รายงานวิจัยฉบับสมบูรณ์

โครงการ “EXPRESSION AND LOCALIZATION OF CALCIUM-SENSING RECEPTOR (CASR) IN BOVINE MAMMARY TISSUE DURING THE PERIPARTURIENT PERIOD”

ผศ.สพ.ญ.ดร.ศิริวรรณ พราพงษ์ คณะสัตวแพทยศาสตร์

มหาวิทยาลัยเกษตรศาสตร์

วันที่ 3 มิถุนายน พ.ศ. 2554

สัญญาเลขที่ PDF/42/2544

รายงานวิจัยฉบับสมบูรณ์

โครงการ "EXPRESSION AND LOCALIZATION OF CALCIUM-SENSING RECEPTOR (CASR) IN BOVINE MAMMARY TISSUE DURING THE PERIPARTURIENT PERIOD"

ผศ.สพ.ญ.ดร.ศิริวรรณ พราพงษ์ คณะสัตวแพทยศาสตร์

มหาวิทยาลัยเกษตรศาสตร์

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

กิตติกรรมประกาศ

ผู้วิจัยขอขอบคุณ สำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ที่ให้การสนับสนุนโครงการวิจัย และขอขอบคุณ สถาบันวิจัยและพัฒนาแห่งมหาวิทยาลัยเกษตรศาสตร์ (KURDI) ที่ให้การสนับสนุนทุนวิจัยบางส่วน ขอขอบคุณ หน่วยงานสัตว์ทดลอง โรงพยาบาลสัตว์หนองโพ และ คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ ที่ให้การสนับสนุนสถานที่ทดลองวิจัย รวมทั้งข้อมูลโคนม และขอขอบคุณนักวิทยาศาสตร์ นางนุช โชติช่วง ภาควิชาสรีรวิทยา สพ.มก. ที่ช่วยรวบรวมข้อมูลโคนม

EXPRESSION AND LOCALIZATION OF CALCIUM-SENSING RECEPTOR (CASR) IN BOVINE MAMMARY TISSUE DURING THE PERIPARTURIENT PERIOD.

Siriwan Prapong[1@] and Timothy A. Reinhardt [2]

ABSTRACT

The goal of our research is to understand the regulation of mammary gland calcium homeostasis. Extracellular calcium has been found to regulate cell functions in tissues involved and uninvolved in mineral ion homeostasis via CaSR. To date, a role for CaSR in mammary gland calcium homeostasis regulation has not been investigated. The objectives of this study were to determine if pregnancy and/or lactation affect the expression of CaSR in mammary gland and to investigate the relationship of the milk calcium secretion with the CaSR expressed in the mammary gland, and also to cellular localization of CaSR in mammary tissues during periods of mammary gland development and lactation. We found that CaSR mRNA expression in bovine mammary tissues increased 2-3 times one week prepartum and remained constant through the experimental period but there was no correlation between cows' plasma calcium and the cows' total first milk calcium to the CaRS expressed in the mammary gland. The CaSR cellular locations were found mainly in mesenchymal cells, fibroblast like cells, fat storage cells, myoepithelial cell, the mammary ductular epithelium, and capillaries and arteries endothelial cells of rat mammary tissue.

Key Words: bovine mammary gland; Calcium-Sensing Receptor; CaSR; calcium; mammary calcium homeostasis; lactation; milk fever; parturition; periparturition.

[1] Department of Physiology, Faculty of Veterinary Medicine, Kasetsart University, BKK 10900, Thailand.

[@] Author for correspondence.

[2] National Animal Disease Center, USDA/ARS, Ames, IA50100.

INTRODUCTION

Controls of transcellular calcium fluxes in the mammary gland during lactation are critical to mammary function, mammary calcium homeostasis, and, also, body calcium homeostasis. It is important to keep the intracellular free calcium ($[Ca^{2+}]_i$) of mammary alveolar secretory cell in micromolar (~10^{-7} M) range, while its extracellular calcium concentration are in millimolar range in both blood compartment (~2mM) and milk compartment (~2 mM free calcium in cow milk) (1). At the same time, sequestration and storage of calcium in the millimolar range in ER and Golgi of the secretory cell is required to maintain milk protein synthesis, modification, and secretion (2, 3). Calcium in Golgi is secreted into milk, as non-diffusible calcium, through casein micelle. The concentration of calcium bound casein in milk is around 20 mM, while around 2mM free calcium is secreted into milk (1).

Calcium transporters, such as Plasma Membrane Ca^{2+}-ATPases (PMCAs), Sarco-Endoplasmic Reticulum Ca^{2+}-ATPases (SERCAs), and Secretory Pathway Ca^{2+}-ATPase (SPCA) are responsible in part for calcium transport in the mammary gland (4-7). The expression and abundance of each calcium-ATPases during pregnancy and lactation, and the subcellular locations of each calcium pumps in mammary tissue suggest a certain function for each pump in mammary gland calcium homeostasis (5-8). For instance, both rat and bovine SPCA, which was found localized in intracellular compartment suspected to be Golgi, increase its mRNA and protein expressions prior but close to parturition (5-7). This information suggests the function of SPCA as Golgi calcium-ATPase for sequestration calcium into intracellular stores (Golgi).

Although several calcium-ATPases are expressed in mammary tissue, it is not clear how calcium homeostasis in this tissue is regulated, especially during the

periparturient period. The systemic variation of several hormones associated with pregnancy, parturition, and lactation, likely, contribute to the regulation of mammary gland calcium homeostasis. However, it seems less likely that calcium transport in this organ is under the influence of calcitropic vitamin D hormone (6, 9). Local modulators, autocrine and paracrine in mammary gland would also have impacts on this regulatory system.

Local modulators such as extracellular calcium have been found to regulate cell function. Extracellular calcium regulates cells functions in tissues involved and uninvolved in mineral ion homeostasis via calcium sensing receptor (CaSR) [Brown, 1999 #192]. For instance, extracellular calcium affects PTH-secreting Chief cells, of the parathyroid gland, activity in PTH secretion through Parathyroid-CaSR (P-CaSR) (11-14).

In the microenvironments of mammary alveolar epithelial cells, there is a large difference in the levels of extracellular calcium in ECF (1-2 mM calcium conc. in serum side) to that on the apical side of cells which are exposed (30 mM calcium conc. in milk compartment). In addition, these microenvironments dramatically change, especially in dairy cow mammary gland, during period of pregnancy to period of lactation, and also during time before milking to time after milking within a day of lactation period. To regulate calcium homeostasis in this microenvironment, it is suggested that CaSR might be involved in regulating mammary gland calcium transport.

To date, a role for CaSR in mammary gland calcium homeostasis regulation has not been investigated. There is one report on the expression of this receptor in human breast (15). We, therefore, hypothesize that the regulation of mammary gland calcium homeostasis involves CaSR, and the mechanism(s) in which this receptor works for this regulation are to be investigated. Our long-term objective is to study the regulatory roles

of Extracellular Calcium Sensing Receptor (CaSR) on dairy cow mammary gland calcium homeostasis, especially during the periparturient period.

Knowledge gained from this study will be critically evaluated with our existing knowledge on the calcium transporting system in mammary tissue in an attempt to complete the story of calcium transport and regulation of its homeostasis in the mammary gland. This information is important for both mammary physiology and animal health, especially for dairy cows. Most dairy cows experience hypocalcemia during parturition period. Around 8% of cows develope severe hypocalcemia leading to be periparturient paresis and, secondary complications such as retained placenta, ketosis, and LAD (Left Abomasal Displacement) (1, 9). Scientists and veterinarians are searching for new bio/molecular-techniques for preventing hypocalcemia in dairy cows and the associated economic looses.

Understanding mechanisms regulating mammary gland calcium homeostasis will provide basic scientific knowledge in mammary gland biology and physiology of lactation. This will enable scientists to apply new or alternative technologies in both Medical field and Agricultural/Veterinary field. The specific aims for this work are:-

- To determine if pregnancy and/or lactation affect the expression of bCaSR in mammary tissue.
- To investigate the relationship of calcium transport in the mammary tissue and into milk with the CaSR expressed in the mammary gland.
- To cellular and sub-cellular localization of CaSR in mammary tissues during periods of mammary gland development and lactation.

LITERATURE REVIEW

An extracellular calcium sensing receptor (CaSR) was originally identified and cloned from bovine parathyroid cells (11). Full length CaSR cDNAs later on have been cloned from human (16), and chicken parathyroid (17), from rat (18), human (19), and rabbit kidney (20), rat C cells (21) and striatrum of rat brain (22). A 5.3 kb cDNA of bovine parathyroid calciun sensing receptor (BoPCaSR) has a 3255 bp ORF encoding 1085 Amino acids (11).

The proposed Bovine parathyroid CaSR (BoPCaSR) protein structure consists of three main domains; 1) a large extracellular hydrophillic N-terminal domain (ECD) which contains potential N-linked glycosylation sites, 2) a central core of seven predicted transmembrane domains (TMDs), and 3) an intracellular C-terminal domain (11).

Clusters of negative charged amino acids, such as aspartate and glutamate, which are located in the CaSR's ECD and/ or in the second extracellular loops (ECL2), might be sites for sensing of Ca_o^{2+} (10) and other polycationic agonists (i.e. Mg_o^{2+}, Ba_o^{2+}, Sr_o^{2+}, La_o^{3+}, and Gd_o^{2+}) (23-27). Studying a chimeric receptor constructed from CaSR's ECD and TMDs, and the mGlu1a intracellular C-terminal domain, Brauner-Osborne et al (28) recently reported that Ser-170 and Ser-147 on ECD of CaSR are involved in agonist-binding activation of the CaSR. The ECD contains both the agonist-binding sites, and sites for dimerization of CaSRs (29-31). Bai et al suggested that intermolecular interactions within the dimeric CaSR are important for receptor's function (31). This was supported by Pace et al's work which demonstrated, by point mutation of Cys residues, that the covalent disulfide bond-mediated dimerization of CaSRs (29). In addition, fourteen Cys residues in CaSR ECD were reported by Fan et al that they are

essential for cell surface expression, dimerization and signal transduction of the receptor (30).

The CaSR ECD also contains multiple N-linked glycosylation sites (11,32). This N-linked glycosylation of the receptor is essential for its expression at the cell surface (32).

Several predicted consensus protein kinase C (PKC) and protein kinase A (PKA) phosphorylation sites were found in intracellular loops and C-terminal intracellular domain of CaSR(11,33). While ECD of the receptor involves in agonist-binding sites, the dimerization and a proper trafficking of CaSRs, C-terminal domain was reported to control the cooperative activity for extracellular calcium activation of huCaSR (34). By using truncated CaSR, Gama and Breitwieser (34) demonstrated that amino acid residues between 868 and 886 are required to the distinct cooperative activity of calcium-mediated activation of G proteins and to CaSR desensitization.

CaSR's predicted structure, from its deduced amino acid sequence, suggested that CaSR is belonged to a large superfamily of guanine nucleotide regulatory (G)-protein-coupled receptors (GPCRs) (11). This family has three groups of receptors; the metabotropic glutamate receptors (mGluR1-8), the calcium sensing receptor (CaSR) and pheromone receptors (VRs or G_oVNs), the $GABA_B$ receptors (10,35).

GPCRs, as well as CaSR, exert their signal transduction downstream through a G-protein coupled process, and a stimulation of phospholipase C (10,35). Phospholipase C, A_2, and D were reported to be activated in bovine parathuroid cells and also in CaSR stably transfected HEK293 cells (36). The G-protein-mediated activation PLC in parathyroid cells, CaSR-transfected HEK cells, and other mammalian cells are believed to be $G_{q/11}$, a pertussis-toxin-nonsensitive G protein (10). A pertussis-toxin-sensitive G

protein, however, is one that mediated CaSR activity to stimulate PLC and subsequence increase inositol 1,4,5-triphosphate (IP_3) in X. Laevis oocytes (10,11). It seems unlikely that extracellular calcium exerts its action through CaSR by one specific signal transduction mechanism, therefore, additional studies are required to identify the signal transduction pathways downstream of Ca_o^{2+}-CaSR activations.

The first known function of CaSR was the regulation of calcium homeostasis by parathyroid cells (11,37). This receptor is capable of detecting minute changes in extracellular calcium concentration and is sensitive to changes in extracellular calcium concentration in the millimolar range. Its action in parathyroid are to modulate the secretion of PTH from PTH secreting cells (11,37), and to control PTH gene expression (38). Distribution of CaSR in tissues involved in the mineral ion homeostasis such as Thyroid C cells, rat kidney tubular cells, bone cells, intestinal epithelial cells, placenta, implis CaSR function in systemic calcium and mineral homeostasis (21, 39-46).

CaSRs are expressed in tissues involved in systemic calcium homeostasis, but it is also in many special tissues not involved in the systemic calcium homeostasis. CaSR expression and its function have been shown in neuron, Glia, and astrocyte cells, gastric mucosal epithelial cells, pancreatic Islet B-cells, lens epithelial cells, bone marrow cells, pituitary adenomas, rat fibroblasts, keratinocytes, ovarian surface epithelial cells, and ductal epithelial cells in human breast (10, 15, 47-57). CaSR's suggested functions in these tissues include to enabling Ca_o^{2+} to serve as an extracellular messenger, and may regulate cellular proliferation and differentiation by the modulation of Ca_o^{2+} (10, 48, 53, 57).

Regulation of CaSR gene expression is of significant interest, though, the promotor of this gene has not been characterized. In the kidney, CaSR gene expression

can be up regulated by 1,25-dihydroxyvitamin D (58), and during developmental in the kidney (59). The developmental effect also has an impact on increasing CaSR expression in rat hippocampus (60). However, controversial results were reported. For instance, Rogers et al did not find any up-regulation of CaSR gene in parathyroid either by 1,25-dihydroxyvitamin D or by raising Ca_o^{2+} (61), Emanuel et al has shown a 2-fold increase in CaSR mRNA in AtT-20 cells by raising the level of Ca_o^{2+} (62). The factor controling CaSR's gene expression is still at an early stage and, yet, required additional studies.

The physiological relevance of CaSR on the mammary calcium homeostasis and its function in milk secretion is not known. As mention earlier that several calcium-ATPasee are expressed in mammary tissue, but it is not clear how calcium homeostasis in this tissue is regulated, especially during the periparturient period. The CaSR is present in the mammary gland but it involvement in regulation of mammary gland calcium homeostasis has not been investigated.

Previously Cheng et al reported the expression and localization of hCaSR in human breast (15), as well as did Reinhardt find its expression in rat mammary tissue (Unpublished data). The lactating mammary gland in dairy cows is considered as an active organ involving in calcium homeostasis. It is suggested a highly expression of CaSR in this tissue. We found that Reverse Transcriptase-PCR (RT-PCR) with specific primers designed from BoPCaSR cDNA sequence has been successfully to produce a correct size of 302 bp PCR product amplified from bovine mammary gland total RNA (Unpublished data). The PCR product was sequenced and it has 99% homology to BoPCaSR cDNA (Unpublished data). The presence of bCaSR in bovine mammary tissue leads us to hypothesize that the regulation of mammary gland calcium homeostasis

involves CaSR, and the mechanism(s) in which this receptor works for this regulation are to be investigated.

MATERIALS AND METHODS

Animals and tissue collection for bovine mammary tissue CaSR expression. Beginning 3 weeks prepartum, fifteen late gestation Jersey cows were fed a diet that increases the incidence of milk fever (63). Mammary tissue biopsies were collected at days -14, -7, 0, +7, +14, with day 0 representing calving day. After calving, all cows were fed a normal lactation diet. Blood samples were collected daily for 2 wk before and 2 wk after calving for monitoring the plasma calcium. Cows were classified as normal cows and periparturient paresis cows (Milk Fever cows), according to their clinical signs and plasma calcium levels during the calving period. Mammary biopsies were immediately frozen in liquid N_2 and stored at –80°C until total RNA was prepared.

Animals and tissue collection for immunohistochemistry. Confirmed pregnant Spraque Dawley rats were purchased from Harlan Spraque Dawley (Madison, WI). Rats were housed individually in hanging basket cages on sawdust bedding. Rats were anesthetized with 50:50 mixture of CO_2 and O_2, and sacrificed by decapitation on –7, 0, +7, and +14 days. Day 0 represents parturition day and + day represents lactation. Mammary tissue was removed, cut into 0.5x0.5x1.0 cm^3 piece and fixed in 10% buffered formalin. The tissue was kept in formalin solution for 24 h. It was then dehydrated through a graded series of ethanol, and embedded in paraffin. The paraffin embedded tissue was sectioned and placed on ProbeOn Plus slide (Fisher Scientific). The slides were kept at room temperature until the immunohistochemistry was performed.

RNA preparation and DNA quantify. Total RNA and DNA were prepared from tissue by the acid guanidinium thiocyanate-phenol-chloroform extraction using TRIzol reagent (GIBCO-BRL, Gaitherburg, MD), with a modified single-step RNA isolation method developed by Chomzynski and Sacchi (64). The total RNA was further precipitated for 2 h on ice with 8 M lithium chloride (LiCl) at 1:10 dilution. The precipitated RNA was washed twice in 70% Ethanol and resuspended in DEPC-H_2O. This LiCl precipitation process is for removal of RT-PCR inhibitors from RNA extracted from tissues. DNA concentration was determined as described by Burton, 1967.

Competitive RT-PCR. Mammary gland CaSR mRNA was quantified by competitive RT-PCR as previously described (65, 66). Primers were designed from bovine CaSR cDNA, accession # S67307, by using MacVector® program, Accelrys Corporate, San Diego, CA, USA. The primers tested are shown in Table I. They were synthesized by KU-VECTOR custom DNA synthesis service unit; Public-Private Technology Development & Transfer Center, Kasetsart University, Thailand. A pair of primer named CaSR302 was selected for competitive RT-PCR. A 302 bp DNA fragment is a PCR product from CaSR302 primer pair.

A CaSR mimic DNA fragments was 389 bp and heterologous sequence to CaSR cDNAs. The mimic was prepared from the Clontech mimic construction kit (Clontech, Palo Alto, CA), according to the manufacturer's instructions.

Six single tube RT-PCR reactions were set up for quantifying CaSR mRNA. Total RNA of 1.0 µg was added in the RT-PCR reaction. The reaction condition was as follows in a total volume of 50 µl: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.8 mM each of dNTP, 50 pmol oligonucleotide primers, 10 U RNasin (Promega), 25 U M-MLV RT (Gibco/BRL), 1.25 U Taq DNA polymerase (Boerhinger). The standard DNA

fragments (DNA mimic) were added to reaction tubes in increasing amounts. The RT reaction was performed at 56°C for 40 min, following by 4 min at 95°C to terminate the M-MLV RT activity. The PCR was conducted in a Perkin-Elmer 9600 thermal cycler for 28 cycles with the following cycle conditions: 94°C (10 sec), 15 sec at 56.8°C annealing temperature, and 72°C for 30 sec.

Electrophoresis was conducted using 18 µl of PCR products from each reaction, and the sample was loaded to a 1.8% agarose gel. The gel was electrophoresed in 40 mM Tris-acetate and 1.0 mM EDTA (pH 8.0) for 40 minutes at 100 volts. Following electrophoresis, the gel was photograph under an UV transilluminator using the Kodak Electrophoresis Documentation and Analysis System (EDAS) 290, Scientific Imaging Systems, Eastman Kodak Company, Rochester, NY, USA. The intensity of the DNA band was analyzed by the 1D Image Anaysis Software®, Scientific Imaging Systems, Eastman Kodak Company, Rochester, NY, USA. The log of the DNA intensity ratio between CaSR gene product and standard DNA mimic product was plotted against the log of mimic concentration. The equation from linear regression of each curve was used for calculating the RNA concentration in each sample.

Plasma and milk calcium measurement. Plasma was collected daily from –14 day to +14 day during periparturient period. Plasma calcium concentration was determined by atomic absorption spectrophotometry as described previously (65).

Milk calcium was determined by atomic absorption spectrophotometry on samples collected at first milk, morning and afternoon milk from day 1, 2, 3, and morning milk from day 7 and day 14 of lactation as described previously (65). Milk was diluted 1:3 with 50% nitric acid and was incubated overnight at room temperature and followed by 2 days incubation at 4°C. The acidified milk sample was further diluted 1:4 with 1.173 g/l

lanthanum solution (La_2O_3). The calcium concentration was measured by atomic absorption spectrophotometry.

Immunohistochemistry. Tissue sections were deparaffinized and rehydrated using a graded series of ethanol. The sections were then washed twice with 50 mM KPBS. Endogeneous peroxidase in tissue sections were neutralized by incubation of the tissue sections in 0.3% H_2O_2 in 50 mM KPBS for 30 min at room temperature. The tissue sections were blocked with; 50 mM KPBS containing 1%BSA, 0.4% v/v Triton-X100 and 1.5% v/v either normal horse serum or normal goat serum. Following the blocking step, a dilution of 1:100 affinity purified polyclonal anti hCaSR antibody (Alpha Diagnostic International, Inc., TX, USA.) was applied on the tissue sections and the sections were further incubated with this primary antibody in a humidified chamber at 4°C for overnight.

The sections were washed with 50 mM KPBS containing 0.02% v/v Triton-X100 for 10 min at room temperature. This wash was repeated 10 times. They were then incubated with either biotinylated Anti-Mouse IgG or biotinylated Anti-Rabbit IgG for 2 h at room temperature, following with 2 washes in 50 mM KPBS containing 0.02% v/v Triton-X100 and one wash in 0.1 M Sodium Acetate. The color development was performed by using Peroxidase Vectastain®Elite ABC standard kit and 3,3'-diaminobenzidine (DAB) substrate kit (Vector Laboratories, Burlingame, CA), as directed by the company. Specific staining yields a brown color at the site of antibody binding. The tissue sections were then counterstained with Hematoxylin (Vector Laboratories, Burlingame, CA).

Statistical analysis. The expression for CaSR mRNA, as effected by time and disease, was analyzed by two-way ANOVA with time as a repeated measure. When time

effects were significant, a Duncan multiple range test was performed to make comparison between time periods. One-tailed t-test was used for proving the direction of comparison in disease effect at specific time point. The strength of a relationship between two continuous variables was analyzed by linear regression and correlation coefficient. All analysis was performed using the SAS® software (SAS Institute, Cary, NC).

RESULTS

Identification of CaSR in bovine mammary tissue. CaSR mRNA in bovine mammary tissue was detected by RT-PCR. Three sets of primer pair, as shown in Table I, were tested. Two primer pairs, CaSR302 and CaSR210, gave PCR products with a correct size. A primer pair of CaSR223 provided no PCR product (data not shown). The 302 PCR product was purified and submitted for sequencing at the DNA facility, ISU. It sequence similarity to bovine CaSR cDNA sequences at position 1029 to 1330 validated the identity of CaSR in mammary tissue.

CaSR mRNA expression in bovine mammary tissue during the periparturient period. To determine whether pregnancy and/or lactation affect the expression of CaSR in bovine mammary tissue, the CaSR expression pattern was examined from mammary biopsies collected prepartum, at calving and one to two weeks postpartum. The CaSR mRNA was quantified by technique competitive RT-PCR. The RNA/DNA ratio, as an indicator of a general transcript, increased two fold at one week prepartum and early lactation compared to two week prepartum (Fig. 1A). The CaSR mRNA expressed 2-3 times one week prepartum ($p \leq 0.05$) and remained quite constant through the experimental period (Fig. 2B).

Effect of late pregnancy and early lactation on calcium metabolism in the cow. During periparturient period, all cows showed hypocalcemia (Fig. 2A). According to levels of hypocalcemia developed in cows, cows were grouped into severe hypocalcemic (milk fever) and mildly hypocalcemic (normal) cows. As shown in Fig. 2A, plasma calcium concentration of milk fever cows was $\leq$ 6 mg% at 24 hr preparturition. On the calving day, these cows' plasma calcium reached 5.0 mg% which is a severe critical level of blood calcium.

On the first day of lactation, milk fever cows secreted more calcium into first milk compared to normal cows (p = 0.55, Table II) while cows from both groups secreted 2-3 less calcium into the second milk ($p \leq 0.05$, Table II). However, cows from both groups had equal of milk calcium concentration through all the period of experiment (Table III).

CaSR expression in bovine mammary tissue in cows that developed milk fever and it relation with calcium metabolism. CaSR expression was examined in cows with milk fever and compared to cows that did not develop milk fever (normal group). As shown in Fig. 2B, the one week mammary gland CaSR mRNA expression in milk fever cows showed tendency to be lower than that expressed in normal cows. Ironically, the expression levels were not different between the 2 groups for all the period examined (Fig. 2B). The degree of hypocalcemia, as indicated by plasma calcium level, on calving day had poor correlation to CaSR mRNA expressed one week prepartum, as well as on the calving day (Fig. 3A and 3B). Neither total calcium secreted into first milk nor first milk calcium concentration shown correlation with CaSR mRNA expressed, either one week prepartum or on the calving day (Fig. 4A & 4B and Fig. 5A & 5B).

Cellular location of CaSR protein during the mammary gland development and parturition. The localizaton of CaSR in the mammary gland during development period was observed in rat mammary tissue obtained on day 14 of pregnancy. The CaSR

was found localized with intense staining at membrane and cytoplasm of fat storage cells (Fig. 6B), macrophage (Fig. 6B), developing alveolar cells (Fig. 6C), and intracellular of developing mammary ductular cell (Fig. 6C).

Slight staining was found intracellular of mammary alveolar epithelium cells at parturition (Fig. 7B). However, fibroblast like cells of tissue at this period were still stained with profound CaSR immunoreative (Fig. 8A, 8B and 8C). The developed ductular system of parturition mammary tissue composed with special ductular epithelium which CaSR protein localized at the apical part of cytoplasm (Fig. 8B and 8C). This special ductular cell was about 10% to total number of normal ductular epithelium. This cell was different from general ductular epithelium such that it had a faint color and a bigger nucleus.

Localization of CaSR in lactating mammary tissue. The CaSR immunoreactivity was detected in mammary tissue 7 and 14 days into lactation (Fig. 9B and Fig. 10B, 10C, 10D). The cellular location of this receptor was found mainly in myoepithelium cell (Fig. 9B and Fig. 10D), fibroblast like cell (Fig. 10B), vascular smooth muscle (Fig. 10C), macrophage (Fig. 10C), and capillary endothelium (Fig. 10D). The CaSR was barely detected from the mammary secreting cells of lactating mammary gland.

DISCUSSION and EXECUTIVE SUMMARY

Transcellular calcium fluxes within an active mammary gland must be vigorously regulated in order to maintain mammary function and to protect calcium cytoxicity of active mammary cells. There is evidence for the importance of mammary gland calcium transport, storage and secretion in the development of hypocalcemia in cows. However, little is known about factors controlling calcium transport in the bovine mammary gland. The Ca^{2+}ATPase isoforms expressed in the mammary gland have been identified (5, 6, 7,

8, 65-68). It was suggested that some of the identified Ca^{2+}ATPases play a significant role in the mammary calcium homeostasis [Reinhardt, 1999 #504] and the large transcellular Ca^{2+} fluxes of lactation. Calcium transporters, such as Plasma Membrane Ca^{2+}-ATPases (PMCAs), Sarco-Endoplasmic Reticulum Ca^{2+}-ATPases (SERCAs) and Secretory Pathway Ca^{2+}-ATPase (SPCA) are responsible in part for calcium transporting in the mammary gland. However, it is not clear how calcium homeostasis in this tissue is regulated. The goal of our research is to understand the regulation of mammary gland calcium homeostasis and to investigate the relationship of the milk calcium secretion with the CaSR expressed in the mammary gland, and also to cellular localization of CaSR in mammary tissues during periods of mammary gland development and lactation. Extracellular calcium has been found to regulate cell functions in tissues involved and uninvolved in mineral ion homeostasis via CaSR. To date, a role for CaSR in mammary gland calcium homeostasis regulation has not been investigated. The objectives of this study are to determine if pregnancy and/or lactation affect the expression of CaSR in mammary gland and to investigate the relationship of the milk calcium secretion with the CaSR expressed in the mammary gland. The CaSR mRNA, quantified by using the competitive RT-PCR technique, was measured in the bovine mammary tissue collected from dairy cows at days -14, -7, 0, +7, +14, with day 0 representing calving day. Total RNA and DNA were prepared from tissue by using the acid guanidinium thiocyanate-phenol-chloroform extraction using Trizol® reagent. The CaSR mRNA was quantified by technique competitive RT-PCR. The intensity of PCR product after electrophoresis was photographed and analyzed by 1D image analysis software® from Kodak. The amount of CaSR mRNA was calculated by comparing to the amount of mimic DNA added in the competitive RT-PCR reaction. Plasma was collected daily from –14 to +14 day during

periparturient period. Milk sample was collected at first milk, second milk, and morning milk from day 1, 7, and 14 of lactation. Plasma calcium concentration and milk calcium concentration were measured by atomic absorption spectrophotometry. The cellular location of CaSR in rat mammary tissues, during the period of development and lactation, was studied by immunohistochemistry technique. A polyclonal anti hCaSR antibody (Alpha Diagnostic International, Inc., TX, USA) was used as a primary antibody. A modified ABC technique with using Peroxidase Vectastain® Elite kit (Vector Laboratories, CA) was performed for detecting the CaSR antigen on the mammary tissue section.

RNA/DNA ratio increased almost two folds at one week prepartum. The CaSR mRNA in the bovine mammary tissue increased 2-3 times one week prepartum and remained constant through the experimental period. During periparturient period, all cows showed hypocalcemia. According to levels of hypocalcemia developed in cows, cows were grouped into severe hypocalcemic (milk fever) and mild hypocalcemic (normal) cows. The milk fever cows' total first milk calcium is higher than that of normal group. While milk fever cows secreted more calcium on the first day of lactation, the one week mammary gland CaSR mRNA expression in these cows tends to be lower than that of normal cows. However, there is not statistically difference between two groups.

The CaSR mRNA level expressed in periparturient cow’s mammary tissue shown similar expression pattern to PMCA1 and bSPCA mRNA expression in bovine mammary tissue (65). However, CaSR mRNA levels in mammary seems not to involve with milk fever status as did in bSPCA mammary tissue (65, 66). Although there was indirect evidence suggesting that increased prepartum mammary gland Calcium storage may contribute to the development of milk fever (65, 66), our results indicate that CaSR may not a direct tool to regulate this metabolic change. There was no correlation between

cows' plasma calcium and the cows' total first milk calcium to the CaRS expressed in the mammary gland. Therefore, results from this study shown that the mammary CaSR may play neither a significant role in regulating whole cow calcium homeostasis nor regulating macro calcium transport in the mammary gland. This speculation was supported by evidences from immuno-histochemistry localization of CaSR protein in rat mammary tissues.

The CaSR immunoreactivity was found mainly in mesenchymal cells, fibroblast like cells, fat storage cells, myoepithelial cell, and capillaries and arteries endothelial cells of the mammary tissue during lactation period. It was found in a less extent around 5-10% in the mammary ductular epithelium, but it was hardly found in the mammary secreting cells unless in the cytosol of the alveolar developing cells of the rat mammary tissue during a pregnancy period.

The pattern of CaSR expression and localization in the mammary tissue during the periparturient period implied that:

- The CaSR may indirectly regulate a macro calcium transport in the mammary tissue and into milk, or
- If it does involve in control a calcium homeostasis in the active mammary gland, this could be a very sensitive and sharply find tune of sensor thus our experimental system is not sensible enough to detect the changes.

However, the CaSR expression in the lactating gland may trigger local mediator(s), such as PTHrp, within the gland, which may subsequently involve(s) in regulating calcium transport in this tissue. Further investigation should be confirmed for this hypothesis.

ACKNOWLEDGEMENTS

We are grateful to the Thailand Research Fund and KURDI for funding. We thank Derrel Hoy and Nuch ChoteChuang for their excellent technical assistance. We also thank the Nongpo animal hospital care unit and the Faculty of Veterinary Medicine, Kasetsart University for providing facilities in this study.

REFERENCES

1. Reinhardt, T. A., Horst, R. L., and Goff, J. P. (1988) *Vet-Clin-North-Am-Food-Anim-Pract* **4**(2)**,** 331-50
2. Duncan, J. S., and Burgoyne, R. D. (1996) *Biochem J* **317**(Pt 2)**,** 487-93
3. Taylor, R. S., Jones, S. M., Dahl, R. H., Nordeen, M. H., and Howell, K. E. (1997) *Mol Biol Cell* **8**(10)**,** 1911-31
4. Neville, M. C., and Watters, C. D. (1983) *J Dairy Sci* **66**(3)**,** 371-80
5. Prapong, S., JP Goff, RL Horst, TA Reinhardt. (1999) in *The Sixth Annual BBMB Symposium*, pp. 21, Dept. of Biochemistry, Biophysics and Molecular Biology, Iowa State University
6. Reinhardt, T. A., and Horst, R. L. (1999) *Am J Physiol* **276**(4 Pt 1)**,** C796-802
7. Reinhardt, T., AG Filoteo, JT Penniston, RL Horst. (1999) in *Experimental Biology'99* Vol. 13, pp. A913, 5 vols., The FASEB Journal, Washington, D.C.
8. Prapong, S., Goff, J. P., Reinhartd, T. A., and Horst, R. L. (1998) in *Experimental Biology' 98* Vol. 12, pp. A1037, 5 vols., FASEB journal, San Francisco, CA
9. Horst RL, J. G., TA Reinhardt. (1997) *J. of Mammary Gland Biology and Neoplasia* **2**(3)**,** 253-263
10. Brown, E. M., Vassilev, P. M., Quinn, S., and Hebert, S. C. (1999) *Vitam Horm* **55,** 1-71

11. Brown, E. M., Gamba, G., Riccardi, D., Lombardi, M., Butters, R., Kifor, O., Sun, A., Hediger, M. A., Lytton, J., and Hebert, S. C. (1993) *Nature* **366**(6455)**,** 575-80
12. Brown, E. M., Pollak, M., Riccardi, D., and Hebert, S. C. (1994) *Nephrol Dial Transplant* **9**(12)**,** 1703-6
13. Mithal, A., Kifor, O., Kifor, I., Vassilev, P., Butters, R., Krapcho, K., Simin, R., Fuller, F., Hebert, S. C., and Brown, E. M. (1995) *Endocrinology* **136**(7)**,** 3087-92
14. Pollak, M. R., Brown, E. M., Chou, Y. H., Hebert, S. C., Marx, S. J., Steinmann, B., Levi, T., Seidman, C. E., and Seidman, J. G. (1993) *Cell* **75**(7)**,** 1297-303
15. Cheng, I., Klingensmith, M. E., Chattopadhyay, N., Kifor, O., Butters, R. R., Soybel, D. I., and Brown, E. M. (1998) *J Clin Endocrinol Metab* **83**(2)**,** 703-7
16. Garrett, J. E., Capuano, I. V., Hammerland, L. G., Hung, B. C., Brown, E. M., Hebert, S. C., Nemeth, E. F., and Fuller, F. (1995) *J Biol Chem* **270**(21)**,** 12919-25
17. Diaz, R., Hurwitz, S., Chattopadhyay, N., Pines, M., Yang, Y., Kifor, O., Einat, M. S., Butters, R., Hebert, S. C., and Brown, E. M. (1997) *Am J Physiol* **273**(3 Pt 2)**,** R1008-16
18. Riccardi, D., Park, J., Lee, W. S., Gamba, G., Brown, E. M., and Hebert, S. C. (1995) *Proc Natl Acad Sci U S A* **92**(1)**,** 131-5
19. Aida, K., Koishi, S., Tawata, M., and Onaya, T. (1995) *Biochem Biophys Res Commun* **214**(2)**,** 524-9
20. Butters, R. R., Jr., Chattopadhyay, N., Nielsen, P., Smith, C. P., Mithal, A., Kifor, O., Bai, M., Quinn, S., Goldsmith, P., Hurwitz, S., Krapcho, K., Busby, J., and Brown, E. M. (1997) *J Bone Miner Res* **12**(4)**,** 568-79
21. Garrett, J. E., Tamir, H., Kifor, O., Simin, R. T., Rogers, K. V., Mithal, A., Gagel, R. F., and Brown, E. M. (1995) *Endocrinology* **136**(11)**,** 5202-11

22. Ruat, M., Molliver, M. E., Snowman, A. M., and Snyder, S. H. (1995) *Proc Natl Acad Sci U S A* **92**(8)**,** 3161-5
23. Mailland, M., Waelchli, R., Ruat, M., Boddeke, H. G., and Seuwen, K. (1997) *Endocrinology* **138**(9)**,** 3601-5
24. Li, L., Kruszewski, F. H., Punnonen, K., Tucker, R. W., Yuspa, S. H., and Hennings, H. (1993) *J Cell Physiol* **154**(3)**,** 643-53
25. Quarles, L. D., Hartle, J. E., 2nd, Middleton, J. P., Zhang, J., Arthur, J. M., and Raymond, J. R. (1994) *J Cell Biochem* **56**(1)**,** 106-17
26. Ruat, M., Snowman, A. M., Hester, L. D., and Snyder, S. H. (1996) *J Biol Chem* **271**(11)**,** 5972-5
27. Ferry, S., Chatel, B., Dodd, R. H., Lair, C., Gully, D., Maffrand, J. P., and Ruat, M. (1997) *Biochem Biophys Res Commun* **238**(3)**,** 866-73
28. Brauner-Osborne, H., Jensen, A. A., Sheppard, P. O., O'Hara, P., and Krogsgaard-Larsen, P. (1999) *J Biol Chem* **274**(26)**,** 18382-6
29. Pace, A. J., Gama, L., and Breitwieser, G. E. (1999) *J Biol Chem* **274**(17)**,** 11629-34
30. Fan, G. F., Ray, K., Zhao, X. M., Goldsmith, P. K., and Spiegel, A. M. (1998) *FEBS Lett* **436**(3)**,** 353-6
31. Bai, M., Trivedi, S., Kifor, O., Quinn, S. J., and Brown, E. M. (1999) *Proc Natl Acad Sci U S A* **96**(6)**,** 2834-9
32. Fan, G., Goldsmith, P. K., Collins, R., Dunn, C. K., Krapcho, K. J., Rogers, K. V., and Spiegel, A. M. (1997) *Endocrinology* **138**(5)**,** 1916-22
33. Chattopadhyay, N., Vassilev, P. M., and Brown, E. M. (1997) *Biol Chem* **378**(8)**,** 759-68
34. Gama, L., and Breitwieser, G. E. (1998) *J Biol Chem* **273**(45)**,** 29712-8
35. Kolakowski, L. F., Jr. (1994) *Receptors Channels* **2**(1)**,** 1-7

36. Kifor, O., Diaz, R., Butters, R., and Brown, E. M. (1997) *J Bone Miner Res* **12**(5)**,** 715-25

37. Brown, A. J., Zhong, M., Ritter, C., Brown, E. M., and Slatopolsky, E. (1995) *Biochem Biophys Res Commun* **212**(3)**,** 861-7

38. Garrett, J. E., Steffey, M. E., and Nemeth, E. F. (1995) *J. Bone Miner. res.* **10(Suppl. 1),** 387 (Abstr.)

39. McGehee, D. S., Aldersberg, M., Liu, K. P., Hsuing, S., Heath, M. J., and Tamir, H. (1997) *J Physiol (Lond)* **502**(Pt 1)**,** 31-44

40. Riccardi, D., Hall, A. E., Chattopadhyay, N., Xu, J. Z., Brown, E. M., and Hebert, S. C. (1998) *Am J Physiol* **274**(3 Pt 2)**,** F611-22

41. Chattopadhyay, N., Mithal, A., and Brown, E. M. (1996) *Endocr Rev* **17**(4)**,** 289-307

42. Quarles, L. D., Hartle, J. E., 2nd, Siddhanti, S. R., Guo, R., and Hinson, T. K. (1997) *J Bone Miner Res* **12**(3)**,** 393-402

43. Chattopadhyay, N., Cheng, I., Rogers, K., Riccardi, D., Hall, A., Diaz, R., Hebert, S. C., Soybel, D. I., and Brown, E. M. (1998) *Am J Physiol* **274**(1 Pt 1)**,** G122-30

44. Gama, L., Baxendale-Cox, L. M., and Breitwieser, G. E. (1997) *Am J Physiol* **273**(4 Pt 1)**,** C1168-75

45. Bradbury, R. A., Sunn, K. L., Crossley, M., Bai, M., Brown, E. M., Delbridge, L., and Conigrave, A. D. (1998) *J Endocrinol* **156**(3)**,** 425-30

46. Kanatani, M., Sugimoto, T., Kanzawa, M., Yano, S., and Chihara, K. (1999) *Biochem Biophys Res Commun* **261**(1)**,** 144-8

47. McNeil, S. E., Hobson, S. A., Nipper, V., and Rodland, K. D. (1998) *J Biol Chem* **273**(2)**,** 1114-20

48. McNeil, L., Hobson, S., Nipper, V., and Rodland, K. D. (1998) *Am J Obstet Gynecol* **178**(2)**,** 305-13

49. Cheng, I., Qureshi, I., Chattopadhyay, N., Qureshi, A., Butters, R. R., Hall, A. E., Cima, R. R., Rogers, K. V., Hebert, S. C., Geibel, J. P., Brown, E. M., and Soybel, D. I. (1999) *Gastroenterology* **116**(1)**,** 118-26
50. Bruce, J. I., Yang, X., Ferguson, C. J., Elliott, A. C., Steward, M. C., Case, R. M., and Riccardi, D. (1999) *J Biol Chem* **274**(29)**,** 20561-8
51. Rasschaert, J., and Malaisse, W. J. (1999) *Biochem Biophys Res Commun* **264**(3)**,** 615-8
52. Romoli, R., Lania, A., Mantovani, G., Corbetta, S., Persani, L., and Spada, A. (1999) *J Clin Endocrinol Metab* **84**(8)**,** 2848-53
53. Rutten, M. J., Bacon, K. D., Marlink, K. L., Stoney, M., Meichsner, C. L., Lee, F. P., Hobson, S. A., Rodland, K. D., Sheppard, B. C., Trunkey, D. D., Deveney, K. E., and Deveney, C. W. (1999) *Am J Physiol* **277**(3 Pt 1)**,** G662-70
54. Tu, C. L., Oda, Y., and Bikle, D. D. (1999) *J Invest Dermatol* **113**(3)**,** 340-5
55. Chattopadhyay, N., Ye, C. P., Yamaguchi, T., Vassilev, P. M., and Brown, E. M. (1999) *Glia* **26**(1)**,** 64-72
56. Adebanjo, O. A., Igietseme, J., Huang, C. L., and Zaidi, M. (1998) *J Physiol (Lond)* **513**(Pt 2)**,** 399-410
57. Brown, E. M., Chattopadhyay, N., Vassilev, P. M., and Hebert, S. C. (1998) *Recent Prog Horm Res* **53,** 257-80
58. Brown, A. J., Zhong, M., Finch, J., Ritter, C., McCracken, R., Morrissey, J., and Slatopolsky, E. (1996) *Am J Physiol* **270**(3 Pt 2)**,** F454-60
59. Chattopadhyay, N., Baum, M., Bai, M., Riccardi, D., Hebert, S. C., Harris, H. W., and Brown, E. M. (1996) *Am J Physiol* **271**(3 Pt 2)**,** F736-43
60. Chattopadhyay, N., Legradi, G., Bai, M., Kifor, O., Ye, C., Vassilev, P. M., Brown, E. M., and Lechan, R. M. (1997) *Brain Res Dev Brain Res* **100**(1)**,** 13-21

61. Rogers, K. V., Dunn, C. K., Conklin, R. L., Hadfield, S., Petty, B. A., Brown, E. M., Hebert, S. C., Nemeth, E. F., and Fox, J. (1995) *Endocrinology* **136**(2)**,** 499-504

62. Emanuel, R. L., Adler, G. K., Kifor, O., Quinn, S. J., Fuller, F., Krapcho, K., and Brown, E. M. (1996) *Mol Endocrinol* **10**(5)**,** 555-65

63. Horst, R.L., Goff, J.P., Reinhardt, T.A., Buxton, D.R., (1997) *J of Dairy Sci* **80**(7), 1269-80

64. Chomczynski, P., and Sacchi, N. (1987) *Anal Biochem* **162**(1)**,** 156-9

65. Prapong, S., (2000) Iowa State University, PhD dissertation.

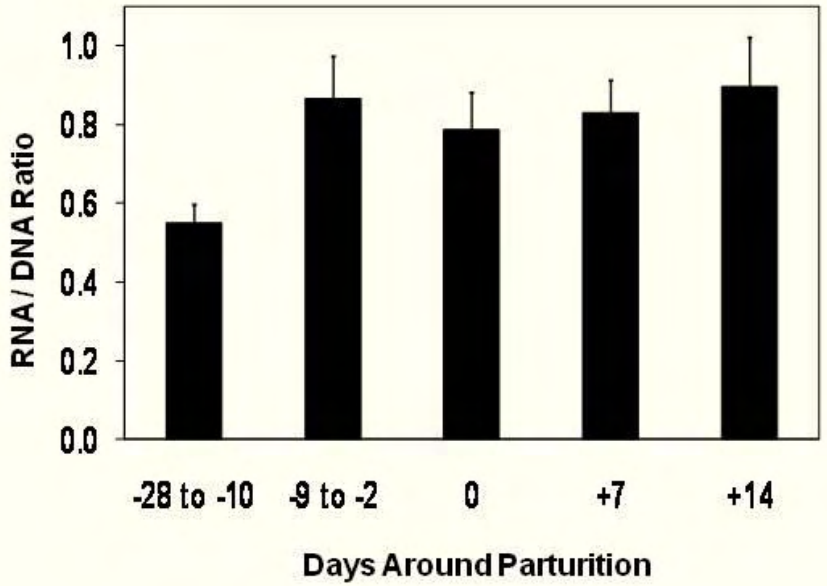
66. Prapong, S., Reinhardt, T.A., Goff, J.P., Horst, R.L., (2005) *J of Dairy Sci* **88**(5), 1741-44

67. Reinhardt, T.A., Filoteo, A.G., Penniston, J.T., Horst, R.L., (2000) *Am J Phtsiol Cell Physiology* **279**, C796-C802

68. Reinhardt, T.A., Lippolis, J.D., Shull, G.E., and Horst, R.L., (2004) *J Biol Chem* **279**, (42369-73)

FIG. 1. Ratio of RNA to DNA (A) and expression of CaSR mRNA (B), as determined by competitive RT-PCR, in bovine mammary tissue during the perparturient period. Data (means $\pm$ SEM) are presented as days around parturition, with 0 as the parturition day: negative days indicate before parturition and positive days indicate lactation. (n = 10-15)

A


1.0
0.8
0.6
0.4
0.2
0.0
RNA / DNA Ratio
-28 to -10
-9 to -2
0
+7
+14
Days Around Parturition

B

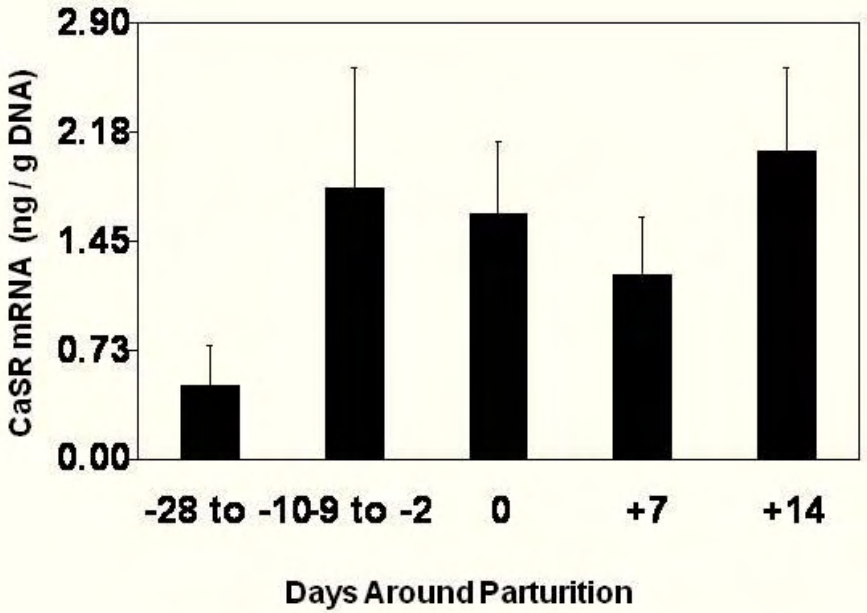

2.90
2.18
1.45
0.73
0.00
CaSR mRNA (ng / g DNA)
-28 to -10
-9 to -2
0
+7
+14
Days Around Parturition

FIG. 2. Plasma Ca^{2+} concentrations on days around parturition (A). Results (means ± SEM) are presented for normal (O, n = 7-8) and milk fever (♦, n = 5-7) cows.The expression of CaSR mRNA in bovine mammary tissue collected from normal and milk fever cows during the periparturient period (B). Data (means ± SEM) are presented as days around parturition, with 0 as the parturition day: negative days indicate before parturition and positive days indicate lactation.

▒ = data from normal cows, n = 4-7; ■ = data from milk fever cows, n = 4-8.

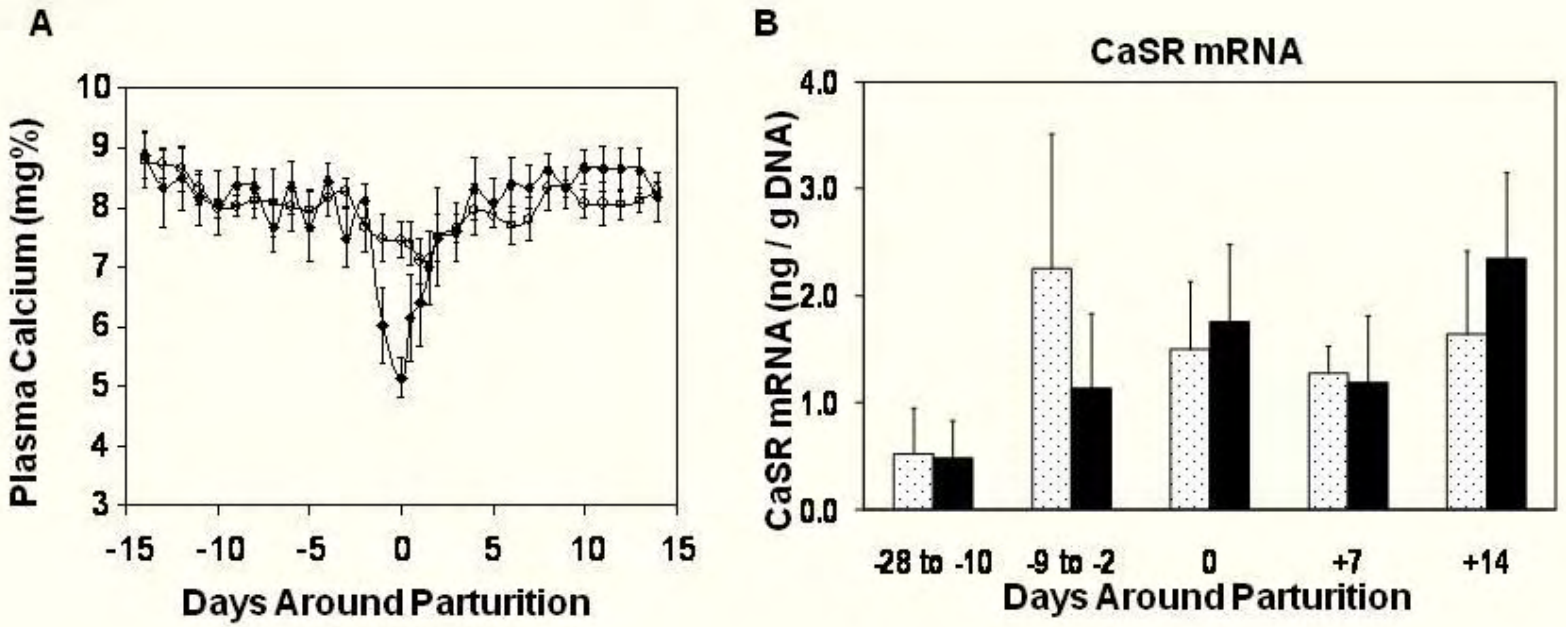

A
Plasma Calcium (mg%)
10
9
8
7
6
5
4
3
-15
-10
-5
0
5
10
15
Days Around Parturition
B
CaSR mRNA
CaSR mRNA (ng / g DNA)
4.0
3.0
2.0
1.0
0.0
-28 to -10
-9 to -2
0
+7
+14
Days Around Parturition

FIG. 3. The relationship between cows plasma calcium concentration on the calving day and CaSR mRNA expression one week prepartum (A) and on the calving day (B).

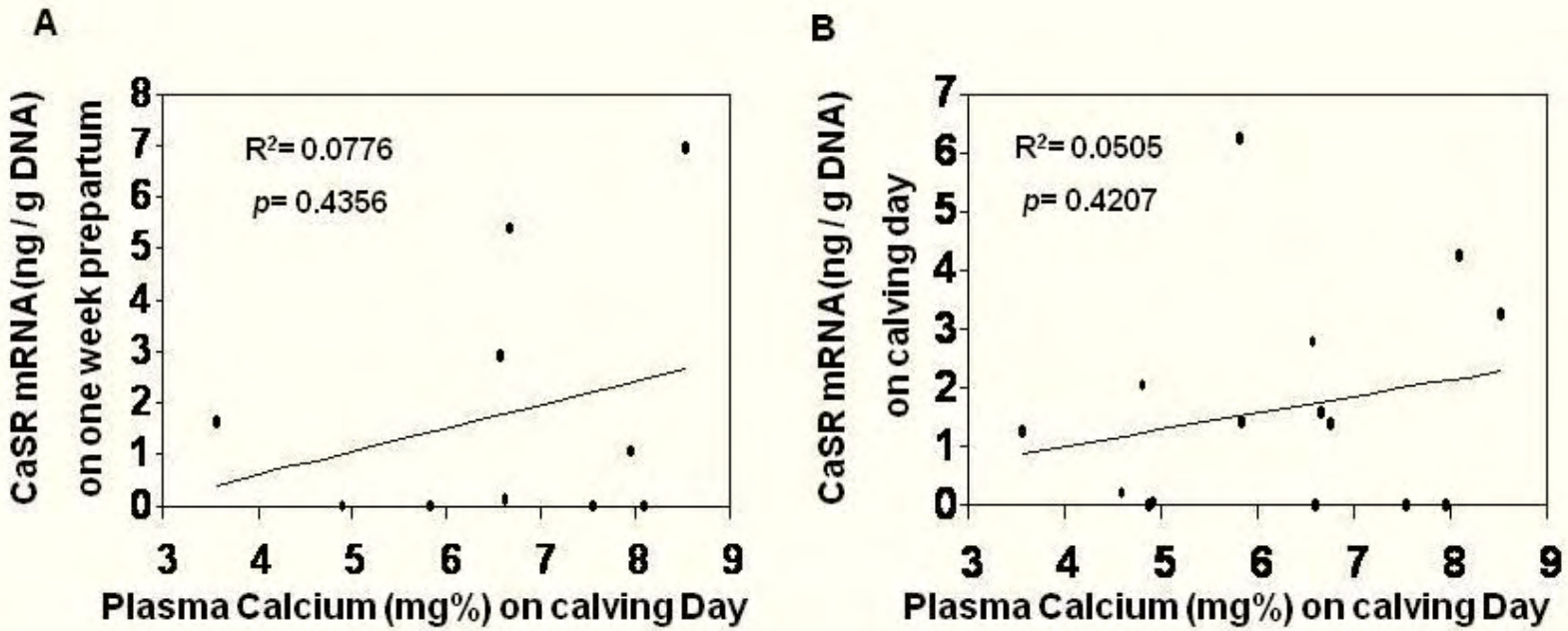

A
CaSR mRNA(ng / g DNA) on one week prepartum
R²= 0.0776
p= 0.4356
Plasma Calcium (mg%) on calving Day
B
CaSR mRNA(ng / g DNA) on calving day
R²= 0.0505
p= 0.4207
Plasma Calcium (mg%) on calving Day

FIG. 4. The relationship between total calcium secreted at the first milk and CaSR mRNA expression one week prepartum (A) and on the calving day (B).

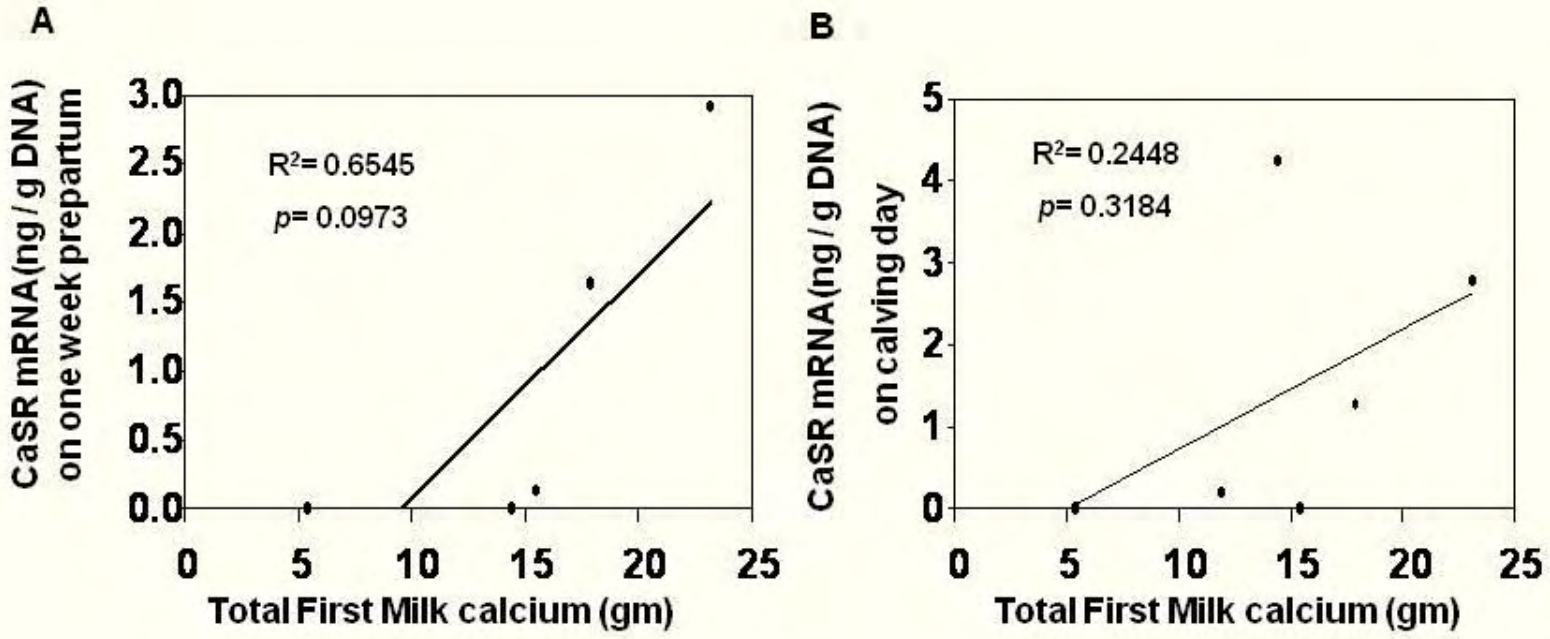

A
CaSR mRNA(ng / g DNA) on one week prepartum
3.0
2.5
2.0
1.5
1.0
0.5
0.0
R²= 0.6545
p= 0.0973
0
5
10
15
20
25
Total First Milk calcium (gm)
B
CaSR mRNA(ng / g DNA) on calving day
5
4
3
2
1
0
R²= 0.2448
p= 0.3184
0
5
10
15
20
25
Total First Milk calcium (gm)

FIG. 5. The relationship between the first milk calcium concentration and CaSR mRNA expression one week prepartum (A) and on the calving day (B).

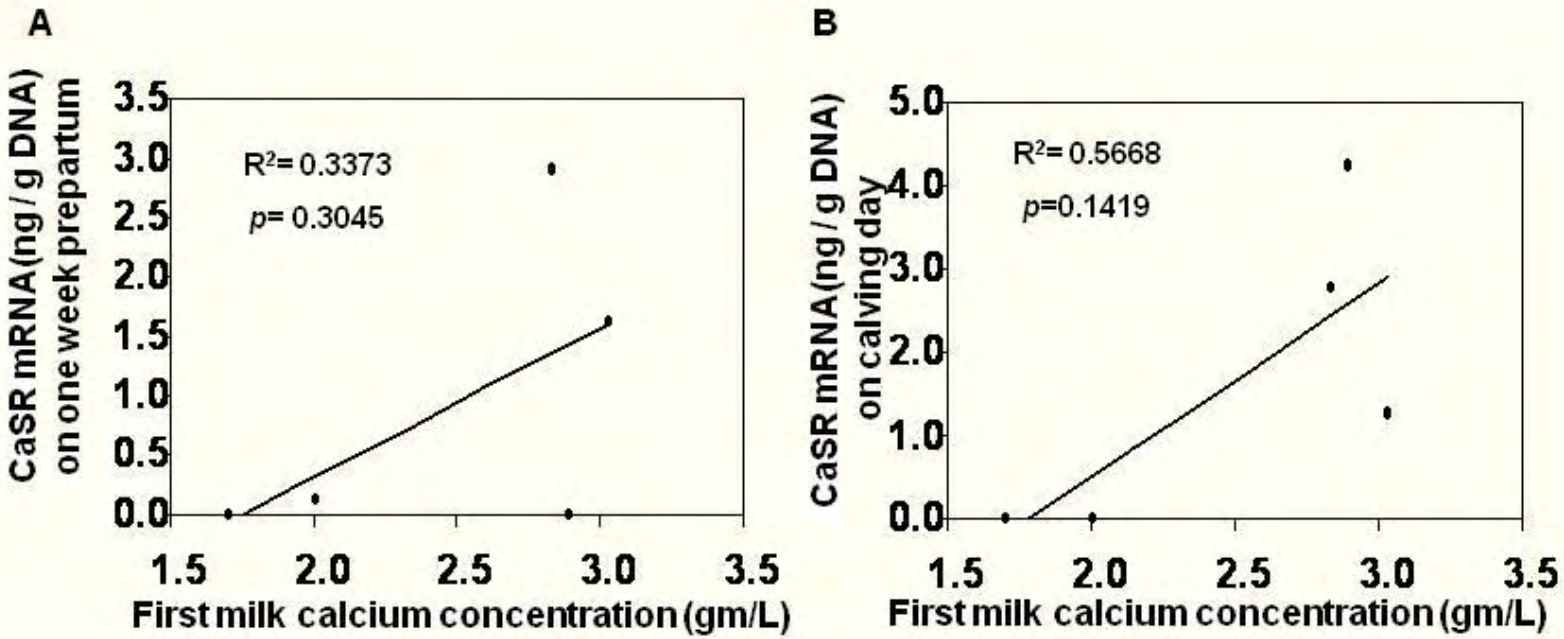

A
CaSR mRNA(ng / g DNA) on one week prepartum
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
R²= 0.3373
p= 0.3045
1.5
2.0
2.5
3.0
3.5
First milk calcium concentration (gm/L)
B
CaSR mRNA(ng / g DNA) on calving day
5.0
4.0
3.0
2.0
1.0
0.0
R²= 0.5668
p=0.1419
1.5
2.0
2.5
3.0
3.5
First milk calcium concentration (gm/L)

FIG. 6. Immunohistochemical localization of CaSR in rat mammary tissue during mammary gland development. Rat mammary tissue sections were obtained on –7 days preparturition. The control section (A) was incubated with normal rabbit IgG as a control antibody. Polyclonal antibody to detect CaSR protein (B and C) and the cellular and intracellular location for CaSR are indicated by the brown color (arrow head). Asterisks indicate the mammary ductal forming unit (B and C). Horizontal red bars represent 50 μm in length.

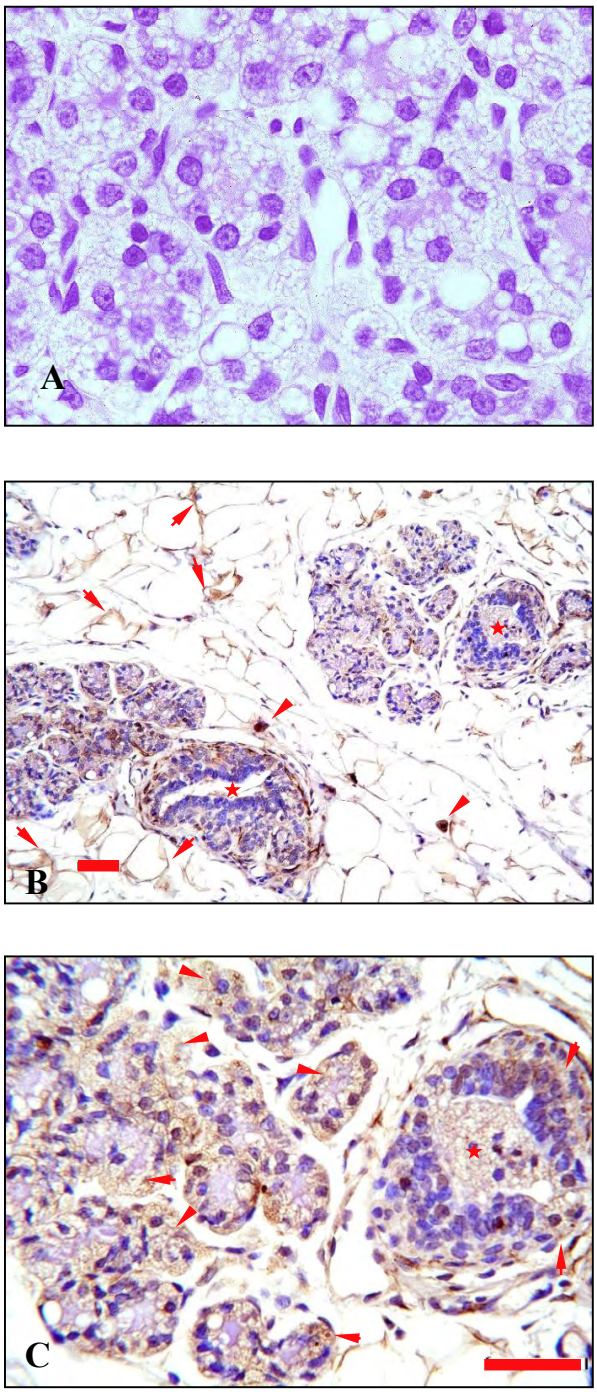


Figure 6

FIG. 7. Detection of CaSR in rat mammary tissue at parturition. The control section (A) and the CaSR antibody incubated section (B) show alveoli with active milk secreting alveolar epithelium. The brown stain indicates the CaSR location (arrow head in B). The horizontal red bar represents a 50 μm in length.

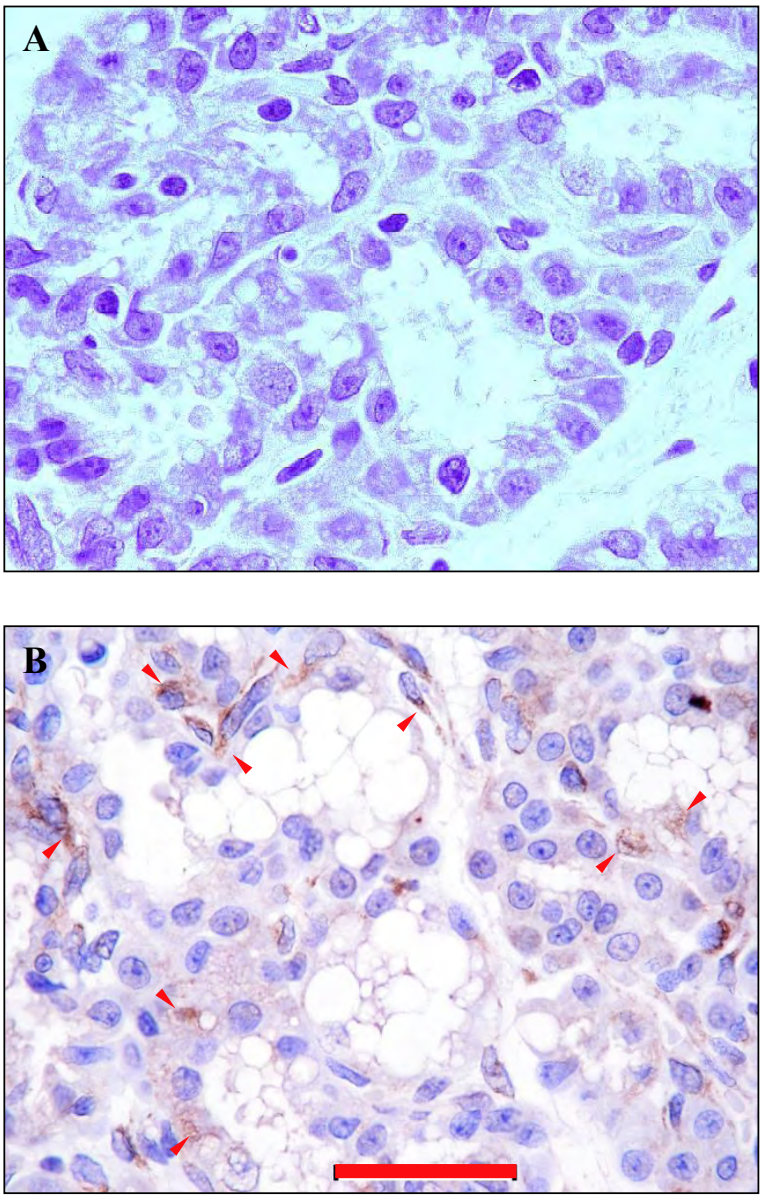


Figure 7

FIG. 8. The cellular location of CaSR in rat mammary tissue at parturition. The tissue section was shown in an area of mammary ductal system (A, B and C). The brown stain indicates the location of CaSR in fibroblast (like) cells (arrow) and in mammary ductal epithelium (arrow head). The horizontal red bar represents a 50 μm in length.

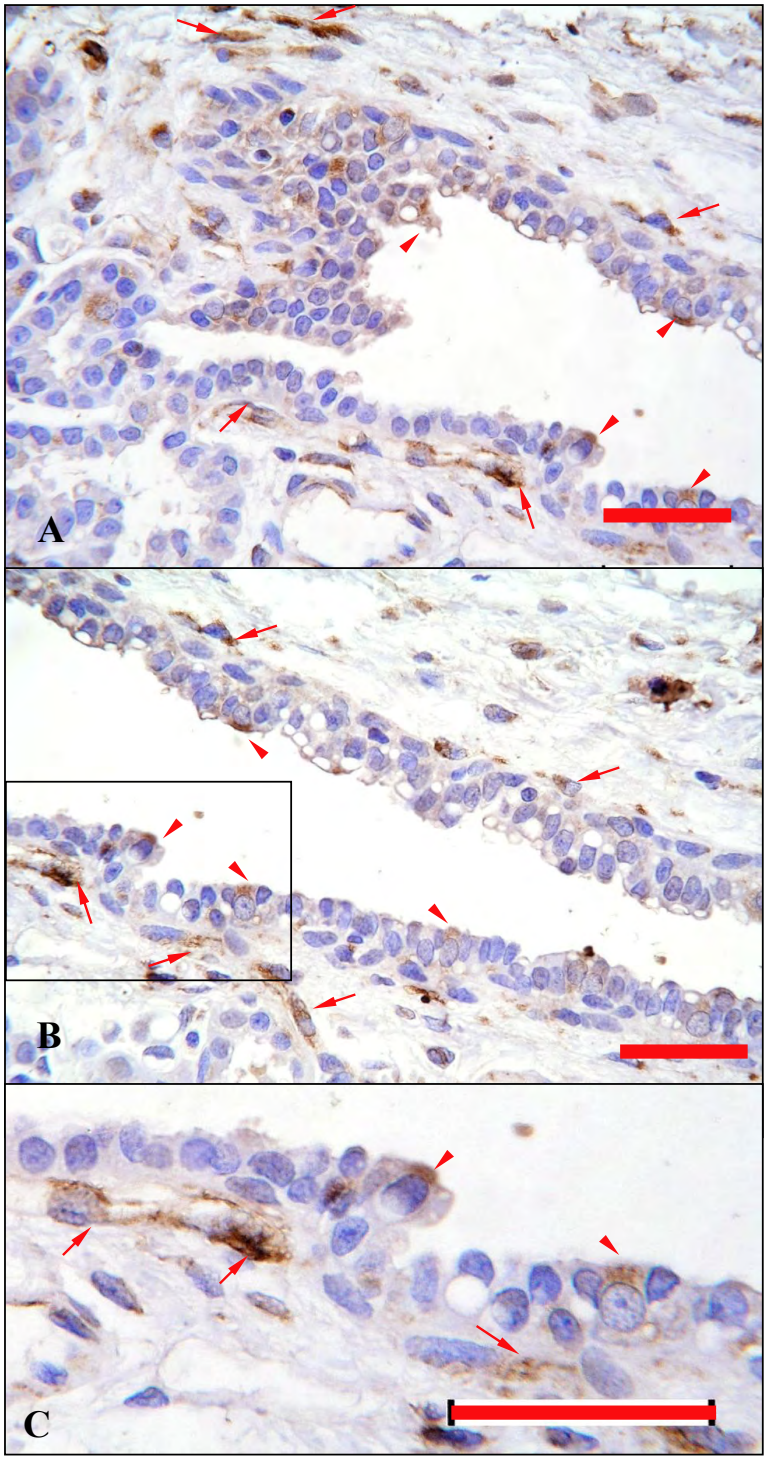
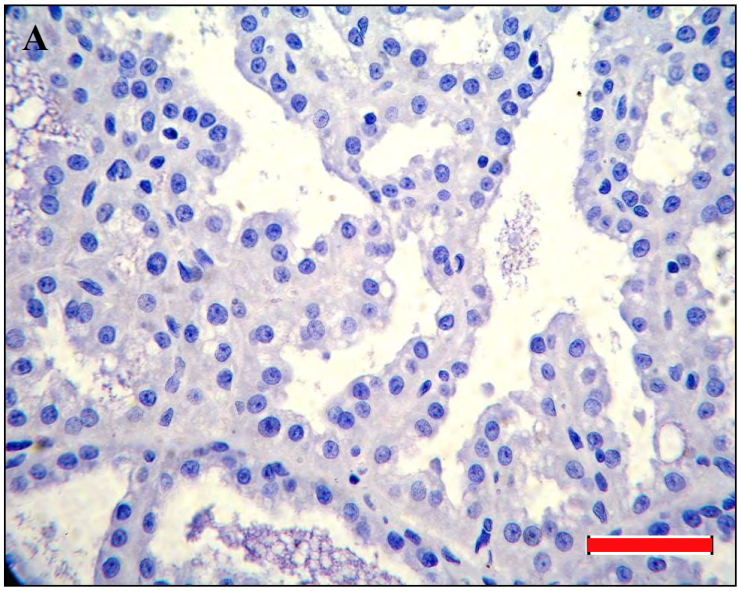


Figure 8

FIG. 9. The cellular location of CaSR in one week lactating rat mammary tissue. (A) Control tissue section; (B) antibody for detecting CaSR. The cells stained with brown color (arrow head) represent cells expressing CaSR. The horizontal red bar represents a 50 μm in length.



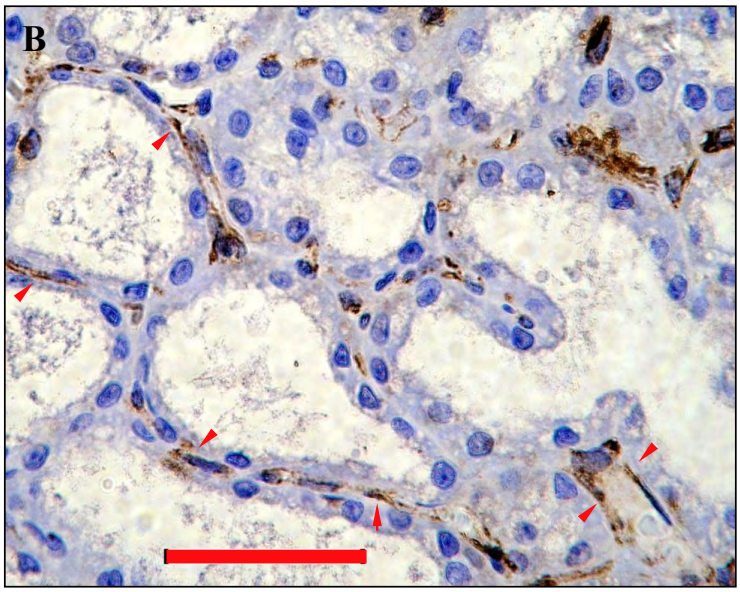


Figure 9

FIG. 10. The cellular location of CaSR in lactating rat mammary tissue section obtained on day 14 of lactation. (A) Control tissue section; (B, C, and D) antibody to detect CaSR. The CaSR location as shown in brown color of fibroblast (like) cells (arrow head in B), macrophage cells (arrow in C), vascular smooth muscle cells (arrow head in C), and capillary epithelium (arrow head in D). The horizontal red bar represents a 50 μm in length.

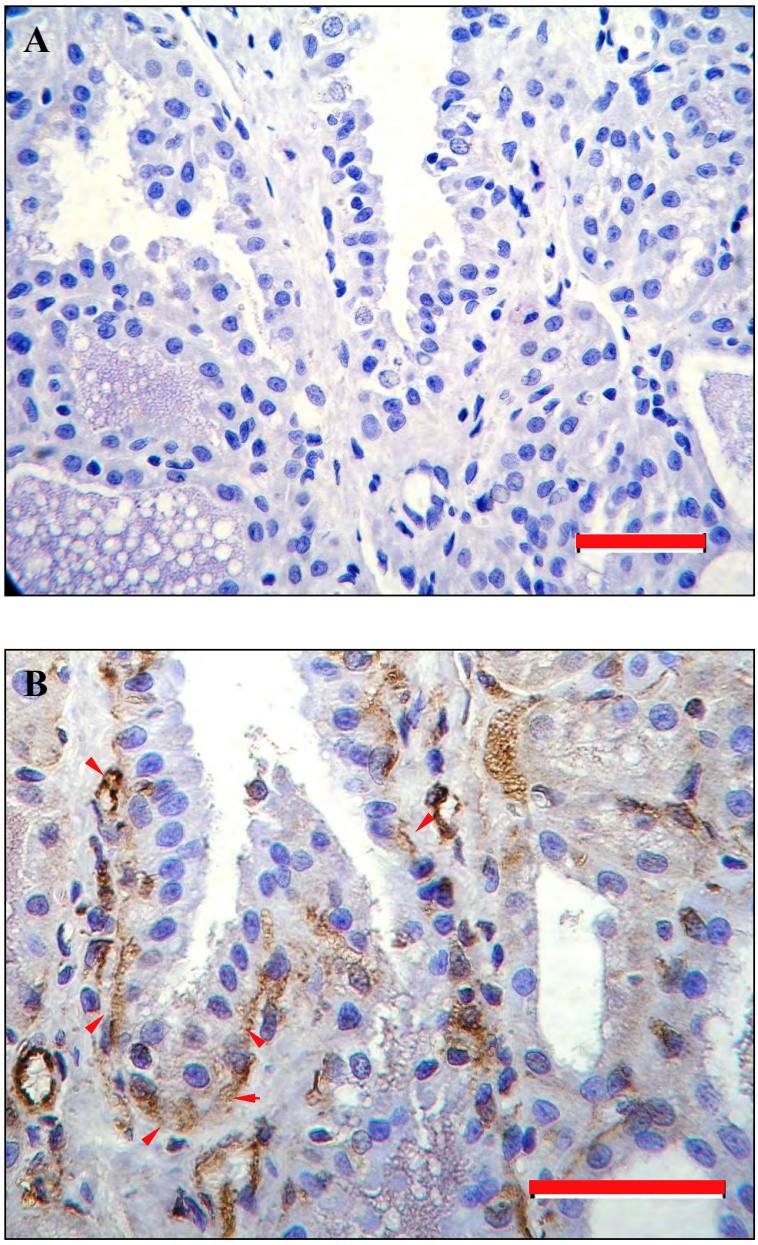


Figure 10

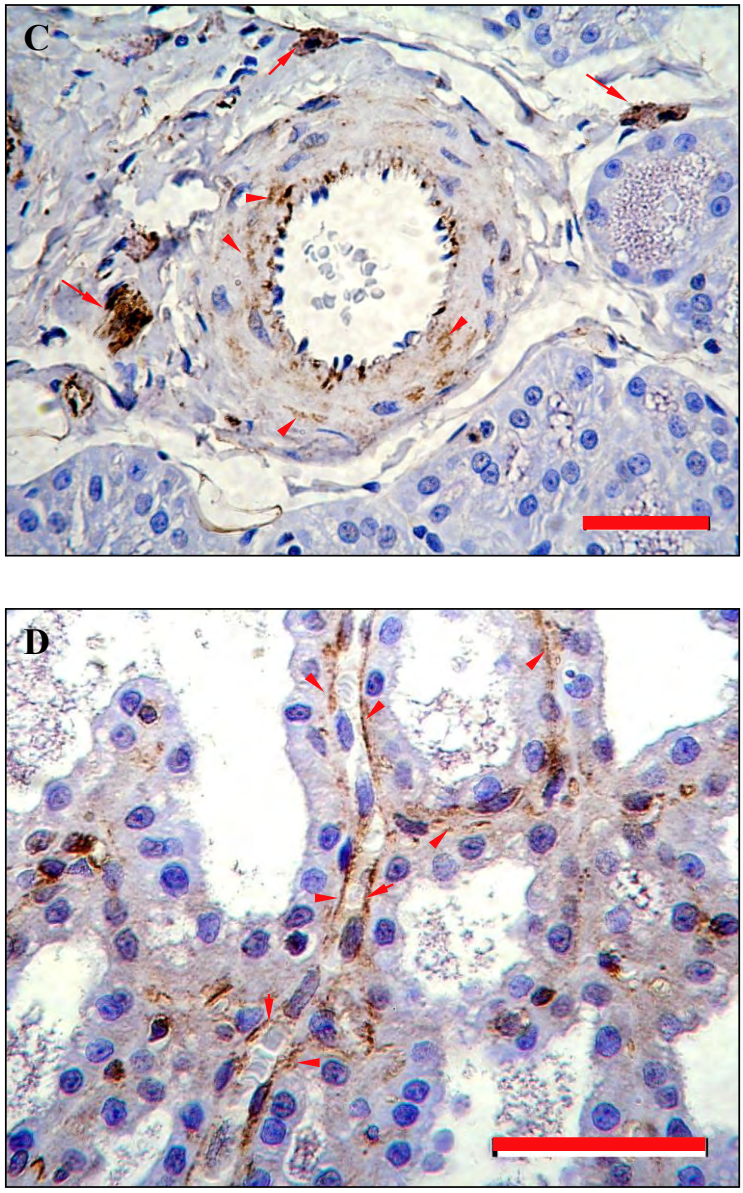


Figure 10 (continued)

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า)
   ไม่สามารถตีพิมพ์ได้เนื่องจากผลทดสอบทางสถิติไม่สามารถบ่งชี้ความสัมพันธ์ได้ และไม่สามารถปรับแก้ไขได้เนื่องจากไม่สามารถเพิ่มจำนวนตัวอย่างเนื้อเยื่อเต้านมวัวได้อีก เพราะการทดลองออกแบบมาเฉพาะช่วงเวลา Periparturient period การเก็บตัวอย่างเนื้อเยื่อเต้านมเพิ่มจึงทำได้ยากเพราะวัวนมมีราคาแพงเกินกว่าที่จะดำเนินการต่อไป จึงต้องทดสอบสถิติด้วยจำนวนตัวอย่างเท่าที่มีอยู่
2. การนำผลงานวิจัยไปใช้ประโยชน์
   - เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป).....ไม่มีการนำไปใช้
   - เชิงนโยบาย (มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบข้อบังคับหรือวิธีทำงาน).....เป็นประสบการณ์ในการออกแบบงานวิจัยที่มีกลุ่มงานวิจัยไม่ใหญ่และมีเงินทุนวิจัยไม่มาก ต้องรอบคอบในการเขียนโครงการวิจัยให้ดี เพื่อป้องกันปัญหาการที่ไม่สามารถทดสอบทางสถิติอย่างเห็นผลได้
   - เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)
     - ทำให้รู้จักนักวิจัยกลุ่ม COCAB ที่มี ศาสตราจารย์ ดร.นทีทิพย์ กฤษณามระ เป็นผู้นำและเกิดความร่วมมือต่อมา
     - การนำเสนอผลงานวิชาการงานประชุมวิชาการประจำปีสมาคมสรีรวิทยาแห่งประเทศไทย วันที่ 8-10 พฤษภาคม 2545
   - เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่).....ใช้เทคนิคการวิจัยในการผลิตบัณฑิตระดับปริญญาโทสองรายคือ
     - นางสาววราภรณ์ แพทย์รักษ์ วิทยาศาสตรมหาบัณฑิต สาขาสรีรวิทยาทางสัตว์ มหาวิทยาลัยเกษตรศาสตร์ วิทยานิพนธ์เรื่อง “Localization of PTHrP and PTH/PTHrP Receptor in Pregnant, Lactating, and Dry Mammary Tissues” / Waraporn Phaet-Rak: 2005: ISBN 974279671
     - นางสาวจิราวรรณ ศิริเวชวิวัฒน์ วิทยาศาสตรมหาบัณฑิต สาขาพันธุวิศวกรรม มหาวิทยาลัยเกษตรศาสตร์ วิทยานิพนธ์เรื่อง “TRPV6 expression in mammary tissues of pregnant AND lactating rats” / Jirawan Siriwetwiwat: 2006: ISBN 9741626088
3. อื่นๆ (เช่น หนังสือ การจดสิทธิบัตร).....ไม่มี