수의학석사학위논문

전압의존성 K^+ 채널,
Kv2.1과 Kv3 아형의 세포 내 분포

Subcellular Localization of the Voltage-gated Potassium Channel, the Kv2.1 and Kv3 subfamily

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변 진 호

Master Thesis

Subcellular Localization of the Voltage-gated Potassium Channel, the Kv2.1 and Kv3 subfamily

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지도교수 이소영

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변 진 호

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ABSTRACT

Many ion channel studies have been performed on the plasma membrane's ion channels. However, intracellular organelles' ion channels have been investigated recently. The number of studies on the intracellular ion channels has increased, and the importance of intracellular ion channels is now being recognized. In addition, there are studies being carried out to identify the function of subcellular localized ion channels. In the present study, the subcellular localization of the voltage-gated potassium (Kv) channel, the Kv2.1 and Kv3 subfamily were investigated. The results revealed that Kv2.1, Kv3.1, Kv3.2, Kv3.3 and Kv3.4 are detected in the nucleus and mitochondria. The alteration in Kv channel expression according to the cell density in A549 cells was also observed. The expression of Kv3.1 and Kv3.4 located in the nucleus was significantly increased in a cell density-dependent manner. Kv2.1 located in the membrane also significantly increased according to cell density. In addition, changes in Kv3.3 channels' expression according to differentiation induced by hemin in K562 cells were observed. The expression level of the nuclear Kv3.3 was increased in the early stage of differentiation.

These results demonstrate that the Kv2.1 and Kv3 subfamily were localized not only in the plasma membrane, but also in the nucleus and mitochondria. Furthermore, the subcellular location where the channel expressions were altered was different each other. Although the function of subcellular localized Kv channels is not clear, expression changes imply that

these subcellular localized Kv channels could be associated with the proliferation and differentiation of cancer cells.

Keyword: Voltage-gated potassium channels; Subcellular localization; Differentiation; Cell density; Mitochondria; Nucleus

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INTRODUCTION

Voltage-gated potassium channels

Voltage-gated potassium (Kv) channels are a large group of channels that can transfer potassium ion and are sensitive to voltage change. Kv channels are related to regulate potassium ion transfer and membrane potential in excitable cells (Armstrong, 2003; Jan and Jan, 1997; Pichon et al., 2004; Yellen, 2002), including neurons (Coleman et al., 1999; Misonou et al., 2005) and cardiac cells (Bijlenga et al., 1998; Grunnet et al., 2008). In addition, Kv channels are present not only in excitable cells but also in non-excitable cells. Kv channels are involved in cell migration, wound healing, proliferation, oxygen sensing, and apoptosis (O'Grady and Lee, 2005). In addition, they could affect the regulation of intracellular Ca^{2+} and cell volume (Bertran et al., 1995; Iliev and Marino, 1993).

Characteristics of the Kv2.1 and Kv3 subfamily

The Shaw gene encodes the Kv3 subfamily in drosophila (Wei et al., 1990), rodents, and humans (Jan and Jan, 1990; Perney and Kaczmarek, 1991; Rudy et al., 1991). The Kv3 subfamily has fast activation and deactivation kinetics with high activation thresholds and channels involved in rapid repolarization in neurons, and they have an important role in the fast-spiking neuronal phenotype (Chow et al., 1999; Rudy and McBain, 2001). When Kv3.1 is selectively blocked by low concentrations of tetra-ethyl-ammonium, the

proliferation of neural progenitor cells is increased (Liebau et al., 2006). Moreover, Kv3.1 channels contribute to oxygen sensing in rabbit pulmonary artery smooth muscle (Osipenko et al., 2000). Kv3.3 has been involved in K562 cells' hemin-induced erythroid differentiation (Song et al., 2016). As a novel target in cancer therapy, the blockage of Kv3.4 using 4-aminopyridine inhibited the growth of oral squamous cell carcinoma (Felipe et al., 2006).

The expression of Kv2.1 could be altered by hypoxia in cultured pulmonary artery smooth muscle cells because of the oxygen-sensing property (Dong et al., 2012; Guo et al., 2010). In addition, Kv2.1/Kv9.3 heteromers are ATP-dependent delayed-rectifier K^+ channels in oxygen-sensitive pulmonary artery myocytes (Patel et al., 1997).

Subcellular localization of ion channels

It is well known that ion channels are localized in the plasma membrane. However, several experimental results have revealed that ion channels, which are generally known as plasma membrane-spanning channels, may exist not only in the plasma membrane but also in the intracellular organelles (Leanza et al., 2013; Mazzanti et al., 1990). Potassium channels are no exception, and there are reports demonstrating their intracellular localizations. For instance, Kv1.3 has been found in the nuclei of cancer cells and human brain tissues (Jang et al., 2015); it has also been identified in the inner mitochondrial membrane of T lymphocytes (Szabo et al., 2005). Ca^{2+}-dependent K^+ channel activities were observed in the nuclei of pancreatic acinar cells (Maruyama et al., 1995), and

Ca^{2+}-independent K^+ channels were found in the envelope of nuclei from a rat's cerebral cortex (Draguhn et al., 1997). An ATP-sensitive K^+ channel (K_{ATP}) exists on the nuclear envelope of pancreatic beta cells (Quesada et al., 2002).

It was also discovered that the Kv channel, which is localized in intracellular organelles, could play its own role—for example, the selective Kv1.3 inhibitor margatoxin induces hyperpolarization of the nuclear membrane. The inhibition of Kv1.3 also induces the activation of transcription factors, such as the phosphorylation of the cAMP response element-binding protein (CREB) and c-Fos activation; the inhibition of the transcription factor Sp1 results in a decrease in Kv1.3 expression (Jang et al., 2015). The Bax-mediated inhibition of mitoKv1.3 could lead to the development of hyperpolarization and reactive oxygen species (ROS) which may play multiple roles in apoptosis (Szabo et al., 2008). A blockade of K_{ATP} provokes a Ca^{2+} increase in the nucleoplasm, and this increase induces CREB phosphorylation, which may activate transcription (Quesada et al., 2002).

Purpose of the present study

Most studies of ion channels have been investigated using patch clamp techniques. As a result, most of the experiments using a patch clamp have focused on channels that are localized in the plasma membrane. This may be the reason intracellular localized channels have been investigated less than the plasma membrane-spanning channels. In recent decades, the functions of intracellular channels have been revealed, but further researches are still required (Gomez-

Ospina et al., 2006; Valenzuela et al., 2000). In the present study, the subcellular localization of the Kv2.1 and Kv3 subfamily was investigated since these channels have been less investigated by electrophysiology than other channels and to find their roles in the cells.

MATERIALS AND METHODS

Cell culture

A549 (lung carcinoma cells), HT-29 (colon adenocarcinoma cells), and K562 (leukemia cells) were maintained with an RPMI 1640 medium (Welgene, Gyeongsan-si, South Korea) containing 10% fetal bovine serum (Welgene, Gyeongsan-si, South Korea) and 1% antibiotic-antimycotic solution (Sigma-Aldrich, MO, USA). SH-SY5Y (neuroblastoma) cells were cultured in an MEM medium (Welgene, Gyeongsan-si, South Korea).

Density-dependent cell seeding

A549 cells were seeded into 6-well plates. Low density represents 20~30% cell confluence (Cell seeding numbers: approximately 2 x 10^4), medium density represents 40~60% cell confluence (approximately 4 x 10^4), and high density represents over 80% cell confluence (approximately 1.2 x 10^5). A549 cells were grown until cells reached the desired confluence for experiments.

Subcellular fractionation

Cells were fractionated using a subcellular protein fractionation kit for cultured cells (Thermo scientific, MA, USA), into cytosol, membrane, and nuclear proteins. The cells suspended in cytoplasmic extraction buffer were incubated at 4°C for 10 m and were gently mixed and centrifuged at 500 × g for 5 m at 4°C. The supernatant was then transferred into a new microcentrifuge tube

(cytoplasmic extract). The membrane extraction buffer was added to the pellets and incubated at 4°C for 10 m; it was then gently mixed. The extracts were centrifuged at 3000 × g for 5 m at 4°C, and the supernatant was transferred into a new microcentrifuge tube (membrane extract). The nuclear extraction buffer was added to the pellets and incubated at 4°C for 30 m; it was then gently mixed. The extracts were centrifuged at 5000 × g for 5 m at 4°C, and the supernatant was transferred into a new microcentrifuge tube (nuclear extract). Finally, subcellular extracts were resolved in a 5× sample buffer to load samples into gels for a western blot analysis.

Mitochondria fractionation

Mitochondria were isolated using a mitochondria isolation kit (Life Technologies, Van Allen Way Carlsbad, CA). The isolation was performed following the manufacturer's instructions, and a reagent-based method was used to isolate mitochondria. Cytosol and mitochondrial fractions were used for the western blot assay right after the isolation. The isolated mitochondrial fraction was confirmed with α-tubulin and COX4 protein expression levels as a reference.

Transfection with small interference RNA (siRNA)

Cells were transfected with siRNA-Kv3.1 using Kv3.1 siRNA (Santa Cruz Biotechnology, Texas, USA) and Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA). Transfection was performed following the manufacturer's instructions. The A549 cells (1 x 10^5) were plated in 6-well plates (SPL Life Sciences, Gyeonggi-do, Korea) immediately prior to the transfection step in

RPMI 1640 (Welgene, Daegu, Korea) containing 10% FBS without any antibiotics. After 24 h, the siRNA-Kv3.1 transfected cells were incubated. The incubation time was 72 h.

Probing with control antigen

Before the probing protein transferred membranes overnight with primary antibody, Kv3.2 control antigen reacted with anti-K3.2 (Alomone, Jerusalem, Israel) according to the manufacturer's instructions. An antigen-antibody reaction was performed in 3% skim milk for 1 h.

Western blot analysis

The total protein concentration was measured by a BCA protein assay kit (Pierce, Rockford, IL). The quantified protein was loaded on a 10% SDS-PAGE and then transferred to nitrocellulose membranes (Whatman, Maidstone, Kent). Blocking was performed using a 1X TBS-Tween 20 containing 5% nonfat milk (Difco, Franklin Lakes, NJ); protein transferred membranes were then probed overnight with target protein primary antibodies, such as anti-Kv2.1, anti-Kv3.1, anti-Kv3.3, $Na^{-}K$-ATPase, lamin A, anti-Kv3.2, and anti-Kv3.4. Primary antibodies probed membranes and were incubated with horseradish peroxidase-conjugated goat, anti-rabbit, or anti-mouse secondary antibody (GenDEPOT, Barker, TX) for 1 h; they were visualized using WesternBright™ Quantum™ (Advansta, Menlo Park, CA).

Immunocytochemistry

A549 cells were grown for 24 h, mitochondria staining with mitotracker solution 1 mM (Life Technologies, Van Allen Way Carlsbad, CA), fixed with 4% PFA, permeabilised with 0.2% Triton X-100 (Sigma-Aldrich, St Louis, MO, USA), and incubated overnight at 4°C with 5% donkey serum (final dilution 1:200) and primary antibodies. Double immunolabelling was done using the appropriate Alexa Fluor secondary antibodies diluted 1:200 (Molecular Probes). Samples were mounted with DAPI staining solution (ImmunoBioScience Corp, Mukilteo, WA).

Hemin-induced erythroid differentiation

K562 cells (5 x 10^5 cells) were cultured into a T75 flask (SPL Life Sciences, Gyeonggi-do, Korea) and incubated for 30 m with 50 μM hemin (Sigma, St. Louis, MO) to induce erythroid differentiation.

Statistical analysis

The values were expressed as mean ± standard errors. For analysis of the density dependent alteration experiment, *t*-test was used to comparing two different groups. Statistical significant for the hemin-induced differentiation experiment was determined using Mann Whitney U test. P-values of less than 0.05 were considered to be statistically significant.

RESULTS

Confirmation of antibody specificity in A549 cells

Before the western blot analysis, it was confirmed that which band represented the target channel proteins in the western blot membrane. To confirm the band size of Kv3.1, siRNA transfection was performed. siRNA-Kv3.1 was transfected into the A549 cells and the degree of siRNA transfection was confirmed by western blot analysis (Fig. 1 A). The results demonstrated that Kv3.1 is 57 kDa in size.

Kv3.2 control antigen was used to verify the Kv3.2 protein band size. It was determined that the specific band that disappeared due to an antigen-antibody reaction represented Kv3.2 proteins. According to western blot, Kv3.2 expression was decreased at 75 kDa in the membrane and 60 kDa in the nucleus (Fig. 1 B). Therefore, it was confirmed that the band size of Kv3.1 is 57 kDa and Kv3.2 is 75 and 60 kDa in protein-transferred membrane.

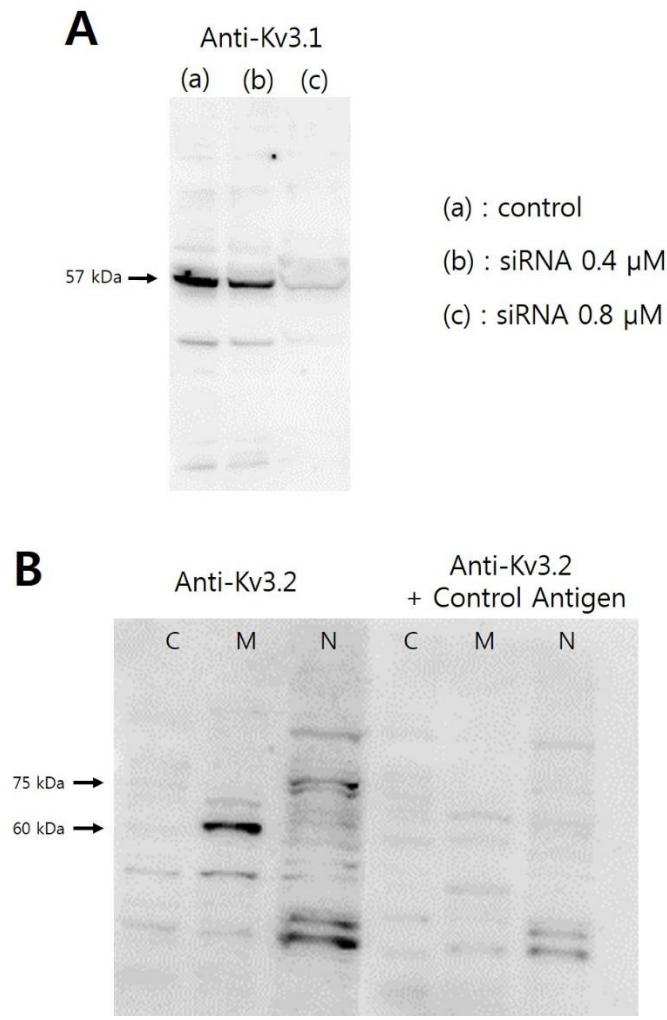


Figure 1. Confirmation of antibody specificity (A) Transfection of siRNA-Kv3.1 in A549 cells. The transfection of small interference RNA was performed on A549 cells to verify the Kv3.1 protein band size. The concentration of siRNA was 0.4 μM and 0.8 μM. Expressions were significantly decreased at 57 kDa, confirming that Kv3.1 antibody was specific to Kv3.1. (B) Probing protein-transferred membranes with control antigen. The control antigen was used to identify the band of Kv3.2 channels. When the control antigen reacted with anti-Kv3.2, the visualized signals of Kv3.2 in the protein-transferred membrane were decreased; it could be confirmed that band size of the Kv3.2 was 60 kDa in the membrane and 75 kDa in the nucleus. (C: cytosol, M: membrane, N: nucleus)

Subcellular localizations of the Kv2.1 and Kv3 subfamily

Cell fractionation (separated into the cytosol, membrane, and nucleus) was performed to identify the subcellular localization of Kv3.1, Kv3.2, Kv3.3, Kv3.4 and Kv2.1 channels in A549, HT-29, K562, and SH-SY5Y cells. Subcellular fractionation was confirmed using Na-K-ATPase (a membrane marker) and lamin A (a nuclear marker) as cell fraction markers. Kv3.1 was observed in the cytosol, membrane and nucleus (Fig. 2). Kv3.2 was localized in the membrane with a band size of 60 kDa and in the nucleus with a band size of 75 kDa (Fig. 3). Kv3.3 was detected in the cytosol, membrane, and nucleus (Fig. 4). Kv3.4 was detected in the cytosol, membrane, and nucleus (Fig. 5). Kv2.1 was also observed in the cytosol, membrane, and nucleus (Fig. 6).

Mitochondria fractionation was also performed (separated into cytosol and mitochondria) to verify the mitochondrial localization of Kv3.1, Kv3.2, Kv3.3, Kv3.4 and Kv2.1 channels in A549 cells. Mitochondria fractionation was confirmed using α-tubulin (a cytosol marker) and COX4 (a mitochondria marker) as the cell fraction marker. According to the data (Fig. 7), Kv3.1, Kv3.2, Kv3.3 Kv3.4 and Kv2.1 channels were observed in the mitochondria.

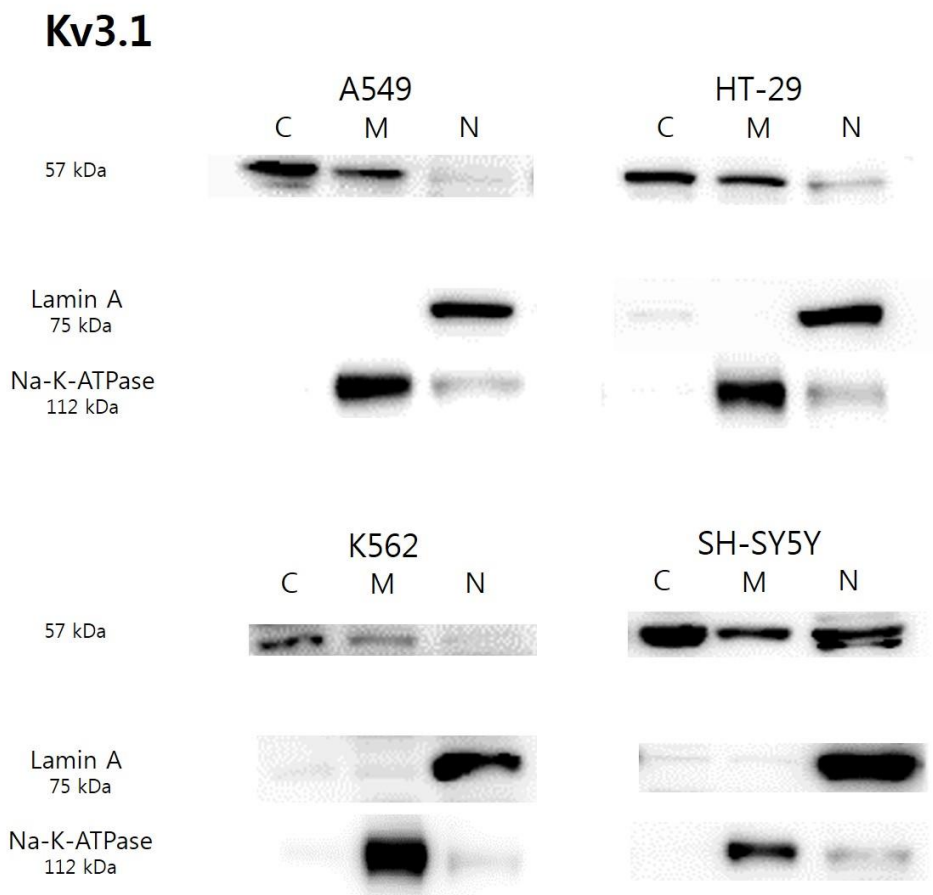


Figure 2. Subcellular localization of Kv3.1 in A549, HT-29, K562, and SH-SY5Y cells. Four cell lines were fractionized to confirm the subcellular localization of Kv3.1. Kv3.1 was detected in the cytosol, membrane, nucleus of A549, HT-29, K562, and SH-SY5Y cells. Cell fractionation was confirmed using Na-K-ATPase (a membrane marker) and lamin A (a nuclear marker). (C: cytosol, M: membrane, N: nucleus)

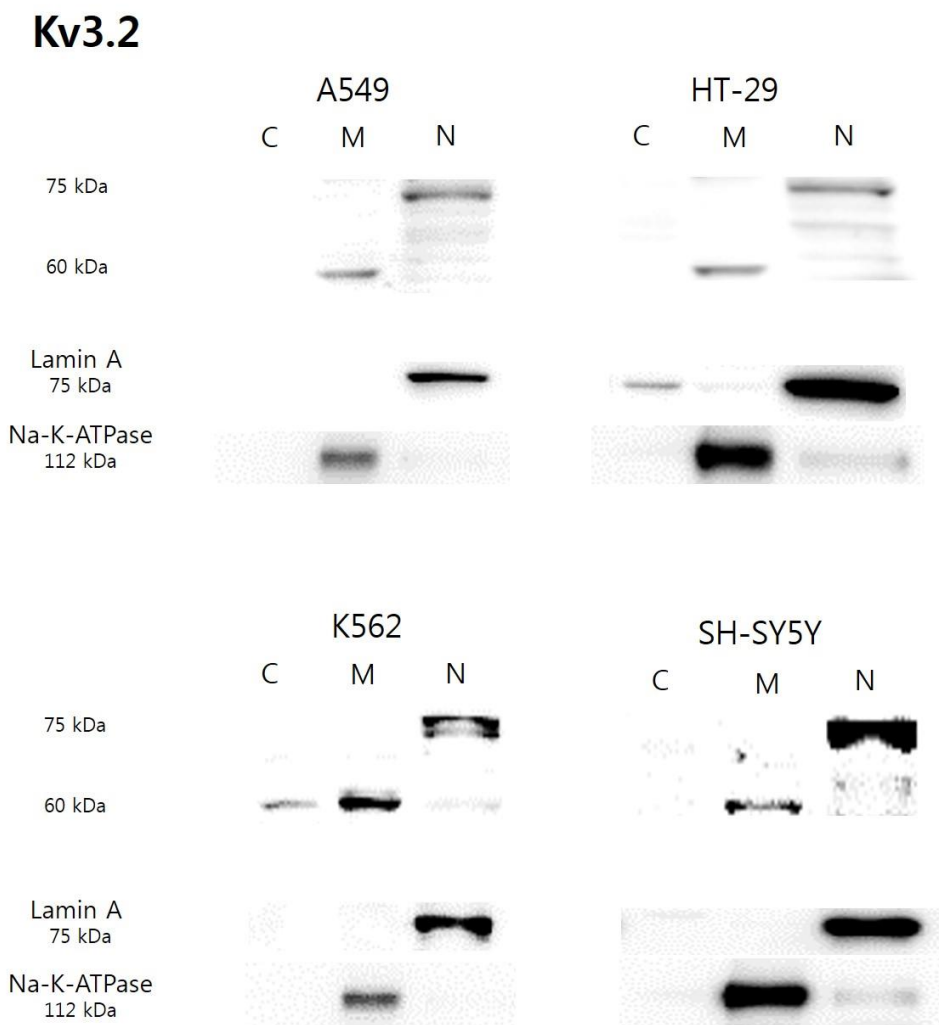


Figure 3. Subcellular localization of Kv3.2 in A549, HT-29, K562, and SH-SY5Y cells. Kv3.2 channels were found in the membrane with the band size of 60 kDa and in the nucleus with the band size of 75 kDa of A549, HT-29, K562, and SH-SY5Y. Cell fractionation was confirmed using Na-K-ATPase (a membrane marker) and lamin A (a nuclear marker). (C: cytosol, M: membrane, N: nucleus)

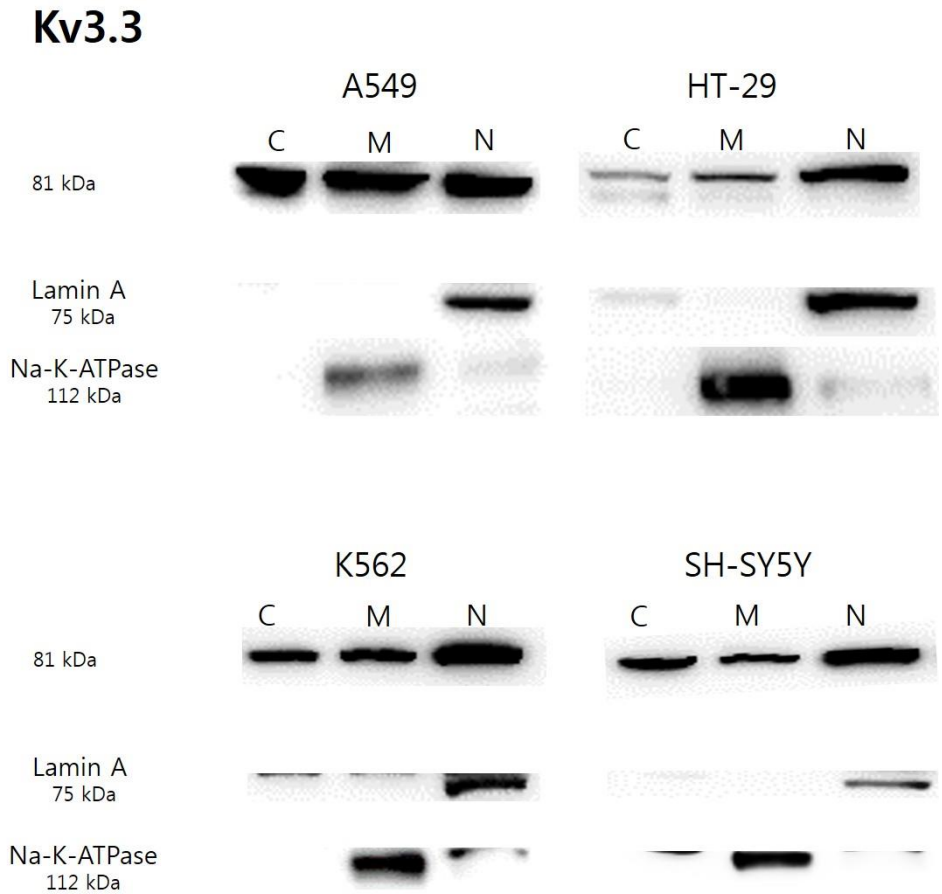


Figure 4. Subcellular localization of Kv3.3 in A549, HT-29, K562, and SH-SY5Y cells. Protein expression of Kv3.3 was detected in the cytosol, membrane, and nucleus of A549, HT-29, K562, and SH-SY5Y. Cell fractionation was confirmed using Na-K-ATPase (a membrane marker) and lamin A (a nuclear marker). (C: cytosol, M: membrane, N: nucleus)

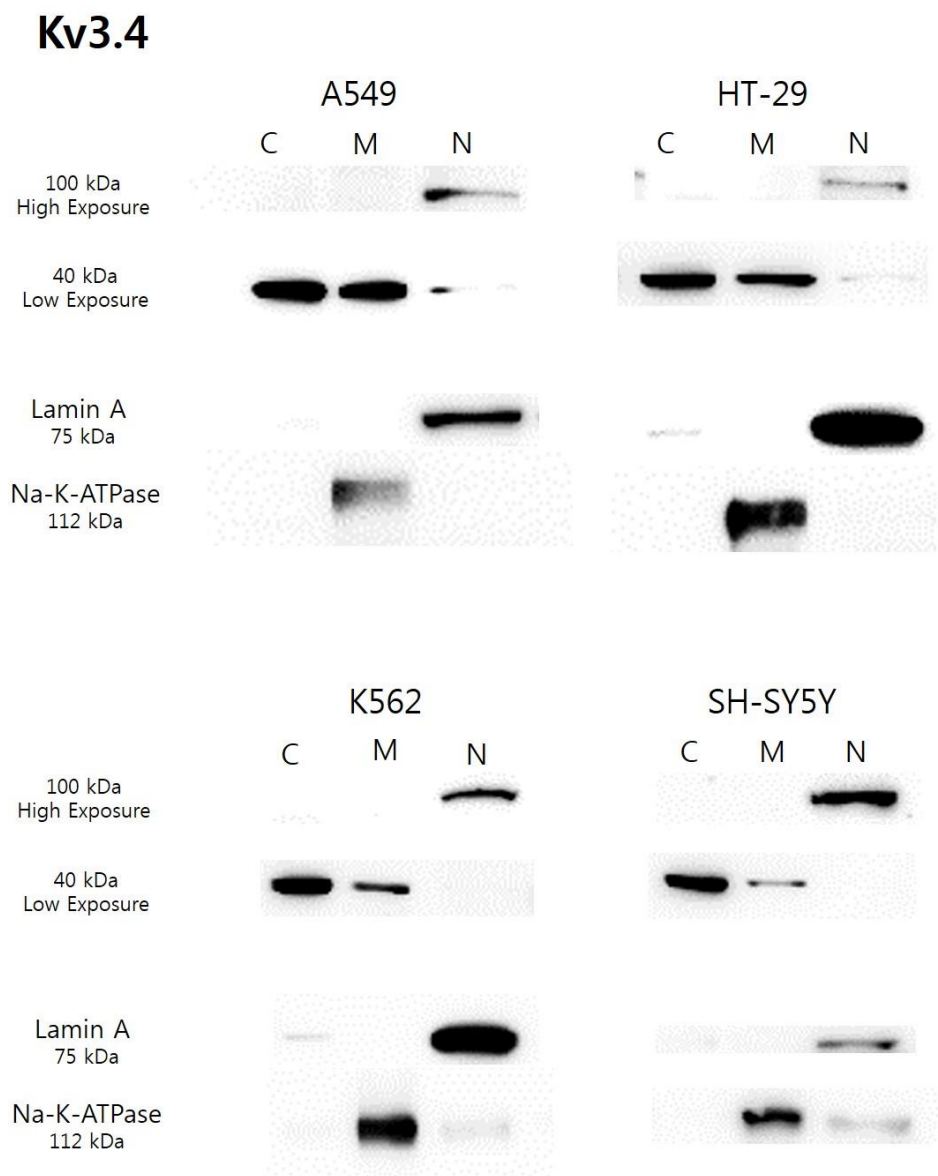


Figure 5. Subcellular localization of Kv3.4 in A549, HT-29, K562, and SH-SY5Y cells. The western blot images of Kv3.4 using subcellular fractionation demonstrate that Kv3.4 is localized in the cytosol, membrane and nucleus of A549, HT-29, K562, and SH-SY5Y. Cell fractionation was confirmed using Na-K-ATPase (a membrane marker) and lamin A (a nuclear marker). (C: cytosol, M: membrane, N: nucleus)

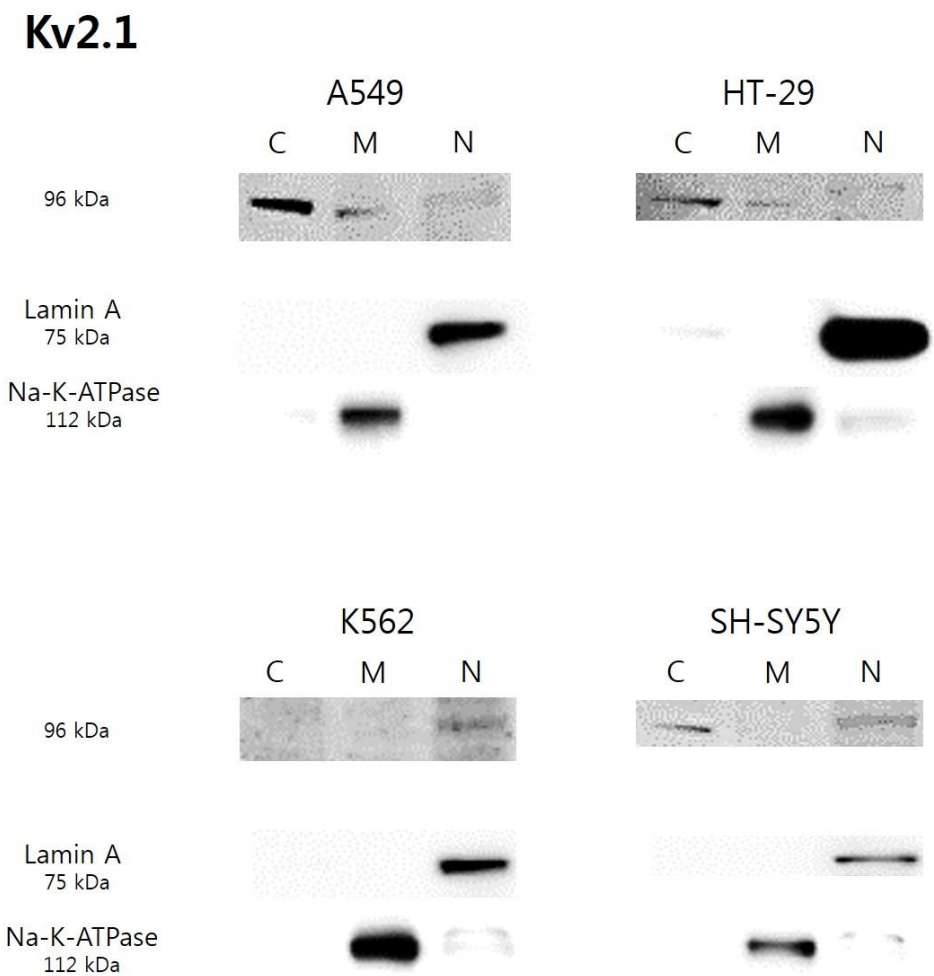


Figure 6. Subcellular localization of Kv2.1 in A549, HT-29, K562, and SH-SY5Y cells. Kv2.1 was observed in the cytosol, membrane and nucleus of A549, HT-29, K562, and SH-SY5Y. Cell fractionation was confirmed using Na-K-ATPase (a membrane marker) and lamin A (a nuclear marker). (C: cytosol, M: membrane, N: nucleus)

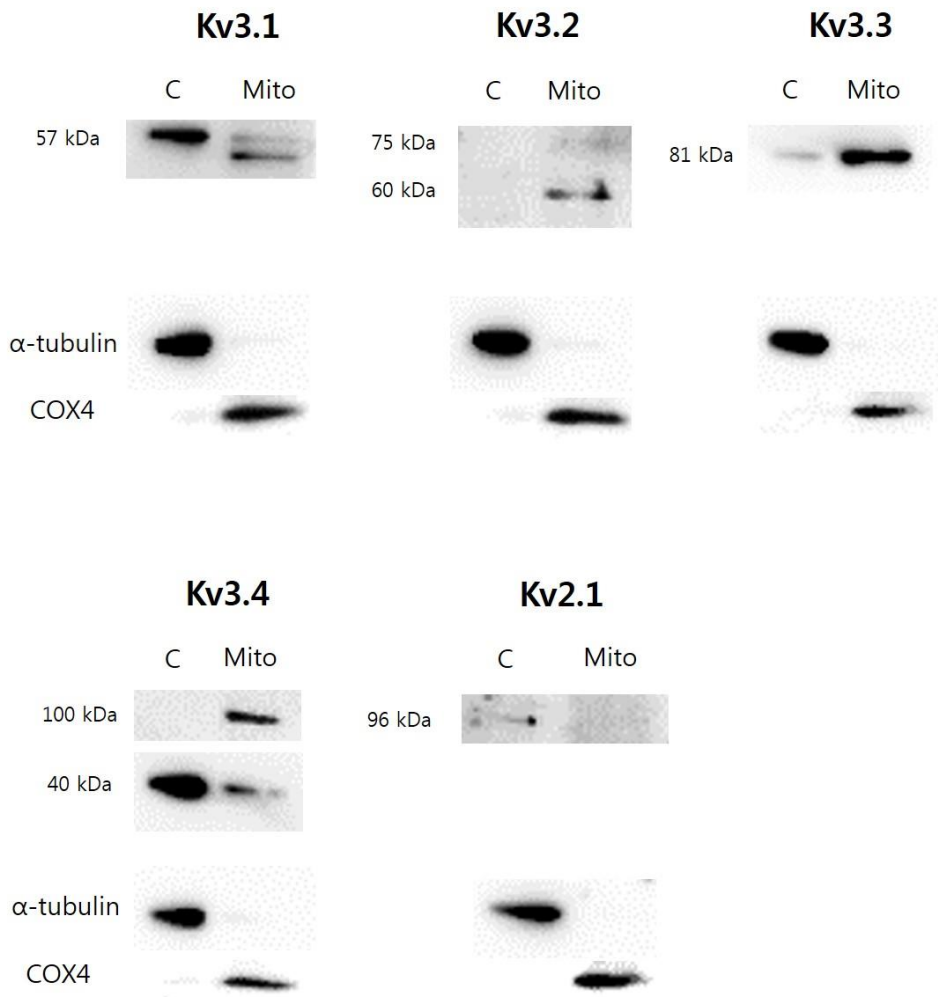


Figure 7. Mitochondria localization of Kv3.1, Kv3.2, Kv3.3, Kv3.4 and Kv2.1 in A549 cells. Kv3.1, Kv3.2, Kv3.3, Kv3.4 and Kv2.1 were observed in the mitochondria. Mitochondria fractionation was confirmed using α-tubulin (a cytosol marker) and COX4 (a mitochondria marker). (C: cytosol, Mito: mitochondria)

Immunocytochemistry

Immunocytochemistry was performed to identify the localization of Kv3.1, Kv3.2, Kv3.3, Kv3.4, and Kv2.1 channels as the target antigen by immunocytochemistry using A549 cells. The immunoreactivity of Kv3.1 (Fig. 8) was most intense in the nucleus region and slightly less in the mitochondria. Similar to Kv3.1, the signal of Kv3.2 (Fig. 9) overlapped the nucleus and mitochondria. Although Kv3.3 (Fig. 10) was mainly observed in the nucleus region, it was detected to some extent in the mitochondria and plasma membrane as well. Kv3.4 (Fig. 11) was detected in the mitochondria, plasma membrane, and nucleus. The signal of Kv2.1 (Fig. 12) was detected in the nucleus, plasma membrane and mitochondria.

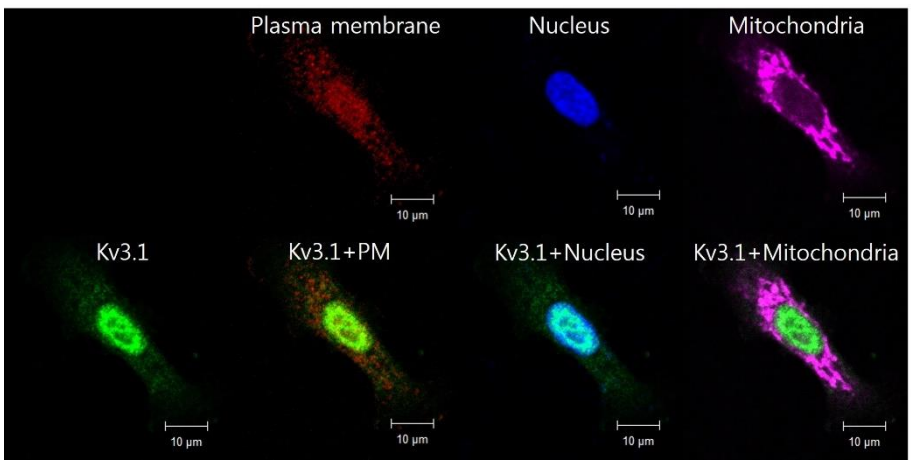


Figure 8. Immunocytochemistry of Kv3.1 in A549 cells. The signal of Kv3.1 channels was co-localized with the signal of the plasma membrane, nucleus and mitochondria. Kv3.1 channels labeled with anti-Kv3.1 antibody are shown in green. The plasma membrane labeled with Na-K-ATPase is shown in red. The mitochondria labeled with mitotracker is in magenta, and the nucleus labeled with DAPI is in blue. (PM: plasma membrane)

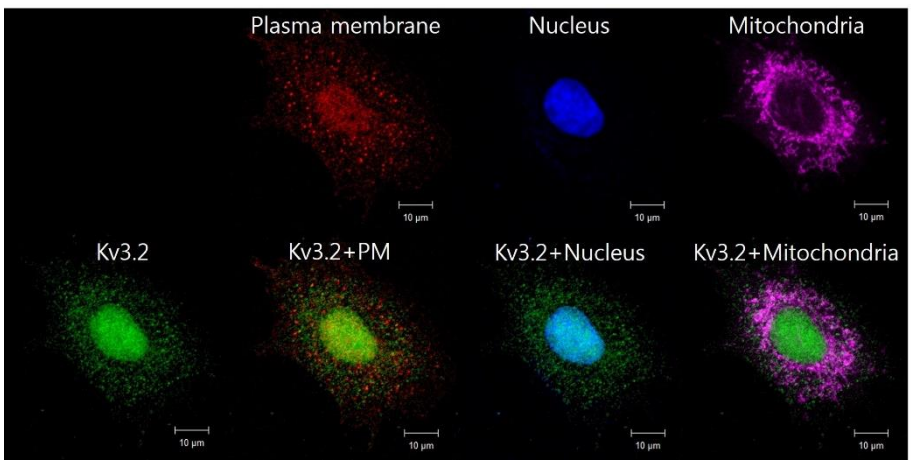


Figure 9. Immunocytochemistry of Kv3.2 in A549 cells. The signal of Kv3.2 channels was co-localized with the signal of the plasma membrane, nucleus and mitochondria. Kv3.2 channels labeled with anti-Kv3.2 antibody are shown in green. The plasma membrane labeled with Na-K-ATPase is in red. The mitochondria labeled with mitotracker is in magenta, and the nucleus labeled with DAPI is in blue. (PM: plasma membrane)

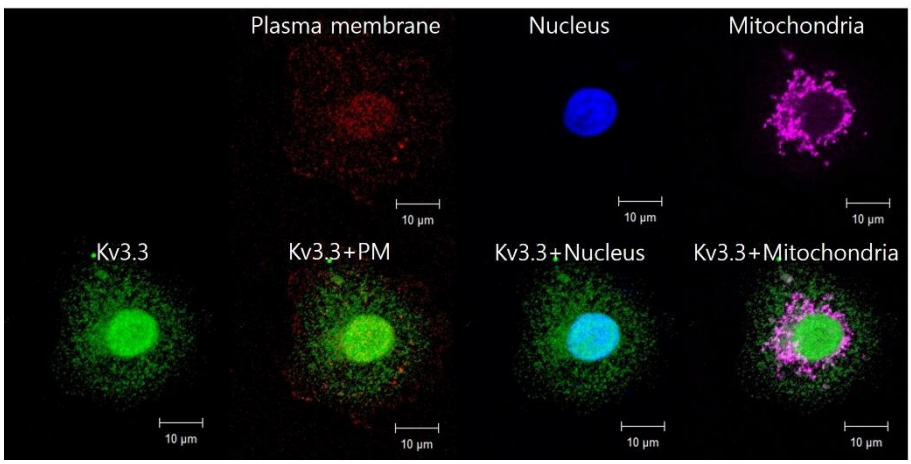


Figure 10. Immunocytochemistry of Kv3.3 in A549 cells. The signal of Kv3.3 channels was co-localized with the signal of the plasma membrane, nucleus and mitochondria. Kv3.3 channels labeled with anti-Kv3.3 antibody are shown in green. The plasma membrane labeled with Na-K-ATPase is in red. The mitochondria labeled with mitotracker is in magenta, and the nucleus labeled with DAPI is in blue. (PM: plasma membrane)

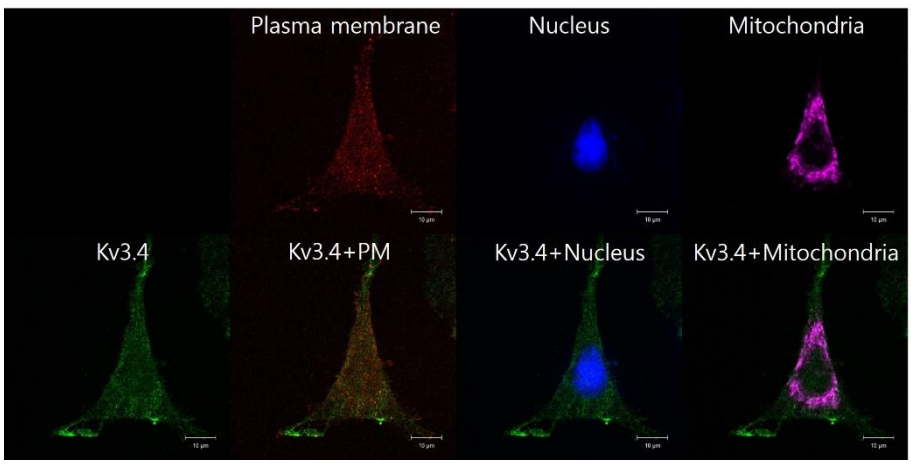


Figure 11. Immunocytochemistry of Kv3.4 in A549 cells. The signal of Kv3.4 channels was co-localized with the signal of the plasma membrane, nucleus and mitochondria. Kv3.4 channels labeled with anti-Kv3.4 antibody are in green. The plasma membrane labeled with Na-K-ATPase is in red. The mitochondria labeled with mitotracker is in magenta, and the nucleus labeled with DAPI is in blue. (PM: plasma membrane)

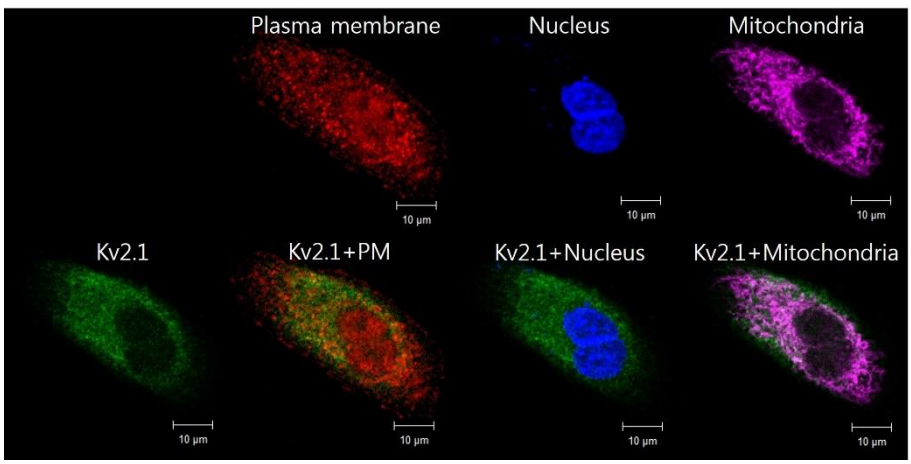
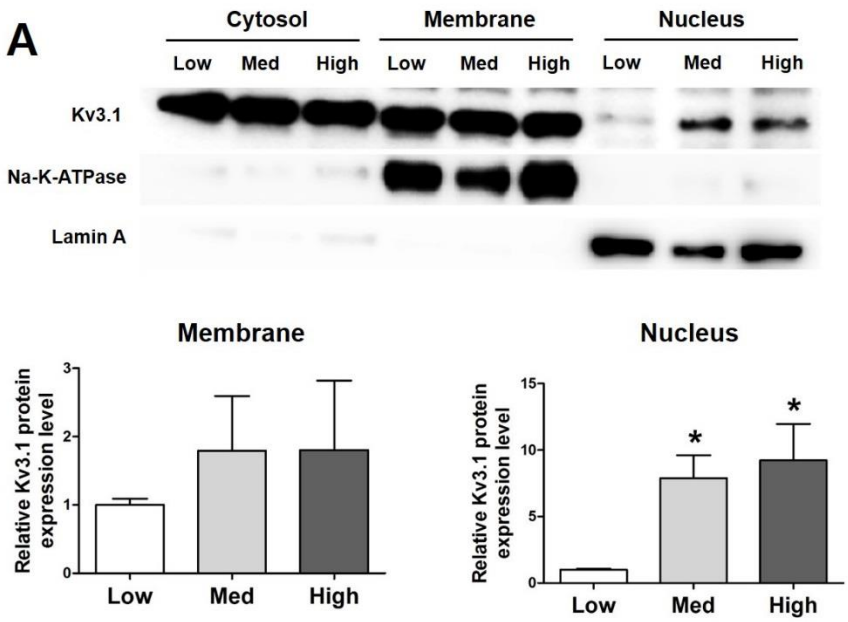
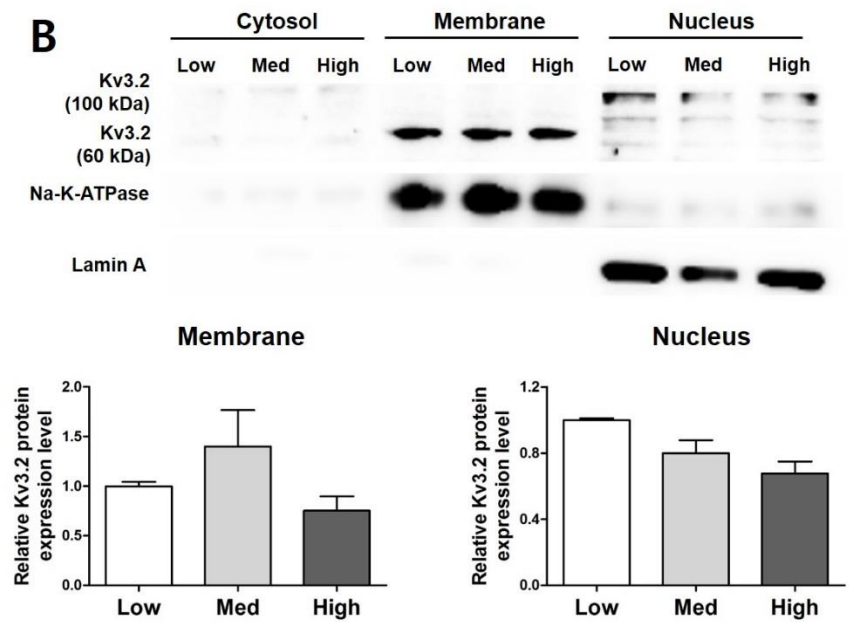


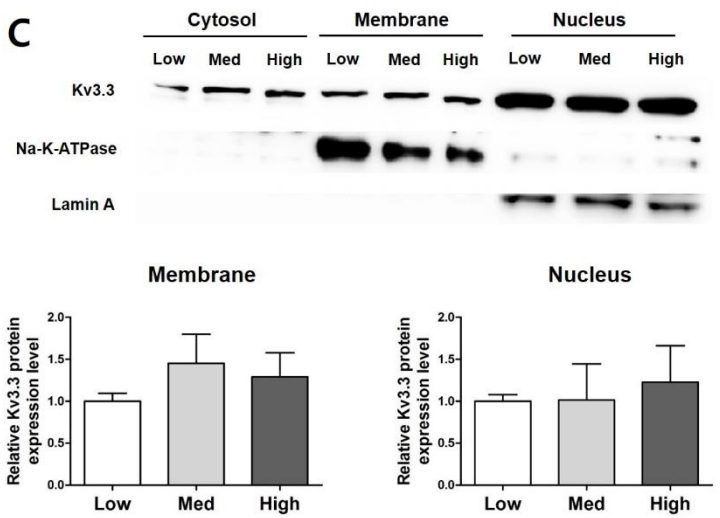
Figure 12. Immunocytochemistry of Kv2.1 in A549 cells. The signal of Kv2.1 channels was co-localized with the signal of the plasma membrane, nucleus and mitochondria. Kv2.1 channels labeled with anti-Kv2.1 antibody are shown in green. The plasma membrane labeled with Na-K-ATPase is in red. The mitochondria labeled with mitotracker is in magenta, and the nucleus labelled with DAPI is in blue. (PM: plasma membrane)

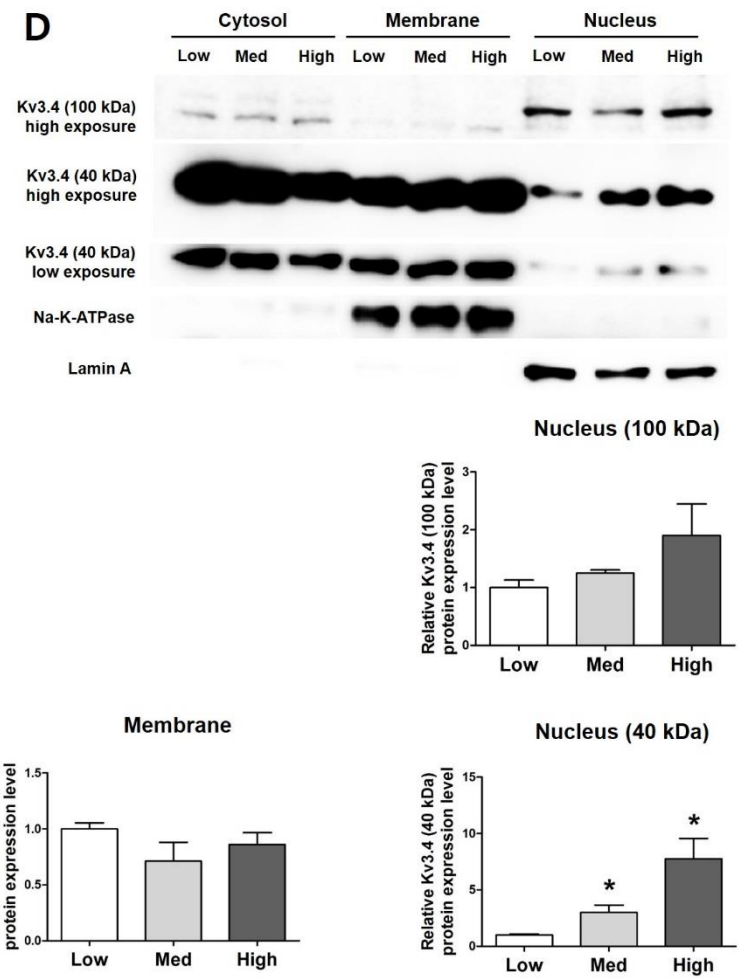
Subcellular localization-dependent alteration of the Kv3 subfamily and Kv2.1 in A549 cells

Next, A549 cells were cultured in three different cell densities (low, med, and high) and subjected cell to fractionation. It was found that Kv3.1 (57 kDa) was cell-density dependently increased in the nuclear fraction (med: 7.89-fold, high: 9.23-fold), whereas Kv3.1 was not altered according to the cell density in the membrane fraction (med: 1.79-fold, high: 1.80-fold). The protein expression of Kv3.2 and Kv3.3 in the membrane and nuclear fraction were not significantly altered during the cell density increment. As with Kv3.1, Kv3.4 (40 kDa) was also increased in the nuclear fraction (med: 2.99-fold, high: 7.77-fold) but not altered according to the cell density in the membrane fraction (med: 0.71-fold, high: 0.86-fold). Interestingly, Kv3.4 (100 kDa), which was observed only in the nuclear fraction, did not show any significant expression changes (med: 1.24-fold, high: 1.90-fold). It was also checked the subcellular expression changes of Kv2.1, which is a well-investigated oxidation-sensitive Kv channel, according to its cell density. It was found that the expression of Kv2.1 in the membrane fraction was increased significantly when the A549 cells were cultured at a high density compared to the cells at a low density (med: 1.87-fold, high: 15.71-fold), whereas Kv2.1 in the nuclear fraction did not show any alterations according to the cell density (med: 1.25-fold, high: 0.98-fold) (Fig. 13).


A
Cytosol
Membrane
Nucleus
Low
Med
High
Kv3.1
Na-K-ATPase
Lamin A
Membrane
Relative Kv3.1 protein expression level
Low
Med
High
Nucleus
Relative Kv3.1 protein expression level
*
*
Low
Med
High


B
Cytosol
Membrane
Nucleus
Low
Med
High
Kv3.2 (100 kDa)
Kv3.2 (60 kDa)
Na-K-ATPase
Lamin A
Membrane
Relative Kv3.2 protein expression level
Low
Med
High
Nucleus
Relative Kv3.2 protein expression level
Low
Med
High


C
Cytosol
Membrane
Nucleus
Low
Med
High
Kv3.3
Na-K-ATPase
Lamin A
Membrane
Nucleus
Relative Kv3.3 protein expression level
2.0
1.5
1.0
0.5
0.0
Low
Med
High


D
Cytosol
Membrane
Nucleus
Low
Med
High
Kv3.4 (100 kDa) high exposure
Kv3.4 (40 kDa) high exposure
Kv3.4 (40 kDa) low exposure
Na-K-ATPase
Lamin A
Nucleus (100 kDa)
Relative Kv3.4 (100 kDa) protein expression level
3
2
1
0
Low
Med
High
Membrane
Relative Kv3.4 (40 kDa) protein expression level
1.5
1.0
0.5
0.0
Low
Med
High
Nucleus (40 kDa)
Relative Kv3.4 (40 kDa) protein expression level
15
10
5
0
*
*
Low
Med
High

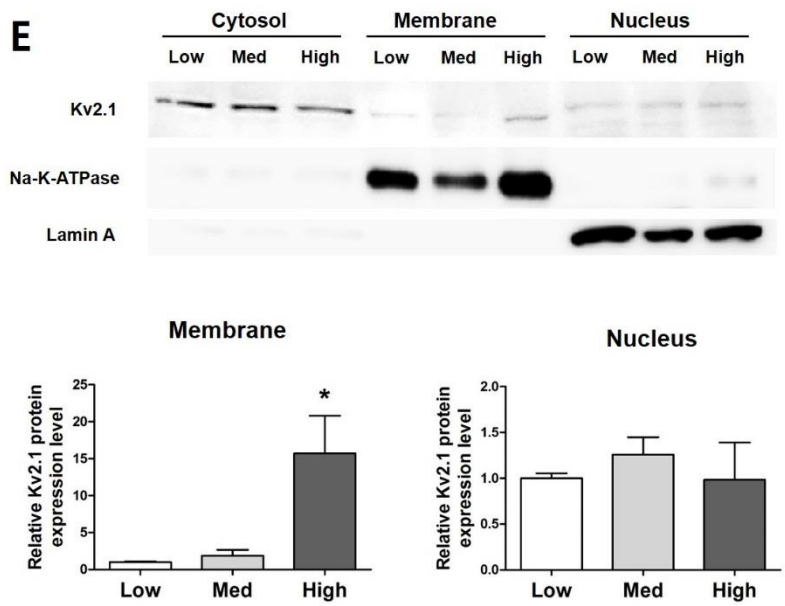


Figure 13. Subcellular expression's alterations of the Kv3 subfamily and Kv2.1 according to the cell density in A549 cells. A549 cells were fractionized into the cytosol, membrane, and nucleus. Kv3.1 (57 kDa) and Kv3.4 (40 kDa) in the nuclear fraction were significantly increased in a cell density-dependent manner, whereas Kv3.1 and Kv3.4 in the membrane fraction did not show any changes in their expression level according to their cell density. Cell density did not have any effect on Kv3.4 (100 kDa), which was only detected in the nuclear fraction. Kv3.2 (60 kDa) in the membrane fraction and Kv3.3 (81 kDa) in the membrane and nuclear fractions were not altered by cell density. Kv2.1 in the membrane fraction increased significantly when the A549 cells were cultured at a high density compared to the cells at a low density. In contrast, Kv2.1 in the nuclear fraction did not show any alterations according to the cell density. Relative protein expressions of the Kv3 subfamily were normalized to the lamin A for the nuclear fraction or the Na-K-ATPase for the membrane fraction, and they were expressed as a fold change relative to the low-density group. All experiments were performed in triplicate, and data represented the mean ± standard error. *$p<0.05$ versus the low-density value.

Hemin-induced K562 differentiation

This investigation was performed to observe the alteration of ion channel expression in the membrane and nucleus according to hemin-induced K562 differentiation. The expression of nuclear Kv3.3 in the hemin-treated group was 1.87-fold higher than in the control group (Fig. 14). On the other hand, the expression of membrane Kv3.3 in the hemin-treated group was 0.87-fold change relative to the control group.

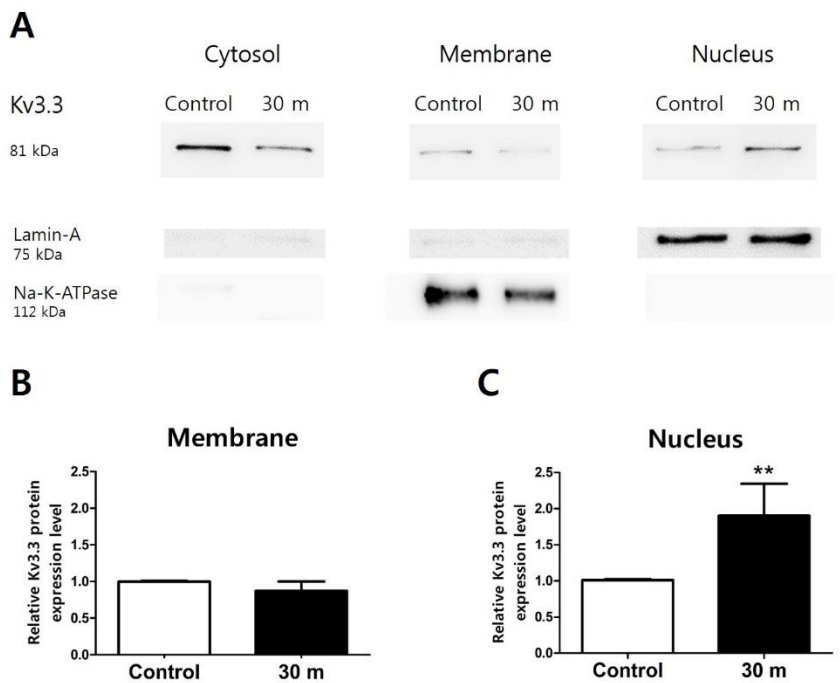


Figure 14. Alteration of Kv3.3 expression in hemin-induced K562 differentiation. The protein expression level of Kv3.3 in the membrane and nucleus was estimated after inducing 30 m of differentiation. In the nucleus, the relative protein expressions of the Kv3.3 were expressed as a 1.87-fold change relative to the control cells. In the membrane, on the other hand, the relative protein expressions of the Kv3.3 were expressed as a 0.87-fold change relative to the control cells. Cell fractionation was confirmed using Na-K-ATPase (a membrane marker) and lamin A (a nuclear marker) All experiments were performed in sextuplicate, and data represented the mean ± standard error. *$p<0.05$ versus the control group value.

DISCUSSION

Ion channels are widely known for existing in the plasma membrane. Since it was found that ion channels exist in the intracellular organelle, studies exploring intracellular channels have been increasing in number, and the function of intracellular channels has been getting more attention (Gomez-Ospina et al., 2006; Jang et al., 2015; Valenzuela et al., 2000; Xu et al., 2015)

To my knowledge, there are no data about the intracellular localization and the nuclear expression of the Kv3 subfamily and Kv2.1, but there are data on the nuclear localization of Kv1.3 and Kv10.1 (Chen et al., 2011; Jang et al., 2015). According to the results, the nuclear localization of the Kv3 subfamily and Kv2.1 in the A549, HT-29, K562, and SH-SY5Y cells was found and the Kv3 subfamily and Kv2.1 were also localized in the mitochondria in the A549 cells. Finally, immunocytochemistry verified the subcellular localization of the Kv3 subfamily and Kv2.1. It was found that Kv3.1 existed in the nucleus and mitochondria; Kv3.2 also existed in the nucleus and mitochondria. Kv3.3 was localized in the nucleus and mitochondria. Kv3.4 was less detected in the nucleus and mainly expressed in the mitochondria and plasma membrane. In western blot analysis, Kv3.4 was observed in the nucleus with protein sizes of 40 and 100 kDa, whereas in the cytosol and membrane, Kv3.4 was found with a protein size of 40 kDa only. The size of Kv3.4 is generally 70 kDa (Kanda et al., 2011; Song et al., 2017), but Kv3.4 could be glycosylated or form tetramers with other Kv channels or other accessory channels. In this case, Kv3.4 has a protein size of 100 kDa

(Baranauskas et al., 2003; Song et al., 2017). Furthermore, the Kv3.4 subunit (which could be assembled with other Kv channels) would be detected with a protein size of 40 kDa. For that reason, various sizes of Kv3.4 may be detected in a western blot (Boda et al., 2012; Guo et al., 2008; Miguel-Velado et al., 2010). Kv2.1 was found in the nucleus and mitochondria.

Kv3 channels Kv3.1, Kv3.2, Kv3.3, and Kv3.4 are encoded in the same *Shaw* genes (Butler et al., 1989; Rettig et al., 1992). In addition, each one of the same subfamily channel α-subunit could be co-assembled, and they consequently make a heteromer (Baranauskas et al., 2003). Although they are encoded in the same genes, the data demonstrated that their localizations are different. Therefore, it would be important to verify the localization of ion channels and to investigate the function of each ion channel in the plasma membrane, mitochondria, and nucleus separately.

Furthermore, according to the data, Kv2.1, Kv3.1, and Kv3.4 showed expression alteration patterns that varied dependent on their localization according to their cell density. Cell density affected Kv2.1 in the membrane fraction, whereas Kv3.1 and Kv3.4 in the membrane fraction were not altered according to their cell density. Only nuclear Kv3.1 and Kv3.4 increased in a cell density-dependent manner. Considering that they are oxidation-sensitive Kv channels and the three Kv channels may respond to the oxidative stress induced by a cell density increment, I could assume that (although some channels have the same oxygen sensing functions), they could play their oxygen-sensing roles in a different subcellular localization. Eukaryotic cells have distinct compartments, such as the nucleus, mitochondria, and cytoplasm, and each

compartment has a different redox potential. They respond differently to microenvironmental changes (Go and Jones, 2008; Go and Jones, 2010; Lopez-Mirabal and Winther, 2008). For instance, EGF-stimulated ROS production affects the cytoplasmic redox system but does not have any effect on the mitochondrial or the nuclear redox system (Halvey et al., 2005). In this scheme, different oxidation-sensitive Kv channels may be involved in a different compartment's redox system; therefore, I assume that Kv2.1 may be involved in cytoplasmic redox regulation, whereas Kv3.1 and Kv3.4 may be involved in the nuclear redox system. Further research detailing the exact mechanism is needed.

It was demonstrated that Kv3.3 is involved in hemin-induced K562 differentiation (Song et al., 2016). In the present study, the alteration of Kv3.3 expression after hemin-induced K562 differentiation in the nucleus and membrane was observed. According to the data, the expression of nuclear Kv3.3 was increased after the hemin-induced differentiation. On the other hand, the expression of membrane Kv3.3 was decreased. The Kv3.3 expression level in the early stage (30 m) of hemin-induced differentiation was estimated, and there was no change in entire Kv3.3 expression level compared to the control cells (Song et al., 2016). However, it is observed that the expression of nuclear Kv3.3 was increased as a 1.87-fold change relative to the control cells and the expression of membrane Kv3.3 was similar as 0.87-fold change relative to the control cells. As a result, I can demonstrate that the ion channels of the nucleus may have an important role in differentiation and ion channels could migrate as the occasion demands.

By understanding the localization of the ion channels, I found that some

ion channels in the intracellular organelles have been poorly investigated. As mentioned above, ion channels have been investigated by recording ion currents, which represent plasma membrane currents (Verkhratsky and Parpura, 2014). Ion channels play important roles in the intracellular organelle (Gulbins et al., 2010; Jang et al., 2015; Leanza et al., 2012; Szabo et al., 2008), and as a result, more studies dealing with the intracellular channels would provide a more advanced understanding of channel-related vital phenomena. In addition, targeting intracellular organelles is essential for drug delivery, and investigations have been done in an attempt to target intracellular organelles using biomaterials such as fluorescent plasmid DNA nanoparticles (Costa et al., 2017) and methods like lipophilic cations, mitochondrial targeting signal peptides, and cell-penetrating peptides to selectively target mitochondria (Chen et al., 2016). Therefore, choosing an appropriate drug according to the localization of an ion channel is also important. In fact, there is a report demonstrating that membrane-permeant Kv1.3 inhibitors induce apoptosis by targeting mitochondrial Kv1.3, whereas membrane-impermeant Kv1.3 inhibitors did not affect cell survival (Leanza et al., 2012). Finally, intracellular ion channels could be novel biomarkers and therapeutic targets (Leanza et al., 2014; Peixoto et al., 2010). Cancer-expressing mitoKv1.3 can be eliminated by a membrane-permeable mitoKv1.3 direct-targeting inhibitor (PAP-1 derivatives), which leads to ROS-induced apoptosis (Leanza et al., 2017).

CONCLUSION

In the present study, the subcellular localization of each Kv3 subfamily and Kv2.1 was identified. It was confirmed that Kv2.1 and Kv3 subfamily were localized in the nucleus and mitochondria. Although the alteration of specific channels' expressions was observed according to cell density, the location of the alteration differed. Kv3.1 and Kv3.4 were altered in the nucleus, and Kv2.1 were altered in the membrane. Based on the results, I found that different Kv channels, which may play similar roles (e.g., oxygen sensor), could be at different subcellular localizations. Furthermore, the expression of Kv3.3 channels, which were located in the nucleus, was altered according to hemin-induced K562 erythroid differentiation in the early stage.

The subcellular localization of Kv channels had not been widely studied. However, the subcellular Kv channels is more investigated currently. These results imply that the investigation of Kv channels' subcellular localizations is important for channel research and that it would provide new insights about Kv channel-related vital phenomena, such as tumor proliferation and differentiation.

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국문초록

전압의존성 K^+ 채널,
Kv2.1과 Kv3 아형의 세포 내 분포

변 진 호

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지도교수 이 소 영

그동안 대부분의 이온 채널 연구는 세포막에 존재하는 이온 채널을 대상으로 이루어졌다. 하지만 최근에는 세포막에 존재하는 이온 채널뿐만 아니라 세포 내에 존재하는 이온 채널도 연구되고 있다. 또한 세포 내에 존재하는 이온 채널에 대한 연구는 갈수록 증가하고 있고 그 중요성은 더욱 커지고 있다. 본 연구는 세포 내에 존재하는 전압의존성 K^+ 채널, Kv2.1과 Kv3 아형을 대상으로 연구하였다. 연구결과, 핵과 미토콘드리아에서 Kv2.1, Kv3 아형이 모두 관찰되었다. A549세포에서 세포 밀도에 따른 Kv채널의 발현 변화를 관찰하였는데, 세포 밀도의 증가에 따라 핵에서의 Kv3.1과 Kv3.4의 발현이 유

의하게 증가하였고 세포막에서 Kv2.1의 발현이 현저하게 증가하였다. 추가적으로, hemin에 의한 K562세포의 분화에 따른 Kv3.3의 발현 변화가 핵에서 특이적으로 관찰되었다.

따라서 본 연구 결과를 통해 Kv2.1과 Kv3 아형은 세포막뿐만 아니라 핵과 미토콘드리아에 존재한다는 것을 알 수 있다. 또한 세포 밀도와 분화에 따른 채널의 발현 변화가 관찰되는 세포 내 위치가 채널 별로 다르다는 것을 알 수 있다. 세포 내에 존재하는 Kv채널들의 기능을 밝히기 위해서는 추가적인 실험이 필요하지만, 본 연구는 세포 내에 존재하는 Kv채널들이 암세포의 증식 및 분화 등의 기능에 관련될 가능성을 시사한다.

주요어: 전압의존성 포타슘 채널; 세포 내 위치; 분화; 세포 밀도; 미토콘드리아; 핵

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