Angiotensin II transduces its signal to focal adhesions via angiotensin II type 1 receptors in vascular smooth muscle cells

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Abstract In cultured vascular smooth muscle cells (VSMCs), angiotensin II (Ang II) stimulated tyrosine phosphorylation of several proteins including a cluster of 70–80-kDa proteins as assessed by anti-phosphotyrosine immunoblotting. These 70–80-kDa proteins were identified as a focal adhesion-associated protein, paxillin, by anti-paxillin immunoprecipitation. Ang II-stimulated tyrosine phosphorylation of paxillin was detectable within 1 min and maximal at around 10 min and was concentration dependent (half-maximal effect at around 1 nM). Ang II also stimulated tyrosine phosphorylation of focal adhesion kinase in a time- and concentration-dependent manner. The Ang II type 1 (AT1) receptor antagonist, CV-11974, but not the Ang II type 2 receptor antagonist, PD123319, inhibited these reactions. These results indicate that Ang II transduces its signal to focal adhesions via AT1 receptors in cultured VSMCs.

Key words: Angiotensin II; Paxillin; Focal adhesion kinase; Tyrosine phosphorylation; Vascular smooth muscle cell

1. Introduction

Angiotensin II (Ang II), a potent vasoconstrictor, plays a pivotal role in the genesis of various vascular lesions which are characterized by vascular smooth muscle cell (VSMC) growth such as vascular remodeling due to chronic hypertension and myointimal proliferation following balloon angioplasty [1,2]. In a cell culture system, Ang II stimulates either hypertrophic [3] or hyperplastic [4,5] growth of VSMCs depending on the conditions. Ang II also has been shown to stimulate VSMC migration which is considered to play a major role in the genesis of intimal thickening following balloon angioplasty [6]. Although Ca^{2+}, calmodulin-dependent phosphorylation of myosin light chain is thought to play a critical role in VSMC contraction, molecular mechanisms responsible for growth-promoting and cell-migrating actions of Ang II have not been fully understood. Recently, several investigators have shown that Ang II stimulates tyrosine phosphorylation of intracellular proteins in VSMCs [7,8]. In past immunoblot experiments with anti-phosphotyrosine antibody, Ang II induces tyrosine phosphorylation of proteins with apparent molecular masses of 190, 100–120, 70–80, 45 and 40 kDa in VSMCs [7]. The 45- and 40-kDa proteins have been identified as mitogen-activated protein kinase isozymes [9]. These may be involved in the signaling mechanism from the Ang II receptor to the nucleus and play crucial roles in the growth-promoting action of Ang II. However, other proteins which are tyrosine-phosphorylated in response to Ang II have not been identified.

Focal adhesions are discrete regions of the plasma membrane in which the actin cytoskeleton is linked to the extracellular matrix via several cytoskeleton and membrane proteins. Focal adhesions are thought to play a central role in the processes that regulate cell adhesion, motility and proliferation [10]. Among a variety of proteins that localize to focal adhesions, paxillin is a 68-kDa protein which binds to vinculin, an actin-capping protein [11]. Paxillin has been shown to be tyrosine-phosphorylated during cell adhesion to the extracellular matrix [12] or in cells transformed by Rous sarcoma virus [13]. This suggests that tyrosine phosphorylation of paxillin may be involved in regulating functions of focal adhesions.

Recently, Rozengurt and his colleagues have shown that paxillin is tyrosine-phosphorylated in Swiss 3T3 cells stimulated with mitogenic neuropeptides such as bombesin, vasopressin or endothelin [14]. Since Ang II and these neuropeptides share the same intracellular signaling pathway including a rapid hydrolysis of phosphoinositides, the present study examined whether the 70–80-kDa proteins which were tyrosine-phosphorylated in response to Ang II may be paxillin. This report shows that these tyrosine-phosphorylated 70–80-kDa proteins are indeed paxillin and that Ang II stimulates paxillin tyrosine phosphorylation viaAng II type 1 (AT1) receptors. Evidence is also provided that Ang II stimulates tyrosine phosphorylation of focal adhesion kinase (FAK), a cytosolic tyrosine kinase which localizes to focal adhesions and may play an important role in the formation of focal adhesions and the actin cytoskeleton [15]. This report provides the first demonstration that Ang II transduces its signal to focal adhesions in VSMCs, one of the most important target cells of this peptide.

2. Materials and methods

2.1. Materials

VSMCs were isolated from rat thoracic aorta by enzymatic dissociation as described previously [7] and used at passage levels 8–16. Ang II and cytochalasin D were obtained from Sigma (St.Louis, MO, USA). The AT1 receptor antagonist, CV-11974, and the Ang II type 2 (AT2) receptor antagonist, PD123319, were gifts from Takeda Pharmaceutical Co., Ltd. (Osaka, Japan) and Parke-Davis, Warner-Lambert Co. (Ann Arbor, MI, USA), respectively. Mouse anti-phosphotyrosine monoclonal antibody (IgM) was prepared as described [7]. Mouse anti-paxillin and anti-FAK monoclonal antibodies were obtained from Transduction Laboratories (Lexington, KY, USA). Sheep anti-mouse IgG conjugated with peroxidase and goat anti-mouse IgM conjugated with peroxidase were from Amersham Life Science (Tokyo, Japan) and Cappel (West Chester, PA, USA), respectively. Protein A-Sepharose 4FF was from Pharmacia (Uppsala, Sweden). Affinity-purified rabbit anti-mouse IgG was from Jackson ImmunoResearch Laboratories, Inc.

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(West Grove, PA, USA). Other materials and chemicals were obtained from commercial sources.

2.2. Preparation of cell extracts and immunoprecipitates

Whole cell extracts were prepared as described previously [7] except that the lysis buffer containing 50 mM Hepes, 1% Triton X-100, 50 mM NaCl, 50 mM NaF, 10 mM sodium pyrophosphate, 5 mM ethylenediaminetetraacetic acid, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 100 kallikrein-inactivating U/ml of aprotinin and 20 μM leupeptin, pH 7.5 was used for FAK immunoprecipitation. They were normalized to protein content (Bio Rad) and divided into two parts. One was directly added to a 1/4 vol. 5× sodium dodecyl sulfate (SDS) sample buffer of Laemmli [16] and boiled at 100°C for 5 min. The other was added to either 1 μg anti-paxillin antibody or 2 μg anti-FAK antibody per 250 μg total proteins and rotated at 4°C overnight. Immunocomplexes were immunoprecipitated for 1 h at 4°C with Sepharose A-linked anti-mouse IgG. Immunoprecipitates were washed three times with lysis buffer, extracted in 100 μl 1× SDS sample buffer of Laemmli, and boiled at 100°C for 5 min.

2.3. Immunoblot analysis

Whole cell extracts and paxillin or FAK immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis (8–16% gradient gel). Each lane corresponded to 150 μg of the cell extract or the immunoprecipitate from 150 μg (paxillin) or 200 μg (FAK) of total proteins. The separated proteins were electrophoretically transferred to nitrocellulose membranes. Immunoblot analysis was performed as described previously [7] except that peroxidase-labeled sheep anti-mouse IgG was used as a second antibody for immunoblot analyses with anti-paxillin antibody or anti-FAK antibody. Stained protein bands were scanned with a Shimadzu Dual-Wavelength TLC Scanner CS-930 for quantification.

2.4. Statistics

Results of densitometric analysis are presented as mean ± standard error of the mean (S.E.M.). Differences between means were evaluated by *t*-test where appropriate. A value of $P < 0.05$ was taken to be significant.

3. Results

As has been reported previously [7], Ang II stimulated tyrosine phosphorylation of several proteins in VSMCs as assessed by anti-phosphotyrosine immunoblotting of whole cell extracts (Fig. 1A, lanes 1,2). To examine whether a cluster of 70–80-kDa proteins which were most heavily stained were related to paxillin, immunoprecipitation analysis with anti-paxillin antiboby was performed (Fig. 1A, lanes 3–6). All of the tyrosine-phosphorylated 70–80-kDa proteins were immunoprecipitated with anti-paxillin antibody. Ang II caused a striking increase in tyrosine phosphorylation of anti-paxillin immunoprecipitable bands (Fig. 1A, lanes 3,4). Parallel immunoblots with anti-paxillin antibody verified that similar amounts of paxillin were immunoprecipitated from VSMCs treated with or without Ang II (Fig. 1B). Based upon these results, it was concluded that most of a cluster of 70–80-kDa proteins which were tyrosine-phosphorylated in response to Ang II were paxillin. A diffuse band on reduced SDS gels may be due to its multiply phosphorylated forms.

Although the basal level of paxillin tyrosine phosphorylation somewhat differed from experiment to experiment, Ang II consistently stimulated this reaction. Ang II-stimulated paxillin phosphorylation was detected within 1 min after the addition of Ang II, reached a maximum at around 10 min and then declined slowly (Fig. 2A). Ang II-stimulated paxillin phosphorylation was concentration dependent with half-maximum and maximum effects obtained at around 1 and 100 nM, respectively (Fig. 2B). Immunoblotting with anti-paxillin antibody confirmed that similar amounts of paxillin were recovered after different times and concentrations of Ang II treatment (data not shown).

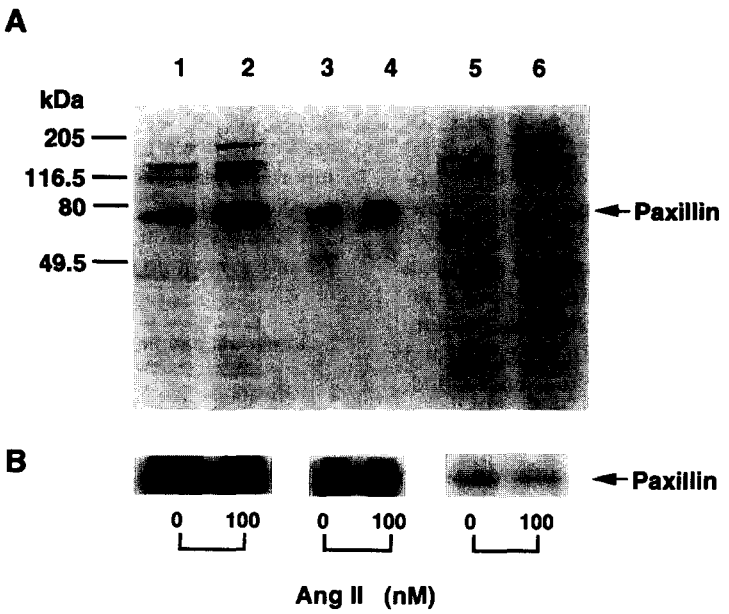

Fig. 1. Ang II-stimulated tyrosine phosphorylation of paxillin. Cultured VSMCs were treated without (lanes 1,3,5) or with 100 nM Ang II (lanes 2,4,6) for 5 min at 37°C. Cell extracts were subjected to immunoprecipitation analysis with anti-paxillin antibody. Whole cell extracts (lanes 1,2), paxillin immunoprecipitates (lanes 3,4) and supernatants after immunoprecipitation (lanes 5,6) were analyzed by immunoblotting with anti-phosphotyrosine (A) or anti-paxillin antibody (B). The experiment shown represents one of three independent trials which gave nearly identical results.

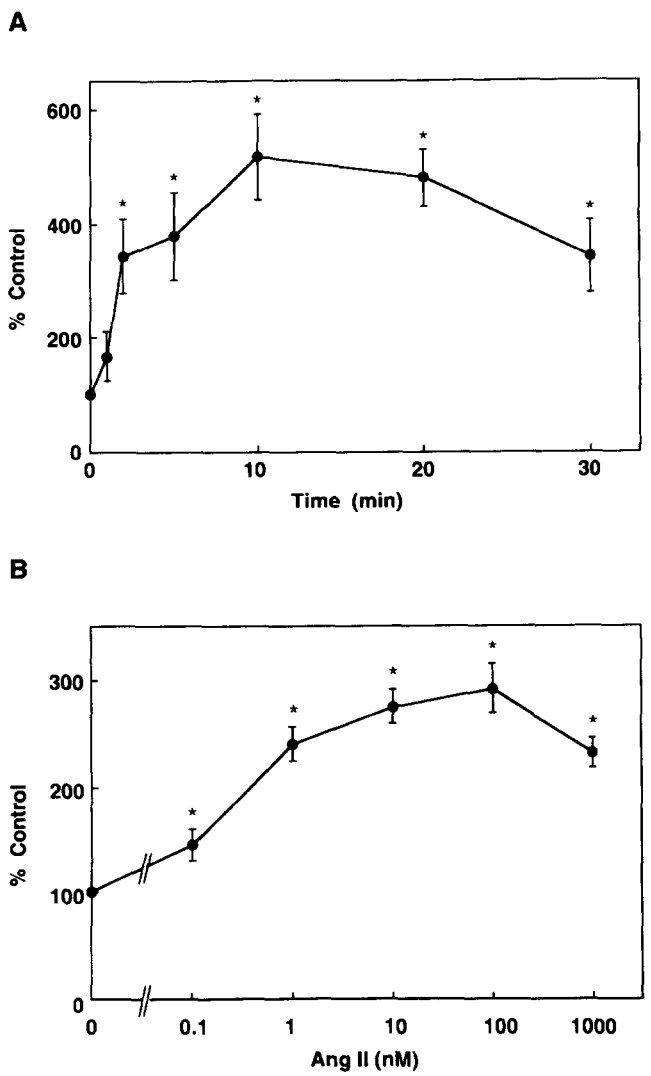

Fig. 2. Time- and concentration-dependent increase of Ang II-stimulated tyrosine phosphorylation of paxillin. Cultured VSMCs were treated for various times with 100 nM Ang II at 37°C (A) or treated for 5 min at 37°C with various concentrations of Ang II as indicated (B). Tyrosine-phosphorylated paxillin was analyzed by immunoblotting of anti-paxillin immunoprecipitates with anti-phosphotyrosine antibody. Quantification of phosphorylation was performed by scanning densitometry. Values shown are the mean ± S.E.M. of four independent trials and are expressed as the percentage of the control unstimulated levels. $*P < 0.05$ vs control.

Ang II receptors have been classified into AT1 and AT2 subtypes [17]. Ang II-stimulated tyrosine phosphorylation of paxillin was completely inhibited by the AT1 receptor antagonist, CV-11974, but not by the AT2 receptor antagonist, PD123319 (Fig. 3). Both antagonists by themselves had no effect on this reaction (data not shown). These results indicate that Ang II stimulates paxillin tyrosine phosphorylation via AT1 receptors.

In the last set of experiments, it was examined whether Ang II stimulated tyrosine phosphorylation of FAK. Ang II rapidly stimulated tyrosine phosphorylation of FAK as assessed by anti-phosphotyrosine immunoblotting of anti-FAK immunoprecipitates. An increase was detected within 1 min after the addition of Ang II and reached a maximum within 5 min (Fig. 4A). The concentrations of Ang II required for the stimulation of FAK tyrosine phosphorylation were roughly the same as those required for the stimulation of paxillin tyrosine phosphorylation (Fig. 4B). Ang II-stimulated tyrosine phosphorylation of FAK was also inhibited by CV-11974 but not by PD123319 (Fig. 5). Both antagonists by themselves had no effect on FAK tyrosine phosphorylation (data not shown).

4. Discussion

The results of the present study clearly demonstrated that Ang II stimulated tyrosine phosphorylation of paxillin and FAK via AT1 receptors in VSMCs. The precise nature of the signaling mechanisms involved have not yet been elucidated. Since the AT1-receptor is coupled to phospholipase C-mediated phosphoinositide hydrolysis [18], it is possible that this signaling pathway is involved in the stimulatory effect of

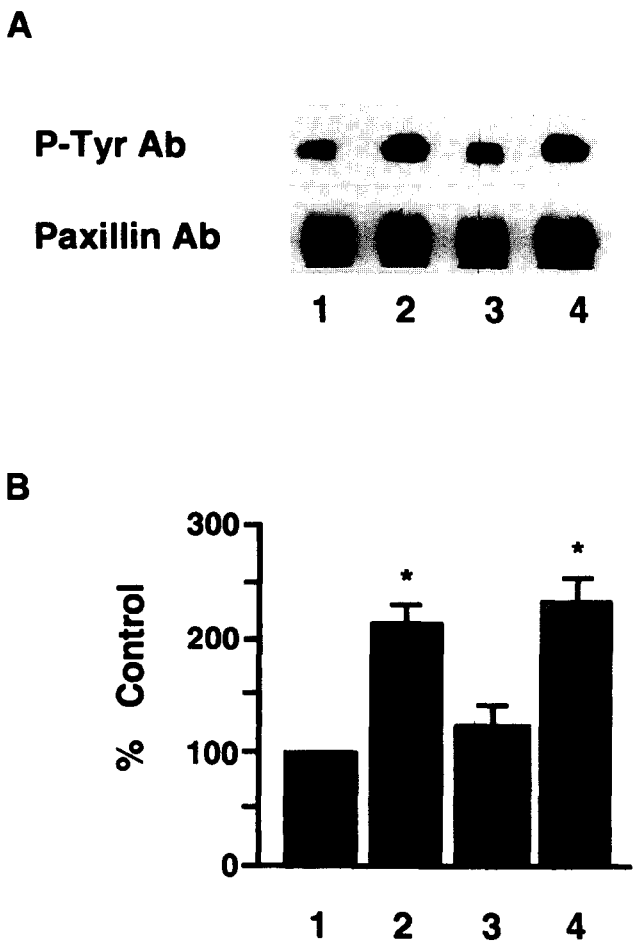

Fig. 3. AT1 receptor-mediated stimulation of paxillin tyrosine phosphorylation. Cultured VSMCs were preincubated without (lanes 1,2) or with 100 nM CV-11974 (lane 3) or 100 nM PD123319 (lane 4) for 2 min at 37°C. Cells were subsequently treated without (lane 1) or with 100 nM Ang II for 5 min (lanes 2–4). Paxillin was analyzed by immunoblotting of anti-paxillin immunoprecipitates with anti-phosphotyrosine antibody (P-Tyr Ab) or anti-paxillin antibody (paxillin Ab) (A). The experiment shown represents one of three independent trials which gave nearly identical results. The bar graph shows densitometric analysis of paxillin tyrosine phosphorylation (B). Values shown are the mean ± S.E.M. of three independent trials and are expressed as the percentage of the control unstimulated levels. $*P < 0.05$ vs control.

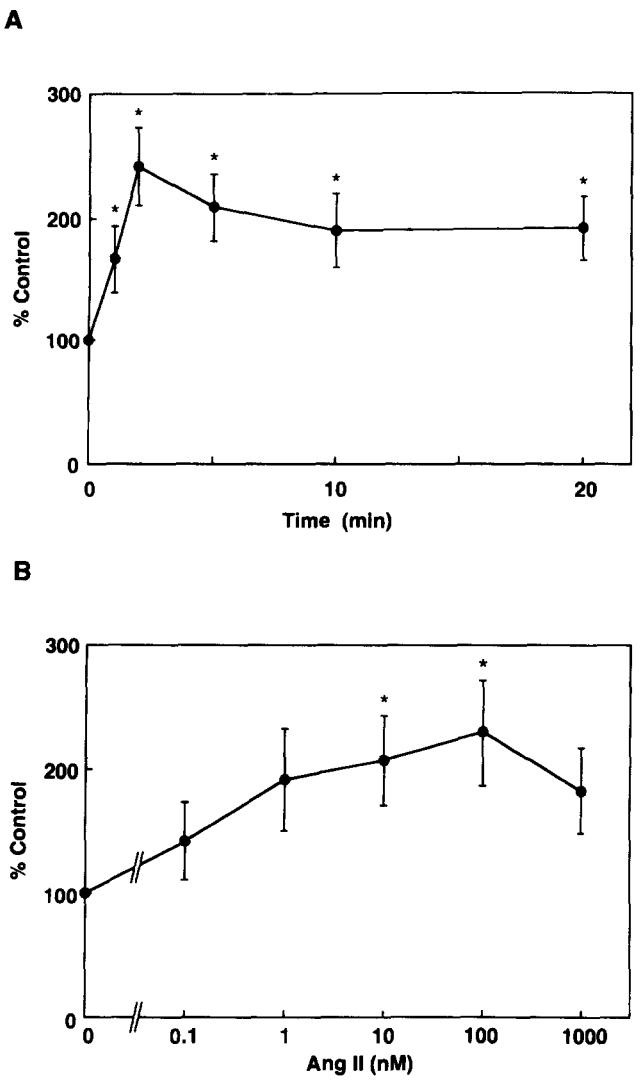

Fig. 4. Time- and concentration-dependent increase of Ang II-stimulated tyrosine phosphorylation of FAK. Cultured VSMCs were treated for various times with 100 nM Ang II at 37°C (A) or treated for 2 min at 37°C with various concentrations of Ang II as indicated (B). Tyrosine-phosphorylated FAK was analyzed by immunoblotting of anti-FAK immunoprecipitates with anti-phosphotyrosine antibody. Quantification of phosphorylation was performed by scanning densitometry. Values shown are the mean ± S.E.M. of three independent trials and are expressed as the percentage of the control unstimulated levels. $^*P < 0.05$ vs control.

Ang II on tyrosine phosphorylation of paxillin and FAK. In agreement with this possibility, other vasoconstrictors, such as vasopressin, 5-hydroxytryptamine, norepinephrine and endothelin-1 which also cause phosphoinositide hydrolysis, stimulated tyrosine phosphorylation of paxillin as judged by immunoblotting with anti-phosphotyrosine antibody of whole cell extracts [7,19]. On the other hand, Zachary et al. [14,20] reported that cytochalasin D, an agent that selectively disrupts the network of actin microfilaments, completely inhibited bombesin-induced tyrosine phosphorylation of paxillin and FAK in Swiss 3T3 cells. *Rho* p21 is a member of the *ras* superfamily of small GTP-binding proteins and has been implicated in the regulation of the actin cytoskeleton [21]. It has been reported that the *Clostridium botulinum* C3 exoenzyme pretreatment, which inactivates *rho* p21 by ADP ribosylation [22], suppressed bombesin- and endothelin-induced tyrosine phosphorylation of paxillin and FAK in Swiss 3T3 cells [23]. Pretreatment of VSMCs with either cytochalasin D or C3 exoenzyme also inhibited Ang II-induced paxillin tyrosine phosphorylation in our system (data not shown). These observations suggest that the integrity of the actin cytoskeleton may be essential for and *rho* p21 may be involved in the signaling mechanisms from the AT1 receptor as well as the bombesin receptor to focal adhesions.

Previously, we reported that Ang II caused tyrosine phosphorylation of proteins with molecular masses of 190, 100–120, 70–80 (paxillin), 45 and 40 kDa (MAP kinases) in VSMCs [7]. We investigated whether there were any proteins tyrosine-

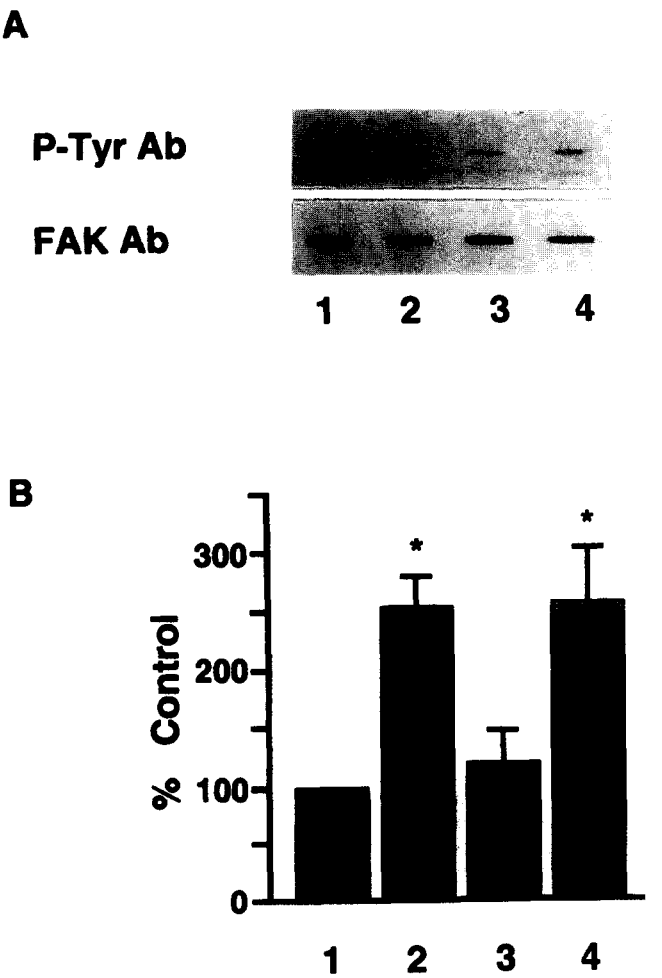

Fig. 5. AT1 receptor-mediated stimulation of FAK tyrosine phosphorylation. Cultured VSMCs were preincubated without (lanes 1,2) or with 100 nM CV-11974 (lane 3) or 100 nM PD123319 (lane 4) for 2 min at 37°C. Cells were subsequently treated without (lane 1) or with 100 nM Ang II for 2 min (lanes 2–4). FAK tyrosine phosphorylation and amounts of FAK recovered after immunoprecipitation were analyzed by immunoblotting of anti-FAK immunoprecipitates with anti-phosphotyrosine antibody (P-Tyr Ab) and anti-FAK antibody (FAK Ab), respectively. The experiment shown represents one of three independent trials which gave nearly identical results. The bar graph shows densitometric analysis of FAK tyrosine phosphorylation (B). Values shown are the mean ± S.E.M. of three independent trials and are expressed as the percentage of the control unstimulated levels. $^*P < 0.05$ vs control.

phosphorylated in response to the AT2 receptor stimulation in VSMCs. As judged by immunoblotting with anti-phosphotyrosine of whole cell extracts, however, tyrosine phosphorylation of all of these proteins was not inhibited by the AT2 receptor antagonist but by the AT1 receptor antagonist, indicating that the AT2 receptor stimulation is not involved in Ang II-induced tyrosine phosphorylation at least in our system.

The physiological significance of tyrosine phosphorylation of paxillin and FAK is unknown at present. The localization of paxillin and its binding to vinculin suggest that paxillin has a potential role in the processes that regulate cell adhesion, motility and proliferation via the rearrangement of the actin cytoskeleton. Tyrosine phosporylation of FAK is also considered to be involved in regulating functions of focal adhesions [12]. Ang II-induced tyrosine phosphorylation of paxillin and/or FAK could be instrumental in triggering the assembly or disassembly of the actin cytoskeleton and contribute to growth promoting and/or cell migrating actions of Ang II. In VSMCs, paxillin also localizes to the dense plaques where the actin cytoskeleton interacts with the surrounding extracellular matrix to transmit force generated during muscle contraction across the membrane [24]. It has been suggested that the transitory rearrangement of the actin filaments is involved in the tonic contraction of VSMCs [25]. Therefore, it is possible that via the rearrangement of the actin cytoskeleton, paxillin tyrosine phosphorylation may play some role in the vasoconstricting action of Ang II. Further studies are clearly needed to elucidate the roles of tyrosine phosphorylation of paxillin and FAK in various actions of Ang II.

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