Role of Ca$^{2+}$-Independent Phospholipase A$_2$ and Store-Operated Pathway in Urocortin-Induced Vasodilatation of Rat Coronary Artery

Tarik Smani, Alejandro Domínguez-Rodríguez, Abdelkrim Hmadcha, Eva Calderón-Sánchez, Angélica Horrillo-Ledesma, Antonio Ordoñez

Abstract—Urocortin has been shown to produce vasodilatation in several arteries, but the precise mechanism of its action is still poorly understood. Here we demonstrate the role of store operated Ca$^{2+}$ entry (SOCE) regulated by Ca$^{2+}$-independent phospholipase A$_2$ (iPLA$_2$) in phenylephrine hydrochloride (PE)-induced vasoconstriction, and we present the first evidence that urocortin induces relaxation by the modulation of SOCE and iPLA$_2$ in rat coronary artery. Urocortin produces an endothelium independent relaxation, and its effect is concentration-dependent (IC$_{50}$=4.5 nmol/L). We show in coronary smooth muscle cells (SMCs) that urocortin inhibits iPLA$_2$ activation, a crucial step for SOC channel activation, and prevents Ca$^{2+}$ influx evoked by the emptying of the stores via a cAMP and protein kinase A (PKA)–dependent mechanism. Lyso phosphatidylcholine and lysophosphatidylinositol, products of iPLA$_2$, exactly mimic the effect of the depletion of the stores in presence of urocortin. Furthermore, we report that long treatment with urocortin downregulates iPLA$_2$ mRNA and proteins expression in rat coronary smooth muscle cells. In summary, we propose a new mechanism of vasodilatation by urocortin which involves the regulation of iPLA$_2$ and SOCE via the stimulation of a cAMP/PKA-dependent signal transduction cascade in rat coronary artery. (Circ Res. 2007;101:1194-1203.)

Key Words: urocortin ■ iPLA$_2$ ■ vasoconstriction ■ store operated Ca$^{2+}$ entry ■ cAMP-PKA

Coronary artery smooth muscle cells (SMCs) regulate vascular tone influencing perfusion of the heart, peripheral resistance, and as a consequence heart function. Agonist induces a contraction of vascular SMCs by a rise in cytosolic free Ca$^{2+}$ concentration$^{1,2}$ because of a rapid Ca$^{2+}$ release by InsP$_3$ from intracellular stores and a transmembrane Ca$^{2+}$ influx through L-type voltage-dependent Ca$^{2+}$ channels or nonvoltage-gated channels such as store-operated Ca$^{2+}$ (SOC) channels. The relative contribution of each channel depends on the smooth muscle type.$^{3-4}$ The use of selective inhibitors of sarcoplasmic reticulum Ca$^{2+}$-ATPase pump, as thapsigargin (TG), to activate SOC channels not only increases Ca$^{2+}$ influx but also enhances tone in a variety of SMCs.$^{3,5}$ Recently we showed Ca$^{2+}$-independent phospholipase A$_2$ (iPLA$_2$) to be a crucial determinant of store-operated Ca$^{2+}$ entry (SOCE). We demonstrated that the emptying of the stores activated iPLA$_2$ and its lysophospholipid products opened the SOC channels in aortic SMCs and nonexcitable cells.$^{7,8}$ Thus iPLA$_2$ became a potential physiological target for regulation and fine-tuning of SOCE by other signaling cascades in SMCs.

A few years ago a new 40-aa peptide, urocortin,$^{9,10}$ related to corticotropin-releasing factor (CRF) was described as a new player in cardiac control,$^{11,12}$ and was proposed to protect cardiac myocytes during ischemia/reperfusion by downregulating iPLA$_2$ expression.$^{13,14}$ Urocortin also emerged as a potent vasodilator peptide, and its mechanism of action appears to be complex, eg, vasodilatation has been reported to be both endothelium-dependent and independent in coronary artery$^{15,16}$ and in other vessels.$^{16-18}$ The vascular effects of urocortin are mediated by the CRF receptors 2 (CRF-R$_2$) which predominates in blood vessels.$^{12,20}$ Binding of urocortin to CRF-R$_2$ increases its affinity for the Gs protein leading to the stimulation of cAMP/PKA pathway (for review see$^{12}$). Furthermore, cAMP-dependent protein kinase seems to modulate SOC channels in rabbit portal vein and in airway smooth muscle.$^{21,22}$

Because urocortin modulates iPLA$_2$ activity and expression in cardiomyocytes, here we test whether urocortin could modulate iPLA$_2$ and in consequence SOCE that might regulate vascular tone of coronary artery. We unveil new important data of the mechanism by which urocortin relaxes rat coronary artery that include cAMP increase, iPLA$_2$ activity and expression modulation, and SOCE regulation in rat coronary SMCs.

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From the Laboratorio de Investigación Cardiovascular (T.S., A.D.-R., E.C.-S., A.O.), Hospital Universitario Virgen del Roció, Universidad de Sevilla, and Centro Andaluz de Biología Molecular & Medicina Regenerativa (A.H., A.H.-L.), Sevilla, Spain.

Correspondence to Tarik Smani, PhD, Laboratorio de Investigación Cardiovascular, Quirófanos Experimentales, Hospital General Universitario Virgen del Rocio, Avenida Manuel Siurot s/n, E-41013 Sevilla, Spain. E-mail tasmani@us.es

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Materials and Methods
Expanded details of all the methods are given in the supplemental data (available online at http://circres.ahajournals.org).

Cells and Arteries Preparation
Isolated rat coronary arteries were dissected from the heart, and SMCs were dispersed acutely or primary cultured as detailed in the online supplemental data. Isometric tension of rat coronary rings was measured in an organ chamber as previously described.23

Measurement of Contractility in Arterial Rings
Rat coronary arteries were obtained from 2-month-old Wistar male rats. Arteries were cleaned of connective tissue, cut in rings (2 mm), and mounted on a small-vessel myograph (JP Trading) to measure isometric tension connected to a digital recorder (Myodataq-2.01, Myodata-2.02 Multi-Myograph System).

Intracellular Ca\(^{2+}\) and Mn\(^{2+}\) Measurement
Dual-excitation imaging with fura-2 was used to measure cytosolic Ca\(^{2+}\) and Mn\(^{2+}\) changes in isolated SMCs as previously shown.6–8

Molecular Studies
The activity of iPLA\(_2\) was performed as described.6–8 The Kinase-Glo Luminescent Kinase Assay (Promega) was used to measure the PKA activity in coronary arteries after the indications of the manufacturers. Q-PCR, Western blot, and immunostaining were used to determine iPLA\(_2\) expression in coronary SMCs.

Statistical Analysis
Group data are presented as mean±SEM. Single or paired Student t test was used to determine the statistical significance of the obtained data. The difference was considered significant at \(P<0.01\) and is marked by * in the figure.

Results
Urocortin Induces Dose-Dependent Relaxation of Rat Coronary Artery
The cumulative addition of urocortin to coronary artery precontracted with phenylephrine (PE) induced a concentration-dependent relaxation with an IC\(_{50}\) ≈ 4.5 nmol/L and maximum relaxation (≈84%) was observed with 10 nmol/L urocortin (Figure 1A). Pretreatment of the arteries with L-NNA (100 μmol/L) to inhibit nitric oxide synthase and endothelial-mediated relaxations induced a small and nonsignificant effect on urocortin dose-dependent relaxation (Figure 1A, right panel). Meanwhile, urocortin (10 to 100...
nmol/L) failed to relax the contraction induced by high K⁺ that involve L-type Ca²⁺ channels pathway (Figure 1B). Furthermore, in presence of 500 nmol/L nifedipine (the selective inhibitor of L-type Ca²⁺ channels), PE was able to produce a small but noticeable contraction that was relaxed by urocortin (Figure 1C) suggesting that urocortin-induced relaxation may occur independently of L-type Ca²⁺ channels pathway.

**Contribution of SOCE and iPLA₂ in PE-Induced Contraction**

Agonist-induced contraction may involve nonvoltage-gated channels such as SOC channels; therefore the contribution of SOCE in coronary vascular tone was examined. Supplementary Figure Ia and Ib shows that PE-induced contraction was relaxed in a dose-dependent manner by 2 aminoethoxydiphenyl borate (2APB) and diethylstilbestrol (DES), inhibitors of SOC channels. Moreover and because we recently demonstrated that iPLA₂ is involved in SOCE, we found that inhibition of iPLA₂ by bromoenol lactone (BEL) relaxed gradually the artery (supplemental Figure Ic). Although BEL is a potent iPLA₂ inhibitor, it also inhibits phosphatidate phosphohydrolase-1 (PAP-1). To block the activity of PAP-1, we pretreated the artery with 50 μmol/L propanolol (PAP-1 specific inhibitor) and observed that propanolol failed to prevent BEL-induced relaxation confirming that PAP-1 is not involved in BEL effect (data not shown). In addition, in isolated fresh and cultured coronary SMCs, 2APB (75 μmol/L), DES (1 μmol/L), and BEL (25 μmol/L) inhibited TG-(2 μmol/L) evoked Ca²⁺ and Mn²⁺ influx (supplemental Figure II), like the data in aorta SMCs. Importantly, when the contraction was induced by depolarization with high K⁺, 2APB and BEL failed to relax the arteries (supplemental Figure III).

**Role of Store-Operated Ca²⁺ Entry in Urocortin-Induced Vasorelaxation**

We then checked the role of SOC channels in urocortin effect on contraction. Figure 1D shows that the activation of SOCE...
with thapsigargin (TG, 10 μmol/L) caused a contraction that was approximately 93% relaxed by urocortin (10 nmol/L). Interestingly, as shown in Figure 1E, TG-induced contraction was also relaxed 69% by nifedipine (1 μmol/L) indicating a possible coactivation of SOC and L-type Ca\(^{2+}\) channels as reported in other SMCs.30 We further performed experiments in acutely dispersed and primary cultured rat coronary SMCs loaded with fura-2 to examine the effect of urocortin on TG-induced SOCE, and we observed that 10 minutes pretreatment with urocortin (10 nmol/L) prevented TG-induced Ca\(^{2+}\) entry in fresh (Figure 2B) and cultured (Figure 2C) SMCs. It is important to note that urocortin effect was specific to Ca\(^{2+}\) influx and did not alter TG-induced Ca\(^{2+}\) release from the stores. This observation was confirmed when low concentration of ionomycin (100 nmol/L) was used to release Ca\(^{2+}\) from the store as described.31 The ratio of ionomycin-induced Ca\(^{2+}\) release was 0.23 ± 0.01 in control versus 0.22 ± 0.02 in urocortin treated SMCs (n=3 cultures). Furthermore, to exclude the influence of mechanisms that remove Ca\(^{2+}\) from the cytoplasm, we used Mn\(^{2+}\)-quench technique considered more direct measurement of ion channel mediated cation influx in intact cells.6,24,32 Indeed, TG-induced Mn\(^{2+}\) influx was also inhibited by urocortin in cultured (Figure 2D) and freshly isolated coronary SMCs (Figure 2E). All together suggest that urocortin modulates agonist-induced contraction of rat coronary artery apparently by SOCE regulation.

Urocortin Modulates iPLA\(_2\) Activity and Expression in Coronary SMCs

Urocortin has been shown to protect the heart against ischemia-reperfusion by iPLA\(_2\) downregulation,13,14 thus we investigated whether urocortin could modulate iPLA\(_2\) activity and expression in coronary artery. First, the activation of iPLA\(_2\) by the depletion of Ca\(^{2+}\) stores was determined in coronary SMCs, and consistent with previous reports,6-8,33 TG enhanced iPLA\(_2\) activity in control coronary SMCs whereas in SMCs treated with urocortin (100 nmol/L) for 10 minutes the iPLA\(_2\) activity was decreased about 80% (Figure 3A). In addition, the expression of iPLA\(_2\) was assessed using real-time quantitative PCR. We observed a concentration-dependent decrease of iPLA\(_2\) mRNA in SMCs treated 24 hours with urocortin and 100 nmol/L urocortin decreased 58% of iPLA\(_2\) mRNA expression (Figure 3B). In addition Western blot study showed that 100 nmol/L urocortin also downregulated about 45% of iPLA\(_2\) protein expression (Figure 3C). These results determine that urocortin can modulate iPLA\(_2\) activity and expression in coronary SMCs that may be crucial for SOCE regulation.

The activation of iPLA\(_2\) releases lysophospholipids and arachidonic acid7,28 that can be metabolized by cyclooxygenase (COX).34 To assess the role of COX pathway in urocortin response, PE was administrated in presence of indomethacin, a widely used COX inhibitor; and consistent with previous results that inhibition of COX decreased vascular tone and this decrease was not observed in urocortin treated SMCs (Figure 3D).
data in aorta.5 PE in presence of 10 μmol/L indomethacin induced a contraction of the same magnitude as in control (Figure 3D) which was relaxed by urocortin. These results discarded the involvement of COX regulation in urocortin-induced relaxation of coronary artery as it was shown previously.15

Urocortin Does Not Prevent SOCE Induced by iPLA2 Products, Lysophosphatidylcholine, and Lysophosphatidylinositol

Lysophospholipids has been shown to activate ionic channels in a membrane-delimited fashion. Here, we checked whether lysophospholipids could mimic the effect of the depletion of the stores as we showed previously in aortic SMCs and rat basophilic leukemia (RBL) cells, independently of urocortin’s negative modulation of iPLA2. In coronary SMCs treated with urocortin, lysophosphatidylcholine (LPC, 300 nmol/L) and lysophosphatidylinositol (LPI, 300 nmol/L) evoked practically the same Ca2+ (Figure 4A,C) and Mn2+ (Figure 4B and 4D) influx as in untreated SMCs, which were inhibited by 2APB consistent with the involvement of the SOC channels. These results suggest that urocortin is not inhibiting directly SOC channels and confirm that its effect is through iPLA2.

![Figure 4. Lysophosphatidylcholine and lysophosphatidylinositol evoke an urocortin insensitive Ca2+ and Mn2+ influx.](image)

Urocortin Inhibits SOCE and Contraction via cAMP and PKA Signaling Pathway

We examined the effect of astressin, specific antagonist of CRF-R2, on contraction and SOCE. We observed that the application of astressin (500 nmol/L) blocked urocortin induced vasodilatation of coronary arteries (n=6, data not shown), and prevented the inhibition of TG-induced Ca2+ and Mn2+ influx by urocortin in SMCs as shown in Figure 2B, 2C, 2D, and 2E. CRF-R2 is functionally linked to the Gs protein that activates adenylate cyclase and cAMP production. We tested the effect of dibutyl-cAMP (db-cAMP, a cell permeable analogue of cAMP) on contraction and on TG-induced Ca2+ and Mn2+ influx. We found that db-cAMP (300 to 500 μmol/L) mimicked the effect of urocortin and induced a vasodilatation of PE- and TG-induced contraction (Figure 5A and 5B), whereas in fura-2 loaded fresh and primary cultured SMCs, db-cAMP (300 to 500 μmol/L) inhibited TG-induced Ca2+ (Figure 5C and 5D) and Mn2+ (Figure 5E and 5F) influx, similar to that observed with urocortin. Importantly, db-cAMP inhibited iPLA2 activity in the same way as urocortin (Figure 6B).

Protein kinase A (PKA) is the major target of cAMP signaling and is known to contribute to its impact on vascular
function. Therefore, we investigated whether PKA is involved in urocortin action on coronary vasorelaxation and on SOCE. Figure 6A shows that urocortin (100 nmol/L) increased PKA activity equally in coronary artery stimulated or not with PE (1 mmol/L). Conversely the inhibition of PKA with KT5720 (1 μmol/L) blocked urocortin-stimulated PKA activity (Figure 6A), and importantly it inhibited iPLA2 activation (Figure 6B). Additionally, PKA blocking prevented urocortin-induced relaxation of PE-induced contraction (Figure 6C) and its inhibition of the store-operated Ca2+ (Figure 5C and 5D) and Mn2+ influx (Figure 5E and 5F). Similar data were observed when H-89 (1 μmol/L) was used to inhibit PKA (data not shown). Importantly, the downregulation of iPLA2 protein expression (Figure 7A) by urocortin was reversed in SMCs pretreated previously with KT5720 (1 μmol/L). The immunostaining approach (Figure 7B) shows the faint fluorescence in urocortin-treated cells and the difference observed in the merged images. These results suggest that urocortin regulates iPLA2 activity and expression via a cAMP/PKA dependent mechanism which modulates SOCE in coronary artery.

All together, our data suggest a new mechanism of urocortin effect on Ca2+ signaling and vascular tone that involves iPLA2 and the SOC pathway summarized in Figure 8.
precontracted rat coronary and human mammary artery, \(15-17\) \(100\) nmol/L of urocortin induced maximum relaxation in U46619 (5 nmol/L). In arteries incubated 5 minutes with PE (1 nmol/L) and then 5 minutes with urocortin (10 nmol/L, \(K+\) + Ucn); and in rings treated 10 minutes with KT5720 (1 \(\mu\)mol/L) and incubated 5 minutes with PE (1 nmol/L), then 5 minutes with urocortin (10 nmol/L) (\(Kt+S\) + Pe + Ucn). \(n=9\) samples from 3 separate experiments. B, Average activity of iPLA\(_2\) in SMCs treated with TG (5 \(\mu\)mol/L) for 10 minutes to deplete the stores in control cells (TG), in cells pretreated 10 minutes with Urocortin (100 nmol/L, + Ucn); in cells treated 15 minutes with KT5720 (1 \(\mu\)mol/L) followed by 10 minutes Urocortin (100 nmol/L, \(Kt+Uc\)); and in cells where db-cAMP was applied 5 minutes (500 \(\mu\)mol/L) + db-cAMP). Activity is normalized to total activity in control SMCs. \(n=3\) cultures. C, Representative recording and average data of the isometric tension showing that KT5720 (1 \(\mu\)mol/L) prevent urocortin (10 nmol/L) but not BEL.

Discussion

The major findings of this study describe a new mechanism for urocortin regulation of vascular tone that involves for the first time \(Ca^{2+}\)-independent phospholipase \(A_2\) and SOCE, which produces a vasoconstriction. We propose that the binding of urocortin to its receptor CRF-R \(2\) stimulates the cAMP/PKA signaling pathway which we showed can negatively modulate iPLA\(_2\), “shut down” the store-operated pathway, and in consequence induces the vasorelaxation of coronary artery.

Furthermore, we presented evidences that urocortin relaxed PE- and TG-activated coronary vasoconstriction by SOCE modulation. Importantly, TG-induced contraction seems to involve SMC depolarization and the consequent coactivation of SOC and \(L\)-type \(Ca^{2+}\) channel as it was reported previously in other SMCs.\(^3,30\) On the other hand, urocortin inhibited TG-induced store operated \(Ca^{2+}\) and \(Mn^{2+}\) influx that we recorded in fresh and primary culture of coronary SMCs. In fact, we confirmed that SOCE is involved in coronary vascular tone regulation. Unfortunately, the progress in the SOCE field has been severely hindered by the absence of relatively specific inhibitors of SOC channels; and despite the lack of specificity of 2APB it remains the most reliable and widely used inhibitor of SOCE.\(^24,25\) Diethylstilbestrol (DES) which has been demonstrated as more specific inhibitor for SOC channels in SMCs, RBL, and Platelets,\(^26,27\) inhibited SOCE in coronary SMCs and induced a very potent relaxation of coronary artery. As reported iPLA\(_2\) activation is absolutely required for the activation of SOC channels and SOCE after depletion of \(Ca^{2+}\) stores.\(^6-8,33\) Here, we brought new and important data concerning the contribution of iPLA\(_2\) and SOCE on coronary vascular tone. Interestingly, urocortin inhibited the activity of iPLA\(_2\) in the same way as iPLA\(_2\) antisense and BEL as described.\(^6,8,33\) This inhibition suggests that the decrease of iPLA\(_2\) products will shut down the \(Ca^{2+}\) influx through SOC channels. Importantly in presence of urocortin LPC and LPI, iPLA\(_2\) products, evoked exactly the same 2APB sensitive scenario, we suggest that an agonist that releases \(Ca^{2+}\) from intracellular stores activates iPLA\(_2\) and SOCE, which produces a vasoconstriction. We propose that the binding of urocortin to its receptor CRF-R\(_2\) stimulates the cAMP/PKA signaling pathway which we showed can negatively modulates iPLA\(_2\), “shut down” the store-operated pathway, and in consequence induces the vasorelaxation of coronary artery.

(25 \(\mu\)mol/L) relaxation of PE- (1 mmol/L) induced contractions. The bar graphs represent the mean±SEM tension expressed in percent of resting tension in each ring (\(n=7\)).
SOCE as in control SMCs demonstrating that even when functional activity of iPLA₂ is inhibited with urocortin its downstream products are capable of activating SOCE in SMCs. The ability of lysophospholipids to activate SOCE in the presence of urocortin ruled out any possibility that urocortin could be inhibiting SOC channels directly. Lysophospholipid activation of SOCE was originally proposed in SMCs and RBL cells and now are shown to activate SOC channels in rat cerebellar astrocytes and some TRP channels in prostate and in Human saphenous vein SMCs.

Furthermore, we established that urocortin via the activation CRF-R₂, stimulated cAMP/PKA pathway. Therefore we showed that db-cAMP mimicked the effects of urocortin and inhibited iPLA₂ activity and SOCE as well as it relaxed TG- and PE-induced vasoconstriction; meanwhile we determined that urocortin regulation of iPLA₂ activity, SOCE, and contractions are mediated by PKA. The precise mechanism(s) by which urocortin modulates iPLA₂ through PKA remains to be determined, but it is known that iPLA₂ possesses numerous potential sites for phosphorylation and its modulation by protein kinase has been proposed in coronary endothelial cells.

Our interests in demonstrating that urocortin could modulate iPLA₂ take us to a very intriguing outcome. Indeed, besides its acute effect on iPLA₂ activation we found that urocortin downregulated iPLA₂ expression. Real-time quantitative PCR, Western blot, and immunohistochemistry results showed that urocortin modulated iPLA₂ expressions in coronary SMCs similar to that described in cardiomyocytes. Here, we gave new data and proof demonstrating that these effects can be observed also in SMCs and are dependent on a mechanism involving cAMP and PKA. The downregulation of iPLA₂ by urocortin can be of major interest, as iPLA₂ is known to participate in several important cell processes such as ischemia/reperfusion syndrome, cellular remodeling, and cell cycle progression.

In summary, our results add further confirmations of the crucial role of the store-operated pathway and iPLA₂ in the regulation of vascular tone and establish iPLA₂ as a potential target for different signaling cascade as the one that involves...
urocortin, cAMP, and PKA. Independent lines of evidence presented here reveal urocortin, via a CAMP/PKA mechanism, as a negative modulator of iPLA₂ activity which leads to the shut down of SOC channels, the inhibition of Ca²⁺ influx that provokes a vasodilatation of rat coronary artery as summarized in Figure 8. We believe that this novel finding may provide a new molecular basis for developing new therapeutic agents for cardiovascular diseases associated with iPLA₂ modulation and Ca²⁺ regulation of vasconstriction.

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Disclosures
None.

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Experimental Procedures

Cells and arteries preparation. Primary culture of coronary arteries from anesthetized Wistar male rats (~500g, 2 months old) was prepared following the same protocol as described previously (1,2). Isolated coronary artery were dissected from the heart, cleaned and cut into pieces, and incubated with 1 mg/ml elastase and 2 mg/ml collagenase in Dublbecco’s Modified Eagle’s Medium (DMEM, PAA labs.) for 30 min at 37°C. Cells were mechanically dispersed, plated on cover slips, and grown in DMEM supplemented with 10% fetal bovine serum (FBS, PAA labs.), 100U/mL penicillin and streptomycin in humidified atmosphere of 5% CO₂ and 95% air at 37°C. Cells exhibited positive fluorescence with antibodies against α-smooth muscle actin. Freshly dispersed cells were obtained following the same protocol as above but dispersion was conducted in a dissociation medium (DM) of the following composition (in mmol/L): 137 NaCl, 5.4 KCl, 0.44 KH₂PO₄, 0.42 NaH₂PO₄, 2 MgCl₂, 4.17 NaHCO₃, 0.2 CaCl₂, 11.11 Glucose, 0.05 EGTA, 10 Hepes. Isolated cells were plated on coverslips in fresh DM and saved in 4°C until their
use. All animal experiments were carried out in accordance with protocols and guidelines established by the US National Institutes of Health.

**Measurement of Contractility in Arterial Rings.** Rat coronary arteries were obtained from 2 month old Wistar male rats. Arteries were cleaned of connective tissue, cut in rings (~2 mm) and mounted on a small-vessel myograph (JP Trading, Aarhus, Denmark) to measure isometric tension connected to a digital recorder (Myodataq-2.01, Myodata-2.02 Multi-Myograph System) as previously described (3). The rings were placed on a chamber filled with the physiological salt solution (PSS) and bubbled with 95% O₂ and 5% CO₂. Before the experiments, the segments were subjected to an optimal tension (90% of the vessels diameter when relaxed and under an intramural pressure of 100 mmHg) and stabilized for at least 1 h following Mulvany’s standard methods (4). Vasoconstriction amplitude was normalized to resting (basal) tone and expressed in percent that was determined in each ring before agonist addition. Summary data presented in bar graphs show the increment of the vasoconstriction normalized to resting tone. Experiments were performed at 37°C. The composition of PSS was (in mmol/L): 118.5 NaCl, 4.7 KCl, 2.5 CaCl₂, 25 NaHCO₃, 1.2 MgSO₄, 1.2 KH₂PO₄, 5 glucose.

**Intracellular Ca²⁺ measurement.** Fluorescence was monitored using a Nikon TS-100 inverted microscope equipped with a 20X fluor objective (0.75 NA) as described previously (1,5). SMC plated on 30 mm round coveslips and mounted in a Teflon chamber were incubated in DMEM with 2 μmol/L fura-2 AM for 30 min at 37°C then the cells were washed. Fluorescence images of 20 to 30 cells were recorded and analyzed with a digital fluorescence imaging system (InCyt Im2, Intracellular Imaging Inc., Imsol, UK) equipped
with a light-sensitive CCD camera (Cooke PixelFly, ASI, Eugene, OR, USA). Changes in intracellular Ca\(^{2+}\) are represented as the ratio of fura-2 fluorescence induced at an emission wavelength of 510 nm due to excitation at 340 nm and 380 nm (ratio = \(F_{340}/F_{380}\)).

Experiments were done in 0Ca\(^{2+}\) solution (in mmol/L: 120 NaCl, 4.7 KCl, 4 MgCl\(_2\), 0.2 EGTA, 10 hepes), and the Ca\(^{2+}\) influx was determined from changes in fura-2 fluorescence after re-addition of Ca\(^{2+}\) (2 mmol/L). \(\Delta\)ratio was calculated as the difference between the peak ratio after extracellular Ca\(^{2+}\) was added and its level right before Ca\(^{2+}\) addition. The basal Ca\(^{2+}\) influx in primary culture of SMC was 0.45 ± 0.1 A.U. \((n = 130)\) and 0.51 ± 0.1 A.U. \((n = 30)\) in acutely dispersed SMC. Basal Ca\(^{2+}\) influx was subtracted from summary data but not from the original traces.

**Mn\(^{2+}\) Influx.** The rate of Mn\(^{2+}\) influx-induced fura-2 quenching was used to estimate SOC influx into SMC as we described previously (1). Fura-2 was measured at 360 nm before and after the addition of 200 \(\mu\)mol/L MnCl\(_2\). SOC entry was evaluated from the rate at which fura-2 fluorescence was quenched by Mn\(^{2+}\), which enters the cell as a Ca\(^{2+}\) surrogate and reduces fura-2 fluorescence on binding to the dye. For each experiment, the fluorescence was normalized to the value measured immediately before addition of Mn\(^{2+}\). \(\Delta F_{360}\) was calculated as the difference between the peaks of the fluorescence measured 1 min after Mn\(^{2+}\) addition and its level right before Mn\(^{2+}\) addition. The basal Mn\(^{2+}\) influx was 10.6 ± 0.6 % \((n = 6)\) and 5.6 ± 4.2 % \((n = 50)\) in fresh and cultured SMC respectively; and was subtracted from summary data but not from the original traces. All the drugs were from Sigma and Fluka.
**Molecular Studies.** The activity and western blotting was performed as previously described (1,5). Coronary SMC were seeded in 6 wells plate and cultured until confluency. After each treatment SMC were collected, homogenized in RIPA buffer supplemented with 1mM PMSF and 10% cocktail of protease inhibitors (Sigma), sonicated and centrifuged at 20,000 g for 15 min at 4 °C. The supernatant was removed and the amount of protein was determined using the Bio-Rad protein dye reagent (Bradford method). The samples were aliquoted (30 μg each), frozen and stored at -80 C until later use.

**Western Blots.** The protein samples were incubated with Laemmli sample buffer at 95 °C for 2 min, and 30 μg of total protein loaded were loaded on a 7.5% SDS-polyacrylamide gel for electrophoresis. Proteins were transferred to nitrocellulose membranes in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at 40 V overnight. The membrane was then blocked in PBST (PBS containing 0.05% Tween 20) with 3% milk for 1 h and then incubated with primary (anti-iPLA₂) antibody (Cayman Chemical) for 2 h at room temperature. The primary antibody was removed, and blots were washed 3 times for 10 min with milk/PBST. Then blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody diluted 1:2000 in milk/PBST, washed 3 times in PBST, and treated with enhanced chemiluminescence reagents (Super ECL, Pierce) for 1 min. Blots were then exposed to photographic films, and the optical density was determined using Image J software. The amount of iPLA₂ protein in SMC is normalized to control cultures. For all experiments, staining with antibody against β-actin was used to demonstrate uniformity of reaction conditions between groups.
**Double immunostaining.** Cells grown in Lab-Tek Chamber glass-bottomed slide system (Nalge Nunc) were fixed with 4% (w/v) fresh paraformaldehyde for 20 min in PBS, treated for 1 h with 1% Heat inactivated donkey serum, and 1% Heat inactivated Goat serum containing 0.1% Triton X-100 in PBS (for blocking) and incubated in a humidified chamber overnight a 4ºC with the primary antibodies solution (Rabbit anti-iPLA2 “Cayman” 1:25 dilution and Goat anti MHC (Y-20) “Santa Cruz” 1:25 dilution). After PBS washes, the slides were incubated for 1 hour in dark with the secondary antibodies solution (Alexa Flúor 568 Donkey anti-goat and Alexa Flúor 568 Goat anti-rabbit “Molecular Probes” 1:100 dilution). The slides were mounted with Vectashield medium with DAPI (Vector Labs) and the fluorescent signal was visualized on a Leica fluorescence microscope (DM-6000; Leica Microsystems Wetzlar GmbH, Germany). As negative controls, the cells were incubated with secondary antibodies only.

**Quantitative Real Time PCR.** Total RNA was extracted using RNeasy® kit (Qiagen). A total of 1 µg RNA was reverse transcribed to cDNA using Super Script™ III according to the manufacturer’s guidelines (Invitrogen life technologies). Gene-specific primers were designed using the Primer Express® software V.3.0 (Applied Biosystems) and were checked for gene specificity using NCBI Blast. In presence of SYBR® GREEN (Applied Biosystems) the primers were used to amplify the expressed cDNA using the Applied Biosystems 7500 Real Time PCR system. The amplification of iPLA2 gene was corrected against the expression level of the housekeeping gene β-actin. The relative quantification of gene expression was calculated using the ΔΔCt comparative method.
iPLA₂ Activity was performed using a modified commercial assay kit originally designed for the cytosolic phospholipase A₂, cPLA₂ (Cayman). To detect the activity of iPLA₂ instead of cPLA₂, the assay buffers were modified to contain no Ca²⁺ (Ca²⁺ is needed for cPLA₂, but not for iPLA₂ activity). Phospholipase activity was assayed by incubating the samples with the substrate, arachidonoyl thio-PC, for 1 hour at room temperature in a modified Ca²⁺-free assay buffer of the following composition (in mmol/L): 300 NaCl, 10 HEPES, 8 Triton X-100, 4 EGTA, 60% glycerol, and 2 mg/ml of BSA (pH 7.4). The generated free thiols were visualized by the addition of DTNB for 5 min, and the absorbance was determined at 405 nm using a standard microplate reader. The background iPLA₂-independent component of basal lipase activity was determined in control samples when all specific iPLA₂ activity was inhibited with BEL (10 μmol/L for 5 min), and was subtracted from all the readings. The specific activity of iPLA₂ was expressed in absorbance/μg protein units.

PKA Activity Assay. The Kinase-Glo® Luminescent Kinase Assay (Promega) was used to measure the PKA activity. Coronary arterial rings were test to assess the activity in the following conditions: untreated rings (control), rings treated 5 min with urocortin (Ucn, 10 nmol/L), rings incubated 5 min with PE (0.5 mmol/L) and then Ucn (10 nmol/L, 5min), and rings pretreated with KT5720 (1 μmol/L for 15min) then treated with PE (0.5 mmol/L for 5 min) followed by Ucn (10nmol/L for 5min). After each treatment the arterial rings were washed in ice-cold PBS, homogenized in RIPA buffer supplemented with 1mM PMSF and 10% cocktail of protease inhibitors (Sigma), sonicated and centrifuged at 20,000 g for 15 min at 4 °C. The supernatant was collected and the amount of protein was determined.
using the Bio-Rad protein dye reagent (Bradford method). The PKA activity was measured in each sample according to the manufacturer’s instruction as follow, 50 μmol/L of each sample were added to 50 μmol/L of reconstituted Kinase-Glo® Reagent and the reaction were run for 15 min at room temperature. Luminescence was recorded on a GloMax™ 96 Microplate Luminometer (Promega). The reaction generates a luminescent signal that is inversely proportional to the amount of kinase activity and is correlated with the amount of ATP present. The activity is expressed in RLU⁻¹ (relative light units)/ amount of protein.

References.


**Online Table 1 Primers**

<table>
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<tr>
<th>Gene</th>
<th>Oligo Sequence</th>
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<td>iPLA₂</td>
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<td>Reverse Primer TCT CCT TCT GCA TCC TGT CAG CAA</td>
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Figure S1. SOC channels inhibition by 2APB and DES; and iPLA₂ blocking with BEL induce a vasodilatation of PE pre-contracted coronary artery. (a), left panel shows representative recording of the isometric tension of PE- (1 mmol/L) induced contractions with dose-dependent relaxation by 2APB in coronary artery rings. Right panel shows average of PE-induced contraction in presence of different concentrations of 2APB as shown in left panel. (b) and (c), shows average of PE-induced contraction in presence of increasing concentrations of DES and BEL respectively. Each data points represent the mean ± s.e.m. tension (per cent of the maximum contraction in each ring). The best fit was generated using Hills equation for 2APB and DES and Sigmoid function was used for BEL. IC₅₀ for 2APB = 15.8 ± 0.3 µmol/L (n = 6-10); IC₅₀ for DES = 0.26 ± 0.1 µmol/L (n = 6-12); and IC₅₀ for BEL = 10.0 ± 2.7 µmol/L (n = 6-12).
Figure S2. TG-induced Ca\(^{2+}\) and Mn\(^{2+}\) influx is inhibited by 2APB, DES, and by iPLA\(_2\) inhibition with BEL in fresh and cultured SMC. (a) left panel: representative traces showing the changes in intracellular Ca\(^{2+}\) concentration in fura-2 loaded fresh rat coronary SMC. TG (2 \(\mu\)mol/L) was applied 4-5 minutes in the absence of extracellular Ca\(^{2+}\) and then 2 mmol/L Ca\(^{2+}\) was added as indicated. Traces are for SMC treated with TG (control), when 2APB (75 \(\mu\)mol/L, +2APB) or DES (1 \(\mu\)mol/L, +DES) were added 1-2 min before Ca\(^{2+}\) as indicated by asterisk; and when cells were pre-treated with BEL to inhibit iPLA\(_2\) (+BEL, 25 \(\mu\)mol/L for 30 min at 37°C). In right panel, summary data of the TG-induced Ca\(^{2+}\) influx (\(\Delta\)ratio ± s.e.m.) in fresh SMC as illustrated in left panel. Each bar summarizes result from 12-40 cells. (b), Same as in (a) but in cultured coronary SMC. Summary data is from 104-260 cells. (c), left panel shows representative traces of Mn\(^{2+}\) influx-induced fura-2 quenching expressed in percent change in F\(_{360}\) fluorescence 1 min after the administration of 200 \(\mu\)mol/L Mn\(^{2+}\) in the absence of extracellular Ca\(^{2+}\). Cultured SMC were treated with TG (2 \(\mu\)mol/L for 5 min before Mn\(^{2+}\) was added) under the same conditions as in (a). Right panel shows graphs illustrating the magnitude of Mn\(^{2+}\) quenching in cultured SMC as shown in left panel (n = 36-156 cells). (d), The average changes in TG-induced Mn\(^{2+}\) influx in freshly dispersed SMC as in (c). n = 5-10 cells.
Figure S3. High K⁺-induced contraction is not relaxed by 2APB neither by iPLA₂ inhibition with BEL in coronary artery. (a) Left panel shows that 2APB (30 μM) doesn’t relax high K⁺ (40 mmol/L) -induced contraction in coronary artery. Nifedipine (3 μmol/L) application induces a complete relaxation of the artery. (b) Left panel shows that BEL (25 μmol/L) doesn’t relax high K⁺ (40 mmol/L) -induced contraction in coronary artery. Right panels show the summary data from 4-5 experiments as presented in left.