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Calcium-Activated Chloride Channel in Rat Pulmonary Artery Smooth Muscle Cells

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Abstract

Calcium-activated chloride channels (CaCC) are crucial regulators of vascular tone by promoting a depolarizing influence on the resting membrane potential of vascular smooth muscle cells. The chloride equilibrium potential (E_{Cl}) of pulmonary artery smooth muscle cells (PASMC) is significantly more positive than the resting potential. The role of CaCC is probably to produce membrane depolarization and consequent opening of voltage-dependent calcium channels (VDCCs) to evoke contraction by influx of extracellular Ca^{2+}. Using the whole cell patch-clamp technique, sustained Ca^{2+}-activated Cl^- currents ($I_{Cl(Ca)}$) evoked by K^+ free pipette solutions containing 500nM Ca^{2+} were recorded in single rat pulmonary artery smooth muscle cell. The electrophysiological characters of CaCC and its possible contributions to the rat pulmonary artery tone were studied. In conclusion, $I_{Cl(Ca)}$ exhibited the typical outwardly rectifying. The steady states currents and activation/inactivation time constant of $I_{Cl(Ca)}$ have obvious voltage dependence when evoked by fixed 500nM $[Ca^{2+}]_i$. However, increasing the duration of a +70 mV test pulse from 200 to 1400 ms didn't significantly augment the amplitude of the outward current relaxation and tail current. The possible reasons are discussed.

Key words: Calcium-activated chloride channel, Pulmonary artery, Smooth muscle cells, Patch clamp, Whole cell, Electrophysiological characters.

1. Introduction

In vascular smooth muscle cells, a variety of cationic channels (Ca^{2+}, K^+, Na^+) in the plasma membrane have been characterized, and their functional roles in controlling vascular tone have been extensively studied. For example, K^+ channel dysfunction plays an important role in the development of pulmonary hypertension [1]. Activity of K^+ channels regulates the membrane potential (E_m) of PASMC and in turn elevates $[Ca^{2+}]_i$ by opening VDCCs which is implicated in stimulating vascular SMC proliferation and inducing vasomotor tone [2]. Analogous to K^+ as the predominant intracellular cation, Cl^- is the most abundant intracellular and extracellular anion under physiological conditions. The contribution of Cl^- channels to the regulation of membrane potential (E_m), cytoplasmic free Ca^{2+} concentration, and vasomotor tone in PASMC is still not completely explored. Membrane depolarization is an important contributor to initiation and maintenance of arterial contraction. The mechanisms responsible for membrane depolarization induced by intracellular Ca^{2+} release and initial Ca^{2+} influx through receptor-operated Ca^{2+} channels are not completely known. However, activation of CaCC and inhibition of delayed rectifier K^+ channels may play a pivotal role in depolarizing the cells when $[Ca^{2+}]_i$ is increased by agonists. Furthermore, CaCC was found playing an important role in regulating the vascular tones. $I_{Cl(Ca)}$ have been recorded from many types of smooth muscle. The majority of experiments studying the properties of $I_{Cl(Ca)}$ in vascular smooth muscle cells have utilized the whole-cell recording technique where the resting intracellular Ca^{2+} concentration and the chloride equilibrium potential (E_{Cl}) is significantly more positive than the resting potential. The role of $I_{Cl(Ca)}$ is probably to produce membrane depolarization and consequent opening of VDCCs to evoke contraction. $I_{Cl(Ca)}$ is activated by free Ca^{2+} acting on the inner surface of the smooth muscle cell membrane. The Ca^{2+} that stimulate $I_{Cl(Ca)}$ release from intracellular Ca^{2+} store or enter through VDCCs from extracellular solution. $I_{Cl(Ca)}$ is manifest not only during the depolarizing steps, but also as an inward "tail" current (I_{tail}) on stepping back to the holding potential. I_{tail} may generate the depolarizing after-potential that is observed following the action potentials occurring in some smooth muscle [3]. Physiologically these after-potentials are likely to have an important influence on membrane

excitability, as they will lead to further opening of VDCCs. The duration of I_{tail} is usually greater than 100 ms, but an interesting observation is that the decay to I_{tail} appears to be vary greatly depending on the tissue used. For example, in rat portal vein and rabbit coronary artery I_{tail} decayed exponentially at negative potentials, and it was postulated that the decline of I_{tail} might represent channel kinetics [3].

$I_{Cl(Ca)}$ has recently been described in smooth muscle cells from coronary artery, portal vein [5] and pulmonary artery [4,5].The present study investigated the electrophysiological properties of CaCC in single rat pulmonary artery's smooth muscle cell such as the activation/inactivation kinetics, voltage-/time-dependence of $I_{Cl(Ca)}$ evoked by fixed 500nM $[Ca^{2+}]_i$.

2. Materials and methods

2.1 Preparation of PASMC

Cells were prepared from the rat main pulmonary artery isolated. After dissection and removal of connective tissue the artery was rubbed with incurvate scissors softly to remove endothelial cells in D-Hanks' balanced salt solution containing (in mg/mL) 0.4 KCl, 0.06 KH_2PO4, 8.0 NaCl, 0.06 Na_2HPO_4, 0.35 $NaHCO_3$, pH7.2. The tissue was then cut into small strips and incubated in 2mL D-Hanks' balanced salt solution containing 2mg/mL collagenase Ⅰ for 1hour and then added 1mL 0.15% trypsinase for 5min at 37℃ to create a single cell suspension. The digestion was stopped by 2mL DMEM supplemented with 10% fetal bovine serum and the cells were released by gentle agitation with a wide bore Pasteur pipette. Single pulmonary artery smooth muscle cells were resuspended and plated onto a glass coverslip and incubated in a humidified atmosphere of 5% CO_2-95% air at 37℃ in 20% fetal bovine serum culture medium for 2-3 days before use.

2.2 Electrophysiology

Conventional whole cell patch-clamp measurements were performed using an EPC-9 patch-clamp amplifier and PULSE software (HEKA, Lambrecht, Germany). In experiments, $I_{Cl(Ca)}$ were evoked by pipette solutions containing 500nM Ca^{2+} as this concentration of Ca^{2+} generated large and robust Cl^- current in pulmonary artery smooth muscle cells [4, 5]. The pipette solution containing (mM): TEA-Cl 20; CsCl 106; HEPES 5; BAPTA 10; MgATP 3; $GTPNa_2$ 0.2; $MgCl_2$ 0.42 and pH was set to 7.2 by adding CsOH. Free $[Ca^{2+}]$ was set at 500nM by the addition of 7.8mM $CaCl_2$ determined by the EQCAL buffer program. The external solution contained (mM): NaCl 126; HEPES 10; pH 7.4, glucose 11; $CaCl_2$ 1.8; $MgCl_2$ 1.2; TEA-Cl 10 and 4-aminopyridine 5. All reagents were purchased from Sigma unless other wise stated. Experiments were performed at room temperature (22-25℃).

2.2 Statistical analysis

Data analysis was carried out using IGOR PRO software (Wavemetrics, LakeOswego, OR). Averaged results were expressed as mean ± SEM. The data fitted by mono-exponential started after 5-10 ms of the pulse to avoid the capacitive current. Comparisons between means were performed using Student's *t* test. Difference between groups were considered significant when $P<0.05$.

3. Results

Rat pulmonary artery smooth muscle cells were dialysed with a K^+ free pipette solution containing 500nM Ca^{2+} (see Methods) in order to activate $I_{Cl(Ca)}$. A stable inward current was evoked at the holding potential of –50 mV (I_{hold}) which had mean steady amplitude of ~97 ± 13 pA (n=16, Figure 1A). This current has been shown to be a Cl^- current that is activated at physiological membrane potentials by intracellular calcium concentrations greater than 100nM [4,5]. $I_{Cl(Ca)}$ elicited in the present study exhibited time-/voltage-dependent properties that were revealed by depolarization from –50 mV to +70 mV. Stepping to +70 mV produced an instantaneous current that was followed by the development of an outward current during the voltage step ($I_{relax+70mV}$). The activation time constant (τ_{act}) of $I_{Cl(Ca)}$ was 156 ± 18 ms (n=16) by mono-exponential fitting.Upon repolarization to –80 mV, a tail current (I_{tail}) was recorded that declined over the course of the step. The time constant of deactivation (τ_{ina}) was 69±8 ms (n=16). After an initial period of stabilization of the amplitude of the current at –50 mV and the kinetics of the voltage-dependent relaxations were reproducible for the duration of the experiment under control conditions.

The pioneers have demonstrated that niflumic acid (NFA) inhibits $I_{Cl(Ca)}$ in micromolar concentrations with an IC_{50} (concentration to reduce the amplitude by 50%) of around 2~5μM for inhibition of spontaneous transient inward currents (STICs). Using the present method of activation of $I_{Cl(Ca)}$ with 500nM Ca^{2+} in the pipette solution, I_{hold} at –50 mV was reduced 30.9% from ~97±13 pA to ~67± 6 pA (n=4, $P<0.05$, Figure 1B,C) after application of 100μM NFA. Fig. 1B shows the great inhibition of 100μM NFA on the current developed at +70 mV with the mean outward relaxation ($I_{relax+70mV}$) decreasing 78.1% from 82±22 pA to 18±7 pA (n=4, $P<0.01$, Figure 1C). The mean amplitude of the inward tail current recorded upon repolarization to –80 mV (I_{tail}) was reduced 40.6% from ~259±29 pA to ~154 ± 13 pA (n=4, $P<0.05$, Fig. 1C). These data suggest that the current we recorded using this method is sensitive to NFA which is known as a special blocker of CaCC.

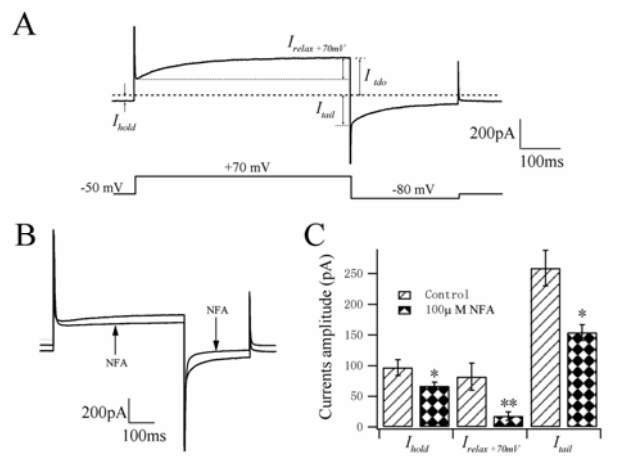

Figure 1. Inhibitory effect of NFA on $I_{Cl(Ca)}$ evoked by 500nM $[Ca^{2+}]_i$. (A) Representative current record elicited by depolarizing cell to +70 mV from a holding potential of -50 mV for 500 ms. Following the depolarizing pulse the cell was stepped to –80 mV for 250 ms to study the tail current. The arrows illustrate the current measurements discussed in the text: inward current at the holding potential of –50 mV (I_{hold}), outward current relaxation at +70 mV ($I_{relax+70mV}$), time-dependent outward current (I_{tdo}) and tail current at –80 mV (I_{tail}). (B) Before (Control) and after application of 100μM NFA, $I_{Cl(Ca)}$ was inhibited obviously. (C) Mean current absolute value showing effects of NFA on I_{hold} , $I_{relax+70mV}$ and I_{tail} .

In order to investigate the voltage-dependence of activation kinetics of $I_{Cl(Ca)}$ at different testing potentials, we used two classical protocols (ramp and steps) to record the whole cell currents. Figure 2A shows the I-V relationship for the Ca^{2+}-activated current was determined by applying 500 ms voltage ramp from –100 mV to +100 mV from the holding potential of –50 mV. I-V curve revealed outward rectification and reversed close to 0 mV (mean reversal potential (E_{rev}) was 3±2 mV, n=5). The theoretical chloride equilibrium potential (E_{Cl}) in these experiments was calculated to be 0.5 mV, suggesting that the Ca^{2+}-activated currents recorded in rat pulmonary artery smooth muscle was $I_{Cl(Ca)}$. The currents amplitude and activation time constants of I_{tdo} by depolarizing voltage steps from –80 mV to +80 mV in 10-mV increments from a holding potential of –50 mV were recorded in freshly isolated cells (Figure 2B). Figure 2C shows the relationship between the potentials and the currents by stepping to different testing potentials, which also exhibited typical outwardly rectifying steady states (n=11). The $I_{relax+70mV}$ could be fitted well by mono-exponential function. The activation time constant is dependent on voltage (n=11, Figure 2D). The testing potentials are higher the activation of CaCC is more quickly. These data indicate that CaCC have distinct voltage dependence in rat pulmonary artery smooth muscle cells when evoked by 500nM $[Ca^{2+}]_i$.

$I_{Cl(Ca)}$ was widely discussed as a tail current in many tissues. We study the tail currents to investigate the inactivation kinetics of CaCC. The PASMC were depolarized initially from –50 mV to +70 mV for 500 ms and then stepped to test potentials from +40 mV to –100 mV in 10-mV increments for 500 ms (Figure 3A). The curve of mean current-voltage relationship was shown in Figure 3B. The measured E_{rev} of I_{tail} was 3.7±3 mV (n=17, Figure 3A,B) that is also close to the theoretical Cl^- equilibrium potential (E_{Cl} =0.5 mV) calculated by using Nernst equation. These data confirmed that I_{tail} carried by Cl^- in deed again. The tail currents were fitted by mono-exponential function and the inactivation time constants were shown in Figure 3C. The inactivation kinetics of $I_{Cl(Ca)}$ is voltage-dependent similar with its activation kinetics. The testing potentials are lower the inactivation of CaCC is more quickly.

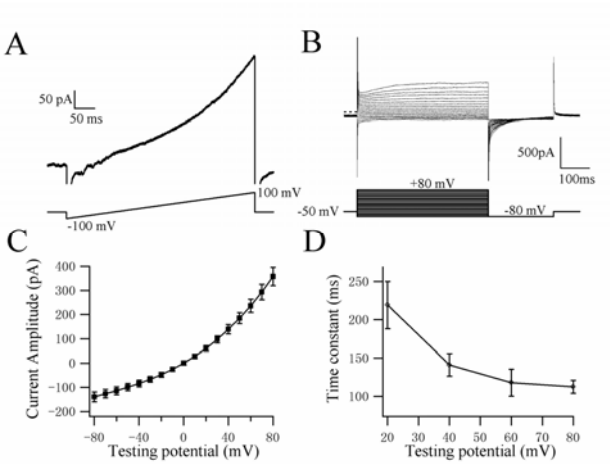

Figure 2. Voltage dependence of $I_{Cl(Ca)}$. (A) I-V relationship for the Ca^{2+}-activated current was determined by applying 500 ms voltage ramp from –100 mV to +100 mV from the holding potential of –50 mV. I-V curve displayed outward rectification and reversed close to 0 mV. (B) A representative family of $I_{Cl(Ca)}$ were recorded by stepping cell to potentials between –80 mV and +80 mV in 10-mV increments for 500 ms from the holding potential of –50 mV. (C) The mean current-voltage relationship also displayed outward rectification. (D) The currents curves were fitted by mono-exponential function well and the activation time constants of $I_{Cl(Ca)}$ displayed voltage dependence.

The time-dependence of CaCC was studied in single rat PASMC. Increasing the duration of +70 mV test pulse from 200 to 1400 ms (in increments of 100 ms) only slightly augmented the amplitude of I_{tdo} and I_{tail} (n=11, Figure 4B,C). With longer periods of prior activation, the time constants for I_{tail} inactivation were also increased a little (n=11, Figure 4B). And, increasing the step duration had no effect on the activation time constant of I_{tdo} at +70 mV (Figure 4A). The activation kinetics followed the same mono-exponential time course as shown in Figure 4A is similar to the results of Figure 1A. The current amplitude of I_{tail} just augmented slightly but not significantly with a settled 500nM $[Ca^{2+}]_i$ which

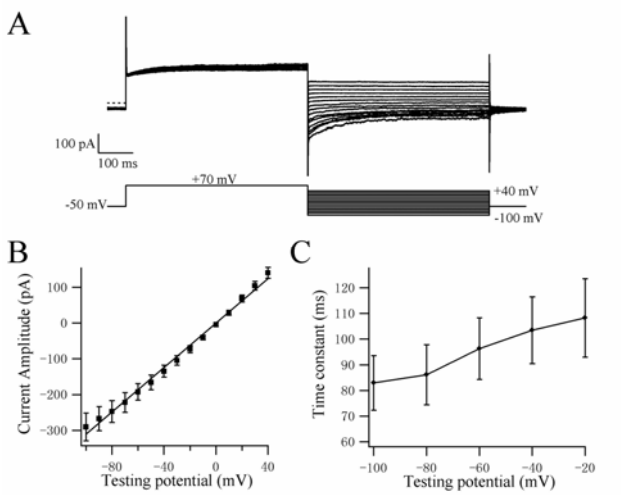

Figure 3. Inactivation characters of $I_{Cl(Ca)}$.
(A) Representative tail currents were recorded by depolarizing to +70 mV for 500 ms followed by a 500 ms test step to different potentials between –100 mV and +40 mV in 10-mV increments from the holding potential of –50 mV. The mean current-voltage relationship of the currents recorded after stepping to the test potentials in panel B. The measured E_{rev} of I_{tail} is close to the theoretical Cl^- equilibrium potential (E_{Cl} =0.5 mV) calculated by using Nernst equation. (C) Inactivation time constants of $I_{Cl(Ca)}$ in different testing potentials. I_{tail} were fitted by mono-exponential. The inactivation time constant displayed voltage dependence.

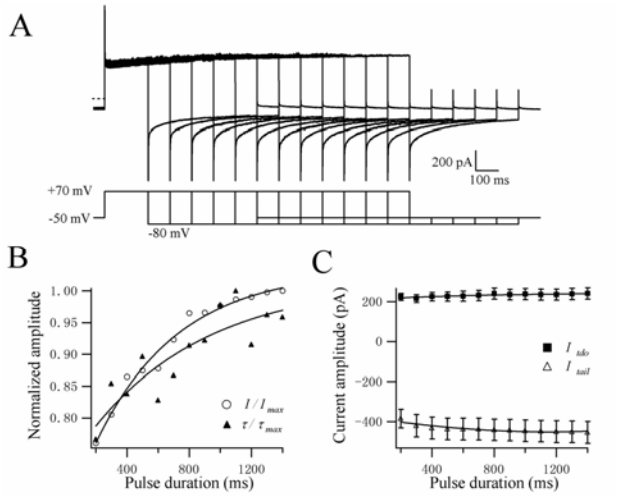

Figure 4. Effect of depolarizing duration of a depolarizing voltage pulse on $I_{Cl(Ca)}$. (A) Representative family of currents elicited by depolarizing the pulmonary artery smooth muscle cell from a holding potential of –50 mV to +70 mV for durations ranging from 200 to 1400 ms in increments of 100 ms. (B) Plot of normalized amplitude (I/I_{max}, ○) and deactivation time constants (τ/τ_{max}, ▲) of I_{tail} as a function of pulse duration (n=11). The current amplitude and inactivation time constants of I_{tail} displayed slightly increasing as increasing of pulse duration. These dots were fitted by mono-exponential. (C) The mean currents amplitude of I_{tdo} and I_{tail}. And the I_{tdo} displayed almost no increasing when increased pulse duration (n=11).

activated the CaCC throughly and stably [4,5]. These results indicate that the activation and inactivation characters of CaCC evoked by a fixed 500nM $[Ca^{2+}]_i$ didn't have obvious time dependence which is reported in Yuan's study. Their results were different that increasing the duration of a +10 mV test pulse significantly augmented the amplitude and inactivation time constants of I_{tail} [12]. The possible reason was that the currents were evoked via a gradual accumulation of Ca^{2+} through Ca^{2+} influx through membrane Ca^{2+} channels and/ or Ca^{2+}-induced Ca^{2+} release from intracellular Ca^{2+} stores when the cells was polarized to +10 mV. However, the concentration of $[Ca^{2+}]i$ in our experiments was an invariable 500nM. So it didn't need time to accumulate of Ca^{2+} to elevate the $[Ca^{2+}]_i$ concentration to evoke $I_{Cl(Ca)}$ progressively.

3. Discussion

3.1 Electrophysiological properties of CaCC

The electrophysiological properties of CaCC are the essential study contents in present experiments. We used three classical voltage protocols to study the activation kinetics, inactivation kinetics of I_{tail}, time dependence of $I_{Cl(Ca)}$ evoked by a fixed 500nM $[Ca^{2+}]_i$ in rat PASMC. In our experiments, we found that the activation kinetics of CaCC in rat PASMC have similar voltage dependent properties as some other cells. And the activation time constants fitted by mono-exponential function decreased following the increasing of voltage. The time constants of I_{tail} were between 70~120 ms at different testing potentials, and inactivation kinetics also had voltage dependent properties. The main finding of the present work is that in rat pulmonary artery smooth muscle cells the $I_{Cl(Ca)}$ when activated by 500nM $[Ca^{2+}]_i$ imposed by dialysis from the pipette solution didn't have obvious time dependent properties. This is a surprising result because in all previous reports it has been shown that CaCC behave an apparent time dependent activation. The most likely explanation for the discrepancy in the results with $[Ca^{2+}]_i$ is a difference in the experimental conditions used. In previous studies, the CaCC were tonically activated by constantly elevated $[Ca^{2+}]_i$ by activating the VDCCs in single cells. The cytoplasmic calcium concentration increased via a gradual accumulation of influx of extracellular Ca^{2+} by depolarizing cell to +10 mV. In the present work $I_{Cl(Ca)}$ was activated by a relatively high 500nM $[Ca^{2+}]_i$ imposed by the patch pipette solution. And the depolarizing potential we used was +70 mV that can't open the VDCCs.

3.2 Activity of CaCC regulates PA vasomotor tone by controlling E_m.

CaCC appear to exist in cells isolated from a variety of types of vessels smooth muscle cells [3, 4, 5, 10]. In PASMC the resting E_m, ranging from –35 mV to –55 mV [5, 6], is more positive than the K^+ equilibrium potential (E_k) but more negative than E_{Cl}.

Accordingly, under these resting conditions, alteration of sarcolemmal Cl^- channel activity would substantially contribute to the regulation of E_m, which dominates the activity of voltage-gated Ca^{2+} channels. Activation of CaCC, by facilitating Cl^- efflux, would thus result in membrane depolarization and activation of voltage-gated Ca^{2+} channels, increasing $[Ca^{2+}]_i$ and causing pulmonary vasoconstriction. The Ca^{2+} concentration threshold for activation of $I_{Cl(Ca)}$ in portal vein smooth muscle cells is 180nM, with full activation at 600nM. In rat PASMC, the resting $[Ca^{2+}]_i$ is 50-100nM, whereas agonist-induced increases in $[Ca^{2+}]_i$ usually range from 200 to 1000nM. Many vasoactive agents mediate vascular contraction in association with an initial transient increase in $[Ca^{2+}]_i$ followed by a sustained $[Ca^{2+}]_i$ plateau. The Ca^{2+} transient is often due to Ca^{2+} release from intracellular stores and serves to trigger contraction [11]. Increase in $[Ca^{2+}]_i$ activates CaCC and elicits inward $I_{Cl(Ca)}$. The resulting Cl^- currents would cause membrane depolarization, thereby open voltage-gated Ca^{2+} channels and leading to additional increase in $[Ca^{2+}]_i$. Although the precise mechanism is not completely known, Ca^{2+}-induced activation of CaCC like 5-TH and PE, in addition to increasing $[Ca^{2+}]_i$, also causes membrane depolarization and sustained vasoconstriction.

Ca^{2+}-activated chloride channels were less studied than other chloride channels, such as volume-regulated chloride channel, or cystic fibrosis trans-membrane conductance regulator (CFTR) chloride channels. Nevertheless, CaCC, in diverse cell types, is clearly an important channel type involved in various physiological functions (cell secretion, anion transport, cell adhesion etc.) [6,7]. The first distinct CaCC was identified in the bovine airway, with an ion selectivity of I>Cl, and sensitive to DIDS [8]. To date, at least ten isoforms of CaCC (found from bovine, human, mouse and porcine) have been identified and published on NCBI GenBank database [8]. The electrophysiological features of CaCC are very similar in the various cell types [9]. The current-voltage relationship described here displayed obvious outward rectification characters observed also in some other cells [7]. The channel kinetics of CaCC in rat PASMC we reported here had similar properties with CaCC demonstrated in cultured rabbit pulmonary artery smooth muscle cells, portal vein smooth muscle cells, sheep lymphatic smooth muscle cells [10]. All of them had typical voltage dependence of activation and inactivation. Thus, CaCC presently tested belongs to the general CaCC family. In smooth muscle cells, the speculated roles of CaCC are regulation of membrane potential and modulation of agonist-induced store-depletion dependent intracellular calcium signaling, including regulation of calcium influx. The calcium influx sequentially regulates the contraction of vascular smooth muscle cells and plays an essential role in controlling vascular tone and other disease such as pulmonary hypertension.

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