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Targeting $G_{\beta\gamma}$ signaling in arterial vascular smooth muscle proliferation: A novel strategy to limit restenosis

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ABSTRACT **Restenosis continues to be a major problem limiting the effectiveness of revascularization procedures. To date, the roles of heterotrimeric G proteins in the triggering of pathological vascular smooth muscle (VSM) cell proliferation have not been elucidated. $\beta\gamma$ subunits of heterotrimeric G proteins ($G_{\beta\gamma}$) are known to activate mitogen-activated protein (MAP) kinases after stimulation of certain G protein-coupled receptors; however, their relevance in VSM mitogenesis *in vitro* or *in vivo* is not known. Using adenoviral-mediated transfer of a transgene encoding a peptide inhibitor of $G_{\beta\gamma}$ signaling (βARKct), we evaluated the role of $G_{\beta\gamma}$ in MAP kinase activation and proliferation in response to several mitogens, including serum, in cultured rat VSM cells. Our results include the striking finding that serum-induced proliferation of VSM cells *in vitro* is mediated largely via $G_{\beta\gamma}$. Furthermore, we studied the effects of *in vivo* adenoviral-mediated βARKct gene transfer on VSM intimal hyperplasia in a rat carotid artery restenosis model. Our *in vivo* results demonstrated that the presence of the βARKct in injured rat carotid arteries significantly reduced VSM intimal hyperplasia by 70%. Thus, $G_{\beta\gamma}$ plays a critical role in physiological VSM proliferation, and targeted $G_{\beta\gamma}$ inhibition represents a novel approach for the treatment of pathological conditions such as restenosis.**

Since its introduction in 1977 (1), percutaneous transluminal coronary angioplasty has represented an alternative to cardiac surgery for revascularization in a series of cardiac diseases, from unstable angina and myocardial infarction, to multivascular diseases (2, 3). However, the major limitation of this procedure is the induction of the accumulation and proliferation of vascular smooth muscle (VSM) cells from the tunica intima to the tunica media of the arterial wall, leading to restenosis in 30–60% of cases within 3–6 months (4, 5). This clinical pathological process is known as intimal hyperplasia and is triggered by the injury of the arterial wall and sustained by the release of humoral and tissue factors. These factors bind specific receptors switching VSM cells from a quiescent to a proliferative phenotype.

In many cell types, proliferative pathways proceed via a cascade of phosphorylation events that transduces mitogenic signals from the extracellular stimuli to the nucleus. The ubiquitous family of mitogen-activated protein (MAP) kinases plays a key role in this type of signaling. A number of enzymes belong to this family, including p42 and p44 MAP kinase (also known as ERK1 and 2). Importantly, the $p21^{ras}$ (Ras)-dependent activation of p42/p44 MAP kinase has been demonstrated to be critical for pathological intimal hyperplasia because its inhibition limits VSM cell proliferation (6). Two classes of receptors can trigger mitogenic pathways in cells: tyrosine kinase receptors and receptors that couple to heterotrimeric G proteins. Both of these receptor-mediated pathways can stimulate MAP kinase cascades via the activation of Ras (7). Elucidating which pathways are most important in stimulating pathological arterial VSM proliferation should make it possible to target more efficaciously specific pathways to limit conditions such as restenosis.

It is becoming increasingly evident that signaling through heterotrimeric G proteins is critically important for regulation of mitogenesis in several cell types (7). Signaling through these G proteins involves the dissociation of the G_α subunit and the $G_{\beta\gamma}$ dimer after receptor activation, and both of these subunits separately can activate a variety of intracellular signaling pathways (8). Included in the importance of G protein signaling in mitogenesis is that both the G_α and $G_{\beta\gamma}$ subunits have been shown to mediate the activation of MAP kinase (7). For example, we have shown in fibroblasts that several G_i-coupled receptors activate the Ras-MAP kinase pathway specifically via the $\beta\gamma$ subunits of G_i (9). This signaling paradigm was mapped out by the use of an exogenous $G_{\beta\gamma}$-binding peptide that can act as a specific $G_{\beta\gamma}$ sequestrant. The inhibitor utilized was the carboxyl-terminal 194 aa of the β-adrenergic receptor kinase (βARKct), which contains a region responsible for the $G_{\beta\gamma}$-mediated membrane translocation of βARK1, a process required for its activation (10, 11). The βARKct peptide has been a powerful reagent both *in vitro* and *in vivo* to specifically identify cellular processes triggered by $G_{\beta\gamma}$ (9, 12–14). The role of $G_{\beta\gamma}$-mediated mitogenesis in either *in vitro* or *in vivo* VSM cell proliferation is not known. Accordingly, in the current study we have utilized adenoviral-mediated gene transfer of the βARKct to investigate whether $G_{\beta\gamma}$ plays a role in this process in response to specific serum mitogens and, importantly, in response to serum itself. Furthermore, we have studied the specific role of $G_{\beta\gamma}$ in pathological VSM proliferation *in vivo* by using a rat carotid model of restenosis after balloon angioplasty (15). Our results indicate a critical role for $G_{\beta\gamma}$ in VSM proliferation and provide support for the idea of using the βARKct as a novel therapeutic approach to limiting pathological intimal hyperplasia.

METHODS

Cell Culture. Arterial VSM cells were obtained from rat aorta by enzymatic digestion, as described (16). Cells were grown on plastic dishes in Medium 199 (M199) supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were studied between passages 4 and 10. Two days before the experiments, cells were incubated 30 min at 37°C with 5 ml

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Abbreviations: VSM, vascular smooth muscle; βARK1, β-adrenergic receptor kinase; βARKct, carboxyl-terminal 194 aa of βARK1; MAP kinase, mitogen-activated protein kinase; β-gal, β-galactosidase; RT, reverse transcription; LPA, lysophosphatidic acid.

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M199 containing adenovirus at a multiplicity of infection (moi) of 100:1, encoding either the βARKct, β-galactosidase (β-gal), as a marker gene or the empty virus as a negative control. These adenoviruses were prepared and expanded as described previously (17, 18).

β-Gal Staining. Forty-eight hours after adenoviral infection, cells were fixed in 0.5% glutaraldehyde in 50% PBS for 5 min at room temperature and then stained with 10 mM $K_4Fe(CN)_6$/10 mm $K_3Fe(CN)_6$/2 mM $MgCl_2$/1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside) in PBS for 30 min at 37°C as described (17). The staining solution then was aspirated and the cells were permanently fixed in 1.5% glutaraldehyde in 50% PBS. To assess β-gal adenoviral transgene delivery *in vivo*, a group of rats ($n = 4$) was sacrificed after 5 days and common carotid artery segments were dissected away and frozen. Arterial segments were cut in cross-section and stained as described (19). Photomicrographs were taken of sections, images were acquired by means of a scanner, and β-gal infected areas were measured with NIH IMAGE 1.61 software. The efficiency of infection was estimated as the percentage of total blue-stained area within the total area of carotid wall.

βARKct Immunoblotting. Forty-eight hours after adenoviral infection, cells were harvested in lysis buffer (5 mM Tris·HCl, pH 7.4/5 mM EDTA) and homogenized with 10 strokes on ice using a dounce homogenizer. Samples were centrifuged at 40,000 × *g* to pellet membranes, and cleared supernatants were concentrated by using a Centricon-10 filtration unit (Amicon) at 5,000 × *g* for 30 min at 4°C. Cytosolic extracts (20–30 μg protein) were electrophoresed on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane, and βARKct expression was visualized by using rabbit polyclonal antiserum raised against the carboxyl terminus of βARK1 and enhanced chemiluminescence (ECL, Amersham) as described (17).

***In Vitro* Measurement of MAP Kinase Activity.** Cells were infected as described and, 24 hr later, were plated in 6-well dishes and incubated in M199 plus 0.5% FBS overnight. On the following day, cells were incubated with agonists for 2 min at 37°C. Cells then were washed twice with ice-cold PBS, homogenized in RIPA buffer (50 mM Tris·HCl, pH 7.5/150 mM NaCl/1% Nonidet P-40/0.25% deoxycholate/9.4 mg/50 ml sodium orthovanadate) and centrifuged as described (20). One milligram of clarified cellular extract was immunoprecipitated in 1 ml of RIPA at 4°C for 1 hr by using an antibody to p42/p44 MAP kinase and protein A-agarose beads (Santa Cruz Biotechnology). The samples then were centrifuged at 18,000 × *g* for 10 min, and the pellets were washed once with 1 ml of RIPA and twice with 1 ml of kinase buffer (20 mM Hepes, pH 7.0/10 mM $MgCl_2$/1 mM DTT). Samples then were resuspended in 40 μl of kinase buffer with myelin basic protein (MBP, 0.25 mg/ml)/20 μM ATP/[γ-^{32}P]ATP (20 μCi/ml) and incubated at 30°C for 15 min. The reactions were quenched with 40 μl of 2× Laemmli buffer and electrophoresed through a 4–20% polyacrylamide/Tris-glycine gel (20). Phosphorylated MBP on dried gels was quantified with a PhosphorImager (Molecular Dynamics).

[^{3}H]Thymidine Incorporation. Twenty-four hours after infection, cells were plated onto 12-well plates (20,000 cells per well) and serum-starved for 36 hr. Serum (5% FBS) then was added in the presence of [^{3}H]thymidine (1 μCi/ml, 1 ml). At the appropriate time points, cells were rinsed twice with PBS and three times with trichloroacetic acid (0.5%) and lysed with 400 μl of 1 M NaOH. Equal volumes of 1 M HCl then were added, and the entire contents of each well (800 μl) were counted by liquid scintillation.

Balloon Injury and Adenovirus Application. Balloon injury in the rat carotid was performed as described previously (6). Briefly, male Wistar rats weighing 280–350 g were anesthetized with ketamine (10 mg/kg) and xylazine (10 mg/kg), and the right common and external carotid arteries were exposed and isolated. Through the external carotid, a 2 French Fogarty balloon catheter was introduced in the common carotid and inflated to 2 atmospheres. Injury was induced with the inflated balloon by moving it back and forth three times. The total time of the balloon inflation was 30 sec. After balloon removal, the common carotid was flushed twice with PBS, and through a 28-gauge plastic cannula, a solution of PBS and adenovirus [5×10^9 plaque-forming unit (pfu)/100 μl] was injected and allowed to incubate in the common carotid in the absence of flow for 30 min. The virus then was removed and the vessel was rinsed twice with PBS. The external carotid was tied and the blood flow was restored through the common carotid. An additional exposure to 5×10^9 pfu of virus was performed by mixing the adenovirus with pluronic gel and applying this mix to the outside of the common carotid before closing the wound in layers.

RNA Preparation and Reverse Transcription–PCR. To assess *in vivo* βARKct transgene delivery to carotid arteries, a group of rats ($n = 4$) was sacrificed after 5 days and the right and left common carotids were harvested, rinsed in PBS, and frozen in liquid nitrogen. Total RNA was isolated by using RNAzol (Biotecx Laboratories, Houston), a one-step guanidium-based extraction solution (21). One microgram of total RNA was used for RT into cDNA by standard methods, and equal aliquots of cDNA then were used as PCR templates for the amplification of a 600-bp βARKct fragment as we have described (24). Primer pairs were a sense primer, 5′-GAATTCGCCGCCACCATGGG-3′ (corresponding to βARKct), and an antisense primer, 5′-GGAACAAAG-GAACCTTTAATAG-3′ [corresponding to the human β-globin sequence attached to the end of the βARKct (9)].

Histological Staining and Restenosis Measurements. Carotid arteries from the experimental groups of rats were treated with either the βARKct virus (βARKct, $n = 9$), empty virus (EV, $n = 7$), or no virus (control, $n = 8$). Animals were sacrificed after 28 days and their carotid arteries were harvested and perfusion-fixed with formalin (19). Arterial segments were embedded in paraffin and cut in cross-section for histological staining and measurements. Cross-sections (5 μm) were taken every 100 μm and stained with Masson trichrome. At least 50 sections were obtained from each carotid, and the 5 sections with maximal intimal hyperplasia were identified and measured. Photomicrographs were taken of these sections, and images were acquired by means of a scanner and measured with NIH IMAGE 1.61 software.

Statistical Analysis. Data are presented as mean ± SE. A paired Student's *t* test was used to compare *in vitro* MAP kinase activation. A repeated-measurements ANOVA was used to evaluate the effect of treatment on [^{3}H]thymidine incorporation. *In vivo* histological findings of intimal hyperplasia and the effects of βARKct treatment were analyzed by ANOVA.

RESULTS

***In Vitro* Adenoviral-Mediated Gene Transfer.** In primary cultures of rat aorta VSM cells, adenovirus infection resulted in nearly 100% infection efficiency as assessed by X-gal staining of cells infected with the β-gal virus (data not shown), which is consistent with our previously published data in cultured ventricular myocytes (17, 18). After βARKct adenovirus infection, protein immunoblotting of cellular extracts revealed robust expression of the ~30-kDa βARKct peptide, which was not present in cells infected with the empty virus (Fig. 1*A*).

***In Vitro* Effects of $G_{\beta\gamma}$ Inhibition on MAP Kinase Activation.** To determine the role of $G_{\beta\gamma}$ in mitogenic signaling in VSM cells, we assessed the effect of the βARKct on p42/p44 MAP kinase activation in response to lysophosphatidic acid

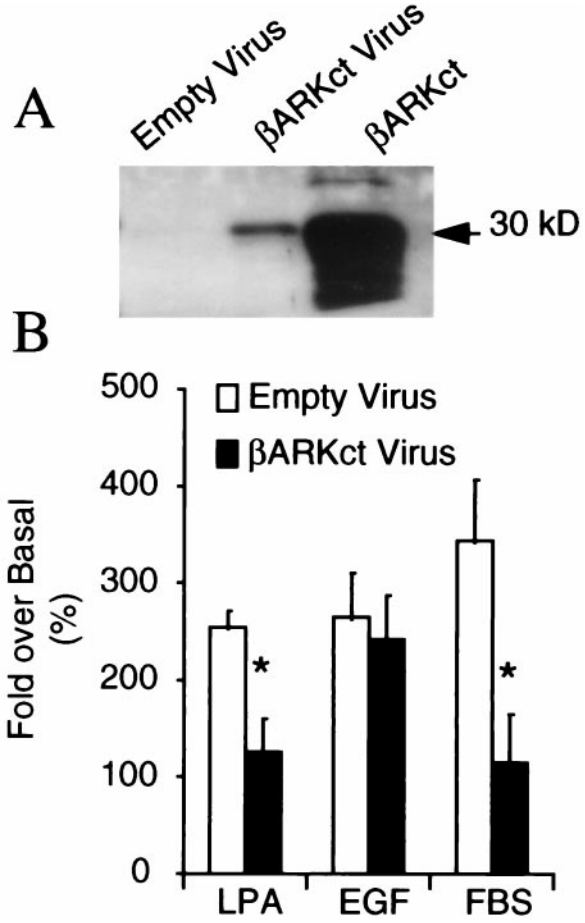

FIG. 1. Effect of βARKct on MAP kinase activation in rat aorta VSM cells. (*A*) Representative Western blot demonstrating that the βARKct peptide is expressed in cellular extracts only after βARKct adenovirus infection. Shown in the right-hand lane of this blot is a βARKct-positive standard that is a COS cell extract overexpressing the ~30-kDa βARKct peptide. (*B*) Histograms displaying MAP kinase activity expressed as the fold over basal activity in rat aorta VSM cells infected with either the βARKct adenovirus or an empty virus as a negative control. MAP kinase activity was induced by LPA (10^{-5} M), EGF (10^{-5} M), or serum (5% FBS). Data shown are the means ± SEM of five separate experiments done in triplicate. *, $P < 0.05$ βARKct versus empty virus (paired *t* test).

(LPA, 10 μM), epidermal growth factor (EGF, 10 μM), and serum (5% FBS) in cultured quiescent rat aorta VSM cells. LPA is a mitogen abundantly expressed in serum (23), which has been shown to activate the Ras-MAP kinase pathway in fibroblasts exclusively through $Gi_{\beta\gamma}$ (7, 9). EGF, on the other hand, is a tyrosine kinase receptor agonist that stimulates MAP kinase independent of $G_{\beta\gamma}$ (7). Fig. 1*B* shows the results of MAP kinase activation in rat aorta VSM cells infected with either an empty adenovirus or the virus containing the βARKct transgene. Our results indicate that in these cells, LPA activation of MAP kinase activity is mediated through $G_{\beta\gamma}$ as the presence of the βARKct significantly inhibited MAP kinase activity (Fig. 1*B*). In contrast and as expected, $G_{\beta\gamma}$ sequestration had no effect on EGF-induced MAP kinase activation (Fig. 1*B*). Interestingly, MAP kinase activation in response to 5% serum replacement was quite robust in control cells, and this response was inhibited significantly in the presence of the βARKct (Fig. 1*B*). In fact, our data suggest that MAP kinase activity in response to serum is mediated primarily via $G_{\beta\gamma}$.

Effects of βARKct Expression on Cellular Proliferation. To verify the relevance of this novel $G_{\beta\gamma}$-mediated MAP kinase activation in response to serum, we assessed the effect of the βARKct on VSM proliferation. This was done by measuring

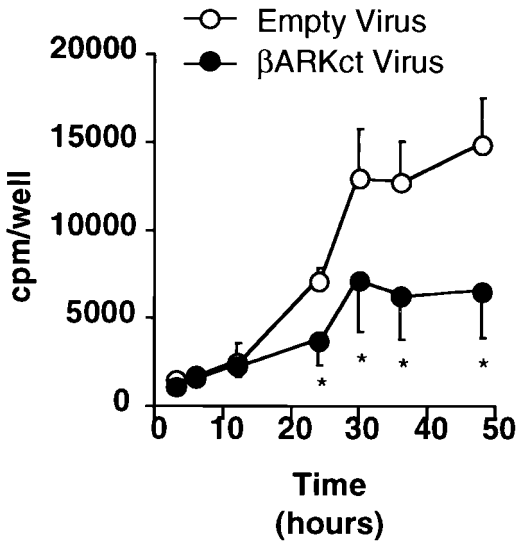

FIG. 2. Effect of βARKct expression on VSM cell proliferation. Cultured VSM cells cultured from rat aorta were infected with either the βARKct adenovirus or an empty virus, and [^{3}H]thymidine incorporation was measured after replacement of serum (5% FBS) to quiescent cells. Data shown represent the mean ± SEM of five experiments done in triplicate. *, $P < 0.05$ βARKct vs. empty virus (repeated-measurements ANOVA with a grouping factor).

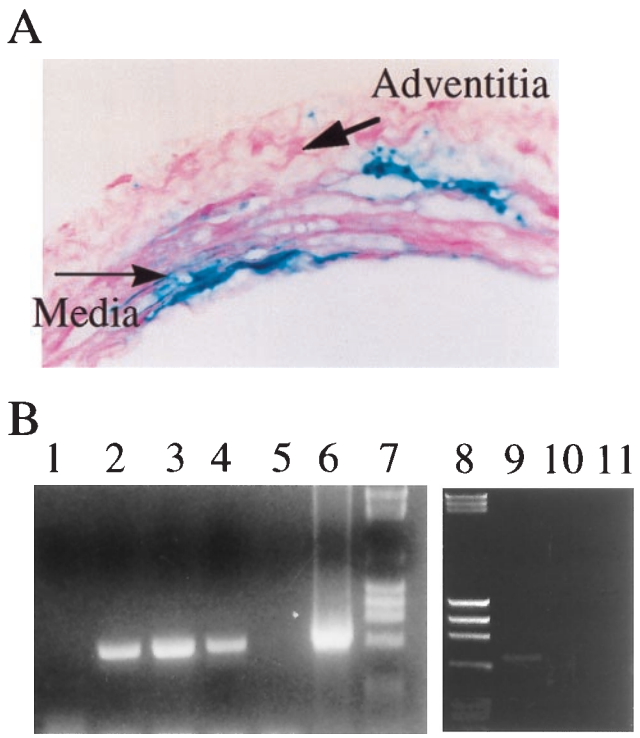

FIG. 3. Adenoviral transgene expression in balloon-injured rat carotid arteries. (*A*) X-gal staining of a representative section of an injured rat carotid artery 5 days after infection with a β-gal adenovirus. (*B*) Demonstration of βARKct mRNA expression by RT-PCR of rat carotid arteries 5 days after balloon injury and infection with the βARKct adenovirus (lanes 2–4 and 9). The ~600-bp-amplified βARKct mRNA fragment is absent from empty virus-infected carotid arteries (lane 1) or a noninjured, nontreated control carotid artery (lane 5). Lane 6 shows the amplified product from a βARKct plasmid, and lanes 7 and 8 contain DNA markers. Lanes 10 and 11 represent negative controls (H_2O) for RT and the PCR, respectively.

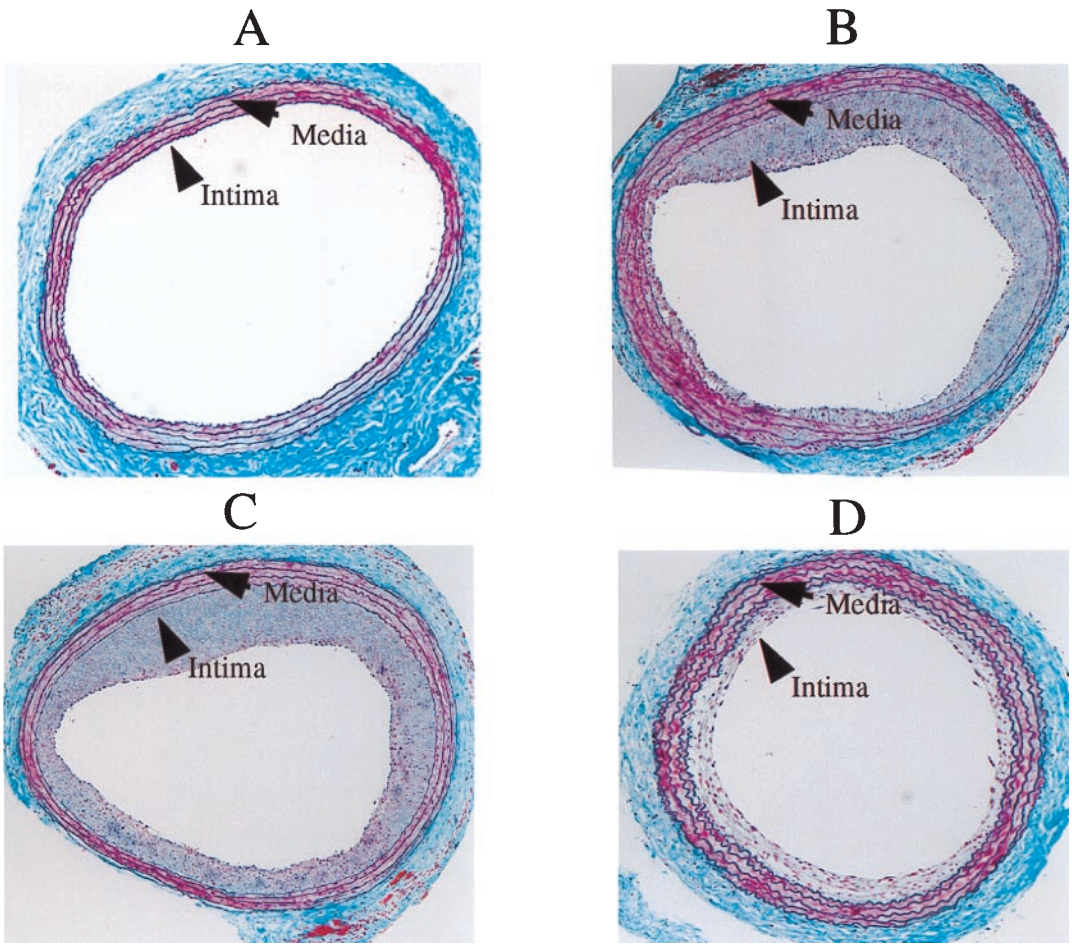

FIG. 4. *In vivo* effects of βARKct expression on balloon-injured rat carotid arteries. (*A*) Histologic staining of a normal, untreated rat carotid artery. (*B–D*) Sections of representative rat carotid segments 28 days after balloon injury. Restenosis and intimal hyperplasia are evident in injured arteries not treated (*B*) or treated with the empty adenovirus (*C*). This proliferative response was attenuated significantly and not as prominent in injured arteries treated with the βARKct adenovirus (*D*).

[^{3}H]thymidine incorporation in cultured rat aorta VSM cells after infection with either the empty or βARKct virus. As shown in Fig. 2, proliferation after 5% serum replacement to quiescent cells was attenuated significantly in the presence of the βARKct at all time points after 12 hr. At 24 hr, serum-induced proliferation was decreased by ~50% when $G_{\beta\gamma}$ signaling was inhibited (Fig. 2) (empty virus, 7,101 ± 757 cpm/well vs. βARKct virus, 3,711 ± 1,420 cpm/well; $P < 0.05$).

Adenoviral-Mediated *in Vivo* Gene Transfer in Balloon-Injured Rat Carotids. Delivery of transgenes via adenoviruses to the rat carotid artery has been achieved in several labs including ours (19, 24). We used the β-gal transgene to examine the extent of adenoviral-mediated gene transfer to the vascular wall of balloon-injured rat carotid arteries. Five days after infection, the presence of the β-gal transgene was visualized throughout the arterial wall, including adventitial and medial layers, with particular and intense localization at the site of maximal injury (Fig. 3*A*). The overall efficiency of infections was estimated to be approximately 25% of the entire vascular wall. To examine whether treatment with the βARKct adenovirus resulted in the expression of the $G_{\beta\gamma}$-inhibitory peptide in infected arteries, βARKct mRNA was amplified by using reverse transcription–PCR (RT-PCR). In injured carotid arteries 5 days after infection with the βARKct adenovirus, βARKct mRNA was observed, which was not the case for carotid arteries treated with the empty virus (Fig. 3*B*).

Effects of $G_{\beta b\gamma}$ Inhibition on Intimal Hyperplasia and Restenosis in Balloon-Injured Rat Carotid Arteries. In rat carotid arteries, a significant proliferative response was observed in the neointima 1 month after balloon injury. The response seen in our work was similar to what has been observed by others (6) and was similar between control (nontreated) arteries (Fig. 4*B*) and those arteries treated with an empty virus (Fig. 4*C*). However, the intimal proliferative response in injured rat carotid arteries treated with the βARKct adenovirus was significantly less (Fig. 4*D*). The restenosis response in several arteries was quantitated, and results are shown in Fig. 5. The intimal areas of injured rat carotid arteries were similar in the two control groups (control, 670 ± 38 μm^2 vs. empty virus, 625 ± 46 μm^2; P = not significant) whereas the area of the intimal layer of βARKct-treated arteries was reduced significantly by ~70% (201 ± 40 μm^2, $P < 0.01$ vs. both controls) (Fig. 5*A*). Medial areas of the injured rat carotid arteries were unchanged in all treatment groups (Fig. 5*A*). The intima-to-media ratio, which is a more sensitive parameter for assessing relative changes in the intima and media areas, also was calculated for the three treatment groups, and there was a significant 70% reduction in the intima-to-media ratio of βARKct-treated injured rat carotid arteries compared with either control treatment group (Fig. 5*B*).

DISCUSSION

The major finding of this study is that $G_{\beta\gamma}$ appears to play a major role in VSM proliferation and that targeted inhibition of $G_{\beta\gamma}$ results in significant reduction of intimal hyperplasia in an *in vivo* restenosis model. This adds a new dimension for the

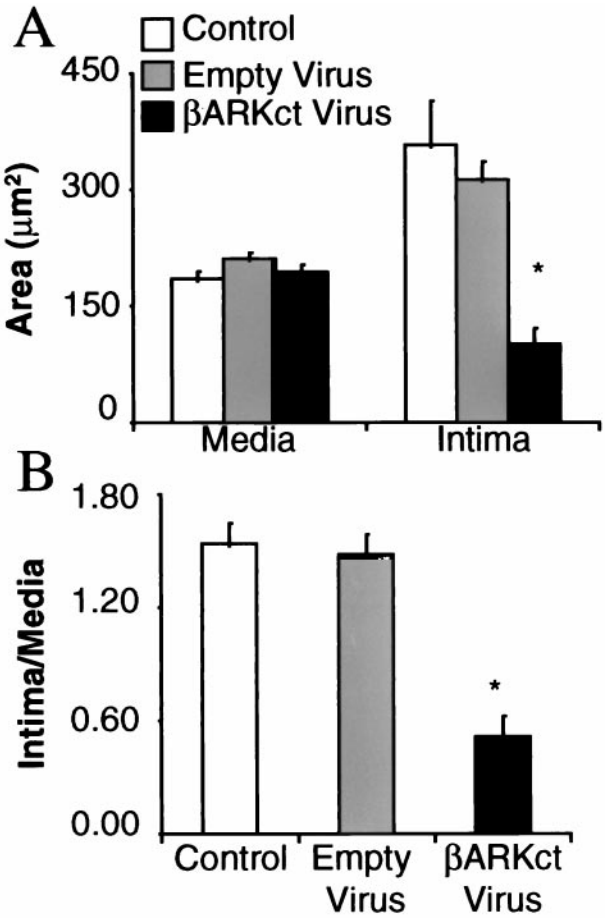

FIG. 5. Effect of adenoviral-mediated gene transfer of the βARKct on intimal hyperplasia in balloon-injured rat carotid arteries. (*A*) Histogram displaying measurements of the areas of the medial and intimal layers of carotid arteries 28 days after balloon injury. The three groups are arteries not treated (control, $n = 8$), treated with an empty adenovirus ($n = 7$), or treated with the βARKct adenovirus ($n = 9$). (*B*) Data expressed as the intima-to-media ratio. Data in both panels represent the mean ± SEM. *, $P < 0.05$ vs. both control and empty virus (ANOVA).

diversity of signals triggered by the βγ subunits of G proteins (25). The inhibition of $G_{\beta\gamma}$ signaling was accomplished by the expression of the last 194 aa of βARK1 (βARKct), which contains a specific $G_{\beta\gamma}$-binding domain (11). Our data shed new light on VSM cell-proliferative processes and provide potential new targets for several pathological processes such as restenosis.

Of significant importance is the unexpected and novel finding that serum-induced VSM proliferation is mediated primarily via $G_{\beta\gamma}$. Using cultured VSM cells, we found that expression of the βARKct via adenovirus infection was able to significantly attenuate p42/p44 MAP kinase activation in response to serum replacement. The degree of inhibition was surprising because it appears that the majority of the mitogenic activity present in serum is attributable to substances that signal via $G_{\beta\gamma}$. An even more compelling finding was that in the presence of the βARKct, VSM cell proliferation in response to serum was markedly attenuated. That $G_{\beta\gamma}$ plays such a prominent role in serum-induced VSM mitogenesis has not been documented previously and indicates that receptors that couple to heterotrimeric G proteins are important serum mitogens.

In these *in vitro* experiments, serum can mimic, at least partially, the complex signaling pathways that stimulate *in vivo* VSM proliferation. Therefore, we hypothesized that $G_{\beta\gamma}$ may also trigger *in vivo* conditions of unchecked intimal VSM proliferation such as arterial restenosis. We chose to study balloon-injured rat carotid arteries, which represent a reliable model of intimal proliferation (15). Furthermore, gene transfer to rat carotid arteries has been achieved in many labs, including ours, and it appears that recombinant adenoviruses are the most efficient vectors currently available for arterial *in vivo* gene transfer (19, 24). It also has been demonstrated that the use of poloxamers such as pluronic gel can improve the efficacy of adenovirus-mediated gene delivery to vessels (26). Thus, we combined adenoviruses with pluronic gel to deliver transgenes to the wall of rat carotid arteries after angioplastic injury with a balloon catheter. After 28 days, the injured carotid arteries displayed significant intimal thickness, which was significantly attenuated by >70% in arteries treated with the βARKct virus, demonstrating that *in vivo* as well as *in vitro* inhibition of $G_{\beta\gamma}$ signaling results in marked attenuation of VSM cell proliferation. Thus, intimal hyperplasia as seen in this restenosis model can be reduced dramatically after βARKct expression and subsequent sequestration of $G_{\beta\gamma}$.

The results of the *in vitro* mitogenesis experiments in cultured VSM cells after serum exposure indicate that, perhaps unexpectedly, agonists to receptors coupled for G proteins are apparently more important than tyrosine kinase receptor agonists. These receptors not only activate G proteins but the data indicate that it is the βγ subunits that also trigger mitogenic signaling. The rat carotid balloon-injury model used in this study, although not a true model of percutaneous transluminal coronary angioplasty in humans, does trigger restenosis in the form of intimal proliferation. In this process, there appears to be a complex set of interactions among different agonists released at the site of injury sustaining mitogenesis in VSM cells. The major question brought to the surface by the results of the current study is: What are the critical local or systemic factors involved in this pathological process that activate receptors leading to $G_{\beta\gamma}$ release? These mitogenic agonists most probably are normal constituents of serum or could be agents released, as a result of the injury, by cells such as platelets, fibroblasts, or VSM cells. Importantly, when taking inventory of candidate factors, the list contains several agents that activate G protein signaling.

Candidate factors such as epinephrine, thrombin, and LPA, which are released by activated platelets, as well as insulin-like growth factor 1 (IGF-1), which can be secreted from VSM cells, all have been shown to activate MAP kinases via $G_{\beta\gamma}$ (7, 9, 27, 29). Previous studies have shown that receptors coupled to G_i primarily lead to $G_{\beta\gamma}$-dependent signaling (7). Specific receptors that have been shown previously to couple to G_i and that may be associated with VSM intimal hyperplasia *in vivo* include the LPA receptor (9), the α_2-adrenergic (9) and β_2-adrenergic (28) receptors, as well as receptors for angiotensin II (29), low density lipoprotein (30), thrombin (27), and endothelin I (31). Interestingly, one classical tyrosine kinase receptor agonist that has been implicated in VSM mitogenesis *in vivo*, IGF-1, has been shown previously to activate the Ras-MAP kinase pathway via $Gi_{\beta\gamma}$ (20).

In experimental settings, it has been shown that single receptor antagonists for some of the above-mentioned factors can result in the attenuation of VSM mitogenesis (32, 33); however, it is likely that several factors contribute to pathological intimal hyperplasia. In fact, several pharmacological approaches that were successful in the laboratory were ineffective in clinical trials at preventing restenosis (34, 35). Thus, targeting inhibition at the common trigger ($G_{\beta\gamma}$) would appear to offer a more efficacious approach by inhibiting signaling through multiple classes of receptors. The release of $G_{\beta\gamma}$ and subsequent mitogenic activation represents the final, common link of all of these signals, and inhibition of $G_{\beta\gamma}$ signaling via βARKct expression severely arrests *in vivo* intimal hyperplasia. Importantly, our *in vitro* results indicate that $G_{\beta\gamma}$ is the primary

trigger of VSM mitogenesis induced by serum. Thus, $G_{\beta\gamma}$ inhibition represents a potential novel therapeutic strategy to prevent pathological VSM-proliferative conditions such as restenosis and vein graft failure because of its ability to block mitogenesis in response to several different agents present *in vivo* in serum. This molecular targeting could be achieved by the genetic transfer of peptides such as the βARKct or novel pharmacological compounds.

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