TOPICAL REVIEW
Crossstalk Between Protein Kinase A and Growth Factor Receptor Signaling Pathways in Arterial Smooth Muscle

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ABSTRACT. Crossstalk between the cyclic AMP-dependent protein kinase (PKA) and growth factor receptor signaling is one of many emerging concepts of crosstalk in signal transduction. Understanding of PKA crosstalk may have important implications for studies of crosstalk between other, less well known, signaling pathways. This review focuses on PKA crosstalk in arterial smooth muscle. Proliferation and migration of arterial smooth muscle cells (SMCs) contribute to the thickening of the blood vessel wall that occurs in many types of cardiovascular disease. PKA potently inhibits SMC proliferation by antagonizing the major mitogenic signaling pathways induced by growth factors in SMCs. PKA also inhibits growth factor-induced SMC migration. An intricate cross-talk between PKA and the mitogen-activated protein kinase (MAPK/ERK) pathway, the p70 S6 kinase pathway and cyclin-dependent kinases has been described. Further, PKA regulates expression of growth regulatory molecules. The result of PKA activation in SMCs is the potent inhibition of cell cycle traverse and SMC migration. In this review, we discuss recent advances in our understanding of the crosstalk between PKA and signaling pathways induced by growth factor receptors in SMCs, and where relevant, in other cell types in which interesting examples of PKA crosstalk have been described.

KEY WORDS. Atherosclerosis, Cyclic AMP, Cyclic AMP-response elements, Cyclins, Mitogen-activated protein kinase, p70 S6 kinase, Proliferation

INTRODUCTION
The second messenger, cyclic adenosine-3',5'-monophosphate (cAMP), was discovered in the late 1950s (reviewed in [1]). It is generated from ATP by adenylate cyclases in essentially all tissues in the body. Currently, at least ten different adenylate cyclase gene families have been characterized (reviewed in [2]). These enzymes are, with some exception, embedded in the plasma membrane and are activated following stimulation of a wide variety of transmembrane receptors that are coupled to trimeric G-proteins (reviewed in [3, 4]). In arterial smooth muscle cells (SMCs) ligands of receptors that increase the formation of cAMP include β-adrenergic agonists, prostacyclin [5, 6], prostaglandin E2 [7, 8], calcitonin gene-related peptide [9], adenosine [10] and possibly dopamine [11]. Modulation of expression of various subtypes of these receptors results in differences in magnitude of the cAMP response to ligand–receptor interaction in different states of SMC differentiation and proliferation.

The vast majority, but not all [12–14], of the cellular effects of cAMP are mediated by cAMP-dependent protein kinase (PKA), a protein kinase that consists of two catalytic subunits and two regulatory subunits (reviewed in [15, 16]). Assembly of the products of several different regulatory and catalytic subunit genes (reviewed in [17]) can generate a number of distinct isoforms of PKA within the cell. PKA is activated when cAMP binds to the regulatory subunits, a process that dissociates the regulatory subunit dimer from the catalytic subunits. The release of the catalytic subunits renders them active and able to phosphorylate target proteins in the cytosol, cytoskeleton, membrane or nucleus on serines in the consensus sequence X-Arg-Arg-X-Ser-X...S (reviewed in [18]). It is now clear that the subcellular localization of PKA is determined by its binding to specific local-
ized A kinase anchoring proteins, AKAPs (reviewed in [19]). These anchoring proteins act as scaffolds that give PKA access to substrates localized in specific subcompartments within the cell, and may also facilitate PKA crosstalk with other signaling molecules that bind to, or in the vicinity of, the same anchoring protein.

Although PKA is a component of the first protein kinase cascade to be identified (reviewed in [20]), numerous factors, including recent discoveries of extensive crosstalk between PKA and other intracellular signals, make PKA signaling the subject of intense studies even today. One such area of research is the crosstalk between PKA and growth factor receptor signal transduction pathways in arterial SMCs in relation to cardiovascular disease. Recent developments in this area are discussed in this review. Where possible, the actions of PKA will be discussed in SMCs. We include other cell types in those instances in which interesting examples of PKA crosstalk are known only from these particular cell types. In these cases, future studies are needed to address the relevance in SMCs.

RESPONSE OF SMCs TO GROWTH FACTORS IN CARDIOVASCULAR DISEASE AND ITS INHIBITION BY cAMP

The arterial SMC has a principal role in the response of the arterial wall to injury in many types of cardiovascular disease. Normally, SMCs are contractile and are not responsive to growth factors or growth regulatory molecules (reviewed in [21]). SMCs in atherosclerotic lesions or SMCs in arteries subjected to acute injury undergo marked structural changes, referred to as a phenotypic modulation. These cells lose their ability to contract, increase their secretion of proteins and become more responsive to growth factors present in the arterial wall after injury, such as platelet-derived growth factor (PDGF) and fibroblast growth factor 2 (reviewed in [22, 23]). The events initiating this phenotypic modulation are not fully understood, but it is known that alteration of the extracellular matrix can modulate the structural and metabolic characteristics of SMCs and increase their responsiveness to growth regulatory molecules (reviewed in [22, 24]) [25].

The thickening of the intima (the innermost layer of the artery) during development and progression of atherosclerotic lesions, restenosis following angioplasty and bypass vein grafting, and as a result of severe pulmonary hypertension, is due to a combination of SMC proliferation, migration and an increased accumulation of extracellular matrix (reviewed in [21]) [26–28]. Growth factors present in the injured blood vessel wall are likely to mediate many of these responses. PDGF has been shown to act as a potent mitogen for SMCs, and to induce directed migration (reviewed in [21]). Although the level of proliferation in most human arteries subjected to various injuries appears to be rather low, animal models suggest that an indolent proliferative response is nevertheless able to produce stenotic lesions (reviewed in [28]). The growing arterial lesions eventually impede blood flow by projecting into the arterial lumen or, in the case of atherosclerotic lesions, by rupturing. The impeded blood flow leads to myocardial infarction, stroke, or gangrene of the extremities, depending on the location of the obstruction. Most of the sudden deaths from myocardial infarction are believed to be due to ruptures or fissures of the lesion, resulting in hemorrhage into the plaque, thrombosis and occlusion of the artery [29]. Thus, if the fibroproliferative response to injury of SMCs could be hindered, a major part of the clinical symptoms would be expected to be delayed, prevented or even reversed.

Uninjured endothelial cells, which conceal the SMCs from the circulating blood, normally synthesize agents that relax and inhibit growth of SMCs. These agents (e.g., prostacyclin) stimulate cAMP generation in the underlying SMCs. Release of other agents, such as nitric oxide, results in cGMP formation in the SMCs. Although cGMP is also known to inhibit SMC proliferation, we focus this review solely on the effects of cAMP. Removal of the endothelium leads to proliferation of the underlying SMCs (reviewed in [30]). Increased levels of cAMP potently inhibit SMC proliferation by arresting the cells primarily in the G1 phase, but also in the G2/M phases of the cell cycle (reviewed in [31]), [32, 33]. Consistent with this notion are studies demonstrating cyclic variations of cAMP throughout the cell cycle in other cell types, in which low levels of cAMP are required during late G1 and G2 in order for the cell to enter S and M, respectively (reviewed in [31]).

In addition to its inhibitory effect on proliferation, cAMP mediates other important processes in SMCs. For example, cyclic AMP appears to mediate cell death under some conditions in SMCs [34], and this increased cell death may contribute to the growth inhibitory effects of cAMP. Cyclic AMP also inhibits migration of SMCs and other cell types [35–37]. Synthesis of some extracellular matrix components is inhibited by cAMP [38, 39]. Based on these findings, elevation of levels of cAMP appears to be able to inhibit most of the responses of SMCs to vascular injury.

CROSSTALK BETWEEN PKA AND MAPK PATHWAYS IN SMC

MAPK/ERK Signaling Pathway

The mitogen-activated protein kinase (MAPK) cascade known as the extracellular signal-regulated kinase (ERK) pathway mediates mitogenic responses induced by a wide variety of growth factor receptors in many cell types (reviewed in [40]), including SMCs [8, 41, 42]. Activation of this pathway can be initiated by ligand binding to a cell surface receptor, activation of the receptor and binding of adapter molecules (such as GRB2 and Shc) to phosphotyrosine residues in the activated receptor, or to proteins phosphorylated by the receptor. These events lead to activation of the small GTP-binding protein Ras by a guanine nucleotide exchange factor (e.g., mammalian son-of-sev- enless; mSOS). With insulin and insulin-like growth factor
I (IGF-I), activation of the MAPK cascade is mediated through the binding of GRB2 or Src to the tyrosine phosphorylated insulin receptor substrate (IRS). Sequential phosphorylation events then lead to activation of the protein kinases, Raf, MAP kinase kinase (MAPKK or MEK), and MAPK (also known as ERK). Two isoforms of MAPK, the p44 MAPK (ERK-1) and the p42 MAPK (ERK-2) are expressed in SMCs, as in most cell types. The substrates of MAPK include nuclear transcription factors, such as Ets proteins [43], non-nuclear substrates, such as the protein serine/threonine kinase p90rk and cytosolic phospholipase A2 (cPLA2) and cytoskeletal proteins (reviewed in [44]). Cytosolic phospholipase A2 catalyzes the release of arachidonic acid from phospholipids in membranes and is one of the rate-limiting steps in the synthesis of prostaglandins (PGs), thromboxanes, leukotrienes and other arachidonic acid metabolites (reviewed in [45, 46]).

PKA and Inhibition of the MAPK/ERK Signaling Pathway

In many cell types [47–51], including human arterial SMCs [52], PKA inhibits the MAPK/ERK cascade. This inhibition is observed after activation of MAPK/ERK by several different growth factor receptors (e.g., the insulin receptor, epidermal growth factor receptor and PDGF receptor), but the receptors themselves do not appear to be targets of PKA-mediated inhibition of the MAPK cascade (reviewed in [53]).

The exact point of inhibition of the MAPK cascade by PKA is still a matter of some controversy, and it is quite possible that PKA might have different specific targets within the MAPK cascade in different cell types. It is, however, clear that whereas PKA potently reduces MAPK activity, the point of inhibition of the MAPK cascade by PKA is upstream of MAPK itself and also of MAPKK. Several studies have shown that the target of PKA is most likely located downstream of Ras activation. This conclusion is based on the findings that the functions of GRB2, Shc or insulin-receptor substrate-1 (IRS-1) do not appear to account for the inhibition of MAPK signaling by PKA [47–49, 54, 55], that PKA does not prevent formation of the active, GTP-loaded form of Ras [47, 54–56] and that activation of PKA inhibits MAPK activity induced by an activated form of Ras [57, 58]. Screening some of the possible targets in the MAPK cascade for typical PKA phosphorylation consensus sites reveals the presence of such sites in human mSOS, Raf-1 and B-Raf, but not in Ras or GRB2.

Several studies suggest that the point of inhibition of the MAPK/ERK cascade by cAMP is at the level of Raf-1 [47], and it has been shown that PKA can phosphorylate Raf-1 mainly on serine 43 [49, 59] and to some extent on serine 621 [60]. Initial studies showed that Raf-1 activity is decreased following elevation of cAMP levels [47, 49, 54]. Interestingly, Ser 43 in Raf-1 is located in close proximity to the Ras-interaction domain, and PKA phosphorylation of Ser 43 reduces the affinity with which Raf-1 binds GTP-loaded Ras [49, 59, 60]. It has also been shown that B-Raf, another isoform, which lacks serine 43 but has two PKA consensus sites on Ser 429 and Ser 446, is phosphorylated by PKA and that its ability to bind GTP-Ras is dramatically reduced by PKA [61]. Phosphorylation of Raf-1 or B-Raf by PKA catalytic subunits in vitro does not inhibit the ability of Raf to activate MAPKK ([61]; Graves, Bornfeldt, and Krebs, unpublished observation). One likely mechanism of PKA inhibition of the MAPK cascade therefore seems to be a reduced ability of GTP-loaded Ras to interact with Raf [50, 51].

Alternative mechanisms of inhibition of Raf by PKA are indicated by the fact that the activity of v-Raf or the isolated Raf-1 kinase domain, each of which lacks the Ras-binding domain, can be inhibited by PKA [51]. One alternative mechanism whereby PKA may inhibit the MAPK cascade is by phosphorylation and activation of the small GTP-binding protein Rap1 [62, 63]. Because the effector domain of Rap1 is homologous to that of Ras, it has been suggested that activation of Rap1 could compete with Ras for binding to Raf [47, 64]. Interestingly, a new group of signaling molecules has recently been discovered [13, 14]. These proteins (termed Epac, exchange protein directly activated by cAMP, or cAMP-GEFs, cAMP-guanine nucleotide exchange factors) bind cAMP and selectively activate Rap1 in a PKA-independent manner by acting as Rap1 guanine nucleotide exchange factors [13, 14]. These findings open the possibility that cAMP-mediated inhibition of the MAPK/ERK pathway may be PKA independent, at least in some cell types.

Yet other mechanisms of PKA inhibition of the MAPK pathway would be an inhibition of kinases that can activate Raf, such as certain isoforms of protein kinase C (PKC) or Src (reviewed in [65]). Further, competition for GTP-loaded Ras by Ras targets other than Raf may potentially result in a reduced MAPK signaling. The precise mechanism(s) of cAMP/PKA crosstalk with the MAPK/ERK cascade in SMCs remains to be defined.

Activation of PKA Can Turn the MAPK/ERK Pathway Into a Growth-Inhibitory Pathway in SMCs

As indicated earlier, activation of the MAPK/ERK pathway by growth factor receptors often leads to proliferation of SMCs. However, we have recently described a complex type of crosstalk between PKA and the MAPK/ERK pathway that results in a growth inhibitory response mediated by the MAPK pathway, as shown in Figure 1 [8]. SMCs that express the inducible form of cyclooxygenase (COX-2) normally secrete large amounts of cAMP-stimulating prostaglandins, such as prostaglandin E2 (PGE2), whereas this is not the case for SMCs that do not express significant levels of COX-2. We observed that an inhibitor of MAPKK (PD 098059) inhibits PDGF-induced proliferation of human arterial SMCs that do not secrete PGE2, as would be expected if the MAPK pathway mediated the mitogenic growth factor response. In striking contrast, in SMCs that express
COX-2, activation of MAPK serves as a negative regulator of proliferation. In these cells, PDGF-induced MAPK activation leads to cPLA₂ activation, PGE₂ release and subsequent activation PKA [66], which acts as a strong inhibitor of SMC proliferation (Fig. 1). A similar loop has been described for bradykinin in airway SMCs [67] and for vascular endothelial growth factor in endothelial cells [68]. Inhibition of either MAPKKK signaling, COX-2 or PKA activation in the human arterial SMCs releases them from the influence of the death-inhibitory prostaglandins and results in the subsequent cell cycle traverse and proliferation [8]. Thus, the growth factor-induced MAPK pathway can mediate either proliferation or growth inhibition in human arterial SMCs depending on the availability of specific downstream enzyme targets and the ability of the MAPK pathway to indirectly activate PKA (Fig. 1). Further studies are needed to clarify why some SMCs express more COX-2 than others. However, COX-2 expression can be induced by a variety of cytokines and growth factors in SMCs in vivo and in culture [69–71], and it is likely that growth factors released from the SMCs themselves are responsible for the increased COX-2 expression. Thus, this mechanism (Fig. 1) constitutes yet another example of the complex crosstalk between PKA and growth factor receptors (in this case PDGF receptor), and emphasizes the importance of considering all the different stimuli affecting a SMC in a specific setting.

PKA Can Activate the MAPK/ERK Pathway Under Certain Circumstances

In some cell types and under certain circumstances, activation of PKA results in an activation, rather than an inhibition, of the MAPK/ERK pathway. This was originally believed to explain how cAMP can provide a mitogenic signal in some cell types, as occurs in Swiss 3T3 cells and thyroid cells (reviewed in [31]). However, it is becoming increasingly clear that PKA can exert a stimulatory effect on the MAPK pathway and still inhibit growth factor-induced proliferation. This is possible because PKA acts on a number of mitogenic signaling pathways, as discussed below. Frödin et al. first described the activation of MAPK/ERK1 by cAMP-elevating agents in PC12 cells [72]. Interestingly, it was subsequently shown that cAMP-elevating agents activate the MAPK pathway in PC12 cells even though they have an inhibitory effect on B-Raf activity, the major Raf isoform in these cells [61]. This activation is synergistic with that of growth factors, and does not seem to be mediated through calcium mobilization or phorbol ester-sensitive isoforms of protein kinase C, but is believed to be upstream of MAPK [61]. Possible explanations of this PKA-mediated MAPK activation could be expression of a PKA-inhibited phosphatase or a PKA-mediated activation of a MAPKK kinase distinct from Raf.

Recently, still another mechanism whereby PKA can activate the MAPK pathway has been described. It was shown that PKA can activate MAPK signaling by an activation of Rap1 and the subsequent activation of B-Raf in PC12 cells [73, 74]. Interestingly, Rap1 acts as an inhibitor of Raf-1 and an activator of B-Raf [73]. This may explain the finding that PKA can activate the MAPK pathway in some cell types despite an inhibition of Raf-1 and Ras activities [56, 75–77]. Thus, it is possible that the effect of PKA on the MAPK cascade is to some extent dependent on the relative amounts of Rap1, B-Raf and Raf-1 expressed by the cell. Further, the PKA-independent activation of Rap1 by cAMP may be relevant here [13, 14].

Taken together, cAMP and PKA appear to be able to activate the MAPK/ERK pathway by at least two different mechanisms, one that is independent of B-Raf and one that is mediated by a Rap1-induced activation of B-Raf. It has recently been shown that the effect of cAMP on the MAPK/ERK pathway depends on both the cell type and the growth factor receptor signaling pathways activated [55]. It is quite likely that under certain conditions, activation of PKA could result in stimulation of the MAPK pathway also in SMCs, as stimulation of cAMP levels can result in an increased DNA synthesis in neonatal but not in adult pulmonary SMCs [78].
PKA Crosstalk with the Stress-Activated MAPK Pathways in SMCs

In addition to the MAPK/ERK pathway, PKA has also been shown to antagonize the stress-activated MAPK/SAPK signaling pathways that lead to activation of c-terminal jun kinase (JNK) and p38 in SMCs from vasculature or airways [79, 80]. The upstream target of PKA in these signaling pathways awaits characterization. However, it has recently been shown that PDGF can activate JNK through phosphatidylinositol 3-kinase (PI3K), a potential target of PKA (see below) in COS-7 cells transfected with the PDGF receptor [81]. The role of the SAPK pathways in SMC proliferation and migration is not yet fully understood.

Recent studies show that the p38 pathway may exert a negative feedback on the MAPK/ERK pathway through inhibition at the level of, or upstream of, MAPKK (MEK) in arterial SMCs [82]. Thus, the cAMP-mediated effects on the ERK and the SAPK pathways may be very complex. In this context, one must bear in mind that the PKA crosstalk is almost certainly determined by the cell type, the signaling components expressed, the subcellular localization of PKA targets, and the combined stimulatory signals.

PKA ANTAGONIZES p70 S6 KINASE AND TRANSLATION INITIATION FACTOR-4E IN SMCs

Although PKA can inhibit the MAPK/ERK cascade, the inhibitory effect of PKA on SMC proliferation is not solely due to inhibition of MAPK/ERK signaling, as discussed below. For example, cAMP levels that are too low to detectably inhibit growth factor-induced MAPK activation cause a strong inhibition of DNA synthesis. Furthermore, elevation of levels of cAMP at a time point when the transient growth factor-induced activation of MAPK/ERK has declined to basal levels still results in potent inhibition of arterial SMC DNA synthesis (our unpublished observations). Although one can argue that the MAPK/ERK pathway may maintain a low activity throughout the cell cycle or have later peaks of activity, the efficiency whereby cAMP elevation inhibits SMC DNA synthesis is nearly identical when initiated prior to growth factor stimulation or 6 h later. The same phenomenon has been observed in other cell types [83]. These observations suggest that PKA inhibits other mitogenic signaling pathways in SMCs. In fact, it has been suggested that PKA may act as a gating signal that can inhibit information flow leading to a mitogenic response within the cell (reviewed in [84]). As discussed below, crosstalk between PKA, the p70 S6 kinase pathway and translation initiation factor-4E may explain some aspects of the inhibition of SMC proliferation by PKA.

The p70 S6 Signaling Pathway

The p70 S6 kinase is a 70-kD serine/threonine protein kinase activated by growth factor receptors through a signaling pathway distinct from the MAPK/ERK kinase pathway. Another isoform of p70 S6 kinase, p85 S6 kinase, is regulated in the same manner as p70 S6 kinase and differs only by a 23 amino acid tail that targets p85 S6 kinase to the nucleus. Activation of p70/p85 S6 kinase has long been thought to be important for protein translation (reviewed in [85]). When activated, p70 S6 kinase phosphorylates the S6 protein of the 40S ribosome, and this has been suggested to allow preferential translation of a family of mRNAs with 5’ polypyrimidine tracts [86, 87]. The mRNAs containing polypyrimidine tracts include all ribosomal protein mRNAs and elongation factor 1A and -2 mRNA (reviewed in [88]). However, p70 S6 kinase also modulates transcription factors and regulates transcription (reviewed in [89]).

The p70/p85 S6 kinase appears to be of major importance for SMC proliferation [25, 90]. This is supported by results from other cell types in which inhibition of p70 S6 kinase activity, either by neutralizing antibodies or by the inhibitor rapamycin severely inhibits cell cycle progression through the G1 phase of the cell cycle [91–93]. Major progress has recently been achieved in our understanding of the signaling events leading to activation of p70/p85 S6 kinase [94–96]. The p70 S6 kinase and the p85 isoform require multiple signaling inputs and phosphorylation events to become fully active [91, 95–97]. First, several serine/threonine sites within the autoinhibitory C-terminus of the enzyme get phosphorylated. This event, which serves to release the autoinhibitory tail from the catalytic site of the enzyme, can be mediated by proline-directed kinases, such as the ERKs, SAPKs and cdc2 [95]. Secondly, a kinase activated by the products of phosphatidylinositol 3-kinase (PI3K) phosphorylates threonine 389, leading to a slight activation (2%–5% activity) of the enzyme. This kinase may be the recently described integrin-linked kinase, ILK [98], and/or other unidentified kinases. The above events are required for PDK1 (3-phosphoinositide-dependent protein kinase 1) to phosphorylate threonine 229, a site for which phosphorylation leads to a fully active p70 S6 kinase [95, 96]. A further complication in the activation of p70 S6 kinase is that mammalian target-of-rapamycin, mTOR (also known as RAFT-1/FRAP/RAFT-1), a member of the phosphatidylinositol kinase-related kinases that possess protein kinase activities (reviewed in [99]) also activates the enzyme. It is believed that mTOR activates p70 S6 kinase by inhibiting a phosphatase that acts to dephosphorylate threonine 389 in the kinase, thereby increasing its ability to be activated by PDK1 [100]. Other upstream activators of p70 S6 kinase include phorbol ester-sensitive PKC isoforms and the small Rho-family G-proteins Rac and cdc42 [101, 102], but it is not yet understood how these signals feed in to the p70 S6 kinase pathway.

Crosstalk Between PKA and the p70 S6 Kinase Pathway

PDGF- and IGF-I-induced p70 S6 kinase phosphorylation/activation are antagonized by agents that elevate levels of cAMP in arterial SMCs [103], a finding consistent with the
response of certain other cell types to cAMP [104]. In rat SMCs, the p70 S6 kinase is inhibited by forskolin, a direct activator of adenylate cyclases, at concentrations that do not result in inhibition of the MAPK/ERK pathway, suggesting that at least in these cells, the p70 S6 kinase pathway is especially sensitive to the inhibitory action of PKA [103]. The p70 S6 kinase is not directly phosphorylated by PKA, and the result of cAMP elevation in SMCs is a dephosphorylation of the enzyme rather than a phosphorylation (reviewed in [53]). It is likely that PKA phosphorylates and inhibits one of the signaling molecules upstream of p70 S6 kinase, or that PKA activates a phosphatase in these cells. One point of crosstalk between PKA and the p70 S6 kinase pathway appears to be at the level of PI3K, since PI3K activity is partially inhibited when cAMP levels are elevated in airway SMCs and other cell types [105, 106]. We have found a similar partial inhibition of PDGF-receptor-associated PI3K activity in human SMCs stimulated with agents that elevate cAMP. However, PKA is likely to inhibit other steps in the p70 S6 kinase pathway as well. This presumption is based on the findings that in some cell types, agents that elevate cAMP levels lead to inhibition of p70 S6 kinase without any appreciable inhibition of PI3K (our unpublished observations). In agreement with these observations is the recently described cAMP-mediated inhibition of mTOR in 3T3 cells [107]. Another recent study places PI3K upstream of mTOR [108], and thus, cAMP may indeed have several targets in the pathway leading to activation of p70 S6 kinase.

Inhibition of PI3K kinase by PKA might be expected to lead to other effects in addition to the effect on p70 S6 kinase activation. For example, because activation of protein kinase B (PKB) is also known to be dependent on PI3K [94] (reviewed in [109]), an inhibition of PKB activity would be expected by PKA. Further, cAMP has been found to abolish tyrosine phosphorylation of the focal adhesion-associated protein, paxillin, in vascular SMCs [110]. Tyrosine phosphorylation of paxillin appears to be mediated by a PI3K-dependent signaling pathway [111]. Phosphorylation of paxillin regulates cell attachment to the matrix, but may also regulate cell migration and play an indirect role in regulation of proliferation through regulation of attachment of the cell to the extracellular matrix (reviewed in [112]). Thus, it is possible that the inhibitory effect of PKA on growth factor-induced tyrosine phosphorylation of paxillin is mediated through inhibition of PI3K, and that this event plays a role in PKA-antagonized SMC migration and/or proliferation.

Regulation of Translation-Initiation Factor-4E

Initiation of mRNA translation in eukaryotic cells is mediated by a number of translation initiation factors (eIFs). The translation initiation factor-4E (eIF-4E) binds to the 5′ cap region of the mRNA, and its actions are believed to be limiting for translation initiation. The ability of eIF-4E to initiate translation is restricted by binding proteins [eIF-4E-BPs or PHAS (Phosphorylated Heat- and Acid-Stable)] that bind eIF-4E. When bound to PHAS-1, eIF-4E can bind to the 5′ cap region of the mRNA, but is unable to bind to another initiation factor, eIF-4G. Phosphorylation of PHAS-1 liberates eIF-4E, and eIF-4E can then participate in complex formation with eIF-4G and eIF-4A to form the complex eIF-4F. eIF-4F, in turn, melts the 5′ mRNA secondary structure, which facilitates binding and/or scanning by the ribosome (reviewed in [113]). It has long been known that PHAS-1 is phosphorylated following stimulation of cells with growth factors or insulin, and this phosphorylation is sufficient to block PHAS-1-binding to eIF-4E.

A number of observations indicate that eIF-4E and PHAS-1 are involved in mitogenic responses. First, overexpression of eIF-4E results in an increased proliferation and even transformation in some cell types [114]. Second, eIF-4E selectively increases translation of mRNAs encoding the proteins c-myc [115] and cyclin D1 [116], both of which regulate cell cycle progression. Thus, it is likely that growth factor-induced phosphorylation of PHAS-1 increases the mitogenic actions of eIF-4E.

The growth factor-induced signaling pathways that lead to phosphorylation of PHAS-1 are not yet fully understood. It is believed that the MAPK/ERK pathway plays a role under certain circumstances. However, IGF-1 stimulation of SMCs leads to phosphorylation of PHAS-1 without a detectable activation of the MAPK pathway [103]. Furthermore, inhibition of the MAPK pathway using the MAPKK inhibitor, PD98059, does not inhibit the ability of angiotensin II to stimulate phosphorylation of PHAS-1 in SMCs [41]. Thus, activation of the MAPK/ERK pathway is not sufficient for phosphorylation of PHAS-1 in SMCs. Instead, mTOR appears to regulate phosphorylation of PHAS-1 in these cells. Growth factor-induced phosphorylation of PHAS-1 can be inhibited by rapamycin, an inhibitor of mTOR, in SMCs and many other cell types (reviewed in [113]). From this follows that phosphorylation of PHAS-1 and activation of p70 S6 kinase share mTOR as a common upstream kinase. The growth factor-sensitive kinase directly upstream of PHAS-1 awaits characterization.

Crosstalk Between PKA and PHAS-1

We have shown that phosphorylation of PHAS-1 induced by PDGF and IGF-1 in rat aortic SMCs is markedly suppressed by agents that elevate cAMP [103]. Similar to the effects on p70 S6 kinase, the effect of PKA on PHAS-1 is evidenced by dephosphorylation rather than increased phosphorylation of the protein. This conclusion is based on the finding that PHAS-1 undergoes a band-shift consistent with the generation of less phosphorylated forms following elevation of cAMP. Consistent with a dephosphorylation of PHAS-1, increased cAMP levels in SMCs also result in an increased binding of PHAS-1 to eIF-4E [103]. As in the case for the p70 S6 kinase pathway, the target for PKA in the PHAS-1 phosphorylation cascade appears to be mTOR [107], and/or PI3K [108]. Because binding of PHAS-1 to
PKA Crosstalk with Other Rapidly Induced Signaling Pathways in SMCs

The Janus family of tyrosine protein kinases consists of at least four members; Jak1, Jak2, Jak3 and Tyk2. These kinases are activated in response to cytokines and growth factors, such as PDGF [117], and they regulate tyrosine phosphorylation and activation of the STAT transcription factors (reviewed in [118]). The Jaks can also signal through the insulin receptor substrates by increasing tyrosine phosphorylation of these proteins and induce subsequent signaling events (reviewed in [119]). It has recently been demonstrated that activation of PKA inhibits interferon-induced tyrosine phosphorylation of Jak1, Tyk2, STAT 1 and STAT2 in a myeloma cell line [120]. In these studies however, tyrosine phosphorylation of the interferon α/β receptor was also decreased following cAMP elevation. Agents that elevate cAMP have been found to abolish tyrosine phosphorylation of Tyk2 in response to angiotensin II in arterial SMCs [110]. The target for PKA in the JAK/STAT signaling pathway in SMCs has not been identified.

The best characterized PKA substrates described are involved in regulation of metabolism (reviewed in [20]); they include enzymes such as phosphorylase kinase, glycogen synthase, pyruvate kinase, phosphofructokinase, phenylalanyl-

PKA AND SMC CONTRACTION

Contraction of SMCs is of crucial physiological importance for many of the functions of the body, including the regulation of blood pressure. SMC contraction also contributes significantly to cardiovascular disease, and an increased blood pressure is a risk factor for cardiovascular disease.

SMC contraction is initiated by a rise in levels of intracellular calcium. The rise in intracellular calcium levels is brought about by an increased mobilization from internal stores and/or by an influx of extracellular calcium through calcium channels of two major types; the transient type (T type) and the long-lasting type (L type). Intracellular calcium binds to calmodulin and the calcium-calmodulin complex binds to, and activates, myosin light-chain kinase (MLCK). MLCK, in turn, phosphorylates Ser 19 on the regulatory chain of myosin, which allows the myosin to be activated by actin and the SMC to contract (reviewed in [125]). It is well known that agents that elevate cAMP levels lead to relaxation of arterial smooth muscle. It has been suggested that the actions of cAMP on SMC relaxation may be mediated by the cGMP-dependent protein kinase-1 (PKG-1), the major PKG isoform in SMCs (reviewed in [126]). However, recent studies on PKG-1 deficient mice indicate that cAMP most likely acts through PKA [127], but the mechanism of action of PKA to induce relaxation is not yet completely understood. Mechanisms suggested to contribute to the effects of PKA on relaxation include phosphorylation of MLCK that results in a reduced affinity between the calcium-calmodulin complex and MLCK (reviewed in [125]), phosphorylation of telokin, which may enhance myosin light-chain phosphatase activity [128], and possibly phosphorylation of the inositol 1,4,5-trisphosphate receptors [129] and/or phospholamban (reviewed in [130]) resulting in increased movement of calcium into internal stores. In this context it is noteworthy that different pools of PKA are likely to be localized to different intracellular structures by the AKAPs, as an AKAP has recently been shown to localize PKA to L-type calcium channels in skeletal muscle [131]. Interestingly, many factors that have mitogenic effects on SMCs act as arterial constrictors [132], possibly by inducing signaling pathways that phosphorylate and enhance calcium currents through the L-type calcium channels [133]. Thus, regulation of SMC contraction-relaxation provides another point of crosstalk between PKA and growth factor signaling.

PKA Antagonizes the Actions of Cyclin-Dependent Kinases in SMCs

Cyclins and their Regulation of Cell Cycle Progression

Cell cycle progression is regulated by a number of cyclin-dependent kinase/cyclin complexes, in which the cyclin-dependent kinase acts as the catalytic subunit and the cyclin acts as the regulatory subunit. Following growth factor stimulation of cells, levels of cyclin Ds (cyclin D1, D2 and D3) increase to form complexes with cyclin-dependent kinase (cdk)-4 and -6. Increased cyclin D-associated kinase activity occurs in early to mid phase of the G1 phase of the cell cycle and is required for G1 to S transition (reviewed in [134]).

The next step in the chain of events in cell cycle progression is an increased activity of cyclin E, which associates with cdk2. In contrast to cyclin Ds, cyclin E expression is usually not induced by growth factors. However, cyclin
E-ck2 activity is markedly increased in mid G1 following growth factor stimulation, and inhibition of cdk2 activity prevents cells from entering the S phase of the cell cycle. When the cell enters S phase, cdk2 forms a complex with cyclin A (reviewed in [134]). The substrates of the cyclin/ck2 complexes have not been characterized in full, but the retinoblastoma protein (Rb) appears to be a likely physiologic substrate of the cyclin D-ck4/-6 complex. Phosphorylation of Rb results in release and activation of the transcription factor E2F, a factor that activates genes required for DNA replication (reviewed in [135, 136]).

Cyclin-dependent kinase inhibitor proteins that bind and inactivate cdkks also regulate the actions of the cyclin-cdk complex. The inhibitor proteins fall into two groups; the Ink4 family and the Kip/Cip family. Inhibitors in the Ink4 family include p16INK4a, p15INK4b, which inhibit the action of cdk4/-6, and inhibitors in the Kip/Cip family include p27Kip1, p21Cip1 or Waf1 and p57Kip2 [137, 138], which inhibit the actions of all cdkks (reviewed in [139, 140]).

Late S phase and the G2 and M phases are regulated by the cyclin-dependent kinase cdc2, which binds to cyclin B in late S and G2 and to both cyclin A and B during M phase.

**PKA Antagonism of Cyclin-Associated Kinases**

PKA not only antagonizes rapidly induced (within minutes) growth factor-receptor protein kinase pathways, such as the MAPK/ERK pathway and the p70 S6 kinase pathway, but also inhibits the actions of cyclins, which are active throughout the cell cycle. This inhibition does not appear to be due to the direct phosphorylation of these proteins by PKA, but may be due to a down-regulation of levels of cyclin D1 mRNA and -protein and cdk2 mRNA in SMCs [141]. In addition, the activity of the cyclin D/cdk4 complex is inhibited by a PKA-mediated induction of the cdk inhibitor, p27Kip1 [142], resulting in inhibition of the transition from the G1 phase of the cell cycle to the S phase. We have observed a similar upregulation of p27Kip1 in response to cAMP elevating agents in human SMCs (unpublished observation).

An interesting link between the cyclins and mTOR signaling has been revealed. In several cell types including SMCs, the mTOR-inhibitor, rapamycin, inhibits phosphorylation and cdk2 activity [90]. This inhibition appears to be due to a loss of growth factor-induced reduction of p27 Kip1 levels [143]. Recently, the effects of rapamycin on cyclin/cdkks have been clarified in more detail in NIH 3T3 cells. Rapamycin has been shown to lead to decreased levels of cyclin D1, which in turn impairs the formation of active cyclin D1/cdk4 complexes and a subsequent re-targeting of p27 Kip1 inhibitor to the cyclin E/cdk2 complex [144]. Thus, inhibition of mTOR by rapamycin may have a similar effect as PKA on the cyclin/cdks. These findings also indicate a direct link between p70 S6 kinase signaling, PHAS-1 phosphorylation and the cyclins.

In addition to its effects on G1 cyclin/cdk activities, PKA has significant actions in the G2 and M phases of the cell cycle. Levels of active PKA catalytic subunit need to be low at the onset of mitosis, or the cell will undergo interphase arrest [145]. This effect of PKA may be due to a loss of cdc2 kinase activity [145]. Cyclic AMP elevating agents have also been shown to down-regulate the protein levels of cdc2, required in the G2 and M phases of the cell cycle in SMCs [141]. Thus, by inhibiting the actions of both G1 and G2/M cyclin-cdk complexes, PKA can efficiently block all parts of the cell cycle (reviewed in [31, 146]).

**IS THE CROSSTALK BETWEEN PKA AND GROWTH FACTOR RECEPTOR SIGNALING DUE TO REGULATION OF PHOSPHATASES?**

Many of the examples of crosstalk between PKA and growth factor receptor signaling described here could potentially be explained by a PKA-mediated activation of phosphatases. For example, elevation of cAMP levels is known to lead to a dephosphorylation of basal and growth factor-stimulated p70 S6 kinase and PHAS-1 phosphorylation (reviewed in [53]). PKA has been shown to regulate both tyrosine- and serine/threonine phosphatases in different cell types [147–149].

Some phosphatases are inhibited by PKA. The best known example may be the PKA-mediated phosphorylation of threonine 35 of protein phosphatase inhibitor-1 [147]. When phosphorylated by PKA, inhibitor-1 acts as a potent and specific inhibitor of the type-1 protein serine/threonine phosphatase (PP1). There are examples of PKA inhibiting protein tyrosine phosphatases as well [149]. Other phosphatases are stimulated by PKA. For example, in Xenopus extracts, PKA appears to stimulate a PIP2-like serine/threonine phosphatase [145]. Yet other studies have observed a stimulation of tyrosine phosphatase activity by PKA [148]. The recent finding that PKA and the calcium-dependent phosphatase calcineurin both bind to the same AKAP anchoring protein in the cell provides another interesting possibility for PKA modulation of phosphatase activity (reviewed in [150]).

Differential expression of PKA stimulated and inhibited phosphatases may explain, in part, the ability of PKA to activate or inhibit a particular signaling pathway under different conditions and/or in different cell types. This possibility needs to be further investigated.

**CYCLIC AMP AND GENE RESPONSES IN SMCs**

Transcriptional regulation by PKA is mediated by a family of cAMP-responsive nuclear factors that act as activators or repressors. The best characterized of the PKA-responsive promoter elements that mediate PKA-regulated transcription is the cAMP-response element, CRE. The CRE-binding proteins, CREB (CRE-binding protein), CREM and ATF-1 are directly phosphorylated and activated by PKA (reviewed in [151]). These transcription factors contain basic domain/leucine zipper motifs and bind as dimers to CRE, found in the promoters of many PKA activated genes. CRE-
Binding proteins may also act as repressors of transcription. One example of such a repressor is ICER (inducible cAMP early repressor) that is generated from an alternative CREM promoter (reviewed in [152]).

**Crosstalk Between Growth Factor Receptor Signaling and PKA at the Level of CREB**

An interesting mode of crosstalk occurs between PKA and growth factor-induced signaling at the level of CREB phosphorylation. Direct activation of gene expression by CREB requires phosphorylation of serine 133. This event can be mediated by PKA [153]. However, Ser 133 phosphorylation can also be brought about by the ERK-regulated kinase Rsk2, also termed CREB-kinase, and this event is stimulated by growth factors [154]. Thus, this is an example of growth factor receptor signaling and PKA signaling converging on the same substrate. PKA or Rsk2 phosphorylation of CREB creates the sequence motif Ser-X-X-X-Ser(P), a consensus site of the glycogen synthase kinase-3 (GSK-3) enzyme. GSK-3 activity can be inhibited by growth factors acting through Rsk [155], but in the major insulin-sensitive tissues, skeletal muscle, fat and liver the regulation of GSK3 is through a PI3K-dependent pathway [156, 157]. Consequently, the crosstalk between growth factor signaling and PKA at the level of CREB is complex. As stated above, MAPK/ERK activation and subsequent Rsk2 activation by growth factors have the same effect as PKA on CREB phosphorylation. Activation of the PI3K pathway by growth factors, on the other hand, may reduce the ability of PKA to activate CREB, as sequential phosphorylation at the PKA and GSK-3 sites of CREB may be essential for cAMP control of CREB activity [158]. Thus, the net effect of growth factor-PKA crosstalk at the level of CREB is likely to depend on the growth factor’s ability to turn on the ERK pathway versus the PI3K pathway. Interestingly, in human arterial SMCs, PDGF is a potent activator of the ERK pathway whereas IGF-I does not activate this pathway [159]. In contrast, both PDGF and IGF-I activate the PI3K pathway in SMCs [37, 103, 160, 161]. The extent and significance of crosstalk between these growth factors and PKA at the level of CREB in SMCs are unknown.

**PKA Regulates Expression of Molecules that Participate in the Process Leading to Cardiovascular Disease**

Many growth factor- and cytokine-induced gene responses are inhibited by cAMP. The suppression by PKA may be mediated either through an inhibition of the kinase pathways that induce gene expression, by a direct effect of PKA on nuclear repressor proteins or by post-translational alterations of specific mRNAs. In addition to examples mentioned earlier, agents that elevate cAMP result in inhibition of transforming growth factor-α- and interleukin-1β-induced expression and transcription of cell adhesion molecules [162]. This may be relevant in formation and progression of atherosclerotic lesions, during which cell adhesion molecules mediate invasion of monocytes into the arterial wall. Expression of the inducible cyclooxygenase COX-2 [71] and of collagen type I [39], both of which have been suggested to play a role in atherosclerosis and/or restenosis, is also suppressed by PKA. IGF-I and EGF-stimulated activity of the transcription factor Elk is inhibited by cAMP-elevating agents without a concomitant inhibition of MAPK/ERK activity [163]. Expression of certain growth factor receptors, such as the angiotensin AT-1 receptor, is decreased by cAMP in SMCs, and this is due to an increased destabilization of the receptor mRNA [164].

PKA also induces expression of genes in SMCs, many of which encode growth inhibitory proteins or proteins that indirectly lead to reduced SMC growth. An interesting example of such an induced gene expression in arterial SMCs is that of vascular endothelial growth factor, VEGF [165]. VEGF is an endothelial growth factor secreted by SMCs, and increased production of VEGF would be predicted to increase re-endothelialization and reduce SMC proliferation after vascular injury.
FIGURE 2. Crosstalk between PKA and mitogenic signal transduction pathways in arterial SMCs. This diagram depicts the points of antagonism between PKA and mitogenic signaling pathways in SMCs, as we know them today. Cyclic AMP is generated following binding of an agonist to a membrane receptor coupled to one of several adenylate cyclases (ACs). The relative activation of PKA is determined by the extent of activation of AC and by the extent of degradation (hydrolysis) of cAMP by cyclic AMP phosphodiesterases (PDEs). The active PKA inhibits mitogenic signaling at several points. The first level of inhibition by PKA is close to the plasma membrane. Thus, signal transduction from a growth factor receptor, for example the PDGF β-receptor, is inhibited at the level of Ras/Raf, at the level of, and/or upstream of, phosphatidylinositol-3′-kinase (PI3K) and the mammalian target-of-rapamycin (mTOR). A second level of inhibition is effective in the nucleus, where PKA inhibits several different cyclin-dependent kinases (Cdks and Cdc2) and induces gene responses through cAMP-responsive transcription factors. The products of these PKA-regulated genes can inhibit SMC proliferation by, for example, reducing the responsiveness of the cell to growth factors.

SMCs should make it feasible to simultaneously stimulate synthesis of cAMP and inhibit its degradation in these cells.

CONCLUSIONS

Our current knowledge on the crosstalk between PKA and growth factor receptor signaling in arterial SMCs in relation to proliferation is schematically shown in Figure 2. It is now clear that PKA inhibits growth factor receptor signaling by interfering with a number of different signaling pathways at different locations in the cell and at different stages of the cell cycle. We have termed the PKA-mediated inhibition of events that occur in close proximity to the plasma membrane and within minutes after growth factor stimulation as the first level of inhibition (see Fig. 2). These events include inhibition of Ras-Raf interaction and subsequent MAPKK activation, inhibition of PI3K and mTOR. The second level of inhibition of proliferation by PKA occurs several hours after growth factor stimulation in the nucleus. These events include inhibition of several of the cyclin/cdk complexes and induction/repression of other gene responses. Although the inhibition of cdk2 activity by PKA may be due to inhibition of mTOR, as described earlier, many of the effects of PKA on the cyclins/cdkks appear to be due to increased or decreased gene expression or regulation of protein stability. Together, the different levels of inhibition by PKA on growth factor receptor signaling in arterial SMCs provide a promising concept for drug intervention in states where SMC proliferation contributes to cardiovascular disease.

As becomes clear from this review, further studies are needed to determine the targets of PKA in many of the growth factor receptor signaling pathways in SMCs. However, the research performed to date gives us a first insight into this truly fascinating crosstalk between PKA and growth factor receptor signaling.

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