

Review

Reciprocal Relationship between Ca^{2+} Signaling and Ca^{2+} -Gated Ion Channels as a Potential Target for Drug Discovery

Yuji Imaizumi

Department of Molecular and Cellular Pharmacology, Graduate School of Pharmaceutical Sciences, Nagoya City University; 3-1 Tanabedori, Mizuho-ku, Nagoya 467-8603, Japan.

Received October 17, 2021

Cellular Ca^{2+} signaling functions as one of the most common second messengers of various signal transduction pathways in cells and mediates a number of physiological roles in a cell-type dependent manner. Ca^{2+} signaling also regulates more general and fundamental cellular activities, including cell proliferation and apoptosis. Among ion channels, Ca^{2+} -permeable channels in the plasma membrane as well as endo- and sarcoplasmic reticulum membranes play important roles in Ca^{2+} signaling by directly contributing to the influx of Ca^{2+} from extracellular spaces or its release from storage sites, respectively. Furthermore, Ca^{2+} -gated ion channels in the plasma membrane often crosstalk reciprocally with Ca^{2+} signals and are central to the regulation of cellular functions. This review focuses on the physiological and pharmacological impact of i) Ca^{2+} -gated ion channels as an apparatus for the conversion of cellular Ca^{2+} signals to intercellularly propagative electrical signals and ii) the opposite feedback regulation of Ca^{2+} signaling by Ca^{2+} -gated ion channel activities in excitable and non-excitable cells.

Key words Ca^{2+} signaling; Ca^{2+} microdomain; ion channel; Ca^{2+} permeable channel; Ca^{2+} -activated K^+ channel; Ca^{2+} -activated Cl^- channel

1. INTRODUCTION

The intracellular concentration of Ca^{2+} ($[\text{Ca}^{2+}]_i$) under resting conditions is generally maintained at 100 nM or lower regardless of the cell type. Ca^{2+} signaling involves increases in $[\text{Ca}^{2+}]_i$, which commonly occur spontaneously or in the early phases of cellular responses to various physiological stimuli.¹⁾ Ca^{2+} signaling functions as a fundamental second messenger in a number of cellular signal cascades in the human body and mediates many cell-specific functions and activities. These functions include contraction in muscles, transmitter release in synapses, hormone release in secretory cells, and the sensing of physical stimuli in sensory nerve endings. Ca^{2+} signaling also regulates basic cellular activities, such as gene expression, cell cycle regulation, cell proliferation, autophagy, focal adhesion, migration, and apoptosis (Fig. 1). However, excessive Ca^{2+} signaling occasionally induces cell damage and even cell death. One of the reasons why Ca^{2+} signaling mediates a large number of cellular functions is its extreme diversity in spatiotemporal features from local Ca^{2+} transients within 100 ms in duration and 500 nm in diameter, such as Ca^{2+} sparks, to sustained or oscillatory increases in $[\text{Ca}^{2+}]_i$ for more than one minute.²⁾

The source of $[\text{Ca}^{2+}]_i$ increases in Ca^{2+} signaling is often Ca^{2+} influx through Ca^{2+} -permeable channels in the plasma membrane (PM). A large Ca^{2+} concentration gradient across the PM produces a strong electromotive force for Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCC) and voltage-independent Ca^{2+} channels on the PM, with Ca^{2+} influx through the latter generally being markedly slower and smaller. VGCCs activated upon depolarization are a predomi-

nant Ca^{2+} influx pathway in excitable cells, but not in non-excitable cells. Another Ca^{2+} source for Ca^{2+} signaling is its release from intracellular storage sites. The main intracellular Ca^{2+} storage/release sites are the endo- and/or sarcoplasmic reticulum (ER and/or SR), on which the Ca^{2+} release channels, ryanodine receptors (RyRs) and inositol 1,4,5-trisphosphate receptors (IP₃Rs) are functional.¹⁾

The present review overviews information accumulated for more than two decades on the physiological and pharmacological impact of i) Ca^{2+} -gated ion channels as an apparatus for signal conversion from changes in $[\text{Ca}^{2+}]_i$ to electrical signals, which are intercellularly propagative, and ii) the opposite feedback regulation of Ca^{2+} signaling in excitable and non-excitable cells by Ca^{2+} -gated ion channels and concomitant membrane potential changes, which are emergent druggable targets.

2. PHYSIOLOGICAL IMPACT OF Ca^{2+} SIGNAL CONVERSION TO ELECTRICAL ACTIVITIES BY Ca^{2+} -GATED ION CHANNELS

2-1. Ca^{2+} -Gated Ion Channels The established classification and nomenclature of ion channels need to be referred to those presented by The International Union of Basic and Clinical Pharmacology (IUPHAR) (<https://www.guidetopharmacology.org/>).³⁾ One of the practical classifications of ion channels may be that based on gating mechanisms. Table 1 proposes a classification of ion channels on the PM based on their gating mechanisms. RyRs and IP₃Rs are channels in ER/SR membranes, but are also listed here because of their close involvement in the issues discussed in this review.

In this classification, “ Ca^{2+} -gated ion channels” refer to channels for which an increase in $[\text{Ca}^{2+}]_i$ is an essential factor for their activation. The Ca^{2+} -gated K^+ channel family consists of three subfamilies classified by channel conduc-

This review of the author's work was written by the author upon receiving the 2018 Pharmaceutical Society of Japan Award.

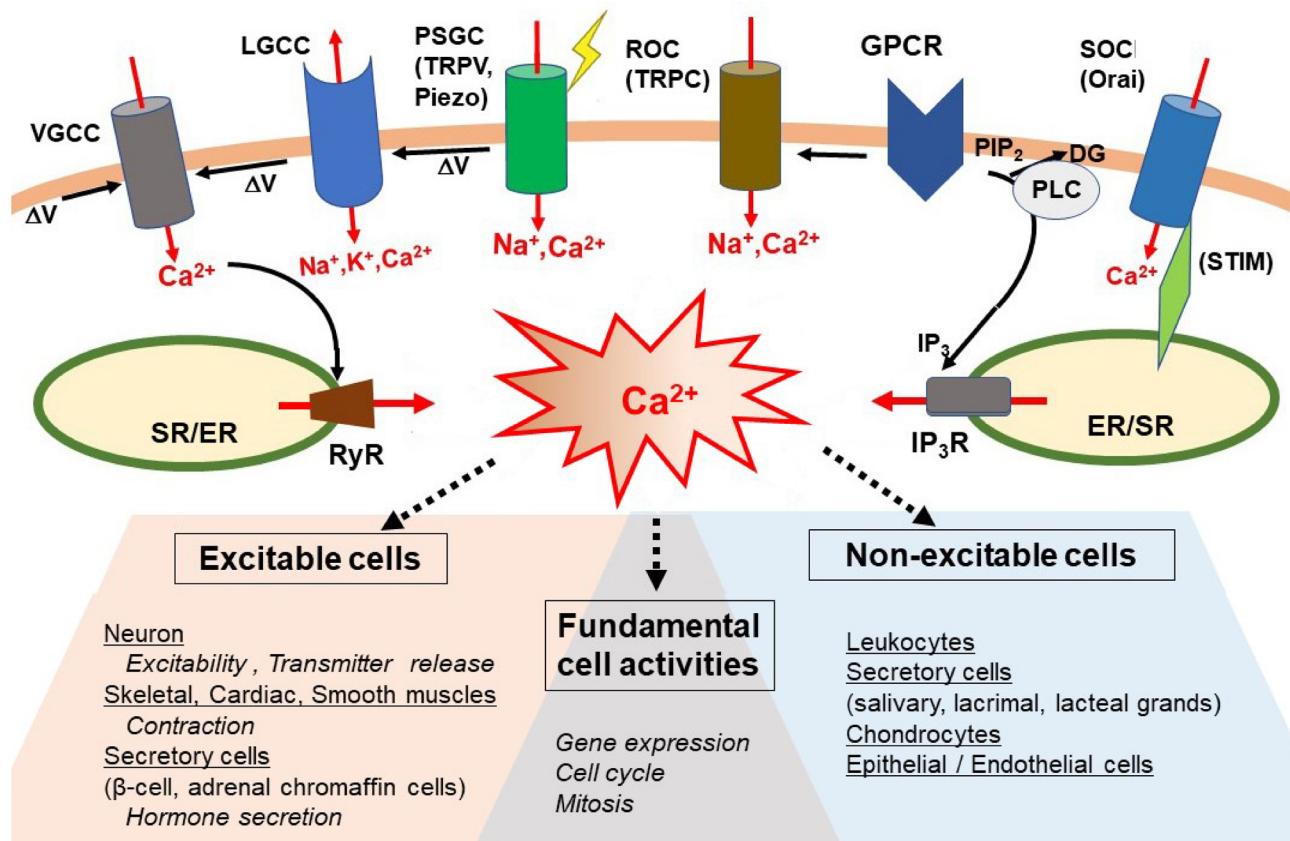


Fig. 1. In a Cell, Ca²⁺ Signaling Is Due to the Influx of Ca²⁺ through Ca²⁺-Permeable Channels in the Plasma Membrane (PM) and/or Ca²⁺ Release from the Endo- and Sarcoplasmic Reticulum (ER/SR)

The activation of G protein-coupled receptors (GPCR) stimulates phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). IP3 diffuses in the cytosol and interacts with IP3 receptors (IP3R) in the ER/SR membrane, which form Ca²⁺ release channels. The Ca²⁺ signal mediates fundamental cell activities, such as gene expression, cell cycle, mitosis, autophagy, and apoptosis. In excitable cells, the primary Ca²⁺ signal is due to Ca²⁺ influx through voltage-gated Ca²⁺ channels (VGCC) by the generation of an action potential. The Ca²⁺ that enters the cell activates the ryanodine receptor (RyR) Ca²⁺ release channel in the ER/SR for Ca²⁺ release. Specific cellular functions driven by Ca²⁺ signaling include transmitter release, contraction, and hormone secretion in excitable cells. Physical stimuli-gated ion channels (PSGC) and ligand-gated ion channels are also permeable to Ca²⁺ but Na⁺ as well. In non-excitable cells, Ca²⁺ influx is mainly due to receptor-operated Ca²⁺ channel (ROC) and store-operated Ca²⁺ channel (SOC). SOC mediated Ca²⁺ entry (SOCE) is activated by the depletion of Ca²⁺ storage sites via IP3 formation and subsequent Ca²⁺ release. (Color figure can be accessed in the online version.)

tance. Ca²⁺-activated K⁺ channels with large conductance (BK, Maxi-K, and K_{Ca}1.1 channels encoded by KCNMA1), intermediate conductance (IK and K_{Ca}3.1 channels encoded by KCNN4), and small conductance (SK) have 100 to 300 pS, 25 to 100 pS, and 2–25 pS, respectively. SK channels are further subclassified by genes into the following three channels: SK1 or K_{Ca}2.1 encoded by KCNN1, SK2 or K_{Ca}2.2 encoded by KCNN2, and SK3 or K_{Ca}2.3 encoded by KCNN3.

In these Ca²⁺-gated channels, intracellular Ca²⁺ binding with an α -subunit protein itself or an auxiliary Ca²⁺-binding protein, such as calmodulin, triggers channel activity. The

direct binding of cellular Ca²⁺ with the α -subunit of BK channels or Ca²⁺-activated Cl⁻ (TMEM16A, Ano-1) channels triggers channel activity. However, BK channel activity also strongly depends on membrane potential, particularly when the γ -subunit is included in the channel complex.^{4–6)} Furthermore, TMEM16A activity is facilitated by membrane depolarization.⁷⁾ IK and SK channels have a constitutive binding site in the α -subunit for the calmodulin molecule.⁸⁾ The channel gating of IK and SK channels is voltage-insensitive and essentially depends upon a conformational change by Ca²⁺ binding to calmodulin in the channel complex.

Biography

Dr. Yuji Imaizumi was born in Tokyo, in 1952. After finishing his master thesis in The University of Tokyo in 1978, he moved with Prof. Minoru Watanabe to Nagoya City University (NCU), Faculty of Pharmaceutical Sciences as an assistant professor. He received his Ph.D. degree from The University of Tokyo in 1981 under the supervision of Prof. Yutaka Kasuya. For two years from 1985, he performed joint research as a visiting scientist at University of Calgary, Canada. After returning to NCU, he was honored by receiving the Japanese Pharmacological Society Encouragement Award in 1992. He was promoted as a full professor in NCU in 1997. Since 2014, he has been a vice president and trustee of NCU. He was honored by receiving the Pharmaceutical Society of Japan Award in 2018. His major research interest is on the Molecular Pharmacology of Ion Channels and related Drug Discovery.



Yuji Imaizumi

Table 1. A List of Ion Channel Groups Roughly Classified Based on Gating Mechanisms

a) Voltage-gated ion channels	
Na ⁺ channel, K ⁺ channel, Ca ²⁺ channel, ★	
Cl ⁻ channels, proton channel	
hyperpolarization-activated cyclic nucleotide-gated channels	
b) Ligand-gated ion channels	
b-1) Extracellular ligand-gated ion channels	
Ion channel receptors: nicotinic acetylcholine, GABA _A , 5-HT _{3A} , glycine, P ₂ X, Acid sensitive, glutamate (AMPA, kainate, NMDA), etc.	
b-2) Cellular ligand-gated ion channels	
ATP-sensitive K ⁺ channel	
<u>Ca²⁺-gated ion channels</u>	
Ca ²⁺ -gated K ⁺ channel (BK, IK, SK) ★	
Ca ²⁺ -gated Cl ⁻ channel (TMEM16A, B) ★	
ryanodine receptor Ca ²⁺ -releasing channel ★	
IP ₃ receptor Ca ²⁺ -releasing channel ★	
Cyclic nucleotide-gated channel	
Lipid-gated ion channel	
c) Physical stimuli-gated ion channels	
Temperature sensitive, pH sensitive,	
Mechanosensitive, Pressure sensitive.	
d) Ca ²⁺ -release activated Ca ²⁺ (CRAC) channel	
Channel for store-operated Ca ²⁺ entry (SOCE)	
(Orai-STIM complex channel) ★	
e) G-protein-gated ion channels	
G protein-coupled inward rectifier K ⁺ channel (GIRK1-4)	
f) Leak or background ion channels	
Two pore domain K ⁺ channels	
Inward rectifier K ⁺ channels	

The stars indicate the channels for which characteristics, physiological functions, and pathophysiological significance in related diseases are discussed in this review. The Ca²⁺-gated ion channels underlined in the list are major issues described. This classification and list shown here are not completely comprehensive, but clearly show ion channel functions. (Color figure can be accessed in the online version.)

In contrast, the voltage-gated K⁺ (K_V) channel interacting protein (KChIP) has been identified as the β -subunit of Kv4.2 and Kv4.3 in cardiac myocytes^{9,10)} and the central nervous system (CNS). KChIP includes an EF hand motif and functions as a type of [Ca²⁺]_i sensor to modulate the activity of Kv4.x in a Ca²⁺-dependent manner.¹¹⁾ Nevertheless, the channel gating of Kv4.x is essentially dependent on voltage. Moreover, ion channel activities are often modulated by phosphorylation in a Ca²⁺-dependent manner, mostly due to Ca²⁺-dependent protein kinases, such as calmodulin-dependent protein kinase II (CaMKII),¹²⁾ while these channels are generally not included in Ca²⁺-gated ion channels.

2-2. The Activation of BK Channels by Ca²⁺ Sparks Functions as a Signal Convertor in the Negative Feedback Regulation of Ca²⁺ Signaling in Smooth Muscle Cells (SMCs) The physiological impact of local Ca²⁺ transients in a cell was initially visualized in 1993 as Ca²⁺ sparks in cardiac myocytes by line-scan microfluorimetry using a laser confocal microscope.¹³⁾ Ca²⁺ sparks have been identified as an

“elementary” Ca²⁺ release event under physiological conditions in cardiac myocytes. They arise from the spontaneous opening of a small number of RyRs acting in concert. In addition, the spontaneous opening of VGCC in the T-tubule of a junctional area closely facing RyRs in SR may elicit a Ca²⁺ spark via the Ca²⁺-induced Ca²⁺ release (CICR) mechanism. Ca²⁺ sparks with similar characteristics have been identified in skeletal muscle.¹⁴⁾ A Ca²⁺ spark, defined as an “elementary Ca²⁺ event of excitation-contraction (E-C) coupling,” has been suggested to comprise the smaller release of Ca²⁺, Ca²⁺ quarks, through a single or few RyRs.¹⁵⁾

Spontaneous transient outward currents (STOCs) were initially observed in isolated voltage-clamped intestinal SMCs in 1986¹⁶⁾ and have since been recorded in almost all types of SMCs. The mechanism underlying STOCs was previously proposed to be BK channel activation by local Ca²⁺ transients.¹⁷⁾ STOCs presumably driven by Ca²⁺ sparks were shown to contribute to muscle relaxation, but not contraction, in vascular SMCs.¹⁸⁾ The simultaneous recording of STOCs using a whole-cell patch clamp technique and Ca²⁺ sparks by two-dimensional Ca²⁺ imaging using fast-scanning confocal fluorescent microscopy (Nikon RCM8000) clearly demonstrated a spatiotemporal relationship between Ca²⁺ sparks and the sequential generation of STOCs in a one-to-one manner in SMCs.¹⁹⁾ Highly localized spontaneous Ca²⁺ release from the SR through RyR2 as a Ca²⁺ spark activates nearby BK channels and elicits STOC, which shifts the membrane potential to a hyperpolarizing direction. The inhibition of Ca²⁺ uptake in the SR using the Ca²⁺ ATPase inhibitor, cyclopiazonic acid, resulted in a transient enhancement in STOCs followed by their suppression.^{20,21)} Ca²⁺ sparks and STOCs randomly occur in multiple cells in smooth muscle tissue and, thus, membrane hyperpolarization by STOCs spreads to electrically connected neighboring SMCs. Membrane hyperpolarization reduces VGCC activity to relax SMCs. The pharmacological blockade of BK channels in isolated arterial vessels depolarized SMCs and increased vessel tone. Therefore, Ca²⁺ sparks as Ca²⁺ signals in SMCs do not induce muscle contraction, but contribute to muscle relaxation via BK channel activation.^{22,23)} The deletion of the BK channel β 1-subunit gene induced hypertension in mice.²⁴⁾ Furthermore, the deletion of the BK α -subunit (BK α) gene resulted in smooth muscle dysfunction, including overactive bladder and incontinence²⁵⁾ as well as erectile dysfunction²⁶⁾ in mice. RyR2 is an essential component of Ca²⁺ sparks in cardiac myocytes and SMCs and RyR1 in skeletal muscle.^{22,27)} The heterozygous knockout of the RyR2 gene (RyR2^{-/+}) reduced the frequency and amplitude of STOCs in isolated SMCs from the urinary bladder and resulted in increased bladder tonus and incontinence.²⁸⁾

Therefore, the physiological impact of Ca²⁺ sparks in SMCs markedly differs from those in cardiac and skeletal muscle cells, in which a Ca²⁺ spark is an elementary event underlying the synchronous release of Ca²⁺ during E-C coupling. In SMCs, the functional coupling between RyR2 in the SR membrane and BK channels in the PM converts local Ca²⁺ sparks to conductive membrane hyperpolarization. This conversion system plays a pivotal role in the negative feedback regulation of Ca²⁺ signaling in SMC tone under resting conditions²⁹⁾ (Fig. 2A, left).

Spatiotemporal analyses of the tow dimensional (2D) Ca²⁺ imaging of single cells with high time resolution using the

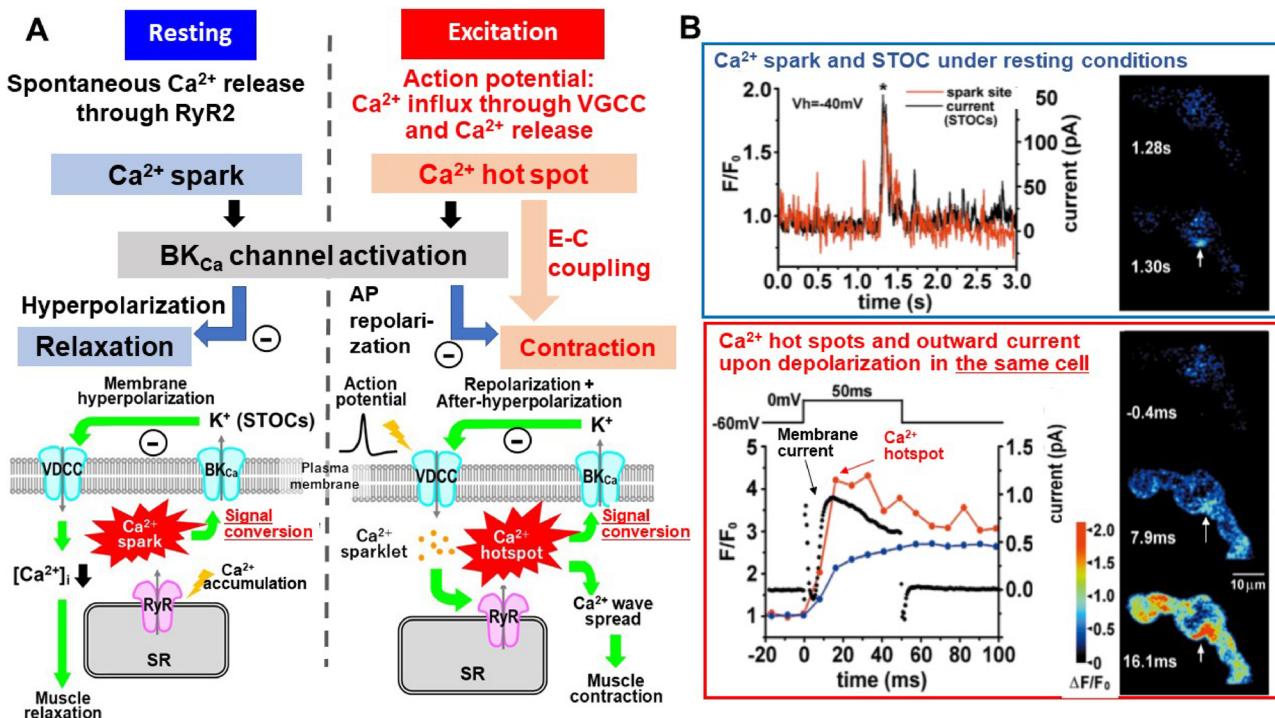


Fig. 2. Negative Feedback Regulation of Ca^{2+} Signaling by BK Channels under Resting and Excitation Conditions in SMCs

A: Under resting conditions, a Ca^{2+} spark occurs as local Ca^{2+} release from the SR by the spontaneous opening of RyRs in the SR membrane. A Ca^{2+} spark signal is converted to membrane hyperpolarization by the activation of BK channels in the PM and suppresses VGCC activity. This reciprocal process forms the “negative feedback regulation of Ca^{2+} signaling” in distinct local areas (see B, upper) in a cell. During excitation with an action potential (AP), Ca^{2+} influx through VGCC elicits a Ca^{2+} hot spot as a larger Ca^{2+} signal by Ca^{2+} -induced Ca^{2+} release from the SR through RyRs (see B, lower). BK channel activation by Ca^{2+} hot spots forms the AP repolarization phase and after-hyperpolarization, which reduce Ca^{2+} influx as “negative feedback regulation.” B: Upper panel (Resting): A transient increase in [Ca^{2+}]_i as a spark and a concomitant spontaneous transient outward current (STOC) were simultaneously recorded in voltage-clamped single urinary bladder SMCs. The Ca^{2+} image was obtained at the peak of the Ca^{2+} spark. Lower panel (Excitation): Ca^{2+} hot spots and associated membrane currents were elicited by depolarization from -60 to 0mV. The initial inward VGCC current and subsequent large outward BK channel current were recorded with a large increase in [Ca^{2+}]_i. The Ca^{2+} image was obtained at the peak of the BK channel current. It is notable that an intensive Ca^{2+} hot spot occurred (lower panel) at the same local area of the Ca^{2+} spark site (upper panel). This research was originally published in *J Physiol* 2001, 534, 313–326,¹⁹ with permission from “John Wiley and Sons.” (Color figure can be accessed in the online version.)

voltage clamp technique have provided important insights into Ca^{2+} spark sites under resting conditions in SMCs. 1) The majority of Ca^{2+} sparks were frequently detected in distinct sites in a cell.¹⁹⁾ 2) Each STOC occurred in a synchronous manner with a Ca^{2+} spark just beneath the PM (Fig. 2B, upper), but did not follow Ca^{2+} sparks located deep inside of the cell.¹⁹⁾ 3) The frequency of STOCs was significantly lower and the resting membrane potential was shallower in SMCs from RyR2^{-/-} mice than from wild-type mice.²⁸⁾

E-C coupling may occur in highly excitable smooth muscle tissues, such as the gastrointestinal tract, arterioles, bladder, vas deferens, ureter, and uterus. In contrast, physiologically quiescent SMCs, including large arteries, airways,^{30–32)} and the iris sphincter,³³⁾ do not elicit action potentials under normal conditions, except when excitability is markedly increased by the blockade of K⁺ channels.³⁴⁾ The membrane excitability of SMCs was previously shown to markedly vary among tissues and was mainly dependent on the density of VGCC in the PM.³⁵⁾ The increase in [Ca^{2+}]_i upon an action potential in highly excitable SMCs originated from a few specific Ca^{2+} spark sites and immediately progressed to larger Ca^{2+} signals, so-called “ Ca^{2+} hot spots.”³⁶⁾ These hot spot sites are located just beneath the PM.^{19,36)} The initial increase in [Ca^{2+}]_i upon an action potential was shown to occur as several Ca^{2+} hot spots and quickly spread throughout the whole cell area.³⁷⁾ The CICR mechanism³⁸⁾ may be induced by the influx of Ca^{2+} via VGCC and following the release of Ca^{2+} through RyRs in SMCs. Therefore, Ca^{2+} signaling following an action po-

tential rapidly occurs by two steps: Ca^{2+} hot spots in discrete Ca^{2+} spark sites (Fig. 2B) that then spread as Ca^{2+} waves throughout a cell to induce contraction. CICR may contribute to both steps of Ca^{2+} signaling.^{37,39)} The Ca^{2+} hot spots that occur upon an action potential strongly activate BK channels to form a repolarizing phase and the after-hyperpolarization of an action potential in order to restore Ca^{2+} signaling and membrane excitability to resting levels (Fig. 2B, lower), indicating the negative feedback regulation of Ca^{2+} signaling by BK channels during excitation (Fig. 2A, right).

A large increase in [Ca^{2+}]_i as a hot spot is sequestered due to extrusion by Na⁺-Ca²⁺ exchangers, pumping in by the SR, and uptake by mitochondria. The alternative confocal imaging of a Ca^{2+} hot spot in the cytosol and Ca^{2+} signaling in a nearby mitochondrion was obtained using the dual and alternative laser excitation system with a confocal fluorescent microscope (Nikon RCM8000). Mitochondrial Ca^{2+} uptake from a nearby Ca^{2+} hot spot was clearly demonstrated following an evoked action potential under the current clamp mode in urinary bladder SMCs.⁴⁰⁾ The mitochondrial increase in Ca^{2+} was markedly slower than the formation of Ca^{2+} hot spots and their spread. Ca^{2+} hot spots facilitate Ca^{2+} uptake by nearby mitochondria and presumably stimulate mitochondrial respiration.

Accumulated evidence suggests that SMCs have a specific Ca^{2+} microdomain for the conversion of intracellular local Ca^{2+} signals into electrical activity.²²⁾ Ca^{2+} hot spots were not clearly detected in SMCs treated with methyl-β-cyclodextrin,⁴¹⁾ which destroys the structure of caveolae by

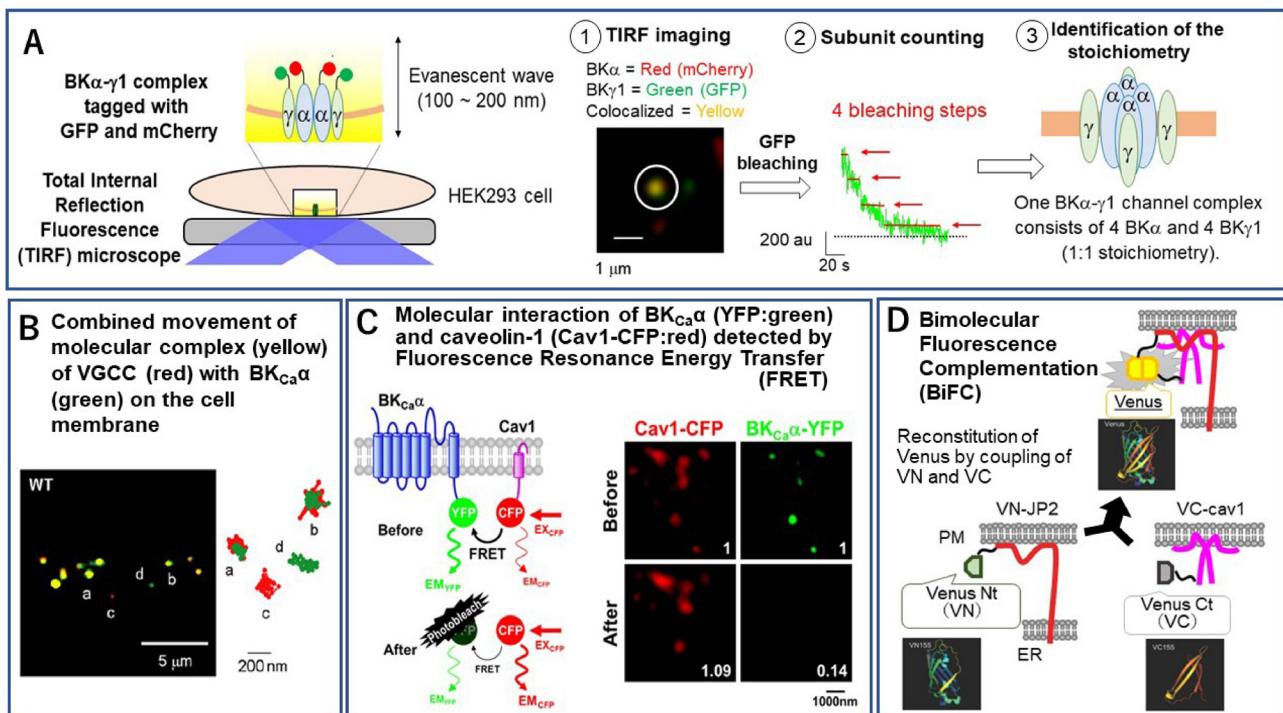


Fig. 3. Single Molecular Imaging by TIRF Microscopy

A: The assembly of ion channel subunits can be examined by the photobleaching method. The BK channel α -subunit forms a functional channel as a tetramer and the γ -subunit interacts with the α -subunit in a one-to-one manner.⁵⁶ **B:** Combined molecular movements suggest molecular complex formation.⁴² **C:** FRET analyses.⁵⁴ **D:** BiFC methods provide indirect, but convincing evidence for the interaction between two molecules in living cells. Two compartments of the Venus fluorescent protein, VN and VC, may be reconstituted when they are located in close proximity (<10nm) and form fluorescent particles in the TIRF field of vision. This strongly suggests a direct molecular interaction between two molecules (JP2 and cav1, in "D") labeled with VN and VC, respectively.⁶³ (Color figure can be accessed in the online version.)

removing cholesterol in the PM. The genetic deletion of caveolin-1 (Cav1), a molecule that is essential for the formation of caveolae, decreased coupling between Ca^{2+} sparks and STOCs in vascular SMCs.⁴² Caveolae on the PM appear to play a vital role in the formation of a Ca^{2+} microdomain presumably composed of VGCC, BK channels, and RyRs in SMCs.

The major RyR type that is functional in SMCs is RyR2. Among the 3 types of RyRs, the expression of RyR3 is widely, but not abundantly, detected in various cell types. However, RyR3 is neither predominant against RyR1 in skeletal myocytes or RyR2 in cardiomyocytes and SMCs.²⁹ The tissue-specific predominant expression of RyR3 has been reported in a number of cell types in rodents, such as CA1 pyramidal neurons,⁴³ some vascular SMCs,⁴⁴ and uterine SMCs.^{45,46} Although the contribution of RyR3 to Ca^{2+} spark generation has been suggested in vascular SMCs,⁴⁷ the co-expression of a RyR3 dominant-negative variant⁴⁸ in SMCs indicates a more complex physiological situation.⁴⁹ The phenotypes of RyR3 gene deletion in mice are CNS symptoms^{50,51} and atypical extraocular muscles⁵²; however, reproductive functions remain unaffected in female rats.⁴⁵ The physiological functions of RyR3 have not yet been elucidated in detail.

2-3. Single Molecular Imaging Analysis as a Potential Tool to Clarify the Molecular Basis for Microdomain Formation in the Ca^{2+} Signal Conversion System The molecular mechanisms underlying the formation of Ca^{2+} microdomains as "an apparatus to convert local Ca^{2+} signals into electrical signals" have been clarified using single molecular imaging with total internal reflection fluorescent (TIRF) microscopy.⁵³

The labeling of a target molecule with a fluorescent protein, such as green/cyan/yellow fluorescent proteins (GFP, CFP, and

YFP, respectively) and mCherry, by genetic engineering allows us to visualize the molecule as a single fluorescent particle in the TIRF field of vision. This visualization makes it possible to analyze the intermolecular relationships responsible for Ca^{2+} signal conversion in living cells as follows (Fig. 3).

(1) Analyses of subunit assembly formation. Functional BK channels are formed by tetrameric BK_{Ca} α -subunits.⁵ When a GFP-labeled BK_{Ca}- α -subunit is expressed in a cell and a single fluorescent particle is continuously exposed to excitation light, photobleaching occurs and quenching is performed in four steps, thereby confirming the tetrameric formation of BK_{Ca}- α .⁵⁴ Moreover, the stoichiometry of a newly identified γ -subunit in the interaction with α -subunits can be assessed using the photobleaching method.^{55,56} The colocalization of α - and γ -subunits labeled with mCherry and GFP, respectively, can be detected as a single yellow particle (Fig. 3A). Based on a distribution analysis of GFP bleaching steps, the 1:1 stoichiometry of two subunits can be identified.⁵⁶

(2) Analysis of interactions between multiple molecules in a living cell. Single molecules of VGCC(Cav1.2)-mCherry and BK_{Ca}- α -GFP often colocalize on the PM and move as a single yellow particle (Fig. 3B). In addition, after cell fixation, the photobleaching method can be used to detect stoichiometry in a molecular complex. It was demonstrated that a VGCC molecule interacts with a BK_{Ca}- α subunit in a one-to-one manner,⁴² and up to four VGCCs with a single BK channel.

(3) Fluorescence resonance energy transfer (FRET) analyses. A FRET analysis is an established method for identifying the extremely close positioning (<40 nm) of two molecules labeled with fluorescent proteins.⁵⁷ A direct interaction between Cav1-CFP and BK_{Ca}- α -YFP was suggested by FRET⁵⁴ (Fig.

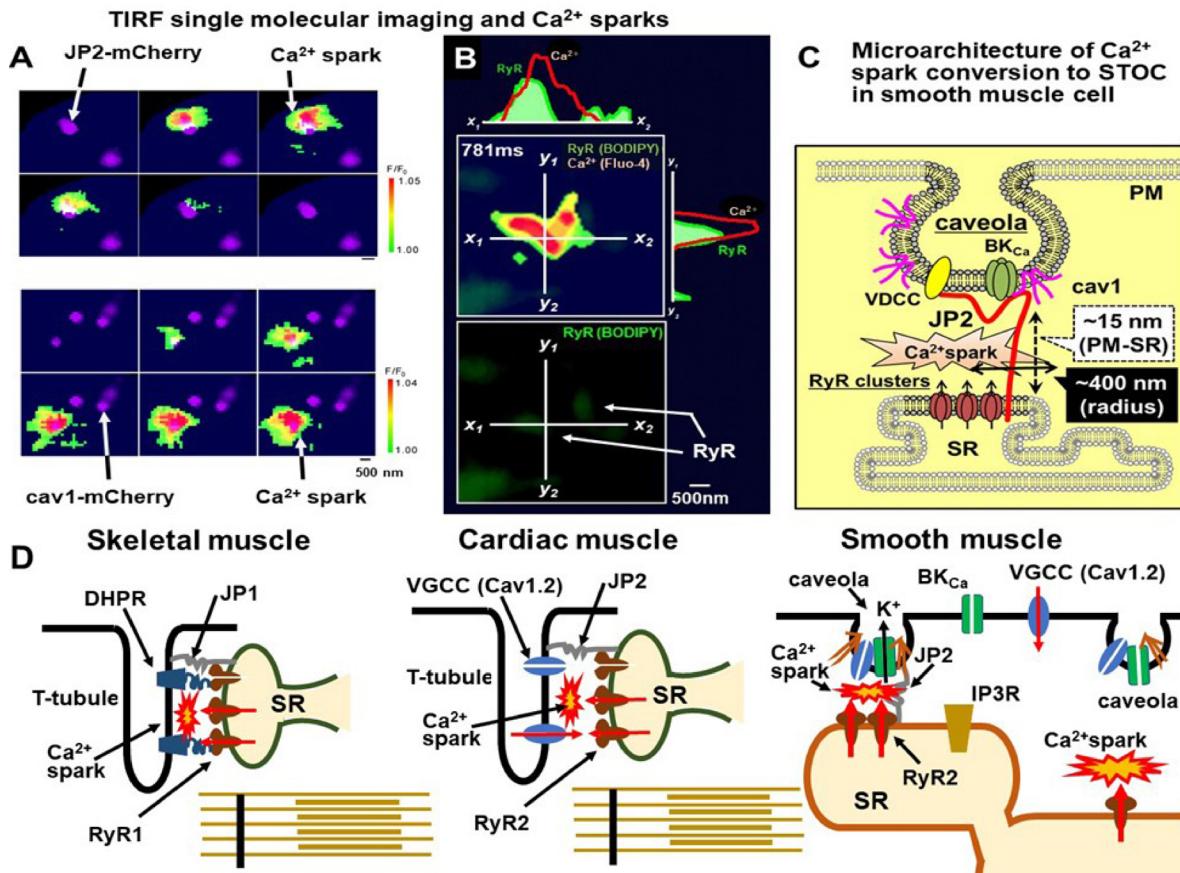


Fig. 4. The 2D Architecture of the Molecular Assembly in a Ca^{2+} Spark Site Can Be Elucidated Using Single Molecular Imaging Methods

A: Ca^{2+} sparks were recorded in a cell, in which mCherry-labeled junctophilin-2 (JP2) or mCherry-labeled caveolin-1 (Cav1) was exogenously expressed. These molecules were carefully expressed at low levels in primary cultured vascular SMCs.⁶³ B: Ca^{2+} sparks were recorded in a cell in which RyRs were stained by BODIPY-ryanodine. Unpublished observation by Dr. Hisao Yamamura, Nagoya City University, with permission from Dr. Yamamura. C: A summarized diagram of the Ca^{2+} microdomain for the effective conversion system from the Ca^{2+} spark signal to the STOC electrical signal.⁶³ D: The different architectures for the Ca^{2+} spark microdomain in cardiac and skeletal myocytes are shown together with that in SMCs. (Color figure can be accessed in the online version.)

3C) and confirmed by co-immunoprecipitation. A single K⁺ channel molecule is composed of four α -subunits, typically as a homotetramer, but occasionally as a heterotetramer within a subfamily. The heterotetramer can also be identified by FRET analyses in a similar manner.⁵⁸

(4) Bimolecular fluorescence complementation (BiFC) as a powerful tool to identify the interaction between two molecules in a living cell. Venus, a GFP-derived fluorescent protein, is divided into two components, VN (the N-terminal fragment of Venus) and VC (the C-terminal fragment of Venus), which are not fluorescent, but may fluoresce by reassembling their contact with each other in close positioning (<10 nm).⁵⁹⁻⁶¹ Junctophilin 2 (JP2), a bridging protein between the PM and SR,⁶² labeled with VN was co-expressed with Cav1 labeled with VC in HEK293 cells. VN-JP2 + VC-cav1 formed fluorescent particles in the TIRF field of vision, strongly suggesting a direct molecular interaction in living cells⁶³ (Fig. 3D).

(5) Simultaneous recording of Ca^{2+} imaging and single molecular imaging. The TIRF method is suitable for Ca^{2+} spark imaging analyses because the correspondence of each spark to a STOC under simultaneous recording is clearer and more exact than that in confocal Ca^{2+} images. Moreover, the spatial configuration of a Ca^{2+} spark and Cav1 and JP2 can be obtained in a vascular SMC in which Cav1 and JP2 are expressed⁶³ (Fig. 4A). Furthermore, after recording Ca^{2+} sparks, RyR2 in SR was stained using BODIPY-ryanodine⁶⁴ (Fig. 4B).

(6) Imaging of Ca^{2+} sparklets and subsequent CICR under voltage clamping. Since the depth of the TIRF field of vision in the vertical direction is up to approximately 200 nm from the bottom of the chamber, it is also suitable for observing the influx of Ca^{2+} through VGCC in the PM. A Ca^{2+} sparklet is a minute local increase in $[\text{Ca}^{2+}]_i$ at the internal opening of a single or clustered VGCC (Cav1.2) in the PM. Ca^{2+} sparklets can be detected upon a short depolarization for 10 ms in voltage-clamped urinary bladder SMCs (not shown in figures).³⁹ A Ca^{2+} sparklet was followed by a rapid Ca^{2+} wave that spread towards the PM from the inside of the cell. The Ca^{2+} wave induced by a Ca^{2+} sparklet was abolished by a treatment with ryanodine. These findings indicate that CICR via the activation of RyRs in the SR by Ca^{2+} sparklets is functional in SMCs.^{37,39}

Based on the findings obtained in single molecular analyses,^{42,63} the molecular interaction of Cav1 with JP2, VGCC, and BK α subunit leads to the accumulation of these molecules in caveolae (Fig. 4C). JP2 in caveolae maintains a close distance between caveolae and SR as the micro-space of a Ca^{2+} spark. Among four junctophilin family members, JP1 is specifically expressed in skeletal muscle to connect T-tubules and SR. JP2 is widely expressed in cardiac and smooth muscles.⁶² JP3 and JP4 are mainly distributed in the brain.^{62,65} It has been demonstrated that approximately 50% of all Ca^{2+} sparks occurred within 400 nm of JP2 or Cav1 in primary cultured SMCs from the mouse mesenteric artery.⁶³ Furthermore, the

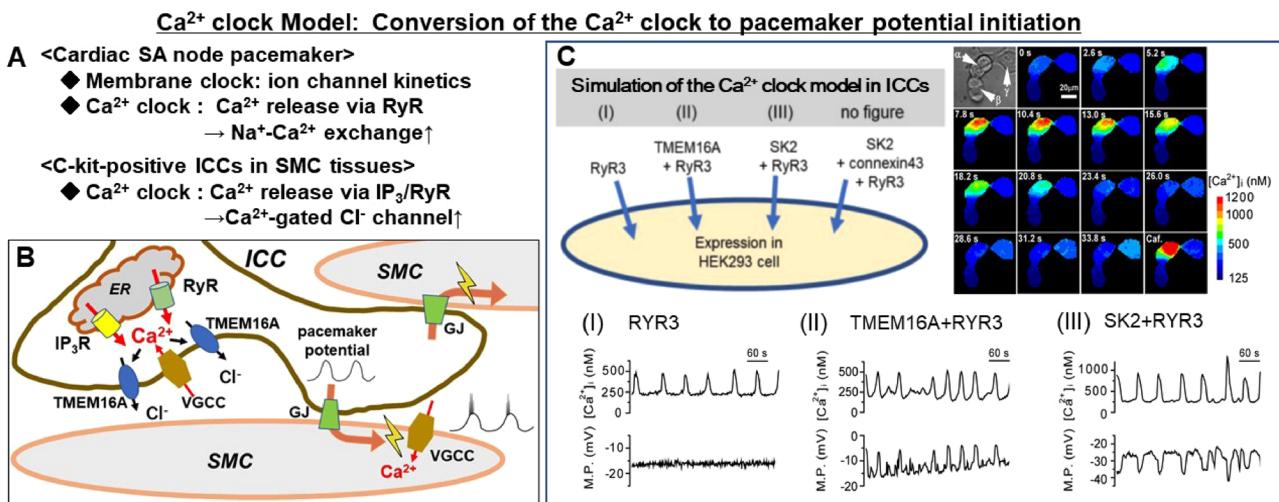


Fig. 5. A: The “Ca²⁺ Clock” Function of Ca²⁺ Release from the SR Has Been Revealed in the Pacemaker Cells of Both the SA Node in the Heart and ICCs (or ICC-Like Cells) in the Gastrointestinal and Urinary Tracts

B: Schematic diagram of the pacemaking mechanism in ICCs. Spontaneous Ca²⁺ release through IP₃Rs and/or RyRs activates the TMEM16A channel to induce a slow wave as the pacemaker potential in ICCs. The slow wave with action potentials spreads along ICCs and connected SMCs. C: The model of ICC pacemaking activity was reconstituted in HEK293 cells. (I) The overexpression of RyR3 induced spontaneous Ca²⁺ oscillations. (II) Co-expression of the TMEM16A channels with RyRs converted Ca²⁺ oscillations to a periodical depolarization-like slow wave. (III) The co-expression of SK2 channels with RyR3 converted Ca²⁺ oscillations to periodical hyperpolarization. (IV) The further expression of connexin 43 with SK2 and RyR3 showed propagated periodical hyperpolarization (Recordings are not shown in this figure). Ca²⁺ imaging represents Ca²⁺ oscillations in HEK293 cells expressing RyR3 + SK2. Following an increase in [Ca²⁺]_i in the initiation cell, the elevation was propagated to the neighboring cell presumably due to the propagation of membrane hyperpolarization, which was blocked using a SK2 channel blocker (not shown). This research was originally published in *Biochemical Biophysical Research Communication*, 2019, 510, 242–247, with permission from Elsevier. (Color figure can be accessed in the online version.)

removal of caveolae^{41,42)} significantly reduced the coupling efficiency between Ca²⁺ sparks and STOCs in SMCs. Similar effects were observed after the knockdown of JP2.⁶³⁾ These findings indicate that the molecular interaction between Cav1 and JP2 is essential for the positioning of VGCC and BK channels near RyRs in spark sites in SMCs.^{63,66)} The molecular assembly of the efficient apparatus for the Ca²⁺ signal converter in SMCs is shown in Fig. 4C. The different architectures of the Ca²⁺ spark microdomains in cardiac and skeletal myocytes are also shown (Fig. 4D). The architecture of the junctional space at which Ca²⁺ sparks occur in SMCs is distinct from those of skeletal and cardiac myocytes⁶⁷⁾ because the T-tubule structure is absent in SMCs. As an alternative, caveolae play pivotal roles in the regulation of Ca²⁺ signaling during rest and excitation by forming Ca²⁺ microdomains in SMCs.²⁹⁾

2-4. The Conversion of Ca²⁺ Signals to Electrical Pacemaker Activity in a Ca²⁺ Clock System The heartbeat and gastrointestinal peristalsis are both driven by electrical signals, which occur in pacemaker cells and spread through specific impulse-conducting systems to muscle cells in these tissues. In the gastrointestinal and urinary systems, spontaneous Ca²⁺ oscillations in the pacemaker cells, c-kit-immunopositive interstitial cells of Cajal (ICC) or ICC-like cells, can be converted to pacemaking electrical oscillations (slow wave). This signal conversion is due to the activation of Ca²⁺-gated Cl⁻ channels, which have been identified as TMEM16A.^{68–70)} “Clockwork Ca²⁺ release” from the ER in ICCs is mediated by IP₃Rs and/or RyRs in a manner that is dependent on the ICC types in tissues.⁷¹⁾

The “Ca²⁺ clock” function of Ca²⁺ release from the SR was originally described as part of the pacemaking mechanism together with the “membrane clock” due to the voltage- and time-dependent ion channel kinetics of the sinoatrial (SA) node cells of the heart.⁷²⁾ In SA node cells, the activation of Na⁺-Ca²⁺ exchange by local Ca²⁺ release from the SR through

RyR2, as Ca²⁺ sparks or similar Ca²⁺ transients, contributes, at least partly, to the slow depolarization that constitutes pacemaker potential⁷³⁾ (Fig. 5A). The Na⁺-Ca²⁺ exchange current in cardiac myocytes clearly exhibits electrogenicity.⁷⁴⁾ While the Na⁺-Ca²⁺ exchange current in SMCs is small and may not have electrophysiological functions,⁷⁵⁾ the contribution of this current in ICC-like cells in the lower urinary tract has been suggested.⁷⁶⁾ The major Ca²⁺ clock mechanism in ICCs in the gastrointestinal tract is considered to be spontaneous Ca²⁺ release through IP₃Rs in the ER membrane.⁷⁷⁾ However, Ca²⁺ release mediated by RyRs has also been suggested to make a significant contribution to the Ca²⁺ clock in ICC-like cells in the lower urinary tract.⁷⁸⁾ In the cardiac SA node and urinary tract, RyRs are major Ca²⁺ release channels for the Ca²⁺ clock, while IP₃Rs may contribute to coupling between the Ca²⁺ clock and membrane clock.⁷³⁾ Previous studies reported that ICCs in the gastrointestinal^{79,80)} and urinary tracts expressed RyR2 and/or RyR3 in addition to IP₃Rs (Fig. 5B). In an organized gut motility system, slow waves in ICCs spread as pacemaker potentials with associated action potentials along ICCs and to electrically connected SMCs. The pacemaking mechanism due to the “Ca²⁺ clock” functions more specifically in the ICCs of the gastrointestinal tract than in those of the SA node, in which a combination with the membrane clock appears to be essential. Moreover, ICC-like cells and telocytes both often exhibit spontaneous or evoked Ca²⁺ oscillations in a number of tissues outside the gastrointestinal tract, including the lower urinary tract.^{78,81,82)}

Pacemaking depolarization by TMEM16A channel activation induces the influx of Ca²⁺ through VGCC, which elicits CICR via RyR activation. This is a positive feedback mechanism that facilitates Ca²⁺ signaling in ICCs.⁷¹⁾ The conversion of discrete Ca²⁺ clock events to the electrical slow wave is an essential step for the initiation and propagation of pacemaker activity through multicellular organs in order to accomplish

synchronized physiological functions, such as peristaltic motion. A simulation model of Ca^{2+} oscillations by clockwork Ca^{2+} release and subsequent signal conversion can be reconstituted in HEK293 cells by the heterologous co-expression of RyR3 and Ca^{2+} -gated ion channels, *i.e.*, the SK2 channel or TMEM16A^{80,83)} (Fig. 5C). The overexpression of RyR3 elicited periodical oscillations to a similar frequency as ICCs. The incorporation of Ca^{2+} -gated ion channels converted Ca^{2+} oscillations to electrical slow waves. The co-expression of TMEM16A or SK2 channels resulted in depolarizing or hyperpolarizing slow waves, respectively. The additional expression of connexin 43, which forms gap junction in ICCs, facilitated multicellular electrical coupling (not shown in Fig. 5).⁸³⁾ This simulation demonstrated that the conversion of Ca^{2+} oscillations to slow waves with cell-to-cell propagation can be reconstituted as a model of Ca^{2+} clock-dependent pacemaker activity by the combined expression of critical elements in the heterologous system and may provide valuable knowledge for fitting to a theoretical simulation.

TMEM16A is highly expressed in ICCs, but is also distributed in SMCs, epithelial cells,⁸⁴⁾ nasal and olfactory neurons,⁸⁵⁾ and the pineal body.⁸⁶⁾ It is often overexpressed in cancer cells.^{87,88)} In some vascular and airway SMCs,⁸⁹⁾ Ca^{2+} sparks couple with both BK and TMEM16A channels. A Ca^{2+} spark elicits a STOC and a spontaneous inward current (STICs).⁹⁰⁾ STICs contribute to leading the resting membrane potential in a depolarizing direction in SMCs. However, the regulation of membrane potential by BK channels is generally more dynamic than that by TMEM16A, presumably due, at least in part, to the single channel conductance of the BK channel (approx. 200 pS), which is approximately 80-fold higher than that of TMEM16A (approx. 2.7 pS).⁸⁶⁾ The cell-dependent reversal potential of Cl^- channels may also be a factor contributing to the involvement of TMEM16A channels in cellular electrical activities, including the resting membrane potential⁹¹⁾ A STOC and a STIC was often recorded as a continuous signal and the STOC always anteceded the STIC,⁸⁹⁾ presumably due to different kinetics of BK and TMEM16A channels activated by a single Ca^{2+} spark. Although the architectural involvement of TMEM16A channels in Ca^{2+} microdomains has not yet been elucidated in detail, an interaction with the actin cytoskeleton⁹²⁾ and a close relationship with RyRs⁹³⁾ have been suggested. The physiological impact of TMEM16A in Ca^{2+} signaling and contraction has been emphasized in vascular SMCs^{94,95)} and other SMC tissues, such as the internal anal sphincter.⁹⁶⁾

Spontaneous and/or nicotine-induced Ca^{2+} oscillations, which are regulated by BK channel activity, have been detected in some primary cultured pineal cells of the rat.^{97,98)} TMEM16A and TMEM16B channels, presumably as a heterodimer, contribute to Ca^{2+} signaling, which regulates melatonin secretion,⁹⁹⁾ suggesting the potential contribution of these channels to circadian rhythms.

3. REGULATION OF Ca^{2+} SIGNALING BY ION CHANNELS AS A POTENTIAL TARGET OF DRUG DISCOVERY

3-1. Opposite Regulation of Ca^{2+} Signaling by the Membrane Potential in Excitable and Non-excitable Cells

The major Ca^{2+} influx pathway in excitable cells is VGCC

activated by membrane depolarization. In many highly excitable cells, an action potential is initiated by voltage-gated Na^+ channel activation. The subsequent activation of VGCC triggers Ca^{2+} signaling and signal cascades. On the other hand, the activation of VGCC is essential for action potentials in SMCs. Na^+ channels are expressed at low levels in some types of SMCs in rodents, including the ureter¹⁰⁰⁾ and uterus¹⁰¹⁾; however, their functional contribution is negligible or small. In any type of excitable cell, membrane depolarization itself facilitates VGCC activity and Ca^{2+} influx, presumably because of the non-inactivating component (window current) of VGCC.¹⁰²⁾ This is also the case in SMCs that are electrically quiescent under physiological conditions, such as those in large arteries and airways.¹⁰³⁾

In non-excitable cells, which do not elicit action potentials, the functional expression of voltage-gated Na^+ channels and VGCC is extremely low or absent. The major Ca^{2+} -permeable channels in these cells are transient receptor potential (TRP) channels and Ca^{2+} release-activated calcium (CRAC) channels, which are abundantly expressed in most cell types, including cancer cells.¹⁰⁴⁾ The TRP channel group consists of 10 family members and more than 50 subfamily members and the distribution of its channels widely varies depending on the cell type.¹⁰⁵⁾ The characteristics of gating mechanisms, ion permeability, and physiological functions also widely vary among TRP channels and are emergent druggable targets.¹⁰⁶⁾ Many TRP channels are non-selective to Na^+ and Ca^{2+} , while CRAC channels shows higher selectivity to Ca^{2+} . TRP and CRAC channels are both gated in non-voltage-dependent manners. The crosstalk of TRP channels with Ca^{2+} -gated ion channels is an emergent issue of importance, particularly in non-excitable cells, but is not discussed in this review.¹⁰⁷⁾

CRAC channels are responsible for store-operated calcium entry (SOCE), which is activated by the depletion of Ca^{2+} stores. Functional CRAC channels consist of two sets of molecules, Orai (Orai 1, 2, or 3) and STIM (STIM 1 or 2).¹⁰⁸⁾ STIM belongs to the CRAC channel family and is distributed on the ER membrane to sense the Ca^{2+} concentration on the luminal side of the ER. The depletion of Ca^{2+} in the ER by Ca^{2+} release through IP₃Rs induces the aggregation of STIM, which results in the formation of oligomers on the ER membrane that directly interact with the Orai protein cluster on the cell membrane. The interaction with STIM activates Orai to function as the Ca^{2+} -permeable channel responsible for SOCE.¹⁰⁹⁾

In *in vitro* experiments, SOCE can be induced by the addition of Ca^{2+} to the extracellular solution after Ca^{2+} store depletion by simultaneous treatments with thapsigargin, a specific inhibitor of SR Ca^{2+} pumps, and an exposure to Ca^{2+} -free solution (Fig. 6A). In t-BBEC177, a cell line derived from the bovine brain capillary endothelium, the increase in $[\text{Ca}^{2+}]_i$ by SOCE, which was activated in this manner, was measured using the fluorescent Ca^{2+} indicator Fura-2 under the voltage clamping. SOCE appeared to be larger at a holding potential of -80mV than at 0mV and was completely blocked by La^{3+} , a non-selective blocker of Ca^{2+} influx.¹¹⁰⁾ Therefore, SOCE through CRAC channels was apparently increased by membrane hyperpolarization. Similar hyperpolarization-induced increases in $[\text{Ca}^{2+}]_i$ have been detected in other non-excitable cells, such as airway epithelial cells¹¹¹⁾ and chondrocytes.¹¹²⁾ Membrane hyperpolarization increases the driving force for Ca^{2+} influx through these non-voltage-gated Ca^{2+} -permeable

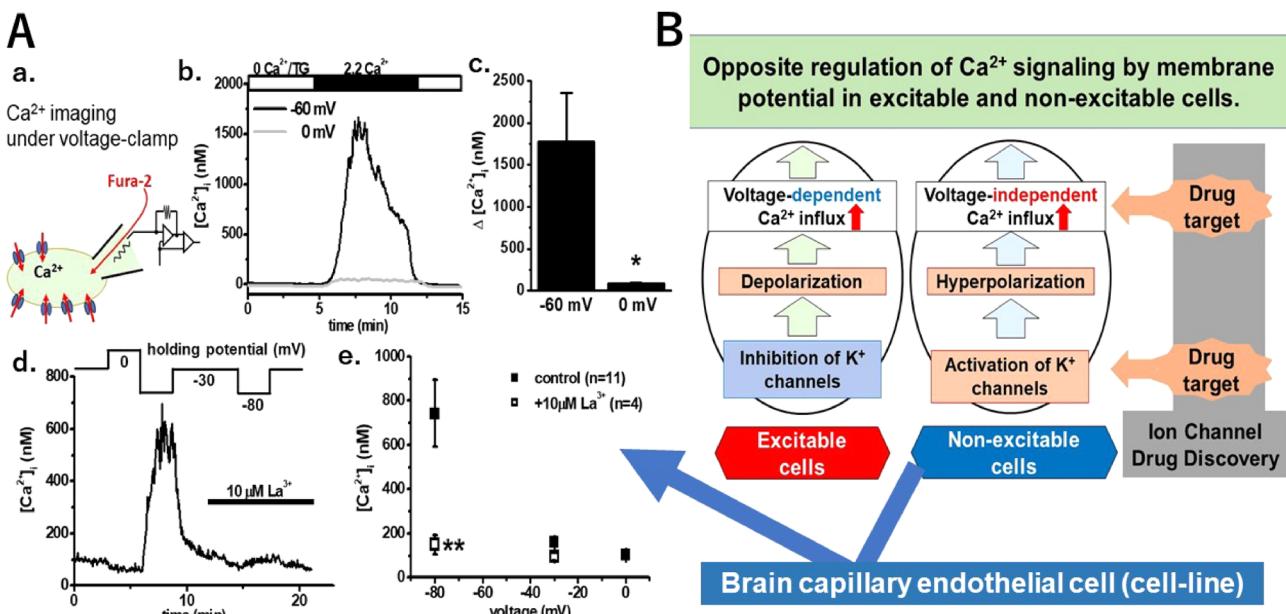


Fig. 6. The Relationship between Membrane Potential and Ca^{2+} Influx

A: SOCE as a Ca^{2+} influx pathway in non-excitable cells was examined in t-BBEC177, a cell line derived from the bovine brain capillary endothelium. The increase in $[\text{Ca}^{2+}]_i$ by SOCE was detected by fura-2 applied from a recording patch pipette under voltage clamping. The addition of Ca^{2+} to the extracellular solution after Ca^{2+} storage depletion by a treatment with thapsigargin in Ca^{2+} -free solution elicited a slow, but large increase in $[\text{Ca}^{2+}]_i$ due to SOCE. The increase in $[\text{Ca}^{2+}]_i$ was strongly dependent on the membrane potential (b, c, d, and e) and completely blocked by La^{3+} (d). Ca^{2+} influx by SOCE was enhanced by membrane hyperpolarization. **B:** The regulation of Ca^{2+} signaling by the membrane potential operated in the opposite directions in excitable and non-excitable cells. To reduce Ca^{2+} signaling for therapy under pathophysiological conditions, the Ca^{2+} influx pathway and regulation of the membrane potential by K^+ channel modulation are both druggable targets; however, the effects of modulators are in opposite directions in excitable and non-excitable cells. This research is a part of Ph.D. Thesis of Dr. Hiroaki Kito at Nagoya City University, 2014, with permission from Dr. Kito. (Color figure can be accessed in the online version.)

channels. The Ca^{2+} influx rate through TRP channels is markedly slower than that through VGCC. Since Ca^{2+} influx through CRAC channels is even slower, it does not induce significant membrane depolarization. TRP and CRAC channels are both potential targets of drug discovery for a number of diseases.^{106,108}

Regardless of whether a cell is excitable or non-excitable, membrane hyperpolarization is generally attributed to K^+ channel activation. In excitable cells, membrane hyperpolarization reduces the open probability of VGCC and suppresses Ca^{2+} signaling and mediated cellular activities. In contrast, membrane hyperpolarization by K^+ channel activation facilitates the influx of Ca^{2+} through TRP and/or CRAC channels in non-excitable cells. Therefore, the regulation of membrane potential by a K^+ channel modulator results in Ca^{2+} signal changes in opposite directions in excitable and non-excitable cells (Fig. 6B). This opposite regulation is a key issue in drug development targeting for K^+ channel modulators with respect to tissue-selective drug actions and side effects.

3-2. Physiological Impact of Ca^{2+} -Gated Ion Channels in the Regulation of Ca^{2+} Signaling in Non-excitable Cells

The functional importance of TRP channels as receptor-operated Ca^{2+} channels has been extensively examined since the middle of 1990's. The Orai-STIM channel complex was identified as a molecular entity of the CRAC channel that is responsible for SOCE in immune cells in 2006.¹¹³ These channels, particularly the CRAC channel, provides a pivotal Ca^{2+} influx pathway in non-excitable cells. It is notable that membrane hyperpolarization facilitates the influx of Ca^{2+} through these non-voltage-gated channels.

In a chondrocyte-like cell line derived from chondrosarcoma, in which VGCC are not functionally expressed, the stimu-

lation of histamine H1 receptors induced oscillatory Ca^{2+} signaling and synchronous membrane hyperpolarization¹¹² (Fig. 7A). Ca^{2+} signaling by histamine consists of biphasic components, namely, initial phasic and smaller sustained components. The phasic component was mainly due to Ca^{2+} release via IP_3 formation. The sustained component was due to the influx of Ca^{2+} through TRP and CRAC channels. The sustained Ca^{2+} signal was extensively enhanced by membrane hyperpolarization from 0 to -60 mV under voltage clamping. Oscillatory membrane hyperpolarization was significantly suppressed by paxilline, a selective blocker of BK channels, indicating the contribution of BK channels to the positive feedback of Ca^{2+} signaling via membrane hyperpolarization.^{61,112} Single molecular imaging using the BiFC method can visualize the process of CRAC channel activation, which involves the oligomerization and translocation of the STIM protein to junctions with the PM and the interaction of STIM with Orai to form a cluster of functional CRAC channels⁶⁰ (Fig. 7B). The architectural interaction between the Orai-STIM channel complex and IP_3 Rs is a hot issue.^{114,115}

The positive feedback regulation of Ca^{2+} influx via CRAC and/or TRP channels by Ca^{2+} -gated K^+ channels has been reported in a wide range of non-excitable cells. In a chondrocyte-like cell line, histamine-induced Ca^{2+} signaling was potentiated by the positive feedback mechanism mainly due to BK channels and SOCE and facilitated extracellular matrix formation and inflammatory prostaglandin production.¹¹⁶ The contribution of Ca^{2+} -gated K^+ channels to positive feedback is not limited to BK channels, it also includes IK and SK channels. IK channels have been shown to play a central role in the positive feedback regulation of Ca^{2+} signaling via IP_3 -induced Ca^{2+} release and CRAC channels in T lymphocytes^{117,118}

Ca²⁺ signaling and Orai1/STIM1 mobilization induced by histamine in chondrocytes

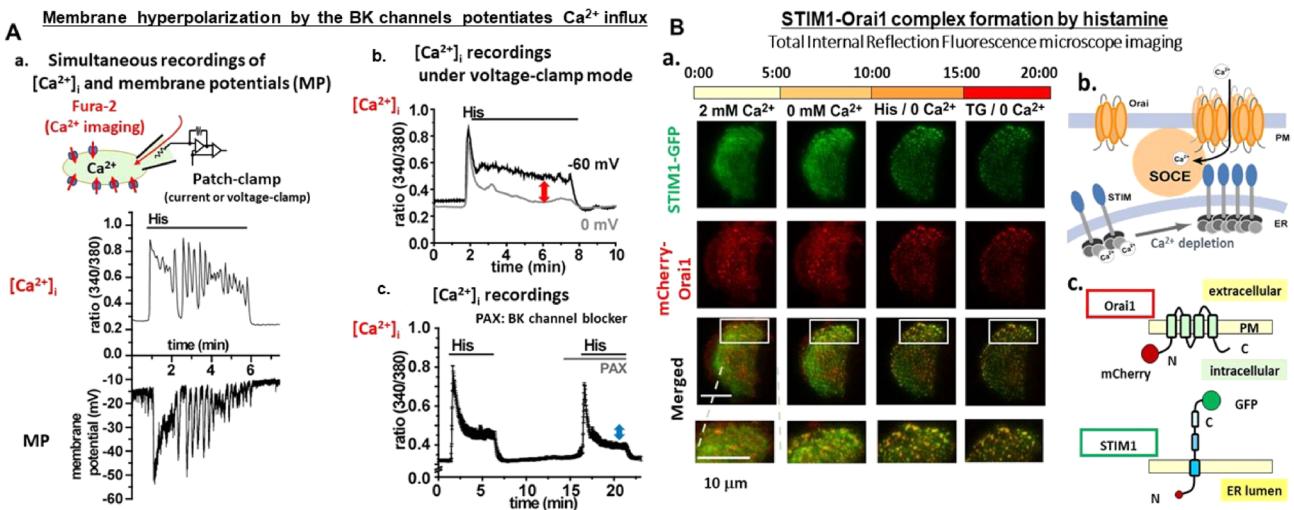


Fig. 7. Relationships between [Ca²⁺]i Changes and the Membrane Potential and Orai1/STIM1 Molecular Complex Formation Were Examined in a Chondrocyte-Like Cell Line Derived from Human Chondrosarcoma

A: The application of 1 μM histamine induced oscillatory Ca²⁺ signaling and synchronous membrane hyperpolarization (a). Ca²⁺ signaling by histamine consisted of biphasic components. The phasic and subsequent tonic components were due to Ca²⁺ release and Ca²⁺ influx, respectively. The sustained component of the Ca²⁺ signal was extensively enhanced by membrane hyperpolarization from 0 to -60 mV under voltage clamping. The BK channel block by paxilline reduced the sustained component, suggesting the contribution of BK channels to the positive feedback regulation of Ca²⁺ signaling.^[112] **B:** The addition of Ca²⁺ after storage depletion by histamine or thapsigargin in Ca²⁺-free solution caused the movement of STIM1-GFP and Orai1-mCherry in single molecular imaging in the TIRF field of vision. They aggregated together and formed clusters of active CRAC channels, as shown by punctate yellow dots. This study was originally published in *Cell Calcium*, 2015, 57, 337–347,^[60] with permission from Elsevier. (Color figure can be accessed in the online version.)

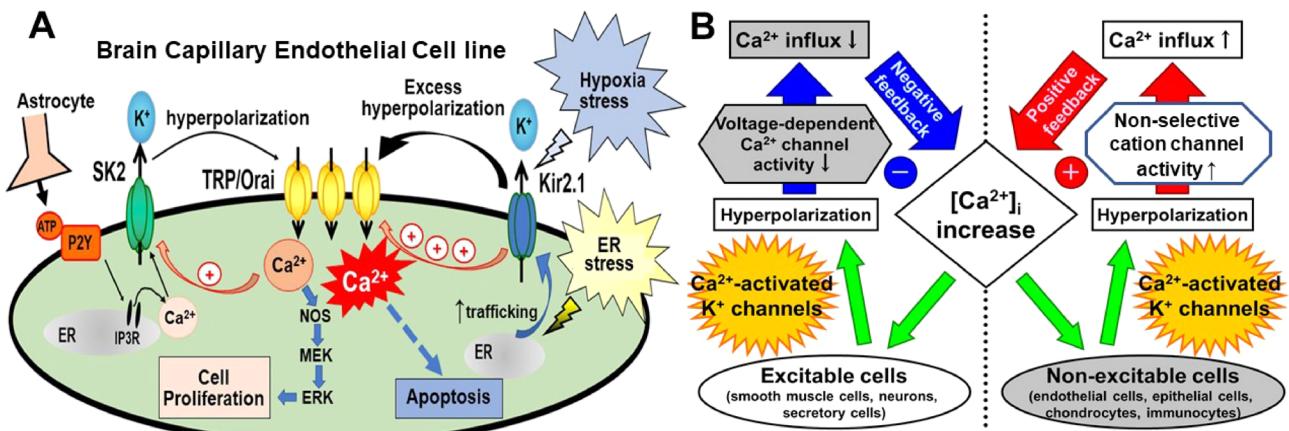


Fig. 8. Ca²⁺ Signaling Regulation Including Ca²⁺-Activated K⁺ Channels in Non-excitable Cells and Excitable Cells

A: In the t-BBEC177 cell line, Ca²⁺ signaling induced by ATP activated SK2 channels and subsequent membrane hyperpolarization, which, in turn, enhanced Ca²⁺ entry through TRP and CRAC channels. This positive feedback regulation of Ca²⁺ signaling by SK2 channels enhanced cell proliferation and presumably contributed to BBB homeostasis. In approximately 25% of ATP-stimulated cells, stable and extensive hyperpolarization to -80 mV was observed. In these hyperpolarized cells, the expression of Kir2.1 channels and rate of apoptosis were high. Net cellular turnover, which results from the balance between cell death and proliferation, may be essential for maintaining healthy BBB functions. **B:** Ca²⁺-gated K⁺ channels play central roles in negative and positive feedback regulation in excitable and non-excitable cells, respectively, and their completely opposite functions are emergent targets for drug discovery. (Color figure can be accessed in the online version.)

while Kv1.3 channels also have obligatory roles in T cell-mediated immune responses.

In the t-BBEC177 cell line, the stimulation of P2Y₁ and P2Y₂ receptors by ATP induced Ca²⁺ release via the formation of IP₃ and also Ca²⁺ influx, presumably via TRPC1 and TRPC2.^[110] This Ca²⁺ signaling activated SK2 channels, and subsequent membrane hyperpolarization enhanced Ca²⁺ entry through TRPC and CRAC channels.^[119] This positive feedback mechanism of Ca²⁺ signaling via SK2 channel activation (Figs. 8A, B) facilitated cell proliferation (Fig. 8A). In addition, some ATP-stimulated cells (approximately 25%) elicited stable and extensive hyperpolarization close to the K⁺ equi-

librium potential (approximately -80 mV). Inward rectifier K⁺ (Kir2.1) channels were highly expressed in these hyperpolarized cells.^[120] Furthermore, apoptotic cell death was frequently observed in these cells. ER stress induced by the application of tunicamycin also resulted in the overexpression of Kir2.1 and apoptosis.^[121] The positive feedback of Ca²⁺ signaling by SK2 was required for the overexpression of Kir2.1. In contrast, milder stress by hypoxia also increased the expression of Kir2.1, but facilitated cell proliferation.^[122] The up-regulated expression of dynamin 2 was shown to be responsible for the enhanced trafficking of Kir2.1 to the PM^[123] (Fig. 8A). Net cellular turnover, which results from the balance between

cell death and proliferation, may be essential for maintaining healthy blood–brain barrier (BBB) functions. The contribution of SK2 channels to the positive feedback regulation of Ca^{2+} signaling may have a physiological impact on the homeostasis of the BBB and CNS.

Therefore, Ca^{2+} -gated K^+ (BK, IK, and SK) channels play central roles in the positive and negative feedback regulation of Ca^{2+} signaling in non-excitable and excitable cells,¹²⁴⁾ respectively (Fig. 8B), under physiological and pathophysiological conditions and, thus, are potential targets of drug discovery.^{124,125)}

4. CA^{2+} -GATED ION CHANNELS AS DRUGGABLE TARGETS

4-1. BK Channel-Related Diseases and Modulator Pharmacology The BK (Maxi-K, slo1, $\text{K}_{\text{Ca}}1.1$) channel is encoded by a single gene (KCNMA1), but is widely expressed across excitable and non-excitable tissues,¹²⁶⁾ and is the most highly expressed in the brain^{127,128)} and muscle, particularly smooth muscle.^{5,129)} BK channelopathy due to gain- and loss-of-function mutations has been identified in 16 mutations, which mainly result in neurological symptoms, such as epilepsy, ataxia, mental retardation, and chronic pain.¹³⁰⁾ A large number of splice variants of α -subunits,^{61,126,131)} variants of $\beta 1-\beta 4$,¹³²⁾ and newly identified γ -subunits¹³³⁾ have contributed to the diversity of the physiological properties of BK channels.^{134,135)}

The BK channel is a factor in the pathogenesis of CNS diseases, including epilepsy.¹³⁶⁾ However, in contrast to several Kv channels, the up-regulation of BK channel activity may be involved not only in decreases, but also in increases in CNS excitability. Therefore, it currently remains unclear whether BK channel modulators may be developed for epilepsy and ataxia therapy.¹²⁸⁾ BK channel openers were shown to exert protective effects against acute ischemic stroke by preventing Ca^{2+} overload and subsequent neuronal cell death in the CNS after ischemic stroke in model animals¹³⁷⁾; however, promising openers were not sufficiently effective in clinical trials. Nevertheless, the therapeutic potential of modulators of BK channels, as well as IK and SK channels, in nervous system disorders has been emphasized.¹²⁴⁾

In contrast, the BK channel has consistently been an attractive drug target for the treatment of diseases due to smooth muscle hypercontractility, including hypertension, overactive bladder, incontinence, asthma, chronic obstructive pulmonary disease, and threatened premature labor.^{138–140)} Similar to ATP-sensitive K^+ channel openers, such as nicorandil,¹⁴¹⁾ minoxidil,¹⁴²⁾ (levo)cromakalim^{143,144)} KRN2391,¹⁴⁵⁾ and KRN4884,¹⁴⁶⁾ the development of BK channel openers targeting these diseases has been extensively challenged, but not yet successful in clinical trials.^{138,147)} The main undesired effects of BK channel openers are facial flushing, migraine, and postural hypotension.¹⁴⁷⁾ Therefore, the BK channel has potential as a target for migraine therapy.¹⁴⁸⁾

Several chemical compounds that exhibit separate bioactivities have been reported as BK channel openers and include nordihydroguaiaretic acid,^{149,150)} tamoxifen, and DiBAC₄(3).¹⁵¹⁾ DiBAC₄(3) and related voltage-sensitive oxonol dyes are specific openers of BK $\alpha\beta 1$ and BK $\alpha\beta 2$, but are not effective for BK α alone or BK $\alpha\beta 2$. Since the tissue distribution of the BK channel mainly depends on the specific expression of the

β -subunits, $\beta 1-\beta 4$,¹³⁵⁾ selectivity to β -subunits is a promising strategy for drug development.

BK channels are not expressed in the PM of the myocardium, while a splice variant of the BK α subunit is functionally expressed in the inner mitochondrial membrane (BK_{mito}).^{131,152)} Among mitochondrial K^+ channels, which are emergent targets of drug discovery,¹⁵³⁾ the protective effects of ATP-sensitive K^+ channels have been well established.¹⁵⁴⁾ The opening of K^+ channels in mitochondria reduces the transmembrane potential and subsequent Ca^{2+} uptake and overload. This mimics an ischemic preconditioning effect and protects the myocardium from injury.¹⁵⁵⁾ Similarly, BK_{mito} channel opening by 17 β -estradiol¹⁵⁶⁾ or pimaric acid (see below) exerts cardioprotective effects.¹⁵⁷⁾ The BK_{mito} channel is a hot target for therapy for cardiac ischemic injury.¹⁵²⁾ 17 β -Estradiol may directly enhance BK channel activity, while BK channel expression is regulated by androgenic hormones in sexual organs¹⁵⁸⁾ and the amygdala.¹²⁷⁾ Various ion channels are expressed in the mitochondrial inner layer and K^+ channels are promising drug targets.¹⁵³⁾

Although none of the BK channel openers assessed to date are currently on the market, endogenous ω -3 polyunsaturated fatty acids, including docosahexaenoic acid and eicosapentaenoic acid, induce the opening of BK channels.¹⁵⁹⁾ Anandamide, an endogenous agonist of cannabinoid (CB) receptors, also opens BK channels,¹⁶⁰⁾ which is not mediated by CB1 or CB2 receptor activation. The modulation of BK channels by lipids,¹⁶¹⁾ cholesterol,¹⁶²⁾ and alcohol¹⁶³⁾ are emergent topics for both health and disease. Some chemical components of natural products, such as dehydrosoyasaponin-I, quercetin magnolol, and maxikdiol, have been shown to induce the opening of BK channels.^{139,147)} Pimaric acid and pimarane compounds from pine resin and seeds have also been identified as potent BK channel openers.^{164,165)} Pimaric acid activates the single channel activity of BK_{mito} channels and exerts protective effects against ischemic cardiac injury.¹⁵⁷⁾ It also potentiates some types of Kv channels^{166,167)} and the TMEM16A channel,¹⁶⁸⁾ presumably through its effects on the voltage dependence of voltage sensors. A previous study reported that pimarane compounds improved special learning in model mice of Alzheimer's disease.¹⁶⁹⁾ However, it has been pointed out that seed compounds for BK channel openers with druggable chemical structures are still poor.¹³⁹⁾

4-2. IK and SK Channel-Related Diseases and Modulator Pharmacology Sickle cell anemia, which is due to a heterozygous gain-of-function mutation in the IK channel (SK4/KCa3.1/KCNN4) in red blood cell membranes, is a well-known channelopathy.¹⁷⁰⁾ Senicapoc, a selective IK channel blocker, was developed as therapy for Sickle cell disease, but is not on market because of insufficient efficacy in patients.¹²⁵⁾ The IK channel contributes to volume regulation by K^+ efflux in red blood cells and also in immune cells, such as B cells, T cells, macrophages, mast cells, and microglia. IK channels play a central role in the positive feedback regulation of Ca^{2+} signaling and subsequent responses in immune cells,¹¹⁷⁾ while voltage-gated Kv1.3 channels are also essential for the activation of immune responses.¹⁷¹⁾ A novel dominant-negative spliced variant of human IK channels, which lacks the N-terminal domain by an alternative splicing event, was shown to be significantly expressed in human lymphoid tissues.¹⁷²⁾ IK channel activity may be modulated by this alternative

splicing event under some inflammatory conditions. Among non-excitable cells other than immune cells, the expression of IK channels has been reported in vascular endothelial cells, osteoblasts,¹⁷³⁾ and cancer cells.¹⁶⁵⁾ IK channel expression has been widely detected in excitable cells, including CNS neurons,¹²⁴⁾ and some types of SMCs.¹⁷⁴⁾

Multiple inflammatory diseases have been targeted by IK channel modulators in animal model diseases, including asthma, atherosclerosis, ischemic stroke, and renal and cardiac fibrosis.¹⁷⁵⁾ Moreover, senicapoc has been provided for investigator-initiated repurposing clinical trials in the US, which include Alzheimer's disease and stroke therapy by the targeting of microglial IK channels.¹⁷⁶⁾

In a mouse model of inflammatory bowel disease (IBD), IK channels were shown to play a role in the enlargement of mesenteric lymph nodes.¹⁷⁷⁾ Pharmacological IK channel blockade significantly attenuated the severity of IBD and reduced increases in the expression of IK channels and the production of Th1 cytokines in CD4⁺ T-lymphocytes.¹⁷⁷⁾ IK channel activity has been shown to regulate inflammatory cytokine production in CD44⁺ T cells *via* epigenetic modifications by histone deacetylases (HDAC2 and HDAC3).¹⁷⁸⁾ A treatment with an IK channel blocker was found to be beneficial in the recovery phase.¹⁷⁹⁾ Although the pharmacological blockade of KCa3.1 may reduce the risk of developing IBD, the possibility of a double-edged sword has also been suggested.¹⁸⁰⁾

IK channels are engaged in the pathogenesis of delayed-type hypersensitivity (DTH) in the auricular lymph node CD44⁺ T lymphocytes of oxazolone-induced DTH model mice.¹⁸¹⁾ The up-regulation of IK channels participates in CD44⁺ T-lymphocyte proliferation in the nodes of DTH model mice. These changes were suppressed by a treatment with an IK channel blocker. Therefore, IK channels have potential as a target for therapeutic interventions for allergy diseases, such as DTH.

An experimental stromal hyperplasia animal model of the rat by the implantation of a urogenital sinus corresponds to clinical benign prostatic hyperplasia (BPH).¹⁸²⁾ IK channels were highly expressed in human BPH samples. Furthermore, an *in vivo* treatment with TRAM-34, another selective IK channel blocker, significantly suppressed increases in implanted urogenital sinus weights. Therefore, IK channel blockers may be a novel therapeutic option for BPH.

In recent years, the IK channel is often referred to as "on-cochannel" or "cancer-associated channel" because its overexpression has been detected in solid tumors,¹⁸³⁾ such as breast cancer, lung adenocarcinoma, oral squamous cell carcinoma, hepatocellular carcinoma, glioblastoma, colorectal cancer, and prostate cancer.¹⁸⁴⁾ The up-regulated expression of the IK channel is not only a marker of cancer progression stages, but is also a therapeutic target because Ca²⁺ signaling in tumor cells is expected to promote proliferation, migration, and invasion. Since the pharmacological blockade of IK channels is often effective in animal models and *in vitro* experiments, this channel is an attractive target for cancer therapy.¹⁷¹⁾ On the other hand, cancer immunity may be modulated by IK channel modulators, suggesting difficulties with assessments of drug efficacy.

SK channels are closely involved in the regulation of action potential firing frequencies in neurons, particularly in the CNS. They are also functionally expressed in the cardiovascular system, and involved in endothelium-derived hyperpolar-

ization in vasculature and, in part, the repolarization of cardiac action potentials.¹⁸⁵⁾ The peptide toxin from bee venom, apamin, is a highly selective blocker of SK (SK1, SK2, and SK3) channels. In addition to senicapoc and TRAM-34, new blockers of IK channels are highly selective against SK channels.¹²⁵⁾ SK channel-selective blockers have also been developed; however, the mechanisms underlying the selectivity among SK channels have not yet been elucidated. The prototype opener, 1-ethylbenzimidazolinone, exhibited low selectivity to IK and SK channels and exerted blocking effects on VGCC.¹⁸⁶⁾ More selective openers of the IK channels, SKA-111 and SKA-121 have recently been developed.¹²⁵⁾ To date, no modulators of IK or SK channels are on the market; however, drug development targeting on IK and SK channels modulators remains a hot issue.

4-3. TMEM16A-Related Diseases and Modulator Pharmacology Among the TMEM16 (anoctamin protein, ANO) family, the main molecular entity of Ca²⁺-gated Cl⁻ channels is TMEM16A, while TMEM16B and F may also be responsible for channel activity, at least in a few types of cells.¹⁸⁷⁾ The functional expression and pathophysiological significance of TMEM16A, known as ANO1, have been described in detail in ICCs and implicated in gastrointestinal motility disorders.⁷⁾ TMEM16A plays important roles in several types of SMCs to maintain tonus, particularly in vascular SMCs, presumably *via* STIC activities. Pharmacological analyses revealed that the application of TMEM16A blockers reduced the tone of isolated vascular preparations and, correspondingly, systemic blood pressure in spontaneously hypertensive rats, indicating the potential of antihypertensive therapy using TMEM16A blockers.^{188,189)}

Since the TMEM16A channel is highly expressed in the epithelium, it is regarded as an emergent therapeutic target for epithelium-originating diseases, such as asthma, diarrhea,¹⁹⁰⁾ and cystic fibrosis.¹⁹¹⁾ TMEM16A and other types of Cl⁻ channels also play key roles in the regulation of resting membrane potential in epithelium-derived t-BBEC177¹⁹²⁾ and its cellular functions.^{193,194)} In a chondrocyte-like cell line as a non-excitable cell, TMEM16A and other types of Cl⁻ channels^{193,195,196)} are essential for the regulation of resting membrane potential and cellular functions. In addition, the overexpression of TMEM16A has been found in various cancers and may have potential as an emergent target of drug discovery.^{197,198)}

Traditional Cl⁻ channel blockers, including niflumic acid and anthracene-9-carboxylic acid, exhibit low selectivity to TMEM16A channels. The BK channel activators NS1619 and isopimaric acid augmented TMEM16A channels as well,¹⁶⁸⁾ indicating the overlap of channel opening effects.¹⁹⁹⁾ A new generation of TMEM16A blockers, CACCinhA01, T16Ainh-A01, and TMinh-23, are sufficiently selective for use as pharmacological tools to investigate the contribution of TMEM16A to physiological functions.^{200,201)} Drug development targeting on activators and blockers of the TMEM16A channel represents another hot issue.²⁰²⁾

5. CONCLUSIONS AND PROSPECTS

The physiological impact of the conversion of Ca²⁺ signals to electrical activity by functional interactions with Ca²⁺-gated ion channels in the PM needs to be emphasized from two aspects. (1) The conversion from Ca²⁺ sparks to STOCs

in SMCs changes the resting membrane potential and, thus, enforces a positive or negative feedback mechanism by enhancing or suppressing, respectively, the further influx of Ca^{2+} in reciprocal relationships. Positive or negative feedback is dependent on the combination of Ca^{2+} -gated ion channels and Ca^{2+} entry pathways. The entry pathway, in turn, depend on excitable or non-excitable cells, in opposite ways. (2) Conversion may initiate pacemaking electrical signals as the Ca^{2+} clock, which propagates from cell to cell for tissue synchronization. Another step in the reciprocal relationship between Ca^{2+} signaling and Ca^{2+} -gated channels is the Ca^{2+} -dependent regulation of Ca^{2+} -gated ion channel expression. These reciprocal relationships are an emergent issue and include promising targets for drug discovery, such as Ca^{2+} entry pathways. The concept of the Ca^{2+} clock has been and will be more extensively incorporated into the long-term periodical regulation system for circadian rhythms, including circadian expression changes in Ca^{2+} -permeable channels and Ca^{2+} -gated ion channels.^{203,204} The reciprocal relationship between Ca^{2+} signaling and Ca^{2+} -gated ion channels is a hot issue in cancer research because these channels are often overexpressed in tumor cells. Therefore, the reciprocal interaction of Ca^{2+} signaling with Ca^{2+} -gated ion channels is a promising target in drug discovery, including microRNAs for the expression control of related ion channels.^{88,205}

Acknowledgments The author expresses sincere gratitude to his supervisors: the late Dr. Minoru Watanabe Emeritus Professor Nagoya City University, the late Dr. Yutaka Kasuya Emeritus Professor The University of Tokyo, Dr. Hideomi Fukuda Emeritus Professor The University of Tokyo, Dr. Tadao Tomita Emeritus Professor Nagoya University, and Dr. Wayne Giles Emeritus Professor University of Calgary Canada. The author deeply thanks all the staff and collaborators in the research group at the Department of Molecular and Cellular Pharmacology, Nagoya City University. Collaborators outside the research group in the department, who took part in the studies described in this review are also appreciated. Studies driven by the author were mainly supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS).

Conflict of Interest The author declares no conflict of interest.

REFERENCES

- 1) Berridge MJ. The inositol trisphosphate/calcium signaling pathway in health and disease. *Physiol. Rev.*, **96**, 1261–1296 (2016).
- 2) Iino M. Spatiotemporal dynamics of Ca^{2+} signaling and its physiological roles. *Proc. Jpn. Acad., Ser. B, Phys. Biol. Sci.*, **86**, 244–256 (2010).
- 3) Alexander SPH, Mathie A, Peters JA, Veale EL, Striessnig J, Kelly E, Armstrong JF, Faccenda E, Harding SD, Pawson AJ, Sharman JL, Southan C, Davies JA. The concise guide to pharmacology 2019/20: ion channels. *Br. J. Pharmacol.*, **176** (Suppl. 1), S142–S228 (2019).
- 4) Noda S, Suzuki Y, Yamamura H, Giles WR, Imaizumi Y. Roles of LRRK26 as an auxiliary $\gamma 1$ -subunit of large-conductance $\text{Ca}(2+)$ -activated $\text{K}(+)$ channels in bronchial smooth muscle cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **318**, L366–L375 (2020).
- 5) Latorre R, Castillo K, Carrasquel-Ursulaez W, Sepulveda RV, Gonzalez-Nilo F, Gonzalez C, Alvarez O. Molecular determinants of BK channel functional diversity and functioning. *Physiol. Rev.*, **97**, 39–87 (2017).
- 6) Noda S, Chikazawa K, Suzuki Y, Imaizumi Y, Yamamura H. Involvement of the $\gamma 1$ subunit of the large-conductance $\text{Ca}(2+)$ -activated $\text{K}(+)$ channel in the proliferation of human somatostatinoma cells. *Biochem. Biophys. Res. Commun.*, **525**, 1032–1037 (2020).
- 7) Hawn MB, Akin E, Hartzell HC, Greenwood IA, Leblanc N. Molecular mechanisms of activation and regulation of ANO1-encoded Ca^{2+} -activated Cl^- channels. *Channels (Austin)*, (2021).
- 8) Berkefeld H, Fakler B, Schulte U. Ca^{2+} -activated K^+ channels: from protein complexes to function. *Physiol. Rev.*, **90**, 1437–1459 (2010).
- 9) Ohya S, Morohashi Y, Muraki K, Tomita T, Watanabe M, Iwatsubo T, Imaizumi Y. Molecular cloning and expression of the novel splice variants of $\text{K}(+)$ channel-interacting protein 2. *Biochem. Biophys. Res. Commun.*, **282**, 96–102 (2001).
- 10) Morohashi Y, Hatano N, Ohya S, Takikawa R, Watabiki T, Takasugi N, Imaizumi Y, Tomita T, Iwatsubo T. Molecular cloning and characterization of CALP/KChIP4, a novel EF-hand protein interacting with presenilin 2 and voltage-gated potassium channel subunit Kv4. *J. Biol. Chem.*, **277**, 14965–14975 (2002).
- 11) Bähring R. Kv channel-interacting proteins as neuronal and non-neuronal calcium sensors. *Channels (Austin)*, **12**, 187–200 (2018).
- 12) Sergeant GP, Ohya S, Reihill JA, Perrino BA, Amberg GC, Imaizumi Y, Horowitz B, Sanders KM, Koh SD. Regulation of Kv4.3 currents by Ca^{2+} /calmodulin-dependent protein kinase II. *Am. J. Physiol. Cell Physiol.*, **288**, C304–C313 (2005).
- 13) Cheng H, Lederer WJ, Cannell MB. Calcium sparks: elementary events underlying excitation-contraction coupling in heart muscle. *Science*, **262**, 740–744 (1993).
- 14) Schneider MF, Klein MG. Sarcomeric calcium sparks activated by fiber depolarization and by cytosolic Ca^{2+} in skeletal muscle. *Cell Calcium*, **20**, 123–128 (1996).
- 15) Brochet DX, Yang D, Cheng H, Lederer WJ. Elementary calcium release events from the sarcoplasmic reticulum in the heart. *Adv. Exp. Med. Biol.*, **740**, 499–509 (2012).
- 16) Benham CD, Bolton TB. Spontaneous transient outward currents in single visceral and vascular smooth muscle cells of the rabbit. *J. Physiol.*, **381**, 385–406 (1986).
- 17) Bolton TB, Imaizumi Y. Spontaneous transient outward currents in smooth muscle cells. *Cell Calcium*, **20**, 141–152 (1996).
- 18) Nelson MT, Cheng H, Rubart M, Santana LF, Bonev AD, Knot HJ, Lederer WJ. Relaxation of arterial smooth muscle by calcium sparks. *Science*, **270**, 633–637 (1995).
- 19) Ohi Y, Yamamura H, Nagano N, Ohya S, Muraki K, Watanabe M, Imaizumi Y. Local $\text{Ca}(2+)$ transients and distribution of BK channels and ryanodine receptors in smooth muscle cells of guinea-pig vas deferens and urinary bladder. *J. Physiol.*, **534**, 313–326 (2001).
- 20) Suzuki M, Muraki K, Imaizumi Y, Watanabe M. Cyclopiazonic acid, an inhibitor of the sarcoplasmic reticulum $\text{Ca}(2+)$ -pump, reduces $\text{Ca}(2+)$ -dependent K^+ currents in guinea-pig smooth muscle cells. *Br. J. Pharmacol.*, **107**, 134–140 (1992).
- 21) Uyama Y, Imaizumi Y, Watanabe M. Effects of cyclopiazonic acid, a novel $\text{Ca}(2+)$ -ATPase inhibitor, on contractile responses in skinned ileal smooth muscle. *Br. J. Pharmacol.*, **106**, 208–214 (1992).
- 22) Imaizumi Y, Ohi Y, Yamamura H, Ohya S, Muraki K, Watanabe M. Ca^{2+} spark as a regulator of ion channel activity. *Jpn. J. Pharmacol.*, **80**, 1–8 (1999).
- 23) Jaggar JH, Porter VA, Lederer WJ, Nelson MT. Calcium sparks in smooth muscle. *Am. J. Physiol. Cell Physiol.*, **278**, C235–C256 (2000).
- 24) Brenner R, Perez GJ, Bonev AD, Eckman DM, Kosek JC, Wiler SW, Patterson AJ, Nelson MT, Aldrich RW. Vasoregulation by the

- beta1 subunit of the calcium-activated potassium channel. *Nature*, **407**, 870–876 (2000).
- 25) Meredith AL, Thorneloe KS, Werner ME, Nelson MT, Aldrich RW. Overactive bladder and incontinence in the absence of the BK large conductance Ca^{2+} -activated K^+ channel. *J. Biol. Chem.*, **279**, 36746–36752 (2004).
- 26) Werner ME, Knorn AM, Meredith AL, Aldrich RW, Nelson MT. Frequency encoding of cholinergic- and purinergic-mediated signaling to mouse urinary bladder smooth muscle: modulation by BK channels. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **292**, R616–R624 (2007).
- 27) Essin K, Gollasch M. Role of ryanodine receptor subtypes in initiation and formation of calcium sparks in arterial smooth muscle: comparison with striated muscle. *J. Biomed. Biotechnol.*, **2009**, 135249 (2009).
- 28) Hotta S, Morimura K, Ohya S, Muraki K, Takeshima H, Imaizumi Y. Ryanodine receptor type 2 deficiency changes excitation-contraction coupling and membrane potential in urinary bladder smooth muscle. *J. Physiol.*, **582**, 489–506 (2007).
- 29) Fan G, Cui Y, Gollasch M, Kassmann M. Elementary calcium signaling in arterial smooth muscle. *Channels (Austin)*, **13**, 505–519 (2019).
- 30) Imaizumi Y, Watanabe M. Effect of procaine on potassium permeability of canine tracheal smooth muscle. *Pflugers Arch.*, **394**, 144–149 (1982).
- 31) Imaizumi Y, Watanabe M. The effect of tetraethylammonium chloride on potassium permeability in the smooth muscle cell membrane of canine trachea. *J. Physiol.*, **316**, 33–46 (1981).
- 32) Imaizumi Y, Watanabe M. Effect of 4-aminopyridine on potassium permeability of canine tracheal smooth muscle cell membrane. *Jpn. J. Pharmacol.*, **33**, 201–208 (1983).
- 33) Imaizumi Y, Banno H, Watanabe M. Anomalous stabilizing action of Ca on sphincter smooth muscle of the rat iris. *Pflugers Arch.*, **400**, 332–334 (1984).
- 34) Muraki K, Imaizumi Y, Kojima T, Kawai T, Watanabe M. Effects of tetraethylammonium and 4-aminopyridine on outward currents and excitability in canine tracheal smooth muscle cells. *Br. J. Pharmacol.*, **100**, 507–515 (1990).
- 35) Watanabe M, Imaizumi Y, Muraki K, Takeda M. A comparative study about voltage-dependent Ca currents in smooth muscle cells isolated from several tissues. *Adv. Exp. Med. Biol.*, **255**, 119–128 (1989).
- 36) Imaizumi Y, Torii Y, Ohi Y, Nagano N, Atsuki K, Yamamura H, Muraki K, Watanabe M, Bolton TB. Ca^{2+} images and K^+ current during depolarization in smooth muscle cells of the guinea-pig vas deferens and urinary bladder. *J. Physiol.*, **510**, 705–719 (1998).
- 37) Morimura K, Ohi Y, Yamamura H, Ohya S, Muraki K, Imaizumi Y. Two-step Ca^{2+} intracellular release underlies excitation-contraction coupling in mouse urinary bladder myocytes. *Am. J. Physiol. Cell Physiol.*, **290**, C388–C403 (2006).
- 38) Iino M. Calcium-induced calcium release mechanism in guinea pig taenia caeci. *J. Gen. Physiol.*, **94**, 363–383 (1989).
- 39) Yamamura H, Imaizumi Y. Total internal reflection fluorescence imaging of $\text{Ca}(2+)$ -induced $\text{Ca}(2+)$ release in mouse urinary bladder smooth muscle cells. *Biochem. Biophys. Res. Commun.*, **427**, 54–59 (2012).
- 40) Yamamura H, Kawasaki K, Inagaki S, Suzuki Y, Imaizumi Y. Local $\text{Ca}(2+)$ coupling between mitochondria and sarcoplasmic reticulum following depolarization in guinea pig urinary bladder smooth muscle cells. *Am. J. Physiol. Cell Physiol.*, **314**, C88–C98 (2018).
- 41) Hotta S, Yamamura H, Ohya S, Imaizumi Y. Methyl-beta-cyclodextrin prevents Ca^{2+} -induced Ca^{2+} release in smooth muscle cells of mouse urinary bladder. *J. Pharmacol. Sci.*, **103**, 121–126 (2007).
- 42) Suzuki Y, Yamamura H, Ohya S, Imaizumi Y. Caveolin-1 facilitates the direct coupling between large conductance Ca^{2+} -activated K^+ (BKCa) and Cav1.2 Ca^{2+} channels and their clustering to regulate membrane excitability in vascular myocytes. *J. Biol. Chem.*, **288**, 36750–36761 (2013).
- 43) Tedoldi A, Ludwig P, Fulgenzi G, Takeshima H, Pedarzani P, Stocker M. Calcium-induced calcium release and type 3 ryanodine receptors modulate the slow afterhyperpolarising current, sIAHP, and its potentiation in hippocampal pyramidal neurons. *PLOS ONE*, **15**, e0230465 (2020).
- 44) Löh M, Jessner W, Furstenau M, Wellner M, Sorrentino V, Haller H, Luft FC, Gollasch M. Regulation of calcium sparks and spontaneous transient outward currents by RyR3 in arterial vascular smooth muscle cells. *Circ. Res.*, **89**, 1051–1057 (2001).
- 45) Matsuki K, Takemoto M, Suzuki Y, Yamamura H, Ohya S, Takeshima H, Imaizumi Y. Ryanodine receptor type 3 does not contribute to contractions in the mouse myometrium regardless of pregnancy. *Pflugers Arch.*, **469**, 313–326 (2017).
- 46) Dabertrand F, Fritz N, Mironneau J, Macrez N, Morel JL. Role of RYR3 splice variants in calcium signaling in mouse nonpregnant and pregnant myometrium. *Am. J. Physiol. Cell Physiol.*, **293**, C848–C854 (2007).
- 47) Dabertrand F, Mironneau J, Macrez N, Morel JL. Full length ryanodine receptor subtype 3 encodes spontaneous calcium oscillations in native duodenal smooth muscle cells. *Cell Calcium*, **44**, 180–189 (2008).
- 48) Jiang D, Xiao B, Li X, Chen SR. Smooth muscle tissues express a major dominant negative splice variant of the type 3 Ca^{2+} release channel (ryanodine receptor). *J. Biol. Chem.*, **278**, 4763–4769 (2003).
- 49) Matsuki K, Kato D, Takemoto M, Suzuki Y, Yamamura H, Ohya S, Takeshima H, Imaizumi Y. Negative regulation of cellular $\text{Ca}(2+)$ mobilization by ryanodine receptor type 3 in mouse mesenteric artery smooth muscle. *Am. J. Physiol. Cell Physiol.*, **315**, C1–C9 (2018).
- 50) Balschun D, Wolfer DP, Bertocchini F, Barone V, Conti A, Zuschratter W, Missiaen L, Lipp HP, Frey JU, Sorrentino V. Deletion of the ryanodine receptor type 3 (RyR3) impairs forms of synaptic plasticity and spatial learning. *EMBO J.*, **18**, 5264–5273 (1999).
- 51) Matsuo N, Tanda K, Nakanishi K, Yamasaki N, Toyama K, Takao K, Takeshima H, Miyakawa T. Comprehensive behavioral phenotyping of ryanodine receptor type 3 (RyR3) knockout mice: decreased social contact duration in two social interaction tests. *Front. Behav. Neurosci.*, **3**, 3 (2009).
- 52) Eckhardt J, Bachmann C, Sekulic-Jablanovic M, Enzmann V, Park KH, Ma J, Takeshima H, Zorzato F, Treves S. Extraocular muscle function is impaired in ryr3 (−/−) mice. *J. Gen. Physiol.*, **151**, 929–943 (2019).
- 53) Fish KN. Total internal reflection fluorescence (TIRF) microscopy. *Curr. Protoc. Cytom.*, **Chapter 12**, Unit12 18 (2009).
- 54) Yamamura H, Ikeda C, Suzuki Y, Ohya S, Imaizumi Y. Molecular assembly and dynamics of fluorescent protein-tagged single $\text{K}_{\text{Ca}}1.1$ channel in expression system and vascular smooth muscle cells. *Am. J. Physiol. Cell Physiol.*, **302**, C1257–C1268 (2012).
- 55) Ulbrich MH, Isacoff EY. Subunit counting in membrane-bound proteins. *Nat. Methods*, **4**, 319–321 (2007).
- 56) Noda S, Suzuki Y, Yamamura H, Imaizumi Y. Single molecule fluorescence imaging reveals the stoichiometry of BK γ 1 subunit in living HEK293 cell expression system. *Biol. Pharm. Bull.*, **43**, 1118–1122 (2020).
- 57) Miyawaki A, Tsien RY. Monitoring protein conformations and interactions by fluorescence resonance energy transfer between mutants of green fluorescent protein. *Methods Enzymol.*, **327**, 472–500 (2000).
- 58) Suzuki Y, Tsutsumi K, Miyamoto T, Yamamura H, Imaizumi Y. Heterodimerization of two pore domain K^+ channel TASK1 and TALK2 in living heterologous expression systems. *PLOS ONE*, **12**, e0186252 (2017).

- 59) Hu CD, Chinenov Y, Kerppola TK. Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. *Mol. Cell.*, **9**, 789–798 (2002).
- 60) Inayama M, Suzuki Y, Yamada S, Kurita T, Yamamura H, Ohya S, Giles WR, Imaizumi Y. Orai1–Orai2 complex is involved in store-operated calcium entry in chondrocyte cell lines. *Cell Calcium*, **57**, 337–347 (2015).
- 61) Suzuki Y, Ohya S, Yamamura H, Giles WR, Imaizumi Y. A new splice variant of large conductance Ca^{2+} -activated K^+ (BK) channel α subunit alters human chondrocyte function. *J. Biol. Chem.*, **291**, 24247–24260 (2016).
- 62) Piggott CA, Jin Y. Junctophilins: key membrane tethers in muscles and neurons. *Front. Mol. Neurosci.*, **14**, 709390 (2021).
- 63) Saeki T, Suzuki Y, Yamamura H, Takeshima H, Imaizumi Y. A junctophilin-caveolin interaction enables efficient coupling between ryanodine receptors and BK(Ca) channels in the $\text{Ca}(2+)$ microdomain of vascular smooth muscle. *J. Biol. Chem.*, **294**, 13093–13105 (2019).
- 64) Yamamura H, Suzuki Y, Imaizumi Y. New light on ion channel imaging by total internal reflection fluorescence (TIRF) microscopy. *J. Pharmacol. Sci.*, **128**, 1–7 (2015).
- 65) Takeshima H, Hoshijima M, Song LS. $\text{Ca}(2+)$ microdomains organized by junctophilins. *Cell Calcium*, **58**, 349–356 (2015).
- 66) Pritchard HAT, Griffin CS, Yamasaki E, Thakore P, Lane C, Greenstein AS, Earley S. Nanoscale coupling of junctophilin-2 and ryanodine receptors regulates vascular smooth muscle cell contractility. *Proc. Natl. Acad. Sci. U.S.A.*, **116**, 21874–21881 (2019).
- 67) Avila G, de la Rosa JA, Monsalvo-Villegas A, Montiel-Jaen MG. $\text{Ca}(2+)$ channels mediate bidirectional signaling between sarcolemma and sarcoplasmic reticulum in muscle cells. *Cells*, **9**, 55 (2019).
- 68) Sanders KM, Zhu MH, Britton F, Koh SD, Ward SM. Anoctamins and gastrointestinal smooth muscle excitability. *Exp. Physiol.*, **97**, 200–206 (2012).
- 69) Drumm BT, Hwang SJ, Baker SA, Ward SM, Sanders KM. $\text{Ca}(2+)$ signalling behaviours of intramuscular interstitial cells of Cajal in the murine colon. *J. Physiol.*, **597**, 3587–3617 (2019).
- 70) Hwang SJ, Blair PJ, Britton FC, O'Driscoll KE, Hennig G, Bayguinov YR, Rock JR, Harfe BD, Sanders KM, Ward SM. Expression of anoctamin 1/TMEM16A by interstitial cells of Cajal is fundamental for slow wave activity in gastrointestinal muscles. *J. Physiol.*, **587**, 4887–4904 (2009).
- 71) Sanders KM. Spontaneous electrical activity and rhythmicity in gastrointestinal smooth muscles. *Adv. Exp. Med. Biol.*, **1124**, 3–46 (2019).
- 72) Capel RA, Terrar DA. The importance of $\text{Ca}(2+)$ -dependent mechanisms for the initiation of the heartbeat. *Front. Physiol.*, **6**, 80 (2015).
- 73) Lang D, Glukhov AV. Functional microdomains in heart's pacemaker: a step beyond classical electrophysiology and remodeling. *Front Physiol*, **9**, 1686 (2018).
- 74) Hinata M, Yamamura H, Li L, Watanabe Y, Watano T, Imaizumi Y, Kimura J. Stoichiometry of $\text{Na}^+/\text{Ca}^{2+}$ exchange is 3:1 in guinea-pig ventricular myocytes. *J. Physiol.*, **545**, 453–461 (2002).
- 75) Yamamura H, Cole WC, Kita S, Hotta S, Murata H, Suzuki Y, Ohya S, Iwamoto T, Imaizumi Y. Overactive bladder mediated by accelerated Ca^{2+} influx mode of $\text{Na}^+/\text{Ca}^{2+}$ exchanger in smooth muscle. *Am. J. Physiol. Cell Physiol.*, **305**, C299–C308 (2013).
- 76) Sergeant GP, Hollywood MA, McHale NG, Thornbury KD. Ca^{2+} signalling in urethral interstitial cells of Cajal. *J. Physiol.*, **576**, 715–720 (2006).
- 77) Suzuki H, Takano H, Yamamoto Y, Komuro T, Saito M, Kato K, Mikoshiba K. Properties of gastric smooth muscles obtained from mice which lack inositol trisphosphate receptor. *J. Physiol.*, **525**, 105–111 (2000).
- 78) Drumm BT, Koh SD, Andersson KE, Ward SM. Calcium signal-
ling in Cajal-like interstitial cells of the lower urinary tract. *Nat. Rev. Urol.*, **11**, 555–564 (2014).
- 79) Liu HN, Ohya S, Wang J, Imaizumi Y, Nakayama S. Involvement of ryanodine receptors in pacemaker Ca^{2+} oscillation in murine gastric ICC. *Biochem. Biophys. Res. Commun.*, **328**, 640–646 (2005).
- 80) Aoyama M, Yamada A, Wang J, Ohya S, Furuzono S, Goto T, Hotta S, Ito Y, Matsubara T, Shimokata K, Chen SR, Imaizumi Y, Nakayama S. Requirement of ryanodine receptors for pacemaker Ca^{2+} activity in ICC and HEK293 cells. *J. Cell Sci.*, **117**, 2813–2825 (2004).
- 81) Radu BM, Banciu A, Banciu DD, Radu M, Cretoiu D, Cretoiu SM. Calcium signaling in interstitial cells: focus on telocytes. *Int. J. Mol. Sci.*, **18**, 397 (2017).
- 82) Huizinga JD, Fausson-Pellegrini MS. About the presence of interstitial cells of Cajal outside the musculature of the gastrointestinal tract. *J. Cell. Mol. Med.*, **9**, 468–473 (2005).
- 83) Saeki T, Kimura T, Hashidume K, Murayama T, Yamamura H, Ohya S, Suzuki Y, Nakayama S, Imaizumi Y. Conversion of $\text{Ca}(2+)$ oscillation into propagative electrical signals by $\text{Ca}(2+)$ -activated ion channels and connexin as a reconstituted $\text{Ca}(2+)$ clock model for the pacemaker activity. *Biochem. Biophys. Res. Commun.*, **510**, 242–247 (2019).
- 84) Rottgen TS, Nickerson AJ, Rajendran VM. Calcium-activated $\text{Cl}(-)$ channel: insights on the molecular identity in epithelial tissues. *Int. J. Mol. Sci.*, **19**, 1432 (2018).
- 85) Oh U, Jung J. Cellular functions of TMEM16/anoctamin. *Pflugers Arch.*, **468**, 443–453 (2016).
- 86) Ji Q, Guo S, Wang X, Pang C, Zhan Y, Chen Y, An H. Recent advances in TMEM16A: structure, function, and disease. *J. Cell. Physiol.*, **234**, 7856–7873 (2019).
- 87) Kunzelmann K, Ousingsawat J, Benedetto R, Cabrita I, Schreiber R. Contribution of anoctamins to cell survival and cell death. *Cancers (Basel)*, **11**, 382 (2019).
- 88) Wang H, Zou L, Ma K, Yu J, Wu H, Wei M, Xiao Q. Cell-specific mechanisms of TMEM16A $\text{Ca}(2+)$ -activated chloride channel in cancer. *Mol. Cancer*, **16**, 152 (2017).
- 89) Henmi S, Imaizumi Y, Muraki K, Watanabe M. Time course of $\text{Ca}(2+)$ -dependent K^+ and Cl^- currents in single smooth muscle cells of guinea-pig trachea. *Eur. J. Pharmacol.*, **306**, 227–236 (1996).
- 90) Henmi S, Imaizumi Y, Muraki K, Watanabe M. Characteristics of caffeine-induced and spontaneous inward currents and related intracellular Ca^{2+} storage sites in guinea-pig tracheal smooth muscle cells. *Eur. J. Pharmacol.*, **282**, 219–228 (1995).
- 91) Zhu MH, Sung TS, Kurahashi M, O'Kane LE, O'Driscoll K, Koh SD, Sanders KM. $\text{Na}^+/\text{K}^+/\text{Cl}^-$ cotransporter (NKCC) maintains the chloride gradient to sustain pacemaker activity in interstitial cells of Cajal. *Am. J. Physiol. Gastrointest. Liver Physiol.*, **311**, G1037–G1046 (2016).
- 92) Ohshiro J, Yamamura H, Suzuki Y, Imaizumi Y. Modulation of TMEM16A-channel activity as Ca^{2+} activated Cl^- conductance via the interaction with actin cytoskeleton in murine portal vein. *J. Pharmacol. Sci.*, **125**, 107–111 (2014).
- 93) Bao R, Lifshitz LM, Tuft RA, Bellve K, Fogarty KE, ZhuGe R, ZhuGe R. A close association of RyRs with highly dense clusters of Ca^{2+} -activated Cl^- channels underlies the activation of STICs by Ca^{2+} sparks in mouse airway smooth muscle. *J. Gen. Physiol.*, **132**, 145–160 (2008).
- 94) Leblanc N, Forrest AS, Ayon RJ, Wiwchar M, Angermann JE, Pritchard HA, Singer CA, Valencik ML, Britton F, Greenwood IA. Molecular and functional significance of $\text{Ca}(2+)$ -activated $\text{Cl}(-)$ channels in pulmonary arterial smooth muscle. *Pulm. Circ.*, **5**, 244–268 (2015).
- 95) Ohshiro J, Yamamura H, Saeki T, Suzuki Y, Imaizumi Y. The multiple expression of Ca^{2+} -activated Cl^- channels via homo- and

- hetero-dimer formation of TMEM16A splicing variants in murine portal vein. *Biochem. Biophys. Res. Commun.*, **443**, 518–523 (2014).
- 96) Zhang CH, Wang P, Liu DH, Chen CP, Zhao W, Chen X, Chen C, He WQ, Qiao YN, Tao T, Sun J, Peng YJ, Lu P, Zheng K, Craige SM, Lifshitz LM, Keaney JF Jr, Fogarty KE, ZhuGe R, Zhu MS. The molecular basis of the genesis of basal tone in internal anal sphincter. *Nat. Commun.*, **7**, 11358 (2016).
- 97) Mizutani H, Yamamura H, Muramatsu M, Kiyota K, Nishimura K, Suzuki Y, Ohya S, Imaizumi Y. Spontaneous and nicotine-induced Ca^{2+} oscillations mediated by Ca^{2+} influx in rat pinealocytes. *Am. J. Physiol. Cell Physiol.*, **306**, C1008–C1016 (2014).
- 98) Mizutani H, Yamamura H, Muramatsu M, Hagihara Y, Suzuki Y, Imaizumi Y. Modulation of Ca^{2+} oscillation and melatonin secretion by BKCa channel activity in rat pinealocytes. *Am. J. Physiol. Cell Physiol.*, **310**, C740–C747 (2016).
- 99) Yamamura H, Nishimura K, Hagihara Y, Suzuki Y, Imaizumi Y. TMEM16A and TMEM16B channel proteins generate $\text{Ca}(2+)$ -activated $\text{Cl}(-)$ current and regulate melatonin secretion in rat pineal glands. *J. Biol. Chem.*, **293**, 995–1006 (2018).
- 100) Muraki K, Imaizumi Y, Watanabe M. Sodium currents in smooth muscle cells freshly isolated from stomach fundus of the rat and ureter of the guinea-pig. *J. Physiol.*, **442**, 351–375 (1991).
- 101) Seda M, Pinto FM, Wray S, Cintado CG, Noheda P, Buschmann H, Cadenas L. Functional and molecular characterization of voltage-gated sodium channels in uteri from nonpregnant rats. *Biol. Reprod.*, **77**, 855–863 (2007).
- 102) Imaizumi Y, Muraki K, Takeda M, Watanabe M. Measurement and simulation of noninactivating Ca current in smooth muscle cells. *Am. J. Physiol.*, **256**, C880–C885 (1989).
- 103) Ohya S, Yamamura H, Muraki K, Watanabe M, Imaizumi Y. Comparative study of the molecular and functional expression of L-type Ca^{2+} channels and large-conductance, Ca^{2+} -activated K^+ channels in rabbit aorta and vas deferens smooth muscle. *Pflugers Arch.*, **441**, 611–620 (2001).
- 104) Tajada S, Villalobos C. Calcium permeable channels in cancer hallmarks. *Front. Pharmacol.*, **11**, 968 (2020).
- 105) Nilius B, Owsianik G, Voets T, Peters JA. Transient receptor potential cation channels in disease. *Physiol. Rev.*, **87**, 165–217 (2007).
- 106) Moran MM, McAlexander MA, Biro T, Szallasi A. Transient receptor potential channels as therapeutic targets. *Nat. Rev. Drug Discov.*, **10**, 601–620 (2011).
- 107) Behringher EJ, Hakim MA. Functional interaction among K_{Ca} and TRP channels for cardiovascular physiology: modern perspectives on aging and chronic disease. *Int. J. Mol. Sci.*, **20**, 1380 (2019).
- 108) Feske S. CRAC channels and disease—From human CRAC channelopathies and animal models to novel drugs. *Cell Calcium*, **80**, 112–116 (2019).
- 109) Lewis RS. Store-operated calcium channels: from function to structure and back again. *Cold Spring Harb. Perspect. Biol.*, **12**, a035055 (2020).
- 110) Yamazaki D, Aoyama M, Ohya S, Muraki K, Asai K, Imaizumi Y. Novel functions of small conductance Ca^{2+} -activated K^+ channel in enhanced cell proliferation by ATP in brain endothelial cells. *J. Biol. Chem.*, **281**, 38430–38439 (2006).
- 111) Ohba T, Sawada E, Suzuki Y, Yamamura H, Ohya S, Tsuda H, Imaizumi Y. Enhancement of $\text{Ca}(2+)$ influx and ciliary beating by membrane hyperpolarization due to ATP-sensitive $\text{K}(+)$ channel opening in mouse airway epithelial cells. *J. Pharmacol. Exp. Ther.*, **347**, 145–153 (2013).
- 112) Funabashi K, Ohya S, Yamamura H, Hatano N, Muraki K, Giles W, Imaizumi Y. Accelerated Ca^{2+} entry by membrane hyperpolarization due to Ca^{2+} -activated K^+ channel activation in response to histamine in chondrocytes. *Am. J. Physiol. Cell Physiol.*, **298**, C786–C797 (2010).
- 113) Feske S, Gwack Y, Prakriya M, Srikanth S, Poppel SH, Tanasa B, Hogan PG, Lewis RS, Daly M, Rao A. A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. *Nature*, **441**, 179–185 (2006).
- 114) Gil D, Guse AH, Dupont G. Three-dimensional model of sub-plasma-membrane $\text{Ca}(2+)$ microdomains evoked by the interplay between orai1 and InsP3 receptors. *Front. Immunol.*, **12**, 659790 (2021).
- 115) Guse AH, Gil Montoya DC, Diercks BP. Mechanisms and functions of calcium microdomains produced by ORAI channels, d-myo-inositol 1,4,5-trisphosphate receptors, or ryanodine receptors. *Pharmacol. Ther.*, **223**, 107804 (2021).
- 116) Suzuki Y, Yamamura H, Imaizumi Y, Clark RB, Giles WRK. K^+ and Ca^{2+} channels regulate Ca^{2+} signaling in chondrocytes: an illustrated review. *Cells*, **9**, 1577 (2020).
- 117) Ohya S, Kito H. $\text{Ca}(2+)$ -activated $\text{K}(+)$ channel $\text{K}_{\text{Ca}}3.1$ as a therapeutic target for immune disorders. *Biol. Pharm. Bull.*, **41**, 1158–1163 (2018).
- 118) Vaeth M, Kahlfuss S, Feske S. CRAC channels and calcium signaling in T cell-mediated immunity. *Trends Immunol.*, **41**, 878–901 (2020).
- 119) Yamazaki D, Ohya S, Asai K, Imaizumi Y. Characteristics of the ATP-induced Ca^{2+} -entry pathway in the t-BBEC 117 cell line derived from bovine brain endothelial cells. *J. Pharmacol. Sci.*, **104**, 103–107 (2007).
- 120) Yamazaki D, Kito H, Yamamoto S, Ohya S, Yamamura H, Asai K, Imaizumi Y. Contribution of $\text{K}(\text{ir})2$ potassium channels to ATP-induced cell death in brain capillary endothelial cells and reconstructed HEK293 cell model. *Am. J. Physiol. Cell Physiol.*, **300**, C75–C86 (2011).
- 121) Kito H, Yamazaki D, Ohya S, Yamamura H, Asai K, Imaizumi Y. Up-regulation of $\text{K}(\text{ir})2.1$ by ER stress facilitates cell death of brain capillary endothelial cells. *Biochem. Biophys. Res. Commun.*, **411**, 293–298 (2011).
- 122) Yamamura H, Suzuki Y, Yamamura H, Asai K, Imaizumi Y. Hypoxic stress up-regulates Kir2.1 expression and facilitates cell proliferation in brain capillary endothelial cells. *Biochem. Biophys. Res. Commun.*, **476**, 386–392 (2016).
- 123) Yamamura H, Suzuki Y, Yamamura H, Asai K, Giles W, Imaizumi Y. Hypoxic stress upregulates $\text{K}(\text{ir})2.1$ expression by a pathway including hypoxic-inducible factor-1 α and dynamin2 in brain capillary endothelial cells. *Am. J. Physiol. Cell Physiol.*, **315**, C202–C213 (2018).
- 124) Kshatri AS, Gonzalez-Hernandez A, Giraldez T. Physiological roles and therapeutic potential of $\text{Ca}(2+)$ activated potassium channels in the nervous system. *Front. Mol. Neurosci.*, **11**, 258 (2018).
- 125) Brown BM, Shim H, Christoffersen P, Wulff H. Pharmacology of small- and intermediate-conductance calcium-activated potassium channels. *Annu. Rev. Pharmacol. Toxicol.*, **60**, 219–240 (2020).
- 126) Toro L, Li M, Zhang Z, Singh H, Wu Y, Stefani E. MaxiK channel and cell signalling. *Pflugers Arch.*, **466**, 875–886 (2014).
- 127) Ohno A, Ohya S, Yamamura H, Imaizumi Y. Gender difference in BK channel expression in amygdala complex of rat brain. *Biochem. Biophys. Res. Commun.*, **378**, 867–871 (2009).
- 128) Contet C, Goulding SP, Kuljis DA, Barth AL. BK channels in the central nervous system. *Int. Rev. Neurobiol.*, **128**, 281–342 (2016).
- 129) Dopico AM, Bukiya AN, Jaggar JH. Calcium- and voltage-gated BK channels in vascular smooth muscle. *Pflugers Arch.*, **470**, 1271–1289 (2018).
- 130) Bailey CS, Moldenhauer HJ, Park SM, Keros S, Meredith AL. KCNMA1-linked channelopathy. *J. Gen. Physiol.*, **151**, 1173–1189 (2019).
- 131) Singh H, Lu R, Bopassa JC, Meredith AL, Stefani E, Toro L. MitoBK(Ca) is encoded by the Kenmali gene, and a splicing sequence defines its mitochondrial location. *Proc. Natl. Acad. Sci. U.S.A.*, **110**, 10836–10841 (2013).
- 132) Ohya S, Fujimori T, Kimura T, Yamamura H, Imaizumi Y. Novel spliced variants of large-conductance $\text{Ca}(2+)$ -activated $\text{K}(+)$ -

- channel $\beta 2$ -subunit in human and rodent pancreas. *J. Pharmacol. Sci.*, **114**, 198–205 (2010).
- 133) Yan J, Aldrich RW. LRRC26 auxiliary protein allows BK channel activation at resting voltage without calcium. *Nature*, **466**, 513–516 (2010).
- 134) Dudem S, Sergeant GP, Thornbury KD, Hollywood MA. Calcium-activated K(+) channels (KCa) and therapeutic implications. *Handb. Exp. Pharmacol.*, **267**, 379–416 (2021).
- 135) Gonzalez-Perez V, Lingle CJ. Regulation of BK channels by beta and gamma subunits. *Annu. Rev. Physiol.*, **81**, 113–137 (2019).
- 136) N'Gouemo P. Targeting BK (big potassium) channels in epilepsy. *Expert Opin. Ther. Targets*, **15**, 1283–1295 (2011).
- 137) Gribkoff VK, Starrett JE Jr, Dworetzky SI, et al. Targeting acute ischemic stroke with a calcium-sensitive opener of maxi-K potassium channels. *Nat. Med.*, **7**, 471–477 (2001).
- 138) Bentzen BH, Olesen SP, Rønn LC, Grunnet M. BK channel activators and their therapeutic perspectives. *Front. Physiol.*, **5**, 389 (2014).
- 139) Kaczorowski GJ, Garcia ML. Developing molecular pharmacology of BK channels for therapeutic benefit. *Int. Rev. Neurobiol.*, **128**, 439–475 (2016).
- 140) Malerba M, Radaeli A, Mancuso S, Polosa R. The potential therapeutic role of potassium channel modulators in asthma and chronic obstructive pulmonary disease. *J. Biol. Regul. Homeost. Agents*, **24**, 123–130 (2010).
- 141) Gribble FM, Reimann F. Pharmacological modulation of K(ATP) channels. *Biochem. Soc. Trans.*, **30**, 333–339 (2002).
- 142) Suchonwanit P, Thammarucha S, Leerunyakul K. Minoxidil and its use in hair disorders: a review. *Drug Des. Devel. Ther.*, **13**, 2777–2786 (2019).
- 143) Matsushita Y, Henmi S, Muraki K, Imaizumi Y, Watanabe M. Cromakalim-induced membrane current in guinea-pig tracheal smooth muscle cells. *Eur. J. Pharmacol.*, **389**, 51–58 (2000).
- 144) Murai T, Muraki K, Imaizumi Y, Watanabe M. Levocromakalim causes indirect endothelial hyperpolarization via a myo-endothelial pathway. *Br. J. Pharmacol.*, **128**, 1491–1496 (1999).
- 145) Muraki K, Sasaoka A, Watanabe M, Imaizumi Y. Effects of KRN2391 on ionic currents in rabbit femoral arterial myocytes. *Br. J. Pharmacol.*, **132**, 1154–1160 (2001).
- 146) Muraki K, Sasaoka A, Ohya S, Watanabe M, Imaizumi Y. Effects of KRN4884, a novel K⁺ channel opener, on ionic currents in rabbit femoral arterial myocytes. *J. Pharmacol. Sci.*, **93**, 289–298 (2003).
- 147) Nardi A, Olesen SP. BK channel modulators: a comprehensive overview. *Curr. Med. Chem.*, **15**, 1126–1146 (2008).
- 148) Al-Karagholi MA, Gram C, Nielsen CAW, Ashina M. Targeting BK_{Ca} channels in migraine: rationale and perspectives. *CNS Drugs*, **34**, 325–335 (2020).
- 149) Nagano N, Imaizumi Y, Hirano M, Watanabe M. Opening of Ca(2+)-dependent K⁺ channels by nordihydroguaiaretic acid in porcine coronary arterial smooth muscle cells. *Jpn. J. Pharmacol.*, **70**, 281–284 (1996).
- 150) Yamamura H, Nagano N, Hirano M, Muraki K, Watanabe M, Imaizumi Y. Activation of Ca(2+)-dependent K(+) current by nordihydroguaiaretic acid in porcine coronary arterial smooth muscle cells. *J. Pharmacol. Exp. Ther.*, **291**, 140–146 (1999).
- 151) Morimoto T, Sakamoto K, Sade H, Ohya S, Muraki K, Imaizumi Y. Voltage-sensitive oxonol dyes are novel large-conductance Ca²⁺-activated K⁺ channel activators selective for betal and beta4 but not for beta2 subunits. *Mol. Pharmacol.*, **71**, 1075–1088 (2007).
- 152) Balderas E, Zhang J, Stefani E, Toro L. Mitochondrial BK_{Ca} channel. *Front. Physiol.*, **6**, 104 (2015).
- 153) Wrzosek A, Augustynek B, Zochowska M, Szewczyk A. Mitochondrial potassium channels as druggable targets. *Biomolecules*, **10**, 1200 (2020).
- 154) Wang Y, Haider HK, Ahmad N, Ashraf M. Mechanisms by which K(ATP) channel openers produce acute and delayed cardioprotection. *Vascul. Pharmacol.*, **42**, 253–264 (2005).
- 155) Zhang J, Li M, Zhang Z, Zhu R, Olcese R, Stefani E, Toro L. The mitochondrial BK_{Ca} channel cardiac interactome reveals BK_{Ca} association with the mitochondrial import receptor subunit Tom22, and the adenine nucleotide translocator. *Mitochondrion*, **33**, 84–101 (2017).
- 156) Ohya S, Kuwata Y, Sakamoto K, Muraki K, Imaizumi Y. Cardioprotective effects of estradiol include the activation of large-conductance Ca(2+)-activated K(+) channels in cardiac mitochondria. *Am. J. Physiol. Heart Circ. Physiol.*, **289**, H1635–H1642 (2005).
- 157) Sakamoto K, Ohya S, Muraki K, Imaizumi Y. A novel opener of large-conductance Ca²⁺-activated K⁺ (BK) channel reduces ischemic injury in rat cardiac myocytes by activating mitochondrial K(Ca) channel. *J. Pharmacol. Sci.*, **108**, 135–139 (2008).
- 158) Ohno A, Ohya S, Yamamura H, Imaizumi Y. Regulation of ryanodine receptor-mediated Ca(2+) release in vas deferens smooth muscle cells. *J. Pharmacol. Sci.*, **110**, 78–86 (2009).
- 159) Hoshi T, Wissuwa B, Tian Y, Tajima N, Xu R, Bauer M, Heinemann SH, Hou S. Omega-3 fatty acids lower blood pressure by directly activating large-conductance Ca(2+)-dependent K(+) channels. *Proc. Natl. Acad. Sci. U.S.A.*, **110**, 4816–4821 (2013).
- 160) Sade H, Muraki K, Ohya S, Hatano N, Imaizumi Y. Activation of large-conductance, Ca²⁺-activated K⁺ channels by cannabinoids. *Am. J. Physiol. Cell Physiol.*, **290**, C77–C86 (2006).
- 161) Dopico AM, Bukiya AN. Lipid regulation of BK channel function. *Front Physiol*, **5**, 312 (2014).
- 162) Bukiya AN, Dopico AM. Regulation of BK channel activity by cholesterol and its derivatives. *Adv. Exp. Med. Biol.*, **1115**, 53–75 (2019).
- 163) Dopico AM, Bukiya AN, Kuntamallappanavar G, Liu J. Modulation of BK channels by ethanol. *Int. Rev. Neurobiol.*, **128**, 239–279 (2016).
- 164) Imaizumi Y, Sakamoto K, Yamada A, Hotta A, Ohya S, Muraki K, Uchiyama M, Ohwada T. Molecular basis of pimarane compounds as novel activators of large-conductance Ca(2+)-activated K(+) channel alpha-subunit. *Mol. Pharmacol.*, **62**, 836–846 (2002).
- 165) Ohwada T, Nonomura T, Maki K, Sakamoto K, Ohya S, Muraki K, Imaizumi Y. Dehydroabietic acid derivatives as a novel scaffold for large-conductance calcium-activated K⁺ channel openers. *Bioorg. Med. Chem. Lett.*, **13**, 3971–3974 (2003).
- 166) Sakamoto K, Suzuki Y, Yamamura H, Ohya S, Muraki K, Imaizumi Y. Molecular mechanisms underlying pimaric acid-induced modulation of voltage-gated K(+) channels. *J. Pharmacol. Sci.*, **133**, 223–231 (2017).
- 167) Ottosson NE, Liin SI, Elinder F. Drug-induced ion channel opening tuned by the voltage sensor charge profile. *J. Gen. Physiol.*, **143**, 173–182 (2014).
- 168) Saleh SN, Angermann JE, Sones WR, Leblanc N, Greenwood IA. Stimulation of Ca²⁺-gated Cl⁻ currents by the calcium-dependent K⁺ channel modulators NSI1619 [1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzo[1,3]diazol-2-one] and isopimaric acid. *J. Pharmacol. Exp. Ther.*, **321**, 1075–1084 (2007).
- 169) Wang F, Zhang Y, Wang L, Sun P, Luo X, Ishigaki Y, Sugai T, Yamamoto R, Kato N. Improvement of spatial learning by facilitating large-conductance calcium-activated potassium channel with transcranial magnetic stimulation in Alzheimer's disease model mice. *Neuropharmacology*, **97**, 210–219 (2015).
- 170) Jensen BS, Strobaek D, Olesen SP, Christoffersen P. The Ca²⁺-activated K⁺ channel of intermediate conductance: a molecular target for novel treatments? *Curr. Drug Targets*, **2**, 401–422 (2001).
- 171) Todesca LM, Maskri S, Brommel K, Thale I, Wunsch B, Koch O, Schwab A. Targeting Kca3.1 channels in cancer. *Cell. Physiol. Biochem.*, **55** (S3), 131–144 (2021).
- 172) Ohya S, Niwa S, Yanagi A, Fukuyo Y, Yamamura H, Imaizumi Y.

- Involvement of dominant-negative spliced variants of the intermediate conductance Ca^{2+} -activated K^+ channel, $\text{K}(\text{Ca})3.1$, in immune function of lymphoid cells. *J. Biol. Chem.*, **286**, 16940–16952 (2011).
- 173) Hirukawa K, Muraki K, Ohya S, Imaizumi Y, Togari A. Electrophysiological properties of a novel $\text{Ca}(2+)$ -activated $\text{K}(+)$ channel expressed in human osteoblasts. *Calcif. Tissue Int.*, **83**, 222–229 (2008).
- 174) Ohya S, Kimura S, Kitsukawa M, Muraki K, Watanabe M, Imaizumi Y. SK4 encodes intermediate conductance Ca^{2+} -activated K^+ channels in mouse urinary bladder smooth muscle cells. *Jpn. J. Pharmacol.*, **84**, 97–100 (2000).
- 175) Wulff H, Castle NA. Therapeutic potential of $\text{KCa}3.1$ blockers: recent advances and promising trends. *Expert Rev. Clin. Pharmacol.*, **3**, 385–396 (2010).
- 176) Staal RGW, Weinstein JR, Nattini M, Cajina M, Chandresana G, Moller T. Senicapoc: repurposing a drug to target microglia $\text{K}_{\text{Ca}}3.1$ in stroke. *Neurochem. Res.*, **42**, 2639–2645 (2017).
- 177) Ohya S, Fukuyo Y, Kito H, Shibaoka R, Matsui M, Niguma H, Maeda Y, Yamamura H, Fujii M, Kimura K, Imaizumi Y. Upregulation of $\text{K}_{\text{Ca}}3.1$ $\text{K}(+)$ channel in mesenteric lymph node CD4(+) T lymphocytes from a mouse model of dextran sodium sulfate-induced inflammatory bowel disease. *Am. J. Physiol. Gastrointest. Liver Physiol.*, **306**, G873–G885 (2014).
- 178) Matsui M, Terasawa K, Kajikuri J, Kito H, Endo K, Jaikhan P, Suzuki T, Ohya S. Histone deacetylases enhance $\text{Ca}(2+)$ -activated $\text{K}(+)$ channel $\text{K}_{\text{Ca}}3.1$ expression in murine inflammatory CD4(+) T cells. *Int. J. Mol. Sci.*, **19**, 2942 (2018).
- 179) Ohya S, Matsui M, Kajikuri J, Endo K, Kito H. Increased interleukin-10 expression by the inhibition of $\text{Ca}(2+)$ -activated $\text{K}(+)$ channel $\text{K}_{\text{Ca}}3.1$ in CD4(+)CD25(+) regulatory T cells in the recovery phase in an inflammatory bowel disease mouse model. *J. Pharmacol. Exp. Ther.*, **377**, 75–85 (2021).
- 180) Ohya S, Matsui M, Kajikuri J. Ca^{2+} -activated K^+ channel $\text{K}_{\text{Ca}}3.1$ as a double-edged sword in the treatment of inflammatory bowel disease. *J. Gastrointestin. Liver Dis.*, **29**, 487–489 (2020).
- 181) Ohya S, Nakamura E, Horiba S, Kito H, Matsui M, Yamamura H, Imaizumi Y. Role of the $\text{K}(\text{Ca})3.1\text{K}^+$ channel in auricular lymph node CD4+ T-lymphocyte function of the delayed-type hypersensitivity model. *Br. J. Pharmacol.*, **169**, 1011–1023 (2013).
- 182) Ohya S, Niwa S, Kojima Y, Sasaki S, Sakuragi M, Kohri K, Imaizumi Y. Intermediate-conductance Ca^{2+} -activated K^+ channel, $\text{K}_{\text{Ca}}3.1$, as a novel therapeutic target for benign prostatic hyperplasia. *J. Pharmacol. Exp. Ther.*, **338**, 528–536 (2011).
- 183) Mohr CJ, Steudel FA, Gross D, Ruth P, Lo WY, Hoppe R, Schroth W, Brauch H, Huber SM, Lukowski R. Cancer-associated intermediate conductance $\text{Ca}(2+)$ -activated $\text{K}(+)$ channel $\text{KCa}3.1$. *Cancers (Basel)*, **11**, 109 (2019).
- 184) Ohya S, Kimura K, Niwa S, Ohno A, Kojima Y, Sasaki S, Kohri K, Imaizumi Y. Malignancy grade-dependent expression of K^+ -channel subtypes in human prostate cancer. *J. Pharmacol. Sci.*, **109**, 148–151 (2009).
- 185) Gu M, Zhu Y, Yin X, Zhang DM. Small-conductance $\text{Ca}(2+)$ -activated $\text{K}(+)$ channels: insights into their roles in cardiovascular disease. *Exp. Mol. Med.*, **50**, 1–7 (2018).
- 186) Morimura K, Yamamura H, Ohya S, Imaizumi Y. Voltage-dependent Ca^{2+} -channel block by openers of intermediate and small conductance Ca^{2+} -activated K^+ channels in urinary bladder smooth muscle cells. *J. Pharmacol. Sci.*, **100**, 237–241 (2006).
- 187) Picollo A, Malvezzi M, Accardi A. TMEM16 proteins: unknown structure and confusing functions. *J. Mol. Biol.*, **427**, 94–105 (2015).
- 188) Lambert M, Capuano V, Olschewski A, Sabourin J, Nagaraj C, Girerd B, Weatherald J, Humbert M, Antigny F. Ion channels in pulmonary hypertension: a therapeutic interest? *Int. J. Mol. Sci.*, **19**, 3162 (2018).
- 189) Cil O, Chen X, Askew Page HR, Baldwin SN, Jordan MC, Myat Thwe P, Anderson MO, Haggie PM, Greenwood IA, Roos KP, Verkman AS. A small molecule inhibitor of the chloride channel TMEM16A blocks vascular smooth muscle contraction and lowers blood pressure in spontaneously hypertensive rats. *Kidney Int.*, **100**, 311–320 (2021).
- 190) Liu Y, Liu Z, Wang K. The $\text{Ca}(2+)$ -activated chloride channel ANO1/TMEM16A: an emerging therapeutic target for epithelium-originated diseases? *Acta Pharm. Sin. B*, **11**, 1412–1433 (2021).
- 191) Danahay H, Gosling M. TMEM16A: An alternative approach to restoring airway anion secretion in cystic fibrosis? *Int. J. Mol. Sci.*, **21**, 2386 (2020).
- 192) Suzuki T, Suzuki Y, Asai K, Imaizumi Y, Yamamura H. Hypoxia increases the proliferation of brain capillary endothelial cells via upregulation of TMEM16A $\text{Ca}(2+)$ -activated $\text{Cl}(-)$ channels. *J. Pharmacol. Sci.*, **146**, 65–69 (2021).
- 193) Yamamura H, Suzuki Y, Imaizumi Y. Physiological and pathological functions of Cl^- channels in chondrocytes. *Biol. Pharm. Bull.*, **41**, 1145–1151 (2018).
- 194) Suzuki T, Yasumoto M, Suzuki Y, Asai K, Imaizumi Y, Yamamura H. TMEM16A $\text{Ca}(2+)$ -activated $\text{Cl}(-)$ channel regulates the proliferation and migration of brain capillary endothelial cells. *Mol. Pharmacol.*, **98**, 61–71 (2020).
- 195) Funabashi K, Fujii M, Yamamura H, Ohya S, Imaizumi Y. Contribution of chloride channel conductance to the regulation of resting membrane potential in chondrocytes. *J. Pharmacol. Sci.*, **113**, 94–99 (2010).
- 196) Kurita T, Yamamura H, Suzuki Y, Giles WR, Imaizumi Y. The CLC-7 chloride channel is downregulated by hypoosmotic stress in human chondrocytes. *Mol. Pharmacol.*, **88**, 113–120 (2015).
- 197) Crottes D, Jan LY. The multifaceted role of TMEM16A in cancer. *Cell Calcium*, **82**, 102050 (2019).
- 198) Chen W, Gu M, Gao C, Chen B, Yang J, Xie X, Wang X, Sun J, Wang J. The prognostic value and mechanisms of TMEM16A in human cancer. *Front. Mol. Biosci.*, **8**, 542156 (2021).
- 199) Greenwood IA, Leblanc N. Overlapping pharmacology of Ca^{2+} -activated Cl^- and K^+ channels. *Trends Pharmacol. Sci.*, **28**, 1–5 (2007).
- 200) Hao A, Guo S, Shi S, Wang X, Zhan Y, Chen Y, An H. Emerging modulators of TMEM16A and their therapeutic potential. *J. Membr. Biol.*, **254**, 353–365 (2021).
- 201) Fedigan S, Bradley E, Webb T, Large RJ, Hollywood MA, Thornbury KD, McHale NG, Sergeant GP. Effects of new-generation TMEM16A inhibitors on calcium-activated chloride currents in rabbit urethral interstitial cells of Cajal. *Pflugers Arch.*, **469**, 1443–1455 (2017).
- 202) Zhong J, Xuan W, Tang M, Cui S, Zhou Y, Qu X, Cao X, Niu B. Advances in anoctamin 1: a potential new drug target in medicinal chemistry. *Curr. Top. Med. Chem.*, **21**, 1139–1155 (2021).
- 203) Cavieres-Lepe J, Ewer J. Reciprocal relationship between calcium signaling and circadian clocks: implications for calcium homeostasis, clock function, and therapeutics. *Front. Mol. Neurosci.*, **14**, 666673 (2021).
- 204) Meredith AL, Wiler SW, Miller BH, Takahashi JS, Fodor AA, Ruby NF, Aldrich RW. BK calcium-activated potassium channels regulate circadian behavioral rhythms and pacemaker output. *Nat. Neurosci.*, **9**, 1041–1049 (2006).
- 205) Santoni G, Morelli MB, Santoni M, Nabissi M, Marinelli O, Amantini C. Targeting transient receptor potential channels by microRNAs drives tumor development and progression. *Adv. Exp. Med. Biol.*, **1131**, 605–623 (2020).