K⁺ efflux through two-pore domain K⁺ channels is required for mouse embryonic development

Chang-Gi Hur^{1,2}, Eun-Jin Kim¹, Seong-Keun Cho³, Young-Woo Cho¹, Sook-Young Yoon⁴, Hyun-Min Tak¹, Chang-Woon Kim^{1,5}, Changyong Choe⁶, Jaehee Han¹ and Dawon Kang¹

¹Department of Physiology, Institute of Health Sciences, Gyeongsang National University School of Medicine, 90 Chilam, Jinju 660-751, South Korea, ²CHO-A Biotechnology Research Institute, CHO-A Pharmaceutical Company, LTD, Seoul 150-992, South Korea, ³Department of Animal Science, Pusan National University, Miryang 627-706, South Korea, ⁴Fertility Center of CHA Gangnam Medical Center, CHA University, Seoul 135-081, South Korea, ⁵Department of Obstetrics and Gynecology, Changwon Samsung Hospital, Sungkyunkwan University School of Medicine, Changwon 630-723, South Korea and ⁶National Institute of Animal Science, RDA, Cheonan 330-801, South Korea

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

C-G Hur and E-J Kim contributed equally to this work

Abstract

Numerous studies have suggested that K⁺ channels regulate a wide range of physiological processes in mammalian cells. However, little is known about the specific function of K⁺ channels in germ cells. In this study, mouse zygotes were cultured in a medium containing K⁺ channel blockers to identify the functional role of K⁺ channels in mouse embryonic development. Voltage-dependent K⁺ channel blockers, such as tetraethylammonium and BaCl₂, had no effect on embryonic development to the blastocyst stage, whereas K_{2P} channel blockers, such as quinine, selective serotonin reuptake inhibitors (fluoxetine, paroxetine, and citalopram), gadolinium trichloride, anandamide, ruthenium red, and zinc chloride, significantly decreased blastocyst formation ($P < 0.05$). RT-PCR data showed that members of the K_{2P} channel family, specifically KCNK2, KCNK10, KCNK4, KCNK3, and KCNK9, were expressed in mouse oocytes and embryos. In addition, their mRNA expression levels, except *Kcnk3*, were up-regulated by above ninefold in morula-stage embryos compared with 2-cell stage embryos (2-cells). Immunocytochemical data showed that KCNK2, KCNK10, KCNK4, KCNK3, and KCNK9 channel proteins were expressed in the membrane of oocytes, 2-cells, and blastocysts. Each siRNA injection targeted at *Kcnk2*, *Kcnk10*, *Kcnk4*, *Kcnk3*, and *Kcnk9* significantly decreased blastocyst formation by ~38% compared with scrambled siRNA injection ($P < 0.05$). The blockade of K_{2P} channels acidified the intracellular pH and depolarized the membrane potential. These results suggest that K_{2P} channels could improve mouse embryonic development through the modulation of gating by activators.

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Introduction

Potassium (K⁺) channels represent the largest family of ion channels. The mammalian K⁺ channels are divided into four superfamilies, including voltage-gated K⁺ (K_v) channels, Ca²⁺-activated K⁺ (K_{Ca}) channels, inwardly rectifying K⁺ (K_{ir}) channels, and two-pore domain K⁺ (K_{2P}) channels, based on the criteria of the International Union of Basic and Clinical Pharmacology (IUPHAR; Shen *et al.* 2009). The primary functions of these K⁺ channels are to maintain resting membrane potential and K⁺ homeostasis. Through these primary functions, a variety of cellular functions, such as the regulation of the neuronal firing rate and heart rate, muscle contraction, hormone secretion, cell proliferation, and apoptosis, are regulated in mammalian cells (Kim 2005, Lang *et al.* 2005, Shen *et al.* 2009,

Enkvetchakul 2010). Although numerous studies have suggested that these K⁺ channels could regulate a wide range of physiological processes in mammalian cells (Buckler 2010, Enyedi & Czirjak 2010), little is known about the specific function of these channels in germ cells.

These K⁺ channels are also expressed in mammalian gametes and embryos, and a small number of studies have reported that the K⁺ channels are involved in a variety of physiological processes, such as maturation, fertilization, and cell cycle regulation (Tosti & Boni 2004, Winston *et al.* 2004, Darszon *et al.* 2007). The cell cycle during mouse embryonic development is regulated by a voltage-dependent inwardly rectifying K⁺ channel that shows high conductance and the ether-a-go-go-related K⁺ channel (Day *et al.* 1993, 1998, Winston *et al.* 2004).

In addition, the K_{2P} channels are responsible for K^+ efflux during apoptotic volume decreases in mouse zygotes (Trimarchi *et al.* 2002).

Considering the expression of K^+ channels in germ cells, we sought to identify the role of K^+ channels during mouse embryonic development because voltage gradients and electric fields regulated by ion transporters or ion channels have been implicated in embryonic development (Morokuma *et al.* 2008). In this study, mouse embryos were cultured in a medium containing K^+ channel blockers, and their development was compared with control embryos that were not treated with K^+ channel blockers. Among the K^+ channels investigated, we found that K_{2P} channels were responsible for the K^+ efflux required for mouse embryonic development.

Results

Requirement of K^+ channels in mouse embryonic development

To investigate whether K^+ channels affect mouse embryonic development, mouse zygotes were cultured in the presence of K^+ channel blockers. Various K^+ channel blockers (25 mM KCl, quinine, tetraethylammonium (TEA), and $BaCl_2$) at the indicated concentrations were added to a culture medium containing mouse zygotes, and embryonic development was compared on day 3.5 following culture. Increasing the extracellular K^+ concentration ($[K^+]_e$) to 25 mM depolarizes the plasma membrane and blocks K^+ channels, as do K^+ channel inhibitors. The application of 25 mM KCl or quinine (100 μ M) to the culture medium significantly decreased development to the blastocyst stage ($94.2 \pm 1.9\%$ in the control, $22.2 \pm 6.0\%$ in 25 mM KCl, and $41.7 \pm 5.2\%$ in 100 μ M quinine; $P < 0.05$), whereas the application of TEA and $BaCl_2$, which are conventional voltage-dependent K^+ channel blockers, had no effect on embryonic development, indicating that voltage-independent K^+ channels are involved in mouse embryonic development (Table 1). The effect of K^+ channel blockers on mouse embryonic development was microphotocaptured at 3.5 days following the beginning of culture (Fig. 1).

In the TEA and $BaCl_2$ treatments, most of the cells developed to either blastocysts or hatched blastocysts (Fig. 1Ab and Ac). However, culturing zygotes in the medium containing 25 mM KCl led to delays in development and the eventual death of the cells in culture (Fig. 1Ad). Most zygotes cultured with quinine (100 μ M and 1 mM) were arrested at the 2- or 4-cell stage (Fig. 1Ae and Af). In the 1 mM quinine-treated culture media, 6% of the embryos developed to the blastocyst stage. In addition, quinine inhibited development to the blastocyst stage in a dose-dependent manner (Table 1 and Fig. 1A). The total cell number in the blastocysts that developed under culture conditions containing different K^+ channel blockers was counted to evaluate the quality of the embryos. The mean cell number per blastocyst was not significantly different among the different K^+ channel blocker treatments (Fig. 1B).

Molecular characterization of K^+ channels involved in mouse embryonic development

To identify the K_{2P} channel mRNA expression in mouse oocytes and embryos, RT-PCR and real-time PCR were performed. The RT-PCR results showed that *Kcnk2*, *Kcnk10*, *Kcnk4*, *Kcnk3*, and *Kcnk9*, which are members of the K_{2P} channel family, were expressed in the mature oocytes (oocytes_{MII}), 2-cell stage embryos (2-cells), 8-cell stage embryos (8-cells), morula-stage embryos (morula), and blastocyst-stage embryos (blastocyst) of mice (Fig. 2A). The mRNA expression levels of these channels changed during mouse embryonic development (Fig. 2B). Compared with oocytes_{MII}, the levels of the entire channel mRNAs tested, except *Kcnk3*, were decreased in 2-cells, progressively increased to high levels in the morula, and then returned to the oocyte_{MII} level (Fig. 2B). In the morula, *Kcnk10* maintained a similar mRNA expression level to its level in oocytes_{MII}, following a reduction in the mRNA level in 2-cells. The *Kcnk3* mRNA level was increased in 2-cells and maintained a constant level of expression throughout embryonic development to the blastocyst stage. The embryo mRNA levels were re-analyzed compared with those in 2-cells (right bottom panel in Fig. 2B). The

Table 1 Effect of K^+ channel blockers on *in vitro* mouse embryonic development.

Treatment (μ M)	No. of zygotes used	Development to			
		2-Cell (%)	4–16 Cell (%)	Morula (%)	Blastocyst (%)
Control	211	211 (100.0) ^a	209 (99.5 $\pm$ 1.0) ^a	206 (97.6 $\pm$ 1.1) ^a	199 (94.2 $\pm$ 1.9) ^a
25 mM KCl	134	133 (99.3 $\pm$ 1.5) ^a	100 (74.5 $\pm$ 3.2) ^b	51 (38.0 $\pm$ 2.4) ^b	30 (22.2 $\pm$ 6.0) ^b
Quinine (10)	60	60 (100.0) ^a	58 (96.7 $\pm$ 2.9) ^a	54 (90.0 $\pm$ 0.0) ^c	54 (90.0 $\pm$ 0.0) ^c
Quinine (50)	40	40 (100.0) ^a	37 (92.6 $\pm$ 1.0) ^c	34 (84.9 $\pm$ 1.1) ^d	34 (84.9 $\pm$ 1.9) ^d
Quinine (100)	53	53 (100.0) ^a	45 (84.9 $\pm$ 3.0) ^d	34 (64.2 $\pm$ 0.9) ^e	22 (41.7 $\pm$ 5.2) ^e
TEA (500)	84	84 (100.0) ^a	84 (100.0) ^a	78 (92.9 $\pm$ 2.5) ^c	75 (89.6 $\pm$ 4.8) ^{c,d}
1 mM TEA	76	76 (100.0) ^a	76 (100.0) ^a	73 (96.3 $\pm$ 2.5) ^a	70 (92.2 $\pm$ 2.6) ^{a,c}
1 mM $BaCl_2$	109	109 (100.0) ^a	107 (98.3 $\pm$ 1.9) ^a	101 (92.7 $\pm$ 2.8) ^{a,c}	99 (90.8 $\pm$ 2.3) ^{a,c}

^{a,b,c,d,e}Values with different superscript letters within each column are significantly different ($P < 0.05$). Percentages are the means $\pm$ s.d. of four independent experiments.

Figure 1 Effect of K^+ channel blockers on mouse embryonic development and total cell number. (A) Photomicrographs of the mouse embryo cultured with different K^+ channel blockers. The zygotes were cultured for 3.5 days under different culture conditions without (control, a) and with 1 mM TEA (b), 1 mM BaCl₂ (c), 25 mM KCl (d), 100 μM quinine (e), or 1 mM quinine (f). Scale bar = 50 μm. (B) The total cell number of blastocysts cultured in the presence and absence of different kinds of K^+ channel blockers. The blastocysts that developed from the zygotes were fixed and stained with Hoechst 33342 at 3.5 days after chemical treatment. NS, no statistical significance.

expression levels were up-regulated up to the morula stage compared with the 2-cell. The expression of the K_{2P} channel member transcripts identified by RT-PCR was subsequently studied at the protein level. The specificity and sensitivity of the antibodies used in these experiments were confirmed in previous studies (Kang *et al.* 2007, Hur *et al.* 2009). Each antibody was specific for its own antigen (Supplementary Figure 1, see section on supplementary data given at the end of this article). Immunocytochemical staining with antibodies against the different K_{2P} channel proteins showed that KCNK2, KCNK10, KCNK4, KCNK3, and KCNK9 were expressed in the plasma membrane of oocytes_{MII}, 2-cells, and blastocysts. Compared with KCNK3 and KCNK9 proteins, KCNK2, KCNK10, and KCNK4 proteins were highly expressed in oocytes_{MII} and blastocysts (Fig. 2C). All of the channel proteins were highly expressed in 2-cells, with the exception of KCNK2. As shown in Fig. 2C, KCNK2, KCNK10, and KCNK4 were easily detectable in blastocyst membranes.

Effect of K_{2P} channel blockers on mouse embryonic development

The K_{2P} channels were expressed in mouse oocytes and embryos (Fig. 2). One of the pharmacological properties of K_{2P} channels is its insensitivity to TEA and BaCl₂ and its sensitivity to quinine. Selective serotonin reuptake

inhibitors (SSRIs) and antipsychotics, which are known to inhibit KCNK2 and KCNK10 channels, decreased mouse embryonic development to the blastocyst stage (Table 2). Concentrations of 10 and 50 μM chlorpromazine (an antipsychotic drug) and SSRIs (fluoxetine, paroxetine, and citalopram) significantly inhibited development to the blastocyst stage by ~31% compared with the control ($P < 0.05$). However, they exhibited dose-independent effects on embryonic development. Gadolinium trichloride (GdCl₃: hexahydrate GdCl₃, 10 μM), a KCNK4 inhibitor, also inhibited blastocyst formation by ~44% compared with control (Table 2). The endocannabinoid anandamide is a direct and selective blocker of KCNK3 and KCNK9. Unlike the other K_{2P} channels, KCNK3 is directly blocked by submicromolar concentrations of anandamide (Maingret *et al.* 2001). Ruthenium red (RR) and zinc chloride (ZnCl₂) are known to block KCNK9 (Czirjak & Enyedi 2003, Clarke *et al.* 2004). As shown in Table 3, the application of anandamide exhibited an inhibitory effect on embryonic development in a dose-dependent manner. Treatment with 100 μM anandamide significantly decreased development to the blastocyst stage (98.3% in the control vs 41.5% in 100 μM anandamide). The application of RR (10 μM) and ZnCl₂ (50 μM) also inhibited development to the blastocyst stage by ~44% compared with the control. However, they had no effect on 2-cell cleavage.

Figure 2 Expression and localization of K_{2P} channels in the mouse oocyte and embryos. (A) The expression of *Kcnk2*, *Kcnk10*, *Kcnk4*, *Kcnk3*, and *Kcnk9* mRNA transcripts in mouse oocytes_{MII}, 2-cells, 8-cells, morula, and blastocysts. The RT-PCR products were separated on a 1.5% standard agarose gel and stained with ethidium bromide. (B) Alterations in the mRNA expression levels of *Kcnk2*, *Kcnk10*, *Kcnk4*, *Kcnk3*, and *Kcnk9* during mouse embryonic development. The mRNA expression of *Kcnk2*, *Kcnk10*, *Kcnk4*, *Kcnk3*, and *Kcnk9* was quantified by real-time PCR in oocytes_{MII}, 2-cells, 8-cells, morula, and blastocysts. The y-axis shows the relative mRNA levels on a log scale. The mRNA expression level was normalized to *Gapdh*. Each bar represents the mean $\pm$ s.d. of five independent experiments. The oocyte_{MII}, 2-cell, 8-cell, morula, and blastocyst indicate mature oocytes arrested at the time metaphase II, 2-cell stage embryo, 8-cell stage embryo, morula-stage embryo, and blastocyst-stage embryo, respectively. Each dataset was summarized in a line graph. The data were normalized to the gene expression levels expressed in the 2-cells. (C) The immunocytochemical analysis of KCNK2, KCNK10, KCNK4, KCNK3, and KCNK9 in mouse oocytes_{MII}, 2-cells, and blastocyst. The 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). Scale bar = 50 μ m. The negative control was checked by omitting the primary antibody, and no staining was observed in any of the negative control cells.

The application of acetylcholine (ACh), an inhibitor of TREK (KCNK2, KCNK10, and KCNK4) and TASK (KCNK3 and KCNK9) channel activities, decreased blastocyst formation by 36% (Fig. 3A). To record the K^+ current in mouse zygotes, the pipette and bath solutions contained 150 and 5 mM K^+ , respectively. The cell membrane potential was held at -60 mV, and a voltage ramp (-80 to $+80$ mV; 1 s duration) was

implemented at room temperature. The current–voltage curves obtained from an individual cell are shown in Fig. 3B. The whole-cell current recorded in mouse zygotes was significantly inhibited by quinine (10 μ M), fluoxetine (10 μ M), and ACh (100 μ M) ($P < 0.05$), suggesting that K_{2P} channels are functionally expressed in mouse zygotes. As shown in Fig. 3B, the application of ACh inhibited whole-cell currents more highly

Table 2 Effect of TREK and TRAAK blockers on mouse embryonic development.

Treatment concentration (μ M)	No. of zygotes used	Development to			
		2-Cell (%)	4–16 Cell (%)	Morula (%)	Blastocyst (%)
Control	94	91 (96.8 $\pm$ 0.2) ^a	87 (92.5 $\pm$ 2.2) ^a	81 (86.2 $\pm$ 0.8) ^a	80 (85.3 $\pm$ 2.5) ^a
Chlorpromazine	101	96 (95.0 $\pm$ 1.9) ^a	89 (88.1 $\pm$ 0.9) ^b	78 (77.3 $\pm$ 3.2) ^b	71 (70.4 $\pm$ 4.3) ^b
	104	102 (98.1 $\pm$ 1.7) ^a	94 (90.4 $\pm$ 1.6) ^{a,b}	82 (78.8 $\pm$ 1.5) ^b	72 (69.2 $\pm$ 3.1) ^b
Fluoxetine	111	107 (96.4 $\pm$ 1.2) ^a	97 (87.5 $\pm$ 2.2) ^b	82 (73.9 $\pm$ 1.3) ^b	74 (66.8 $\pm$ 3.7) ^b
	110	102 (92.7 $\pm$ 1.5) ^{a,b}	94 (85.5 $\pm$ 2.9) ^b	80 (72.7 $\pm$ 1.5) ^b	69 (62.7 $\pm$ 2.9) ^b
Paroxetine	93	85 (91.4 $\pm$ 1.7) ^{a,b}	73 (78.5 $\pm$ 1.7) ^c	62 (66.8 $\pm$ 3.2) ^c	59 (63.5 $\pm$ 3.1) ^b
	107	98 (91.6 $\pm$ 2.6) ^{a,b}	88 (82.3 $\pm$ 3.0) ^{b,c}	70 (65.4 $\pm$ 4.7) ^c	64 (59.8 $\pm$ 2.6) ^{b,c}
Citalopram	110	100 (90.9 $\pm$ 3.0) ^{a,b}	96 (87.3 $\pm$ 1.4) ^{b,c}	86 (78.2 $\pm$ 4.4) ^b	78 (70.9 $\pm$ 1.9) ^b
	92	83 (90.2 $\pm$ 3.3) ^{a,b}	78 (84.8 $\pm$ 1.7) ^{b,c}	62 (67.3 $\pm$ 4.3) ^c	54 (58.8 $\pm$ 2.1) ^c
GdCl ₃	90	81 (90.0 $\pm$ 3.3) ^{a,b}	75 (83.3 $\pm$ 3.4) ^{b,c}	60 (66.7 $\pm$ 3.4) ^c	48 (53.3 $\pm$ 3.4) ^c
	90	79 (88.9 $\pm$ 1.9) ^b	72 (80.0 $\pm$ 3.3) ^{b,c}	57 (63.3 $\pm$ 6.7) ^c	43 (47.8 $\pm$ 5.1) ^{c,d}

^{a,b,c,d}Values with different superscript letters within each column are significantly different ($P < 0.05$). Percentages are the means $\pm$ s.d. of three independent experiments. TREK and TRAAK indicate KCNK2, KCNK10, and KCNK4.

Table 3 Effect of TASK inhibitors on mouse embryonic development.

Treatment (μ M)	No. of zygotes used	Development to			
		2-Cell (%)	4–16 Cell (%)	Morula (%)	Blastocyst (%)
Control	60	60 (100 ± 0) ^a	59 (98.3 ± 2.9) ^a	59 (98.3 ± 2.9) ^a	59 (98.3 ± 2.9) ^a
Anandamide (10)	60	60 (100 ± 0) ^a	58 (96.7 ± 2.9) ^a	54 (93.3 ± 2.9) ^a	54 (93.3 ± 2.9) ^a
Anandamide (50)	40	40 (100 ± 0) ^a	37 (92.2 ± 1.9) ^{a,b}	34 (84.5 ± 3.9) ^b	34 (84.5 ± 3.9) ^b
Anandamide (100)	53	53 (100 ± 0) ^a	45 (84.9 ± 3.5) ^c	34 (64.2 ± 2.8) ^c	22 (41.5 ± 2.8) ^c
RR (10)	70	70 (100 ± 0) ^a	60 (86.0 ± 3.5) ^{b,c}	45 (64.7 ± 5.0) ^c	39 (56.0 ± 4.0) ^d
ZnCl ₂ (50)	70	70 (100 ± 0) ^a	58 (83.0 ± 2.6) ^{b,c}	43 (61.7 ± 2.9) ^c	38 (54.7 ± 2.7) ^d

a,b,c,d Values with different superscript letters within each column are significantly different ($P < 0.05$). Percentages are the means $\pm$ s.d. of three independent experiments. TASK indicates KCNK3 and KCNK9.

compared with the other blockers (2735 ± 449 pA in the control vs 1111 ± 471 pA in ACh, $P < 0.05$). The ion selectivity of the whole-cell current recorded in 2-cells was further studied in large outside-out patches by recording the reversal potentials at various $[K^+]_e$ after applying voltage ramps from -120 to $+60$ mV (Fig. 3C). The reversal potential shifted to the right as the $[K^+]_e$ in the bath solution increased. These results showed that the whole-cell current recorded in 2-cells is a K^+ -selective channel. In 2- and 8-cells, the voltage ramp produced large currents with reversal potentials near -70 mV, indicating that the currents formed a functional K^+ channel in the plasma membrane of 2- and 8-cells (Fig. 3D). The ramp pulses of 1 s durations applied from -120 to $+60$ mV showed a mildly outwardly rectifying K^+ current. These currents were

inhibited by K^+ channel blockers except TEA (1 mM) and Ba^{2+} (1 mM). Quinine (10 μ M), chlorpromazine (10 μ M), fluoxetine (10 μ M), GdCl₃ (10 μ M), anandamide (50 μ M), RR (10 μ M), and ZnCl₂ (50 μ M) inhibited the K^+ currents by $\sim 50\%$ (Fig. 3D). The currents in the 8-cells were slightly decreased compared with those in the 2-cells, indicating that the decline of currents is likely to be affected by the reduction in the density of K^+ channels following cell division. The currents were recorded in each blastomere.

Gene silencing of K_{2P} channel genes inhibits blastocyst formation

To confirm the involvement of K_{2P} channels in embryonic development, knockdown experiments

Figure 3 Effect of K_{2P} channel blockers on whole-cell current in mouse embryos. (A) The effect of ACh on blastocyst formation. The zygotes were cultured for 3.5 days in KSOM medium with ACh (100 μ M). (B) The whole-cell K^+ currents recorded in mouse zygotes. Whole-cell currents were elicited by ramp pulses from -80 to $+80$ mV before and after the application of ACh (100 μ M). The magnitudes of currents at $+80$ mV were determined and plotted as a bar graph. The control currents were recorded in the absence of K^+ channel blockers. Each bar represents the mean $\pm$ s.d. of five independent experiments. * $P < 0.05$ compared with the control value. (C) The whole-cell currents recorded at different $[K^+]_e$ are shown. Note the rightward shift of the reversal potential as $[K^+]_e$ is increased. (D) The whole-cell currents recorded at different stage embryos. The currents were recorded from 2- to 8-cells. The cell membrane potential was held at -80 mV, and voltage ramps from -120 to 60 mV were applied for 1 s durations. The pipette and bath solutions contained 150 and 5 mM K^+ , respectively. In addition, the bar graphs show the effects of various K_{2P} channel blockers on the background K^+ channel activity in 2- and 8-cells. Each bar represents the mean $\pm$ s.d. of five independent experiments. * $P < 0.05$ compared with the control value. pA, picoampere.

were performed. *Kcnk2*, *Kcnk10*, *Kcnk4*, *Kcnk3*, or *Kcnk9* small interfering RNA (siRNA) was microinjected into the cytoplasm of zygotes. Scrambled siRNA was also microinjected as a negative control in a separate zygote population. As shown in Table 4, the microinjection of the K_{2P} channel siRNA into the cytoplasm of zygotes significantly decreased development to the blastocyst stage compared with sham controls microinjected with scrambled siRNA. The sham controls showed a significant inhibition of development to the 2-cell and blastocyst stages ($P < 0.05$), indicating that microinjection alone induces cell damage. All of the K_{2P} channels tested affected blastocyst formation, but they did not affect 2-cell cleavage. In particular, *Kcnk4* and *Kcnk9* knockdown resulted in a larger inhibition of development to the blastocyst stage than the other channels (*Kcnk2*, *Kcnk10*, and *Kcnk3*).

Changes in pH and membrane potential affect mouse embryonic development

Changes in the intracellular or extracellular pH (pH_i or pH_e) modulate KCNKs (KCNK2, KCNK10, KCNK4, KCNK3, and KCNK9) channel activities. The measurement of pH_i using the fluorescent pH indicator SNARF-1 showed that K^+ channel blockers acidified the cytoplasm. Quinine (10 μ M), fluoxetine (10 μ M), RR (10 μ M), and $GdCl_3$ (10 μ M) acidified the pH_i to 6.2 ± 0.3 , 6.5 ± 0.4 , 6.1 ± 0.4 , and 6.2 ± 0.5 , respectively (Fig. 4A). After determining the changes in pH_i using K_{2P} channel blockers, we changed the pH_e because changing the pH_i is not easy. The changes in pH_i are dependent on the changes in pH_e (Fellenz & Gerweck 1988, Kaba *et al.* 2008). As shown in Fig. 4B, zygotes cultured at low pH_e levels (pH 5.3 and 6.3) exhibited decreased development to the blastocyst stage than the controls (pH 7.3), whereas zygotes cultured at pH 8.3 exhibited a higher blastocyst formation than the controls. However, the highest pH tested (pH 9.3) failed to increase development to the

blastocyst stage. Moreover, the application of quinine (10 μ M), fluoxetine (10 μ M), $GdCl_3$ (10 μ M), and RR (10 μ M) significantly depolarized the membrane potential ($P < 0.05$; Fig. 4C).

Discussion

This study demonstrates, for the first time, that K_{2P} channels are involved in mouse embryonic development through maintaining the pH_i and membrane potential by the modulation of K_{2P} channel gating. Quinine-sensitive and TEA-insensitive K^+ channels, showing the characteristic properties of K_{2P} channels, were expressed in the plasma membranes of mouse oocytes_{MII} and embryos, and their expression levels changed during embryonic development.

Expression of K_{2P} channels in the mammalian reproductive organ and cells

K_{2P} channels are thought to be responsible for setting the resting membrane potential of excitable and non-excitable cells. Recent studies have found that K_{2P} channels are expressed in the mammalian reproductive organ and cells, such as in human cytotrophoblast cells, placental villous tissue and trophoblast cells, myometrium, the placental vascular system, and uterine smooth muscle cells (Bai *et al.* 2005a, 2005b, 2006, Tichenor *et al.* 2005, Wareing *et al.* 2006, Chow *et al.* 2007). In addition, a previous study from our laboratory also found these channels to be expressed in bovine ovaries, testis, and germ cells (Hur *et al.* 2009). Furthermore, they were also present in monkeys, where their expression was restricted to sperm (Chow *et al.* 2007). In this study, the patterns of each K_{2P} channel mRNA expression showed embryo stage-specific effects. Their mRNA expression levels were relatively high in the morula compared with the other stage embryos. The 8-cells undergo compaction to reach the morula stage of development and the morula then undergoes differentiation to the blastocyst stage (Johnson & McConnell 2004, Wang *et al.* 2008). The high expression of the K_{2P} channel in the morula is likely to be a key factor for blastocyst formation. The blockade of K_{2P} channel function could interrupt compaction and blastocyst formation. The results of the application of the K_{2P} channel blockers and siRNAs to mouse zygotes support the hypothesis that K_{2P} channels are involved in embryonic development. These results indicated that K_{2P} channel activity and their expression levels play a role in embryonic development.

Modulation and involvement of K_{2P} channels in mouse embryonic development

K_{2P} channels, which are known to be regulated by a variety of biologically relevant stimuli, regulate a variety

Table 4 Inhibition of blastocyst formation by silencing K_{2P} channels.

siRNA	No. of zygotes used	Development to	
		2-Cell (%)	Blastocyst (%)
Control	112	107 (95.6 $\pm$ 1.5) ^a	92 (82.4 $\pm$ 3.8) ^a
Sham	126	108 (85.8 $\pm$ 5.7) ^b	80 (63.3 $\pm$ 8.6) ^b
<i>Kcnk2</i>	143	118 (82.3 $\pm$ 5.4) ^b	69 (48.0 $\pm$ 4.8) ^c
<i>Kcnk10</i>	140	121 (86.3 $\pm$ 2.0) ^b	70 (49.4 $\pm$ 5.3) ^c
<i>Kcnk4</i>	142	111 (77.5 $\pm$ 11.6) ^b	59 (41.4 $\pm$ 8.7) ^c
<i>Kcnk3</i>	53	42 (78.9 $\pm$ 4.5) ^b	26 (48.1 $\pm$ 8.2) ^c
<i>Kcnk9</i>	55	43 (78.0 $\pm$ 6.9) ^b	22 (39.7 $\pm$ 6.8) ^c

^{a,b,c}Values with different superscript letters within each column are significantly different ($P < 0.05$). Percentages are the means $\pm$ s.d. of four independent experiments.

Figure 4 Effect of K_{2P} channel blockers on pH_i and membrane potential. (A) K_{2P} channel blockers induced changes in pH_i . The 2-cells were exposed to the indicated K^+ channel blockers. The pH_i was analyzed by methods previously described in the Materials and Methods section. (B) The effect of changes in pH_e on mouse embryonic development. The zygotes were exposed to various pH_e conditions (pH 5.3 to 9.3) for 3.5 days. The percentage of development to the blastocyst stage was summarized. * $P < 0.05$ compared with the pH 7.3. (C) The changes in membrane potential due to K_{2P} channel blockers. The 2-cells were exposed to the indicated K^+ channel blockers. The membrane potential change was normalized to the control. Each bar represents the mean $\pm$ s.d. of five independent experiments. (A and C) * $P < 0.05$ compared with the control value. The difference in the amount of changes in pH_i and membrane potential by K_{2P} channel blockers resulted from the difference in the calculation methods. The pH_i was displayed as the pH value, but the membrane potential was displayed as the relative value normalized to the control. The pH changes showing a small change will result in a large effect because altering the pH above or below its optimum pH reduces the enzyme's activity.

of cellular functions in the cells expressing them. K^+ efflux has apoptotic and protective effects due to the dysfunction in maintaining cell volume and hyperpolarization of the membrane, respectively (Yu *et al.* 1997, Wang *et al.* 1999). Therefore, K^+ efflux represents a very

important determinant of the turning point between cell survival and the death of normal cells. Alterations in K^+ channel activity induce changes in membrane potential, and these changes in membrane potential may modulate cellular physiological functions, such as gamete maturation, fertilization, cell division, and early embryonic development. The activity of high-conductance K^+ channels has been shown to be decreased during mouse embryonic development with associated membrane depolarization (Day *et al.* 1993, Kang *et al.* 2006). Additionally, the activity of K^+ channels can be affected by a variety of factors produced by *in vitro* culture. These factors could also be altered *in vivo*. The reduction in temperature and an increase in reactive oxygen species during *in vitro* maturation (IVM), IVF, and development could induce the closing and opening of KCNK2, KCNK10, and KCNK4 channels, respectively (Maingret *et al.* 2000, Kang *et al.* 2005, 2007, Kim *et al.* 2007). In addition, the changes in membrane potential could affect the pH_i or could be affected by changes in pH_i . Furthermore, these changes could act together or separately to regulate important physiological and pathological events.

KCNK3 and KCNK9 are involved in pH and nutrient/metabolic sensing in a variety of cells (Buckler 2010). The cellular and organismal pH levels function as signals to modulate various cellular events (Tresguerres *et al.* 2010). The pH_i regulation is an important mechanism for early embryo development (Baltz 1993). Mammalian preimplantation embryos are well equipped with pH_i regulation machineries, such as Na^+/H^+ and HCO_3^-/Cl^- exchangers that are essential for embryo development (FitzHarris & Baltz 2009). The Na^+/H^+ exchanger is usually activated when the pH_i falls and exchanges external Na^+ for internal H^+ . In this study, the application of K^+ channel blockers acidified the pH_i . We hypothesized that the K^+ channel blockers are likely to interrupt Na^+/H^+ exchange activity. However, we did not observe acidosis correction by the combined treatment of K^+ channel blockers and Na^+/H^+ exchanger activators (calyculin and epidermal growth factor) despite many trials. Earlier studies have shown that pharmacological manipulation of the MEK/MAPK signaling pathway had no effect on HCO_3^-/Cl^- exchange activity during meiosis; however, the direct manipulation of MAPK activity, such as constitutively active and dominant negative forms of MEK, had a positive effect on HCO_3^-/Cl^- exchange activity (Phillips *et al.* 2002, Zhou *et al.* 2009). Thus, further study is needed to find a way to treat cells with K^+ channel blockers to elicit similar effects as the manipulation of MAPK. In renal epithelial cells, aldosterone-induced H^+ secretory fluxes are paralleled by K^+ secretion, and an increase in pH_i activates K^+ channels (Oberleithner *et al.* 1988). In addition, H^+/K^+ exchanger dysfunction by K^+ channel blockers is likely to modulate pH_i and pH_e . One well-known property of KCNK2, KCNK10,

KCNK4, KCNK3, and KCNK9 is their regulation by pH (Kim 2003). KCNK2 and KCNK10 are activated by low pH_i , whereas KCNK4 is activated by high pH_i . KCNK3 and KCNK9 are inhibited by low pH_e . Under acidic conditions, which reduce KCNK3 and KCNK9 channel activities, embryonic development was reduced, but severe alkalization also inhibited blastocyst formation, suggesting that severe changes in pH may affect other physiological functions and ion channel activity. Treatment with $\text{K}_{2\text{P}}$ channel blockers acidified the pH_i in this study, and KCNK2 and KCNK10 could be activated under these conditions. The percentage of blastocyst formation observed in this study was $\sim 50\%$ and could partially account for the compensatory effect of KCNK2 and KCNK10 in response to the blockade of KCNK3 and KCNK9 channels and vice versa. The compensatory mechanisms in *in vivo* and *in vitro* systems are likely to be employed during various physiological processes.

Based on these reports, KCNK2, KCNK10, KCNK4, KCNK3, and KCNK9 may be modulated by *in vitro* culture and by environmental conditions during embryonic development, and therefore, their blockades may induce the delay or inhibition of blastocyst formation due to dysfunction in sensing pH levels, in maintaining cellular volume, and in regulating membrane potential. Normal embryonic development to the blastocyst stage is important for the initiation of implantation and pregnancy. Any defects in blastocyst formation result in defective implantation and infertility (Wang *et al.* 2008) through changes in the ion transport and channel systems (Barcroft *et al.* 2003). The KCNQ protein or mRNA is required in *Drosophila* early embryonic development (Wen *et al.* 2005).

In conclusion, the blockade of $\text{K}_{2\text{P}}$ channels reduces mouse embryonic development by causing cell depolarization and acidification. Our pharmacological and gene silencing experiments suggested that the modulation of $\text{K}_{2\text{P}}$ channels serves as a switch to induce embryonic development by contributing to the background K^+ conductance of germ cells. The $\text{K}_{2\text{P}}$ channels expressed in mouse germ cells could be a good target for the regulation of embryonic development in an *in vitro* culture system and *in vivo*.

Materials and Methods

Chemicals

All chemicals with reagent grade or better were purchased from Sigma Chemical Co., unless specified. All components of the medium were of tissue culture grade. To determine the effect of K^+ channel blockers on mouse embryonic development, KCl, TEA, BaCl_2 , quinine, anandamide, GdCl_3 , ACh, chlorpromazine, fluoxetine, paroxetine, citalopram, RR, and ZnCl_2 were treated to the culture medium containing zygotes. All chemicals were treated all the time during culture. Stock solutions of KCl (2 M), TEA (1 M), BaCl_2 (1 M), chlorpromazine (100 mM), fluoxetine (10 mM), citalopram (10 mM), RR (20 mM), ZnCl_2

(1 M), GdCl_3 (100 mM), ACh (100 mM), and $(\text{NH}_4)_2\text{SO}_4$ were prepared in distilled water; quinine (1 M) and paroxetine (20 mM) in dimethyl sulfoxide (DMSO); and anandamide (arachidonylethanolamide, 719 mM) in ethanol, respectively, and then diluted in KSOM medium to a working concentration. When DMSO or ethanol was used as a solvent, a solution containing an equivalent concentration was used as a control. Final DMSO and ethanol concentrations were $\sim 0.1\%$.

Animals

Female mice of the ICR strain were purchased from Koatech Co. (Animal Breeding Center, Pyeongtaek, South Korea). Mice, which were 6 to 8 weeks old, were superovulated by an i.p. injection of 5 IU of pregnant mare's serum gonadotrophin, followed 48 h later by an injection of 5 IU hCG (Yuhan, Seoul, Korea). Females were either left unmated or paired with fertile males. Evidence of successful mating between the female and paired male was confirmed by observation of the presence of a copulation plug. Mice were used in accordance with the Guide for the Care and Use of Laboratory Animals (Gyeongsang National University, GLA-090520-M0061 and GLA-100310-M0024).

Oocyte or embryo collection

To isolate oocytes or embryos, mice were killed by cervical dislocation. Oocytes_{MII} were obtained by tearing the oviducts with fine forceps at 14 h post-hCG injection. Zygotes were obtained at 20 h after hCG injection and mating. Mating was performed after hCG injection. The zygotes were flushed from the oviduct using a HEPES-buffered flushing holding medium (FHM), which is based on KSOM medium with HEPES buffer (20 mM) and decreased NaHCO_3 (4 mM), that had been prewarmed to 37 °C. The FHM medium for embryo collection was supplemented with 6 mg/ml polyvinyl alcohol (PVA). Cumulus cells of oocytes_{MII} were removed by exposure to 0.01% hyaluronidase (Type I-S) for 2 min. For recording K^+ current, the zona pellucida of zygotes was subsequently removed by exposure to 0.5% protease (Type XXIV) for 1 min in FHM, followed by washing in FHM medium with 10% FBS (Gibco BRL). The protease exposure time was carefully and consistently maintained in the repetitive experiments to prevent experimental error such as the damage of the cell membrane by the protease used for removal of the zona pellucida.

Zygote culture

Embryo culture was performed in KSOM medium with amino acids (KSOMaa). The addition of amino acids to KSOM medium enables the blastocysts to synthesize mRNA of several types of proteins at *in vivo* levels (Ho *et al.* 1995). The components of the home-made KSOMaa medium used here (Lawitts & Biggers 1993) were (in mM) NaCl (95), KCl (2.5), CaCl_2 (1.7), KH_2PO_4 (0.4), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2), NaHCO_3 (25), sodium lactate (10), sodium pyruvate (0.2), glucose (0.2), glutamine (1.0), EDTA (0.01), BSA (1 mg/ml), MEM NEAA (0.5 $\times$), and MEM EAA (0.5 $\times$), penicillin-G (100 units/ml), streptomycin (50 mg/ml), and phenol red. The osmolarity and pH of the KSOMaa medium were adjusted to 280 mOsm and

7.3, respectively. For culture experiments, 10–40 embryos with intact zona pellucida and normal morphology were transferred to each well in four-well dishes (Nunc, Roskilde, Denmark), which contained 500 μ l of KSOMaa medium and the treatment compounds. In this study, four-well dishes were used instead of microdrops to prevent the oil effect against chemicals containing DMSO. The zygotes were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 5 days post-hCG. The embryos were observed every 24 h under an Olympus stereo zoom microscope (SZ60, Tokyo, Japan). The cleavage and development of the zygotes were evaluated by determining the proportion of embryos reaching to the 2-cell (day 1 post-culture) and blastocyst stage (day 3.5 post-culture), respectively. Blastocyst formation was compared between the control and the treatment of K^+ channel blockers or siRNA against each K^+ channel.

Total cell number counting

The blastocysts obtained on day 3.5 post-culture were washed twice in PBS and were fixed with 4% (wt/vol) paraformaldehyde. The blastocysts were incubated in KSOMaa medium containing 10 μ g/ml Hoechst 33342 at 37 °C with 5% CO_2 for 30 min. The blastocysts were placed on ethanol pre-cleaned slide and were mounted with a coverslip. The cell numbers of these cultured blastocysts were enumerated under an inverted fluorescent microscope (Olympus, IX-50).

RT-PCR analysis

First-strand cDNAs were synthesized from total RNA isolated from mouse oocyte_{MII}, 2-cells, 8-cells, morula, and blastocyst

using oligo dT (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 5 lists the DNA sequences of primers used to detect the expression of each K_{2P} channel. PCR conditions were as follows: initial denaturation at 94 °C for 4 min, then 35 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 germ cells by RT-PCR were directly sequenced with the ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA).

Real-time PCR analysis

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>). The DNA sequences of primers used to detect the expression of each K_{2P} channel are listed in Table 5. PCR conditions 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 1.5% (wt/vol) agarose gel to check the product size.

Table 5 Mouse primer sequences used for PCR.

Gene name (channel name)	GenBank acc. nos	Primer sequences	Application
<i>Gapdh</i>	NM_008084	Forward: 5'-CTGAAGGGCATCTTGGG-3' Reverse: 5'-TACTCCTTGGAGGCCATG-3'	RT-PCR
		Forward: 5'-CATGGCCTTCCGTGTTT-3' Reverse: 5'-CTGCTTCAACACCTTCTT-3'	Real-time PCR
<i>Kcnk2</i> (TREK-1)	NM_010607	Forward: 5'-TGTGGTATCACTCTGACGA-3' Reverse: 5'-ATGACAGCTATGTCCTCACC-3'	RT-PCR
		Forward: 5'-CCACCATCATCTTCATCCTG-3' Reverse: 5'-CCAGCCTTCTATGTGCTTGA-3'	Real-time PCR
<i>Kcnk10</i> (TREK-2)	DQ185134	Forward: 5'-CAAAAGAAGAGGTTGGTGAG-3' Reverse: 5'-GAGTAATCCGGAAGGTTTT-3'	RT-PCR
		Forward: 5'-GAAAGCAGGTGAAGTGGGAT-3' Reverse: 5'-GAGTCGAGGAACAGGGTGAT-3'	Real-time PCR
<i>Kcnk4</i> (TRAAK)	NM_008431	Forward: 5'-CACCCTGTAGGCTTGGCGATTATG-3' Reverse: 5'-ACTCTGCGTGTCTGAGGACTCGTCG-3'	RT-PCR
		Forward: 5'-ATGGAGAGCTGGAGCAAGTT-3' Reverse: 5'-GCTGGTAGGCTGGAGAGTTC-3'	Real-time PCR
<i>Kcnk3</i> (TASK-1)	NM_010608	Forward: 5'-TGTCTGCATGTTCTACGCG-3' Reverse: 5'-TGGAGTACTGCAGCTTCTCG-3'	RT-PCR
		Forward: 5'-GCTCCTTCTACTTCGCCATC-3' Reverse: 5'-GAACATGCAGAACACCTTGC-3'	Real-time PCR
<i>Kcnk9</i> (TASK-3)	DQ185133	Forward: 5'-TGACTACTATAGGGTTCGGCG-3' Reverse: 5'-AAGTAGGTGTTCTCAGCAGC-3'	RT-PCR
		Forward: 5'-GACGCTGGTTATGTTCCAGA-3' Reverse: 5'-CGGTCAACATGTTCTCCATA-3'	Real-time PCR

Immunostaining of germ cells

Oocytes_{MII} and embryos were washed in PBS containing 0.1% (wt/vol) PVA and fixed with 4% (wt/vol) paraformaldehyde in 0.1 M PBS for 2 h. The cells were washed in PBS, permeabilized in PBS containing 0.1% (vol/vol) Triton X-100 (Fisher Scientific, Pittsburgh, PA, USA) for 10 min, and preincubated in a blocking buffer containing 2% (vol/vol) normal goat serum (NGS) for 2 h at room temperature under gentle rotation. The oocytes_{MII} and embryos were incubated with affinity-purified polyclonal antibodies for KCNK2, KCNK10, KCNK4, KCNK3, and KCNK9, which were diluted 1:200 in PBS containing 0.1% (wt/vol) gelatin, 0.05% (wt/vol) NaN₃, and 1% (vol/vol) NGS for 2 h at room temperature. The germ cells were washed in PBS containing 0.05% (vol/vol) Tween-20 (Bio-Rad), which was followed by a 2-h incubation with FITC-conjugated anti-rabbit IgG that was diluted 1:200 in PBS containing 1.0% (vol/vol) NGS in the dark and at room temperature. The cells were washed in PBS, placed on pre-cleaned slides coated with gelatin, and wet-mounted on glass slides with ProLong Gold Antifade Reagent (Invitrogen). The stained cells were observed using a confocal laser scanning microscope (CLSM) equipped with a 40× objective (IX-81, Olympus). The primary antibodies were purchased from Chemicon (Billerica, MA, USA; KCNK9) and Alomone Labs (Jerusalem, Israel; KCNK3, KCNK2, TREK2, and KCNK4). The negative and positive controls (NC and PC) were checked by omitting the primary antibody and by transfection of the plasmid DNA, respectively. No staining of green fluorescence was observed on any of the NC cells (Supplementary Figure 1 and Figure 2, see section on supplementary data given at the end of this article).

Recording of the whole-cell current

Recording of whole-cell currents was performed using a patch clamp amplifier (Axopatch 200, Axon Instruments, Union City, CA, USA). The patch pipettes had a resistance of 4–5 MΩ when filled with a pipette solution. The membrane potential was held at −80 mV and depolarizing voltage pulses of 1 s were then applied to voltages between −120 and +60 mV. All of the recordings were performed at room temperature. The pipette solution contained (mM): 150 KCl, 1 MgCl₂, 5 EGTA, and 10 HEPES, and the bath solution contained (mM): 135 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 5 glucose, and 10 HEPES. The pH was adjusted to 7.3 using HCl or NaOH (KOH), and the whole-cell currents were recorded after canceling the capacitive transients. The whole-cell currents were analyzed with the pCLAMP program (Version 8).

Gene silencing with siRNA of mouse K_{2P} channels

The siRNA against K_{2P} channels (ON-TARGETplus SMARTpool siRNA) was purchased from Thermo Scientific Dharmacon (Lafayette, CO, USA). The siRNA sequences are shown in Table 6. The gene silencing experiments were performed using a micromanipulation system (Narishige, Tokyo, Japan). The microinjection procedures were performed according to previously described methods (Wu *et al.* 2001). The zygotes were placed in a 50 µl drop of HEPES-supplemented M2

Table 6 siRNA target sequences of mouse K_{2P} channels.

Gene name (channel name)	Sequences
<i>Kcnk2</i> (TREK-1)	5'-GUGGAUCAGACAUUGAAUA-3' 5'-UCAGCUAGGAACUAUUAUU-3' 5'-GAGCAUCUAUCUGAACGGU-3' 5'-GCAAAUAGUGGCAGCAAUA-3'
<i>Kcnk10</i> (TREK-2)	5'-CAAUCGAGACGCCAAGAAA-3' 5'-GCAGUCAGAAGAACAUAU-3' 5'-GGAGGCCUGCAAACAGUUA-3' 5'-UAAACGGGCAUCACCCUGUU-3'
<i>Kcnk4</i> (TRAAK)	5'-CAAUAUAGUCUUACACACA-3' 5'-GGGCAUCGGCCACAUCGAA-3' 5'-GCACAGUGACAGCGCGAGU-3' 5'-CUACUUUGUUUAUAGUGACU-3'
<i>Kcnk3</i> (TASK-1)	5'-CAACGUCUAUGCCGAGGUG-3' 5'-GGGCAUCGGUUUCGUGUCG-3' 5'-GGGCAUCCCGCUCACACUA-3' 5'-CCGAGGACGAGAAGCGUGA-3'
<i>Kcnk9</i> (TASK-3)	5'-GAUAACCGAUGAGGAGCUA-3' 5'-AUGAUGAUGUCGUAUA-3' 5'-GGAGAAACUUAAGCCGAA-3' 5'-GCGCAACACUGAAGUUUCU-3'

medium under paraffin oil. A picoliter injector (PLI-100; Harvard Medical Systems Corp., Greenvale, NY, USA) was used to inject the siRNAs into the cytoplasm of zygotes by pneumatic pressure. The zygotes were injected with ~10 pl of each siRNA (5 nM) and were cultured in the KSOMaa medium for 5 days. The scrambled siRNA (NC, ON-TARGET Non-targeting Pool, Thermo Scientific Dharmacon) was also injected and compared. After a 6-h incubation, the zygotes were changed with fresh medium and grown for additional days. The effects of gene silencing were determined by immunocytochemistry and by whole-cell current recording in mouse embryos (Supplementary Figure 2). In addition, western blot analysis in a B35 mouse neuronal cell line was also performed (Supplementary Figure 1). The siRNA against each K_{2P} channel did not affect the expression of the other K_{2P} channels examined in this study.

Western blot analysis

B35 cells were cultured in DMEM supplemented with 10% FBS and antibiotics for 2 days. The B35 cells were homogenized in a protein extraction solution (PRO-PREP; iNtRON Biotechnology, Inc., Seongnam, Korea) containing 50 mM Tris–Cl (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol, 0.5% NP-40, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM EDTA, 1 µM leupeptin, 1 µM pepstatin A, 1 mM phenylmethylsulfonyl fluoride, and 0.1 µM aprotinin. These mixtures were then incubated for 30 min on ice with intermittent vortexing. The extracts were clarified by centrifugation at 13 000 r.p.m. (16 609 g, Micro 17TR; Hanil, Inchun, Korea) for 20 min at 4 °C. The resulting supernatant was separated using a 10% SDS–polyacrylamide gel and was transferred onto a PVDF membrane for 30 min via semi-dry transfer (Bio-Rad). The membranes were then blocked with 5% fat-free dry milk and incubated with K_{2P} channel polyclonal primary antibodies (1:200 dilution) and α-tubulin MAB (1:10 000 dilution).

The membranes were subsequently incubated with a secondary peroxidase-conjugated anti-rabbit or anti-mouse antibody at 1:10 000. Immunopositive bands were visualized by ECL (ECL Plus kit; ELPIS, Taejon, Korea) and performed according to the manufacturer's instructions.

Measurement of pH_i

The 2-cells were incubated directly with 20 μ M SNARF-1 (a pH indicator, carboxylic acid acetate succinimidyl ester; Invitrogen) for 30 min, and fluorescence was detected using single-excitation/dual-emission fluorescence ratios under a CLSM (Olympus). The wavelength settings were excitation at 488 nm and emission at 540–590 nm for channel 1 and 610–700 nm for channel 2. A cytoplasmic region of the cells was randomly chosen, and the ratio of channel 2/channel 1 in this region was calculated. To standardize the pH_i, the cells were incubated with 10 μ M nigericin (a K^+ /H⁺ exchanger that results in the equilibration of pH_e and pH_i) in addition to SNARF-1 in PBS with pH values at 0.5 unit intervals, and the ratio of fluorescence to pH was obtained. The intracellular pH can be calculated as a linear relationship between the pH and fluorescence intensity ratios over a pH range of 6.0–8.0.

Measurement of membrane potential changes

The 2-cells on a coverglass-bottom dish were labeled for 20 min by incubating in FBS-free culture media containing the membrane fluorescent probe 3,3'-dilinoylethoxycarbocyanine perchlorate (FAST DiO; Invitrogen) at 37 °C. A stock solution was prepared by mixing DiO and octadecylamine in a 5:1 (wt/wt) ratio. The solution was dissolved in chloroform, heated to 50 °C, precipitated on ice with two volumes of methanol, and pelleted by centrifugation. The pellet was dried and then dissolved in dimethylformamide at 5% (wt/vol), heated to 50 °C, and centrifuged. The cell-labeling solution was added to the FBS-free culture media (5 μ l/ml). The relative fluorescence of intracellular DiO was quantified by the CLSM (Olympus) using the excitation and emission wavelengths of 488 and 530 nm, respectively.

Data analysis and statistical analysis

LightCycler Software 4.0 (Roche) and Fluoview (FV1000, Ver. 1.5, Olympus) were used for the real-time PCR data and immunofluorescence analysis, respectively. The differences among the groups were analyzed using the one-way ANOVA test and *post hoc* comparisons using Tukey's test (SPSS18 software; SPSS, Inc., Chicago, IL, USA). The data are represented as mean $\pm$ s.d. A $P < 0.05$ was considered to be significant.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-11-0225>.

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.

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