Articles

Phosphorylation Site-Specific Inhibition of Platelet-Derived Growth Factor β-Receptor Autophosphorylation by the Receptor Blocking Tyrphostin AG1296

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ABSTRACT: The mechanism of action of AG1296, a potent and specific inhibitor of the platelet-derived growth factor (PDGF) receptor tyrosine kinase [Kovalenko, M., Gazit, A., Böhmer, A., Rorsman, Ch., Rönnstrand, L., Heldin, C.-H., Waltenberger, J., Böhmer, F. D., & Levitzki, A. (1994) *Cancer Res. 54*, 6106−6114] was investigated. This quinoxalin-type tyrphostin neither interferes with PDGF-BB binding to the PDGF β-receptor nor has any effect on receptor dimerization. Kinetic analysis of the inhibition was carried out using a synthetic peptide substrate (KY751) corresponding to the sequence around tyrosine 751 autophosphorylation site of the PDGF receptor. It revealed purely competitive inhibition vis-à-vis ATP, mixed competitive inhibition vis-à-vis the peptide substrate for the non-activated receptor, and mixed competitive inhibition vis-à-vis both substrates for the activated receptor. Thus, the type of inhibition apparently changes upon receptor activation, indicating conformational changes at the ATP-binding site. The high degree of selectivity for the tyrphostin AG1296 might result from the complex type of interaction with the active center of the receptor as revealed by the kinetic analysis. Dose−response curves for inhibition of the phosphorylation of individual autophosphorylation sites of the PDGF β-receptor by AG1296 were different, phosphorylation of tyrosine 857 being the most susceptible to inhibition. Thus, phosphorylation of tyrosine 857 in the PDGF receptor kinase domain seems dispensable for partial kinase activation. The findings are discussed in relation to current models of receptor tyrosine kinase activation.

PDGF[1] and its receptor have been functionally implicated in numerous pathological conditions involving chronic inflammation and connective tissue overgrowth such as atherosclerosis and restenosis (Ross, 1990, 1993; Cercek et al., 1991; Pickering et al., 1993; Tanizawa et al., 1996; Uchida et al., 1996), glomerular nephritis (Floege & Johnson, 1995), rheumatoid arthritis (Rubin et al., 1988; Endresen & Forre, 1992), and fibrotic diseases (Crystal et al., 1984; Pinzani et al., 1996). Furthermore, data are being accumulated which demonstrate the involvement of PDGF in various malignancies. These include glioma and glioblastoma (Hermanson et al., 1992; Shamah et al., 1993; Nistér et al., 1994; Nitta & Sato, 1994; Strawn et al., 1994; Guha et al., 1995) and soft tissue tumors (Wang et al., 1994), where PDGF might contribute to tumor growth in an autocrine manner, as well as mammary carcinoma (Seymour et al., 1993; Bhardwaj et al., 1996) and colon carcinoma (Lindmark et al., 1993), where a paracrine role of PDGF for tumor growth has been suggested.

The involvement of the activation of the PDGF receptor in various disease states has prompted the search for PDGF receptor antagonists. The design of low molecular weight inhibitors of the PDGF receptor tyrosine kinase represents a promising strategy toward the goal of a specific interference with unwanted PDGF receptor signaling (Bilder et al., 1991; Bryckaert et al., 1992; Levitzki & Gazit, 1995). Two groups of compounds which inhibit the PDGF receptor activation in intact cells potently and with a high degree of specificity have recently been described: CGP 53716, a phenylaminopyrimidine derivative (Buchdunger et al., 1995), and AG1295 and 1296, tyrophostins with a quinoxalin core structure, developed in our laboratories (Kovalenko et al., 1994; Gazit et al., 1996). Both compounds abrogate the autocrine PDGF receptor activation in *sis*-transformed NIH 3T3 cells and reverse the transformed phenotype. Also, AG1295 inhibits selectively the outgrowth of smooth muscle

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[1] Abbreviations: BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; DSS, disuccinimidyl suberate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; EGTA, (ethylenebis(oxyethylenenitrilo))tetraacetic acid; FCS, fetal calf serum; FPLC, fast protein liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PAE, porcine aortic endothelial cells; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PI3-kinase, phosphoinositide 3-kinase; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TLC, thin-layer chromatography; WGA, wheat germ agglutinin.

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cells from arterial explants (Banai et al., 1996) and is therefore currently being tested for its effectiveness in preventing restenosis in experimental models *in vivo* (S. Banai and A. Levitzki, personal communication).

In the present study, the mechanism of interference of AG1296 with the activity of the PDGF receptor tyrosine kinase has been investigated. We find that AG1296 is an ATP-competitive inhibitor of the receptor kinase; however, the exact kinetic type of inhibition depends on the receptor activation state. Receptor autophosphorylation is affected by AG1296 differently at different phosphorylation sites. The data raise interesting questions with respect to the mechanism of PDGF receptor kinase activation and may prove to be useful for further development of specific receptor tyrosine kinase inhibitors.

EXPERIMENTAL PROCEDURES

Cells and Reagents. Canine kidney epithelial (TRMP) cells stably expressing human PDGF β-receptors (a generous gift of Dr. A. Kazlauskas, Denver) have been described previously (Valius & Kazlauskas, 1993) and were grown in DMEM, supplemented with 4 g/L glucose, glutamine, antibiotics, and 10% FCS. Porcine aortic endothelial (PAE) cells stably expressing human PDGF β-receptors were cultured in Ham's F-12 medium supplemented with geneticin (0.4 mg/mL) and 10% FCS. All cell culture reagents were from Gibco. PDGF was the recombinant human BB-homodimer and was obtained from Biomol (Hamburg). The anti-PDGF receptor antiserum DIG1 was raised against a peptide corresponding to amino acid residues 1075−1089 in the human PDGF α-receptor but recognized PDGF α- and β-receptors equally well (L. J. Gonez, unpublished data). The antiserum PDGFR3 against PDGF receptor has been described (Claesson-Welsh et al., 1989). [γ^{32}P]ATP was purchased from DuPont/NEN (Dreieich, Germany). Peptides KY751 and KY857 were synthesized by U. Engström (Uppsala). KY751 was alternatively obtained from BioTeZ GmbH (Berlin). DSS, *N*-acetylglucosamine, phenylphosphate, and anti-phosphotyrosine agarose were from Sigma (Deisenhofen). WGA-Sepharose was from Pharmacia (Uppsala).

PDGF Binding Assay. PDGF binding measurements using PDGF β-receptor-expressing PAE cells were performed as described earlier (Sorkin et al., 1991). Cells were grown in 12-well plates (Falcon) and pre-incubated with vehicle (DMSO) or different concentrations of AG1296. Thereafter, the cells were washed once with binding buffer (PBS containing 1 mg/mL BSA), and ^{125}I-labeled PDGF-BB (38 000 cpm/well, approximately 1 ng of PDGF/mL) was added in a new portion of binding buffer (450 μL) together with DMSO or different concentrations of AG1296. After 1 h of incubation at 4 °C with occasional shaking, the cells were washed five times with 1 mL of binding buffer and lysed in 0.5 mL/well lysis buffer containing 20 mM Tris-HCl, pH 7.5, 1% Triton X-100, and 10% glycerol by 30 min incubation at room temperature. The lysates were then transferred to scintillation tubes, and the radioactivity was measured with a γ-counter.

Preparation of Membranes from TRMP Cells. Membranes were prepared from confluent cultures of TRMP cells grown in 625-cm^2 square plates (Nunc). The cells were pre-incubated twice in serum-free DMEM for 30 min (at 37 °C), washed twice with buffer A (40 mM HEPES, pH 7.4, 150 mM NaCl), and scraped off in 15−20 mL of buffer A per plate. After centrifugation for 5 min at 1000*g*, the cells were suspended in buffer A (2−4 mL/plate), placed on ice, and disrupted by ultrasound. Immediately after sonication, EGTA (1 mM), DTT (0.5 mM), and protease inhibitors (1 mM PMSF, 1 μg/mL leupeptin, 5 mM benzamidine, and 1% Trasylol) were added. The homogenate was centrifuged for 30 min at 30000*g*. After the supernatant had been discarded, the pellet was transferred to a Potter-Elvehjem homogenizer, suspended in cold buffer B (10−15 mL/plate) containing 40 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and then centrifuged. The pellet was washed again with cold buffer B and finally suspended in 1−2 mL of buffer B per plate. After protein determination (Bradford, 1976), glycerol was added to a final concentration of 20%, and the membranes were stored at −80 °C.

Partial Purification of the PDGF β-Receptor. The purification procedure was based on that described by Rönnstrand et al. (1987) with some modifications. At all stages, the PDGF receptor was monitored by autophosphorylation assay (see below), by immunoblotting with DIG1 antiserum, and by SDS−PAGE with subsequent gel silver staining. The whole procedure was performed at 0−4 °C. TRMP cell membranes (200−250 mg of protein, from thirty 625-cm^2 plates of confluent cells) were thawed and washed with buffer B (total volume 260 mL). After centrifugation at 30000*g* for 30 min, the membrane pellet was solubilized in 35−40 mL of buffer containing 40 mM HEPES, pH 7.4, 0.5 M NaCl, 1% Triton X-100, 1 mM EGTA, 1 mM DTT, 10% glycerol, and protease inhibitors (1 mM PMSF, 1 μg/mL leupeptin, 5 mM benzamidine, 1% Trasylol) by incubation for 40 min with constant end-over-end rotation and subsequent centrifugation at 100000*g* for 30 min. The supernatant was filtered through nylon gauze and applied to a 1.5-mL WGA-Sepharose column. The column was washed with 6−8 mL of 40 mM HEPES, pH 7.4, 0.5 M NaCl, 0.05% Triton X-100, 1 mM EGTA, 1 mM DTT, 10% glycerol, and protease inhibitors (see above) and eluted with 4 mL of the same buffer containing 0.3 M *N*-acetylglucosamine. A 2-mL FPLC MonoQ column was equilibrated with buffer C (20 mM HEPES, pH 7.4, 0.2% Triton X-100, 1 mM EGTA, 1 mM DTT, 1% glycerol). The eluate from the WGA-Sepharose column was diluted with 4 vol of buffer C and applied to the MonoQ at a flow rate of 0.75 mL/min. The column was washed with 5 vol (10 mL) of buffer C and eluted with a gradient of NaCl (0−0.5 M) in the same buffer (total gradient volume 12 mL, flow rate 0.75 mL/min, fraction volume 0.4 mL). Fractions were analyzed for the PDGF receptor content by PDGF-stimulated autophosphorylation. For this, aliquots of the PDGF receptor-containing fractions (5 μL) were incubated with 2 μg/mL PDGF-BB in 40 mM HEPES, pH 7.4, 5 mM $MnCl_2$, 0.5 mM DTT, for 20 min on ice. The phosphorylation was then started by the addition of [γ^{32}P]ATP (2 μM, 5 μCi), continued for 10 min, and terminated with SDS−PAGE sample buffer. The samples were analyzed by SDS−PAGE and autoradiography.

PDGF Receptor Dimerization. Partially purified PDGF receptor was pre-incubated with DMSO or 50 μM AG1296 (final DMSO concentration 1%) for 15 min at room temperature in 20 mM HEPES, pH 7.4, 3 mM $MnCl_2$, and 0.5 mM DTT. Thereafter, the samples were transferred onto ice, PDGF-BB was added to a final concentration of 0.5 μg/

mL where necessary, and the incubation was continued for another 20 min. Kinase reaction was then started by the addition of [γ^{32}P]ATP (2.5 μCi; 15 μM). After 10 min of phosphorylation on ice, nonlabeled ATP and phenylphosphate were added (final concentrations 1.5 and 4 mM, respectively) to terminate ^{32}P incorporation, and the samples were treated with 0.2 mM DSS or vehicle (DMSO, final concentration 4%) for 30 min at room temperature. Crosslinking was stopped with 50 mM methylamine, and the samples were treated with SDS−PAGE sample buffer and subjected to SDS−PAGE and autoradiography. For immunoblotting with DIG1 antiserum, the procedure was done in the same way except the phosphorylation was carried out in the presence of unlabeled ATP only (15 μM).

Establishing the System for Kinetic Analysis. We tested a peptide corresponding to residues 744−759 from the kinase insert of PDGF β-receptor containing the autophosphorylation site Y751 for its suitability for kinetic studies. Four lysine residues were added to the N-terminus of the peptide to enable its binding to phosphocellulose paper. The resulting peptide KKKKSKDESVDYVPMLDMKG was designated KY751. The peptide KY751 was readily phosphorylated by partially purified PDGF β-receptor. Phosphorylation was stimulated by PDGF, and AG1296 blocked the phosphorylation of the peptide completely (not shown). According to these results, the peptide KY751 was considered to be an exogenous substrate for the PDGF receptor suitable for the kinetic analysis. Another question addressed concerned the suitability of partially purified PDGF β-receptor for the kinetic experiments. This preparation was obtained from membranes of canine kidney epithelial cells expressing human PDGF β-receptor as described above. We checked for the possible presence of other kinase(s) in the preparation which could also be inhibited by AG1296. The reversibility of AG1296 inhibition prompted us to apply it in an analytical purification procedure designed in such a way that only kinase(s) inhibited by the compound would appear in the final preparation. This purification includes the following steps: (i) Phosphorylation of pooled PDGF receptor-containing fractions from the MonoQ column in the presence of AG1296 (under these conditions autophosphorylation of kinases not inhibited by AG1296 can occur); (ii) affinity chromatography over an anti-phosphotyrosine agarose column (tyrosine-phosphorylated proteins will bind, PDGF receptor is in the flow-through); (iii) phosphorylation of dialyzed flow-through from step ii in the absence of AG1296 (now PDGF receptor autophosphorylates); (iv) step ii repeated and eluate containing autophosphorylated PDGF receptor collected. In this way, the PDGF receptor (as identified by molecular weight, immunoblotting, and PDGF-stimulated autophosphorylation) was purified to near-homogeneity (data not shown, details of this method to be published elsewhere). This indicates that the PDGF receptor preparation obtained after the MonoQ step does not contain significant amounts of other kinase(s) which is (are) subject to inhibition by the compound except the PDGF receptor itself. Thus, we considered it possible to use easily obtainable and highly active partially purified PDGF receptor for studying the mechanism of its inhibition by AG1296.

Kinetics of the Inhibition of KY751 and KY857 Phosphorylation. Partially purified PDGF β-receptor was preincubated with or without 1 μg/mL PDGF-BB in 20 mM HEPES, pH 7.4, 5 mM $MnCl_2$, 0.5 mM DTT, 0.05% Triton X-100 for 20 min on ice. The assay was initiated by the addition of 8.5 μL of the activated PDGF receptor solution to a 6.5-μL reaction mixture containing 100 mM HEPES, pH 7.4, different concentrations of AG1296 or vehicle (DMSO; final concentration 2%), the substrate peptide KY751 or KY857, ATP, and 10 μCi of [γ^{32}P]ATP. When concentration of the peptide was varied, ATP was taken at 400 μM (2.4K_m) for nonstimulated receptor and at 200 μM (4K_m) for the PDGF-stimulated receptor. In the assays with variable ATP concentrations, KY751 was always 3 mM (2−3K_m). The assay was performed for 10 min on ice and terminated by the addition of 15 μL of EDTA/BSA solution (final concentrations 5 mM and 0.5 mg/mL, respectively). Thereafter, 10 μL of 40% TCA was added (final concentration 10%), and the samples were left for 1 h on ice and then centrifuged for 10 min in a microfuge (high speed). Aliquots of the supernatant (25 μL) were applied to phosphocellulose paper. After extensive washing in 75 mM H_3PO_4, the phosphocellulose sheets were rinsed with acetone and dried, and the radioactivity was quantified with a phosphorimager. Data were analyzed with the Enzyme Kinetics program (Trinity Software, Campton, NH) (best-fitting of curves, Lineweaver−Burk plots and calculation of the kinetic parameters) and with the EKI-Programm, described by Bisswanger (1994) (determination of the type of inhibition).

Tryptic Phosphopeptide Mapping of the PDGF Receptor. Aliquots of the partially purified PDGF receptor were preincubated for 15 min with DMSO or different concentrations of AG1296 (final DMSO concentration 1%) in 40 mM HEPES, pH 7.4, 5 mM $MnCl_2$, 0.5 mM DTT (in the absence or presence of 0.1 mM sodium orthovanadate, three experiments and one experiment, respectively) and were then stimulated with 1 μg/mL PDGF-BB for 20 min and phosphorylated in the presence of [γ^{32}P]ATP (50 μM, 15 μCi) for 10 min on ice. The reaction was stopped by SDS−PAGE sample buffer. The samples were subjected to SDS−PAGE (6.5% polyacrylamide gel) and transferred to Hybond C nitrocellulose (Amersham). The position of phosphorylated PDGF receptor bands was visualized with a phosphorimager. The bands were excised and processed as described by Boyle et al. (1991). Tryptic phosphopeptides were separated by thin-layer electrophoresis (first dimension: 40 min at 2000 V) and ascending chromatography (second dimension: overnight in isobutyric acid:*n*-butanol:pyridine:acetic acid:H_2O, 1250:38:96:58:558, v/v/v/v/v). Incorporation of ^{32}P was analyzed with the phosphorimager.

PI3-Kinase Activity Assays. PAE cells expressing human PDGF β-receptor were grown in 25 cm^2 flasks (Nunc) to confluency and incubated overnight in serum-free Ham's F-12 medium. AG1296 was added at concentrations ranging from 0 to 50 μM (final DMSO concentration 0.5%) and the incubation was continued for 4 h. The cells were then stimulated with 100 ng/mL PDGF-BB for 5 min at room temperature, washed twice with ice-cold PBS and lysed in 0.3 mL of lysis buffer containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10 mM sodium pyrophosphate, 50 mM NaF, 2 mM sodium orthovanadate, 10 mM EDTA, 2 mM EGTA, 1 mM PMSF, and 5 μg/mL leupeptin per flask. The cell lysates were clarified by centrifugation, and PDGF β-receptor was immunoprecipitated with PDGFR3 antiserum for 1 h at 4 °C. The immunocomplexes were collected with 15 μL of protein A-Sepharose and washed 4 times with lysis buffer and once with PI3-kinase assay buffer,

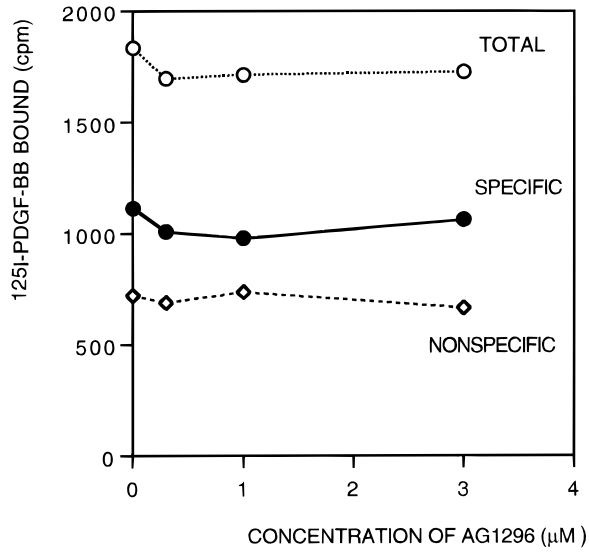

FIGURE 1: Effect of tyrphostin AG1296 on the binding of PDGF-BB to the PDGF β-receptor. PAE cells expressing PDGF β-receptor were preincubated with DMSO or different concentrations of AG1296. Cells were then incubated with ^{125}I-labeled PDGF-BB in the absence (total, ○) or presence (nonspecific, ◇) of 500 ng/mL of unlabeled PDGF-BB. Thereafter, the cells were washed and cell-associated radioactivity was measured. Specific PDGF binding (●) was calculated as the difference between total and nonspecific binding.

which contained 20 mM Tris-HCl, pH 7.4, 4 mM $MgCl_2$, and 100 mM NaCl. The immunoprecipitates were suspended in the PI3-kinase assay buffer, and the kinase reaction was performed in the presence of 130 μM sonicated phosphatidylinositol (Sigma) and 10 μCi [γ^{32}P]ATP (240 μM). The total reaction volume was 75 μL. To test the direct effect of AG1296 on the PI3-kinase activity, 1 ng of pure recombinant p110α (kindly provided by Dr. M. Waterfield, London) was incubated in the same reaction mixture in the presence of different concentrations of the inhibitor (from 0 to 50 μM; final DMSO concentration 1%). The reaction was continued for 15 min at room temperature and terminated by the addition of 150 μL of 1 M HCl. Lipids were then extracted from the reaction mixture with 450 μL of chloroform:methanol (1:1, v/v). The organic phase was washed twice with 200 μL of 1 M HCl, and 100-μL aliquots were loaded onto a TLC plate (silica gel, from Merck). TLC was run for 2 h in [2 M acetic acid]:[*n*-propanol] (35:65, v/v), and the plate was dried and analyzed by autoradiography.

RESULTS

Effect of AG1296 on PDGF Binding. The tyrphostin AG1296 is known to potently inhibit PDGF receptor signaling in membrane preparations and in intact cells; however, its exact mechanism of action is not known. We first addressed the question whether it would interfere with binding of PDGF-BB to the PDGF β-receptor. PDGF binding assays were performed on porcine aortic endothelial (PAE) cells transfected with PDGF β-receptor cDNA and expressing the receptor on the cell surface. The cells were preincubated with different concentrations of AG1296 and then equilibrated with ^{125}I-labeled PDGF-BB at 4 °C. Nonspecific binding was determined in the presence of 100-fold excess of unlabeled PDGF. As shown in Figure 1, cell-associated radioactivity did not change with increasing AG1296 concentrations, indicating that the ability of the

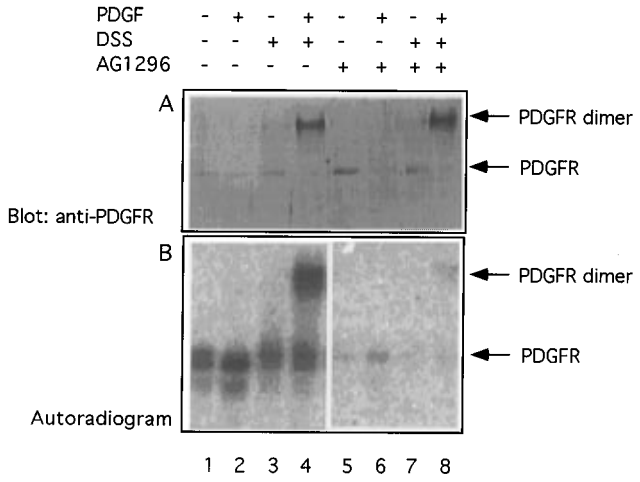

FIGURE 2: Effect of tyrphostin AG1296 on dimerization of the PDGF receptor. Partially purified PDGF β-receptor was incubated in the absence or in the presence of 50 μM AG1296 (lanes 4−8), 0.5 μg/mL PDGF-BB (lanes 2, 4, 6, and 8), and DSS (lanes 3, 4, 7, and 8), added sequentially. Samples were analyzed by SDS−PAGE and immunoblotting with anti-PDGF receptor antiserum DIG1 (A) or by autoradiography (B). Positions of monomeric and dimeric, cross-linked, PDGF receptor are indicated.

PDGF β-receptor to bind PDGF-BB was not affected by the compound.

Effect of AG1296 on Dimerization of PDGF Receptor. Upon PDGF stimulation two receptor molecules dimerize and the receptor dimer can be visualized after covalent crosslinking as a high molecular weight band in SDS−PAGE (Heldin et al., 1989). To analyze the effect of AG1296 on PDGF receptor dimerization, partially purified PDGF β-receptor was incubated either in the absence or in the presence of 50 μM AG1296, 1 μg/mL PDGF-BB, and 0.2 mM DSS, added one after another. Two series of samples were prepared in parallel in this way. One series was phosphorylated in the presence of [γ^{32}P]ATP before cross-linking to monitor receptor autophosphorylation, and another was phosphorylated with non-radioactive ATP and analyzed by Western blotting with anti-PDGF receptor antiserum DIG1. On the autoradiogram (Figure 2B), in the absence of AG1296, the PDGF-induced dimerization of the receptor was clearly indicated by shifting a radioactivity-containing band from 185 kDa (lane 3) to the higher molecular weight region after cross-linking (lane 4). In parallel, the anti-PDGF receptor immunoreactive band was shifted in the same manner, as detected by immunoblotting (Figure 2A, lanes 3 and 4), confirming that it contained PDGF receptor. In the presence of AG1296, only traces of radioactivity were detected in either the 185-kDa or the high molecular weight band, indicating that autophosphorylation of the receptor was blocked by the compound (Figure 2B, lanes 7 and 8). However, the high molecular weight band of the cross-linked PDGF receptor dimer was still readily detected by immunoblotting with DIG1 antiserum (Figure 2A, lane 8). The band had the same intensity as in the absence of the inhibitor (lane 4). So, AG1296, while abolishing tyrosine kinase activity of the PDGF receptor, did not affect PDGF-induced receptor dimerization.

Kinetic Analysis of PDGF Receptor Tyrosine Kinase Inhibition. Since AG1296 does not interfere with PDGF binding, nor with PDGF receptor dimerization while abrogating receptor autophosphorylation, we concluded that it is solely a direct inhibitor of the PDGF receptor tyrosine kinase.

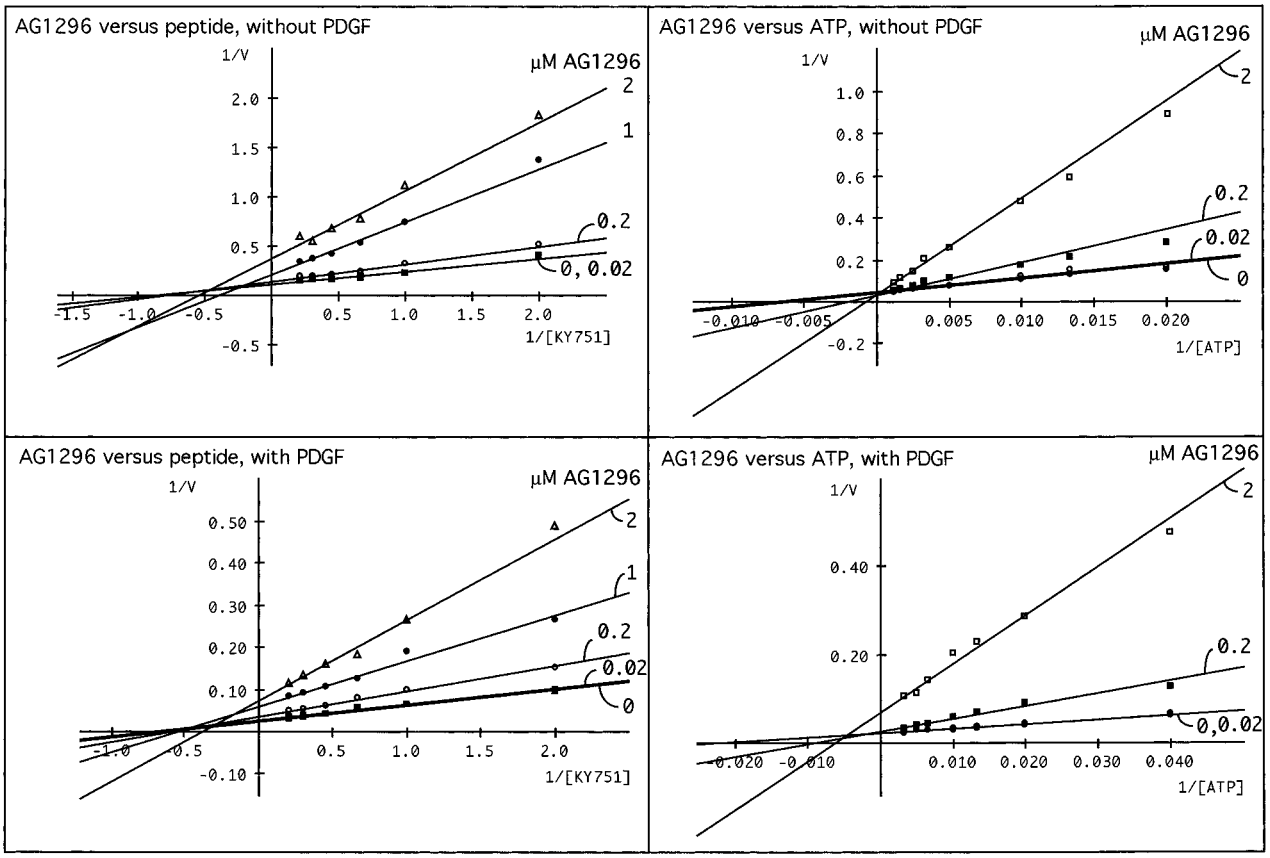

FIGURE 3: Lineweaver−Burk plots of the inhibition of phosphorylation of the synthetic peptide KY751 by AG1296. The kinase reaction was performed using partially purified PDGF β-receptor, in the presence of different concentrations of AG1296, with either KY751 or ATP as variable substrate. Details are given under Experimental Procedures.

We, therefore, performed kinetic experiments to determine the mode of inhibition exerted by AG1296, relative to ATP and phosphoacceptor substrate. The system for kinetic analysis was established as described under Experimental Procedures. The activity of partially purified PDGF β-receptor was measured first as a function of the synthetic substrate peptide KY751 concentration at five different concentrations of AG1296 and with fixed excess of ATP, and then as a function of ATP concentration at four concentrations of the inhibitor and with fixed excess of the peptide. The employed concentration ranges were based on the known K_m for ATP of about 320 and 80 μM in the absence and presence of PDGF, respectively (Rönnstrand et al., 1990), and an approximate K_m for the KY751 peptide of about 1.2 mM derived from preliminary titrations. Both series of measurements were performed in the absence and in the presence of PDGF-BB. The obtained Lineweaver−Burk plots are shown in Figure 3. The K_m and V_{max} values were computed using the Lineweaver−Burk method, and the results are presented in Table 1A and B. For the nonstimulated receptor, increase of AG1296 concentration leads to a significant (7.8-fold at 2 μM inhibitor) increase of the K_m value for ATP, indicating that the inhibitor apparently lowers the affinity of PDGF receptor to this substrate, competing for its binding. The absence of a decrease in V_{max} (Table 1A) demonstrates that AG1296 is purely competitive with ATP in the absence of PDGF, i.e., for the nonactivated receptor. However, with the PDGF-stimulated receptor, the increase of K_m was less pronounced (3.2-fold at 2 μM AG1296), and it was accompanied by a decrease in V_{max} (Table 1A). These findings indicate a change of the type of inhibition from the pure competitive type to the mixed competitive type. This is also illustrated by the Lineweaver−Burk plots (Figure 3). Activation of the receptor by PDGF leads to a decrease in the K_m for ATP (Table 1), as observed before (Rönnstrand et al., 1990). Interestingly, the K_i value for AG1296 also decreased in the presence of PDGF (Table 1).

As for the mode of AG1296 inhibition regarding peptide substrate, a purely competitive inhibition mechanism could be excluded, because the inhibitor markedly decreased the V_{max} of both PDGF-stimulated and nonstimulated receptor (Table 1B). To discriminate between noncompetitive and mixed competitive types of inhibition, we employed comparative curve-fitting with the enzyme kinetics software "EKI" (Bisswanger, 1994). By this method, the type of inhibition proved to be mixed competitive both with PDGF-stimulated and nonstimulated receptor. Another criterion for differentiating between mixed competitive and noncompetitive inhibition was the comparison of K_i and K_{ii} values, where K_i is the dissociation constant for the complex of inhibitor and free enzyme, and K_{ii} is the dissociation constant of the inhibitor from the enzyme−substrate complex. In case of noncompetitive inhibition, K_i is equal to K_{ii}, indicating that the substrate does not interfere with inhibitor binding, whereas for mixed competitive inhibition these values are different. In our experiments, K_i and K_{ii} differed approximately 2-fold vis-à-vis the peptide substrate for both, the nonactivated and the PDGF-activated receptor. K_i and K_{ii} differ by a factor of 3.5 vis-à-vis ATP for the PDGF-activated receptor. Taking these data together, we conclude

Table 1: Kinetic Parameters of the Inhibition of PDGF β-Receptor Kinase Activity by AG1296

	− PDGF				+ PDGF			
AG1296 (μM)	K_m	V_{max} (%)	K_i	K_{ii}	K_m	V_{max} (%)	K_i	K_{ii}
			(A) ATP as Variable Substrate[a]					
0	168 μM	100			51 μM	100		
0.02	141 μM	82	0.44	na[b]	46 μM	93	0.25	0.9
0.2	470 μM	120			114 μM	80		
2.0	1324 μM	116			166 μM	30		
			(B) Synthetic Peptide KY751 as Variable Substrate					
0	1.09 mM	100			1.62 mM	100		
0.02	1.05 mM	97			1.44 mM	90		
0.2	1.21 mM	80	0.47	0.87	1.66 mM	65	0.51	1.15
1.0	2.53 mM	55			1.85 mM	40		
2.0	1.77 mM	30			2.64 mM	32		
			(C) Synthetic Peptide KY857 as Variable Substrate					
0	1.40 mM	100			1.30 mM	100		
0.2	2.36 mM	92			1.05 mM	83		
0.5	1.93 mM	65	0.31	0.62	1.20 mM	64	0.82	1.00
1.0	2.26 mM	39			1.25 mM	47		
2.0	2.98 mM	30			1.25 mM	32		

[a] KY751 was used as peptide substrate in this analysis. [b] na, not applicable.

that AG1296 displays the following types of inhibition of the PDGF receptor kinase:

	− PDGF (non-activated receptor)	+ PDGF (activated receptor)
toward ATP	competitive	mixed competitive
toward exogenous peptide substrate KY751	mixed competitive	mixed competitive

Differential Inhibition of PDGF β-Receptor Autophosphorylation Sites by AG1296. PDGF receptor is phosphorylated on multiple tyrosine residues upon PDGF stimulation [reviewed in Claesson-Welsh (1994)]. Studying the effect of AG1296 on the PDGF receptor, we were interested to know whether the compound inhibits all autophosphorylation sites with equal efficacy. To test this, we performed two-dimensional tryptic phosphopeptide mapping of the PDGF β-receptor which was phosphorylated after PDGF stimulation in the presence of [γ^{32}P]ATP at different concentrations of AG1296. Figure 4A shows four selected phosphopeptide maps of the receptor phosphorylated at 0, 0.1, 0.5, and 2.5 μM AG1296. A more rapid decrease of the intensity of the R4 spot as compared with the other spots is well noticeable. To estimate the effect of the inhibitor on phosphorylation of different sites quantitatively, radioactivity incorporated into the phosphopeptides designated R1, R2, R3, and R4, as well as total radioactivity in all spots, was quantified and plotted against the inhibitor concentration (Figure 4B). The graph clearly shows preferential inhibition of phosphorylation of the R4 peptide as compared with the inhibition of R1, R2, R3, and total receptor phosphorylation. At the highest inhibitor concentration used (2.5 μM), incorporation of ^{32}P into phosphopeptide R4 was almost completely abolished, whereas phosphorylation of the other residues examined was inhibited by 50−75%.

R4 contains phosphorylated tyrosine 857, a major autophosphorylation site of the PDGF β-receptor located in the kinase domain. Quantification revealed that AG1296 inhibited phosphorylation of R4 with an IC_{50} of about 0.5 μM, whereas R1 phosphorylation was inhibited with a lower potency ($IC_{50} \sim 1$ μM), phosphorylation of R3, corresponding to the autophosphorylation site 751 decreased together with total receptor phosphorylation ($IC_{50} \sim 2$ μM), and R2 phosphopeptide was the least affected one ($IC_{50} \sim 2.5$ μM). The observed differential inhibition was not related to protein tyrosine phosphatase activity in the preparations since identical results were obtained in the presence or absence of the protein tyrosine phosphatase inhibitor sodium orthovanadate (not shown).

We wondered whether such differential inhibition would be observed also using synthetic peptide substrates corresponding to different autophosphorylation sites. Given the pronounced inhibition of the phosphorylation of peptide R4 harboring tyrosine 857, we examined the inhibition of phosphorylation of a corresponding synthetic peptide, KKKKRDIMRDSNYISKG (KY857), and compared the data with those obtained for KY751. In agreement with previous observations (Rönnstrand et al., 1990), KY857 was readily phosphorylated and this phosphorylation was susceptible to inhibition by AG1296 (Table 1C). The data analysis revealed a mixed competitive type of inhibition in the absence and an almost purely noncompetitive inhibition in the presence of PDGF (Table 1C and not shown). The K_i values obtained by Lineweaver−Burk plot were found to be not significantly different from those found with peptide KY751 (Table 1), revealing a similar sensitivity of the phosphorylation of both synthetic peptides to inhibition by AG1296 by PDGF β-receptor *in vitro*.

Effect of AG1296 on the PDGF Receptor-Associated and Intrinsic PI3-Kinase Activity. Inhibition of the PDGF receptor autophosphorylation by AG1296 is expected to abrogate receptor downstream signaling, leading finally to inhibition of PDGF-induced cellular responses. While the latter has been demonstrated before (Kovalenko et al., 1994), the effects of AG1296 on intermediate steps in the signaling cascade have not yet been investigated. We chose to measure the activity of PI3-kinase, a key intracellular mediator of the PDGF signaling (Coughlin et al., 1989). PI3-kinase binds to phosphorylated tyrosines 740 and 751 of the PDGF β-receptor (Kazlauskas et al., 1992). PDGF β-receptor was immunoprecipitated from lysates of PAE cells which were preincubated with different AG1296 concentrations and stimulated with PDGF. Thereafter, PI3-kinase activity was measured in the immunoprecipitates. The effect of AG1296

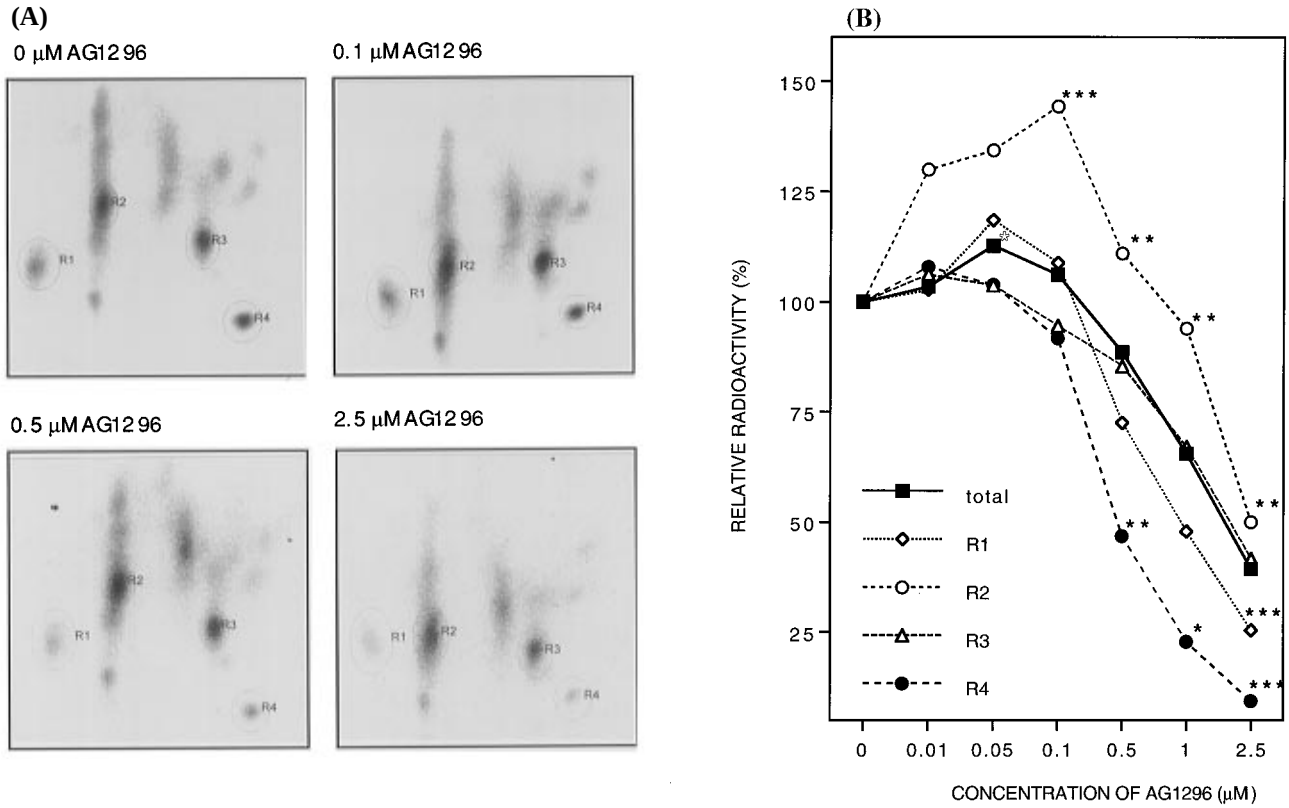

FIGURE 4: (A) Effect of tyrphostin AG1296 on the phosphorylation of different tyrosine residues of PDGF β-receptor and preferential inhibition of Tyr 857 phosphorylation by AG1296. Partially purified PDGF β-receptor was preincubated with DMSO or different concentrations of AG1296 as indicated, stimulated with PDGF-BB, and phosphorylated in the presence of [γ^{32}P]ATP. Samples were subjected to SDS−PAGE and blotted to Hybond C nitrocellulose membrane. The bands of phosphorylated PDGF receptor were excised and processed as described in Experimental Procedures to obtain two-dimensional tryptic phosphopeptide maps. Four selected maps from a typical experiment are shown. Phosphopeptides investigated in this work are encircled. They contain the following autophosphorylation sites: R1 and R2, Tyr 763, Tyr 771, Tyr 775, Tyr 778 in different phosphorylation states (Rönnstrand et al., unpublished data); R3, Tyr 751; R4, Tyr 857 (Kazlauskas & Cooper, 1989). (B) Radioactivity in the phosphopeptides R1, R2, R3, and R4, as well as in all phosphopeptides (total) was quantified with the phosphorimager and plotted against AG1296 concentration. Radioactivity at 0 μM AG1296 was taken as 100%. The graph represents the mean of four experiments. The relative signal intensity of selected points for R1, R2, R3, and R4 was statistically tested against the corresponding relative values for total radioactivity using the paired *t* test (closed asterisks). Also, the difference between total relative radioactivity values at 0.05 and 0 μM AG1296 was tested (open asterisk). Significance levels are <0.01 (***), <0.05 (**), and <0.1 (*).

on PDGF receptor-associated PI3-kinase activity is shown in Figure 5. Receptor-associated activity of PI3-kinase decreased by 50% at 1 μM of AG1296 and was completely abolished at 25 μM. To test whether the inhibitor could directly affect PI3-kinase, the activity of pure recombinant PI3-kinase (p110α subunit) was measured *in vitro* after preincubation with different concentrations of AG1296. The corresponding dose-dependence curve is shown for comparison (Figure 5). Recombinant PI3-kinase appeared to be inhibited with an IC_{50} of about 5 μM. This is at least 1 order of magnitude higher than the IC_{50} for the inhibition of PDGF receptor kinase in intact cells [0.3−0.5 μM; Kovalenko et al. (1994)] and 5-fold higher than that for the inhibition of receptor-associated PI3-kinase activity. Moreover, pure PI3-kinase was not completely blocked even at 50 μM AG1296, whereas PI3-kinase activity in PDGF receptor immunoprecipitates was undetectable already at 25 μM (Figure 5). This suggests that the decrease of receptor-associated PI3-kinase activity occurred due to the inhibition of PDGF receptor autophosphorylation and subsequent PI3-kinase binding, rather than due to the inhibition of PI3-kinase itself. These data provide evidence that blocking of PDGF receptor autophosphorylation by AG1296 abrogates PDGF-induced downstream signaling exemplified by the PI3-kinase.

DISCUSSION

The mechanism of inhibition of PDGF receptor signaling activity by the potent and selective receptor blocker AG1296 (Kovalenko et al., 1994; Gazit et al., 1996) was investigated. Although AG1296, being structurally classified as "tyrphostin", was expected to interact directly with the receptor kinase, it seemed important to evaluate whether it interferes with the steps of receptor activation preceding elevated kinase activity, i.e., ligand binding and receptor dimerization. Examples of low molecular weight compounds inhibiting ligand binding to the PDGF receptor are known (Betsholtz et al., 1986; Kuratsu & Ushio, 1990; Mullins et al., 1994). As shown here, AG1296 interferes neither with PDGF binding nor with PDGF receptor dimerization while it abolishes PDGF receptor autophosphorylation. Thus, AG1296 is a pure inhibitor of the catalytic activity of the receptor tyrosine kinase. Furthermore, our data support the existing model of PDGF-stimulated activation of the receptor, according to which autophosphorylation occurs after, and is not required for, receptor dimerization (Heldin et al., 1989).

We furthermore investigated the effect of AG1296 on a downstream event of PDGF receptor signaling, namely, PDGF-stimulated binding of PI3-kinase to the PDGF receptor. Receptor-associated PI3-kinase activity was blocked by

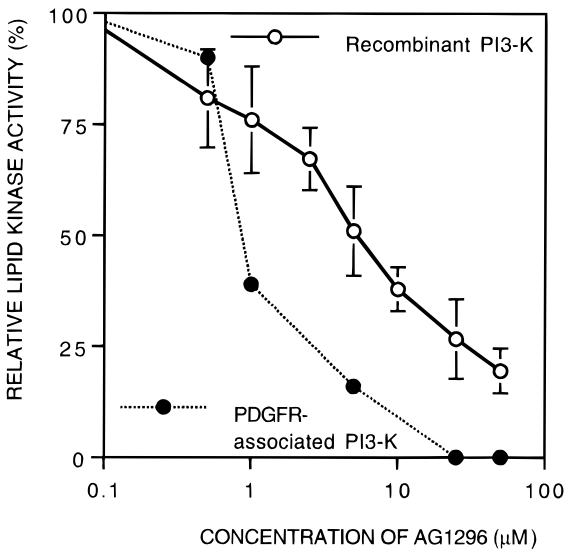

FIGURE 5: Effect of tyrphostin AG1296 on PDGF receptor-associated and intrinsic PI3-kinase activity. PAE cells expressing PDGF β-receptors were treated with DMSO or different concentrations of AG1296, stimulated with PDGF, and lysed, and PDGF receptor was immunoprecipitated with PDGFR3 anti-PDGF receptor antiserum. PI3-kinase was measured in the immunoprecipitates as PI4,5-bisphosphate phosphorylating activity as described under Experimental Procedures. The activity in the immunoprecipitates from DMSO-treated cells was taken as 100%. For comparison, the effect of AG1296 on the activity of pure recombinant PI3-kinase α, measured in parallel, is also presented.

the tyrphostin with an IC_{50} of 1 μM, a value close to that for the inhibition of PDGF receptor autophosphorylation (about 0.5 μM) in intact cells and also for the inhibition of phosphorylation of Tyr 751 (Figure 4) which is involved in PI3-kinase binding to the receptor. We also found that AG1296 was able to inhibit pure recombinant PI3-kinase. However, the inhibition of the pure enzyme was much less efficient. Still, the inhibitory effect of AG1296 on PI3-kinase(s) at high concentration could explain the loss of selectivity with respect to inhibition of cell proliferation induced by different growth factors, which was observed at high inhibitor concentrations (Kovalenko et al., 1994).

The kinetic mechanism of receptor kinase inhibition by AG1296 was studied using a peptide corresponding to Tyr 751 autophosphorylation site as exogenous substrate. In these experiments, AG1296 exhibited purely competitive inhibition vis-à-vis ATP for the nonstimulated PDGF receptor, indicating that the binding of ATP and of AG1296 is mutually exclusive in the nonactivated receptor. After PDGF treatment, a more than 3-fold decrease of the K_m for ATP [Table 1, reported also by Rönnstrand et al. (1990)] reflected higher affinity of the stimulated PDGF receptor toward this substrate. This was accompanied by an approximately 2-fold increase of affinity toward AG1296. These changes suggest that PDGF binding induces a conformational change affecting the ATP binding domain. Interestingly, the mode of inhibition by AG1296 relative to ATP changed from purely competitive to mixed competitive upon PDGF stimulation, suggesting that, in the activated receptor, the ATP binding site and inhibitor binding site are still overlapping but no longer identical. One could speculate that the PDGF-induced conformational change brings ATP-binding amino acid residues (in the first part of the split PDGF receptor kinase domain) and the catalytic residues (in the second part of the kinase domain) closer to each other. As a consequence, the inhibitor interacts with additional residues in the receptor catalytic domain and thus interferes with the step of phosphate transfer. A conformational change leading to close proximity of the ATP binding and the catalytic amino acid residues in the activated enzyme has been reported for the cAMP-dependent protein kinase (Zheng et al., 1993) and was suggested for the insulin receptor tyrosine kinase (Hubbard et al., 1994). When we studied the kinetics of PDGF receptor kinase inhibition by titrating substrate peptide and AG1296 against each other, we observed a mixed competitive type of inhibition. No significant change of the affinity of the PDGF receptor to KY751 peptide nor of the mode of AG1296 inhibition vis-à-vis the peptide occurs upon PDGF stimulation. This finding suggests that AG1296 interferes with both peptide binding and phosphorylation and that the peptide binding site does not undergo substantial conformational changes upon PDGF stimulation.

AG1296 appears to compete with ATP binding more effectively than with the binding of the peptide substrate. In the nonactivated receptor, increasing inhibitor concentration causes an about 8-fold increase of the K_m for ATP, whereas the K_m for the peptide substrate does not increase more than 2-fold over the same concentration range of AG1296 (Table 1). In the PDGF-stimulated receptor, this difference is less pronounced but still significant (Table 1). Since ATP-binding domains are highly conserved among tyrosine kinases (Hanks et al., 1988), much doubt has been expressed about the feasibility of finding selective inhibitors among ATP competitors [for review see Levitzki and Gazit (1995)]. However, more and more data indicate that some ATP-competitive blockers are able to discriminate even between closely related tyrosine kinases. Quercetin, known as a nonspecific ATP-competitive tyrosine phosphorylation blocker (Graziani et al., 1983), was found to inhibit $p60^{c-src}$ and $p60^{v-src}$ kinases with different efficacy, although they have highly homologous amino acid sequences of their kinase domains. Benzenemalononitrile tyrphostins AG825 and AG494, both competitive with ATP, could discriminate between very closely related HER-1/EGF receptor and HER2/neu kinases (Osherov et al., 1993). Staurosporine, another ATP-competitive blocker, nonselectively inhibits many protein kinases (Ruegg & Burgess, 1989). However, staurosporine-related compounds, dianilinophthalimides, retaining ATP-competitiveness, showed a high degree of selectivity toward EGF receptor kinase (Trinks et al., 1994; Furet et al., 1995). Recently discovered quinazoline derivatives inhibiting EGF receptor tyrosine kinase (PD 153035, AG1478, and AG1517) (Fry et al., 1994; Ward et al., 1994; Osherov & Levitzki, 1994; Wakeling et al., 1996) have also been shown to be ATP-competitive and to exhibit pronounced selectivity and potency on isolated receptors as well as in intact cells. Taken together, these findings suggest that the ATP binding sites of different tyrosine kinases are sufficiently structurally divergent to allow for such an inhibitor selectivity. Additional interactions with other parts of the catalytic machinery, as suggested here by the change in the mode of AG1296 inhibition of the PDGF receptor subsequent to its activation, might contribute to this selectivity. Also for other tyrosine kinases, there is indirect evidence that binding of substrates and inhibitors to a kinase may be affected by domains other than the ATP-binding and catalytic ones. The normal and transforming *abl* family kinases ($p140^{c\text{-}abl}$, $p160^{gag\text{-}abl}$, $p185^{bcr\text{-}abl}$, and $p210^{bcr\text{-}abl}$) have identical

amino acid sequences of their catalytic domains, yet they interact differently with ATP, peptide substrates, and tyrphostins (Anafi et al., 1992), suggesting that the conformation of substrate- and inhibitor-binding sites can be regulated by allosteric effects. So, the ATP-binding site of each tyrosine kinase may possess unique features, making it possible to design highly selective ATP-competitive inhibitors.

Comparing the efficacy of inhibition of PDGF receptor autophosphorylation sites by AG1296 in a cell-free system, we obtained different dose−inhibition curves for different phosphorylation sites (Figure 4B). PDGF-stimulated phosphorylation of Tyr 857 was preferentially inhibited by AG1296 as compared to other sites. Tyrosine 857 is a major autophosphorylation site in PDGF-stimulated human PDGF β-receptor (Kazlauskas & Cooper, 1989). It is located in the second part of the split PDGF receptor kinase domain. Phosphorylation of the homologous residue in the insulin receptor (Tyr 1162) leads to an increase in kinase activity (Hubbard et al., 1994) and precedes phosphorylation of other sites (Wei et al., 1995). For tyrosine 857 in the PDGF receptor, a similar role was suggested, although the precise mechanism of PDGF receptor activation is yet unknown (Heldin, 1995). Our data suggest that low activation of the PDGF receptor kinase and phosphorylation of tyrosines 763, 771, 775 or 778 (phosphopeptides R1 and R2), and 751 (R3) is possible in the absence of the phosphorylation of Tyr 857. This is in agreement with the work of Kazlauskas et al. (1991), who reported that the Y857F mutant of human PDGF β-receptor was still phosphorylated on tyrosine after PDGF stimulation, bound PI3-kinase, and retained about 50% of its ability to mediate PDGF-induced mitogenesis. Earlier, Morrison et al. (1990) showed that a Y857F mutant PDGF receptor bound PLCy and mediated its phosphorylation, albeit at a lower level compared with the wild-type receptor, indicating the ability of the mutant PDGF receptor to autophosphorylate at least tyrosine residues 1009 and 1021 (PLCγ binding sites). Together with our results, these data suggest that phosphorylation of tyrosine 857 may not be absolutely required for kinase activation.

Why does AG1296 inhibit phosphorylation of different receptor tyrosine residues with different efficacy? To evaluate whether differential competition of AG1296 with the different receptor peptide sequences might be the basis for this phenomenon, we examined kinetically the inhibition of phosphorylation of a synthetic peptide corresponding to the autophosphorylation site 857 and compared the data with those for KY751 phosphorylation. Although the kinetics of inhibition of KY857 phosphorylation differed slightly from the one for KY751 phosphorylation in that inhibition was almost purely noncompetitive in the presence of PDGF, the overall susceptibility of phosphorylation of both peptides to the inhibition by AG1296 was very similar. Taken together, the synthetic peptide phosphorylation data do not explain the differential inhibition observed at the level of receptor autophosphorylation. One possible alternative explanation for the latter phenomenon might be that AG1296 in the intact receptor kinase can sterically prevent the access of Tyr 857 to the active site, thus inhibiting its phosphorylation, but interferes less efficiently with access of Tyr 751 and other tyrosine residues to the active site. Still, the exact mechanism of the PDGF-stimulated receptor kinase activation and the role of different tyrosine residues in this process await investigation. Crystal structure of the PDGF receptor kinase domain in the presence and in the absence of the inhibitor would provide much information about the mechanism of activation as well as of its inhibition by AG1296. Since this is not available yet, comparison of the kinetics of phosphorylation of different sites, both in the absence and in the presence of functional Tyr 857, could be informative.

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REFERENCES

Anafi, M., Gazit, A., Gilon, C., Ben, N. Y., & Levitzki, A. (1992) *J. Biol. Chem. 267*, 4518−4523.

Banai, S., Schneider, A., Pearle, A., Gertz, S. D., Golomb, G., Levitzki, A., & Walff, Y. (1996) *J. Am. Coll. Cardiol. 27*, 255A, 976−980.

Betsholtz, C., Johnsson, A., Heldin, C.-H., & Westermark, B. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6440−6444.

Bhardwaj, B., Klassen, J., Cossette, N., Sterns, E., Tuck, A., Deeley, R., Sengupta, S., & Elliott, B. (1996) *Clin. Cancer Res. 2*, 773−782.

Bilder, G. E., Krawiec, J. A., McVety, K., Gazit, A., Gilon, C., Lyall, R., Zilberstein, A., Levitzki, A., Perrone, M. H., & Schreiber, A. B. (1991) *Am. J. Physiol. 260*, C721−C730.

Bisswanger, H. (1994) *Enzymkinetik. Theorie und Methoden*, VCH Verlagsgesellschaft mbH, Weinheim.

Boyle, W. J., van der Geer, P., & Hunter, T. (1991) *Methods Enzymol. 201*, 110−149.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.

Bryckaert, M. C., Eldor, A., Fontenay, M., Gazit, A., Osherov, N., Gilon, C., Levitzki, A., & Tobelem, G. (1992) *Exp. Cell Res. 199*, 255−261.

Buchdunger, E., Zimmermann, J., Mett, H., Meyer, T., Muller, M., Regenass, U., & Lydon, N. B. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 2558−2562.

Cercek, B., Sharifi, B., Barath, P., Bailey, L., & Forrester, J. S. (1991) *Am. J. Cardiol. 68*, 24C−33C.

Claesson-Welsh, L. (1994) *J. Biol. Chem. 269*, 32023−32026.

Claesson-Welsh, L., Eriksson, A., Westermark, B., & Heldin, C.-H. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4917−4921.

Coughlin, S. R., Escobedo, J. A., & Williams, L. T. (1989) *Science 243*, 1191−1194.

Crystal, R. G., Bitterman, P. B., Rennard, S. I., Hance, A. J., & Keogh, B. A. (1984) *N. Engl. J. Med. 310*, 154−166.

Endresen, G. K., & Forre, O. (1992) *Clin. Exp. Rheumatol. 10*, 181−187.

Floege, J., & Johnson, R. J. (1995) *Mineral. Electrolyte Metab. 21*, 271−282.

Fry, D. W., Kraker, A. J., McMichael, A., Ambroso, L. A., Nelson, J. M., Leopold, W. R., Conners, R. W., & Bridges, A. J. (1994) *Science 265*, 1093−1095.

Furet, P., Caravatti, G., Lydon, N., Priestle, J. P., Sowadski, J. M., Trinks, U., & Traxler, P. (1995) *J. Comput. Aided Mol. Des. 9*, 465−472.

Gazit, A., App, H., McMahon, G., Chen, J., Levitzki, A., & Böhmer, F. D. (1996) *J. Med. Chem. 39*, 2170−2177.

Graziani, Y., Erikson, E., & Erikson, R. L. (1983) *Eur. J. Biochem. 135*, 583−589.

Guha, A., Dashner, K., Black, P. M., Wagner, J. A., & Stiles, C. D. (1995) *Int. J. Cancer 60*, 168−173.

Hanks, S. K., Quinn, A. M., & Hunter, T. (1988) *Science 241*, 42−52.

Heldin, C.-H. (1995) *Cell 80*, 213−223.

Heldin, C.-H., Ernlund, A., Rorsman, C., & Rönnstrand, L. (1989) *J. Biol. Chem. 264*, 8905−8912.
Hermanson, M., Funa, K., Hartman, M., Claesson-Welsh, L., Heldin, C.-H., Westermark, B., & Nistér, M. (1992) *Cancer Res. 52*, 3213−3219.
Hubbard, S. R., Wei, L., Ellis, L., & Hendrickson, W. A. (1994) *Nature 372*, 746−754.
Kazlauskas, A., & Cooper, J. A. (1989) *Cell 58*, 1121−1133.
Kazlauskas, A., Durden, D. L., & Cooper, J. A. (1991) *Cell Reg. 2*, 413−425.
Kazlauskas, A., Kashishian, A., Cooper, J. A., & Valius, M. (1992) *Mol. Cell. Biol. 12*, 2534−2544.
Kovalenko, M., Gazit, A., Böhmer, A., Rorsman, C., Rönnstrand, L., Heldin, C.-H., Waltenberger, J., Böhmer, F. D., & Levitzki, A. (1994) *Cancer Res. 54*, 6106−6114.
Kuratsu, J., & Ushio, Y. (1990) *J. Neurosurg. 73*, 436−440.
Levitzki, A., & Gazit, A. (1995) *Science 267*, 1782−1788.
Lindmark, G., Sundberg, C., Glimelius, B., Pahlman, L., Rubin, K., & Gerdin, B. (1993) *Lab. Invest. 69*, 682−689.
Morrison, D. K., Kaplan, D. R., Rhee, S. G., & Williams, L. T. (1990) *Mol. Cell. Biol. 10*, 2359−2366.
Mullins, D. E., Hamud, F., Reim, R., & Davis, H. R. (1994) *Arterioscler. Thromb. 14*, 1047−1055.
Nistér, M., Enblad, P., Bäckström, G., Söderman, T., Persson, L., Heldin, C.-H., & Westermark, B. (1994) *Br. J. Cancer 69*, 952−956.
Nitta, T., & Sato, K. (1994) *Neurosurgery 34*, 309−314.
Osherov, N., & Levitzki, A. (1994) *Eur. J. Biochem. 225*, 1047−1053.
Osherov, N., Gazit, A., Gilon, C., & Levitzki, A. (1993) *J. Biol. Chem. 268*, 11134−11142.
Pickering, J. G., Weir, L., Jekanowski, J., Kearney, M. A., & Isner, J. M. (1993) *J. Clin. Invest. 91*, 1469−1480.
Pinzani, M., Milani, S., Herbst, H., Defranco, R., Grappone, C., Gentilini, A., Caligiuri, A., Pellegrini, G., Ngo, D. V., Romanelli, R. G., & Gentilini, P. (1996) *Am. J. Pathol. 148*, 785−800.
Rönnstrand, L., Beckmann, M. P., Faulders, B., Östman, A., Ek, B., & Heldin, C.-H. (1987) *J. Biol. Chem. 262*, 2929−32.
Rönnstrand, L., Sorokin, A., Engström, U., & Heldin, C.-H. (1990) *Biochem. Biophys. Res. Commun. 167*, 1333−1340.
Ross, R. (1990) *Adv. Nephrol. Necker Hosp. 19*, 79−86.
Ross, R. (1993) *Nature 362*, 801−809.
Rubin, K., Terracio, L., Rönnstrand, L., Heldin, C.-H., & Klareskog, L. (1988) *Scand. J. Immunol. 27*, 285−294.
Ruegg, U. T., & Burgess, G. M. (1989) *Trends Pharmacol. Sci. 10*, 218−220.
Seymour, L., Dajee, D., & Bezwoda, W. R. (1993) *Breast Cancer Res. Treat. 26*, 247−252.
Shamah, S. M., Stiles, C. D., & Guha, A. (1993) *Mol. Cell. Biol. 13*, 7203−7212.
Sorkin, A., Westermark, B., Heldin, C.-H., & Claesson-Welsh, L. (1991) *J. Cell Biol. 112*, 469−478.
Strawn, L. M., Mann, E., Elliger, S. S., Chu, L. M., Germain, L. L., Niederfellner, G., Ullrich, A., & Shawver, L. K. (1994) *J. Biol. Chem. 269*, 21215−21222.
Tanizawa, S., Ueda, M., Vanderloos, C. M., Vanderwal, A. C., & Becker, A. E. (1996) *Heart 75*, 549−556.
Trinks, U., Buchdunger, E., Furet, P., Kump, W., Mett, H., Meyer, T., Muller, M., Regenass, U., Rihs, G., Lydon, N., et al. (1994) *J. Med. Chem. 37*, 1015−1027.
Uchida, K., Sasahara, M., Morigami, N., Hazama, F., & Kinoshita, M. (1996) *Atherosclerosis 124*, 9−23.
Valius, M., & Kazlauskas, A. (1993) *Cell 73*, 321−334.
Wakeling, A. E., Barker, A. J., Davies, D. H., Brown, D. S., Green, L. R., Cartlidge, S. A., & Woodburn, J. R. (1996) *Breast Cancer Res. Treat. 38*, 67−73.
Wang, J., Coltrera, M. D., & Gown, A. M. (1994) *Cancer Res. 54*, 560−564.
Ward, W. H., Cook, P. N., Slater, A. M., Davies, D. H., Holdgate, G. A., & Green, L. R. (1994) *Biochem. Pharmacol. 48*, 659−666.
Wei, L., Hubbard, S. R., Hendrickson, W. A., & Ellis, L. (1995) *J. Biol. Chem. 270*, 8122−8130.
Zheng, J., Knighton, D. R., Xuong, N. H., Taylor, S. S., Sowadski, J. M., & Ten, E. L. (1993) *Protein Sci. 2*, 1559−1573.

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