수의학석사학위논문

핵에서의 전압의존성 포타슘 채널의 기능적 특성

Functional Roles of Voltage-gated Potassium Channels in the Nucleus

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변 준 규

Master's Thesis

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ABSTRACT

It is widely known that voltage-gated potassium (Kv) channels play a crucial role in the transmission of electrical signals by regulating the potassium that passes through the plasma membrane in excitable cells, such as neurons and cardiac muscle cells. However, recent studies have reported that several Kv channel subunits, including Kv1.3, influence cell proliferation in non-excitable cancer cells.

Herein, Kv1.3 was detected at the nuclear membrane of A549 cancer cells. The effect of nuclear membrane potential with Kv1.3 selective inhibition was changed by collapse in the potassium gradient in isolated nuclei of A549 cells. The membrane-permeable Kv1.3 blocker, PAP-1, was shown to induce the phosphorylation of the cAMP response element-binding (CREB) protein and c-Fos activation in A549 cells. Chromatin immunoprecipitation assay revealed that the Sp1 transcription factor is directly bound to the promoter region of the Kv1.3 gene, and Sp1 regulated Kv1.3 expression in the nucleus of A549 cells. Furthermore, nuclear Kv1.3 was shown to form complexes with nuclear proteins including UBF1. The amino-acid sequence-based predictors demonstrated putative subcellular localization and functional domains of Kv channels and the location of Kv channels was confirmed in A549, MCF7, K562 cells, and human brain tissues using subcellular protein fractionation.

These results demonstrate that nuclear Kv channels could be associated with proliferation of cancer cells by regulating nuclear membrane potential and specific transcription factors such as pCREB and c-Fos. Moreover, nuclear Kv1.3 channels mediated with Sp1 transcription factor may have unknown biological function through interaction of nuclear proteins.

Keywords: Kv1.3, nucleus, Sp1, UBF1, subcellular localization

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CONTENTS

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ABBREVIATIONS

ChIP	Chromosome immunoprecipitation
CRAC	Ca^{2+} release-activated Ca^{2+} channels
CREB	cAMP response element-binding
DBD	DNA-binding domain
KLF	Krüppel-like factor
Kv	Voltage-gated potassium channels
LC-MS/MS	Liquid chromatography tandem mass spectrometry
MgTX	Margatoxin
mTP	Mitochondria targeting peptide
NES	Nuclear export signal
NLS	Nuclear localization signal
PAP-1	5-(4-Phenoxybutoxy) psoralen
pCREB	Phosphorylated cAMP response element-binding
siRNA	Small interfering RNA
Sp1	Specificity protein 1
UBF1	Upstream binding factor 1

INTRODUCTION

Voltage-gated potassium channels and cancer

Voltage-gated potassium K^+ (Kv) channels are known to regulate membrane potential and a variety of cell signals in neuron and muscle fibers, including cardiac cells, and thus, are involved in electrical excitability (Hille, 2001). Expression of Kv channels is not only limited to excitable cells but is also present in epithelial cells contributing to cell functions including electrolyte/nutrient transport, cell proliferation, apoptosis, cell migration, wound healing, modulation of cell volume/pH, and O_2 sensing (Kunzelmann, 2005; O'Grady and Lee, 2005). In addition, it has been previously reported that several subfamilies of Kv channels are associated with the mitotic cell cycle, cell growth, and tumorigenesis in cancer (Felipe et al., 2006). Kv1.1 and Kv4.1 have been found in lung and gastric cancer cell lines (Jang et al., 2011b; Kim et al., 2010) respectively and the inhibition of Kv1.1 in A549 lung cancer cell lines and silencing of Kv4.1 in MKN-45 and SNU-638 gastric cancer cell lines suppress cell proliferation through cell-cycle arrest in the G1-phase (Jang et al., 2011b; Kim et al., 2010). Kv1.3 is overexpressed in lung, prostate, and colon cancers (Abdul and Hoosein, 2002a; Abdul and Hoosein, 2002b; Jang et al., 2011a) and the gene expression of Kv1.3 in human prostate cancer may be used to be biomarker for malignancy grade (Ohya et al., 2009). Kv11.1 is expressed in various cancer cell lines—epithelial, leukemic, and connective tissue—where it affects cell proliferation and apoptosis (Jehle et al., 2011).

Subcellular localization and biological functions of Kv1.3 channels

Kv1.3 channels are located in the plasma membrane of T lymphocytes, where they regulate membrane potential, Ca^{2+} signaling, cell volume, proliferation, and autoimmune diseases (Hu et al., 2012; Matheu et al., 2008). Kv1.3 channels contribute to olfactory development/function (Fadool et al., 2004) and participate in the control of insulin-stimulated glucose uptake in adipose tissue and skeletal muscle (Xu et al., 2004) through their effects on membrane potential. It is noteworthy that Kv1.3 has been identified in the mitochondrial inner membrane of lymphocytes, hippocampus, and cancer cells (Bednarczyk et al., 2010; Leanza et al., 2012; Szabo et al., 2005). Szabo et al. (2008) demonstrated that expression of Kv1.3 in mitochondria contributes to apoptotic signaling by binding pro-apoptotic Bax protein, resulting in alteration of mitochondrial membrane potential. We have been previously confirmed that Kv1.3 channels exist in the nuclei of A549, MCF7, SNU484 cancer cells, and human brain tissues. Furthermore, Kv1.3 blocker, MgTX suppresses A549 proliferation by inhibiting the G1-S cell cycle transition (Jang et al., 2011a). Because Kv1.3 channels are involved in cell proliferation, apoptosis, and the progression of cancer (Jang et al., 2011a; Leanza et al., 2012), Kv1.3 in the nuclear membrane as well as the plasma membrane may lead to biological signal mechanisms related to cell proliferation, and thus, could be used as a novel target protein for anticancer therapy.

Sp1 transcription factor and Kv channel expression regulators

Specificity protein 1 (Sp1) is a ubiquitous nuclear protein that is a member of the Sp1/KLF (Krüppel-like factor) family of transcription factors (Wierstra, 2008). Sp1 serves as an activator of housekeeping genes and TATA-less genes; it has a DNA-

binding domain (DBD) with three Cys2His2-type zinc fingers so that it binds to GC-rich promoter regions (Wierstra, 2008). Sp1 is closely related to cell growth, proliferation, and apoptosis through regulation of basal constitutive genes (Black et al., 1999; Deniaud et al., 2006; Grinstein et al., 2002). In particular, Sp1 is associated with invasive and metastatic tumors, and tumor growth and Sp1 expression levels control lung tumor progression (Black et al., 2001; Hsu et al., 2012). In the early stage of lung tumor growth, Sp1 is highly upregulated; in later stages, Sp1 decreases with metastasis and invasiveness.

It has been reported that Sp1 upregulates Kv1.5 (Fountain et al., 2007) and Kv4.3 (Qi et al., 2012), which have been known to have GC-rich nucleotide sequences in promoter regions. Sp1 transcription factor is bound to the Kv1.5 promoter region CACCC box motif, which is considered an important factor in Kv1.5 expression regulation in cardiovascular physiology (Fountain et al., 2007). HEK-293T cells transformed by large T-antigen increase Kv4.3 channels through upregulation of Sp1 (Qi et al., 2012).

Purpose of the present study

Although the functions of ion channels at the nucleus have been suggested (Matzke et al., 2010), it is not clearly demonstrated whether several types of Kv channels are in the nucleus and these channels could be involved in various signaling pathways. Thus, this study focuses on the biological and functional characteristics of Kv1.3 in the nuclear membrane, which include 1) regulation of nuclear Kv1.3 expression, 2) activation of transcription factors, and 3) interaction between nuclear Kv1.3 and nuclear proteins. In addition, we investigated subcellular localization of Kv channels using computational prediction tools and subcellular fractionation in A549 and MCF7 cancer cells, K562 leukemia cells, and human brain tissues (Fig. 1).

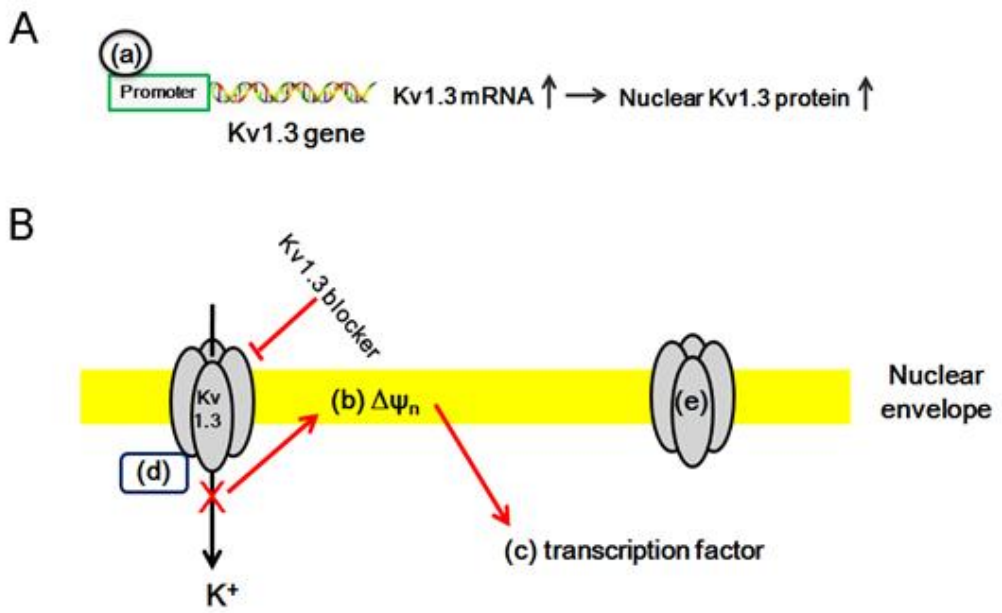


Figure 1. Purpose of the study. We investigated (a) what transcription factor regulates Kv1.3 gene expression, how the inhibition of Kv1.3 channels affects (b) nuclear membrane potential and (c) regulation of transcription factors, (d) whether nuclear Kv1.3 channels are bound to nuclear proteins, and (e) subcellular localization of Kv channels.

MARTERIALS AND METHODS

Cell culture

A549 (human lung adenocarcinoma cell line), MCF7 (human breast adenocarcinoma cell line), and K562 (human myeloid leukemia cell line) cells were purchased from the Korean Cell Line Bank and maintained with RPMI 1640 medium containing 10% fetal bovine serum and 1% antibiotic-antimycotic solution (Sigma) supplemented with 5% CO_2.

Nuclear isolation and nuclear membrane purification

The nucleus of A549 cells was isolated using the method previously reported (Franco-Obregon et al., 2000). A549 cells were incubated in 4 ml of hypotonic solution containing (in mM) 10 KCl, 1.5 $MgCl_2$, 10 HEPES free acid, 0.5 D,L-dithiothreitol, pH 7.9 for 10 min on ice. The cells were harvested by centrifuge for 10 min at 400 X g, 4℃ and homogenized with a ~20 ml Dounce homogenizer in 4 ml of hypotonic solution. Nuclei were deposited by centrifuge for 10 min at 400 X g, 4℃.

Nuclear membrane was purified by high-ionic-strength methods (Kaufmann et al., 1983) from isolated nuclei. Isolate nuclei were suspended in nuclear suspension medium containing (in mM) 250 sucrose, 5 $MgCl_2$, 50 Tris-Cl, pH 7.4, including DNase I and RNase for 1 hr at 4℃. The nuclei were spun at 1000 X g, 4℃ for 10 min and resuspened in the incubation buffer containing (in mM) 0.2 $MgCl_2$, 10 Tris-Cl, pH 7.4 for 15 min at 4℃, adding high-NaCl buffer containing (in mM) 2000 NaCl, 0.2 $MgCl_2$, 10 Tris-Cl, pH 7.4, and 2-mercaptoethanol. The nuclear membrane was harvested by centrifugation at 1600 X g, 4℃ for 30 min.

Measurement of nuclear membrane potential by flow cytometry

Isolated nuclei in 5 ml polystyrene round-bottom tubes (BD Bioscience) were resuspended in intracellular solution containing (in mM) 125 KCl, 2 K_2PO_4, 40 HEPES, 0.1 $MgCl_2$, pH 7.2/100 nM Ca^{2+} with 10.2 EGTA and 1.65 $CaCl_2$ or low K^+ solution containing (in mM) (65, 95, or 125 mM) NMDG-Cl, (60, 30, or 0 mM) KCl, 2 K_2PO_4, 40 HEPES, 0.1 $MgCl_2$, pH 7.2/100 nM Ca^{2+} with 10.2 EGTA and 1.65 $CaCl_2$. And then, the nuclei were treated with 1 nM MgTX and stained with 200 nM 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) (Molecular Probes) at room temperature for 10 min (Quesada et al., 2002). $DiOC_6(3)$ is a lipophilic dye, that can be used to label organelle membranes including nuclei (Koning et al., 1993). The intensity of $DiOC_6(3)$ fluorescence signal was measured using BD FACSCalibur™ (BD Bioscience) and analyzed by Cell quest software.

Western blot analysis

The phosphorylation of CREB and activation of c-Fos were measured in A549 cells treated with 10 nM PAP-1 (Sigma) for 10 min and 30 min, respectively, using western blot analysis. Human brain tissue extracts were purchased from Abcam. Cellular extracts were resolved on a 10% polyacrylamide gel and transferred to PVDF membranes. Membranes were probed with Na,K-ATPase (1:1000), GRP78 BiP (1:1000), lamin A (1:500), Sp1 (1:500), PARP (1:400), Emerin (1:500) (Abcam), Kv1.3 (1:500) (Alomone laboratories), CREB (1:200), phospho-CREB (Ser133) (1:200) (Millipore), c-Fos (K-25) (1:200) and β-actin (1:2000) (Santa Cruz Biotechnology) followed by a horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody (1:5000) (Santa Cruz Biotechnology).

Chromatin Immunoprecipitation

ChIP-IT® Express Enzymatic kit (Activemotif) was used to perform chromatin immunoprecipitation (ChIP) according to a modification of the manufacturer's instructions using A549 cells. Briefly, approximately 1.3×10^7 cells were fixed with 1% formaldehyde for 5 min at room temperature and washed in ice-cold 1 x PBS. Isolated chromatins were cleaved into DNA fragments of 100-250 bp by enzymatic shearing. Sheared chromatins were incubated with 2 μg of anti-Sp1 antibody or normal rabbit IgG (Santa Cruz Biotechnology) at 4℃ for 4 hours. After washing, elution, reverse cross-linking, and proteinase K digestion, the collected DNA was analyzed using RT-PCR with Kv1.3 promoter specific-primers. The sequences of primers were: Kv1.3 promoter (-910/-728) forward 5'-AAC AAC TAG AGC GCT GCA AA-3', reverse 5'-GCG GGA AAT AAG AGG AAA A-3'; Kv1.3 promoter (-702/-498) forward 5'-AAG AGG CTG TCA CTG CT-3', reverse 5'-CTT CTG CTA GGA TGC GAA GC-3'; Kv1.3 promoter (-542/-325) forward 5'-CCA TCA GCA CCA TTC TCC-3', reverse 5'-TCC ACA CCC TAG GTA CAG C-3' ; Kv1.3 promoter (-344/-102) forward 5'-GCT GTA CCT AGG GGT GTG GA-3', reverse 5'-CGA AAG AGC CCT CAT GTT GT-3'; Kv1.3 promoter (-121/+61) forward 5'-ACA ACA TGA GGG CTC TTT CG-3', reverse 5'CCT CCT CCC TCC TTC TCG-3'.

Total RNA extraction and RT-PCR

Total RNA was extracted using RNeasy Micro Kit (Qiagen) following the manufacturer's instructions. Total RNA (1 μg) was reverse transcriptased using a random hexamer and M-MLV reverse transcription kit (Promega). The PCR reaction was carried out with Sp1 primer (forward 5'-TGC AGC AGA ATT GAG TCA CC-3', reverse 5'-CAC AAC ATA CTG CCC ACC AG-3') or β-actin primer (forward 5'-GGA

CTT CGA GCA AGA TGG-3', reverse 5'-AGC ACT GTG TTG GCG TAC AGC ACT GTG TTG GCG TAC AG-3'), 2 μl of cDNA, and 1× GoTaq® green master mix (Promega) under the following conditions: initial denaturation at 94℃ for 5 min, cycling (30-37 cycles) at 94℃ for 40 s, 55-60℃ for 40 s, 72℃ for 1 min, and a final extension at 72℃ for 7 min.

Transient transfection of Sp1 siRNA and a Sp1 inhibitor mithramycin A

Approximately, 2.4 x 10^5 A549 cells per well were plated in six-well plates with RPMI 1640 medium containing 10% fetal bovine serum before transfection. The next day, cells were transfected with Sp1 small interfering RNA (siRNA) or control siRNA (Santa Cruz Biotechnology) using Lipofectamine™ 2000 reagent (Invitrogen) following the manufacturer's instructions or treated with 100 nM, 250 nM mithramycin A (Sigma) for 24 hours.

Mass Spectrometry and co-immunoprecipitation

Detection of Kv1.3 protein complex in nucleus of A549 cells was performed using the Nuclear Complex Co-IP kit (Activemotif) following a modification of the manufacturer's instructions. Nuclear extracts (400-500 μg) of A549 cells were incubated with 2 μg of Kv1.3 antibody (Alomone laboratories) or rabbit IgG (Santa Cruz Biotechnology) overnight at 4℃. Magnetic beads (Invitrogen) were conjugated with the antibody by incubating for 1 hour at 4℃ and washed six times with washing buffer. After being eluted and denatured, bounded proteins were resolved on a 10% polyacrylamide gel. Bands detected by silver staining were identified using the liquid chromatography tandem mass spectrometry (LC-MS/MS) (Applied Biosystems).

MS/MS data were analyzed using a SEQUEST search. Furthermore, a loaded gel was tranferred, blocked, and incubated with anti-UBF1 (1:1000) (Abcam) or anti-Kv1.3 (1:500) (Alomone laboratories) for western blot analysis.

Subcellular fractionation

Cells were fractionated, using Qproteome cell compartment kit (Qiagen), into cytosol, membrane, and nuclear protein. All cell extraction buffers contained 1 × protease inhibitor solution. The subcellular fractions isolated were resolved in 5 × sample buffer for a western blot assay.

Statistical analysis

Statistical significance for the nuclear membrane potential experiments was determined using a Wilcoxon signed rank test and unpaired *t*-test. For analysis of western blot band density, a Mann-Whitney U test was used and all tests were performed using the SAS program (Version 9.1).

RESULTS

Kv1.3 channels exist in the nuclear membranes of A549 cells

To investigate whether Kv1.3 is located in the nuclear membrane, we performed a nuclear membrane isolation. A western blot assay indicated that Kv1.3 is found in the nuclear membrane (Fig. 2). To confirm that there was no cross-contamination, we used the inner nuclear membrane marker, Emerin, and the nucleopalsmic protein Sp1 antibodies.

Effects of valinomycin and concentration gradients of K^+ on changes in the nuclear membrane potential induced by MgTX

In order to identify how collapse in the K^+ gradient affects changes in the nuclear membrane hyperpolarization induced by MgTX, the K^+ ionophore, valinomycin, was added to the intracellular medium. Treatment with MgTX (1 nM) in the presence of valinomycin for 5 or 10 min decreased nuclear membrane potential compared to no MgTX treatment (Fig. 3A). We also measured changes in nuclear membrane potential while varying the K^+ concentration gradient across the nuclear membrane. MgTX (1 nM) had little effect on the nuclear membrane potential in 0 mM K^+ solution (Fig. 3B). However, when the K^+ concentration outside the nucleus was increased (to 30, 60, and 125 mM), the effect of MgTX was significantly greater. These results demonstrate that the effects of MgTX are dependent on the magnitude of the K^+ gradient across the nuclear membrane.

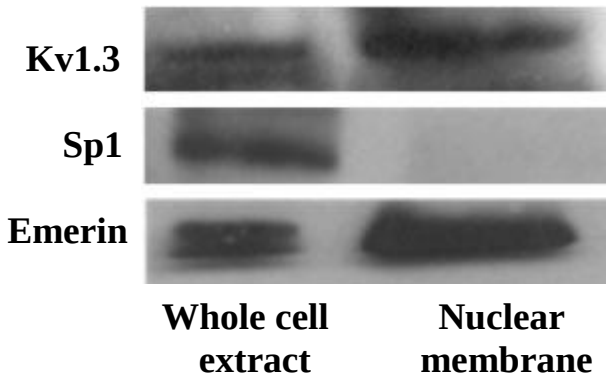


Figure 2. Kv1.3 exists in the nuclear membrane of A549 cells. Western blot analysis indicates that Kv1.3 is located in the nuclear membrane. Nuclear membrane fractionation is confirmed using Sp1 (a nucleoplasmic protein) and Emerin (an inner nuclear membrane) antibodies.

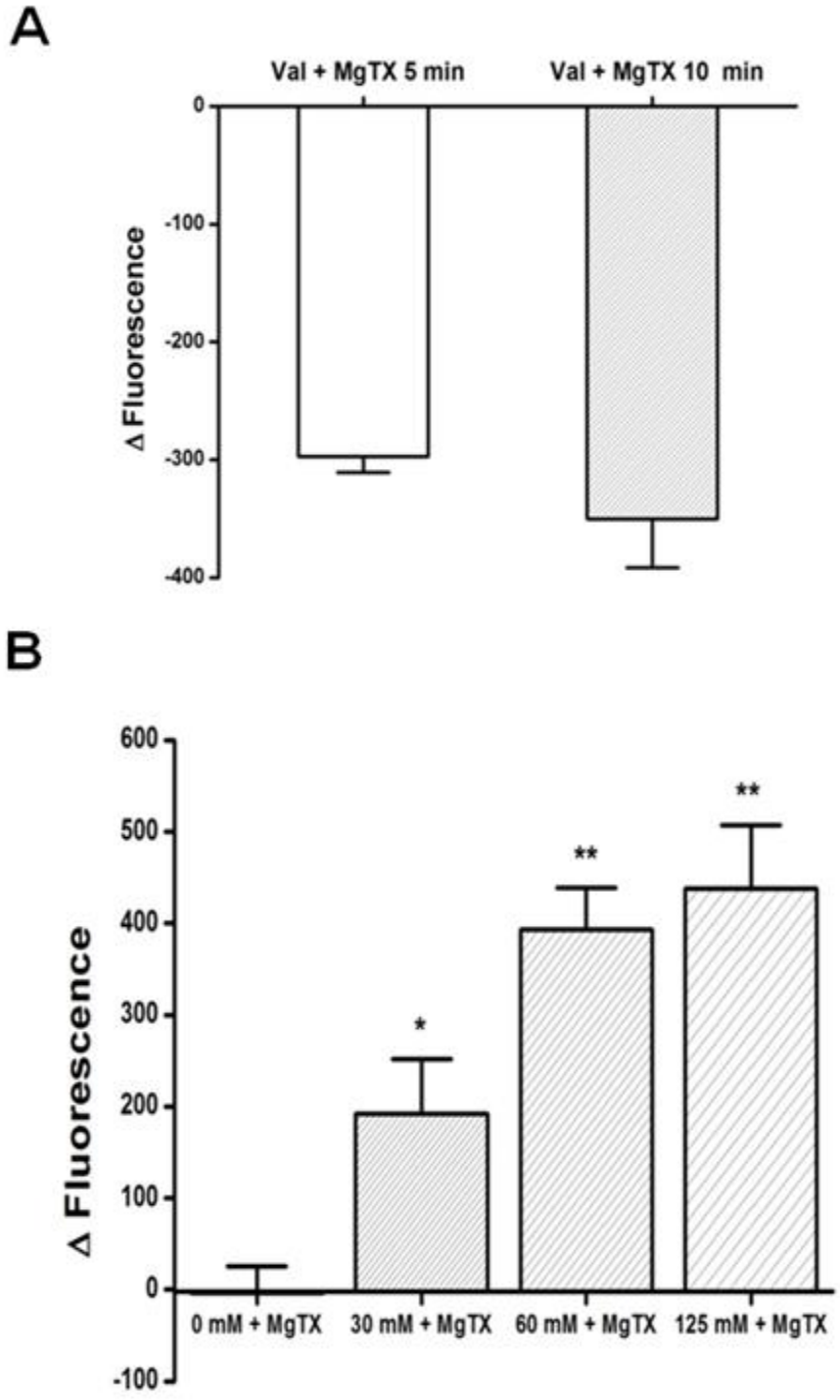

A
Val + MgTX 5 min
Val + MgTX 10 min
0
-100
-200
-300
-400
Δ Fluorescence
B
600
500
400
300
200
100
0
-100
Δ Fluorescence
*
**
**
0 mM + MgTX
30 mM + MgTX
60 mM + MgTX
125 mM + MgTX

Figure 3. The effect of MgTX on nuclear membrane potential of isolated nuclei from A549 cells. (A) The nuclear membrane potential shifts by treatment of 1 nM MgTX in isolated A549 nuclei resuspended in intracellular solution containing 10 μM valinomycin (n=6). (B) Isolated nuclei were resuspended in 0 mM [K^+] solution for 30 min and then transferred to defined extranuclear [K^+] solutions (0, 30, 60, or 125 mM) for 5 min. Effects of MgTX on nuclear membrane potential was measured with different extranuclear [K^+] solutions. ΔFluorescence refers to subtraction of the value of when isolated nuclei was suspended in only defined [K^+] solution from in the same [K^+] solution with 1 nM MgTX (n=4). Results were normalized as the value of controls (without MgTX) and presented as mean ± SE (* $p<0.05$; ** $p<0.001$).

Kv1.3 blocker PAP-1 induces phosphorylation of CREB and c-Fos activation

We detected that the inhibition of Kv1.3 channels using membrane-permeable Kv1.3 blocker PAP-1 (10 nM) increased phosphorylation of transcription factor CREB (Fig. 4A) and c-Fos, an immediate early response transcription factor (Fig. 4B) in A549 cells. In contrast, CREB total protein level was similar between control and PAP-1 treatment conditions.

Sp1 transcription factor binds to the Kv1.3 promoter

The Kv1.3 promoter sequence contains TATA-less and GC-rich (Simon et al., 1997). Therefore, we speculated that Sp1 could bind to the Kv1.3 promoter and regulate the expression of Kv1.3 in the nucleus. Through computational analysis using the JASPAR database and TFSEARCH, several potential Sp1 binding sites were found in the human Kv1.3 promoter (Accession number 3180). To determine whether Sp1 is bound to the Kv1.3 promoter, we performed a ChIP assay using A549 cells. PCR analysis using primers divided into the five primers in the Kv1.3 promoter showed that two region (-910/-728) (-702/-498) of the Kv1.3 promoter interacts with Sp1 protein (Fig. 5A). The ChIP assay demonstrates that Sp1 interacts with the human Kv1.3 promoter and based on computational analysis, three putative Sp1 binding site was identified in the promoter region of human Kv1.3 (Fig. 5B).

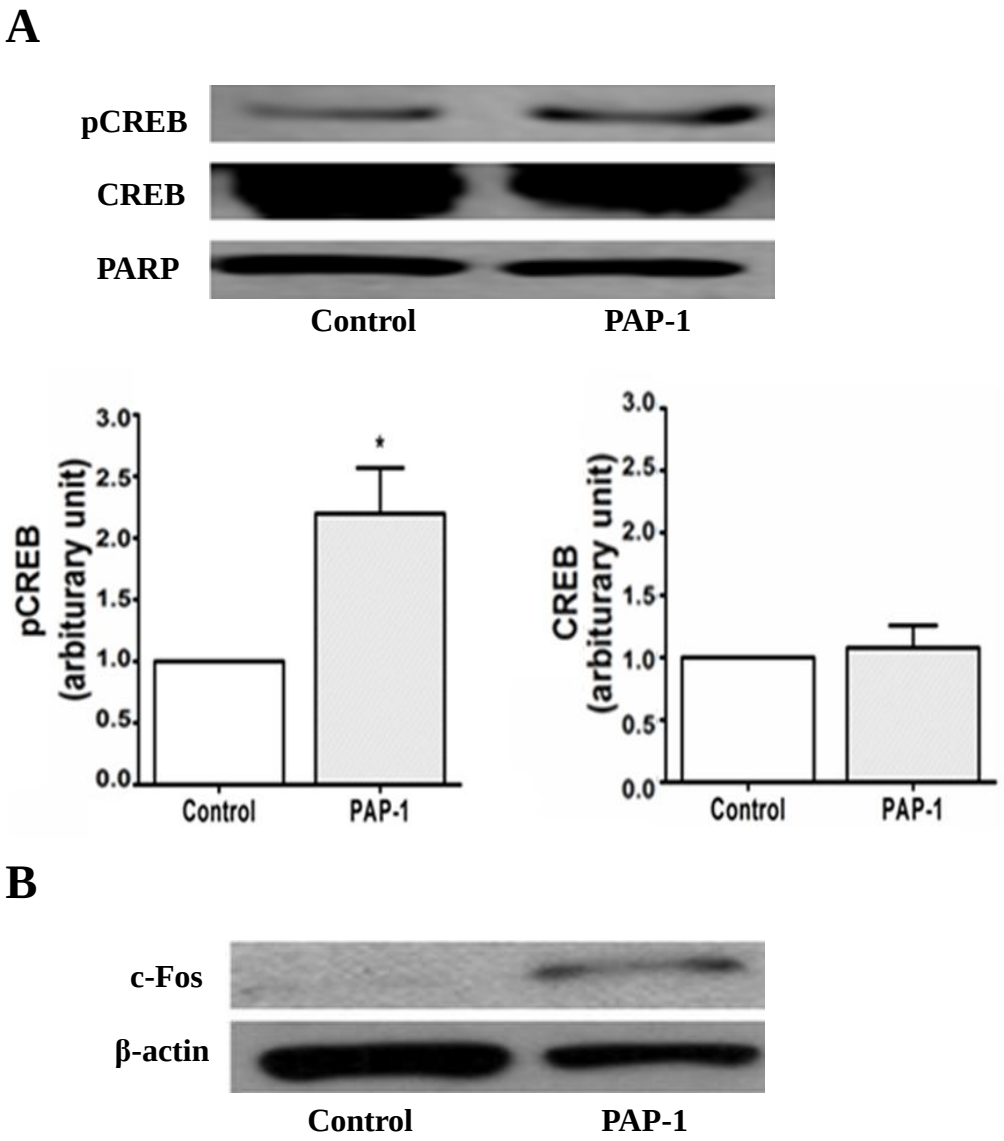
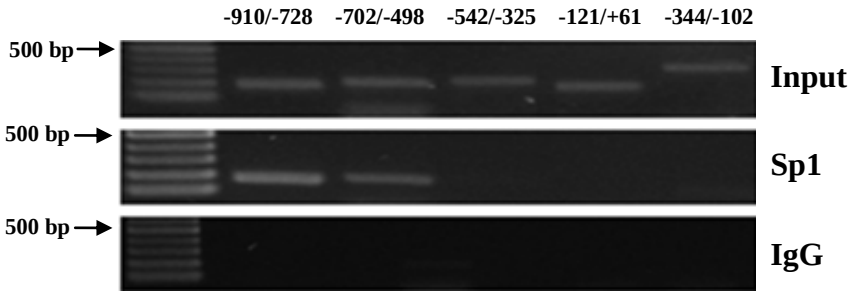


Figure 4. Up-regulation of phosphorylated CREB and c-Fos induced by PAP-1, membrane-permeable Kv1.3 blocker. Representative gel images of western blots and the relative expression level of pCREB, CREB (A), and c-Fos (B) induced by 10 nM PAP-1 in A549 cells. The data were normalized as the value of controls and presented as mean ± SE (n=3) (* $p<0.05$).

A



B

AACAACTAGAGCGCTGCAAATTCCCTACCACAAGGGCAGTGCCAAAA -864

TATTAAGTAAAAAGTCTAGGTACAGGTCCTGCTCTCATCCCTTCCACG -816

Sp1

ACAGGCTTGAGTCATAAAGCTGGACTTTCTAACCACACAACTTCGACT -768

TCGTGAATTTCCTTTCCCATTTTTCCTCTTATTTCCCCGCCAACAGGAA -719

Sp1

ACCTGTGTAGTGGATAAAGACCAGGCTGTCACTGCTAATCCCAATCAGA -670

AACTTGGTCCTTGATTCTTCCCAGTGGCCGACATCTCCGCTGCGAAAA -622

Sp1

Figure 5. Sp1 transcription factor interaction with the Kv1.3 promoter. (A) A ChIP assay showing Sp1 binding to the Kv1.3 promoter in A549 cells. Input DNA was used as a control and rabbit IgG was used as a negative control. (B) Identification of three putative Sp1 binding site within the nucleotide sequence of the human Kv1.3 promoter. The numbers indicate location of the Kv1.3 promoter sequence. We selected +1 as the first exon start site of Kv1.3.

Sp1 controls Kv1.3 protein expression in the nucleus

To determine whether Sp1 was involved in regulating Kv1.3 expression, mithramycin A, a selective inhibitor of transcription factor binding to the GC-rich promoter, and RNA interference were used to inhibit interaction of Sp1 with the Kv1.3 promoter. Reducing Sp1 protein-promotor interactions using mithramycin A decreased Kv1.3 channel expression compared to control in A549 cell extracts (Fig. 6A(a)) and nuclear extracts (Fig. 6A(b)). Furthermore, silencing Sp1 mRNA expression by transfection with Sp1 siRNA (Fig. 6B(a)) also reduced Kv1.3 protein expression in whole cell extracts (Fig. 6B(b)) and nuclear extracts (Fig. 6B(c)) of transfected A549 cells.

Physical interaction of Kv1.3 with proteins in the nucleus

To investigate whether Kv1.3 interacts with other proteins in the nucleus of A549 cells, we purified Kv1.3 channel complexes using LC-MS/MS. The transcription factors, receptors, and some proteins interacted with nuclear Kv1.3 were identified (Table 1). As shown in the MS/MS spectrum (Fig. 7A) and western blot image (Fig. 7B), the Upstream Binding Factor 1 (UBF1) was co-purified with Kv1.3 using an antibody that selectively binds Kv1.3 protein.

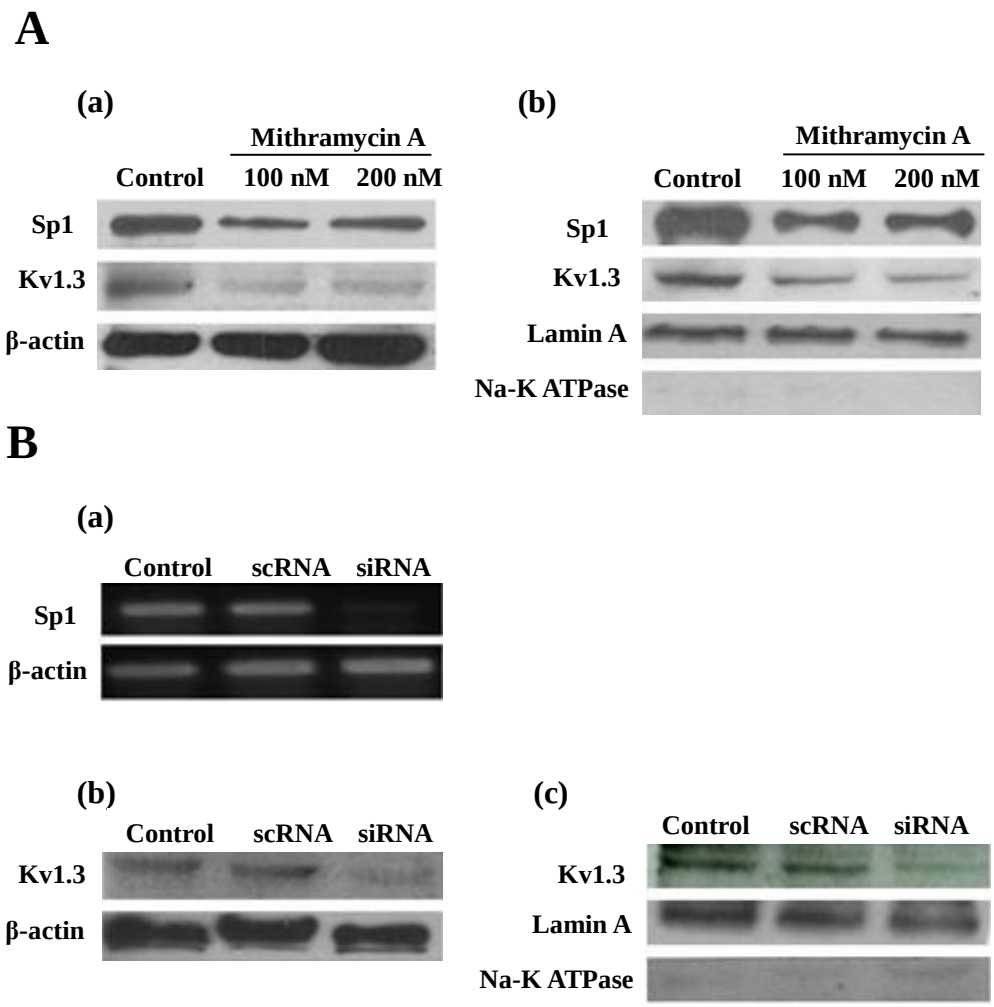
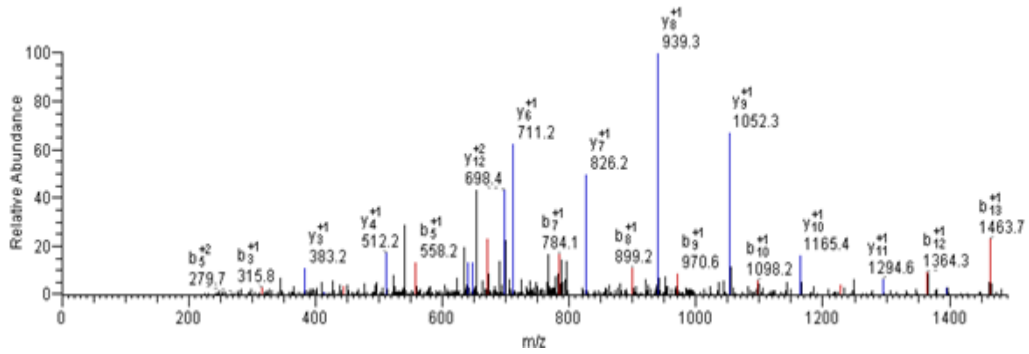


Figure 6. Down-regulated Kv1.3 protein expression by inhibition of Sp1. (A) Western blot analysis of whole cell extracts (a) and nuclear extracts (b) of A549 cells treated with 100 and 200 nM mithramycin A for 24 hours. (B) A549 cells were transfected with Sp1 siRNA. RT-PCR (a) and Western blot (b, c) were used for detecting Sp1 mRNA and Kv1.3 protein expression levels in whole cell extracts (b) and nuclear extracts (c), respectively.

Table 1. Proteins purified with Kv1.3 from isolated nuclei of A549 cells using LC-MS/MS.

Peptide	P(prep)	Score
WD repeat protein 3	3.62E-11	198.29
Isoform 1 of ATPase family AAA domain-containing protein 2	9.95E-07	96.19
Isoform 1 of Bcl-2-associate transcription factor 1	9.97E-06	84.15
Isoform UBF1 of nuclear transcription factor 1	1.04E-07	52.20
TATA-binding protein-associated factor 172	9.55E-04	48.09
Serine/threonine-protein kinase TBK1	1.00E-04	42.11
Isoform 1 of homeodomain-interacting protein kinase 3	7.20E-04	42.08
FERM and PDZ domain-containing protein 3	5.20E-04	40.09
Transcription factor-like nuclear regulator	1.74E-04	36.09
Isoform Short of TATA-binding protein-associated factor 2N	6.09E-04	26.09
Lamin-B receptor	8.41E-09	20.21
Isoform 2 of transcription factor RFX3	5.49E-04	20.10

A



#	1	2	3	4	5	6	7	8	9	10	11	12	13	14
b ion	102	214	316	445	558	671	784	899	970	1098	1227	1364	1463	-
Peptide	T	L	T	E	L	I	L	D	A	Q	E	H	V	K
y ion	-	1508	1395	1294	1165	1052	939	826	711	640	512	383	246	147
#	14	13	12	11	10	9	8	7	6	5	4	3	2	1

B

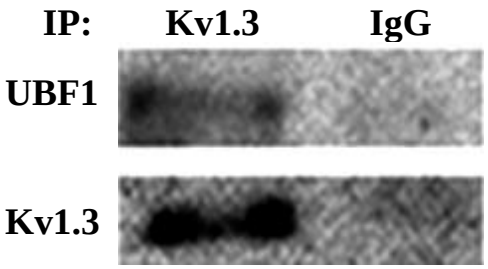


Figure 7. Coassembly of UBF1 and nuclear Kv1.3 channel. (A) MS/MS spectra of the UBF1-specific peptide obtained by mass spectrometry. The b ions originate from cleavage of the peptide backbone with N-terminal charge retention and the y ions indicate peptide fragments with C-terminal charge retention. The b and y ions matched to the UBF1 peptide are indicated by red and blue, respectively. (B) Co-immunoprecipitation of Kv1.3 with UBF1 from A549 nuclear extracts. Rabbit IgG was used as a negative control.

Subcellular localization of Kv channels and functional sites in Kv1.3 structure using computational prediction tools

We used the amino-acid sequence-based structure predictors TargetP 1.1, Golgi Predictor, PredictNLS, NucPred, and NetNES. TargetP 1.1 is an N-terminal sequence-based predictor that provides mitochondria targeting peptide (mTP) that is peptide sequences directing to the mitochondrial matrix (Table 2). As previously reported (Szabo et al., 2005), it is confirmed that Kv1.3 is present in mitochondria. Golgi Predictor predicts whether proteins have Golgi retention signals (Lys-Asp-Glu-Leu) within the transmembrane helix. Nuclear Localization Signal (NLS) is a nuclear target signal that is recognized by the nuclear import pathway-related proteins, importin α/β or importin β. The NucPred tool comparing proteins with similar NLS motif and the PredictNLS tool confirming known NLS sequences are used to predict nuclear localization. NucPred predicts that Kv2.2 is relatively possible to be localized in the nucleus, but PredictNLS provides that Kv1.4 and Kv3.2 containing NLS sequences is present in the nucleus. In contrast to NLS, leucine-rich nuclear export signal (NES) sequences are related to transport into cytoplasm. This can be predicted using the NetNES program. It demonstrates that Kv1.2 and Kv1.3 do not possess NES sequences. CBS prediction servers providing post-translation modifications, protein function and structure demonstrate that Kv1.3 channels include many functional amino acids, including phosphorylation, glycosylation, and a PDZ binding domain (Fig. 8).

Table 2. Predicted subcellular localization signals of Kv channels.

	mTP	Golgi retention (Threshold : 20.005)	NucPred score (total point: 1)	NLS	NES
Kv1.1	X	X (17.786)	0.26	X	O
Kv1.2	X	X (18.429)	0.56	X	X
Kv1.3	O	O (22.677)	0.49	X	X
Kv1.4	X	O (22.677)	0.67	O	O
Kv2.1	X	X (18.173)	0.86	X	O
Kv2.2	X	X (18.108)	0.94	X	O
Kv3.1	X	O (20.830)	0.20	X	O
Kv3.2	X	O (20.830)	0.30	O	O
Kv4.1	X	O (21.821)	0.57	X	O
Kv4.2	X	O (21.821)	0.64	X	O
Kv4.3	X	O (21.821)	0.71	X	O
Kv9.3	X	X (16.921)	0.07	X	O

mTP means the signals targeting to mitochondria matrix and golgi retention (threshold) indicates the possibility for proteins to exist in Golgi. NucPred score suggests that higher score means higher possibility present in the nuclues. NLS sequences for nuclear retention and NES sequences for trafficking to outer nuclear are confirmed using PredictNLS and NetNES tools respectively. O and X indicate the existence or absence of signals in Kv channels

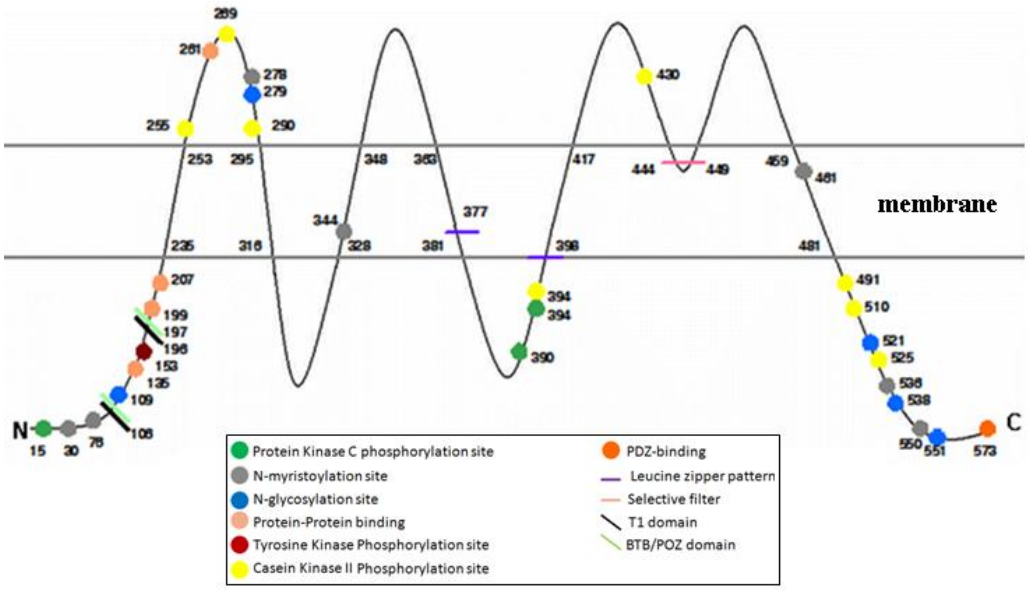


Figure 8. Putative functional sites in Kv1.3 channels. Kv1.3 structure contains several functional positions, such as phosphorylation, protein binding, and PDZ-binding, predicted using computational tools. Numbers of Kv1.3 structure indicate the location of amino-acid.

Kv channels were found in the nucleus of A549, MCF7, K562, and human brain tissues

To determine the subcellular localization of other Kv channels (Kv1.2, Kv1.4, Kv4.1, and Kv9.3), we performed cell fractionation, divided into cytosol, membrane, and nucleus. Na, K-ATPase (a plasma membrane marker), GRP78 BiP (an endoplasmic reticulum marker), and lamin A (a nuclear marker) antibodies were used as cell fractionation markers. Kv1.2 channels were detected in the membranes of A549, MCF7, and K562 cells (Fig. 9). However, as shown in Fig. 10, Kv1.4 channels were confirmed in the cytosol and nucleus of A549 cells, but in the cytosol and membrane of MCF7 and K562 cells. Kv1.4, from human brain tissues, is shown to be present in the membrane and nucleus. Kv4.1 was observed in the membrane of MCF7 and K562 cells (Fig. 11). Kv9.3 channels were detected in the cytosol and membrane of A549 and K562, but in the membrane of MCF7. In the nuclear region of A549, MCF7, and K562 cells, Kv9.3 was detected as high molecular weight proteins, suggesting that Kv9.3 can be modified in the nuclear region. In addition, Kv9.3 exists in the membrane and nucleus of human brain tissues (Fig. 12).

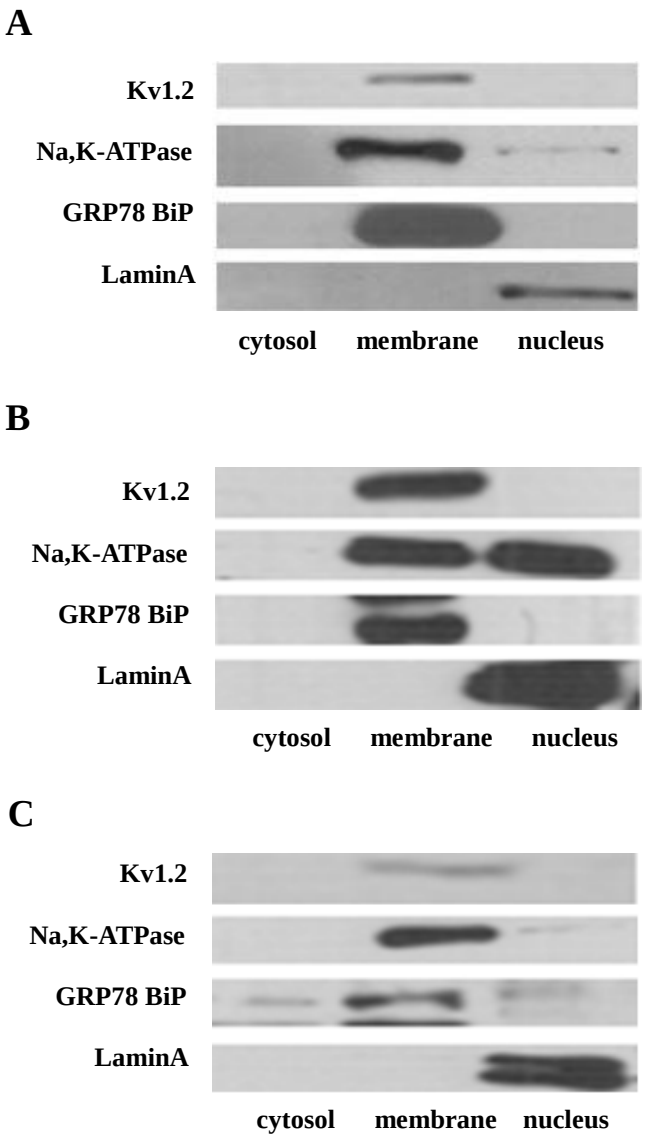
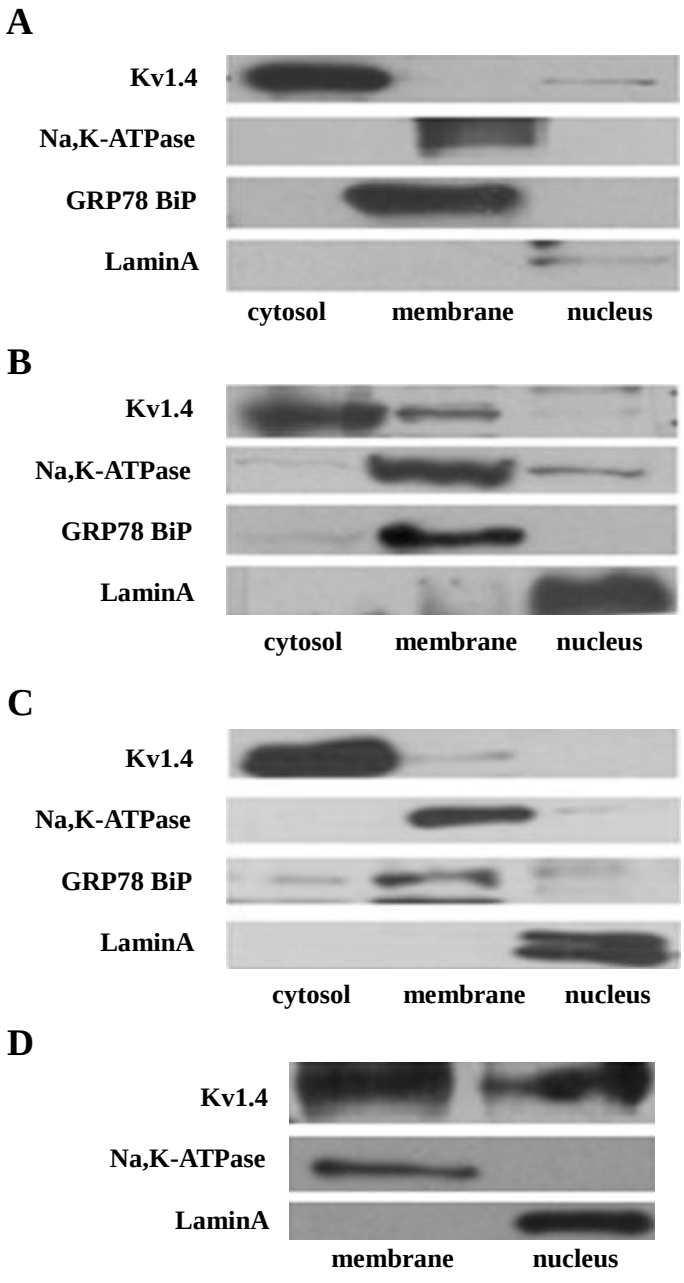


Figure 9. Subcellular localization of Kv1.2 in A549, MCF7, and K562 cells. The western blot images of Kv1.2 using subcellular fractionation demonstrate that Kv1.2 is localized in the membrane of A549 (A), MCF7 (B), and K562 (C) cells. Cell fractionation is confirmed using Na,K-ATPase (a plasma membrane marker), GRP78 BiP (an endoplamic reticulum marker), and lamin A (a nuclear marker) antibodies.


A
Kv1.4
Na,K-ATPase
GRP78 BiP
LaminA
cytosol
membrane
nucleus
B
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GRP78 BiP
LaminA
cytosol
membrane
nucleus
C
Kv1.4
Na,K-ATPase
GRP78 BiP
LaminA
cytosol
membrane
nucleus
D
Kv1.4
Na,K-ATPase
LaminA
membrane
nucleus

Figure 10. Subcellular localization of Kv1.4 in A549, MCF7, K562, and human brain tissues. Protein expression of Kv1.4 is detected in the cytosol and nucleus of A549 cells (A), but in the cytosol and plasma membrane of MCF7 (B) and K562 (C) cells. Kv1.4 proteins were detected in the membrane and nuclear region of human brain tissues (D). Cell fractionation is confirmed using Na,K-ATPase (a plasma membrane marker), GRP78 BiP (an endoplamic reticulum marker), and lamin A (a nuclear marker) antibodies.

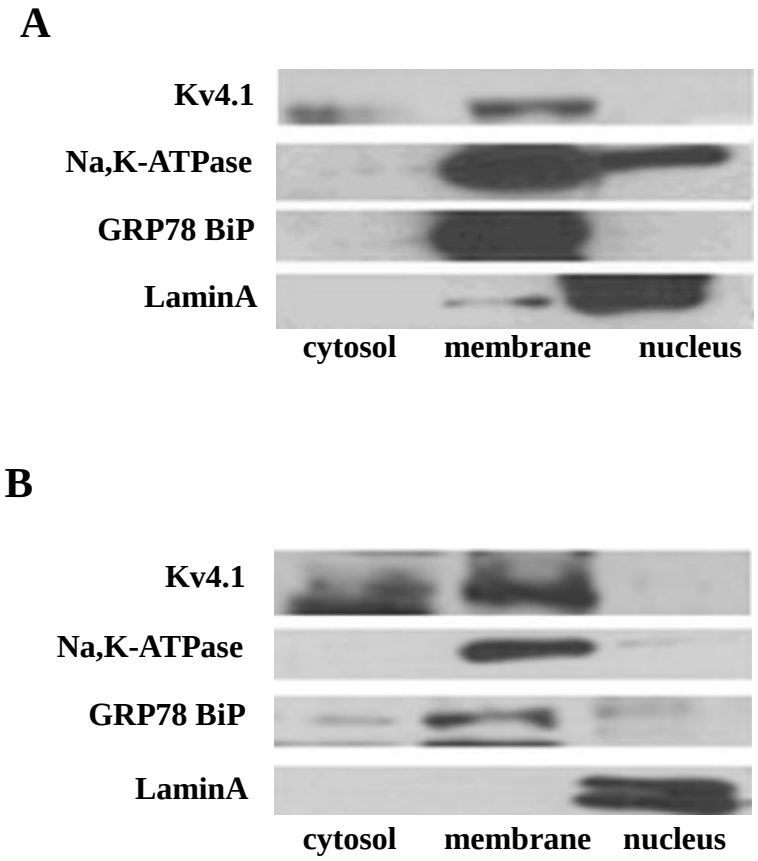
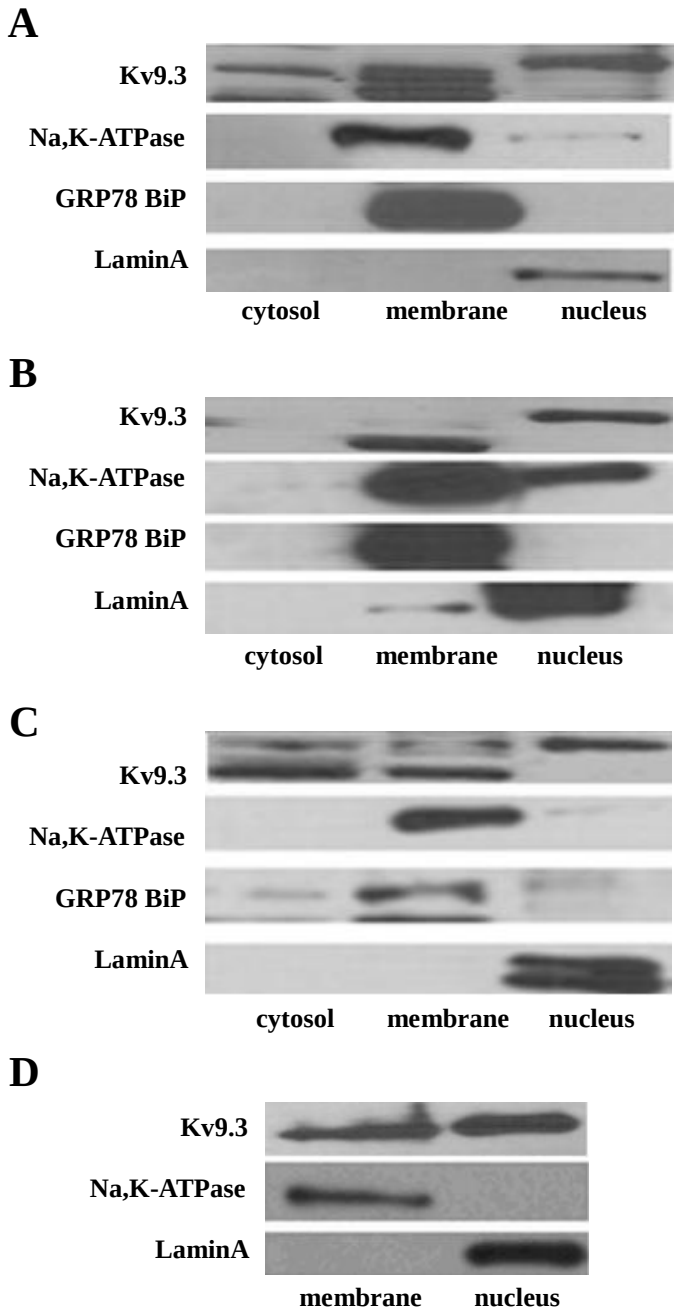


Figure 11. Subcellular localization of Kv4.1 in MCF7 and K562. Kv4.1 was founded in the membrane region of MCF7 (A) and K562 (B) cells. Cell fractionation is confirmed using Na,K-ATPase (a plasma membrane marker), GRP78 BiP (an endoplamic reticulum marker), and lamin A (a nuclear marker) antibodies.


A
Kv9.3
Na,K-ATPase
GRP78 BiP
LaminA
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membrane
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GRP78 BiP
LaminA
cytosol
membrane
nucleus
C
Kv9.3
Na,K-ATPase
GRP78 BiP
LaminA
cytosol
membrane
nucleus
D
Kv9.3
Na,K-ATPase
LaminA
membrane
nucleus

Figure 12. Subcellular localization of Kv9.3 in A549, MCF7, K562, and human brain tissues. Kv9.3 channels were detected in the cytosol, plasma membrane, and nucleus of A549 (A) and K562 (C) cells, but in the plasma membrane and nucleus of MCF7 (B). Human brain tissues express Kv9.3 proteins in the membrane and nucleus (D). Cell fractionation is confirmed using Na,K-ATPase (a plasma membrane marker), GRP78 BiP (an endoplamic reticulum marker), and lamin A (a nuclear marker) antibodies.

DISCUSSION

The results revealed that the Kv1.3 channels localized in the nuclear membrane in A549 cells are capable of participating in the regulation of nuclear membrane potential, and selective inhibition of nuclear Kv1.3 channel activity can trigger gene expression by activation of specific transcription factors, including pCREB and c-Fos. The expression of nuclear Kv1.3 channels is controlled by Sp1, a transcription factor that directly binds to the Kv1.3 promoter, and nuclear Kv1.3 forms a complex with nuclear protein UBF1. Furthermore, we confirmed that several Kv channels are detected in the nucleus of several types of cells and human brain tissues

Functional roles of Kv1.3 in the nucleus

Selective Kv1.3 inhibitor, MgTX-induced nuclear membrane potential response is eliminated by blocking K^+ flow across the nuclear membrane by a potassium concentration of 0 mM between the inner- and outer-nucleus and treatment with valinomycin. These results are similar to K^+ movement through Kv1.3 channels located in the mitochondria (Szabo et al., 2005). As proposed in the mitochondrial membrane (Szabo et al., 2005), Kv1.3 channels in the nuclear membrane may be partly responsible for basal K^+ conductance in the nucleus.

Another possibility is that Kv1.3 may function as an exchange pathway, since the concentration of K^+ ions existing in the nucleus and cytoplasm is similar. Kotturi et al. (2006) and Nicolaou et al. (2009) suggested that Kv1.3 and Ca^{2+} release-activated Ca^{2+} channels (CRAC) are involved in Ca^{2+} influx. In hyperpolized cells, Ca^{2+} influx across the nuclear membrane could induce depolarization, and therefore, would drive K^+ out of the nucleus through Kv1.3 channels. This response will compensate for the

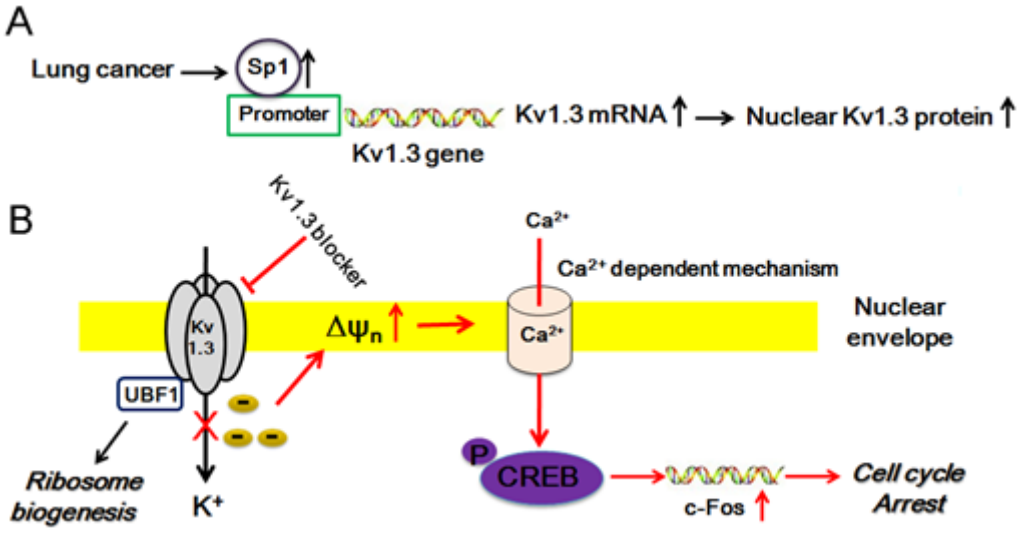

Figure 13. Kv1.3 in the nuclear membranes. (A) Sp1 overexpressed in the lung cancer cells binds to Kv1.3 promoter regions and increases the transcription of Kv1.3 genes. (B) Selective Kv1.3 blockers (MgTX or PAP-1) hyperpolarize the nuclear membrane potential. The hyperpolarization by Kv1.3 blockers may activate Ca^{2+} channels and facilitate calcium inflow inducing Ca^{2+} dependent mechanism (Quesada et al., 2002), which then phosphorylates CREB; pCREB leads to the expression of downstream gene, such as c-Fos, which could result in cell cycle arrest (Jang et al., 2011a). In addition, the interaction of Kv1.3 and UBF1 in the nucleus may be related to ribosome biogenesis

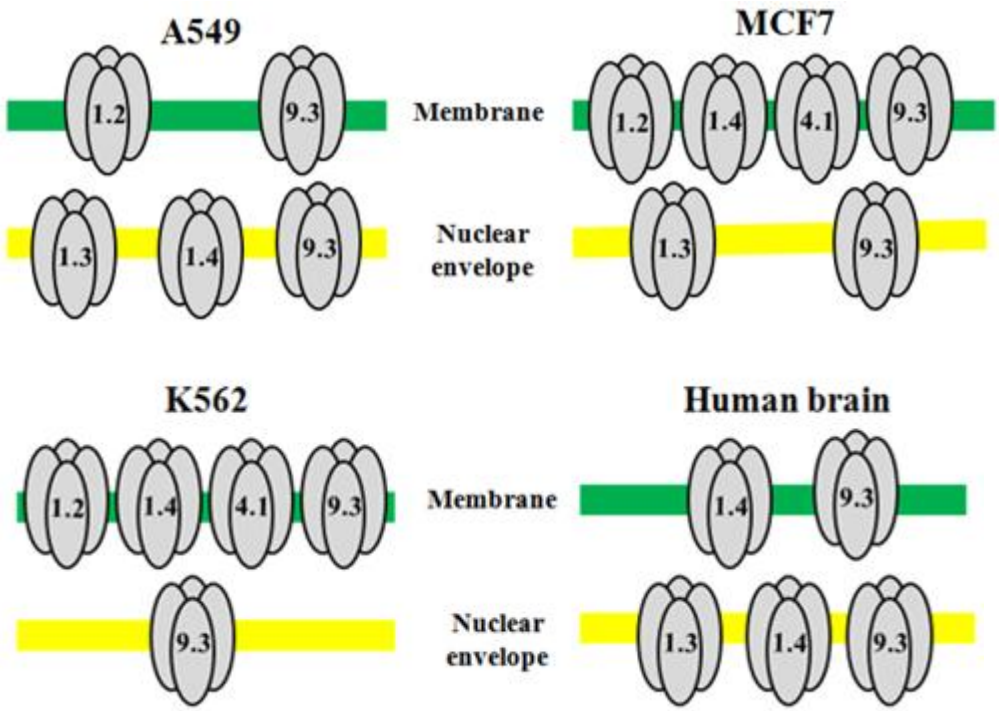

Figure 14. Subcellular localization of Kv channels in A549, MCF7, K562, and human brain tissues. Subcellular location of Kv1.2, Kv1.4, Kv4.1, and Kv9.3 based on Figure 9, 10, 11, and 12.

depolarization by Ca^{2+} influx, and thereby, facilitate the driving force for Ca^{2+} entry. Therefore, nuclear Kv1.3 could play a crucial role in Ca^{2+} signaling by cross-talking with Ca^{2+} channels.

Activation of transcription factors, CREB and c-Fos, by selective inhibition of Kv1.3 using PAP-1

We found that the membrane permeable Kv1.3 channel blocker, PAP-1, phosphorylated CREB in A549 cells. CREB phosphorylation has been reported to promote tumorigenesis (Conkright and Montminy, 2005; Linnerth et al., 2005). CREB phosphorylation is also associated with cell cycle arrest (Glass et al., 2004). Similar to our findings, blockers of K_{ATP} channels have been reported to increase Ca^{2+} concentration in the nucleus and induce phosphorylation of CREB (Quesada et al., 2002). Additionally, membrane hyperpolarization with 4-aminopyridine, a nonselective voltage-gated K^+ channel blocker, also increases Ca^{2+} influx through L-type voltage-dependent Ca^{2+} channels, which leads to CREB phosphorylation in vascular smooth muscle myocytes (Coussin et al., 2003).

Along with CREB, c-Fos was found to be increased by PAP-1 in A549 cells. The c-Fos proto-oncoprotein is a primary component of the AP-1 transcription factor complex that is known to be involved in cell growth, differentiation, and the G0/G1 transition (Balsalobre and Jolicoeur, 1995; Okada et al., 1999; Thomas et al., 2000). It has been reported that expression of c-Fos inhibits cell cycle progression and subsequent proliferation (Balsalobre and Jolicoeur, 1995; Garrido et al., 1993). These reports have shown that the inhibitory effects on cell cycle transition occur at the G0/G1 boundary as a result of downregulated cell cycle-associated proteins. This observation corresponds with our previous report that silencing Kv1.3 channel

expression or inhibiting channel activity with MgTX induces anti-proliferative effects through inhibition of the G1-S transition (Jang et al., 2011a). Previously, it was reported that MgTX treatment leads to increased c-Fos mRNA transcription and protein in the striatum, which triggers long-term neuronal plasticity (Saria et al., 2000).

These results, along with our data from the present study, strongly suggest that ion channels, such as Kv1.3, located in the nuclear membrane can influence the regulation of gene expression by modulating the activation of certain transcription factors.

Sp1-mediated Kv1.3 expression

We investigated the regulation of nuclear Kv1.3 channel expression in A549 cells by identifying the transcription factor that mediates gene transcription. The Kv1.3 gene has been reported to have GC-rich and TATA-less promoter regions (Simon et al., 1997). Previous studies have shown that the transcription factor, Sp1, notably binds to GC-rich motifs but also regulates the transcription of other Kv channel genes, such as Kv1.5 (Fountain et al., 2007) and Kv4.3 (Qi et al., 2012). We showed that Sp1 affects Kv1.3 expression through interaction with the Kv1.3 promoter and that silencing Sp1 expression using siRNAs or the Sp1 blocker, mithramycin A, reduces Kv1.3 channel expression in the nucleus. This is the first report demonstrating that Sp1 plays a crucial role in Kv1.3 gene expression. Furthermore, considering the report that Sp1 regulates lung cancer cell proliferation and metastasis (Hsu et al., 2011), nuclear Kv1.3 channel expression appears to be associated with tumorigenesis.

Nuclear Kv1.3 complexes

A protein binding assay using the nuclear extracts indicated that the nuclear Kv1.3 channel binds to nuclear proteins, including UBF1 transcription factor, which participates in RNA polymerase I activity, mediating ribosomal RNA synthesis. It was reported that the NR1 subunit of the NMDA receptor is found in the nucleoli of hydra cells, which could serve as a regulator for ribosomal subunit production (Kass-Simon et al., 2009). Therefore, it is possible that nuclear Kv1.3 channels play the role of regulators in ribosome biogenesis. Further studies are needed to determine the function of nuclear Kv1.3 channel and UBF1 complexes. Also, other than UBF1, our MS analysis revealed that many other proteins could bind directly to nuclear Kv1.3. The roles of other possible binding proteins need to be confirmed in future work.

Nuclear trafficking of Kv1.3

Our search using the PredictNLS algorithms indicates that known NLS sequences are not present in Kv1.3 channels. Thus, Kv1.3 channels may contain an NLS that has not been previously described. It is also worth noting that Kv1.3 channels contain a PDZ domain sequence at the end of the C-terminal sequence (Marks and Fadool, 2007), which could potentially affect the localization of the channel and allow for interactions with signaling molecules within the nucleus. Although it has been suggested that alternative splicing or post-translational protein modifications (Schulz et al., 2008) could influence the localization of proteins within the cell, our previous data indicated that the Kv1.3 coding region in A549 cells was unaffected by alternative splicing, suggesting that, at least, this potential differential trafficking mechanism does not account for the nuclear localization of Kv1.3 channels.

Nuclear localization of Kv channels

In addition to Kv1.3, Kv1.4 and Kv9.3 were detected in the nucleus. Previously, Mazzanti et al. (1990) suggested that K^+ selective channels are detected in the isolated nucleus using the patch-clamp technique and these channels regulate the nuclear membrane potential through a conductive pathway between nuclear membrane. It has been reported that nuclear Kv channels immunoreactivities are detected in the paraventricular nucleus projecting into the rostral ventrolateral medulla (Sonner and Stern, 2007). Recently, eag I channels are found to be localized on the inner nuclear membrane of Kv10.1-transfected NIH3T3 and CHO cells, MCF7 cells, and rat cerebellum and hippocampus. Therefore, based on our results and previous reports, several Kv channels could be present in nuclear membrane and the nuclear membrane potential regulated by Kv channels could contribute to the activation of other voltage-gated calcium and chloride channels in the nuclear membrane (Bkaily et al., 2009), which may play a crucial role in the nucleus similar to the nuclear functions of Kv1.3. Additional studies regarding the roles of Kv channels expressed in the nucleus are warranted in the future.

CONCLUSION

In the present study, we confirmed that Kv1.3 is localized in the nuclear membrane of A549 cancer cells. Based on the results, we determined that inhibition of nuclear Kv1.3 channels activates transcription factor pCREB and c-Fos, which are associated with cell survival and proliferation. Endogenous Sp1 transcription factors known to be required for lung tumor growth directly bind to Kv1.3 promoter regions and influence the expression of nuclear Kv1.3 channels. Moreover, nuclear Kv1.3 is complexed with nuclear proteins, including the UBF1 protein. According to our results, Kv1.3 may have unidentified function in the nuclear region, in addition to regulating nuclear membrane potential. The subcellular localization prediction analysis and subcellular protein fractionation analysis show that several Kv channels other than Kv1.3 can be localized in the nuclei of several cancer cells, K562, and human brain tissues. The results of this study, taken together, provide novel biochemical and functional characteristics of nuclear Kv channels; this mechanism could be related to the recently revealed Kv channel functions of proliferation, apoptosis, and differentiation, and also contribute to expanding our knowledge about the functional roles of ion channels at the nucleus.

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국문초록

핵에서의 전압의존성 포타슘 채널의 특징

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전압의존성 포타슘 (Kv) 채널은 흥분성 세포인 뉴론과 심근세포에서 포타슘의 세포막 이동을 조절함으로서 전기신호를 전달하는 중요한 역할을 한다고 알려져 있다. 하지만 최근 연구에 따르면 Kv1.3을 포함한 여러 Kv 채널들은 비흥분성 세포인 암세포의 세포증식에 관여함을 보였다.

본 실험결과, A549 암세포에서 Kv1.3이 핵막에 존재하였고 핵막전위에 영향을 주었다. A549 세포에서 막을 통과할 수 있는 Kv1.3 억제제인 PAP-1이 CREB을 인산화시키고 c-Fos를 활성화시켰다. 염색질 면역침전법은 Sp1 전사요소가 Kv1.3 프로모터에 결합하고 이는 A549 세포의 핵에 존재하는 Kv1.3의 발현을 조절하였다. 그리고 핵에 있는 Kv1.3이 UBF1을 포함한 핵단백질들과 복합체를 형성함을 보여주었다. 아미노산 서열을 기반한 예측프로그램은 Kv 채널들의 세포 내 위치와 기능적인 도메인을 보여주고 세포 내 분리를 이용하여 A549, MCF7, K562 세포와 뇌조직에서 Kv 채널의 위치가 확인되었다.

이번 결과는 핵에 존재하는 Kv 채널들이 핵막전위와 pCREB과 c-Fos 같은 전사요소들을 조절하여 암세포 증식에 관여할 수 있음을 보여준다. 또한 Sp1 전사요소에 의해 조절되는 핵에 위치한 Kv1.3은 핵단백질과 결합을 통해 지금까지 알려지지 않은 생물학적 기능적 역할을 가질 수 있을 것으로 예측된다.

주요어: Kv1.3, 핵, Sp1, UBF1, 세포 내 위치
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