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Activation of $Ca_v1.2$ and BK_{Ca} is involved in the downregulation of caffeine-induced contraction in mice mesenteric arteries

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ABSTRACT

Aims: Caffeine is a methylxanthine with multiple actions in vascular smooth muscle cells (VSMCs), including the increase in the intracellular Ca^{2+} ($_iCa^{2+}$) concentration by the activation of ryanodine receptors (RyRs). The present study aimed at investigating the participation of Ca^{2+}-influx through different Ca^{2+}-channels on the transient contraction (TC) induced by caffeine in mice mesenteric arteries.

Main methods: Second-order of mesenteric arteries was isolated from male Swiss mice. Vessels without functional endothelium were stimulated with caffeine (10 mM). The caffeine-induced TC was evaluated after the incubation of artery rings for 30 min with the following drugs: nifedipine (10 μM), a $Ca_v1.2$ blocker; 2-aminoethoxydiphenyl borate (2-APB; 10 μM) and ruthenium red (RuR; 10 μM), transient receptor potential (TRPs) channels blockers; capsazepine (10 μM) and HC067047 (10 μM), TRPV1 and TRPV4 antagonists, respectively; paxilline (1 μM), a selective BK_{Ca} blocker; and SKF-96365 (30 μM), an Orai blocker. Ca^{2+}-fluorescence measurements were also performed on the investigated arteries.

Key findings: The TC induced by caffeine was partially dependent on Ca^{2+}-influx. However, the blockage of $Ca_v1.2$ increased the TC while reduced the $_iCa^{2+}$ signal. Similar results were observed after the blockage of TRPs or BK_{Ca}. Therefore, caffeine promoted Ca^{2+}-influx via TRPs and $Ca_v1.2$, and hyperpolarization through the activation of BK_{Ca}, inducing negative feedback of TC.

Significance: Our results indicate an alternative mechanism for the control of VSMCs contraction in resistance arteries. The evidence of the negative feedback of contraction via TRP-$Ca_v1.2$-BK_{Ca} provides a new perspective for understanding the mechanism involved in the vascular responses triggered by caffeine.

1. Introduction

Caffeine is one of the most regularly consumed drugs in the world [1,2]. It is a methylxanthine, which multiple beneficial and deleterious actions in the cardiovascular system are still under debate [1,3]. The main issue is if the consumption of caffeine could be related to a higher incidence of coronary heart disease, and in which case, its consumption should be avoided. Therefore, the investigation of the mechanism involved in the control of the effect of caffeine in vascular smooth muscle (VSM) of healthy arteries is the first step towards the understanding of its effect in pathophysiological conditions.

Vascular smooth muscle cells (VSMCs) are one of the targets of caffeine in the cardiovascular system, being the increase of the intracellular Ca^{2+} ($_iCa^{2+}$) concentration the mostly reported effect [4,5]. The increase in the $_iCa^{2+}$ initially occurs through the activation of ryanodine receptors (RyRs) of the sarcoplasmic reticulum (SR), which also generates a transitory contraction [6]. Besides the relationship between the Ca^{2+}-release from the SR and induction of Ca^{2+}-influx being well described in VSMC [7], the contribution of the Ca^{2+}-influx in the contraction induced by caffeine in VSM is not well established.

Ca^{2+}-influx through the plasma membrane (PM) and Ca^{2+}-release from SR are critical regulators of VSMCs contraction [8,9]. Voltage-gated Ca^{2+}-channels (VGCCs) and non-voltage-gated Ca^{2+}-channels represent essential PM structures responsible for the control of Ca^{2+}-entry in VSMCs. VGCC isoforms go from $Ca_v1.1$ to 1.4, but $Ca_v1.2$ is the dominant isoform found in VSMCs [10,11]. RyRs are Ca^{2+}-release channels present in the SR membrane. It promotes Ca^{2+}-release through small (Ca^{2+}-sparks) or large (Ca^{2+}-waves) $_iCa^{2+}$ elevations

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[12,13]. Non-voltage gated Ca^{2+}-channels, represented by transient receptor potential (TRP) channels ($P_{Ca}/P_{Na} < 10$), play an essential role in the regulation of vascular tonus. Canonical TRP (TRPC), vanilloid TRP (TRPV) and melastatin TRP (TRPM) contribute to Ca^{2+} signaling in resistance arteries. TRPC1, TRPC3, TRPC6, TRPV1, TRPV2, and TRPM4 are the main TRPs expressed in mesenteric arteries [14,15].

TRPC1 is a store-operated Ca^{2+} channel (SOC) [16], which forms a complex with stromal interaction molecule (STIM) after store depletion and stimulates phospholipase C (PLC) activity [17], but it is also gated by diacylglycerol (DAG) and phosphorylated by protein kinase C (PKC), suggesting a versatility of gating [18,19]. TRPC3 and TRPC6 contribute to vascular tone [14,20,21]. TRPC3 has been described to control the vascular contractility in mouse mesenteric arteries and may function as both receptor-operated and SOC channel [22–25]. TRPC6 has multiple functions and can act as SOC, receptor-operated (ROC), stretch-activated (SAC) and osmotically-activated (OAC) cation channels in VSMCs [26]. TRPV1 is involved in the contractility of several vascular beds [14,27], while TRPV2 has participation in mechanotransduction [28] and may be activated by osmotic cell swelling [29,30]. Moreover, some TRPs have ankyrin domains at the NH_2-termini that facilitate the interaction between them and give TRPs multiple functions according to the complex formed [31].

VGCCs and TRPs may promote Ca^{2+} influx triggering K^+ channels activated by Ca^{2+} (BK_{Ca}) [32,33], that contribute to the maintenance of the membrane potential (*Em*) of VSMCs between −35 to −50 mV. BK_{Ca} channels are densely expressed in VSMCs working as buffers of the depolarization of PM [34]. The property of BK_{Ca} indicates that to be active, it needs 1–10 μM of calcium at −50 mV [35]. It has been described that the co-localization of BK_{Ca} with Ca^{2+} channels, or regulatory subunits, modulates its Ca^{2+} sensitivity or voltage [32].

This work hypothesizes that caffeine promotes transient contraction (TC) that is modulated via Ca^{2+}-influx and shows that Ca^{2+}-influx through $Ca_v1.2$ and TRPs promote negative feedback of the TC, as a control mechanism of the contraction in mesenteric arteries and may have a relevant role in controlling the blood flow of resistance arteries.

2. Materials and methods

2.1. Ethical approval

Protocols were submitted and approved by the ethics committee (CETEA) - [protocol #86/2015] of the Universidade Federal de Minas Gerais (UFMG). Male Swiss mice (8 to 12 weeks old) were used. All animals were obtained from the animal facility of the Institute of Biological Sciences - UFMG. Mice were maintained at five per cage at constant temperature (23 °C), with 12 h dark/light cycle. Free access was allowed to standard chow and filtered water ad libitum.

2.2. Small mesenteric artery preparation and mounting

Small mesenteric arteries were prepared [36]. Briefly, male Swiss mice were euthanized by decapitation. A proximal segment of the small bowel was removed and pinned in a dissecting dish containing a physiological salt solution (PSS) after the viscera be exposed. The PSS composition was (mmol/L): NaCl, 119; KCl, 4.7; KH_2PO_4, 0.4; $MgSO_4$, 1.17; $NaHCO_3$, 14.9; $CaCl_2$, 2.5; glucose, 5.5. Second order branches of resistance arteries were cleaned of fat and connective tissue and segmented into rings of 1.6 to 2.0 mm in length. The endothelial layer was removed immediately after dissection by the gentle friction of the lumen with tungsten wire. Then, the segment was mounted on a wire myograph, as previously described [37]. The absence of relaxation response to acetylcholine (ACh) was taken as evidence that vessel segments were without functional endothelium. For experiments with Ca^{2+}-free solution, vessels were stimulated immediately after the solution exchange with caffeine (10 mM), the composition of Ca^{2+}-free solution (in mmol/L): NaCl, 119; KCl, 4.7; KH_2PO_4, 0.4; $MgSO_4$, 1.17; $NaHCO_3$, 14.9; glucose, 5.5; EGTA, 10. After the first stimulation with caffeine, the bath solution was changed three times with normal PSS and a period of 30 min was allowed to pass to refill the SR. Finally, vessels were stimulated again with caffeine (for control experiments) or caffeine in the presence of drugs.

2.3. Measurement of $_iCa^{2+}$ by fluorescence microscopy in mesenteric arteries

The fluorescent dye Fluo 4-AM (Invitrogen) was used to measure the $_iCa^{2+}$ signal. Arteries were isolated and placed in HEPES-PSS (mmol/L): NaCl 130; HEPES 10; glucose 6; KCl 4; $NaHCO_3$ 4; $CaCl_2$ 1.8; $MgSO_4$ 1.2; KH_2PO_4 1.18; EDTA 0.03, pH 7.4 [36,38]. Rings were placed in a light protected bath containing Fluo 4-AM (5 μM) plus pluronic acid (5 μM) and HEPES-PSS, for 30 min. After incubation, a stainless steel wire (40 μM diameter) was used to fix the arterial ring (1.6 to 2 mm in length) on a mounting plate. After recording the basal fluorescence, the arterial ring was stimulated with caffeine (10 mM).

For experiments with Ca^{2+}-free, the HEPES-PSS solution was changed immediately before the stimulus with caffeine (10 mM) by HEPES-PSS without Ca^{2+}, composition (in mmol/L): NaCl, 130; HEPES, 10; glucose, 6; KCl, 4; $NaHCO_3$, 4; EGTA, 10; $MgSO_4$, 1.2; KH_2PO_4, 1.18; EDTA, 0.03, pH 7.4. Then, mesenteric arteries rings were pre-incubated for 30 min with drugs. F_0 was used as basal fluorescence before the stimulation, and F as maximal fluorescence after the stimulation, obtaining (F/F_0). Images were acquired with a fluorescence microscope NIKON Eclipse Ti and analyzed with ImageJ 1.51j8.

2.4. Em measurement

Segments of mesenteric artery (1.6 to 2 mm in length) were removed as described above and then, mounted on a mounting plate with HEPES-PSS (mM) solution: NaCl 130; HEPES 10; glucose 6; KCl 4; $NaHCO_3$ 4; $CaCl_2$ 1.8; $MgSO_4$ 1.2; KH_2PO_4 1.18; EDTA 0.03, pH 7.4. In these experiments, the endothelial layer was removed immediately after dissection by the gentle friction of the lumen with a steel wire. Transmembrane potentials were recorded with a glass microelectrode filled with KCl 3 M (tip resistance 30–50 MΩ). The microelectrode was mounted on a sliding micromanipulator (Leitz; St Gallen, Switzerland). The *Em* was amplified using an Axopatch 200B and digitalized with a Digidata 1200 (Axon Instruments, Foster City, U.S.A.). The signal was monitored and acquired in the scope mode of pClamp7 software. Impalements were accepted when a sudden negative change in voltage was kept constant for at least 1 min at the point the *Em* had stabilized and returned instantaneously to the previous voltage level after the withdrawal of microelectrode. Impalements were performed from the adventitial side for experiments in endothelium-denuded vessels.

2.5. Drugs

Fluo-4 AM, pluronic acid, nifedipine, capsazepine, HC067047, paxilline, and SKF-96365 were solubilized in DMSO. The DMSO final concentration did not exceed 0.01%. 2-aminoethoxydiphenyl borate (2-APB) was solubilized in methanol. Phenylephrine, acetylcholine, caffeine, and ruthenium red (RuR) were solubilized in water. Drugs were acquired from Sigma-Aldrich.

2.6. Data analysis

Results are expressed as means ± SEM. GraphPad Prism 7.01 was used to analyze data. Paired Student's *t*-test analyzed TC and Ca^{2+}-fluorescence parameters such as peak response and area under the curve (AUC). *Em* measurements were analyzed by two-way ANOVA, followed by Fisher's LSD post-test. Data were considered significantly different when $P < 0.05$. The area under the curve was performed using GraphPad Prism 6 and was created by connecting the responses

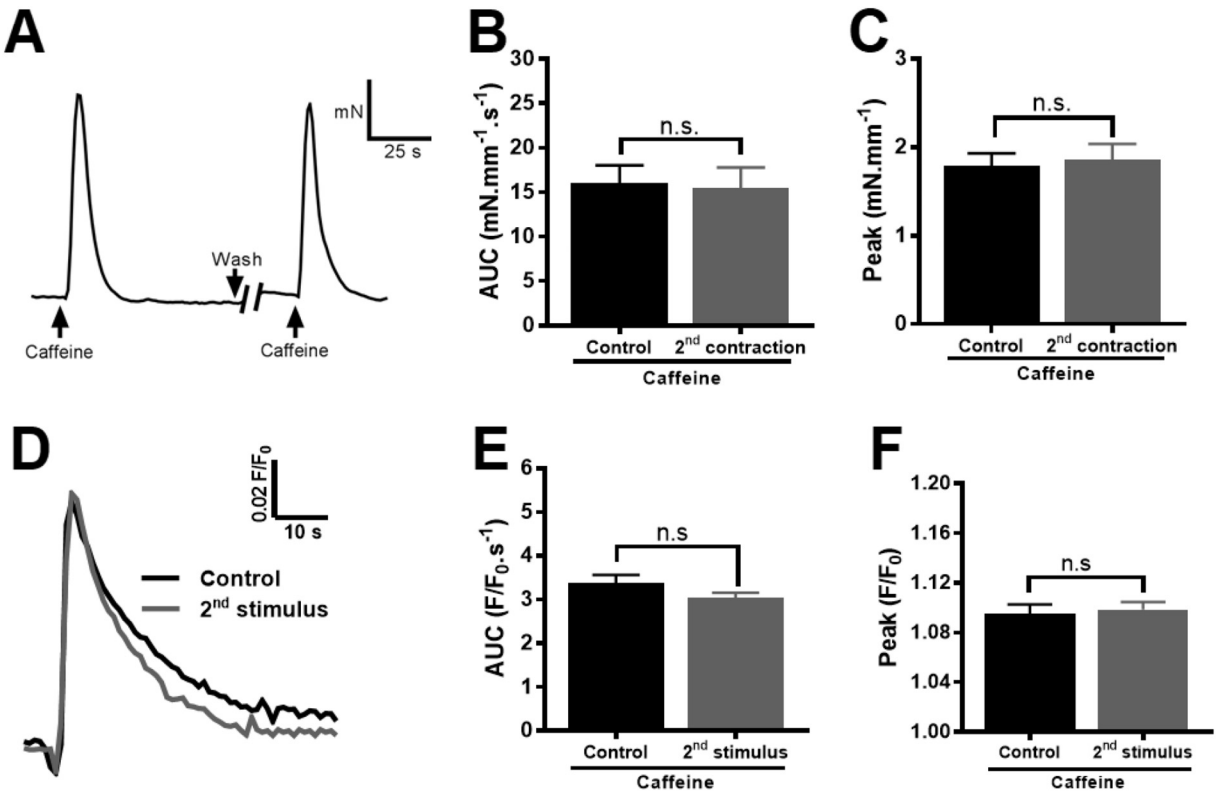

Fig. 1. TC and $_{i}Ca^{2+}$ signal stimulated with caffeine (10 mM) in mouse mesenteric artery. A to C show trace, AUC, and the peak of the contraction, respectively. D to F show trace, AUC, and the peak of the $_{i}Ca^{2+}$ signal. Data expressed as mean ± S.E.M. of 6 and 3 mice for contraction and $_{i}Ca^{2+}$ signal, respectively. N.S. (non-significant).

with straight lines. Its units are the X units (seconds) times the Y units (mN/mm and F/F_0, for contraction and calcium, respectively). We considered Y = 0 for baseline for the contractile response and Y = 1 for F/F_0.

3. Results

3.1. Caffeine induces a TC dependent on Ca^{2+}-influx

Application of caffeine (10 mM) in mesenteric arteries caused TC and increased the $_{i}Ca^{2+}$ signal (Fig. 1). The entire duration of the TC was 26.3 ± 2.7 s. A similar effect was observed after the 2nd stimulus with caffeine (Fig. 1). In Ca^{2+}-free solution, the peak and the AUC of the TC, and the $_{i}Ca^{2+}$ signal were significantly reduced (Fig. 2).

3.2. Ca^{2+}-influx through $Ca_v1.2$ downregulates caffeine-induced TC

To investigate the participation of $Ca_v1.2$ channels in the caffeine-induced TC, arterial rings were pre-treated with nifedipine (10 μM), a $Ca_v1.2$ blocker. In this experimental condition, the AUC and the peak of TC were surprisingly increased (Fig. 3A–C) despite the reduction in the $_{i}Ca^{2+}$ signal (Fig. 3D–F). It is important to note that these results suggest that the Ca^{2+}-influx through $Ca_v1.2$ does not contribute to the upregulation of the TC induced by caffeine. Instead it seems to downregulate the contraction.

Consequently, the next step was directed to the investigation of the mechanism involved in the modulation of the TC associated with Ca^{2+}-influx through $Ca_v1.2$ channel. In this sense, paxilline (1 μM), a BK_{Ca} blocker, also increased the AUC but did not change the peak of the TC (Fig. 4).

3.3. The caffeine-induced TC activates TRPs

The blockage of TRPCs with 2-APB (10 μM), a non-selective TRPC blocker, increased the AUC and the peak of TC, despite reducing the AUC and peak of $_{i}Ca^{2+}$ signal (Fig. 5). Also, the inhibition of TRPVs with RuR (10 μM), a non-selective TRPV blocker, increased the AUC of the TC while reduced the peak of $_{i}Ca^{2+}$signal (Fig. 6). The selective blockade of TRPV1 with capsazepine (10 μM) increased the AUC but did not change the peak of the TC induced by caffeine (Fig. 7A–C), while the selective blockage of TRPV4 with HC-067047 (10 μM) did not change the TC (Fig. 8D–F).

3.4. Caffeine modulates Em through cross-talk between $Ca_v1.2$-TRP-BK_{Ca} channels

The caffeine-induced TC stimulates a hyperpolarization of PM on the VSMCs from mice mesenteric arteries. In the presence of nifedipine, the hyperpolarization was blunted in vessels stimulated with caffeine (Fig. 8A–B). Similar inhibition of the caffeine-induced hyperpolarization was observed in vessels pre-treated with 2-APB and paxilline (Fig. 8C). These results suggest the existence of a relationship between $Ca_v1.2$, TRPs, and BK_{Ca} associated with the caffeine-induced TC.

3.5. The blockage of Orai decreases the TC induced by caffeine

To evaluate the participation of STIM-Orai system, vessels were pre-treated with SKF-96365 (30 μM), a non-selective Orai/TRPC blocker. In the presence of SKF-96365, the AUC and the peak of TC were blunted (Fig. 9A–C). The AUC and peak of the $_{i}Ca^{2+}$ signal were also reduced (Fig. 9D–E), but not to the same extent as the TC. These results indicate the importance of Ca^{2+}-entry via Orai to refilling the Ca^{2+} stores participating in the TC induced by caffeine.

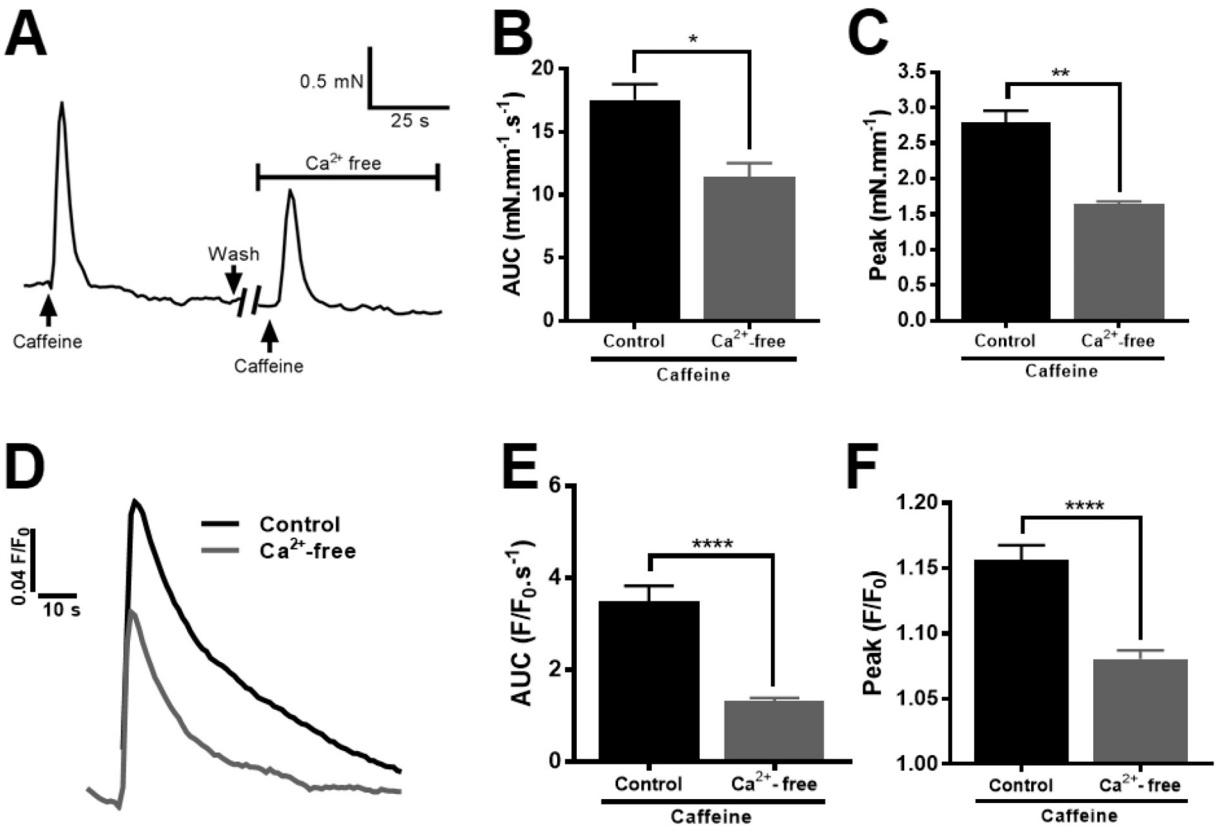

Fig. 2. TC and $_{i}Ca^{2+}$ stimulated with caffeine (10 mM) in mouse mesenteric artery kept in Ca^{2+}-free solution. A to C show trace, AUC, and peak of the contraction, respectively. D to F show the trace, AUC, and peak of the $_{i}Ca^{2+}$ signal. Data expressed as mean ± S.E.M. of 5 mice and 3 mice for contraction and $_{i}Ca^{2+}$ signal, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{****}P < 0.0001$ versus control.

4. Discussion

In the present study, the mechanism involved in the control of the TC induced through the activation of RyRs by caffeine in VSMCs of mesenteric arteries was investigated. The results demonstrate that caffeine stimulated negative feedback of the TC, involving Ca^{2+}-influx via $Ca_v1.2$ and TRP channels, followed by BK_{Ca} activation (Fig. 10).

Caffeine is a well-known activator of RyRs that, in millimolar concentrations, induces a TC and transient increase in the $_{i}[Ca^{2+}]$ via Ca^{2+}-wave in VSMCs [5,13]. The present work showed that the caffeine-induced TC is dependent of Ca^{2+}-influx in mice mesenteric arteries, as confirmed by the reduction of the TC and $_{i}Ca^{2+}$ signal in the absence of extracellular Ca^{2+}. This is a surprising observation, considering that the contraction induced by caffeine is regarded as dependent only on the release of $_{i}Ca^{2+}$ stores. Therefore, the next step was to investigate the channels involved in the Ca^{2+}-influx induced by caffeine.

Ca^{2+}-influx through $Ca_v1.2$ is essential to myogenic tone and contraction. Previous works demonstrated that myogenic tone was markedly reduced in the presence of $Ca_v1.2$ blockers [39]. The contraction induced by other agents is also dependent on Ca^{2+}-influx through $Ca_v1.2$ and TRPs channels [36,38]. It is well established in the literature that $Ca_v1.2$ may colocalize with Orai1 and TRPC1. However, the SR stimulation with thapsigargin enhances this co-localization in aorta from mice [40]. Our results with nifedipine suggest that Ca^{2+}-release after stimulation of RyR induced a Ca^{2+}-influx via $Ca_v1.2$, which did not contribute to the global iCa^{2+} involved in the upregulation of TC induced by caffeine. Actually, the global cytosolic Ca^{2+} is reduced in the presence of nifedipine, while the TC induced by caffeine was increased, suggesting the participation of the Ca^{2+}-influx through $Ca_v1.2$ on a negative feedback mechanism modulating the level of TC. A cross-talk involving the activation of $Ca_v1.2$ channels and a negative feedback mechanism has previously been described in SMC from resistance arteries [41]. In a previous report, Ca^{2+}-influx via $Ca_v1.2$ channel activates RyR, inducing Ca^{2+}-sparks with consequent stimulation of BK_{Ca} channels [41]. However, in the present work, the activation of RyR stimulated Ca^{2+}-influx through $Ca_v1.2$ triggering a negative feedback mechanism on caffeine-induced TC. The next question is if other channels could contribute to Ca^{2+} influx involved in the contraction induced by caffeine.

Ca^{2+}-influx through TRPs is essential for the contraction of VSMCs. TRPC family is identified to be regulated by store depletion [42,43]. TRPC1 and BK_{Ca} are colocalized on the same subcellular regions in VSMCs, where Ca^{2+} influx through TRPC1 activates BK_{Ca} to induce hyperpolarization [44]. In this sense, our results showed an increased TC in the presence of a TRPC blocker, while a reduction in the $_{i}Ca^{2+}$ occurred. Moreover, Ca^{2+}-influx through TRPCs can activate BK_{Ca}, since 2-APB, a TRPC blocker, abolished the membrane hyperpolarization induced by caffeine. Another hypothesis is that Na^{+}-influx via TRPC may induce membrane depolarization [27], activating $Ca_v1.2$, and the mechanism of negative feedback of the contraction. Although our recordings did not show a caffeine-induced depolarization followed by hyperpolarization, we believe that this may be a consequence of the frequency of acquisition of our *Em* recordings (about 2 ms). Therefore, TRPCs may contribute to the Ca^{2+}-influx induced by caffeine in SMC from mesenteric arteries, but did not participate in the upregulation of the contractile response. Actually, the Ca^{2+}-influx through TRPCs seems to be downregulating the contractile response to caffeine.

TRPV channels are important sensors of physiological and biochemical stimuli [31]. TRPV4 forms a complex Ca^{2+} signaling with RyRs and BK_{Ca} channels promoting smooth muscle hyperpolarization and arterial relaxation via Ca^{2+}-release [45]. The present work

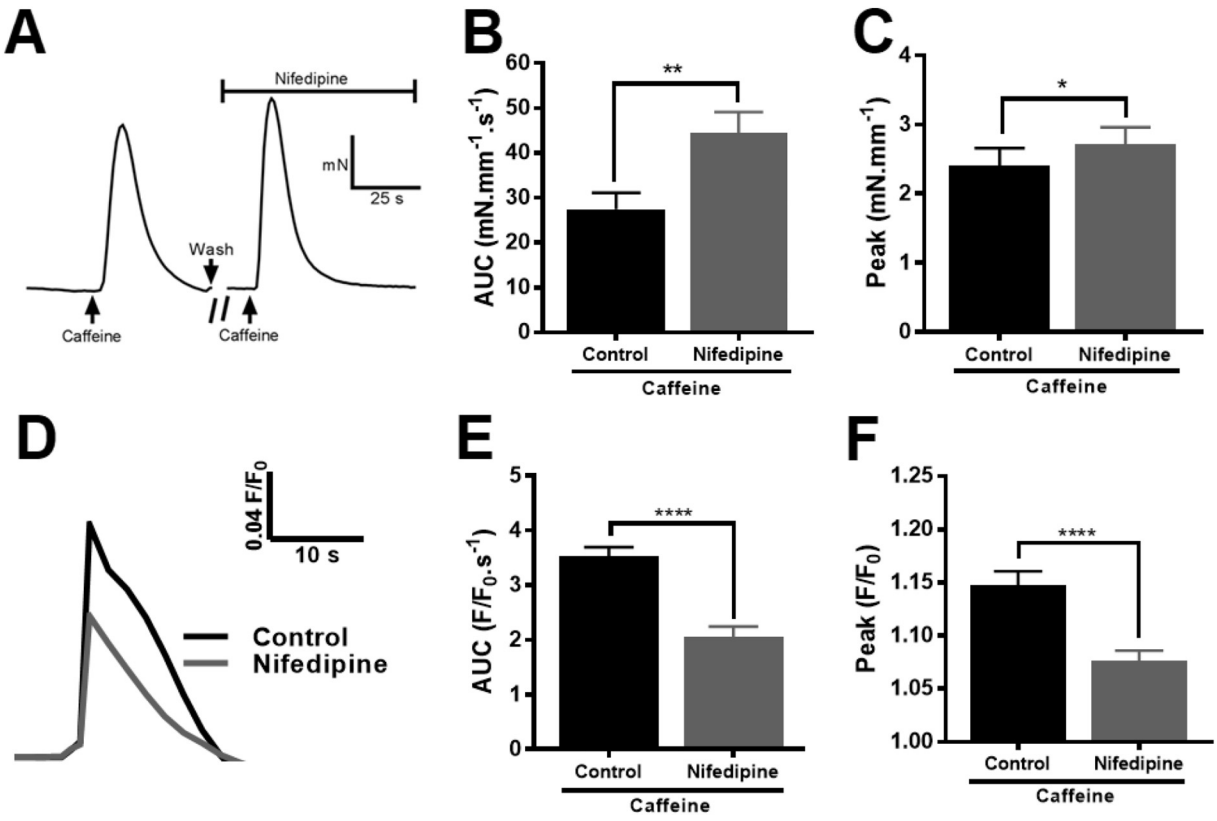

Fig. 3. TC and Ca^{2+}-fluorescence stimulated by caffeine (10 mM) in the presence of nifedipine (10 μM) in mouse mesenteric artery. A to C show the trace, AUC, and peak of the contraction, respectively. D to F show the trace, AUC, and peak of the $_{i}Ca^{2+}$ signal. Data expressed as mean ± S.E.M. of 6 and 3 mice for contraction and $_{i}Ca^{2+}$ signal, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{****}P < 0.0001$ versus control.

demonstrated that the TRPV blocker (RuR) increased the AUC of the TC, while reduced the $_{i}Ca^{2+}$ signal. These results suggest that Ca^{2+}-influx via TRPVs was activated, but not involved in the induction of the contractile response, in a similar way as observed for the Ca^{2+}-influx via $Ca_v1.2$ and TRPCs. In addition, the selective blockage of TRPV1 with capsazepine also increased the AUC of the TC induced by caffeine, while the selective blockage of TRPV4 did not change the TC. Therefore, TRPV1 seems to be the channel involved in the activation of the negative feedback of the TC induced by caffeine. Consequently, the next question was what mechanism could be involved in the downregulation of the contraction induced by caffeine where the Ca^{2+}-influx through Cav1.2 and TRPs could activate. Thus, the next step was to investigate the participation of Ca^{2+}-activated potassium channels.

The activation of BK_{Ca} by TRPs and $Ca_v1.2$ has been previously reported in VSMC of resistance arteries [33,41,43]. Results in the present work led us to hypothesize that after stimulation of RyR, $Ca_v1.2$- and TRPs-dependent Ca^{2+}-influx are responsible for BK_{Ca} activation. The increased AUC of the TC in the presence of paxilline, in a similar way as observed with nifedipine, 2-APB, and RuR confirmed this hypothesis. Moreover, paxilline, nifedipine, and 2-APB abolished the hyperpolarization induced by caffeine. These findings highlight that Ca^{2+} release via RyR indirectly activated $Ca_v1.2$ and TRPs, which in turn activated BK_{Ca} and the mechanism of negative feedback of the TC induced by caffeine (Fig. 10). This complex mechanism might be explained by iCa^{2+} microdomains in which the proximity and Ca^{2+} sensitivity of individual elements lead to stringent regulation of Ca^{2+} influx and changes in global cytosolic Ca^{2+} [46]. Also, it could be explained by the frequency and amplitude of Ca^{2+} release via RyR that is different when we compare Ca^{2+}-sparks or Ca^{2+}-waves [12]. In this sense, discrete events as Ca^{2+}-sparks (small elevation of Ca_i^{2+} through

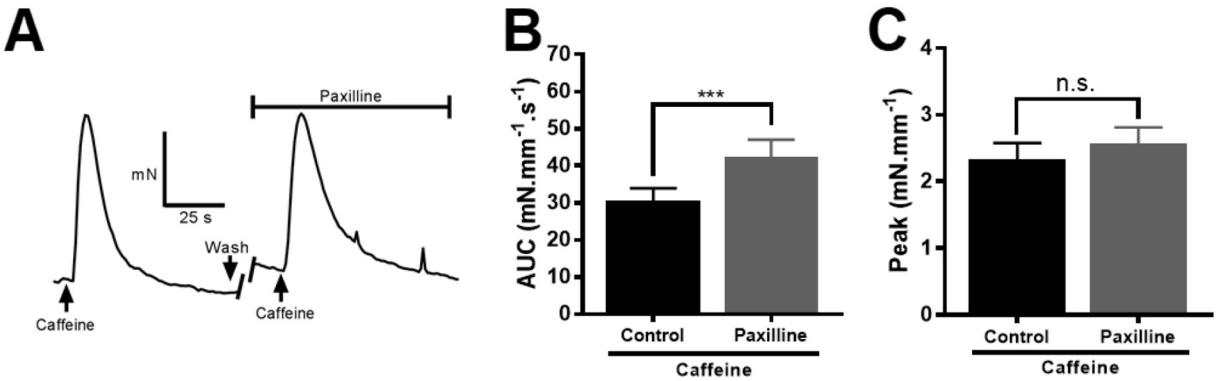

Fig. 4. TC stimulated by caffeine (10 mM) in the presence of paxilline (1 μM). A to C show trace, AUC, and peak of the contraction, respectively - Data expressed as mean ± S.E.M. of 8 mice. N.S. (non-significant); $^{***}P < 0.001$ versus control.

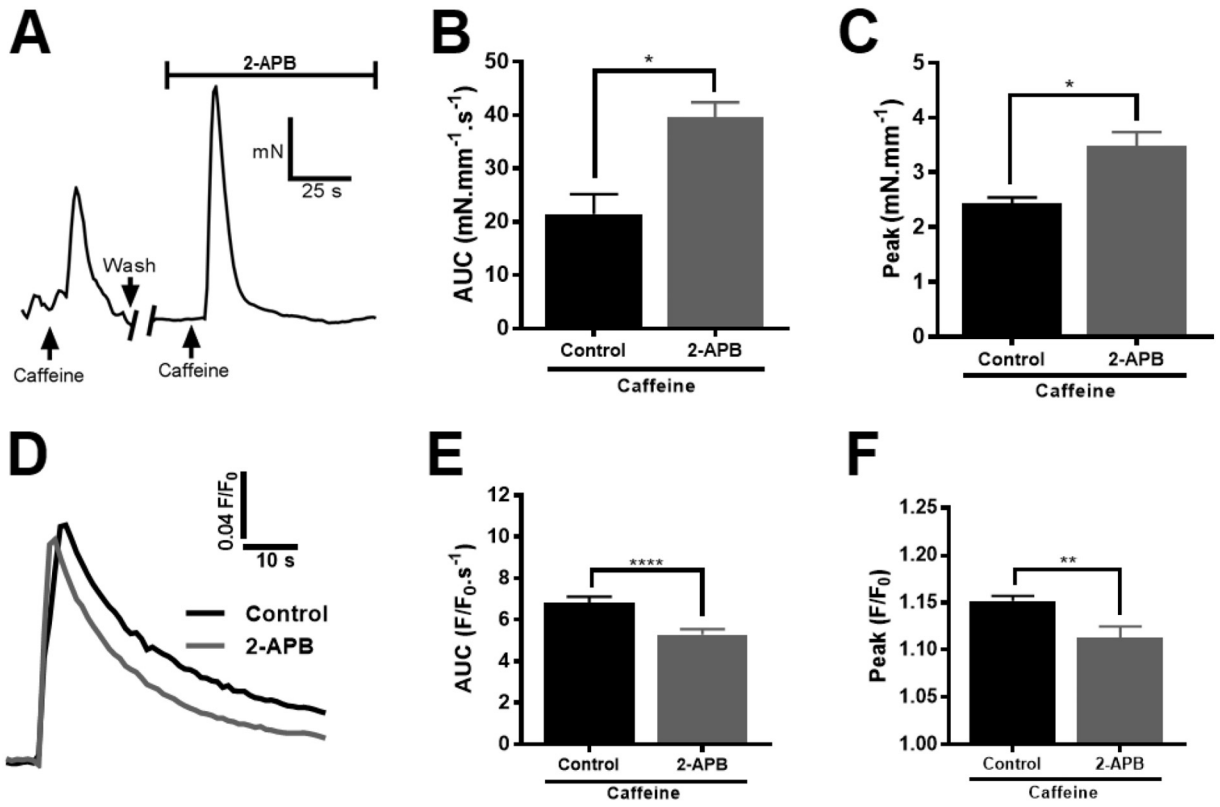

Fig. 5. TC and $_{i}Ca^{2+}$-stimulated by caffeine (10 mM) in the presence of 2-APB (10 μM) in mouse mesenteric artery. A to C show the trace, AUC, and peak of the contraction, respectively. D to F show the trace, AUC, and peak of the $_{i}Ca^{2+}$ signal. Data expressed as mean ± S.E.M. of 5 and 3 mice for contraction and $_{i}Ca^{2+}$ signal, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ versus control.

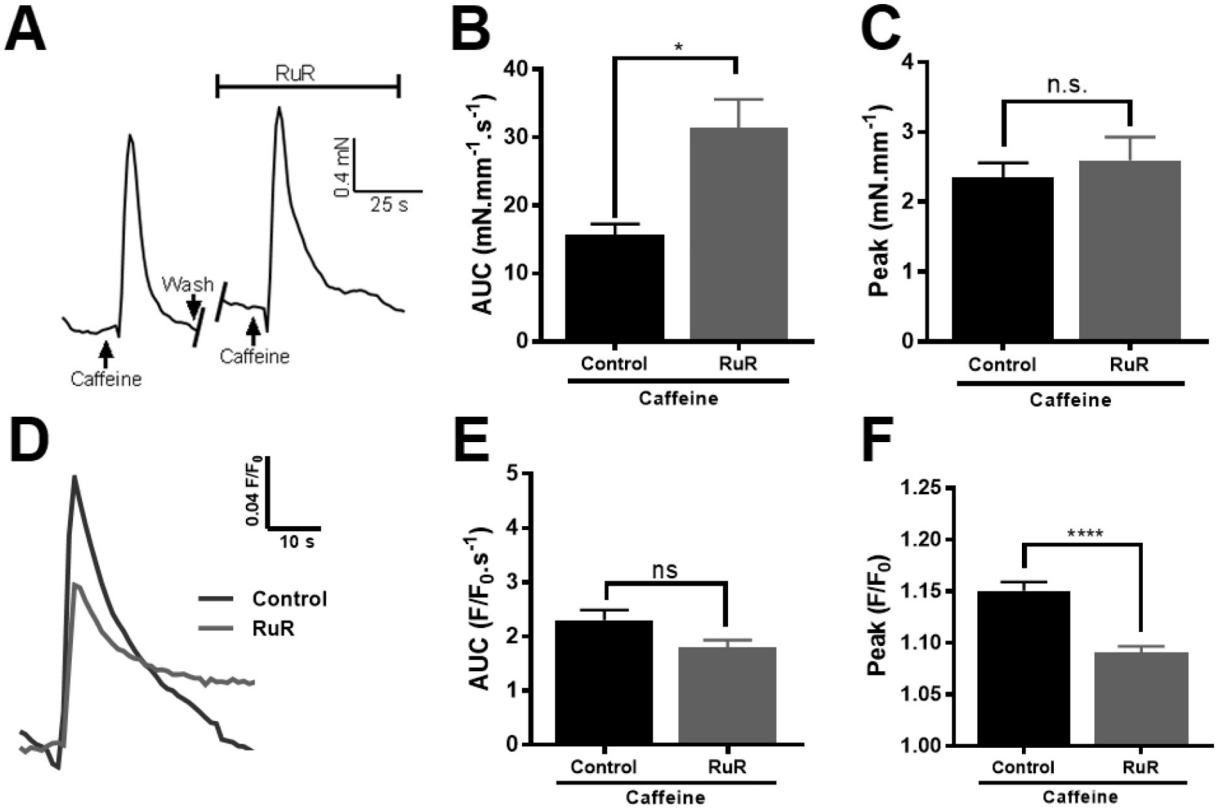

Fig. 6. TC and $_{i}Ca^{2+}$ stimulated by caffeine (10 mM) in the presence of RuR (10 μM) in mouse mesenteric artery. A to C show trace AUC and peak of the contraction, respectively. D to F show trace, AUC, and peak of $_{i}Ca^{2+}$ signal, respectively. Data expressed as mean ± S.E.M. of 5 and 3 mice for contraction and $_{i}Ca^{2+}$ signal, respectively. N.S. (non-significant); $^{*}P < 0.05$, $^{****}P < 0.0001$ versus control.

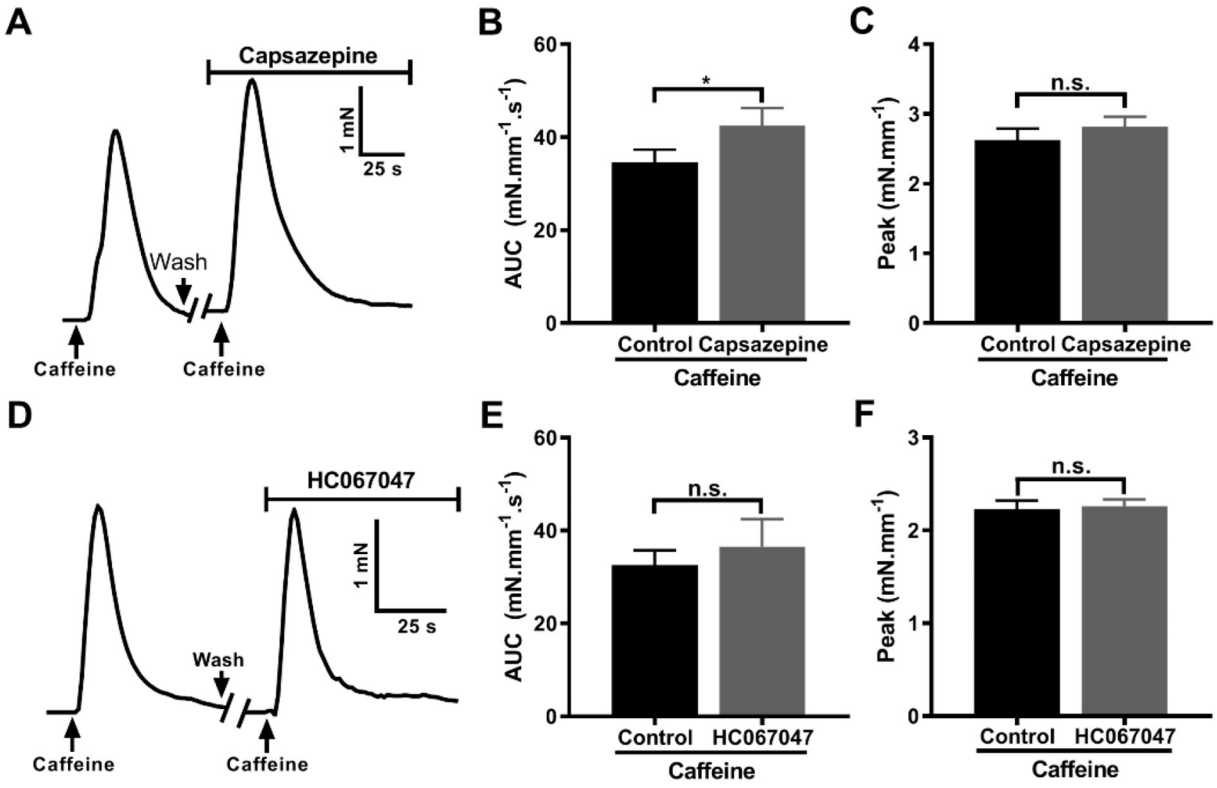

Fig. 7. TC stimulated by caffeine (10 mM) in the presence of capsazepine (10 μM) and HC067047 (10 μM) in mouse mesenteric artery. A to C show the respective trace, AUC, and the peak of the contraction in the absence and presence of capsazepine. D to F show the respective trace, AUC, and the peak of the contraction in the absence and presence of HC067047. Data expressed as mean ± S.E.M. of 5 mice. N.S. (non-significant); $^{*}P < 0.05$ versus control.

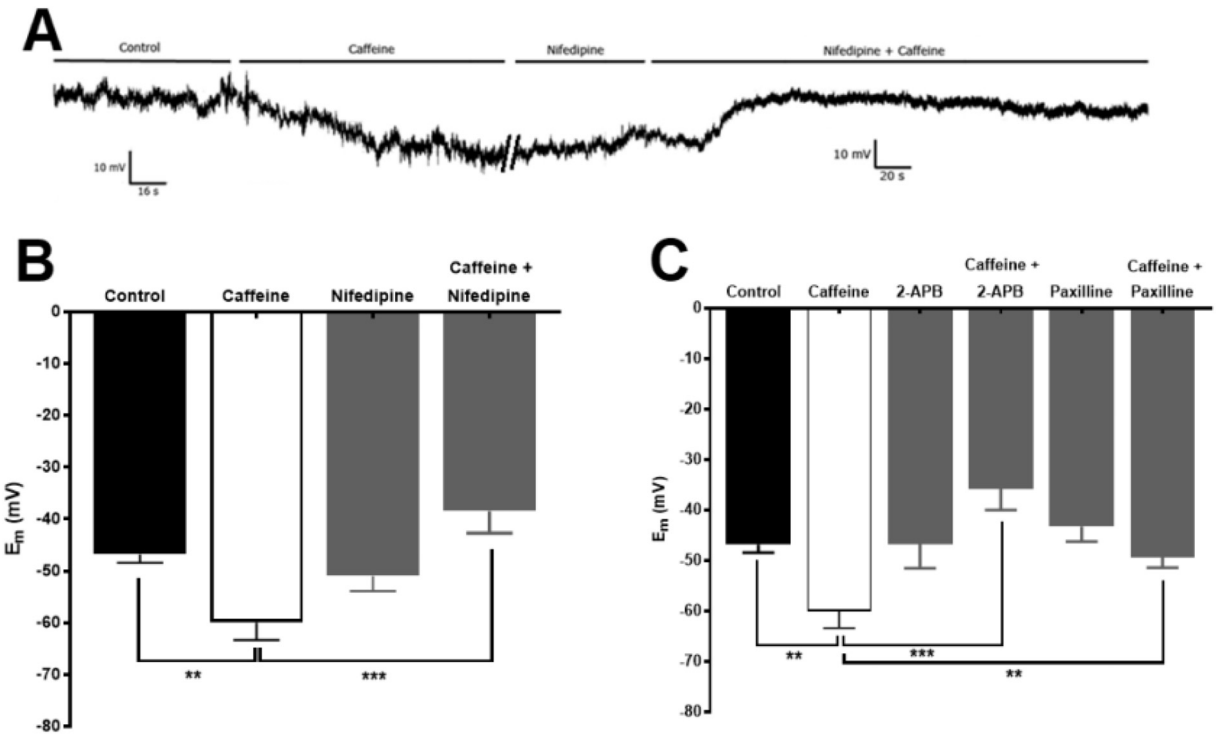

Fig. 8. *Em* in the presence of caffeine (10 mM) with nifedipine (10 μM), paxilline (1 μM) or 2-APB (10 μM) in mouse mesenteric artery. Data expressed as mean ± S.E.M. of 4 mice. $^{**}P < 0.01$ versus control. $^{**}P < 0.01$, and $^{***}P < 0.001$ versus caffeine.

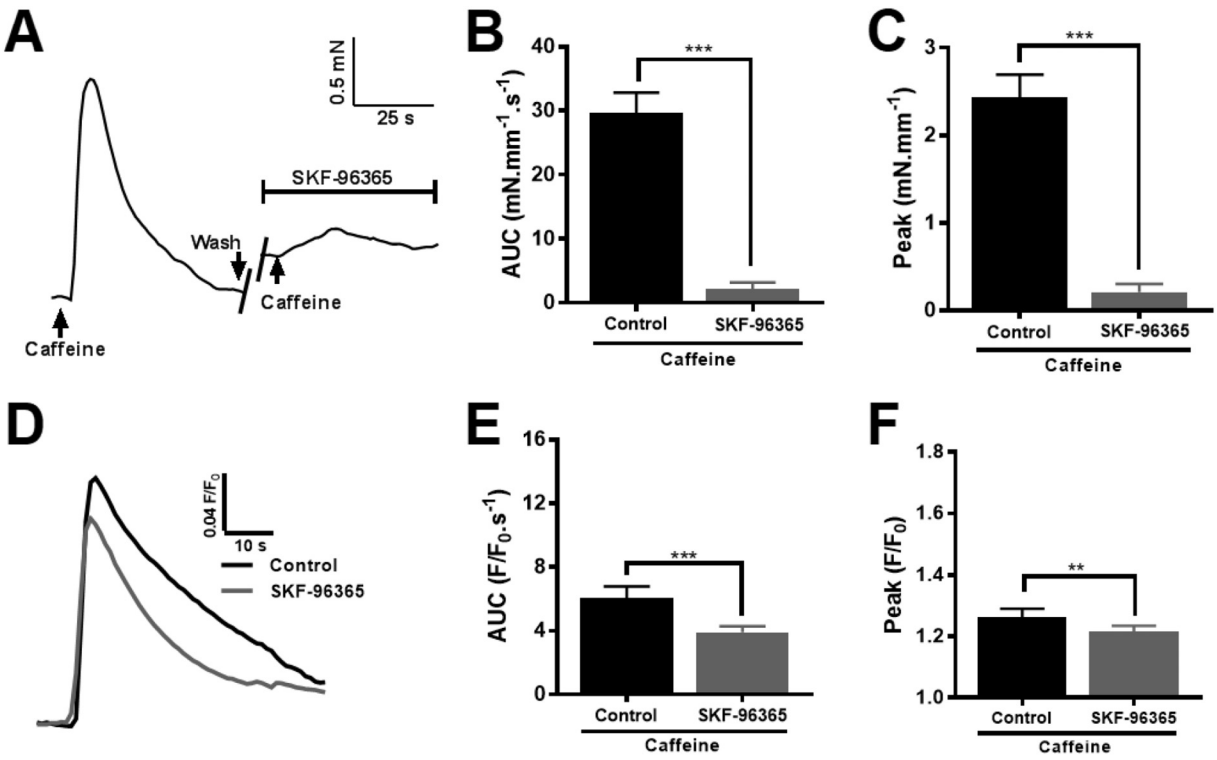

Fig. 9. TC and $_{i}Ca^{2+}$ stimulated by caffeine (10 mM) in the presence of SKF-96365 (30 μM) in mouse mesenteric artery. A to C show trace, AUC, and peak of the contraction, respectively. D to F show trace, AUC, and peak of $_{i}Ca^{2+}$ signal, respectively. Data expressed as mean ± S.E.M. of 5 and 3 mice for contraction and $_{i}Ca^{2+}$ signal, respectively. $^{**}P < 0.01$, $^{***}P < 0.001$ versus control.

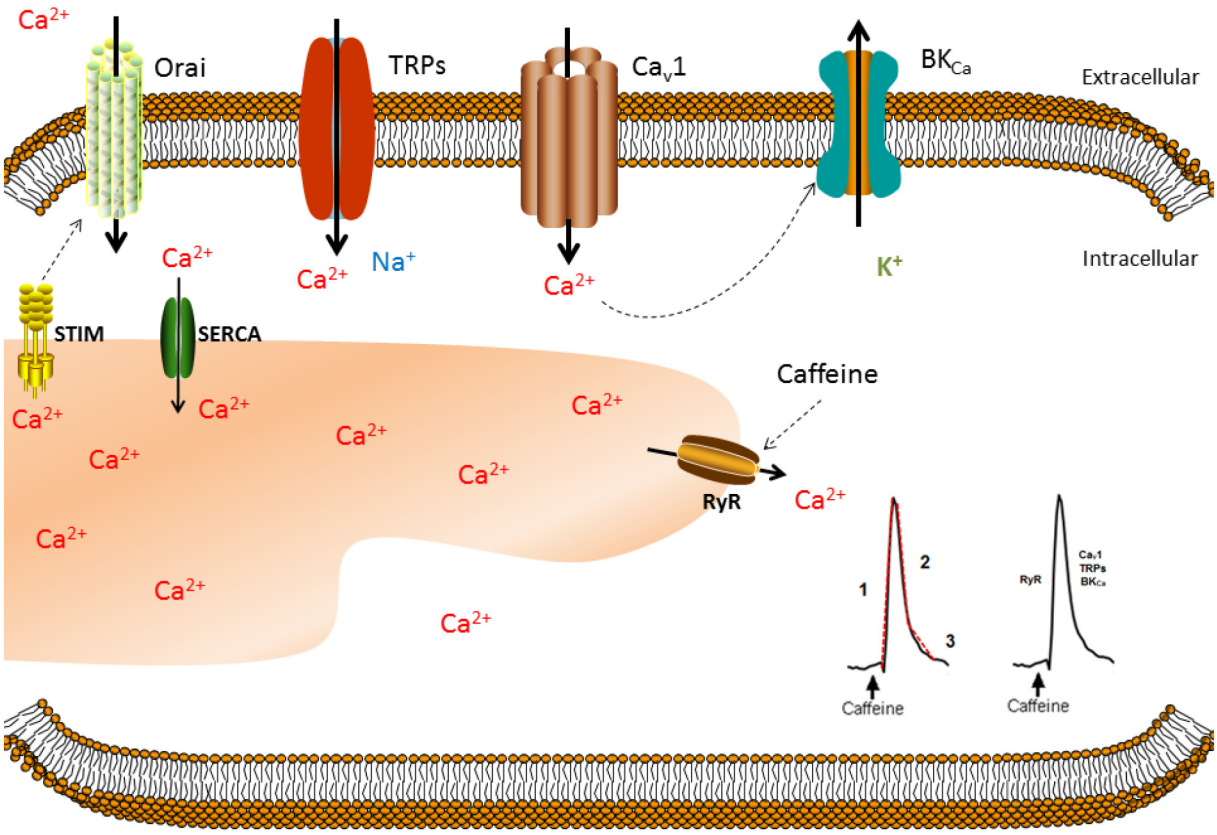

Fig. 10. Mechanism of contraction triggered by caffeine in VSMCs. Caffeine is a RyR agonist that promotes Ca^{2+}-release from SR activating the contractile machinery to induces TC (phase 1). SR stimulation promotes Ca^{2+}-influx via Ca_v1 and transient receptor potential (TRPs) increasing the global Ca^{2+} and been responsible for BK_{Ca} channel activation on crosstalk that prevents excessive contraction (phases 2 and 3).

Ca^{2+} release) or Ca^{2+}-sparklets (small elevation of Ca_i^{2+} through Ca^{2+} influx) could be able to activate BKCa [33,45,47], unlike large events as Ca^{2+}-waves.

STIM-Orai system is responsible for refilling the Ca^{2+} stores in the SR when Ca^{2+} release is evoked by the stimulation of inositol trisphosphate receptor (IP_3R) or RyR [48]. Our results showed that after the blockage of Orai with SKF-96365, the TC was blunted, while the $_iCa^{2+}$ was reduced. It is interesting to note that the inhibition of the contraction induced by caffeine was intensely higher than the reduction in the cytosolic Ca^{2+}. This observation suggests that the refilling of the SR via the STIM-Orai system is essential for the release of Ca^{2+} involved in the contraction induced by caffeine. It reinforces the significance of results obtained in a Ca^{2+}-free solution that showed the importance of extracellular Ca^{2+} for the TC induced by caffeine in VSMCs.

5. Conclusion

This work demonstrates that the activation of Ca^{2+}-influx via $Ca_v1.2$ and TRP channels modulate the contractile response to caffeine in mesenteric arteries. The activation of these channels is involved in an intricate mechanism of downregulation of the contraction through the activation of BK_{Ca}. The evidence of downregulation of contraction via TRP-$Ca_v1.2$-BK_{Ca} provides a new perspective for understanding the mechanism involved in the vascular responses triggered by caffeine (Fig. 10).

Author contributions

The contributions of each author to the study were: Daniela C. G. Garcia[a,b,c,d], Miguel J. Lopes[a,c], Ulrich C. Mbiakop[a,c], Virgínia S. Lemos[b,d], Steyner F. Côrtes[b,d].

a. Acquisition of data; b. Substantial contribution to conception and design; c. Analysis and interpretation of data; d. Drafting the article, revising it critically for important intellectual content and final approval of the version to be published.

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Declaration of Competing Interest

The authors state that there are no conflicts of interest.

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