Membrane-Bound Protein Kinase A Inhibits Smooth Muscle Cell Proliferation In Vitro and In Vivo by Amplifying cAMP–Protein Kinase A Signals

Ciro Indolfi,* Eugenio Stabile,* Carmela Coppola, Adriana Gallo, Cinzia Perrino, Giovanna Allevato, Luigi Cavuto, Daniele Torella, Emilio Di Lorenzo, Giancarlo Troncone, Antonio Feliciello, Enrico Vittorio Avvedimento, Massimo Chiariello

Abstract—cAMP-dependent protein kinase is anchored to discrete cellular compartments by a family of proteins, the A-kinase anchor proteins (AKAPs). We have investigated in vivo and in vitro the biological effects of the expression of a prototypic member of the family, AKAP75, on smooth muscle cells. In vitro expression of AKAP75 in smooth muscle cells stimulated cAMP-induced transcription, increased the levels of the cyclin-dependent kinase-2 inhibitor p27kip1, and reduced cell proliferation. In vivo expression of exogenous AKAP75 in common carotid arteries, subjected to balloon injury, significantly increased the levels of p27kip1 and inhibited neointimal hyperplasia. Both the effects in smooth muscle cells in vitro and in carotid arteries in vivo were specifically dependent on the amplification of cAMP-dependent protein kinase (PKA) signals by membrane-bound PKA, as indicated by selective loss of the AKAP75 biological effects in mutants defective in the PKA anchor domain or by suppression of AKAP effects by the PKA-specific protein kinase inhibitor. These data indicate that AKAP proteins selectively amplify cAMP-PKA signaling in vitro and in vivo and suggest a possible target for the inhibition of the neointimal hyperplasia after vascular injury. (Circ Res. 2001;88:319-324.)

Key Words: A-kinase anchor proteins • p27 • protein kinase A • smooth muscle cells

Cyclic AMP controls growth and differentiation in a variety of organisms and cell types.1,2 In eukaryotes, cAMP binds the regulatory subunit of cAMP-dependent protein kinases (PKAs). This releases the catalytic subunit (C-PKA), which phosphorylates a wide variety of substrate proteins. A fraction of the C-PKA migrates to the nucleus and phosphorylates nuclear proteins and transacting factors.3 PKA is targeted to certain subcellular locations by specific anchor proteins (A-kinase anchor proteins, AKAPs). Localized PKA holoenzymes might in vivo perform important aspects of cAMP-activated signal transduction. We have previously provided evidence that links membrane targeting of PKAII to cAMP-dependent gene transcription in differentiated and nondifferentiated cells.4,5 In thyroid, neuronal, and kidney cells, displacement of immobilized PKAII from perinuclear sites to the cytoplasm impairs cAMP-induced transcription.4 Conversely, overexpression of AKAP75, a prototype AKAP that targets PKA to the membranes, enhanced the propagation of cAMP signals to the nucleus.5 However, the biological effects of AKAPs on smooth muscle cell (SMC) growth in vitro and in vivo are not known.

Therefore, we performed 2 experimental models to assess the effects of AKAP expression on SMC proliferation both in vitro and in vivo. In the first model, we have transfected stabilized SMCs derived from aorta with expression vectors encoding the prototypic member of AKAP family, AKAP75 and its derivatives. The same vectors were also used in vivo in the rat model of angioplasty. After balloon injury, the cells in the media of the arterial wall are potently stimulated and proliferate vigorously, thus generating the so-called neointima.6 This system is ideally suited for assessing nontransformed SMC proliferation in vivo7–10 and testing innovative strategies to prevent vascular proliferative disorders such as atherosclerosis and restenosis.

Here we report that overexpression in vitro and in vivo of AKAP75 potently inhibits SMC proliferation and prevents neointima formation after balloon injury. These effects are specifically dependent on the amplification of cAMP signals by membrane-bound PKA and suggest a potential target site for therapeutic control of balloon angioplasty–induced neointimal formation.

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From the Division of Cardiology (C.I.) and Dipartimento di Medicina Sperimentale e Clinica (E.V.A.), “Magna Graecia” University, Catanzaro, and Division of Cardiology (E.S., C.C., C.P., L.C., D.T., E.D.L., M.C.) and Dipartimento di Biologia e Patologia Molecolare e Cellulare (A.G., G.A., A.F., E.V.A.), Centro di Endocrinologia ed Oncologia Sperimentale del Consiglio Nazionale delle Ricerche, University Federico II, Naples, Italy.

*Both authors contributed equally to this study.
Correspondence to Enrico V. Avvedimento or Ciro Indolfi, Department of Patologia Molecolare e Cellulare, Centro di Endocrinologia ed Oncologia Sperimentale, CNR, Via S. Pansini, 5 80131, Naples, Italy. E-mail avvedim@unina.it

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Materials and Methods
DNA Plasmids, Cell Culture, and Transfections
The plasmids used in this study are (1) cAMP-responsive element (CRE)-chloramphenicol acetyltransferase (CAT), carrying the Escherichia coli CAT gene under the control of the somatostatin promoter; (2) CMV-AKAP75, containing the bovine brain RIβ anchor protein under the control of cytomegalovirus (CMV) promoter; (3) CMV-AKAP45, a truncated mutant of AKAP75 and a derivative of AKAP45 mutated in the RII binding site (AKAP45mut); (4) RSV-LacZ, the E. coli LacZ gene driven by the long terminal repeats of Rous sarcoma virus (RSV); (5) RSV-p27kip1 IgG1 (0.25 mg/mL, dilution 1:1000, Santa Cruz Biotechnology), after 24 hours of incubation in nonselective medium, the plates were plated in the selective medium with a neomycin analogue (G418, 500 µg/mL).

Western Immunoblot Analysis
Dishes were transfected with 5 µg each of CRE-CAT, RSV-Lac Z, AKAP75, AKAP45, AKAP45mut, and protein kinase inhibitor (PKI), as well as up to 21 µg of salmon sperm DNA. CAT activity was performed 48 hours after transfection before and after 4 hours of 8-bromo-cAMP (8-Br-cAMP) incubation.

SMC Cultures
To assess the effects of AKAP75 transfection on SMC proliferation in vitro, the cultures were synchronized by starvation for 48 hours in serum-free DMEM and then serum-restimulated in the presence of 10% FCS, and DNA transfections were carried out by the calcium phosphate procedure. For stable transformed lines (CMV-AKAP75, RSV-Neo, and AKAP45mut), after 24 hours of incubation in nonselective medium, the cells were plated in the selective medium with a neomycin analogue (G418, 500 µg/mL).

CAT Assay
Dishes were transfected with 5 µg each of CRE-CAT, RSV-Lac Z, AKAP75, AKAP45, AKAP45mut, and protein kinase inhibitor (PKI), as well as up to 21 µg of salmon sperm DNA. CAT activity was performed 48 hours after transfection before and after 4 hours of 8-bromo-cAMP (8-Br-cAMP) incubation.

Localization of AKAP75 and p27kip1 in the Vessel Wall
Two days after balloon injury, additional animals from the control and AKAP75 groups were euthanized by administration of an overdose of pentobarbital, and the arteries were perfusion-fixed in 4% formaldehyde. Arteries were divided into 2-mm-thick segments, overlaid with OCT compound, and frozen in liquid nitrogen. Cryostat sections (7 µm) were mounted on poly-L-lysine–coated slides. To detect AKAP75 and p27kip1 immunoreactivity, sections were washed twice with Tris-buffered saline (TBS), blocked with rabbit serum, diluted 1:5 in TBS for 45 minutes, incubated overnight with polyclonal murine anti-AKAP75 or anti-p27kip1 IgG1 antibody (2 µg/mL, Santa Cruz Biotechnology, Inc), and incubated for 1 hour with a rabbit anti-mouse IgG conjugated with horseradish peroxidase (dilution 1:50; preabsorbed overnight at 4°C with 10% preimmune rat serum and 3% BSA). Antibody binding in the vessel wall was visualized with 3,3′-diaminobenzidine (Sigma Chemical Co) in 0.1 mol/L Tris buffer (pH 7.2) containing 0.01% H2O2. Sections were counterstained with Harris hematoxylin, dehydrated, and mounted with dePex mounting medium. The number of cells expressing p27kip1 was quantified as the ratio of nuclear-positive cells on total cells in each field.

Morphology
At the time of the final experiment, the arteries were dissected free from the surrounding tissues, and rats were euthanized for structural and immunohistochemical analyses. All data are mean±SEM. ANOVA for repeated measures was performed using the SPSS program, version 10.0; the Tukey test was used to compare single mean values.17

Results
We first determined whether the expression of AKAP75, a prototypic member of the AKAP family, influences cAMP signaling in cultured aortic SMCs, by measuring the transcriptional activity of a cAMP-inducible promoter driving the expression of the bacterial enzyme, CAT (CRE-CAT) (Figure 1). Expression of AKAP75 induced CRE-CAT in the absence of cAMP stimulation (Figure 1). This effect was mediated by PKA, given that it was reversed by the simultaneous transfection of the PKA nuclear inhibitor PKI, but not by a single-point PKI mutant that did not inhibit PKA (data not shown). These effects were specific to the CRE promoter because RSV-CAT under the same conditions was not activated by AKAP75 or cAMP (Figure 1). Moreover, expression of AKAP45, a derivative of AKAP75 carrying a deletion in the membrane-anchoring domain (residues 1 to 180; Reference 12), did inhibit CRE-CAT both in the presence and the absence of cAMP (Figure 1). Because AKAP45 binds the endogenous PKA and translocates it to the cytosol,13 the inhibition of CRE-CAT transcription is apparently dependent on the loss of cellular membrane-bound PKA. Mutation of the PKA binding site in AKAP45 (A45mut) completely eliminated the negative effect of AKAP45 on cAMP-induced transcription, indicating that both the positive (AKAP75) and the
negative (AKAP45) effects on CRE-CAT transcription were mediated by the PKA binding domain of AKAP (Figure 1). This finding also indicates the high degree of specificity of AKAP75 in cAMP signal transduction, because AKAP75 binds other signaling molecules, such as PKC and calcineurin, that might be activated by injury.18

To test the biological consequences of AKAP75 expression, we analyzed the DNA synthesis in AKAP75-transfected cells. Figure 2 shows that the DNA synthesis of AKAP75-expressing cells was dramatically reduced. As control we used cells transfected with AKAP45mut indicated above.

Mock or RSV-Neo–expressing cells show a profile of DNA synthesis and proliferation comparable with those of AKAP45mut-expressing cells (data not shown). Because levels of p27kip1, a specific cyclin-dependent kinase-2 (cdk2) inhibitor, are stimulated by cAMP,19 we determined p27kip1 concentration in AKAP75-expressing cells by Western blotting analysis. Figure 3 shows that cells expressing AKAP75 contained higher p27kip1 levels relative to control cells. Moreover, these cells responded very efficiently to cAMP by increasing p27 levels. PKI but not the mutated variant reduced cAMP-induced p27 levels in AKAP75-expressing cells. These data replicate the effects of AKAP75 on CRE-CAT transcription and indicate that p27 levels are tightly controlled by cAMP-PKA.

To translate these results in vivo, we determined the biological consequences of AKAP75 expression in rat carotid arteries (n=64), subjected to vascular injury as previously described.7 Rat carotid arteries subjected to balloon injury

Figure 1. a, Representative CAT activity after transfection of aortic rat SMCs with CRE-CAT, RSV-CAT, AKAP75 (A75), and A75+PKI. CRE-CAT and RSV-CAT+AKAP75–transfected cells were treated (+) with 500 μmol/L 8-Br-cAMP or not treated. As expected, cAMP induced CAT activity. This effect was specific to the CRE promoter, given that RSV-CAT under the same conditions was not activated by AKAP75 and cAMP. Expression of AKAP75 induced CRE-CAT in the absence of cAMP stimulation. This effect was mediated by PKA, given that it was reversed by the simultaneous transfection of the PKA nuclear inhibitor (PKI). B, Quantitative CAT activity (expressed as percentage of acetylated chloramphenicol) in SMCs transfected with vectors indicated. Cells were treated for 4 hours with 500 μmol/L 8-Br-cAMP (+) or not treated (–). Results shown are derived from 4 independent experiments. All transfections were normalized with β-galactosidase activity expressed by RSV-LacZ gene. *P<0.01 vs respective basal activity; #P<0.01 vs all basal activity; P<0.02 vs A45+.

Figure 2. DNA synthesis expressed as fold induction over basal conditions in A75 (–)–expressing cells and in A45mut (–)–expressing cells (P<0.05). To assess the effects of AKAP75 transfection on SMC proliferation in vitro, cultures were synchronized by starvation for 48 hours in serum-free DMEM and then serum-restimulated in the presence of [3H]thymidine. DNA synthesis cells were measured by [3H]thymidine incorporation. DNA synthesis of AKAP75–expressing cells was dramatically reduced compared with cells transfected with AKAP45mut indicated above.

Figure 4. a, Representative photomicrographs of rat common carotid arteries fixed 2 days after balloon injury and stained with anti-AKAP75 polyclonal antibody. a, Rat treated with RSV-Neo transfection. b, Rat treated with CMV-AKAP75 transfection.

Figure 3. a, Bar graphs showing induction of p27kip1 protein after transfection of aortic rat SMCs (A10 cells). p27 levels determined by Western blot analysis with specific antibodies in A10 cells transiently transfected with the vectors are indicated. CON indicates control cells (expressing RSV-LacZ). Forty-eight hours after transfection, cells were treated (+) with cAMP (500 μmol/L 8-Br-cAMP) or not treated (–). Data are shown as fold induction over basal conditions (control cell level, 1). Data were derived from 4 independent experiments. *P<0.05 vs all basal conditions; #P<0.05 vs all cAMP-stimulated conditions. b, Representative Western blot analysis of exogenous AKAP75 and endogenous p27kip1 after transient transfection of aortic rat SMCs with expression vectors indicated. Control cells, expressing only RSV-LacZ, were treated with 500 μmol/L 8-Br-cAMP for 4 hours.
were “transfected” in vivo with plasmid vectors expressing AKAP75, AKAP45, and PKI wild-type or its mutated variant (PKImut). We first determined, 48 hours after the injury, the expression of exogenous AKAP75 gene in the arterial walls with antibodies specific to AKAP75. Cross sections of the arterial walls showed the specific AKAP75 signal in the SMCs of the tunica media of the vessel only in AKAP75-treated animals (Figure 4). The expression was maximal 48 hours after the injury and steadily decreased thereafter (data not shown).

In parallel experiments, the animals were euthanized 14 days after injury to determine the effects of AKAP75 expression on in vivo SMC proliferation. Figure 6 shows that AKAP75 expression reduced significantly the formation of neointima 14 days after arterial balloon injury. DNA expressing vectors containing different viral (RSV-CMV) or eukaryotic (transgenic) promoters driving the expression of bacterial or animal proteins did not inhibit the formation of the neointima.8

The arteries treated with AKAP75 in vivo were also stained with p27 antibody. Figure 5 shows that AKAP75-treated arteries contained a significant fraction of cells that expressed p27k⁺ 48 hours after the arterial injury (33% versus 18% of positive cells per field, P<0.05) and were not present in arteries transfected with control vectors.

Figure 6 shows representative cross sections of each group of animals studied. The inhibition of neointimal hyperplasia after balloon injury produced by AKAP75 transfection was totally abolished by the wild-type PKA-specific inhibitor PKI but not by its mutated version. The neointima area was 0.129±0.040 mm² in the group transfected with AKAP75 and PKI (n=5) compared with 0.079±0.017 mm² in the group treated with AKAP75 alone (n=12) (P<0.01 versus AKAP75+PKI). Similarly, the neointima/media ratio was 0.621±0.123 in AKAP75 treated arteries compared with 1.118±0.316 in the group treated with AKAP75 and PKI (P<0.01 versus AKAP75). On the other hand, after balloon injury in the group treated with AKAP75 and PKI mutant, no difference was observed either in neointimal area (n=4) (0.073±0.014 mm²; P=NS versus AKAP75) or in neointima/media ratio (0.717±0.142; P=NS versus AKAP75) compared with the AKAP75 group.

The AKAP75 mutant, AKAP45, carrying the deletion of the membrane-anchoring domain, did not inhibit SMC proliferation in vitro (Figure 2) nor the neointimal formation after balloon injury (Figure 6). In the AKAP45-treated group (n=7), at 14 days after balloon injury the neointimal area was 0.129±0.024 mm² (P<0.01 versus AKAP75). Similarly, the neointima/media ratio was 0.621±0.123 (P<0.01 versus AKAP75). These findings indicate that AKAP75 effects are tightly dependent on the PKA anchoring domain and that amplification of cAMP signaling reduces neointimal hyperplasia after balloon injury.
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AKAPs and Smooth Muscle Cell Proliferation

Discussion

The major findings of the present study are that AKAP75 expression in cultured SMCs in vitro and in arteries subjected to balloon injury amplifies and stimulates cAMP-PKA signaling resulting in activation of cAMP-induced transcription, increased levels of p27kip1, and suppression both of SMC growth in vitro and of neointimal hyperplasia after balloon injury.

These effects in vitro and in vivo were dependent on selective stimulation of AKAP-targeted PKAII. Indeed, a PKA-specific inhibitor, PKI, but not its inactive mutant, inhibits the effects of AKAP both in vitro and in vivo. AKAP75 binds and anchors the regulatory subunit of PKA to discrete subcellular membranes. Apparently, the membrane system where AKAP-PKA localizes can vary and depends on the cell type.5,20,21 We have shown that independently on the specific localization of the complex AKAP75-PKA in different cell types (kidney, thyroid, and neuronal cells), the invariant result of AKAP75 expression is the amplification of cAMP signaling to the nucleus. PKA in AKAP75-expressing cells appears to be more stable.5,11,12 Although we did not explore the specific localization of the complex AKAP-PKAII in SMCs, the net effect of AKAP75 expression in cultured SMCs in vitro and in arteries in vivo was the amplification of cAMP signaling.

The selective inhibition of AKAP75 effects by selected mutants provides important information about the mechanism(s) underlying the AKAP75 function. A mutant of AKAP75, AKAP45, which lacks the targeting domain, does not prevent neointimal hyperplasia after balloon injury, inhibits CRE-CAT transcription, and decreases p27kip1. This finding indicates that the RII binding domain is essential for the effects of AKAP75 on cAMP signaling. Moreover, it suggests a function for the endogenous membrane-bound PKA in SMCs. Thus, AKAP45 inhibits basal and cAMP-stimulated transcription, whereas it has no effect on the formation of neointima (Figure 1 and Figure 6). Because this mutant binds endogenous PKA and translocates it to the cytosol,11,12 the inhibition of CRE-CAT transcription and the loss of growth-inhibitory effect suggest that in SMCs endogenous membrane-bound PKA amplifies the effects of cAMP signaling on transcription and inhibits proliferation. We suggest that cytosolic PKA bound to AKAP45 is readily dissociated by basal cAMP, and it is degraded faster. Cells expressing AKAP45 do not transcribe efficiently cAMP-induced genes, contain less p27, and grow faster.12 Furthermore, the effects of AKAP45 are critically dependent on the interaction with PKA, given that the derivative mutant in the RII binding site (AKAP45mut) did not inhibit CRE-CAT transcription or SMC growth (Figures 1 and 2). These data further support the notion that the effects of AKAP on neointima formation after balloon injury are mediated by PKA. Interestingly, AKAP75 binds other signaling molecules, such as PKC or calcineurin, which might independently be activated by arterial balloon injury and might contribute to the regulation of neointima formation as well. Our data suggest that the mechanism of SMC growth inhibition in vivo and in vitro by AKAP75 is related to the amplification of cAMP signals, which can inhibit transcription of cyclin D1 expression13 and increase p27 levels. We have data indicating that cAMP-PKA signals influence the degradation and not the synthesis of p27.12 Under these conditions, even a small quantitative effect (≈3-fold) on p27 levels may have a dramatic effect on DNA synthesis, because p27 levels in G1 are in equilibrium with cdk2, and any variation in this ratio can trigger cdk2 activity.12

The present study further supports the hypothesis that ras pathway inhibition and cAMP stimulation are powerful means to inhibit neointimal formation after balloon injury.8,10 In conclusion, expression of AKAP75 in vitro stimulated cAMP-induced transcription, increased the levels of the cdk2 inhibitor p27kip1, and reduced SMC proliferation. In an in vivo model of vascular injury, AKAP75 significantly increased p27kip1 levels and inhibited neointimal hyperplasia. These effects on SMCs in vitro and on injured arteries in vivo were specifically dependent on the amplification of cAMP-PKA signals by membrane-bound PKA. These data indicate that AKAP proteins selectively amplify cAMP-PKA signaling in vitro and in vivo and support a possible target for the inhibition of the neointimal hyperplasia after vascular injury.

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