Isosforms of the serine-threonine kinase Akt coordinate multiple cell survival pathways in response to stimuli such as platelet-derived growth factor (PDGF). Activation of Akt is a multistep process, which relies on the production of 3'-phosphorylated phosphoinositide (PI) lipids by PI 3-kinases. To quantitatively assess the kinetics of PDGF receptor/PI 3-kinase/Akt signaling in fibroblasts, a systematic study of this pathway was performed, and a mechanistic mathematical model that describes its operation was formulated. We find that PDGF receptor phosphorylation exhibits positive cooperativity with respect to PDGF concentration, and its kinetics are quantitatively consistent with a mechanism in which receptor dimerization is initially mediated by the association of two 1:1 PDGF/PDGF receptor complexes. Receptor phosphorylation is transient at high concentrations of PDGF, consistent with the loss of activated receptors upon endocytosis. By comparison, Akt activation responds to lower PDGF concentrations and exhibits more sustained kinetics. Further analysis and modeling suggest that the pathway is saturated at the level of PI 3-kinase activation, and that the p110α catalytic subunit of PI 3-kinase contributes most to PDGF-stimulated 3'-PI production. Thus, at high concentrations of PDGF, the kinetics of 3'-PI production are limited by the turnover rate of these lipids, while the Akt response is additionally influenced by the rate of Akt deactivation.

Platelet-derived growth factor (PDGF) is a polypeptide mitogen of broad specificity, one of the earliest and most potent serum factors to be isolated (1). Beyond signaling of proliferation, PDGF acts as a strong chemoattractant during wound healing and can mediate protection from apoptosis in response to serum withdrawal and certain stress stimuli (2, 3). Three forms of PDGF have been studied extensively. They are composed of disulfide-bonded homo- and heterodimers of A and B chains, of which PDGF-BB is the best characterized. There are two structurally related PDGF receptors, α and β, which exhibit different affinities for the A chain but roughly equivalent affinities for the B chain (4, 5). More recently, PDGF-C and -D isoforms, which form homodimers, have also been identified; these too exhibit different affinities for PDGF α- and β-receptors (6–8).

PDGF receptors belong to the well studied class of signal transducers collectively known as receptor-tyrosine kinases (9, 10). As with other receptors of this class, ligand-induced dimerization of PDGF receptors activates their intrinsic kinase domains, which catalyze the autophosphorylation of the receptors on multiple intracellular tyrosine residues in trans (11–13). The phosphorylated receptor may then act as a scaffold for specific binding interactions with cytosolic signal transduction enzymes and adaptor proteins (14). Among the most important of these are isoforms of phosphoinositide (PI) 3-kinase, which phosphorylate phosphatidylinositol (PtdIns) lipids in cell membranes to produce the 3'-PI second messengers PtdIns(3)P, PtdIns(3,4)P2, and PtdIns(3,4,5)P3 (15, 16). The 110 kDa α and β isoforms of the PI 3-kinase catalytic subunit are involved in receptor-tyrosine kinase signaling, and they almost exclusively phosphorylate PtdIns(4,5)P2 to form PtdIns(3,4,5)P3, with PtdIns(3,4)P2 generated from the subsequent dephosphorylation of PtdIns(3,4,5)P3. The 85 kDa regulatory subunit coordinates binding of PI 3-kinase to PDGF receptors through dual SH2 domains, transmitting a conformational change that activates the catalytic subunit (17–20). Equally if not more important is the induced localization of PI 3-kinase in proximity to its plasma membrane-associated substrate (21).

Cellular roles for 3'-PIs are now appreciated (22, 23). Their general mechanism of action is the membrane recruitment and activation of signaling proteins containing pleckstrin homology (PH) domains, such as the serine-threonine kinase Akt. Akt isoforms are activated in response to PDGF and other factors in a strictly PI 3-kinase-dependent manner (24, 25) and have been strongly implicated in multiple cell survival pathways (26, 27). The recruitment of Akt to the plasma membrane, through transient interaction of its PH domain with PtdIns(3,4,5)P3 or PtdIns(3,4)P2, is a necessary first step in Akt activation; sequential phosphorylation of Thr308 and Ser473 (residue positions in human Akt-1) completes the process (28–31). 3-Phosphoinositide-dependent protein kinase-1 (PDK-1) is recruited by PtdIns(3,4,5)P3 and catalyzes phosphorylation of Akt on Thr308, and a second, yet to be identified 3'-PI-dependent kinase (dubbed PDK-2) phosphorylates the critical Ser473 residue (32–35).

A detailed molecular level understanding of intracellular signal transduction, including the PDGF receptor/PI 3-kinase/Akt pathway, has thus emerged, yet our knowledge base is largely qualitative. To examine complexities such as the timing and duration of signal activation, which have been implicated...
as important factors governing cell function (36, 37), a more quantitative approach is warranted. To this end, we have made a reasonably precise measurements of PDGF-stimulated activation of the PDGF receptor/PI 3-kinase/Akt signaling pathway in NIH 3T3 fibroblasts, at various times and over a range of PDGF concentrations. We were thus able to assess the sensitivity of each step in the pathway, with respect to both the magnitude and kinetics of the response. Accompanying this analysis is a proposed model that describes the pathway in mathematical terms, with a minimum number of rate parameters.

We find that activation of Akt is saturated with respect to PDGF receptor phosphorylation, apparently at the level of activating PI 3-kinases. Thus, at higher concentrations of PDGF the kinetics of 3'-PI production and activation of Akt are sustained and largely limited by the rate of 3'-PI turnover. Although we found that both p110α and p110β catalytic subunits of PI 3-kinase are recruited by PDGF receptors in our cells, our results suggest that p110α contributes most to 3'-PI production and Akt activation. Another primary result of our modeling and analysis concerns the mechanism of PDGF receptor dimerization. We report that a model in which dimeric PDGF ligand binds to one receptor molecule and then cross-links a second, unbound receptor is neither quantitatively nor qualitatively consistent with our data. On the other hand, our data is completely consistent with a model in which dimerization requires the association of two 1:1 ligand-receptor complexes as an initial step, perhaps with formation of a stable 1:2 complex thereafter.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—All tissue culture reagents were purchased from Invitrogen. Human recombinant PDGF-BB was from Peprotech, and LYT294002 was from Calbiochem. Antibodies against the extracellular domain of PDGF β-receptor were from Oncogene Research Products (PC-17, without bovine serum albumin), and horseradish peroxidase-conjugated Fab fragments recognizing phosphotyrosine (RC20) were from Transduction Laboratories. Antibodies against the Akt 1/2 N terminus and the peptide substrate for Akt were from Santa Cruz Biotechnology, and γ-32P-ATP was from PerkinElmer Life Sciences.

Porphyspecific antibodies against Akt (pSer473) and PDGF β-receptor (pTyr787) were from Cell Signalling Technologies, antibodies against PI 3-kinase p110α and p110β isoforms were from Upstate Biotechnology, and protein A-Sepharose was from Zymed Laboratories Inc. Unless otherwise noted, all other reagents were from Sigma.

Cell Culture and Preparation of Detergent Lysates—NIH 3T3 fibroblasts (American Type Culture Collection) were subcultured in 10-cm tissue culture dishes with Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum, 2 mM l-glutamine, and the antibiotics penicillin and streptomycin. Dishes to be processed on the same day were plated with equal numbers of cells and allowed to reach at least 90% confluency. The cells were incubated for 4 h in DMEM containing 2 mM l-glutamine, the antibodies penicillin and streptomycin, and 1 mg/ml fatty acid-free bovine serum albumin (BSA). At various times, PDGF-BB and other treatments were added to each plate at the final concentration indicated and incubated for the time interval specified at 37 °C in 5% CO₂. At the end point of the experiment, each plate was washed once with ice-cold Dulbecco’s phosphate-buffered saline (PBS) and then lysed in 500 μl of ice-cold buffer containing 50 mM HEPES, pH 7.4, 100 mM NaCl, 10% v/v glycerol, 1% v/v Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 50 mM β-glycerophosphate, pH 7.3, 5 mM sodium fluoride, 1 mM EGTA, and 10 μg/ml each aprotinin, leupeptin, pepstatin A, and chymostatin. After scraping insoluble debris and transferring to an Eppendorf tube, the lysates were vortexed briefly, incubated on ice for 20 min, and clarified by centrifugation. The supernatants were collected and stored frozen at −80 °C until use. Protein assays (Micro BCA, Pierce) were used to confirm that lysates collected on the same day contained roughly equivalent protein concentrations.

Enzyme-linked Immunosorbent Assay (ELISA) of PDGF β-Receptor Phosphorylation—Opaque, high protein binding microtiter plates (Corning) were coated overnight with at least 375 ng of capture antibody recognizing the PDGF β-receptor extracellular domain per well.

The wells were then incubated with ELISA blocking buffer (10% v/v human serum, 0.05% v/v Tween-20 in PBS) for 1 h at room temperature. After washing once with the same buffer, each well was incubated with 50 μl of ELISA blocking buffer plus 50 μl of cell lysate for 90 min with agitation at room temperature, followed by extensive washing with high salt buffer (10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Tween-20, and 0.1% SDS). Wells were then incubated with horseradish peroxidase-conjugated anti-phosphotyrosine Fab fragments at 0.2 μg/ml in ELISA blocking buffer for 1 h at room temperature, followed by more washes with high salt buffer. Finally, substrate solution (ELISA Femto, Pierce) was added, and the relative light signals were acquired using a microplate luminometer (Wallac MR 2002).

Akt Kinase Activity Assay—High protein binding microtiter plates (Corning) were incubated overnight with 500 ng/well protein G in carbonate buffer, followed by washes with carbonate buffer alone. The wells were then incubated with anti-Akt antibodies at 500 ng/well in carbonate buffer for 2 h with agitation at room temperature. This solution was removed, and BSA blocking buffer (10 mg/ml BSA, 0.05% Tween 20 in PBS) was added for 1 h at room temperature. After washing once with BSA blocking buffer, 25 μl of BSA blocking buffer and 50 μl of cell lysate were added to each well and incubated for 90 min with agitation at room temperature. After washing three times with BSA blocking buffer and twice with reaction buffer (20 mM Tris-HCl, pH 7.5, 5 mM β-glycerophosphate, pH 7.3, 1 mM EGTA, 0.2 mM dithiothreitol, and 0.1 mg/ml fatty acid-free BSA), each well was incubated with 80 μl of reaction buffer supplemented with 1 μg of peptide substrate, 1 μM ATP, 2 μM γ-32P-ATP, and 15 mM MgCl₂ for 1 h at agitation at room temperature. The reaction was stopped by adding 80 μl of 100 mM H₃PO₄ to each well. From each well 100 μl was carefully transferred to the corresponding well in a phosphocellulose filter-bottom plate (Millipore), pre-equilibrated with 100 mM H₃PO₄. After extensive washing with 75% H₃PO₄, and then 75% ethanol, the filter plate was dried and counted with 40 μl of scintillation fluid per well in a microplate scintillation counter (Wallac MR 2002).

Quantitative Immunoblotting—Pooled cell lysates were subjected to SDS-PAGE in 20 cm-wide gels using standard techniques. When immunoprecipitations were performed, each lysate was first incubated with capture antibodies and 25 μl of protein A-Sepharose at 4 °C, followed by extensive washing with lysis buffer. After gel electrophoresis, proteins were transferred to PVDF membrane (Immobilon, Millipore) and probed with the indicated antibodies. The blots were incubated with chemiluminescence substrates (Pierce) and imaged using a high sensitivity CCD camera (BioRad Fluor S-Max). All pixel intensities were within the dynamic range.

Total Internal Reflection Fluorescence (TIRF) Microscopy—This technique was performed essentially as described (38). Our microscope was equipped with a Melles Griot tunable wavelength laser (60 mW at 488 nm), Zeiss upright stand, Lumon emission filter wheel with Chroma filters, and Hamamatsu ORCA ER digital CCD. The TIRF microscope was cloned into pEGFP-C1 (Clontech) to express the GFP-AktPH construct in mammalian cells. Cells were plated onto glass cover slips coated with poly-1-lysine and later transfected with GFP-AktPH using LipofectAMINE Plus (Invitrogen). The following day, the cells were incubated in serum-free medium for 4 h and then visualized on the microscope. The stage was enclosed in a chamber maintained at 37 °C, and the imaging buffer was composed of 20 mM HEPES pH 7.4, 125 mM NaCl, 5 mM KCl, 1.5 mM MgCl₂, 1.5 mM CaCl₂, 10 mM glucose, and 2 mM fatty acid-free BSA, to which PDGF-BB and other treatments were added at the times indicated. Images were acquired and analyzed using Metamorph software (Universal Imaging).

Estimation of Integrated Responses—As an estimation of the integral of a measured variable with respect to time, the trapezoidal rule in Eq. 1 was employed.

\[ \int_0^t y(t) dt = \frac{1}{2} \sum_{i=0}^{N-1} (y_{i+1} + y_i)(t_{i+1} - t_i) \]  

Dividing the time integral by the total duration of the time course \( t_N \), yields the time-averaged value of the measurement. This was found to be a robust way of normalizing data from time course experiments collected on different days.

Model Computation—The coupled ordinary differential equations were solved by numerical integration using Excel. Parameter optimization was performed using the Solver tool, minimizing the sum of absolute deviations (least-squares minimization showed bias toward agreement with data for higher PDGF concentrations). Numerical accuracy was confirmed by comparing model output with different time
Kinetic Model of PDGF Receptor Signaling

Fig. 1. PDGF receptor phosphorylation shows positive cooperativity with respect to PDGF concentration. A, time courses of PDGF β-receptor tyrosine phosphorylation in NIH 3T3 fibroblasts, stimulated with various concentrations of PDGF-BB, were quantified by sandwich ELISA as described under “Experimental Procedures.” After subtracting the average signal from lysates of unstimulated cells, each data point was normalized by the time-integrated average for 10 nM stimulation measured on the same day. The symbols are averages of experiments performed on 5 different days (mean ± S.E., n = 5). PDGF-BB concentrations are: ▲, 0.05 nM; ◆, 0.1 nM; △, 0.2 nM; ■, 0.5 nM; ■, 1 nM; ○, 3 nM; ●, 10 nM. B, the peak level of PDGF receptor phosphorylation achieved with respect to time, from the data in A, is plotted as a function of PDGF dose. A fit to the Hill function, \(y(x) = \frac{y_{\text{max}}}{1 + (x/K^n)^n}\), yielded a Hill coefficient of 1.55 ± 0.16 \((R^2 > 0.99)\), indicating positive cooperativity (solid curve). The function with a Hill coefficient of 1 passing through the same half-maximal value is shown for comparison (dashed curve).

Results

Quantitative Measurements of PDGF β Receptor Autophosphorylation Kinetics Reveal a Positively Cooperative Activation Mechanism—Tyrosine phosphorylation of the PDGF β receptor, the hallmark of receptor dimerization and kinase activation, was measured in detergent lysates of NIH 3T3 fibroblasts using a quantitative sandwich ELISA assay. In control experiments, it was confirmed that this measurement is linear with respect to the amount of lysate protein incubated in the wells under the conditions of our assay. Tissue culture plates containing equal numbers of cells were stimulated with PDGF-BB concentrations of 0.05, 0.1, 0.2, 0.5, 1, 3, or 10 nM for durations of 2, 5, 10, or 20 min on the same day. Lysates from two unstimulated plates were also prepared. Each lysate was assayed in duplicate, and the procedure was replicated on five different days. The phosphorylation signal at each condition was normalized by the time-averaged 10 nM phosphorylation signal, integrated numerically over the 20-min time course, obtained on the same day. The means of the five experiments are displayed in Fig. 1A, as a function of time for the various doses of PDGF.

At low concentrations of PDGF-BB (below 0.5 nM), tyrosine phosphorylation of the PDGF β-receptor achieves a steady state, and the plateau value is sensitive to PDGF-BB concentra-

tration in this regime. At higher ligand concentrations (above 0.5 nM), receptor phosphorylation is clearly transient, exhibiting a maximum value at 2–5 min. As the concentration of PDGF-BB is increased, the peak phosphorylation level becomes less sensitive to ligand concentration, and the peak occurs at increasingly earlier times. Another feature observed in the data is the presence of positive cooperativity with respect to PDGF concentration. At low concentrations of PDGF, doubling the ligand concentration yielded gains in receptor phosphorylation of 3–4-fold. Consistent with this, the peak receptor phosphorylation levels over the entire range of PDGF doses exhibit an observed Hill coefficient of 1.6, as shown in Fig. 1B. Receptor activation from 10 to 90% maximum is achieved within roughly one log of PDGF concentration, rather than the two logs predicted from single-site ligand-receptor binding.

The Activities of PI 3-Kinase and Akt Are Saturated with Respect to the Number of Phosphorylated PDGF Receptors, with No Apparent Cooperativity—From the same NIH 3T3 lysates used to measure PDGF β-receptor phosphorylation at various PDGF concentrations and times, we assessed the activation of Akt by in vitro kinase assay. As in the PDGF receptor phosphorylation ELISA, great care was taken here to ensure that the measurement was sensitive to dilution of the lysate applied to the wells coated with anti-Akt antibodies, and all measurements were made in duplicate. The Akt activation time courses, displayed in Fig. 2A, are clearly distinct from the kinetics of PDGF β-receptor phosphorylation. In terms of the dose response, half-maximal Akt activation is observed between 0.1 and 0.2 nM PDGF, and at PDGF concentrations above 0.5 nM, the Akt activation kinetics are insensitive to PDGF dose and more sustained in relation to receptor phosphorylation. These observations suggest that the ability of the cell to activate Akt is saturated with respect to phosphorylated PDGF receptors.

To further assess the relationship between receptor phosphorylation and Akt activity, the two responses were correlated. When a signal is integrated over time, the resulting quantity is sensitive to both the magnitude and kinetics of the response. In Fig. 2B, the time-integrated Akt activity is plotted as a function of the associated time-integral of PDGF receptor phosphorylation for each concentration of PDGF-BB to assess the sensitivity of this relationship, which we term the receptor-signal response curve. In terms of time integrals, Akt activation is 50% maximal when receptor phosphorylation is only ~10% of its maximum value. In addition, the relationship does not exhibit apparent positive cooperativity; a Hill coefficient of 1 fits the data well, far better than values of 1.5 or higher.

In previous work with the same cells, the PDGF dose response and kinetics of 3’-PI production were reported (38), showing the same essential features observed here for Akt activation. Half-maximal 3’-PI production was stimulated in the range of 0.1–0.3 nM PDGF-BB, and at PDGF-BB concentrations of 1 nM and above, the kinetics were sustained and insensitive to PDGF concentration. These observations indicate that the pathway is saturated upstream of Akt, at the level of PI 3-kinase activation. The lack of cooperativity in Fig. 2B further suggests a roughly linear relationship between the 3’-PI level and Akt activation.

Akt Activation Correlates with Receptor-mediated Recruitment of PI 3-Kinase p110α; Higher Levels of PDGF Receptor Phosphorylation Are Required to Recruit PI 3-Kinase p110α—We sought to confirm the relationship between PDGF receptor phosphorylation and Akt activation using alternative assays, and to further explore the saturation of the pathway at the level of PI 3-kinase activation. Lysates of cells stimulated with PDGF-BB for 10 min were pooled from 3 days of experiments. As shown in Fig. 3A, anti-phosphotyrosine immunoblotting of
FIG. 2. Akt activation does not exhibit positive cooperativity with respect to PDGF receptor phosphorylation. A, time courses of Akt activation in NIH 3T3 fibroblasts, stimulated with various concentrations of PDGF-BB, were quantified by in vitro kinase assay as described under “Experimental Procedures.” After subtracting the average signal from lysates of unstimulated cells, each data point was normalized by the time-integrated average for 10 nM stimulation measured on the same day. The symbols are averages of experiments performed on 5 different days (mean ± S.E., n = 5). PDGF-BB concentrations are: ▼, 0.05 nM; ▲, 0.1 nM; △, 0.2 nM; □, 0.5 nM; ■, 1 nM; ○, 3 nM; ●, 10 nM. B, receptor-signal response curve for PDGF-stimulated Akt activation in NIH 3T3 fibroblasts. The time-integral of Akt activation, from the data in A, is plotted versus that of PDGF receptor phosphorylation (Fig. 1A). Time-integrals were normalized by the 10 nM PDGF point for each day of experiments, and the values shown are mean ± S.E., n = 5. The solid curve is the best fit assuming no cooperativity, y(x) = ymax x 0.5 K + x).

cell lysate proteins yielded intense bands at just below 200 kDa, attributed to the phosphorylation of PDGF β- as well as α-receptors dimerized in the plasma membrane. The band intensity is half-maximal at roughly 0.5 nM PDGF-BB, in accord with the ELISA measurements (Fig. 1). A similar pattern was observed when the same lysate proteins were blotted with an antibody recognizing pTyr751 of PDGF β-receptor (residue in the human receptor), one of the PI 3-kinase p85 binding sites. Akt activities in the same lysates were assessed by immunoblotting with phospho-Akt-specific antibodies. Half-maximal activation was observed between 0.1–0.2 nM PDGF-BB, in agreement with our in vitro Akt kinase assays.

In parallel, the same lysates were subjected to immunoprecipitation with antibodies against the p110α or p110β catalytic subunit of PI 3-kinase, and the recovered proteins were probed for phosphotyrosine (Fig. 3B). Bands corresponding to PDGF receptors were readily detected in both cases, presumably reflecting the PDGF receptor-mediated recruitment of p85-p110 complexes; however, recruitment of p110α and p110β followed markedly different patterns. Whereas p110α recruitment responded to low levels of receptor phosphorylation and reached saturation at 0.5 nM PDGF-BB and above, significant p110β recruitment required higher receptor phosphorylation levels.

To the extent that formation of receptor-p85-p110 complexes is indicative of PI 3-kinase activation, these results suggest that Akt activation is more sensitive to recruitment of complexes containing p110α.

The Kinetics of Akt Deactivation, in Relation to Changes in the 3′-PI Level, Reveal the Influences of 3′-PI Turnover and Akt Dephosphorylation—To address the relative rates of processes that influence the activation and deactivation of Akt, 3′-PI generation and Akt activity were measured in separate experiments designed to introduce rapid increases and decreases in 3′-PI 3-kinase activity. Total internal reflection fluorescence microscopy was used to monitor the kinetics of 3′-PI production in individual cells transfected with the fluorescent probe GFP-AktPH (38), and Akt activation was measured by in vitro kinase assay as in Fig. 2. Fig. 4A shows the aggregate 3′-PI response of individual fibroblasts maximally stimulated with PDGF at 37 °C. A peak value is achieved in about 5 min, after which some cells show a slight decay, consistent with the downstream activation of Akt shown in Fig. 2A. The same cells were then treated with a high dose of wortmannin, which rapidly blocks PI 3-kinase activity and allows the kinetics of 3′-PI turnover to be assessed in isolation, as shown in Fig. 4B; quantitatively similar results were achieved using LY294002 instead of wortmannin (results not shown). From the initial value at the time of wortmannin addition, the decay in 3′-PI level followed first-order kinetics. Fitting the aggregate response in Fig. 4B yielded an apparent value of the 3′-PI turnover rate constant (0.95 min⁻¹).

The time course of Akt activation and deactivation in such experiments is displayed in Fig. 4C. The data in both the stimulation and inhibition periods exhibited approximately the same observed first-order rate constant values (0.55 and 0.56 min⁻¹, respectively), and quantitatively similar results were observed using LY294002 instead of wortmannin (data not shown). The similarity of these rate constant values is consistent with activation of only a small fraction of cellular Akt; if this was incorrect, and the majority of Akt was activated, then the characteristic time required to achieve Akt activation in
The derivation of the model equations and their underlying assumptions are described in detail in the online Supplemental Materials.

| Kinetic model equations |  |
|-------------------------|--|
| $C_1 = [L]/K_{D,L}$ |  |
| $d[R + C_1]/{dt} = -2(k_{-c}C_2 - k_Cc_1^2)$ |  |
| $dC_2/{dt} = k_CC_1^2 - (k_a + k_h)C_2$ |  |
| $R(0) = R_0; C_0(0) = C_0(0) = 0$ |  |

**PI 3-kinase activation (p*), 3'-PI generation (s), and Akt activation (a**)**

$$p_1^* = 2C_2/[R_{DF,PI} + 2C_2]; p_2^* = 2C_2/[R_{OF,PI} + 2C_3]$$

$$\frac{dx}{dt} = k_1(p_1^* + \gamma p_2^* - x)$$

$$\frac{da}{dt} = k_{dasso}(x - a^*)$$

$x(0) = a^*(0) = 0$

**PDGF, at concentration [L], binds to a free receptor R to form a 1:1 complex C_1; this interaction is assumed to be at pseudo-equilibrium, with dissociation constant $K_{D,L}$. A receptor dimer, C_2, is formed through the interaction of two C_1 (rate constant $k_C$). Dimers dissociate either at the cell surface or upon endocytosis (rate constants $k_a$ and $k_h$, respectively). Receptor species levels are in units of number/cell, and the initial surface receptor expression level is R_0.

Two isofoms of PI 3-kinase are activated by PDGF receptor dimers (active fractions $p_1^*$ and $p_2^*$); pseudo-equilibrium relations are assumed here. 3'-PI is produced by the PI 3-kinase activity and is consumed with first-order kinetics (rate constant $k_{dasso}$); the dimensionless variable $x$ is the appropriately scaled 3'-PI level. The rate of Akt activation is assumed to be proportional to the 3'-PI level, and Akt is deactivated with first-order kinetics (rate constant $k_{dasso}$). The dimensionless variable $a^*$ is the appropriately scaled Akt activity.

In the receptor activation portion of our model, PDGF-BB ligand is added at time 0 to yield a constant extracellular solution concentration [L]. One of two binding surfaces of the ligand engages a free surface receptor R to produce a 1:1 complex C_1, a requisite first step in receptor activation, while the active (phosphorylated) species C_2 consists of two dimerized receptors. The manner in which this dimerization occurs is the pivotal feature of our model, as illustrated in Fig. 5. It is generally accepted that one dimeric PDGF is able to bridge two PDGF receptor molecules (5, 39, 40), and thus it is tempting to apply previous theoretical models of monovalent receptors engaging a homodimeric ligand (41). In such a receptor cross-linking model, C_2 is formed from the lateral association of a C_1 complex and a receptor R in the plasma membrane (Fig. 5A), yielding a number of important predictions. Chief among them is that when the ligand concentration is increased such that the majority of binding sites are occupied, dimer formation and therefore PDGF-mediated signaling is inhibited. However, to our knowledge, this has not been observed for PDGF or other ligands that engage receptor tyrosine kinases. PDGF-BB binds to cell membranes or immobilized PDGF receptor extracellular domains with an observed $K_p = 0.1-1$ nM (11, 39, 42–44), yet inhibition of cell responses at much higher concentrations has not been observed. The receptor cross-linking model further predicts that, at relatively low ligand concentrations [L], the steady state number of dimerized receptors is proportional to [L]; i.e. such a model does not by itself predict positive cooperativity in receptor activation. Thus, the model depicted in Fig. 5A fails to capture multiple characteristics of our pTyr-PDGF

response to PDGF would be significantly shorter than that of Akt deactivation in response to inhibition by wortmannin. The value of this rate constant, which reflects both 3'-PI turnover and deactivation of Akt, is significantly less than (but within a factor of 2 of) the apparent 3'-PI turnover rate constant from Fig. 4B, suggesting that 3'-PI turnover and Akt deactivation occur at similar rates.

Measurements of PDGF β receptor phosphorylation from the same lysates, shown in Fig. 4D, confirm that wortmannin treatment does not adversely affect receptor activation. The data are consistent with the corresponding kinetics shown in Fig. 1A, and if anything, receptor activation as measured by ELISA may transiently increase upon wortmannin addition.

**Mathematical Modeling Reveals Important Features of the Apparent PDGF Receptor Activation Mechanism—Ordinary differential equations were formulated to simulate the observed kinetics of PDGF receptor phosphorylation and activation of Akt (Table I). The utility of kinetic modeling is that it may be used to distinguish between candidate molecular mechanisms and to synthesize the conclusions derived from the experimental analyses. Starting with a more complex model, the goal was to construct a simplified model with the minimum number of kinetic parameters required to capture all salient features of our experimental results (a detailed description of the full model and its simplification is provided in the online Supplemental Material).**

![Graphs](image-url)
receptors are rapidly dephosphorylated, in agreement with other recent work using NIH 3T3 cells (47). As shown in Fig. 5C, a best fit of this model agrees with our pTyr-PDGF receptor data set in every respect. The resulting estimates of the rate constants, listed in Table II, are reasonable in comparison with other growth factor-receptor systems (41). Their values suggest that the dimerized receptor complex is very stable, with an intrinsic lifetime of ~14 min (1/\(k_{-d}\)). However, the signal is strongly regulated by endocytosis, which apparently reduces the mean lifetime of a dimer at the cell surface to about 4 min (1/(\(k_{-d} + k_d\))). In the absence of endocytosis, the model predicts that steady-state ligand binding to the cell surface would be half-maximal at roughly 0.5 nM PDGF-BB, in approximate agreement with the aforementioned PDGF-BB binding studies.

**Insights from Modeling PI 3-KinaseAkt Activation**—In our model, PI 3-kinases are activated through receptor-mediated recruitment to the plasma membrane, and the fraction of each PI 3-kinase in this state is termed \(p^*\). Two PI 3-kinase isoforms were included in the model based on the detection of both of the relevant PI 3-kinases, p110\(\alpha\) and p110\(\beta\), in complex with PDGF receptors (Fig. 3B). The number of available PI 3-kinase-binding sites is assumed to be proportional to the number of PDGF receptors in dimers, and the extent of binding is controlled by the dissociation constant \(K_{D2}\) for each isoform. The parameter \(\gamma\) is the ratio of the two PI 3-kinase activities in the limit of \(p_1^* = p_2^* = 1\). The net increase in the 3'-PI level is scaled to give the dimensionless variable \(x\), which is subject to the PI 3-kinase activity and turnover by first order reaction. The rate of 3'-PI turnover is determined by the rate constant \(k_{d3}\), and its value was set to 0.95 min\(^{-1}\) from Fig. 4B. The PDGF-stimulated increase in activated Akt was also scaled appropriately, giving the dimensionless variable \(a^*\). The forward rate of Akt activation in the simplified model is simply proportional to the 3'-PI level, based on the lack of positive cooperativity noted in Fig. 2B, and Akt deactivation is modeled as a first-order process with rate constant \(k_{dA}^*\).

The values of the remaining adjustable rate parameters (\(K_{D2,p1}, K_{D2,p2}, \gamma\), and \(k_{dA}^*\)) were estimated by fitting the model output to our Akt activation data set (Fig. 2A); the best fit is displayed in Fig. 6A, with parameter values listed in Table II. The parameters are such that the two PI 3-kinase isoforms make different contributions to the activation of Akt. Our experimental analysis indicated that the saturation in Akt activation (Fig. 2B) occurred at the level of PI 3-kinase, and this is reflected in the model by the low dissociation constant of the first PI 3-kinase isoform, \(K_{D2,p1}\). Its value predicts that half-maximal recruitment of this isoform occurs when less than 1% of the receptors initially at the cell surface are phosphorylated. In contrast, binding of the second isoform is not saturable, such that this isoform is only effective at higher concentrations of PDGF. With the binding of the second isoform roughly proportional to receptor phosphorylation, the same gain in its lipid kinase activity can be produced with various combinations of the parameters \(K_{D2,p2}\) and \(\gamma\). Nevertheless, the model fits to the data reproducibly predicted that the second isoform contributes a maximum of 20–25% of the total PI 3-kinase activity. Despite the subtle role of the second isoform, eliminating it from the model yielded a poorer fit to the data (results shown in the Supplemental Material). These predictions concerning the first and second isoforms are consistent with our analysis of the apparent recruitment of PI 3-kinase p110\(\alpha\) and p110\(\beta\), respectively.

With saturation of PI 3-kinase activation, the kinetics of 3'-PI accumulation at higher concentrations of PDGF are controlled by the turnover of 3'-PIs in the membrane (rate con-
Kinetic Model of PDGF Receptor Signaling

**TABLE II**

Best-fit values of kinetic model parameters

| Parameter        | Definition                                      | Value       |
|------------------|-------------------------------------------------|-------------|
| $K_{D,L}$        | PDGF single-site dissociation constant           | 1.47 nM     |
| $k_c$            | Receptor dimerization rate constant              | (0.290 min$^{-1}$)$/R_0$a | |
| $k_x$            | Dimer uncoupling rate constant                   | 0.0703 min$^{-1}$ | |
| $k_h$            | Dimer internalization rate constant              | 0.193 min$^{-1}$ | |
| $k_{d,P1}$       | PI 3-kinase-receptor dissociation constant, p110α | 0.00711$R_0$ | |
| $K_{D,P2}$       | PI 3-kinase-receptor dissociation constant, p110β | 0.40$R_0$b | |
| $\gamma$         | Ratio of p110α/p110β activation, infinite $C_2$ limit | 0.67c | |
| $k_{PI}$         | 3'-PI turnover rate constant                     | 0.95 min$^{-1}$ | |
| $k_{d,act}$      | Akt deactivation rate constant                   | 1.31 min$^{-1}$ | |

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*This value is 2 orders of magnitude below the diffusion-limited value, assuming a receptor expression level $R_0 \sim 10^7$ cell and a receptor mobility coefficient of $\sim 10^{-10}$ cm$^2$/s.

b The values of these two parameters can be adjusted together to yield a similar fit, provided that the value of $K_{D,P2}$ is sufficiently high.

c Value taken from an exponential fit to the data in Fig. 4B.

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![Diagram A](image1.png)

**Fig. 6. Comparison of the Akt activation model with experiment.** The plots in A and B are adapted from Fig. 2A and 4C, respectively. A, the solid curves through the data are the best fit of the differential equations listed in Table I, with $2C_2/R_0$ as a function of time from Fig. 5C. The best-fit rate parameters are listed in Table II. The model output ($\phi^* \hat{x}$) was multiplied by a scaling constant, which was adjusted along with the rate parameters for alignment with the data. B, the solid curve is the model prediction; the only fitted parameter here was a scaling constant for alignment with the data.

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**DISCUSSION**

Intracellular signal transduction, even through a single receptor, is remarkably complex. To quantitatively predict the strength and duration of biochemical signals, under normal and perturbed stimulation conditions, it is becoming clear that signaling processes need to be described in mathematical terms. Many such models are now appearing, but the challenge is that a large set of experimental data is needed to validate them. With validated models of signaling pathways, it will be possible to identify the kinetically rate-limiting steps and perhaps, through a comparison of competing models, infer aspects of the molecular mechanisms involved.

Through the measurement and modeling of PDGF receptor phosphorylation, we have proposed a mechanism in which receptor dimerization is mediated by the association of two 1:1 ligand-receptor complexes. Supporting such a mechanism is the evidence that PDGF-mediated dimerization of PDGF receptor extracellular domains requires receptor sequences that apparently do not contribute to PDGF binding (40, 48, 49). The proposed mechanism is sufficient for positive cooperativity to be observed with respect to PDGF concentration. To maintain the degree of cooperativity observed (Hill coefficient of 1.6), we predict that only about 40% of the receptors initially at the cell surface are phosphorylated under maximal stimulation conditions (Fig. 5C). This value is similar in magnitude to previous quantitative measurements, using a cell line with a similar number of receptors (46). In light of these studies, along with the aforementioned prediction of observed PDGF-BB binding affinity, we conclude that our interpretation of PDGF/PDGF receptor dynamics is conceptually and quantitatively consistent with previous findings.

Yet another mechanism that can produce the degree of positive cooperativity observed is the presence of preassembled receptor dimers or oligomers, to which two ligand molecules must bind in sequence to activate the complex for signaling. Evidence for preassembled PDGF receptor oligomers on cells has indeed been reported (50), and we have formulated and analyzed mathematical models of this mechanism as well. When such models were fit to our data set, the level of agreement approached (but did not match) that of the model in Table I (results shown in the Supplemental Material).

With an adequate model of PDGF receptor phosphorylation, we wished to describe the kinetics of PI 3-kinase/Akt activation. Compared with the PDGF receptor phosphorylation response, 3'-PI production and Akt activation responded to lower PDGF concentrations and were more sustained at higher concentrations of PDGF. Sustained activation of the PI 3-kinase/Akt pathway may be important for cell function, e.g., protection from programmed cell death, and indeed the timing of PDGF-
stimulated PI 3-kinase activation has previously been implicated in the control of different cell responses (37). The kinetics of Akt activation relative to PDGF receptor phosphorylation are completely consistent with saturation at the level of PI 3-kinase activation. Previous models have also been used to conclude that saturation in signaling can produce sustained responses even when the input is transient (51, 52). Together with positive cooperativity introduced at the level of PDGF responses even when the input is transient (51, 52). The extreme differences predicted in both the recruitment and activation of the two PI 3-kinase isoforms are consistent with differential regulation of p110α and p110β, perhaps through differences in their interactions with p85 subunit isoforms and/or Ras-GTP (61).

Progress in the area of cellular modeling will come with the expansion, refinement, and integration of models such as the one formulated here, accommodating more complex interactions to make the models more predictive. To do this in a manner that is fully validated by experiment, it is clear that we must not only make quantitative measurements of the key signaling intermediates, but also reproducibly manipulate the levels and/or activities of the molecules involved.

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