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NAME: Miss Jirawan Siriwetwiwat

THIS THESIS HAS BEEN ACCEPTED BY

Siriwan Prapong

THESIS ADVISOR

(Assistant Professor Siriwan Prapong, Ph.D.)

T. Toojinda

COMMITTEE MEMBER

(Mr. Theerayut Toojinda, Ph.D.)

Surin Peyachoknagul

COMMITTEE MEMBER

(Associate Professor Surin Peyachoknagul, Ph.D.)

Amornrat Promboon

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(Assistant Professor Amornrat Promboon, Ph.D.)

APPROVED BY THE GRADUATE SCHOOL ON September 1st, 2006

Vinai Artkongharn

DEAN

(Associate Professor Vinai Artkongharn, M.A.)

THESIS

TRPV6 EXPRESSION IN MAMMARY TISSUES OF PREGNANT AND LACTATING RATS

JIRAWAN SIRIWETWIWAT

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Transient receptor potential vanilloid channel 6 (TRPV6) is a highly selective Ca^{2+} channel. It plays an important function as gatekeeper of transepithelial Ca^{2+} transport in many tissues. The mammary secretory cells during the transition from pregnancy to lactation require essentially large transcellular Ca^{2+} fluxes into milk. In this study, the expression profile of TRPV6 mRNA was investigated by using semiquantitative RT-PCR analysis. TRPV6 mRNA expression was found in rat mammary during pregnancy, partum and lactation (-7, -1, 0, +1, +7 and +14). The expression of TRPV6 mRNA increased 5 and 3 folds at +7 and +14 days of lactation periods compared with that at pregnant, partum and early lactating periods ($p \leq 0.01$). Its expression profile was correlated to level of total RNA present in mammary tissue and calcium concentrations in rat milk at various periods.

Jirawan Siriwetwiwat Siriwan Prapong Aug 17, 2006

Student's signature Thesis Advisor's signature

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LIST OF ABBREVIATIONS

TRP	=	Transient receptor potential channel
TRPV	=	Transient receptor potential vanilloid channel
CaT1	=	Calcium transporter type 1
ECaC	=	Epithelial calcium channel
PMCA	=	plasma membrane Ca^{2+}-ATPase
SERCA	=	Sarco(endo)plasmic reticulum Ca^{2+}-ATPase
SPCA	=	Golgi Ca^{2+}-ATPase
SOC	=	Store-operated channels
PLC	=	Phospholipase C
IP_3	=	Inositol-1, 4, 5-triphosphate
IP_3R	=	Inositol-1, 4, 5-triphosphate receptor
DAG	=	Diacylglycerol
GPCR	=	G protein-coupled receptor
PIP_2	=	Phosphatidylinositol 4, 5-bisphosphate
µl	=	Micro-litre
µg	=	Micro-gram
M	=	Molar
°C	=	Degree Celsius

TRPV6 EXPRESSION IN MAMMARY TISSUES OF PREGNANT AND LACTATING RATS

INTRODUCTION

Rat transient receptor potential vanilloid channel 6 (rTRPV6), renamed after CaT1 or ECaC2 (Montell *et al.*, 2002), have been cloned from rat and shown to mediate calcium influx when expressed in Xenopus oocytes (Peng *et al.*, 1999). This channel is all or part of the Ca^{2+} release-activated Ca^{2+} channel (CRAC), which its major characteristic is an activation following store depletion (Petersen and Fedirko, 2001). The TRPV6 is a highly selective calcium channel belonging to the transient receptor potential vanilloid channel (TRPV) family (Benham *et al.*, 2002). Its structure is similar to a Ca^{2+} channel TRPV5's structure. TRPV6 shares 75.6% amino acid sequence identity with TRPV5 which is one of the first members of the TRP superfamily of ion channels (Peng *et al.*, 2001a). TRPV6 activity is $[Ca^{2+}]_i$- (Bodding, 2005; Bodding and Flockerzi, 2004) and pH-sensitive (Hirnet *et al.*, 2003). It is activated by low levels of $[Ca^{2+}]_i$ (Bodding, 2005; Bodding and Flockerzi, 2004) and it is inactivated by high $[Ca^{2+}]_i$ (Bodding, 2005; Bodding and Flockerzi, 2004) and by low pH (Hirnet *et al.*, 2003). The TRPV6 channel is the principal mechanism for Ca^{2+} entry into enterocytes as part of the transcellular pathway of calcium absorption in the intestine (Hoenderop *et al.*, 1998). Its apical location on membrane of mouse and human gastrointestinal tract was reported (Zhuang *et al.*, 2002). This supported its physiological function in intestinal calcium absorption (Barley *et al.*, 2001).

Milk is a calcium-rich fluid which the mammary gland during lactation extracts large quantities of calcium from plasma to meet the requirements of the growing neonate. The plasma calcium significantly decreased on one day before parturition which occurred in conjunction with the rat's preparation to give birth and initiate lactation (Reinhardt and Horst, 1999). Calcium is secreted into milk in several chemical forms such as free ionized calcium, casein-bound calcium, and calcium complexed to inorganic anions (Neville *et al.*, 1994). Several calcium transporters have been reported in mammary tissue, for example PMCA1-4 (Reinhardt and Horst, 1999), SPCA (Reinhardt *et al.*, 2000), SERCA (Reinhardt and Horst, 1999). A calcium transport from plasma component to milk compartment required a high capacity mechanism rather than only a passive transport

mechanism. The high capacity mechanism of Ca^{2+} uptake across the basolateral membrane has not been precisely identified, however it could be through a Ca^{2+} channel mechanism. In addition, a stretch-activated Ca^{2+} channel may also provide a pathway for Ca^{2+} uptake (Shennan and Peaker, 2000).

Since the TRPV6 is a Ca^{2+}-selective channel displaying constitutive activity and saturable kinetics in the submillimolar range (Peng *et al.*, 1999; Lambers *et al.*, 2004), it would be an ideal facilitator of mammary epithelial Ca^{2+} transport during lactation. However, this channel has not been widely studied in the mammary gland, though its expression has been reported in mammary tumor (Zhuang *et al.*, 2002). Therefore, this TRPV6 Ca^{2+}-selective channel is likely to be a candidate Ca^{2+} channel for supplying the mammary gland Ca^{2+} transport for lactational needs.

This study was designed to examine the expression of TRPV6 mRNA in the normal rat mammary tissue. In addition, this study also examined profile of TRPV6 mRNA expression in rat mammary tissues at various stages of pregnancy, parturition and lactation. Knowledge gained from this study would fulfill a basic physiology of calcium transport in the mammary biology.

LITERATURE REVIEW

The transient receptor potential (TRP) channel family

Many transient receptor potential (TRP) ion channels have been identified in invertebrates and vertebrates, one in yeast, none in bacteria or plants (Harteneck *et al.*, 2000; Zhou *et al.*, 2003; Montell, 2003). The mammalian transient receptor potential (TRP) ion channels are named after the role of the channels in *Drosophila* phototransduction (Montell *et al.*, 2002; Vennekens *et al.*, 2002). The TRP ion channels are a large family of plasma membrane non-selective and selective cationic channels that are either specifically or ubiquitously expressed in excitable and non-excitable cells (Elliott, 2001). The TRP family of ion channels now comprises more than 30 cation channels (Harteneck, 2003). These proteins have been categorized into seven main subfamilies on the basis of sequence homology; the TRPC (Canonical) subfamily, the TRPV (Vanilloid) subfamily, the TRPM (Melastatin) subfamily, the TRPP (Polycistin) subfamily, the TRPML (Mucolipin) subfamily, the TRPA (Ankyrin) subfamily, and the TRPN (no mechanoreceptor potential C, or NOMPC) subfamily (Birnbaumer *et al.*, 2003). The TRPC (Canonical) and TRPM (Melastatin) subfamilies consist of seven and eight different channels, respectively (i.e. TRPC1-TRPC7 and TRPM1-TRMP8) (Vennekens *et al*., 2002). The TRPV (Vanilliod) subfamily presently comprises six members (TRPV1-TRPV6) (Gunthorpe *et al.*, 2002). The TRPML (Mucolipin) subfamily comprises three members, and the TRPP (Polycystin) subfamily comprises three members. The TRPA (Ankyrin) has only one mammalian member, TRPA1 (Clapham, 2003). Finally, the TRPN (no mechanoreceptor potential C) subfamily has so far only been detected in *Drosophila*, and zebra fish (Figure 1) (Pederson *et al.*, 2005). The TRP ion channels are gated by responding to temperature, touch, pain, osmolarity, pheromones, taste, and other stimuli (Clapham, 2003; Wissenbach *et al.*, 2004). Although research into TRP ion channels is still in its infancy, they have received particular attention within the field of Ca^{2+} signalling because they are the most likely candidates for store-operated Ca^{2+} channels (Petersen and Fedirko, 2001). Transient receptor potential vanilloid channel 6 (TRPV6) is an example channel for a highly selective calcium channel of these TRP ion channels (Hirnet *et al.*, 2003).

Role of the transient receptor potential (TRP) channel family in Phospholipase C-Dependent Ca^{2+} Influx (PLC)

In virtually all eukaryotic cells, the major Ca^{2+}-storing, buffering and signalling compartment with in cells is endoplasmic/sarcoplasmic reticulum (ER/SR), or derived organelles. In lumen of the ER, Ca^{2+} is buffered by proteins with high binding capacity and low affinity, calreticulin, which may have additional signalling functions (Bootman *et al*., 2002; Zitt *et al*., 2002; Berridge *et al*., 2003; Hofmann *et al*., 2002). Stimulus-induced Ca^{2+} released from the ER is thought to be mediated by two main types of Ca^{2+} release channels, ryanodine receptor (RyR) and inositol-1, 4, 5-triphosphate receptor (IP_3R) (Williams, 1998; Petersen *et al.*, 2001; Petersen *et al.*, 2005).

Activation of phospholipase-coupled membrane receptors, G protein-coupled receptor (GPCR) or tyrosine kinase receptor, by hormones, neurotransmitters, growth factors, and other ligands leads to an increase in intracellular Ca^{2+} (Mikoshiba, 1997; Hofer and Brown, 2003; Sternfeld *et al*., 2005). Activation of PLC hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PIP_2) to generate inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DAG). The increase in intracellular Ca^{2+} is initially produced by release of Ca^{2+} from intracellular IP_3-sensitive stores and is followed by an influx of Ca^{2+} from the extracellular space through plasma membrane Ca^{2+}-permeable channels (Spassova *et al*., 2004; Vassilev *et al*., 2001). The entry of Ca^{2+} from the extracellular space is important for refilling intracellular stores. The mechanism for Ca^{2+} entry has been studied extensively. In one mechanism, termed "store-operated Ca^{2+} entry", the emptying of intracellular stores activates surface membrane Ca^{2+} channels via either diffusible factors or a direct interaction between IP_3 receptors and surface Ca^{2+} channels (Figure 2) (Clapham, 1995; Putney, 2001; Holda *et al*., 1998; Venkatachalam *et al*., 2002). These Ca^{2+}-permeable channels on the cell surface are called store-operated channels (SOC). Not all Ca^{2+} entry after PLC activation is dependent on store depletion. Ca^{2+} entry may also be due to activation of surface Ca^{2+} channels by other intracellular second messengers, such as DAG activating protein kinase C (PKC) (Clapham, 1995; Hardie, 2003).

However, most TRP channels are non-selectively mediate influx of cations but some members are very selective, for example, TRPM4b and TRPM5 are permeant only to monovalent cations, whereas TRPV5 and TRPV6 are highly selective calcium channels (Montell, 1997).

The Ca^{2+} influx channels of the TRP family comprise all TRPCs, all TRPVs, TRPM1, 2, 3, 6, 7, and 8, TRPA1, TRPP2, 3, and 5 and TRPML1, 2, and, 3 (Pedersen *et al*., 2005). The permeability ratios P_{Ca}/P_{Na} for these channels vary considerably, ranging from 0.3 to >100. For example, the permeability ratios P_{Ca}/P_{Na} is 0.3 for TRPM2 and >100 for TRPV5 and TRPV6 (Pederson *et al.*, 2005).

The transient receptor potential vanilloid (TRPV) channel

The transient receptor potential vanilliod (TRPV) subfamily, is consisted of 6 members; TRPV1 (VR1), TRPV2 (VRL-1), TRPV3 (VRL-3), TRPV4 (OTRPC4), TRPV5 (ECaC, ECaC1 or CaT2) and TRPV6 (CaT1 or ECaC2) (Gunthorpe *et al.*, 2002; Kedei *et al.*, 2001; Wissenbach et al., 2000; Smith *et al.*, 2002). The TRPV subfamily, on the basis of structure and function, comprises four groups of mammalian TRPVs: TRPV1/TRPV2, TRPV3, TRPV4 and TRPV5/TRPV6 (Peng *et al.*, 2001a). Moreover, TRPV1, TRPV2, TRPV3 and TRPV4 are modestly permeable for Ca^{2+} with a permeability ratio P_{Ca}/P_{Na} between ~3 and ~12 so these channels are non-selective Ca^{2+} channels, whereas TRPV5 and TRPV6 are the only highly Ca^{2+} selective ion channels (P_{Ca}/P_{Na}>100) within the TRPV family (Meir, 2002; Suzuki *et al.*, 2000). The TRPV5 and TRPV6 exhibit a similar ion permeation sequence for divalent cations (Ca^{2+}> Ba^{2+}~ Sr^{2+}> Mn^{2+}) (Peng *et al.*, 2003b). Like voltage-gate Ca^{2+} channels, both of epithelial calcium channels exhibit permeation for monovalent cations in the absence of divalent cations with the permeation sequence of Na^{+}~ Li^{+}> K^{+}> Cs^{+} (Vennekens *et al.*, 2000). In addition, both channels have recently been shown to have the biophysical characteristics of the Ca^{2+}-release activated Ca^{2+} channel (CRAC) that refills internal Ca^{2+} stores following agonist-elicited release (Yue *et al.*, 2001; Prakriya and Lewis, 2003). The TRPV6 function acts likely as Ca^{2+}-sensing Ca^{2+} channel to depletion Ca^{2+} (Bodding *et al.*, 2002; Schindl *et al.*, 2002). The Ca^{2+} channels are inhibited by an increase in the cytosolic Ca^{2+} concentration. This negative feedback protects against cytosolic Ca^{2+} overloading. Ca^{2+} entry therefore only occurs when Ca^{2+} can be effectively removed from the inner aspect of the plasma membrane (Prakriya and Lewis, 2003).

All members of the TRPV (Vanilliod) channels family display a membrane topology of six putative transmembrane segments (TM1-TM6), with a re-entrant pore loop (P-loop) between TM5 and TM6, and cytosolic amino and carboxyl termini (Gunthorpe *et al.*, 2002). All TRPV channels form cation channels that are either non-selective or Ca^{2+}-selective. TRPV channels have four linked domains of six transmembrane segments so they are though to form tetramers (Figure 3) (Hellwig *et al.*, 2005).

The TRPV subfamily of calcium-permeable channels is widely distributed in sensory and non-sensory cells from nematodes to mammals (Benham *et al.*, 2002). Members of the TRPV subfamily are activated by a diverse range of stimuli, including heat, proton, lipid, phorbols, phosphorylation, changes in extracellular osmolarity and/or pressure, and depletion of intracellular Ca^{2+} stores (Table 1) (Benham *et al.*, 2002; O'Neil and Brown, 2003).

Transient receptor potential vanilloid channel 6

Transient receptor potential vanilloid channel 6 (TRPV6) was first identified from rat duodenum using an expression cloning strategy in *Xenopus laevis* (Peng *et al.*, 1999), and formerly named Calcium transporter type 1 (CaT1) (Peng *et al.*, 1999). It is synonymous with Epithelial calcium channel 2 (ECaC2) (Montell *et al*., 2002). This protein belongs to the TRPV (vanilliod) subfamily, which is one of the seven subfamilies of the transient receptor potential (TRP) ion channels. TRPV6 is highly selective calcium channel which was supposed to mediate calcium absorption in rat intestine (Peng *et al.*, 1999). In 2000, Peng *et al.* reported that a human orthologue (hTRPV6) of rat TRPV6 (rTRPV6) was cloned from small intestine. Moreover, hTRPV6 expression was studied by using Northern blot analysis. It was reported that hTRPV6 transcripts were most abundantly expressed in duodenum at lower levels, in esophagus, stomach and jejunum, and the signal was absent in ileum (Peng *et al.*, 2000a). It shows that TRPV6 constitutes the rate-limiting influx step of transepithelial Ca^{2+} transport in the gastrointestinal tract (Barley *et al.*, 2001). Qiu and Hogstrand (2004) reported an orthologue to the mammalian epithelial calcium channel, TRPV6, was cloned and characterized from pufferfish (*Fugu rubripes*). They demonstrated that this gene predates the evolution of land-living vertebrates. The TRPV6 has been proposed to be responsible for apical calcium entry in the vitamin D-regulated transcellular pathway of Ca^{2+} absorption (Hoenderop *et al.*, 1998).

Genomic of transient receptor potential vanilloid channel 6

Genes for the TRP ion channels subunits were first defined in the *Drosophila* visual system (Macpherson *et al*., 2001). In the *trp* mutation, the light response (receptor potential) decays during prolonged exposure to light. TRP-deficient files are blinded by intense light because sustained Ca^{2+} entry via TRP ion channels and subsequent Ca^{2+}-dependent adaptation is disrupted (Clapham *et al.*, 2001; Scott and Zuker, 1998).

The *trpv6* gene encoding TRPV6 protein is juxtaposed on rat chromosome 4 (4q22), mouse chromosome 6 (Peng *et al.*, 2001a), and human chromosome 7 (7q33-34) (Peng *et al.*, 2000a). This gene comprises 15 exons, extends over 15.7 kb, and encodes protein of about 730 amino acid residues. Chromosomal organization analysis revealed that two TRPV members are found as direct repeats; TRPV6 follows TRPV5 (Peng *et al.*, 2001a). This duplication appear to be recent that TRPV5 and TRPV6 are more similarity to each other than to other members of TRPV subfamily (Birnbaumer *et al.*, 2003). Therefore, it is quite possible that gene duplication events occurred during evolution that gave rise to these two highly homologous pairs of genes. The various gene products have subsequently acquired their function diversities over the course of evolution.

Human TRPV6 (hTRPV6) shares 30% amino acid sequence identity with the other four proteins (TRPV1-4), whereas hTRPV5 and hTRPV6 share 75.6% overall amino acid identity with one another (Peng *et al.*, 2001a). The main sequence differences are located in the N- and C-terminal tails. However, significant differences exist in the N- and C-termini of TRPV5 and TRPV6, which may account for distinct functional and regulatory features (Nijenhuis *et al.*, 2003). The largest exon of TRPV5 and TRPV6 is the region within the TM domains that encodes part of TM5, the pore region, and TM6 (Peng *et al.*, 2001a). Within the sequence encoded by this exon, TM6 is highly conserved in 20 different proteins belonging to various families of channels of divergent structure and function (Birnbaumer *et al*., 2003). The conservation of TM6 may suggest a role for it as a key structural element for this diverse group of TM proteins (Peng *et al.*, 2001a). However, the detailed structure of the TM1-TM4 segments of TRPV channels is not available; mutagenesis data provided some clues about their functions (Gunthorpe *et al.*, 2002; Clapham *et al.*, 2001). The TM4 segment in voltage changes across the membrane into movement of the helix, somehow gating the pore by twisting this TM4 helix (Clapham *et al.*, 2001). The TRP channels are very weakly voltage-dependent

and lack the full complement of charged amino acids in the TM4 domain (Clapham *et al.*, 2001). Furthermore, the pore regions of TRPV5 and TRPV6 are quite distinct from those of these other channels. The less-conserved pore region, in contrast, may be one determinant of the characteristic Ca^{2+}-selectivity of TRPV5 and TRPV6 (Ellinor *et al*., 1995; Peng *et al.*, 2001a).

Structure of transient receptor potential vanilloid channel 6

In 1999, Peng *et al*. reported that the 2995 base pair of rat TRPV6 cDNA contains an open reading frame of 2181 base pairs that encodes a protein 727 amino acid residues with a predicted relative molecular mass of 83,245 Da. rTRPV6 is polytopic protein containing six transmembrane domains (TMs). A short hydrophobic stretch between TM5 and TM6 was predicted to be the pore-forming region (Peng *et al.*, 1999). The TM2-TM3 loop is a critical determinant of fast Ca^{2+}-dependent inactivation in TRPV6 (Nilius *et al.*, 2002). The amino-terminal hydrophilic segment (326 amino acid residues) of TRPV6 contains three ankyrin repeat domains, which are 33-residue motifs that mediate cytoskeleton anchoring or protein–protein interaction. Thus, TRPV6 may be linked to the cytoskeleton via its ankyrin repeats, enabling anchorage to the plasma membrane and channel regulation via actin filaments (Erler *et al.*, 2004). The carboxyl terminus (150 amino acid residues) contains a highly conserved 25 amino acids, called the TRP domain (Huang, 2004). Putative phosphorylation sites for protein kinase C are present in the cytoplasm domains, suggesting that transport activity could be regulated by phosphorylation. The N-and C-terminal tails of TRPV6 each contain an internal PDZ motif (Hoenderop *et al.*, 2002) which can function as part of a molecular scaffold via interaction with PDZ-domain containing proteins. Via this interaction, transporters, ion channels and receptors are coupled to intracellular signalling molecules, which regulate their activity, and position them to specialized regions in a cell (Figure 4) (Kornau *et al.*, 1997).

Chang and coworkers reported that N-tail (residues 64-77) and C-tail (residues 569-601) of TRPV5 are important for channel subunit assembly, subsequent trafficking of the TRPV5 channel complex to the plasma membrane, and channel activity (Chang *et al.*, 2004). Moreover, Nilius *et al*. (2003b) identified two domains in the carboxyl terminus of TRPV5 that control Ca^{2+}-dependent inactivation, whereas hTRPV6 lacks the carboxyl terminal domain (residues 695-725) for Ca^{2+}-calmodulin binding site; as a result, it does not form constitutive active channel (Bodding and Flockerzi, 2004).

Architecture of transient receptor potential vanilloid channel 5/6

By using co-immuno precipitations and molecular mass determination of TRPV5/6 complexes using sucrose gradient sedimentation, it was showed that TRPV5 and TRPV6 form homo- and heterotetrameric channel complexes (Hoenderop *et al*., 2003c). Since TRPV5 and TRPV6 are co-expressed in several tissues (Table 2), that allow oligomerization of these channels could occur *in vivo* (Hoenderop *et al*., 2003c). The oligomerization of TRPV5 and TRPV6 might influence the functional properties of the Ca^{2+} channel. TRPV5 and TRPV6 exhibit different channel kinetics with respect to Ca^{2+}-dependent inactivation, Ba^{2+} selectivity and sensitivity for inhibition by ruthenium red (Hoenderop *et al.*, 2001a). The influence of the heterotetramer composition on channel properties was investigated.

Concatemers were constructed consisting of four TRPV5 and/or TRPV6 subunits, which configured in a head-to-tail fashion. A different number of TRPV5 and TRPV6 subunits in these concatemers showed that the phenotype resembled the mixed properties of TRPV5 and TRPV6 (Hoenderop *et al*., 2003c). An increased number of TRPV5 subunits in such a concatemeric displayed more TRPV5-like properties, indicating that the stoichiometry of TRPV5/6 heterotetramers influences the channel properties. The clustering of four subunits is assumed to create an aqueous pore centered at the four-fold symmetry axis (Figure 5) (Hellwig *et al.*, 2005; Hoenderop *et al.*, 2003c).

Nilius and coworkers studied point mutation of the aspartate residue, D542A of TRPV5. They showed that an aspartate-to-alanine mutation at position D^{542} of TRPV5, which corresponds to D^{541} of TRPV6, abolishes Ca^{2+} permeation, and Ca^{2+}-dependent current decay, whereas permeation of monovalent cation basically remains intact. It established that D^{542}/D^{541} is a crucial determinant of the conductive properties of TRPV5/TRPV6 and a key element of the selectivity filter (Nilius *et al*., 2001).

A characteristic feature of TRPV5/TRPV6 channels is the open block by intracellular Mg^{2+} (Nilius *et al*., 2001). In the absence of extracellular divalent cations, the monovalent currents display characteristic voltage-dependent gating (Voets *et al.*, 2003). Mg^{2+} blocks the channel by binding to a single aspartate residue D^{541} within TRPV6 (corresponding to D^{542} of TRPV5), where it interacts with permeant cations (Voets and Nilius, 2003; Voets *et al.*, 2001; Martinez *et al.*, 2000). The pore helix is followed by the selectivity filter, which has a diameter of approximately 5.4 A (Voets *et al.*, 2004).

These data indicate that selectivity and permeation properties in TRPV5 and TRPV6 are mainly determined by a ring of four aspartate residues in the channel pore. Sequence similarity is weak between the pore regions of the ring of voltage-dependent gating Ca^{2+} channels and TRPV5/TRPV6 Ca^{2+} channels (Peng *et al.*, 2001a). It was suggested that the highly similarity functional properties are due to the occurrence of a ring of negative charges that structure is a high affinity binding site for cationic molecules at the extracellular entry way (Wood *et al.*, 2001; Owsianik *et al.*, 2006; Vennekens *et al.*, 2000).

Tissue distribution of transient receptor potential vanilloid channel 5/6

The tissue distribution of TRPV5 and TRPV6 has been studied extensively by Northern blot, RT-PCR analysis and immunohistochemistry (Table 2). TRPV5 and TRPV6 are co-expression in the organs that mediate transcellular Ca^{2+} transport such as duodenum, jejunum, colon and kidney (Barley *et al.*, 2001; Weber *et al.*, 2001; Hoenderop *et al.*, 2001a; Zhuang *et al.*, 2002). The transport of Ca^{2+} across polarized Ca^{2+} transporting epithelial within organs such as the intestine, kidney and placenta is a coordinate and active process essential for dietary Ca^{2+} absorption, renal reabsorption and maternal-to-fetal transfer (Peng *et al.*, 2003a).

(Re)absorption of Ca^{2+} ion in kidney and intestine is essential for homeostasis of Ca^{2+} ion in the body. Transepithelial transport of Ca^{2+} in the (re)absorptive tissues occurs via paracellular as well as transcellular routes. Paracellular transport is passive, nonsaturable ways of Ca^{2+} (re)absorption (Larsson and Nemere, 2002). Transcellular (re)absorption of Ca^{2+} is mediated by Ca^{2+} entry across the apical membrane through the speciallized epithelial Ca^{2+} channels, TRPV5 and TRPV6, intracellular buffering and facilitated diffusion bound to Ca^{2+}-binding proteins (calbindins) and extrusion across the basolateral membrane by Na^{+}/ Ca^{2+} exchanger (NCX1) and/or by plasma membrane Ca^{2+}-ATPases (Larsson and Nemere, 2002). Immunohistological studied demonstrated the localization of TRPV6 in the brush border membrane of intestinal absorptive cells (Kutuzova and DeLuCa, 2004). This finding hints at a role of this protein as the rate apical influx pathway of active Ca^{2+} transport (Zhuang *et al.*, 2002; Kutuzova and DeLuca, 2004). TRPV6 was identified as the apical channel responsible for intestinal absorption of Ca^{2+} (Forsell *et al.*, 2006; Kutuzova and DeLuca, 2004; Slepchenko and Bronner, 2001; van Abel *et al*., 2003; Walters *et al*., 2004), whereas TRPV5 was initially identified as the apical Ca^{2+} entry channel in kidney (Hoenderop *et al*., 2003b).

The TRPV5 was localized to the Ca^{2+}-reabsorptive distal convoluted tubules (DCT) and connecting tubules (CNT) (Hoenderop *et al.*, 1998). Recent studies, however, have found that both TRPV5 and TRPV6 are expressed in the DCT and CNT of kidney, where they may form heteromultimers (Peng and Hediger, 2002; Peng *et al*., 2000b).

The epithelial calcium channels (TRPV5 and TRPV6) present in placenta (Moreau *et al.*, 2002b) might be involved in placental transfer of maternal Ca^{2+}. This is a crucial process in fetal development, which is carried out by the placental syncytiotrophoblast layer. The finding that mRNAs of TRPV5 and TRPV6 are expressed in this layer might indicate that these Ca^{2+} channels account for the basal Ca^{2+} uptake by these cells (Belkacemi *et al.*, 2005; Moreau *et al.*, 2002a; Moreau *et al.*, 2002b; Bernucci *et al.*, 2006).

TRPV6 is not only expressed in (re)absorptive epithelial cells but also in exocrine tissue such as pancreas, prostate, sweat gland, and mammary gland (Weber *et al.*, 2001; Hoenderop *et al.*, 2001a; Zhuang *et al.*, 2002). Moreover, the increasing expression of TRPV6 was found in prostate cancer and correlatative with tumor grade. It was also found in carcinoma colon and carcinomas of other tissues such as breast, thyroid, and ovary (Wissenbach *et al.*, 2001; Abeele *et al.*, 2003a; Abeele *et al.*, 2003b; Peng *et al.*; 2001b; Prevarskaya *et al.*, 2004; Zhuang *et al.*, 2002).

Regulation of epithelial calcium channel TRPV5/6

Transcellular Ca^{2+}transport is a highly coordinated and regulated process, which can be fine-tuned to the body's specific requirements. TRPV5/6 expression and channel activity have to be conspicuously controlled. This regulation occurs at different levels, including transcriptional and translational regulation, the tetrameric channel stoichiometry, trafficking of channels to and from the plasma membrane and modulation of channel activity at the plasma membrane (Nijenhuis *et al.*, 2005; Hoenderop *et al.*, 2005).

Transcriptional and translational regulation

Parathyroid hormone (PTH) and the biologically active form of vitamin D [1,25$(OH)_2D_3$] are the main calciotropic hormones controlling Ca^{2+} balance (Hoenderop *et al.*, 2005). PTH, which is secreted into the circulation when the extracellular Ca^{2+} level decreases (hypocalcaemia), stimulates the activity of 25-hydroxyvitamin-D_3-1α-hydroxylase (1α-OHase), a crucial enzyme in the biosynthesis of 1,25-$(OH)_2D_3$ in kidney (Brown *et al*., 1999). Studies in several cell models suggested that 1,25-$(OH)_2D_3$ enhances epithelial Ca^{2+} channel expression (Hoenderop, 2001b; van Abel *et al*., 2002; van Abel *et al*., 2003; van de Graaf *et al.*, 2004; Brown *et al*., 2005). In vitamin D receptor (VDR) knockout mice, duodenal TRPV6 mRNA levels were significantly down-regulated and associated with decreased intestinal Ca^{2+} absorption and hypocalcemia (Weber *et al.*, 2001; Cromphaut *et al*., 2001; Ferrari *et al*., 1998). Analysis of putative promoter region of human and murine *TRPV5* and *TRPV6* genes revealed potential vitamin D-response elements (Weber *et al.*, 2001; Nijenhuis *et al.*, 2003) so the expression of TRPV5 and TRPV6 is tightly controlled by 1,25-$(OH)_2D_3$. The increasing 1,25-$(OH)_2D_3$ affects absorption of Ca^{2+} in the small intestine and kidney (Nijenhuis *et al.*, 2003; Wood *et al.*, 2001). However, the transcriptional and translational regulation of TRPV6 by 1,25-$(OH)_2D_3$ was consistently established.

Tetrameric channel stoichiometry

In 2003, Hoenderop *et al*. revealed that TRPV5 and TRPV6 can indeed form homo- and heterotetrameric channel complexes. The concatemeric channels were constructed consisting of four TRPV5 and/or TRPV6 subunits, differences in the ratio of TRPV5 and TRPV6 subunits resulted in mixed properties of TRPV5 and TRPV6 channels because TRPV5 and TRPV6 exhibit different channel kinetics (Hoenderop *et al.*, 2003a). Recent studies showed that, whereas the other TRPV channels preferentially form homotetramers, TRPV5 and TRPV6 seem to be unique in forming heterotetramers (Hellwig *et al.*, 2005). Thus, regulation of the relative expression levels of TRPV5 and TRPV6 may be a mechanism to fine-tune the Ca^{2+} transport kinetics in TRPV5/6 co-expressing tissues. Indeed, several studies indicated that certain tissues co-express TRPV5 and TRPV6, including kidney and small intestine, which would allow oligomerization of these channels *in vivo* (Hoenderop *et al.*, 2005).

Trafficking of channels to the plasma membrane

Accurate trafficking of channels and transporters to the plasma membrane is essential for transcellular ion transport. van de Graaf and his coworkers provided the first evidence of a regulatory role for the S100A10-annexin 2 heterotetramer in the trafficking of TRPV5 and TRPV6 (van de Graaf *et al.*, 2003). S100A10, a 97-amino acid protein member of the S100 superfamily, is found tightly associated with annexin 2. S100A10-annexin 2 is a member of the Ca^{2+} and phospholipids-binding proteins which is implicated in numerous biological processes including exocytosis, endocytosis and membrane-cytoskeleton interactions (Gerke and Moss, 2002; Gerke *et al*., 2005). The association of S100A10 with TRPV5 and TRPV6 was restricted to a short conserved peptide sequence located in the C-tail of these channels (van de Graaf *et al.*, 2003). The first threonine of this sequence was identified as a crucial amino acid for binding and channel function. When this particular threonine was mutated, the activity of TRPV5 and TRPV6 was abolished accompanied by a major disturbance in their subcellular localization (Gerke *et al*., 2005). This indicated that the S100A10-annexin 2 heterotetramer facilitates the translocation of TRPV5 and TRPV6 channels towards the plasma membrane. Interestingly, S100A10 as well as annexin 2 appear to be regulated by 1,25-$(OH)_2D_3$, further supporting the importance of the S100A10-annexin-2 complex in the regulation of 1,25-$(OH)_2D_3$-dependent TRPV5/6-mediated Ca^{2+} influx (van de Graaf *et al.*, 2003).

Modulation of channel activity at the plasma membrane

It is well known that metabolic acidosis and alkalosis influence Ca^{2+} homeostasis. Therefore, pH might directly influence TRPV5/6 channel activity at the apical plasma membrane. In addition, extracellular pH also affected current kinetics including extracellular Mg^{2+} blockade and Ca^{2+} affinity. The mean current density decreased at acidic pH and increased at alkaline pH (Peng *et al.*, 1999). Recent studies showed that mutation of the Glu residue at position 522 to Gln near the pore helix decreased the inhibition of TRPV5 by extracellular acidification (Yeh *et al*., 2003). This Glu-522 in the extracellular loop between TM5 and the pore region, appears to mediate pH sensitivity and, therefore, acts as the pH sensor in TRPV5 but the exact mechanism explaining these effects is unknown (Hoenderop *et al.*, 2005). Alternatively, these data suggest that the luminal pH directly regulates Ca^{2+} entry through the epithelial Ca^{2+} channels *in vivo*.

Milk calcium and its transportation in mammary epithelial cell

The mammary gland can extract large quantities of Ca^{2+} from the plasma during lactation, to ensure that milk is concentrated with sufficient Ca^{2+}. Ca^{2+} is secreted into milk as free ionized Ca^{2+}, Ca^{2+} bound to citrate or phosphate and as Ca^{2+} complexed with milk caseins by exocytotic pathway (McManaman and Neville, 2003; Neville, 2005). Ionized Ca^{2+} concentration within human milk is approximately 3 mM, total milk Ca^{2+} concentration is around 8 mM, whereas total plasma Ca^{2+} concentration, of which half is ionized, is 3 mM. Thus, the mammary gland is required to transport large amounts of Ca^{2+}, presumably in an active process due to this concentration gradient. Mammary gland Ca^{2+} transport is transcellular and overall unidirectional. The overall direction of transcellular calcium transport is unidirectional but note the presence of two ionized calcium gradients; one across the basolateral membrane and the other across the apical membrane. The Ca^{2+} transporters including the plasma membrane Ca^{2+}-ATPases (PMCAs), sarco(endo)plasmic reticulum Ca^{2+} ATPases (SERCAs), Golgi pumps or secretory pathway Ca^{2+}-ATPases (SPCAs) as well as Na^{+}/ Ca^{2+} exchangers being the predominant mechanism for Ca^{2+} efflux of the plasma membrane are also likely to be important mechanisms of calcium transport during lactation (Reinhardt and Horst, 1999; Reinhardt *et al.*, 2000). These P-type Ca^{2+}-transporting ATPases are likely to be the major pathways for lowering $[Ca^{2+}]_i$ in mammary gland epithelial cells and due to the high demand for Ca^{2+} translocation from plasma to milk during lactation, they may also be important regulators of macro-Ca^{2+} homeostasis in the mammary gland (Figure 6) (Reviewed by Lee *et al.*, 2006).

Although large transcellular Ca^{2+} influxes are required to support the transfer of Ca^{2+} from plasma to milk during lactation, uncertainty exists as to the exact mechanisms responsible for the transport of Ca^{2+} across the basolateral membranes of secretory mammary gland epithelial cells. Ideally, processes for Ca^{2+} transport across the basolateral membrane would have to be of large capacity, unidirectional and inducible. These make Ca^{2+} channels likely candidates to perform this role (Reviewed by Lee *et al.*, 2006). TRPV6, a Ca^{2+} channels with properties suggesting potential roles in mammary gland epithelial cells Ca^{2+} transport, may involve these processes.

Table 1 Functional properties of members of TRPV subfamily

Name	P_{Ca}/P_{Na}	Proposed regulation	Function	Tissue distribution	Reference
TRPV1 (VR-1)	3-9	Capsaicin, Heat, Acidic pH	Heat sensor	Dorsal root ganglia	(Kedei *et al.*, 2001)
TRPV2 (VRL-1)	3	Heat, Growth factors	Heat sensor	Spinal cord, Brain, spleen	(Vennekens *et al.*, 2002)
TRPV3 (VRL-3)	12	Heat	Heat sensor	Skin, spinal cord, testis	(Smith *et al.*, 2002)
TRPV4 (OTRPC4)	6	Osmolarity, phorbol derivatives	Osmoreceptor	Kidney, salivary gland	(Nilius *et al.*, 2003a)
TRPV5 (CaT2 or ECaC1)	>100	Low $[Ca^{2+}]_i$	Apical Ca^{2+} transport	kidney, intestine	(Peng *et al.*, 2003a)
TRPV6 (CaT1 or ECaC2)	>100	Store-operated, Low $[Ca^{2+}]_i$	Apical Ca^{2+} transport	Intestine, placenta, kidney	(Hoenderop *et al.*, 2003a)

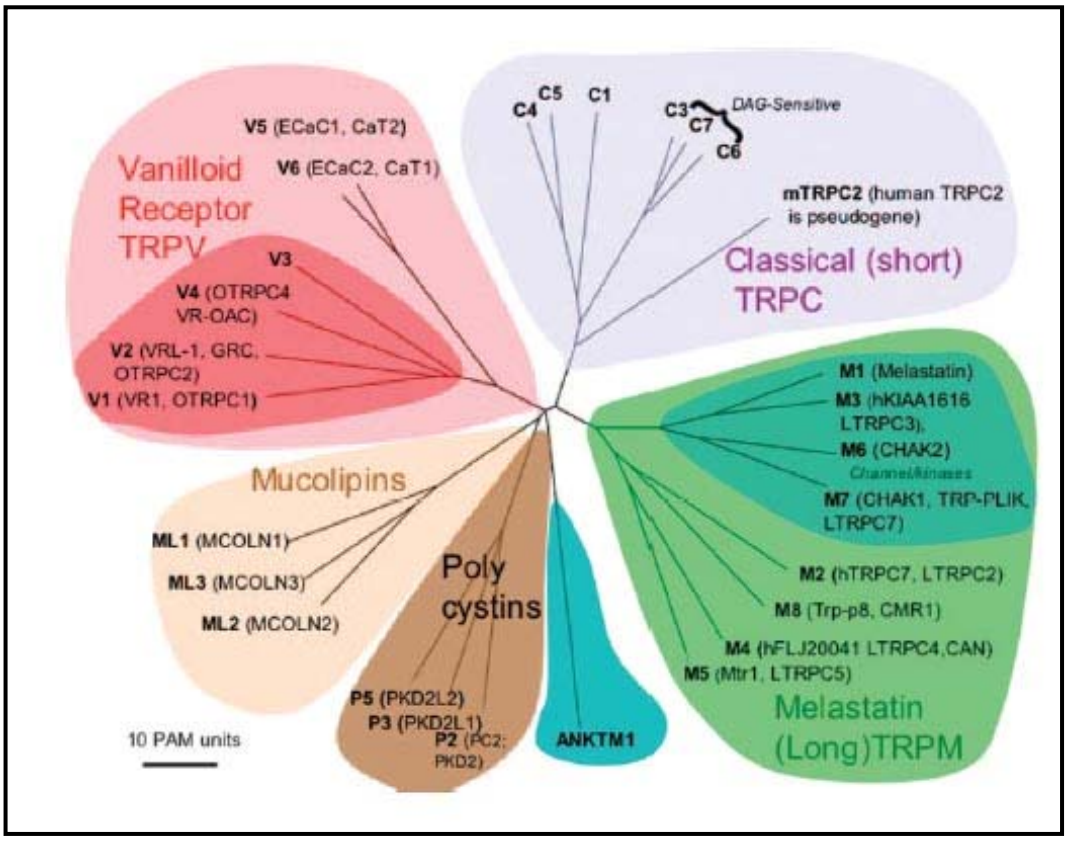


Figure 1 Mammalian TRP family tree. The evolutionary distance between the TRP channels is shown by the total branch lengths in point accepted mutations (PAM) units, which is the mean number of substitutions per 100 residues. The tree was calculated using the neighbor-joining method for human, rat, and mouse sequences.

Source: Huang (2004)

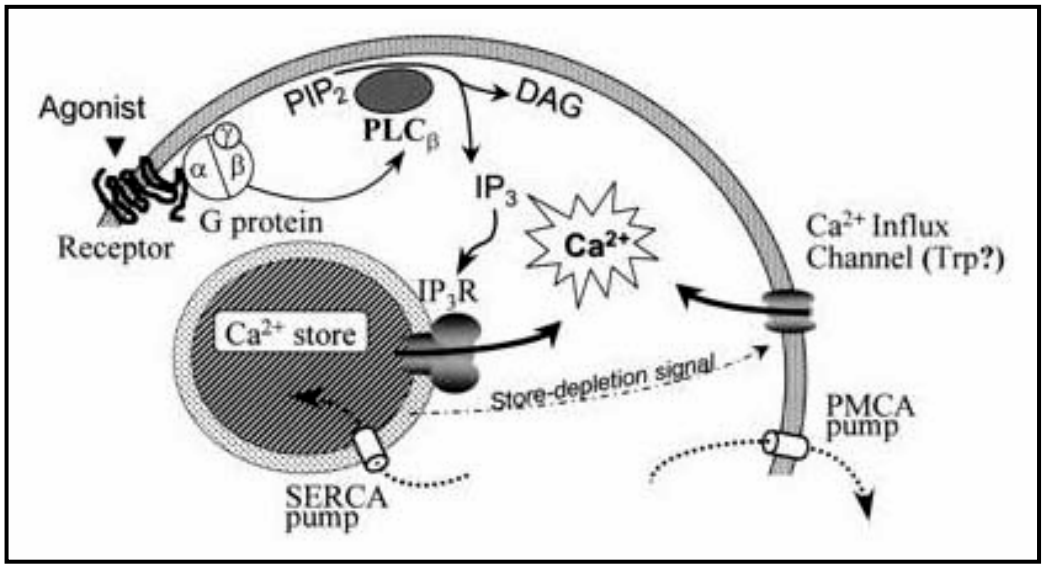


Figure 2 Diagram of stimulation of phospholipase C (PLC) and activation of Ca^{2+} signaling cascade. PLC-β can be activated by βγ-subunits of heterotrimeric G proteins following receptor activation. Two sources contribute to the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increase. On inositol 1,4,5-trisphosphate (IP_3) binding, Ca^{2+} in the internal store is released via an intracellular Ca^{2+} release channel, the IP_3 receptor (IP_3R). Ca^{2+} also comes in from extracellular space via undefined Ca^{2+} influx channels. Influx channel is activated by an undefined store depletion signal. Ca^{2+} pumps located on the endoplasmic reticulum (ER) [sarcoplasmic endoplasmic reticulum Ca^{2+}-ATPase (SERCA)] and plasma membrane [plasma membrane Ca^{2+}-ATPase (PMCA)] actively remove Ca^{2+} from cytosol into the ER or external space. PIP_2, phosphatidylinositol 4, 5-bisphosphate; DAG, 1, 2-diacylglycerol.

Source: Zhu and Birnbaumer (1998)

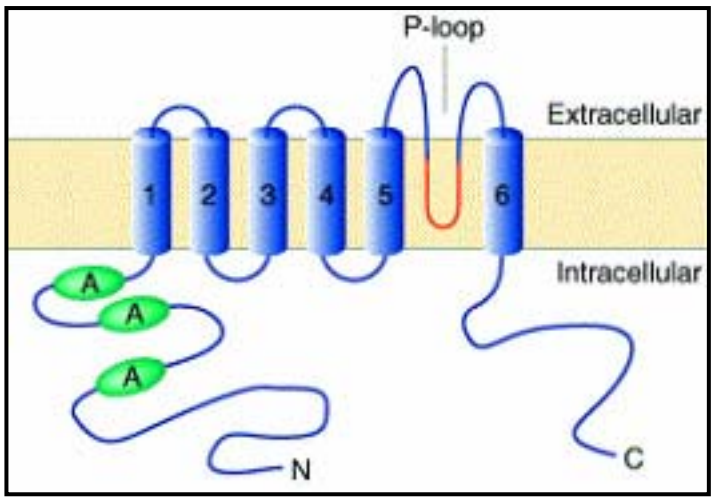


Figure 3 The predicted topology of TRPV receptor. TRPV contains six transmembrane domains (6TMs) and a pore loop (P-loop). Three intracellular ankyrin repeats (A) are also present in the N-terminal region.

Source: Gunthorpe *et al.* (2002)

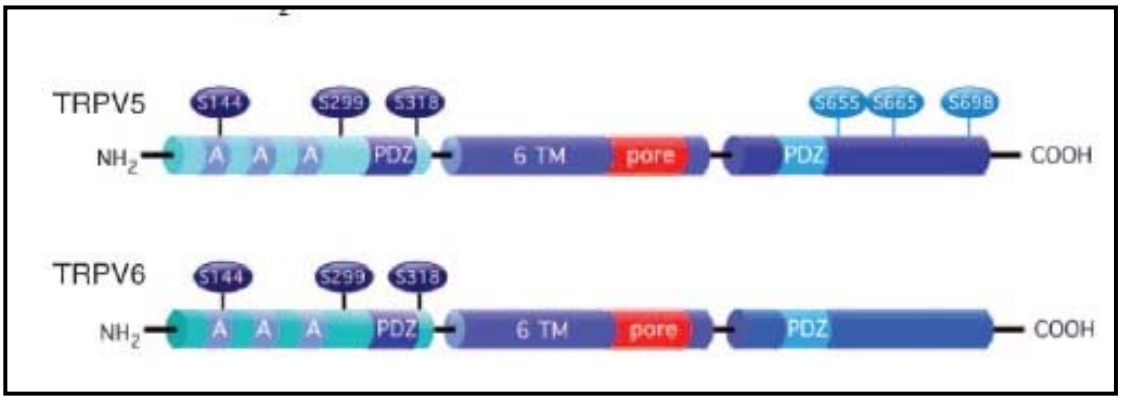
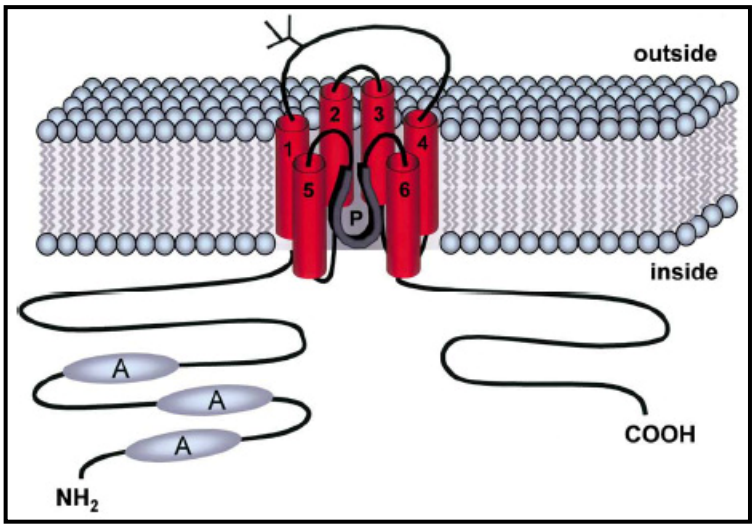


Figure 4 Structural organization of TRPV5 and TRPV6. Potential regulatory sites in the amino and carboxyl tail of TRPV5 and TRPV6 including ankyrin repeats and PDZ motifs and conserved PKC phosphorylation sites.

Source: Hoenderop *et al*. (2005)

A



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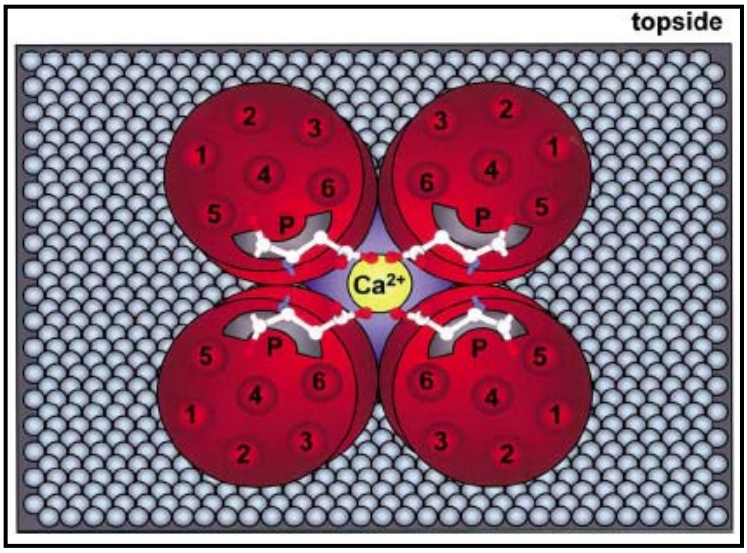


Figure 5 The predicted topology of the TRPV5/6 monomer and architecture of (homo/hetero) tetrameric channel complex.

(A) Schematic representation of TRPV5 and TRPV6 topology. Transmembrane segments are numbered, the pore region is depicted in grey, and ankyrin repeats in the N-terminal tail are indicated with A.

(B) Top view of the TRPV5/6 (hetero) tetrameric channel shows four monomeric TRPV5/6 subunits forming a tetrameric complex to be the Ca^{2+}-binding site within the pore of the channel

Source: den Dekker *et al*. (2003)

Table 2 Tissue expression of TRPV5 and TRPV6

Tissue	Mouse		Human		Rat	
	TRPV5	TRPV6	TRPV5	TRPV6	TRPV5	TRPV6
Adrenal gland	–	+	ND	ND	ND	ND
Bone	–	+	ND	ND	ND	ND
Brain	–	+	+	–	–	ND
Caecum	–	+	–	–	–	+
Colon	–	+	+	(–)(+)	ND	ND
Duodenum	–	+	+	+	–	+
Heart	–	–	–	–	ND	ND
Ileum	ND	+	–	(–)(+)	ND	ND
Jejunum	–	+	+	+	ND	ND
Kidney	+	+	+	(–)(+)	+	–
Leukocyte	ND	ND	ND	–	ND	ND
Liver	–	+	–	–	–	ND
Lung	–	+	–	–	–	ND
Mammary gland	ND	ND	ND	+	ND	ND
Oesophagus	ND	+	–	(–)(+)	ND	ND
Osteoblast	–	+	ND	ND	ND	ND
Ovary	–	+		–	ND	ND
Pancreas	–	+	+	+	+	ND
Placenta	–	+	+	+	ND	ND
Prostate	–	+	+	+	ND	ND
Rectum	ND	ND	–	–	ND	ND
Salivary gland	ND	ND	ND	+	ND	ND
Skeletal muscle	ND	ND	–	–	ND	ND
Skin	–	+	ND	ND	ND	ND
Small intestine	ND	ND	–	(–)(+)	ND	ND
Spleen	–	+	–	–	–	ND
Stomach	ND	+	–	+	ND	ND
Sweat gland	ND	ND	ND	+	ND	ND
Testis	–	+	+	(+)(–)	–	ND
Thymus	–	+	ND	–	–	ND

TRPV5/6 expression or lack of expression is indicated with + and -, respectively. ND: not determined.

Source: den Dekker *et al.* (2003)

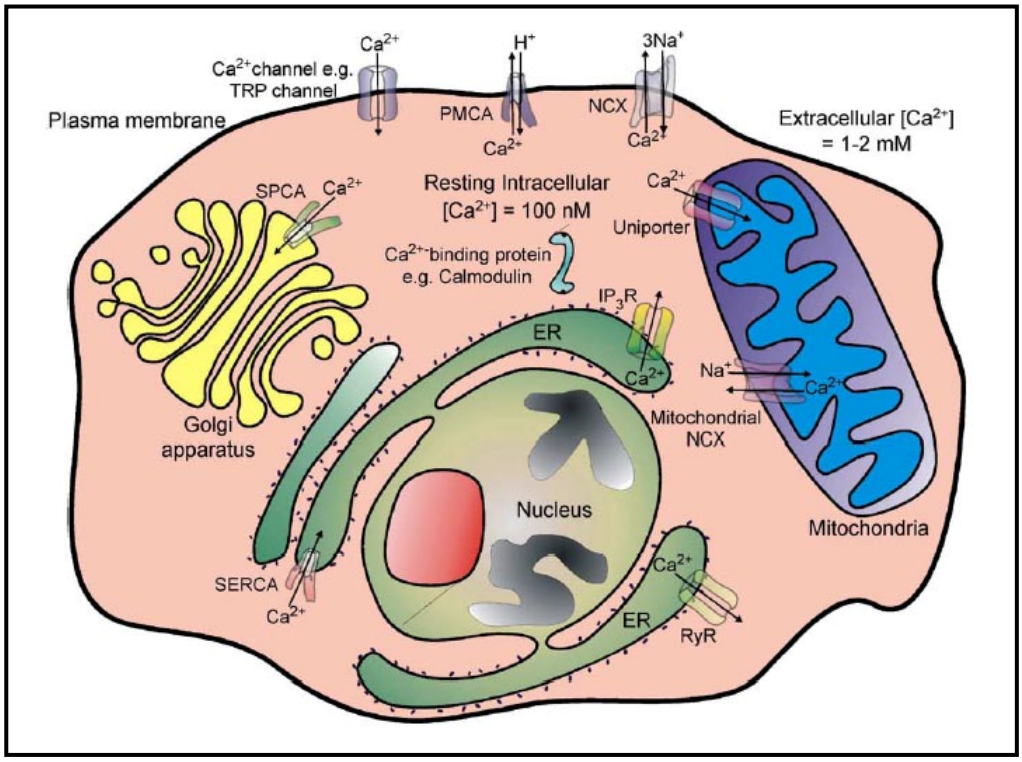


Figure 6 Calcium regulation in mammary gland epithelial cells. The calcium transporters, channels and binding proteins are likely to contribute to calcium homeostasis and signaling in cells of the mammary gland.

Source: Lee *et al*. (2006)

MATERIALS AND METHODS

Animal models and tissue samples

Twenty-four confirmed pregnant Sprague Dawley rats were purchased from the Animal Facility of National Laboratory Animal Centre (NLAC), Mahidol University. Rats were individually housed in basket cages on sawdust bedding. Throughout pregnancy and lactation the dams were fed standard rat chow. The first day after parturition was assigned as the first day of lactation and the number of pups in each dam was reduced to seven. Rats were randomized block designed into 6 groups assigned as -7, -1, 0, +1, +7, and +14 for totally 4 blocks. Rats in -7 and -1 groups were sacrificed on 7 and 1 day prepartum. Rats in 0 group were sacrificed on parturition day, and rats in +1, +7, and +14 groups were sacrificed on 1, 7, and 14 day of lactation. Rats were sacrificed by 3 times overdose of Sodium Barbiturate (Nembutal®) on the date assigned. A 5 ml of blood was collected into sodium citrate anticoagulant tube (BD Vacutainer®, USA). Mammary and intestinal tissues were removed from the animal immediately after sacrification and tissues were flash frozen on ethanol-dry ice bath and stored at -80°C until total RNA was prepared.

Total RNA preparation and quantitation

Total RNA was prepared from tissues by the acid guanidine isothiocyanate-phenol-chloroform extraction using TRIzol reagent (Invitrogen, USA.) according to the manufacturer's protocol with slight modifications of single-step isolation method developed by Chomczynski and Sacchi (Chomczynsk and Sacchi, 1987). A 1 gram tissue was homogenized in 15 ml TRIzol reagent at 15 seconds each for 3 times. A 3 ml chloroform was added into the homogenized sample. Let the homogenized sample stand undisturbed for 5 minutes before a 15 seconds of shaking and following with a standing undisturbed for 3 minutes at room temperature. The mixed solution was then centrifuged at 9,500 xg for 25 minutes at 4°C, and an aqueous phase of colorless top layer was collected into a new tube containing 7.5 ml isopropanol, mixed gentlely 3 times, and let stand undisturbed on ice for 10 minutes before a centrifugation at 11,800 xg for 15 minutes at 4°C. The supernatant was discarded and precipitated RNA pellet was washed with 15 ml 75% iced cold ethanol for 5 minutes at room temperature. The precipitated RNA pellet was then air dry for 10 minutes before being dissolved in diethylpyrocarbonate treated H_2O (DEPC-ddH_2O).

Total RNA was further precipitated for 2 hours on ice with 8M lithium chloride (LiCl) at 1:10 dilution. The LiCl precipitated RNA was collected after centrifugation at 14,000 xg for 20 minutes at 4°C. The LiCl precipitated RNA was washed twice in 75% ethanol and resuspended in DEPC–ddH_2O.

To quantify the extracted total RNA, the RNA concentration was calculated from an UV260 and 280 nm absorbent measurement by spectrophotometer and its concentration was calculated by a following equation:-

μg/μl of total RNA concentration = A_{260} X (0.04 μg/μl) X DF;

DF represents the dilution factor.

A ratio of A_{260} to A_{280} was calculated in order to specify a quality of extracted RNA.

TRPV6 primer design

TRPV6 primers were designed by using MacVector 7.2.3 software from conserved regions of rat *TRPV6* gene (Accession number NM_053686). Primers were always chosen according to following parameters: length between 18 and 25 bases; Tm in between 55 - 80°C; length of amplification product between 400 and 1000 bp; G+C content between 45-55 %. All primer sequences were shown in Table 3. To determine specificity, all primer sequences were similarly compared against previously reported sequences at the GenBank database located at an URL: http://www.ncbi.nlm.nih.gov by using BLAST program version 2.0. Primers with sequences similar to sequence of genes other than TRPV6, they would be not selected. The selected TRPV6 primers were synthesized by KU Vector, Kasetsart University.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Polymerase Chain Reaction (PCR) for primer screening

The Reverse Transcription Polymerase Chain Reaction (RT-PCR) conditions were as follows in a total volume of 50 μl reaction: 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.8 mM each of dNTPs, 50 pmol oligonucleotide primers, 50 U of SuperScript™ III Reverse Transcriptase (Invitrogen, USA.), 10 U RNaseOut™ (Invitrogen, USA.), 1.25 U *Taq* polymerase (Invitrogen, USA.), and 1 μg total RNA.

The Polymerase Chain Reaction (PCR) contained similar components as RT-PCR reaction except for SuperScript™ III Reverse Transcriptase and RNaseOut™, which were excluded.

The RT reaction was conducted in a Thermocycler (Biometra-UNO II, Germany) starting at 55°C for 50 minutes, following by 4 minutes at 95°C. The PCR was performed 35 cycles of; 94°C for 15 seconds (denaturing), 55-58°C (primer specific annealing temperature depending on each primer set) for 20 seconds, and 72°C for 40 seconds (extension). The reaction was completed with a 25 minutes extension step at 72°C, and then was cooled to 4°C.

RT-PCR and PCR products were examined by horizontal electrophoresis. Electrophoresis was conducted by prestaining 9 μl of RT-PCR or PCR products with 9 μl of 1:5000 diluted SYBR® Gold (Molecular Probes, Eugene, USA) for 20 minutes in the dark. Prestained sample was loaded to a 2 % agarose gel. The gel was electrophoresed in 1X TAE buffer for 1 hour at 110 watts. A 100 bp DNA ladder molecular weight marker (Fermentas) was used as standard maker. The RT-PCR and/or PCR product bands were visualized under UV-transilumainator (Spectroline®). Images of the RT-PCR and PCR product were acquired with an EDAS 290 Kodak camera.

Primer set which gave a correct RT-PCR product size with >95% homology to rat TRPV6 gene and gave an undetectable of PCR product would be chosen for further experiment.

Sequencing of PCR products

A DNA fragment, product from the RT-PCR using TRPV6 primer set, was recovered from the gel by using QIAquick Gel Extraction Kit (QIAGEN) as the manufacturer's protocol. The recovered DNA fragment was used as template for PCR reaction using the same primer set to increase a copy number of DNA fragment. The PCR product was confirmed its size by agarose gel electrophoresis, and then it was purified by QIAquick PCR Purification Kit (QIAGEN). The purified PCR product was submitted with its primer set for sequencing at BSU Bio Unit, Thailand. Sequencing was carried out for both forward and reverse primers. Nucleotide sequences of PCR product were analyzed for homology with sequences reported in GenBank database by BLAST program available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). A multiple sequence alignment was performed to compared with NCBI reported TRPV6 genes.

TRPV6 and GAPDH RT-PCR efficiency test

TRPV6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs in mammary tissues were detected by RT-PCR technique using TRPV6 primer set 3 and GAPDH primers reported by Gottwald *et al*. (Gottwald *et al.*, 2001), respectively. The GAPDH forward and reverse primer sequences were (5' TGA CCT TGC CCA CAG CCT TG 3') and (5' CAT CAC CAT CTT CCA GGA GCG 3') (Gottwald *et al.*, 2001). The RT-PCR conditions for each gene were analyzed for optimal $MgCl_2$ concentration, optimal annealing temperature, and optimal number of PCR cycle. The number of PCR cycle was 18-35 and 15-30 cycles for TRPV6 and GAPDH RT-PCR, respectively. The RT-PCR products of TRPV6 and GAPDH mRNAs were electrophoresed and products' band intensity were analyzed by Kodak 1D Image Analysis software®.

RT-PCR products image analysis

An image of SYBR® Gold prestained DNA band was acquired with an EDAS 290 Kodak camera. The bands' intensity was quantified by KODAK 1D Image Analysis Software 1D. The image file to be analyzed must be saved in TIFF file format.

In order to analyze band intensity, the image file was opened and adjusted by using "Magnification Tool" on Toolbar to increase to 1.50X magnification. An area in image was selected by using "Image Selection Tool". Lanes in a selected area were defined and bands in each lane were defined and adjusted. The band sensitivity and percentage of the profile with were adjusted to "0" and 80%. The band peak parameter was selected to display band intensity.

Semiquantitation of *TRPV6* gene expression

TRPV6 gene expression was semiquantitated by comparing amount of RT-PCR products from TRPV6 mRNA RT-PCR reaction to housekeeping gene (GAPDH) mRNA RT-PCR reaction, at the number of PCR cycle at exponential phase (Marone *et al*., 2001). The 50 μl RT-PCR reaction of 1 μg total RNA was set, as previously described with TRPV6 primer set 3 (as shown in Table 3) and GAPDH forward-backward primers (as reported by Gottwald *et al*., 2001) for TRPV6 mRNA and GAPDH mRNA, respectively. The PCR reactions were run totally 35 and 19 cycles for TRPV6 and GAPDH reactions, respectively. Their 10 μl RT-PCR products were electrophoresed and products' band intensity was evaluated by Kodak 1D Image Analysis software®. A ratio of band intensity of TRPV6 RT-PCR product to GAPDH RT-PCR product was calculated. Duplicate to triplicate reactions were performed for each total RNA sample.

Statistical analysis

The expression of *TRPV6* gene in rat mammary tissues during pregnant, parturition and lactation was shown as mean of TRPV6/GAPDH band intensity ratio ± SEM. The statistical analysis was done by using SAS analysis software (SAS Institute, Cary, NC). Differences in TRPV6 mRNA expression in each period were analyzed by One-way ANOVA and it was considered significant when $p \leq 0.05$. When expression was significant difference, Duncan's Multiple Range Test was performed and $p \leq 0.05$ was accepted.

Table 3 Sequences of TRPV6 primer set designed by using MacVector software

Primer set	PCR product size (bp)	Annealing temperature (°C)	Primer sequences
1	615	57-56	5' AAC AAA GCA GGA GCC CTC TTC G 3' 5' AGC ACG GAC CAA GTT CAC ATT C 3'
2	455	57-56	5' GAG AAC ATC CTT TGT CCT TTG CTG 3' 5' TTC CTT CAC AGG TGT TCT GGT CC 3'
3	570	56.5	5' CCC TTG AAC TTG TGC CCA ATA AC 3' 5' GAA TGG TCT GCC CAA AAA ATC G 3'
4	626	56	5' CTT ATT GTT ACC ACC AAG AAG CGG 3' 5' CCC AAG ATT ACC ACA GCC ATC AG 3'
5	564	58	5' TGG ACC AGA CAC CTG TGA AGG AAC 3' 5' GCC AGC AGA ATC GCA TCA AGT C 3'
6	475	55	5' ATC TTC AGG TTG GGG GTC ACT C 3' 5' TGA TGG CAA AGG CAG CGT AG 3'
7	706	55	5' TCT CAA CAG ACA ACG CAT CCG 3' 5' GAA ATG ACT TTA CTG GAA GGT GGC 3'
8	771	56	5' GAG AAC ATC CTT TGT CCT TTG CTG 3' 5' GAA TGG TCT GCC CAA AAA ATC 3'
9	379	56	5' TGG ACC AGA CAC CTG TGA AGG AAC 3' 5' GAA TGG TCT GCC CAA AAA ATC 3'
10	339	58	5' ATC TTC AGG TTG GGG GTC ACT C 3' 5' GAA TGG TCT GCC CAA AAA ATC 3'

RESULT

Twenty-four confirmed pregnant Sprague Dawley rats were divided into four blocks. Each block contained six dams in a period of 7 days prepartum (-7), 1 day prepartum (-1), partum (0), 1 day lactation (+1), 7 days lactation (+7), and 14 days lactation (+14). Each block was experimentally designed as described in materials and methods.

Quality and quantity of total RNA from mammary tissues

Total RNA from rat mammary tissues on 7 days prepartum (-7), 1 day prepartum (-1), partum (0), 1 day lactation (+1), 7 days lactation (+7), and 14 days lactation (+14) was expressed in μg total RNA per 1 gram tissue as shown in Figure 7. The quality of total RNA before LiCl precipitation was poor as A_{260}/A_{280} ratio being around 1.4-1.6. However, LiCl precipitated total RNA had a better quality as A_{260}/A_{280} ratio being around 1.6-1.8, and RT-PCR reaction of this RNA was not inhibited (data not shown). As shown in Figure 7, concentration of total RNA, extracted from mammary tissues during prepartum, partum, and early lactation increased gradually during early lactation. It was 2-3 folds total RNA concentration extracted from 14 days lactating samples compared to samples from pregnancy, partum and early lactation periods.

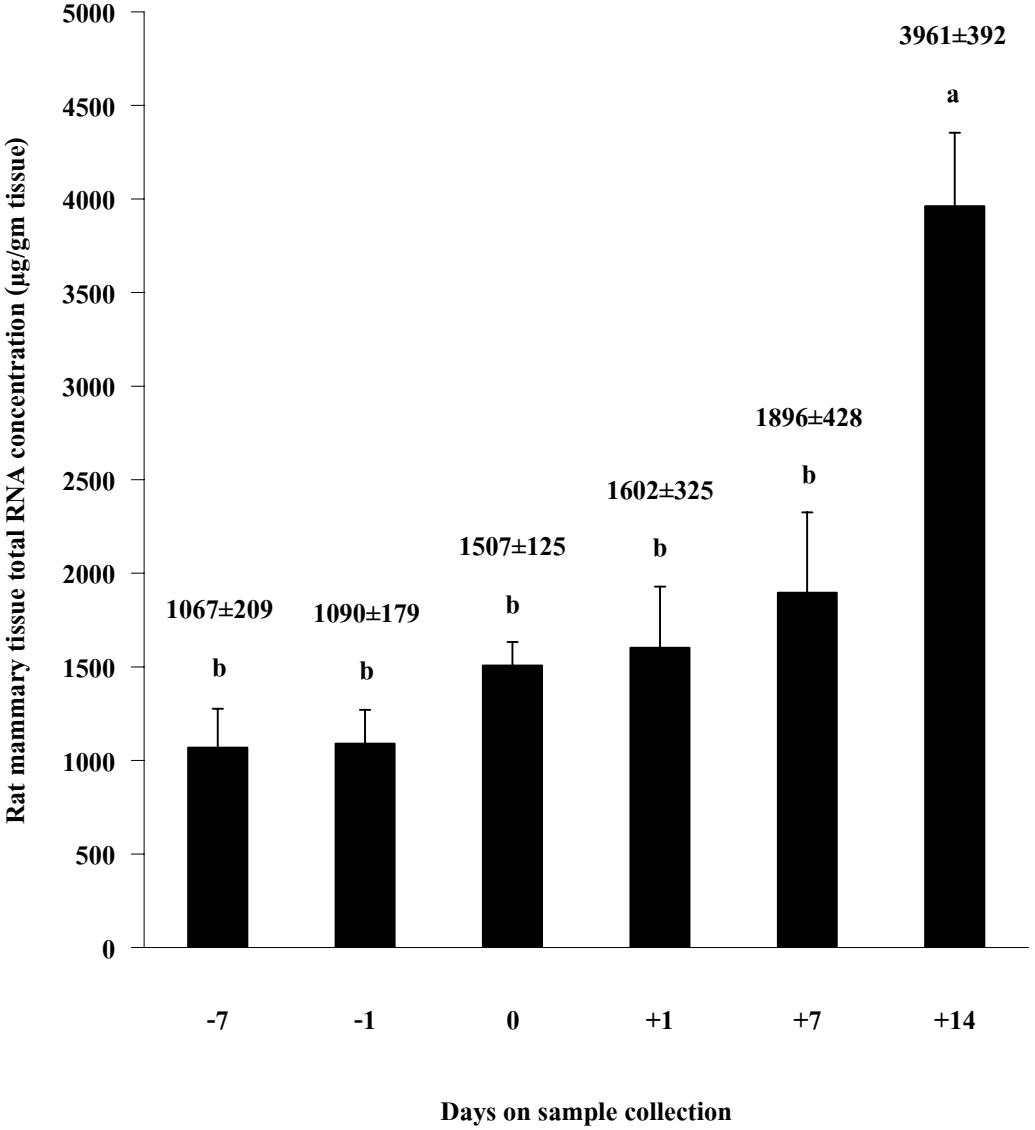


Figure 7 Total RNA concentration extracted from rat mammary tissues. –7; 7 days prepartum, -1; 1 day prepartum, 0; partum day, +1; 1 day lactation, +7; 7 days lactation, +14; 14 days lactation. (means ± SEM, n=4)

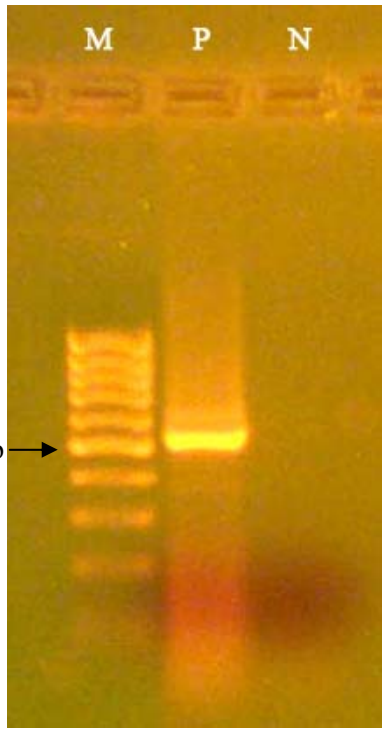
a, b; Significant differences ($p \leq 0.05$)

Primer sequences and specific TRPV6 primer selection by RT-PCR

The TRPV6 primers were designed from conserved regions of rat *TRPV6* gene (Accession number NM_053686) by MacVector 7.2.3 software. All primer sequences were shown in Table 3. Their specificity to TRPV6 mRNA was tested by RT-PCR with rat intestinal tissue total RNA. There were only primer set 3 and set 7 (Figure 8) which their RT-PCR products were corresponded to the expected size as indicated in Table 3. Therefore, primer set 3 and 7 were further tested by RT-PCR and PCR with mammary tissue total RNA.

TRPV6 RT-PCR product sizes were 570 bp and 706 bp when RT-PCR were performed with rat mammary total RNA by primer set 3 and 7, respectively (data not shown). However, primer set 7 gave positive PCR products when PCR reaction was performed without reverse transcriptase enzyme (Figure 9). Primer set 3 was selected for further test by RT-PCR and PCR with total RNA extracted from rat mammary tissues for the whole period of experiment (-7, -1, 0, +1, +7, and +14 days). Figure 10 shown results from RT-PCR and PCR products of primer set 3. As shown in Figure 10, RT-PCR products amplified by primer set 3 were 570 bp and there was no PCR product shown off in PCR reactions of total RNA extracted from mammary tissue of all periods. Therefore, RT-PCR product of primer set 3 was selected for further study by sequencing technique.

A



B

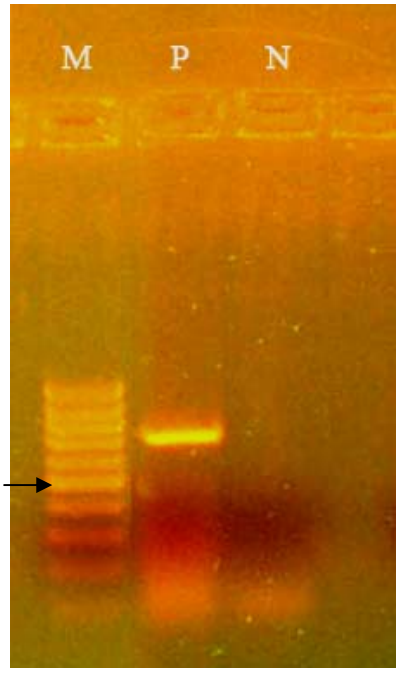
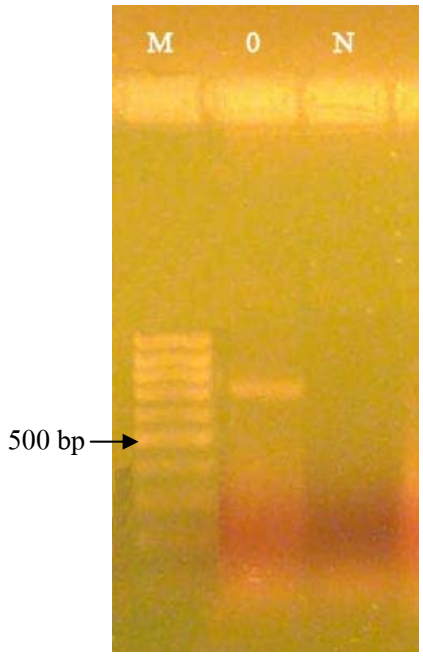


Figure 8 RT-PCR product of TRPV6, as amplified by primer set 3 (A) and primer set 7 (B) with rat intestinal tissue total RNA. M; 100 bp DNA ladder molecular weight maker (Fermentas), P; rat intestinal tissue, and N; negative control.

A



B

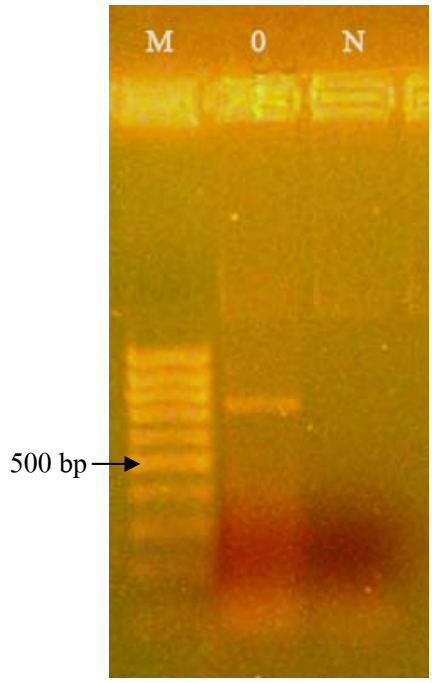
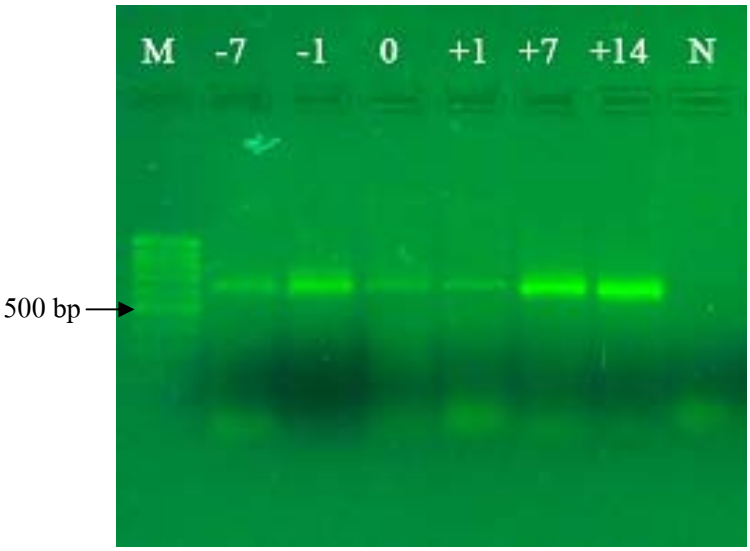


Figure 9 RT-PCR product (A) and PCR product (B) of TRPV6, as amplified by primer set 7, in rat mammary tissue on partum day. M; 100 bp DNA ladder molecular weight maker (Fermentas), 0; partum day, and N; negative control.

A



B

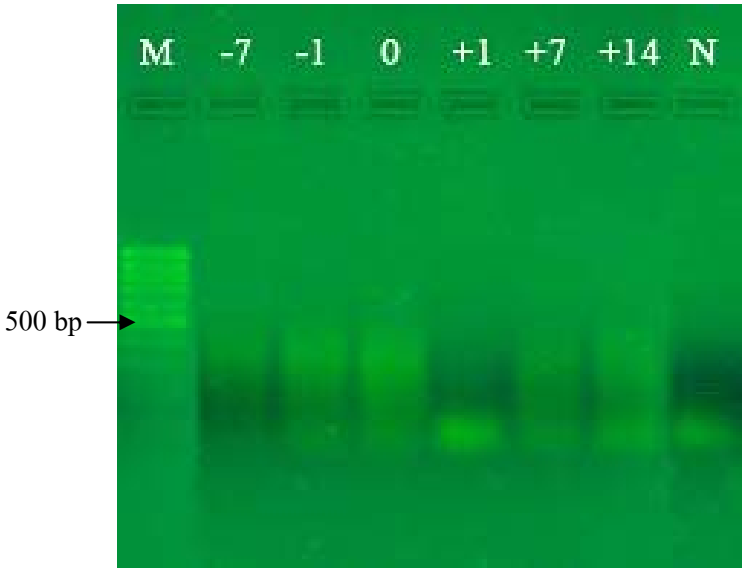


Figure 10 RT-PCR product (A) and PCR product (B) of TRPV6, as amplified by primer set 3, in rat mammary tissue during pregnant, partum, and lactation. M; 100 bp DNA ladder molecular weight maker (Fermentas), -7; 7 days prepartum, -1; 1 day prepartum, 0; partum, +1; 1 day lactation, +7; 7 days lactation, +14; 14 days lactation, and N; negative control.

Sequencing and analysis of primer set 3 RT-PCR product

A 570 bp RT-PCR product, amplified by primer set 3, of total RNA from mammary tissue on +14 days lactation was recovered and reamplified by PCR with the same primer set. The PCR product was then purified and confirmed its correct size by agarose gel electrophoresis. The purified PCR product was submitted for sequencing. Figure 11 shown sequencing result of RT-PCR product of primer set 3.

A 532 base sequences, shown in Figure 12, starting from base no. 1 to no. 532 was analyzed by multiple sequence alignment with bovine, human, mouse, and rat TRPV6 sequences by using Clustal W Alignment MacVector 7.2.3 software. The multiple sequence alignment result was shown in Figure 12. As shown in Figure 12, there was 98%, 93%, 85%, and 53% homology between RT-PCR product sequence to sequences of TRPV6 from rat, mouse, human, and bovine, respectively.

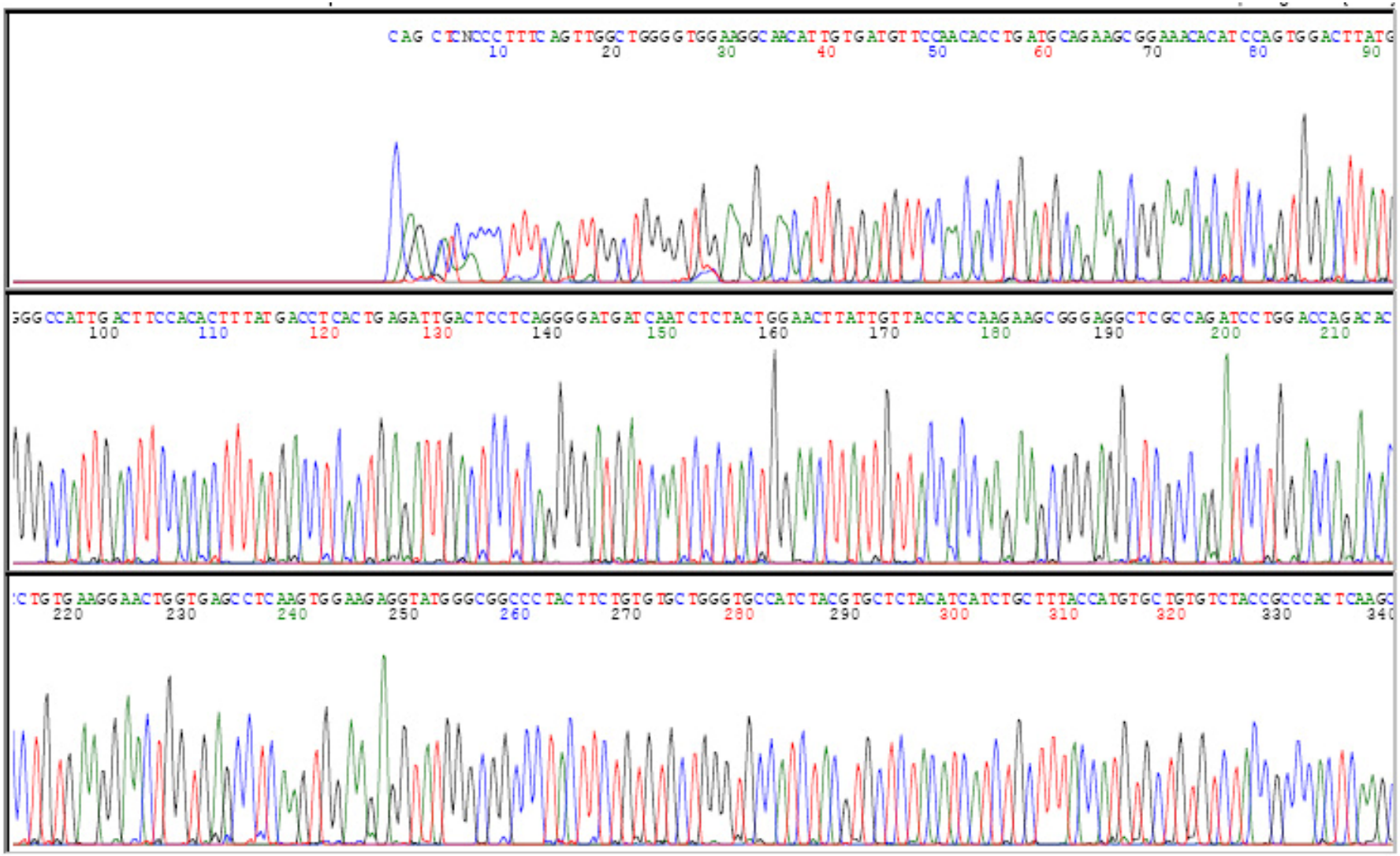


Figure 11 Nucleotide sequences of primer set 3 RT-PCR product as sequencing by primer set 3 forward primer.

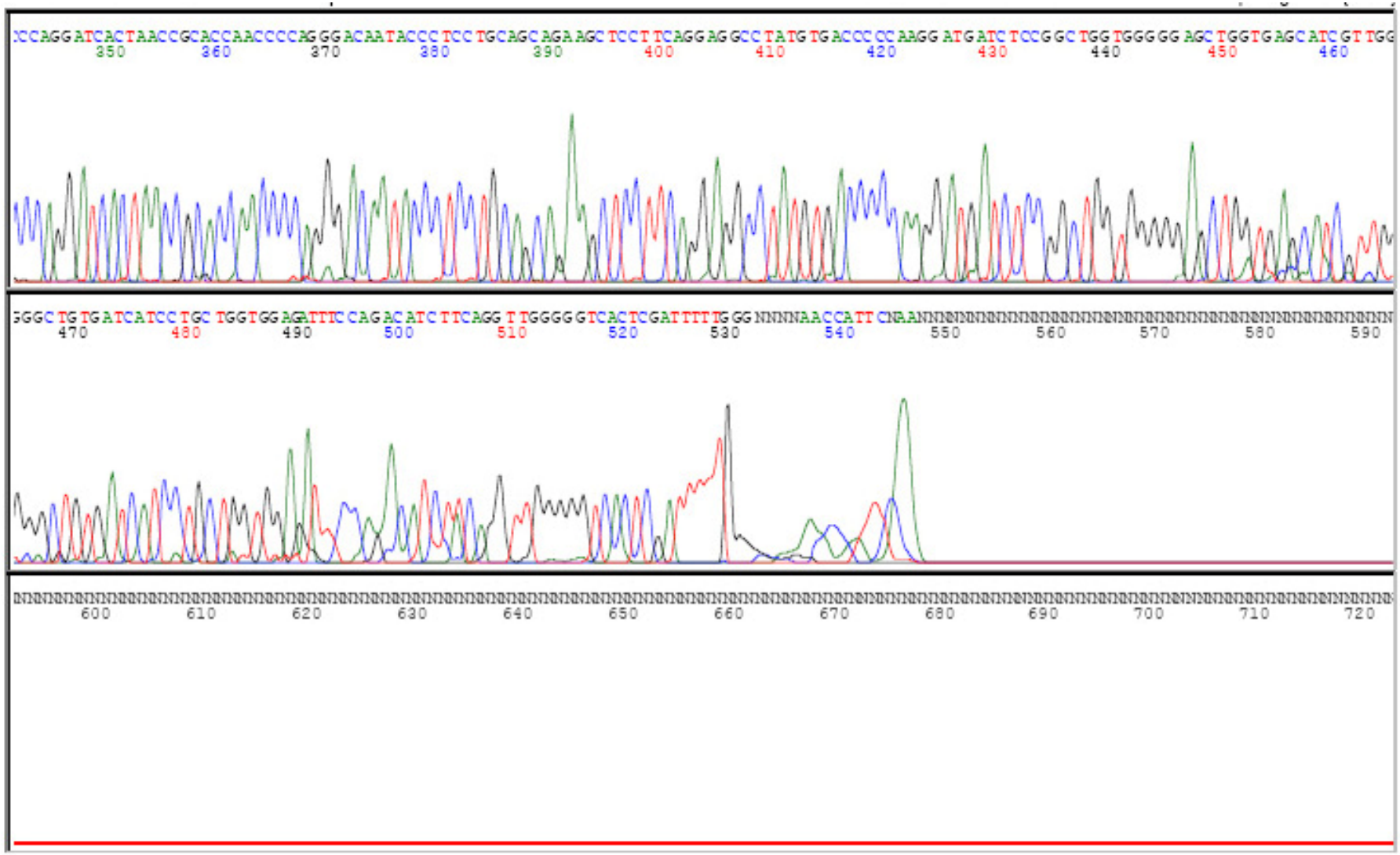


Figure 11 (Continued)

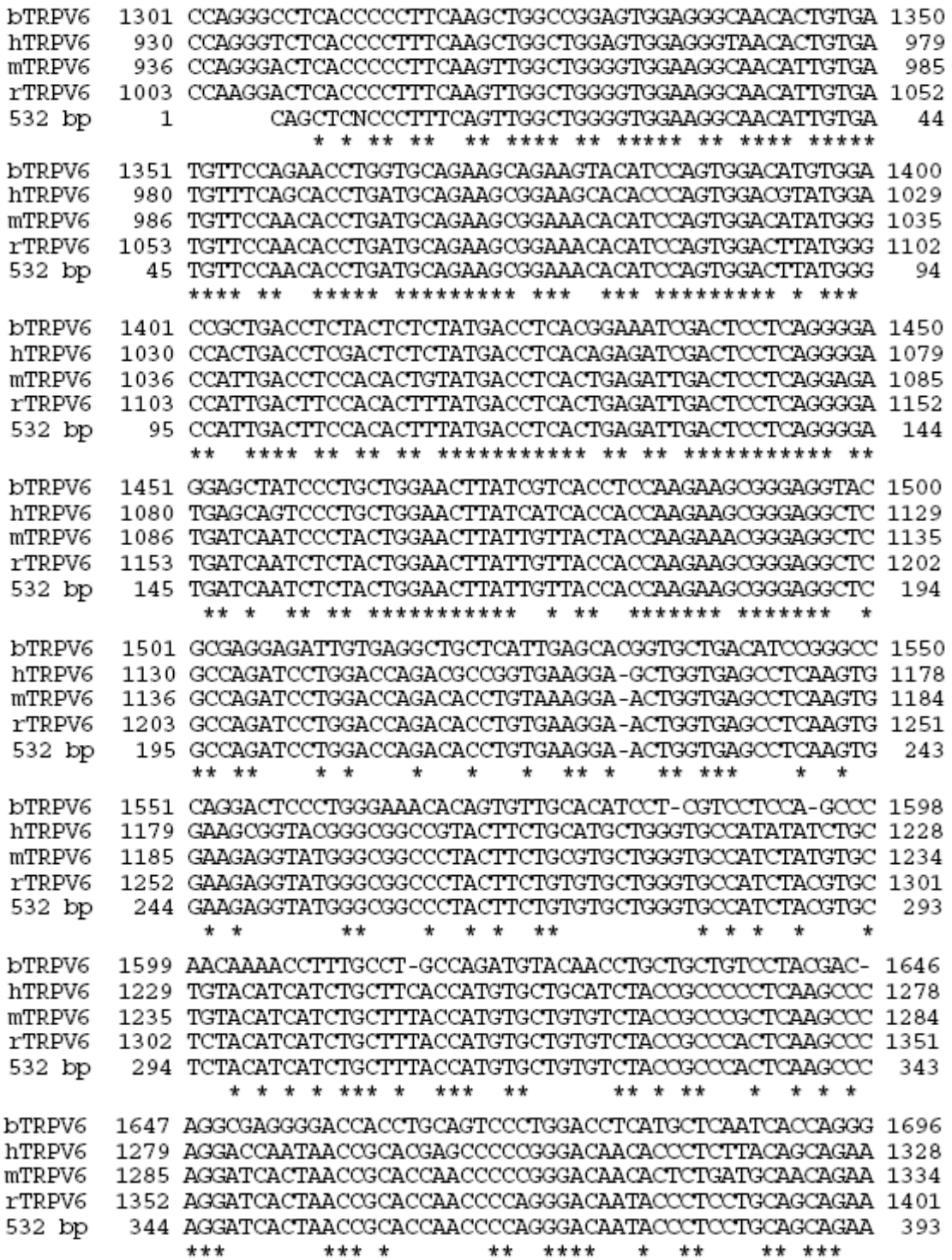


Figure 12 Multiple alignment of the 532 bases RT-PCR product nucleotide sequences with bTRPV6 (NM_018646), hTRPV6 (NM_595413), mTRPV6 (NM_022413), and rTRPV6 (NM_053686) by using Clustal W Alignment MacVector 7.2.3 software.

```
bTRPV6   1697 CCTCACCCCCTTCAAGCTGGCCGGAGTGGAGGGCAACACTGTGATGTTCC 1746
hTRPV6   1329 GCTACTTCAGGAAGCCTACATGACCCCTAAGGACGATATCCGGCTGGTCG 1378
mTRPV6   1335 GCTCCTTCAGGAGGCCTATGTGACCCCTAAGGATGACCTCCGGTTGGTGG 1384
rTRPV6   1402 GCTCCTTCAGGAGGCCTATGTGACCCCCAAGGATGATCTCCGGCTGGTGG 1451
532 bp    394 GCTCCTTCAGGAGGCCTATGTGACCCCCAAGGATGATCTCCGGCTGGTGG  443
               **    *                  ***   *      * ** *

bTRPV6   1747 AGCACCTGATGCAGAAGCGGAAGTACATCCAGTGGACGTGCGGGCCGCTG 1796
hTRPV6   1379 GGGAGCTGGTG-ACTGTCATTGGGGC-TATCATCATCCTGCTGGTAGAGG 1426
mTRPV6   1385 GGGAACTCGTG-AGCATCGTTGGGGC-TGTGATCATCCTGCTGGTGGAGA 1432
rTRPV6   1452 GGGAGCTGGTG-AGCATCGTTGGGGC-TGTGATCATCCTGCTGGTGGAGA 1499
532 bp    444 GGGAGCTGGTG-AGCATCGTTGGGGC-TGTGATCATCCTGCTGGTGGAGA  491
               * * **  ** *    *    *  * *    *   * *** **  *

bTRPV6   1797 ACCTCCACTCTCTACGACCTCACAGAGATTGACTCCTGGGGAGAAGACGT 1846
hTRPV6   1427 TT-CCAGACATCTTCAGAATGGGGGTCACTCGCTTCTTTGGACAGACCAT 1475
mTRPV6   1433 TT-CCAGACATCTTCAGGTTGGGGGTCACTCGATTCTTTGGACAGACCAT 1481
rTRPV6   1500 TT-CCAGACATCTTCAGGTTGGGGGTCACTCGATTTTTTGGGCAGACCAT 1548
532 bp    492 TTTCCAGACATCTTCAGGTTGGGGGTCACTCGATTTTTGGG           532
                  *     *** *    *    *  * *   *  *  **
```

Figure 12 (Continued)

TRPV6 and GAPDH RT-PCR efficiency test

Semiquantitative RT-PCR analysis was chosen to assess expression levels of TRPV6 transcript from rat mammary tissue during pregnant, partum, and lactation. This method is based on the use of an internal control, which is a housekeeping gene. A highly level expression housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as an internal control in this experiment. A number of parameters could affect RT-PCR results and were extremely important for the reaction to be quantitative.

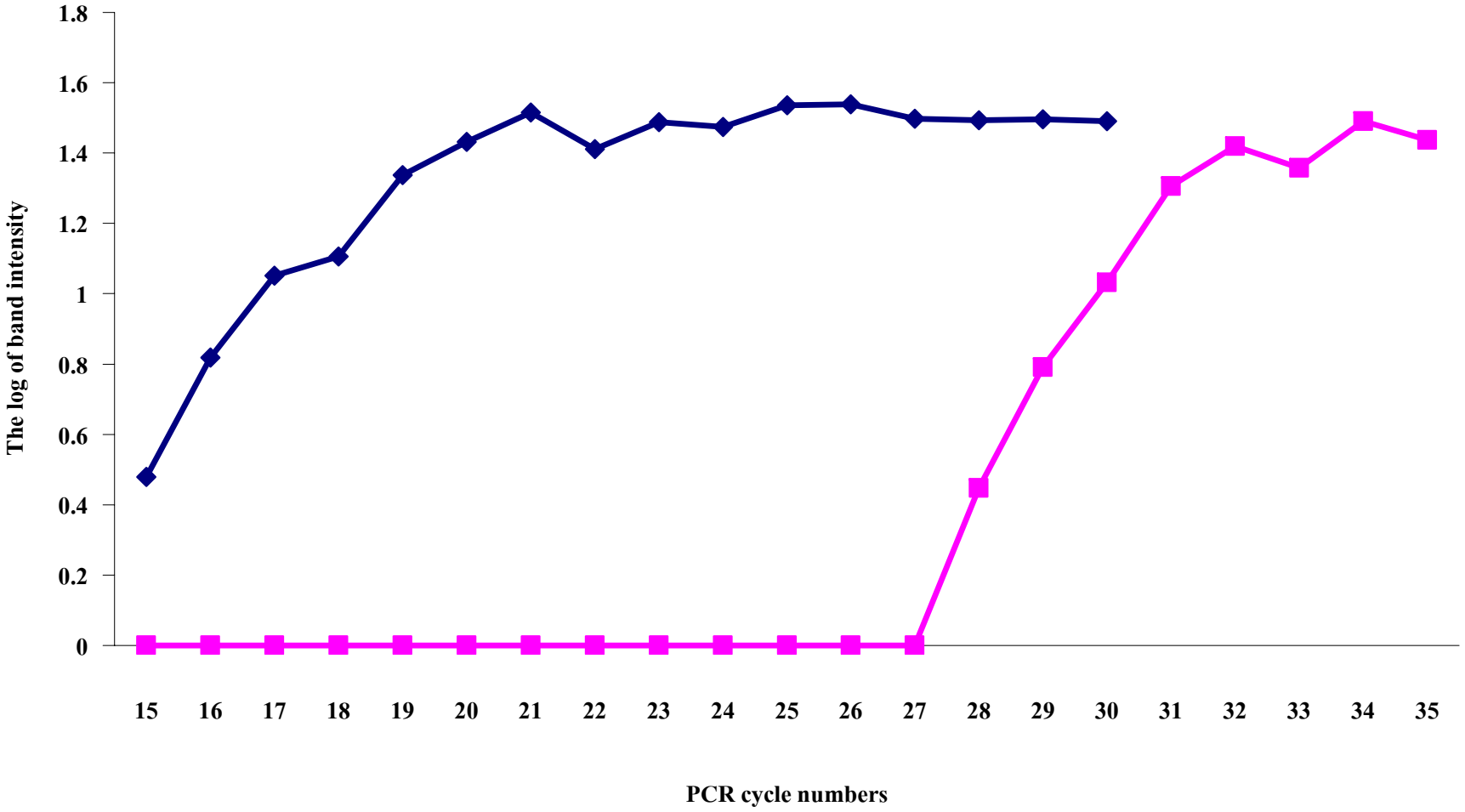
An optimal concentration of $MgCl_2$ and optimal primer annealing temperature were tested for both TRPV6 and GAPDH RT-PCR. There were 1.5 mM $MgCl_2$ concentration and 56.5°C optimal annealing temperature that gave the best results for both TRPV6 and GAPDH (data not shown).

The optimal PCR cycle numbers was evaluated by choosing cycle numbers that provided product band intensity in exponential phase. As shown in Figure 13, exponential phases for TRPV6 RT-PCR and GAPDH RT-PCR were during 28-31 cycle and 16-19 cycle numbers, respectively. However, 31 PCR cycle numbers for TRPV6 RT-PCR of total RNA of samples at prepartum and partum days gave a very poor to undetectable result, hence, a 35 cycle numbers was selected for TRPV6 RT-PCR rather than 31 cycle numbers. A 19 and 35 PCR cycle numbers were used in GAPDH RT-PCR and TRPV6 RT-PCR reactions in next experiments of semiquantitation.

Figure 13 Determination of a amplification power for TRPV6 and GAPDH. The intensity of TRPV6 and GAPDH RT-PCR products were shown in log band intensity.

GAPDH RT-PCR

TRPV6 RT-PCR

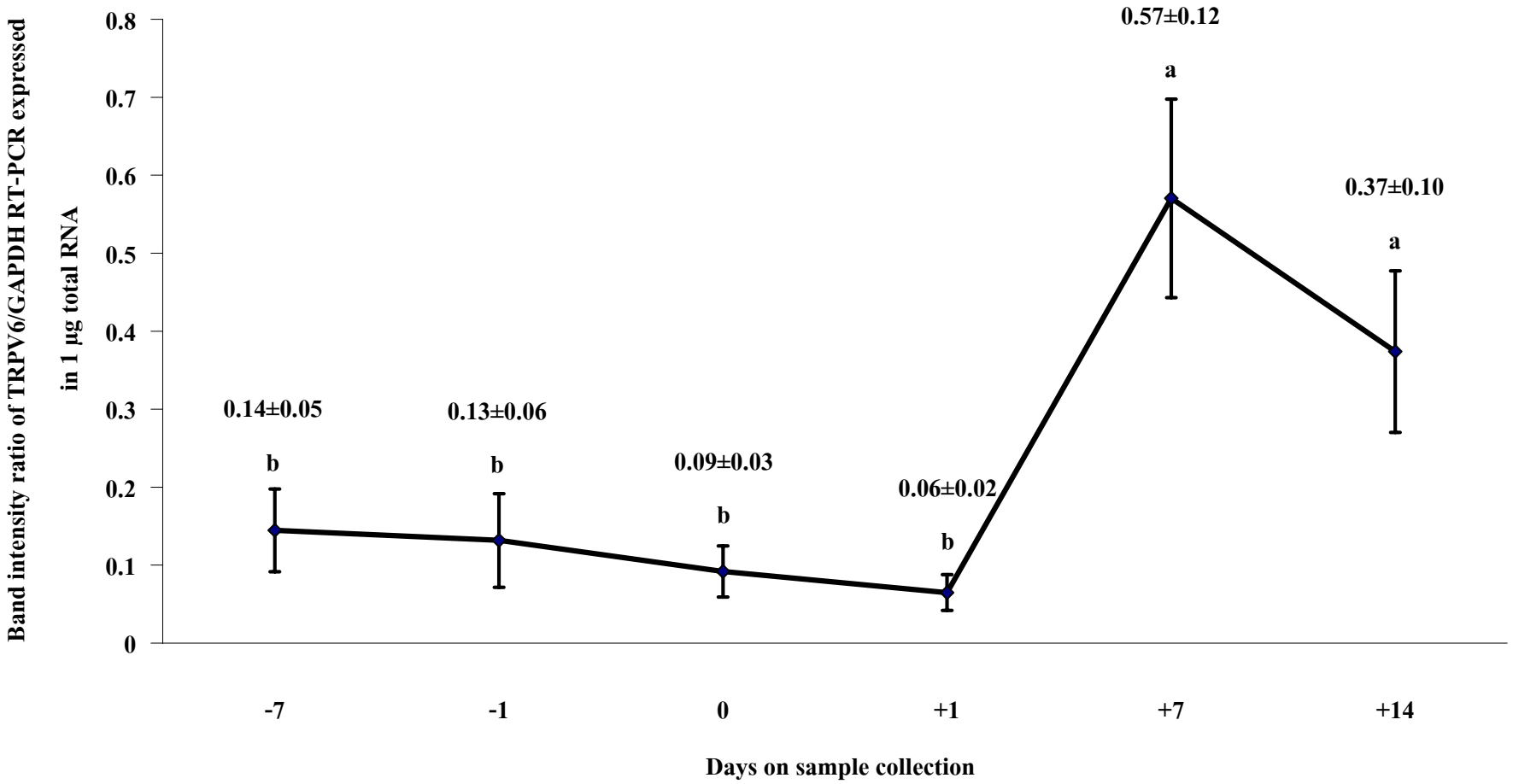

1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
The log of band intensity
15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35
PCR cycle numbers

Semiquantitative of TRPV6 gene expression in rat mammary tissues during pregnancy, partum, and lactation

An expression of TRPV6 mRNA in mammary tissues was evaluated during periods of pregnancy, parturition, and lactation. Figure 14 shown band intensity ratio of TRPV6/GAPDH RT-PCR products over a period of -7, -1, 0, +1, +7, and +14 of prepartum, partum and lactation days. During -7, -1, 0, and +1 days, TRPV6 mRNA was expressed at low basal level. The expression of TRPV6 transcript increased to 5 and 3 folds at +7 and +14 days lactation periods ($p \leq 0.01$), Figure 14. This expression profile was evaluated in 1 μg total RNA. If one calculated the expression profile of TRPV6 transcripts in mammary tissue per gram tissue, expressions of mammary TRPV6 would be 9 and 11 folds in mammary tissue during +7 and +14 days of lactation compared to TRPV6 level in mammary tissue during prepartum, partum and early lactation (data not shown).

Figure 14 The band intensity ratio of TRPV6/GAPDH on days during pregnancy, partum, and lactation of rat mammary tissue. –7; 7 days prepartum, -1; 1 day prepartum, 0; partum day, +1; 1 day lactation, +7; 7 days lactation, +14; 14 days lactation. (means ± SEM, n=4)

a, b; Significant differences ($p \leq 0.01$)


Band intensity ratio of TRPV6/GAPDH RT-PCR expressed in 1 µg total RNA
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.14±0.05
b
0.13±0.06
b
0.09±0.03
b
0.06±0.02
b
0.57±0.12
a
0.37±0.10
a
-7
-1
0
+1
+7
+14
Days on sample collection

DISCUSSION

TRPV6 protein in many organs, such as intestine, kidney, placenta, and mammary gland, has been reported by several investigators (Peng *et al.*, 1999; Nijenhuis *et al.*, 2003; Bernucci *et al.*, 2006; Zhuang *et al.*, 2002). However, there have been no evidences on the changes of TRPV6 mRNA expression in rat mammary gland under different physiological condition. The purpose of this study was to investigate TRPV6 mRNA expression profile in rat mammary tissues during pregnancy, parturition and lactation (-7, -1, 0, +1, +7, and +14) by using semiquantitative RT-PCR analysis.

Quality and quantity of total RNA from mammary tissues

The total RNA of rat intestine and mammary tissues were isolated by TRIzol reagent (Invitrogen, USA). The concentration of total RNA extracted from these mammary tissues increased gradually from pregnancy to early lactation but significantly increased on 14 days of lactation ($p \leq 0.05$) as shown in Figure 7. The increasing total RNA concentration during lactation correlated with an increasing of total protein concentrations in rat milk throughout lactation (Yamamoto *et al.*, 1992). In this experiment, the total RNA extracted from rat mammary tissues must be precipitated with 8 M lithium chloride (LiCl) since non LiCl precipitated total RNA from mammary tissue gave a negative result in RT-PCR reaction (data not shown). LiCl precipitated total RNA had a better quality than the quality of total RNA before LiCl precipitation because RT-PCR inhibitors were removed from RNA extracted from mammary tissue (Mathy *et al*., 1996).

The specific TRPV6 primer selection by RT-PCR and sequencing

To select specific TRPV6 primer, the LiCl precipitated total RNA of rat mammary tissue was used as template in RT-PCR reaction with primer sets as shown in Table 3. The RT-PCR product from primer set 1, 2, 4, 5, 6, 8, and 10 showed that their RT-PCR product sizes were not corresponded to the expected size as shown in Table 3, when compare with RT-PCR product (positive control) amplified from total RNA of rat intestine as template (data not shown). It might be alternatively RNA splicing forms of this gene in mammary tissue, evidences in mammalian plasma membrane Ca^{2+}-ATPases, PMCA1-4, encoded by a multigene family. Additional diversity of PMCA1-4 is generated by alternative RNA splicing (Filoteo *et al*., 1997; Reinhardt and Horst, 1999).

Further studies to investigate some spliced forms of this *TRPV6* gene in mammary tissues, we need to clone full length of *TRPV6* gene in rat mammary tissue.

There are two primer sets, set 3 and set 7, that could generate positive correct size RT-PCR products, but primer set 7 gave positive PCR products when PCR reaction was performed without reverse transcriptase enzyme (Figure 9). From this result, it was suggested that it might be DNA contaminated in total RNA sample. In addition, the forward and reverse primers of primer set 7 are on the same exon 15 (Peng *et al.*, 1999; Peng *et al*., 2001a; Hirnet *et al*., 2003), so PCR gave positive result with correct size of PCR product. Therefore, RT-PCR product primer set 3 was selected to be confirmed by sequencing as shown in Figure 11. The obtained sequences were then analyzed by multiple sequence alignment with bovine, human, mouse, and rat TRPV6 sequences (Figure 12). It had 98%, 93%, 85%, and 53% homology between RT-PCR product sequence to sequences of TRPV6 from rat, mouse, human, and bovine, respectively, so this primer set 3 was selected to assess expression levels of TRPV6 transcript in further study.

TRPV6 gene expression in rat mammary tissue during pregnancy, partum, and lactation

Results from experiment showed that TRPV6 mRNA expressed in rat mammary tissues during pregnancy, parturition and lactation. The expressions of TRPV6 increased 5 and 3 folds at +7 and +14 days lactation periods compared with that at prepartum, and partum periods ($p \leq 0.01$). The expressions of TRPV6 transcripts in mammary tissue per gram tissue were 9 and 11 folds during +7 and +14 days of lactation compared to TRPV6 level in mammary tissue during prepartum, partum and early lactation. Its expression profile was correlated to level of total RNA present in mammary tissue at various periods (Figure 7 and Figure 14). Calcium concentrations in rat milk increased only during early stages of lactation and remained stable after 7 days lactation (Yamamoto *et al.*, 1992), so this TRPV6 expression profile increase paralleled to milk calcium secretion. An increasing expression of TRPV6 mRNA in lactating mammary tissues suggested that TRPV6 plays important role(s) in calcium transport during lactation and milk production.

Rat milk has calcium concentration in excess of 60 mM. In human and cows, changes in milk calcium appear to be regulated by the amount of casein and citrate rather that ionized calcium [Ca^{2+}] (Neville *et al*., 1994). In most milk, calcium is associated with casein, citrate, phosphate,

bicarbonate, and carbonate in the sacculars of the terminal Golgi apparatus and is secreted via the Golgi-secretory vesicle system from the mammary alveolar cell (Neville and Peaker, 1979). It seems that the mammary gland does not transport calcium directly from the blood-to-milk compartments direction since calcium cannot cross the apical membrane or tight junctions (Neville and Peaker, 1979). It can be inferred that calcium transport into milk is transcellular (McManaman and Neville, 2003; Neville, 2005). The mechanism by which calcium is secreted into milk should be complex but, in fact, they are simple (Figure 15). During the transition from pregnancy to lactation, calcium transport mediates from blood across the basolateral membrane of mammary epithelial cell to cytoplasm by passive transport. This mechanism is not enough for large transcellular Ca^{2+} fluxes which is required to support the transfer of Ca^{2+} from plasma to milk during lactation. This profiling expression of TRPV6 in mammary tissue during pregnancy and lactation supported that TRPV6 Ca^{2+} channel involves in macro calcium in this active mammary tissue. However, the exact mechanism(s) responsible for the transport of Ca^{2+} across the basolateral membranes of mammary secretory cells has not been determinated (Shennan and Peaker, 2000).

Intracellular calcium is pumped from the cytoplasm into Golgi compartment by the Golgi Ca^{2+}-ATPase, SPCA (Taylor *et al*., 1997). Reinhardt *et al.*, 2000 reported SPCA protein expression increased 1 week before parturition and increased further as lactation proceeded. During lactation, the Golgi compartment requires large amount of calcium concentration for casein synthesis and micelle formation (Neville *et al*., 1994). The expression of Ca^{2+}-ATPases (PMCA1b, PMCA4b, SERCA2, and SERCA3) mRNA and their protein targeted at basolateral membrane and endoplasmic reticulum (ER), respectively, showed the lowest levels of expression during pregnancy and early lactation (Reinhardt and Horst, 1999; Reinhardt *et al.*, 2000). Their levels became significantly elevated on days 14 of lactation (Reinhardt and Horst, 1999). These Ca^{2+}-ATPases play an important role in maintaining low Ca^{2+} concentration in cytoplasm while facilitating a large transcellular calcium flux. Another Ca^{2+}-ATPase, PMCA2b, is located at apical membrane of mammary secretory cell (Reinhardt *et al.*, 2000). The expression of PMCA2b mRNA and protein increased continuously from a starting of lactation and they increased on 21 days of lactation while a macro amount of Ca^{2+} was transported into milk. PMCA2b is important for macro- Ca^{2+} homeostasis in lactating tissue. (Reinhardt and Horst, 1999; Reinhardt *et al.*, 2000). The large amount of calcium required during lactation correlated with TRPV6 mRNA expression that its mRNA significantly increased ($p \leq 0.01$) at +7 and +14 days lactation periods. This evidence implied that TRPV6 channel might be the

channel mediate calcium transport from blood into mammary epithelial cell during a requirement of large amount of calcium.

However, to evaluate role(s) of TRPV6 Ca^{2+}-selective channel as a channel to mediate blood calcium into mammary secretory cell, further studies will be required to identify the localization of TRPV6 protein in the mammary gland. Whether apical or basolateral locations are required for physiological explanations.

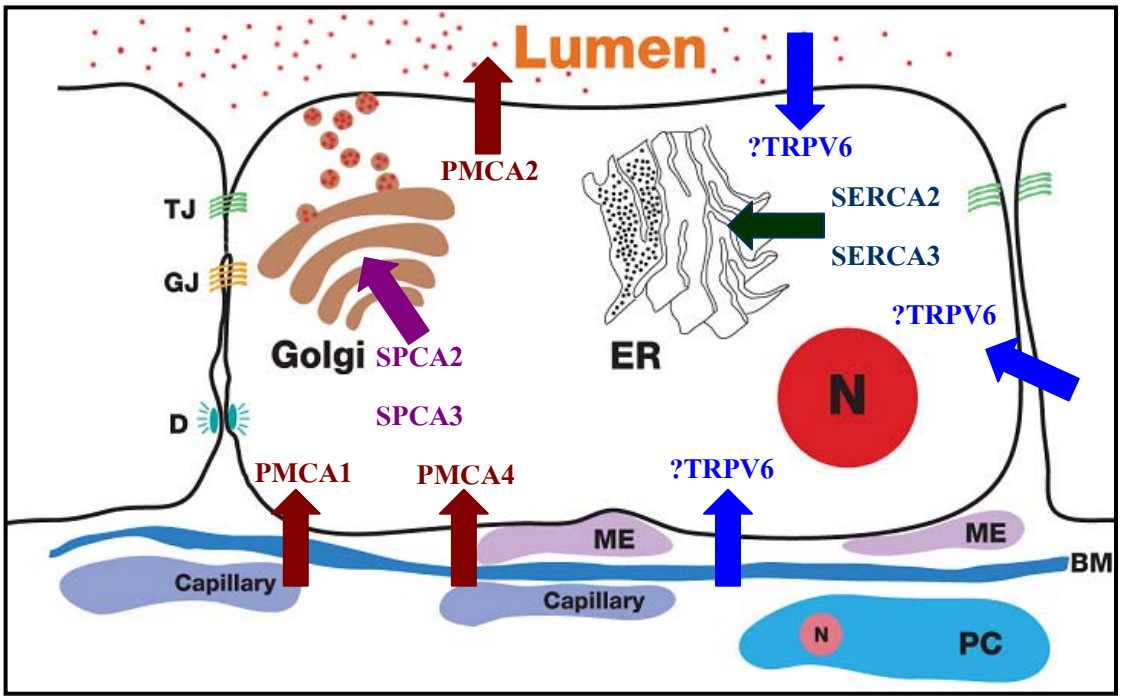


Figure 15 Diagram of calcium transport in mammary epithelial cell. PMCA; plasma membrane Ca^{2+}-ATPase, SERCA; Sarco(endo)plasmic reticulum Ca^{2+}-ATPase, and SPCA; Golgi Ca^{2+}-ATPase.

CONCLUSION

By using semiquantitative RT-PCR analysis, the expression of TRPV6 mRNA was observed in pregnant, parturition and lactating rat mammary tissues (-7, -1, 0, +1, +7, and +14). Measurement of TRPV6 mRNA expression in rat mammary tissues during pregnancy, parturition and lactation indicated that TRPV6 mRNA levels did not change appreciably between pregnancy, parturition, and early lactation, but increased significantly ($p \leq 0.01$) on 7 days lactation and 14 days lactation. This result suggested that the increasing TRPV6 mRNA expression during 7 days lactation until 14 days lactation might respond to calcium requirement of mammary secretory cells during lactation.

Lactating mammary tissue is an organ in which large amounts of calcium are transported. Therefore, it is tempting to hypothesize that TRPV6 channel in the mammary gland contributes to the massive transport of calcium from blood to milk.

However, in order to understand role(s) of TRPV6 protein in rat mammary tissue during pregnancy, parturition and lactation, an immunohistochemistry technique was required to investigate localization of TRPV6 protein in rat mammary tissue.

LITERATURE CITED

Abeele, F.V., M. Roudbaraki, Y. Shuba, R. Skryma and N. Prevarskaya. 2003a. Store-operated Ca^{2+} current in prostate cancer epithelial cells. **J. Biol. Chem.** 278: 15381-15389.

_____, Y. Shuba, M. Roudbaraki, L. Lemonnier, K. Vanoverberghe, P. Mariot, R. Skryma and N. Prevarskaya. 2003b. Store-operated Ca^{2+} channels in prostate cancer epithelial cells: function, regulation, and role in carcinogenesis. **Cell Calcium** 33: 357-373.

Barley, N.F., A. Howard, D. O'Callaghan, S. Legon and J.R. Walters. 2001. Epithelial calcium transporter expression in human duodenum. **Am. J. Physiol. Gastrointest. Liver Physiol.** 280: 285-290.

Belkacemi, L., I. Bedard, L. Simoneau and J. Lafond. 2005. Calcium channels, transporters and exchangers in placenta: a review. **Cell Calcium** 37: 1-8.

Benham, C.D., J.B. Davis and A.D. Randall. 2002. Vanilloid and TRP channels: a family of lipid-gated cation channels. **Neuropharmacology** 42: 873-888.

Bernucci, L., M. Henriquez, P. Diaz and G. Riquelme. 2006. Diverse calcium channel types are present in the human placental syncytiotrophoblast basal membrane. **Placenta** In Press

Berridge, M.J., M.D. Bootman and H.L. Roderick. 2003. Calcium signalling: dynamics, homeostasis and remodelling. **Mol. Cell Biol.** 4: 517-529.

Birnbaumer, L., E. Yidirim and J. Abramowitz. 2003. A comparison of the genes coding for canonical TRP channels and their M, V and P relatives. **Cell Calcium** 33: 419-432.

Bodding, M. 2005. Voltage-dependent changes of TRPV6-mediated Ca^{2+} currents. **J. Biol. Chem.** 280: 7022-7029.

Bodding, M. and V. Flockerzi. 2004. Ca^{2+} dependence of the Ca^{2+}-selective TRPV6 channel. **J. Biol. Chem.** 279: 36546-36552.

_____, U. Wissenbach and V. Flockerzi. 2002. The recombinant human TRPV6 channel functions as Ca^{2+} sensor in human embryonic kidney and rat basophilic leukemia cells. **J. Biol. Chem.** 277: 36656-36664.

Bootman, M.D., M.J. Berridge and H.L. Roderick. 2002. Calcium Signalling: More Messengers, More Channels, More Complexity. **Curr. Biol.** 12: 563-565.

Brown, A.J., A. Dusso and E. Slatopolsky. 1999. Vitamin D. **Am. Physiol. Soc.** 157-175.

_____, I. Krits and H.J. Armbrecht. 2005. Effect of age, vitamin D, and calcium on the regulation of rat intestinal epithelial calcium channels. **Arch. Biochem. Biophys.** 437: 51-58.

Chang, Q., E. Gyftogianni, S.F. van de Graaf, S. Hoefs, F.A. Weidema, R.J. Bindels and J.G. Hoenderop. 2004. Molecular determinants in TRPV5 channel assembly. **J. Biol. Chem.** 279: 54304-54311.

Chomczynsk, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. **Anal. Biochem.** 162: 156-159.

Clapham, D.E. 1995. Calcium signalling. **Cell** 80: 259-268.

_____. 2003. TRP channels as cellular sensors. **Nature** 426:517-524.

_____, L.W. Runnels and C. Struebing. 2001. The TRP ion channel family. **Nature Rev.** 2: 387-396.

Cromphaut, S.J., M. Dewerchin, J.G. Hoenderop, I. Stockmans, E. van Herck, R. Kato, R. Bindels, J. M., D. Collen, G. Carmeliet, R. Bouillon and G. Carmeliet. 2001. Duodenal calcium absorption in vitamin D receptor–knockout mice: Functional and molecular aspects. **Pro. Natl. Acad. Sci. USA** 98: 13324–13329.

den Dekker, E., J.G. Hoenderop, B. Nilius and R.J. Bindels. 2003. The epithelial calcium channels, TRPV5 & TRPV6: from identification towards regulation. **Cell Calcium** 33: 497-507.

Ellinor, P.T., J. Yang, W.A. Sather, J.F. Zhang and W. Tsien. 1995. Ca^{2+} channel selectivity at a single locus for high-affinity Ca^{2+} interactions. **Neuron** 15: 1121-1132.

Elliott, A.C. 2001. Recent developments in non-excitable cell calcium entry. **Cell Calcium** 30: 73-93.

Erler, I., D. Hirnet, U. Wissenbach, V. Flockerzi and B.A. Niemeyer. 2004. Ca^{2+}-selective transient receptor potential V channel architecture and function require a specific ankyrin repeat. **J. Biol. Chem.** 279: 34456-34463.

Ferrari, S., J.P. Bonjour and R. Rizzoli. 1998. The vitamin D receptor gene and calcium metabolism. **Trends in Endocrinology and Metabolism.** 9: 259-265.

Filoteo, A. G., N. L. Elwess, A. Enyedi, A. Caride, H. H. Aung and J. T. Penniston. 1997. Plasma membrane Ca^{2+} pump in rat brain. Patterns of alternative splices seen by isoform-specific antibodies. **J. Biol. Chem.** 272: 23741-23747.

Forsell, P., M. Eberhardson, H. Lennernas, T. Knutson and L. Knutson. 2006. Rapid modulation of Ca^{2+} uptake in human jejunal enterocytes. **Biochem. and Biophys. Res. Commun.**340: 961-966.

Gerke, V., C.E. Creutz and S.E. Moss. 2005. Annexins: linking Ca^{2+} signalling to membrane dynamics. **Mole. Cell Biol.** 6: 448-461.

Gerke, V. and S.E. Moss. 2002. Annexins: from structure to function. **Physiol. Rev.** 82:331–371

Gottwald, E., O. Mueller and A. Polten. 2001. Semiquantitative reverse transcription-polymerase chain reaction with the Agilent 2100 Bioanalyzer. **Electrophoresis** 22: 4016-4022.

Gunthorpe, M.J., C.D. Benham, A. Randall and J.B. Davis. 2002. The diversity in the vanilloid (TRPV) receptor family of ion channels. **Trends Pharmacological Sci.** 23: 183-191.

Hardie, R. C. 2003. TRP channels in *Drosophila* photoreceptors: the lipid connection**. Cell Calcium** 33: 385–393

Harteneck, C. 2003. Proteins modulating TRP channel function. **Cell Calcium** 33: 303-310.

_____, T.D. Plant and G. Schultz. 2000. From worm to man: three subfamilies of TRP channels. **Trends Neurosci.** 23: 159-166.

Hellwig, N., N. Albrecht, C. Harteneck, G. Schultz and M. Schaefer. 2005. Homo- and heteromeric assembly of TRPV channel subunits. **J. Cell Sci.** 118: 917-928.

Hirnet, D., J. Olausson, C. Fecher-Trost, M. Bodding, W. Nastainczyk, U. Wissenbach, V. Flockerzi and M. Freichel. 2003. The *TRPV6* gene, cDNA and protein. **Cell Calcium** 33: 509-518.

Hoenderop, J.G., B. Nilius and R.J. Bindels. 2005. Calcium absorption across epithelia. **Physiol. Rev.** 85: 373-422.

_____, _____ and R.J. Bindels. 2003a. Epithelial calcium channels: from identification to function and regulation. **Pflugers Arch.** 446: 304-308.

_____, A.W. Van Der Kemp, A. Hartog, S.F. van de Graaf, C.H. Van Os, P.H. Williams and R.J. Bindels. 1998. Molecular identification of the apical Ca^{2+} channel in 1,25-dihydroxyvitaminD_3-responsive epithelia. **J. Biol. Chem.** 274: 8375-8378.

Hoenderop, J.G., J.P. van Leeuwen, B.C. van der Eerden, F.F. Kersten, A.W. van der Kemp, A.M. Merillat, J.H. Waarsing, B.C. Rossier, V. Vallon, E. Hummler and R.J. Bindels. 2003b. Renal Ca^{2+} wasting, hyperabsorption, and reduced bone thickness in mice lacking TRPV5. **J. Clin. Invest.** 112: 1906-1914.

_____, R. Vennekens, D. Mueller, J. Prenen, G. Droogmans, R.J. Bindels and B. Nilius. 2001a. Function and expression of mammalian ECaC1 and ECaC2. **J. Physiol.** 537: 747-761.

_____, T. Voets, S. Hoefs, F. Weidema, J. Prenen, B. Nilius and R.J. Bindels. 2003c. Homo- and heterotetrameric architecture of the epithelial Ca^{2+} channels TRPV5 and TRPV6. **EMBO J..** 22: 776-785.

_____, D. Mueller, A.W.C.M. Van De Kemp, A. Hartog, M. Suzuki, K. Ishibashi, M. Imai, F. Sweep, P.H.G.M. Willems, C.H. Van OS and R. Bindels, J. M. 2001b. Calcitriol controls the epithelial calcium channel in kidney. **J. Am. Soc. Nephrol.** 12: 1342-1349.

_____, B. Nilius and R.J.M. Bindels. 2002. ECaC: the gatekeeper of transepithelial Ca^{2+} transport. **Biochim. Biophys. Acta** 1600: 6-11.

Hofer, A.M. and E.M. Brown. 2003. Extracellular calcium sensing and signalling. **Nature** 4: 530-538.

Hofmann, T., M. Schaefer, G. Schultz and T. Gudermann. 2002. Subunit composition of mammalian transient receptor potential channels in living cells. **Pro. Natl. Acad. Sci. USA** 99: 7461-7466.

Holda, J.R., A. Klishin, M. Sedova, J. Hueser and L.A. Blatter. 1998. Capacitative calcium entry. **News Physiol. Sci.** 13: 157-163.

Huang, C.L. 2004. The transient receptor potential superfamily of ion channels. **J. Am. Soc. Nephrol.** 15: 1690-1699.

Kedei, N., T. Szabo, J.D. Lile, J.J. Treanor, Z. Olah, M.J. Iadarola and P.M. Blumberg. 2001. Analysis of the native quaternary structure of vanilloid. **J. Biol. Chem.** 276: 28613-28619.

Kornau, H.C., P.H. Seeburg and M.B. Kennedy. 1997. Interaction of ion channels and receptors with PDZ domain protiens. **Curr. Opin. Nephrol. Hypertens.** 7: 368-373.

Kutuzova, G.D. and H.F. DeLuca. 2004. Gene expression profiles in rat intestine identify pathways for 1,25-dihydroxyvitamin D_3 stimulated calcium absorption and clarify its immunomodulatory properties. **Arch. Biochem. Biophys.** 432: 152-166.

Lambers, T.T., A.F. Weidema, B. Nilius, J.G. Hoenderop and R.J. Bindels. 2004. Regulation of the mouse epithelial Ca^{2+} channel TRPV6 by the Ca^{2+}-sensor calmodulin. **J. Biol. Chem.** 279: 28855-28861.

Larsson, D. and I. Nemere. 2002. Vectorial transcellular calcium transport in intestine: integration of current models. **J. Biomed. Biotechnol**. 2: 117-119

Lee, W.J., G.R. Monteith and S.J. Roberts-Thomson. 2006. Calcium transport and signalling in the mammary gland: Targets for breast cancer. **Biochim. Biophys. Acta** 1765: 235-255.

Macpherson, M.R., V.P. Pollock, K.E. Broderick, L. Kean, F.C. O'Connell, A.T. Dow and S.A. Davies. 2001. L-type calcium channels regulate epithelial fluid transport in *Drosophila melanogaster*. **Am. J. Physiol. Cell physio.** 280: 394-407.

Mikoshiba, K. 1997. The $InsP_3$ receptor and intracellular Ca^{2+} signalling. **Curr. Opin. Neurobiology.** 7: 339-345

Marone, M., S. Mozzetti, D.D. Ritis, L. Pierelli and G. Scambia. 2001. Semiquantitative RT-PCR analysis to assess the expression levels of multiple transcripts from the same sample. **Biol. Proced. Online.** 3: 19-25.

Martinez, X.G., C.M. Palao, R.P. Cases, J.M. Merino and A.F. Monteil. 2000. Identification of an Aspartic residue in the P-loop of the vanilliod receptor that modulates pore properties. **J. Biol. Chem.** 275: 32552-32558.

Mathy, N. L., R. P. Lee and J. Walker. 1996. Removal of RT-PCR inhibitors from RNA extracts of tissues. **Biotechniques** 21: 770-774.

McManaman, J.L. and M.C. Neville. 2003. Mammary physiology and milk secretion. **Advanced Drug Delivery Rev.** 55: 629-641.

Meir, A. 2002. The molecular diversity of TRP channels and related proteins. **Modulator** 16: 1-7.

Montell, C. 2003. The venerable inveterate invertebrate TRP channels. **Cell Calcium** 33: 409–417

_____. 1997. New light on TRP and TRPL. **Mole. Pharmacology.** 52: 755-763.

_____, L. Birnbaumer, V. Flockerzi, R.J. Bindels, E.A. Bruford, M.J. Caterina, D.E. Clapham, C. Harteneck, S. Heller and D. Julius. 2002. A unified nomenclature for the superfamily of TRP cation channels. **Mole. Cell** 9: 229-231.

Moreau, R., G. Daoud, R. Bernatchez, L. Simoneau, A. Masse and J. Lafond. 2002a. Calcium uptake and calcium transporter expression by trophoblast cells from human term placenta. **Biochim. Biophys. Acta** 1564: 325-332.

_____, A. Hamel, G. Daoud, L. Simoneau and J. Lafond. 2002b. Expression of calcium channels along the differentiation of cultured trophoblast cells from human term placenta. **Biol. Reprod.** 67: 1473-1479.

Neville, M. C. 2005. Calcium secretion into milk. **J. Mammary Gland Biol. Neoplasia.** 10: 119-128.

_____, R. P. Keller and C. Casey. 1994. Calcium partitioning in human and bovine milk. **J. Dairy Sci.** 77: 1964-1975.

_____ and M. Peaker. 1979. The secretion of calcium and phosphorus into milk. **J. Biol. Chem.** 290: 59-67.

Nijenhuis, T., J.G. Hoenderop and R.J. Bindels. 2005. TRPV5 and TRPV6 in Ca^{2+} (re)absorption: regulating Ca^{2+} entry at the gate. **Pflugers Arch.** 451: 181-192.

_____, _____, A.W. van der Kemp and R.J. Bindels. 2003. Localization and regulation of the epithelial Ca^{2+} channel TRPV6 in the kidney. **J. Am. Soc. Nephrol.** 14: 2731-2740.

Nilius, B., J. Prenen, J.G. Hoenderop, R. Vennekens, S. Hoefs, A.F. Weidema, G. Droogmans and R.J. Bindels. 2002. Fast and slow inactivation kinetics of the Ca^{2+} channels ECaC1 and ECaC2 (TRPV5 and TRPV6). Role of the intracellular loop located between transmembrane segments 2 and 3. **J. Biol. Chem.** 277: 30852-30858.

_____, R. Vennekens, J. Prenen, J.G. Hoenderop, G. Droogmans and R.J. Bindels. 2001. The single pore residue Asp^{542} determines Ca^{2+} permeation and Mg^{2+} block of the epithelial Ca^{2+} channel. **J. Biol. Chem.** 276: 1020-1025.

_____, H. Watanabe and J. Vriens. 2003a. The TRPV4 channel: structure-function relationship and promiscuous gating behaviour. **Eur. J. Physiol.** 446: 298-303.

_____, A.F. Weidema, J. Prenen, J.G. Hoenderop, R. Vennekens, S. Hoefs, G. Droogmans and R. Bindels, J. M. 2003b. The carboxyl terminus of the epithelial Ca^{2+} channel ECaC1 is involved in Ca^{2+}-dependent inactivation. **Eur. J. Physiol.** 445: 584-588.

O'Neil, R.G. and R.C. Brown. 2003. The vanilloid receptor family of calcium permeable channels: molecular integrators of microenvironmental stimuli. **News Physiol. Sci.** 18: 226-231.

Owsianik, G., K. Talavera, T. Voets and B. Nilius. 2006. Permeation and selectivity of trp channels. **Annu. Rev. Physiol.** 68: 685-717.

Pedersen, S.F., G. Owsianik and B. Nilius. 2005. TRP channels: An overview. **Cell Calcium** 38: 233-252.

Peng, J.B., E.M. Brown and M.A. Hediger. 2003a. Apical entry channels in calcium-transporting epithelia. **News Physiol. Sci.** 18: 158-163.

_____, _____ and _____. 2003b. Epithelial Ca^{2+} entry channels: transcellular Ca^{2+} transport and beyond. **J. Physiol.** 551: 729-740.

_____, _____ and _____. 2001a. Structural conservation of the genes encoding CaT1, CaT2, and related cation channels. **Genomics** 76: 99-109.

_____, H.C. Chen, U.V. Berger, S. Weremowicz, C.C. Morton, P.M. Vassilev, E.M. Brown and M.A. Hediger. 2000a. Human calcium transport protein CaT1. **Biochem. Biophys. Res. Commun.** 278: 326-332.

_____, X.Z. Chen, U.V. Berger, P.M. Vassilev, E.M. Brown and M.A. Hediger. 2000b. A rat kidney-specific calcium transporter in the distal nephron. **J. Biol. Chem.** 275: 28186-28194.

_____, _____, _____, _____, H. Tsukaguchi, E.M. Brown and M.A. Hediger. 1999. Molecular cloning and characterization of a channel-like transporter mediating intestinal calcium absorption. **J. Biol. Chem.** 274: 22739-22746.

_____ and M.A. Hediger. 2002. A family of calcium-permeable channels in the kidney: distinct roles in renal calcium handling. **Curr. Opin. Nephrol. Hypertens.** 11: 555-561.

_____, L. Zhuang, U.V. Berger, R.M. Adam, B.J. Williams, E.M. Brown, M.A. Hediger and M.R. Freeman. 2001b. CaT1 expression correlates with Tumor grade in prostate cancer. **Biochem. Biophys. Res. Commun.** 282: 729-734.

Petersen, O.H. and N.V. Fedirko. 2001. Calcium signalling: Store-operated channel found at last. **Curr. Biol.** 11: 520-523.

_____, M. Michalak and A. Verkhratsky. 2005. Calcium signalling: Past, present and future. **Cell Calcium** 38: 161-169.

Prakriya, M. and R.S. Lewis. 2003. CRAC channels: activation, permeation, and the search for a molecular identity. **Cell Calcium** 33: 311-321.

Prevarskaya, N., R. Skryma and Y. Shuba. 2004. Ca^{2+} homeostasis in apoptotic resistance of prostate cancer cells. **Biochem. Biophys. Res. Commun.** 322: 1326-1335.

Putney, J.W. 2001. Channelling calcium. **Nature** 410: 648-649.

Qiu, A. and C. Hogstrand. 2004. Functional characterisation and genomic analysis of an epithelial calcium channel (ECaC) from pufferfish, Fugu rubripes. **Gene** 342: 113-123.

Reinhardt, T.A., A.G. Filoteo, J.T. Penniston and R.L. Horst. 2000. Ca^{2+}-ATPase protein expression in mammary tissue. **Am. J. Physiol. Cell physiol.** 279: 1595-1602.

_____ and R.L. Horst. 1999. Ca^{2+}-ATPase and their expression in the mammary gland of pregnant and lactating rats. **Am. J. Physiol.** 276: 796-802.

Schindl, R., H. Kahr, I. Graz, K. Groschner and C. Romanin. 2002. Store depletion-activated CaT1 currents in rat basophilic leukemia mast cells are inhibited by 2-aminoethoxydiphenyl borate. Evidence for a regulatory component that controls activation of both CaT1 and CRAC (Ca^{2+}) release-activated Ca^{2+} channel channels. **J. Biol. Chem.** 277: 26950-26958.

Scott, K. and C. Zuker. 1998. TRP, TRPL and trouble in photoreceptor cells. **Curr. Opin. Neurobiol.** 8: 383-388.

Shennan, D.B. and M. Peaker. 2000. Transport of milk constituents by the mammary gland. **Physiol. Rev.** 80: 925-951.

Slepchenko, B. and F. Bronner. 2001. Modeling of transcellular Ca^{2+} transport in rat duodenum points to coexistence of two mechanisms of apical entry. **Am. J. Physiol. Cell Physiol.** 281: 270-281.

Smith, G.D., M.J. Gunthorpe, R.E. Kelsell, P.D. Hayes, P. Reilly, P. Facer, J.E. Wright, J.C. Jerman, J.P. Walhin, L. Ooi, J. Egerton, K.J. Charles, D. Smart, A.D. Randall, P. Anand and J.B. Davis. 2002. TRPV3 is a temperature-sensitivity vanilloid receptor-like protein. **Nature** 418: 186-190.

Spassova, M.A., J. Soboloff, L.P. He, T. Hewavitharanaa, w. Xu, K. Venkatachalam, D.B. Van Rossum, R.L. Patterson and D.L. Gill. 2004. Calcium entry mediated by SOCs and TRP channels: variations and enigma. **Biochim. Biophys. Acta** 1742: 9-20.

Sternfeld, L., E. Krause, A. Schmid, I. Anderie, A. Latas, H. Al-Shaldi, A. Kohl, K. Evers, H.W. Hofer and I. Schulz. 2005. Tyrosine phosphatase PTP1B interacts with TRPV6 in vivo and plays a role in TRPV6-mediated calcium influx in HEK293 cells. **Cellular Signalling** 17: 951-960.

Suzuki, M., K. Ishibashi, G. Ooki, S. Tsuruoka and M. Imai. 2000. Electrophysiologic characteristics of the Ca-permeable channels, ECaC and CaT, in the kidney. **Biochem. Biophys. Res. Commun.** 274: 344-349.

Taylor, R. S., S. M. Jones, R. H. Dahl, M. H. Nordeen and K. E. Howell. 1997. Characteriazation of Golgi complex cleared of proteins in transit and examination of calcium uptake activities. **Mol. Biol. Cell** 8: 1911-1931.

van Abel, M., J.G. Hoenderop, O. Dardenne, R. Arnaud, C. van Os, J.P. van Leeuwen and R. Bindels, J. M. 2002. 1,25-Dihyroxyvitamin D_3-independent stimulatory effect of estrogen on the expression of ECaC1 in the kidney. **J. Am. Soc. Nephrol.** 13: 2102-2109.

_____, _____, A.W. van der Kemp, J.P. van Leeuwen and R.J. Bindels. 2003. Regulation of the epithelial Ca^{2+} channels in small intestine as studied by quantitative mRNA detection. **Am. J. Physiol. Gastrointest. Liver Physiol.** 285: G78-85.

van de Graaf, S.F., J.G. Hoenderop, D. Gkika, D. Lamers, J. Prenen, U. Rescher, V. Gerke, O. Staub, B. Nilius and R.J. Bindels. 2003. Functional expression of the epithelial Ca^{2+} channels (TRPV5 and TRPV6) requires association of the S100A10-annexin 2 complex. **EMBO J..** 22: 1478-1487.

_____, I. Boullart, J.G.J. Hoenderop and R.J.M. Bindels. 2004. Regulation of the epithelial Ca^{2+} channels TRPV5 and TRPV6 by 1[alpha],25-dihydroxy Vitamin D3 and dietary Ca^{2+}. **J. Steroid Biochem. Mol. Biol.** 89-90: 303-308.

Vassilev, P.M., J.B. Peng, J. Johnson, M.A. Hediger and E.M. Brown. 2001. Inhibition of CaT1 channel activity by a noncompetitive IP_3 antagonist. **Biochem. Biophys. Res. Commun.** 280: 145-150.

Venkatachalam, K., D. van Rossum, R.L. Patterson, H.T. Ma and D.L. Gill. 2002. The cellular and molecular basis of store-operated calcium entry. **Nature cell biol.** 4: 263-272.

Vennekens, R., J.G. Hoenderop, J. Prenen, M. Stuiver, P.H. Willems, G. Droogmans, B. Nilius and R.J. Bindels. 2000. Permeation and gating properties of the novel epithelial Ca^{2+} channel. **J. Biol. Chem.** 275: 3963-3969.

_____, T. Voets, R.J.M. Bindels, G. Droogmans and B. Nilius. 2002. Current understanding of mammalian TRP homologues. **Cell Calcium** 31: 253-264.

Voets, T., A. Janssens, G. Droogmans and B. Nilius. 2004. Outer pore architecture of a Ca^{2+}-selective TRP channel. **J. Biol. Chem.** 279: 15223-15230.

_____, _____, J. Prenen, G. Droogmans and B. Nilius. 2003. Mg^{2+}-dependent gating and strong inward rectification of the cation channel TRPV6. **J. Gen. Physiol.** 121: 245-260.

_____ and B. Nilius. 2003. The pore of TRP channels: trivial or neglected?. **Cell Calcium** 33: 299-302.

Voets, T., J. Prenen, A. Fleig, R. Vennekens, H. Watanabe, J.G. Hoenderop, R.J. Bindels, G. Droogmans, R. Penner and B. Nilius. 2001. CaT1 and the calcium release-activated calcium channel manifest distinct pore properties. **J. Biol. Chem.** 276: 47767-47770.

Walters, J.R., N.F. Barley, M. Khanji and O. Rhodes-Kendler. 2004. Duodenal expression of the epithelial calcium transporter gene TRPV6: is there evidence for Vitamin D-dependence in humans?. **J. Steroid. Biochem. Mol. Biol.** 89-90: 317-319.

Weber, K., R.G. Erben, A. Rump and J. Adamski. 2001. Structure and regulation of murine epithelial calcium channels ECaC1 and 2. **Biochem. Biophys. Res. Commun.** 289: 1287-1294.

Williams, R.J.P. 1998. Calcium: outside/inside homeostasis and signalling. **Biochim. Biophys. Acta** 1448: 153-165.

Wissenbach, U., M. Bodding, M. Freichel and V. Flockerzi. 2000. Trp12, a novel Trp related protein from kidney. **FEBS Lett.** 485: 127-134.

_____, B. Niemeyer, N. Himmerkus, T. Fixemer, H. Bonkhoff and V. Flockerzi. 2004. TRPV6 and prostate cancer: cancer growth beyond the prostate correlates with increased TRPV6 Ca^{2+} channel expression. **Biochem. Biophys. Res. Commun.** 322: 1359-1363.

_____, B.A. Niemeyer, T. Fixemer, A. Schneidewind, C. Trost, A. Cavalie, K. Reus, E. Meese, H. Bonkhoff and V. Flockerzi. 2001. Expression of CaT-like, a novel calcium-selective channel, correlates with the malignancy of prostate cancer. **J. Biol. Chem.** 276: 19461-19468.

Wood, R.J., L. Tchack, S. Taparia, O.H. Petersen, N.V. Fedirko, J.B. Peng, L. Zhuang, U.V. Berger, R.M. Adam, B.J. Williams, E.M. Brown, M.A. Hediger and M.R. Freeman. 2001. 1,25-Dihydroxyvitamin D_3 increases the expression of the CaT1 epithelial calcium channel in the Caco-2 human intestinal cell line. **BMC Physiol.** 1: 11-19.

Yamamoto, M., J. E. Fisher, M. A. Thiede, M. P. Caulfield, M. Rosenblatt and L. T. Duong. 1992. Concentrations of Parathyroid Hormone-related Protein in rat milk change with duration of lactation and interval from previous sucking, but not with milk calcium. **Endocrinology** 130: 741-747.

Yeh, B.I., T. J. Sun, J. Z. Lee, H. H. Chen, C. L. Huang. 2003. Mechanism and molecular determinant for regulation of rabbit transient receptor potential type 5 (TRPV5) channel by extracellular pH. **J Biol. Chem**. 278: 51044–51052

Yue, L., J.B. Peng, M.A. Hediger and D.E. Clapham. 2001. CaT1 manifests the pore properties of the calcium-release-activated calcium channel. **Nature** 410: 705-709.

Zhou, X.Z., A.F. Batiza, S.H. Loukin, C. Kung and Y. Saimi. 2003. The transient receptor potential channel on the yeast vacuole is mechanosensitive. **Pro. Natl. Acad. Sci. USA** 100: 7105-7110.

Zhu, X. and L. Birnbaumer. 1998. Calcium channels formed by mammalian Trp homologues. **News Physiol. Sci.** 13: 211-217

Zhuang, L., J.B. Peng, L. Tou, H. Takanaga, R.M. Adam, M.A. Hediger and M.R. Freeman. 2002. Calcium-selective ion channel, CaT1, is apically localized in gastrointestinal tract epithelia and is aberrantly expressed in human malignancies. **Lab Invest.** 82: 1755-1764.

Zitt, C., C. R. Halaszovich and A. Lückhoff. 2002. The TRP family of cation channels: probing and advancing the concepts on receptor-activated calcium entry. **Prog. Neurobiol.** 66: 243–264

APPENDIX

Appendix A

Preparation of chemical reagents

1 litre 0.1% diethylpyrocarbonate treated water (DEPC-ddH_2O)

1 ml of diethylpyrocarbonate (BIO BASIC INC,)

1 litre deionized distilled water

Add 1 ml DEPC into 1 litre deionized distilled water and then mixed by using stirrer and incubate overnight at 42°C. Then autoclave 45 mins, 121°C at 15lb/sq liquid cycle before use.

20 ml 8 M Lithium chloride (8 M LiCl)

6.78 g LiCl

20 ml DEPC-ddH_2O DEPC

Add 6.78 g. LiCl into 20 ml DEPC-ddH_2O and shaked well before use

1 litre 50X TAE buffer

242 g Tris base

57.1 ml glacial acetic acid

100 ml 0.5 M EDTA (pH 8)

1 litre distilled water

Add 242 g. Tris Base, 57.1 ml glacial acetic acid, and 100 ml 0.5 M EDTA (pH 8) into 500 ml distilled water. Then mixed by using stirrer, and add distilled water until 1 liter.

Appendix B

The band intensity ratios of TRPV6/GAPDH on days during pregnancy, partum, and lactation of rat mammary tissues

Blocks	Days	Replication I			Replication II		
		TRPV6	GAPDH	Ratio I	TRPV6	GAPDH	Ratio II
1st	-7	18.0700	44.5800	0.40534	4.3451	42.2800	0.10277
	-1	0.0001	52.0200	0.00000	8.4275	48.9400	0.17220
	0	7.7370	45.1900	0.17121	5.7659	42.1600	0.13676
	+1	3.0648	49.8900	0.06143	4.9020	43.5000	0.11269
	+7	7.1171	41.2500	0.17254	30.9300	45.9200	0.67356
	+14	28.4000	43.8100	0.64825	16.5600	37.8400	0.43763
2nd	-7	9.8803	33.3200	0.29653	0.0001	23.6900	0.00000
	-1	0.0001	28.9200	0.00000	0.0001	4.2014	0.00002
	0	0.0001	28.3500	0.00000	0.0001	19.9000	0.00001
	+1	4.5787	33.9400	0.13491	0.0001	32.3500	0.00000
	+7	22.9000	33.4400	0.68481	19.2000	19.9200	0.96386
	+14	5.4804	37.4000	0.14653	0.0001	16.8300	0.00001
3rd	-7	15.4000	72.6600	0.21195	6.9032	49.3300	0.13994
	-1	12.4700	65.2100	0.19123	19.9400	52.7100	0.37830
	0	4.3039	46.9300	0.09171	6.1608	44.7300	0.13773
	+1	0.0001	51.4700	0.00000	0.0001	45.7800	0.00000
	+7	10.9800	43.7500	0.25097	17.2800	52.3100	0.33034
	+14	2.9940	31.8500	0.09400	26.3500	36.0800	0.73032
4th	-7	0.0001	69.2200	0.00000	0.0001	62.7500	0.00000
	-1	5.7130	44.0400	0.12972	8.7078	47.9600	0.18156
	0	11.5000	67.2300	0.17105	1.8192	67.6200	0.02690
	+1	0.0001	65.0000	0.00000	15.3000	72.9800	0.20965
	+7	26.8400	32.9400	0.81481	23.5400	35.0500	0.67161
	+14	29.8200	58.9300	0.50602	24.9700	58.3400	0.42801

CURRICULUM VITAE

NAME : Miss Jirawan Siriwetwiwat

BIRTH DATE : October 15, 1980

BIRTH PLACE **:** Nakhonsawan, Thailand

EDUCATION **:** B.S. (Biology), Naresuan University (2002)

SCHOLARSHIP **:** The KING PRAJADHIPOK and QUEEN RAMPHAI BARNI MEMORIAL FOUDATION