Distinct cAMP signaling pathways differentially regulate α2C-adrenoceptor expression: role in serum induction in human arteriolar smooth muscle cells

Maqsood A. Chotani, Srabani Mitra, Ali H. Eid, Seon A. Han, and Nicholas A. Flavahan. Distinct cAMP signaling pathways differentially regulate α2C-adrenoceptor expression: role in serum induction in human arteriolar smooth muscle cells. Am J Physiol Heart Circ Physiol 288: H69–H76, 2005. First published September 2, 2004; doi:10.1152/ajpheart.01223.2003. —The physiological role of α2-adrenoceptors (α2-ARs) in cutaneous, arteriolar, vascular smooth muscle cells (VSMs) is to mediate cold-induced constriction. In VSMs cultured from human cutaneous arterioles, there is a selective increase in α2C-AR expression after serum stimulation. In the present study, we examined the cellular mechanisms contributing to this response. Serum induction of α2C-ARs was paralleled by increased expression of cyclooxygenase-2 (COX-2), increased release of prostaglandins, and increased intracellular concentration of cAMP. Inhibition of COX-2 by acetyl salicylic acid (1 mM), NS-398 (5 μM), or celecoxib (3 μM) abolished the increase in cAMP and markedly reduced α2C-AR induction in response to serum stimulation. The cAMP agonists, forskolin (10 μM), isoproterenol (10 μM), and cholera toxin (0.1 μg/ml) each dramatically increased expression of α2C-ARs in human cutaneous VSMs. The A-kinase inhibitor H-89 (2 μM) inhibited phosphorylation of cAMP response element binding protein, but not the increase in α2C-AR expression in response to these agonists. cAMP-dependent but A-kinase independent signaling can involve activation of guanine nucleotide exchange factors for the GTP-binding protein, Rap. Indeed, pull-down assays demonstrated Rap1 activation by serum and forskolin in VSM. Transient transfections using α2C-AR promoter-luciferase reporter construct demonstrated that Rap1 increased reporter activity, whereas the A-kinase catalytic subunit decreased reporter activity. These results indicate that cAMP signaling can have dual effects in cutaneous VSMs: activation of α2C-AR transcription mediated by Rap1 GTPase and suppression mediated by A-kinase. The former effect predominates in serum-stimulated VSMs leading to a COX-2, cAMP, and Rap 1-dependent increase in α2C-AR expression. Such increased expression of α2C-ARs may contribute to enhanced cold-induced vasoconstriction and Raynaud’s phenomenon.

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RESULTS

Role of COX-2 in α2C-AR expression. Dermal arteriolar VSM α2C-AR expression was dramatically increased by serum (6.68 ± 0.81-fold increase in message after 12 h of serum stimulation [n = 19], P < 0.001; Ref. 8). The increase in α2C-ARs was paralleled by a serum-induced increase in COX-2 protein expression, which peaked between 4 and 8 h after initiation of serum stimulation (Fig. 1A; n = 3). This serum effect was not observed for COX-1, which showed constitutive expression (Fig. 1, and data not shown). The...
A robust increase in COX-2 was associated with release of PGE2 (1.47 ± 0.25 ng/ml at 5 h, n = 8) and 6-keto-PGF1α (1.26 ± 0.11 ng/ml at 5 h, n = 8), the stable metabolite of prostacyclin (Fig. 1B). The nonselective COX inhibitor acetylsalicylic acid (1 mM) reduced serum-induced α2C-AR message by 81.2 ± 3.8% (n = 7, P < 0.01; Fig. 2). To further distinguish between COX-1- and COX-2-mediated effects, selective COX-2 inhibitors NS-398 and celecoxib were utilized. NS-398 (5 μM) and celecoxib (3 μM) each showed effects similar to acetylsalicylic acid, reducing serum-induced α2C-AR by 76.0 ± 4.3% (n = 5, P < 0.01) and 75.1 ± 2.9% (n = 4, P < 0.01), respectively. These results suggest that COX-2 plays a critical role in serum-mediated increase in α2C-ARs in arteriolar VSM.

**COX-2, cAMP, and α2C-ARs.** Serum stimulation of quiescent arteriolar VSM caused a time-dependent robust increase in intracellular concentration of cAMP, which paralleled the expression of COX-2, and prostaglandin release (Fig. 3). The serum-stimulated increase in cAMP was ablated by acetylsalicylic acid (1 mM, n = 6), NS-398 (5 μM, n = 4), or celecoxib (3 μM, n = 4; Fig. 3, P < 0.0001 for control vs. treated groups).

To directly examine the role of cAMP in α2C-AR expression, VSMs were treated with forskolin (10 μM; n = 4), isoproterenol (agonist for the Gs-coupled β-ARs, 10 μM; n = 3–4), or cholera toxin (activator of Gs, 0.1 μg/ml; n = 3). Each stimulus increased α2C-AR message (Figs. 4 and 5, A and B). Forskolin and cholera toxin had the greatest effect, causing 12.65 ± 1.38-fold and 16.82 ± 4.15-fold increases in α2C-AR message, respectively, after 6 h of treatment (Figs. 4 and 5B). The profile of α2C-AR induction with isoproterenol was distinct, with peak message observed after 3 h (5.62 ± 1.16-fold increase; Fig. 5A), with the effect rapidly declining thereafter, falling below baseline level after 12 h.

The selective A-kinase inhibitor H-89 (5, 29, 31), at a concentration of 2 μM (~42-fold above the inhibitor constant; Figs. 4 and 5, A and B), did not affect the increase in α2C-AR expression evoked by forskolin, isoproterenol, or cholera toxin. However, phosphorylation of the A-kinase substrate CREB was increased by forskolin (10 μM, n = 4, P < 0.01) or cholera toxin (0.1 μg/ml, n = 4; P < 0.01), and this effect was markedly inhibited by H-89 (2 μM; Fig. 6). These results suggest that in arteriolar VSMs, the increase in α2C-AR expression is not mediated by A-kinase-dependent signaling.

**Rap GTPase and α2C-ARs.** cAMP can also signal through Rap1 and Rap2, members of the Ras GTPase superfamily binding proteins (12, 20). Western blot analyses demonstrated expression of both Rap1 and Rap2 in arteriolar VSMs. Rap pull-down assays were performed to assess the activation state of Rap proteins on forskolin treatment of quiescent VSM. Indeed, Rap1 was activated within 5 min after forskolin exposure (Fig. 7; 260.9 ± 23.5% increase, n = 3, P < 0.05). In contrast, Rap2 showed constitutive activity, and was not affected by forskolin (Fig. 7; 127 ± 34%, n = 3, P = not significant [NS]). Serum also activated Rap1, but with a slower time course compared with forskolin (peaked after 1 h of serum stimulation, Fig. 8A, 358.7 ± 58.8% increase, n = 3; P < 0.05), consistent with the slower increase in α2C-AR expression. There was no significant change in Rap2 activation by serum (Fig. 8B).

Transient cotransfections were performed in arteriolar VSM to assess the role of serum in transcriptional regulation of α2C-ARs. Indeed, serum increased α2C-AR-promoter-driven reporter activity by 3.5 ± 0.3-fold (n = 8; P < 0.001) at 12 h of serum stimulation. This effect of serum was not seen with

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**Fig. 2.** Effect of COX-2 inhibitors on α2C-AR induction. Representative autoradiograph illustrating serum induction of α2C-ARs in the absence or presence of the COX-inhibitor aspirin (acetyl salicylic acid; ASA). Yeast tRNA denotes control lane for background signal. Suppression of COX-2 activity by pharmacological inhibitors significantly reduced α2C-AR serum induction (ASA, 1 mM; 81.2 ± 3.8% inhibition, n = 7, P < 0.01, compared with FBS), NS-398 (5 μM; 76.0 ± 4.3% inhibition, n = 5, P < 0.01, compared with FBS), and celecoxib (3 μM; 75.1 ± 2.9% inhibition, n = 4, P < 0.01, compared with FBS). Q, quiescent cells; FBS, serum-induced cells (12 h).

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**Fig. 3.** COX-2 and cAMP. Intracellular concentration of cAMP was measured over various time intervals (0 to 24 h) of serum (FBS) treatment, in the absence (control, n = 6), or presence of pharmacological inhibitors for COX, ASA (1 mM, n = 6), NS-398 (5 μM, n = 4), and celecoxib (3 μM, n = 4). The concentration of cAMP in the control samples was significantly higher than the concentrations in the treated groups (P < 0.0001).
the α2A-AR-promoter driven reporter (0.8 ± 0.1-fold, n = 4, P = NS). Experiments were performed to examine the role of A-kinase and Rap GTPase in transcriptional regulation of α2C-ARs. The catalytic subunit of A-kinase transcriptionally activated a luciferase reporter driven by four copies of CRE that responds to the A-kinase substrate CREB (increasing reporter activity from 28.7 ± 4 × 10^{-3} to 97.25 ± 21.3 × 10^{-3} firefly/Renilla luciferase ratio, P < 0.004; Fig. 9). In contrast, the A-kinase catalytic subunit suppressed the α2C-AR promoter-driven reporter activity (decreasing from 66.05 ± 11 × 10^{-3} to 7.1 ± 0.9 × 10^{-3} firefly/Renilla luciferase ratio, P < 0.0014; Fig. 9). The constitutively active Rap 1 (Rap 1-CA) did not activate the CRE luciferase reporter (P = NS) but activated the α2C-AR promoter-driven luciferase reporter (increasing reporter activity from 66.05 ± 11 × 10^{-3} to 149.6 ± 37.2 × 10^{-3} firefly/Renilla luciferase ratio, P < 0.04; Fig. 9).

DISCUSSION

α2C-ARs, expressed on VSMs, contribute to cold-induced vasoconstriction in the cutaneous circulation (7). Indeed, within the human circulation, α2C-ARs have high expression in cutaneous VSMs but are not expressed by aortic medial VSMs (8). Furthermore, α2C-AR expression in human cutaneous...
VSMs is dramatically and selectively increased by serum stimulation (8). The present study identifies an unusual signaling role for cAMP, independent of the classic A-kinase signaling cascade, in mediating this serum response. Indeed, although A-kinase inhibits transcription of the human α2C-AR (29, this study), the alternate cAMP-responsive Rap1 signaling pathway actually increased α2C-AR transcription. The present results suggest that serum increases α2C-AR expression in human cutaneous VSMs by a COX-2, cAMP, and Rap1-dependent increase in transcription. Pathological activation of this pathway could contribute to the cold-induced vasospasm occurring in Raynaud’s phenomenon.

A crucial step in the response to serum was increased expression of COX-2. This enzyme converts arachidonic acid to prostaglandin H2 leading to the subsequent production of the prostaglandins thromboxane and prostacyclin (3). Indeed, serum stimulation caused a slow increase in cAMP accumulation in VSMs that paralleled induction of the COX-2 protein and prostacyclin release. Inhibition of COX-2 activity by acetyl salicylic acid, NS-398, celecoxib, markedly reduced the serum-induced increases in cAMP and α2C-AR expression. Likely candidates for coupling COX-2 to elevations in cAMP are the Gs coupled, E-prostanoid (EP) receptors (subtypes 2 and 4) and prostacyclin (IP) receptor (3, 4). This COX-2:cAMP signaling pathway was primarily responsible for serum induction of α2C-ARs and could be mimicked by elevating cAMP levels in the absence of serum using: forskolin, a direct stimulant of adenyl cyclase, isoproterenol, a β-AR agonist, or by cholera toxin, a direct activator of the Gs protein. Each of these stimuli caused dramatic increases in expression of α2C-AR, which occurred more rapidly than the response to serum. This would be consistent with the slow COX-2-dependent increase in cAMP in response to serum stimulation. Furthermore, with forskolin and with cholera toxin, the level of α2C-AR expression was over twofold that achieved after serum stimulation. All of the stimuli examined in the present study had a transient effect on α2C-AR expression, which mimicked the induction caused by serum (8). The transient nature of induction was especially notable with isoproterenol. Unlike the response to forskolin or cholera toxin, which peaked at ~6 h, the response to isoproterenol reached a maximum at ~3 h and had declined to basal levels by 12 h. Consistent with this abbreviated time course, the maximal effect of isoproterenol was significantly less than that achieved with forskolin or cholera toxin. The magnitude and kinetics of α2C-AR induction by isoproterenol are consistent with desensitization of β-ARs, and degradation of cAMP by β-arrestin-mediated recruitment of phosphodiesterases (27).

The powerful effect of cAMP agonists on the expression of α2C-ARs suggests that increases in cAMP are not only necessary but also sufficient for serum-induction of the receptor. This was somewhat surprising considering that cAMP, through

![Graph](http://example.com/graph.png)

**Fig. 6.** Effect of the A-kinase inhibitor H-89 (2 μM) on cAMP response element (CRE) binding protein (CREB) phosphorylation in forskolin (10 μM, 10 min, n = 4) or cholera toxin (CTX; 0.1 μg/ml, 10 min, n = 4) treated arteriolar VSM. H-89 inhibited phosphorylation of CREB in response to forskolin, or cholera toxin. Q, quiescent VSM. Forskolin caused a 4.2 ± 0.7-fold increase in phosphorylation, n = 4, P < 0.01, and cholera toxin a 2.2 ± 0.3-fold increase, n = 4, P < 0.01.

![Graph](http://example.com/graph2.png)

**Fig. 7.** cAMP and Rap GTPase activation in arteriolar VSM. Activation of small GTPases Rap1 and Rap2 was examined by pull-down assays (0, 5, 10, 30 min, n = 3). Forskolin activated Rap1 (A; maximum activation at 5 min, P < 0.05), whereas Rap2 (B) showed constitutive activity in arteriolar VSM, and was not affected by forskolin; P = NS.
The a-kinase signaling pathway, known to inhibit the expression of human α2C-ARs, Schaal et al. (29) demonstrated an inhibitory role for cAMP and A-kinase signaling on transcription of α2C-ARs in the human hepatocarcinoma cell line HepG2. Indeed, in the present study, the A-kinase inhibitor H-89 did not affect the increase in α2C-AR expression evoked by forskolin, cholera toxin, or isoproterenol. Therefore, the classic cAMP-dependent A-kinase signaling pathway does not mediate the increase in α2C-AR expression in response to these stimuli in human cutaneous VSM. When the A-kinase catalytic subunit was expressed in human cutaneous VSMs, it inhibited the transcriptional activity of the α2C-AR gene. Furthermore, the A-kinase substrate protein CREB (23) was phosphorylated in response to cAMP stimuli in VSMs, and the A-kinase inhibitor H-89 inhibited this effect. Therefore, these results suggest that A-kinase was activated in the VSMs and had the potential to inhibit α2C-AR expression. Although no functional effects of endogenous A-kinase could be observed on α2C-AR expression, overexpression of the catalytic subunit of A-kinase markedly reduced the transcriptional activity of the α2C-AR gene. Furthermore, the A-kinase substrate protein CREB (23) was phosphorylated in response to cAMP stimuli in VSMs and the A-kinase inhibitor H-89 inhibited this effect. Therefore, these results suggest that A-kinase was activated in the VSMs and had the potential to inhibit α2C-AR expression. This suggests that these divergent cAMP-signaling cascades may be tightly controlled within discrete signaling compartments. For example, A-kinase an-

effect interactions (2, 11). Results from the present study demonstrated that both Rap1 and Rap2 are expressed in cutaneous VSM. However, although the cAMP stimulus forskolin activated Rap1, Rap2 had constitutive activity and was unresponsive to the agonist. Transient transfections confirmed a direct role of Rap1 in transcriptional regulation of α2C-ARs. These results suggest that the Rap1 subtype may have a discrete role in α2C-AR expression in human cutaneous VSM. Altogether, these findings indicate a new and distinct role of cAMP-Rap1 signaling pathway in facilitating α2C-AR expression in cutaneous VSMs.

Therefore, cAMP can apparently have opposite effects on the transcriptional activity and expression of human α2C-ARs: inhibition, mediated by A-kinase-dependent signaling, and augmentation, mediated by Rap1-dependent signaling. The former effect was dominant in HepG2 cells (29), whereas the latter effect is dominant in cutaneous VSMs. Inhibition of A-kinase in VSMs did not enhance α2C-AR expression in response to cAMP stimuli. This suggests that the effect of cAMP in these cells is dominated by Rap1 signaling and is not restrained by the inhibitory effects of A-kinase-dependent signaling.

Although no functional effects of endogenous A-kinase could be observed on α2C-AR expression, overexpression of the catalytic subunit of A-kinase markedly reduced the transcriptional activity of the α2C-AR gene. Furthermore, the A-kinase substrate protein CREB (23) was phosphorylated in response to cAMP stimuli in VSMs, and the A-kinase inhibitor H-89 inhibited this effect. Therefore, these results suggest that A-kinase was activated in the VSMs and had the potential to inhibit α2C-AR expression. This suggests that these divergent cAMP-signaling cascades may be tightly controlled within discrete signaling compartments. For example, A-kinase an-

![Fig. 8. Time course (0, 0.5, 1, 2, 3, 4, 5, 6 h, n = 3–4) of activation of Rap GTPases by serum. Serum activated Rap1 (A), which peaked at 1 h (P < 0.05), whereas Rap2 (B) did not show a significant response to serum (P = NS).](http://ajpheart.physiology.org/)

![Fig. 9. Role of A-kinase and Rap1 GTPase in transcriptional activation of α2C-AR promoter. The data are expressed as ratio of firefly luciferase reporter activity to the internal control Renilla luciferase activity and allow normalization for transfection efficiency. The empty cytomegalovirus (CMV)-driven expression plasmid (10–20 ng) was cotransfected with CRE and α2C-AR luciferase reporters, giving baseline reporter activity. The catalytic subunit of A-kinase (20 ng) activated the luciferase reporter gene driven by four copies of CRE (n = 6, P < 0.004) but inhibited basal α2C-AR expression (1.915 ± 0.33) reporter activity (n = 6, P < 0.0014). The constitutively active Rap1–63E (Rap1-CA, 20 ng) increased α2C-AR reporter activity (n = 6, P < 0.04) but showed no effect on CRE reporter activity (n = 6, P = NS).](http://ajpheart.physiology.org/)
chor proteins play an important role in cAMP:A-kinase signaling by targeting the A-kinase to specific subcellular compartments (18, 24). Therefore, A-kinase may be targeted to a subcellular location that diminishes its inhibitory effect on α2C-AR expression. The mechanism(s) underlying the inhibitory effect of A-kinase on α2C-AR transcription has not been defined (29).

Previous studies (8) identified a crucial role of the p38 MAPK in α2C-AR expression in human cutaneous VSMs. The p38 MAPK was necessary but not sufficient for α2C-AR expression. Indeed, p38 MAPK can mediate COX-2 induction and prostaglandin synthesis in non-VSMs (9, 10, 16, 21, 25). Similarly, in cutaneous VSMs, the p38 MAPK may augment the effect of COX-2 on α2C-AR expression.

In summary, the serum-mediated increase in α2C-AR expression by cutaneous VSMs involves COX-2-cAMP signaling. Increasing intracellular cAMP is sufficient to increase α2C-AR expression, and a cAMP-A-kinase-independent pathway preferentially triggers a robust increase in α2C-AR expression. The results identify an alternate, Rap1-dependent pathway for cAMP signaling in arteriolar VSM, facilitating acute α2C-AR expression. The α2C-ARs have been implicated in mediating the cold-induced vasospasm of Raynaud’s phenomenon (12), and selective increase in α2C-AR responsiveness is observed in Raynaud’s phenomenon (15) and in arterioles from scleroderma patients versus controls (14). Indeed, vasospastic attacks in Raynaud’s phenomenon are believed to contribute to cycles of ischemia and reperfusion, known to involve COX-2 and postischemic inflammation, ultimately leading to local vascular injury (1, 13, 17). Conversely, vascular injury (vibration-induced mechanical, chemotherapeutic, or autoimmune disease related), may trigger an inflammatory response, leading to increased α2C-AR expression and secondary Raynaud’s phenomenon (1). Signaling cascades identified in this study may facilitate increased expression of α2C-ARs, further contributing to the severity of the condition.

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