A LONGITUDINAL STUDY OF THE PROLACTIN-STIMULATED TRANSEPITHELIAL CALCIUM TRANSPORT IN THE DUODENUM OF PREGNANT AND LACTATING RATS

KAMONSHANOK KRAIDITH

**A THESIS SUMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE
(PHYSIOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2009**

COPYRIGHT OF MAHIDOL UNIVERSITY

Copyright by Mahidol University

Thesis
Entitled

A LONGITUDINAL STUDY OF THE PROLACTIN-STIMULATED TRANSEPITHELIAL CALCIUM TRANSPORT IN THE DUODENUM OF PREGNANT AND LACTATING RATS

Kamonshanok Kraidith
.....
Miss Kamonshanok Kraidith
Candidate

.....
Asst. Prof. Narattaphol Charoenphandhu, M.D., Ph.D.
Major-Advisor

.....
Prof. Nateetip Krishnamra, Ph.D.
Co-Advisor

.....
Assoc. Prof. Suthasinee Poonyachoti, D.V.M., Ph.D.
Co-Advisor

B. Mahaisavariya
.....
Prof. Banchong Mahaisavariya, M.D.
Dean
Faculty of Graduate Studies

.....
Assoc. Prof. Varanuj Chatsudthipong, Ph.D.
Chair
Master of Science Programme in Physiology
Faculty of Science

Copyright by Mahidol University

Thesis
Entitled

A LONGITUDINAL STUDY OF THE PROLACTIN-STIMULATED TRANSEPITHELIAL CALCIUM TRANSPORT IN THE DUODENUM OF PREGNANT AND LACTATING RATS

was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Master of Science (Physiology)
on
March 24, 2009

Kamonshanok Kraidith
Miss Kamonshanok Kraidith
Candidate

Lect. Jantarima Pandaranandaka, Ph.D.
Chair

Asst. Prof. Narattaphol Charoenphandhu, M.D., Ph.D.
Member

Assoc. Prof. Sutthasinee Poonyachoti, D.V.M., Ph.D.
Member

Prof. Nateetip Krishnamra, Ph.D.
Member

Prof. Banchong Mahaisavariya, M.D.
Dean
Faculty of Graduate Studies
Mahidol University

Prof. Skorn Mongkolsuk, Ph.D.
Dean
Faculty of Science
Mahidol University

Copyright by Mahidol University

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and sincere appreciation to my major advisor, Asst. Prof. Narattaphol Charoenphandhu for his exellent advice, ideas for improvement, encouragement, financial and mental support throughout my study, all of which enabled me to complete this thesis.

The success of this thesis is also attributed to the extensive support and assistance from my co-advisors and examining committee, Prof. Nateetip Krishnamra, Assoc. Prof. Sutthasinee Poonyachoti, Dr. Jantarima Pandaranandaka who provided valuable advice and guidance for my research.

My sincere appreciation and gratitude are also expressed to Miss Walailak Jantarajit, Mr. Kukiat Tudpor, Dr. Narongrit Thongon and Mrs. Wassana Saengamnart for their excellent technical advice and assistance during my research.

I would like to thank Mr. Supreecha Jaroonsuck and Mr. Somkid Khanmune, the faculty engineers, for their helps in Ussing chamber maintenance.

I would like to express my gratitude to the Graduate Studies of Mahidol University Alumni Association for the scholarship.

I would like to thank the staffs at the Central Animal Facility for their helps and facilitation. I also wish to extend my appreciation to all COCAB members and friends at the Department of physiology for their kind supports.

Finally, I am grateful to my family for their financial support, care, and love. This thesis, I dedicate to my parents and all teachers who have thaught me since my childhood.

Kamonshanok Kraidith

Copyright by Mahidol University

A LONGITUDINAL STUDY OF THE PROLACTIN-STIMULATED TRANSEPITHELIAL CALCIUM TRANSPORT IN THE DUODENUM OF PREGNANT AND LACTATING RATS

KAMONSHANOK KRAIDITH 4936450 SCPS/M

M.Sc. (PHYSIOLOGY)

THESIS ADVISORY COMMITTEE: NARATTAPHOL CHAROENPHANDHU, M.D., Ph.D., NATEETIP KRISHNAMRA, Ph.D., SUTTHASINEE POONYACHOTI, D.V.M., Ph.D.

ABSTRACT

Prolactin (PRL) is known to stimulate the duodenal calcium absorption in nonmated rats. However, the effects of PRL on intestinal calcium absorption in pregnant, lactating, and suckling rats as well as its mechanisms were not well understood. The present study, therefore, aimed to *(i)* investigate the PRL-enhanced duodenal calcium transport during pregnancy and lactation, *(ii)* to determine the PRL-induced changes in the charge selectivity of the duodenal epithelium, and *(iii)* to investigate the physiological significance of the suckling-induced PRL surge in the regulation of duodenal calcium absorption in lactating rats. The direct stimulatory effect of PRL on the cecum which has the highest rate of calcium absorption was also investigated. Rats were divided into 7 groups, i.e., day-14 and-21 pregnancy (P14 and P21), day-7, -14, and -21 lactation (L7, L14 and L21), and 15-day postweaning. Calcium flux and charge selectivity were determined by Ussing chamber and dilution potential techniques, respectively.

The results showed that chronic PRL exposure was responsible for the elevated “baseline” of the duodenal calcium absorption during pregnancy and lactation, especially in P21 and L7 groups, while the transient PRL surge during suckling rapidly enhanced the transepithelial calcium transport to the maximal capacity above the newly elevated baseline. The chronic effect of endogenous PRL on calcium transport was abolished by 7-day bromocriptine administration, which inhibited the pituitary PRL release. Such an effect was restored by exogenous PRL administration. PRL altered the charge selectivity of the duodenal epithelium during pregnancy and lactation and made it more cation-selective by increasing P_{Na}, without changing in P_{Cl}. The milk volume ingested by pups during suckling correlated well with the enhanced total calcium fluxes. PRL also directly stimulated the transepithelial calcium absorption in the cecum and increased its cation selectivity.

It could be concluded that PRL elevated the “baseline” of duodenal calcium absorption during pregnancy and lactation, and also altered the epithelial charge selectivity. A further increase in the duodenal calcium absorption resulted from the suckling-induced PRL surge. Moreover, the cecal calcium absorption was directly stimulated by PRL.

KEY WORDS: CALCIUM ABSORPTION/PROLACTIN/TIGHT JUNCTION

65 pages.

Copyright by Mahidol University

การศึกษาผลต่อเนื่องระยะยาวของโพรแลกตินที่กระตุ้นการขนส่งแคลเซียมผ่านเยื่อบุผนังลำไส้เล็กส่วนต้นของหนูขาวที่ตั้งท้องและให้นม

A LONGITUDINAL STUDY OF THE PROLACTIN-STIMULATED TRANSEPITHELIAL CALCIUM TRANSPORT IN THE DUODENUM OF PREGNANT AND LACTATING RATS

กมลชนก ไกรดิษฐ์ 4936450 SCPS/M

วท.ม. (สรีรวิทยา)

คณะกรรมการที่ปรึกษาวิทยานิพนธ์: นรัตถพล เจริญพันธุ์, พ.บ., ปร.ด., นทีทิพย์ กฤษณามระ, ปร.ด., สุทธาสินี ปุญญโชติ, สพ.บ., ปร.ด.

บทคัดย่อ

โพรแลกตินกระตุ้นการดูดซึมแคลเซียมในลำไส้เล็กส่วนต้นในหนูขาวปกติที่ไม่ได้ผสมพันธุ์ อย่างไรก็ตาม ผลของโพรแลกตินและกลไกการดูดซึมแคลเซียมในลำไส้เล็กของหนูขาวช่วงตั้งท้อง ให้นม และขณะลูกดูดนม ยังไม่เป็นที่เข้าใจนัก งานวิจัยนี้มีวัตถุประสงค์เพื่อ *(1)* ศึกษาผลของโพรแลกตินต่อการขนส่งแคลเซียมในช่วงท้องและให้นม *(2)* ศึกษาผลของโพรแลกตินต่อการเปลี่ยนคุณสมบัติการคัดเลือกประจุของช่องระหว่างเซลล์ของเยื่อบุผนังลำไส้*(3)* ศึกษาผลทางสรีรวิทยาของโพรแลกตินที่หลั่งขณะดูดนมที่มีผลต่อการดูดซึมแคลเซียมในผนังลำไส้เล็กส่วนต้น นอกจากนี้ยังศึกษาผลโดยตรงของโพรแลกตินต่อลำไส้ใหญ่ส่วนซีกัมซึ่งในภาวะปกติจะมีอัตราการดูดซึมแคลเซียมสูงสุดเมื่อเทียบกับลำไส้ส่วนอื่น กลุ่มการทดลองมี 7 กลุ่ม คือ ท้องวันที่ 14 และ 21 หนูให้นมวันที่ 7, 14 และ 21, หนูที่ลูกหย่านมแล้ว 15 วันและหนูปกติกลุ่มควบคุม อัตราการขนส่งแคลเซียมวัดโดยเทคนิคอูสซิงส่วนคุณสมบัติการคัดเลือกประจุตรวจสอบโดยเทคนิคไดลูชั่น

การวิจัยพบว่าผลระยะยาวของโพรแลกตินทำให้อัตราการดูดซึมแคลเซียมในลำไส้เล็กส่วนต้นของหนูท้องและให้นมเพิ่มสูงขึ้น โดยเฉพาะหนูท้องวันที่ 21 และให้นมวันที่ 7 ขณะที่โพรแลกตินที่หลั่งออกมาในระหว่างลูกหนูดูดนมกระตุ้นการขนส่งแคลเซียมในแม่อย่างเฉียบพลัน ผลระยะยาวของโพรแลกตินสามารถยับยั้งได้ด้วยการฉีดโบรโมคริบตินต่อเนื่องนาน 7 วัน ส่วนการฉีดโพรแลกตินเสริมสามารถลดผลของโบรโมคริปตินได้ โพรแลกตินยังเพิ่มความสามารถในการคัดเลือกประจุบวกของเยื่อบุผนังลำไส้ซึ่งวัดจากการยอมให้โซเดียมผ่านมากขึ้น แต่ไม่เพิ่มการยอมให้คลอไรด์ผ่าน ปริมาตรน้ำนมมีความสัมพันธ์เชิงบวกกับอัตราการดูดซึมแคลเซียมของลำไส้ นอกจากนี้โพรแลกตินยังมีผลโดยตรงต่อการดูดซึมแคลเซียมของซีกัมและเพิ่มการคัดเลือกประจุบวกในลำไส้ใหญ่ส่วนนี้ได้

จากผลการวิจัยสรุปได้ว่าโพรแลกตินสามารถเพิ่มระดับการดูดซึมแคลเซียมให้สูงกว่าปกติในช่วงตั้งท้องและให้นม อีกทั้งยังเปลี่ยนคุณสมบัติการคัดเลือกประจุบริเวณเยื่อบุผนังลำไส้เล็กส่วนต้น นอกจากนี้ขณะลูกหนูดูดนมจะเพิ่มผลของโพรแลกตินต่อการดูดซึมแคลเซียมได้ดียิ่งขึ้น อนึ่งการดูดซึมแคลเซียมในลำไส้ใหญ่ส่วนซีกัมยังถูกกระตุ้นได้โดยตรงจากโพรแลกติน

65 หน้า

Copyright by Mahidol University

CONTENTS

		Page
ACKNOWLEDGEMENTS		iii
ABSTRACT		iv
LIST OF TABLES		vii
LIST OF FIGURES		viii
LIST OF ABBREVIATIONS		xii
CHAPTER I	INTRODUCTION	1
CHAPTER II	LITERATURE REVIEW	4
CHAPTER III	MATERIALS AND METHODS	16
CHAPTER IV	RESULTS	25
CHAPTER V	DISCUSSION	43
CHAPTERVI	CONCLUSIONS	50
REFERENCES		51
APPENDIX		61
BIOGRAPHY		65

Copyright by Mahidol University

LIST OF TABLES

Table		Page
1.1	Serum and plasma PRL concentrations in humans and rats.	11
3.1	Average cow milk weight and milk volume from 10 independent samples.	22
4.1	Epithelial electrical parameters in the duodenum	32
4.2	Electrical parameters in the cecum directly exposed to 800 ng/mL PRL. Mounted tissue was bathed on both sides with 1.25 mmol/L calcium-containing solution. The apical side was negative with respect to the basolateral side.	42

Copyright by Mahidol University

LIST OF FIGURES

Figure		Page
1.1	Schematic diagram shows the components of the total calcium transport in the duodenum of female rats.	6
1.2	Intestinal calcium absorption is composed of the paracellular and transcellular calcium transport (Charoenphandhu et al., 2001). TRPV6, transient receptor potential vanilloid family; PMCA, plasma membrane Ca^{2+}-ATPase; SGLT1, sodium-dependent glucose transporter 1; GLUT2, glucose transporter 2.	7
1.3	Signal transduction of PRL. PRLRs mediate their signaling pathways mainly through the JAK/STAT. Schematic representation of PRLRs activation.	14
3.1	Modified Ussing chamber was composed of 2 hemichambers and the mounted epithelium in the middle between two hemichambers. PD (or V_T)-sensing electrodes were placed near the epithelium, whereas the current-supplying electrodes were placed at both ends.	15
3.2	Experimental design of the group of animal.	23
4.1	The total active duodenal calcium fluxes in the apical to basolateral direction in P14 rats. In some experiments, P14 rats were administered for 7 days bromocriptine (Br7d) or bromocriptine plus exogenous PRL supplentation (Br7d+PRL7d).	26
4.2	The total active duodenal calcium fluxes in the apical to basolateral direction in P21 rats. In some experiments, P21 rats were administered for 7 days with bromocriptine (Br7d) or bromocriptine plus exogenous PRL supplementation (Br7d+PRL7d).	27

Copyright by Mahidol University

LIST OF FIGURES (cont.)

Figure		Page
4.3	The total active duodenal calcium fluxes in the apical to basolateral direction in L7 rats. In some experiments, L7 rats were administered for 7 days with bromocriptine (Br7d) or bromocriptine plus exogenous PRL supplementation (Br7d+PRL7d) to determine the chronic (genomic) PRL effects. The acute (Nongenomic) PRL effects were demonstrated in suckling rats injected with bromocriptine 1 h (Br1h) prior to the calcium flux study, with or without single-dose PRL supplementation (PRL1h).	29
4.4	The total active duodenal calcium fluxes in the apical to basolateral direction in L14 rats. In some experiments, L14 rats were administered for 7 days with bromocriptine (Br7d) or bromocriptine plus exogenous PRL supplementation (Br7d+PRL7d). The acute (Nongenomic) PRL effects were demonstrated in suckling rats injected with bromocriptine 1 h (Br1h) prior to the calcium flux study, with or without single-dose PRL supplementation (PRL1h).	30
4.5	The total active duodenal calcium fluxes in the apical to basolateral direction in L21 rats. In some experiments, L21 rats were administered for 7 days with bromocriptine (Br7d) or bromocriptine plus exogenous PRL supplementation (Br7d+PRL7d). The acute (Nongenomic) PRL effects were demonstrated in suckling rats injected with bromocriptine 1 h (Br1h) prior to the calcium flux study, with or without single-dose PRL supplementation (PRL1h).	31

Copyright by Mahidol University

LIST OF FIGURES (cont.)

Figure		Page
4.6	Correlation between the total calcium flux and milk volume in suckling rats.	33
4.7	Correlation between the total calcium flux and milk volume in single-dose bromocriptine(Br)-treatd suckling rats.	34
4.8	The total active duodenal calcium fluxes in the apical to basolateral direction in 15-day postweaning.	35
4.9	Charge-selective property of the duodenal tissue in P21 rats. Permeability ratio of sodium and chloride (P_{Na}/P_{Cl}), absolute sodium permeability (P_{Na}), and absolute chloride permeability (P_{Cl}) obtained from the dilution potential experiments are demonstrated. In some experiments, P21 rats were administered for 7 days with bromocriptine (Br7d) or bromocriptine plus exogenous PRL supplementation (Br7d+PRL7d).	37
4.10	Charge-selective property of the duodenal tissue in L7 rats. Permeability ratio of sodium and chloride (P_{Na}/P_{Cl}), absolute sodium permeability (P_{Na}), and absolute chloride permeability (P_{Cl}) obtained from the dilution potential experiments are demonstrated. In some experiments, L7 rats were administer red for 7 days with bromocriptine (Br7d) or bromocriptine plus exogenous PRL supplementation (Br7d+PRL7d).	38
4.11	The total active cecal calcium flux in the cecum directly exposed to 800 ng/mL PRL.	40

Copyright by Mahidol University

LIST OF FIGURES (cont.)

Figure		Page
4.12	Charge-selective property of the cecal tissue. Permeability ratio of sodium and chloride (P_{Na}/P_{Cl}), absolute sodium permeability (P_{Na}), and absolute chloride permeability (P_{Cl}) obtained from the dilution potential experiments are presented.	41
5.1	The role of the breast in controlling skeletal deminerization during lactation.	47

Copyright by Mahidol University

LIST OF ABBREVIATIONS

$1,25(OH)_2D_3$	1α,25-dihydroxyvitamin D_3
%	percent
α	alpha
β	beta
μ	micro
μA cm^{-2}	micro-ampere per centimeter-square
μM	micromolar
Ω cm^2	ohm centimeter-square
ANOVA	analysis of variance
°C	degree celsius
Ca^{2+}	calcium
Ca^{2+}-ATPase	calcium-adenosine triphosphatase
Cl^-	chloride
Cm	centimeter
Cm^2	centimeter-square
cm•s^{-1}	centimeter per second
CO_2	carbon dioxide
G	conductance
g	gram
h	hour
H^+	hydrogen ion
GH	growth hormone
Isc	short-circuit current
K^+	potassium ion
mg	milligram
mg/day	milligram per day

Copyright by Mahidol University

LIST OF ABBREVIATIONS (cont.)

min	minute
mM	millimolar
mV	millivolt
ng/mL	nanogram per milliliter
nM	nanomolar
P_{Ca}	calcium permeability
PD	potential difference
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PMCA	plasma membrane calcium-adenosine triphosphatase
P_{Na}/P_{Cl}	relative permeability ratio of sodium and chloride
PRL	prolactin
PRLR	prolactin receptor
r^2	coefficient of determination
s	second
SE	standard error
STAT	signaling transducer and activator of transcription
s.c.	subcutanouse
TER	transepithelial electrical resistance
TRPV	transient receptor potential vanilloid family

Copyright by Mahidol University

CHAPTER I
INTRODUCTION

During pregnancy and lactation, 200–300 mg Ca/d is transferred via the placenta to the fetus or secreted from mammary gland as milk, (King et al., 1992; Prentice et al., 2000). Several investigators provided evidence that, to mitigate the stress on maternal calcium homeostasis, the small intestine which is the major organ supplying calcium to the body must increase its capability to absorb calcium from meals (Boass et al., 1997; Halloran et al., 1980). Besides the small intestine, additional calcium may be absorbed in the cecum, which normally has the highest rate of calcium transport (Karbach et al., 1993).

PRL has been postulated to be a calcium-regulating hormone during these reproductive periods because the intestine of pregnant and lactating rats are not well responsive to $1,25(OH)_2D_3$ (Boass et al., 1981; Pahuja and DeLuca, 1981). Concurrently, plasma PRL levels are elevated during pregnancy and lactation, especially during suckling. In non-mated rats, normal plasma PRL concentrations are 7–10 ng/mL, and are usually 10 or 20 times (i.e., ~ 75–100 and 200–300 ng/mL) higher during pregnancy and lactation, respectively (Boass et al., 1992; Piyabhan et al., 2000). PRL secretion is abruptly increased (i.e., PRL surge) in response to nipple stimulation during breastfeeding, i.e., approximately 400–650 ng/mL for 60 min after suckling (Arbogast and Voogt, 1998). However, the suckling-induced PRL surge mainly occurs during early lactation, but not late lactation (Subramanian et al., 1975).

Physiological significance of the basal PRL levels was demonstrated in growing rats as bromocriptine which could inhibit endogenous PRL secretion decreased the intestinal calcium absorption (Piyabhan et al., 2000). The acute stimulatory effect of PRL on the fractional intestinal calcium absorption was also reported in pregnant and lactating rats (Lotinun et al., 1998). Moreover, Jantarajit and co-workers (2007) demonstrated that 400, 600 and 800 ng/mL PRL directly added in Ussing chamber rapidly stimulated the duodenal calcium transport in a dose-dependent

Copyright by Mahidol University

manner, with the maximal effective dose being 800 ng/mL. Since PRL concentrations of 400–600 ng/mL were comparable to the maternal PRL levels during suckling, it was possible that, after suckling, the maternal calcium absorption could be markedly increased by the suckling-induced PRL surge, presumably to compensate for calcium loss in milk. Although direct PRL action on the intestine was well established, the signal transduction of PRL in the intestine is not completely understood. Recently, Thongon and co-workers (2008) found that inhibitors of protein kinase C (PKC; GF-109203X) and RhoA-associated coiled-coil forming kinase (ROCK; Y-27632) abolished the stimulatory effect of PRL on the transcellular calcium transport, while only ROCK inhibitor diminished the PRL effect on the paracellular calcium transport in Caco-2 monolayer. However, the target calcium transporters regulated by these signaling proteins remain unknown.

Because animals are usually subjected to long-term exposure to PRL during pregnancy and lactation (approximately 30 days in rats and at least 9 months in humans), an animal model was previously developed to simulate this long-term PRL exposure by anterior pituitary transplantation. Two pituitary glands obtained from 2 donors were implanted under the left renal capsule of a recipient rat. After transplantation, sustained hyperprolactinemia of 75–100 ng/mL (comparable to the levels during pregnancy in rats) was observed within 15 days in the absence of the hypothalamic dopaminergic inhibition. (Piyabhan et al., 2000). By, using the anterior pituitary-grafted rats, Tudpor and co-workers (2005) demonstrated that chronic PRL exposure led to a significant increase in the solvent drag-induced calcium transport in young female rats. The effect of long-term PRL exposure on calcium absorption was long-lasting and showed plasticity, since the increased calcium fluxes in mounted duodenal segments were observed in Ussing chamber for at least 60 min despite the bathing solution having no PRL (Tudpor et al., 2005). Therefore, in pregnant and lactating rats, chronic hyperprolactinemia may sustainedly alter the transporting machinery at the cellular and/or molecular levels to elevate the “baseline” calcium absorption. However, during suckling, an abrupt increase in PRL may transiently induce a surge of calcium absorption above the newly elevated baseline to match excessive calcium loss in milk. Furthermore, Tanrattana and co-workers (2004) also demonstrated that an increase in the PRL-enhanced solvent drag was not due to

Copyright by Mahidol University

widening of the tight junction because cytochalasin E, a substance that induced tight junction widening, did not increase the solvent drag-induced paracellular calcium transport in the PRL-exposed duodenum. In other words, changes in the charge selectivity of the tight junction were possibly much more important for the regulation of paracellular calcium transport in rats. Although the exact mechanism of the PRL-enhanced solvent drag remains unclear, it may be related to the claudin-modulated charge and/or ion-selective property of the tight junction rather than the size-selective property (Tanrattana et al., 2004; Thongon et al., 2008).

In addition to the small intestine, PRL may also stimulate calcium transport in the cecum. Previous investigation by Jantarajit and co-workers (2007) showed that cecal epithelial cells strongly expressed PRL receptors. Gennerally, cecum is the site with the highest rate of transepithelial calcium transport, and may help absorb additional calcium released from the luminal contents during intraluminal fermentation (Karbach et al., 1993; Levrat et al., 1991).

The objectives of the present study were:

1. To investigate the PRL-enhanced duodenal calcium transport during pregnancy and lactation.
2. To determine the PRL-induced changes in the charge selectivity of the duodenal epithelium during pregnancy and lactation.
3. To investigate a physiological significance of the suckling-induced PRL surge in the regulation of duodenal calcium absorption in lactating rats.
4. To demonstrate a direct stimulatory effect of PRL on the cecal calcium absorption.

The hypotheses of the present study were:

1. PRL stimulates the duodenal calcium transport in pregnant and lactating rats.
2. PRL alters charge selectivity of the duodenal epithelium in pregnant and lactating rats.
3. The suckling-induced PRL surge further increases the duodenal calcium absorption in lactating rats.
4. PRL directly stimulates cecal calcium absorption.

Copyright by Mahidol University

CHAPTER II
LITERATURE REVIEW

A. Calcium homeostasis

Calcium plays a crucial role in several biological processes, such as bone metabolism, neuromuscular function, blood coagulation and signal transduction. Therefore, maintenance of extracellular calcium concentrations is important for several vital functions of the body. In face of large variations in calcium input and output, the organisms are equipped with a set of regulatory systems to keep the total plasma calcium levels within the range of 2.4–2.6 mM, and plasma free-ionized calcium between 1.1–1.3 mM (Hoenderop et al., 2005).

Calcium balance is controlled by the interplay amoung the three organ systems, i.e., gastrointestinal tract, bone and kidney (Boron, 2002). A number of factors, such as pH, extracellular calcium concentration, and various hormones, have been shown to influence calcium absorption across the epithelia (Hoenderop et al., 2005). The intestine is the organ that supplies calcium to the body, while the kidney excretes excess calcium from the body. Some calcium is deposited in bone as hydroxyapatite crystals. All processes are tightly regulated by the three classical calcium-regulating hormones, namely parathyroid hormone (PTH), $1,25(OH)_2D_3$ and calcitonin.

B. Calcium metabolism during pregnancy and lactation

Pregnancy and lactation are periods of high calcium demand. In human, 200–300 mg Ca/d is transferred via the placenta to the fetus in pregnancy or for milk production in lactation (Prentice et al., 2000; King et al., 1992). The provision of calcium during these reproductive periods requires physiological adaptation of calcium homeostatic mechanisms, including intestinal calcium absorption, urinary calcium reabsorption, and maternal bone calcium turnover. Studies of calcium homeostatic responses during

Copyright by Mahidol University

pregnancy and lactation in women with relatively high calcium intakes (> 1,000 mg/d) showed increases in both intestinal calcium absorption and urinary calcium excretion (King et al., 1992; Kovacs et al., 1997; Prentice et al., 2000). If the mother does not have adequate calcium intake before and during pregnancy, significant maternal bone loss may occur, possibly putting her at risk for osteopenia and/or osteoporosis later in life (Prentice et al., 2000). In addition, low calcium intake can retard the fetal skeletal development and lead to inadequate calcium concentrations in breast milk (Prentice et al., 2000).

C. Intestinal calcium absorption

In general, calcium transport is mediated by a complex array of passive and passive transport processes that are regulated by hormones, such as $1,25(OH)_2D_3$, PRL and estrogen (Pérez et al., 2008).

Intestinal calcium absorption occurs via two routes, i.e., the **transcellular pathway** that mainly takes place in the duodenum, proximal jejunum and cecum, and the **paracellular pathway** that occurs throughout the small and large intestine. The duodenum is the most effective site for calcium transport (Freeman et al., 1995; Kabach et al., 1991) because of its high capability to extract and absorb luminal calcium by passive and active mechanisms to mitigate a negative calcium balance, particularly, during pregnancy, lactation, and low calcium intake (Boass et al., 1992; Brown et al., 2005; Nellans et al., 1990). However, the cecum has the highest “rate” of the transepithelial calcium transport (Karbach et al., 1993).

As shown in Figure 1.1, the duodenal calcium transport is divided into active and passive calcium transport. Active calcium transport is a cellular energy-dependent process, consisting of transcellular, solvent drag-induced paracellular, and voltage-dependent paracellular transport. In contrast, the passive calcium transport is energized by a free energy generated by the transepithelial calcium gradient between the luminal calcium concentration (~5 mM) and 1.25 mM free calcium in the plasma.

Calcium absorption across the cecal epithelium is approximate 4, 9, and 20 times higher than that in the ascending colon, duodenum, and descending colon, respectively (Nellans and Goldsmith, 1981; Favus and Angeid-Backman 1985; Karbach and Feldmeier, 1993). Moreover, in the cecum, ascending and descending

Copyright by Mahidol University

colon, approximately 40% of the total mucosa-to-serosa calcium flux measured across the short-circuited tissue is transcellular, compose to 30% in the small intestine (Karbach et al., 1993). In other words, the major part of the transepithelial calcium fluxes across the intestinal epithelia occurs via the paracellular pathway. The paracellular calcium flux in mucosa-to-serosa direction across the cecum is highest when compared to other segments (Karbach and Feldmeier, 1993).

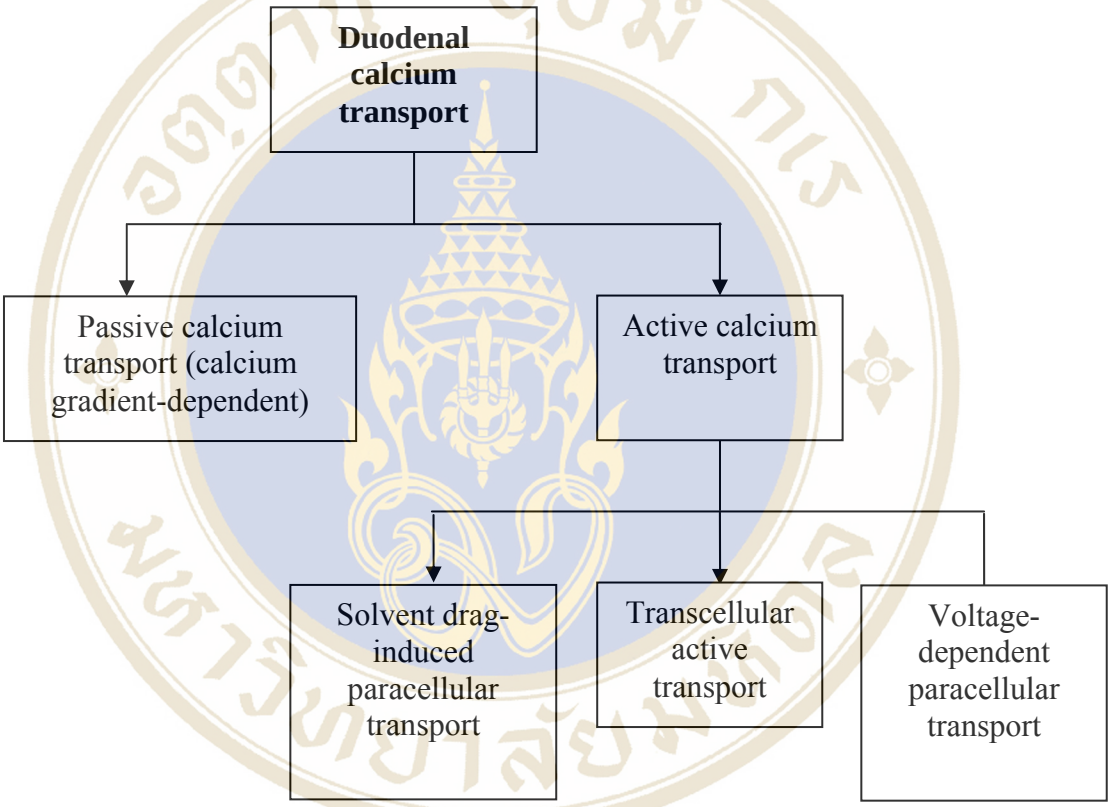


Figure 1.1 Schematic diagram shows the components of the total calcium transport in the duodenum of rats (Charoenphandhu et al., 2001).

Copyright by Mahidol University

2. Calcium gradient-dependent passive transport

Passive calcium movement is down the concentration gradient (Figure 1.2), via the paracellular pathway. It is absent when calcium gradient is eliminated (Bronner et al., 1999; Charoenphandhu et al., 2001). Paracellular transport is determined by size- and charge-selective properties of the tight junction or epithelia. These properties can be altered by the activities of perijunctional actomyosin ring complex and charge-selective tight junction proteins of the claudin family, respectively (Nusrat et al., 2000; Van Itallie et al., 2006). In female rats, the paracellular passive calcium transport is significant when luminal calcium concentration exceeds 5 mM (Figure 1.2).

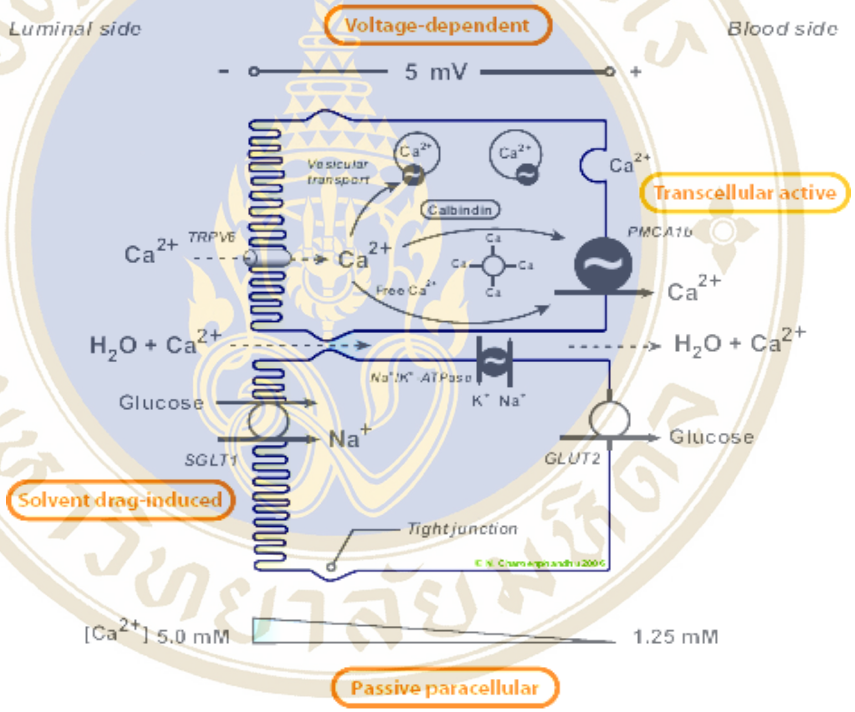


Figure 1.2 Intestinal calcium absorption is composed of the paracellular and transcellular calcium transport (Charoenphandhu et al., 2001). TRPV6, transient receptor potential vanilloid family; PMCA, plasma membrane Ca^{2+}-ATPase; SGLT1, sodium-dependent glucose transporter 1; GLUT2, glucose transporter 2.

Copyright by Mahidol University

2. Active calcium transport

Duodenal active calcium transport consists of three major metabolically energized components (Figure 2), i.e., transcellular, solvent drag-induced paracellular and voltage-dependent paracellular transport (Charoenphandhu et al., 2001).

2.1 Transcellular active calcium transport

Under physiological conditions, ~20–30% of the total active intestinal calcium transport occurs through the active transcellular pathway. The transcellular active transport is $1,25(OH)_2D_3$-dependent. It is a three-step process that consists of apical calcium uptake via the transient receptor potential vanilloid family Ca^{2+} channel 6 (TRPV6) (Hoenderop et al., 2005), cytoplasmic calcium translocation in a calbindin-D_{9k}-bound form (Feher et al., 1992), and basolateral calcium extrusion via the plasma membrane Ca^{2+}-ATPase isoform 1b ($PMCA_{1b}$) (Hoenderop et al., 2005). In addition, cytoplasmic calcium can be extruded by another transporter named Na^+/Ca^{2+} exchanger (NCX1). The transporting capacity of NCX1 is only 20% compared to that of $PMCA_{1b}$ (Wasserman et al., 1992). $1,25(OH)_2D_3$, a hormone known to stimulate the transcellular active calcium transport in the small intestine, upregulates the expression of TRPV6, calbindin-D_{9k} and $PMCA_{1b}$ as well as their activities (Fleet et al., 1999; Hoenderop et al., 2005; Jones et al., 1998).

2.2 Solvent drag-induced paracellular transport

Solvent drag-induced calcium transport depends on the paracellular sodium gradient generated by Na^+/K^+-ATPase lining the lateral membrane (Contreras et al., 1999; Diamond et al., 1967; Larsen et al., 2000). Therefore, it can be abolished by reducing the transepithelial sodium uptake, which maintains the paracellular hyperosmotic environment (Charoenphandhu et al., 2001; Tanrattana et al., 2004). It was previously reported that the solvent drag component accounted for ~70–80% of the total active calcium transport (Tanrattana et al., 2004).

Tight junction has been shown to play an important role in the paracellular permeability of ions by creating the size- and charge-selective barriers for ions, such as Ca^{2+}, Na^+ and Cl^- (Anderson et al., 2004). Factors known to stimulate the solvent drag induced calcium transport in the small intestine, e.g., chronic metabolic acidosis, were also found to upregulate expression of the tight junction proteins (Charoenphandhu et al., 2006). Widening of the tight junction as well as changes in the charge-selective

Copyright by Mahidol University

property of the tigh junction may also affect the solvent drag-induced paracellular transport of small ions and nutrients (Fihn et al., 2000; Madara et al., 1986; 1998; Van Itallie et al., 2006). Therefore, the size and charge selectivities are important for the solvent drag transport.

2.3 Voltage-dependent paracellular transport

This transport mechanism is also paracellular, and can be classified as secondary active transport since it is cellular energy-dependent (Charoenphandhu et al., 2001). The voltage (transepithelial potential difference) is mostly generated by Na^+/K^+-ATPase, which creates unequal distribution of charges between the two sides of the epithelial sheet (Contreras et al., 1989). Calcium transport due to the voltage-dependent mechanism is considered negligible in the duodenum (Charoenphandhu et al., 2001), but in the thick ascending limb of the loop of Henle, this mechanism is important for the renal calcium and magnesium reabsorption (Hou et al., 2005).

D. Hormones affecting the intestinal calcium transport

Duodenal calcium transport is tightly regulated by a number of calcium-regulating hormones. **$1,25(OH)_2D_3$** is widely known to stimulate the intestine calcium absorption at both cellular and molecular levels. However, $1,25(OH)_2D_3$ has no effect on the net calcium transport in the large intestine (Nellans and Kimberg, 1978). Binding of $1,25(OH)_2D_3$ to its classical vitamin D receptors (VDR) in the nuclei of enterocytes activates the genomic actions to enhance the transcription of TRPV6, calbindin-D_{9K}, and PMCA, thereby increasing the transcellular calcium absorption (Brown et al., 2005; Buitrago et al., 2002). Besides the genomic actions, nongenomic or acute actions of $1,25(OH)_2D_3$ have been demonstrated in many types of cells, such as chick intestinal absorptive cells, osteoblasts and myoblasts (Van et al., 2003, Vertino et al., 2005; Zhao et al., 2002). Nemere and co-workers (2004) reported that in isolated chick enterocytes, interaction between $1,25(OH)_2D_3$ and the membrane-associated rapid response steroid binding receptors (MARRS) rapidly stimulated apical calcium uptake and vesicular transport of phosphate across the cytoplasm. Recently, Tudpor and co-workers (2008) found that $1,25(OH)_2D_3$ used the nongenomic signaling pathways involving phosphoinositide 3kinase (PI3K), protein kinase C (PKC), and

Copyright by Mahidol University

mitogen-activated protein kinase (MAPK)/extracellular signal–regulated kinase (MEK) to rapidly enhance the solvent drag-induced calcium transport.

Direct stimulatory effect of **parathyroid hormone (PTH)** on the calcium transport in the small intestine was demonstrated by perfusion experiments in isolated duodenal loops (Nemere et al., 1986), although it was previously believed that PTH had only indirect effect on intestinal absorption through conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$. Moreover, **estrogen** also exerts a physiological role in the stimulation of intestinal calcium absorption (Nordin et al., 1991). The impairment of intestinal calcium absorption following ovarectomy is not the result of decreased circulating $1,25(OH)_2D_3$ or reduced intestinal responsiveness to $1,25(OH)_2D_3$. Estrogen stimulates intestinal calcium absorption probably by a direct effect on the intestine (O' Loughin et al., 1998).

E. Prolactin

Prolactin (PRL) is a 23-kDa polypeptide hormone, which is mainly synthesized by the lactotrophs of the anterior pituitary gland. PRL is involved in several biological processes, e.g., gonadal functions, maternal behavior, neuronal function, and water and mineral metabolism. Amino acid sequence of PRL is similar to that of two other polypeptide hormones, namely growth hormone (GH) and placental lactogen (PL) (Goffin et al., 2002).

The gene encoding PRL is conserved and is found in all vertebrates. In humans, it contains five exons and four introns, for overall length of 10 kb (Goffin et al., 2002). After removal of signal peptide (28 residues), the mature form of the PRL contains 199 amino acid residues (23 kDa) (Goffin et al., 2002).

PRL secretion is episodic and follows a diurnal variation in non-lactating male and female rats (Hill et al., 1999). Milk also contains PRL and PRL-binding proteins. It was evident that 16% of milk PRL molecules were transferred from the intestinal lumen to the circulation of suckling pups (Dusanter-Fourt et al., 1992).

Copyright by Mahidol University

Table 1.1 Serum and plasma PRL concentrations in humans and rats.

	Serum PRL (ng/mL)	References
Humans		
Children	6.8±1.2	Tolis, 1980
Male	6.2±0.6	Tolis, 1980
Female		
Non-pregnant	9.0±0.6	Jacob et al., 1972
Pregnant	>20 times of normal level	Jacob et al., 1972
Lactating	252.8±47.4	Noel et al., 1974
Female rats		
Diestrus	7.5±3.4	Boass et al., 1992
Proestrus-estrus-metestrus	19.7±6.6	Boass et al., 1992
Pregnant	115.8±20.3	Boass et al., 1992
Lactating	201.3±37.0	Boass et al., 1992
Suckling	400–650	Arbogast and Voogt, 1998
	Plasma PRL (ng/mL)	**References**
Female rats		
Control	5.34±0.70	Piyabhan et al., 2000
Anterior pituitary-grafted rats*	91.69±2.88	Piyabhan et al., 2000

***Note** Rats were transplanted with two pituitary glands under the renal fascia for 1 month to induce hyperprolactinemia.

Tyson and co-workers (1972) showed that, in pregnant women, elevated PRL levels first appeared at about week 8 of gestation and continued to raise steadily to a mean level of about 200 ng/ml at term. In another study, basal PRL concentrations during pregnancy increased gradually by approximately 4-fold and 8-fold as observed at weeks 24 and 36, respectively, compared with levels observed at week 12 (Bohnet et al., 1985). However, milk production does not occur during pregnancy because of a high level of progesterone that inhibits lactogenesis (Hill et al., 1999). After delivery,

Copyright by Mahidol University

progesterone and estrogen levels fall over 5 to 6 days. Without suckling, PRL levels also decline over 14 to 21 days postpartum (Hill et al., 1999).

In lactating women with suckling, PRL levels are 10 to 20 times higher than non-pregnant levels (Jacobs et al., 1972). PRL secretion in lactating women can be divided into 3 phases. Phase 1 occurs in immediate puerperium when there is a high basal PRL level followed by a surge within 10 to 30 min after nursing or mechanical nipple stimulation (Hill et al., 1999). In phase 2, i.e., between 8 and 60 days postpartum, basal PRL levels are still elevated compared to the non-lactating levels. Nevertheless, PRL surge in response to suckling peaks approximately 15 to 30 min after the onset of suckling with levels being 50 times higher than the non-lactating concentration (Tyson et al., 1972). In phase 3, i.e., 60 days postpartum, inconsistent basal PRL levels and post-suckling PRL surge are present (Tyson et al., 1972). Diaz and co-workers (1989) suggested that plasma PRL levels increased and decreased proportionately to frequency, intensity, and duration of nipple stimulation. Mean serum PRL was significantly higher during night time than during day time in non-lactating as well as in lactating women (Diaz et al., 1989).

Generally, secretion of PRL from the anterior pituitary gland is under the inhibitory control of the hypothalamus. The major hypothalamic factor responsible for this inhibition is dopamine released from the tuberoinfundibular dopaminergic (TIDA) neurons in the arcuate nucleus (Andrews et al., 2001). Dopamine activates D_2 dopamine receptors on the lactotrophs to tonically inhibit the release of PRL. Drugs that inhibit PRL release are primarily dopaminergic agonist, such as bromocriptine, whereas drugs that activate PRL release are thyrotropin-releasing hormone (TRH), estrogen, metoclopramide and phenothiazines.

F. Prolactin receptors

PRL receptors (PRLR) are specific, high-affinity, saturable membrane-bound proteins of class I cytokine receptor superfamily (Goffin et al., 2002). Three isoforms of PRLR, namely short, intermediate and long isoforms, were identified in rats, based on their cytoplasmic lengths. The gastrointestinal tract, including the duodenum and cecum, expresses both short- and long-form PRLRs (Jantarajit et al., 2007). Indeed, PRLRs are virtually expressed in several organs and/or tissues, e.g., brain, kidney,

Copyright by Mahidol University

bone, intestine and ovary. Their expression has been shown to vary as a function of the stage of the estrous cycle, pregnancy and lactation (Gofffin et al., 2002).

PRLRs are widely distributed along the intestine. Duodenum and jejunum are the major sites of PRLR gene expression and PRL-binding sites (Dusanter-Fourt et al., 1992). The duodenum which is the main site of the active calcium transport is also highly responsive to PRL by increasing calcium absorption (Krishnamra et al., 1998). The jejunum and, to a lesser extent, the ileum also exhibited the PRL-stimulated calcium transport (Krishnamra et al., 1998). In the large intestine, PRLRs were detected in both cecum and colon (Jantarajit et al., 2007).

G. Prolactin signaling pathways

PRL uses STAT proteins to regulate the transcription of specific genes through the JAK/STAT pathway (Figure 1.3) (Bole-Feysot et al., 1998). Ligand binding triggers dimerization of PRLRs. Thereafter, receptor-associated tyrosine kinases, namely Janus kinases (JAK), then phosphorylate each other as well as the tyrosine residues on PRLRs. Subsequently, SH2-containing cytoplasmic proteins from the STAT family are recruited to the receptor complex and phosphorylated by JAKs. Two STAT proteins then undergo dimerization, translocate into the nucleus, and activate gene transcription. PRL was reported to activate STATs 1, 3 and 5. Furthermore, in the mammary gland, STAT 5a and STAT 5b were activated by PRL and probably also by placental lactogen (Hennighausen et al., 1997).

Besides JAK/STAT system, the PRL-activated PI3K and MAPK were demonstrated in the islets of Langerhan of pregnant rats (Amaral et al., 2004). It was found that PRL stimulated islets insulin receptor substrates (IRSs)/PI3K to convey information through their downstream signals, i.e., AKT and p70s6k, to promote cell growth and glucose uptake. In the duodenum, PRL also used the PI3K pathway to stimulate calcium absorption (Jantarajit et al., 2007).

Copyright by Mahidol University

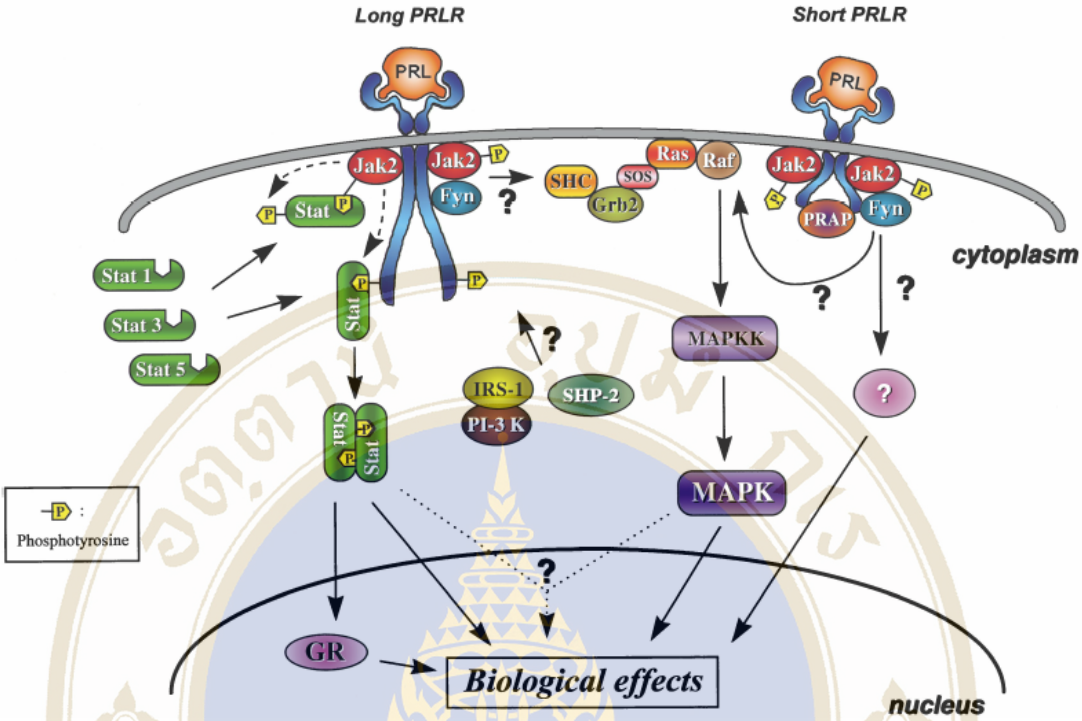


Figure 1.3 Signal transduction of PRL. PRLRs mediate their signaling pathways mainly through the JAK/STAT. Other possible pathways, e.g., MAPK and PI3K, are also depicted (modified from Bole-Feysot et al., 1998).

H. Prolactin and intestinal calcium transport

As mentioned earlier, PRL is an important hormone during pregnancy and lactation, and its levels are dramatically increased more than 20 folds during those periods (Table 1.1). At the peak lactation, PRL may be associated with excessive loss of 200–400 mg calcium per day for milk production, which is presumably later compensated by the PRL-enhanced intestinal calcium absorption (Charoenphandhu and Krishnamra, 2007).

Amenomori and co-workers (1970) demonstrated that the change in plasma PRL was parallel to the change in the active intestinal calcium absorption. PRL was found to increase the active component of the duodenal calcium transport in rats, possibly by increasing the apical calcium uptake and basolateral PMCA activity in the duodenal enterocytes (Charoenphandhu et al., 2006). In situ perfusion experiments in rat duodenum suggested that PRL increased the paracellular passive calcium flux in the presence of 20 mM luminal calcium (Krishnamara et al., 1998). By using in vitro

Copyright by Mahidol University

Ussing chamber technique, PRL was demonstrated to directly and acutely stimulate the active calcium absorption through both the transcellular active (Charoenphandhu et al., 2001) and the solvent drag-induced mechanisms (Tanrattana et al., 2004). Long-term exposure to PRL for 15 days also stimulated the transcellular active duodenal calcium transport in both young and adult non-mated rats, but enhancing the solvent drag-induced calcium transport only in young rats (Tudpor et al., 2005).

The large intestine also has capability to absorb calcium (Charoenphandhu 2007), but its physiological significance is still controversial. It was evident that PRL had some osmoregulatory functions in the large intestine (Krishnamra et al., 2001), such as enhancement of colonic water transport. Recently, Puntheeranurak and colleagues (2007) found that calcium-dependent potassium and chloride transport in the proximal and distal colon of mice were also inhibited by PRL. However, by using in situ intestinal loop technique, intraperitoneal injection of 0.2 mg/kg PRL 1 h before the experiment significantly increased the net gradient-dependent passive calcium absorption in the cecum by 38% (Krishnamra et al., 1993).

Copyright by Mahidol University

CHAPTER III
MATERIALS AND METHODS

1. Animals

Female Sprague-Dawley rats (pregnant/lactating, 9–14 weeks old), weighing 200–250 g, were obtained from the National Laboratory Animal Centre, Mahidol University. They were housed in the laboratory animal husbandry unit under 12:12 h light-dark cycle for at least 7 days before the beginning of the experiment. The room had temperature in the range of 20–25°C and humidity of 50−60%. Animals were fed regular pallets containing 1.0 % calcium (CP Co., Ltd, Thailand), and distilled water ad libitum. All animals were cared for in accordance with the principles and guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Science, Mahidol University.

2. Experimental procedures

Ussing chamber technique was used to investigate the duodenal and cecal calcium transport. The chamber consisted of two functional halves, i.e., the chamber itself and the electrical circuitry (Figure 3.1). The chamber was separated into two compartments (mucosal and serosal sides) by the mounted tissue. Each half of the chamber was filled with physiological bathing solution which contained substances of interest, e.g., ^{45}Ca or inhibitors. The electrical circuitry allowed measurement of the potential difference (PD), transepithelial electrical resistance (TER) and short-circuit current (Isc), all of which were indicative of epithelial electrogenic transport activity, viability and membrane integrity.

Copyright by Mahidol University

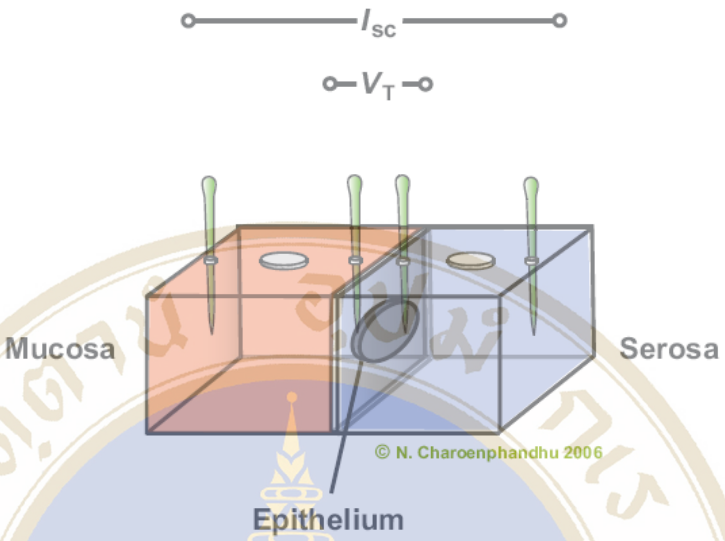


Figure 3.1 Modified Ussing chamber was composed of 2 hemichambers and the mounted epithelium in the middle between two hemichambers. PD (or V_T)-sensing electrodes were placed near the epithelium, whereas the current-supplying electrodes were placed at both ends.

2.1 Tissue preparation

After the animal was anesthetized with 50 mg/kg sodium pentobarbitone i.p., the duodenum (2–10 cm distal to pylorus) and cecum were removed. After being rinsed with an ice-cold physiological bathing solution pre-gassed with 5% CO_2 in 95% O_2, each intestinal segment was cut longitudinally along the mesentery Gorder, and rinsed again to remove the luminal contents. The tissue was then mounted in Ussing chamber with an exposed surface area of 0.69 cm^2 and bathed on both sides with 3 mL solution buffer maintained at 37°C, and continuously gassed with 5% CO_2 in 95% O_2. The tissue was firmly affixed to the chamber with adhesive silicone. Gas bubbles were fine but vigorous enough to minimize the unstirred water layer and to provide good mixing. The tissue was incubated for 15–20 min before 50-min experiment was performed.

Copyright by Mahidol University

2.2 Solution

Physiological bathing solution contained in mM: 118 NaCl, 4.7 KCl, 1.1 $MgSO_4$, 1.25 $CaCl_2$, 23 $NaHCO_3$, 12 D-glucose, and 2 mannitol. The solution was be continuously gassed with humidified 5% CO_2 in 95% O_2, maintained at 37°C, pH 7.4, and had osmolality of 290–293 mmol/kg water.

2.3 Unidirectional flux measurement

Calcium fluxes were determined by the modified methods of Karbach (1991) and Charoenphandhu et al. (2001). After 20-min incubation, the bathing solution was replaced with fresh solution at both sides. One side of the chamber was replaced with bathing solution containing ^{45}Ca. Seven samples were collected from each setup to calculate the unidirectional calcium flux from the hot side (H) to cold side (C) using the following equations.

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

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

^{45}Ca was analyzed by liquid scintillation spectrophotometer (model 1219, LKB Wallac, Turku, Finland).

2.4 Electrical measurement

Electrical parameters were continuously measured after 20-min incubation. Two pairs of agar bridges (4 M KCl per 4 g% agar) were used to measure the transepithelial potential difference (PD) and to apply short-circuit current (Isc). All electrodes were made of Ag/AgCl half cells, and were connected to the chamber via agar bridges. Two PD-sensing electrodes located near the tissue surface were connected to a pre-amplifier (model EVC-4000, World Precision Instruments, Sarasota, FL, USA) and finally to a PowerLab 4/30 (ADInstruments, Colorado Springs, CO, USA). Another pair of Ag/AgCl electrodes was placed at the distant end of each hemichamber to supply Isc, which was also measured by a PowerLab 4/30

Copyright by Mahidol University

connected in series to the EVC-4000 current-generating unit. PowerLab 4/30 operated with Chart 5.2.2 for Mac OS X (ADInstruments). Transepithelial resistance (TER) was calculated by Ohm's equation, as follow.

$$\text{TER } (\Omega\ \text{cm}^2) = \frac{\text{PD (mV)} \times 10^{-3}}{\text{Isc } (\mu\text{A cm}^{-2})} \quad (3)$$

2.5 Determination of charge selectivity

Permeability of sodium (P_{Na}) and chloride (P_{Cl}) ions determined charge selectivity, and were measured by the dilution potential technique. This method was performed by removing a half of NaCl in one side of the chamber before monitoring an immediate change of PD. Permeability ratio (P_{Na}/P_{Cl}) and absolute P_{Na} and P_{Cl} were calculated from the changed PD and conductance (G; G=1/TER).

The ion permeability ratio (ρ) was calculated from the dilution potential (V_δ) by using the Goldman-Hodgkin-Katz equation, as followed:

$$V = \frac{RT}{F} \ln \frac{P_{Na} C_m + P_{Cl} C_s}{P_{Na} C_s + P_{Cl} C_m} \quad (4)$$

where P_{Na} is the absolute permeability of sodium; P_{Cl} is the absolute permeability of chloride; C_m is the mucosal NaCl concentration; C_s is the serosal NaCl concentration; R,T and F are gas constant, temperature and Faraday constant, respectively.

When given $\rho = P_{Na}/P_{Cl}$, $\phi = C_s/C_m$, and $v = F V_\delta/RT$, then Equation 4 can be rewritten as,

$$\rho = (\phi - e^v)/(\phi e^v - 1) \quad (5)$$

The absolute epithelial permeability to sodium and chloride were calculated from the conductance (G) and permeability ratio by using the simplified Kimizuka-Koketsu equation (Kahle et al., 2004), as followed:

$$P_{Na} = \frac{GRT}{C_m F^2} \times \frac{\rho}{1+\rho} \quad (6)$$

$$P_{Cl} = P_{Na}/\rho \quad (7)$$

Copyright by Mahidol University

3. Experimental protocols

3.1 Protocol I: To investigate the PRL-enhanced duodenal calcium transport in pregnant and lactating rats.

Hypothesis: PRL stimulated the duodenal calcium transport on pregnant day 14 (P14) and day 21 (P21), and lactating day 7 (L7), day 14 (L14) and day 21 (L21).

Experimental procedure:

Ussing technique was used to determine the transepithelial calcium flux. In some experiments, bromocriptine was administered to abolish endogenous PRL secretion in the presence or absence of exogenous PRL supplementation. The ages of animals were 10, 11, 12, 13, and 14 week for P14, P21, L7, L14 and L21 rats, respectively. Animals were divided as followed:

1. Pregnant groups

1.1 Age-matched nulliparous controls

1.2 Pregnancy: P14 and P21

1.3 Pregnancy+4 mg/kg/day bromocriptine s.c.: PB14 and PB21

1.4 Pregnancy+4 mg/kg/day bromocriptine s.c.+exogenous PRL (0.4 mg/kg/day PRL s.c.): PBP14 and PBP21

2. Lactating groups

2.1 Age-matched nulliparous controls

2.2 Lactation: L7, L14 and L21

2.3 Lactation+4 mg/kg/day bromocriptine s.c.: LB7, LB14 and LB21

2.4 Lactation+4 mg/kg/day bromocriptine s.c.+exogenous PRL (0.6 mg/kg/day PRL s.c.): LBP7, LBP14 and LBP21

As for the lactating groups, the litter size was adjusted to 8 pups per dam. In PB, PBP, LB, and LBP groups, the animals were injected with bromocriptine (B) to abolish pituitary PRL secretion with or without PRL (P) supplementation for 7 days prior to calcium flux measurement.

3. 15-day post-weaning group

Maternal calcium flux was also determined at 15 days post-weaning.

Measured parameters for groups 1–3 were:

1. The electrical (biophysical) parameters: PD, Isc and TER
2. Transepithelial calcium flux

Copyright by Mahidol University

3. Body weight of pups

3.2 Protocol II: To determine the PRL-induced changes in the charge selectivity in the duodenum of pregnant and lactating rats.

Hypothesis: PRL altered charge selectivity of the duodenal epithelium during pregnancy and lactation.

Experimental procedure:

The dilution potential technique was used to determine charge selectivity. Animals were divided as shown in Protocol I.

Measured parameters were:

1. P_{Na}/P_{Cl}
2. P_{Na}
3. P_{Cl}

3.3 Protocol III: To investigate physiological significance of suckling-induced PRL surge in the regulation of duodenal calcium absorption in lactating rats.

Hypothesis: Suckling-induced PRL surge further increased the duodenal calcium absorption in lactating rats.

Experimental procedure:

Transepithelial calcium flux was determined by Ussing chamber technique. Control and lactating rats were divided, as followed:

1. Age-matched nulliparous controls
2. Lactation: without suckling: L7, L14 and L21
3. Lactation+suckling on specific lactating day: LS7, LS14 and LS21
4. Lactation without suckling+exogenous PRL (single-dose, 0.8 mg/kg s.c. 1 h before calcium flux measurement to mimic PRL surge): LSP7, LSP14 and LSP21
5. Lactation+suckling+bromocriptine (single-dose, 4 mg/kg i.p. 1 h before suckling): LSB7, LSB14 and LSB21
6. Lactation+suckling+single dose bromocriptine+single-dose exogenous PRL: LSBP7, LSBP14 and LSBP21

As for suckling protocol, the pups were separated from the dam for 2 h on the experimental day. Thereafter, pups were placed back to allow suckling for 30 min. In the lactating group without suckling, the pups were not returned to the dams. Maternal

Copyright by Mahidol University

calcium transport measurement was performed at 60 min after the start of suckling. Single-dose bromocriptine administered 15 min prior to suckling was used to abolish the suckling-induced PRL surge, but neither the "baseline" PRL levels nor baseline calcium transport was interfered.

Measured parameters were:

1. Electrical parameters: PD, Isc and TER
2. Transepithelial calcium flux
3. Epithelial charge selectivity
4. Milk volume per suckling, performed by measuring the weight of pups before and after suckling. Volume was calculated from body weight (i.e., milk weight) and milk density. The milk density of 1.61 g/mL was the ratio of average cow milk weight and milk volume (Table 3.1) with an assumption that cow milk and rat milk have similar density.

Table 3.1 Average cow milk weight and milk volume from 10 independent samples.

Milk weight (g)	Volume (mL)
1.22	1
1.33	1
1.85	1
1.85	1
1.86	1
1.84	1
1.85	1
1.84	1
1.28	1
1.18	1
Average 1.61	1

Copyright by Mahidol University

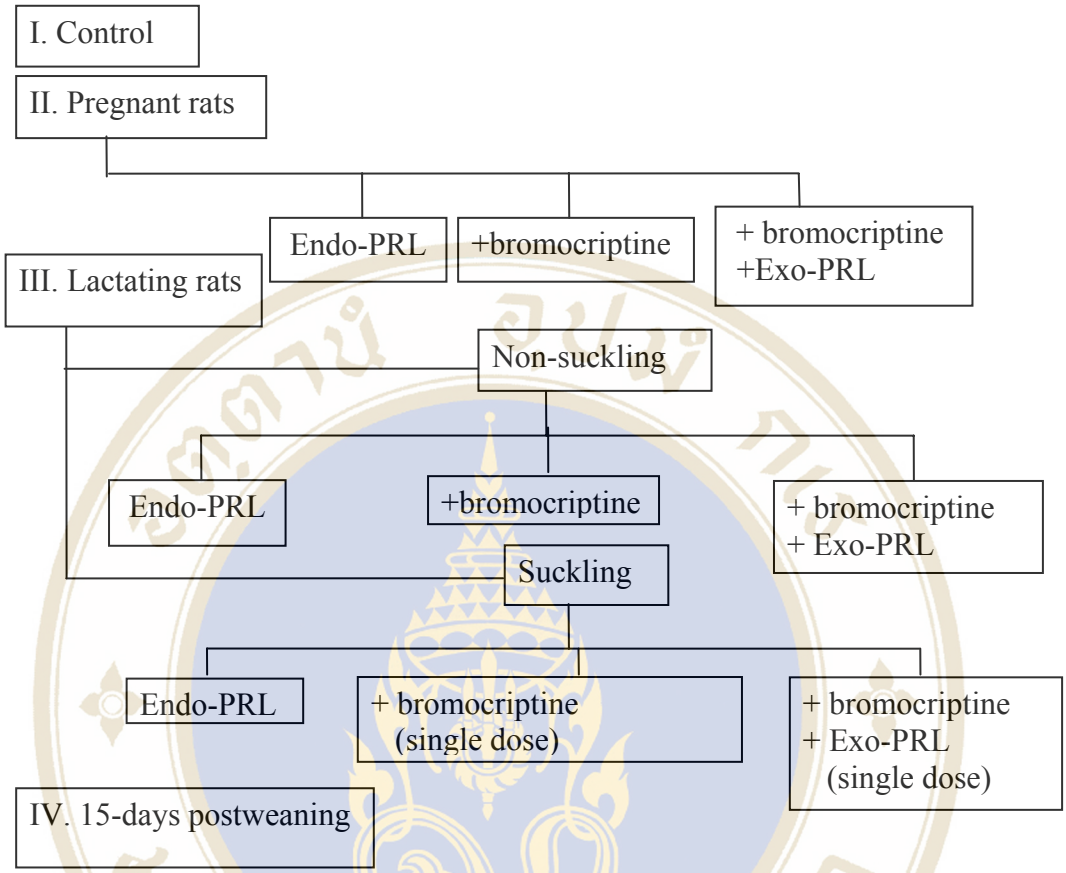


Figure 3.2 Experimental design.

3.4 Protocol IV: To demonstrate the PRL-induced changes in calcium transport and charge selectivity in the cecum.

Hypothesis: PRL directly stimulated the cecal calcium absorption and altered charge selectivity of the cecal epithelium

Experimental procedure:

Transepithelial calcim flux and charge selectivity were determined by Ussing chamber technique and dilution potential technique, respectively. Animals were randomly divided into 2 groups:

1. Control
2. 800 ng/mL Prolactin-treated (PRL was directly added into the chamber)

Copyright by Mahidol University

Measured parameters were:

1. Electrical parameters: PD, Isc and TER
2. Transepithelial calcium flux
3. P_{Na}/P_{Cl}
4. P_{Na}
5. P_{Cl}

4. Statistical analysis

The results were expressed as means ± SE. Multiple comparisons were made by one-way analysis of variance (ANOVA). The differences in pairs of means were determined by Dunnett post-test. The level of significance for statistical tests was $P<0.05$. Data were analyzed by GraphPad Prism 4.03 for Windows XP (GraphPad Software, San Diego, CA, USA).

Copyright by Mahidol University

CHAPTER IV
RESULTS

Part 1: Effects of PRL on the duodenal calcium transport during pregnancy.

The aim of this experiment was to investigate the PRL-enhanced duodenal calcium transport in pregnant and lactating rats. The exogenous bromocriptine was administered to abolish endogenous PRL secretion with or without exogenous PRL supplementation.

As shown in Figures 4.1 and 4.2, pregnancy significantly increased the total active calcium fluxes from the control value of 36.46 ± 1.89 to 47.61 ± 2.58 nmol h^{-1} cm^{-2} at mid-pregnacy (P14), and 32.67 ± 2.03 to 48.44 ± 3.17 nmol h^{-1} cm^{-2} at late pregnancy. The pregnancy-induced increase in duodenal calcium transport was abolished by bromocriptine, while exogenous PRL supplementation restored calcium fluxes in bromocriptine-treated rats to the normal pregnant levels. The results suggested PRL was responsible for the enhanced duodenal calcium transport during pregnancy

Copyright by Mahidol University

P14

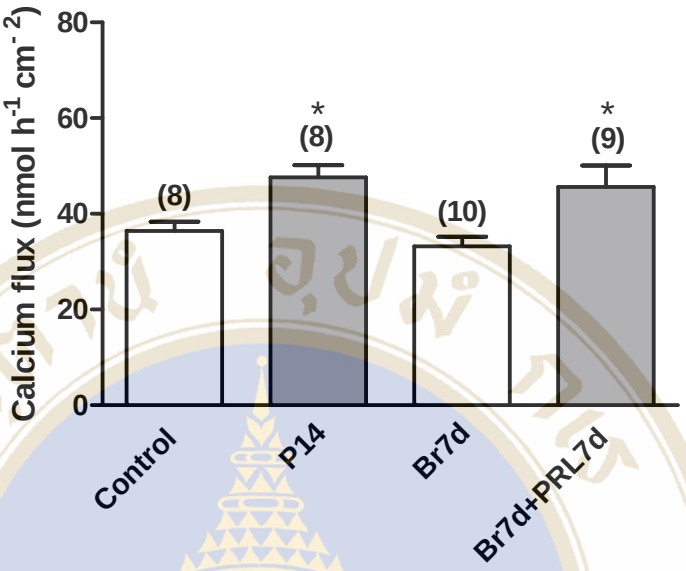


Figure 4.1 The total active duodenal calcium fluxes in the apical to basolateral direction in P14 rats. In some experiments, P14 rats were administered for 7 days bromocriptine (Br7d) or bromocriptine plus exogenous PRL supplementation (Br7d+PRL7d). Numbers in parentheses represent the number of animals.

* $P<0.05$ compared with control group.

Copyright by Mahidol University

P21

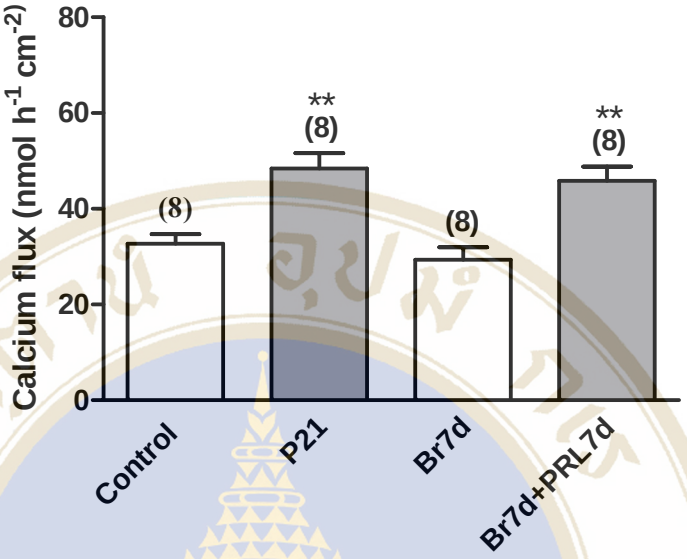


Figure 4.2 The total active duodenal calcium fluxes in the apical to basolateral direction in P21 rats. In some experiments, P21 rats were administered for 7 days with bromocriptine (Br7d) or bromocriptine plus exogenous PRL supplementation (Br7d+PRL7d). Numbers in parentheses represent the number of animals.

** $P<0.01$ compared with control group.

Copyright by Mahidol University

Part 2: Effect of PRL on the duodenal calcium transport during lactation and suckling-induced PRL surge.

As shown in Figures 4.3, 4.4 and 4.5, lactation induced an increase in the duodenal calcium transport in lactating rats, i.e., from the control value of 17.16 ± 2.26 to 41.81 ± 2.52 nmol h^{-1} cm^{-2} in L7 rats, 27.04 ± 1.49 to 39.20 ± 2.87 nmol h^{-1} cm^{-2} in L14 rats, and 26.47 ± 2.51 to 39.38 ± 2.18 nmol h^{-1} cm^{-2} in L21 rats. Administration of bromocriptine for 7 days completely abolished the lactation-induced increase in calcium transport, while 7-day exogenous PRL administration to 7-day bromocriptine-treated lactating rats restored calcium fluxes to 7-day bromocriptine-treated lactating rats restored calcium fluxes to the "baseline" lactating levels. In addition to calcium flux, lactation also induced a decrease in TER (Table 4.1), suggesting that the paracellular calcium transport may be changed during lactation.

Interestingly, during suckling (acute PRL surge) in L7, L14 and L21 rats, the total active duodenal calcium absorption further increased above the "baseline" lactating levels (Figures 4.3, 4.4 and 4.5). Single-dose exogenous bromocriptine injected 1 h before the experiment 1 h abolished the effect of PRL surge. Single-dose PRL administration to single-dose bromocriptine-treated rats could mimic the effect of suckling-induced PRL surge on calcium transport. Moreover, the suckling-enhanced calcium transport was well correlated with milk volume (Figure 4.6). The degree of correlation became lower in single-dose bromocriptine-injected rats.

At 15-day postweaning, there was no change in the duodenal calcium transport in the dams when compared to the age-match nulliparous control rats (Figure 4.8).

Copyright by Mahidol University

L7

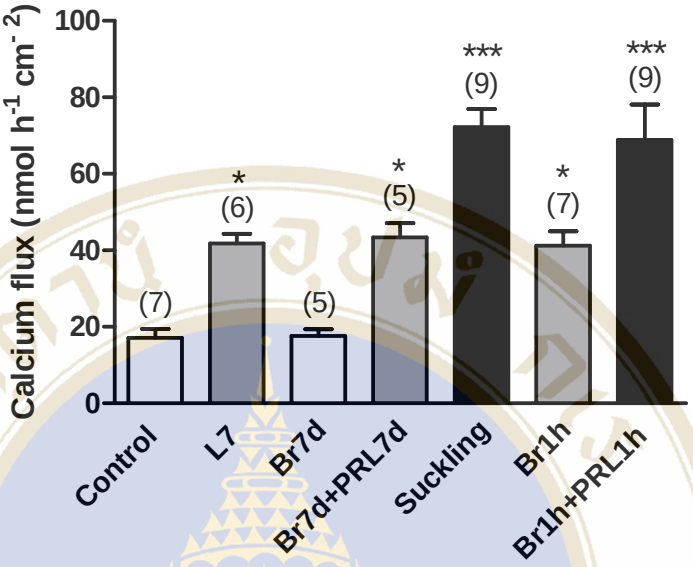


Figure 4.3 The total active duodenal calcium fluxes in the apical to basolateral direction in L7 rats. In some experiments, L7 rats were administered for 7 days with bromocriptine (Br7d) or bromocriptine plus exogenous PRL supplementation (Br7d+PRL7d) to determine the chronic (genomic) PRL effects. The acute (Nongenomic) PRL effects were demonstrated in suckling rats injected with bromocriptine 1 h (Br1h) prior to the calcium flux study, with or without single-dose PRL supplementation (PRL1h). Numbers in parentheses represent the number of animals.

* $P<0.05$, *** $P<0.001$ compared with control group.

Copyright by Mahidol University

L14

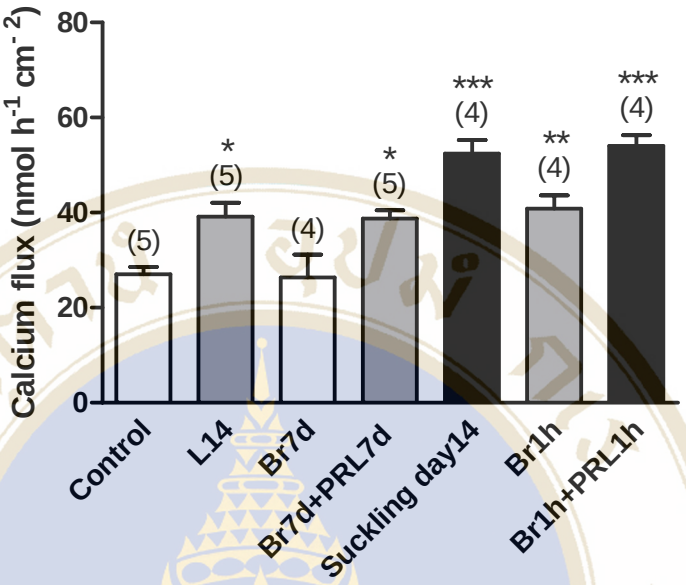


Figure 4.4 The total active duodenal calcium fluxes in the apical to basolateral direction in L14 rats. In some experiments, L14 rats were administered for 7 days with bromocriptine (Br7d) or bromocriptine plus exogenous PRL supplementation (Br7d+PRL7d). The acute (Nongenomic) PRL effects were demonstrated in suckling rats injected with bromocriptine 1 h (Br1h) prior to the calcium flux study, with or without single-dose PRL supplementation (PRL1h). Numbers in parentheses represent the number of animals. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared with control group.

Copyright by Mahidol University

L21

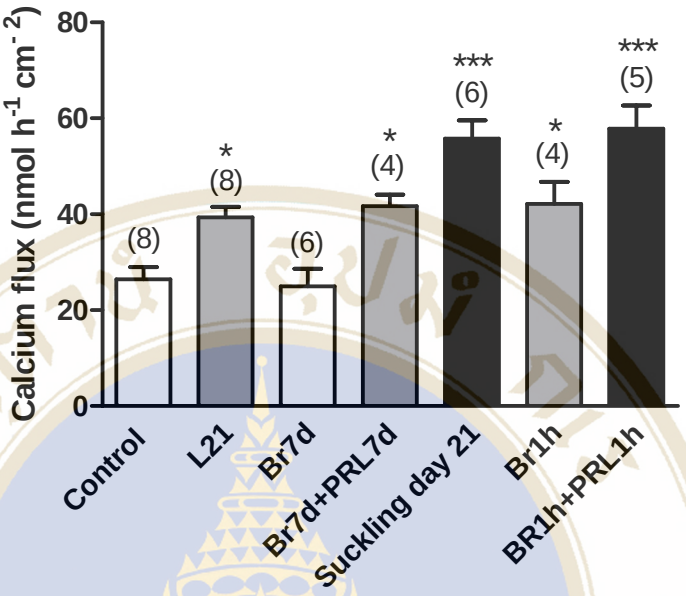


Figure 4.5 The total active duodenal calcium fluxes in the apical to basolateral direction in L21 rats. In some experiments, L21 rats were administered for 7 days with bromocriptine (Br7d) or bromocriptine plus exogenous PRL supplementation (Br7d+PRL7d). The acute (Nongenomic) PRL effects were demonstrated in suckling rats injected with bromocriptine 1 h (Br1h) prior to the calcium flux study, with or without single-dose PRL supplementation (PRL1h). Numbers in parentheses represent the number of animals.

* $P<0.05$, *** $P<0.001$, compared with control group.

Copyright by Mahidol University

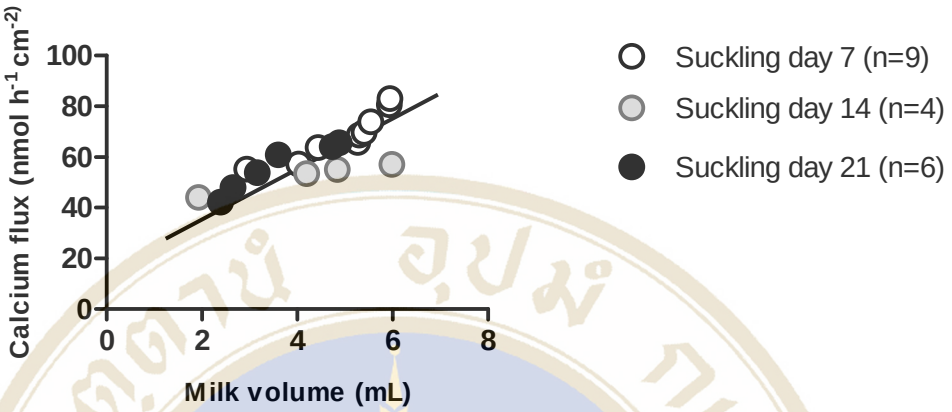
Table 4.1 Epithelial electrical parameters consisting of transepithelial potential difference (PD), short-circuit current (Isc), and transepithelial resistance (TER). Mounted tissue was bathed on both sides with 1.25 mmol/L calcium-containing solution. The apical side was negative with respect to the basolateral side. Values are means ± SE.

Conditions	n	Electrical parameters		
		PD (mv)	*Isc* (μA cm^{-2})	TER (Ω cm^2)
Control	7	3.93±0.11	28.95±1.26	146.10±3.73
L7	6	4.55±0.29	40.30±1.62*	108.00±8.08**
Br7d	5	4.14±0.15	34.25±1.24	131.40±6.23
Br7d+PRL7d	5	4.53±0.63	40.89±6.62*	115.50±11.31*
Suckling	9	4.44±0.09	43.29±1.61***	105.30±5.36***
Br1h	7	4.03±0.10	36.61±1.78	118.40±6.37*
Br1h+PRL1h	9	4.36±0.09	41.77±1.57***	115.10±6.64**

*P < 0.05 , ** P < 0.01, *** P < 0.001 compared with the control group.

Copyright by Mahidol University

Without Bromocriptine



$r^2 = 0.71$

$\hat{y} = (7.37 \pm 1.14)x + (28.85 \pm 5.22)$

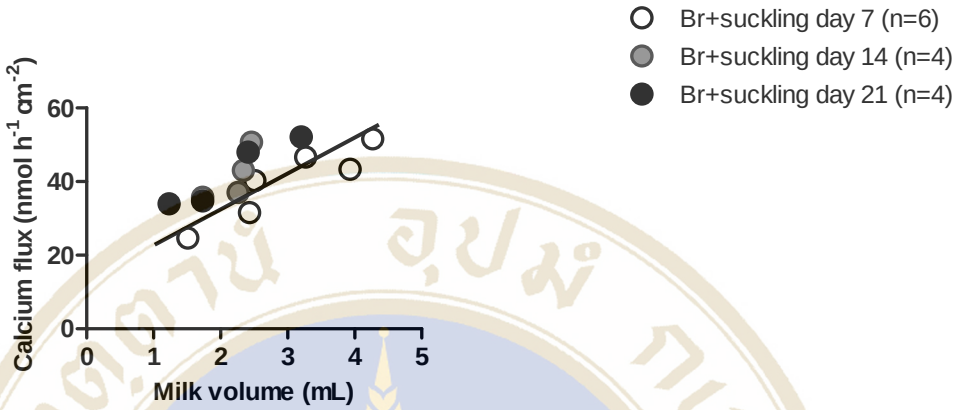
$\hat{y}$ = calcium flux

x = milk volume

Figure 4.6 Correlation between the total calcium flux and milk volume in suckling rats.

Copyright by Mahidol University

With Bromocriptine



$r^2 = 0.51$

$\hat{y} = (6.75 \pm 1.91)x + (23.92 \pm 5.90)$

$\hat{y}$ = calcium flux

x = milk volume

Figure 4.7 Correlation between the total calcium flux and milk volume in single-dose bromocriptine(Br)-treated suckling rats.

Copyright by Mahidol University

Postweanning

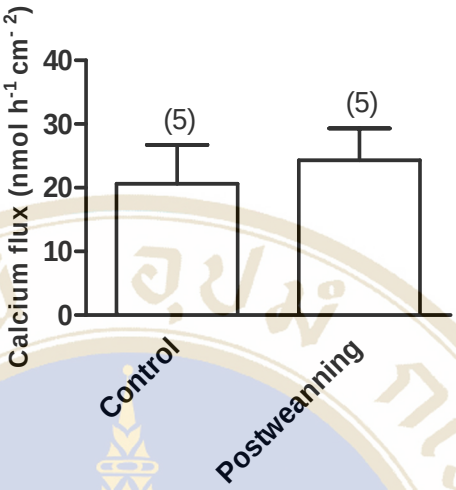


Figure 4.8 The total active duodenal calcium fluxes in the apical to basolateral direction in 15-day postweaning. Number in parentheses represent the number of animals.

Copyright by Mahidol University

Part 3: Effects of PRL on the charge-selective properties of the duodenal epithelial during pregnant and lactating rats

Dilution potential technique was used to determine the PRL-induced changes in the charge selectivity in the duodenum of pregnant and lactating rats. As shown in Figure 4.9, during the late pregnancy (P21), PRL was the responsible hormone that increased P_{Na}/P_{Cl} from a control value of 2.06 ± 0.09 to 3.75 ± 0.15. The increased P_{Na}/P_{Cl} resulted from an increase in P_{Na} from14.98 ± 1.87 to 30.78 ± 4.51 × 10^{-6} cm/s, without change in P_{Cl}.

During lactation (Figure 4.10), PRL also increased P_{Na}/P_{Cl} from a control value of 2.02 ± 0.09 to 3.72 ± 0.30, PRL also which resulted from an increase in P_{Na} from 20.53 ± 2.47 to 40.85 ± 4.15×10^{-6} cm/s without change in P_{Cl}. The present data indicated that PRL increased cation selectivity of the duodenal epithelium, which, in tern, allowed more calcium transport through the paracellular pathway.

Copyright by Mahidol University

P21

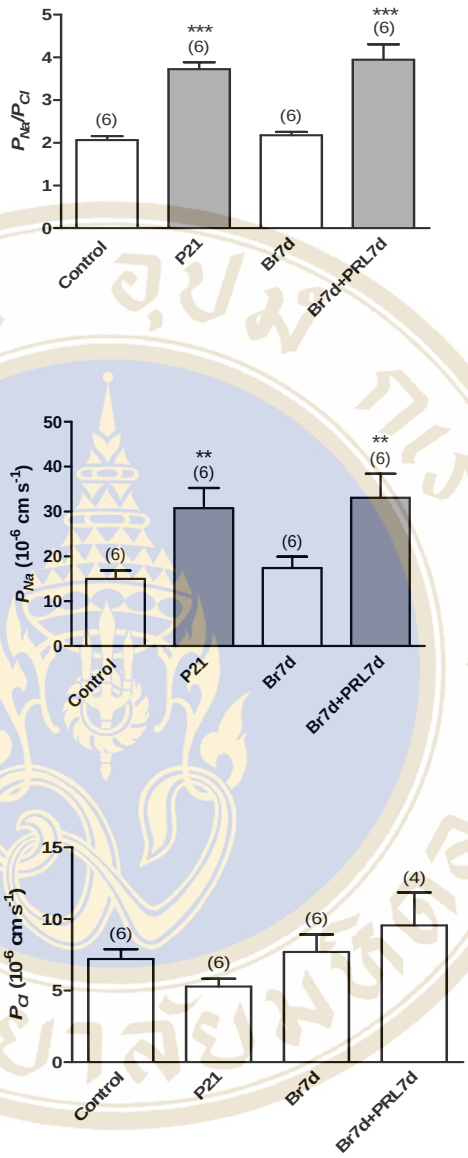


Figure 4.9 Charge-selective property of the duodenal tissue in P21 rats. Permeability ratio of sodium and chloride (P_{Na}/P_{Cl}), absolute sodium permeability (P_{Na}), and absolute chloride permeability (P_{Cl}) obtained from the dilution potential experiments are demonstrated. In some experiments, P21 rats were administered for 7 days with bromocriptine (Br7d) or bromocriptine plus exogenous PRL supplementation (Br7d+PRL7d). Numbers in parentheses represent the number of animals. ** P<0.01 compared with control group, *** P<0.001 compared with control group.

Copyright by Mahidol University

L7

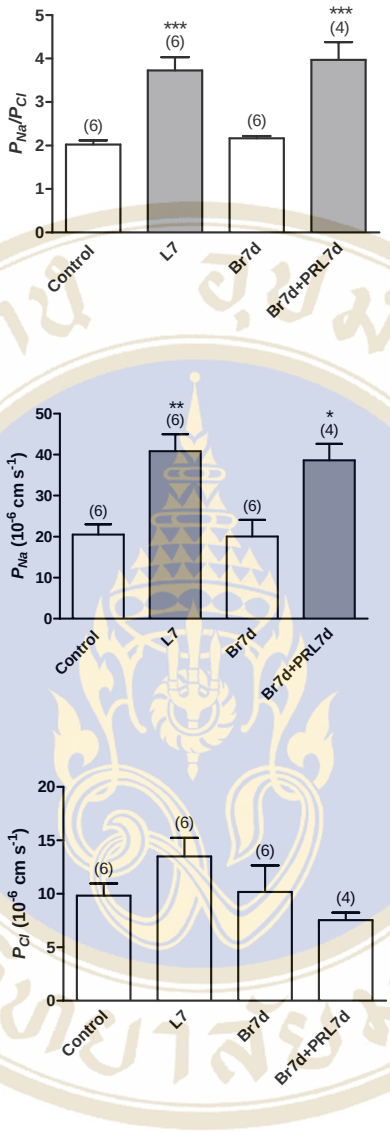


Figure 4.10 Charge-selective property of the duodenal tissue in L7 rats. Permeability ratio of sodium and chloride (P_{Na}/P_{Cl}), absolute sodium permeability (P_{Na}), and absolute chloride permeability (P_{Cl}) obtained from the dilution potential experiments are demonstrated. In some experiments, L7 rats were administerred for 7 days with bromocriptine (Br7d) or bromocriptine plus exogenous PRL supplementation (Br7d+PRL7d), Numbers in parentheses represent the number of animals. * P<0.05, ** P<0.01, *** P<0.001 compared with control group.

Copyright by Mahidol University

Part 4: PRL directly stimulated the total active calcium transport and altered charge selectivity in the cecum.

To demonstrate the direct stimulatory effect of PRL on the cecal calcium absorption, the mounted cecal tissues from non-mated rats were exposed to PRL which was added directly into the Ussing chamber. The results showed that 800 ng/mL PRL significantly increased the cecal calcium flux from the control value of 71.44 ± 6.36 to 141.90 ± 38.79 nmol $h^{-1}cm^2$ (Figure 4.11). TER of the cecal epithelium was also decreased (Table 4.2).

Dilution potential technique was used to determine the PRL-induced changes in the cecal charge selectivity. As shown in Figure 4.12, PRL increased P_{Na}/P_{Cl} from a control value of 1.99 ± 0.13 to 3.92 ± 0.15. Similar to that seen in the duodenums of pregnant and lactating rats, the increased P_{Na}/P_{Cl} resulted from an increase in P_{Na} from 19.60 ± 1.66 to 50.44 ± 8.09×10^{-6} cm/s, without change in P_{Cl}.

Copyright by Mahidol University

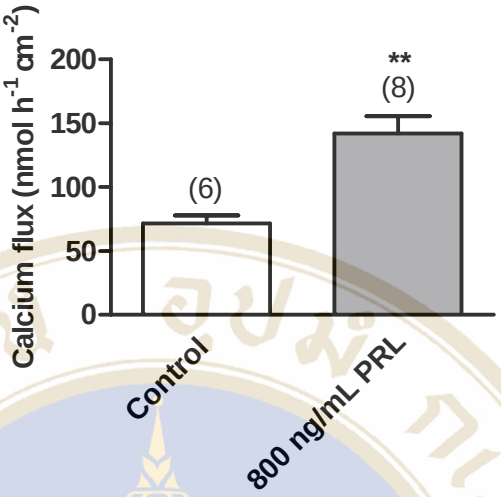


Figure 4.11 The total active cecal calcium flux in the cecum directly exposed to 800 ng/mL PRL. Numbers in parentheses represent the number of experimental animals. ** P<0.01 compared with control group.

Copyright by Mahidol University

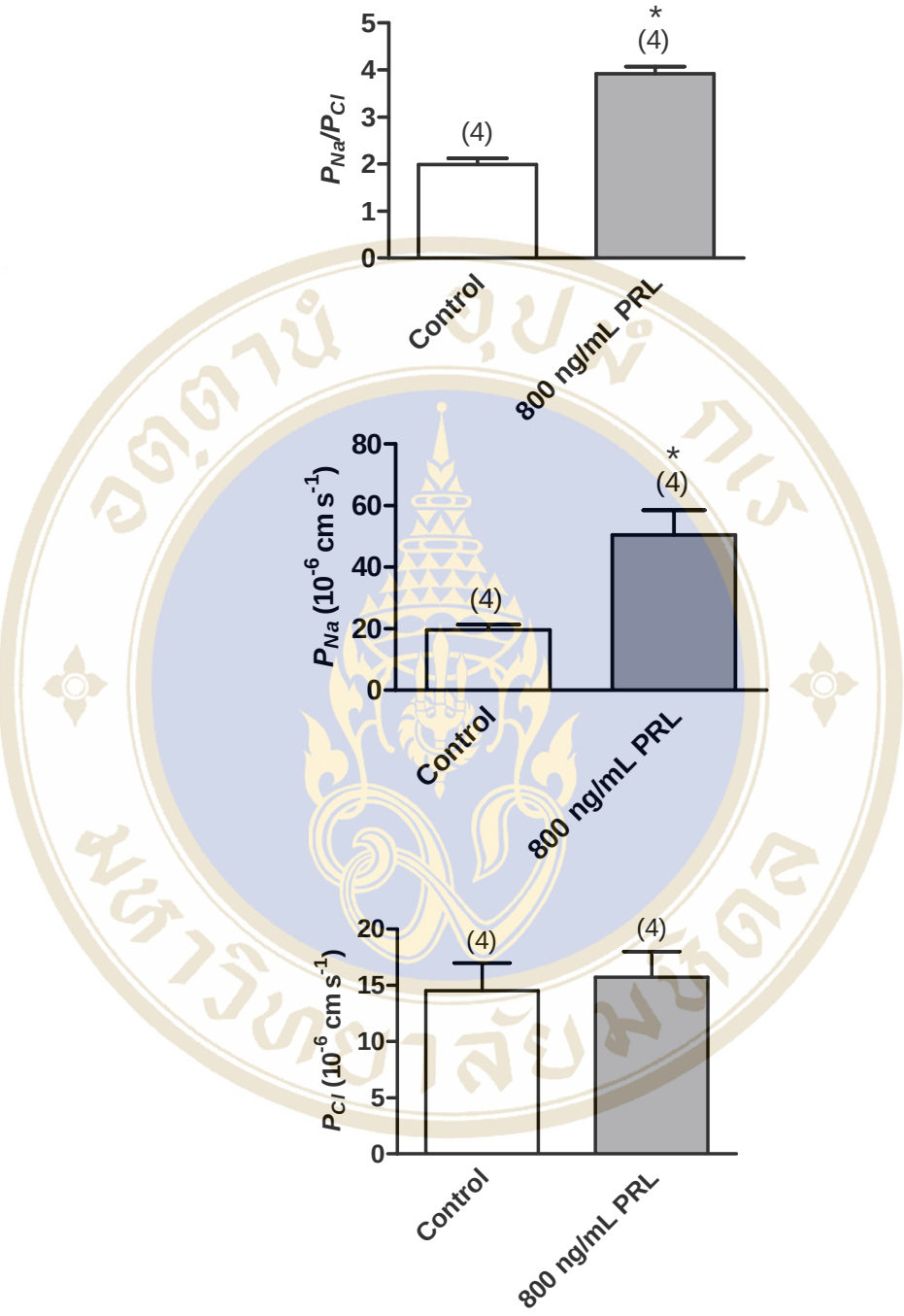


Figure 4.12 Charge-selective property of the cecal tissue. Permeability ratio of sodium and chloride (P_{Na}/P_{Cl}), absolute sodium permeability (P_{Na}), and absolute chloride permeability (P_{Cl}) obtained from the dilution potential experiments are presented. Numbers in parentheses represent the number of animals. * $P<0.05$ compared with control group.

Copyright by Mahidol University

Table 4.2 Electrical parameters in the cecum directly exposed to 800 ng/mL PRL. Mounted tissue was bathed on both sides with 1.25 mmol/L calcium-containing solution. The apical side was negative with respect to the basolateral side.

Conditions		Electrical parameters		
	n	PD (mv)	*Isc* (μA cm^{-2})	TER (Ω cm^2)
Control	7	5.43±0.67	55.23±4.15	102.90±11.53
800 ng/mL PRL	8	4.26±0.18	59.52±1.22	69.41±3.27*

Values are means ± SE. * $P < 0.05$ compared with the control group.

Copyright by Mahidol University

CHAPTER V
DISCUSSION

The negative calcium balance in mother during pregnancy and lactation leads to an increase in intestinal calcium absorption. PRL, as a calcium-regulating hormone during these reproductive periods, has been demonstrated to increase calcium transport in the rat duodenum especially via the active mechanism (Boass et al., 1992; Krishnamra and Taweerathitam, 1995). However, the cellular mechanisms and responsible signaling pathways of the PRL-stimulated calcium absorption during pregnancy and lactation have never been investigated.

The large intestine also has capability to absorb calcium (Charoenphandhu 2007), but its physiological significance is still controversial. It was evident that PRL had some osmoregulatory functions in the large intestine, such as enhancement of colonic water transport (Krishnamra et al., 2001). Recently, Puntheeranurak and colleagues (2007) found that the calcium-dependent potassium and chloride transport in the proximal and distal colons of mice were inhibited by PRL. Moreover, by using in situ intestinal loop technique, intraperitoneal injection of 0.2 mg/kg PRL 1 h before the experiment significantly increased the gradient-dependent passive calcium absorption in the cecum by 38% (Krishnamra et al., 1993). However, direct effects of PRL on cecum, which was the sites of highest calcium absorption rate, were not known. The present study aimed to *(i)* investigate the PRL-enhanced duodenal active calcium transport pregnancy and lactation, *(ii)* to determine the PRL-induced changes in the charge selectivity of the duodenal epithelium, *(iii)* to investigate the physiological significance of the suckling-induced PRL surge in the regulation of duodenal active calcium absorption in lactating rats, and *(iv)* to demonstrate direct stimulatory effect of PRL on the cecal active calcium transport.

Copyright by Mahidol University

A. Methodology

To study ion transport in the duodenal and cecal epithelia, Ussing chamber technique was employed because (*i*) informations on the transport function, biophysical characteristics and the intregrity of the tissue were obtained simultaneously, (*ii*) the set up could mimick the physiological condition i.e., with temperature of 37°C, pH of 7.4, and osmolality of 289–292 mmol/kg H_2O, and (*iii*) the in vitro set up allowed measurement of calcium flux in the absence of interference from intrinsic neural and hormonal regulation. Since the electrical parameters, e.g., PD and Isc, could not be used to represent the transepithelial calcium transport due to the relatively small magnitude of calcium transporter, radioactive calcium was used to demonstrate calcium fluxes.

The charge selectivity of the tight junction, which was a barrier in the paracellular space, was determined by the dilution potential technique. Immediate changes of PD (i.e., dilution potential) and conductance were used to calculate the permeability of the sodium and chloride. Although most epithelia and their tight junctions usually allow paracellular transport Na^+ and Cl^- as well as other trace elements, such as Ca^{2+} and Mg^{2+}. However, little has been known regarding the paracellular regulation and properties. Charge selectivity is experimentally determined by a ratio absolute permeability to sodium and chloride (P_{Na}/P_{Cl}). For cation-selective epithelia, their P_{Na}/P_{Cl} values are greater than 1. It was assumed that sodium was a representative of cations with comparable size, including calcium.

B. Effect of PRL on calcium transport in the duodenum and cecum

PRL could be regarded as a calcium-regulating hormone in non-pregnant, pregnant, and lactating rats, base on the fact that (*i*) it is secreted by the anterior pituitary gland and exerts action on the organs that maintain calcium homeostasis (i.e., small intestine, bone, and kidney) (Piyaphan et al., 2000), (*ii*) its functional receptors are present on the intestinal absorptive cells (Ouhtit et al., 1994), (*iii*) exogenous PRL administration is able to mimick the effects of physiologically elevated plasma PRL on calcium transport in several animal models (Charoenphandhu et al., 2001; Krishnamra et al., 1998), and (*iv*) the effects of endogenous PRL on calcium transport are abolished

Copyright by Mahidol University

by bromocriptine which inhibits pituitary PRL release and restored by exogenous PRL administration (Piyaphan et al., 2000).

The total active duodenal calcium transport consists of 2 major components, i.e., the transcellular active and solvent drag-induced calcium transport, while the voltage-dependent calcium transport is negligible in the rat duodenum (Charoenphandhu et al., 2001). The physiological significance of the transcellular active calcium transport in non-mated animals was doubtful, because the transcellular transport contributed less than 15% of the total active calcium transport (Charoenphandhu et al., 2001). Besides that TRPV6 knock out mice still exhibited normal rate of intestinal calcium absorption (Bianco et al., 2004). In addition, calbindin-D_{9k} knockout mice were also normocalcemic (Kutuzova et al., 2004). However, under certain conditions, such as pregnancy and lactation, the transcellular calcium transport may play a significant role in the extraction of extra calcium from the luminal contents, perhaps under the regulation of PRL.

Expression of calbindin-D_{9k} has also been shown to be regulated by PRL. Bruns and colleagues (1983) found that PRL administered to female rats at 10 μg/day for 9 days increased the amount of duodenal calbindin-D_{9k} protien by 25%–27%. However, the effects of PRL on the expression and functions of other important proteins involved in the transcellular and paracellular calcium transport such as TRPV5, TRPV6 and tight junction proteins (e.g., claudins and occludin) remain to be investigated.

Physiological significance of the different PRL concentrations in the regulation of calcium absorption is still inconclusive. It is likely that low basal PRL levels of 7–10 ng/mL in young or non-mated rats are important for the maintenance of the basal duodenal calcium absorption (Amnattanakul et al., 2005). Higher physiological PRL concentrations of 100–200 ng/mL (Mazor et al., 1996), 200–400 ng/mL (Noel et al., 1974) and 400–650 ng/mL (Arbogast and Voogt, 1998) found during pregnancy, lactation, and suckling, respectively, may further enhance calcium absorption and may help alleviate negative calcium balance that occurs during these reproductive periods.

Differential responses to a very high PRL concentration might be due to binding of PRL to other receptors of the type 1 cytokine receptor superfamily such as growth hormone receptor (GHR). PRL and growth hormone are classified as the

Copyright by Mahidol University

hematopoietin family of cytokines, and both can directly bind to PRLRs as well as GHRs to create functional receptor dimers (Forsyth and Wallis, 2002). It was reported that changes in amino acids on the long loop between helices I and II of PRL molecule promoted binding of PRL to GHRs (Cunningham et al., 1990). However, in general, PRL exerts its actions through the long- and/or intermediate-form receptors, whereas the short-form receptors seem to have a fine-tuning or counterbalancing role (Berlanga et al., 1997; Jahn et al., 1997). Physiological significance of each intestine-expressed isoform has not been demonstrated. Both short- and long-form PRLR mRNA transcripts were found in mucosal scrapings collected from the duodenum, jejunum, ileum, cecum, and colon (Jantarajit et al., 2007).

The present investigation showed that endogenous PRL during the late pregnancy (P21) and early lactation (L7) markedly enhanced the total active calcium fluxes, i.e., the "baseline" calcium flux was elevated. Interestingly, PRL surge during suckling additionally increased calcium flux above the elevated "baseline" flux. It was previously shown that serum PRL levels increased by ~10-fold between 18 and 20 days of pregnancy, and remained at a high level until day 7 of lactation. During the lactating period, plasma PRL was further increased to ~200–300 ng/mL without suckling, and ~400–600 ng/mL with suckling (Boass et al., 1992; Arbogast and Voogt, 1998). Therefore, chronic PRL exposure was responsible for the elevated "baseline" of the duodenal calcium absorption during pregnancy and lactation, while transient PRL surge during suckling acutely and rapidly enhanced the transepithelial calcium transport presumably to match calcium loss in milk. In the present study, the biphasic action of PRL was not seen because *(i)* PRL concentration was not high enough, *(ii)* and PRLR number may be increased during pregnancy and lactation compared to the control condition, thus allowing more PRLR to form functional dimmers.

The volume of milk consumed by pups showed a strong correlation with the total calcium fluxes, whereas bromocriptine-treated dams exhibited an impairment of this relationship (i.e., r^2=0.71 vs. 0.51). The role of neuroendocrine that regulate milk volume and calcium transport in the present study, may be explained by the hormonal response to calcium loss in milk. Suckling may induce release of PRL which subsequently stimulated calcium transporters and/or other hormones ,e.g., PTH-related protein (PTHrP). PTHrP production released from breast is known to be controlled by

Copyright by Mahidol University

several factors, including suckling, PRL, and calcium-sensing receptor (Kovacs 2005). Suckling and PRL both inhibit the hypothalamic GnRH pulse center, which can suppress gonadotropin (LH, FSH) release, thus in turn lowering the plasma levels of ovarian sex steroids (estradiol and progesterone) (Kovacs 2005). PTHrP enters blood stream and combines with systemically low estradiol levels to markedly enhance bone resorption (Kovacs 2005). Increased bone resorption releases calcium and phosphate into the blood stream, for milk production. Calcitonin (CT) may also help modulate the skeletal responsiveness to prevent excessive bone loss (Kovacs et al., 1997).

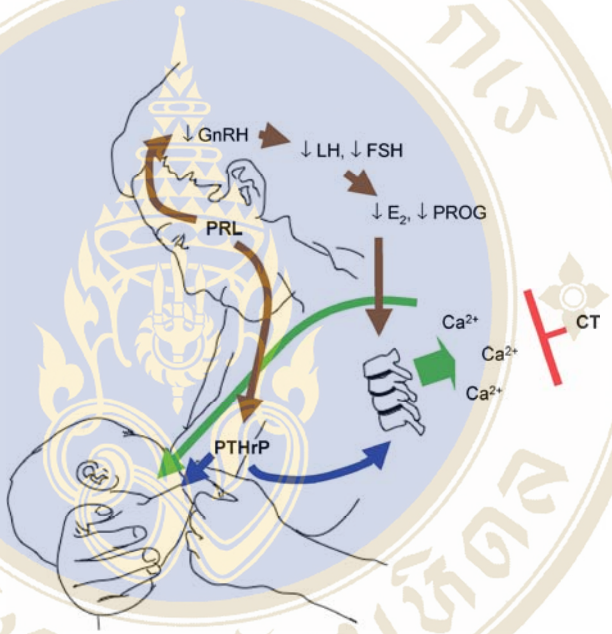


Figure 5.1 The role of the breast in controlling skeletal demineralization during lactation (Kovacs 2005).

Cellular mechanism of PRL in the duodenal epithelial cells remain elusive. Zhu and co-workers (1998) reported that the intestinal Ca^{2+}-ATPase and calbindin mRNA levels were not changed in day-7 and day-14 pregnant rats. However, at day 21 of pregnancy, Ca^{2+}-ATPase and calbindin mRNA levels increased 2–3-fold and remained elevated until day 7 of lactation. PRL may require the presence of 1,25-$(OH)_2D_3$ to exert its long-term stimulatory effect on intestinal calcium absorption (Tudpor et al., 2005). Therefore, it could be implied that the expressions of vitamin D-dependent genes such as TRPV6, calbindin-D9k, or $PMCA_{1b}$ are probably require for PRL-enhanced calcium absorption. Although most reported signal transductions of PRL were genomic and involved de novo gene transcription, nongenomic effects of

Copyright by Mahidol University

PRL on the duodenal calcium transport, which were seen within 60 min after PRL exposure, were also reported (Jantarajit et al., 2007). Moreover, the PRL-enhanced apical calcium uptake in duodenal enterocytes occurred as rapidly as 8 min, while the PRL-activated $PMCA_{1b}$ and Na^+/K^+-ATPase activities were detected within 30 min (Charoenphandhu et al., 2006). Nongenomic actions of PRL have been proposed to mediate through the ligand-induced modification of protein or re-localization of proteins rather than alteration of protein expression. PRL may exert its action on the transcellular active calcium transport by activating apical calcium uptake by increasing TRPV6 and $PMCA_{1b}$ activities. Moreover, it was possible that PRL increased cation-selective activity of claudins at the tight junction, thus in turn stimulating the paracellular passive calcium transport.

During postweaning, the unaltered total active calcium flux may result from the cessation of suckling-induced PRL surge. In other words, the enhanced calcium absorption was reversible and present only during these reproductive periods.

Besides the duodenum, PRL could also stimulate calcium absorption in the cecum. Since cecum is the site that absorbs more calcium released by bacterial fermentation, the PRL-enhanced cecal calcium transport may also help alleviate negative calcium balance during high calcium demand.

C. The effects of PRL on the charge-selective property of the duodenal epithelia

It was found, herein, that PRL was an important regulator that increased cation selectivity in the duodenum of pregnant and lactating rats. The enhanced capability to move cations through the tight junction helped explain the PRL-stimulated paracellular calcium transport. The barrier function of the paracellular space is generally controlled by two important properties of the tight junction, i.e., charge-and size-selective properties. It has been shown that the charge residues on the extracellular domains of claudins, which are tight junction-associated proteins, can regulate ion diffusion through the paracellular space (Colegio et al., 2003). Paracellular transport through the tight junctions of the small intestine generally prefers cations (Van Itallie and Anderson, 2004a). The P_{Na}/P_{Cl} value greater than 1.0, observed in the present study, confirmed that the duodenal epithelium was cation-selective. This paracellular transport behavior of the leaky epithelia may be associated with differential patterns of

Copyright by Mahidol University

claudin expression. For example, MDCK II, a cation-selective epithelium, became more restrictive to positive charges after an overexpression of claudin-4 (Van Itallie et al., 2001), whereas overexpression of claudin-2 in MDCK I monolayer increased cationic selectivity (Colegio et al., 2003). Moreover, the thick ascending limb of the loop of the Henle used paracellin-1 (also known as claudin-16) to increase cation movement (i.e., Na^+) from the renal parenchyma to the lumen, thereby facilitating the paracellular calcium and magnesium reabsorption in a voltage-dependent manner (Goodenough et al., 1999). In the duodenum, $1,25(OH)_2D_3$, a major hormone for the regulation of transcellular and paracellular calcium transport, has been reported to control the expression of claudin-3 (Kutuzova et al., 2004). It was possible that PRL may be a factor that regulated the expression and/or functions of claudins in the intestinal epithelium. However, further investigations are required to elucidate in depth the mechanisms of the PRL-stimulated paracellular calcium transport during pregnant and lactating periods.

Copyright by Mahidol University

CHAPTER VI
CONCLUSIONS

The present study aimed to *(i)* investigate the PRL-enhanced duodenal active calcium transport during pregnancy and lactation, *(ii)* to determine the PRL-induced changes in the charge selectivity of the duodenal epithelium, *(iii)* to investigate the physiological significance of suckling-induced PRL surge in the regulation of duodenal active calcium absorption in lactating rats, and *(iv)* to demonstrate direct stimulatory effect of PRL on the cecal active calcium transport.

The conclusions could be drawn as followed:

1. Chronic PRL exposure was responsible for the elevated “baseline” of the duodenal calcium absorption during pregnancy and lactation, especially on pregnant day 21 and lactation day 7, while the transient PRL surge during suckling rapidly enhanced the transepithelial calcium transport to match calcium loss in milk.

2. The chronic effect of endogenous PRL on calcium transport was abolished by 7-day bromocriptine administration, which inhibited pituitary PRL release. Such effect was restored by exogenous PRL supplementation.

3. PRL altered the charge selectivity of the duodenal epithelium during pregnancy and lactation to be more cation-selective by increasing P_{Na}, without change in P_{Cl}.

4. Milk volume ingested by pups during suckling correlated well with the enhanced total calcium fluxes, whereas 1-h bromocriptine treatment which inhibited PRL surge impaired this relationship.

5. PRL could also directly stimulate the transepithelial calcium absorption in the cecum, and increase cation selectivity of the cecal epithelium.

Copyright by Mahidol University

REFERENCES

Amaral ME, Cunha DA, Anhê GF, Ueno M, Carneiro EM, Velloso LA, Bordin S, Boschero AC. Participation of prolactin receptors and phosphatidylinositol 3-kinase and MAP kinase pathways in the increase in pancreatic islet mass and sensitivity to glucose during pregnancy. J Endocrinol. 2004;183(3):469–476.

Amenomori Y, Chen CL, Meites J. Serum prolactin levels in rats during different reproductive states. Endocrinology. 1970;86:506−510.

Anderson JM, Van Itallie CM, Fanning AS. Setting up a selective barrier at the apical junction complex. Curr Opin Cell Biol. 2004;16(2):140−145.

Andrews Z, Kokay I, Grattan D. Dissociation of prolactin secretion from tuberoinfundibular dopamine activity in late pregnant rats. Endocrinology. 2001;142:2719−2724.

Amnattanakul S, Charoenphandhu N, Limlomwongse L, Krishnamra N. Endogenous prolactin modulated the calcium absorption in the jejunum of suking rats. Can J Physiol Pharmacol. 2005;83(7):595–604.

Arbogast LA, Voogt JL. Endogenous opioid peptides contribute to suckling-induced prolactin release by suppressing tyrosine hydroxylase activity and messenger ribonucleic acid levels in tuberoinfundibular dopaminergic neurons. Endocrinology 1998;139(6):2857–2862.

Berlanga JJ, Gualillo O, Buteau H, Applanat M, Kelly PA, Edery M. Prolactin activates tyrosyl phosphorylation of insulin receptor subatrate 1 and phosphatidylinositol-3-OH kinase. J Biol Chem. 1997;272:2050–2052.

Copyright by Mahidol University

Bianco SD, Peng JB, Takanaga H, Suzuki Y, Crescenzi A. Marked distriburbance of calcium homeostasis in mice with targeted distribution of TRPV6 calcium channel gene. J. Bone Miner. Res. 2007;22:274–285.

Boass A, Toverud SU, Pike JW, Haussler MR. Calcium metabolism during lactation: Enhance intestinal calcium absorption in vitamin-D deprived hypocalcemic rats. Endocrinology. 1981;109:900−907.

Boass A, Lovdal J. Pregnancy- and lactation-induced changes in active intestinal calcium transport in rat. Am J Physiol. 1992;263:G127–G134.

Boass A, Toverud SU. Enhanced nonsaturable calcium transport in the jejunum of rats during lactation, but not during pregnancy. J Bone Miner Res. 1997;12:1577−1583.

Bohnet HG, Kato K. Prolactin secretion during pregnancy and puerperium: Response to metoclopramide and interactions with placental hormones. Obstet Gynecol. 1985;5:789−792.

Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA. Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. Endocr Rev. 1998;19:225−268.

Bronner F, Pansu D. Nutritional aspects of calcium absorption. J Nutr. 1999;129:9–12.

Brown AJ, Krits I, Armbrecht HJ. Effect of age, vitamin D, and calcium on the regulation of rat intestinal epithelial calcium channels. Arch Biochem Biophys. 2005;437:51–58.

Buitrago C, Pardo VG, de Boland AR. Nongenomic action of 1α,25$(OH)_2$-vitaminD_3. Activation of muscle cell PLC through the tyrosine kinase c-Src and PtIdlns 3-kinase. Eur J Biochem. 2002;269:2506–2515.

Charoenphandhu N, Limlomwongse L, Krishnamra N. Prolactin directly stimulates transcellular active calcium transport in the duodenum of femals rats. Can J Physiol Pharmacol. 2001;79(5):430–438.

Charoenphandhu N, Limlomwongse L, Krishnamra N. Prolactin directly enhanced Na^+/K^+- and Ca^{2+}-ATPase activities in the duodenum of female rats. Can J Physiol Pharmacol. 2006;84:555–563.

Copyright by Mahidol University

Charoenphandhu N, Tudpor K, Pulsook K, Krishnamra N. Chronic metabolic acidosis stimulated trascellular and solvent drag-induced calcium transport in the duodenum of female rats. Am J Physiol Gastrointest Liver Physiol. 2006;291: G446–G455.

Charoenphandhu N, Krishnamra N. Prolactin is an important regulator of intestinal calcium transport. Can J Physiol Pharmacol . 2007;85:569–581.

Colegio OR, Van Itallie CM, Rahner C, Anderson JM. Claudin extracellular domains determine paracellular charge selectivity and resistance but not tight junction fibril architecture. Am J Physiol Cell Physiol. 2003;284(6):C1346–C1354.

Contreras RG, Shoshani L, Flores-Maldonado C, Lazaro A, Cereijido M. Relationship between Na^+, K^+-ATPase and cell attachment. J Cell Sci. 1989;112:4223–4232.

Cunningham BC, Henner DJ, Wells JA. Engineering human prolactin to bind to the human growth hormone receptor. Science. 1990;247:1461-1465.

Diamond JM, Bossert WH. Standing-gradient osmotic flow. A mechanism for coupling of water and solute transport in epithelia. J Gen Physiol. 1967;50:2061–2083.

Diaz S, Seron-Ferre M, Cardenas H, Schiappacasse V, Brandeis A, Croxatto HB. Circadian variation of basal plasma prolactin, prolactin response to suckling, and length of amenorrhea in nursing women. J Clin Endocrinol Metab. 1989;68:946−955.

Dusanter-Fourt I, Belair L, Gespach C. and Djiane J. Expression of prolactin (PRL) receptor gene and PRL-binding sites in rabbit intestinal epithelial cells. Endocrinology. 1992;130:1305−2877.

Feher JJ, Fullmer CS, Wasserman RH. Role of facilitated diffusion of calcium by calbindin in intestinal calcium absorption. Am J Physiol Cell Physiol. 1992;262:C517–C526.

Favus MJ, Angeid-backman E. Effect of $1,25(OH)_2D_3$ and and calcium channel blockers on cecal calcium transport in the rat. Am J Physiol. 1985;248:G676–G681.

Copyright by Mahidol University

Feher JJ, Fullmer CS, Wasserman RH. Role of facilitated diffusion of calcium by calbindin in intestinal calcium absorption. Am J Physiol Cell Physiol. 1992;262:C517–C526.

Fihn BM, Sjöqvist A, Jodal M. Permeability of the rat small intestinal epithelium along the villus-crypt axis: effects of glucose transport. Gastroenterology. 2000;119:1029–1036.

Forsyth IA, Willis M. Growth hormone and prolactin-molecular and functional evolution. J Mammary Gland Biol Neoplasia. 2002;7:291−312.

Freeman TC, Howard A, Bentsen BS, Legon S, Walters JR. Cellular and regional expression of transcripts of the plasma membrane calcium pump PMCA1 in rabbit intestine. Am J Physiol Gastrointest Liver Physiol. 1995;269:G126–G131.

Fleet JC, Wood RJ. Specific $1,25(OH)_2D_3$-mediated regulation of transcellular calcium transport in Caco-2 cells. Am J Physiol Gastrointest Liver Physiol. 1999;276: G958−G964.

Goffin V, Binart N, Touraine P, Paul A. Prolactin: the new biology of an old hormone. Annu Rev Physiol. 2002;64:47−67.

Halloran BP, DeLuca HF. Calcium transport in small intestine during pregnancy and lactation. Am J Physiol. 1980;239:64−68.

Hennighausen L, Robinson GW, Wagner KU, Lui X. Prolactin signaling in mammary gland development. J Biol Chem. 1997;272:7567−7569.

Heringhausen J, Kristen S. Maternal calcium intake and metabolism during pregnancy and lactation. J Perinat Educ. 2005;14:52−57.

Hill PD, Chatterton RT, Aldag JC. Serum prolactin in breastfeeding: state of the sience. Biol Res Nurs. 1999;1:65–75.

Hoenderop JG, Nilius B, Bindels RJ. Calcium absorption across epithelia. Physiol Rev. 2005; 85:373–422.

Hou J, Paul DL, Goodenough DA. Paracellin-1 and the modulation of ion selectivity of tight junctions. J Cell Sci. 2005;118:5109–5118.

Hou J, Renigunta A, Konrad M, Gomes AS, Schneeberger EE, Paul DL, Waldegger S, Goodenough DA. Claudin-16 and claudin-19 interact

Copyright by Mahidol University

and form a cation-selective tight junction complex. J Clin Invest. 2008;118(2):619–628.

Jacob SL, Mariz IK, Daughaday WH. A mixed heterogenous radioimmunoassay for human prolactin. J Clin Endocrinol Metab. 1972;34:4844−4890.

Jahn GA, Daniel N, Jolivet G, Belair L, Bole-Feysot C, Kelly PA, Djiane J. In vivo study of prolactin (PRL) intracellular signalling during lactogenesis in the rat: JAK/STAT pathway is activated by PRL in the mammary gland but not in liver. Biol Reprod. 1997;57:894−900.

Jantarajit W, Thongon N, Pandaranandaka J, Teerapornpuntakit J, Krishnamra N, Charoenphandhu N. Prolactin-stimulated transepithelial calcium transport in duodenum and Caco-2 monolayer are mediated by the phosphoinositide 3-kinase pathway. Am J Physiol Endocrinol Metab. 2007;293(1):E372−E384.

Jones G, Strugnell SA, DeLuca HF. Cerrent understanding of the molecular actions of vitamin D. Physiol Rev. 1998;78:1193−1231.

Karbach U. Segmental heterogeneity of cellular and paracellular calcium transport across the rat duodenum and jejunum. Gastroenterology. 1991;100:47−58.

Karbach U. Paracellular calcium transport across the small intestine. J Nutr. 1992; 122:672–677.

Karbach U, Feldmeier H. The cecum is the site with the highest calcium absorption in rat intestine. Dig Dis Sci. 1993;38(10):1815−1824.

King JC, Halloran BP, Huq N, Diamond T, Buckendahl PE. Calcium metabolism during pregnancy and lactation. In: Picciano MF, Lonnerdal B, eds. Mechanisms regulating lactation and infant nutrient utilization. New York: Wiley-Liss. 1992;129−146.

Kovacs CS, Kronenberg HM. Maternal-fetal calcium and bone metabolism during pregnancy, puerperium, and lactation. Endocr Rev. 1997;18:832–872.

Kovacs CS. Calclium and bone metabolism during pregnancy and lactation. J Mammary Gland Biol Neoplasia. 2005;10(2):105–118.

Copyright by Mahidol University

Krishnamra N, Lotinun S, Limlomwongse L. Acute effect and mechanism of action of prolactin on the in situ passive calcium absorption in rat. Bone and Mineral. 1993;23:253−266.

Krishnamra N, Taweerathitam P. Acute effect of prolactin on active calcium absorption in rats. Can J Physiol Pharmacol. 1995;73:1185−1189.

Krishnamra N, Wirunrattanakij Y, Limlomwongse L. Acute effects of prolactin on passive calcium absorption in small intestine by in vivo perfusion technique. Can J Physiol Pharmacol. 1998;76:161−168.

Krishnamra N, Ousingsawat J, Limlomwongse L. 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 Pharmacol. 2001;79(5):415−421.

Kutuzova GD, DeLuca HF. Gene expreesion profiles in rat intestine identify pathways for 1,25-dihydroxyvitamin D_3 stimulated calcium absorption and clarify its immunomodulatory properties. Arch Biochem Biophys. 2004;432:152−166.

Larsen EH, Nedergaard S, Ussing HH. Role of lateral intercellular space and sodium recirculation for isotonic transport in leaky epithelia. Rev Physiol Biochem Pharmacol. 2000;141:153–212.

Levrat MA, Rémésy C, Demigné C. High propionic acid fermentations and mineral accumulation in the cecum of rats adapted to different levels of inulin. Am J Clin Nutr. 1991;91:1730–1737.

Lotinun S, Limlomwongse L, Krishnamra N. The study of a physiological significance of prolactin in the regulation of calcium metabolism during pregnancy and lactation in rats. Can J Physiol Pharmacol. 1998;76:218−228.

Madara JL, Barenberg D, and Carlson S. 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. 1986;102:2125–2136.

Copyright by Mahidol University

Mazor M, Hershkowitz R, Ghezzi F, Cohen J, Chaim W, Wiznitzer A, Levy J, Leiberman JR, Glezerman M. Prolactin concentrations in preterm and term pregnancy and labour. Arch Gynecol Obstet. 1996;258(2):69–74.

Nellans HN, Kimberg DV. Cellular and paracellular calcium transport in rat ileum: effects of dietary calcium. Am J Physiol. 1978;235(6):E726–E737.

Nellans HN, Goldsmith RS. Transepithelial calcium transport by rat cecum: high-efficiency absorptive site. Am J Physiol. 1981;240(6):G424–G431.

Nellans HN. Intestinal calcium absorption. Interplay of paracellular and cellular pathways. Miner Electrolyte Metab. 1990;16:101–108.

Nemere I, Norman A. Parathyroid hormone stimulates calcium transport in perfused duodena from normal chicks: comparison with the rapid (transcaltachic) effect of 1,25-dihydroxyvitamin D_3. Endocrinology. 1986;119:1406–1408.

Nemere I, Farach-Carson MC, Rohe B, Sterling TM, Norman AW, Boyan BD, Safford SE. Ribozyme knockdown functionally links a 1,25-dihydroxyvitamin D_3 membrane binding protien ($1,25D_3$-MARRS) and phosphate uptake in intestinal cells. Proc Natl Acad Sci USA. 2004;101:739−747.

Noel GL, Suh HK, Frantz AG. Prolactin release during nursing and breast stimulation in postpartum and nonpostpartum subjects. J Clin Endocrinol Metab. 1974;38:413−423.

Nordin B, Need A, Morris H, Horowitz M, and Robertson W. Evidence for a renal calcium leak in postmenopausal women. J Clin Endocrinol Metab. 1991;72:401–407.

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. 2000;279:G851–G857.

O'Loughlin PD, Morris HA. Oestrogen deficiency impairs intestinal calcium absorption in the rat. J Physiol. 1998;511.1:313–322.

Copyright by Mahidol University

Ouhtit A, Kelly PA, Morel G. Visualization of gene expression of short and long forms of prolactin receptor in rat digestive tissues. Am J Physiol. 1994;266:G807−G815.

Pérez AV, Picotto G, Carpentieri AR, Rivoira MA, Peralta López ME, Tolosa de Talamoni NG. Minireview on regulation of intestinal calcium absorption. Digestion. 2008;77:22–34.

Pahuja D, DeLuca HF. Stimulation of intestinal calcium transport and bone calcium mobilization by prolactin in vitamin D-deficient rats. Science. 1981;214:1038–1039.

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. 2000;78:757−765.

Prentice A. Calcium in pregnancy and lactation. Annu Rev Nutr. 2000;20:249–272.

Puntheeranurak S, Schreiber R, Spitzner M, Ousingsawat J, Krishnamra N, Kunzelmann K. Cell Physiol Biochem. 2007;19:77–78.

Subramanian MG and Reece RP. Anterior pituitary and plasma prolactin in rats after 2 to 90 minutes of suckling. Proc Soc Exp Biol Med. 1975;149(3):754–756.

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. 2004;1655:81−91.

Tolis G. Prolactin, physiology and pathology. Hosp Pract. 1980;(15):85−95.

Tongon N, Nakkrasae L, Thongbunchoo J, Krishnamra N, Charoenphandhu N. Prolactin stimulates transepithelial calcium transport and modulates paracellular permselectivity in Caco-2 monolayer mediation by PKC and ROCK pathways. Am J Physiol. 2008;294:C1−C11.

Tudpor K, Charoenphandhu N, Saengamnart W, Krishnamra N. Long-term prolactin exposure differentially stimulated the transcellular and

Copyright by Mahidol University

solvent drag-induced calcium transport in the duodenum of ovariectomized rats. Exp Biol Med. 2005;230:836−844.

Tudpor K, Teerapornpuntakit J, Jantarajit W, Krishnamra, Charoenphandhu N. 1,25-dihydroxyvitamin D_3 rapidly stimulates the solvent drag-induced paracellular calcium transport in the duodenal of female rats. J Physiol Sci. 2008;58:297–307.

Tyson JE, Friesen HG, Anderson MS. Human lactational and ovarian response to endogenous prolactin release. Science. 1972;177:897−900.

Van Abel M, Hoenderop JG, van der Kemp AW, van Leeuwen JP, Bindels RJ. Regulation of epithelial Ca^{2+} channels in small intestine as studied by quantitative mRNA detection. Am J Physiol Gastrointest Liver Physiol. 2003;285:G78−G85.

Van Itallie C, Rahner C, Anderson JM. Regulated expression of claudin-4 decreases paracellular conductance through a selective decrease in sodium permeability. J Clin Invest. 2001; 107(10):1319−1327.

Van Itallie CM, Anderson JM. The role of claudins in determining paracellular charge selectivity. Proc Am Soc. 2004;1:38−41.

Van Itallie CM, Anderson JM. Claudins and epithelial paracellular transport. Annu Rev Physiol. 2006;68:403–429.

Vertino AM, Bula CM, Chen JR, Almeida M, Han L, Bellido T, Kousteni S, Norman AW, Manolagas SC. Nongenomic, ati-apoptotic signaling of $1\alpha,25(OH)_2$-vitamin D_3 and analog through the ligand binding domain of vitamin D receptor in osteoblasts and osteocytes. Mediation by Src, phosphatidylinositol 3-, and JNK kinase. J Biol Chem. 2005;280:14130−14137.

Wasserman RH, Chandler JS, Meyer SA, Smith CA, Brindak ME, Fullmer CS. Intestinal calcium transport and calcium extrusion process at the basolateral membrane. J Nutr. 1992;122:662−671.

Zhao B, Nemere I. $1,25(OH)_2D_3$-mediate phosphate uptake in isolated chick intestinal cells: effect of $24,25(OH)_2D_3$, signaling transduction activators, and age. J Cell Biochem. 2002;86:4975−5008.

Copyright by Mahidol University

Zhu Y, Goff JP, Reinhardt TA, Horst RL. Pregnancy and lactation increase vitamin D-dependent intestinal membrane calcium adenosine triphosphatase and calcium binding protein messenger ribonucleic acid expression. Endocrinology. 1998; 139:3520−3524.

Copyright by Mahidol University

APPENDIX

Copyright by Mahidol University

RADIOACTIVE CALCIUM MEASUREMENT

A. Principle

The radioactive calcium, ^{45}Ca, decays with the production of β-particles, as shown in the following equation, with maximum energy of 255 KeV and a half life of 165 days.

$$_{20}Ca^{45} \longrightarrow {}_{21}Sc^{45} + \beta^{-}$$

To determine Ca^{45} radioactivity, the liquid scintillation cocktail is used for dissolving the samples. The scintillation cocktail contains solvents and solutes, also known as fluors. The solutes, usually aromatic substances such as toluene or xylene, quench energy from ^{45}Ca and transmit energy to the solutes as molecular excitation. They must also be transparent so that photons can be emitted by solutes, and provide an adequate solubility for solvents and samples. The cocktail contain primary and secondary solutes. After being excited, the primary solute converts the excitation energy into visible-light·photon. However, the emission spectrum of the high energy photons (short wavelength) does not match and cannot be detected by the photodetector. The secondary solute or wavelength shifter takes up the photons emitted by the primary solute, and re-emits as lower energy photons (long wavelength) which is detected by the photodetector.

B. Reagents of the liquid scintillation cocktail

1. 2,5-biphenyloxazole (PPO, primary solute which emits photons at 360 nm)

Copyright by Mahidol University

2. 1,4-bis(5-phenyloxazole-2-yl)benzene (POPOP, secondary solute which emits photons at 420 nm)
3. Triton X-100 (detergent)
4. Toluene (solvent)

C. Instrument

Liquid scintillation spectrophotometer (LKB-Wallac model 1219, LKB Wallac, Finland)

D. Liquid scintillation cocktail preparation

300 mL of toluene is added to 500 mL beaker containing 5 g PPO and 0.3 g POPOP. The solution is mixed using magnetic stirrer for 30 min. Thereafter, the solution is placed in a 1000-mL volumic flask. 200 mL of toluene and 500 mL of Triton X-100 are added to the solution, which is later mixed with magnetic stirrer. Finally, the solution is transferred into a light protection bottle.

E. Procedure

1. Aliquot 500 μL of scintillation cocktail into 2 mL transparent microcentrifuge tubes.
2. Pipette 100 mL of sample into a microcentrifuge tube.
3. Mix well by a vortex.
4. Load each microcentrifuge tube into 25-mL transparent vial which is already checked for radioactivity background.

F. References

Mesquita CH, Duarte CL, Hamada MM. Radiation physical chemistry effects on organic detectors. Nucle Instru Method Physic Research 2003; 505:385–388.

Copyright by Mahidol University

Osterman LA, Isotopes, scintillator and scintillation counters. In: Methods of protein and nucleic research 1984. Germany: Springer-Verlag, 67–78.

Copyright by Mahidol University

BIOGRAPHY

NAME	Miss Kamonshanok Kraidith
DATE OF BIRTH	18 January 1982
PLACE OF BIRTH	Pattanee, Thailand
INSTITUTION ATTENDED	Mahidol University, 2002–2005: Bachelor of Science (Physical Therapy) Mahidol University, 2006–2008: Master of Science (Physiology)
HOME ADDRESS	39, Soi Sadsana 2, Rama 6 Road Bangkok 10400 Thailand
SCHOLARSHIP	2008: The Graduate Studies of Mahidol University Alumni Association

Copyright by Mahidol University