Metabotropic Regulation of RhoA/Rho-Associated Kinase by L-type Ca\(^{2+}\) Channels

New Mechanism for Depolarization-Evoked Mammalian Arterial Contraction

Miguel Fernández-Tenorio, Cristina Porras-González, Antonio Castellano, Alberto del Valle-Rodríguez, José López-Barneo, Juan Ureña

Background: Sustained vascular smooth muscle contraction is mediated by extracellular Ca\(^{2+}\) influx through L-type voltage-gated Ca\(^{2+}\) channels (VGCC) and RhoA/Rho-associated kinase (ROCK)-dependent Ca\(^{2+}\) sensitization of the contractile machinery. VGCC activation can also trigger an ion-independent metabotropic pathway that involves G-protein/phospholipase C activation, inositol 1,4,5-trisphosphate synthesis, and Ca\(^{2+}\) release from the sarcoplasmic reticulum (calcium channel-induced Ca\(^{2+}\) release). We have studied the functional role of calcium channel-induced Ca\(^{2+}\) release and the inter-relations between Ca\(^{2+}\) channel and RhoA/ROCK activation.

Methods and Results: We have used normal and genetically modified animals to study single myocyte electrophysiology and fluorimetry as well as cytosolic Ca\(^{2+}\) and diameter in intact arteries. These analyses were complemented with measurement of tension and RhoA activity in normal and reversibly permeabilized arterial rings. We have found that, unexpectedly, L-type Ca\(^{2+}\) channel activation and subsequent metabotropic Ca\(^{2+}\) release from sarcoplasmic reticulum participate in depolarization-evoked RhoA/ROCK activity and sustained arterial contraction. We show that these phenomena do not depend on the change in the membrane potential itself, or the mere release of Ca\(^{2+}\) from the sarcoplasmic reticulum, but they require the simultaneous activation of VGCC and the downstream metabotropic pathway with concomitant Ca\(^{2+}\) release. During protracted depolarizations, refilling of the stores by a residual extracellular Ca\(^{2+}\) influx through VGCC helps maintaining RhoA activity and sustained arterial contraction.

Conclusions: These findings reveal that calcium channel-induced Ca\(^{2+}\) release has a major role in tonic vascular smooth muscle contractility because it links membrane depolarization and Ca\(^{2+}\) channel activation with metabotropic Ca\(^{2+}\) release and sensitization (RhoA/ROCK stimulation). (Circ Res. 2011;108:1348-1357.)

Key Words: calcium channels • sarcoplasmic reticulum • vascular smooth muscle • vasoconstriction

Cardiovascular diseases secondary to alterations in the arterial diameter constitute an important cause of morbidity and mortality in humans; however, the mechanisms that determine vascular smooth muscle (VSM) contractility are not fully understood. Myocyte contraction is triggered by the increase of cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) because of Ca\(^{2+}\) influx through ion channels in the plasmalemma, Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR), or both. Besides exerting direct control of myosin light chain phosphorylation, cytosolic Ca\(^{2+}\) also influences VSM contraction through enhancement of the sensitivity of the contractile apparatus, a process called Ca\(^{2+}\) sensitization that is mediated by the small monomeric G-protein RhoA and its target Rho-associated kinase (ROCK). The RhoA/ROCK pathway, presumed to be involved in the pathogenesis of major vascular disorders, is turned on by excitatory agonists through G-protein-coupled receptor stimulation and it also seems to be activated by membrane depolarization. Nonetheless, the relationships between membrane voltage changes, cellular Ca\(^{2+}\) regulation, and RhoA activation remain obscure. We have hypothesized that activation of voltage-dependent Ca\(^{2+}\) channels and RhoA could share some common mechanisms.

Ca\(^{2+}\) influx through voltage-gated L-type Ca\(^{2+}\) channels (VGCC) is a major event in VSM excitation–contraction coupling. Depolarization is also known to induce Ca\(^{2+}\) release from the SR independently of extracellular Ca\(^{2+}\) influx (depolarization-induced Ca\(^{2+}\) release) by means of a metabotropic pathway that involves G-protein/phospho-

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lipase C (PLC) activation and inositol 1,4,5-trisphosphate (InsP₃) synthesis. Although other membrane proteins in the receptor/G-protein/PLC cascade have been proposed to be voltage-sensitive, it has been shown that L-type Ca²⁺ channels are necessary for depolarization-induced Ca²⁺ release in vascular and skeletal muscle. Therefore, plasmalemmal Ca²⁺ channels in VSM have been suggested to act as voltage sensors that mediate ion-independent coupling between membrane depolarization and SR Ca²⁺ release (CCICR). Treatment of the arteries with cyclopiazonic acid, an ionotropic (Ca²⁺) channel inhibitor, selectively reduced the tonic component of contractions evoked by either high K⁺ or ATP (Figure 1A and 1B). These phasic and tonic components were also observed in contractions evoked by application of ATP, a vasoactive agent that induces activation of VGCC.14,18 The high K⁺ solution shifted the membrane potential of basilar VSM cells from -50 mV to -20 mV. Depolarization with 70K triggered an isometric contraction that showed an initial fast and transient (phasic) component, followed by a slowly increasing (tonic) component, which was generally maintained until the end of the stimulus (Figure 1A and 1B). These phasic and tonic components were also observed in contractions evoked by application of ATP, a vasoactive agent that induces activation of VGCC14,18. Treatment of the arteries with cyclopiazonic acid (CPA), an SR Ca²⁺ ATPase inhibitor that depletes Ca²⁺ stores without effects on VGCC (Figure IIA and IIB), selectively reduced the tonic component of contractions evoked by either high K⁺ or ATP (Figure 1A and 1B). Similar results (decrease of the sustained phase of arterial contraction) were observed with two other drugs (thapsigargin and ryanodine) that also reduce Ca²⁺ storage in SR (Online Figure IIC and IID). These data indicated that the sustained component of depolarization-evoked VSM contraction depends on Ca²⁺ release from intracellular stores, possibly as a consequence of CCICR. CCICR is inhibited by organic VGCC antagonists14,18 hence, we tested the effect of nifedipine on depolarization-evoked contractions. Nifedipine was more potent to inhibit the tonic than the phasic components of contractions. Nifedipine (10 nmol/L) produced a marked reduction of the high K⁺-evoked or ATP-evoked tonic contraction leaving intact the phasic period. At higher concentrations of nifedipine (100 nmol/L), the two phases of contraction were abolished (Figure 1C and 1D; Online Figure IIE and IIF). These results suggest that VSM cell contraction elicited by cell depolarization depends on both ionotropic (Ca²⁺ entry) and metabotropic (CCICR) actions of plasmalemmal Ca²⁺ channels.

To test whether depolarization-evoked sustained VSM contraction does involve metabotropically induced Ca²⁺ release,
arterial rings were exposed to U73122, an inhibitor of PLC. This treatment left unaffected the phasic component of the 70K-evoked contraction but markedly reduced the tonic component. U73343, a structurally closely related compound that does not inhibit PLC, did not significantly affect VSM contractility (Figure 2A). Similar results were obtained in basilar arterial rings contracted with ATP and treated with U73122 (Figure 2B). The effect of U73122 was mediated by changes in $[Ca^{2+}]_{i}$, because in isolated myocytes U73122 decreased the sustained phase of the 70K-induced $[Ca^{2+}]_{i}$ signal, with no significant effect.
on the transient component (Online Figure IIIA through IIID). Further support for the notion that the G-protein/PLC pathway participates in depolarization-induced tonic contractions came from experiments on intact arteries in which myocytes had been reversibly permeabilized in the presence of GDP\textsubscript{S} to inhibit G-protein activation.\textsuperscript{14,23,24} In these arteries, the tonic component of the 70K-evoked VSM contraction was also selectively reduced, whereas the phasic component of contraction and the mechanical signal induced by caffeine (a drug that directly elicits Ca\textsuperscript{2+} release from the SR) were unaltered (Figure 2C and 2D). Together, these results further supported the notion that stimulation of the G-protein/PLC cascade subsequent to membrane depolarization and Ca\textsuperscript{2+} channel activation leads to the SR Ca\textsuperscript{2+} release necessary for maintained VSM contraction.

**Sustained Depolarization-Evoked Contraction Requires G-Protein-Dependent and InsP\textsubscript{3}-Dependent RhoA/ROCK Activation**

In VSM, a Ca\textsuperscript{2+} sensitization mechanism mediated by the RhoA/ROCK pathway seems to participate in the depolarization-evoked contraction; however, the underlying mechanisms are not well-known.\textsuperscript{6–10} We tested in immunostained isolated myocytes the redistribution of activated RhoA and its displacement toward the plasma membrane in response to high K\textsuperscript{+} induced depolarization (Figure 3A through 3C).\textsuperscript{9,27} Because ROCK is a RhoA downstream effector, we studied in intact pressurized arteries the effect of Y27632, a ROCK inhibitor,\textsuperscript{1} on the cytosolic Ca\textsuperscript{2+} and mechanical responses to high K\textsuperscript{+}. ROCK blockade did not modify the Ca\textsuperscript{2+} signal elicited by depolarization but selectively inhibited the tonic component of the contraction (Figure 3D). Qualitatively similar results (inhibition of the tonic phase of contraction) were obtained in arterial rings treated with HA1077, another ROCK inhibitor structurally unrelated to Y27632\textsuperscript{1} (Online Figure IIIE and IIIF). These experimental observations further suggested that the depolarization-evoked sustained phase of contraction requires, at least in part, RhoA/ROCK activation.

To show the parallel metabotropic regulation of tonic contraction and RhoA activation, we performed experiments in reversibly permeabilized arteries (Figure 2C and 2D) that were frozen at the end of the depolarization pulse (≈10 minutes after exposure to high K\textsuperscript{+}) to directly measure RhoA activity. Application of GDP\textsubscript{S} and heparin (inhibitors of G-protein activation and InsP\textsubscript{3} receptors, respectively) selectively reduced the tonic component of the 70K-evoked contractions and also decreased RhoA activity induced by depolarization (Figure 4; Online Figure IC). These observations supported the view that metabotropic Ca\textsuperscript{2+} release from the SR elicited by membrane depolarization maintains tonic contractions through activation of the RhoA/ROCK pathway.
Essential Role of L-type Ca\(^{2+}\) Channel Activation and Ca\(^{2+}\) Channel-Induced Ca\(^{2+}\) Release in Depolarization-Evoked RhoA Activation and Sustained Myocyte Contraction

Because depolarization-evoked tonic myocyte contraction is paralleled by metabotropic RhoA/ROCK activation, we tested to see whether blockade of tonic contractility with nifedipine (Figure 5A and 1D) also altered RhoA activity. As expected, 70K-induced contractions and RhoA activation in arterial rings were fully prevented by nifedipine (Figure 5A, top and bottom), thus suggesting that Ca\(^{2+}\) channel activation is required to increase RhoA activity. The critical role of VGCC activation (independently of membrane depolarization) in RhoA regulation was further supported by two sets of experiments. First, the application of FPL64176 (FPL), a selective L-type Ca\(^{2+}\) channel agonist with minor effects on the resting membrane potential,\(^{14,18}\) induced a contraction that was fully blocked by nifedipine (Figure 5B and 5C, top panels; Online Figure ID and IE). RhoA activity, which increased by FPL in parallel with the mechanical response, was also almost abolished by nifedipine application (Figure 5B and 5C, bottom panels). Second, in a conditional Cav1.2 (L-type) Ca\(^{2+}\) channel knockout mice model,\(^{22,20}\) contractile responses to 70K and FPL were greatly reduced in parallel with inhibition of RhoA activity (Figure 6). In these genetically modified animals, contractions induced by application of either phenylephrine or caffeine (because of Ca\(^{2+}\) release from InsP\(_3\) or ryanodine-sensitive stores, respectively\(^{28,29}\)) were unaffected, thus indicating that VGCC deficiency did not alter other physiological features of the VSM cells. The RhoA/ROCK pathway was maintained functional in Cav1.2 null myocytes because it was activated by phenylephrine with similar potency as in control cells (Figure 6D). Moreover, the sustained contraction evoked by phenylephrine was blocked by pharmacological ROCK inhibition (Online Figure IV).

Figure 4. Participation of G-proteins and inositol 1,4,5-trisphosphate (InsP\(_3\)) receptors in the sustained component of the depolarization-induced contraction and RhoA activation in rabbit femoral arteries. A, Effect of GDP\(_{S}\) (10 mmol/L) on contraction induced by depolarization (70K) and caffeine (10 mmol/L) in reversibly permeabilized arteries. B, Quantitative summary of relative isometric force developed in response to the different stimuli, 70K or caffeine (n=11). C, Effect of GDP\(_{S}\) on RhoA activation (relative to resting condition in the absence of any stimulus) during 70K-induced depolarization (n=5). D, Effect of heparin (10 mg/mL) on contractions induced by 70K and caffeine (10 mmol/L) (control).

Figure 5. Effect of nifedipine on 70K-induced and FPL64176 (FPL)-induced contraction and RhoA activation in rabbit basilar artery. A, Statistical analysis of isometric contraction (top) and RhoA activity (bottom) measured 10 minutes after stimulation induced by 70K in the presence or absence of nifedipine (1 \(\mu\)mol/L, n=3). RhoA activity is normalized to basal condition (control). B, Time-dependent changes in contraction (top) and RhoA activation (bottom) in basilar arterial rings stimulated with FPL (0.5 \(\mu\)mol/L). The vertical arrows indicate the time at which arterial rings were frozen (F) for posterior measurement of RhoA activity (2 minutes, n=3; 10 minutes, n=10; 30 minutes, n=3). C, Statistical analysis of isometric contraction (top) and RhoA activity (bottom, measured at 10 minutes after stimulation) induced by FPL (0.5 \(\mu\)mol/L) in the presence (n=32) or absence (n=3) of 1 \(\mu\)mol/L nifedipine. Values are presented as mean±SEM. *P<0.01.
To distinguish between the effects of Ca\(^{2+}\) channel activation (and the downstream metabotropic pathway) and Ca\(^{2+}\) entry/release on RhoA activation, we performed experiments in arteries bathed in Ca-free solutions (0Ca plus 1 mmol/L EGTA; estimated [Ca\(^{2+}\)]\(_{i}\) <100 mmol/L) to prevent any transmembrane Ca\(^{2+}\) influx. In these conditions, caffeine induced a transient Ca\(^{2+}\) release signal that triggered arterial contraction (Online Figure VA). These effects of caffeine decreased progressively as the SR stores were depleted of Ca\(^{2+}\) (Figure 7A). RhoA activity measured 10 minutes after caffeine application was similar to control values (Figure 7B and 7C), thus indicating that caffeine-induced Ca\(^{2+}\) release and contraction occur without involvement of the RhoA/ROCK pathway. Pharmacological inhibition of ROCK did not produce any effect on the mechanical responses to caffeine (Online Figure VB). Application of a membrane-permeant InsP\(_3\) derivative also failed to induce contraction and RhoA activation in arterial rings (Online Figure VI). Therefore, SR Ca\(^{2+}\) release per se (bypassing Ca\(^{2+}\) channel-induced metabotropic activation) does not seem to be sufficient to activate RhoA. In contrast to caffeine, FPL (that also induced a transient Ca\(^{2+}\) signal and arterial contraction; online Figure VC) elicited a marked RhoA activation that decreased with time as the SR was depleted of Ca\(^{2+}\) (Figure 7D). On exposure to FPL, RhoA activity increased to a maximal value that started to decrease 10 minutes after treatment (Figure 7E and 7F). These findings contrast with the results shown before in the presence of extracellular Ca\(^{2+}\), a condition in which FPL induced sustained contraction and RhoA activation (Figure 5B). In accord with these observations, FPL-induced contraction was inhibited by pharmacological blockade of ROCK (Online Figure VD).

It is known that phosphorylation of myosin light chain in KCl-stimulated myocytes is highly sensitive to [Ca\(^{2+}\)]\(_{i}\),\(^8\) hence, we sought to determine whether changes in extracellular [Ca\(^{2+}\)] can modify RhoA activation. Experiments were performed to compare the data described before in arteries bathed in 0Ca plus EGTA solutions (estimated [Ca\(^{2+}\)]\(_{i}\) <100 mmol/L) with preparations exposed to 0Ca solutions without EGTA added (estimated [Ca\(^{2+}\)]\(_{i}\) ~7 μmol/L) for more than 60 minutes. In this last solution, both caffeine-evoked contraction and FPL-induced RhoA activation remained similar to the values observed in control conditions, before application of any stimulus (Figures 8A, 8B, 7A, and 7D). FPL elicited a sustained contraction paralleled by an increase in RhoA activity that, after reaching a maximal value, remained almost constant or declined slowly during treatment (Figures 8C, 8D, 7E, and 7F). FPL-induced sustained contraction was decreased by pharmacological ROCK inhibition, whereas the cytosolic Ca\(^{2+}\) signal was not affected (Online Figure VE). Interestingly, the level of FPL-evoked RhoA activation, after 10 minutes of treatment, was not significantly different in arterial rings bathed in either =7 μmol/L (136%±8%; n=8; Figure 8D) or 2.5 mmol/L (143%±6%; n=10; Figure 5B) Ca\(^{2+}\) (P=0.18), thus suggesting that VSM cells have a highly effective Ca\(^{2+}\) uptake system that can maintain refilling the intracellular stores even when extracellular [Ca\(^{2+}\)] is reduced to μmol/L levels.

Altogether, these data suggest that depolarization-evoked sustained RhoA activation and myocyte contraction do not depend on the change in the membrane potential per se or the simple release of Ca\(^{2+}\) from the SR, but they require the simultaneous activation of VGCC and the downstream stimulation of a metabotropic pathway leading to InsP\(_3\) synthesis and Ca\(^{2+}\) release. Therefore, CCICR has a major role in tonic VSM contractility because it links Ca\(^{2+}\) channel activation with metabotropic Ca\(^{2+}\) release and sensitization (RhoA/ROCK stimulation).

**Discussion**

In this article, we describe that besides their classical ionotropic role responsible for Ca\(^{2+}\) entry and the Ca\(^{2+}\)/calmodulin-dependent activation of myosin light chain kinase, L-type Ca\(^{2+}\) channels have other unexpected functions required for maintenance of depolarization-evoked contraction in VSM cells. We show that L-type Ca\(^{2+}\) channel activation constitutes the key...
event that triggers depolarization-dependent metabotropic Ca\(^{2+}\) release from SR and smooth muscle sensitization through activation of the RhoA/ROCK pathway. It had been described before that in the absence of extracellular Ca\(^{2+}\) influx, Ca\(^{2+}\) channels of VSM cells act as voltage sensors that couple membrane depolarization to InsP\(_3\) synthesis and Ca\(^{2+}\) release from SR (CCICR). We now show that in normal conditions, CCICR mediates Ca\(^{2+}\) release and sensitization of the contractile apparatus necessary to generate sustained VSM contractions.

**Maintenance of Depolarization-Evoked Vascular Myocyte Contraction Requires Functional Ca\(^{2+}\) Channels and Metabotropic Ca\(^{2+}\) Release From the SR**

In agreement with previous studies on other arteries, high K\(^+\)-evoked contraction in basilar arterial rings exhibited a time course characterized by an early transient phase followed by a tonic sustained component. Transient and sustained phases in vascular myocyte contraction were also observed after application of agonists that activate membrane VGCC. In this article, we provide strong evidence indicating that, besides transmembrane Ca\(^{2+}\) influx, the sustained phase of contraction also depends on Ca\(^{2+}\) release from internal stores. Therefore, CCICR, a mechanism described in arteries bathed without external Ca\(^{2+}\), seems to have a major functional role in arterial contraction under normal conditions. Exposure to high KCl is often used as a tool to bypass G-protein–coupled receptor stimulation and activate smooth muscle by changing the K\(^+\) equilibrium potential and clamping membrane potential above resting level. However, it is well-established that in several cell types (including VSM cells) membrane depolarization can induce direct activation of the G-protein-PLC cascade, leading to InsP\(_3\) synthesis and Ca\(^{2+}\) release. In vascular, as well as in skeletal muscle cells, a great deal of experimental evidence exists supporting the view that VGCC are the voltage sensors linking membrane depolarization to G-protein activation, InsP\(_3\) synthesis, and Ca\(^{2+}\) release. In bronchial smooth muscle and megakaryocytes lacking VGCC, other membrane proteins have been proposed to also have intrinsic voltage sensitivity.

In the present set of experiments, the role of CCICR on VSM contraction, studied before in isolated myocytes, has been investigated in arterial rings and reversibly “permeabilized” arteries. These last preparations better-represent the in vivo physiological conditions yet permit pharmacological modifications of the extracellular and intracellular media. The data show that sustained depolarization-evoked vascular contraction depends on G-protein/PLC/InsP\(_3\)-mediated Ca\(^{2+}\) release from the SR, a metabotropic pathway triggered by VGCC activation. We find particularly conclusive the experiments performed using the conditional L-type Ca\(^{2+}\) channel knockout mouse, in which we have recently shown the strict dependence of depolarization-evoked Ca\(^{2+}\) release on the maintenance of functional Ca\(^{2+}\) channels. In this animal model, protracted VSM contraction induced by either membrane depolarization (exposure to high K\(^+\)) or pharmacological Ca\(^{2+}\) channel activation (application of FPL) was reduced in parallel with the decrease of channel expression. However, contractions induced by either caffeine, which directly activates Ca\(^{2+}\) release from stores, or phenylephrine, which activates the G-protein/PLC cascade and ROCK, were unaltered. Therefore, it seems that VGCC have two different roles in the contractile response to depolarization. At first instance, membrane depolarization causes massive Ca\(^{2+}\) entry, myosin light chain kinase activation, and a phasic contraction. During maintained depolarization, channels undergo a conformational change (switching between inactive and open states) that triggers the metabotropic cascade, resulting in Ca\(^{2+}\) release from SR and tonic contraction. Ca\(^{2+}\) channel inactivation and the displacement of the membrane potential toward the Ca\(^{2+}\) equilibrium potential drastically reduce transmembrane Ca\(^{2+}\) influx. However, a small steady-state Ca\(^{2+}\) channel activity and Ca\(^{2+}\) current have been described in sustained depolarized myocytes.
Therefore, it is possible that a residual transmembrane \( \text{Ca}^{2+} \) influx, without effect on the contractile machinery itself, contributes to refilling of peripherally located SR, in which membranes of the plasmalemma and the superficial SR come close together. Accumulation of \( \text{Ca}^{2+} \) in the SR would then permit subsequent release of enough \( \text{Ca}^{2+} \) to activate or sensitize the contractile apparatus. This proposal explains why both the phasic and tonic components of contraction depend on extracellular \( \text{Ca}^{2+} \). The higher sensitivity of the tonic phase of contraction to nifedipine could be explained because it is known that this drug has more affinity for the inactivated state of the \( \text{Ca}^{2+} \) channels than for channels that are in the closed or open states. Because before stimulation channels are mainly in the closed state, a higher dose of the antagonist is required to block the phasic component of the contraction.

**Sustained Depolarization-Evoked Contraction Requires L-type \( \text{Ca}^{2+} \) Channel-Dependent Metabotropic RhoA/ROCK Activation**

In accord with previous observations, we have shown in immunostained isolated myocytes the redistribution of activated RhoA and its displacement toward the plasma membrane after high K\(^+\) induced depolarization. In intact arteries, pharmacological inhibition of ROCK (a downstream effector of RhoA) selectively inhibited the tonic phase of contraction without altering the \( \text{Ca}^{2+} \) signal. Therefore, the data suggested that besides metabotropic \( \text{Ca}^{2+} \) release, activation of the RhoA/ROCK pathway is necessary for tonic myocyte contraction. In reversibly permeabilized arteries, RhoA activity was found increased in sustained depolarized arteries, and both contraction and RhoA activation decreased in parallel after G-protein blockade with GDP\(\beta\)S or inhibition of InsP\(_3\).
receptors with heparin. Hence, high K\(^+\)-induced sustained contraction requires G-protein-mediated RhoA activation and Ca\(^{2+}\) sensitization, a phenomenon that was previously thought to be restricted only to receptor-mediated forms of smooth muscle activation.\(^1\)

Because RhoA/Rho kinase is activated by G-protein-coupled receptor stimulation,\(^4,5\) the apparent high K\(^+\) induced activation of RhoA/ROCK could, in fact, be attributable to activation of a G-protein-coupled receptor with intrinsic voltage sensitivity rather than to membrane depolarization.\(^36\) This possibility was discarded because FPL, a Ca\(^{2+}\) channel agonist that can activate Ca\(^{2+}\) channels without affecting membrane potential,\(^14,18\) induced arterial contraction and RhoA activation in a qualitatively similar way to the changes in the same parameters evoked by KCl. Moreover, in conditional Cav1.2 knockout mice, the absence of FPL-induced or depolarization-induced L-type Ca\(^{2+}\) channels activation was paralleled by the lack of RhoA activation and contraction. In this animal model, contraction induced by caffeine, which bypassed plasma membrane depolarization, was unchanged. Similarly, RhoA activation induced by phentolamine, which is G-protein-mediated, was also unaltered. Therefore, these data strongly suggest that functional L-type Ca\(^{2+}\) channels are required for the high K\(^+\)-evoked or FPL-evoked RhoA activation. This novel role for VGCC on Ca\(^{2+}\) sensitization in VSM helps explain the KCl-evoked Rho/ROCK activation profusely described in the literature and whose underlying mechanisms were unknown.\(^6\)–\(^10\) Our experimental data also indicate that InsP\(_3\)-mediated Ca\(^{2+}\) release is necessary for depolarization-evoked or FPL-evoked RhoA activation, because it was markedly decreased in arterial rings in which the internal Ca\(^{2+}\) stores had been previously depleted. Interestingly, caffeine-induced Ca\(^{2+}\) release did not induce RhoA activation, thus suggesting that SR Ca\(^{2+}\) release per se, bypassing activation of Ca\(^{2+}\) channels and the downstream metabolic pathway, is not sufficient to induced RhoA activation. Therefore, RhoA/ROCK activation and VSM sensitization requires both Ca\(^{2+}\) channel activation and CCICR from SR.

In conclusion, the findings reported here suggest that CCICR links Ca\(^{2+}\) channel activation with RhoA/ROCK stimulation. They indicate that depolarization-evoked sustained RhoA activation and myocyte contraction do not depend on the change in the membrane potential itself or the mere release of Ca\(^{2+}\) from the SR, but they require the simultaneous activation of VGCC and the downstream stimulation of a metabotropic pathway, leading to InsP\(_3\) synthesis and Ca\(^{2+}\) release. An explanatory summary of the ionotropic and metabotropic functions of VGCC regulating VSM contraction is given in Figure 8E. Our results could have a wide functional relevance for the pathogenesis of vasospasms mediated by vasoactive agents, such as noradrenaline, endothelin, or ATP, that can activate VGCC.\(^18,37,38\) Because sustained VSM depolarization and VGCC activation mediate numerous pathophysiological processes,\(^39\)–\(^42\) the data could also help to optimize therapeutic treatment for clinical conditions such as hypertension, angina pectoris, and cardiac arrhythmias, in which Ca\(^{2+}\) channels antagonist are recommended.\(^43\)

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**Disclosures**

None.

**References**


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**Novelty and Significance**

Although a wide number of investigations have revealed the important contribution of L-type Ca\(^{2+}\) channels and RhoA/Rho kinase in the pathophysiology of vascular smooth muscle, the inter-relationships between both elements are unknown. We show for the first time to our knowledge that the depolarization-evoked sustained contraction in these cells is mediated, at least in part, by Ca\(^{2+}\) channel-induced metabotropic Ca\(^{2+}\) release from the SR. We also demonstrate that sarcocremal Ca\(^{2+}\) channels not only mediate the Ca\(^{2+}\) influx that triggers contraction but also metabolically regulate SR Ca\(^{2+}\) release and RhoA/Rho kinase activation responsible for sustained contraction. These novel findings assign to L-type channels a role in the regulation of vascular smooth muscle tonus that is much broader than previously thought and help in the understanding of the role of Ca\(^{2+}\) channels and the RhoA/Rho kinase sensitization pathway in sustained contraction. Our results could have special relevance for understanding the pathogenesis of vasospasms mediated by vasoactive agents that can activate VGCC, such as noradrenaline, endothelin, or ATP. Because sustained vascular smooth muscle depolarization and VGCC activation participate in numerous pathophysiological processes, our data could also help to optimize therapeutic treatment for clinical conditions such as hypertension, angina pectoris, and cardiac arrhythmias, in which Ca\(^{2+}\) channels antagonist are recommended.
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Metabotropic regulation of RhoA/ROCK by L-type Ca^{2+} channels: New mechanism for depolarization-evoked mammalian arterial contraction

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MATERIALS AND METHODS

Confocal microscopy and immunocytochemistry. Isolated cells were placed on coverslips and fixed with 4% paraformaldehyde in PBS solution for 20 minutes. Subsequently, cells were rinsed in this solution and incubated 1 hour in non specific binding solution (PBS, 10% FBS, 1 mg/mL BSA, 0.1% Triton X-100). We used mouse anti-RhoA antibody (Santa Cruz, USA) and rabbit anti-mouse FITC (Sigma-Aldrich, USA) secondary antibody to stain RhoA. Cells were incubated for 1 hour at room temperature in mouse anti-RhoA diluted 1:200 in non specific binding solution. Cells were rinsed in PBS and incubated for 1 hour in rabbit anti-mouse FITC diluted 1:1000 in non specific binding solution. The cell nucleus was marked using propidium iodide (1 µg/mL). Finally coverslips were rinsed in PBS and mounted onto slides with DPX. Fluorescence was observed with the confocal microscope (Leica, TCS SP2). Excitation was done with an Argon-Kripton laser (488 nm) and the emitted fluorescence (500-535 nm) was recorded through an oil immersion objective (40x1.25). To measure RhoA cellular distribution, six serial z sections were obtained across the width of a stained myocyte, being numbers 3 and 4 central sections. From each section, two line scans were analyzed. From the corresponding surface plots of these line scans, the peak pixel intensities over the outer 15% of each cell width were averaged and taken as an estimate of peripheral staining. The peak pixel intensities of the remaining central 70% of the stained cell were averaged and taken as an estimate of the cytosolic staining.

RhoA activation assay. Arterials rings that were contracted isometrically were quickly frozen by immersion in liquid nitrogen. RhoA activity was determined from these arterial rings using the colorimetric G-LISA RhoA activation assay biochemistry kit (Cytoskeleton, USA). Frozen tissues were homogenized in lysis buffer. Protein concentration was determined according to the manufacturer’s protocol, and tissue extracts were equalized to contain protein concentration of 1 mg/mL for the assay. Active RhoA was detected using indirect immunodetection followed by a colorimetric reaction measured by absorbance at 490 nm. As measurement of RhoA activity by G-LISA requires a relatively large amount of tissue, due to the small size of mouse and rat basilar arteries we did these experiments either in rabbit basilar artery or in mouse aorta arterial rings. Several arterial rings were obtained from a mouse aorta and pooled together for a single G-LISA measurement. Each experiment was repeated in at least 5 animals. In the case of rabbit basilar arteries, four to six arterial rings from at least two animals, were used in each individual G-LISA measurement (non-stimulated, or treated with 70K/FPL). In addition, G-LISA measurements in the various experimental conditions were repeated at least three times and presented as percentage of control. Each set of data were expressed as mean ± SE of the determinations done in duplicate or triplicate.

Reversible permeabilization. To load rat basilar arteries with GDPβS (10 mmol/L), arteries were incubated in the following sequence of solutions kept at 4 ºC (in mmol/L): 1) 10 EGTA, 120 KCl, 5 ATP, 2 MgCl₂, 20 TES (pH 6.8, 20 minutes); 2) 120 KCl, 5 ATP, 2 MgCl₂, and 20 TES and 10 mmol/L GDPβS (pH 6.8, 3 hours); and 3) 120 KCl, 5 ATP, 10 MgCl₂, and 20 TES and 10 mmol/L GDPβS (pH 6.8, 30 minutes). Subsequently, arteries were bathed in a fourth solution containing (in mmol/L): 140 NaCl, 5 KCl, 10 MgCl₂, 5 glucose, and 2 MOPS (pH 7.1, 22°C) in which [Ca²⁺] was gradually increased from 0.01 to 0.1 to 1.8 mmol/L every 15 minutes. Qualitatively similar protocol was done for reversible permeabilization of rabbit arteries, keeping arterial ring in solution 2 for 18 hours. As for unknown reasons rabbit basilar arteries did not seem to respond well to the relatively aggressive treatment required for rabbit artery permeabilization, we used femoral arteries instead. The method of reversible permeabilization was also used to introduce heparin (10 mg/mL).

Solutions. The composition of Hank’s solution (in mmol/L) was: 125 NaCl, 5.36 KCl, 5 NaHCO₃, 0.34 Na₂HPO₄, 0.44 KH₂PO₄, 10 glucose, 1.45 sucrose, and 10 Heps, pH 7.4. The
composition of PBS solution (in mmol/L) was: 137 NaCl, 2.68 KCl, 4.02 Na$_2$HPO$_4$, 1.76 KH$_2$PO$_4$, pH 7.4. The composition of Kreb’s solution (in mmol/L) was: 119 NaCl, 4.7 KCl, 1.17 KH$_2$PO$_4$, 24 NaHCO$_3$, 2.5 CaCl$_2$, 1.17 MgSO$_4$, 5.5 glucose. The 70K$^+$ solution was obtained by replacing 70 mmol/L of NaCl with KCl. The 0Ca$^{2+}$ solution was obtained by substituting Mg$^{2+}$ for Ca$^{2+}$ and with 1 mmol/L EGTA added (estimated [Ca$^{2+}$]i < 100 nmol/L). In nominally 0Ca$^{2+}$ solution CaCl$_2$ was omitted (estimated [Ca$^{2+}$]i = 7 µmol/L). [Ca$^{2+}$] contained in these solutions was measured using an electrode (Orion Calcium Electrode, Thermo, Electron Corp., USA). To exclude the potential confounding effects on G-proteins of neurotransmitters released from nerves innervating the arterial rings we performed the appropriate experimental tests (measurement of tension and RhoA activity) using a 70K/FPL solution containing a cocktail of receptor antagonists previously used for the same purpose in the literature (Walch, et al. 2000, 2001; Sakurada, et al. 2003; Yang, et al. 2009; del Valle-Rodriguez, et al. 2006). The composition of this cocktail was as follows: ACh receptor antagonist (atropine, 5 µmol/L), angiotensin AT1 receptor antagonist (losartan, 1 µmol/L), β-adrenergic receptor blocker (propanolol, 10 µmol/L), purinergic P2 receptor blocker (suramin, 25 µmol/L), and thromboxane receptor antagonist (AM6809, 1 µmol/L). Arterial ring contraction and RhoA activity were not affected by the presence of the indicated drugs (Online Figure I). To record whole-cell Ca$^{2+}$ channel currents the solutions used were as follows (in mmol/L): external (140 NaCl, 2.7 KCl, 10 BaCl$_2$, 10 HEPES, pH 7.4); internal (100 CsCl, 30 CsF, 2 MgCl$_2$, 4 ATP-Mg, 10 EGTA, 10 HEPES, pH 7.2).
**ADDITIONAL FIGURES**

**Online Figure I.** Effect of a cocktail of different inhibitors of neurotransmitter receptors on Ca\(^{2+}\) channel-induced contraction and RhoA activation in basilar artery. A, 70K-induced contraction in the presence or absence of the cocktail. B, Quantitative summary of isometric contractions induced by 70K solution in the presence of inhibitors of neurotransmitter receptors (n=8). C, Effect of the inhibitory cocktail on depolarization-induced RhoA activation. D, FPL-evoked contraction in the presence or absence of the cocktail (n=6).

**Online Figure II.** Effect of depletion of intracellular Ca\(^{2+}\) stores on Ca\(^{2+}\) channel currents and isometric force. A, Effect of CPA (10 µmol/L) on Ca\(^{2+}\) currents recorded from voltage-clamped dispersed myocytes using the whole cell configuration of the patch clamp technique. B, Effect of CPA on the current/voltage relations of Ca\(^{2+}\) channels (n=4). Arrow indicates that I\(_{\text{Ca}}\) is not reduced in the presence of CPA at membrane potentials close to that obtained in cells exposed to 70K (~20 mV). C, Effect of thapsigargin (2 µmol/L), an inhibitor of SR Ca\(^{2+}\) pump, on the 70K-induced contraction. The lack of contraction induced by caffeine (Caf) after treating the arterial rings with thapsigargin indicates that intracellular stores were depleted. D, Statistical analysis of isometric contractions induced by 70K solution in the presence of CPA (n=6), thapsigargin (measured during the first exposure to 70K in the presence of the inhibitor, n=4) and ryanodine (n=6) to deplete intracellular Ca\(^{2+}\) stores. Force was measured at 10 minutes after stimulus application. E, Cumulative effect of nifedipine (expressed in nmol/L) on the tonic component of the 70K-evoked contraction. F, Dose-response curve of the effect of nifedipine on the amplitude of the tonic component of the 70K-evoked contraction. Data points relating relative isometric force to nifedipine concentration were fitted to a double exponential function (K\(_d\) = 6.62 nmol/L) (n=6). Values are presented as means ±SEM. *P<0.05.

**Online Figure III.** Effect of Phospholipase C and ROCK inhibitors on [Ca\(^{2+}\)]i and the contraction induced by 70K and caffeine. A, Effect of U73122 (1 µmol/L), a phospholipase C (PLC) inhibitor, on cytoplasmic calcium increase induced by 70K. B, Statistical analysis of the effect of U73122 on intracellular calcium in rat basilar myocytes during both, the phasic and tonic components of [Ca\(^{2+}\)]i (n=3). C, Arterial ring contractions induced by repeated exposures to caffeine (10 mmol/L) in the absence or presence of U73122 (1 µmol/L). Arrows indicate caffeine applications. D, Quantitative summary of the action of PLC inhibitor (U73122) on caffeine-evoked contractions in rat basilar arterial rings (n=5). The data show that in our preparation U73122 had no significant effect on refilling of stores (see, however, Macmillan and McCarron, 2010). E, Effect of the ROCK inhibitor HA 1077 (5 minutes treatment) on the 70K-evoked contraction. F, Quantitative summary of the inhibition of the relative isometric force with two ROCK inhibitors (HA 1077, 2.5 µmol/L, n=6; Y27632, 2.5 µmol/L, n=6), relative to the phasic component of the 70K-induced contraction. Values are presented as mean±SEM. *P<0.05, **P<0.01.

**Online Figure IV.** Effect of Y27632 on the phenylephrine-evoked contraction in mouse femoral arterial rings. A, Isometric force developed in response to phenylephrine (1 µmol/L) in the presence of the ROCK inhibitor Y27632 (2.5 µmol/L). B, Statistical analysis of the effect of Y27632 (2.5 µmol/L, n=6) on phenylephrine-induced contraction in mouse femoral arterial rings. Values are presented as mean±SEM. *P<0.05.

**Online Figure V.** Role of extracellular [Ca\(^{2+}\)] and ROCK inhibitors in FPL- and caffeine-evoked contractions in rat basilar arteries. A and C, Representative recordings of simultaneous measurements of [Ca\(^{2+}\)] (fluorescence ratio, F\(_{340}/F_{380}\)) and force (arterial diameter) in Fura-2 loaded intact basilar arteries in response to 10 mmol/L caffeine (A) (F\(_{340}/F_{380}\) increased 0.15±0.02 units, diameter decreased 68.63±9.11 µm, n=8) and 5 µmol/L FPL (C) (F\(_{340}/F_{380}\)
increased 0.071±0.019 units, diameter decreased 47±19.7 μm, n=4) in external solutions where Ca^{2+} was omitted and 1 mmol/L EGTA was added ([Ca^{2+}] < 100 nmol/L). B, Lack of effect of HA 1077 (1 μmol/L) on caffeine-induced arterial ring contraction. D, Effect of Y27632 (2.5 μmol/L) on the FPL-evoked contraction (41.1±1.3% inhibition at 5 minutes, n=3). E, Representative example of three similar experiments of simultaneous measurements of [Ca^{2+}] (fluorescence ratio, F340/F380) and force (arterial diameter) in Fura-2 loaded intact basilar arteries in response to FPL (5 μmol/L) and HA 1077 (2.5 μmol/L) in an external solution where Ca^{2+} was omitted ([Ca^{2+}] ~7 μmol/L).

**Online Figure VI.** Effect of a permeant InsP₃–derivative on cytosolic Ca^{2+} and RhoA activity in arterial myocytes. A. Oscillations of cytosolic [Ca^{2+}] induced by Bt-InsP₃ AM (5 μmol/L) in dispersed rat basilar myocytes. Note that the larger Ca^{2+} signal induced by high K⁺ was unaffected by application of the drug (representative example of 5 similar experiments). B and C, Quantitative summary of the effect of Bt-InsP₃ AM (5 μmol/L) on RhoA activation in intact (B, n=3; basilar artery) and in reversibly permeabilized (C, n=3; femoral artery) rabbit arterial rings. Bt-InsP₃ AM (5 μmol/L) did not induce contraction in any of these arterial preparations (data not shown).
Online Figure I
Online Figure II
Online Figure III
Online Figure IV
Online Figure V
Online Figure VI
REFERENCES


