Store-Operated Ca\textsuperscript{2+} Entry Activates the CREB Transcription Factor in Vascular Smooth Muscle

Renee A. Pulver, Patricia Rose-Curtis, Michael W. Roe, George C. Wellman, Karen M. Lounsbury

Abstract—Ca\textsuperscript{2+}-regulated gene transcription is a critical component of arterial responses to injury, hypertension, and tumor-stimulated angiogenesis. The Ca\textsuperscript{2+}/cAMP response element binding protein (CREB), a transcription factor that regulates expression of many genes, is activated by Ca\textsuperscript{2+}-induced phosphorylation. Multiple Ca\textsuperscript{2+} entry pathways may contribute to CREB activation in vascular smooth muscle including voltage-dependent Ca\textsuperscript{2+} channels and store-operated Ca\textsuperscript{2+} entry (SOCE). To investigate a role for SOCE in CREB activation, we measured CREB phosphorylation using immunofluorescence, intracellular Ca\textsuperscript{2+} levels using a fluorescence resonance energy transfer (FRET)–based Cameleon indicator, and c-fos transcription using RT-PCR. In this study, we report that SOCE activates CREB in both cultured smooth muscle cells and intact arteries. Depletion of intracellular Ca\textsuperscript{2+} stores with thapsigargin increased nuclear phospho-CREB levels, intracellular Ca\textsuperscript{2+} concentration, and transcription of c-fos. These effects were abolished by inhibiting SOCE through lowering extracellular Ca\textsuperscript{2+} concentration or by application of 2-aminoethoxydiphenylborate and Ni\textsuperscript{2+}. Inhibition of Ca\textsuperscript{2+} influx through voltage-dependent Ca\textsuperscript{2+} channels using nimodipine partially blocked intact artery responses, but was without effect in cultured smooth muscle cells. Our findings indicate that Ca\textsuperscript{2+} entry through store-operated Ca\textsuperscript{2+} channels leads to CREB activation, suggesting that SOCE contributes to the regulation of gene expression in vascular smooth muscle. (Circ Res. 2004;94:1351-1358.)

Key Words: SERCA ■ gene transcription ■ calcium channels ■ arteries

Vascular smooth muscle cells (VSMCs) possess an ability to transition between differentiated and proliferative phenotypes in response to environmental cues.\textsuperscript{1} Although the proliferative phenotype is essential for vasculogenesis, uncontrolled proliferation and migration caused by changes in VSMC gene transcription are associated with the development of vascular pathologies such as atherosclerosis, hypertension, postangioplasty restenosis, and tumor-stimulated angiogenesis.\textsuperscript{2,3} Disease-related variations in VSMC phenotype correlate with atypical Ca\textsuperscript{2+} signaling, elevated intracellular Ca\textsuperscript{2+}, and gene transcription.\textsuperscript{4–6} As yet, the interrelationships between Ca\textsuperscript{2+} signaling and transcriptional control of gene expression in VSMCs remain unresolved.

Regulation of gene expression by Ca\textsuperscript{2+} can be mediated by Ca\textsuperscript{2+}-dependent phosphorylation of the transcription factor CREB (Ca\textsuperscript{2+}/cAMP-response element binding protein). Regulation of c-fos and other immediate early genes is in part Ca\textsuperscript{2+}-dependent and requires CREB.\textsuperscript{7,8} CREB activation requires phosphorylation at \textsuperscript{133}Serine to facilitate formation of an active transcriptional complex including recruitment of CREB binding protein (CBP300) and other cofactors to the Ca\textsuperscript{2+}/cAMP-response element (CRE) in the promoter of many genes.\textsuperscript{9–11} CREB phosphorylation can be mediated by multiple kinases including cAMP-dependent protein kinase, ribosomal S6 kinase, mitogen- and stress-activated protein kinases, and calmodulin-dependent protein kinase (CaMK).\textsuperscript{9}

We have previously determined that membrane depolarization increases phosphorylated CREB (P-CREB) levels and c-fos transcription in VSMCs.\textsuperscript{12} This effect is dependent on Ca\textsuperscript{2+} influx through L-type voltage-dependent Ca\textsuperscript{2+} channels (VDCCs) and CaMK activation.\textsuperscript{8,12} In addition, cerebral arteries from hypertensive rats exhibit elevated intracellular Ca\textsuperscript{2+} and an increased level of basal P-CREB and c-fos transcription.\textsuperscript{6}

Multiple sources of Ca\textsuperscript{2+} may participate in regulation of gene expression in VSMCs. Elevation of Ca\textsuperscript{2+} in smooth muscle cells can result from entry of extracellular Ca\textsuperscript{2+} as well as release from Ca\textsuperscript{2+} sequestered within organelles such as the sarcoplasmic reticulum (SR).\textsuperscript{13–15} Ca\textsuperscript{2+} influx across the plasma membrane is mediated by voltage-dependent Ca\textsuperscript{2+} channels, and voltage-independent cation channels including store-operated Ca\textsuperscript{2+} channels. Store-operated calcium entry (SOCE), also known as capacitative Ca\textsuperscript{2+} entry, has been detected in VSMCs\textsuperscript{16,17} and is thought to play an essential role in the regulation of contraction, cell proliferation, and apoptosis.\textsuperscript{18,19} Activation of Ca\textsuperscript{2+} influx through store-
operated Ca\(^{2+}\) channels is triggered by a reduction in SR Ca\(^{2+}\) concentration.\(^{17,18}\) Transient discharge of SR Ca\(^{2+}\) occurs during the course of signaling events that activate inositol 1,4,5-trisphosphate receptors (IP\(_3\)R) or ryanodine receptors in the SR membrane.\(^{15,20}\) SR Ca\(^{2+}\) stores also can be depleted by inhibiting sarcoendoplasmic reticulum Ca\(^{2+}\) ATPases (SERCA) with thapsigargin or cyclopiazonic acid.\(^{21,22}\)

A role for SOCE in the regulation of gene expression in VSMCs is unclear. In the present study, we examined the signaling pathway linking SR Ca\(^{2+}\) store depletion to CREB phosphorylation in cultured VSMCs and intact arterial myocytes. Our findings indicate that Ca\(^{2+}\) entry through SOCE contributes to Ca\(^{2+}\) homeostasis and induces CREB activation, suggesting a novel mechanism for the regulation of gene expression by Ca\(^{2+}\) in VSMCs.

**Materials and Methods**

**Cell Culture, Animals, and Reagents**

Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, 1985) following protocols approved by the University of Vermont IACUC. Female Sprague-Dawley rats (Harlan, Indianapolis, Ind) (~12 weeks, 200 g) were euthanized (pentobarbital 150 mg/kg IP), and the aorta, middle, and posterior cerebral arteries were dissected in cold HBS (HEPES buffered saline). Rat VSMCs were cultured from aorta explants, maintained in DMEM containing 10% fetal bovine serum as detailed previously.\(^{12}\) Human VSMCs were obtained from human cerebral artery explants (IRB No. CHRMS 01-195; informed consent) and cultured in SMGM2 media (Cambrex). VSMCs were used between passages 2 and 4.

Thapsigargin (TG), 1,2-bis(o-aminophenoxy)ethane-N\(_2\),N\(_3\),N\(_6\),N\(_8\)tetraacetic acid (BAPTA), nimodipine (Nim), and ionomycin were purchased from Calbiochem, and 2-aminoethoxydiphenylborate (2-APB) was from Tocris Cookson, Inc. Cell culture reagents were purchased from Gibco. All other chemicals were purchased from Sigma.

**P-CREB Immunofluorescence**

Immunofluorescence was performed using anti-P-CREB antibodies (Cell Signaling Technology) [1:250] and Cy3-anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Labs) [1:500] as described,\(^{12}\) with the following exceptions. VSMCs were fixed with 4% formaldehyde, and 0.1% Triton X-100 was added to blocking and antibody dilution solutions. YOYO-1 (Molecular Probes) [1:10,000] containing 250 g/mL RNase was added for 30 minutes at 37°C to counterstain the cell nuclei. For immunolabeling of intact arteries, Triton X-100 concentration was 0.2%, and Cy5 goat anti-rabbit IgG (Jackson ImmunoResearch Labs) [1:500] was used as a counterstain.

**Figure 1.** Thapsigargin induces a dose-dependent transient increase in CREB phosphorylation in cultured VSMCs. A, Cells were incubated with increasing concentrations of thapsigargin (TG) for 5 minutes, and P-CREB was detected by immunofluorescence. Top, Representative images of P-CREB immunofluorescence in nuclei of VSMCs. Bottom, Graph represents quantification of nuclear P-CREB immunofluorescence intensities (±SEM, n=3). Bar=100 μm. B, Cells were incubated with 100 nmol/L TG over a time period of 1 minute to 24 hours followed by detection of P-CREB by immunofluorescence. Time points for TG addition were staggered such that all cells were simultaneously fixed at 24 hours. Top, Representative images of P-CREB for untreated control and 5-minute and 120-minute time points. Bottom, Graph represents quantification of nuclear P-CREB immunofluorescence intensities normalized by the untreated control P-CREB intensity (±SEM, n=3).
the secondary antibody. Images were captured using a Bio-Rad 1000 laser scanning confocal microscope with a 40× objective. Fluorescence intensities from 30 to 90 nuclei were determined per condition from at least three independent experiments as described.23

RNA Isolation, RT-PCR, and Quantitative RT-PCR

Total RNA was extracted from treated hcVSMCs using TriZol reagent and quantified using the NanoDrop spectrophotometer. For c-fos detection by RT-PCR, RNA (25 ng) was reverse-transcribed using Sensiscript RT kit (Qiagen) according to the manufacturer’s instructions, and cDNA was amplified using c-fos and β-actin primers. PCR products were separated by agarose gel electrophoresis and quantified using Quantity One software. β-Actin was used as an internal standard. For c-fos detection by quantitative RT-PCR, RNA (2 μg) was reverse-transcribed using Omniscript RT kit (Qiagen) according to the manufacturer’s instructions. The resulting cDNA was amplified using Assays-on-Demand gene expression products kits (Applied Biosystems) and analyzed with a 7900HT Sequence Detection System (TaqMan, Applied Biosystems). Assays were run

Figure 2. Thapsigargin-mediated CREB phosphorylation requires Ca2⁺ influx and is reduced by blockers of SOCE in cultured VSMCs. A, VSMCs were incubated in HBS with normal Ca2⁺ (2 mmol/L), 100 nmol/L Ca2⁺, or preincubated with 5 mmol/L BAPTA for 30 minutes then treated with 100 nmol/L thapsigargin (TG) for 5 minutes. P-CREB was detected by immunofluorescence, and shown are histograms of nuclear P-CREB intensities normalized by the untreated control P-CREB intensity (∼SEM, n = 3). ***P<0.001 compared with 2 mmol/L Ca2⁺. B, Where indicated, VSMCs were preincubated with 100 nmol/L nimodipine (Nim) for 15 minutes (n = 3), 100 μmol/L 2-APB for 10 minutes (n = 4), or 500 μmol/L nickel for 10 minutes (n = 4) then treated with 100 nmol/L TG for 5 minutes. Detection and quantification of P-CREB were performed as in A. ***P<0.001 compared with TG alone.

Figure 3. Thapsigargin promotes transcription of c-fos that is insensitive to nimodipine and inhibited by the SOCE blocker 2-APB in cultured VSMCs. A and B, hcVSMCs were incubated in HBS containing 100 nmol/L thapsigargin (TG) or with isotonic elevation of K⁺ to 120 mmol/L for 30 minutes followed by extraction of RNA and RT-PCR using primers recognizing c-fos. Shown are representative agarose gel (A) of the resulting PCR products and histogram (B) of c-fos band intensities relative to β-actin (∼SEM, n = 5). **P<0.01, ***P<0.001. C, hcVSMCs were treated as described, and where indicated, cells were preincubated with 100 nmol/L nimodipine (Nim) for 15 minutes or 100 μmol/L 2-APB for 10 minutes. RNA was isolated and c-fos transcript levels were determined using quantitative RT-PCR. Histograms represent data normalized to the TG response (average ±SEM) of duplicate determinations from 2 independent experiments. *P<0.05.
in duplicate for each independent experiment according to manufacturer's recommendations. HPRT was used as the internal standard. Data analysis was fully automated and performed using Sequence Detection 2.1 software (Applied Biosystems).

**Determination of Intracellular Ca\(^{2+}\)**

VSMCs were grown on coverslips and growth media was replaced with DMEM containing 0.1% fetal bovine serum. Cells were transfected with 1 µg/mL of yellow cameleon2.1 (YC2.1), a fluorescence resonance energy transfer (FRET)–based calcium biosensor\(^{24}\) using Lipofectamine 2000 (Invitrogen). After 2 hours, the media was replaced and cells were maintained in a humidified incubator 24 hours before use. Coverslips were placed into a SA-NIK chamber (Warner Instruments) mounted on a Nikon Diaphot 200 inverted microscope equipped for epifluorescence. Cells were superfused with HBS at room temperature. YC2.1 excitation was 440 nm and emission recorded with an ORCA-ER charge-coupled device (Hamamatsu) at 480 nm (CFP) and 535 nm (YFP). Data were analyzed using Metafluor 3.0 Imaging Software (Universal Imaging Corporation). FRET emission ratio (ratio 535/480) was used as a measure of intracellular Ca\(^{2+}\) concentration. Normalized ratio was obtained by dividing the Ratio 535/480 by the starting baseline ratio value. To calculate the percent maximal FRET ratio change, data were converted to a range of 100% using maximum, Ionomycin (10 µmol/L), and minimum, EGTA (2 mmol/L), values obtained in each experiment. Area under the curve (AUC) ± SEM was calculated using Sigma Plot.

**Statistical Analysis**

Student t test and Student-Newman-Keuls multiple comparisons test were used to determine statistical significance between treatment groups.

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

**Results**

**SERCA Inhibition Promotes CREB Phosphorylation and c-fos Transcription Independent of L-Type Ca\(^{2+}\) Channel Activity in Cultured VSMCs**

Ca\(^{2+}\) entry through L-type VDCCs has been shown to increase phosphorylation and activation of the transcription factor CREB.\(^{8,12}\) To determine whether elevations in Ca\(^{2+}\) triggered by depletion of SR Ca\(^{2+}\) stores can also initiate CREB phosphorylation, SERCA was irreversibly inhibited with thapsigargin. CREB phosphorylation at Ser\(^{133}\) was detected by immunofluorescence using P-CREB antibodies and quantified using nuclear pixel intensity. P-CREB nuclear fluorescence increased in response to thapsigargin in a concentration-dependent manner (Figure 1A). The EC\(_{50}\) (28.2 ± 10.7 nmol/L, n = 3) was similar to the EC\(_{50}\) for thapsigargin-induced arterial contraction (Wellman and Phillips, unpublished observation, 2004) and is consistent with SERCA inhibition.\(^{21}\) Cyclopiazonic acid (CPA), a reversible...
reduce thapsigargin-induced Ca\(^{2+}\) (C), a blocker of SOCE, significantly reduced
intracellular Ca\(^{2+}\) without effect, whereas nickel (Ni\(^{2+}\)) significantly decreased thapsigargin-induced P-CREB levels (D).

To confirm that the observed effects on P-CREB correlated with changes in its transcriptional activity, we measured the effect of thapsigargin on transcription of the CRE-regulated gene, c-fos, using both standard and quantitative RT-PCR. Transcription of c-fos was induced by thapsigargin, and this effect was insensitive to nimodipine, but blocked by 2-APB, closely paralleling the data profile for CREB phosphorylation (Figure 3). Together, these results provide evidence that depletion of SR Ca\(^{2+}\) using thapsigargin leads to CREB activation through a mechanism requiring Ca\(^{2+}\) influx and functional store-operated Ca\(^{2+}\) channels.

**Thapsigargin-Induced Ca\(^{2+}\) Signaling Involves Store-Operated Ca\(^{2+}\) Channels**

The mechanism of CREB activation by thapsigargin was consistent with Ca\(^{2+}\) entry through store-operated Ca\(^{2+}\) channels. To support these data using single cell measurements of Ca\(^{2+}\), we expressed the Cameleon YC2.1, a calmodulin-based FRET biosensor in VSMCs.\(^{24}\) Because this indicator has not been previously characterized in VSMCs, we first examined FRET ratio changes over a range of Ca\(^{2+}\) concentrations. Cells were incubated with buffers of known Ca\(^{2+}\) concentration\(^{21}\) in the presence of the Ca\(^{2+}\) ionophore, ionomycin. A 20% FRET ratio change occurred at 200 mmol/L Ca\(^{2+}\) and the indicator appeared saturated at concentrations above 10 mmol/L (Figure 4A).

Administration of serum, known to stimulate IP\(_3\)-mediated Ca\(^{2+}\) release from thapsigargin-sensitive stores,\(^{28-30}\) resulted in a transient rise in Ca\(^{2+}\). Thapsigargin caused a rapid and more sustained increase in Ca\(^{2+}\) and its administration inhibited subsequent serum responses, indicating that it effectively diminished SR Ca\(^{2+}\) (Figure 4B through 4D). Similar to our results related to CREB phosphorylation, the increase in Ca\(^{2+}\) initiated by thapsigargin was significantly attenuated by BAPTA or 2-APB (Figure 5). These findings indicate that the source of Ca\(^{2+}\) leading to CREB activation by thapsigargin is likely through SOCE.

**Store-Operated Ca\(^{2+}\) Entry Leads to CREB Phosphorylation in Intact Arteries**

VSMCs maintained in culture undergo multiple phenotypic changes.\(^{3}\) It is therefore possible that SOCE and Ca\(^{2+}\) signaling responses may be different in smooth muscle cells present in intact arteries. To measure the effect of SR Ca\(^{2+}\) store depletion on CREB phosphorylation in arterial myocytes, rat cerebral arteries were isolated and treated in vitro with thapsigargin, followed by detection of P-CREB using immunofluorescence. Thapsigargin induced an increase in P-CREB fluorescence that colocalized with nuclei (Figure 6A). In agreement with our previous findings,\(^{4}\) induction of CREB phosphorylation following membrane depolarization by elevated K\(^{+}\) was prevented by nimodipine or reducing extracellular Ca\(^{2+}\). The thapsigargin-induced CREB phosphorylation was partially inhibited by nimodipine, but was ablated by reducing extracellular Ca\(^{2+}\) (Figure 6A and 6B). Furthermore, the nimodipine-insensitive CREB phosphorylation was eliminated by treatment with 2-APB or Ni\(^{2+}\) (Figure 6C), suggesting that thapsigargin-mediated CREB activation is accomplished by Ca\(^{2+}\) signaling through voltage-dependent Ca\(^{2+}\) channels and SOCE in intact arteries.

**Discussion**

The gene expression profile of arterial smooth muscle cells is a critical determinant of the differentiated versus proliferative...
phenotype. CREB is implicated in both promoting VSMC proliferation and conversely in the protection of arteries from smooth muscle cell dedifferentiation. P-CREB levels and c-fos transcription are increased in smooth muscle cells of hypertensive arteries, and inhibition of CREB activity through expression of dominant negative CREB prevents apoptosis and augments mitogenesis of VSMCs. However, CREB content of vascular tissues inversely correlates with VSMC proliferation and migration. In light of its regulation by multiple pathways, CREB likely has pleiotropic effects on smooth muscle cell functions that may explain its regulation of opposing events, depending on the signal source and duration.

The underlying Ca\(^{2+}\)-dependent signaling mechanisms involved in CREB activation and VSMC gene transcription are not completely understood. In this study, we used pharmacological tools and measurements of intracellular Ca\(^{2+}\) to establish a role for SOCE in the activation of CREB in VSMCs. We report that influx of Ca\(^{2+}\), caused by thapsigargin-induced depletion of SR Ca\(^{2+}\), results in transient phosphorylation of CREB and transcription of c-fos. Ca\(^{2+}\) influx through VDCCs did not affect thapsigargin-induced CREB phosphorylation or c-fos transcription in cultured VSMCs derived from vascular explants, but did contribute to P-CREB formation in intact arteries. The effect of SOCE on CREB activation suggests that SR Ca\(^{2+}\) store homeostasis is important in regulating gene expression in vivo and supports the hypothesis that store-operated Ca\(^{2+}\) influx pathways are involved in CREB-mediated transcriptional events in both physiological arterial signaling and in pathological growth changes associated with the development of hypertension and atherosclerosis.

Although Ca\(^{2+}\) is a ubiquitous signaling ion affecting many aspects of VSMC physiology, the relative contribution of different modes of Ca\(^{2+}\) entry or intracellular Ca\(^{2+}\) release in the induction of gene transcription is uncertain. Coupling of Ca\(^{2+}\) influx and intracellular Ca\(^{2+}\) mobilization pathways to CREB activation has been observed in neurons. Our work suggests that similar mechanisms are present in VSMCs. Results in intact arteries indicate that influx of Ca\(^{2+}\) through
either VDCCs or store-operated Ca\(^{2+}\) channels can contribute to regulation of CREB, and suggest that P-CREB formation occurs after global increases in Ca\(^{2+}\). The simplest explanation for the discrepancy between VSMCs from aortic explants and intact arterial myocytes is the reduction in L-type VDCC expression in the cultured cells and indirect effects of thapsigargin on membrane potential\(^3\).\(^5\) The VDCC-independent component of CREB phosphorylation was sensitive to inhibition of SOCE, supporting the hypothesis that SR Ca\(^{2+}\) and SOCE regulate Ca\(^{2+}\)-dependent gene expression in intact arterial myocytes. Consistent with SOCE playing a role in the change between the differentiated and proliferative VSMC phenotypes, previous studies have demonstrated up-regulation of store-operated channels in vascular smooth muscle during proliferation\(^7\) and growth arrest of smooth muscle cells after loss of SERCA expression\(^38\).

The kinases activated downstream of SOCE were not identified in this study. In neurons, CaM kinases have been implicated in the immediate phase of Ca\(^{2+}\)-activated CREB phosphorylation, whereas the Ras/MAP kinase pathway has been linked to sustained CREB phosphorylation\(^39\). CaM kinase activity has also been shown to play an important role in CREB phosphorylation after membrane depolarization in vascular smooth muscle.\(^8\) The transient nature of CREB phosphorylation after SERCA inhibition that we observed in the present study suggests that SOCE activates the immediate pathway involving CaM kinases.

CREB phosphorylation has been established as an important molecular switch to control gene transcription driven by CREs. In this study, we have identified changes in c-fos transcription that correlate with SOCE-induced CREB phosphorylation. It is likely that the interplay between SR Ca\(^{2+}\) homeostasis and SOCE contributes to transcriptional regulation of multiple genes through CREB phosphorylation and interactions with other proteins in transcriptional complexes.\(^40\)\(^–\)\(^42\) Moreover, different spatial and temporal patterns of Ca\(^{2+}\) gradients in VSMCs may add another level of transcriptional regulation.

In summary, we have established that SOCE stimulates phosphorylation of CREB, an essential step in the activation of this transcription factor. Future studies that determine the relative contributions of Ca\(^{2+}\) signals arising from multiple sources to the diverse patterns of CRE-mediated gene expression will contribute greater understanding of Ca\(^{2+}\) regulation of VSMC phenotype and development of vascular pathologies.

Acknowledgments

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Expanded Materials and Methods

Cell culture, animals, reagents, and solutions

All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH PUBLICATION 85-23, 1985) following protocols approved by the Institutional Animal Use and Care Committee of the University of Vermont. Female Sprague Dawley rats (~12 weeks, 200 g) were euthanized (pentobarbital 150 mg/kg intraperitoneal) and the middle cerebral artery, posterior cerebral artery and descending aorta were dissected in cold HBS (HEPES buffered saline) or 100 nmol/L Ca²⁺ HBS. Cerebral arteries were used for intact artery immunofluorescence, and the aorta was used to generate explants of cultured VSMCs as described previously¹. Briefly, rat explants were cultured in DMEM containing 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 1000 units/ml penicillin, and 1 mg/ml streptomycin. VSMCs that migrated within 3 to 5 days were passaged with trypsin/EDTA and cells were used between passages 2 and 4.

Early passage hc VSMCs (passages 2-4) were obtained from human cerebral artery explants and used for RNA isolation. 2 mm slices of human cerebral artery (IRB# CHRMS 01-195; informed consent) were applied to scored 60 mm culture dishes, cultered in SMGM2 media (Cambrex) and passaged with trypsin/EDTA.

Thapsigargin (TG), cyclopiazonic acid (CPA), BAPTA (1,2-bis(o-Aminophenoxy)ethane-N,N,N',N'-tetraacetic Acid), Nimodipine (Nim), and
Ionomycin were purchased from CalBiochem, San Diego, CA. 2-APB (2-aminoethoxydiphenylborate) and levcromakalim (Lev) were purchased from Tocris Cookson, Inc., Ellisville, MO. Cell culture reagents were obtained from Gibco, Grand Island, NY. All other chemicals were obtained from Sigma, St. Louis, MO. The composition of PSS was (in mmol/L): 119 NaCl, 4.7 KCL, 1.2 KH$_2$PO$_4$, 1.2 MgSO$_4$, 1.6 CaCl$_2$, 1.2 MgCl$_2$, 0.023 EDTA, 11 glucose and 24 NaHCO$_3$ (pH = 7.4 with NaOH). The composition of HBS was (in mmol/L): 10 HEPES, pH 7.4, 6 KCl, 140 NaCl, 2 CaCl$_2$, 1 MgCl$_2$, and 10 glucose and the composition of 100 nmol/L Ca$^{2+}$ HBS was (in mmol/L): 10 HEPES, pH 7.4, 6 KCl, 140 NaCl, 0.1 CaCl$_2$ (effectively 100 nmol/L due to EGTA), 1 MgCl$_2$, 10 glucose, and 1.1 mmol/L EGTA. 60 mmol/L K$^+$ HBS consisted of HBS with isotonic replacement of NaCl by KCl (final composition in mmol/L: 60 KCl and 86 NaCl).

**Immunofluorescence of cultured VSMCs**

VSMCs were grown to approximately 60% confluence on glass coverslips in 6-well culture dishes and serum starved in media containing 0.1% FBS 24-48 hr prior to treatment. Following treatment the cells were washed with ice-cold phosphate-buffered saline (PBS), fixed with 4% formaldehyde, and blocked in PBS containing 2% milk and 0.1% Triton X-100. Coverslips were incubated consecutively for 1 hr at 37°C with rabbit anti-P-CREB antibody (Cell Signaling Technology, Beverly, MA) [1:250] and Cy3 goat anti-rabbit IgG (Jackson ImmunoResearch Labs) [1:500] in PBS containing 2% bovine serum albumin
(BSA) and 0.1% Triton X-100. YOYO-1 (Molecular Probes) [1:10,000] containing 250 µg/ml RNase was then added for 30 min at 37°C to counterstain the cell nuclei. Coverslips were washed with PBS and mounted with Aqua Poly/Mount (Polysciences, Inc.). Images were captured using a Bio-Rad 1000 laser scanning confocal microscope with a 40X objective. For statistical analysis, at least 30 cells were quantified from at least 3 independent experiments. P-CREB nuclear fluorescence intensity was quantified using Corel Photopaint™ by calculating mean nuclear pixel intensity after background subtraction1. Data were plotted in histograms as mean values ±SEM and a Student’s Neuman-Keuls test for multiple comparisons was used to determine statistical significance. Means were considered significantly different at * p < 0.05, ** p < 0.01, and ***p<0.001.

RNA Isolation, RT-PCR, and Quantitative RT-PCR

Total RNA was extracted from treated hc VSMCs using TriZol reagent and chloroform. RNA was precipitated using isopropanol with glycoblu (Ambion) as a carrier, washed with 75% ethanol, dissolved in RNAse free water and quantified using the NanoDrop® spectrophotometer.

For c-fos mRNA analysis using RT-PCR, RNA (25 ng) was reverse-transcribed using a Sensiscript™ RT kit (Qiagen) according to the manufacturer’s instructions and cDNA was amplified using c-fos (5’- TTATCTGTGCGTGAAACACACC; 3’-CCATGCGTTTTGCTACATCTC, 104 bp product, Sigma Genosys) and β-actin (5’-ATGATATCGCCGCGCTCGTCGTC;
3’-CCGCTCGGCCGTGGTGGTGAA, 611 bp product, Operon) primers. PCR products were separated by agarose gel electrophoresis and quantified using Quantity One™ software. The c-fos product level was corrected by levels of β-actin amplified by the same procedure. Data were plotted in histograms as mean values ±SEM and a Student’s Neuman-Keuls test for multiple comparisons was used to determine statistical significance. Means were considered significantly different at ** p < 0.01 and ***p<0.001.

For c-fos mRNA analysis by quantitative RT-PCR, RNA (2 µg) was reverse-transcribed using Omniscript™ RT kit (Qiagen) according to the manufacturer’s instructions. cDNA was amplified by real time RT-PCR using Assays-on-Demand™ gene expression product kits (Applied Biosystems) and a 7900HT Sequence Detection System (TaqMan, Applied Biosystems). HPRT was used as the internal standard. Data analysis was fully automated and carried out using Sequence Detection 2.1 software (Applied Biosystems). Standard curves were run to validate comparing the threshold cycle (C_T) between c-fos and HPRT for data analysis. RNA was isolated from at least two independent experiments and duplicate assays were performed. Data were plotted in histograms as mean values ±SEM and a Student’s Neuman-Keuls test for multiple comparisons was used to determine statistical significance. Means were considered significantly different at * p < 0.05.

**Ca^{2+}** imaging using a FRET-based Cameleon Ca^{2+} indicator
VSMCs were grown on coverslips in 60 mm dishes and maintained in DMEM containing 0.1% fetal bovine serum. Cells were transiently transfected with 1 µg/ml of yellow cameleon2.1 (YC2.1), a fluorescence resonance energy transfer (FRET)-based calcium biosensor using Lipofectamine 2000™ (Invitrogen). After 2 hr, the media was replaced and cells were maintained in a humidified incubator 24 hr before use.

Coverslips were placed into a SA-NIK chamber (Warner Instruments) mounted on a Nikon Diaphot 200 inverted microscope equipped for epifluorescence. Cells were superfused with HBS at room temperature. YC2.1 excitation was 440 nm and emission recorded with an ORCA-ER™ charge-coupled device (Hamamatsu) at 480 nm (FRET donor, enhanced cyan fluorescent protein, ECFP) and 535 nm (FRET acceptor, enhanced yellow fluorescent protein, EYFP). FRET emission ratio (Ratio 535/480) was recorded every 10 sec with an acquisition time of 200 msec and was used as a measure of intracellular Ca²⁺ concentration Ca²⁺. Normalized ratio was obtained by dividing the Ratio 535/480 by the starting baseline ratio value. The data were converted to a range of 100% using maximum and minimum values for each experiment to calculate the percent maximal FRET ratio change. Ionomycin (10 µmol/L) and EGTA (2 mmol/L) were used to determine the maximal and minimal values, respectively. Data were analyzed using Metafluor 3.0 Imaging Software (Universal Imaging Corporation, West Chester, PA). Area under the curve (AUC) ± SEM was calculated using Sigma Plot™. Student’s t test and Student-
Neuman-Keuls multiple comparisons test were used where appropriate to determine statistical significance.

**Immunofluorescence of intact arteries**

Following dissection, arteries were immobilized with insect pins in sylgard-coated dishes and bathed in cold HBS for 15 min before exposure to treatments. Treatments were performed at room temperature then arteries were fixed in 4% formaldehyde. Immunofluorescence was performed following the same protocol as for cultured VSMCs with the following exceptions. Triton X-100 was increased to 0.2% in the blocking and antibody dilution solutions. Cy5 goat anti-rabbit IgG (Jackson ImmunoResearch Labs) [1:500] was used as the secondary antibody to reduce background auto-fluorescence from the elastic lamina. Following the final wash, arteries were transferred to slides and mounted using Aqua Poly/Mount. Specificity of the P-CREB fluorescence was confirmed by loss of signal in the presence of a P-CREB blocking peptide (Cell Signaling Technology) (not shown). Images were captured using a Bio-Rad 1000 laser scanning confocal microscope with a 40X objective. P-CREB nuclear fluorescence intensity was quantified as for cultured VSMCs measuring fluorescence in at least 90 cells per condition from at least 3 independent experiments. Data were plotted in histograms as mean values ±SEM and a Student-Neuman-Keuls test for multiple comparisons was used to determine statistical significance. Means were considered significantly different at * or # p < 0.05, ** or ## p < 0.01, and *** or ### p<0.001.
Reference List
