Integrative Physiology

Coupling of Ca$^{2+}$ to CREB Activation and Gene Expression in Intact Cerebral Arteries From Mouse

Roles of Ryanodine Receptors and Voltage-Dependent Ca$^{2+}$ Channels

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Abstract—Pathological changes of the vasculature are characterized by changes in Ca$^{2+}$ handling and alterations in gene expression. In neurons and other cell types, [Ca$^{2+}$], often drives changes in gene expression. However, the relationship between Ca$^{2+}$ signaling and gene expression in vascular smooth muscle is not well understood. This study examines the ability of Ca$^{2+}$ influx through voltage-dependent, L-type Ca$^{2+}$ channels (VDCCs) and Ca$^{2+}$ release through ryanodine receptors (RyRs) to activate the transcription factor, cAMP-responsive element binding protein (CREB), and increase c-fos levels in intact cerebral arteries. Membrane depolarization increased the fraction of nuclei staining for phosphorylated CREB (P-CREB) and levels of c-fos mRNA in intact mouse cerebral arteries. Ryanodine, which inhibits RyRs, increased P-CREB staining and c-fos levels. Forskolin, an activator of adenylyl cyclase, and sodium nitroprusside, an NO donor, increased P-CREB and c-fos levels. Nisoldipine, an inhibitor of VDCCs, reversed the effects of depolarization and ryanodine on P-CREB and c-fos levels, but not the effects of forskolin or sodium nitroprusside. Inhibition of Ca$^{2+}$/calmodulin-dependent protein kinase (CaM kinase) blocked increases in P-CREB and c-fos levels seen with membrane depolarization, suggesting that CaM kinase has an important role in the pathway leading from Ca$^{2+}$ influx to CREB-mediated changes in c-fos levels. Our data suggest that membrane depolarization increases [Ca$^{2+}$], through activation of VDCCs, leading to increased P-CREB and c-fos, and that RyRs have a profound effect on this pathway by indirectly regulating Ca$^{2+}$ entry through VDCCs. These results provide the first evidence of Ca$^{2+}$ regulation of CREB and c-fos in arterial smooth muscle. (Circ Res. 2000;86:760-767.)

Key Words: Ca$^{2+}$ sparks ■ CREB ■ gene expression ■ receptors, ryanodine ■ arterial smooth muscle

Intracellular Ca$^{2+}$ plays an important role in regulating many cellular functions in vascular smooth muscle, including cellular contraction and proliferation. The proliferative response in smooth muscle is important for injury recovery and plays a role in the pathogenesis of hypertension and atherosclerosis, which are often associated with changes in gene expression. Furthermore, intracellular Ca$^{2+}$ is chronically elevated in the smooth muscle cells of arteries in hypertension and thus may contribute to alterations in gene expression. In neurons and neuronal cell lines, there is evidence that Ca$^{2+}$ is an important mediator of gene expression, including the induction of the immediate-early gene, c-fos. However, little is known about the role of Ca$^{2+}$ in mediating changes in gene expression in arterial smooth muscle.

In neurons, transcription of c-fos is increased in response to membrane depolarization through phosphorylation of the transcription factor, cAMP-responsive element binding protein (CREB). Phosphorylation of CREB on Ser133 enables CREB to modulate transcription of genes containing upstream cAMP/ Ca$^{2+}$ response elements, including the c-fos gene. Several kinases are capable of phosphorylating CREB, including cAMP-dependent protein kinase (protein kinase A [PKA]), PKC, Ras-dependent p105 kinase, Rsk-2, and Ca$^{2+}$/calmodulin-dependent protein kinase (CaM kinase). The signaling pathways to CREB in arterial smooth muscle are not known.

In hippocampal neurons, the mode of Ca$^{2+}$ entry is critical in transducing changes in excitability to changes in gene expression. Ca$^{2+}$ ions bind calmodulin shortly after entering neurons through dihydropyridine-sensitive, voltage-dependent (“L-type”) Ca$^{2+}$ channels (VDCCs). This Ca$^{2+}$/calmodulin signal ultimately activates nuclear Ca$^{2+}$/CaM kinase IV to phosphorylate CREB. Other Ca$^{2+}$ channels, including N-type Ca$^{2+}$ channels in the cell membrane, do not communicate with CREB, indicating that the nature of the Ca$^{2+}$ signal is critical for the coupling of Ca$^{2+}$ to CREB phosphorylation.

The primary Ca$^{2+}$ entry pathway in arterial smooth muscle is the dihydropyridine-sensitive VDCC. In addition to VDCCs in the plasma membrane, ryanodine-sensitive Ca$^{2+}$ release channels (ryanodine receptors [RyRs]) in the membrane of the sarcoplasmic reticulum (SR) can deliver large local increases in Ca$^{2+}$ into the cytoplasm. These localized Ca$^{2+}$ release events from RyRs, termed “Ca$^{2+}$ sparks,” arise from the coordinated opening of a number of RyRs. In
arterial myocytes, Ca\(^{2+}\) sparks activate sarcocellular large-conductance, Ca\(^{2+}\)-sensitive K\(^{+}\) (K\(_{Ca}\)) channels, causing an outward K\(^{+}\) current\(^\text{13,14}\). A Ca\(^{2+}\) spark contributes little to overall cytoplasmic [Ca\(^{2+}\)]\(_i\) (~2 nmol/L)\(^\text{13,14}\) but acts as a negative feedback element on the membrane potential of smooth muscle cells in cerebral arteries. Inhibition of Ca\(^{2+}\) sparks leads to an increase in global cytoplasmic [Ca\(^{2+}\)]\(_i\), through a decrease in K\(_{Ca}\) channel activity, resulting in membrane depolarization and activation of VDCCs.\(^\text{13}\) The ability of Ca\(^{2+}\) sparks to modulate gene expression has not been explored.

The goal of this study was to identify pathways leading to CREB phosphorylation and c-fos expression and to explore the roles of VDCCs and RyRs in regulating CREB activation in intact arterial smooth muscle. Our results indicate that Ca\(^{2+}\) influx through VDCCs, activation of cAMP- and cGMP-dependent protein kinases (PKA and PKG, respectively), and inhibition of Ca\(^{2+}\) sparks with ryanodine all increase levels of phosphorylated CREB (P-CREB) and c-fos transcripts. Our results also indicate that signaling through CaM kinase is an essential step in the pathways leading from an increase in intracellular Ca\(^{2+}\) to an increase in P-CREB and c-fos levels. Our data are consistent with the following pathway: VDCC\(\rightarrow\) ↑[Ca\(^{2+}\)]\(_i\)\(\rightarrow\)CaM kinase\(\rightarrow\) ↑P-CREB\(\rightarrow\) ↑c-fos. Our data are also consistent with the idea that RyRs can indirectly modulate this pathway through VDCCs (Figure 8).

**Materials and Methods**

CD-1 mice (8 to 10 weeks, \(\approx\)20 g) were euthanized with pentobarbital (150 mg/kg IP). The brain was removed and intact isolated midcerebral and posterior arteries were dissected and placed in physiological saline solution (PSS containing, in mmol/L, NaCl 119, KCl 3, KH\(_2\)PO\(_4\) 1.7, MgSO\(_4\) 1.2, NaHCO\(_3\) 25, EDTA 0.02, CaCl\(_2\) 1.6, and glucose 11.0; pH 7.4).

**Immunofluorescence**

Arteries were treated with test stimuli (high K\(^{+}\), forskolin, sodium nitroprusside [SNP], and ryanodine) for 15 minutes at 37°C unless otherwise stated. When indicated, arteries were incubated with inhibitors for 15 to 60 minutes before the test stimuli, and inhibitors were present during the simulation period (preincubation periods: nisoldipine, 15 minutes; H-89, 15 minutes; calmidazolium, 1 hour; and KN-93 and KN-92, 30 minutes). After the test stimulus, arteries were incubated in PSS for 30 minutes at 37°C, flash-frozen, and embedded. Mouse cerebral arteries were cut in cross section to a thickness of 10 \(\mu\)m, fixed with \(-20^\circ\)C methanol, and blocked with 2% milk in PBS. Sections were treated with primary antibody, rabbit anti–P-CREB (1:250 dilution in 0.2% Triton-X/2% BSA/PBS), followed by secondary antibody, Cy3–anti-rabbit IgG (1:500 dilution in 2% BSA/PBS). Nuclei were stained by treating sections with the DNA dye YOYO-1 to identify nuclei. Control sections contained YOYO-1–stained nuclei; however, few nuclei displayed P-CREB positive staining (Figures 2A and 2B). Membrane depolarization increases P-CREB staining (Figures 2A and 2B). Membrane

**RNA Isolation and Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)**

Arteries were stimulated as described for immunofluorescence. Total RNA was then extracted using TriZol reagent and quantified using a spectrophotometer. First-strand cDNA was synthesized using Superscript II RNase H reverse transcriptase. cDNA was amplified using c-fos and GAPDH primers. PCR products were separated by gel electrophoresis and quantified using Adobe Photoshop software. Equal amounts of cDNA were loaded on the gel; the c-fos DNA concentration was normalized to GAPDH expression. All results are expressed as mean±SEM.

**Arterial Wall Ca\(^{2+}\), Diameter, and Ca\(^{2+}\) Spark Measurements**

Cannulated arteries were loaded with the ratiometric Ca\(^{2+}\)-sensitive dye fura-2-acetoxymethyl ester (fura-2-AM; 2 \(\mu\)mol/L) and imaged as previously described.\(^\text{13}\) Arterial [Ca\(^{2+}\)]\(_i\), was calculated as previously described.\(^\text{15}\) Calcium sparks were measured as previously described in Jaggar et al.\(^\text{14}\)

**SDS-PAGE and Western Blotting**

Mouse cerebral arteries were homogenized and resuspended in SDS/PAGE sample buffer, followed by SDS/PAGE. Proteins were transferred onto polyvinylidene difluoride membranes and incubated in 5% milk/PBS. The membrane was incubated in primary antibodies (anti–CaM kinase II, 1:5000, and anti–CaM kinase IV, 1:2000), followed by secondary antibody (goat anti-mouse IgG–horseradish peroxidase, 1:10 000). Protein was detected using an enhanced chemiluminescence procedure.

**Statistics**

Results are expressed as mean±SEM where applicable. Statistical significance was determined using a paired or unpaired \(t\) test where appropriate.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

**Results**

**Membrane Depolarization Increases Arterial Wall [Ca\(^{2+}\)]\(_i\) and Constricts Intact Mouse Cerebral Arteries**

Intracellular Ca\(^{2+}\) was measured in intact mouse cerebral arteries, with the fluorescent dye fura-2. Membrane potential depolarization with high K\(^{+}\) caused an increase in [Ca\(^{2+}\)]\(_i\), from 123.0±5.5 to 210.0±25.7 nmol/L and caused a 39.7±2.0% vasoconstriction, which was reversed by washout (n=4) (Figure 1). The depolarization-induced Ca\(^{2+}\) increase and vasoconstriction were blocked by the dihydropyridine inhibitor of VDCCs, nisoldipine (100 nmol/L) (Figure 1), indicating that the increase in [Ca\(^{2+}\)]\(_i\), and the vasoconstriction result from Ca\(^{2+}\) influx through VDCCs.

**Membrane Depolarization Increases P-CREB and c-fos Levels in Mouse Cerebral Arteries Through Activation of VDCCs**

In other preparations, CREB is activated by phosphorylation in response to depolarization-induced Ca\(^{2+}\) influx.\(^\text{2,3}\) However, the signaling pathways leading to CREB phosphorylation in intact arterial smooth muscle are unknown. To explore CREB phosphorylation in native smooth muscle, intact cerebral arteries were treated with 30 and 60 nmol/L KCl for 15 minutes, and tissue sections were analyzed for P-CREB by immunostaining with an antibody specific for CREB phosphorylation (Figure 8).
potential depolarization with 30 and 60 mmol/L KCl caused an increase in the percentage of nuclei with P-CREB staining, which was prevented by pretreatment with nisoldipine (100 nmol/L, 15 minutes, 37°C) (Figure 2B).

To establish that the phosphorylation of CREB correlates with an increase in c-fos, RT-PCR was performed on RNA extracted from intact mouse cerebral arteries. Membrane depolarization with KCl (30 and 60 mmol/L) resulted in an increase in c-fos levels (3.2-fold and 3.7-fold, respectively) when compared with control (6 mmol/L KCl) or pretreatment with nisoldipine (Figure 2C).

**Ca²⁺ Sparks in Intact Mouse Cerebral Arteries**

In addition to VDCCs, Ca²⁺ can enter the cytoplasm through RyRs. This local Ca²⁺ release through RyRs, termed Ca²⁺ sparks, has been measured in several types of smooth muscle.² In rat cerebral arteries, ryanodine (10 μmol/L) has been shown to inhibit Ca²⁺ sparks leading to membrane depolarization of cerebral artery smooth muscle.¹³,¹⁷ Membrane depolarization activates VDCCs, which elevates global [Ca²⁺].¹⁵,¹⁷ Therefore, ryanodine eliminates a source of local Ca²⁺ release, which leads to an elevation of global [Ca²⁺], (see Figure 8).

Here, we provide the first measurements of Ca²⁺ sparks in the smooth muscle cells of intact mouse cerebral arteries. Membrane depolarization with KCl increased global [Ca²⁺], and increased Ca²⁺ spark frequency ~2-fold (Figure 3). Global arterial wall Ca²⁺ has been estimated to be 120 nmol/L in these cerebral arteries at low pressure as measured using fura-2 (see Figure 1). Therefore, the increase in fractional fluorescence observed with membrane depolarization (1.6-fold) corresponds to an elevation of global Ca²⁺ from 120 to 192 nmol/L. Diltiazem (60 μmol/L), in the presence of 30 mmol/L KCl, decreased, based on fractional fluorescence, global [Ca²⁺], from 192 to 115 nmol/L and decreased Ca²⁺ spark frequency from 2.3±0.2 to 0.6±0.1 Hz (n=5 paired arteries). These results are consistent with the idea that membrane depolarization activates VDCCs, which leads to an elevation of global [Ca²⁺], and an increase in Ca²⁺ spark frequency.

Ryanodine, an inhibitor of the RyR in the SR membrane, inhibits Ca²⁺ sparks and the associated K_Ca currents in...
isolated cerebral myocytes and Ca\(^{2+}\) sparks in intact rat cerebral arteries. 13,14 Ca\(^{2+}\) sparks were not observed in the presence of ryanodine (10 \(\mu\)mol/L) (n=4 arteries). Application of ryanodine increased global [Ca\(^{2+}\)]\(_i\) from 120 to 170 nmol/L (1.4-fold increase in fractional fluorescence). The ability of ryanodine to inhibit Ca\(^{2+}\) sparks and increase global [Ca\(^{2+}\)]\(_i\) is consistent with results from previous studies on rat cerebral arteries. 13,14,17

Regulation of P-CREB and c-fos Levels by RyRs

To explore the role of RyRs (Ca\(^{2+}\) sparks) in the regulation of CREB, the effects of RyR inhibition by ryanodine were examined on the levels of P-CREB and c-fos. Ryanodine increased levels of P-CREB (Figure 4A) and c-fos (Figures 4B and 4C). If the ryanodine-induced increases in P-CREB and c-fos levels are dependent on Ca\(^{2+}\) entry through VDCCs, inhibition of VDCCs with nisoldipine should prevent the responses seen with ryanodine. Indeed, nisoldipine abolished the ryanodine-induced increase in P-CREB and c-fos levels (Figure 4). These results suggest that the ryanodine-induced elevation in global [Ca\(^{2+}\)]\(_i\) by activation of VDCCs has a dominant effect over inhibition of local Ca\(^{2+}\) release on the activation of CREB and c-fos in native smooth muscle (Figure 8).

Activation of CREB Phosphorylation and c-fos Expression by Forskolin and SNP

In other cell types, CREB is activated by stimulation of adenylyl cyclase (AC) and guanylyl cyclase (GC). 4,18 Therefore, the ability of AC and GC stimulation to activate CREB phosphorylation in intact arterial smooth muscle was examined. Forskolin (1 \(\mu\)mol/L), an activator of AC, and SNP (10 \(\mu\)mol/L), a NO donor, increased P-CREB (Figure 4A) and c-fos (Figures 4B and 4C). However, the forskolin- and SNP-induced increases in P-CREB and c-fos were not affected by nisoldipine, suggesting that forskolin and SNP are able to activate CREB and c-fos independently of VDCC activity.
SNP, which elevates levels of cGMP through NO, may act through PKG or by "cross activation" of PKA to influence levels of gene expression. To examine the possibility that SNP could affect P-CREB and c-fos levels by cross activation of PKA, experiments were performed using H-89, a cell-permeable inhibitor of PKA. Pretreatment of cerebral arteries with H-89 (1 μmol/L) blocked forskolin-induced increases in P-CREB (Figures 5A and 5B) and c-fos (Figures 5C and 5D) but had no effect on the SNP-induced changes in the levels of P-CREB or c-fos (Figure 5). These results suggest that SNP is not activating CREB and c-fos through the stimulation of PKA.

Depolarization-Induced Increases in P-CREB and c-fos Levels Involve CaM Kinase

To determine the signaling pathways leading to CREB phosphorylation and activation of c-fos in intact smooth muscle, the possible roles of calmodulin and CaM kinase were examined. Arteries were treated with calmidazolium (10 μmol/L, 30 minutes, 37°C), a calmodulin inhibitor, or KN-93 (30 μmol/L, 1 hour, 37°C), a CaM kinase inhibitor, followed by membrane depolarization with 60 mmol/L K⁺ for 15 minutes. Calmidazolium blocked the increase in P-CREB levels observed with membrane depolarization (Figure 6A) and the increase in c-fos levels (Figure 6B). KN-93 also blocked the depolarization-induced increases seen in P-CREB and c-fos levels (Figures 6A and 6B). KN-92 (10 μmol/L), an inactive analogue of KN-93, had no effect on P-CREB or c-fos levels when compared with the depolarization response (Figures 6A and 6B).

To establish that the effects of KN-93 and calmidazolium on P-CREB and c-fos levels were not due to inhibition of VDCCs, diameter measurements were performed on intact mouse cerebral arteries. Membrane depolarization with high K⁺ caused similar constrictions before and after application of 30 μmol/L KN-93 (high K⁺, 36.5±3.8% constriction; high K⁺+KN-93, 37.8±3.2% constriction). Therefore, KN-93 does not inhibit VDCCs in intact mouse cerebral arteries, because depolarization-induced constrictions were unaffected (Figure 7A). However, calmidazolium (10 μmol/L) did cause an irreversible inhibition of depolarization-induced arterial constrictions (n=3) (Figure 7B).

Calmidazolium could have blocked high K⁺-induced constriction by inhibiting Ca²⁺/calmodulin-dependent myosin light chain kinase. Therefore, the effects of calmidazolium were also tested on high K⁺-induced increases in arterial wall [Ca²⁺]. Under control conditions, membrane depolarization with high K⁺ increased levels of [Ca²⁺], similar to those seen in Figure 1. In the presence of calmidazolium, membrane depolarization with high K⁺ did not cause an increase in [Ca²⁺], (Figure 7B). After several hours, high K⁺ was still unable to increase [Ca²⁺], suggesting that calmidazolium is inhibiting VDCCs. Direct assessment of the role of calmodulin in CREB phosphorylation or c-fos activation is not possible, given the effects of calmidazolium on VDCCs. However, the KN-93 and KN-92 results support the role of CaM kinase in the depolarization-induced activation of CREB.

Because the increases in P-CREB and c-fos were blocked by the addition of a CaM kinase inhibitor, we sought to establish the presence of CaM kinase in intact arterial smooth muscle. Western analysis using mouse cerebral artery extracts indicated the presence of both CaM kinases II and IV (n=4) (Figure 6C).
The mechanisms by which Ca\(^{2+}\) controls gene expression in arterial smooth muscle are not well understood. Alterations in these mechanisms and signaling pathways play a critical role in the development of various pathogenic states, including hypertension and atherosclerosis.\(^{20}\) We provide the first information on the regulation of Ca\(^{2+}\)-dependent transcription factors in native smooth muscle.

In this study, we explored the roles of VDCCs and RyRs in the regulation of CREB phosphorylation and c-fos levels in intact vascular smooth muscle. Our results indicate that Ca\(^{2+}\) influx through VDCCs regulates P-CREB and c-fos transcript levels. RyRs also regulate P-CREB and c-fos levels. However, the regulation of P-CREB and c-fos levels by RyRs appears to be indirect, through alterations in Ca\(^{2+}\) influx through VDCCs. The ability of forskolin and SNP to elevate P-CREB and c-fos levels occurs through mechanisms independent of Ca\(^{2+}\) influx through VDCCs. Figure 8 illustrates proposed mechanisms to explain CREB activation by VDCCs, RyRs, PKA, and PKG in cerebral artery smooth muscle.

**DHP-Sensitive, Voltage-Dependent Ca\(^{2+}\) Channels Regulate CREB Activation**

In hippocampal neurons, certain Ca\(^{2+}\) entry pathways (L-type Ca\(^{2+}\) channels and N-methyl-D-aspartate receptors) are capable of causing rapid translocation of calmodulin to the nucleus, which is important for CREB phosphorylation.\(^{9}\) Other Ca\(^{2+}\) entry pathways (N- and P/Q-type Ca\(^{2+}\) channels) are not able to cause CREB phosphorylation in hippocampal neurons;\(^{9}\) however, P/Q-type Ca\(^{2+}\) channels have been shown to activate syntaxin-1A expression via CREB phosphorylation in HEK293 cells.\(^{21}\) These results suggest that the activation of gene expression is highly dependent on the Ca\(^{2+}\) entry pathway and may be cell-type specific. We were interested in the roles of various Ca\(^{2+}\) signaling pathways in smooth muscle, including Ca\(^{2+}\) influx through VDCCs and local Ca\(^{2+}\) release through RyRs (Ca\(^{2+}\) sparks), and their ability to influence levels of P-CREB and c-fos by membrane depolarization.

Raising external K\(^+\) to 60 mmol/L depolarizes pressurized rat cerebral arteries to \(-25\) mV and raises [Ca\(^{2+}\)].\(^{15}\) In mouse cerebral arteries held at low pressure (<20 mm Hg), raising external K\(^+\) to 60 mmol/L elevated [Ca\(^{2+}\)], in mouse cerebral arteries and increased levels of both P-CREB and c-fos. Nisoldipine blocked the depolarization-induced elevation of global [Ca\(^{2+}\)], and vasoconstriction, as well as the increases in P-CREB and c-fos, suggesting that Ca\(^{2+}\) influx through VDCCs is necessary for the activation of CREB and
activity, by Ca$^{2+}$ spark (RyR)–mediated activation of K$_{ca}$ channels (Figure 8). However, this study does not exclude the possibility that Ca$^{2+}$ sparks may directly modulate gene expression. It is conceivable that RyRs or inositol (1,4,5)-triphosphate receptors could directly communicate to CREB under different conditions. These possibilities remain to be explored.

**PKA and PKG Act Independently of VDCCs to Cause CREB Phosphorylation**

Activators of PKA (forskolin) or PKG (SNP) increased CREB phosphorylation and c-fos expression independently of VDCC activity. The ability of PKA to phosphorylate CREB is well established.1-4 It has been suggested that cGMP can exert effects through “cross talk” activation of PKA.19 Our data provide evidence against cross activation of PKA by cGMP in intact arterial smooth muscle, because increases in CREB phosphorylation and c-fos expression were not blocked by H-89, an inhibitor of PKA (Figure 5). The idea that PKG can directly alter gene expression is supported by studies showing that NO regulates gene expression via cGMP activation of PKG.18

**Signaling Pathway Leading From Ca$^{2+}$ to Gene Expression in Intact Smooth Muscle**

Various kinases are capable of phosphorylating CREB, including CaM kinases II and IV.24-25 Phosphorylation of CREB on Ser133 by nuclear CaM kinase IV promotes the activity of genes containing an upstream cAMP-responsive element.10,26 CaM kinase II is capable of phosphorylating CREB on Ser133, as well as Ser142. Phosphorylation on Ser142 is believed to block the Ser133 phosphorylation–dependent activation of CREB, suggesting a possible mechanism for the regulation of CREB activity.24 CaM kinases II and IV are present in native smooth muscle (Figure 6C). The inhibition of CaM kinases abolished the depolarization–induced increase in P-CREB and c-fos levels. These results suggest that a CaM kinase plays a role in the signaling pathway from Ca$^{2+}$ entry to an increase in P-CREB and c-fos levels in intact smooth muscle.

**Conclusions**

One interesting issue that arises from our results is the ability of vasoconstrictors (membrane depolarization) and vasodilators (forskolin, SNP) to activate CREB phosphorylation and increase c-fos expression. These results are paradoxical, but smooth muscle may have the ability to differentially process signals leading to alterations in gene expression. Similar differential processing exists in hippocampal neurons, which have the ability to differentiate between Ca$^{2+}$ signals, depending on the source or duration of Ca$^{2+}$ influx.9,27

The present study provides the first evidence for the coupling of Ca$^{2+}$ and gene expression in arteries. Our data are consistent with the following pathway: VDCC→↑[Ca$^{2+}$]→CaM kinase→↑P-CREB→↑c-fos levels; these data suggest that RyRs are able to modulate this pathway through VDCCs (Figure 8).

**Acknowledgments**

This work was supported by NIH Grant HL-44455 and the Totman Medical Research Fund. We thank Drs Joseph Brayden, David

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**Figure 8.** Proposed mechanisms of Ca$^{2+}$-dependent and Ca$^{2+}$-independent activation of CREB and c-fos in intact arterial smooth muscle. Membrane depolarization with elevated potassium increases [Ca$^{2+}$] through activation of nisoldipine-sensitive VDCCs (Figure 1). This elevation in [Ca$^{2+}$] activates a CaM kinase, based on the effects of the CaM kinase inhibitor KN-93 (see Figure 6). CaM kinase then phosphorylates CREB, which leads to increased transcription of c-fos. An elevation of global [Ca$^{2+}$] leads to an increase in Ca$^{2+}$ sparks frequency through activation of RyR by cytoplasmic and SR Ca$^{2+}$. Ca$^{2+}$ sparks activate K$_{ca}$ channels, which causes membrane potential hyperpolarization, leading to closure of VDCCs. Inhibition of RyR (Ca$^{2+}$ sparks) by ryanodine (Figure 4) leads to an elevation in global Ca$^{2+}$, which is blocked by an inhibitor of VDCCs. These results are consistent with our previous studies on rat cerebral arteries, ie, that Ca$^{2+}$ sparks exert a negative feedback control over the smooth muscle membrane potential through activation of K$_{ca}$ channels.12,16 Forskolin and SNP are also able to increase P-CREB and c-fos levels through PKA and PKG, respectively, independently of VDCCs. The roles of PKA and PKG are based on the literature, the observed selective block of the actions of forskolin by H-89, and the lack of effects of nisoldipine.

c-fos by membrane depolarization (Figures 1 and 2; see also Figure 8).

**RyRs Regulate CREB Phosphorylation Through VDCCs**

RyRs in the SR are another potential source of Ca$^{2+}$ that could lead to CREB activation. However, RyRs have been shown to lower global Ca$^{2+}$ through the activation of K$_{ca}$ channels.13,14,16,22 Therefore, we tested the effects of inhibiting RyRs on P-CREB and c-fos levels. If local Ca$^{2+}$ release through RyRs dominates the activation of CREB, as L-type channels do in hippocampal neurons, then inhibition of RyRs should decrease levels of P-CREB and c-fos. However, if negative feedback control dominates, then the inhibition of RyRs should lead to an elevation of global [Ca$^{2+}$], and activation of CREB and increased c-fos levels. In cerebral myocytes and intact arteries from rat, Ca$^{2+}$ spark inhibition by ryanodine leads to membrane depolarization (~8 to 10 mV, rat cerebral arteries11,17) and elevates global [Ca$^{2+}$], ~45 nmol/L by increasing the open probability of VDCCs.13,17,23 In this study, ryanodine inhibited Ca$^{2+}$ sparks and caused an increase in global [Ca$^{2+}$], as well as an increase in levels of P-CREB and c-fos (Figure 4). Nisoldipine abolished the effects of ryanodine on levels of P-CREB and c-fos, suggesting that RyRs are capable of mediating CREB phosphorylation and c-fos levels through regulation of VDCCs (Figure 8).

These results are consistent with a dominant role of negative feedback control of membrane potential, and hence VDCC
References

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Circ Res. 2000;86:760-767
doi: 10.1161/01.RES.86.7.760

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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