Ca²⁺/calmodulin-dependent protein kinase IV activates cysteine-rich protein 1 through adjacent CRE and CArG elements

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Najwer, Ida, and Brenda Lilly. Ca²⁺/calmodulin-dependent protein kinase IV activates cysteine-rich protein 1 through adjacent CRE and CArG elements. Am J Physiol Cell Physiol 289: C785–C793, 2005. First published May 25, 2005; doi:10.1152/ajpcell.00098.2005.—Smooth muscle-specific transcription is controlled by a multitude of transcriptional regulators that cooperate to drive expression in a temporospatial manner. Previous analysis of the cysteine-rich protein 1 (CRP1/Csrp) gene revealed an intronic enhancer that is sufficient for expression in arterial smooth muscle cells and requires a serum response factor-binding CArG element for activity. The presence of a CArG box in smooth muscle regulatory regions is practically invariant; however, it stands to reason that additional elements contribute to the modulation of transcription in concert with the CArG. Because of the potential importance of other regulatory elements for expression of the CRP1 gene, we sought to identify additional motifs within the enhancer that are necessary for expression. In this effort, we identified a conserved cAMP response element (CRE) that, when mutated, diminishes the expression of the enhancer in cultured vascular smooth muscle cells. Using transfection and electrophoretic mobility shift assays, we have shown that the CRE binds the cAMP response element-binding protein (CREB) and is activated by Ca²⁺/calmodulin-dependent protein kinase IV (CaMKIV), but not by CaMKII. Furthermore, our data demonstrate that CaMKIV stimulates CRP1 expression not only through the CRE but also through the CArG box. These findings represent evidence of a functional CRE within a smooth muscle-specific gene and provide support for a mechanism in which CREB functions as a smooth muscle determinant through CaMKIV activation.

smooth muscle; cAMP response element-binding protein; serum response factor

ONE OF THE MOST CHALLENGING ISSUES to be confronted in the understanding of tissue specificity is how universal signals are deciphered into distinct profiles of gene expression. Smooth muscle-specific transcription has presented a particularly formidable challenge because these cells can readily switch from a differentiated/quiescent phenotype to a proliferative or synthetic one (36). The signaling events that dictate these phenotypes have offered limited clues to the downstream activators that control gene expression. Studies of smooth muscle-restrictive gene regulation have established the serum response factor (SRF) and its cognate binding site, the CArG box, as essential components in differentiation-specific expression (32, 36, 38). Although the CArG box is clearly an important motif, it seems unlikely that it is solely responsible for deciphering the multitude of regulatory signals. Indeed, mutations in a TGF-β control element (TCE) as well as E-box elements have been shown to abrogate expression of smooth muscle genes (24, 27, 36). For this reason, we set out to identify additional elements within the cysteine-rich protein 1 (CRP1) gene that are critical for expression in vascular smooth muscle.

CRP1 is a member of the cysteine-rich protein (CRP) family, which includes CRP1, CRP2/smLIM (20, 29), and CRP3/MLP (1, 29). CRP1 is predominately expressed in smooth muscle cells and is transcriptionally activated upon differentiation (15). Previous regulatory analysis of CRP1 identified an enhancer within an intron (CRP1-5.0) that is sufficient for expression in arterial smooth muscle cells (25). The enhancer requires a CArG element that binds SRF for its differentiation-specific activity. In this study, we further characterized the CRP1-5.0 enhancer and identified a new binding element that is important for expression. Herein we demonstrate that a conserved cyclic AMP-response element (CRE) within the CRP1 gene is critical for enhancer activity.

The CRE is an 8-bp motif with the consensus sequence TGAACGTCA (34). As its name implies, CRE serves as a transcriptional conduit for cyclic AMP-stimulated processes, but it also responds to a variety of other stimuli, including intracellular Ca²⁺ through the activation of Ca²⁺/calmodulin-dependent protein kinases (30, 31, 49). The primary factors that bind CRE are the cAMP element-binding protein (CREB) and the related proteins activating transcription factor (ATF)-1 and CRE modulator (CREM). The activity of CREB on the CRE is dependent largely on phosphorylation of a Ser133 residue. This phosphorylation event transforms CREB into a potent transcriptional activator and facilitates interactions with additional regulators, namely, CREB-binding protein (CBP) (31, 49). With respect to function, CREB has been implicated in governing a host of cellular processes and adaptive responses, including differentiation, metabolic changes, cell survival, and proliferation (3, 18, 19, 28, 37, 44, 53). In vascular smooth muscle cells, the role of CREB is a matter of debate (42). Several studies have demonstrated a negative correlation with proliferation (23, 43, 58, 59); however, others have suggested that it has a mitogenic effect (10, 56). In mature quiescent blood vessels, CREB levels are high, whereas in disease states such as atherosclerosis and diabetes, CREB levels decrease when smooth muscle cells begin to proliferate (23, 43). Moreover, CREB has been shown to diminish the expression of numerous cell cycle-regulated genes and growth factor receptors (43). Taken together, these data suggest that CREB functions as a molecular determinant of a quiescent differentiated phenotype. Despite this evidence, the ability of CREB to activate differentiation-specific smooth muscle genes has not been tested directly.
In this study, we have identified a conserved CRE in the CRP1-5.0 enhancer that binds the transcriptional activator CREB. Mutational analysis revealed that this element is important for the activity of the CRP1 gene and, furthermore, is uniquely activated by a Ca\(^{2+}\)-dependent pathway. Ca\(^{2+}\) signaling is mediated principally by calmodulin, which promotes the activation of many downstream effectors, including the Ca\(^{2+}\)/calmodulin (CaM)-dependent protein kinases (17, 50). The CaM kinase (CaMK) family consists of three members: CaMKI, CaMKII, and CaMKIV. All are multifunctional Ser/Thr kinases with a variety of substrates. In particular, CaM kinases have been shown to stimulate Ca\(^{2+}\)-dependent transcription through phosphorylation of CREB and SRF (8, 30, 32). Our results have shown that CaMKIV, but not CaMKII, activates CRP1, and this response is mediated through the CRE as well as the adjacent CARG element. These findings suggest that the smooth muscle-determining properties of CREB may be attributed to its ability to control directly the expression of differentiation-specific genes.

**EXPERIMENTAL PROCEDURES**

**Cell culture.** Smooth muscle cells were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Hyclone) and maintained in humidified 5% CO\(_2\) at 37°C. Pulmonary aortic smooth muscle cells (PAC1) (46) were a gift from Joe Miano (University of Rochester). Primary cultures of bovine aortic smooth muscle cells (BASMCs) were purchased from Cambrex (BioWhittaker). Cells were stimulated with 60 mM KCl, 2.5 mM MgCl\(_2\), 200 mM dNTP, a 15 μM concentration of each primer, and 1 U of Taq DNA polymerase. CRP1 primers were designed to amplify a 360-bp fragment of CRP1 mRNA. Primer sequences were RFV8–5′-CTATTGCAAGTCTAGTTATGG-3′ and RFV19–5′-CTCTGAA-ATGGACCAAGGCCG-3′. GAPDH primers (BD Biosciences;Clontech) were used as an internal control for RNA integrity. GAPDH sequences were 5′-ACCCACTTGTTGCTGTTGTA-3′ and 5′-TCACCCACCTTGTTGCTGTTGTA-3′. PCR products were sequenced using a Kodak Gel Logic 100 imaging system to measure band intensity.

**Transfection and luciferase assays.** Cells in a 24-well plate were transiently transfected at 80% confluence using Lipofectamine 2000 (Invitrogen) and harvested 48 h after the start of transfection. One microgram of each reporter construct was introduced into cells; for cotransfection experiments, 0.5 μg of the designated expression construct was added with the luciferase reporters. To normalize for transfection efficiency, 0.2 μg of heat shock protein 68 (HSP68)-LacZ reporter construct was cotransfected and luciferase activities were calculated on the basis of equivalent amounts of LacZ activity. Luciferase assays were performed as described previously using Steady-Glo reagent (Promega) (2) and quantified using a Turner Diagnostics luminometer. LacZ activities were measured as described previously (35). Each experiment was repeated a minimum of three times, and LacZ and luciferase assays were measured in duplicate. The expression constructs were pcDNA1.1 containing the cytomegalovirus promoter without insert (Novagen) used as a control, VP16-CREB (60), CaMKII and CaMKIV (54), dominant-negative CREB (A-CREB) (61), dominant-negative SRF (5), and CREBm1 (11, 44).

**Immunoblotting.** Protein extract isolation and Western blot analyses were performed as described previously (14). Protein samples were run on 10% SDS-PAGE gels, transferred to Immobiline-NC (Amersham) and subjected to consecutive incubations with primary and secondary antibodies. Proteins were detected using enhanced chemiluminescence (ECL) with horseradish peroxidase (HRP)-conjugated secondary antibodies (Pierce). Primary antibodies used were CaMKII (1:1,000 dilution; Cell Signaling), CaMKIV (1:1,000 dilution; Cell Signaling), and GAPDH (1:1,000 dilution; Novus Biologicals). Secondary antibodies used were sheep anti-mouse Ig-HRP (1:8,000 dilution) and donkey anti-rabbit Ig-HRP (1:8,000 dilution; Amersham).

**RESULTS**

**Identification of CRE in CRP1-5.0 enhancer.** Multiple transcription factor binding sites exist within the CRP1-5.0 arterial enhancer, which we identified using the TESS (47a) and MatInspector (40) computer algorithms to screen for consensus elements (25). In proximity to the essential CARG box, we identified a CRE. The presence of a CRE was intriguing because the function of CREB in vascular smooth muscle cells is controversial (42). Therefore, we became interested in determining whether this CRE might regulate expression of the CRP1 gene in a CREB-dependent fashion. Comparative analysis of the CRE sequence from the mouse, rat, and human revealed that this site is evolutionarily conserved across species (Fig. 1A), suggestive of a functional role for this CRE in the control of CRP1. To evaluate which protein complexes were bound to this sequence in smooth muscle cells, we performed EMSAs with nuclear extracts isolated from PAC1 cells (46). CRE sequences from mouse and human were used as probes, and each bound a single prominent complex that was competed by cold competitor but not by a 3-bp mutated version of this site (Fig. 1B). To determine whether this complex contained leukemia virus reverse transcriptase (Invitrogen) with 0.5 μg of RNA according to the manufacturer’s instructions. For each 25-μl PCR, 2 μl of cDNA were added to 1× PCR buffer (Invitrogen), 2 mM MgCl\(_2\), 200 μM dNTP, a 15 μM concentration of each primer, and 1 U of Taq DNA polymerase. Primer sequences were RFV8–5′-CTATTGCAAGTCTAGTTATGG-3′ and RFV19–5′-CTCTGAAATGGACCAAGGCCG-3′. GAPDH primers (BD Biosciences;Clontech) were used as an internal control for RNA integrity. GAPDH sequences were 5′-ACCCACTTGTTGCTGTTGTA-3′ and 5′-TCACCCACCTTGTTGCTGTTGTA-3′. PCR products were sequenced using a Kodak Gel Logic 100 imaging system to measure band intensity.

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CREB-related proteins, did not alter the complex (Fig. 1). Addition of antibodies against CREM and ATF-1, two smooth muscle extracts bound to the antibody abolished the complex, indicating that CREB from that recognizes CREB. The addition of the CREB-specific CREB, we incubated extracts in the presence of an antibody that recognizes CREB. The addition of the CREB-specific antibody abolished the complex, indicating that CREB from smooth muscle extracts bound to the CRP1-5.0 CRE. Furthermore, addition of antibodies against CREM and ATF-1, two CREB-related proteins, did not alter the complex (Fig. 1C). These data reveal that the CRE within the CRP1-5.0 enhancer is capable of binding CREB and may serve to mediate CREB-dependent transcription.

To evaluate the role of the CRE in the activity of the CRP1-5.0 enhancer, we introduced a mutation into the CRE site and examined expression by luciferase reporter assays in PAC1 cells and primary cultures of BASMCs (Fig. 2). The wild-type enhancer (CRP1-5.0-Luc) is activated between 5- and 10-fold above basal levels; however, with the CRE mutation (CRP1-5.0mutCRE-Luc), enhancer expression was considerably diminished, resulting in a 50% reduction in activity. The CRE mutation was identical to that tested in EMSAs, which was unable to compete for or bind to CREB (Fig. 1 and data not shown). To test whether the CREB protein bound to this site in smooth muscle cells, we cotransfected an activated form of CREB (VP16-CREB) (60) with the wild-type and mutated CRE luciferase constructs. VP16-CREB activated the CRP1-5.0 wild-type enhancer, whereas the mutant CRE construct was not substantially stimulated by VP16-CREB. Taken together, these results show that the CRE within the CRP1-5.0 enhancer is important for expression in both PAC1 and BASMCs and that CREB is capable of binding to this site to activate expression of the CRP1 gene.

CRP1-5.0 enhancer is activated by CREB-dependent pathways. The importance of the CRE for expression of the CRP1 gene prompted us to ask which upstream pathways might activate CRP1 through this site. In vascular smooth muscle, CREB can be phosphorylated by both cAMP and Ca2+ -dependent pathways (4, 26). Treatment of cells with forskolin activates adenylyl cyclase, causing cAMP-dependent protein kinase A (PKA) to phosphorylate CREB. In addition, a rise in cytoplasmic Ca2+ by K+ -induced membrane depolarization also promotes CREB phosphorylation. To ascertain whether either or both of these pathways might activate CRP1 expression, we treated cells with forskolin to activate PKA and KCl to promote Ca2+ influx. Expression of the endogenous CRP1 gene under these conditions was monitored using semiquantitative RT-PCR to detect changes in transcription levels. As shown in Fig. 3, treatment of cells with 10 μM forskolin and 60 mM KCl resulted in an average threefold increase in the CRP1 transcripts, demonstrating that both stimuli promote expression of CRP1. Forskolin treatment resulted in a spike of CRP1 transcript that was evident as early as 8 h (data not shown) and leveled off at the 48-h time point. The leveling of forskolin-dependent activation might reflect a refractory period in which cAMP is unable to cause CREB phosphorylation (34), resulting in a decrease in transcription. In contrast, addition of KCl produced a steady increase in CRP1 transcripts. These distinct transcript profiles reflect that these two agents use separate downstream effectors to stimulate CRP1 expression.

We next wanted to examine whether the CRP1-CRE mediated the response to either of these stimuli. To do so, we directly tested the ability of KCl and forskolin to induce expression of the CRP1-5.0 enhancer. These results, shown in Fig. 4, revealed that the luciferase reporter activity of the CRP1-5.0 enhancer was enhanced by KCl but not by forskolin. Furthermore, enhancer activity by KCl was attenuated when the CRE was mutated (Fig. 4A). To verify that CRE was...
functionally responsive to these stimuli outside the context of the CRP1 enhancer, we tested a multimerized CRE luciferase construct (3X-CRE-Luc) harboring three CRP1-CREs in tandem. The multi-CRE showed robust activity in response to both forskolin and KCl (Fig. 4A). These data suggest that while forskolin and KCl can activate the CRP1 gene as determined by performing RT-PCR (Fig. 3), only KCl uses sites within the CRP1-5.0 enhancer. Moreover, the failure of KCl to elicit a strong response from the CRP1-5.0mutCRE-Luc reporter indicated that its activity was mediated through the CRE.

The ability of KCl to induce CRP1-5.0 enhancer activity implied that Ca\textsuperscript{2+} was a likely mediator of this effect. Consistent with this hypothesis, treatment of cells with the Ca\textsuperscript{2+} ionophore ionomycin also stimulated CRP1-5.0 activity (data not shown). To confirm that KCl-dependent Ca\textsuperscript{2+} influx was responsible for this action, we treated cells with nifedipine, an L-type Ca\textsuperscript{2+} channel antagonist (41). As shown in Fig. 4B, nifedipine completely blocked KCl-induced enhancer activity, verifying the role of Ca\textsuperscript{2+} in this process. A primary mode of Ca\textsuperscript{2+}-dependent transcriptional control is through activation of the CaM kinase cascade, which phosphorylates CREB (17, 50). To test the hypothesis that CaM kinases were important for KCl stimulation, we measured luciferase activity in the presence of the CaM kinase inhibitor KN-93 (Fig. 4B). With the addition of 30 \mu M KN-93, which is reported to block all CaM kinase activity (17, 21), the CRP1-5.0 enhancer exhibited a reduced response to KCl. The activity of the enhancer without KCl stimulation was also diminished by KN-93, possibly reflecting inhibition of basal levels of CaM kinases or a nonspecific inhibition.

To continue to explore the role of CaM kinases in CRP1 activation, we used an inhibitor of CaM kinase (CaMKK), STO-609 (55). As part of the CaM kinase cascade, CaM KKs uniquely phosphorylate and activate CaMKI and CaMKIV in response to Ca\textsuperscript{2+} (6, 17, 50). In the presence of STO-609, KCl stimulation of the CRP1-5.0 enhancer was diminished (Fig. 4B), further supporting the role of CaM kinases in this process and particularly implicating CaMKI and CaMKIV as primary mediators. Overall, these data are consistent with the hypothesis that KCI stimulation of the CRP1-5.0 enhancer is mediated by Ca\textsuperscript{2+} and dependent on CaM kinase activation.

CaMKIV activates the CRP1-5.0 enhancer. The ability of KCl to activate the CRP1-5.0 enhancer led us to consider the
role of Ca\(^{2+}\)/calmodulin-dependent kinases in this activity. Indeed, the CaM kinase inhibitor KN-93 blunted KCl stimulation, linking these protein kinases to the activation of CRP1 (Fig. 4B).

Moreover, STO-609, the CaM kinase kinase inhibitor also blocked activation. In an attempt to clarify the potential role of CaM kinases, we used constitutively active forms of CaMKII and CaMKIV (54) to measure directly the ability of these activators to stimulate transcription of the CRP1-5.0 enhancer. These two kinases, which both translocate to the nucleus, are known to phosphorylate CREB (17, 50) and are expressed in smooth muscle cells (4). Western blot analysis of protein extracts revealed detectable levels of both CaMKII and CaMKIV in PAC1 cells (Fig. 5A). Cotransfection experiments with luciferase reporters showed that constitutive CaMKIV, but not constitutive CaMKII, activated the CRP1 enhancer (Fig. 5B). The response of the enhancer in the presence of CaMKII was diminished, suggesting that activated CaMKII had an inhibitory function. CaMKIV, on the other hand, robustly stimulated expression from the CRP1-5.0 enhancer as well as the multimerized CRE. In addition to CaMKIV’s established role in augmenting CREB phosphorylation and CRE activation, it has also been shown to stimulate transcription through the SRF-binding CArG box (8, 33). The CArG box within the CRP1 enhancer sits adjacent to the CRE (Fig. 1A).

To examine the response of the CArG box, we monitored activity of a multimerized CArG element in the presence of the CaM kinase activators. The multi-CArG displayed a similar profile of expression compared with the wild-type and multimerized CRE reporters, being inhibited by CaMKII, and strongly activated by CaMKIV (Fig. 5B). These data demonstrate that CaMKIV, but not CaMKII, can augment expression of the CRP1-5.0 enhancer, and this activity is likely through the CRE and CArG elements.

The ability of CaMKIV to activate expression through these two distinct binding sites indicated that stimulation by Ca\(^{2+}\) might use both motifs to amplify a response. Examination of KCl activation of the CRP1-5.0 enhancer showed that induc-
tion of expression by KCl was not entirely abolished by a CRE mutation (Fig. 4A), implying that other sites are also KCl responsive. Given that CaM kinase inhibitors abolished all KCl-dependent activation, together with the presence of a CaMKIV-responsive CArG box, these data strongly suggest that the CRE and CArG elements facilitate Ca\(^{2+}\)-dependent transcription through CaMKIV. We therefore tested the ability of CaMKIV to activate these individual sites in two ways: first by examining mutations in the individual sites and second by using dominant-negative SRF and mutant CREB expression constructs (Fig. 6). In the presence of a CRE mutation or a CArG box mutation, the activity of the CRP1-5.0 enhancer was attenuated but not abolished in response to CaMKIV (Fig. 6A). However, the enhancer harboring both mutations exhibited a complete loss of CaMKIV responsiveness. These results reveal that the CRE and CArG box are direct downstream targets of CaMKIV. Furthermore, in the presence of dominant-negative SRF (5) or a mutant CREB protein (CREBm1) (11, 44), the activation of SRF (5) or a mutant CREB protein (CREBm1) (11, 44), the activation of CaMKIV (30), thus demonstrating the importance of this event for downstream activation. These data directly show that CaMKIV uses two distinct binding sites within the CRP1-5.0 enhancer to promote transcription of the CRP1 gene.

To explore the role of Ca\(^{2+}\) and CaMKIV on the endogenous CRP1 gene more directly, we examined expression using RT-PCR in the presence of selective activators and inhibitors. Consistent with the enhancer studies, nifedipine and STO-609 repressed CRP1 induction by KCl (Fig. 7A), substantiating the conclusion that KCl regulates CRP1 expression through Ca\(^{2+}\) and a CaM kinase-dependent pathway. Likewise, introduction of dominant-negative CREB and dominant-negative SRF also diminished CRP1 expression in response to KCl as observed with the enhancer studies. Moreover, constitutively active CaMKIV induced expression from the CRP1 locus, and this induction was not blocked by the upstream inhibitors nifedipine or STO-609, but CaMKIV activity was attenuated by both dominant-negative CREB and dominant-negative SRF. In conclusion, these findings suggest that expression of the CRP1 gene is governed by intracellular Ca\(^{2+}\) through the ability of CaMKIV to stimulate both CREB and SRF activation.

**DISCUSSION**

Studies of smooth muscle-specific transcription have focused primarily on the role of SRF and the CArG box. Although these two important players have an obvious role in cell-restrictive gene expression, other cis-acting elements and trans-acting factors likely play significant roles in governing cell-specific signaling (36). In an effort to define novel elements that control expression of the CRP1 gene, we identified a conserved CRE within the CRP1-5.0 intronic enhancer. The CRE is positioned in a region of the enhancer that shows high homology among mouse, rat, and human sequences. The CRE is capable of binding CREB, and by using a constitutively active VP16-CREB construct, we showed that CREB is able to activate the CRP1-5.0 enhancer through this motif. Furthermore, a mutation in the CRE caused the CRP1-5.0 enhancer to exhibit reduced activity. Expression from the CRE mutant enhancer was \(\sim50\%\) less than wild type but displayed residual activity above the pGL3 control, particularly in BASMCs. This persistent expression, albeit less than that of the wild type, indicates the existence of CRE-independent regulatory mechanisms. The requirement of the CRE for gene expression in smooth muscle cells has been reported for the Cyr61/CCN1 (13) and Hex genes (48); however, CREs have not been identified previously in differentiation-specific smooth muscle genes such as CRP1. In addition to the CRP1-CRE, putative CREs were found in a limited sequence search of the SM-22\(\alpha\) and smooth muscle myosin heavy-chain promoter regions. The
determination of whether these CREs and possibly others are functionally important for expression of these genes is of particular interest for future studies. Perhaps the CRE, like the CArG element, represents a common motif that is used to promote differentiation-specific gene expression.

With the identification of a CRE that binds CREB, we sought to determine the pathways that activate the CRP1 gene through this element. We tested the ability of CRP1 to be activated by two CREB-dependent pathways. Both PKA activation by forskolin and Ca\(^{2+}\) influx through membrane depolarization resulted in an increase in CRP1 transcripts. This result prompted us to explore the specific role of the CRE within the CRP1-5.0 enhancer in response to these stimuli. Surprisingly, while KCl was capable of eliciting a response, forskolin could not activate this enhancer element. These results imply that while both pathways augment expression of the endogenous CRP1 gene, only KCl used sites within the CRP1-5.0 enhancer. The reason for this is not clear; however, PKA-activated transcription of CREB in certain cases has been shown to be dependent on other promoter elements (45, 62). The ability of the multimerized CRP1-CRE to respond to forskolin offers evidence that the context of the CRE is important for its response. Moreover, evidence exists that PKA inhibits SRF function (7, 16). Given the juxtaposition of the CRE and CArG element in the enhancer, PKA inhibition of SRF might possibly override any stimulatory effect of CREB.

The capacity of KCl to stimulate the phosphorylation of CREB in vascular smooth muscle cells was demonstrated previously (39, 51, 52), and this response was shown to be achieved through CaM kinases (4, 9). Consistent with this finding, treatment of PAC1 cells with the CaM kinase inhibitor KN-93, and CaM kinase kinase inhibitor STO-609 abolished KCl stimulation of the CRP1-5.0 enhancer. Furthermore, constitutively active CaMKIV, but not CaMKII, activated the enhancer. The failure of CaMKII to turn on transcription likely reflects an inhibitory effect on CREB due to the constitutive phosphorylation of Ser142. In contrast, CaMKIV stimulates CREB activity through specific phosphorylation of Ser133 in addition to phosphorylating the CREB-interacting protein CBP (17, 54).

KCl stimulation of the CRP1-5.0 enhancer was dependent on an intact CRE element; however, some activity remained in the presence of the CRE mutation. This indicated that other elements were also mediating the KCl response. This was not entirely a surprise, because CaMKIV can also regulate transcription through the CArG box (8, 33). Indeed, the multimerized CRP1-CArG element was activated by expression of constitutive CaMKIV. We evaluated the capacity of the individual sites to facilitate CaMKIV's response to the enhancer. CaMKIV activation was decreased, but not abolished, in the presence of either the CRE or CArG mutation, while a double mutation completely abolished CaMKIV activity. Furthermore, using the CREB mutant (CREBm1) and dominant-negative SRF constructs, we showed that KCl and CaMKIV activation is attenuated on the CRP1-5.0 enhancer. The data garnered from the enhancer analysis were corroborated by examination of CRP1 transcripts, which showed similar results. Thus these data directly show that CaMKIV modulates the CRP1 enhancer through adjacent CRE and CArG elements.

Our experiments reveal that the CRP1 gene is controlled by a Ca\(^{2+}\)-dependent pathway, which involves the activation of...
CaMKIV. CaM kinases can phosphorylate a range of substrates, but CaMKIV is best characterized for its nuclear functions, particularly the phosphorylation of CREB (17, 50). Our studies are not the first to link CaM kinase activity and gene expression in smooth muscle cells (4); however, it does demonstrate the utilization of two conserved binding sites within the CRP1-5.0 enhancer: a newly identified CRE and the CArG box. One question that arises from these studies is why CaMKIV uses two sites to activate transcription. We speculate that these two sites might serve to amplify a response. Given that these two elements are separated by only 14 bp, CREB and SRF could cooperate by assisting in the recruitment of CBP, both of which bind to CBP’s NH2 terminus. We hypothesize that this dual recruitment strategy may help to maintain the expression of CREB over extended periods. In smooth muscle cells, Ca2+/CaM plays a vital role in governing contraction, which is directly linked to a differentiated phenotype. Therefore, it is interesting to determine whether other smooth muscle differentiation genes also respond to Ca2+/CaM through a CaMKIV pathway and whether they use both CRE and CArG elements to do so.

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GRANTS

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CamK IV REGULATES EXPRESSION OF CRP1

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