\( \alpha_1 \) Adrenergic Receptors Activate Phosphatidylinositol 3-Kinase in Human Vascular Smooth Muscle Cells

ROLE IN MITOGENESIS*

(Received for publication, December 7, 1995, and in revised form, January 29, 1996)

Zhuo-Wei Hu†, Xiao-You Shi, Richard Z. Lin§, and Brian B. Hoffman
Department of Medicine, Stanford University School of Medicine, Stanford, California 94305 and Veterans Affairs Medical Center, Palo Alto, California 94304

Activation of \( \alpha_1 \) adrenergic receptors stimulates mitogenesis in human vascular smooth muscle cells (HVSMCs). To examine signaling pathways by which activation of \( \alpha_1 \) receptors may induce mitogenesis in HVSMCs, we have found that \( \alpha_1 \) receptor-stimulated DNA synthesis and activation of mitogen-activated protein (MAP) kinase are blocked by wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI 3-kinase). To determine directly if activation of \( \alpha_1 \) receptors stimulated PI 3-kinase, in vitro assays of kinase activity were performed in immunocomplexes precipitated by an antibody against the \( \alpha_1 \) subunit of PI 3-kinase. Noradrenaline stimulated a time- and concentration-dependent activation of PI 3-kinase in the presence of a \( \beta \) adrenergic receptor antagonist. Noradrenaline-stimulated PI 3-kinase activation was blocked by antagonists of \( \alpha_1 \) receptors and by pertussis toxin, suggesting that \( \alpha_1 \) receptors activate PI 3-kinase via a pertussis toxin-sensitive G protein. Direct activation of protein kinase C by a phorbol ester did not stimulate PI 3-kinase; also, a \( \mathrm{Ca}^{2+} \) L-channel blocker did not inhibit noradrenaline-stimulated PI 3-kinase activity. Increased PI 3-kinase activity was detected in both anti-Ras and anti-phosphotyrosine immunoprecipitates from noradrenaline-stimulated HVSMCs. Moreover, noradrenaline stimulated formation of active Ras GTP complexes. Because blockade of PI 3-kinase by wortmannin inhibited formation of this complex, this result suggests that Ras might be a target of PI 3-kinase. Noradrenaline stimulated tyrosine phosphorylation of the p85 subunit of PI 3-kinase and a phosphorylated tyrosine protein could be co-immunoprecipitated with anti-p85 of PI 3-kinase. These results demonstrate that stimulation of \( \alpha_1 \) receptors activates PI 3-kinase in HVSMCs and that \( \alpha_1 \) receptor-activated PI 3-kinase is associated with an increase in active Ras-GTP and activation of tyrosine protein phosphorylation. These pathways may contribute to \( \alpha_1 \) receptor-stimulated mitogenic responses including activation of MAP kinase and DNA synthesis in HVSMCs.

\( \alpha_1 \) adrenergic receptors are members of the superfamily of G protein-coupled membrane receptors; these pathways mediate many of the important physiological effects of catecholamines such as noradrenaline and adrenaline. \( \alpha_1 \) adrenergic receptors play a particularly important role in control of blood pressure via induction of vascular smooth muscle contraction (Minnemann and Esbenshade, 1994). Also, activation of \( \alpha_1 \) adrenergic receptors stimulates cardiac and vascular smooth muscle growth and hypertrophy (Milano et al., 1994; Nakafuku et al., 1990; and Okazaki et al., 1994). However, signaling pathways utilized by \( \alpha_1 \) receptors in promoting mitogenic effects, such as growth-related gene expression and DNA synthesis, are unclear.

It is generally accepted that activation of \( \alpha_1 \) receptors stimulates phospholipase C, leading to increased hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate and 1,2-diacylglycerol. Both inositol 1,4,5-trisphosphate and 1,2-diacylglycerol play important roles as intracellular second messengers that increase intracellular \( \mathrm{Ca}^{2+} \) concentrations and activate various isoforms of protein kinase C, respectively. These coupling mechanisms are typically mediated by pertussis toxin-insensitive G proteins, likely in the Gq11 family (Perez et al., 1993; Schwinn et al., 1995). Additionally, stimulation of \( \alpha_1 \) receptors activates phospholipase D and phospholipase A2 via pertussis toxin-insensitive/sensitive G proteins (Minnemann and Esbenshade, 1994; Perez et al., 1993).

Although this predominant view of \( \alpha_1 \) receptor signaling provides substantial insight into \( \alpha_1 \) receptor-mediated responses in various cells, there are clear indications that these mechanisms may not explain all aspects of \( \alpha_1 \) receptor signaling. For example, recent evidence demonstrates that \( \alpha_1 \) receptor-stimulated mitogenic responses in myocytes may be due to activation of tyrosine protein kinases (TPKs)\(^1\) and MAP kinases (Thorburn et al., 1994), suggesting that \( \alpha_1 \) adrenergic receptors may share common signal pathways with tyrosine kinase receptors in the stimulation of mitogenesis.

There has been considerable recent interest in lipid kinases that phosphorylate the 3-position of the inositol ring of inositol phospholipids; this has led to the identification of the enzyme PI 3-kinase (for reviews see Carpenter and Cantley (1990), Divecha and Irvine (1995), Fry (1994), and Valius and Kazlauskas (1993). PI 3-kinase is a lipid kinase that has been implicated in the regulation of cell growth and proliferation by receptor tyrosine kinases (Ruderman et al., 1990; Valius and Kazlauskas, 1993), nonreceptor tyrosine kinases (Ding et al., 1995), cytokine receptors (Karnitz et al., 1995) and oncogene products (Fukui et al., 1991). Stimulation of cells with mitogens

---

\(^{1}\) The abbreviations used are: TPK, tyrosine protein kinase; IGF-I, insulin-like growth factor I; MAP kinase, mitogen-activated protein kinase; PI 3-kinase, phosphatidylinositol 3-kinase; SH2, SRC homology 2; TLC, thin layer chromatography; HVSMC, human vascular smooth muscle cell.
such as platelet-derived growth factor and many other peptide growth factors leads to accumulation of the lipid products phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate (Carpenter and Cantley, 1990; Divo et al., 1995). Although the function of these lipids has not yet been determined, increasing evidence suggests that they may serve as intracellular second messengers. PI 3-kinase is a heterodimer consisting of a p85 regulatory subunit with SRC homology domains (SH2 and SH3) and a p110 catalytic subunit. A major mode of activation by growth factors likely involves docking of PI 3-kinase through SH2 domains of the p85 subunit to phosphorylated tyrosine residues(s) of tyrosine kinase receptors (Rordorf-Nikolic et al., 1994). Moreover, activation of PI 3-kinase by growth factors may occur via either Ras-dependent ( Kodaki et al., 1994 ) or Ras-independent ( Rodriguez-Viciana et al., 1994 ) pathways. In either situation, activation of Ras is sufficient to activate mitogenic responses in a variety of cells. In other important cases, such as G protein-coupled receptors, PI 3-kinase has been shown to be directly activated by \( \beta_2 \) subunits released from activated G proteins (Zhang et al., 1995).

In the present study, we have found that \( \alpha_2 \) adrenergic receptor-stimulated mitogenic responses, such as DNA synthesis and activation of MAP kinase in HVS MSCs, are inhibited by wortmannin, a specific inhibitor of PI 3-kinase receptors (Rordorf-Nikolic et al., 1994). Moreover, activation of PI 3-kinase by growth factors may occur via either Ras-dependent (Kodaki et al., 1994) or Ras-independent (Rodriguez-Viciana et al., 1994) pathways. In either situation, activation of Ras is sufficient to activate mitogenic responses in a variety of cells. In other important cases, such as G protein-coupled receptors, PI 3-kinase has been shown to be directly activated by \( \beta_2 \) subunits released from activated G proteins (Zhang et al., 1995).

In the present study, we have found that \( \alpha_2 \) adrenergic receptor-stimulated mitogenic responses, such as DNA synthesis and activation of MAP kinase in HVS MSCs, are inhibited by wortmannin, a specific inhibitor of PI 3-kinase receptors (Rordorf-Nikolic et al., 1994). Moreover, activation of PI 3-kinase by growth factors may occur via either Ras-dependent (Kodaki et al., 1994) or Ras-independent (Rodriguez-Viciana et al., 1994) pathways. In either situation, activation of Ras is sufficient to activate mitogenic responses in a variety of cells. In other important cases, such as G protein-coupled receptors, PI 3-kinase has been shown to be directly activated by \( \beta_2 \) subunits released from activated G proteins (Zhang et al., 1995).

In the present study, we have found that \( \alpha_2 \) adrenergic receptor-stimulated mitogenic responses, such as DNA synthesis and activation of MAP kinase in HVS MSCs, are inhibited by wortmannin, a specific inhibitor of PI 3-kinase receptors (Rordorf-Nikolic et al., 1994). Moreover, activation of PI 3-kinase by growth factors may occur via either Ras-dependent (Kodaki et al., 1994) or Ras-independent (Rodriguez-Viciana et al., 1994) pathways. In either situation, activation of Ras is sufficient to activate mitogenic responses in a variety of cells. In other important cases, such as G protein-coupled receptors, PI 3-kinase has been shown to be directly activated by \( \beta_2 \) subunits released from activated G proteins (Zhang et al., 1995).

In the present study, we have found that \( \alpha_2 \) adrenergic receptor-stimulated mitogenic responses, such as DNA synthesis and activation of MAP kinase in HVS MSCs, are inhibited by wortmannin, a specific inhibitor of PI 3-kinase receptors (Rordorf-Nikolic et al., 1994). Moreover, activation of PI 3-kinase by growth factors may occur via either Ras-dependent (Kodaki et al., 1994) or Ras-independent (Rodriguez-Viciana et al., 1994) pathways. In either situation, activation of Ras is sufficient to activate mitogenic responses in a variety of cells. In other important cases, such as G protein-coupled receptors, PI 3-kinase has been shown to be directly activated by \( \beta_2 \) subunits released from activated G proteins (Zhang et al., 1995).

In the present study, we have found that \( \alpha_2 \) adrenergic receptor-stimulated mitogenic responses, such as DNA synthesis and activation of MAP kinase in HVS MSCs, are inhibited by wortmannin, a specific inhibitor of PI 3-kinase receptors (Rordorf-Nikolic et al., 1994). Moreover, activation of PI 3-kinase by growth factors may occur via either Ras-dependent (Kodaki et al., 1994) or Ras-independent (Rodriguez-Viciana et al., 1994) pathways. In either situation, activation of Ras is sufficient to activate mitogenic responses in a variety of cells. In other important cases, such as G protein-coupled receptors, PI 3-kinase has been shown to be directly activated by \( \beta_2 \) subunits released from activated G proteins (Zhang et al., 1995).

In the present study, we have found that \( \alpha_2 \) adrenergic receptor-stimulated mitogenic responses, such as DNA synthesis and activation of MAP kinase in HVS MSCs, are inhibited by wortmannin, a specific inhibitor of PI 3-kinase receptors (Rordorf-Nikolic et al., 1994). Moreover, activation of PI 3-kinase by growth factors may occur via either Ras-dependent (Kodaki et al., 1994) or Ras-independent (Rodriguez-Viciana et al., 1994) pathways. In either situation, activation of Ras is sufficient to activate mitogenic responses in a variety of cells. In other important cases, such as G protein-coupled receptors, PI 3-kinase has been shown to be directly activated by \( \beta_2 \) subunits released from activated G proteins (Zhang et al., 1995).

In the present study, we have found that \( \alpha_2 \) adrenergic receptor-stimulated mitogenic responses, such as DNA synthesis and activation of MAP kinase in HVS MSCs, are inhibited by wortmannin, a specific inhibitor of PI 3-kinase receptors (Rordorf-Nikolic et al., 1994). Moreover, activation of PI 3-kinase by growth factors may occur via either Ras-dependent (Kodaki et al., 1994) or Ras-independent (Rodriguez-Viciana et al., 1994) pathways. In either situation, activation of Ras is sufficient to activate mitogenic responses in a variety of cells. In other important cases, such as G protein-coupled receptors, PI 3-kinase has been shown to be directly activated by \( \beta_2 \) subunits released from activated G proteins (Zhang et al., 1995).

In the present study, we have found that \( \alpha_2 \) adrenergic receptor-stimulated mitogenic responses, such as DNA synthesis and activation of MAP kinase in HVS MSCs, are inhibited by wortmannin, a specific inhibitor of PI 3-kinase receptors (Rordorf-Nikolic et al., 1994). Moreover, activation of PI 3-kinase by growth factors may occur via either Ras-dependent (Kodaki et al., 1994) or Ras-independent (Rodriguez-Viciana et al., 1994) pathways. In either situation, activation of Ras is sufficient to activate mitogenic responses in a variety of cells. In other important cases, such as G protein-coupled receptors, PI 3-kinase has been shown to be directly activated by \( \beta_2 \) subunits released from activated G proteins (Zhang et al., 1995).

In the present study, we have found that \( \alpha_2 \) adrenergic receptor-stimulated mitogenic responses, such as DNA synthesis and activation of MAP kinase in HVS MSCs, are inhibited by wortmannin, a specific inhibitor of PI 3-kinase receptors (Rordorf-Nikolic et al., 1994). Moreover, activation of PI 3-kinase by growth factors may occur via either Ras-dependent (Kodaki et al., 1994) or Ras-independent (Rodriguez-Viciana et al., 1994) pathways. In either situation, activation of Ras is sufficient to activate mitogenic responses in a variety of cells. In other important cases, such as G protein-coupled receptors, PI 3-kinase has been shown to be directly activated by \( \beta_2 \) subunits released from activated G proteins (Zhang et al., 1995).

In the present study, we have found that \( \alpha_2 \) adrenergic receptor-stimulated mitogenic responses, such as DNA synthesis and activation of MAP kinase in HVS MSCs, are inhibited by wortmannin, a specific inhibitor of PI 3-kinase receptors (Rordorf-Nikolic et al., 1994). Moreover, activation of PI 3-kinase by growth factors may occur via either Ras-dependent (Kodaki et al., 1994) or Ras-independent (Rodriguez-Viciana et al., 1994) pathways. In either situation, activation of Ras is sufficient to activate mitogenic responses in a variety of cells. In other important cases, such as G protein-coupled receptors, PI 3-kinase has been shown to be directly activated by \( \beta_2 \) subunits released from activated G proteins (Zhang et al., 1995).

In the present study, we have found that \( \alpha_2 \) adrenergic receptor-stimulated mitogenic responses, such as DNA synthesis and activation of MAP kinase in HVS MSCs, are inhibited by wortmannin, a specific inhibitor of PI 3-kinase receptors (Rordorf-Nikolic et al., 1994). Moreover, activation of PI 3-kinase by growth factors may occur via either Ras-dependent (Kodaki et al., 1994) or Ras-independent (Rodriguez-Viciana et al., 1994) pathways. In either situation, activation of Ras is sufficient to activate mitogenic responses in a variety of cells. In other important cases, such as G protein-coupled receptors, PI 3-kinase has been shown to be directly activated by \( \beta_2 \) subunits released from activated G proteins (Zhang et al., 1995).

In the present study, we have found that \( \alpha_2 \) adrenergic receptor-stimulated mitogenic responses, such as DNA synthesis and activation of MAP kinase in HVS MSCs, are inhibited by wortmannin, a specific inhibitor of PI 3-kinase receptors (Rordorf-Nikolic et al., 1994). Moreover, activation of PI 3-kinase by growth factors may occur via either Ras-dependent (Kodaki et al., 1994) or Ras-independent (Rodriguez-Viciana et al., 1994) pathways. In either situation, activation of Ras is sufficient to activate mitogenic responses in a variety of cells. In other important cases, such as G protein-coupled receptors, PI 3-kinase has been shown to be directly activated by \( \beta_2 \) subunits released from activated G proteins (Zhang et al., 1995).
receptor-selective antagonist terazosin. To determine whether paired effects were compared by one-way analysis of variance or Student's adrenoreceptor antagonist timolol (1 μM) for 20 h in the presence of a β adrenoreceptor antagonist timolol (1 μM) (some cells had been preincubated with 100 ng/ml pertussis toxin (PTx) for 12 h). Cells were then incubated with [3H]thymidine (0.1 μCi) for another 4 h. Potential inhibitors were added to the cell dishes 1 h before the addition of noradrenaline (Nor) as indicated. Incorporation of [3H]thymidine into cells was performed as described under “Experimental Procedures.” The data are an average ± S.E. of three experiments.

**RESULTS AND DISCUSSION**

To characterize signaling pathways involved in α₁ receptor stimulation of mitogenic responses in vascular smooth muscle cells, we tested the capacity of Wortmannin, a specific inhibitor of PI 3-kinase, to inhibit DNA synthesis. Wortmannin (10 nM) completely blocked noradrenaline-stimulated DNA synthesis as well as platelet-derived growth factor-induced DNA synthesis (Fig. 1A). Noradrenaline's action was also blocked by the α₁ receptor-selective antagonist terazosin. To determine whether the α₁ receptor-mediated increase in [3H]thymidine incorporation was mediated via pertussis toxin-sensitive G proteins, cells were preincubated with pertussis toxin (100 ng/ml) for 12 h before stimulation with noradrenaline. Noradrenaline induced an 89% increase of [3H]thymidine incorporation under control conditions; preincubation with pertussis toxin markedly inhibited the noradrenaline-induced increase in DNA synthesis in these cells (Fig. 1A). Since MAP kinase has been postulated to play a key role in mediating mitogenic responses of many receptors, including α₁ adrenergic receptors in myocytes, we also examined α₁ adrenergic receptor-mediated activation of MAP kinase in HVSMCs (Fig. 1B). Noradrenaline (1 μM) stimulated an approximately 2-fold increase in MAP kinase activity in the presence of a β receptor antagonist timolol. The α₁ receptor antagonist terazosin (1 μM) blocked noradrenaline-activated MAP kinase activity (Fig. 1B). Noradrenaline-stimulated activation of MAP kinase was significantly attenuated by a 12-h preincubation of cells with pertussis toxin (100 ng/ml) (about 30% increase over basal) and partially blocked by inhibitor of PI 3-kinase wortmannin (about 43% increase over basal) (Fig. 1B). Increased MAP kinase activity was not inhibited by the protein kinase C inhibitor H7. These results suggest that activation of PI 3-kinase as well as activation of MAP kinases are involved in mediating catecholamine-induced mitogenesis in human vascular smooth muscle cells. In addition, we found that IGF-I increased MAP kinase activity in these cells and that this response was inhibited by wortmannin but not by pertussis toxin; this result suggests that pertussis toxin was not having nonspecific effects (Fig. 1B).

Several lines of evidence indicate that PI 3-kinase plays an important role in growth regulation and transformation. Analysis of mutations in the binding site for PI 3-kinase on the polypoma virus middle T antigen, which leads to either failure to associate with PI 3-kinase or impaired capacity to elevate the concentrations of PI 3-kinase products, have been found to result in a transformation-defective phenotype (Fanti et al., 1992). Similarly, point mutations in the PI 3-kinase binding sites of platelet-derived growth factor receptors impair this receptor's ability to stimulate DNA synthesis (Valius and Kazlauskas, 1993). Roche et al. (1994) have shown that microinjection of antibodies specific for the p110 subunit of the PI 3-kinase into quiescent fibroblasts inhibited platelet-derived growth factor-induced DNA synthesis. Finally, a number of studies have demonstrated that inhibition of PI 3-kinase by wortmannin, a specific PI 3-kinase inhibitor, results in blockade of growth factors or serum-induced cell proliferation (Panyiatou and Waterfield, 1993; Varticovski et al., 1994; Vemuri and Rittenhouse, 1994), inhibition of protein kinase cascades (Ding et al., 1995), and suppression of growth factor-induced blockade of apoptosis (Yao and Cooper, 1995). Consequently, the present results demonstrate that wortmannin can block noradrenaline-stimulated DNA synthesis and activation of MAP kinase, suggesting that PI 3-kinase plays an important role in α₁ receptor-mediated metaplasia in HVSMCs.

The capacity of a whole range of tyrosine kinase receptors or TPKs (Cantley et al., 1991; Rordorf-Nikolic et al., 1995; Van der Geer and Hunter, 1991) to activate PI 3-kinase has been extensively studied; the activated PI 3-kinase is believed to play an important role in signal transduction pathways of peptide growth factors (Fry, 1994). On the other hand, there is an increasing experimental evidence indicating that PI 3-kinase may also be involved in signaling pathways of G protein-coupling receptors; most of this evidence has been derived from investigations of thrombin receptors in platelets (Stephens et al., 1993; Zhang et al., 1995) and chemoattractant receptors in neutrophils (Bokoch, 1995; Varticovski et al., 1994). To deter-
mine directly if activation of \(\alpha_1\) receptors expressed in HVSMCs activated PI 3-kinase, we implemented an in vitro assay of PI 3-kinase activity in immunocomplexes precipitated by an antibody against the p85 subunit of PI 3-kinase (Yano et al., 1993). The results demonstrated that noradrenaline stimulated a time-dependent activation of PI 3-kinase at concentration of 10 \(\mu\text{M}\) in the presence of a \(\beta\) adrenergic receptor antagonist timolol (1 \(\mu\text{M}\)) (Fig. 2A and Table I). Noradrenaline stimulated a very rapid and significant activation of PI 3-kinase that occurred as early as 1 min after activation of \(\alpha_1\) receptors, with the peak activity at 5 min. The activity of PI 3-kinase was specifically noradrenaline-stimulated by noradrenaline; as indicated in Fig. 3, lane 5.

### Table I

Noradrenaline-stimulated time- and concentration-dependent activation of PI 3-kinase

| NE treatment time (min) | PI 3-kinase activity -fold of control | NE concentration (\(\mu\text{M}\)) | PI 3-kinase activity -fold of control |
|------------------------|--------------------------------------|----------------------------------|--------------------------------------|
| 0                      | 1                                    | 0                               | 1                                    |
| 1                      | 1.7 ± 0.3\(^a\)                      | 1                               | 2.9 ± 0.5\(^a\)                      |
| 2.5                    | 3.5 ± 0.8\(^b\)                      | 10                              | 4.3 ± 0.7\(^b\)                      |
| 5                      | 4.0 ± 0.5\(^b\)                      | 100                             | 1.8 ± 0.3\(^a\)                      |
| 10                     | 2.8 ± 0.4\(^a\)                      |                                 |                                      |

\(^a\) \(p < 0.05\) compared with control.

\(^b\) \(p < 0.01\) compared with control.

Thrombin and several chemoattractants stimulate cell responses in several different cell types via activation of pertussis toxin-sensitive G proteins leading to activation of PI 3-kinase. There are several signaling pathways that are utilized by G protein-coupled to thrombin and chemoattractant receptors in the activation of PI 3-kinase (Bokoch, 1995; Stephens et al., 1993). One pathway involves pertussis toxin-sensitive G proteins including P21 heterologous small G proteins such as Ras or Rho (Zhang et al., 1995). Another pathway occurs via activation of the traditional TPKs such as SRC kinases (Cantley et al., 1991). For the latter pathway, activation of thrombin and chemoattractant receptors leads to phosphorylation of cytosol TPKs, which in turn may provide phosphorylated site(s) for binding of the p85 subunit of PI 3-kinase through SH2 to TPKs. Additionally, a recent study has identified a novel p110 isoform of the catalytic subunit of PI 3-kinase that is activated without association with the p85 subunit of the originally described PI 3-kinase heterodimer (Stoyanov et al., 1995); interestingly, \(\gamma\) subunits released from receptor-activated G proteins directly activate this newly described PI 3-kinase catalytic moiety (Stephens et al., 1993). Although it has been generally accepted that \(\alpha_1\) receptor-stimulated responses are likely predominantly mediated by \(\alpha_1\) subunits released by pertussis toxin-insensitive G proteins, likely in the Gq/11 family (Schwinn et al., 1995), increasing evidence suggests that pertussis toxin-sensitive G proteins may also be utilized to transduce the signals of \(\alpha_1\) receptor stimulation (Perez et al., 1993). To test if pertussis toxin-sensitive G proteins are involved in \(\alpha_1\) receptor-mediated activation of PI 3-kinase, HVSMCs were preincubated with pertussis toxin (100 ng/ml for 12 h) and then stimulated by noradrenaline; as indicated in Fig. 3, lane 5, pertussis toxin completely blocked activation of PI 3-kinase in

### Fig. 2. Noradrenaline-stimulated time- and concentration-dependent activation of PI 3-kinase.

A, cells were treated with vehicle or noradrenaline (10 \(\mu\text{M}\)) for the indicated times. The whole cell lysates (1 mg of protein) were subjected to immunoprecipitation with a rabbit polyclonal antibody against the p85 subunit of PI 3-kinase. PI 3-kinase activity in immunoprecipitates from noradrenaline-treated or control cells was determined as described under "Experimental Procedures." Change in activity of PI 3-kinase is presented as production of phosphatidylinositol phosphate (PIP). The autoradiogram of TLC of PI 3-kinase was exposed for 20 h. Experiments were repeated 3 times with essentially identical results. B, cells were treated with vehicle or the indicated concentrations of noradrenaline for 5 min, and cell lysates were prepared and immunoprecipitated as described above. The autoradiogram of TLC of PI 3-kinase was exposed for 24 h. Experiments were repeated 3 times with essentially identical results.

### Fig. 3. Activation of PI 3-kinase inhibited by \(\alpha_1\) adrenergic receptor antagonists and pertussis toxin.

Cells were pretreated with \(\alpha_1\) receptor-selective antagonist terazosin (10 \(\mu\text{M}\)) or the \(\alpha_1\) receptor-selective antagonist idazoxan (10 \(\mu\text{M}\)), an inhibitor of PI 3-kinase wortmannin (10 nm) for 2 h or with pertussis toxin (PTx, 100 ng/ml) for 12 h. Cells were then treated with vehicle, 10 \(\mu\text{M}\) of noradrenaline or 100 ng/ml of IGF-I for 5 min. Cell lysates (1 mg of protein) were prepared, immunoprecipitated with anti-p85 of PI 3-kinase antibody, and subjected to determination of PI 3-kinase as described above. The autoradiogram of TLC of PI 3-kinase was exposed for 24 h. Experiments were repeated 3 times with similar results.
these cells. However, IGF-I (100 ng/ml), a well-known activator of a tyrosine kinase receptor, increased PI 3-kinase activity in these cells, but the response was not attenuated by pertussis toxin (Fig. 3, lanes 1 and 2), anti-phosphotyrosine (anti-Tyr) (lanes 3 and 4), or anti-p85 of PI 3-kinase (lanes 5 and 6). PI 3-kinase activity in immunoprecipitates from noradrenaline-treated or control cells was determined as described in Fig. 2. The autoradiogram of TLC of PI 3-kinase was exposed for 16 h. Experiments were repeated twice with essentially identical results. B, HVSMCs were metabolically labeled with [32P]Pi for 12 h and treated with noradrenaline (10 μM) in the presence of timolol (1 μM) and idazoxan (1 μM) for the indicated times. Cell lysates were prepared and immunoprecipitated with anti-Ha-Ras antibody. TLC was used to separate GTP and GDP; autoradiograms of TLC plates were exposed for 10 h. Ras-bound GTP (percentage of GTP + GDP) was calculated with a PhosphorImager system (Molecular Dynamics), and percentages are shown at the bottom. Experiments were repeated 3 times with essentially identical results. C, HVSMCs were labeled as in B. Inhibitors as indicated were added to dishes for 2 or 12 h (pertussis toxin; PTX) before stimulation of cells with noradrenaline for 10 min. Changes in Ras-bound GTP were measured as in B. The autoradiogram of TLC was exposed for 10 h. Experiments were repeated twice with essentially identical results. D, HVSMCs were metabolically labeled with [32P]Pi for 12 h and treated with noradrenaline (10 μM) in the presence of timolol (1 μM) and idazoxan (1 μM) for the indicated times. Cell lysates were prepared and immunoprecipitated with anti-p85 of PI 3-kinase and then resolved by SDS-polyacrylamide gel electrophoresis. The autoradiogram of the film was exposed for 16 h. Experiments were repeated twice with essentially identical results. This result indicates that noradrenaline treatment stimulated a phosphorylation of the p85 of PI 3-kinase as indicated by the arrow. E, cell lysates from control or noradrenaline-treated cells were immunoprecipitated with anti-p85 of PI 3-kinase and resolved by SDS-polyacrylamide gel electrophoresis. Blots were probed with an anti-phosphotyrosine antibody. Experiments were repeated twice with essentially identical results. This result suggests that noradrenaline stimulates tyrosine phosphorylation of p85 as indicated by the arrow.
Table II

PI 3-kinase activity in immunocomplexes precipitated by anti-p85α, anti-phosphotyrosine, and anti-Ras antibodies

| Immunoprecipitation by     | PI 3-kinase activity         |
|----------------------------|------------------------------|
|                            | Basal (10 μg)                |
| Anti-H-Ras                 | 1.5 ± 0.2                    |
| Anti-phosphotyrosine       | 1.3 ± 0.1                    |
| Anti-p85α                  | 2                            |

PI 3-kinase activity could be detected in both anti-Ras- and anti-phosphotyrosine immunocomplexes from noradrenaline-stimulated cells (Fig. 4A and Table II). These results demonstrate that both Ras protein and tyrosine proteins are associated with α1 receptor-activated PI 3-kinase in HVSMSs. However, these data do not provide the sequence of activation of PI 3-kinase, Ras protein, or tyrosine kinases by α1 receptors. It is known that PI 3-kinase may act at either downstream (Kodaki et al., 1994) or upstream of Ras protein (Rodriguez-Viciana et al., 1994) in other cells. We further investigated the interaction of PI 3-kinase and Ras protein in noradrenaline-stimulated HVSMSs; as illustrated in Fig. 4B, noradrenaline stimulated a time-dependent increase in Ras-bound GTP in the presence of antagonists of α2 and β receptors, suggesting that activation of α1 receptors stimulates an increase in the active Ras-GTP. On analysis of the temporal relationship between activation of PI 3-kinase (Fig. 2A) and increased active Ras protein (Fig. 4B), the active Ras-GTP appeared later than activation of PI 3-kinase, suggesting that Ras protein might function as a target of PI 3-kinase after stimulation of cells with noradrenaline. This possibility was supported by the fact that noradrenaline-stimulated increase in the active Ras-GTP could be partially blocked by the specific inhibitor of PI 3-kinase wortmannin as was terazosin, pertussis toxin, and genistein (Fig. 4C). We postulate that Ras protein is localized downstream of PI 3-kinase and functions as a target of PI 3-kinase. The definite interaction between the two important protein molecules in HVSMSs by activation of α1 receptors will require further investigation. Since increased activity of PI 3-kinase had been found in anti-phosphotyrosine protein immunocomplexes, cells were metabolically labeled with [32P]Pi, and stimulated with or without noradrenaline. Cell lysates from control and noradrenaline-treated cells were immunoprecipitated with antibodies against the p85 subunit of PI 3-kinase and then resolved by SDS-polyacrylamide gel electrophoresis. Noradrenaline-stimulated phosphorylation of the p85 subunit of PI 3-kinase (Fig. 4D, lanes 1-3). To determine if noradrenaline stimulated tyrosine phosphorylation of PI 3-kinase, cell lysates from control and noradrenaline-treated cells were immunoprecipitated with anti-p85 antibody and then detected by anti-phosphotyrosine antibody. Results indicated that noradrenaline stimulated a tyrosine phosphorylation of p85 itself (Fig. 4E, lanes 1-3).

In summary, the results demonstrate that α1 receptors expressed in HVSMSs are coupled to stimulation of PI 3-kinase via pertussis toxin-sensitive G proteins. Activation of PI 3-kinase by noradrenaline leads to association of the kinase with activation of Ras proteins and TPKs. The results highlight the potential importance of α1 receptors in the activation of PI 3-kinase, particularly concerning activation of mitogenes in vascular smooth muscle cells. Moreover, these results broaden concepts relating to interaction and cross-talk of α1 adrenergic receptors with families of tyrosine kinases.

Acknowledgments—We thank Robert J. Lefkowitz’s laboratory for providing anti-α1a and α1c receptor antibodies.

REFERENCES

Bokoch, G. M. (1995) Blood 86, 1649–1660
Carpenter, C. L., and Cantley, L. C. (1990) Biochemistry 29, 11147–11156
Cantley, L. C., Auger, K. R., Carpenter, C. L., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S. (1991) Cell 64, 281–302
Ding, J., Vlahos, C. J., Liu, R., Brown, R. F., and Badwey, J. A. (1995) J. Biol. Chem. 270, 11684–11691
Divecha, N., and Irvine, R. F. (1995) Cell 80, 269–278
Fanti, W. J., Escobedo, J. A., Martin, G. A., Turk, C. W., del Rosario, M., McCormick, F., and Williams, L. T. (1993) Cell 71, 413–423
Fry, M. J. (1994) Biochem. Biophys. Acta 1126, 237–268
Fukui, Y., Saltiel, A. R., and Hanauska, H. (1991) Oncogene 6, 407–411
Greenberg, S. M., Koo, E. H., Selkoe, D. J., Qiu, W. Q., and Kosik, K. S. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 10109–10113
Minneman, K. P., and Ebbesen, H. (1994) Ann. Rev. Pharmacol. Toxicol. 34, 117–133
Nakafuku, M., Satoh, T., and Kaziro, Y. (1992) J. Biol. Chem. 267, 19448–19454
Nakaki, T., Nakayama, M., Yamamoto, Y., and Kato, R. (1996) Mol. Pharmacol. 49, 30–36
Okazaki, M., Hu, Z. W., Fujinaga, M., and Hoffman, B. B. (1994) J. Clin. Invest. 94, 210–218
Panayotou, G., and Waterfield, M. D. (1993) Bioessays 15, 171–179
Perz, D. M., De’Young, M. B., and Graham, R. M. (1993) Mol. Pharmacol. 44, 784–795
Roche, S., Koepl, M., and Courtneidge, S. A. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 9185–9189
Rordo-Nikolic, T., Van Horn, D. J., Chen, D., White, M. F., and Backer, J. M. (1995) J. Biol. Chem. 270, 3662–3666
Rodriguez-Viciana, P., Warne, P. H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M. J., Waterfield, M. D., and Doward, J. (1994) Nature 370, 527–532
Ruderman, N. B., Kapeller, R., White, M. F., and Cantley, L. C. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 1411–1415
Sato, T., Endo, M., Nakafuku, M., Nakamura, S., and Kaziro, Y. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 5993–5997
Schwinn, D. A., Johnston, G. I., Page, S. O., Mosley, M. J., Wilson, K. H., Worman, N. P., Campbell, S., Fidock, M. D., Furness, L. M., Parry-Smith, D. J., Peter, B., and Bailey, D. S. (1995) J. Pharmacol. Exp. Ther. 272, 134–142
Stephens, L., Gquinou, A., Corey, S., Jackson, T., and Hawkins, P. T. (1993) EMBO J. 12, 2265–2273
Stoyanov, B., Vdinia, S., Hanck, T., Loubtchenkov, M., Malek, D., Stoyanova, S., Vanhaesebroeck, B., Dhand, R., Nürnberg, B., Gierschik, P., Seedor, K., Hsu, J. S., Waterfield, M. D., and Wetzker, R. (1995) Science 269, 690–693
Thorburn, J. G., Frost, J. A., and Thorburn, A. (1994) J. Cell Biol. 126, 1556–1572
Valius, M., and Kazlauska, A. (1993) Cell 73, 321–334
Van der Geer, P., and Hunter, T. (1991) Mol. Cell. Biol. 11, 4698–4709
Vartiovuori, L., Harrison-Findik, D., Keeler, M. L., and Suss, M. (1994) Biochem. Biophys. Acta 1226, 1–11
Vemuri, G. S., and Rittenhouse, S. E. (1994) Biochem. Biophys. Res. Commun. 202, 1619–1623
Yao, R., and Cooper, G. M. (1995) Science 267, 2003–2006
Yano, H., Nakashishi, K., Hanai, N., Satoh, Y., Fukui Y., Nomura Y., and Matsuda, Y. (1993) J. Biol. Chem. 268, 25846–25856
Zhang, J., Zhang, J., Benovic, J. L., Sugai, M., Wetzker, H., Gout, J., Rittenhouse, S. E. (1995) J. Biol. Chem. 270, 6589–6594