

Role of iPLA₂ and store-operated channels in agonist-induced Ca²⁺ influx and constriction in cerebral, mesenteric, and carotid arteries

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Park KM, Trucillo M, Serban N, Cohen RA, Bolotina VM. Role of iPLA₂ and store-operated channels in agonist-induced Ca²⁺ influx and constriction in cerebral, mesenteric, and carotid arteries. *Am J Physiol Heart Circ Physiol* 294: H1183–H1187, 2008. First published December 21, 2007; doi:10.1152/ajpheart.01148.2007.—Store-operated channels (SOC) and store-operated Ca²⁺ entry are known to play a major role in agonist-induced constriction of smooth muscle cells (SMC) in conduit vessels. In microvessels the role of SOC remains uncertain, in as much as voltage-gated L-type Ca²⁺ (Ca_L²⁺) channels are thought to be fully responsible for agonist-induced Ca²⁺ influx and vasoconstriction. We present evidence that SOC and their activation via a Ca²⁺-independent phospholipase A₂ (iPLA₂)-mediated pathway play a crucial role in agonist-induced constriction of cerebral, mesenteric, and carotid arteries. Intracellular Ca²⁺ in SMC and intraluminal diameter were measured simultaneously in intact pressurized vessels in vitro. We demonstrated that 1) Ca²⁺ and contractile responses to phenylephrine (PE) in cerebral and carotid arteries were equally abolished by nimodipine (a Ca_L²⁺ inhibitor) and 2-aminoethyl diphenylborinate (an inhibitor of SOC), suggesting that SOC and Ca_L²⁺ channels may be involved in agonist-induced constriction of cerebral arteries, and 2) functional inhibition of iPLA₂β totally inhibited PE-induced Ca²⁺ influx and constriction in cerebral, mesenteric, and carotid arteries, whereas K⁺-induced Ca²⁺ influx and vasoconstriction mediated by Ca_L²⁺ channels were not affected. Thus iPLA₂-dependent activation of SOC is crucial for agonist-induced Ca²⁺ influx and vasoconstriction in cerebral, mesenteric, and carotid arteries. We propose that, on PE-induced depletion of Ca²⁺ stores, nonselective SOC are activated via an iPLA₂-dependent pathway and may produce a depolarization of SMC, which could trigger a secondary activation of Ca_L²⁺ channels and lead to Ca²⁺ entry and vasoconstriction.

store-operated calcium entry; smooth muscle cells; constriction

IN CEREBRAL ARTERIES and other small-diameter vessels, voltage-gated L-type Ca²⁺ (Ca_L²⁺) channels are known to be the primary channels responsible for agonist-induced constriction, in as much as their inhibition was shown to fully relax most microvessels (19, 20). On the other hand, depletion of intracellular Ca²⁺ stores by different agonists [or by inhibitors of sarco(endo)plasmic reticulum Ca²⁺-ATPase] activates store-operated (SOC) channels and store-operated Ca²⁺ entry (SOCE) in isolated smooth muscle cells (SMC) (for review see Refs. 1, 3, 26). In conduit vessels, nonselective cation SOC (rather than Ca_L²⁺ channels) play a major role in agonist-induced constriction and nitric oxide-induced relaxation (8, 27), but the role of SOC in constriction of microvessels, especially in the cerebral circulation, remains unclear. In SMC

of cerebral arterioles, activation of SOCE increases Ca²⁺ but fails to generate constriction (11, 12), suggesting that SOC may be mediating Ca²⁺ influx into restricted space (or cellular compartments), which may be spatially separated from the contractile apparatus. In interlobular arteries, SOC are present but do not significantly contribute to Ca²⁺ entry after agonist stimulation (10). In numerous other types of SMC, Ca²⁺ store depletion was reported to increase Ca²⁺ and cause constriction, although it was studied only using poorly selective SOC inhibitors (for review see Refs. 3 and 14). Thus the following questions remain. 1) By what mechanism might SOC be involved in the physiological regulation of agonist-induced constriction in microvessels? 2) How might their role be related to activation of Ca_L²⁺ channels. Because of the nonselective cation permeability of SOC in SMC, SOC activation should cause a significant membrane depolarization, which may result in cross talk between SOC and Ca_L²⁺ channels (7); this theory is supported by studies in isolated SMC from guinea pig gallbladder (18) and in the A7r5 cell line (21), but little is known about their possible cross talk in intact vessels.

Recently, the Ca²⁺-independent phospholipase A₂ (iPLA₂) β (group VI) (2, 30) was found to be a crucial determinant of SOCE activation in isolated aortic SMC, but the role of iPLA₂β in intact cerebral and other microvessels is unknown. Functional inhibition of iPLA₂ with its suicidal substrate bromoenol lactone (BEL) or specific antisense oligonucleotides against iPLA₂β has been shown to produce identical impairment of SOCE in isolated SMC (24, 25), as well as astrocytes (23), keratinocytes (22), skeletal muscle (4), fibroblasts (16), prostate cancer (28), and other cell types (24, 25). The crucial role of iPLA₂β in SOCE activation was recently confirmed in a screen of *Drosophila melanogaster* genes (29), where molecular knockdown of the CG6718 gene (a *Drosophila* homolog of human iPLA₂β) significantly affected SOCE. In isolated vascular SMC, we discovered that single-cation SOC and SOCE can be activated by lysophospholipids, which are produced by the plasma membrane-bound iPLA₂β (24). Activation of iPLA₂β can be physiologically achieved by Ca²⁺ influx factor, which is produced in endo(sarco)plasmic reticulum on depletion of Ca²⁺ stores (5) and is capable of displacing inhibitory calmodulin from its binding site on iPLA₂β. Thus agonist-induced depletion of the stores can activate SOC and SOCE in SMC via an iPLA₂β-dependent pathway (24).

Discovery of iPLA₂β as a crucial component of the SOCE mechanism in SMC and development of a chiral-specific functional inhibitor of iPLA₂β (9, 15) have opened new avenues for

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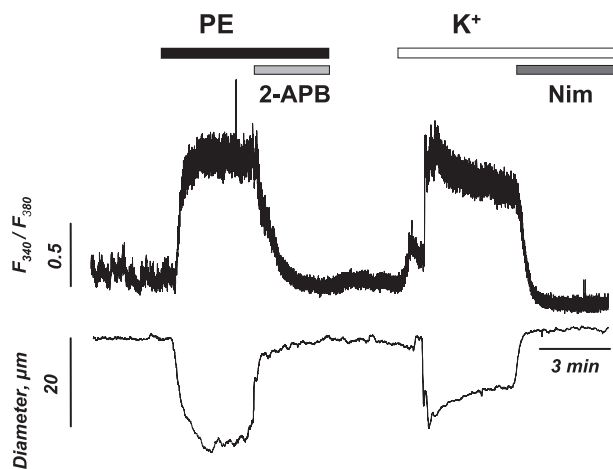


Fig. 1. Simultaneous Ca^{2+} rise in smooth muscle cells (SMC) and constriction caused by phenylephrine (PE) and high K^+ in intact cerebral artery. Representative traces show changes in intracellular Ca^{2+} [as ratio of fura 2-AM fluorescence at 340 nm to 380 nm (*top trace*)] and intraluminal diameter of intact pressurized vessel (*bottom trace*) during bath application of $10 \mu\text{M}$ PE, $75 \mu\text{M}$ 2-aminoethyl diphenylborinate (2-APB), high (60 mM) K^+ , and $5 \mu\text{M}$ nimodipine (Nim). Vessel was washed for 3 min between PE and K^+ applications.

studying the role of the SOCE mechanism in vascular constriction. In the present study, we have examined the roles of SOC and Ca_L^{2+} channels in microvessels and tested the hypothesis that agonist-induced $i\text{PLA}_2$ -dependent activation of SOC may be required for secondary activation of Ca_L^{2+} and Ca^{2+} entry, which trigger contractile responses in cerebral, mesenteric, and carotid arteries. Using simultaneous recording of Ca^{2+} in SMC and vessel diameter, we demonstrate that phenylephrine (PE)-induced constriction fully depends on the functional activity of $i\text{PLA}_2\beta$ and SOC, as well as Ca_L^{2+} channels. These new findings establish the crucial role of $i\text{PLA}_2\beta$ -dependent SOC in vascular responses to agonist stimulation and provide a new important mechanism of regulation of tone in different microvascular beds.

MATERIALS AND METHODS

Drugs and materials. Unless otherwise indicated, all drugs were purchased from Sigma. BEL was purchased from Biomol; S- and R-BEL from Cayman Chemicals; and fura 2-AM from Invitrogen. Physiological salt solution (PSS) consisting of (in mM) 119 NaCl, 4.7 KCl, 24 NaHCO_3 , 1.2 KH_2PO_4 , 1.6 CaCl_2 , 1.2 MgSO_4 , 0.023 EDTA, and 11 glucose was bubbled with 95% O_2 -5% CO_2 to ensure pH 7.4.

Isolation of mouse cerebral and mesenteric and rabbit carotid arteries. Posterior cerebral arteries (PCA) and mesenteric arteries were obtained from male mice (C57BL/6, 8–10 wk old). First-order PCA and second-order mesenteric arteries ($\sim 120 \mu\text{m}$ diameter) were dissected free from the mesenteric arterial bed or brain and connective tissue in ice-cold PSS. Dissected arterial segments (200 μm long) were cannulated in a chamber connected to a pressure myograph (Living Systems Instrumentation, Burlington, VT) and immediately used for experiments.

Carotid arteries were obtained from male New Zealand rabbits (3.1 ± 0.4 kg body wt), cleaned of all connective tissue in cold PSS, and mounted onto a myograph (see below).

All research was performed in accordance with the American Physiological Society "Guiding Principles in the Care and Use of Animals" and with Institutional Animal Care and Use Committee approval.

Simultaneous recording of Ca^{2+} and vessel diameter. Microvessels were maintained at 37°C throughout the experiment by superfusion with prewarmed, bubbled PSS at a rate of 5 ml/min. Before the experiments, vessels were equilibrated for 20 min at 10 mmHg, then pressure was raised to 60 mmHg; intravascular pressure was controlled by a pressure servo and peristaltic pump (Living Systems Instrumentation). The chamber with the microvessel was mounted on an Olympus microscope equipped with the IonOptix data acquisition system (IonOptix, Milton, MA), which allowed simultaneous measurement of 1) intraluminal diameter using the Softedge Acquisition Subsystem and 2) intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) using a ratiometric photomultiplier system. Fura 2-AM loading of SMC was accomplished by incubation of the mounted arteries in external PSS containing $5 \mu\text{M}$ fura 2-AM for 50 min at room temperature in the dark. The fura 2-AM ratio (ratio of fura 2 fluorescence at 340-nm excitation to that at 380-nm excitation, with emission at 510 nm) was recorded at a sampling rate of 5 Hz. Arterial diameter and fluorescence ratio were simultaneously recorded and plotted over time.

Isometric tension measurements. Isometric tension was measured as previously described (6, 8). Briefly, 5-mm-long rings of rabbit carotid artery segments were cleaned of connective tissue and mounted on metal stirrups in organ chambers, and isometric tension was recorded. Rings were maintained at 37°C in bubbled (95% O_2 -5% CO_2) PSS. Rings were equilibrated and stretched to an optimal tension of 7 g. Pharmacological agents were added to the bath as indicated.

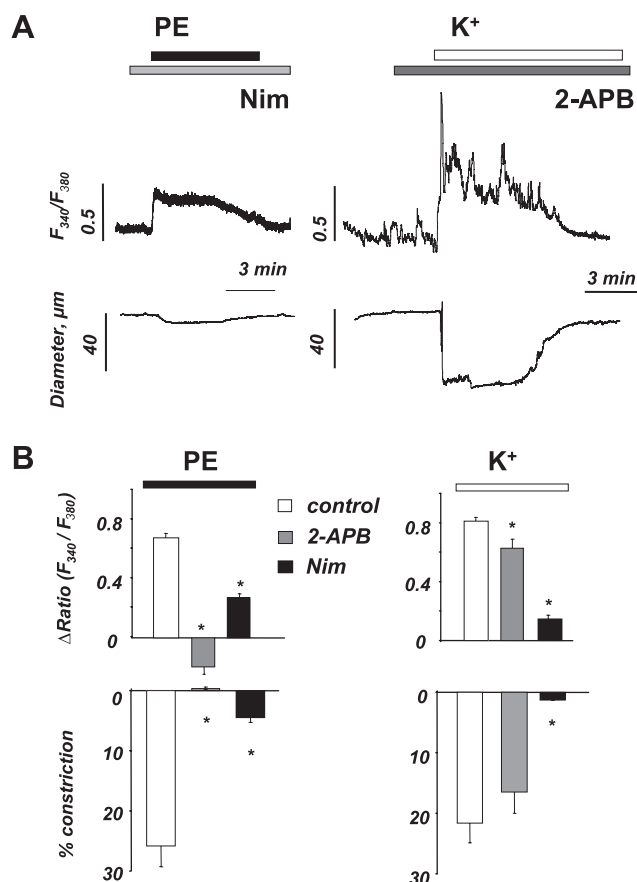


Fig. 2. Effects of 2-APB and Nim on PE- and high- K^+ -induced simultaneous Ca^{2+} rise and constriction in intact cerebral arteries. *A*: changes in intracellular Ca^{2+} (*top trace*) and intraluminal diameter (*bottom trace*) during application of $10 \mu\text{M}$ PE, $5 \mu\text{M}$ Nim, 60 mM K^+ , and $75 \mu\text{M}$ 2-APB. *B*: changes in Ca^{2+} (*top*) and simultaneous changes in intraluminal vessel diameter (*bottom*). Values are means \pm SE from 8 experiments. * $P < 0.05$ vs. control.

Statistical analysis. Summary data are presented as means ± SE. Student's *t*-test was used to determine the statistical significance of the data. *P* < 0.05 was considered significant.

RESULTS

To investigate the role of SOC in contractile responses of microvessels, we first tested and compared the effects of 2-aminoethyl diphenylborinate (2-APB, a widely used SOC inhibitor) and nimodipine (Nim, an inhibitor of Ca_L²⁺ channels) on responses to PE or high K⁺. [Ca²⁺]_i in SMC was measured simultaneously with intraluminal vessel diameter in PCA (Fig. 1). The high-K⁺-induced Ca²⁺ rise and vessel constriction were inhibited by 5 μM Nim but were not affected by 75 μM 2-APB [which is known to fully inhibit SOC and SOCE in SMC (12)], consistent with the involvement of only Ca_L²⁺ channels in depolarization-induced [Ca²⁺]_i rise and vessel constriction (Figs. 1 and 2). On the other hand, PE (10 μM)-induced [Ca²⁺]_i rise and vessel constriction were inhibited by Nim and 2-APB (Figs. 1 and 2), suggesting the involvement of SOC and Ca_L²⁺ channels in agonist-induced responses.

To provide new mechanistic insights into the role of SOC activation and the iPLA₂-mediated SOCE pathway in microvessel constriction, the effect of functional inhibition of iPLA₂ on PE- and K⁺-induced Ca²⁺ influx and constriction of cerebral, mesenteric, and carotid arteries was examined. Simultaneous changes in [Ca²⁺]_i and vessel diameter in response to consecutive application of K⁺ (60 mM) and PE (10 μM) to a cerebral artery are shown in Fig. 3. After the responses were tested under control conditions, the vessels were treated for 30 min with 25 μM BEL, which was previously shown to irreversibly impair iPLA₂ function in SMC (25). After the vessels were treated with BEL and washed for 10 min, K⁺ and PE were reapplied (Fig. 3), and the vessels retained a normal response to high K⁺, but not PE. As shown in Fig. 4A, inhibition of iPLA₂ did not affect Ca²⁺ influx and vessel constriction induced by high K⁺ (and mediated by Ca_L²⁺ channels) but dramatically impaired agonist-induced responses in cerebral arteries. Similar results in mouse mesenteric arteries

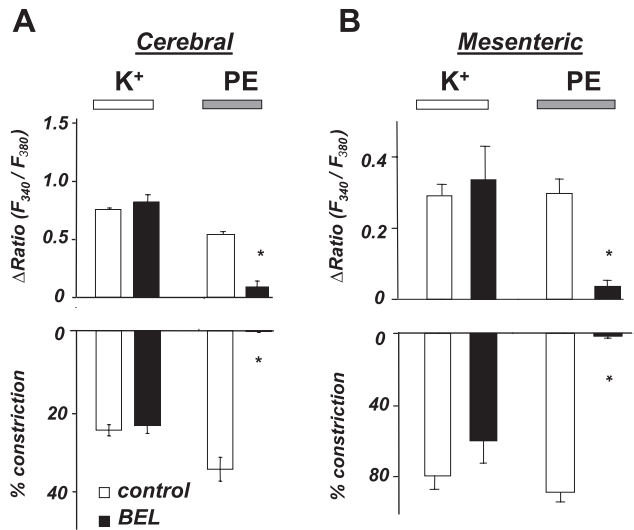


Fig. 4. Inhibition of iPLA₂ by BEL results in inhibition of Ca²⁺ influx and vessel constriction on stimulation by agonist, but not high K⁺, in cerebral (A) and mesenteric (B) arteries. Summary data are from experiments similar to those described in Fig. 3. Values are means ± SE for 6 (A) and 8 (B) experiments. **P* < 0.05 vs. control.

(Fig. 4B) and rabbit carotid artery (Fig. 5) extended our findings to these additional microvascular beds as well as different types of animals. Figure 5 also demonstrates that inhibition of iPLA₂ not only could prevent agonist-induced constriction but also could relax the precontracted artery.

To confirm that iPLA₂β, and not iPLA₂γ, is required for agonist-induced Ca²⁺ entry and constriction in microvessels, we compared the effects of *S*-BEL and *R*-BEL [chiral enantiomers of this suicidal substrate that can discriminate these iPLA₂ isoforms (9, 15)] on PE-induced responses in cerebral arteries. Although vessels pretreated with *R*-BEL (which is specific to iPLA₂γ) responded normally to PE, *S*-BEL (which is specific to iPLA₂β) totally impaired PE-induced Ca²⁺ entry and constriction in the same vessels (Fig. 6).

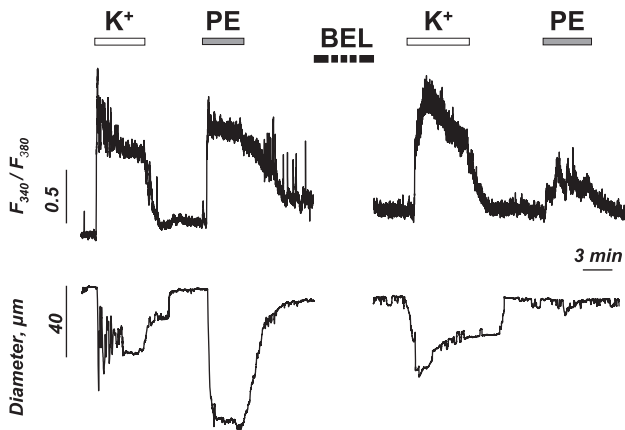


Fig. 3. Effect of irreversible Ca²⁺-independent phospholipase A₂ (iPLA₂) inhibition by bromoenol lactone (BEL) on K⁺- and PE-induced responses in intact cerebral artery. Results from a representative experiment show effects of application of high (60 mM) K⁺ and 10 μM PE on Ca²⁺ (top trace) and vessel diameter (bottom trace) before and after 30 min of treatment with 25 μM BEL. Addition of PE after BEL treatment dramatically reduced intracellular Ca²⁺ rise and completely abolished constriction; K⁺ did not affect Ca²⁺ or vessel diameter. Traces represent results from 4 experiments.

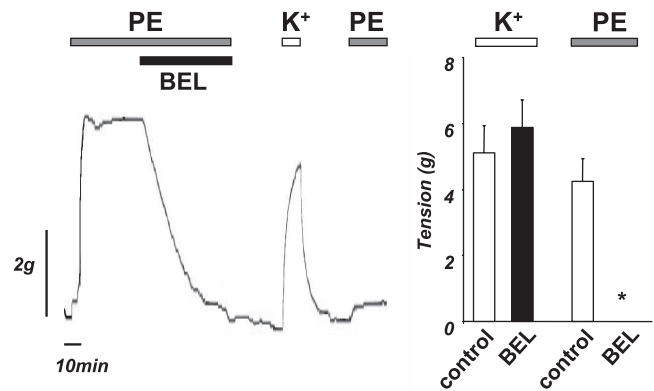


Fig. 5. Inhibition of iPLA₂ by BEL results in vessel relaxation and irreversible inhibition of agonist-induced, but not depolarization-induced, constriction in carotid artery SMC. Carotid artery ring was constricted with 0.1 μM PE, and 25 μM BEL was added in the continuous presence of PE. After complete vessel relaxation, PE and BEL were washed out for 20 min, and vessel was treated with high (30 mM) K⁺ and then with PE. Values are means ± SE from 4 experiments. **P* < 0.05 vs. control.

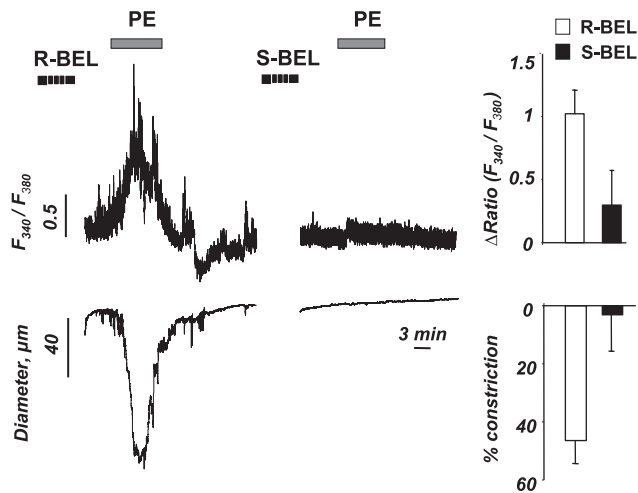


Fig. 6. Inhibition of $iPLA_2\beta$ by S -BEL results in inhibition of agonist-induced Ca^{2+} influx and vessel constriction, whereas inhibition of $iPLA_2\gamma$ by R -BEL has no effect on agonist responses. Representative traces and summary data show PE (10 μ M)-induced Ca^{2+} (top trace) and diameter (bottom trace) responses of cerebral vessels after treatment with R -BEL (25 μ M for 25 min) and then again after treatment with S -BEL (25 μ M for 25 min).

DISCUSSION

Our data in intact cerebral, mesenteric, and carotid arteries demonstrate for the first time that $iPLA_2$ -dependent activation of SOC is essential for agonist-induced Ca^{2+} entry and constriction in a wide variety of arteries, suggesting that this is a widespread phenomenon in microvessels.

To account for our finding of the equally important role of $iPLA_2\beta$, as well as SOC and Ca_L^{2+} channels, we propose a model for agonist-induced responses in microvessels (Fig. 7). On the basis of our earlier findings in isolated SMC (8, 24), we propose that agonist stimulation causes depletion of Ca^{2+} stores and activation of $iPLA_2$, which produces lysophospho-

lipids that activate SOC. Nonspecific cation SOC (27) can produce significant membrane depolarization and secondary activation of Ca_L^{2+} channels, which cause Ca^{2+} entry and, in turn, lead to vessel constriction. In this way, Ca_L^{2+} channels and SOC may be equally important for agonist-induced Ca^{2+} entry in SMC and constriction, as we found in microvessels. In contrast to smaller arteries, in the aorta we found that Ca_L^{2+} channels are not essential for agonist-induced Ca^{2+} influx and constriction (inhibition of Ca_L^{2+} has little to no effect on aortic tone) (8, 27). The difference may be explained by an ability of Ca^{2+} entering through SOC to directly cause contraction in aortic SMC and/or SOC-induced depolarization being insufficient for secondary activation of Ca_L^{2+} channels in SMC of these vessels. There is little doubt about the role of Ca_L^{2+} channels in responses of resistance vessel to agonists, in as much as selective inhibition of Ca_L^{2+} channels in all vessels tested in the present study impaired Ca^{2+} entry and constriction. This result was consistent with Ca_L^{2+} channels, not SOC, mediating Ca^{2+} entry, which triggers vessel constriction. However, SOC still plays a crucial role in agonist-induced responses, if not as a major Ca^{2+} entry path (as in aortic SMC), then as a depolarizing trigger for secondary activation of Ca_L^{2+} channels. Interestingly, Figs. 3 and 4 show that PE-induced constriction may be fully abolished, even though some residual Ca^{2+} rise in SMC could be detected after BEL treatment. Stronger inhibition of contractile responses may reflect the involvement of $iPLA_2$ in agonist-induced Ca^{2+} sensitization of contractile proteins (13), which may amplify the effect of the reduction on agonist-induced Ca^{2+} influx.

The use of functional inhibition of $iPLA_2\beta$ in our study (in addition to a widely used SOC inhibitor) introduces a new effective way to selectively impair the SOCE pathway in microvascular SMC and provides a more reliable method for assessing the role of SOC in physiology and pathology of microcirculation. Indeed, direct SOC inhibitors (e.g., 2-APB) may not exclude the possibility of involvement of additional

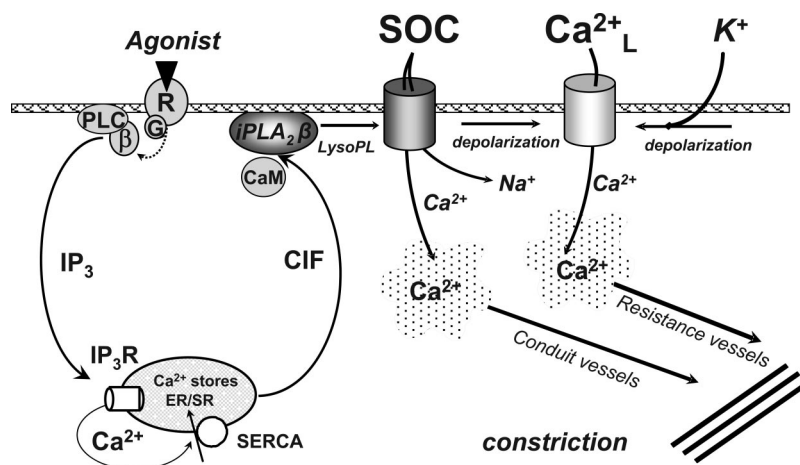


Fig. 7. A model for agonist-induced Ca^{2+} entry and constriction of conduit and microvessels. Agonist stimulation triggers inositol trisphosphate (IP_3) production and depletion of Ca^{2+} stores, leading to production of Ca^{2+} influx factor (CIF). CIF displaces inhibitory calmodulin (CaM) from $iPLA_2$, causing its activation and production of lysophospholipids (LysoPL) and, in turn, activates SOC. Nonspecific cation SOC in SMC allow Ca^{2+} and Na^+ entry. In conduit vessels, SOC-mediated Ca^{2+} entry may cause vessel constriction; in microvessels, this pool of Ca^{2+} may be spatially separated from the contractile apparatus. Instead, in microvessels, SOC-induced depolarization may trigger activation of L-type Ca^{2+} (Ca_L^{2+}) channels, which allow Ca^{2+} entry and, in turn, lead to vessel constriction. Thus SOC may play an important, but slightly different, role in constriction of different vessels: in conduit vessels, SOC allow Ca^{2+} influx, which is sufficient for constriction; in resistance vessels, SOC serve as a trigger for secondary activation of Ca_L^{2+} channels, which mediate Ca^{2+} influx and, in turn, cause vessel constriction. IP_3R , IP_3 receptor; PLC, phospholipase C; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; SERCA, sarco(endo)plasmic reticulum Ca^{2+} -ATPase. R, G:G-coupled receptor.

non-SOC, in as much as 2-APB is known to inhibit agonist-induced Ca²⁺ release (17), as well as other non-SOC, which could also participate in agonist-induced responses. Recently, we demonstrated (31) that a TRPC1-encoded inositol triphosphate (IP₃) receptor-operated channel (which can be clearly distinguished from SOC) may be activated by agonist-induced IP₃ rise and, potentially, may be involved in SMC and vessel responses to agonists. Importantly, because 2-APB is able to block both channels (SOC and IP₃ receptor-operated channels), it is impossible to distinguish their role in agonist-induced responses solely on the basis of 2-APB-induced inhibition. In our present study, in addition to 2-APB, we used BEL-induced inhibition of iPLA₂ as a selective way to impair SOC activation, in as much as only SOC require its functional activity (4, 9, 16, 22–25, 28). BEL (and its chiral enantiomers) provides new specific tools for assessing the role of iPLA₂β and SOC activation in intact vessels, in which molecular knockdown of proteins poses significant difficulties.

In summary, we have demonstrated that iPLA₂-dependent activation of SOC, as well as Ca_L²⁺ channels, is crucial for agonist-induced Ca²⁺ entry and constriction in conduit and microvessels (Fig. 7).

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GRANTS

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