia. The rats fed a high-calcium diet (2.0% w/w Ca) of each age group were divided into two groups: ovariectomized (OVX), and AP-grafted ovariectomized (OVX+AP) rats. Calcium fluxes and three electrical parameters were measured and compared with those of their respective controls: OVX and OVX+AP, fed with normal calcium diet (1.0% w/w Ca).

Statistical Analyses. Results were expressed as mean ± SEM. Two sets of data were compared using the unpaired Student's *t* test. Multiple comparisons were performed by one-way ANOVA. The level of significance for statistical tests was $P < 0.05$. Data were analyzed by GraphPad Prism 4.03 for Microsoft Windows (GraphPad Software Inc., San Diego, CA).

Results

Long-Term Prolactin Exposure Markedly Stimulated the Transcellular Active Duodenal Calcium Transport in Both Young and Adult Rats, Whereas Ovariectomy Had No Effect. Effects of bilateral ovariectomy, which led to long-term estrogen depletion on the transcellular active duodenal calcium transport, were studied as described in Protocol 1. As illustrated in Figure 1, the transcellular active calcium fluxes in young and adult rats were compared. Both young and adult rats fed with normal calcium diet showed no change in the transcellular active duodenal calcium fluxes after long-term estrogen depletion by bilateral ovariectomy (i.e., 7.81 ± 0.75 [Sham, $n = 9$] vs. 7.56 ± 0.79 [OVX, $n = 9$], and 8.99 ± 1.20 [Sham, $n = 8$] vs. 9.78 ± 0.72 [OVX, $n = 8$] nmol·hr^{-1}·cm^{-2}, respectively).

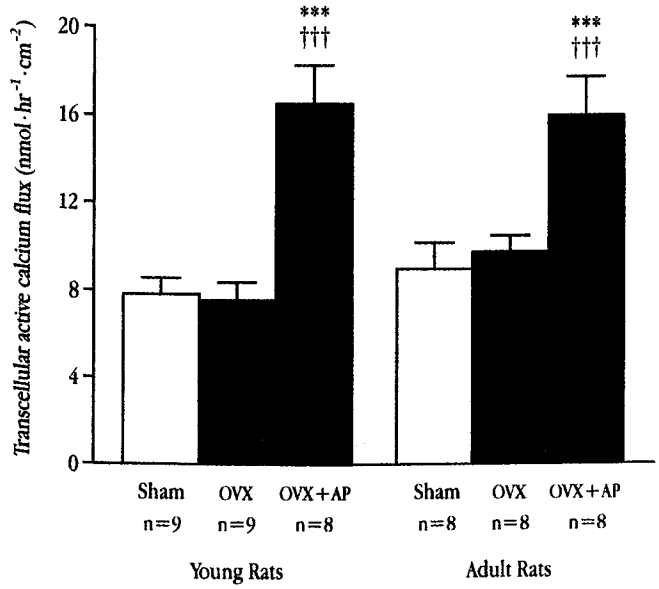


Figure 1. Transcellular active calcium fluxes in the duodenum of sham-operated (Sham), ovariectomized (OVX), and anterior pituitary-grafted ovariectomized (OVX+AP) rats of two different age groups: 9-week-old (young) and 22-week-old (adult) rats. All rats were fed with normal calcium diet (1.0% w/w Ca). The mucosal glucose of 12 mM was replaced with an equivalent amount of mannitol to abolish the solvent drag-induced calcium transport. ***$P < 0.001$ compared with its respective sham-operated rats. †††$P < 0.001$ compared with its respective ovariectomized rats. *n* denotes the number of experimental animals.

However, the transcellular active calcium transport of young AP-grafted ovariectomized rats was markedly enhanced from 7.56 ± 0.79 (OVX, $n = 9$) to 16.54 ± 2.05 (OVX+AP, $n = 8$) nmol·hr^{-1}·cm^{-2} ($P < 0.001$). Similar results were obtained in the adult AP-grafted ovariectomized rats, that is, transcellular active calcium transport increased from 9.78 ± 0.72 (OVX, $n = 8$) to 15.99 ± 1.75 (OVX+AP, $n = 8$) nmol·hr^{-1}·cm^{-2} ($P < 0.001$). The results suggested that the effect of prolactin on the transcellular active calcium transport was independent of estrogen.

Stimulatory Effect of Prolactin on the Transcellular Active Calcium Transport Was Diminished by a High-Calcium Diet. When rats were fed with high-calcium diet (2.0% w/w Ca, Protocol 3), the stimulatory effect of the long-term prolactin exposure on the transcellular active duodenal calcium transport was markedly diminished (Fig. 2), that is, from 16.54 ± 2.05 ($n = 8$) to 7.49 ± 0.83 ($n = 8$) nmol·hr^{-1}·cm^{-2} ($P < 0.001$) in young rats, and 15.99 ± 1.75 ($n = 8$) to 9.85 ± 1.45 ($n = 8$) nmol·hr^{-1}·cm^{-2} ($P < 0.001$) in adult rats. The transcellular active duodenal calcium transport in AP-grafted ovariectomized rats fed with 2.0% w/w calcium-containing diet in both age groups returned toward the levels found in the respective sham-operated rats. However, the high-calcium diet did not affect transcellular active calcium transport in sham-operated and ovariectomized rats (data not shown).

Ovariectomy Decreased the Solvent Drag-Induced Duodenal Calcium Transport in Adults, Whereas Long-Term Hyperprolactinemia Stimulated It in Young Rats. According to Protocol 1, the effect of ovariectomy on the solvent drag-induced duodenal calcium transport was also investigated. Figure 3 shows that ovariectomy significantly decreased the solvent drag-induced calcium transport in the duodenum only in the adult rats fed with normal calcium from a control value of 75.50 ± 10.12 (Sham, $n = 7$) to 55.75 ± 4.77 (OVX, $n = 8$) nmol·hr^{-1}·cm^{-2} ($P < 0.05$). Comparison of the results in Figure 1 and Figure 3 also demonstrated that the solvent drag-induced calcium fluxes in both age groups were approximately 10-fold higher than the transcellular active calcium fluxes ($P < 0.001$).

In contrast, the solvent drag-induced calcium transport in the duodenum of young AP-grafted ovariectomized rats was significantly increased by long-term hyperprolactinemia from a control value of 95.51 ± 10.64 (OVX, $n = 7$) to 163.20 ± 18.03 (OVX+AP, $n = 9$) nmol·hr^{-1}·cm^{-2} ($P < 0.001$), whereas there was no change in the adult AP-grafted ovariectomized rats from ovariectomy alone.

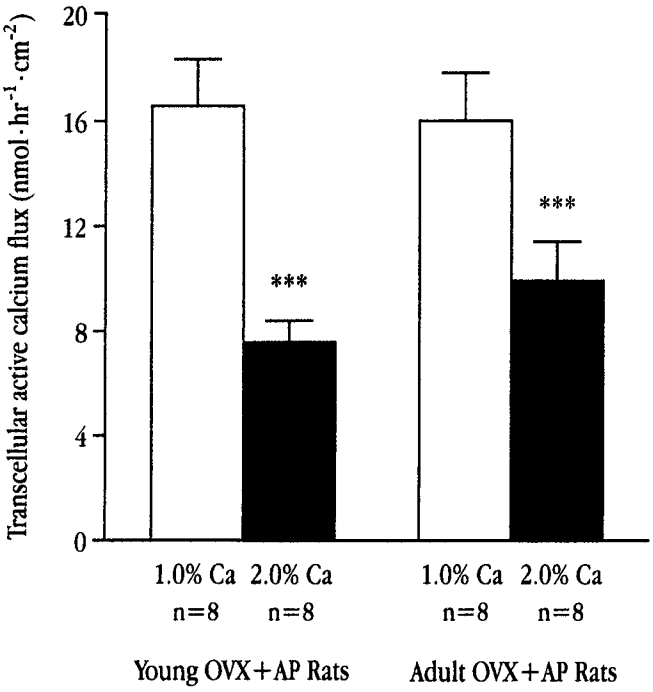


Figure 2. Transcellular active calcium fluxes in the duodenum of 9-week-old (young) and 22-week-old (adult) anterior pituitary-grafted ovariectomized (OVX+AP) rats fed with normal calcium diets (1.0% w/w Ca) or high-calcium diets (2.0% w/w Ca). ***$P < 0.001$ compared with its respective sham-operated rats. *n* denotes the number of experimental animals.

Solvent Drag-Induced Calcium Transport in Ovariectomized Rats Was Not Affected by the High-Calcium Diet. As shown in Figure 4, a high-calcium diet did not alter the magnitude of the solvent drag-induced duodenal calcium transport in both young and adult ovariectomized rats (i.e., 95.51 ± 10.64 [Young OVX 1.0% w/w Ca, $n = 7$] vs. 86.19 ± 9.36 [Young OVX 2.0% w/w Ca, $n = 8$] and 55.75 ± 4.77 [Adult OVX 1.0% w/w Ca, $n = 8$] vs. 43.56 ± 7.27 [Adult OVX 2.0% w/w Ca, $n = 7$] nmol·hr^{-1}·cm^{-2}). However, young rats, whether receiving a 1.0% or 2.0% w/w calcium-containing diet, showed higher solvent drag-induced calcium fluxes than those in adult rats ($P < 0.01$).

Stimulatory Effect of Prolactin on Solvent Drag-Induced Calcium Transport Was Diminished by a High-Calcium Diet. Figure 5 shows that the enhancing effect of long-term prolactin on the solvent drag-induced calcium transport in young rats was markedly decreased by high calcium diet, that is, from 163.20 ± 18.03 ($n = 9$) to 67.86 ± 10.97 ($n = 8$) nmol·hr^{-1}·cm^{-2} ($P < 0.001$). Interestingly, the solvent drag-induced calcium fluxes in adult AP-grafted ovariectomized rats fed with high-calcium diets were further decreased from 47.77 ± 5.42 ($n = 8$) to 14.96 ± 0.86 ($n = 8$) nmol·hr^{-1}·cm^{-2} ($P < 0.001$).

Electrical Parameters Were Unchanged After Ovariectomy and Prolactin Exposure. Electrical parameters of young and adult rats are shown in Tables 1 and 2, respectively. In both young and adult rats, no significant change in the electrical parameters during transcellular or solvent drag experiments was observed. However, the

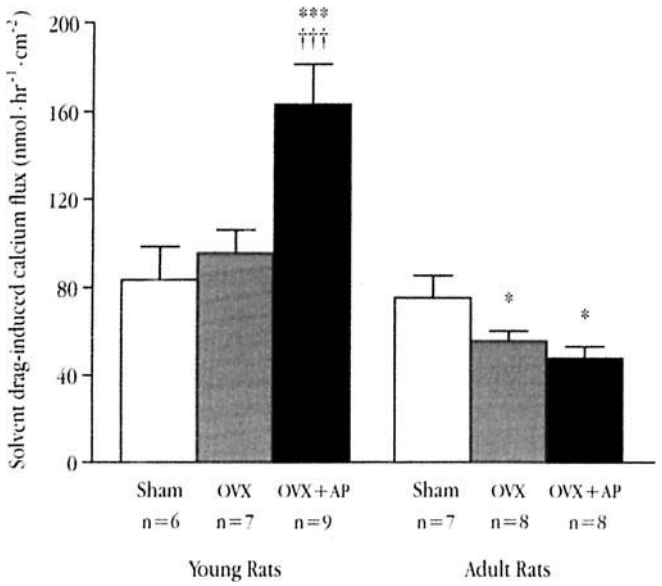


Figure 3. Solvent drag-induced calcium fluxes in the duodenum of sham-operated (Sham), ovariectomized (OVX), and anterior pituitary-grafted ovariectomized (OVX+AP) rats of two different age groups: 9-week-old (young) and 22-week-old (adult) rats. All rats were fed with normal calcium diets (1.0% w/w Ca). The experiment was performed in the presence of 0.1 mM trifluoperazine, which abolished the transcellular active calcium transport. *$P < 0.05$, ***$P < 0.001$ compared with its respective sham-operated rats. †††$P < 0.001$ compared with young ovariectomized rats. *n* denotes the number of experimental animals.

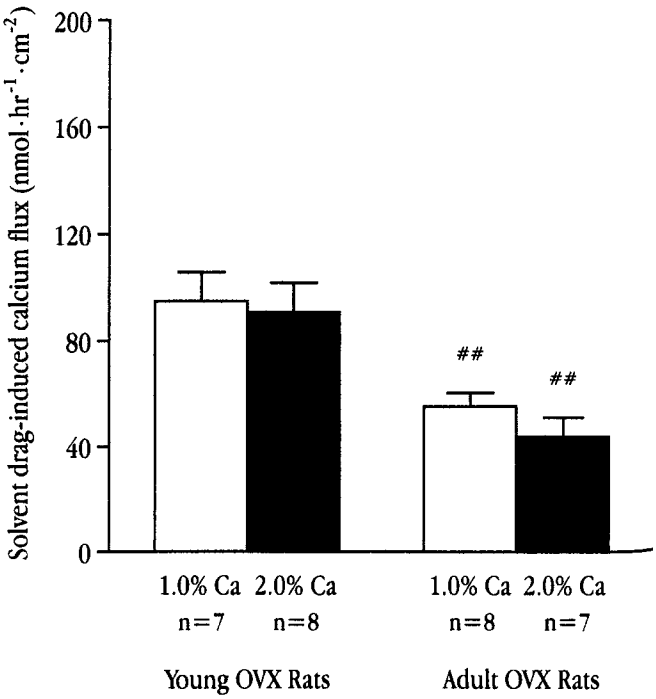


Figure 4. Solvent drag-induced calcium fluxes in the duodenum of 9-week-old (young) and 22-week-old (adult) ovariectomized (OVX) rats fed normal calcium diets (1.0% w/w Ca) or high-calcium diets (2.0% w/w Ca). ##$P < 0.01$ compared with young ovariectomized rats fed with the same diets. *n* denotes the number of experimental animals.

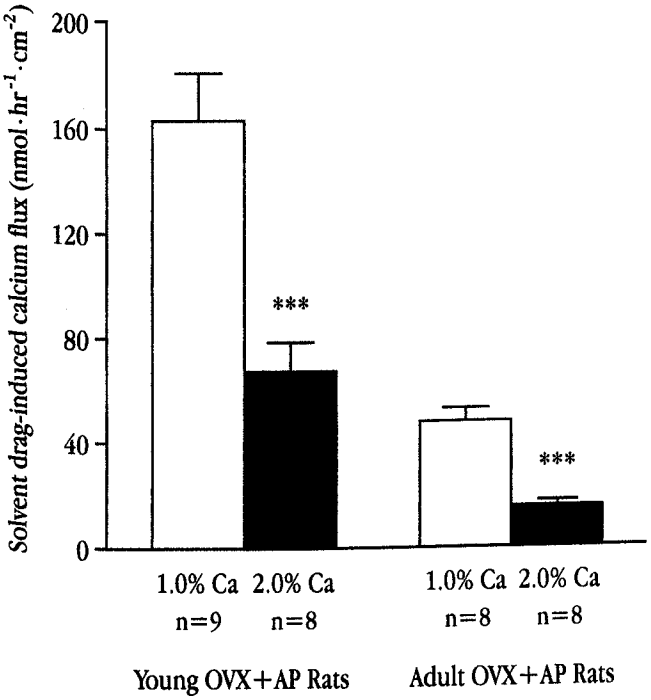


Figure 5. Solvent drag-induced calcium fluxes in the duodenum of 9-week-old (young) and 22-week-old (adult) anterior pituitary-grafted ovariectomized (OVX+AP) rats fed normal calcium diets (1.0% w/w Ca) or high-calcium diets (2.0% w/w Ca). ***$P < 0.001$ compared with respective 1.0% w/w calcium-fed rats. *n* denotes the number of experimental animals.

transepithelial potential difference and the short-circuit current of the solvent drag experiments were significantly higher than those of the transcellular experiments ($P < 0.001$), whereas the epithelial resistance remained unaltered.

Discussion

The present study demonstrated, for the first time, that long-term prolactin exposure significantly stimulated the transcellular active duodenal calcium transport in both young and adult ovariectomized rats. Interestingly, it differentially enhanced the solvent drag-induced calcium transport in the duodenum of young, but not adult, ovariectomized rats. The present results thus confirmed our previously reported stimulatory action of prolactin on the intestinal calcium absorption (4–6).

Transcellular active calcium transport in the duodenum is primarily a metabolically energized process consisting of apical entry via TRPV6 channel (31, 32), cytoplasmic translocation in a calbindin D_{9k}-bound form (33), and basolateral extrusion by $PMCA_{1b}$ (34). Solvent drag-induced calcium transport, on the other hand, is a paracellular calcium movement secondary to the Na^+/K^+-ATPase-mediated transcellular active sodium transport (35). The Na^+/K^+-ATPase lining the lateral membrane (36, 37) creates about 15 mM hyperosmotic milieu within the intercellular space (38) which, in turn, induced the solvent drag (35). The solvent drag-induced calcium transport was approximately 10-fold greater than the transcellular active calcium transport (4). This was confirmed by the present finding of a similar ratio (Figs. 1 vs. 3). Whereas the solvent drag-induced calcium transport represented the major portion of duodenal calcium transport (5), the transcellular active calcium transport was more significant in the presence of a very low luminal calcium concentration (26) or when the calcium requirement was increased, such as during pregnancy and lactation (2).

Because the effects of ovariectomy, which represented oophorectomy or menopause in humans, on intestinal calcium absorption was unclear, we investigated the components of the duodenal active calcium transport in young and adult rats after bilateral ovariectomy that resulted in a 2-week estrogen deficiency. We found no change in the duodenal active calcium transport in young ovariectomized

Table 1. Electrical Parameters[a] in the Duodenum of Young Rats in Five Experimental Groups[b]

	Transcellular experiments			Solvent drag experiments		
Young rats	PD (mV)	Isc ($\mu A \cdot cm^{-2}$)	R ($\Omega \cdot cm^2$)	PD (mV)	Isc ($\mu A \cdot cm^{-2}$)	R ($\Omega \cdot cm^2$)
Sham	1.87 ± 0.15 ($n = 9$)	32.48 ± 1.60 ($n = 9$)	56.98 ± 1.98 ($n = 9$)	4.03 ± 0.67* ($n = 6$)	69.98 ± 12.32** ($n = 6$)	59.03 ± 3.36 ($n = 6$)
Normal calcium diet						
OVX	1.48 ± 0.09 ($n = 9$)	29.24 ± 1.96 ($n = 9$)	51.22 ± 1.70 ($n = 9$)	4.94 ± 0.28* ($n = 7$)	71.67 ± 5.49** ($n = 7$)	69.92 ± 3.51 ($n = 7$)
OVX+AP	1.39 ± 0.04 ($n = 8$)	29.64 ± 2.21 ($n = 8$)	47.95 ± 2.23 ($n = 8$)	4.86 ± 0.31* ($n = 9$)	85.01 ± 4.16** ($n = 9$)	57.27 ± 2.90 ($n = 9$)
High-calcium diet						
OVX	1.71 ± 0.18 ($n = 8$)	33.34 ± 2.84 ($n = 8$)	50.81 ± 2.33 ($n = 8$)	4.53 ± 0.33* ($n = 8$)	69.22 ± 5.46** ($n = 8$)	66.61 ± 4.19 ($n = 8$)
OVX+AP	1.95 ± 0.16 ($n = 8$)	35.33 ± 0.95 ($n = 8$)	55.24 ± 3.78 ($n = 8$)	4.17 ± 0.26* ($n = 8$)	73.26 ± 4.49** ($n = 8$)	57.46 ± 3.11 ($n = 8$)

[a] Electrical parameters consisted of transepithelial potential difference (PD), short-circuit current (Isc), and epithelial resistance (R); *n* denotes the number of animals.
[b] Five experimental groups were sham operation (Sham), ovariectomy (OVX), and ovariectomy plus anterior pituitary transplant (OVX+AP)-induced hyperprolactinemia, and the rats were fed either a normal calcium (1.0% w/w Ca) or a high-calcium diet (2.0% w/w Ca).

Table 2. Electrical Parameters[a] in the Duodenum of Adult Rats in Five Experimental Groups[b]

	Transcellular experiments			Solvent drag experiments		
Adult rats	PD (mV)	Isc ($\mu A \cdot cm^{-2}$)	R ($\Omega \cdot cm^2$)	PD (mV)	Isc ($\mu A \cdot cm^{-2}$)	R ($\Omega \cdot cm^2$)
Sham	2.02 ± 0.35 ($n = 8$)	37.94 ± 2.48 ($n = 8$)	52.58 ± 5.45 ($n = 8$)	4.57 ± 0.41* ($n = 7$)	90.57 ± 10.09** ($n = 7$)	59.03 ± 3.36 ($n = 7$)
Normal calcium diet						
OVX	1.79 ± 0.09 ($n = 8$)	37.17 ± 1.87 ($n = 8$)	47.81 ± 1.50 ($n = 8$)	4.47 ± 0.22* ($n = 8$)	77.42 ± 4.61** ($n = 8$)	58.13 ± 1.42 ($n = 8$)
OVX+AP	1.79 ± 0.16 ($n = 8$)	39.67 ± 1.83 ($n = 8$)	44.75 ± 2.87 ($n = 8$)	4.70 ± 1.78* ($n = 8$)	79.74 ± 2.10** ($n = 8$)	58.89 ± 0.79 ($n = 8$)
High-calcium diet						
OVX	1.48 ± 0.05 ($n = 8$)	31.59 ± 1.89 ($n = 8$)	46.94 ± 1.39 ($n = 8$)	5.04 ± 0.14* ($n = 7$)	95.80 ± 3.78** ($n = 7$)	52.92 ± 1.30 ($n = 7$)
OVX+AP	1.54 ± 0.11 ($n = 8$)	35.40 ± 1.87 ($n = 8$)	43.44 ± 1.23 ($n = 8$)	4.61 ± 0.14* ($n = 8$)	84.43 ± 2.69** ($n = 8$)	54.68 ± 1.26 ($n = 8$)

[a] Electrical parameters consisted of transepithelial potential difference (PD), short-circuit current (Isc), and epithelial resistance (R); *n* denotes the number of animals.
[b] Five experimental groups were sham operation (Sham), ovariectomy (OVX), and ovariectomy plus anterior pituitary transplant (OVX+AP)-induced hyperprolactinemia, and the rats were fed either a normal calcium (1.0% w/w Ca) or a high-calcium diet (2.0% w/w Ca).
*$P < 0.001$ compared with the PD of transcellular experiments. **$P < 0.001$ compared with the Isc of transcellular experiments.

rats (Fig. 1). In contrast, ovariectomized adult rats exhibited a 26% decrease in the solvent drag-induced calcium transport (Fig. 3). These results were consistent with that reported by Colin *et al.* in 1999 (28). On the other hand, Miller *et al.*, in 1991, using ligated intestinal loop technique (39), and Ten Bolscher *et al.*, in 1999, using single pass duodenal perfusion (40), found no change in the calcium absorption in adult ovariectomized rats. It was possible that a small change in calcium transport may not be detected by the *in vivo* and *in situ* techniques. By investigating each component of the active calcium transport separately with the *in vitro* Ussing chamber technique, the present study was able to detect a decrease of 19.75 $nmol \cdot hr^{-1} \cdot cm^{-2}$ in the solvent drag-induced duodenal calcium transport in ovariectomized adult rats. Nevertheless, although previous investigators documented intestinal calcium absorption controversially as being unchanged (39, 40) or decreased (9) after ovariectomy, the reports pertaining to the effect of sustained estrogen administration were consistent: an increase in intestinal calcium transport (28).

Despite the presence of estrogen receptor mRNA expression (41), and functional estrogen receptors in the intestinal epithelium (41, 42), Van Cromphaut *et al.* failed to demonstrate any change in the duodenal gene expression of TRPV6, calbindin D_{9k}, or $PMCA_{1b}$ after ovariectomy (43). These findings, together with the present results, indicate that the action of estrogen on duodenal calcium absorption does not involve the transcellular component but is, more likely, on the paracellular calcium transport. Because estrogen potentiated $1,25(OH)_2D_3$ synthesis (12) as well as intestinal responsiveness to $1,25(OH)_2D_3$ (10, 11), estrogen-induced increases in intestinal calcium transport in adult rats used to be regarded as being secondary to the $1,25(OH)_2D_3$-mediated paracellular calcium transport (13). However, suppression of $1,25(OH)_2D_3$ production by a high-calcium diet in the present study did not further alter the solvent drag-induced calcium fluxes in adult ovariectomized rats (Fig. 4), suggesting that this component of duodenal calcium transport does not require $1,25(OH)_2D_3$, and estrogen might exert its action through some other undefined $1,25(OH)_2D_3$-independent mechanisms. Unlike adult rats, ovariectomy had no effect on the solvent drag-induced calcium transport in young rats. It is possible that, like other estrogen-responsive organs, the duodenal responsiveness to estrogen is not fully developed in young rats (44, 45).

A number of studies from our laboratory previously showed a stimulatory effect of prolactin on duodenal active calcium transport, be it a single physiological injected dose (3), direct exposure in the Ussing chamber (4), or long-term hyperprolactinemia induced by anterior pituitary transplantation (18). The present results, therefore, confirmed that prolactin could markedly enhance the transcellular active calcium transport in the duodenum of rats.

Regarding the solvent drag-induced calcium transport, the duodenum appeared to differentially respond to prolactin depending on the duration of the exposure. An acute stimulatory effect of 200 to 800 ng/ml prolactin on this component of the duodenal calcium transport in adult rats was demonstrated when prolactin was directly added into the serosal solution (5). The present study, on the other hand, showed that long-term exposure to prolactin stimulated the solvent drag-induced calcium transport only in young rats (Fig. 3). Age-variant effects of prolactin could be explained by age-dependent unequal distribution of prolactin receptor isoforms (46, 47) and the different rate of endocytic internalization after long-term prolactin exposure, that is, internalization of the long form was faster than that of the short form (48). However, the relevancy of prolactin action on the intestinal calcium absorption depended not

only on the time of exposure or the age of the animals but also on the physiological condition related to luminal calcium availability and the body requirement, which would dictate the dominant component of calcium transport.

In the final part of the present study (Protocol 3), the effect of fortified calcium supplement was investigated. It was not surprising to find that a high-calcium diet abolished the stimulatory effect of long-term prolactin exposure on the transcellular active calcium transport (Fig. 2) because a 2.0–3.0% calcium diet was known to suppress $1,25(OH)_2D_3$ production by 86% (26, 49). Thus, the abolishment of prolactin action in young and adult ovariectomized rats fed a high-calcium diet was probably because of a low serum level of $1,25(OH)_2D_3$. Because we previously showed that the action of prolactin on intestinal calcium absorption observed within 15–20 mins after exposure to prolactin was not dependent on $1,25(OH)_2D_3$ (3, 4, 6), it was likely that acute and long-term effect of prolactin utilized different mechanisms of action. The prolonged $1,25(OH)_2D_3$ deficiency probably affected the essential components of the transcellular active calcium transport that, in turn, prevented duodenal response to prolactin action. Several investigators provided evidence that TRPV6, calbindin D_{9k}, and PMCA1b expressions were reduced after vitamin D receptor knockout (50) or a high-calcium diet (32, 51). Therefore, calcium supplementation was effective for improving the calcium balance but, at the same time, reduced the effectiveness of prolactin action on duodenal calcium absorption.

It was noted that a high-calcium diet further decreased the solvent drag-induced calcium transport in long-term prolactin-exposed ovariectomized adult rats whereas diminishing that in young rats to the control value (Fig. 5). It is possible that the paracellular duodenal calcium transport under normal condition is also under the regulation of $1,25(OH)_2D_3$, as previously proposed by Karbach in 1992 (52) and Chirayath *et al.* in 1998 (53). Although the underlying cellular mechanism of $1,25(OH)_2D_3$ on this component of calcium transport has not been elucidated, it might involve a change in the architecture and proteins of the tight junction (54). The $1,25(OH)_2D_3$ dependency obviously differed in young and adult rats and probably contributed to the stimulatory action of prolactin.

The electrical parameters, simultaneously measured with calcium fluxes, were not affected by ovariectomy, prolactin exposure, or the combination of both. This was because, unlike the active sodium transport (28), the active duodenal calcium flux was too small to alter the transepithelial potential difference. Unchanged epithelial resistance during the enhanced solvent drag-induced duodenal calcium transport in young rats also supported our data interpretation that there was no widening of the tight junction (5), which is necessary for paracellular transport of some ions and nutrients (55, 56). The lower transepithelial potential difference and short-circuit current in the transcellular experiments was a result of the abolishment of the solvent drag-induced calcium transport.

It was concluded that, first, bilateral ovariectomy did not affect either the transcellular active or solvent drag-induced duodenal calcium transport in young rats, but significantly decreased the solvent drag-induced duodenal calcium transport in adult rats. Second, long-term prolactin exposure had a beneficial effect on both young and adult rats by stimulating the active duodenal calcium transport. It stimulated the transcellular active duodenal calcium transport in rats of both age groups, but differentially enhanced solvent drag-induced duodenal calcium transport only in young rats. Third, a high-calcium diet totally abolished the stimulatory effect of prolactin, possibly as a consequence of a decrease in serum $1,25(OH)_2D_3$.

We thank Supreecha Jaroonsuck, Somkid Khanmune, and Wilaiwan Thipsing for their excellent technical assistance.

1. Pahuja DN, DeLuca HF. Stimulation of intestinal calcium transport and bone calcium mobilization by prolactin in vitamin D-deficient rats. Science 214:1038–1039, 1981.
2. Boass A, Lovdal JA, Toverud SU. Pregnancy- and lactation-induced changes in active intestinal calcium transport in rat. Am J Physiol 263: G127–G134, 1992.
3. Krishnamra N, Taweerathitam P. Acute effect of prolactin on active calcium absorption in rats. Can J Physiol Pharmacol 73:1185–1189, 1995.
4. Charoenphandhu N, Limlomwongse L, Krishnamra N. Prolactin directly stimulates transcellular active calcium transport in the duodenum of female rats. Can J Physiol Pharmacol 79:430–438, 2001.
5. Tanrattana C, Charoenphandhu N, Limlomwongse L, Krishnamra N. Prolactin directly stimulated the solvent drag-induced calcium transport in the duodenum of female rats. Biochim Biophys Acta 1665:81–91, 2004.
6. Krishnamra N, Wirunrattanakij Y, Limlomwongse L. Acute effects of prolactin on passive calcium absorption in the small intestine by in vivo perfusion technique. Can J Physiol Pharmacol 76:161–168, 1998.
7. Fiore CE, Falcidia E, Foti R, Motta M, Tamburino C. Differences in the time course of the effects of oophorectomy in women on parameters of bone metabolism and interleukin-1 levels in the circulation. Bone Miner 20:79–85, 1993.
8. Seeman E. Pathogenesis of osteoporosis. J Appl Physiol 95:2142–2151, 2003.
9. Kalu DN, Liu CC, Hardin RR, Hollis BW. The aged rat model of ovarian hormone deficiency bone loss. Endocrinology 124:7–16, 1989.
10. Francis RM, Peacock M, Taylor GA, Storer JH, Nordin BE. Calcium malabsorption in elderly women with vertebral fractures: evidence for resistance to the action of vitamin D metabolites on the bowel. Clin Sci (Lond) 66:103–107, 1984.
11. Gennari C, Agnusdei D, Nardi P, Civitelli R. Estrogen preserves a normal intestinal responsiveness to 1,25-dihydroxyvitamin D_3 in oophorectomized women. J Clin Endocrinol Metab 71:1288–1293, 1990.
12. Baksi SN, Kenny AD, Galli-Gallardo SM, Pang PK. Vitamin D metabolism in bullfrogs and Japanese quail: effects of estradiol and prolactin. Gen Comp Endocrinol 35:258–262, 1978.
13. Karbach U. Segmental heterogeneity of cellular and paracellular calcium transport across the rat duodenum and jejunum. Gastroenterology 100:47–58, 1991.
14. Manson JE, Martin KA. Clinical practice. Postmenopausal hormone-replacement therapy. N Engl J Med 345:34–40, 2001.
15. Lindsay R. Hormones and bone health in postmenopausal women. Endocrine 24:223–230, 2004.

16. Compston JE. The risks and benefits of HRT. J Musculoskelet Neuronal Interact 4:187–190, 2004.
17. Krishnamra N, Lotinun S, Limlomwongse L. Acute effect and mechanism of action of prolactin on the in situ passive calcium absorption in rat. Bone Miner 23:253–266, 1993.
18. Piyabhan P, Krishnamra N, Limlomwongse L. 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 Pharmacol 78:757–765, 2000.
19. Lotinun S, Limlomwongse L, Sirikulchayanonta V, Krishnamra N. Bone calcium turnover, formation, and resorption in bromocriptine- and prolactin-treated lactating rats. Endocrine 20:163–170, 2003.
20. Adler RA, Farrell ME, Krieg RJ, Deiss WP. Hypogonadism does not mediate urinary calcium loss in pituitary-grafted rats. Metabolism 38: 805–809, 1989.
21. Obal F, Jr., Kacsoh B, Bredow S, Guha-Thakurta N, Krueger JM. Sleep in rats rendered chronically hyperprolactinemic with anterior pituitary grafts. Brain Res 755:130–136, 1997.
22. Kau MM, Chang LL, Kan SF, Ho LT, Wang PS. Stimulatory effects of hyperprolactinemia on aldosterone secretion in ovariectomized rats. J Investig Med 50:101–109, 2002.
23. Ben-Jonathan N, Hnasko R. Dopamine as a prolactin (PRL) inhibitor. Endocr Rev 22:724–763, 2001.
24. Reid IR, Ames RW, Evans MC, Gamble GD, Sharpe SJ. Effect of calcium supplementation on bone loss in postmenopausal women. N Engl J Med 328:460–464, 1993.
25. Duflos C, Bellaton C, Pansu D, Bronner F. Calcium solubility, intestinal sojourn time and paracellular permeability codetermine passive calcium absorption in rats. J Nutr 125:2348–2355, 1995.
26. Bronner F. Calcium absorption—a paradigm for mineral absorption. J Nutr 128:917–920, 1998.
27. Pansu D, Duflos C, Bellaton C, Bronner F. Solubility and intestinal transit time limit calcium absorption in the rat. J Nutr 123:1396–1404, 1993.
28. Colin EM, Van Den Bemd GJ, Van Aken M, Christakos S, De Jonge HR, Deluca HF, Prahl JM, Birkenhager JC, Buurman CJ, Pols HA, Van Leeuwen JP. Evidence for involvement of 17beta-estradiol in intestinal calcium absorption independent of 1,25-dihydroxyvitamin D_3 level in the Rat. J Bone Miner Res 14:57–64, 1999.
29. Schultz SG, Zalusky R. Ion transport in isolated rabbit ileum. I. short-circuit current and Na fluxes. J Gen Physiol 47:567–584, 1964.
30. Favus MJ, Angeid-Backman E, Breyer MD, Coe FL. Effects of trifluoperazine, ouabain, and ethacrynic acid on intestinal calcium transport. Am J Physiol 244:G111–G115, 1983.
31. Peng JB, Chen XZ, Berger UV, Vassilev PM, Tsukaguchi H, Brown EM, Hediger MA. Molecular cloning and characterization of a channel-like transporter mediating intestinal calcium absorption. J Biol Chem 274:22739–22746, 1999.
32. Song Y, Peng X, Porta A, Takanaga H, Peng JB, Hediger MA, Fleet JC, Christakos S. Calcium transporter 1 and epithelial calcium channel messenger ribonucleic acid are differentially regulated by 1,25 dihydroxyvitamin D_3 in the intestine and kidney of mice. Endocrinology 144:3885–3894, 2003.
33. Feher JJ, Fullmer CS, Wasserman RH. Role of facilitated diffusion of calcium by calbindin in intestinal calcium absorption. Am J Physiol 262:C517–C526, 1992.
34. Wasserman RH, Fullmer CS. Vitamin D and intestinal calcium transport: facts, speculations and hypotheses. J Nutr 125:1971S–1979S, 1995.
35. Diamond JM, Bossert WH. Standing-gradient osmotic flow: a mechanism for coupling of water and solute transport in epithelia. J Gen Physiol 50:2061–2083, 1967.
36. Contreras RG, Shoshani L, Flores-Maldonado C, Lazaro A, Cereijido M. Relationship between Na^+, K^+-ATPase, and cell attachment. J Cell Sci 112:4223–4232, 1999.
37. Cereijido M, Shoshani L, Contreras RG. The polarized distribution of Na^+, K^+-ATPase, and active transport across epithelia. J Membr Biol 184:299–304, 2001.
38. Chatton JY, Spring KR. The sodium concentration of the lateral intercellular spaces of MDCK cells: a microspectrofluorimetric study. J Membr Biol 144:11–19, 1995.
39. Miller SC, Bowman BM, Miller MA, Bagi CM. Calcium absorption and osseous organ-, tissue-, and envelope-specific changes following ovariectomy in rats. Bone 12:439–446, 1991.
40. Ten Bolscher M, Netelenbos JC, Barto R, Van Buuren LM, Van der Vijgh WJ. Estrogen regulation of intestinal calcium absorption in the intact and ovariectomized adult rat. J Bone Miner Res 14:1197–1202, 1999.
41. Arjmandi BH, Salih MA, Herbert DC, Sims SH, Kalu DN. Evidence for estrogen receptor-linked calcium transport in the intestine. Bone Miner 21:63–74, 1993.
42. Thomas ML, Xu X, Norfleet AM, Watson CS. The presence of functional estrogen receptors in intestinal epithelial cells. Endocrinology 132:426–430, 1993.
43. Van Cromphaut SJ, Rummens K, Stockmans I, Van Herck E, Dijcks FA, Ederveen AG, Carmeliet P, Verhaeghe J, Bouillon R, Carmeliet G. Intestinal calcium transporter genes are upregulated by estrogens and the reproductive cycle through vitamin D receptor-independent mechanisms. J Bone Miner Res 18:1725–1736, 2003.
44. Blanchard O, Tsagris L, Rappaport R, Duval-Beaupere G, Corvol M. Age-dependent responsiveness of rabbit and human cartilage cells to sex steroids in vitro. J Steroid Biochem Mol Biol 40:711–716, 1991.
45. Diep N, Constantinou CE. Age dependent response to exogenous estrogen on micturition, contractility and cholinergic receptors of the rat bladder. Life Sci 64:279–289, 1999.
46. Royster M, Driscoll P, Kelly PA, Freemark M. The prolactin receptor in the fetal rat: cellular localization of messenger ribonucleic acid, immunoreactive protein, and ligand-binding activity and induction of expression in late gestation. Endocrinology 136:3892–3900, 1995.
47. Freemark M, Driscoll P, Maaskant R, Petryk A, Kelly PA. Ontogenesis of prolactin receptors in the human fetus in early gestation—implications for tissue differentiation and development. J Clin Invest 99:1107–1117, 1997.
48. Lu JC, Scott P, Strous GJ, Schuler LA. Multiple internalization motifs differentially used by prolactin receptor isoforms mediate similar endocytic pathways. Mol Endocrinol 16:2515–2527, 2002.
49. Papakonstantinou E, Flatt WP, Huth PJ, Harris BS. High dietary calcium reduces body fat content, digestibility of fat, and serum vitamin D in rats. Obes Res 11:387–394, 2003.
50. Song Y, Kato S, Fleet JC. Vitamin D receptor (VDR) knockout mice reveal VDR-independent regulation of intestinal calcium absorption and ECaC2 and calbindin D_{9k} mRNA. J Nutr 133:374–380, 2003.
51. Armbrecht HJ, Boltz MA, Bruns ME. Effect of age and dietary calcium on intestinal calbindin D-9k expression in the rat. Arch Biochem Biophys 420:194–200, 2003.
52. Karbach U. Paracellular calcium transport across the small intestine. J Nutr 122:672–677, 1992.
53. Chirayath MV, Gajdzik L, Hulla W, Graf J, Cross HS, Peterlik M. Vitamin D increases tight-junction conductance and paracellular Ca^{2+} transport in Caco-2 cell cultures. 274:G389–G396, 1998.
54. Kutuzova GD, Deluca HF. Gene expression profiles in rat intestine identify pathways for 1,25-dihydroxyvitamin D_3 stimulated calcium absorption and clarify its immunomodulatory properties. Arch Biochem Biophys 432:152–166, 2004.
55. Madara JL, Moore R, Carlson S. Alteration of intestinal tight junction structure and permeability by cytoskeletal contraction. Am J Physiol 253:C854–C861, 1987.
56. Nusrat A, Turner JR, Madara JL. Molecular physiology and pathophysiology of tight junctions, IV—regulation of tight junctions by extracellular stimuli: nutrients, cytokines, and immune cells. Am J Physiol Gastrointest Liver Physiol 279:G851–G857, 2000.