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의학석사 학위논문

**Rescue effects of small conductance
Ca²⁺-activated K⁺ channel (SK4)
activator on the Ca²⁺ overload
induced death of head and neck
cancer cells**

칼슘과부하에 의한 두경부암세포 사멸현상에
대한 SK4 포타슘 이온통로 활성화제의 세포사멸
방지효과

2016년 02월

서울대학교 대학원
의과학과 의과학전공

윤 명 철

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A thesis of the Master's degree

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February, 2016

The Department of Biomedical Sciences,

Seoul National University

College of Medicine

Yin Ming Zhe

칼슘과부하에 의한 두경부암세포 사멸현상에 대한
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지도 교수 김 성 준

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의과학과 의과학전공

윤 명 철

윤 명 철의 의학석사 학위논문을 인준함
2015년 12월

위 원 장 _____ (인)
부위원장 _____ (인)
위 원 _____ (인)

**Rescue effects of small conductance
Ca²⁺-activated K⁺ channel (SK4)
activator on the Ca²⁺ overload induced
death of head and neck cancer cells**

by

Yin Ming Zhe

**A thesis submitted to the Department of Biomedical Sciences
in partial fulfillment of the requirements for the Degree of
Master of Science in Medicine (Physiology) at Seoul National
University College of Medicine**

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Approved by Thesis Committee:

Professor _____ Chairman

Professor _____ Vice chairman

Professor _____

ABSTRACT

Ion channels in carcinoma and their roles in cell proliferation are drawing attention. Intracellular Ca^{2+} ($[\text{Ca}^{2+}]_c$)-dependent signaling affects the fate of cancer cells. K^+ channels play critical roles in proliferation and apoptosis in many types of cancer cells. Small conductance Ca^{2+} activated K^+ channel (SK4) is founded to be functional expressed in head and neck cancer cell carcinoma cells. The role of Ca^{2+} -activated K^+ channel (SK4) in head and neck squamous cell carcinoma (HNSCC) were investigated different cell lines; SNU-1076, OSC-19 and HN5 . Treatment with 1 μM ionomycin (Ca^{2+} ionophore) induced cell death in all of the three cell lines. Whole-cell patch clamp study suggested common expressions of Ca^{2+} -activated Cl^- channels (Ano-1) and Ca^{2+} -activated nonselective cation channels (CAN). 1-EBIO, an activator of SK4, induced outward K^+ current (I_{SK4}) in SNU-1076 and OSC-19. In HN5, I_{SK4} was not observed or negligible. The 1-EBIO-induced current was abolished by TRAM-34, a selective SK4 blocker. Interestingly, the ionomycin-induced cell death was effectively prevented by 1-EBIO in SNU-1076 and OSC-19, and the rescue effect was annihilated by combined TRAM-34. Consistent with the lower level of I_{SK4} , the rescue by 1-EBIO was least effective in HN5. The results demonstrate the role of SK4 in the fate of HNSCC under the Ca^{2+} overloaded condition. Pharmacological modulation of SK4 might provide an intriguing novel tool for the anti-cancer strategy in HNSCC.

Key words

Squamous cell cancer, Ionomycin, Ca²⁺-activated K⁺ channel, 1-EBIO,

Proliferation

Student number: 2014-22132

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LIST OF ABBREVIATIONS

HNSCC: Head and neck squamous cell carcinoma

K_{Ca} : Ca^{2+} -activated K^+ (K_{Ca}) channels

CAN: Ca^{2+} -activated nonselective cation

Cl_{Ca} : Ca^{2+} -activated Cl^- (Cl_{Ca}) channels

1-EBIO: 1-ethyl-2-benzimidazolinone

TRAM-34: 1-[(2-chlorophenyl) diphenylmethyl]-1*H*-pyrazole

T16A : T16Ainh-A01

SK4: Small conductance Ca^{2+} -activated K^+ channel type 4

Ano-1: Anoctamin 1

I_{SK4} : SK4 current

AVD: Apoptotic volume decrease

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) are challenging diseases. The cancer itself and its treatments impair the quality of life. In addition to the changes of the physical appearance, it causes deficits in speech, swallowing, taste and olfaction. To preserve the organ and its function, chemotherapy and radiation therapy are preferred to surgical resection in many patients with locally advanced diseases [1]. However, the chemotherapeutic agents are usually unspecific to the cancer cells, causing various complications damaging the normal cells and tissues. The efficacy of the molecular targeted agents for HNSCC is still very limited and the conventional chemotherapeutic agents such as cisplatin are still used. Therefore, further investigation of chemical agents affecting the proliferation and death of HNSCC is still requested.

Ion channels are critical players of physiological functions and pathophysiological processes [2]. Ion channels are activated by a variety of physicochemical factors and intracellular second messengers such as Ca^{2+} ion. The changes in cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_c$) are important and influence a number of ion channel activities. The representative Ca^{2+} -activated ion channels are, 1) two subfamily of Ca^{2+} -activated K^+ (K_{Ca}) channels (e.g. BK_{Ca} and SK_{Ca} (SK1 - 4), (2)) Ca^{2+} -activated nonselective cation (CAN) channels (e.g. TRPM4 and 5), and Ca^{2+} -activated Cl^- (Cl_{Ca}) channels (e.g. Ano1/TMEM16A) [3-7].

The Cl_{Ca} current equivalent to functional expression of Ano-1 is well known in squamous epithelial cells such as keratinocytes [8-9]. In the HNSCC

studies, Ano-1 has been suggested to play a role in metastasis and proliferation. Efflux of Cl^- is accompanied by water flux and subsequent cell volume changes. Such changes are thought to underlie the migration through narrow intercellular spaces and tumor metastasis. In fact, genomic amplification and protein expression of Ano-1 have been suggested as strong predictors of poor outcome in HNSCC [10-12].

Secretory types of epithelial cells express various K_{Ca} as well as Cl_{Ca} channels [13-17]. However, studies on K_{Ca} channels are rare in the squamous epithelial cells [18], and lacking in HNSCCs. The K^+ channel activation is generally responsible for hyperpolarization of membrane potential. The K^+ channel-dependent negative membrane voltage provides electrical driving force for the concomitant transport of other ions along with essential nutrients such as glucose and amino acids. In addition, the level of membrane potential affects cell cycle regulation and survival [19]. In some types of apoptotic conditions, excessive activation of K^+ efflux is regarded to be responsible for apoptotic volume decrease due to accompanied Cl^- and water efflux [20-22].

Recent evidence indicates that that potassium channels are involved in the proliferation of various cells. Decoursey and Cahalan first demonstrated that voltage gated potassium regulate T lymphocyte proliferation, in 1984 [37]. Small conductance Ca^{2+} activated K^+ channel (SK4) also play an important role in regulating cell proliferation. SK4 are widely expressed in exciting and non-exciting cells, has been implicated in endothelial cells, T lymphocytes, vascular smooth muscle cells, and various types of cancer cells [31-35, 38, 39]. SK4 are voltage insensitive and high sensitive to intracellular Ca^{2+} can be

activated by low concentration of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$). The intracellular Ca^{2+} act via binding to calmodulin, and then calmodulin interact with calmodulin binding site in C-terminal region. I_{SK4} is selectively blocked by clotrimazole and TRAM-34, both of which have no effect on SK1-3 and BK channels [4].

Sustained increase in $[\text{Ca}^{2+}]_c$ and subsequent Ca^{2+} overload in intracellular organelles (e.g. mitochondria) are generally thought to be harmful for cells and would induce cell death depending on the level of $[\text{Ca}^{2+}]_c$ and on the cell types. In fact, an application of ionophore such as ionomycin has been used as a cell death inducing condition in cancer cells [23-25]. Under the sustained increase in $[\text{Ca}^{2+}]_c$, ion channels would be also activated. Interestingly, Ano-1 has been reported to play roles in the migration and proliferation of HNSCCs and prostate cancer cells [10, 26]. However, previous studies have not paid attention to the role of K_{Ca} channels of Ca^{2+} -overloaded cancer cells, especially in HNSCCs.

On these backgrounds, we initially investigate the effects of ionomycin on the ion channel currents including I_{SK4} in HNSCCs. Secondly, the effects of pharmacological activators and inhibitor of the SK4 on the ionomycin-induced cell death are compared between the HNSCC cell lines. The present results demonstrate that pharmacological activation of SK4 effectively rescues HNSCCs from the ionomycin-induced cell death.

MATERIALS AND METHODS

1. Cell culture and proliferation assay

Three kinds of HNSCC cell lines were used in this study; SNU-1076, OSC-19, and HN5. SNU-1076 cells are originated from laryngeal cancer. OSC-19 and HN5 are originated from oral squamous cell carcinoma. SNU-1076, OSC-19, HN5 cells were maintained in RPMI-1640 media with 10% (v/v) fetal bovine serum (GIBCO) , 1% antibiotic/antimycotic (GIBCO) and 0.1% Gentamicin Reagent solution (GIBCO) at 37°C in a humidified, 5% CO₂, 95% air atmosphere and routinely sub-cultured using trypsin-EDTA. For proliferation assay, cells were seeded in culture plates and incubated for the specific time at 37°C prior to the treatment with specific drugs for indicated time. After treatment, Cell Counting Kit-8 (Dojindo Lab., Tokyo, Japan) was used to measure cell proliferation according to the manufacturer's instructions.

2. Electrophysiology

Whole-cell patch clamp experiments were performed on HN5, OSC-19, and SNU-1076 cells at room temperature (22–25 °C). Ionic currents and membrane potential were recorded in voltage-clamp and current-clamp mode, respectively, using Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster, CA, USA). Recorded traces were digitized, sampled at 10 kHz, and low-pass filtered at 2 kHz using Digidata 1440A converter (Axon Instrument).

Sampled data were stored on a computer using Clampex of pClamp 10.1 software (Axon Instrument). The patch-clamp data was analyzed by using Clampfit 10.3 and Origin 7.0 software (Microcal Inc., Northampton, MA, USA). Glass patch pipettes (World Precision Instruments, Sarasota, FL, USA) were pulled with PP-830 (Narishige, Tokyo, Japan) to make tip resistance 2.5~3 M Ω when filled with KCl or CsCl in pipette solution.

3. Solutions and Drugs

For the whole-cell patch clamp experiment, low Ca²⁺-buffered KCl pipette solution contained (in mM): 140 KCl, 10 HEPES, 5 NaCl, 3 Mg-ATP, 0.1 EGTA, 1 MgCl₂ and was adjusted to pH 7.2 with KOH. Ca²⁺-clamped KCl pipette solution contained (in mM) 140 KCl, 1 MgCl₂, 3 Mg-ATP, 10 HEPES, 5 EGTA, 5 NaCl, and 300 nM free Ca²⁺ calculated using the free WebMax software (www.stanford.edu/~cpatton/webmaxcS), and was adjusted to pH 7.25 with KOH. CsCl pipette solution contained (in mM): 140 CsCl, 1 MgCl₂, 3 Mg-ATP, 10 HEPES, 5 EGTA, 5 NaCl, and 600 nM free Ca²⁺ calculated using the free WebMax software and was adjusted to pH 7.25 with CsOH. Normal Tyrode's (NT) bath solution contained (in mM): 145 NaCl, 3.6 KCl, 1 MgCl₂, 1.3 CaCl₂, 10 HEPES, 5 glucose, 10 sucrose and was adjusted to pH 7.4 with NaOH. NMDG-Cl bath solution contained: 150 NMDG-Cl, 10 HEPES, 10 glucose, 1 MgCl₂, 1.3 CaCl₂ and was adjusted to pH 7.4 with NMDG-OH. Ionomycin, 1-ethyl-2-benzimidazolinone (1-EBIO) and 1-[(2-chlorophenyl) diphenylmethyl]-1*H*-pyrazole (TRAM-34) were purchased

from Sigma-Aldrich. T16Ainh-A01, a specific inhibitor of Ano-1, was purchased from TOCRIS Biosciences. The chemicals were initially dissolved in dimethyl sulfoxide (DMSO), and were diluted to the bath solution for experiments. The amount of DMSO in the bath solution was kept below 0.1 %.

4. $[Ca^{2+}]_i$ measurement

HNSCC cells were loaded with fura-2 acetoxymethylester (5 mM, 30 min, 25°C) and washed twice with HEPES-buffered NT solution. Fluorescence was monitored in a stirred quartz microcuvette (1 ml), and stirring in a temperature-controlled (37°C). The cell holder of a fluorescence spectrophotometer (Photon Technology International) at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. At the end of each experiment, 5 μ M ionomycin was applied to produce a maximum fluorescence ratio (R_{max} , 340/380 nm). Subsequently, 10 mM EGTA was added to confirm a minimum value of fluorescence ratio (R_{min}). In each cell, $R_{340/380}$ were normalized against the R_{max} and R_{min} [$(R_{340/380} - R_{min})/R_{max}$].

5. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cancer cells using the Trizol (Invitrogen, CA, USA), and 2 μ g of total RNA was reverse transcribed into first-strand cDNA. Reverse transcription was performed with RT pre-mix (Intron Bio, Korea) according to the manufacturer's instructions. The cDNA was amplified by 35 cycles of PCR with PCR PreMix (Intron, Korea) in a C1000 Thermal

Cycler (bio-rad, CA, USA). The SK1-4 primers were obtained from Bioneer (Bioneer, Korea). The expected product sizes from the customized primer sets were 155, 162, 150, and 154 bp for SK1 (product No.P243222), SK2 (P243234), SK3 (P114469) and SK4 (P249299), respectively. GAPDH was used as control. RT-PCR products were resolved by 1.2% agarose gel electrophoresis and visualized with ethidium bromide staining.

6. Statistical Analysis

Data are presented as the mean \pm standard deviation (SD) of at least triplicates, or as a representative of 3 separate experiments. Significance was determined between treated and untreated groups by two-sided Student's *t*-test. *P* values <0.05 were considered statistically significant.

RESULTS

Ionomycin induced currents (Ca^{2+} -activated Channels) in HNSCCs.

Firstly, ionomycin-induced changes in membrane conductance were investigated in; SNU-1076, OSC-19, and HN5. Low Ca^{2+} -buffered KCl pipette solution (0.1 mM EGTA) was used to allow $[\text{Ca}^{2+}]_i$ increase in response to the ionomycin treatment. Ramp-like voltage change from -100 to 60 mV was repetitively applied at every 10 s. During inter-pulse period, the cell membrane voltage was held at -60 mV (Fig. 1A, C, E). Before ionomycin application, membrane conductance was commonly low in the three cell lines; small amplitudes at both negative and positive voltages (Fig. 1B, D, F; black I/V curves labelled (1) in the right panels). Application of ionomycin gradually increased the membrane conductance (i.e. the slope of I/V curve) in the three cell lines. However, the dominant type of ionic conductance at the earlier period appeared different between the cell lines. In SNU-1076, outward K^+ current was initially activated as indicated from the upward deflection of basal current trace at the holding voltage of -60 mV (Fig. 1A, red I/V curve labelled (2)). Consistently, the I/V curve showed outward currents that reversed its direction at around -80 mV, close to the K^+ equilibrium potential (Fig. 1B, I/V curve in red). The weakly inwardly rectifying shape of I/V curve was consistent with the property of SK4. In the later phase of ionomycin application, however, the outward current disappeared with concomitant activation of inward current. Also the I/V curve at the later

period (ca. 4 - 5 min of ionomycin treatment) showed linear shape with reversal potential close to 0 mV (Fig. 1B, blue I/V curve labelled (3)). Different from SNU-1076, OSC-19 and HN5 cells did not show discernible SK4-like current but only showed inward currents at the negative clamp voltage (Fig. 1C, E). The I/V curves showed linear shapes with reversal potentials close to 0 mV (Fig. 1D, F, red I/V curves). A measurement of intracellular $[Ca^{2+}]_c$ with fura-2 fluorometry consistently showed increase in $[Ca^{2+}]_c$ to 500 -800 nM by ionomycin (n=5, data not shown).

Common expression of Ca^{2+} -activated Cl^- channels (Ano-1) and Ca^{2+} -activated nonselective cation channels (CAN) in the three cell lines

The I/V curves of ionomycin-induced currents in their steady-state with 0 mV of reversal potential suggested that either Cl_{Ca} (Ano-1) or CAN channels were activated. To evaluate the relative contribution of Cl_{Ca} and CAN channels in HNSCC, CsCl pipette solution with fixed level of high $[Ca^{2+}]_c$ (600 nM) was adopted in the next whole-cell patch clamp experiment (Fig. 2). K^+ current was excluded by replacing the intracellular K^+ with Cs^+ that are impermeable to K^+ channels. The membrane current increased slowly after making the whole-cell configuration. After confirmed a steady-state (I/V curves in black, Fig. 2A-C), 10 μ M T16A, a selective inhibitor of Ano-1, commonly decreased the conductance in the three cell lines (I/V curves in red, Fig. 2A-C). Then, in the presence of T16A, all the extracellular Na^+ was

replaced by NMDG⁺, a large organic cation impermeable to cation channels. On the NMDG⁺ replacement, additional decrease of inward current was observed at the negative membrane voltages (I/V curves in green, Fig. 2A-C). The current density normalized to the steady-state control current (%) showed that about half of the current was inhibited by T16A in all three cell types (Fig. 2D). In OSC-19, most of the remaining inward current was abolished by replacing extracellular Na⁺ with NMDG⁺ (Fig. 2B, D). In SNU-1076 and HN5, however, 10 - 20 % of the conductance was remained even after the removal of Na⁺. The nature of T16A- and NMDG⁺-resistant conductance was not further investigated here.

Ionomycin (1 μ M, 48 h) induces cell death of HNSCCs, effectively rescued by 1-EBIO.

To investigate the role of Ano-1 and SK4 in the Ca²⁺-overloaded HNSCCs, viability was assessed in the ionomycin-treated cells with T16A (Ano-1 inhibitor) or TRAM-34 (SK4 inhibitor). The number of live cells was counted at 48 h after 1 μ M ionomycin treatment, and the numbers were commonly decreased in all the three cell lines. However, neither T16A nor TRAM-34 changed the tendency of ionomycin-induced suppression of proliferation in all the three cell lines (Fig. 3A). Then we tested the effect of 1-EBIO, an activator of SK channels, on the ionomycin-treated HNSCCs. Interestingly, the co-treatment of 1-EBIO with ionomycin effectively recovered the live cell numbers close to the control levels in SNU-1076 and OSC-19. The rescue

effect of 1-EBIO was observed already from the relatively low range of concentration (3 μM and 10 μM), and was completely reversed by the SK4 channel specific inhibitor TRAM-34 (Fig. 3B). However, 1-EBIO rescued the ionomycin-treated HN5 cells only to a small extent (Fig. 3B). The treatment with 1-EBIO or TRAM-34 without ionomycin did not significantly affect the viability of HNSCCs. Representative data for SNU-1076 is show in Fig. 3C.

Molecular identification of SK4 channels in three cell lines.

The rescue effect of 1-EBIO on OSC-19 was unexpected since ionomycin did not directly induce noticeable I_{SK4} in OSC-19 (Fig. 1C, D). Therefore we were attempted to evaluate whether 1-EBIO actually induces I_{SK4} in OSC-19. Here we used KCl pipette solution containing 300 nM of free Ca^{2+} activity. With the moderate level of $[\text{Ca}^{2+}]_c$ alone, i.e. 300 nM, Ca^{2+} -activated ion channel current was not significantly observed. However, the treatment with 1-EBIO induced outward K^+ currents with weakly inward rectifying property in the three cell lines (Fig. 4). Interestingly, the current density of 1-EBIO-induced I_{SK4} was the largest in OSC-19 and smallest in HN5 (Fig. 4D). Then we performed RT-PCR analysis for SK1-4 in the three cell lines, as was detected from the pharmacological assay of membrane current (Fig. 4D), SK4 transcript was conformed in SNU-1076 and OSC-19 while not in HN5 (Fig. 4E). However, unexpectedly, all the three cell lines showed positive signal for the mRNA of SK1. Since the 1-EBIO-activated current was negligible in

HN5, and the K^+ current induced by 1-EBIO completely inhibited by the SK4 channel specific inhibitor TRAM-34 in SNU-1076 and OSC-19 cell lines, we interpreted that the functional SK1 channel proteins not expressed in the three cell lines (Fig. 4E).

1-EBIO additionally activates SK4 current in the ionomycin-treated cancer cells

Then we investigated whether 1-EBIO also affect the K^+ conductance of ionomycin-treated cells with unclamped $[Ca^{2+}]_i$ conditions, i.e. 0.1 mM EGTA in KCl pipette solution. As shown in Fig. 1, application of 1 μ M ionomycin induced outward K^+ current in SNU-1076 whereas inward currents in OSC-19 and HN5. Interestingly, addition of 50 μ M 1-EBIO induced large outward current in both OSC-19 and SNU-1076. Subsequent application of TRAM-34 effectively abolished the 1-EBIO-induced K^+ currents (Fig. 5A-D). In HN5 cells, however, application of 1-EBIO still induced only minute increase of membrane conductance (Fig. 5E-F).

1-EBIO activated SK4 channel induces membrane hyperpolarization.

The activation of I_{SK4} by 1-EBIO implied membrane hyperpolarization as suspected from the negative shift of reversal potentials (Fig. 5B and D). To confirm the actual effects on membrane potential, zero-current clamp recording was performed. Ionomycin, 1-EBIO and TRAM-34 were serially

applied to HNSCCs with 0.1 mM EGTA/145 mM KCl in the pipette solution. All the three cell lines showed depolarized membrane potential between -15 and 0 mV at the steady-state of whole-cell configuration. In SNU-1076, ionomycin induced initial negative shift of membrane potential, which was further hyperpolarized by 1-EBIO (Fig. 6A). In OSC-19, ionomycin induced a slight depolarization, which was reversed to strong hyperpolarization by 1-EBIO (Fig. 6B). The final application of TRAM-34 depolarized the membrane potential in both SNU-1076 and OSC-19. In HN5, however, neither ionomycin nor 1-EBIO induced consistent changes in the membrane potential (Fig. 6C).

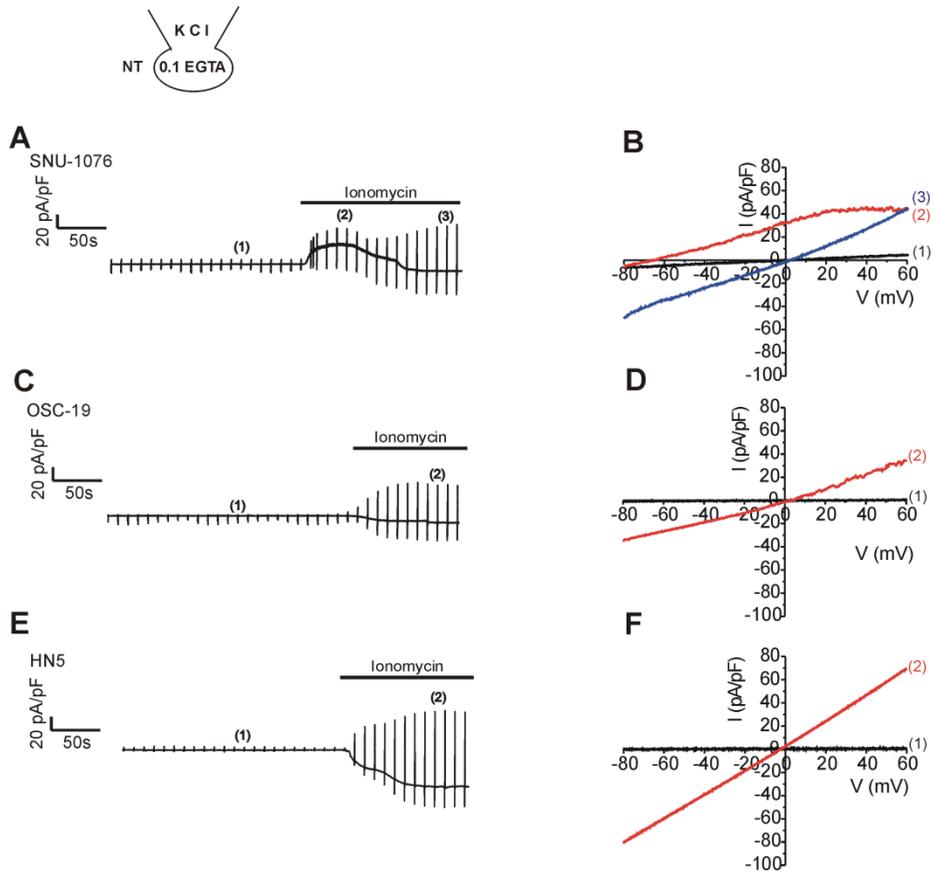


Figure 1. Ionomycin-induced membrane currents in HNSCCs

(A, C, E) Representative original current traces in response to repetitive ramp-like pulses (from -100 to 60 mV, holding voltage, -60mV) in whole-cell voltage clamped cells. The type of cell line is indicated above each trace. KCl pipette solution with 0.1 mM EGTA was commonly used. The numbers in parenthesis indicate the corresponding current to voltage relations (I/V curves) depicted in the right panels (B, D, F). After achieving the whole-cell configuration, the steady-state conductance was very small; black I/V curves that are almost indistinguishable from the horizontal axis. On applying 1 μ M ionomycin, membrane conductance increased gradually, and the addition of 5

μM TRAM-34 inhibited outward currents in SNU-1076 (A, B). In contrast, the ionomycin-treated OSC-19 (time break, 1min) and HN5 showed negligible inhibition by TRAM-34 (C, D).

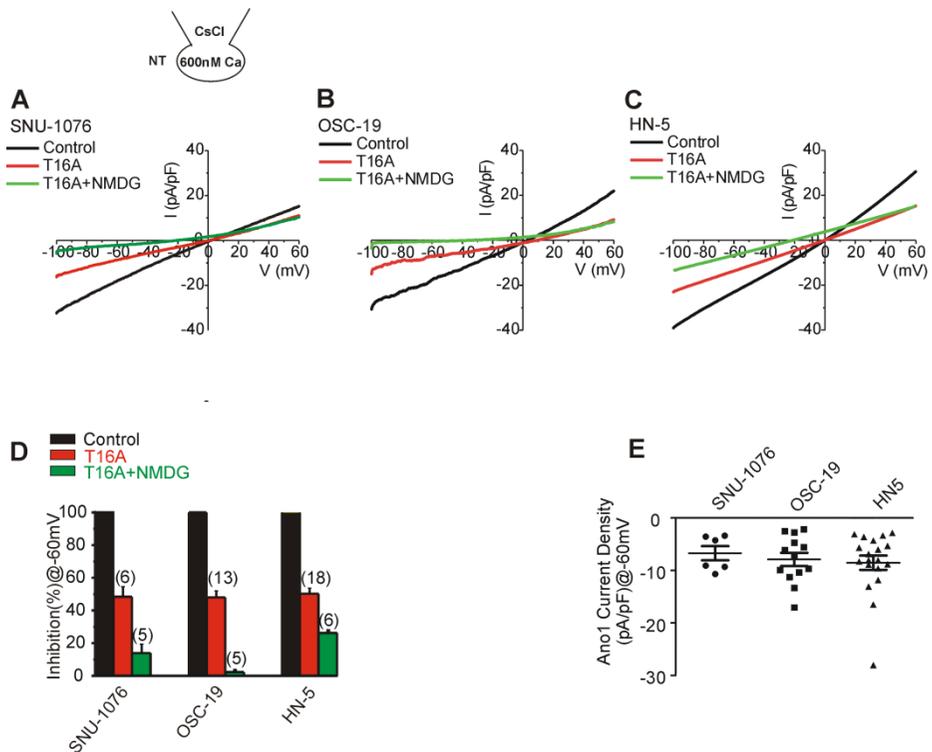


Figure 2. Ca^{2+} -activated Cl^- current and Ca^{2+} -activated nonselective cationic current in HNSCCs

(A-C) Representative I/V curves obtained by ramp-like pulses used CsCl pipette solution with 600nM free Ca^{2+} activity in the three types of cell lines. Steady-state Ca^{2+} -activated conductance (black I/V curves in each panel) was decreased by about half after applying 10 μM T16A, a selective inhibitor of Ano-1 (I/V curves in red). The remained conductance, especially the inward current, was further decreased by replacing extracellular Na^+ with NMDG^+ (T16A+NMDG, I/V curves in green). (D) Summary of normalized inward current densities at -60 mV. The inward current density (pA/pF) was normalized (%) to the control steady-state in each cell. The remained current after T16A (red) and Na^+ removal (green) are averaged. (E) Summary Ano-1

current densities (pA/pF) at -60mV in HNSCCs. Ano-1 current was obtained by subtracting T16A-resistant current from control in each cell, and the plotted dots indicate data from individual cell.

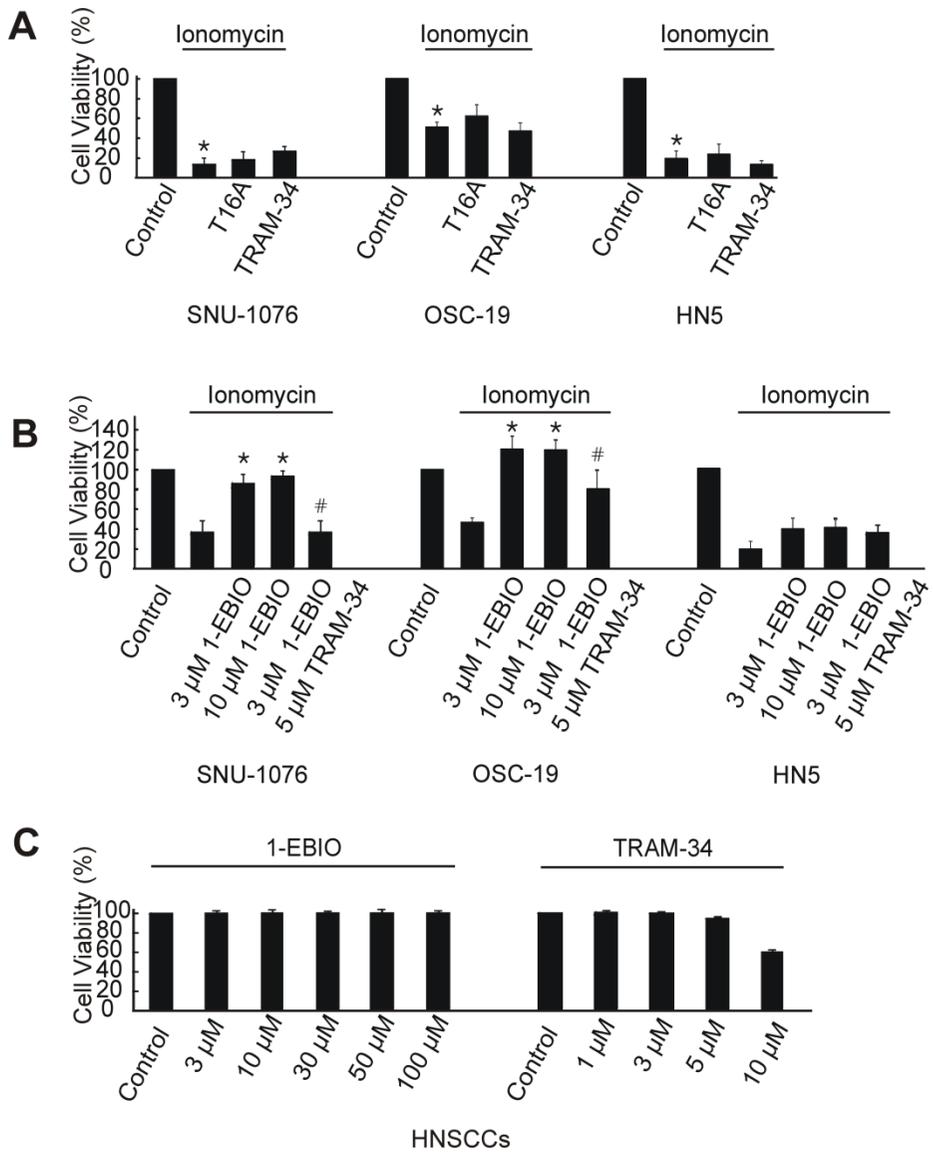


Figure 3. Iononycin-induced cell death and rescue effects of 1-EBIO in HNSCCs

(A) Cells from each cell line were divided into four groups; non-treated (control), treated with 1 μM iononycin, iononycin + 0.1 μM T16A and iononycin + 10 μM TRAM-34. Cells were subjected to proliferation assay at 48h and normalized to the control (cell viability). Summary of MTT

experiments are displayed. *P* values were based on comparison with control (*, $P < 0.001$).

(B) Cells from each cell line were divided into five groups; non-treated (control), treated with 1 μM ionomycin, ionomycin + 3 μM 1-EBIO, ionomycin + 10 μM 1-EBIO, and ionomycin/3 μM 1-EBIO + 5 μM TRAM-34. Cell numbers at 48 h were counted and normalized to the control (n= 6). *P* values were based on comparison with ionomycin-treated group (*, $P < 0.001$) and ionomycin plus 1-EBIO (3 μM)-treated group (#, $P < 0.005$).

(C) Effects of various concentrations of 1-EBIO or TRAM-34 on the cell viability of SNU-1076 without ionomycin treatment at 48 h.

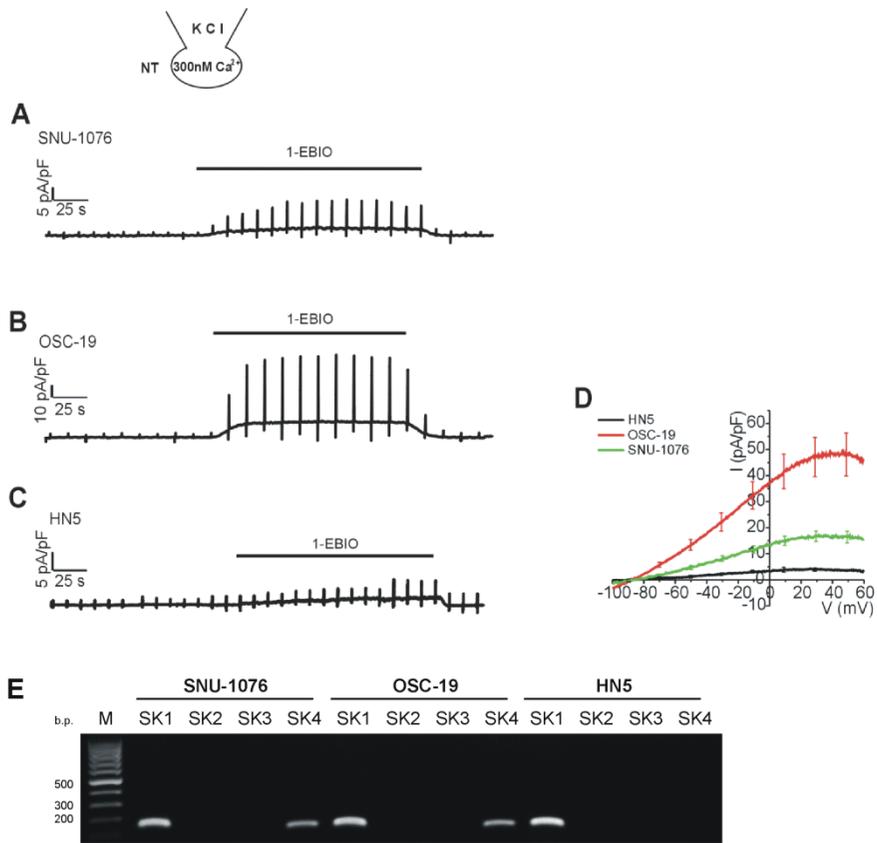


Figure 4. Effects of 1-EBIO on the membrane conductance of HNSCCs with clamped $[Ca^{2+}]_i$

(A-C) Representative current traces in response to ramp pulses (from -100 to 60 mV, holding voltage, -60mV) and to the application of 50 μ M 1-EBIO in HNSCCs. The Ca^{2+} activity of KCl pipette solution was equilibrated to 300 nM with 5 mM EGTA. (D) Summary of I/V curves of the 1-EBIO-induced currents in each cell line (mean \pm SEM, n=7, respectively). (E) Identification of SK mRNA in HNSCC cancer cells. mRNA was detected for SK1 and SK4 by RT-PCR.

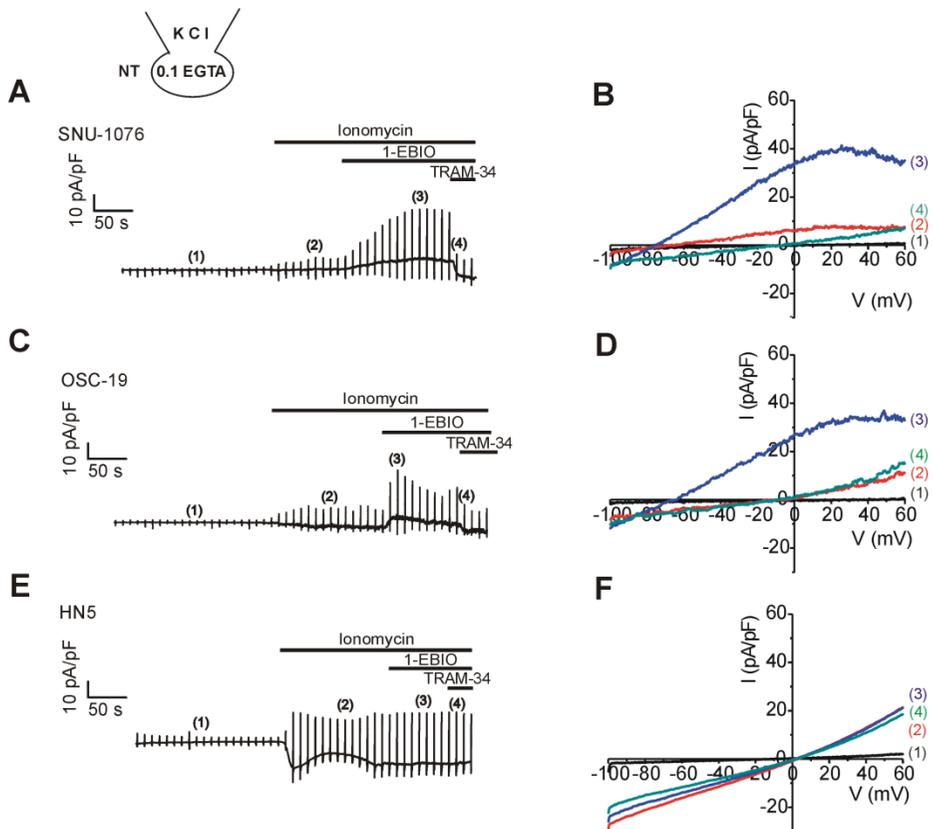


Figure 5. Effects of 1-EBIO on the membrane conductance of HNSCCs pretreated with ionomycin under Ca^{2+} -unclamped condition

Left panels (A, C, E) representative current traces of HNSCCs in response to the depolarizing ramp pulses. 1 μM ionomycin, 50 μM 1-EBIO, and 5 μM TRAM-34 were applied as indicated above each trace. Right panels (B, D, F); I/V curve obtained by the ramp pulses in each cell line of HNSCCs as indicated at the corresponding left panels. The specific timing of I/V curve is indicated by the number in parenthesis. Note the weakly inward rectifying outward currents induced by 1-EBIO in SNU-1076 and OSC-19.

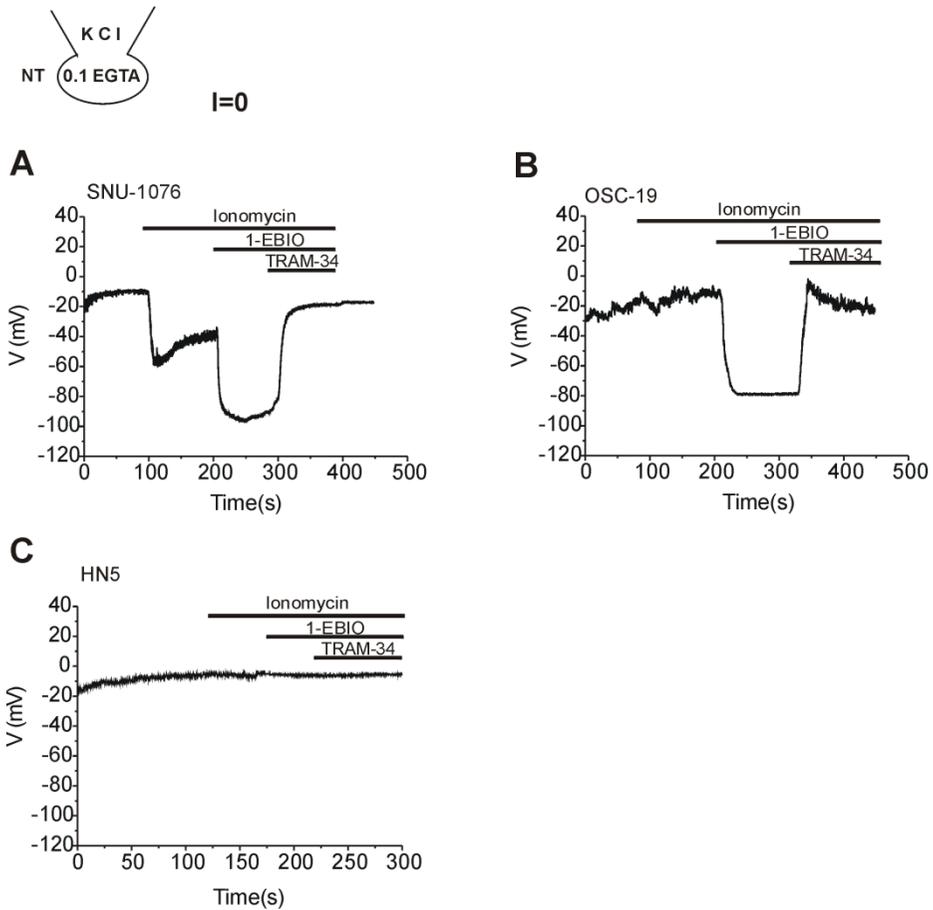


Figure 6. Membrane voltage changes in response to ionomycin, 1-EBIO and TRAM-34 in HNSCCs

Traces of membrane potential in HNSCCs obtained under zero-current clamp condition with 0.1 mM EGTA KCl pipette solution. 1 μ M ionomycin, 50 μ M 1-EBIO, and 5 μ M TRAM-34 were applied as indicated in each panel. Representative results from more than five cells from each cell line are shown. Ionomycin alone hyperpolarized the membrane potential in SNU-1076 (A) while the combined application of 1-EBIO induces strong hyperpolarization in both SNU-1076 and OSC-19 (A, B). Final addition of TRAM-34 depolarized the membrane potential in SNU-1076 and OSC-19 (A, B).

Neither ionomycin nor 1-EBIO induced significant change in the membrane potential of HN5 (C).

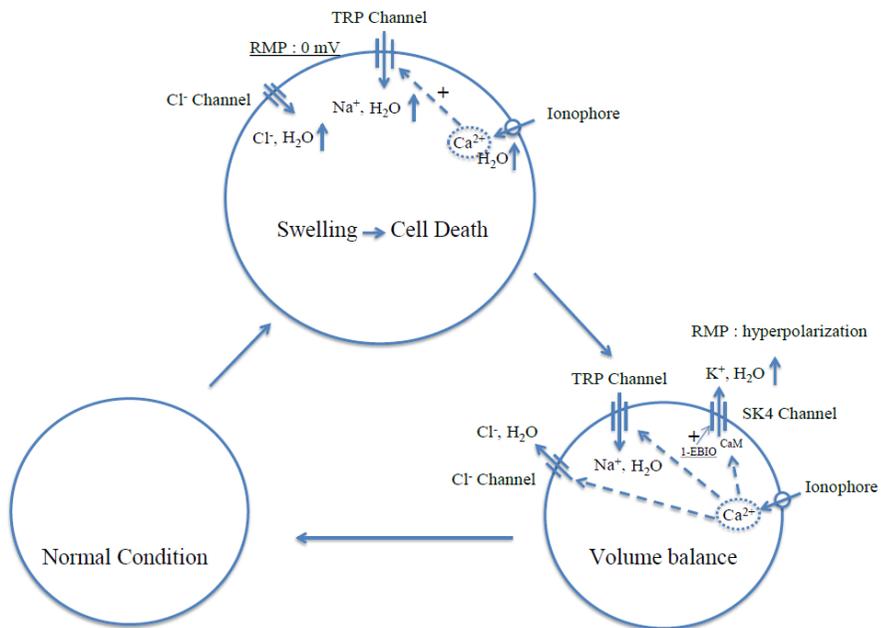


Figure 7. Hypothesis for Ca^{2+} dependent channels in cell death and rescue effect by cell volume regulation.

A rise of $[\text{Ca}^{2+}]_i$ activates Ca^{2+} -activated nonselective cation channels (CAN) together with Cl^- channels to increase intracellular Na^+ and Cl^- ions. Resulting osmotic stress and water influx cause cell swelling, which might be induce cell death when excessive. Activation of SK4 by 1-EBIO, membrane can be plasma membrane hyperpolarization and intracellular Ca^{2+} may activate K^+ and Cl^- ions efflux along with the water, which might be helpful to maintain the cell volume balance.

DISCUSSION

Our present study demonstrates that HNSCCs differentially express functional SK4 channels as revealed by the amplitudes of 1-EBIO-induced I_{SK4} . The pharmacological activation of I_{SK4} rescued the HNSCCs from ionomycin-induced cell death. The abolishment of 1-EBIO effects by TRAM-34 is also the evidence indicating specific role of SK4. The activation of SK4, i.e. K^+ efflux and membrane hyperpolarization appear to be the critical conditions for the rescue effect. In fact, the rescue effect was least significant in HN5 cells where the current density of SK4 was smaller than the other cell lines.

Ca²⁺-activated ion channels in HNSCCs

In the resting HNSCCs without raised $[Ca^{2+}]_c$, little background ionic conductance was observed; the slopes of I/V curves were very shallow in all the three cell lines tested. In contrast, experimental measures increasing $[Ca^{2+}]_c$ consistently activated multiple types of ion channels permeating K^+ (SK4), Cl^- (Cl_{Ca}) and Na^+ (CAN) with differential contributions depending on the cell types. Molecular identity of the Cl_{Ca} has been recently discovered as Ano-1/TMEM16A, and found in HNSCCs [10, 27]. The significant decrease of Ca^{2+} -activated inward current by T16A indicates the expression of Ano-1 in the three lines of HNSCCs investigated here. In addition to Ano-1, the incomplete inhibition by T16A and further decrease by replacing Na^+ with NMDG⁺ indicate that CAN channels are expressed in HNSCCs (Fig. 2). In the

present study, the precise molecular identity of CAN was not investigated. The molecular identity of CAN is generally ascribed to TRPM4 and TRPM5, members of TRP channel superfamily [5, 6].

The activation of Cl_{Ca} and CAN commonly induces depolarization of HNSCC close to their Nernst equilibrium voltages (V_{eq}) of Cl^- or monovalent cations in the present experimental conditions. Therefore large increase in $[Ca^{2+}]_c$ inevitably set the membrane potential of HNSCCs close to 0 mV. Since the HNSCCs do not show background K^+ conductance, the resting membrane potentials are depolarized. The lack of voltage changes by ionomycin alone in OSC-19 and HN5 seem to be due to the already depolarized resting membrane potentials of HNSCCs (Fig. 6). In other words, application of ionomycin would further stabilize the depolarized states. Even in 1076 cells where the activation by SK4 by ionomycin was evident, application of ionomycin induces only a moderate hyperpolarization (Fig. 6A).

A finding of present study was the requirement of 1-EBIO for the activation of SK4 in OSC-19. Although the final amplitude of 1-EBIO-induced I_{SK4} was comparable or even larger in OSC-19 than SNU-1076, the application of ionomycin did not effectively activate I_{SK4} in OSC-19. Such results might imply that the activation of SK4 require, in addition to $[Ca^{2+}]_c$ increase, unknown factors or molecular components that are selectively modulated by 1-EBIO in HNSCCs.

Role of SK4 in cancer cells

The effects of 1-EBIO on the cell viability of ionomycin-treated HNSCCs can be associated with the level of I_{SK4} because TRAM-34 effectively reversed the rescue effect by 1-EBIO (Fig. 3). Thus one can suggest that the full activation of SK4 and strong hyperpolarization are critical for the effects of 1-EBIO. What is the precise target or mechanism of SK4 activation in the cell proliferation of HNSCCs? Unfortunately, we do not have concrete answers to that question yet but could only provide general inquiry of previous studies.

The importance of K^+ channel activity in cell cycle regulation and proliferation/death has been investigated in various aspects and the responses are widely variable from proliferation to cell death [22, 28, 29]. In the studies of apoptosis, it is proposed that the excessive activation of K^+ channel and hyperpolarization would induce continuous efflux of Cl^- as well as K^+ , which is accompanied by the loss of cytoplasmic water and cell volume, causing apoptotic volume decrease (AVD) [22, 30]. In this respect, the excessive activation of SK4 in line with Ano-1 may also induce cell volume decrease in SNU-1076 and OSC-19. However, the outcome of the cell viability was opposite to the K^+ -channel dependent AVD suggested by Bortner's group [22]. Thus the final effects of K^+ efflux on cell proliferation seem to be variable or could even show opposite results depending on the context of intracellular signals and the cell types.

Without K^+ channel activation, the CAN activated by Ionomycin induced intracellular Ca^{2+} influx causes Na^+ influx, depolarization of the cell membrane, and Ano-1 dependent Cl^- influx. In excessive activation of CAN and Ano-1, results osmotic increase cause rapid cell excessive swelling and induced cell death. Activation of SK4 by 1-EBIO, hyperpolarization of the membrane would induce Cl^- efflux, which would be helpful to maintain cell volume balance. Maintenance of the volume balance contributes to carcinoma cells metabolic homeostasis, which might rescue ionomycin induced cell death (Fig. 8).

Partly consistent with our present results, the previous studies of breast, prostate and pancreatic cancer cells commonly report the inhibition of cell proliferation by SK4 inhibitor [31, 32]. In another study, the proliferation of human prostate cancer cell lines (LNCaP and PC-3) was increased by 1-EBIO, and this effect was again reversed by SK4 inhibitors [33]. Regarding to the mechanism, it has been proposed that the hyperpolarization and augmented Ca^{2+} influx promotes the transition of cell cycle phases and thereby proliferation [32, 34]. Since the treatment of HNSCCs with ionomycin alone would induce significant increase in $[Ca^{2+}]_c$, the theory of augmented Ca^{2+} influx by I_{SK4} -induced hyperpolarization seems unlikely for the rescue effect of 1-EBIO. In fact, a measurement of intracellular $[Ca^{2+}]_c$ with fura-2 fluorometry consistently showed large increase in $[Ca^{2+}]_c$ by ionomycin alone, that was not significantly augmented by 1-EBIO in SNU-1076 and OSC-19 (n=5, data not shown). Recently, Millership *et al* proposed that SK4 may directly activate ERK1/2 and JNK signaling pathways in a voltage-

independent way, which promotes proliferation [35]. The putative non-electrical role of SK4 directly interacting with the intracellular signaling molecules might have to be also considered in HNSCCs. Apart from the Ca²⁺-activated K⁺ channels, background type K⁺ channels such as TREK-2 could also regulate the cancer cell proliferation [36].

CONCLUSION

In summary, this study firstly demonstrates the expression of SK4 and functional activation by 1-EBIO in HNSCC. Although the precise mechanisms are not known yet, the activation of I_{SK4} and membrane hyperpolarization induced by 1-EBIO effectively rescue the ionomycin-induced cell death in the HNSCCs expressing SK4. Pharmacological modulation of SK4 might provide a hint for the development of more effective anti-cancer agent or better strategy for cancer treatment.

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국문 초록

암세포에 존재하는 이온통로들에 대한 연구는 암세포 증식/사멸에 대한 조절 여부 및 그 기전과 관련하여 주목받고 있다. 세포 내 칼슘증가와 관련된 신호전달은 암세포 증식과 사멸에 중요하다. 이 연구를 통해 칼슘 의존성 포타슘 이온통로들 중 하나인 SK4 를 두경부 암세포주 (HNSCCs) 에서 발견하였다. HNSCC의 세 가지 세포주들인 HN5, OSC-19, SNU-1076에서 SK4의 역할을 분석하였다. 세포내 칼슘농도를 크게 높여주는 약물인 Ca^{2+} Ionophore인 Ionomycin을 투여하면 세포주들 모두에서 세포사멸이 일어났다. 전세포 막전압고정법(whole cell patch clamp)을 적용하여 세가지 cell line 에서 모두 Ca^{2+} -activated Cl^- channels (Ano-1) 과 Ca^{2+} -activated nonselective cation channels (CAN)의 활성화를 발견하였다. 하지만 SK4를 통한 K^+ 전류는 SNU-1076과 OSC-19 세포주에서만 관찰되었다. SK4 활성화제인 1-EBIO를 투여하더라도 SNU-1076과 OSC-19에서는 커다란 외향전류가 유도되었지만, HN5 세포주에서는 이러한 외향 전류가 거의 존재하지 않거나 약간의 증가만 관찰될 뿐이었다. 1-EBIO에 의하여 유도 되었던 외향 전류는 SK4 channel의 특정한

억제제인 TRAM-34에 의하여 억제되었다. Ionomycin에 의한 세포사멸은 1-EBIO를 함께 투여하면 방지되었는데, 이 현상은 SNU-1076과 OSC-19 세포주에서만 관찰되었다. 이 세포들에서도, SK4 차단제인 TRAM-34를 추가하면 세포사멸이 또다시 관찰되었다. 1-EBIO 의 세포사멸 방지현상은 HN5 세포주에서는 관찰되지 않았다. 이러한 실험 결과는 두경부 암세포주가 과도한 세포내 칼슘에 의해서 세포사멸이 일어나는 과정에서 K^+ 이온통로가 일으키는 작용을 처음으로 보여주었다. 두경부 암에서 이러한 SK4 channel을 조절해주는 약리학적 현상은 항암 치료제를 개발하는데 참고할 수 있는 흥미로운 작용기전을 시사한다.

주요어 : 편평 세포암, Ionomycin, Ca^{2+} -activated K^+ channel, 1-EBIO, 세포 증식

학 번: 2014-22132



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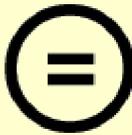
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**Rescue effects of small conductance
Ca²⁺-activated K⁺ channel (SK4)
activator on the Ca²⁺ overload
induced death of head and neck
cancer cells**

칼슘과부하에 의한 두경부암세포 사멸현상에
대한 SK4 포타슘 이온통로 활성화제의 세포사멸
방지효과

2016년 02월

서울대학교 대학원
의과학과 의과학전공

윤 명 철

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서울대학교 대학원
의과학과 의과학전공
윤 명 철

<p style="text-align: center;">Rescue effects of small conductance Ca²⁺-activated K⁺ channel (SK4) activator on the Ca²⁺ overload induced death of head and neck cancer cells</p>				
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A thesis of the Master's degree

**Rescue effects of small conductance
Ca²⁺-activated K⁺ channel (SK4)
activator on the Ca²⁺ overload
induced death of head and neck
cancer cells**

칼슘과부하에 의한 두경부암세포 사멸현상에
대한 SK4 포타슘 이온통로 활성화제의 세포사멸
방지효과

February, 2016

The Department of Biomedical Sciences,

Seoul National University

College of Medicine

Yin Ming Zhe

칼슘과부하에 의한 두경부암세포 사멸현상에 대한
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지도 교수 김 성 준

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윤 명 철

윤 명 철의 의학석사 학위논문을 인준함
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위 원 장 _____ (인)
부위원장 _____ (인)
위 원 _____ (인)

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Ca²⁺-activated K⁺ channel (SK4)
activator on the Ca²⁺ overload induced
death of head and neck cancer cells**

by

Yin Ming Zhe

**A thesis submitted to the Department of Biomedical Sciences
in partial fulfillment of the requirements for the Degree of
Master of Science in Medicine (Physiology) at Seoul National
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Approved by Thesis Committee:

Professor _____ Chairman

Professor _____ Vice chairman

Professor _____

ABSTRACT

Ion channels in carcinoma and their roles in cell proliferation are drawing attention. Intracellular Ca^{2+} ($[\text{Ca}^{2+}]_c$)-dependent signaling affects the fate of cancer cells. K^+ channels play critical roles in proliferation and apoptosis in many types of cancer cells. Small conductance Ca^{2+} activated K^+ channel (SK4) is founded to be functional expressed in head and neck cancer cell carcinoma cells. The role of Ca^{2+} -activated K^+ channel (SK4) in head and neck squamous cell carcinoma (HNSCC) were investigated different cell lines; SNU-1076, OSC-19 and HN5 . Treatment with 1 μM ionomycin (Ca^{2+} ionophore) induced cell death in all of the three cell lines. Whole-cell patch clamp study suggested common expressions of Ca^{2+} -activated Cl^- channels (Ano-1) and Ca^{2+} -activated nonselective cation channels (CAN). 1-EBIO, an activator of SK4, induced outward K^+ current (I_{SK4}) in SNU-1076 and OSC-19. In HN5, I_{SK4} was not observed or negligible. The 1-EBIO-induced current was abolished by TRAM-34, a selective SK4 blocker. Interestingly, the ionomycin-induced cell death was effectively prevented by 1-EBIO in SNU-1076 and OSC-19, and the rescue effect was annihilated by combined TRAM-34. Consistent with the lower level of I_{SK4} , the rescue by 1-EBIO was least effective in HN5. The results demonstrate the role of SK4 in the fate of HNSCC under the Ca^{2+} overloaded condition. Pharmacological modulation of SK4 might provide an intriguing novel tool for the anti-cancer strategy in HNSCC.

Key words

Squamous cell cancer, Ionomycin, Ca²⁺-activated K⁺ channel, 1-EBIO,

Proliferation

Student number: 2014-22132

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LIST OF ABBREVIATIONS

HNSCC: Head and neck squamous cell carcinoma

K_{Ca} : Ca^{2+} -activated K^+ (K_{Ca}) channels

CAN: Ca^{2+} -activated nonselective cation

Cl_{Ca} : Ca^{2+} -activated Cl^- (Cl_{Ca}) channels

1-EBIO: 1-ethyl-2-benzimidazolinone

TRAM-34: 1-[(2-chlorophenyl) diphenylmethyl]-1*H*-pyrazole

T16A : T16Ainh-A01

SK4: Small conductance Ca^{2+} -activated K^+ channel type 4

Ano-1: Anoctamin 1

I_{SK4} : SK4 current

AVD: Apoptotic volume decrease

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) are challenging diseases. The cancer itself and its treatments impair the quality of life. In addition to the changes of the physical appearance, it causes deficits in speech, swallowing, taste and olfaction. To preserve the organ and its function, chemotherapy and radiation therapy are preferred to surgical resection in many patients with locally advanced diseases [1]. However, the chemotherapeutic agents are usually unspecific to the cancer cells, causing various complications damaging the normal cells and tissues. The efficacy of the molecular targeted agents for HNSCC is still very limited and the conventional chemotherapeutic agents such as cisplatin are still used. Therefore, further investigation of chemical agents affecting the proliferation and death of HNSCC is still requested.

Ion channels are critical players of physiological functions and pathophysiological processes [2]. Ion channels are activated by a variety of physicochemical factors and intracellular second messengers such as Ca^{2+} ion. The changes in cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_c$) are important and influence a number of ion channel activities. The representative Ca^{2+} -activated ion channels are, 1) two subfamily of Ca^{2+} -activated K^+ (K_{Ca}) channels (e.g. BK_{Ca} and SK_{Ca} (SK1 - 4), (2)) Ca^{2+} -activated nonselective cation (CAN) channels (e.g. TRPM4 and 5), and Ca^{2+} -activated Cl^- (Cl_{Ca}) channels (e.g. Ano1/TMEM16A) [3-7].

The Cl_{Ca} current equivalent to functional expression of Ano-1 is well known in squamous epithelial cells such as keratinocytes [8-9]. In the HNSCC

studies, Ano-1 has been suggested to play a role in metastasis and proliferation. Efflux of Cl^- is accompanied by water flux and subsequent cell volume changes. Such changes are thought to underlie the migration through narrow intercellular spaces and tumor metastasis. In fact, genomic amplification and protein expression of Ano-1 have been suggested as strong predictors of poor outcome in HNSCC [10-12].

Secretory types of epithelial cells express various K_{Ca} as well as Cl_{Ca} channels [13-17]. However, studies on K_{Ca} channels are rare in the squamous epithelial cells [18], and lacking in HNSCCs. The K^+ channel activation is generally responsible for hyperpolarization of membrane potential. The K^+ channel-dependent negative membrane voltage provides electrical driving force for the concomitant transport of other ions along with essential nutrients such as glucose and amino acids. In addition, the level of membrane potential affects cell cycle regulation and survival [19]. In some types of apoptotic conditions, excessive activation of K^+ efflux is regarded to be responsible for apoptotic volume decrease due to accompanied Cl^- and water efflux [20-22].

Recent evidence indicates that that potassium channels are involved in the proliferation of various cells. Decoursey and Cahalan first demonstrated that voltage gated potassium regulate T lymphocyte proliferation, in 1984 [37]. Small conductance Ca^{2+} activated K^+ channel (SK4) also play an important role in regulating cell proliferation. SK4 are widely expressed in exciting and non-exciting cells, has been implicated in endothelial cells, T lymphocytes, vascular smooth muscle cells, and various types of cancer cells [31-35, 38, 39]. SK4 are voltage insensitive and high sensitive to intracellular Ca^{2+} can be

activated by low concentration of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$). The intracellular Ca^{2+} act via binding to calmodulin, and then calmodulin interact with calmodulin binding site in C-terminal region. I_{SK4} is selectively blocked by clotrimazole and TRAM-34, both of which have no effect on SK1-3 and BK channels [4].

Sustained increase in $[\text{Ca}^{2+}]_c$ and subsequent Ca^{2+} overload in intracellular organelles (e.g. mitochondria) are generally thought to be harmful for cells and would induce cell death depending on the level of $[\text{Ca}^{2+}]_c$ and on the cell types. In fact, an application of ionophore such as ionomycin has been used as a cell death inducing condition in cancer cells [23-25]. Under the sustained increase in $[\text{Ca}^{2+}]_c$, ion channels would be also activated. Interestingly, Ano-1 has been reported to play roles in the migration and proliferation of HNSCCs and prostate cancer cells [10, 26]. However, previous studies have not paid attention to the role of K_{Ca} channels of Ca^{2+} -overloaded cancer cells, especially in HNSCCs.

On these backgrounds, we initially investigate the effects of ionomycin on the ion channel currents including I_{SK4} in HNSCCs. Secondly, the effects of pharmacological activators and inhibitor of the SK4 on the ionomycin-induced cell death are compared between the HNSCC cell lines. The present results demonstrate that pharmacological activation of SK4 effectively rescues HNSCCs from the ionomycin-induced cell death.

MATERIALS AND METHODS

1. Cell culture and proliferation assay

Three kinds of HNSCC cell lines were used in this study; SNU-1076, OSC-19, and HN5. SNU-1076 cells are originated from laryngeal cancer. OSC-19 and HN5 are originated from oral squamous cell carcinoma. SNU-1076, OSC-19, HN5 cells were maintained in RPMI-1640 media with 10% (v/v) fetal bovine serum (GIBCO) , 1% antibiotic/antimycotic (GIBCO) and 0.1% Gentamicin Reagent solution (GIBCO) at 37°C in a humidified, 5% CO₂, 95% air atmosphere and routinely sub-cultured using trypsin-EDTA. For proliferation assay, cells were seeded in culture plates and incubated for the specific time at 37°C prior to the treatment with specific drugs for indicated time. After treatment, Cell Counting Kit-8 (Dojindo Lab., Tokyo, Japan) was used to measure cell proliferation according to the manufacturer's instructions.

2. Electrophysiology

Whole-cell patch clamp experiments were performed on HN5, OSC-19, and SNU-1076 cells at room temperature (22–25 °C). Ionic currents and membrane potential were recorded in voltage-clamp and current-clamp mode, respectively, using Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster, CA, USA). Recorded traces were digitized, sampled at 10 kHz, and low-pass filtered at 2 kHz using Digidata 1440A converter (Axon Instrument).

Sampled data were stored on a computer using Clampex of pClamp 10.1 software (Axon Instrument). The patch-clamp data was analyzed by using Clampfit 10.3 and Origin 7.0 software (Microcal Inc., Northampton, MA, USA). Glass patch pipettes (World Precision Instruments, Sarasota, FL, USA) were pulled with PP-830 (Narishige, Tokyo, Japan) to make tip resistance 2.5~3 M Ω when filled with KCl or CsCl in pipette solution.

3. Solutions and Drugs

For the whole-cell patch clamp experiment, low Ca²⁺-buffered KCl pipette solution contained (in mM): 140 KCl, 10 HEPES, 5 NaCl, 3 Mg-ATP, 0.1 EGTA, 1 MgCl₂ and was adjusted to pH 7.2 with KOH. Ca²⁺-clamped KCl pipette solution contained (in mM) 140 KCl, 1 MgCl₂, 3 Mg-ATP, 10 HEPES, 5 EGTA, 5 NaCl, and 300 nM free Ca²⁺ calculated using the free WebMax software (www.stanford.edu/~cpatton/webmaxcS), and was adjusted to pH 7.25 with KOH. CsCl pipette solution contained (in mM): 140 CsCl, 1 MgCl₂, 3 Mg-ATP, 10 HEPES, 5 EGTA, 5 NaCl, and 600 nM free Ca²⁺ calculated using the free WebMax software and was adjusted to pH 7.25 with CsOH. Normal Tyrode's (NT) bath solution contained (in mM): 145 NaCl, 3.6 KCl, 1 MgCl₂, 1.3 CaCl₂, 10 HEPES, 5 glucose, 10 sucrose and was adjusted to pH 7.4 with NaOH. NMDG-Cl bath solution contained: 150 NMDG-Cl, 10 HEPES, 10 glucose, 1 MgCl₂, 1.3 CaCl₂ and was adjusted to pH 7.4 with NMDG-OH. Ionomycin, 1-ethyl-2-benzimidazolinone (1-EBIO) and 1-[(2-chlorophenyl) diphenylmethyl]-1*H*-pyrazole (TRAM-34) were purchased

from Sigma-Aldrich. T16Ainh-A01, a specific inhibitor of Ano-1, was purchased from TOCRIS Biosciences. The chemicals were initially dissolved in dimethyl sulfoxide (DMSO), and were diluted to the bath solution for experiments. The amount of DMSO in the bath solution was kept below 0.1 %.

4. $[Ca^{2+}]_i$ measurement

HNSCC cells were loaded with fura-2 acetoxymethylester (5 mM, 30 min, 25°C) and washed twice with HEPES-buffered NT solution. Fluorescence was monitored in a stirred quartz microcuvette (1 ml), and stirring in a temperature-controlled (37°C). The cell holder of a fluorescence spectrophotometer (Photon Technology International) at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. At the end of each experiment, 5 μ M ionomycin was applied to produce a maximum fluorescence ratio (R_{max} , 340/380 nm). Subsequently, 10 mM EGTA was added to confirm a minimum value of fluorescence ratio (R_{min}). In each cell, $R_{340/380}$ were normalized against the R_{max} and R_{min} [$(R_{340/380} - R_{min})/R_{max}$].

5. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cancer cells using the Trizol (Invitrogen, CA, USA), and 2 μ g of total RNA was reverse transcribed into first-strand cDNA. Reverse transcription was performed with RT pre-mix (Intron Bio, Korea) according to the manufacturer's instructions. The cDNA was amplified by 35 cycles of PCR with PCR PreMix (Intron, Korea) in a C1000 Thermal

Cycler (bio-rad, CA, USA). The SK1-4 primers were obtained from Bioneer (Bioneer, Korea). The expected product sizes from the customized primer sets were 155, 162, 150, and 154 bp for SK1 (product No.P243222), SK2 (P243234), SK3 (P114469) and SK4 (P249299), respectively. GAPDH was used as control. RT-PCR products were resolved by 1.2% agarose gel electrophoresis and visualized with ethidium bromide staining.

6. Statistical Analysis

Data are presented as the mean \pm standard deviation (SD) of at least triplicates, or as a representative of 3 separate experiments. Significance was determined between treated and untreated groups by two-sided Student's *t*-test. *P* values <0.05 were considered statistically significant.

RESULTS

Ionomycin induced currents (Ca^{2+} -activated Channels) in HNSCCs.

Firstly, ionomycin-induced changes in membrane conductance were investigated in; SNU-1076, OSC-19, and HN5. Low Ca^{2+} -buffered KCl pipette solution (0.1 mM EGTA) was used to allow $[\text{Ca}^{2+}]_i$ increase in response to the ionomycin treatment. Ramp-like voltage change from -100 to 60 mV was repetitively applied at every 10 s. During inter-pulse period, the cell membrane voltage was held at -60 mV (Fig. 1A, C, E). Before ionomycin application, membrane conductance was commonly low in the three cell lines; small amplitudes at both negative and positive voltages (Fig. 1B, D, F; black I/V curves labelled (1) in the right panels). Application of ionomycin gradually increased the membrane conductance (i.e. the slope of I/V curve) in the three cell lines. However, the dominant type of ionic conductance at the earlier period appeared different between the cell lines. In SNU-1076, outward K^+ current was initially activated as indicated from the upward deflection of basal current trace at the holding voltage of -60 mV (Fig. 1A, red I/V curve labelled (2)). Consistently, the I/V curve showed outward currents that reversed its direction at around -80 mV, close to the K^+ equilibrium potential (Fig. 1B, I/V curve in red). The weakly inwardly rectifying shape of I/V curve was consistent with the property of SK4. In the later phase of ionomycin application, however, the outward current disappeared with concomitant activation of inward current. Also the I/V curve at the later

period (ca. 4 - 5 min of ionomycin treatment) showed linear shape with reversal potential close to 0 mV (Fig. 1B, blue I/V curve labelled (3)). Different from SNU-1076, OSC-19 and HN5 cells did not show discernible SK4-like current but only showed inward currents at the negative clamp voltage (Fig. 1C, E). The I/V curves showed linear shapes with reversal potentials close to 0 mV (Fig. 1D, F, red I/V curves). A measurement of intracellular $[Ca^{2+}]_c$ with fura-2 fluorometry consistently showed increase in $[Ca^{2+}]_c$ to 500 -800 nM by ionomycin (n=5, data not shown).

Common expression of Ca^{2+} -activated Cl^- channels (Ano-1) and Ca^{2+} -activated nonselective cation channels (CAN) in the three cell lines

The I/V curves of ionomycin-induced currents in their steady-state with 0 mV of reversal potential suggested that either Cl_{Ca} (Ano-1) or CAN channels were activated. To evaluate the relative contribution of Cl_{Ca} and CAN channels in HNSCC, CsCl pipette solution with fixed level of high $[Ca^{2+}]_c$ (600 nM) was adopted in the next whole-cell patch clamp experiment (Fig. 2). K^+ current was excluded by replacing the intracellular K^+ with Cs^+ that are impermeable to K^+ channels. The membrane current increased slowly after making the whole-cell configuration. After confirmed a steady-state (I/V curves in black, Fig. 2A-C), 10 μ M T16A, a selective inhibitor of Ano-1, commonly decreased the conductance in the three cell lines (I/V curves in red, Fig. 2A-C). Then, in the presence of T16A, all the extracellular Na^+ was

replaced by NMDG⁺, a large organic cation impermeable to cation channels. On the NMDG⁺ replacement, additional decrease of inward current was observed at the negative membrane voltages (I/V curves in green, Fig. 2A-C). The current density normalized to the steady-state control current (%) showed that about half of the current was inhibited by T16A in all three cell types (Fig. 2D). In OSC-19, most of the remaining inward current was abolished by replacing extracellular Na⁺ with NMDG⁺ (Fig. 2B, D). In SNU-1076 and HN5, however, 10 - 20 % of the conductance was remained even after the removal of Na⁺. The nature of T16A- and NMDG⁺-resistant conductance was not further investigated here.

Ionomycin (1 μM, 48 h) induces cell death of HNSCCs, effectively rescued by 1-EBIO.

To investigate the role of Ano-1 and SK4 in the Ca²⁺-overloaded HNSCCs, viability was assessed in the ionomycin-treated cells with T16A (Ano-1 inhibitor) or TRAM-34 (SK4 inhibitor). The number of live cells was counted at 48 h after 1 μM ionomycin treatment, and the numbers were commonly decreased in all the three cell lines. However, neither T16A nor TRAM-34 changed the tendency of ionomycin-induced suppression of proliferation in all the three cell lines (Fig. 3A). Then we tested the effect of 1-EBIO, an activator of SK channels, on the ionomycin-treated HNSCCs. Interestingly, the co-treatment of 1-EBIO with ionomycin effectively recovered the live cell numbers close to the control levels in SNU-1076 and OSC-19. The rescue

effect of 1-EBIO was observed already from the relatively low range of concentration (3 μ M and 10 μ M), and was completely reversed by the SK4 channel specific inhibitor TRAM-34 (Fig. 3B). However, 1-EBIO rescued the ionomycin-treated HN5 cells only to a small extent (Fig. 3B). The treatment with 1-EBIO or TRAM-34 without ionomycin did not significantly affect the viability of HNSCCs. Representative data for SNU-1076 is show in Fig. 3C.

Molecular identification of SK4 channels in three cell lines.

The rescue effect of 1-EBIO on OSC-19 was unexpected since ionomycin did not directly induce noticeable I_{SK4} in OSC-19 (Fig. 1C, D). Therefore we were attempted to evaluate whether 1-EBIO actually induces I_{SK4} in OSC-19. Here we used KCl pipette solution containing 300 nM of free Ca^{2+} activity. With the moderate level of $[Ca^{2+}]_c$ alone, i.e. 300 nM, Ca^{2+} -activated ion channel current was not significantly observed. However, the treatment with 1-EBIO induced outward K^+ currents with weakly inward rectifying property in the three cell lines (Fig. 4). Interestingly, the current density of 1-EBIO-induced I_{SK4} was the largest in OSC-19 and smallest in HN5 (Fig. 4D). Then we performed RT-PCR analysis for SK1-4 in the three cell lines, as was detected from the pharmacological assay of membrane current (Fig. 4D), SK4 transcript was conformed in SNU-1076 and OSC-19 while not in HN5 (Fig. 4E). However, unexpectedly, all the three cell lines showed positive signal for the mRNA of SK1. Since the 1-EBIO-activated current was negligible in

HN5, and the K^+ current induced by 1-EBIO completely inhibited by the SK4 channel specific inhibitor TRAM-34 in SNU-1076 and OSC-19 cell lines, we interpreted that the functional SK1 channel proteins not expressed in the three cell lines (Fig. 4E).

1-EBIO additionally activates SK4 current in the ionomycin-treated cancer cells

Then we investigated whether 1-EBIO also affect the K^+ conductance of ionomycin-treated cells with unclamped $[Ca^{2+}]_i$ conditions, i.e. 0.1 mM EGTA in KCl pipette solution. As shown in Fig. 1, application of 1 μ M ionomycin induced outward K^+ current in SNU-1076 whereas inward currents in OSC-19 and HN5. Interestingly, addition of 50 μ M 1-EBIO induced large outward current in both OSC-19 and SNU-1076. Subsequent application of TRAM-34 effectively abolished the 1-EBIO-induced K^+ currents (Fig. 5A-D). In HN5 cells, however, application of 1-EBIO still induced only minute increase of membrane conductance (Fig. 5E-F).

1-EBIO activated SK4 channel induces membrane hyperpolarization.

The activation of I_{SK4} by 1-EBIO implied membrane hyperpolarization as suspected from the negative shift of reversal potentials (Fig. 5B and D). To confirm the actual effects on membrane potential, zero-current clamp recording was performed. Ionomycin, 1-EBIO and TRAM-34 were serially

applied to HNSCCs with 0.1 mM EGTA/145 mM KCl in the pipette solution. All the three cell lines showed depolarized membrane potential between -15 and 0 mV at the steady-state of whole-cell configuration. In SNU-1076, ionomycin induced initial negative shift of membrane potential, which was further hyperpolarized by 1-EBIO (Fig. 6A). In OSC-19, ionomycin induced a slight depolarization, which was reversed to strong hyperpolarization by 1-EBIO (Fig. 6B). The final application of TRAM-34 depolarized the membrane potential in both SNU-1076 and OSC-19. In HN5, however, neither ionomycin nor 1-EBIO induced consistent changes in the membrane potential (Fig. 6C).

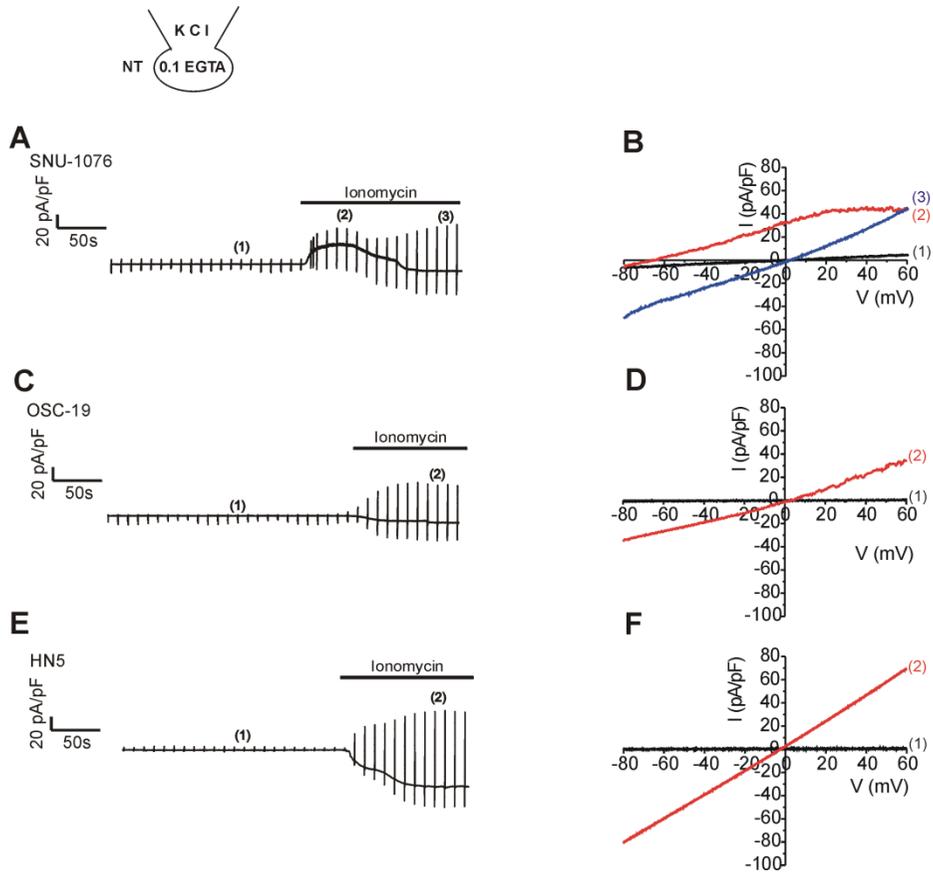


Figure 1. Ionomycin-induced membrane currents in HNSCCs

(A, C, E) Representative original current traces in response to repetitive ramp-like pulses (from -100 to 60 mV, holding voltage, -60mV) in whole-cell voltage clamped cells. The type of cell line is indicated above each trace. KCl pipette solution with 0.1 mM EGTA was commonly used. The numbers in parenthesis indicate the corresponding current to voltage relations (I/V curves) depicted in the right panels (B, D, F). After achieving the whole-cell configuration, the steady-state conductance was very small; black I/V curves that are almost indistinguishable from the horizontal axis. On applying 1 μ M ionomycin, membrane conductance increased gradually, and the addition of 5

μM TRAM-34 inhibited outward currents in SNU-1076 (A, B). In contrast, the ionomycin-treated OSC-19 (time break, 1min) and HN5 showed negligible inhibition by TRAM-34 (C, D).

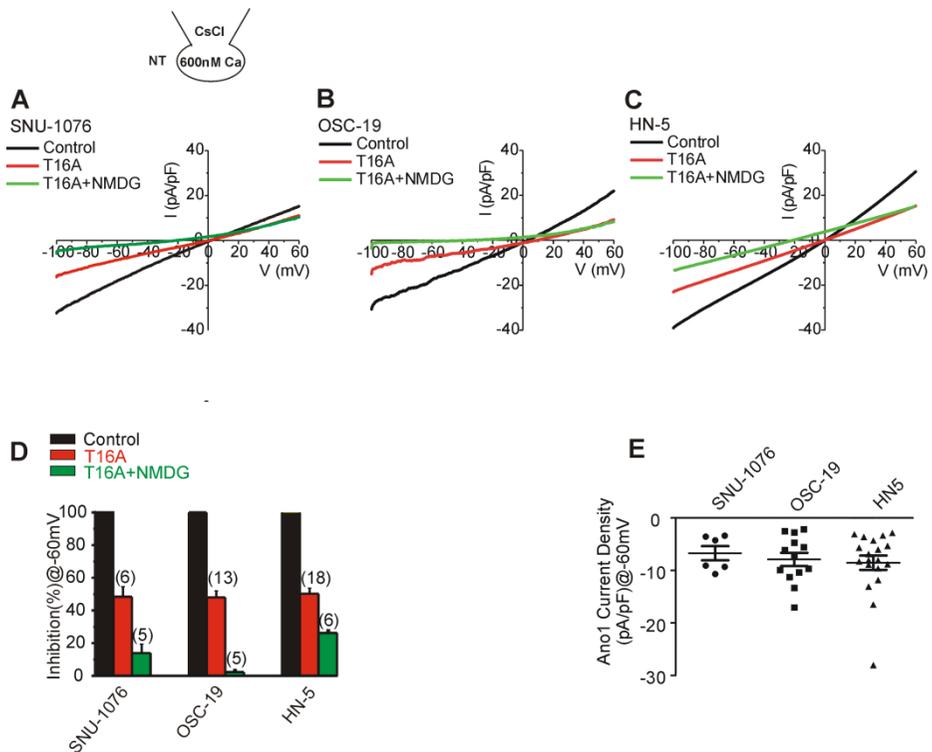


Figure 2. Ca^{2+} -activated Cl^- current and Ca^{2+} -activated nonselective cationic current in HNSCCs

(A-C) Representative I/V curves obtained by ramp-like pulses used CsCl pipette solution with 600nM free Ca^{2+} activity in the three types of cell lines. Steady-state Ca^{2+} -activated conductance (black I/V curves in each panel) was decreased by about half after applying 10 μM T16A, a selective inhibitor of Ano-1 (I/V curves in red). The remained conductance, especially the inward current, was further decreased by replacing extracellular Na^+ with NMDG^+ (T16A+NMDG, I/V curves in green). (D) Summary of normalized inward current densities at -60 mV. The inward current density (pA/pF) was normalized (%) to the control steady-state in each cell. The remained current after T16A (red) and Na^+ removal (green) are averaged. (E) Summary Ano-1

current densities (pA/pF) at -60mV in HNSCCs. Ano-1 current was obtained by subtracting T16A-resistant current from control in each cell, and the plotted dots indicate data from individual cell.

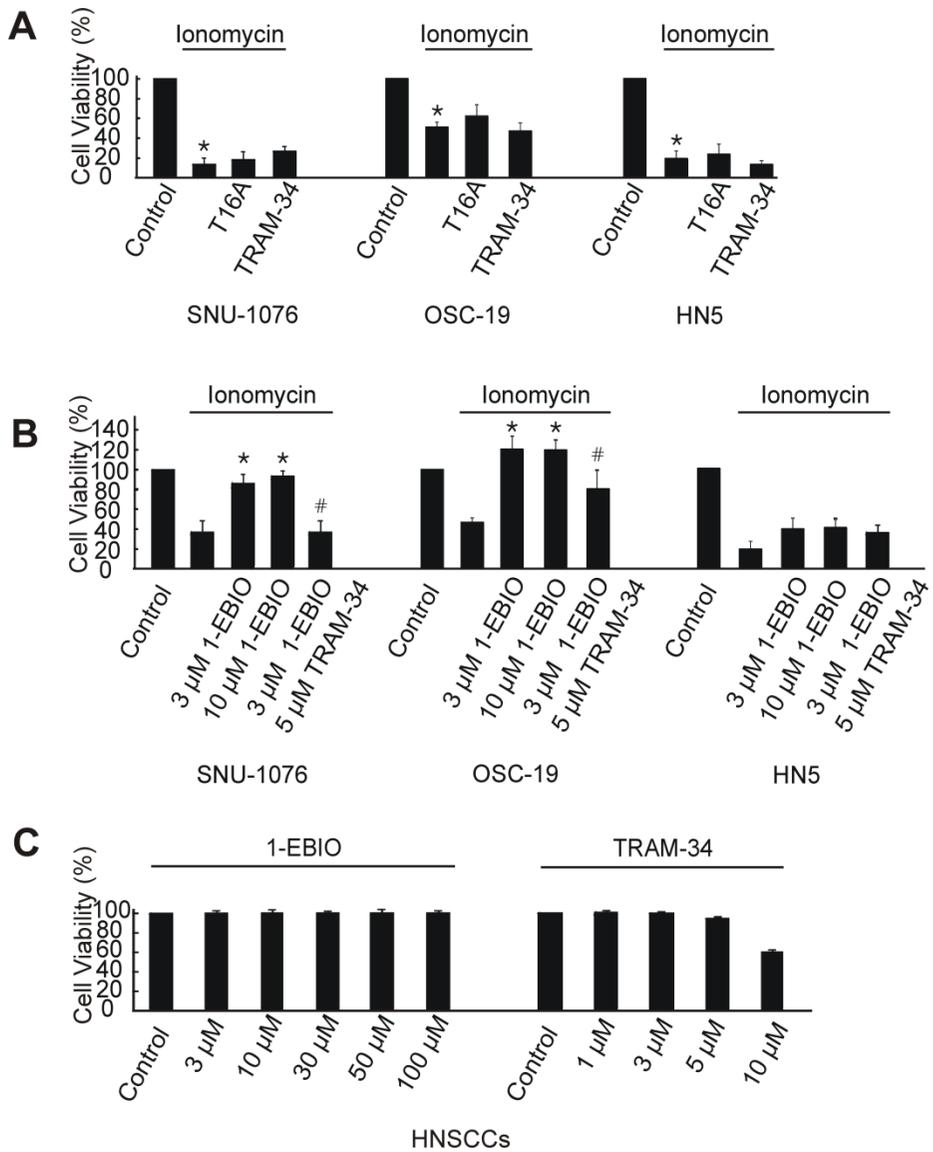


Figure 3. Iononycin-induced cell death and rescue effects of 1-EBIO in HNSCCs

(A) Cells from each cell line were divided into four groups; non-treated (control), treated with 1 μ M iononycin, iononycin + 0.1 μ M T16A and iononycin + 10 μ M TRAM-34. Cells were subjected to proliferation assay at 48h and normalized to the control (cell viability). Summary of MTT

experiments are displayed. *P* values were based on comparison with control (*, $P < 0.001$).

(B) Cells from each cell line were divided into five groups; non-treated (control), treated with 1 μM ionomycin, ionomycin + 3 μM 1-EBIO, ionomycin + 10 μM 1-EBIO, and ionomycin/3 μM 1-EBIO + 5 μM TRAM-34. Cell numbers at 48 h were counted and normalized to the control ($n= 6$). *P* values were based on comparison with ionomycin-treated group (*, $P < 0.001$) and ionomycin plus 1-EBIO (3 μM)-treated group (#, $P < 0.005$).

(C) Effects of various concentrations of 1-EBIO or TRAM-34 on the cell viability of SNU-1076 without ionomycin treatment at 48 h.

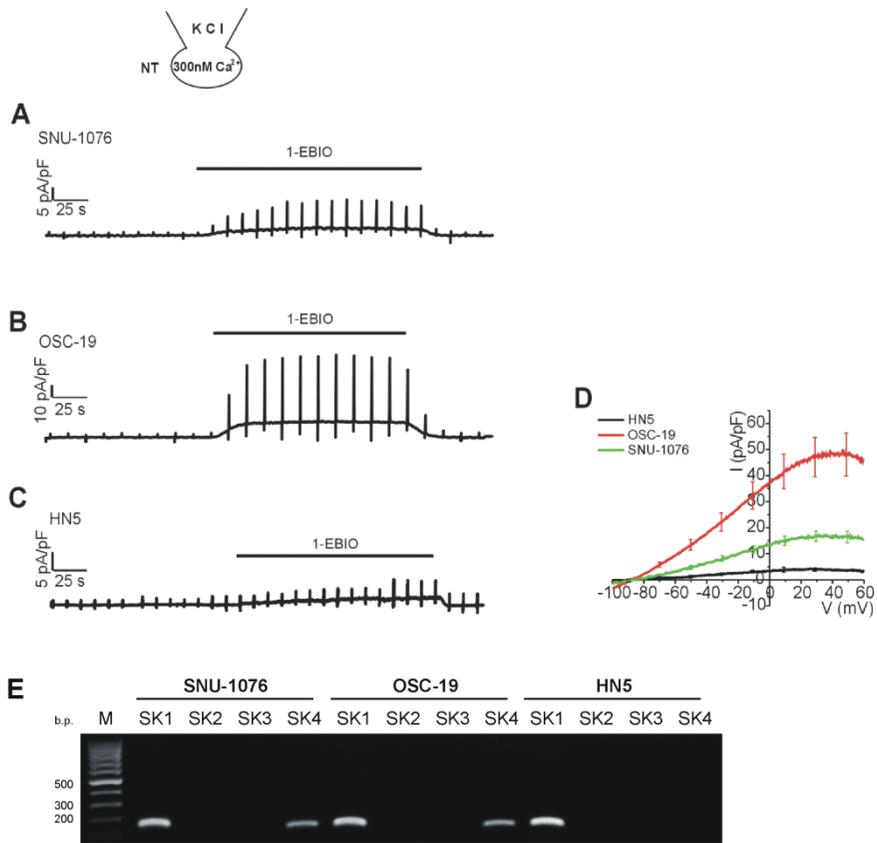


Figure 4. Effects of 1-EBIO on the membrane conductance of HNSCCs with clamped $[Ca^{2+}]_i$

(A-C) Representative current traces in response to ramp pulses (from -100 to 60 mV, holding voltage, -60mV) and to the application of 50 μ M 1-EBIO in HNSCCs. The Ca^{2+} activity of KCl pipette solution was equilibrated to 300 nM with 5 mM EGTA. (D) Summary of I/V curves of the 1-EBIO-induced currents in each cell line (mean \pm SEM, n=7, respectively). (E) Identification of SK mRNA in HNSCC cancer cells. mRNA was detected for SK1 and SK4 by RT-PCR.

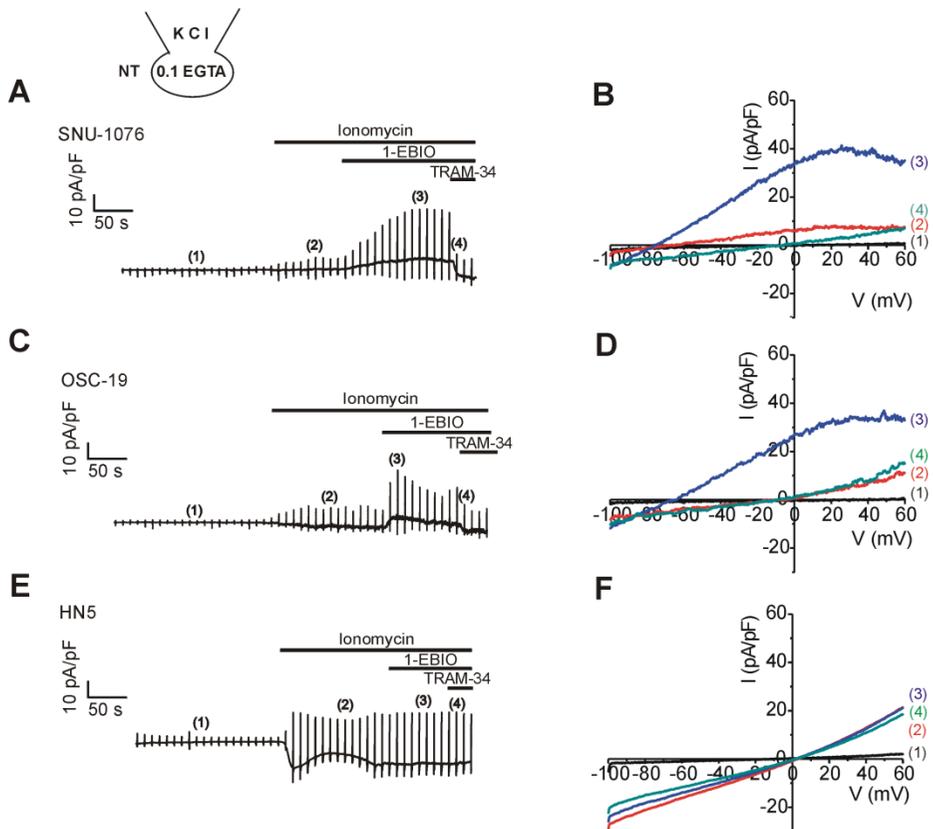


Figure 5. Effects of 1-EBIO on the membrane conductance of HNSCCs pretreated with ionomycin under Ca^{2+} -unclamped condition

Left panels (A, C, E) representative current traces of HNSCCs in response to the depolarizing ramp pulses. 1 μM ionomycin, 50 μM 1-EBIO, and 5 μM TRAM-34 were applied as indicated above each trace. Right panels (B, D, F); I/V curve obtained by the ramp pulses in each cell line of HNSCCs as indicated at the corresponding left panels. The specific timing of I/V curve is indicated by the number in parenthesis. Note the weakly inward rectifying outward currents induced by 1-EBIO in SNU-1076 and OSC-19.

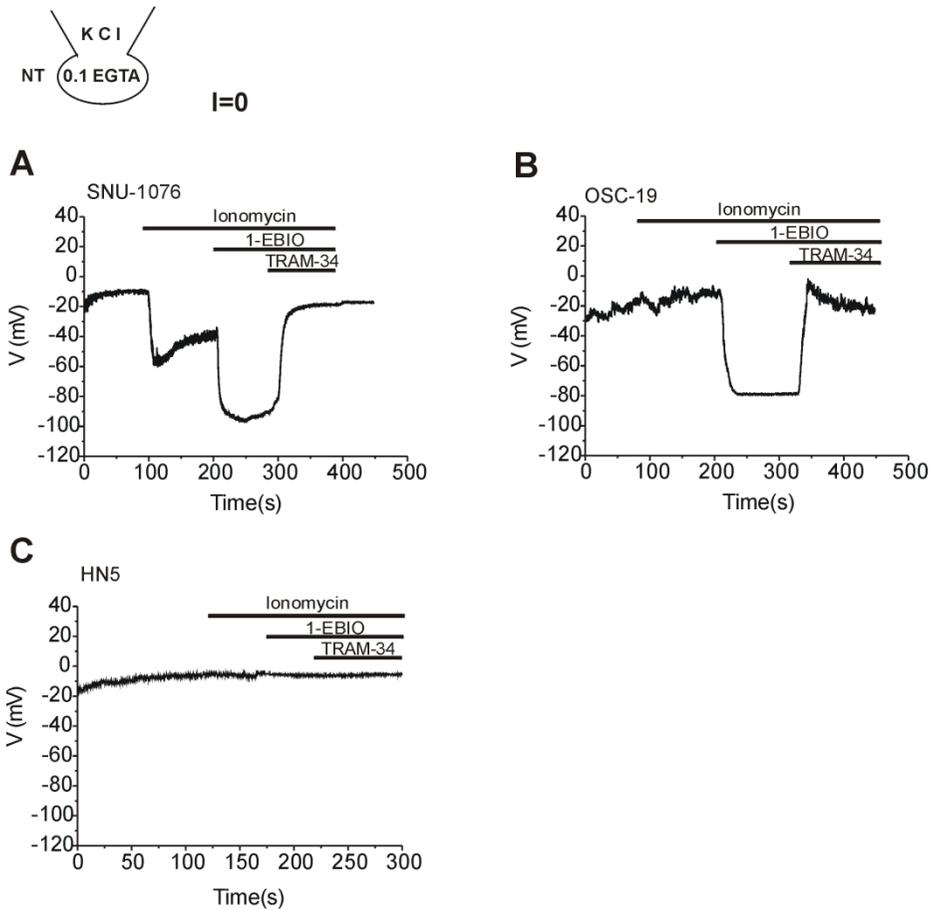


Figure 6. Membrane voltage changes in response to ionomycin, 1-EBIO and TRAM-34 in HNSCCs

Traces of membrane potential in HNSCCs obtained under zero-current clamp condition with 0.1 mM EGTA KCl pipette solution. 1 μ M ionomycin, 50 μ M 1-EBIO, and 5 μ M TRAM-34 were applied as indicated in each panel. Representative results from more than five cells from each cell line are shown. Ionomycin alone hyperpolarized the membrane potential in SNU-1076 (A) while the combined application of 1-EBIO induces strong hyperpolarization in both SNU-1076 and OSC-19 (A, B). Final addition of TRAM-34 depolarized the membrane potential in SNU-1076 and OSC-19 (A, B).

Neither ionomycin nor 1-EBIO induced significant change in the membrane potential of HN5 (C).

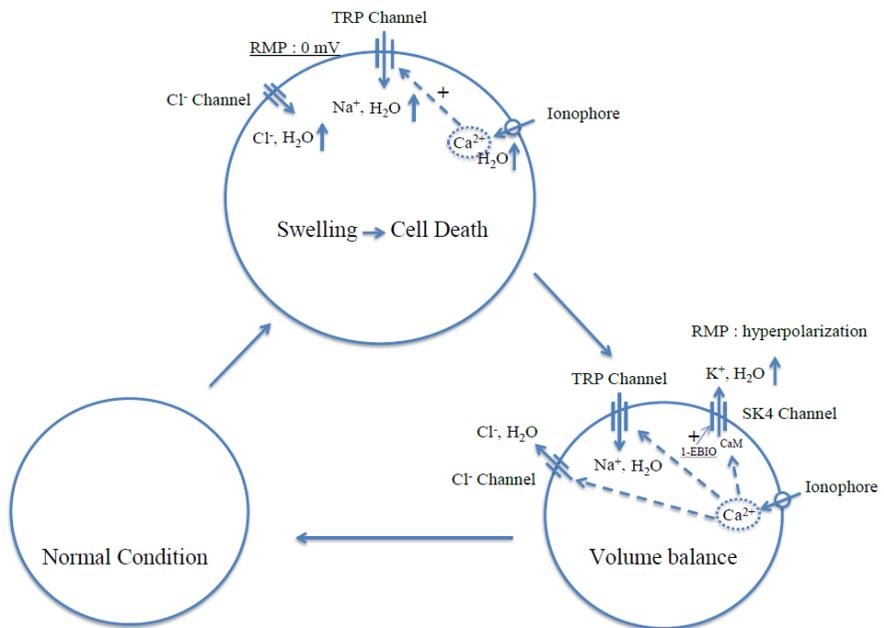


Figure 7. Hypothesis for Ca^{2+} dependent channels in cell death and rescue effect by cell volume regulation.

A rise of $[\text{Ca}^{2+}]_i$ activates Ca^{2+} -activated nonselective cation channels (CAN) together with Cl^- channels to increase intracellular Na^+ and Cl^- ions. Resulting osmotic stress and water influx cause cell swelling, which might induce cell death when excessive. Activation of SK4 by 1-EBIO, membrane can be plasma membrane hyperpolarization and intracellular Ca^{2+} may activate K^+ and Cl^- ions efflux along with the water, which might be helpful to maintain the cell volume balance.

DISCUSSION

Our present study demonstrates that HNSCCs differentially express functional SK4 channels as revealed by the amplitudes of 1-EBIO-induced I_{SK4} . The pharmacological activation of I_{SK4} rescued the HNSCCs from ionomycin-induced cell death. The abolishment of 1-EBIO effects by TRAM-34 is also the evidence indicating specific role of SK4. The activation of SK4, i.e. K^+ efflux and membrane hyperpolarization appear to be the critical conditions for the rescue effect. In fact, the rescue effect was least significant in HN5 cells where the current density of SK4 was smaller than the other cell lines.

Ca²⁺-activated ion channels in HNSCCs

In the resting HNSCCs without raised $[Ca^{2+}]_c$, little background ionic conductance was observed; the slopes of I/V curves were very shallow in all the three cell lines tested. In contrast, experimental measures increasing $[Ca^{2+}]_c$ consistently activated multiple types of ion channels permeating K^+ (SK4), Cl^- (Cl_{Ca}) and Na^+ (CAN) with differential contributions depending on the cell types. Molecular identity of the Cl_{Ca} has been recently discovered as Ano-1/TMEM16A, and found in HNSCCs [10, 27]. The significant decrease of Ca^{2+} -activated inward current by T16A indicates the expression of Ano-1 in the three lines of HNSCCs investigated here. In addition to Ano-1, the incomplete inhibition by T16A and further decrease by replacing Na^+ with NMDG⁺ indicate that CAN channels are expressed in HNSCCs (Fig. 2). In the

present study, the precise molecular identity of CAN was not investigated. The molecular identity of CAN is generally ascribed to TRPM4 and TRPM5, members of TRP channel superfamily [5, 6].

The activation of Cl_{Ca} and CAN commonly induces depolarization of HNSCC close to their Nernst equilibrium voltages (V_{eq}) of Cl^- or monovalent cations in the present experimental conditions. Therefore large increase in $[Ca^{2+}]_c$ inevitably set the membrane potential of HNSCCs close to 0 mV. Since the HNSCCs do not show background K^+ conductance, the resting membrane potentials are depolarized. The lack of voltage changes by ionomycin alone in OSC-19 and HN5 seem to be due to the already depolarized resting membrane potentials of HNSCCs (Fig. 6). In other words, application of ionomycin would further stabilize the depolarized states. Even in 1076 cells where the activation by SK4 by ionomycin was evident, application of ionomycin induces only a moderate hyperpolarization (Fig. 6A).

A finding of present study was the requirement of 1-EBIO for the activation of SK4 in OSC-19. Although the final amplitude of 1-EBIO-induced I_{SK4} was comparable or even larger in OSC-19 than SNU-1076, the application of ionomycin did not effectively activate I_{SK4} in OSC-19. Such results might imply that the activation of SK4 require, in addition to $[Ca^{2+}]_c$ increase, unknown factors or molecular components that are selectively modulated by 1-EBIO in HNSCCs.

Role of SK4 in cancer cells

The effects of 1-EBIO on the cell viability of ionomycin-treated HNSCCs can be associated with the level of I_{SK4} because TRAM-34 effectively reversed the rescue effect by 1-EBIO (Fig. 3). Thus one can suggest that the full activation of SK4 and strong hyperpolarization are critical for the effects of 1-EBIO. What is the precise target or mechanism of SK4 activation in the cell proliferation of HNSCCs? Unfortunately, we do not have concrete answers to that question yet but could only provide general inquiry of previous studies.

The importance of K^+ channel activity in cell cycle regulation and proliferation/death has been investigated in various aspects and the responses are widely variable from proliferation to cell death [22, 28, 29]. In the studies of apoptosis, it is proposed that the excessive activation of K^+ channel and hyperpolarization would induce continuous efflux of Cl^- as well as K^+ , which is accompanied by the loss of cytoplasmic water and cell volume, causing apoptotic volume decrease (AVD) [22, 30]. In this respect, the excessive activation of SK4 in line with Ano-1 may also induce cell volume decrease in SNU-1076 and OSC-19. However, the outcome of the cell viability was opposite to the K^+ -channel dependent AVD suggested by Bortner's group [22]. Thus the final effects of K^+ efflux on cell proliferation seem to be variable or could even show opposite results depending on the context of intracellular signals and the cell types.

Without K^+ channel activation, the CAN activated by Ionomycin induced intracellular Ca^{2+} influx causes Na^+ influx, depolarization of the cell membrane, and Ano-1 dependent Cl^- influx. In excessive activation of CAN and Ano-1, results osmotic increase cause rapid cell excessive swelling and induced cell death. Activation of SK4 by 1-EBIO, hyperpolarization of the membrane would induce Cl^- efflux, which would be helpful to maintain cell volume balance. Maintenance of the volume balance contributes to carcinoma cells metabolic homeostasis, which might rescue ionomycin induced cell death (Fig. 8).

Partly consistent with our present results, the previous studies of breast, prostate and pancreatic cancer cells commonly report the inhibition of cell proliferation by SK4 inhibitor [31, 32]. In another study, the proliferation of human prostate cancer cell lines (LNCaP and PC-3) was increased by 1-EBIO, and this effect was again reversed by SK4 inhibitors [33]. Regarding to the mechanism, it has been proposed that the hyperpolarization and augmented Ca^{2+} influx promotes the transition of cell cycle phases and thereby proliferation [32, 34]. Since the treatment of HNSCCs with ionomycin alone would induce significant increase in $[Ca^{2+}]_c$, the theory of augmented Ca^{2+} influx by I_{SK4} -induced hyperpolarization seems unlikely for the rescue effect of 1-EBIO. In fact, a measurement of intracellular $[Ca^{2+}]_c$ with fura-2 fluorometry consistently showed large increase in $[Ca^{2+}]_c$ by ionomycin alone, that was not significantly augmented by 1-EBIO in SNU-1076 and OSC-19 (n=5, data not shown). Recently, Millership *et al* proposed that SK4 may directly activate ERK1/2 and JNK signaling pathways in a voltage-

independent way, which promotes proliferation [35]. The putative non-electrical role of SK4 directly interacting with the intracellular signaling molecules might have to be also considered in HNSCCs. Apart from the Ca^{2+} -activated K^+ channels, background type K^+ channels such as TREK-2 could also regulate the cancer cell proliferation [36].

CONCLUSION

In summary, this study firstly demonstrates the expression of SK4 and functional activation by 1-EBIO in HNSCC. Although the precise mechanisms are not known yet, the activation of I_{SK4} and membrane hyperpolarization induced by 1-EBIO effectively rescue the ionomycin-induced cell death in the HNSCCs expressing SK4. Pharmacological modulation of SK4 might provide a hint for the development of more effective anti-cancer agent or better strategy for cancer treatment.

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국문 초록

암세포에 존재하는 이온통로들에 대한 연구는 암세포 증식/사멸에 대한 조절 여부 및 그 기전과 관련하여 주목받고 있다. 세포 내 칼슘증가와 관련된 신호전달은 암세포 증식과 사멸에 중요하다. 이 연구를 통해 칼슘 의존성 포타슘 이온통로들 중 하나인 SK4 를 두경부 암세포주 (HNSCCs) 에서 발견하였다. HNSCC의 세 가지 세포주들인 HN5, OSC-19, SNU-1076에서 SK4의 역할을 분석하였다. 세포내 칼슘농도를 크게 높여주는 약물인 Ca^{2+} Ionophore인 Ionomycin을 투여하면 세포주들 모두에서 세포사멸이 일어났다. 전세포 막전압고정법(whole cell patch clamp)을 적용하여 세가지 cell line 에서 모두 Ca^{2+} -activated Cl^- channels (Ano-1) 과 Ca^{2+} -activated nonselective cation channels (CAN)의 활성화를 발견하였다. 하지만 SK4를 통한 K^+ 전류는 SNU-1076과 OSC-19 세포주에서만 관찰되었다. SK4 활성화제인 1-EBIO를 투여하더라도 SNU-1076과 OSC-19에서는 커다란 외향전류가 유도되었지만, HN5 세포주에서는 이러한 외향 전류가 거의 존재하지 않거나 약간의 증가만 관찰될 뿐이었다. 1-EBIO에 의하여 유도 되었던 외향 전류는 SK4 channel의 특정한

억제제인 TRAM-34에 의하여 억제되었다. Ionomycin에 의한 세포사멸은 1-EBIO를 함께 투여하면 방지되었는데, 이 현상은 SNU-1076과 OSC-19 세포주에서만 관찰되었다. 이 세포들에서도, SK4 차단제인 TRAM-34를 추가하면 세포사멸이 또다시 관찰되었다. 1-EBIO 의 세포사멸 방지현상은 HN5 세포주에서는 관찰되지 않았다. 이러한 실험 결과는 두경부 암세포주가 과도한 세포내 칼슘에 의해서 세포사멸이 일어나는 과정에서 K^+ 이온통로가 일으키는 작용을 처음으로 보여주었다. 두경부 암에서 이러한 SK4 channel을 조절해주는 약리학적 현상은 항암 치료제를 개발하는데 참고할 수 있는 흥미로운 작용기전을 시사한다.

주요어 : 편평 세포암, Ionomycin, Ca^{2+} -activated K^+ channel, 1-EBIO, 세포 증식

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