

Ca²⁺ Channel Activation by Platelet-derived Growth Factor–induced Tyrosine Phosphorylation and Ras Guanine Triphosphate–binding Proteins in Rat Glomerular Mesangial Cells

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Abstract

We investigated the signaling pathways mediating 1-pS Ca²⁺ channel activation by PDGF in cultured rat mesangial cells. In cell-attached patches, intrapipette PDGF-BB (PDGF B chain homodimer isoform) (50 ng/ml) dramatically stimulates channel activity ($P < 0.003$, $n = 6$). Tyrosine kinase inhibition (100 μ M genistein or 10 μ M tryphostin 9) abolished PDGF-induced channel activation ($P < 0.02$, $n = 6$). In excised patches, the effect of tyrosine kinase inhibition could be reversed by 200 μ M GTP γ S ($P < 0.02$, $n = 4$). In contrast, 200 μ M GDP β S inhibited PDGF-induced channel activity ($P < 0.04$, $n = 6$). Pertussis toxin (250 ng/ml) had no effect on PDGF-induced channel activity ($P = 0.45$, $n = 6$). When excised patches were exposed to anti-Ras antibody (5 μ g/ml), PDGF-induced channel activity was abolished ($P < 0.002$, $n = 11$). Western immunoblots revealed that PDGF-BB binding stimulates the formation of a membrane-bound complex consisting of growth factor receptor–binding protein 2, son of sevenless, and the PDGF- β receptor. Complex formation was abolished by genistein. In mesangial cells, the intrinsic tyrosine kinase activity of the PDGF- β receptor stimulates the formation of a membrane-bound growth factor receptor–binding protein 2/son of sevenless/PDGF- β receptor complex and activation of the pertussis toxin-insensitive GTP-binding protein, p21-Ras, which leads to the opening of 1-pS Ca²⁺ channels. (*J. Clin. Invest.* 1996; 97: 2332–2341.) Key words: growth factor receptors • Ca²⁺ channels • G-proteins • tyrosine kinase • Ras

Introduction

PDGF is an important stimulator of proliferation, matrix deposition, chemotaxis, and contraction in glomerular mesangial cells (1–6). PDGF stimulates DNA synthesis and proliferation of cultured mesangial cells and, when infused into rats, induces mesangial proliferation. Conversely, inhibition of PDGF re-

duces mesangial proliferation in rat models of experimental nephritis. Proliferation and extracellular matrix expansion by mesangial cells likely contributes to the destructive and sclerotic responses observed in many forms of glomerulonephritis. Mesangial cells express significant levels of PDGF- β receptors and show mitogenic and chemotactic responses to the B chain homodimer (BB) isoform of PDGF.

The influx of extracellular Ca²⁺ is an important prerequisite for the initiation of cellular DNA synthesis and proliferation in response to PDGF (7). Conversely, inhibition of PDGF-induced Ca²⁺ entry blocks mitogenic responses (8). Evidence in nonexcitable tissues, including mesangial cells, suggests that this Ca²⁺ entry pathway is voltage independent and directly receptor operated or ligand gated (9–12). Our previous patch-clamp studies have shown that cultured rat mesangial cells possess low conductance, 1-pS Ca²⁺ channels in high abundance (3,000–4,000 channels per cell) (4, 13). This channel has the following properties: (a) baseline channel activity is very low, but dramatic activation occurs when PDGF-BB is applied inside the patch pipette; (b) channel kinetics are only slightly voltage dependent; and (c) the channel is cation nonselective, but with a higher permeability to divalents (e.g., Ca²⁺ or Mn²⁺) than to monovalents (e.g., Na⁺ or K⁺). All of these features make this PDGF-induced Ca²⁺ channel a likely candidate for the PDGF- β receptor–operated Ca²⁺ entry pathway proposed for glomerular mesangial cells.

Our previous observation that channel activation occurs only when PDGF-BB is directly applied to the surface of the membrane patch (inside, but not outside, the patch pipette), suggests that the signal transduction pathways linking the PDGF- β receptor and this 1-pS Ca²⁺ channel must be tightly coupled (4, 13). Similar to other growth factor receptors, the PDGF- β receptor possesses intrinsic tyrosine kinase activity which upon ligand binding leads to receptor autophosphorylation and the activation of multiple mitogenic signaling pathways (1–3, 14). An important signaling pathway downstream of PDGF- β receptor binding involves the translocation of growth factor receptor–binding protein 2 (GRB-2)¹ and son of sevenless (SOS), which exists as a cytoplasmic complex, to the plasma membrane where it binds to the PDGF- β receptor itself (15). This membrane-bound complex functions as a guanine nucleotide-releasing or -exchange factor (GNRF or GEF) and activates the small GTP-binding protein, p21-Ras (15–22). Ras is an important component for initiating cellular growth

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1. Abbreviations used in this paper: GEF, guanine nucleotide-exchange factor; GNRF, guanine nucleotide-releasing factor; GRB-2, growth factor receptor-binding protein 2; NP_o, number of channels \times the open probability; PTX, pertussis toxin; SOS, son of sevenless.

and differentiation by multiple growth factors, including PDGF.

The regulation of receptor-operated Ca^{2+} entry by tyrosine kinases has been reported in several cell types (23–25). In addition, multiple ion channel types are regulated by tyrosine kinases (e.g., nonselective cation, Cl^- , or Na^+ channels) (26–31) and *Ras* (e.g., Na^+ , Ca^{2+} , or K^+ channels) (32–36) in a variety of cell types. Therefore, we investigated the role of PDGF- β -receptor tyrosine kinase activity, guanine nucleotides, and *Ras* in the regulation of the 1-pS Ca^{2+} channel in glomerular mesangial cells.

Methods

Preparation and maintenance of glomerular rat mesangial cell cultures. Glomerular mesangial cell cultures were prepared and maintained as previously described (13, 37, 38). Briefly, male Sprague-Dawley rats (Harlan Sprague-Dawley Co., Indianapolis, IN) weighing 75–100 g were decapitated. The kidneys were quickly removed into iced Hank's solution and decapsulated. Cortical tissue was minced and successively filtered through a 149- μm and then a 74- μm nylon sieve. The isolated glomeruli were then washed and plated into RPMI 1640 medium supplemented with 17% FBS, 2 mM glutamine, 5 $\mu\text{g}/\text{ml}$ insulin, 5 $\mu\text{g}/\text{ml}$ transferrin, 5 ng/ml selenium, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 100 U/ml penicillin. The suspension was incubated in humidified air containing 5% CO_2 . Only the third to seventh mesangial cell subpassages were used and the cells were exposed to serum-free medium for 6 h before each experiment.

Patch-clamp recording. Glass pipettes (TW150F-6; World Precision Instruments Inc., New Haven, CT) were prepared with a patch-pipette puller and a pipette polisher (PP-83 and MF-83, respectively; Narishige, Tokyo, Japan) (13, 37, 38). Pipettes with resistances of > 10 megaohms were used for the experiments. Single-channel patch clamp configurations, including the cell-attached and inside-out modes, were established on the membrane of mesangial cells cultured on glass coverslips. In the cell-attached mode, single-channel currents were recorded without applying potential to the pipette (i.e., resting membrane potential). In the excised inside-out mode, single-channel recordings were obtained with +60 mV applied pipette voltage (i.e., $V_m = -60$ mV). All experiments were conducted with an amplifier (Axopatch-ID, Axon Instruments Inc., Foster City, CA) at room temperature, 22–23°C. Only patches with a seal resistance > 30 gigaohms and containing currents without baseline drift were used for experiments. Data were digitally recorded with a pulse code modulator (DAS 601; Dagan Corp., Minneapolis, MN) and a VCR (SL-HF860D; Sony Corp. of America, Park Ridge, NJ), and acquired using an 8-pole Bessel filter (902LFP; Frequency Devices Inc., Haverhill, MA), TL-2 acquisition hardware, and Axotape software (Axon Instruments Inc.). Single channel traces were sampled at 2 ms/point and software filtered at 100 Hz. Inward Ca^{2+} or Mn^{2+} current (pipette to cell) is represented by downward channel transitions.

Single channel and statistical analysis. Data analysis was performed on a computer (MicroVax II; Digital Equipment Corp., Maynard, MA) using a locally developed software program (Douglas C. Eaton, Ph.D.; Emory University School of Medicine, Atlanta, GA) (13, 37, 38). As a measure of channel activity, number of channels \times the open probability (NP_o) was calculated.

$$\text{Eq. 1: } \text{NP}_o = \sum_{N=0}^N \frac{n \cdot t_n}{T}$$

T was the total record time, n was the number of channels open, t_n was the record time during which n channels are open, and N was the total number of functional channels in the patch (estimated by observing the number of peaks detected on current amplitude histo-

grams). Therefore, NP_o could be calculated without making assumptions about the total number of channels in a patch or the open probability of individual single channels.

All NP_o values were calculated from 3 min of single channel recording and are reported as mean $\text{NP}_o \pm \text{SEM}$. Whenever possible, experiments were conducted in a paired fashion, with each patch acting as its own control. In these cases, the average change in NP_o for a group of experiments, compared before and after an experimental manipulation, was analyzed using a paired t test or ANOVA for multiple comparisons. Significance was $P < 0.05$. Statistical analysis was performed using SigmaStat (Jandel Scientific, San Rafael, CA).

Preparation of mesangial cell-membrane fractions for Western analysis. Cultured mesangial cells were washed twice with serum-free RPMI 1640 (Irvine Scientific, Santa Ana, CA) as previously described (31). Briefly, cells were incubated in 5 ml of serum-free RPMI 1640 (pH 7.4) for 24 hr, and then the dishes were exposed to 50 ng/ml PDGF-BB for timed incubation periods ranging up to 60 min. To terminate the incubation, the cells were washed three times with ice-cold PBS plus 1 mM Na_3VO_4 . Hypotonic buffer (50 mM Tris-HCl, pH 7.4, 2 mM Na_3VO_4 , 50 mM NaF, 10 mM $\text{Na}_2\text{P}_2\text{O}_7$, 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ leupeptin, and 10 $\mu\text{g}/\text{ml}$ aprotinin) was added and the cells were then placed on ice to thaw for 30 min. The thawed cells were scraped from the plate and centrifuged (Ultracentrifuge, J2-21 with JA-21 Rotor; Beckman Instruments, Inc., Fullerton, CA) for 20 min at 6,000 g at 4°C. The supernatant was discarded. 1 ml of ice cold RIPA buffer (hypotonic buffer plus 10% NP-40, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, and 2.5 mM EDTA) was added to the pellet, followed by incubation for 1 h at 4°C. After centrifugation for another 20 min at 6,000 g at 4°C, the supernatant was used for Western analysis. Protein concentrations were determined by the method of Lowry et al. (39).

Western immunoblot analysis. Electrophoresis in the presence of SDS (0.1%) was conducted on 1.5-mm slabs with a stacking gel of 4% polyacrylamide and a running gel of 10% polyacrylamide. We used the Protean II apparatus (Bio-Rad Laboratories, Hercules, CA) according to the method of Laemmli (40). Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes (Amersham Corp., Arlington Heights, IL) using a Trans-blot cell apparatus (Bio-Rad Laboratories) as previously described (31). The nitrocellulose membranes were incubated for 2 h in buffer A (20 mM Tris buffer, 150 mM NaCl, and 0.05% Tween 20, pH 7.5) and BSA (5% wt/vol), and thereafter for 3 h in buffer A plus BSA (5% wt/vol) containing monoclonal antibody against GRB-2 (1:1,000) or SOS (1:250). After two 5-min washes in buffer A plus BSA (5% wt/vol), the membranes were incubated for 2 h in buffer A plus BSA (5% wt/vol) containing horseradish peroxidase conjugated to sheep anti-mouse IgG. The membranes were washed once for 15 min in buffer A and four more times in buffer A for 5 min. Proteins were then detected using an enhanced chemiluminescence kit (Amersham Corp.).

Immunoprecipitation of PDGF- β Receptor-SOS complex. Mesangial cells, grown in 100-mm dishes to near confluence, were growth arrested in 5 ml of serum-free RPMI 1640 for 24 h. Cultures were again exposed to 50 ng/ml PDGF-BB and incubated for timed periods ranging up to 15 min. To terminate the incubation, the cells were washed three times with ice-cold PBS plus 1 mM Na_3VO_4 . Hypotonic buffer (see above) was added and the cells were then placed on ice to thaw for 30 min. The thawed cells were scraped from the plate and centrifuged for 20 min at 6,000 g at 4°C. The supernatant was discarded and to the pellet was added 1 ml of ice-cold RIPA buffer (hypotonic buffer plus 10% NP-40, 150 mM NaCl, 2.5 mM EDTA), followed by incubation for 1 h at 4°C. After centrifugation for another 20 min at 6,000 g at 4°C, the supernatant was collected, anti-PDGF- β receptor antibody (5 $\mu\text{l}/\text{ml}$ lysate) was added, followed by incubation overnight at 4°C. The next day, protein A/G plus 30 μl of buffer (2 mM Na_3VO_4 , 50 mM NaF, 10 mM $\text{Na}_2\text{P}_2\text{O}_7$, 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ leupeptin, and 10 $\mu\text{g}/\text{ml}$ aprotinin) was added while rocking at 4°C for 2–3 h. The immunoprecipitates were then recovered by centrifugation (Microfuge 11; Beckman Instruments, Inc.) and washed three times with ice-cold wash buffer (50 mM Tris-HCl, pH 7.4, 0.1% NP-40,

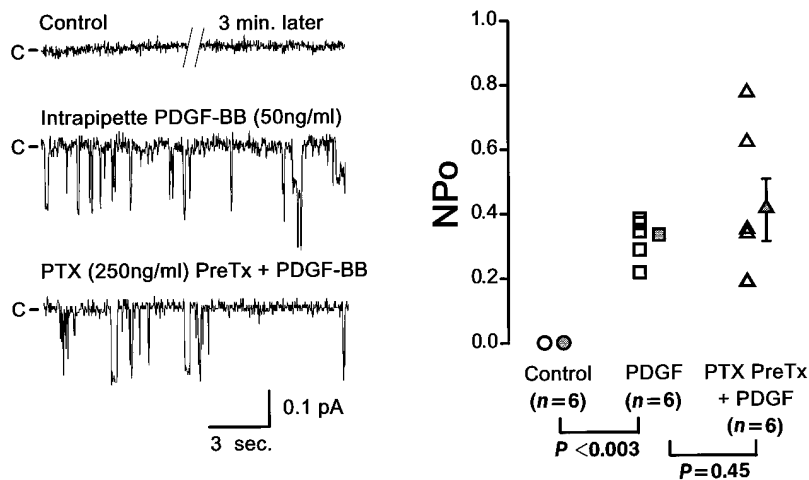


Figure 1. Activation of 1-pS Ca^{2+} channel by PDGF-BB in rat glomerular mesangial cells. (Left) Top trace shows scarce spontaneous channel activity under basal conditions with saline in a cell-attached patch pipette (Control). Middle trace shows a dramatic increase in channel activity in a cell-attached patch with PDGF-BB (50 ng/ml) in the pipette. Bottom trace shows channel activity from another patch pipette again containing PDGF-BB. However, the mesangial cell was pretreated with PTX for 4 h before patching. (Right) Summary plot of NP_0 from individual cell-attached patches without intrapipette PDGF-BB (Control, $n = 6$), with intrapipette PDGF-BB ($n = 6$), and with intrapipette PDGF-BB after PTX pretreatment ($n = 6$).

150 mM NaCl, and 1 mM Na_3V_0_4). Immunoprecipitated proteins were dissolved in 80 μl of Laemmli buffer, boiled for 5 min at 95°C, and analyzed on 10% gels by SDS-PAGE. Proteins were then transferred to nitrocellulose membranes and blotted with anti-SOS antibody. The proteins were detected using enhanced chemiluminescence.

Solutions and chemicals. For cell-attached patch experiments, the extracellular bath solution (outside the patch pipette) contained (mM): 140 NaCl (after adjusting to pH 7.4 with NaOH), 4 KCl, 1 CaCl_2 , 1 MgCl_2 , and 10 Hepes, while the intrapipette solution contained (mM): 110 MnCl_2 , 4 KCl (after adjusting to pH 7.4 with KOH), 1 CaCl_2 , 1 MgCl_2 , and 10 Hepes. When excised inside-out patch experiments were performed, the cytoplasmic bath solution contained (mM): 140 KCl (after adjusting to pH 7.4 with KOH), 10 NaCl, 1 MgCl_2 , 2 EGTA, and 10 Hepes, while the intrapipette solution contained (mM): 110 CaCl_2 , 4 KCl (after adjusting to pH 7.4 with KOH), 1 MgCl_2 , and 10 Hepes. The final free Ca^{2+} activity in the cytoplasmic bath was 10^{-7} M. Using known stability constants, a computer program developed by Fabiato (41) calculated the total amount of Ca^{2+} to add to a solution (of known pH, univalent ion, divalent ion, and EGTA concentration) to achieve the desired free Ca^{2+} activity. PDGF-BB, $\text{GTP}\gamma\text{S}$, GTP, $\text{GDP}\beta\text{S}$, GDP, and pertussis toxin (PTX) were purchased from Sigma Chemical Co. (St. Louis, MO), tryphostin 9 and genistein from Biomol Research Laboratories (Plymouth Meeting, PA), SDS from Bio-Rad Laboratories, prestained mol wt standards from Life Technologies Inc. (Grand Island, NY), monoclonal anti-GRB-2, -SOS, -PDGF- β receptor, and Rap1 antibodies from Transduction Laboratories (Lexington, KY), protein A/G plus agarose from Santa Cruz Biotechnology (Santa Cruz, CA), and polyclonal anti-Rho A and anti-Rho B antibodies from Santa Cruz Biotechnology. Anti-Ras (Transduction Laboratories) is a mouse IgG

mAb produced using the entire 21-kD Ras (Ha-ras) protein as immunogen and has been shown to immunoprecipitate p21-Ras in human, dog, rat, mouse, and chick cells. Anti-v-H-ras (Santa Cruz Biotechnology) is a rat monoclonal IgG antibody derived by fusion of spleen cells from an immunized rat with Y3Ag 1.2.3. rat myeloma cells. The latter antibody has been shown to immunoprecipitate v-H-ras and v-K-ras p21s, react with the p21 translational products of the H-, K-, and N-ras human oncogenes, and neutralize the biological and biochemical activities of H-, K-, and N-ras p21 of vertebrates.

Results

Effect of PDGF-BB on 1-pS Ca^{2+} channel activity. As we have previously reported (4, 13), 1-pS Ca^{2+} channel activity was extremely low under basal, unstimulated conditions in cell-attached patches, NP_0 was 0.0002 ± 0.0001 ($n = 6$) (Fig. 1). In contrast, channel activity was dramatically increased when human recombinant PDGF-BB (50 ng/ml) was present in the patch pipette, NP_0 was 0.33 ± 0.02 ($n = 6$) (Fig. 1). Intrapipette PDGF significantly increased channel activity over basal values ($P < 0.003$). Consistent with our previous studies (4, 13), channel activation required that PDGF-BB be in close proximity to the channel (i.e., intrapipette) since channel stimulation was not observed when PDGF-BB was added to the extracellular bath, outside the patch pipette (data not shown).

Role of tyrosine kinase activity in channel activation by PDGF-BB. Upon recognition and binding to their respective ligands, growth factor receptors, including the PDGF- β recep-

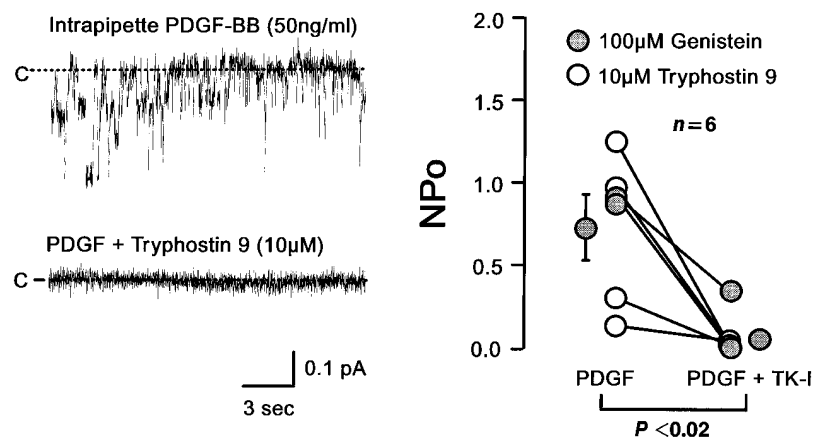


Figure 2. Effect of tyrosine kinase inhibitors on PDGF-induced channel activity. (Left) Top trace shows channel activity in a cell-attached patch with PDGF-BB (50 ng/ml) in the pipette. Bottom trace shows channel activity from the same cell-attached patch after the addition of a tyrosine kinase inhibitor, 10 μM tryphostin 9, to the extracellular bath. (Right) Summary plot of NP_0 from individual cell-attached patch pipettes containing PDGF-BB, before and after the addition of a tyrosine kinase inhibitor to the extracellular bath (\bullet , 100 μM genistein; \circ , 10 μM tryphostin 9). Lines that connect symbols represent data from the same cell-attached patch.

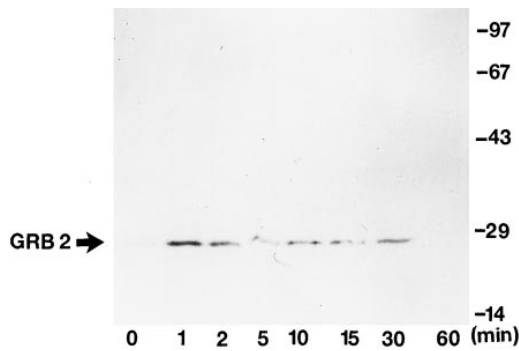


Figure 3. Membrane translocation of GRB-2 by PDGF. Mesangial cells were treated for the times indicated with 50 ng/ml PDGF-BB and then the membrane fraction was separated after hypotonic cellular lysis. Membrane-associated proteins were immunoprecipitated with a monoclonal anti-GRB-2 antibody ($n = 3$). A representative experiment shows a significant increase in membrane-associated GRB-2 within 1 min of PDGF exposure. The molecular mass reported for purified GRB-2 is 24 kD.

tor, activate intrinsic tyrosine kinase domains, which in turn lead to receptor autophosphorylation (14, 15). Signaling proteins with Src homology 2 domains then bind to these tyrosine phosphorylated residues and initiate multiple intracellular signaling cascades. We investigated PDGF-induced 1-pS Ca^{2+} channel activation in response to two agents which inhibit tyrosine kinase activity via different mechanisms (Fig. 2). Channel activity was first recorded from cell-attached patches with 50 ng/ml intrapipette PDGF-BB. Using each cell-attached patch as its own control, tryptostin 9 (10 μ M) or genistein (100 μ M) was then added to the extracellular bath. Despite the continued presence of intrapipette PDGF, exposure to either tyrosine kinase inhibitor abolished 1-pS Ca^{2+} channel activity, decreasing NP_o from 0.75 ± 0.18 to 0.08 ± 0.06 ($P < 0.02$; $n = 6$). Our previous (13) and present observation that channel activation occurs only when PDGF-BB is directly applied (i.e., intrapipette) to the surface of cell-attached or excised inside-out patch membranes, indicated that the signal transduction pathways linking PDGF- β receptor tyrosine kinase activity and the 1-pS Ca^{2+} channel must be tightly coupled and membrane delimited.

Role of GRB-2 and SOS in PDGF-induced mesangial cell responses. One membrane-delimited signaling pathway that is promoted by PDGF-receptor tyrosine kinase activity involves translocation of the cytoplasmic GRB-2-SOS complex to the plasma membrane where it binds, directly or via a docking protein, to the PDGF- β receptor itself (15). We investigated PDGF-induced membrane translocation of GRB-2-SOS complex components in glomerular mesangial cells. Mesangial cells were incubated in PDGF-BB (50 ng/ml) for various exposure times and then were subjected to hypotonic cellular lysis, followed by isolation of the membrane fraction. Western immunoblot analysis with anti-GRB-2 antibody revealed that significant recruitment of GRB-2 (24 kD) to the membrane occurred within 1 min of PDGF exposure ($n = 3$) (Fig. 3). Similarly, probing membrane fractions with anti-SOS antibody showed that translocation of SOS (170 kD) was also observed within 1 min of PDGF exposure ($n = 3$) (Fig. 4).

Mesangial cells were again incubated in PDGF-BB (50 ng/ml) for various time periods, but this time we immunoprecipi-

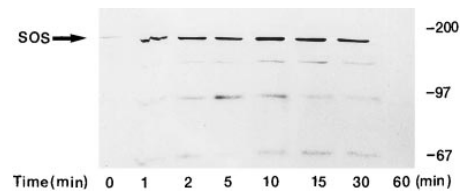


Figure 4. Membrane translocation of SOS by PDGF. Mesangial cells were treated for the times indicated with 50 ng/ml PDGF-BB, and the membrane fraction was separated after hypotonic cellular lysis. Membrane-associated proteins were immunoprecipitated with a monoclonal anti-SOS antibody ($n = 3$). A representative experiment shows a significant increase in membrane-associated SOS within 1 min of PDGF exposure. The molecular mass reported for purified SOS is 170 kD.

tated the isolated membrane fraction with anti-PDGF- β receptor antibodies. We then probed with anti-SOS antibodies and identified a membrane-associated complex between SOS and the PDGF- β receptor itself ($n = 3$) (Fig. 5, top left). A different set of mesangial cells was preincubated with the tyrosine

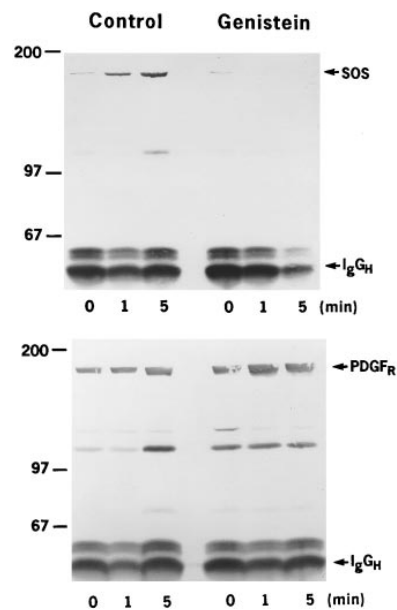


Figure 5. PDGF-BB stimulates membrane complex formation between SOS and the PDGF- β receptor. Mesangial cells were treated for the times indicated with 50 ng/ml PDGF-BB and then the membrane fraction was separated after hypotonic cellular lysis. Proteins were first immunoprecipitated with an anti-PDGF- β receptor antibody and developed with an anti-SOS antibody. (Top left) A representative experiment shows a significant increase in PDGF- β receptor/SOS complex formation within 1 min of PDGF exposure. Another set of mesangial cells were pretreated with 100 μ M genistein for 1 h before timed exposures to 50 ng/ml PDGF-BB. Membrane fractions were again separated, immunoprecipitated with an anti-PDGF- β receptor antibody, and then developed with an anti-SOS antibody. (Upper right) A representative experiment shows that in the presence of a tyrosine kinase inhibitor, no PDGF- β receptor/SOS complex formation is observed in response to PDGF exposure. (Bottom) These same membranes were stripped and reprobed with anti-PDGF- β receptor antibody, showing roughly equivalent amounts of PDGF- β receptor being brought down with the original immunoprecipitations.

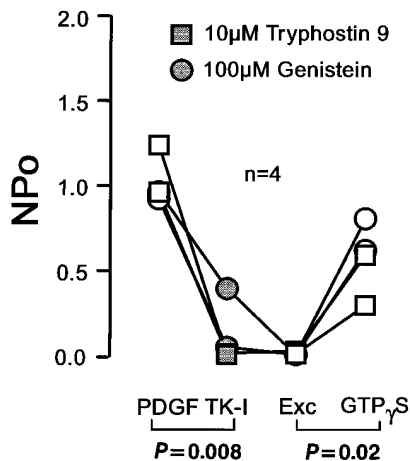
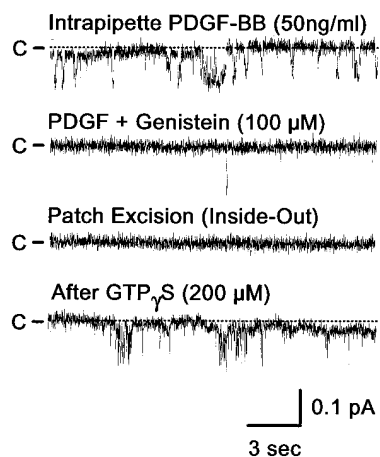


Figure 6. Nonhydrolyzable GTP analogue reverses the effects of tyrosine kinase inhibitors. (Left) Top two traces show channel activity from the same cell-attached patch pipette containing PDGF-BB (50 ng/ml), before and after addition of 100 μ M genistein to the extracellular bath. Bottom two traces show channel activity from the same patch after excision. Exposing the inside-out patch to cytoplasmic bath saline had no effect on channel activity. However, exposure to 200 μ M GTP γ S in the cytoplasmic bath dramatically activated channel activity. (Right) Summary plot of NP_o from individual cell-attached patch pipettes containing PDGF-BB, before and after the addition of a tyrosine kinase inhibitor to the extracellular bath (circles, 100 μ M genistein; squares, 10 μ M tryphostin 9). Patches after excision are shown before and after exposure to 200 μ M GTP γ S in the cytoplasmic bath. Lines connecting symbols represent data from the same patch membrane.

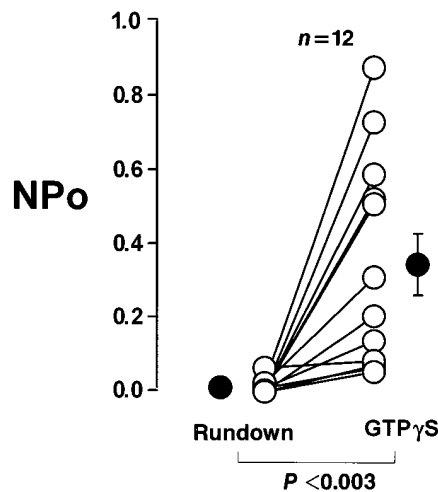
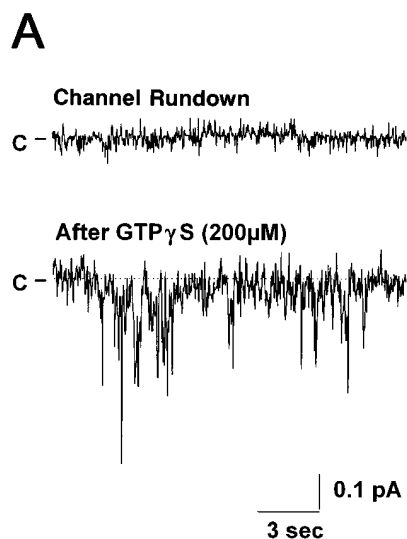
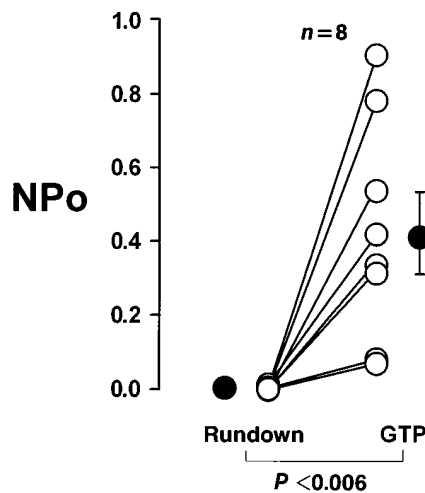
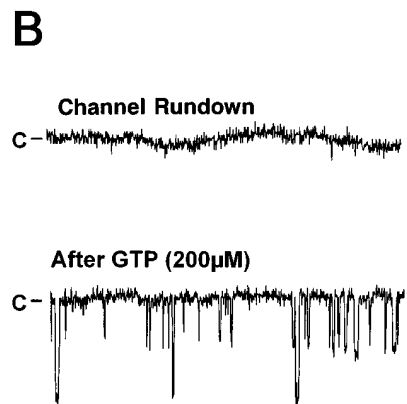


Figure 7. GTP analogues reverse channel run-down after prolonged patch excision. (A) Top trace shows channel rundown from an inside-out patch pipette containing PDGF-BB (50 ng/ml) which was maintained for > 30 min after excision. Bottom trace shows channel activation after exposure to 200 μ M GTP γ S in the cytoplasmic bath. Summary plot of NP_o from individual inside-out patch pipettes containing PDGF-BB, after channel rundown and after the subsequent addition of 200 μ M GTP γ S to the cytoplasmic bath. Lines connecting symbols represent data from the same patch membrane. (B) Top trace shows channel rundown from an inside-out patch pipette containing PDGF-BB (50 ng/ml) which was maintained for > 30 min after excision. Bottom trace shows channel activation after exposure to 200 μ M GTP in the cytoplasmic bath. Summary plot of NP_o from individual inside-out patch pipettes containing PDGF-BB, after channel rundown and after the subsequent addition of 200 μ M GTP to the cytoplasmic bath. Lines connecting symbols represent data from the same patch membrane.



kinase inhibitor, genistein (100 μM), for 1 h before timed exposures to PDGF-BB. Membrane fractions were again immunoprecipitated with anti-PDGF- β receptor antibodies and then probed with anti-SOS antibodies. Under these conditions, formation of the PDGF- β receptor-SOS complex was not observed ($n = 3$) (Fig. 5, *top right*). Finally, these same membranes were stripped and reprobed with anti-PDGF- β receptor antibodies to demonstrate that roughly equivalent amounts of PDGF- β receptor were being brought down with our immunoprecipitations (Fig. 5, *bottom*). These studies suggested that tyrosine kinase activity was essential to PDGF-induced membrane recruitment of the cytoplasmic GRB-2-SOS complex and its association with the PDGF- β receptor itself in glomerular mesangial cells.

Role of guanine nucleotides in channel activation by PDGF. The membrane-bound GRB-2-SOS complex is known to function as a GNR or a GEF (16). Therefore, we also investigated the effects of guanine nucleotides on PDGF-induced 1-pS Ca^{2+} channel responses (Fig. 6). Channel activity was again recorded from cell-attached patch pipettes containing 50 ng/ml PDGF-BB. As before, PDGF-induced channel activity was inhibited by the addition of tyrosine kinase inhibitors

(10 μM tryphostin 9 or 100 μM genistein) to the extracellular bath. NP_o decreased from 1.02 ± 0.07 to 0.11 ± 0.09 ($P < 0.008$, $n = 4$). The patches were then excised. Using each inside-out patch as its own control, channel inhibition by either tyrosine kinase inhibitor was consistently reversed by the addition of a nonhydrolyzable GTP analogue (200 μM GTP γS) to the cytoplasmic bath. NP_o increased from 0.006 ± 0.005 to 0.57 ± 0.11 ($P < 0.02$, $n = 4$).

In our previous studies of the PDGF-induced 1-pS Ca^{2+} channel (13), we observed rundown of channel activity after excised patches were maintained for a prolonged period of time (> 30 min) (unpublished data). This suggested the possibility that maintenance of channel activity required some cytoplasmic stimulatory factor that was lost with patch excision. Based on our studies above, GTP appeared to be a likely candidate factor to examine. We first allowed 1-pS Ca^{2+} channel activity to rundown in inside-out patches maintained for > 30 min after excision. Subsequent application of 200 μM GTP γS to the cytoplasmic bath consistently increased NP_o from 0.012 ± 0.006 to 0.34 ± 0.08 ($P < 0.003$, $n = 12$) (Fig. 7 A). Similarly, stimulation of rundown channel activity with direct application of 200 μM GTP to excised patch membranes was also

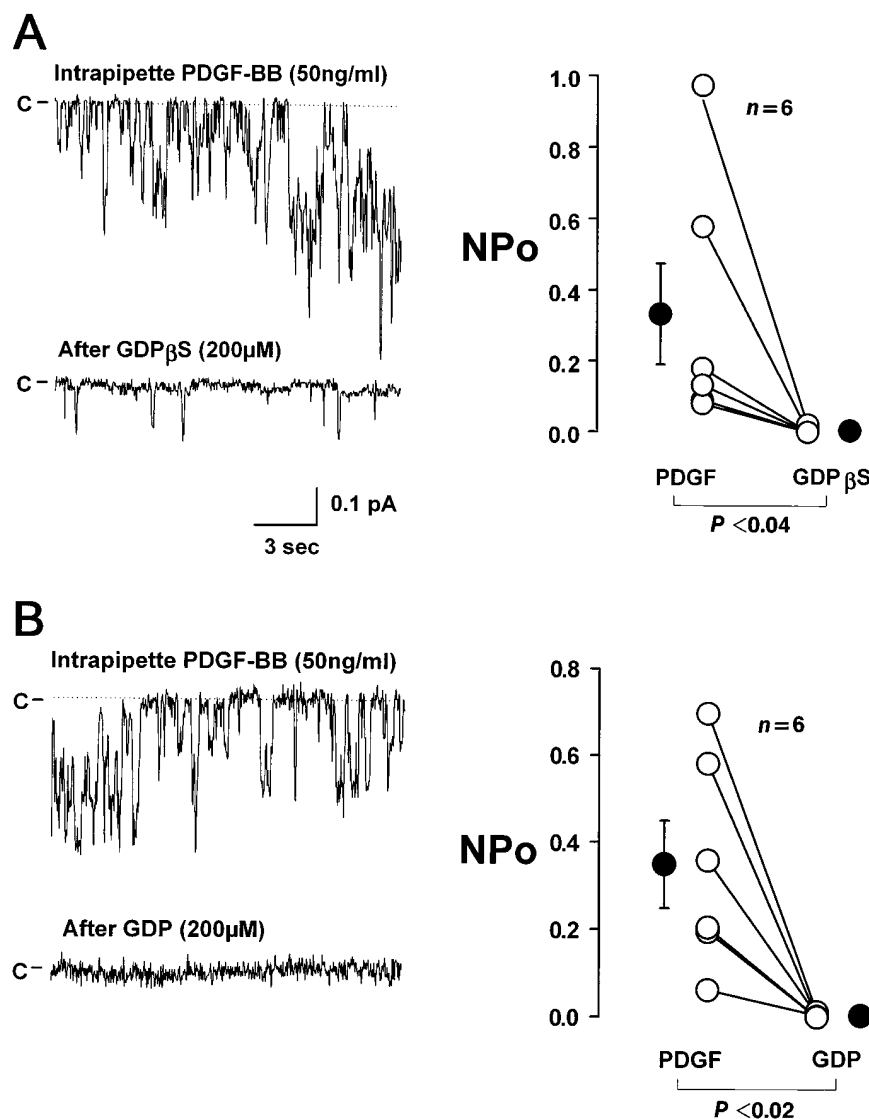


Figure 8. Inhibition of PDGF-induced channel activity by GDP analogues. (A) Top trace shows channel activity from an inside-out patch pipette containing PDGF-BB (50 ng/ml) immediately after patch excision. Bottom trace shows channel inhibition after exposure to 200 μM GDP βS in the cytoplasmic bath. Summary plot of NP_o from individual inside-out patch pipettes containing PDGF-BB, before and after the addition of 200 μM GDP βS to the cytoplasmic bath. Lines connecting symbols represent data from the same patch membrane. (B) Top trace shows channel activity from an inside-out patch pipette containing PDGF-BB (50 ng/ml) immediately after patch excision. Bottom trace shows channel inhibition after exposure to 200 μM GDP in the cytoplasmic bath. Summary plot of NP_o from individual inside-out patch pipettes containing PDGF-BB, before and after the addition of 200 μM GDP to the cytoplasmic bath. Lines connecting symbols represent data from the same patch membrane.

observed. NP_o increased from 0.011 ± 0.004 to 0.43 ± 0.11 after GTP exposure ($P < 0.006$, $n = 8$) (Fig. 7 B).

In another set of cultured mesangial cells, 1-pS Ca^{2+} channel activity was recorded immediately following excision and formation of inside-out patches using patch pipettes containing 50 ng/ml PDGF-BB. In contrast to the stimulatory effect of GTP analogues, subsequent addition of a nonhydrolyzable GDP analogue ($200 \mu\text{M}$ GDP βS) to the cytoplasmic bath decreased NP_o from 0.33 ± 0.14 to 0.004 ± 0.003 ($P < 0.04$, $n = 6$) (Fig. 8 A). Similarly, $200 \mu\text{M}$ GDP also decreased NP_o from 0.35 ± 0.10 to 0.004 ± 0.002 ($P < 0.02$, $n = 6$) (Fig. 8 B).

Given the above evidence, suggesting the involvement of a GTP-binding protein in the activation of the 1-pS Ca^{2+} channel, we examined mesangial cells pretreated with 250 ng/ml PTX for 4 h before cell-attached patching. When compared with controls, channel activation by 50 ng/ml intrapipette PDGF-BB ($NP_o = 0.41 \pm 0.10$, $n = 6$) was still significant despite the presence of PTX ($P < 0.003$), and was not different from PDGF-induced channel activity in the absence of PTX ($P = 0.45$) (Fig. 1). Therefore, the intrinsic tyrosine kinase activity of the PDGF- β receptor, a PTX-insensitive GTP-binding protein contained in excised membrane patches, and a cytoplasmic source of GTP all appeared to be important for the activation of 1-pS Ca^{2+} channels by PDGF-BB.

Role of Ras in channel activation by PDGF. A small PTX-insensitive guanine nucleotide-binding protein, which is implicated in the mitogenic effects of PDGF-BB, is Ras (15, 17–20). We examined the role of Ras in PDGF-induced Ca^{2+} channel

activation (Fig. 9). With 50 ng/ml intrapipette PDGF, cell-attached patches containing 1-pS Ca^{2+} channel activity were excised to form inside-out patches. In control experiments, we recorded for at least 3 min immediately after patch excision into cytoplasmic bath saline (see Methods). Under these conditions, NP_o was 0.29 ± 0.07 , $n = 16$. In the experimental group, we also recorded channel activity immediately after patch excision, but this time the cytoplasmic bath contained one of two different mAbs against Ras (final concentration $5 \mu\text{g/ml}$) (see Methods). PDGF-induced channel activity was abolished in the presence of either anti-Ras antibody ($NP_o = 0.017 \pm 0.005$; $P < 0.002$, $n = 11$). In the continued presence of anti-Ras antibody (anti-v-H-Ras), subsequent application of $200 \mu\text{M}$ GTP γS to the cytoplasmic bath failed to restore NP_o (0.006 ± 0.003 ; $P = 0.18$, $n = 5$). When patches from another set of mesangial cells were excised into a cytoplasmic bath containing heat-inactivated anti-Ras antibodies (60°C for 15 min), PDGF-induced channel activation was not inhibited ($NP_o = 0.26 \pm 0.04$; $P = 0.71$, $n = 4$).

In addition to Ras, the Rho family of small GTP-binding proteins has recently been shown to be membrane associated with the PDGF- β receptor upon binding to PDGF-BB (42). We again recorded channel activity immediately after patch excision, but this time the cytoplasmic bath contained polyclonal anti-Rho A or anti-Rho B antibody (final concentration $5 \mu\text{g/ml}$) (Fig. 9). However, under these latter conditions, channel activation by 50 ng/ml intrapipette PDGF was still intact despite the presence of anti-Rho antibodies ($NP_o =$

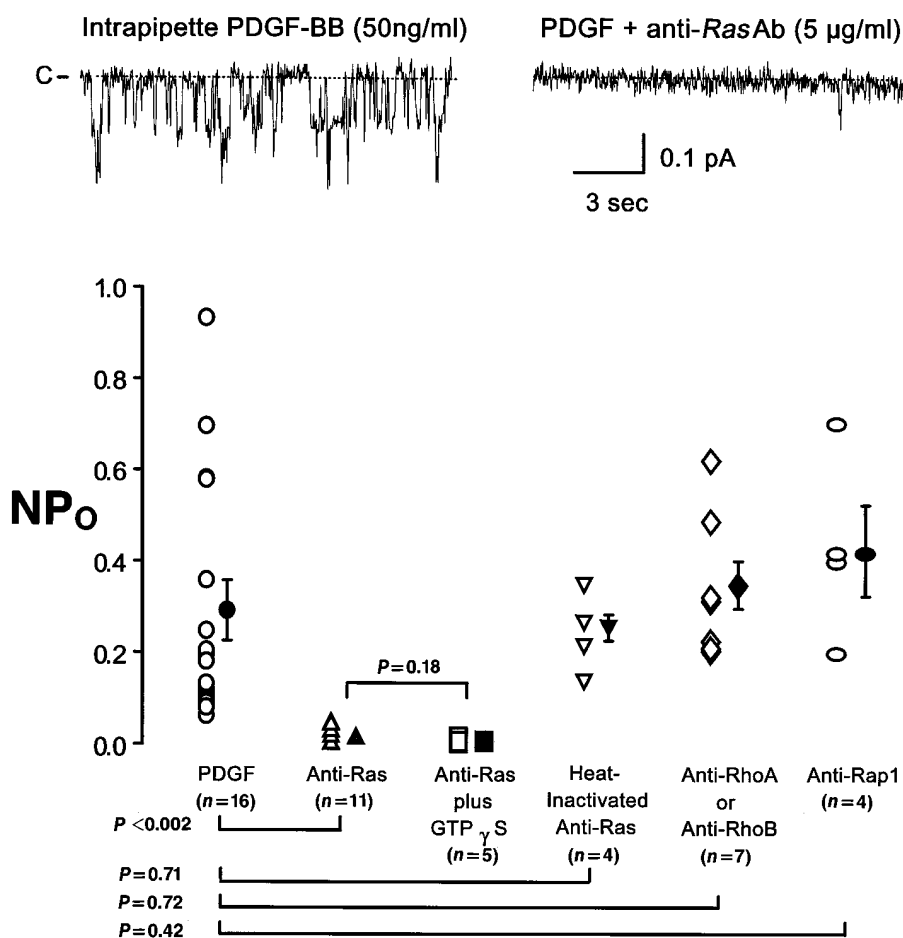


Figure 9. Blockage of PDGF-induced channel activity by anti-Ras antibody. (Top) Left trace shows channel activity from an inside-out patch pipette containing PDGF-BB (50 ng/ml) immediately after patch excision into cytoplasmic bath saline. Right trace shows channel activity in another inside-out patch excised into a cytoplasmic bath containing anti-Ras antibody (final concentration $5 \mu\text{g/ml}$). (Bottom) Summary plot of NP_o from individual inside-out patch pipettes all containing PDGF-BB (50 ng/ml). The patches were excised into a cytoplasmic bath containing saline alone (PDGF; $n = 16$), anti-Ras antibody ($n = 11$), anti-Ras antibodies plus $200 \mu\text{M}$ GTP γS ($n = 5$), heat-inactivated anti-Ras antibody ($n = 4$), anti-Rho A or anti-Rho B antibody ($n = 7$), or anti-Rap1 antibody ($n = 4$).

0.35±0.06; $P = 0.72$, $n = 4$ with anti-*Rho A* and $n = 3$ with anti-*Rho B*).

Finally, we examined the effect of *Rap1*, a small GTP-binding protein that differs in its cellular localization from *Ras* and *Rho* (42). While *Ras* and *Rho* are found on the inner surface of the plasma membrane, *Rap1* is associated with the Golgi region. We again recorded channel activity immediately after patch excision, but this time the cytoplasmic bath contained anti-*Rap1* antibody (final concentration 5 µg/ml) (Fig. 9). Channel activation by 50 ng/ml intrapipette PDGF was unaffected by the presence of anti-*Rap1* antibody (NPo = 0.43±0.11; $P = 0.42$, $n = 4$).

These experiments suggested that the effects of anti-*Ras* antibodies were not due simply to nonspecific effects on other members of the *Ras* superfamily of small GTP-binding proteins, and that p21-*Ras* does mediate PDGF-induced 1-pS Ca²⁺ channel activation.

Discussion

Receptor-operated or ligand-gated Ca²⁺ channels have been proposed as a voltage-independent pathway for Ca²⁺ entry, which mediates a variety of cellular functions in nonexcitable cells (9–12). We have previously characterized a PDGF-BB-induced 1-pS Ca²⁺ channel in the plasma membrane of cultured rat glomerular mesangial cells (4, 13, 30). Combining our previous single channel and whole cell electrophysiologic data, we calculated that mesangial cells contain 3,000–4,000 channels per cell. With normal extracellular monovalent cation and Ca²⁺ concentrations, this channel has a unit conductance of 0.01 pS and a total Ca²⁺ current (I_{Ca}) of ~0.6 pA or 3×10^{-18} mol Ca²⁺/s. Assuming a cell volume of 20 pL, this I_{Ca} would raise intracellular Ca²⁺ at a rate of 150 nM/s. Even with strong intracellular Ca²⁺ buffering, this PDGF-induced channel could easily explain the increases in mesangial cell Ca²⁺ entry observed in response to PDGF-BB binding.

In the present study, we find that 1-pS Ca²⁺ channel activation by PDGF-BB is dependent on the intrinsic tyrosine kinase activity of the PDGF-β receptor, the cytosolic ratio of GTP/GDP, and the PTX-insensitive guanine nucleotide-binding protein, *Ras*. Unlike the subtype of receptor-operated Ca²⁺ channels, which are controlled by the Ca²⁺ content of the intracellular stores (9, 10), the signal transduction pathway we describe for PDGF-induced activation of the 1-pS Ca²⁺ channel can occur independently of intracellular Ca²⁺ release (i.e., in excised patches).

1-pS Ca²⁺ channel activation is mediated via PDGF-β-receptor tyrosine kinase. The PDGF-BB isoform mediates mitogenic and chemotactic responses in mesangial cells through binding to PDGF-β receptors (1–4, 30). Similar to other growth factor receptors, upon ligand binding, the PDGF-β-receptor tyrosine kinase domain promotes receptor autophosphorylation of multiple tyrosine residues leading to the activation of multiple mitogenic signaling pathways (14, 15). In cultured rat glomerular mesangial cells, we find that PDGF-induced Ca²⁺ channel activation is significantly reduced (89%) by two structurally different tyrosine kinase inhibitors, tryphostin 9 and genistein, in cell-attached patches. Tryphostin 9 is a selective inhibitor of the PDGF-receptor tyrosine kinase (43), while genistein inhibits tyrosine kinase activity through competition for the ATP binding site (23–29). In human fibroblasts, mouse osteoblast-like cells, and rat pancreatic acinar cells, receptor-

operated Ca²⁺ influx is also blocked by genistein and tryphostin (23–25). Regulation of other ion channel types (e.g., nonselective cation, Cl⁻, and Na⁺ channels) by tyrosine phosphorylation has also been reported in ileal, vascular smooth muscle, epithelial, fibroblast, and mesangial cells (26–31). In glomerular mesangial cells, our data suggest that protein tyrosine phosphorylation in response to PDGF-BB binding is an essential step for the activation of the PDGF-β receptor-operated, 1-pS Ca²⁺ channel.

1-pS Ca²⁺ channel activation by PDGF-BB is GTP-dependent. In many cell types, an important signaling pathway downstream of PDGF-β receptor binding involves the translocation of GRB-2 and SOS, which coexist as a cytoplasmic complex, to the plasma membrane where it binds to the PDGF-β receptor itself (15). Our Western immunoblots show that in glomerular mesangial cells, PDGF-BB also stimulates the recruitment of GRB-2 and SOS to the membrane fraction, forming a complex with the PDGF-β receptor itself.

The membrane-bound GRB-2/SOS complex is known to function as a GNRG or a GEF (15). We find that the membrane-associated complex formation between SOS and the PDGF-β receptor is abolished when we inhibit tyrosine kinase activity with genistein. However, the reduction in PDGF-induced channel activity that we observe with tyrosine kinase inhibition can be reversed by direct exposure of the cytoplasmic surface of the patch membrane to GTP analogues. In contrast, PDGF-induced channel activation is abolished by GDP analogues in the cytoplasmic bath. Intracellular Ca²⁺ signaling pathways (e.g., phospholipase C) are often coupled to a PTX-sensitive G-protein; PTX promotes ADP ribosylation and disconnects G_i-like proteins from their ligand receptor (44). However, the GTP-dependent step in 1-pS Ca²⁺ channel activation by PDGF was insensitive to mesangial cell pretreatment with pertussis toxin. Our data suggest that the intrinsic tyrosine kinase activity of the PDGF-β receptor stimulates membrane translocation of GRB-2/SOS which in turn forms a complex with the PDGF-β receptor itself. The GRB-2-SOS complex, acting as a GEF, then activates a PTX-insensitive guanine nucleotide binding protein which in turn mediates Ca²⁺ channel activation.

We also find that rundown of PDGF-induced channel activity after prolonged patch excision (> 30 min) is reversed by the addition of GTP analogues to the cytoplasmic bath. The latter observation suggests that the presence of the PTX-insensitive G-protein alone is not enough to sustain channel activity. Cytosolic GTP is a stimulatory factor for 1-pS Ca²⁺ channels and in its absence (i.e., patches excised into an artificial cytoplasm), channel activity cannot be sustained for prolonged periods. These experiments in excised patch membranes indicate that this guanine nucleotide-binding protein is also membrane bound, in close proximity to both the PDGF-β receptor and the 1-pS Ca²⁺ channel.

1-pS Ca²⁺ channel activation by PDGF is mediated via Ras. A candidate PTX-insensitive guanine nucleotide-binding protein is *Ras* (15). *Ras* proteins are located on the inner face of the plasma membrane and are known to be activated by receptor tyrosine kinases, including the PDGF receptor, in different cell types (15, 17–20). The regulation of multiple ion channel types (e.g., Na⁺, Ca²⁺, K⁺ channels) by *Ras* has also been reported in fibroblast, pituitary, atrial, and neuronal cells (32–36). In excised patches from glomerular mesangial cells, we find that the activation of 1-pS Ca²⁺ channels by PDGF is

abolished in the presence of either of two different anti-*Ras* antibodies in the cytoplasmic bath (see Methods). Zubiaur et al. (42) have recently shown that, upon auto tyrosine phosphorylation, the PDGF- β receptor can also form a complex with the *Rho* family of small GTP-binding proteins. However, in excised patches from glomerular mesangial cells, we find that PDGF-induced activation of 1-pS Ca^{2+} channels was not affected by the presence of either anti-*Rho A* or *B* antibodies in the cytoplasmic bath. Neither was PDGF-induced Ca^{2+} channel activation affected by an antibody against a nonplasma membrane-associated GTP-binding protein, *Rap1*. These experiments show that the effects of anti-*Ras* antibodies are not due to nonspecific effects on other members of the *Ras* superfamily of small GTP-binding proteins, and that PDGF-induced 1-pS Ca^{2+} channel activation is mediated via p21-*Ras*.

Consistent with our finding that PDGF-induced channel activation requires GTP and is inhibited by GDP, *Ras* is active in its GTP-bound state and is inactive in its GDP-bound state (15, 16). *Ras* becomes active when GDP is released and replaced by GTP. However, this exchange reaction is extremely slow unless it is accelerated by the interaction of *Ras* with one of the family of GNRFs or GEFs (e.g., SOS). We and others have shown that PDGF- β receptor binding leads to the membrane association and activation of the GRB-2/SOS complex (15).

Pathological and physiological significance of the PDGF-induced channel. The 1-pS Ca^{2+} channel that we have characterized provides a voltage-independent, PDGF receptor-operated Ca^{2+} entry pathway. The initiation of cellular DNA synthesis and proliferation in response to PDGF requires the influx of extracellular Ca^{2+} (7). Conversely, inhibition of PDGF-induced Ca^{2+} entry blocks mitogenic responses (8). Therefore, our study of PDGF-induced Ca^{2+} channel regulation provides information regarding potential signal transduction mechanisms contributing to PDGF-induced mesangial proliferation. We have shown that PDGF-receptor tyrosine kinase and *Ras* activity is required for Ca^{2+} channel activation in glomerular mesangial cells. The importance of *Ras* activation by receptor tyrosine kinases in controlling cellular growth and differentiation is now well established (15, 21, 22). In mammalian cells, there are four true *Ras* proteins (encoded by *Ha-ras*, *N-ras*, *Ki-rasA*, and *Ki-rasB*) which, upon mutational activation, can function as independent oncogenes. These *Ras* proteins relay signals from tyrosine kinases at the plasma membrane to a cascade of serine/threonine kinases which subsequently lead to a myriad of growth factor responses.

Evidence strongly implicates PDGF in mediating the mesangial cell proliferation associated with many experimental and human glomerulonephritides (1–3, 5, 6). PDGF-BB stimulates proliferation, matrix deposition, chemotaxis, and contraction in glomerular mesangial cells in vitro (1–4, 30). Increases in PDGF A and B chain as well as in PDGF- β receptor mRNA expression have been demonstrated in experimental models of mesangial proliferative glomerulonephritis. Conversely, administration of anti-PDGF antibody reduced mesangial cell proliferation in experimental nephritis (2). Glomerular cell proliferation and PDGF expression precede glomerulosclerosis in the remnant kidney model of chronic renal failure (45). Therefore, PDGF-mediated proliferation and extracellular matrix expansion by mesangial cells likely contributes to the destructive and sclerotic responses observed in many forms of glomerular disease.

Conclusions. In either cell-attached or excised patches, the 1-pS Ca^{2+} channel is only activated when PDGF-BB is in direct contact (i.e., intrapipette) with the patch membrane containing the channel protein (13). This suggests that the signaling pathway linking PDGF-BB and the channel protein itself are tightly coupled and membrane-delimited in glomerular rat mesangial cells. Our studies provide a candidate pathway involving: (a) PDGF-BB binding to PDGF- β receptors; (b) PDGF-receptor tyrosine kinase activity mediating translocation of the cytosolic GRB-2/SOS complex to the plasma membrane where the complex binds to the receptor itself; (c) GRB-2/SOS mediating *Ras* GTP/GDP exchange; and (d) activation of the 1-pS Ca^{2+} channel by the active GTP-bound *Ras*. These results provide a convergent signaling pathway for the mesangial proliferation and expansion observed in response to PDGF, tyrosine kinases, *Ras* oncogenes, and receptor-operated Ca^{2+} channels.

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