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Phosphorylation of β-catenin by PKA promotes ATP-induced proliferation of vascular smooth muscle cells

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Abstract

Extracellular ATP stimulates proliferation of vascular smooth muscle cells (VSMC) through activation of G protein-coupled P2Y purinergic receptors. We have previously shown that ATP stimulates a transient activation of protein kinase A (PKA), which, together with the established mitogenic signaling of purinergic receptors, promotes proliferation of VSMC (Hogarth DK, Sandbo N, Taurin S, Kolenko V, Miano JM, Dulin NO. *Am J Physiol Cell Physiol* 287: C449–C456, 2004). We also have shown that PKA can phosphorylate β-catenin at two novel sites (Ser552 and Ser675) in vitro and in overexpression cell models (Taurin S, Sandbo N, Qin Y, Browning D, Dulin NO. *J Biol Chem* 281: 9971–9976, 2006). β-Catenin promotes cell proliferation by activation of a family of T-cell factor (TCF) transcription factors, which drive the transcription of genes implicated in cell cycle progression including cyclin D1. In the present study, using the phosphospecific antibodies against phospho-Ser552 or phospho-Ser675 sites of β-catenin, we show that ATP can stimulate PKA-dependent phosphorylation of endogenous β-catenin at both of these sites without affecting its expression levels in VSMC. This translates to a PKA-dependent stimulation of TCF transcriptional activity through an increased association of phosphorylated (by PKA) β-catenin with TCF-4. Using the PKA inhibitor PKI or dominant negative TCF-4 mutant, we show that ATP-induced cyclin D1 promoter activation, cyclin D1 protein expression, and proliferation of VSMC are all dependent on PKA and TCF activities. In conclusion, we show a novel mode of regulation of endogenous β-catenin through its phosphorylation by PKA, and we demonstrate the importance of this mechanism for ATP-induced proliferation of VSMC.

Keywords

purinergic; protein kinase A; cyclin D1

EXTRACELLULAR ATP IS RELEASED from sympathetic nerves, activated platelets, inflammatory cells, and endothelial cells to stimulate vascular smooth muscle cell (VSMC) contraction and proliferation (16). The levels of ATP rise dramatically during vascular injury, hypoxia, or inflammatory cell activation (9, 14). Under these conditions, ATP stimulates proliferation of VSMC and adventitial fibroblasts, which contributes to vascular hypertrophy during atherosclerosis, restenosis, or pulmonary hypertension (6, 12, 14). The mitogenic signaling of ATP in VSMC is mediated by P2Y purinergic receptors and includes activation of phospholipase C, release of inositol trisphosphate, and diacyl glycerol, Ca^{2+} mobilization, and activation of various protein kinases (Ca^{2+}/calmodulin-dependent kinase, protein kinase C, and mitogen-activated protein kinases) (4, 12, 15, 20). We and others have previously

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shown that ATP stimulates a transient cAMP production and PKA activation, which is required for maximal DNA synthesis in response to ATP (19, 31). However, the molecular mechanism by which PKA may promote VSMC proliferation upon purinergic receptor stimulation remains elusive.

β-Catenin is a multifunctional protein that controls cell-cell adhesion and cell proliferation. In the latter function, β-catenin stimulates T-cell factor (TCF)/lymphoid enhancer factor transcription factors to induce transcription of a variety of growth-promoting genes, including c-*myc* (3) and cyclin D1 (29). In quiescent cells, β-catenin is maintained at low levels in the cytoplasm through phosphorylation by casein kinase-1 at Ser45 and by glycogen synthase kinase-3 (GSK-3) at Ser33/Ser37/Thr41 sites, respectively (23), and its subsequent ubiquitination and degradation by the proteosome (2, 7). Inhibition of GSK-3 through Wnt signaling results in a decrease in phosphorylation of β-catenin at Ser33/Ser37/Thr41 sites, its stabilization, and activation of TCF-dependent gene transcription (30). Mutations of β-catenin or of its regulatory proteins, resulting in the accumulation of β-catenin and the activation of TCF-dependent gene transcription, are frequently found in various types of cancers (5, 21). β-Catenin signaling is also implicated in VSMC proliferation in vitro and in vivo during vascular injury (22, 25).

We have recently discovered that PKA can phosphorylate β-catenin at Ser552 and Ser675 sites, and this phosphorylation by PKA promotes transcriptional activity of β-catenin in over-expression cell models (28). In the present study, we sought to examine whether ATP, through PKA, can stimulate phosphorylation of endogenous β-catenin at Ser552 and Ser675 sites and how this translates to ATP-induced proliferation of VSMC.

MATERIALS AND METHODS

Cell culture

The rat VSMC were isolated from Wistar-Kyoto rat aortas by enzymatic digestion and maintained as described previously (10). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml streptomycin, 250 ng/ml amphotericin B, and 100 U/ml penicillin. Twenty-four hours before stimulation, the cells were serum deprived using Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin and 2 mM L-glutamine. Transient transfections were performed by using LipofectAMINE-PLUS reagent (Invitrogen) following the standard manufacturer's protocol. Adenovirus-meditated gene transduction was performed as described previously (19). This study was approved by the University of Chicago Biosafety and Animal Care and Use Committees.

Reagents

The cDNA for Flag-tagged β-catenin and its mutants were described previously (28). The cyclin D1 promoter (−1,745 base pairs) luciferase reporter was from Dr. Richard Pestell. The TCF/lymphoid enhancer factor luciferase reporter (TOP) and its negative control (FOP) plasmids were from Upstate Biotechnology. The dominant negative PKA plasmid (dnPKA) was described previously (8). The dominant negative TCF-4 plasmid (dnTCF-4) was from Dr. Tong-Chuan He. Adenovirus encoding protein kinase inhibitor PKI (Ad-PKI) was described previously (19). Adenovirus encoding the dominant negative TCF-4 mutant was from Vector Biolabs. Antibodies against β-catenin, phospho-S552-β-catenin, and phospho-S675-β-catenin were from Cell Signaling Technology. Antibodies against Flag and β-actin were from Sigma-Aldrich. Antibodies against Flag and β-actin were from Sigma Aldrich. antibodies against TCF-4 and cyclin D1 were from Santa Cruz Biotechnology. Antibodies against ERK1/2 were from Dr. Michael Dunn.

Immunoprecipitation and Western blot analysis

Cells were lysed in a buffer containing 150 mM NaCl, 20 mM TRIS (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, protease inhibitors (1 mg/ml leupeptin, 1 mg/ml aprotinin, and 1 mM PMSF), and phosphatase inhibitors (1 mM NaF, 200 mM Na-orthovanadate). The lysates were cleared by centrifugation at 14,000 *g* for 10 min. Immunoprecipitation of Flag-tagged β-catenin proteins was performed using agarose-conjugated mouse anti-Flag antibodies (Sigma-Aldrich). The immunoprecipitation of endogenous β-catenin was performed using agarose-conjugated goat anti-β-catenin antibodies (Santa Cruz Biotechnology). Immunoprecipitation of TCF-4 was performed by incubating cleared cell lysates with 10 μg/ml rabbit polyclonal TCF-4 antibodies (Santa Cruz Biotechnology) at 4°C overnight, followed by incubation with protein A/protein G-conjugated agarose beads. The immune complexes were washed three times with 1 ml lysis buffer, boiled in Laemmli buffer, and subjected to polyacrylamide gel electrophoresis and Western blotting with the desired primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies, and developed by enhanced chemiluminescence reaction (Pierce). The digital chemiluminescence images were taken by a Luminescent Image Analyzer LAS-3000 (Fujifilm).

Nonradioactive in vitro assay for PKA activity

Following stimulation with desired agonists, the cells (grown in 12-well plates) were lysed in 0.1 ml/well lysis buffer containing 25 mM HEPES (pH 7.5), 0.5% NP-40, protease inhibitors (1 mg/ml leupeptin, 1 mg/ml aprotinin, and 1 mM PMSF), and phosphatase inhibitors (1 mM NaF, 200 mM Na-orthovanadate) (19). The lysates were cleared from insoluble material by centrifugation at 20,000 *g* for 10 min, and 5 μl cleared lysates were subjected to a kinase reaction with the fluorescence-labeled PKA substrate kemptide (Promega) following the manufacturer's protocol. The reaction was stopped by boiling the samples for 10 min. The phosphorylated kemptide was separated from the non-phosphorylated one by 0.8% agarose electrophoresis. The fluorescent images were taken by Luminescent Image Analyzer LAS-3000.

Luciferase reporter assay

Cells were transfected with desired luciferase reporter plasmid, thymidine kinase (TK)-driven renilla plasmid (transfection efficiency control), and cDNA encoding a gene of interest or an empty plasmid, serum starved overnight, followed by stimulation with 30 μM ATP for 12 h. The cells were washed twice with PBS, lysed in protein extraction reagent, and assayed for luciferase and renilla activity using the corresponding assay kits (Promega, Madison, WI). To account for differences in transfection efficiency, luciferase activity of each sample was normalized to renilla activity.

The [^{3}H]thymidine uptake assay was performed as described previously (19). Serum-starved VSMC were stimulated with 30 μM ATP for 24 h. [^{3}H]thymidine (1 μCi/ml) was added 6 h after cell stimulation for 18 h. The cells were then washed twice with ice-cold PBS, precipitated with 10% trichloroacetic acid (TCA) for 30 min, washed once with 5% TCA, and lysed in a solution containing 0.1% NaOH and 0.1% SDS for 15 min. The lysates were analyzed for radioactivity by scintillation spectrometry.

RESULTS

To examine whether ATP stimulates phosphorylation of β-catenin at Ser552 and Ser675 sites that are the PKA substrates in vitro (28), we used the phosphospecific antibodies generated against the corresponding phosphorylation sites of β-catenin. We first characterized the specificity of antibodies by assessing phosphorylation of overexpressed (in

Cos-7 cells) wild-type (WT) β-catenin or its phosphorylation-deficient mutants. As shown in Fig. 1, both antibodies recognized WT-β-catenin after PKA stimulation by forskolin. The S552A mutation abolished the recognition by phospho-Ser552 antibodies, but not by phospho-Ser675 antibodies. The S675A mutation abolished the recognition by phospho-Ser675 antibodies, but not by phospho-Ser552 antibodies. The double S552A/S675A mutation abolished the recognition by either antibodies.

Having confirmed the specificity of these antibodies, we then examined the phosphorylation of endogenous β-catenin in VSMC stimulated by ATP. As shown in Fig. 2, ATP stimulated phosphorylation of β-catenin at both Ser-552 (Fig. 2*B*) and Ser-675 (Fig. 2*C*) sites, which was abolished by PKA inhibition through adenovirus-mediated overexpression of PKI. The effectiveness of PKI was confirmed by the inhibition of PKA activity as assessed by in vitro PKA assay of cell lysates (Fig. 2*A*). The specificity of PKI was confirmed by showing that it had no effect on ATP-induced phosphorylation of ERK1/2, as detected by electrophoretic mobility shift assay of phosphorylated ERK1/2 (Fig. 2*E*). It is noteworthy that β-catenin was also significantly phosphorylated at Ser675 site at the basal state, but probably not by PKA, because PKI expression did not attenuate this basal phosphorylation (Fig. 2*C*).

We then examined how the PKA-dependent phosphorylation of β-catenin by ATP affects its transcriptional activity in VSMC by using a luciferase reporter driven by TCF-binding elements. As shown in Fig. 3*A*, ATP stimulated a profound induction of TCF-luciferase activity. This increase was inhibited by overexpression of dominant negative PKA mutant (dnPKA), the efficiency and specificity of which were confirmed in our previous studies (8, 19). Given that the interaction between TCF transcription factors and β-catenin is critical for TCF-dependent gene transcription, we examined the effect of ATP (and the role of PKA) on β-catenin binding to the TCF-4 isoform that is expressed in VSMC. Figure 3*B* shows that β-catenin binding to TCF-4 was significantly increased on stimulation with ATP, as assessed by coimmunoprecipitation. This increased β-catenin/TCF-4 interaction was abolished by overexpression of PKI. Together, these data show that endogenous β-catenin is phosphorylated by PKA in VSMC stimulated by ATP and suggest that ATP-induced phosphorylation of β-catenin by PKA stimulates TCF-dependent gene transcription by promoting association of β-catenin with TCF-4.

The β-catenin/TCF transcription complex drives the expression of several genes critical for cell cycle progression including cyclin D1. Figure 4*A* shows that ATP stimulates a profound induction of cyclin D1 promoter activity as assessed by luciferase reporter driven by – 1,745 cyclin D1 promoter. Overexpression of dominant negative mutant of TCF-4 (dnTCF-4) significantly attenuated ATP-induced cyclin D1 promoter activation (Fig. 4*A*). The efficiency of dnTCF-4 was confirmed by its inhibition of TCF-luciferase reporter activity (Fig. 4*B*). The specificity of dnTCF-4 was confirmed by showing no effect of dnTCF-4 on ATP-induced cAMP response element (CRE) reporter activity (Fig. 4*C*). Furthermore, the stimulation of cyclin D1 promoter by ATP was also significantly decreased by dnPKA (Fig. 4*D*). Together, these data suggest that activation of cyclin D1 promoter by ATP in VSMC is mediated, at least in part, by PKA and TCF-4.

We next examined whether the above experiments translate functionally to the expression of the endogenous cyclin D1 protein and to proliferation of VSMC. As shown in Fig. 5*A*, ATP stimulated a time-dependent expression of cyclin D1 with the maximum at 12 h of exposure. Adenovirus-mediated transduction of either PKI (Fig. 5*B*), or dnTCF-4 (Fig. 5*C*) inhibited ATP-induced cyclin D1 expression without affecting the levels of housekeeping β-actin protein. Furthermore, having previously demonstrated that ATP-induced VSMC proliferation is partially mediated by PKA (19), and given the activation of β-catenin/TCF-4 signaling by PKA established in the present study and before (28), we examined the role of

TCF-4 in ATP-induced proliferation of VSMC. As shown in Fig. 6, adenovirus-mediated transduction of dnTCF-4 significantly decreased DNA synthesis in response to ATP. Together, these results demonstrate the importance of PKA-mediated β-catenin/TCF-4 signaling in ATP-induced proliferation of VSMC.

DISCUSSION

The present study describes three major findings: *1*) endogenous β-catenin can be phosphorylated by PKA at Ser552 and Ser675 in response to ATP in VSMC; *2*) ATP-induced phosphorylation of endogenous β-catenin by PKA promotes its interaction with TCF-4, resulting in an increased transcriptional activity of TCF-4; and *3*) ATP stimulates cyclin D1 expression and proliferation of VSMC in a manner dependent on PKA and TCF-4 activities. Even though the present study focused on VSMC and ATP as an agonist, to our knowledge, these three findings have not been previously reported for endogenous β-catenin regulation in other cells or by other stimuli.

It is accepted that the activity of β-catenin is controlled at the level of its stability through regulated proteolysis. As such, phosphorylation of β-catenin by GSK-3 at Ser33, Ser37, and Thr41 targets it for proteasomal degradation; whereas inhibition of GSK-3 through Wnt signaling results in accumulation of unphosphorylated β-catenin (7). Previous studies by others and us showed that GSK-3 activity can be inhibited through its phosphorylation by Akt (17) or by PKA (1, 13, 28). In our additional experiments, ATP stimulated a PKA-dependent phosphorylation of GSK-3α in VSMC (data not shown). However, this did not lead to accumulation of β-catenin, the levels of which were already easily detected in quiescent VSMC cells (Fig. 2*D*). Instead, we show for the first time that endogenous β-catenin can be directly activated (Fig. 3*A*) through its phosphorylation by PKA at Ser552 and Ser675 sites (Fig. 2). This represents an additional new mode of β-catenin regulation that was previously not appreciated. Furthermore, we provide a molecular mechanism for PKA-dependent activation of β-catenin by showing for the first time that phosphorylation of β-catenin by PKA promotes its association with TCF-4 in response to ATP (Fig. 3*B*).

β-Catenin and TCF-4 stimulate transcription of many genes implicated in cell cycle progression, including cyclin D1, c-*myc*, *c-jun*, and others (3, 11, 27, 29). In the present study, we show that cyclin D1 promoter activation and protein expression in response to ATP are dependent on both PKA and TCF-4 activities in VSMC (Figs. 4 and 5). It is noteworthy that cyclin D1 promoter also contains CREs known to be activated by PKA (18). However, even though ATP stimulated activation of artificial CRE reporter (Fig. 4*C*), the ATP-induced cyclin D1 promoter activation was not dependent on CRE, because mutation of CRE in cyclin D1 promoter did not affect its activation by ATP (data not shown). Thus we believe that in ATP responses, PKA activates cyclin D1 promoter not through CRE, but through β-catenin/TCF-4 axis.

Finally, we show for the first time that ATP-induced proliferation of VSMC is dependent on the activity of TCF-4 (Fig. 6). Proliferation of VSMC induced by other growth stimuli, such as serum, β-cellulin (epidermal growth factor receptor ligand), or platelet-derived growth factor, is also dependent on β-catenin/TCF-4 activity (22, 26, 32). In these cases, TCF-4 stimulation likely occurs through activation of Akt, inhibition of GSK-3, and stabilization of unphosphorylated (at GSK-3 sites) β-catenin (26). As discussed above, ATP stimulated TCF-4 through a different mechanism, i.e., through a direct phosphorylation of β-catenin by PKA (at sites distinct from those that are phosphorylated by GSK-3), resulting in increased association of β-catenin with TCF-4.

Regarding PKA, its role in cell proliferation depends on the cell type as well as on the stimulus. In VSMC, stimulation of PKA through β-adrenergic signaling inhibits proliferation, whereas PKA activation through purinergic or endothelin signaling promotes VSMC proliferation and hypertrophy, respectively (19, 28, 31). This agonist-specific role of PKA can be explained at least in part by *1*) differential duration of PKA activation by these agonists [sustained PKA activation through β-adrenergic stimulation vs. transient PKA activation through purinergic or endothelin stimulation (8)]; and *2*) inability of β-adrenergic stimuli to induce mitogenic signaling (MAP kinase, etc.), which is activated by purinergic or endothelin stimulation (19, 24). The transient nature of PKA activation by ATP is likely due to *1*) relatively small cAMP increase in response to ATP compared with β-adrenergic stimulation; and *2*) simultaneous activation of Ca^{2+}/calmodulin-dependent phosphodiesterases by ATP, but not by β-adrenergic stimulation (data not shown). Thus we favor a notion that transient PKA activation works together with the established mitogenic signaling to promote VSMC proliferation in response to ATP. The present study describes one potential mechanism by which PKA may contribute to ATP-induced proliferation of VSMC through phosphorylation of β-catenin and activation of TCF-dependent gene transcription.

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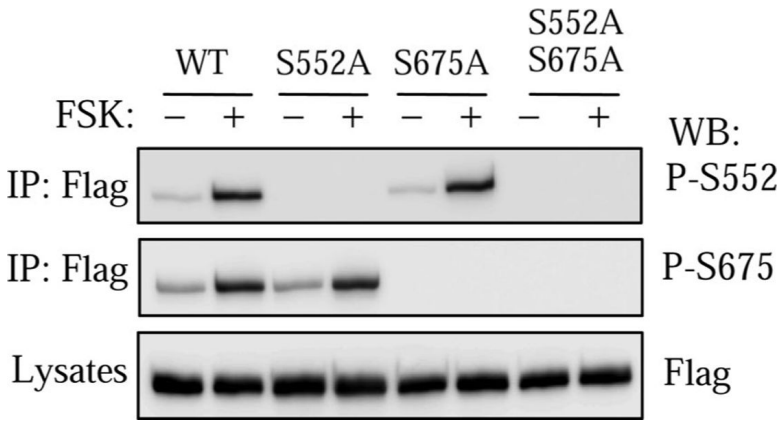

Fig. 1.

Specificity of phospho-S552 and phospho-S675 β-catenin antibodies. Cos-7 cells were transfected with Flag-tagged β-catenin or the corresponding mutants of β-catenin and stimulated with 10 μM forskolin (FSK) for 5 min. The proteins were immunoprecipitated (IP) with Flag antibodies followed by Western blotting (WB) with phospho-S552 or phospho-S675 β-catenin antibodies as indicated. The equal amounts of immunoprecipitated β-catenin or the corresponding mutants were confirmed by Western blotting with Flag antibodies. WT, wild-type.

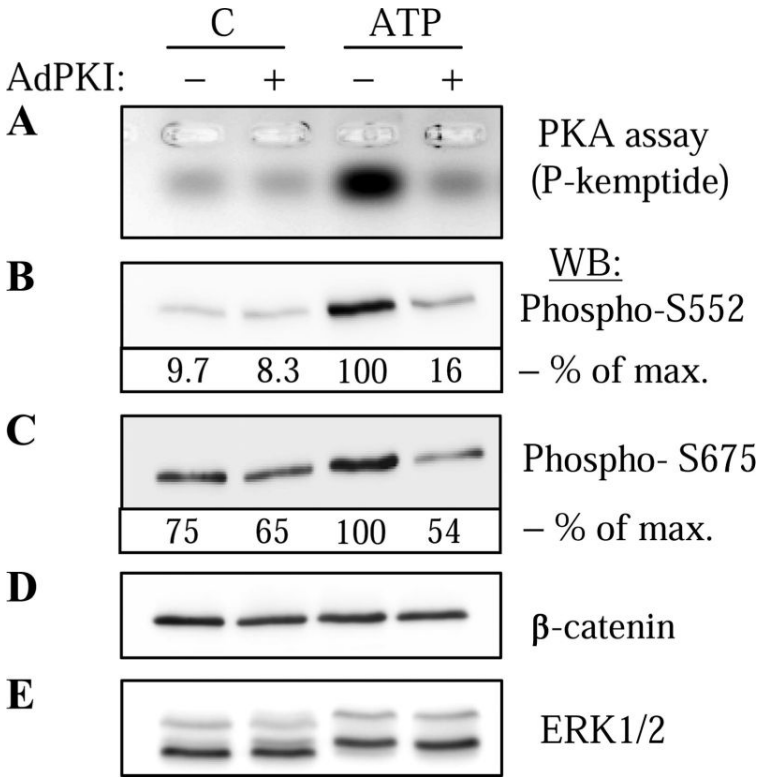

Fig. 2.

ATP-induced phosphorylation of endogenous β-catenin by PKA in vascular smooth muscle cells (VSMC). VSMC were transduced with control adenovirus (−) or adenovirus encoding PKA inhibitor PKI (Ad-PKI), stimulated with 30 μM ATP for 5 min and lysed. Cell lysates were analyzed for PKA activity (*A*) or were subjected to Western blotting with phospho-S552 (*B*) or phospho-S675 (*C*) β-catenin antibodies as indicated. The equal amounts of endogenous β-catenin were confirmed by Western blotting with β-catenin antibodies (*D*). For control purposes, ERK1/2 phosphorylation was assessed by electrophoretic mobility shift assay following Western blotting with ERK1/2 antibodies (*E*). The densitometry of selected blots is shown as % of maximal (max) response to ATP.

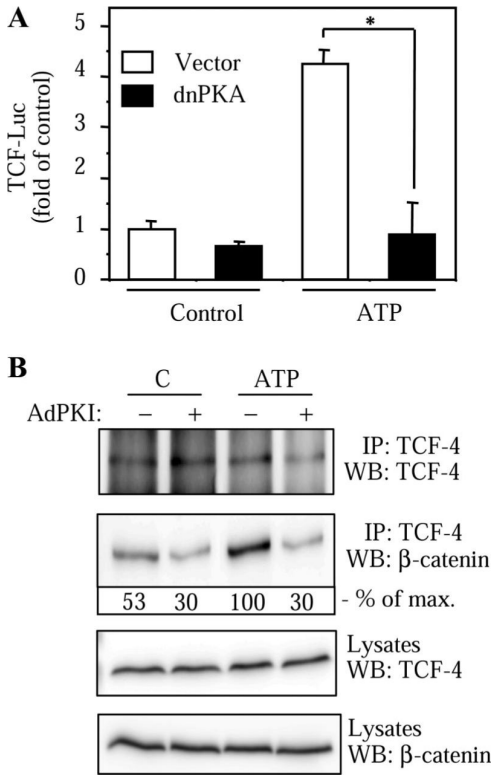

Fig. 3.

ATP-induced, PKA-mediated activation of T-cell factor (TCF)-dependent gene transcription in VSMC. *A*: PKA-dependent activation of TCF reporter by ATP. VSMC were transfected with cDNA for TCF-luciferase (Luc) reporter (TOPflash) or the mutated control reporter (FOPflash) along with a renilla reporter driven by thymidine kinase promoter (TK-RL), and with an empty vector or cDNA for PKA dominant negative mutant (dnPKA). Following the stimulation of cells with 30 μM ATP for 12 h, luciferase activity was measured and normalized to a corresponding renilla activity. The TOP-Luc/TK-RL values were subtracted from the FOP-Luc/TK-RL values. Data represent means ± SD from a representative of three experiments performed in triplicate. **P* < 0.01. *B*: PKA-dependent interaction between endogenous TCF-4 and β-catenin in response to ATP. VSMC were transduced with Ad-PKI, stimulated with ATP for 5 min, and lysed. Endogenous TCF-4 was immunoprecipitated from cell lysates, and the immune complexes or total cell lysates were examined by Western blotting with desired antibodies as indicated. The densitometry of a selected blot is shown as % of maximal response to ATP.

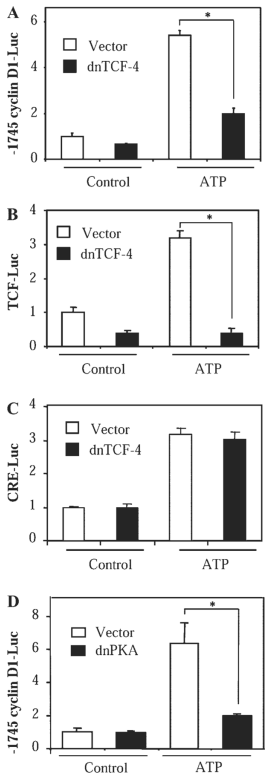

Fig. 4.

ATP-induced activation of cyclin D1 promoter in VSMC is mediated by TCF-4 and PKA. VSMC were transfected with a control TK-RL DNA, a desired luciferase reporter for cyclin D1 promoter (*A* and *D*), TCF (Top/Fop) (*B*), or cAMP response element (CRE; *C*), along with the empty vector or the cDNAs for the TCF-4 dominant negative mutant (dnTCF-4; *A*–*C*), or dnPKA (*D*). Following the stimulation of cells with 30 μM ATP for 12 h, luciferase activity was measured and normalized to a corresponding renilla activity. Data represent means ± SD from a representative of three experiments performed in triplicate. **P* < 0.01.

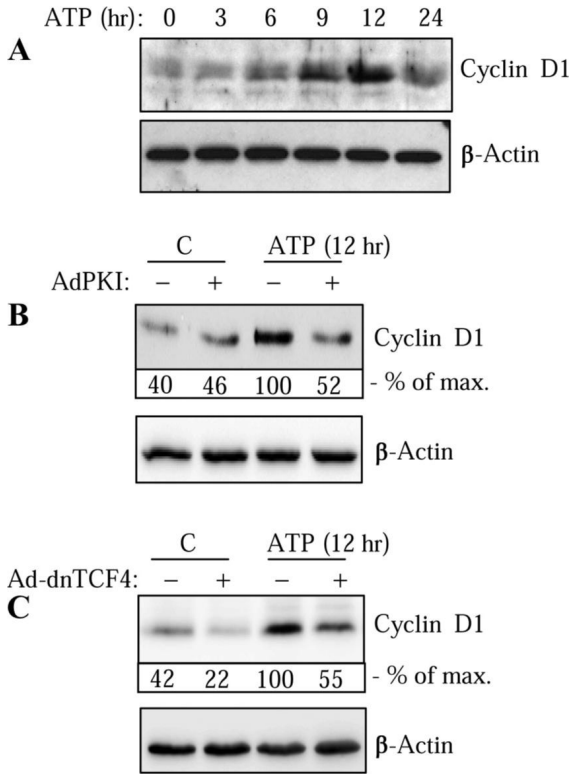

Fig. 5.

ATP-induced activation of cyclin D1 protein expression in VSMC is mediated by TCF-4 and PKA. *A*: time course of cyclin D1 protein expression in response to 30 μM ATP as assessed by Western blotting with cyclin D1 antibodies. *B* and *C*: VSMC were transduced with control adenovirus or with Ad-PKI (*B*) or with Ad-dnTCF-4 (*C*). Cells were stimulated with 30 μM ATP for 12 h, and cell lysates were analyzed by Western blotting with antibodies against cyclin D1 and β-actin. The densitometry of selected blots is shown as % of maximal response to ATP.

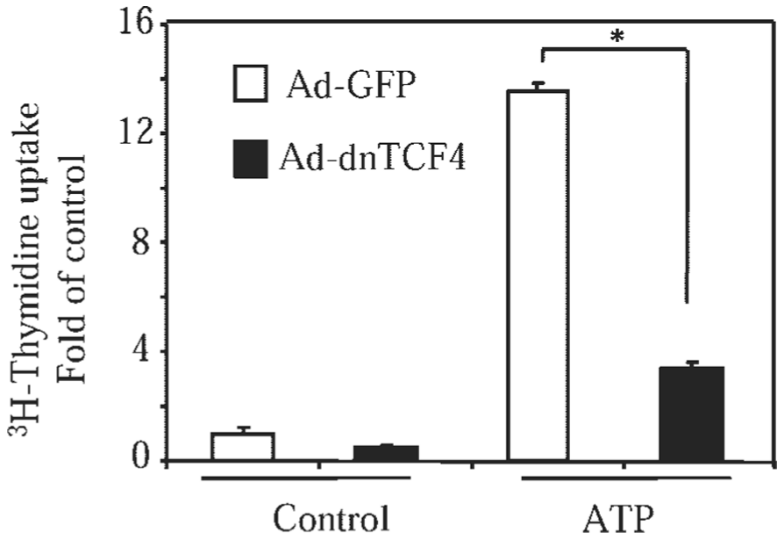

Fig. 6.

ATP-induced DNA synthesis in VSMC is mediated by TCF-4. VSMC were transduced with control adenovirus [AD-green fluorescent protein (GFP)] or adenovirus encoding dnTCF-4 (Ad-dnTCF-4), followed by stimulation with 30 μM ATP for 12 h. The [^{3}H]thymidine uptake assay was then performed as described in MATERIALS AND METHODS. Data represent means ± SD from a representative of three experiments performed in triplicate. *$P < 0.01$.