Expression and localization of two-pore domain K⁺ channels in bovine germ cells

Chang-Gi Hur¹, Changyong Choe², Gyu-Tae Kim, Seong-Keun Cho¹, Jae-Yong Park, Seong-Geun Hong, Jaehee Han and Dawon Kang

Department of Physiology, College of Medicine and Institute of Health Sciences, Medical Research Center for Neural Dysfunction, Gyeongsang National University, 90 Chilam-dong, Jinju, Gyeongnam 660-751, South Korea, ¹CHO-A Biotechnology Research Institute, CHO-A Pharmaceutical Company Ltd, Seoul 150-992, South Korea and ²Animal Genetic Resources Station, National Institute of Animal Science, RDA, Namwon 590-832, South Korea

*Correspondence should be addressed to D Kang; Email: dawon@gnu.ac.kr;
J Han; E-mail: jheehan@gnu.ac.kr*

Abstract

Two-pore domain K⁺ (K_{2P}) channels that help set the resting membrane potential of excitable and nonexcitable cells are expressed in many kinds of cells and tissues. However, the expression of K_{2P} channels has not yet been reported in bovine germ cells. In this study, we demonstrate for the first time that K_{2P} channels are expressed in the reproductive organs and germ cells of Korean cattle. RT-PCR data showed that members of the K_{2P} channel family, specifically KCNK3, KCNK9, KCNK2, KCNK10, and KCNK4, were expressed in the ovary, testis, oocytes, embryo, and sperm. Out of these channels, KCNK2 and KCNK4 mRNAs were abundantly expressed in the mature oocytes, eight-cell stage embryos, and blastocysts compared with immature oocytes. KCNK4 and KCNK3 were significantly increased in eight-cell stage embryos. Immunocytochemical data showed that KCNK2, KCNK10, KCNK4, KCNK3, and KCNK9 channel proteins were expressed at the membrane of oocytes and blastocysts. KCNK10 and KCNK4 were strongly expressed and distributed in oocyte membranes. These channel proteins were also localized to the acrosome sperm cap. In particular, KCNK3 and KCNK4 were strongly localized to the post-acrosomal region of the sperm head and the equatorial band within the sperm head respectively. These results suggest that K_{2P} channels might contribute to the background K⁺ conductance of germ cells and regulate various physiological processes, such as maturation, fertilization, and development.

Reproduction (2009) 137 237–244

Introduction

Potassium (K⁺) channels, which form the largest family of ion channels, play a pivotal role in a variety of physiological processes in mammalian germ cells (Darszon *et al.* 1999, Everill & Kocsis 1999, Trimarchi *et al.* 2002, Tosti & Boni 2004, Winston *et al.* 2004, Yang *et al.* 2004). Ion channels are involved in electrical modification of plasma membrane of gametes. In bovine oocytes, the levels of K⁺ and L-type Ca²⁺ channels that are preponderant at the germinal vesicle stage are decreased at the metaphase II (MII) stage oocytes. At fertilization, sperm induce Ca²⁺ release from the intracellular stores that gate the Ca²⁺-activated K⁺ channels (Tosti & Boni 2004). These ion channels are also involved in electrical modification of sperm plasma membrane during chemotaxis and acrosome reaction processes (Tosti & Boni 2004). A voltage-dependent inward rectifying K⁺ channel that shows high conductance and the ether-a-go-go-related K⁺ channels control the cell

cycle during mouse embryonic development (Day *et al.* 1993, 1998, Winston *et al.* 2004). The high-conductance K⁺ channels are active in oocytes, but their activity was decreased during mouse embryonic development with membrane depolarization (Day *et al.* 1993, Kang *et al.* 2006). Other K⁺ channels could be also involved in reproductive physiology. Two-pore domain K⁺ (K_{2P}) channels are responsible for K⁺ efflux during apoptotic volume decreases (AVD) in mouse zygotes (Trimarchi *et al.* 2002). Quinine sensitive-, TEA insensitive-, and calcium independent-K⁺ channels regulate the volume and motion of human sperm (Yeung & Cooper 2001), indicating that these channels possess K_{2P} channels. K_{2P} channels are sensitive to quinine and insensitive to TEA and calcium, and a K⁺ channel reported by Day *et al.* (1993) also shows similar single-channel kinetics and pharmacological properties to KCNK10, a member of the K_{2P} channel family. These reports give rise to study of the physiological roles and function of K⁺ channels.

Among K^+ channels, K_{2P} channels set and stabilize the resting membrane potential and behave as leak K^+ channels when they are expressed in *Xenopus* oocytes and mammalian cells (Kim 2005). These channels are expressed in both excitable and nonexcitable cells and regulated by a variety of biologically relevant stimuli, such as receptor ligands, temperature, lipids, pressure, oxygen tension, volatile anesthetic agents, pH, and neurotransmitters (Kim 2003). Recent studies using RT-PCR, immunostaining, and western blotting have demonstrated that K_{2P} channels are expressed in mammalian reproductive systems. KCNK3, KCNK5, KCNK17, KCNK15, and KCNK2 are detected in human cytotrophoblast cells, placental villous tissue and trophoblast cells, myometrium, and placental vascular system (Bai *et al.* 2005a, 2005b, 2006). KCNK2 and KCNK4 are also expressed in human uterine smooth muscle, and KCNK2 expression is significantly increased in pregnant term tissues (Tichenor *et al.* 2005). KCNK5, KCNK2, and KCNK4 are expressed by and localized specifically to monkey sperm (Chow *et al.* 2007).

Numerous studies have suggested that the K_{2P} channels could regulate cell excitability and a wide range of physiological processes in mammalian cells (Kim 2003, 2005, Talley *et al.* 2003, Besana *et al.* 2005, Kang & Kim 2006, Sanders & Koh 2006). In reproductive cell types, the presence and function of K_{2P} channels were also reported (Bai *et al.* 2005a, 2005b, 2006, Tichenor *et al.* 2005, Wareing *et al.* 2006, Chow *et al.* 2007). However, the expression and localization of the K_{2P} channels in bovine germ cells have not yet been reported. In this study, we identified the expression and localization of the K_{2P} channels in germ cells of Korean cattle, known as Hanwoo.

Results

Alterations in two-pore domain K^+ (K_{2P}) channel mRNA expression during bovine embryonic development

To identify whether the K_{2P} channels are expressed in bovine germ cells, RT-PCR was performed. RT-PCR data showed that *KCNK2*, *KCNK10*, *KCNK4*, *KCNK3*, and *KCNK9*, members of the K_{2P} channel family, were expressed in ovaries, immature and mature (metaphase II, MII) oocytes, eight-cell stage embryos, and blastocysts of Hanwoo (Fig. 1A). The mRNA levels of these channels were quantified using real-time PCR. In testis, the mRNA levels of *KCNK2*, *KCNK10*, *KCNK4*, *KCNK3*, and *KCNK9* were significantly higher than those in the ovary ($P < 0.05$, Fig. 1B).

To identify whether the mRNA expression of K_{2P} channels changes during bovine embryonic development, real-time PCR was also performed (Fig. 1C). In female germ

Figure 1 Expression of the K_{2P} channels in bovine reproductive organs and female germ cells. (A) The expression of *KCNK2*, *KCNK10*, *KCNK4*, *KCNK3*, and *KCNK9* in ovary, oocytes, and embryos. RT-PCR products were separated on a 1.2% standard agarose gel and stained with ethidium bromide. (B) mRNA levels in the ovary and testis. Each bar is the mean $\pm$ S.E.M. of 10 repeated experiments. The y-axis shows relative mRNA levels on a log scale. The mRNA expression level was normalized to *GAPDH*. (C) Alteration in mRNA levels of *KCNK2*, *KCNK10*, *KCNK4*, *KCNK3*, and *KCNK9*, during bovine embryonic development. The mRNA expression of *KCNK2*, *KCNK10*, *KCNK4*, *KCNK3*, and *KCNK9* was quantified by means of real-time PCR in immature oocytes, MII oocytes, eight-cell stage oocytes, and blastocysts. The mRNA expression level was normalized to *GAPDH*. Each data point is the mean $\pm$ S.E.M. of 10 repeated experiments. *Significantly different from the corresponding control value obtained in immature (IM) oocytes ($P < 0.05$). MI, eight cell, and BL indicate mature oocytes arrested at the time metaphase II, eight-cell stage embryo, and blastocyst respectively.

cells, *KCNK2* and *KCNK4* mRNA levels increased by approximately fourfold in MII oocytes compared with immature oocytes. *KCNK4* and *KCNK3* were increased in MII oocytes and eight-cell stage embryos but decreased in blastocysts, while *KCNK2*, *KCNK10*, and *KCNK9* were slightly increased in MII oocytes but did not show significant changes in eight-cell stage embryos and blastocysts.

K_{2P} channel expression and localization in bovine germ cells

The presence of mRNA in cells, however, does not necessarily imply the translation of mRNA into protein. The expression of K_{2P} channels identified by RT-PCR was further studied at the protein level. The specificity and sensitivity of antibodies used in this study were previously confirmed and proved in this study. Each antibody specifically detected its own antigen (Kang *et al.* 2007a, 2007b, Supplementary Figures 1 and 2, which can be viewed online at www.reproduction-online.org/supplemental/). Immunocytochemical data stained with specific antibodies for the K_{2P} channels showed that KCNK2, KCNK10, KCNK4, KCNK3, and KCNK9 were expressed and localized at the plasma membrane of oocytes and blastocysts. Oocytes tended to express highly KCNK10 and KCNK4 proteins, compared with other channels (Fig. 2). However, KCNK10 showed low expression in dorsal root ganglion (DRG) neurons, human breast cancer cells, and HaCaT cells, indicating that anti-KCNK10 antibody could specifically detect

KCNK10 antigen in the bovine oocytes (Supplementary Figure 2). As shown in Fig. 2, KCNK4 was significantly decreased in MII oocytes and blastocysts compared with immature oocytes, while KCNK10 was increased in MII oocytes but decreased in blastocysts. KCNK9 was increased in MII oocytes and blastocysts. KCNK2 and KCNK3 were decreased in MII oocytes but increased in blastocysts.

In addition to female germ cells, K_{2P} channel expression was analyzed in mature sperm, a male germ cell. KCNK2, KCNK10, KCNK4, KCNK3, and KCNK9 were detected in bovine sperm by immunocytochemistry. Negative and positive controls were checked by omitting the primary antibody and transfecting the K_{2P} channel cDNA respectively. No staining was observed on any of the negative control cells. These channel proteins were distributed at sperm head, including acrosomal cap (Fig. 3). Specifically, KCNK3 and KCNK4 were predominantly expressed in the post-acrosomal region of the sperm head and at the equatorial band within the sperm head respectively.

Figure 2 Expression and localization of KCNK2, KCNK10, KCNK4, KCNK3, and KCNK9 in bovine oocytes and blastocysts. (A) Immunocytochemical analysis of KCNK2, KCNK10, KCNK4, KCNK3 and KCNK9. Fluorescent images labeled with channel-specific antibodies and FITC-conjugated anti-rabbit IgG showed channel expression at the cell membrane (left panel, fluorescent images; right panel, differential interference contrast images). (B) Immunofluorescence intensity of KCNK2, KCNK10, KCNK4, KCNK3 and KCNK9. Each data point is the mean $\pm$ S.E.M. of 10 repeated experiments. *Significantly different from the corresponding control value obtained in IM (immature oocytes; $P < 0.05$). KCNK10 and KCNK4 proteins were significantly decreased in the blastocyst stage. MI and BL indicate mature oocytes arrested at the time metaphase II and blastocyst respectively.

Figure 3 Expression and localization of the K_{2P} channel proteins in bovine sperm. Immunocytochemical analysis of KCNK2, KCNK10, KCNK4, KCNK3 and KCNK9 expression. Fluorescent images labeled with channel-specific antibody and FITC (fluorescence isothiocyanate)-conjugated anti-rabbit IgG, showed channel expression at the cell membrane (left panel, fluorescent images; right panel, differential interference contrast images). Scale bars, 10 μ m.

Discussion

K_{2P} channels are expressed in many kinds of cells and tissues and regulated by various biophysical and chemical stimuli, and thus they could be involved in a variety of cellular functions. This study demonstrates for the first time that bovine reproductive tissues (ovary and testis) and germ cells (oocyte, embryo, and sperm) express the K_{2P} channels, and suggests that these channels could be involved in the regulation of oocyte maturation, fertilization, and development. These channels were distributed on the membranes of oocytes and blastocysts, and were localized to a special region of the bovine sperm.

As shown in Fig. 1, the mRNA levels of *KCNK3* and *KCNK4* were dramatically increased in eight-cell stage embryos after fertilization. On average, *KCNK3* showed low expression in immature and MII oocytes. In eight-cell stage embryos, the increase of *KCNK3* mRNA might be the result of a fertilization event induced by sperm *KCNK3*, which is strongly expressed in the post-acrosomal region. A marked increase of *KCNK4* in eight-cell stage embryos may also include *KCNK4* originating from the fertilizing sperm. Sperm depends on ion channels to rapidly exchange information with the outside world and to fertilize the MII oocyte (Darszon *et al.* 2007). The equatorial band and post-acrosomal region of sperm head, which in the study predominantly expressed *KCNK4* and *KCNK3*, are important during and after fertilization (see Fig. 3). Specifically, the equatorial band that is located on the middle of the sperm head is first bound with the oocyte membrane at fertilization

(Mahony & Gwathmey 1999, Travis *et al.* 2001, Turner 2003). We further compared the channel expression pattern between intact sperm and acrosome-reacted sperm. In the acrosome reacted sperm, *KCNK3* and *KCNK4* proteins were still expressed at the post-acrosomal and equatorial regions respectively, although the levels of the K_{2P} channel proteins expressed in the acrosome cap were reduced (Supplementary Figure 3, which can be viewed online at www.reproduction-online.org/supplemental/). Further studies are needed to analyze the expression pattern of the K_{2P} channel proteins in more detail. However, one of the limitations of dual staining is the difference in fluorescence intensity. The intensity of TRITC-conjugate antibody (red) is lower than that of FITC-conjugated PSA (green). This limitation of dual staining should be considered in the comparison of protein expression. Nonetheless, the expression and distribution of the K_{2P} channel proteins in mammalian sperm give insight into the physiological role of these channels in acrosome reaction and fertilization.

As shown in Figs 1 and 2, the K_{2P} channel expression is changed during embryonic development, although the alteration in mRNA and protein levels showed different patterns among the channels. In mRNA expression, the channels were generally increased in MII oocytes compared with immature oocytes, and *KCNK4* and *KCNK3* were significantly increased in eight-cell stage embryos ($P < 0.05$), suggesting that the expression of these channels may affect maturation, fertilization, and development. In another study, we observed that inhibitors of the K_{2P} channels significantly

reduced maturation, fertilization, and development rate in bovine and mouse oocytes, and embryos (data not shown).

Voltage-dependent K^+ and Ca^{2+} channels are widely recognized as critical players in the physiological functions of gametes and embryos, such as maturation, fertilization, cell cycle, and development (Mitani 1985, Day *et al.* 1993, 1998, Tosti & Boni 2004, Winston *et al.* 2004, Boni *et al.* 2007). In addition to voltage-dependent channels, voltage-independent K_{2P} channels could control reproductive cell functions as a modulator that controls voltage-dependent mechanisms in reproductive cells. Gametes and embryos are excitable cells in which transmembrane ion currents cause rapid metabolic responses (Tosti & Boni 2004, Cuomo *et al.* 2006). Various biological and physiological events due to a dramatic ion flux appear or disappear during gamete maturation, fertilization, cell division, and early embryonic development. The formation of the blastocyst, which is required for implantation and establishment of pregnancy, requires the ion transport and channel system (Barcroft *et al.* 2003). K_{2P} channels were also expressed in bovine blastocysts. However, the expression levels of these channels can be affected by *in vitro* culture conditions, thus the developmental rate to blastocyst can change during *in vitro* culture or *in vivo*. The KCNK2, KCNK10, and KCNK4 channels are regulated by the changes in temperature (Maingret *et al.* 2000, Kang *et al.* 2005, 2007a, 2007b) and reactive oxygen species (Kim *et al.* 2007) that may occur during *in vitro* maturation (IVM), fertilization (IVF), and development. Increased ambient temperature (heat shock) during IVM and/or IVF significantly reduced oocyte maturation, fertilization rates, and subsequent embryonic development (de Castro & Hansen 2007, Sugiyama *et al.* 2007). During *in vitro* manipulation including IVF procedures, germ cells tend to exposure to lower temperature than 37 °C or 39 °C. The low temperature induces close of KCNK2, KCNK10, and KCNK4 channels. The changes in membrane potential by thermosensitive channels are likely to affect to *in vitro* embryonic development. Also, oxygen (O_2) concentration is a factor that modulates the K_{2P} channels. Earlier studies have shown that *in vitro* cultured embryos could not develop normally because O_2 concentration in *in vitro* is higher than that in oviduct (Dalvit *et al.* 2005, Nagai *et al.* 2006). Hydrogen peroxide (H_2O_2) and nitric oxide that may be induced by high O_2 concentration activate KCNK2 and KCNK10 channels (Koh *et al.* 2001, Kim *et al.* 2007). These culture conditions are likely to increase or reduce the activity of K_{2P} channels that may affect *in vitro* embryonic development.

The identification of the K_{2P} channels expressed in reproductive organs and germ cells may help the understanding of ion channel-related function in reproductive physiology. Further study is needed to study the function and current recording of the K_{2P} channels

expressed in mammalian germ cells. This should be helpful for research to improve bovine embryo technologies.

Materials and Methods

Oocyte recovery and in vitro embryo production

Bovine ovaries were collected from a slaughterhouse and transported to the laboratory in PBS at 35–39 °C. All experiments were performed with the approval of Animal Ethics Committee of Gyeongsang National University. Cumulus-oocyte-complexes (COCs) were aspirated from antral follicles of 2–8 mm diameter with an 18 G needle fitted to a 10 ml syringe. After washing twice with Dulbecco's PBS (D-PBS) containing 1% (w/v) polyvinyl alcohol (PVA), oocytes were examined under a 40× stereomicroscope (SZ60, Olympus, Tokyo, Japan). Oocytes with multiple layers of compact cumulus cells and evenly granulated cytoplasm were selected for this study. Fifteen COCs were transferred into a 50 µl drop of maturation medium under mineral oil. The COCs were then incubated at 39 °C in a humidified atmosphere with 5% (v/v) CO_2 in air. The medium used to mature COCs was TCM-199 containing Earle's salts, 10% (v/v) fetal bovine serum (FBS, Invitrogen), 25 mmol/l HEPES, 2.5 mmol/l sodium pyruvate, 1 mmol/l L-glutamine, and 0.1% (w/v) penicillin–streptomycin (Pen–Strep, Invitrogen). The pH and osmolality of the media were adjusted to 7.4 and 285 mOsm/kg respectively. At 22 h of maturation, cumulus cells were partially removed from the oocytes by vortexing for 10 s in Tyrode's albumin lactate pyruvate medium containing 2% (w/v) BSA (Fraction V) and 10 mM HEPES (washing-TALP medium). Fifteen oocytes were transferred into a 50 µl fertilization-TALP droplet containing 10 µg/ml heparin, 2.5 mmol/l caffeine, and 0.3% (w/v) BSA. The oocytes were then inseminated with sperm at a final concentration of 2×10^6 sperm/ml. The cumulus cells were removed after 6 h of culturing post-insemination by vortexing for 2 min in washing-TALP medium. Cumulus-free zygotes were washed several times in washing-TALP medium and TCM-199 medium supplemented with 10% (v/v) FBS. After transferring 15 presumptive zygotes into a 50 µl droplet of TCM-199 medium, the embryos were cultured for 9 days.

Sperm isolation

The testes of Korean cattle were collected from a slaughterhouse and transported to the laboratory within 1 h on ice. Sperm collected from cauda epididymis were treated with the swim-up procedure. Briefly, sperm were washed once in 10 ml washing-TALP medium by centrifugation at 300 g for 10 min. From 0.5 ml sperm pellet, five 0.1 ml aliquots were transferred into five 2-ml round-bottom test tubes; then, each was overlaid with 0.5 ml fertilization-TALP medium. With the tubes tilted at a 45° angle, incubation at 39 °C in a humidified atmosphere of 5% CO_2 in air for 1 h allowed the motile sperm to swim-up. The top 0.4 ml of the supernatant was harvested and further centrifuged at 300 g for 10 min to yield a sperm pellet, which was resuspended in 0.6 ml fertilization-TALP medium. The resuspended sperm were used for immunostaining.

Table 1 Bovine primer sequences used for reverse transcriptase PCR.

Gene name	Primer sequence (5'–3')	GenBank accession numbers
<i>GAPDH</i>	Forward: CAGCGACACTCACTCTTCTAC Reverse: GGAAGTCAGGAGATTCTCAGT	NM_001034034
<i>KCNK2</i>	Forward: CTGATTGCTGGATCCTAAG Reverse: CTGATTGATTGGAGGTGTT	NM_174686
<i>KCNK10</i>	Forward: GCAGCCAAAAGAACACTATC Reverse: AGATGAACAGGATGGTTGAG	XM_603455
<i>KCNK4</i>	Forward: GACACCAACTCAACCAGTAA Reverse: GTCACCACCACGAAGTAGAT	XM_869390
<i>KCNK3</i>	Forward: ACACCTTCGTGAAGTACCTG Reverse: GGATGTAGACGAAGCTGAAG	XM_597401
<i>KCNK9</i>	Forward: CTACGTGGCCTTTAGCTTTA Reverse: GTCGGTAAAGCTGTGTAACC	XM_588194

Reverse transcriptase (RT-PCR) analysis

First-strand cDNAs were synthesized from total RNA isolated from Hanwoo ovary and testis using oligo dT (SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen). *In vitro* produced cells (60 immature oocytes, 60 mature oocytes, 35 eight-cell stage embryos, and 15 blastocysts) were lysed, and the lysates were used for cDNA synthesis. cDNA was generated from the lysate in a single tube using the SuperScript III CellsDirect cDNA Synthesis System (Invitrogen). First-strand cDNA was used as a template for PCR amplification. Specific primers for each K_{2P} channel were used for PCR with *Taq* polymerase (Takara, Otsu, Japan). Table 1 lists the DNA sequences of primers used to detect the expression of each K_{2P} channels. PCR conditions were initial denaturation at 94 °C for 4 min, then 30 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 60 s, and a final extension step at 72 °C for 10 min. The DNA fragments obtained from the ovary, testis, and germ cells by RT-PCR were directly sequenced with the ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems, CA, USA).

Real-time PCR

K_{2P} channel expression identified by RT-PCR was quantified by means of real-time PCR with FastStart DNA Master SYBR Green I (Roche Applied Science) using the LightCycler System (LightCycler 2.0 instrument, Roche). The mRNA expression of each of the K_{2P} channels was normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Real-time PCR primers were designed by using GenScript

(<https://www.genscript.com/ssl-bin/app/primer>). Table 2 lists the DNA sequences of primers used to detect the expression of each K_{2P} channel. PCR conditions were consisted of a denaturing cycle (95 °C for 10 min), 50 cycles of PCR (95 °C for 7 s, 56 °C for 7 s, and 72 °C for 10 s), a melting cycle (95 °C for 0 s and 65 °C for 60 s) including a step cycle (starting at 65 °C up to 95 °C with a 0.1 °C/s transition rate), and a cooling cycle (40 °C for 30 s). Melt-curve analysis was conducted to confirm the homogeneity of each product, and the products were electrophoresed on a 1.2% (w/v) agarose gel to check product size.

Immunostaining of germ cells

Germ cells (oocytes, embryo, and sperm) were washed in PBS containing 0.1% PVA and fixed with 4% (w/v) paraformaldehyde in 0.1 M PBS for 2 h. The germ cells were washed in PBS, permeabilized in PBS containing 0.1% (v/v) Triton X-100 (Fisher Scientific, Pittsburgh, PA, USA) for 10 min, and preincubated in a blocking buffer containing 2.0% (v/v) normal goat serum (NGS) for 3 h at room temperature under gentle rotation. The oocytes and embryos were incubated with affinity-purified polyclonal antibodies for KCNK2, KCNK10, KCNK4, KCNK3, and KCNK9, which were diluted 1:150 in PBS containing 0.1% (w/v) gelatin, 0.05% (w/v) NaN₃, and 1% (v/v) NGS for 3 h at room temperature. Sperm were smeared on slides and then fixed in –20 °C methanol for 30 min. Fixed slides were exposed to affinity-purified polyclonal antibodies for KCNK2, KCNK10, KCNK4, KCNK3, and KCNK9 that were diluted 1:150 in PBS containing 0.1% gelatin (w/v), 0.05%

Table 2 Bovine primer sequences used for real-time PCR.

Gene name	Primer sequence (5'–3')	GenBank accession numbers
<i>GAPDH</i>	Forward: ATGATCTACATGTTCCAG Reverse: AAGATGGTGATGGCCTTT	NM_001034034
<i>KCNK2</i>	Forward: AGATACGAATCATCTCC Reverse: ATAAATGGCGTCCAGGG	NM_174686
<i>KCNK10</i>	Forward: GGAAACGCTGGCATCAA Reverse: TCCGATCATGCTGAGGACA	XM_603455
<i>KCNK4</i>	Forward: GGTGCTCTTCCTGCTGA Reverse: CACCACCACGAAGTAGAT	XM_869390
<i>KCNK3</i>	Forward: CAGGCCTACTACTACTGCT Reverse: GGCCCCGTGAGGATGTATA	XM_597401
<i>KCNK9</i>	Forward: CTACTACTGCTTCATCACGTTG Reverse: CCCACCAGGATATACATAAAGCTA	XM_588194

(w/v) NaN_3 , and 1% (v/v) NGS overnight at 4 °C. The germ cells were washed in PBS containing 0.05% (v/v) Tween-20 (Bio-Rad), which was followed by 2 h incubation with FITC-conjugated anti-rabbit IgG that was diluted 1:150 in PBS containing 1.0% (v/v) NGS in the dark and at room temperature. The cells were washed in PBS containing 0.05% (v/v) Tween-20. After washing, oocytes and embryos were placed on precleaned slides coated with Antifade (Invitrogen) and wet mounted on glass slides. Stained cells were observed with a confocal laser scanning microscope equipped with a 40× objective (IX-81, Olympus). Primary antibodies were purchased from Chemicon (Temecula, CA, USA; KCNK9) and Alomone Labs (Jerusalem, Israel; KCNK3, KCNK2, KCNK10, and KCNK4). All other chemicals were purchased from Sigma Chemical Co., unless specified.

Capacitation and the acrosome reaction of bovine sperm

Fresh sperm (10×10^6) isolated by a swim-up preparation were incubated at 39 °C and 5% CO_2 for 4 h in washing-TALP medium containing heparin (15 µg/ml) to achieve capacitation. After capacitation, the acrosome reaction was induced by the addition of 10 µM ionomycin (Calbiochem, CA, USA) for 20 min. Sperm suspension isolated by a swim-up preparation was used as the control sample (without heparin and ionomycin).

Detection of an acrosome reaction of capacitated bovine sperm

Sperm smeared onto a slide were dried and fixed with cold methanol (−20 °C), then washed thrice in PBS. The sperm smears were incubated with FITC-conjugated *Pisum sativum* agglutinin (FITC-PSA, 100 µg/ml) in a dark chamber for 30 min at room temperature. After washing thrice in distilled water, the sperm were subjected to propidium iodide (PI, 50 µg/ml) staining for the nuclei. In some experiments, the sperm smears were incubated with both TRITC-conjugated goat anti-rabbit IgG against affinity-purified polyclonal antibodies for KCNK3 and KCNK4, and FITC-PSA. The sperm smears were rinsed and wet mounted, and the stained signals were immediately evaluated.

Statistical analysis

Light Cycler Software 4.0 (Roche) and Fluoview (FV1000, v. 1.5, Olympus) were used for real-time PCR data and immunofluorescence analysis respectively. Student's *t*-test was used with $P < 0.05$ as the criterion for significance. Data are represented as mean $\pm$ S.E.M.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was carried out with support of 'On-Site Cooperative Agriculture Research Project (No. 20070401-080-057-001-01-00)', RDA, and with partial support by grant no. R13-2005-012-01002-0 from the Basic Research Program of the Korea Science & Engineering Foundation and the Korea Research Foundation Grant (KRF-2006-005-J04204).

Acknowledgements

We thank Dr Choong-Saeng Park for the critical reading of the manuscript.

References

- Bai X, Bugg GJ, Greenwood SL, Glazier JD, Sibley CP, Baker PN, Taggart MJ & Fyfe GK 2005a Expression of TASK and TREK, two-pore domain K^+ channels, in human myometrium. *Reproduction* **129** 525–530.
- Bai X, Greenwood SL, Glazier JD, Baker PN, Sibley CP, Taggart MJ & Fyfe GK 2005b Localization of TASK and TREK, two-pore domain K^+ channels, in human cytotrophoblast cells. *Journal of the Society for Gynecologic Investigation* **12** 77–83.
- Bai X, Lacey HA, Greenwood SL, Baker PN, Turner MA, Sibley CP & Fyfe GK 2006 TASK channel expression in human placenta and cytotrophoblast cells. *Journal of the Society for Gynecologic Investigation* **13** 30–39.
- Barcroft LC, Offenberg H, Thomsen P & Watson AJ 2003 Aquaporin proteins in murine trophectoderm mediate transepithelial water movements during cavitation. *Developmental Biology* **256** 342–354.
- Besana A, Robinson RB & Feinmark SJ 2005 Lipids and two-pore domain $\text{K}(+)$ channels in excitable cells. *Prostaglandins & Other Lipid Mediators* **77** 103–110.
- Boni R, Gualtieri R, Talevi R & Tosti E 2007 Calcium and other ion dynamics during gamete maturation and fertilization. *Theriogenology* **68** (Supplement 1) S156–S164.
- de Castro EPLA & Hansen PJ 2007 Interactions between oxygen tension and glucose concentration that modulate actions of heat shock on bovine oocytes during *in vitro* maturation. *Theriogenology* **68** 763–770.
- Chow GE, Muller CH, Curnow EC & Hayes ES 2007 Expression of two-pore domain potassium channels in nonhuman primate sperm. *Fertility and Sterility* **87** 397–404.
- Cuomo A, Silvestre F, De Santis R & Tosti E 2006 Ca^{2+} and Na^+ current patterns during oocyte maturation, fertilization, and early developmental stages of *Ciona intestinalis*. *Molecular Reproduction and Development* **73** 501–511.
- Dalvit GC, Cetica PD, Pintos LN & Beconi MT 2005 Reactive oxygen species in bovine embryo *in vitro* production. *Biocell* **29** 209–212.
- Darszon A, Labarca P, Nishigaki T & Espinosa F 1999 Ion channels in sperm physiology. *Physiological Reviews* **79** 481–510.
- Darszon A, Trevino CL, Wood C, Galindo B, Rodriguez-Miranda E, Acevedo JJ, Hernandez-Gonzalez EO, Beltran C, Martinez-Lopez P & Nishigaki T 2007 Ion channels in sperm motility and capacitation. *Society of Reproduction and Fertility Supplement* **65** 229–244.
- Day ML, Pickering SJ, Johnson MH & Cook DI 1993 Cell-cycle control of a large-conductance K^+ channel in mouse early embryos. *Nature* **365** 560–562.
- Day ML, Johnson MH & Cook DI 1998 Cell cycle regulation of a T-type calcium current in early mouse embryos. *Pflügers Archiv* **436** 834–842.
- Everill B & Kocsis JD 1999 Reduction in potassium currents in identified cutaneous afferent dorsal root ganglion neurons after axotomy. *Journal of Neurophysiology* **82** 700–708.
- Kang D, Choe C & Kim D 2005 Thermosensitivity of the two-pore domain K^+ channels TREK-2 and TRAAK. *Journal of Physiology* **564** 103–116.
- Kang D & Kim D 2006 TREK-2 (K2P10.1) and TREK1 (K2P18.1) are major background K^+ channels in dorsal root ganglion neurons. *American Journal of Physiology. Cell Physiology* **291** C138–C146.

- Kang D, Hur CG, Choi CR, Park JY, Hong SG & Han J 2006 Ca^{2+} and K^{+} concentration change during early embryonic development in mouse. *Journal of Embryo Transfer* **21** 35–43.
- Kang D, Kim ES, Yang HY, Choe CY & Han J 2007a Expression of two-pore domain K^{+} channels in endometrial cells of Korean cattle. *Journal of Embryo Transfer* **22** 149–154.
- Kang D, Kim SH, Hwang EM, Kwon OS, Yang HY, Kim ES, Choi TH, Park JY, Hong SG & Han J 2007b Expression of thermosensitive two-pore domain K^{+} channels in human keratinocytes cell line HaCaT cells. *Experimental Dermatology* **16** 1016–1022.
- Kim D 2003 Fatty acid-sensitive two-pore domain K^{+} channels. *Trends in Pharmacological Sciences* **24** 648–654.
- Kim D 2005 Physiology and pharmacology of two-pore domain potassium channels. *Current Pharmaceutical Design* **11** 2717–2736.
- Kim Y, Lee SH & Ho WK 2007 Hydrogen peroxide selectively increases TREK-2 currents via myosin light chain kinases. *Frontiers of Bioscience* **12** 1642–1650.
- Koh SD, Monaghan K, Sergeant GP, Ro S, Walker RL, Sanders KM & Horowitz B 2001 TREK-1 regulation by nitric oxide and cGMP-dependent protein kinase. An essential role in smooth muscle inhibitory neurotransmission. *Journal of Biological Chemistry* **276** 44338–44346.
- Mahony MC & Gwathmey T 1999 Protein tyrosine phosphorylation during hyperactivated motility of cynomolgus monkey (*Macaca fascicularis*) spermatozoa. *Biology of Reproduction* **60** 1239–1243.
- Maingret F, Lauritzen I, Patel AJ, Heurteaux C, Reyes R, Lesage F, Lazdunski M & Honore E 2000 TREK-1 is a heat-activated background $\text{K}(+)$ channel. *EMBO Journal* **19** 2483–2491.
- Mitani S 1985 The reduction of calcium current associated with early differentiation of the murine embryo. *Journal of Physiology* **363** 71–86.
- Nagai T, Funahashi H, Yoshioka K & Kikuchi K 2006 Up date of *in vitro* production of porcine embryos. *Frontiers of Bioscience* **11** 2565–2573.
- Sanders KM & Koh SD 2006 Two-pore-domain potassium channels in smooth muscles: new components of myogenic regulation. *Journal of Physiology* **570** 37–43.
- Sugiyama S, McGowan M, Phillips N, Kafi M & Young M 2007 Effects of increased ambient temperature during IVM and/or IVF on the *in vitro* development of bovine zygotes. *Reproduction in Domestic Animals* **42** 271–274.
- Talley EM, Sirois JE, Lei Q & Bayliss DA 2003 Two-pore-domain (KCNK) potassium channels: dynamic roles in neuronal function. *Neuroscientist* **9** 46–56.
- Tichenor JN, Hansen ET & Buxton IL 2005 Expression of stretch-activated potassium channels in human myometrium. *Proceedings of the Western Pharmacology Society* **48** 44–48.
- Tosti E & Boni R 2004 Electrical events during gamete maturation and fertilization in animals and humans. *Human Reproduction Update* **10** 53–65.
- Travis AJ, Jorgez CJ, Merdiushev T, Jones BH, Dess DM, Diaz-Cueto L, Storey BT, Kopf GS & Moss SB 2001 Functional relationships between capacitation-dependent cell signaling and compartmentalized metabolic pathways in murine spermatozoa. *Journal of Biological Chemistry* **276** 7630–7636.
- Trimarchi JR, Liu L, Smith PJ & Keefe DL 2002 Apoptosis recruits two-pore domain potassium channels used for homeostatic volume regulation. *American Journal of Physiology. Cell Physiology* **282** C588–C594.
- Turner RM 2003 Tales from the tail: what do we really know about sperm motility? *Journal of Andrology* **24** 790–803.
- Wareing M, Bai X, Seghier F, Turner CM, Greenwood SL, Baker PN, Taggart MJ & Fyfe GK 2006 Expression and function of potassium channels in the human placental vasculature. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* **291** R437–R446.
- Winston NJ, Johnson MH, McConnell JM, Cook DI & Day ML 2004 Expression and role of the ether-a-go-go-related (MERG1A) potassium-channel protein during preimplantation mouse development. *Biology of Reproduction* **70** 1070–1079.
- Yang EK, Takimoto K, Hayashi Y, de Groat WC & Yoshimura N 2004 Altered expression of potassium channel subunit mRNA and alpha-dendrotoxin sensitivity of potassium currents in rat dorsal root ganglion neurons after axotomy. *Neuroscience* **123** 867–874.
- Yeung CH & Cooper TG 2001 Effects of the ion-channel blocker quinine on human sperm volume, kinematics and mucus penetration, and the involvement of potassium channels. *Molecular Human Reproduction* **7** 819–828.

Received 25 January 2008

First decision 26 February 2008

Revised manuscript received 8 October 2008

Accepted 5 November 2008