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Platelet-Derived Growth Factor Receptor Is a Novel Modulator of Type A γ-Aminobutyric Acid-Gated Ion Channels

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SUMMARY

Platelet-derived growth factor (PDGF) and PDGF receptors (PDGFRs) are ubiquitously expressed in the mammalian central nervous system, where they exert trophic actions on both neuronal and glial cells. However, the acute actions of PDGF on synaptic transmission are unknown. We report a novel regulatory action of PDGF/PDGFR. Activation of PDGFRs inhibited the function of native type A γ-aminobutyric acid ($GABA_A$) receptors ($GABA_A$-Rs) in rat hippocampal CA1 pyramidal neurons and mouse brain membrane vesicles. The mechanism of this inhibition was studied with a panel of mutant PDGFRs-β coexpressed with cloned human $GABA_A$-Rs in *Xenopus* oocytes. These experiments revealed that phospholipase C-γ is the protein that relays the inhibitory signal from PDGFRs to $GABA_A$-Rs. Experiments with microinjected EGTA and inositol-1,3,4-triphosphate demonstrated that inhibition of $GABA_A$-Rs depended on a phospholipase C-γ-mediated increase in intracellular Ca^{2+}-levels. The PDGFR-induced inhibitory effect was independent of the subunit composition of $GABA_A$-Rs. Moreover, $GABA_A$-Rs composed of $\alpha 1\beta 1_{S409A}$ subunits, which do not contain any known protein kinase C phosphorylation sites, were inhibited by PDGF to the same extent as wild-type $GABA_A$-Rs. Inhibitors of protein kinase C, Ca^{2+}/calmodulin-dependent protein kinase II, calcineurin, and tyrosine phosphatases did not affect the modulatory actions of PDGFR. In conclusion, our results suggest that PDGFRs exert potent modulatory actions on $GABA_A$-R-dependent inhibitory synaptic transmission. These regulatory actions of PDGF could play important roles in the function of the mammalian central nervous system during physiological and pathophysiological conditions.

PDGF is a molecule that exerts important trophic actions in a wide range of tissues, including the mammalian CNS. PDGFs are dimeric molecules (PDGF-AA, -BB, and -AB) that differentially bind to two types of tyrosine kinase receptors, denoted as PDGFR-α and -β (1). *In situ* hybridization and immunostaining studies have determined that PDGF-A and -B subunits are widely expressed in the CNS (2, 3). Similar studies have also shown that PDGFR-β and -α are expressed in both neuronal and glial cells, respectively, of virtually all CNS regions (4, 5). Because of its ubiquitous expression in the CNS, the actions of PDGF on both glial and neuronal cell types have been the focus of intense research. Research on PDGF has been centered primarily on its trophic actions because of the potential uses of growth factors in the treatment of neurodegenerative diseases of the CNS (6). It is well established that PDGF is important for the proliferation of glial cells during physiological and pathophysiological conditions (5, 7, 8). In addition, PDGF exerts trophic effects on neuronal cells. Exposure of cultured newborn rat neurons to PDGF-BB increases survival, promotes neurite outgrowth, and induces the transcription factor c-*fos* (4). Because dopaminergic and GABAergic neurons are targets for the neurotrophic actions of PDGF (9, 10), this growth factor could be useful in the treatment of Parkinson's disease and, perhaps, other degenerative diseases of the CNS.

Neurotrophic growth factors appear to be important not

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ABBREVIATIONS: PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; CNS, central nervous system; PLC-γ, phospholipase C-γ; $GABA_A$-R, type A γ-aminobutyric acid receptor; IP_3, inositol-1,3,4-triphosphate; PKC, protein kinase C; PKA, protein kinase A; PKG, cGMP-dependent protein kinase; CAM-kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; IPSC, inhibitory postsynaptic current; PI3K, phosphatidylinositol-3-kinase; GAP, *ras*-GTPase activating protein; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; DMSO, dimethylsulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ANOVA, analysis of variance; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

only for neuronal growth and differentiation but also for the normal function of the adult CNS. It has been recently reported that members of the nerve growth factor family of neurotrophic factors potentiate synaptic transmission in cortical and hippocampal neurons (11, 12, 13). However, little is known about the effects of other growth factors, such as PDGF, on neuronal function. Because of the wide expression of PDGF in the CNS, it is possible that this growth factor exerts important modulatory actions on synaptic transmission. Because PDGF is a trophic factor for GABAergic neurons (10), we decided to examine the effects of PDGFR activation on the function of the $GABA_A$-R, which is the major inhibitory neurotransmitter-gated ion channel in the CNS (for review, see Ref. 14). Experiments with rat hippocampal slices and mouse brain membrane vesicles (brain microsacs) indicate that PDGFR activation reduces $GABA_A$-R function. In addition, experiments with *Xenopus* oocytes coexpressing wild-type and mutant PDGF and $GABA_A$ receptors revealed several details of the mechanism of the PDGFR-induced modulation of GABAergic responses.

Experimental Procedures

Materials. The sources of the materials used in this study were as follows: mature male Sprague-Dawley rats, Sasco (Omaha, NE); adult *Xenopus laevis* female frogs, Xenopus I (Ann Arbor, MI); ICR mice, Harlan Laboratories (Indianapolis, IN); $^{36}Cl^-$, ICN Radiochemicals (Irvine, CA); GF109203X (bisindolylmaleimide) and IP_3, Calbiochem (La Jolla, CA); BAPTA-AM, Molecular Probes (Eugene, OR); PKC inhibitor peptide, GIBCO-BRL (Gaithersburg, MD); deltamethrin, Alomone Laboratories (Jerusalem, Israel); and KN-62, Biomol Research Laboratories (Plymouth Meeting, PA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). PDGF-BB was generously provided by Synergen (Boulder, CO).

Electrophysiological recording from hippocampal slices. Transverse brain slices (400 μM) were obtained from mature male Sprague-Dawley rats (120–160 g) as described previously (15). Whole-cell patch recordings were made from hippocampal CA1 neurons at 32° in a superfusion chamber. Aerated artificial cerebrospinal fluid contained (in mM) NaCl 126, KCl 3, NaH_2PO_4 1.2, $CaCl_2$ 2.4, $MgCl_2$ 1.5, $NaHCO_3$ 25.9, and glucose 11, pH 7.4 (300 ± 5 mOsm). The patch pipette solution contained (in mM) potassium gluconate 125, KCl 15, HEPES 10, $CaCl_2$ 0.1, K·EGTA 1, Mg·ATP 2, and Tris·GTP 0.2, pH 7.25 adjusted with KOH (290 ± 5 mOsm).

Pharmacologically isolated $GABA_A$ IPSCs were evoked in the presence of the glutamate receptor blockers DL-2-amino-5-phosphonovaleric acid (50 μM) and 6,7-dinitroquinoxaline-2,3(1*H*,4*H*)-dione (10 μM) at a holding potential of −45 mV. Synaptic stimulation was delivered using a bipolar, twisted tungsten wire electrode (0.1-msec pulses of 5–20 V) every 20 sec. IPSCs were collected in the continuous voltage-clamp mode with an Axoclamp-2A amplifier (Axon Instruments, Foster City, CA), stored on a hard drive, and analyzed on- and off-line using the computer program NEUROPRO (R. C. Electronics, Goleta, CA). Concentrated PDGF-BB (stored in 10 mM acetic acid plus 0.1% bovine serum albumin at −20°) was diluted 1:1250 in artificial cerebral spinal fluid immediately before each experiment.

$^{36}Cl^-$ uptake experiments. $^{36}Cl^-$ uptake experiments in mouse brain microsacs were performed as described elsewhere (16). Microsac aliquots (200 μl) were incubated at 34° for 30 min with or without PDGF-BB (6 nM). Assay buffer (200 μl at 34°) containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.5, 1–10 μM muscimol, and $^{36}Cl^-$ (0.2 μCi) was added to the microsacs to initiate the reaction, which then was stopped after 3 sec by the addition of assay buffer containing 100 μM picrotoxin plus 100 μM bicuculline methiodide. In some cases, microsacs were incubated for 30 min at 34° with 200 nM GF109203X or for 15 min at 34° in assay buffer without Ca^{2+} plus 10 μM BAPTA-AM before the addition of PDGF. Stock solutions of GF109203X and BAPTA-AM were dissolved in 100% DMSO, and the final concentrations of DMSO in the assay mixture was 0.1%. Equivalent concentrations of DMSO were added to the controls when appropriate.

Expression vectors and *in vitro* transcription. The $GABA_A$ receptor subunits cDNAs were cloned on the eukaryotic expression vector pCDM8 (Invitrogen Corp., San Diego, CA); the cloning of these subunits is described elsewhere (17, 18). Human wild-type PDGFR-α subunits were on the *Not*I/*Bam*HI site of pBluescript II SK$^+$ (Stratagene Cloning Systems, La Jolla, CA). Human wild-type PDGFR-β subunits were on the *Eco*RI/*Pst*I site of pBluescript$^-$ (Stratagene) modified to knockout the *Sph*I and *Hin*dIII sites in the polylinker. The construction of the F5 and the F5 "add-back" PDGFR-β mutants has been described elsewhere (19). Briefly, the F5 PDGFR mutant was generated by mutating tyrosines Y740, Y751, Y771, Y1009, and Y1021 to phenylalanine residues. The F5 "add-back" mutants were generated by selectively replacing phenylalanines back to tyrosines in the F5 PDGFR-β mutant. For example, in the Y40/51 PDGFR-β mutant, Tyr^{740} and Tyr^{751} have been restored, which permits the binding and activation of PI3K. The complete cDNA construct of the F5 and "F5 add-back" PDGFR-β mutants was excised as a 4.2 *Eco*RI/*Sal*I fragment from pUC13 and subcloned into pBluescript II KS+ for subsequent *in vitro* transcription. The PDGFR Y579 mutant was constructed as described elsewhere (20). The PDGFR Y579 mutant in pSELECT (also known as pALTER; Promega Corp., Madison, WI) was generously provided by Dr. Lena Claesson-Welsh, Ludwig Institute for Cancer Research, Uppsala, Sweden.

Wild-type and mutant cRNAs were synthesized *in vitro* by using the mRNA capping kit from Stratagene. The wild-type PDGFR α subunit and the PDGFR Y579 mutant were linearized with *Hin*dIII and transcribed with T3 and T7 RNA polymerase, respectively. The wild-type PDGFR β subunit and the F5 series of PDGF mutant receptors were linearized with *Sal*I and transcribed with T7 RNA polymerase. Expression of mutant PDGFRs that activate PLC-γ was confirmed electrophysiologically. Expression of mutant PDGFRs that do not detectably activate PLC-γ was confirmed by Western blot analysis, as described elsewhere (21, 22, 23).

Microinjection and electrophysiological recording of *Xenopus* oocytes. The methods used for oocyte preparation and cRNA/cDNA microinjection are essentially the same as those described elsewhere (24). Isolated oocytes were placed in modified Barth's saline that contained (in mM) NaCl 88, KCl 1, HEPES 10, $MgSO_4$ 0.82, $NaHCO_3$ 2.4, $CaCl_2$ 0.91, and $Ca(NO_3)_2$ 0.33 adjusted to pH 7.5. A mixture of $GABA_A$ receptor subunit cDNAs (1.5 ng/30 nl) was injected into the animal pole of oocytes by the "blind" method. Wild-type and mutant PDGFR cRNAs were injected (100 ng/30 nl) into the vegetal pole near the equator by using a sterile glass pipette. The injected oocytes were cultured at 15–19° in sterile modified Barth's saline supplemented with 10 mg/l streptomycin, 10,000 units/l of penicillin, 50 mg/l of gentamicin, 90 mg/l theophylline, and 220 mg/l pyruvate. Oocytes were used for recording on days 1–4 after injection. A 100-μl rectangular chamber was used to hold the oocytes during recording. The animal poles of oocytes were impaled with two glass electrodes (0.5–10 MΩ) filled with 3 M KCl and voltage-clamped at −70 mV using an Axoclamp II amplifier (Burlingame, CA) or a Warner oocyte clamp OC-725B apparatus (Hampden, CT). Currents were continuously plotted on a strip-chart recorder.

GABA and PDGF-BB were dissolved in modified Barth's saline and bath applied for 20 sec. A 5–20-min washout period was allowed between drug applications, except for PDGF, which required a 45–90-min washout period for resensitization. Drugs were microinjected at least 15 min before recording (30–50 nl/oocyte) to the following final concentrations (assuming an oocyte volume of 1 μl): EGTA = 500 μM (stock 10 mM, pH 8.0), IP_3 = 20–30 nM (stock 1 μM), PKC inhibitor peptide Arg^{19}-Asn^{36} = 300 ng (PKCI; stock 10 mg/ml), deltamethrin = 2 nM (stock 66.6 nM in 0.06% DMSO), sodium orthovanadate 100 μM (stock 2 mM), and KN-62 = 10–15 μM (stock 500

μM/1% DMSO). After microinjection of these inhibitors, a stable baseline of $GABA_A$ responses was obtained before testing the effects of PDGFR activation. To determine the PDGFR-induced maximum percent inhibition of $GABA_A$-R independent of direct effects of these drugs on GABA currents, all values were calculated relative to the average of these baseline responses.

Statistical analysis. In most cases, currents were expressed as percentages of control responses due to the variability from oocyte and microsac preparation to preparation. Control responses in oocytes correspond to at least two or three $GABA_A$-R currents determined before PDGF-BB application. In all cases, the maximal percent PDGFR-induced inhibition was calculated relative to the average of these control responses. All values are given as mean ± standard error. Except where noted, values in parentheses refer to the number of different oocytes used in the statistical analysis. Statistical analyses were performed using both parametric (*t* tests or one-way ANOVA) and nonparametric (Sign, Wilcoxon, Mann-Whitney, or Kruskal-Wallis tests) methods by using the *Solo* program (BMPD statistical software, Los Angeles, CA).

Results

Effects of PDGFR activation on IPSCs in hippocampal slices and on $GABA_A$-R function in brain microsacs. We examined the effects of PDGFR activation on $GABA_A$-R-mediated inhibitory synaptic transmission in the hippocampus, a brain region where these two types of receptors are known to coexist (4, 25). Application of PDGF-BB (6 nM for 3 min) significantly reduced the amplitude of $GABA_A$-mediated IPSCs (by 31 ± 5%; $p < 0.03$ by *t* test [$n = 10$]) with no apparent change in the decay of these responses (Fig. 1). The inhibition began soon after the onset of PDGF-BB application and recovered only partially after a 35-min wash. To characterize the effect of PDGF-BB on $GABA_A$-R function in other brain regions, we measured the effect of PDGF on muscimol-activated $^{36}Cl^-$ uptake in mouse forebrain and cerebellar microsacs. Preincubation of microsacs with PDGF-BB (6 nM) for 30 min produced a significant decrease ($p < 0.05$ by one-sample Sign test) of ~20–30% in the muscimol-stimulated $^{36}Cl^-$-uptake in forebrain and cerebellar microsacs (Table 1). The inhibitory effect of PDGF was independent of the concentration of muscimol (Table 1, $p > 0.09$ by ANOVA and Kruskal-Wallis tests). The inhibitory actions of PDGF were blocked by preincubation with the membrane permeable Ca^{2+} chelator BAPTA-AM in Ca^{2+}-free buffer (Table 1, $p < 0.02$ by *t* and Wilcoxon tests). The inhibitory actions of PDGF in cerebellar microsacs were not blocked by the PKC inhibitor GF109203X (bisindolylmaleimide; Table 1; $p > 0.1$ by *t* and Wilcoxon tests). It should be noted that activation of PDGFR did not affect the basal $^{36}Cl^-$ uptake.

Effects of PDGFR activation on GABA-activated chloride currents in *Xenopus* oocytes. To study in greater detail the mechanism of the PDGFR-induced inhibition of $GABA_A$-Rs, PDGFR α or β subunit cRNAs and $GABA_A$-R subunit cDNAs were coinjected into *Xenopus* oocytes. Bath application of PDGF-BB (6 nM) to *Xenopus* oocytes expressing PDGFR-α or -β produced inward currents with a transient phase followed by a long-lasting oscillatory phase (Fig. 2A). These currents correspond to Ca^{2+}-activated Cl^- currents because they were reduced 70% by microinjection of 500 μM EGTA and 56% by treatment with a Cl^- channel inhibitor (150 μM niflumic acid) (26). In this batch of oocytes, activation of homomeric PDGFR-β or -α with PDGF-BB (6 nM) inhibited $GABA_A$-R (α1β1γ2L) currents in a reversible and time-dependent manner by ~35% (Fig. 2, A and B). The PDGFR-induced inhibition of $GABA_A$-R reached a maximum at 22 ± 2 min ($n = 33$) and lasted for >90 min. Importantly, PDGF-BB application did not inhibit

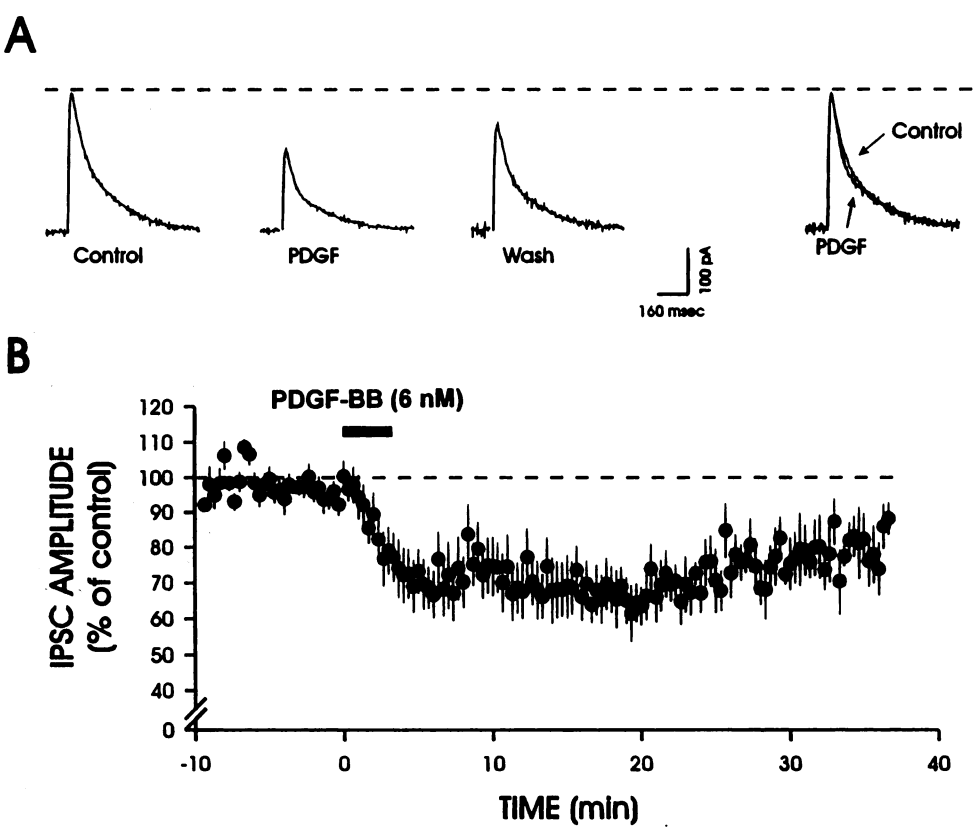

Fig. 1. Effect of PDGFR activation on $GABA_A$-R-mediated IPSCs in hippocampal CA1 pyramidal neurons. A. Shown are averages of 8–12 IPSCs recorded from a representative cell before (*Control*), 5–7 min after (*PDGF*), and 35–37 min after (*Wash*) a 3-min bath application of 6 nM PDGF-BB. The superimposed current traces (*far right*) represent the PDGF-inhibited IPSCs normalized to the control response. Note that no change in decay was associated with the PDGF-mediated decrease in IPSC amplitude. Synaptic stimulation was delivered with a twisted tungsten wire electrode (0.1-msec pulses of 5–20 V) every 20 sec. The clamping voltage was −45 mV. B, Summary of the effect of a 3-min application of PDGF-BB (6 nM) on the amplitude of $GABA_A$-receptor-mediated IPSCs. Each point represents the mean ± standard error of the IPSCs amplitude of 4–10 cells recorded at the times indicated on the abscissa. Responses for each cell were normalized with respect to the point immediately before PDGF-BB application.

TABLE 1

Effect of PDGF on the function of native $GABA_A$-R in mouse brain microsacs

Shown is the PDGF-induced percent inhibition of muscimol-stimulated $^{36}Cl^-$ uptake in mouse forebrain and cerebellar microsacs. $^{36}Cl^-$ uptake was measured in forebrain microsacs that had been preincubated for 30 min with or without PDGF-BB (6 nM) at 34° in regular assay buffer. In some cases, the $^{36}Cl^-$ uptake was measured in Ca^{2+}-free assay buffer in the presence or absence of 10 μM BAPTA-AM (preincubated for 15 min). Also shown is the effect of PDGF on cerebellar microsacs that were incubated for 30 min with and without the PKC inhibitor GF109203X (200 nM) in regular assay buffer. Values are given as mean ± standard error. Numbers in parentheses indicate the number of determinations, which were each performed in quadruplicate.

Region	Condition	Muscimol	Inhibition
		μM	%
Forebrain	Regular	1	33 ± 4 (3)
	Regular	3	37 ± 10 (5)
	Regular	10	19 ± 2 (6)
	Ca^{2+}-free	10	15 ± 2 (8)
	Ca^{2+}-free + BAPTA-AM	10	3 ± 5 (8)[a]
Cerebellum	Regular	10	30 ± 10 (4)
	Regular + GF109203X	10	23 ± 4 (4)

[a] $p < 0.02$ relative to control microsacs in Ca^{2+}-free assay buffer by t and Wilcoxon tests.

$GABA_A$-Rs in oocytes expressing only α1β1γ2L subunits, which indicates that the observed effects required PDGFR activation.

We next determined the concentration dependence of the PDGFR-mediated inhibition of $GABA_A$-R function. This was done in two steps. First, concentration-response curves for the PDGFR-induced Ca^{2+}-dependent Cl^- currents were obtained in *Xenopus* oocytes expressing PDGFR-β (Fig. 3A). Nonlinear regression analysis of these curves yielded an EC_{50} of ~1 nM and a Hill coefficient of 2.5. It should be noted that the K_D value for PDGF-BB binding to PDGFR-β is ~0.5 nM (1). Second, we assessed the effects of the activation of PDGFR-β with 0.06–6 nM PDGF-BB on $GABA_A$-R function. In this batch of oocytes, activation of PDGFR-β with 0.06 nM, 1 nM, and 6 nM PDGF-BB inhibited GABA-gated Cl^- currents by 0%, 19 ± 8%, and 54 ± 8%, respectively (Fig. 3B).

We also examined the effect of PDGFR activation on $GABA_A$-R concentration-response curves (Fig. 4). In this batch of oocytes, PDGFR activation produced a 75% decrease in the $GABA_A$-R (α1β1γ2L) E_{max} with no significant change in EC_{50} ($p > 0.15$ by t test); EC_{50} values before and during PDGFR activation were 47 ± 6 μM and 36 ± 5 μM ($n = 9$). The Hill coefficients were minimally but significantly ($p < 0.02$ by t test) changed by PDGFR activation from 1.3 ± 0.1 to 1.0 ± 0.03.

Finally, we measured the effect of PDGFR activation on $GABA_A$-R current-voltage relationships. PDGFR activation inhibited $GABA_A$-R currents independent of the membrane holding potential (Fig. 5A). The reversal potentials for the GABA-activated Cl^- currents before, during, and after PDGFR activation were not significantly different from each other ($p > 0.6$ by one-way ANOVA) (Fig. 5B).

Role of SH2-domain proteins and intracellular Ca^{2+} on the PDGFR-induced $GABA_A$-R inhibition. A panel of PDGFR mutants (19, 20) was used to determine which PDGFR-associated SH2-domain protein mediates the PDGFR inhibitory action on $GABA_A$-R. We used the F5 mutant PDGFR-β (19) where Tyr^{740}, Tyr^{751}, Tyr^{771}, Tyr^{1009}, and Tyr^{1021} have been mutated to phenylalanines. This mutant PDGFR-β possesses intact intrinsic tyrosine kinase activity

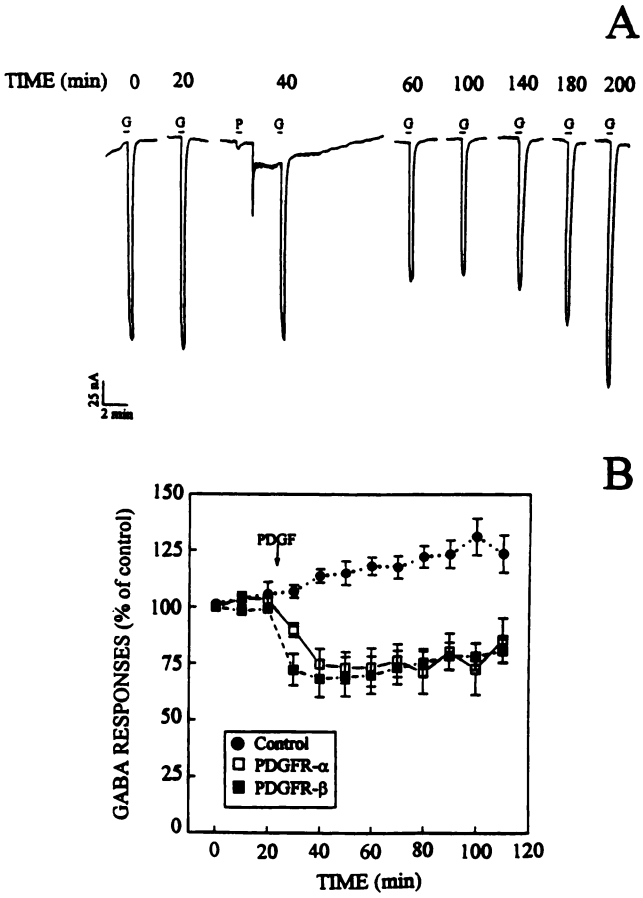

Fig. 2. Inhibition of $GABA_A$ receptor responses by PDGF receptors in *Xenopus* oocytes. A, Representative tracing of the Cl^- currents recorded continuously over a 200-min period from *Xenopus* oocytes coexpressing α1β1γ2L $GABA_A$-R subunits and PDGFR-α. A baseline of 50 μM GABA-gated (*G*) responses was obtained before the application of 6 nM PDGF-BB (P). PDGFR activation produced inward currents that correspond to Ca^{2+}-activated Cl^- currents. B, Effect of PDGF-BB (6 nM) application on 5–50 μM $GABA_A$-R responses in *Xenopus* oocytes that had been injected with α1β1γ2L subunit cDNA alone (●) or α1β1γ2L subunit cDNA plus either PDGFR-α (□) or PDGFR-β (■) subunit cRNA. Currents are expressed as percentages of the average of at least two or three control $GABA_A$-R responses obtained before PDGF-BB application. Each point represents the mean ± standard error of 8–23 tracings similar to the one shown in A.

but does not bind or activate the following SH2-domain proteins: PI3K, GAP, the protein tyrosine phosphatase Syp, or phospholipase C-γ. We also used the F5 series of "add-back" mutants, where selected tyrosine residues were mutated back from phenylalanine to tyrosine. This panel of "add-back" mutants possesses restored activation sites for one of the SH2-domain proteins described above. In addition, we used a PDGFR mutant where Tyr^{579} was mutated to phenylalanine, which impairs the binding and activation of SRC family kinase (20). These mutants are schematically defined in Fig. 6 (*top*). The F5 PDGFR-β mutant inhibited $GABA_A$-R responses significantly less ($p < 0.001$ by one-way ANOVA and Kruskal-Wallis tests) than wild-type PDGFR-β (Fig. 6). Moreover, the Y40/51, Y771, and Y1009 "F5 add-back" PDGFR-β mutants that possess restored binding sites for PI3K, GAP, and Syp, respectively, also inhibited $GABA_A$-Rs significantly less than wild-type PDGFR-β. However, the Y1021 "F5 add-back" PDGFR-β mutant, with restored acti-

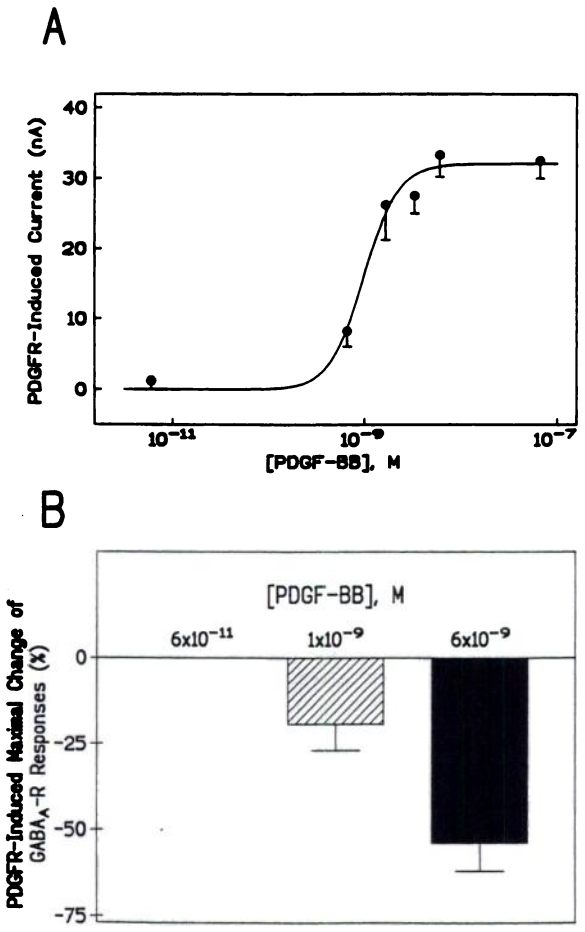

Fig. 3. Effect of various concentrations of PDGF on $GABA_A$-R responses. A, Shown is a concentration-response curve for the PDGFR-induced Ca^{2+}-dependent Cl^- currents in *Xenopus* oocytes expressing PDGFR-β. Each point represents the average minus standard error of six different oocytes. The data was fitted to a four-parameter logistic equation (sigmoid) by using GraphPad computer program (San Diego, CA) that yielded an EC_{50} of 1 nM and a Hill coefficient of 2.5. B, Shown is the maximal change of 50 μM GABA responses in oocytes expressing $\alpha1\beta1\gamma2L$ subunits produced by the activation of PDGFR-β at various concentrations of PDGF-BB. Each bar represents the average ± standard error of eight oocytes. A concentration of 6×10^{-11} M PDGF-BB produced no detectable inhibition in any of the oocytes.

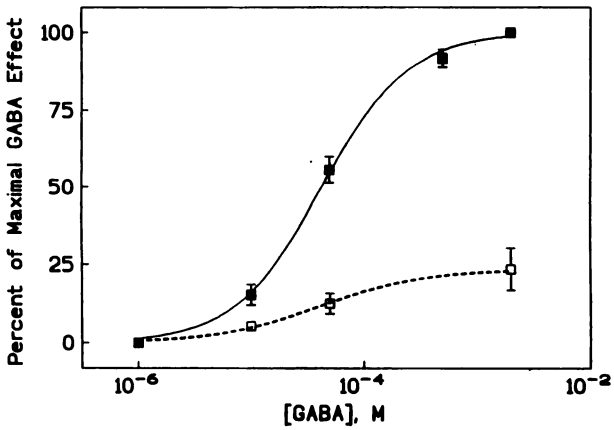

Fig. 4. Effect of PDGFR activation on $GABA_A$-R concentration-response curves. Curves were obtained by application of increasing GABA concentrations to oocytes expressing $\alpha1\beta1\gamma2L$ GABA subunits before (■) and during (□) PDGFR-induced inhibition. PDGFR were activated with 6 nM of PDGF-BB for 20 sec. The data was fitted to a four-parameter logistic equation (sigmoid) by using GraphPad computer program that yielded EC_{50} values of 47 ± 6 μM and 36 ± 5 μM, respectively. Each point represents the average ± standard error of nine oocytes.

vation sites for PLC-γ, "rescued" the inhibitory actions of PDGFR on $GABA_A$-R responses. The Y579 mutant inhibited $GABA_A$-Rs to the same extent as wild-type PDGFR-β.

Because the activation of PLC-γ results in an IP_3-dependent release of intracellular Ca^{2+} stores, the role of intracellular Ca^{2+} in the PDGFR-induced inhibition of $GABA_A$-R was assessed (Fig. 7A). Microinjection of the Ca^{2+} chelator EGTA (500 μM) significantly reduced (~6-fold) the PDGFR-induced maximal inhibition of $GABA_A$-R responses ($p < 0.02$ by t and Wilcoxon tests [$n = 9$]). In addition, we tested the effect of microinjection of IP_3 into *Xenopus* oocytes expressing $GABA_A$-Rs. IP_3 (20–30 nM) inhibited GABA-gated Cl^- currents by 26 ± 3% ($n = 9$). This value is not significantly different ($p > 0.2$ by t and Wilcoxon tests) from the PDGF-induced inhibition of $GABA_A$-R in control oocytes (Fig. 7A).

Effect of $GABA_A$-R subunit composition and of kinase and phosphatase inhibitors on the PDGFR-mediated inhibition of GABAergic responses. Because Ca^{2+} can activate protein kinases and phosphatases and phosphorylation has been reported to modulate $GABA_A$-R function (16, 27–29), the role of this process on the PDGFR-induced inhibition of GABA responses was investigated. This investigation was done in three steps. First, we examined the role of phosphorylation sites on specific $GABA_A$-R subunits (27, 28, 30, 31) by expressing $GABA_A$-R with different subunit compositions. PDGFR inhibited equally well wild-type $GABA_A$-R composed of either $\alpha1\beta1\gamma2L$, $\alpha1\beta1$, or $\beta1\gamma2L$ subunits (Fig. 7B). Homomeric $GABA_A$-R composed of $\beta1$ subunits appear to be inhibited to a lesser extent than receptors composed of two or three subunit types. However, the magnitude of inhibition for $GABA_A$-R composed of $\beta1$ subunits was not significantly different ($p > 0.1$ by Kruskal-Wallis test) from the inhibition of heteromeric $GABA_A$-Rs.

Second, the effect of PDGFR activation on $GABA_A$-Rs composed of wild-type $\alpha1$ plus mutant $\beta1_{(S409A)}$ subunits was examined to determine whether this key phosphorylation site of the $\beta1$ subunit (27, 28) was required for the inhibitory actions of PDGFR. The PDGFR-induced inhibition of $GABA_A$-Rs composed of $\alpha1\beta1_{(S409A)}$ subunits was not statistically different ($p > 0.2$ by t and Wilcoxon tests) from the inhibition of $GABA_A$-R composed of wild-type $\alpha1\beta1$ subunits (Fig. 7B).

Finally, we tested the effects of specific inhibitors of kinases and phosphatases that could play a role on this modulatory process. We used the specific PKC inhibitor PKCI (32), the CAM kinase II inhibitor KN-62 (33), the calcineurin inhibitor deltamethrin (34), and the tyrosine phosphatase inhibitor sodium orthovanadate. None of these inhibitors, at concentrations up to 20-fold their published IC_{50} values (32–34), significantly affected the PDGFR-induced inhibition of $GABA_A$-R (Table 2) ($p > 0.07$ by t and Mann-Whitney tests).

Discussion

PDGF plays important roles in a myriad of physiological and pathophysiological processes, including embryonic and placental development, wound healing, atherosclerosis, and cancer (1, 35). We report a novel modulatory action of PDGF.

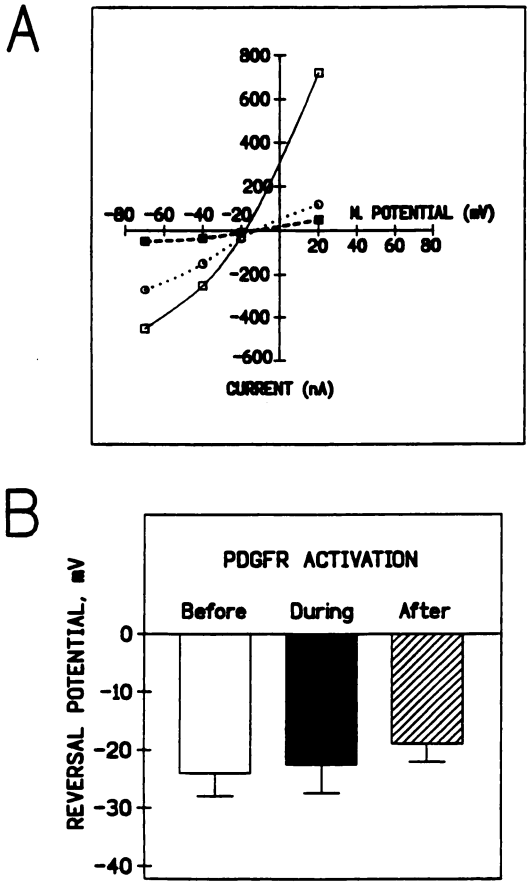

Fig. 5. Effect of PDGFR activation on the $GABA_A$-R reversal potential. A, Representative current-voltage relationships for $GABA_A$-Rs ($\alpha1\beta1\gamma2L$) obtained from one oocyte before (□), during (■), and after (○) PDGFR-induced inhibition. B, Each bar represents the average ± standard error values for the $GABA_A$-R reversal potential calculated from current-voltage relationships obtained before, during, and after PDGFR-induced inhibition in six different oocytes. PDGF-BB and GABA concentrations were 6 nM and 50 μM, respectively.

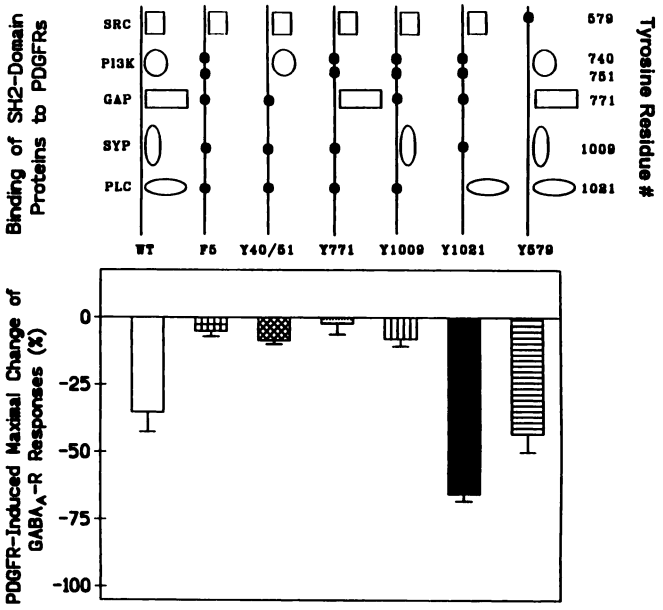

Fig. 6. Effect of PDGFR mutants on the PDGFR-induced modulation of $GABA_A$-R. *Top*, schematic representation of the PDGFR mutants used in this study. *Hollow shapes*, association of the indicated SH2-domain proteins to intact autophosphorylation tyrosine sites on the intracellular segment of the PDGFR-β. For reference, wild-type PDGFR-β is depicted (*left*). In the F5 mutant PDGFR, Tyr^{740}, Tyr^{751}, Tyr^{771}, Tyr^{1009}, and Tyr^{1021} have been mutated to phenylalanine (●). This mutant has intact intrinsic tyrosine kinase activity but does not associate or activate PI3K, GAP, Syp, or PLC-γ. Mutants Y40/50, Y771, Y1009, and Y1021 were constructed by mutating specific phenylalanines back to tyrosines in the F5 mutant. This "add-back" mutations selectively restore the association of the SH2-domain proteins represented by the hollow shapes. The Y579 mutant was constructed by mutating Tyr^{579} to phenylalanine, which impairs the binding and activation of SRC family kinase. *Bottom*, PDGFR-induced percent maximal inhibition of 5–50 μM GABA responses in oocytes coexpressing the respective PDGFR mutants depicted immediately above with $\alpha1\beta1\gamma2L$ $GABA_A$-R subunits. Mutant and wild-type PDGFRs were activated with 6 nM of PDGF-BB for 20 sec. Statistical analysis was performed by one-way ANOVA and Kruskal-Wallis tests that yielded a $p < 0.001$. For a description of statistical significant differences among groups, see Results.

We used electrophysiological and biochemical techniques to demonstrate that a brief activation of PDGFRs, with nanomolar concentrations of PDGF-BB, produces a long-lasting (30–90 min) and reversible inhibition of $GABA_A$-R function in hippocampal CA1 neurons, brain microsacs, and *Xenopus* oocytes expressing cloned human receptors. Because of the widespread expression of both PDGF and $GABA_A$ receptors in practically all regions of the CNS, this finding suggests that PDGF may be an important modulator of inhibitory synaptic transmission in the mammalian brain.

We chose to study the details of the mechanism of interaction between PDGF and $GABA_A$ receptors in *Xenopus* oocytes because this system allows the coexpression of high numbers of wild-type and mutant receptors in a single cell, and it is particularly suited for the study of intracellular signal transduction cascades. These experiments revealed several important details about the mechanism by which this growth factor receptor relays its inhibitory signal to $GABA_A$-Rs. Present results indicate that (a) the intrinsic tyrosine kinase activity of the PDGFR is not sufficient to inhibit $GABA_A$-Rs; (b) the SRC family kinases, GAP, PI3K, and Syp signal transduction pathways do not play a role in this process; and (c) PLC-γ is the molecule that transduces the inhibitory signal from PDGFRs to $GABA_A$-Rs (schematically shown in Fig. 8). These findings are in agreement with those of a previous study (36) where it was demonstrated that fibroblast growth factor receptor inhibits the function of voltage-gated K^+ channels in a PLC-γ-dependent manner. Consequently, activation of PLC-γ by growth factor receptors appears to be an important modulatory signal transduction pathway for the function of both voltage- and ligand-gated ion channels. Interestingly, inhibition of $GABA_A$-R by G protein-coupled $GABA_B$ (37) or 5-hydroxytryptamine$_{2C}$ receptors[1] also appears to depend on the activation of PLC. Because a different isoform of PLC (PLC-β) is activated by these G protein-coupled receptors, it would be interesting to determine differences or similarities between the inhibition of $GABA_A$-responses produced by G protein-coupled versus growth factor receptors.

The results discussed above, taken together with the finding that the effects of PDGFR were blocked by EGTA and were mimicked by IP_3 in *Xenopus* oocytes, indicate that the PDGFR-induced inhibition of GABA responses depends on a

[1] R. A. Harris, J. P. Huidobro-Toro, and C. F. Valenzuela, unpublished observations.

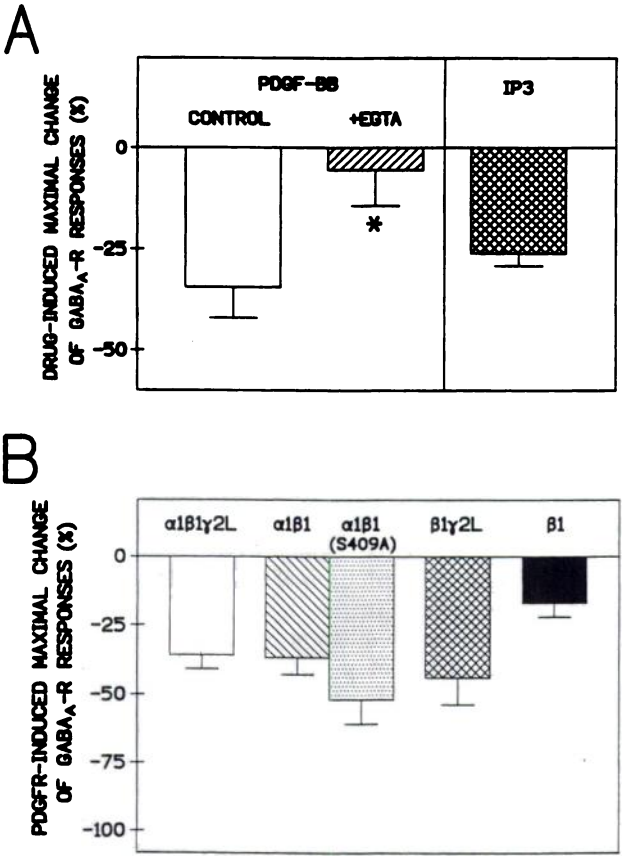

Fig. 7. Effect of EGTA and subunit composition on the PDGFR-induced inhibition of $GABA_A$-R and of IP_3 on $GABA_A$-R function. A, Each bar represents the PDGFR-induced maximal inhibition of $GABA_A$-R ($\alpha1\beta1\gamma2L$) in oocytes microinjected with 500 μM EGTA (nine). PDGF-BB was applied at a concentration of 6 nM. Also shown is the maximal effect of microinjected 20–30 nM IP_3 on $\alpha1\beta1\gamma2L$ $GABA_A$-Rs ($n = 9$). *, $p < 0.02$ by t test. GABA concentration was 50 μM. B, Shown is the PDGFR-induced percent maximal inhibition of 50 μM GABA responses in oocytes microinjected with equimolar concentrations of the indicated $GABA_A$-R subunit cDNAs. Also shown is the effect of PDGFR on $GABA_A$-R composed of wild-type $\alpha1$ plus mutant (S409A) $\beta1$ subunits. Each bar represents the mean ± standard error of 8–35 oocytes. Kruskal-Wallis test yielded a $p > 0.1$. For details, see Results.

TABLE 2

Effect of inhibitors on kinases and phosphatases on the PDGFR-induced percent maximal inhibition of $GABA_A$-R

Values represent the maximum percent inhibition produced by PDGFR activation and are given as mean ± standard error. Values in parentheses represent the number of oocytes studied. All experiments were performed with oocytes coexpressing WT PDGFR-β and $\alpha1\beta1\gamma2L$ $GABA_A$-R, except for the experiments with KN-62, which were performed with oocytes coexpressing Y1021 mutant PDGFR-β and $\alpha1\beta1\gamma2L$ $GABA_A$-R. The inhibitors were microinjected in the oocytes to give the following final concentrations (assuming a 1-μl oocyte volume): PKC inhibitor peptide, 300 ng/oocyte; deltamethrin, 2 nM; KN-62, 10–15 μM; and sodium orthovanadate (Na_3 VO_4), 100 μM. Student's t and Mann-Whitney tests did not reveal any significant differences ($p > 0.07$) between control and inhibitor-microinjected oocytes. For more details, see Experimental Procedures. GABA concentrations were 50 or 200 μM, and PDGF concentration was 6 nM.

Inhibitor	Target	Control	+ Inhibitor
PKCI peptide	PKC	36 ± 5 (31)	47 ± 12 (6)
Deltamethrin	Calcineurin	35 ± 7 (12)	33 ± 8 (6)
KN-62	CAM-kinase II	64 ± 6 (24)	57 ± 8 (11)
Na_3 VO_4	Tyrosine phosphatases	35 ± 7 (12)	53 ± 8 (8)

PLC-γ/IP_3-mediated elevation of intracellular Ca^{2+} levels (Fig. 8). Moreover, this mechanism of inhibition is not exclusive to the *Xenopus* oocyte expression system because we

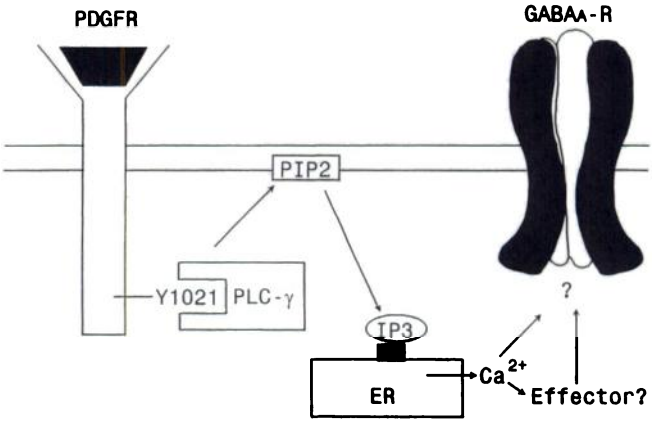

Fig. 8. Model of the steps necessary for the inhibition of $GABA_A$-Rs by PDGFR activation. On activation with PDGF, PDGFRs dimerize and autophosphorylate on tyrosine residues. PLC-γ binds to phosphotyrosine 1021 and becomes activated. PLC-γ catalyzes the breakdown of phosphatidylinositol-4,5-bisphosphate (*PIP2*) and produces IP_3 (*IP3*). IP_3 binds to its receptor and releases Ca^{2+} from the endoplasmic reticulum (*ER*). The slow onset of the PDGFR-induced inhibition of $GABA_A$-Rs suggests that a Ca^{2+}-dependent cascade, and not a direct action of Ca^{2+}, is involved in this inhibitory process. Our results suggest that Ca^{2+}-dependent enzymes, such as PKC, CAM-kinase II, or calcineurin, do not play a role in this modulatory cascade.

obtained similar results with brain microsacs, where the PDGFR-induced effects were significantly blocked by the Ca^{2+} chelator BAPTA-AM. Thus, one possible explanation for our results is that the PDGFR/PLC-γ/IP_3 signaling cascade induces an elevation of cytoplasmic Ca^{2+} concentration, which directly inhibits $GABA_A$-R function. A direct inhibitory action of intracellular Ca^{2+} on $GABA_A$-Rs has not been demonstrated yet because it is extremely difficult to purify $GABA_A$-Rs without contamination from Ca^{2+}-dependent enzymes or other Ca^{2+}-activated molecules. Even detailed patch-clamp studies have been unable to determine whether Ca^{2+} affects the $GABA_A$ channel directly or indirectly by activating an intracellular effector (38). Consequently, it cannot be ruled out that Ca^{2+} inhibits GABA-gated responses directly; however, the slow onset of the $GABA_A$-R inhibition observed on PDGFR-activation argues against this possibility. It has been demonstrated that activation of IP_3 signaling by an agonist in *Xenopus* oocytes produces a rapid increase in intracellular Ca^{2+} levels across wide areas of the cell and that this elevation is maximal within ~60 sec (39). One would, therefore, expect a rapid onset of inhibition of $GABA_A$-R responses if Ca^{2+} was acting directly on the receptor. Thus, we hypothesize that the slow onset of the PDGFR-induced inhibition of $GABA_A$-R is due to the activation of a Ca^{2+}-dependent enzyme or Ca^{2+}-dependent process that inhibits the GABA-gated Cl^- currents.

One Ca^{2+}-dependent enzyme that can be activated during the PDGFR/PLC-γ/IP_3/Ca^{2+} signal transduction cascade is PKC. We reasoned that PKC could be involved in this process because activation of this enzyme with phorbol esters has been shown to inhibit the function of the $GABA_A$-R (16, 28). Surprisingly, our experiments with $GABA_A$-Rs with different subunit compositions suggest that PKC does not play a role in this process. We found that $GABA_A$-Rs composed of $\alpha1\beta1\gamma2L$ subunits were inhibited to the same extent as receptors composed of $\alpha1\beta1$ subunits. This finding indicates that the two PKC phosphorylation sites that have been iden-

tified to date in γ2L subunits (S327 and S343) are not required for the PDGFR-induced modulation of $GABA_A$-Rs (28). Moreover, $GABA_A$-Rs composed of α1 plus mutant $\beta1_{(S409A)}$ subunits, which lack any known PKC phosphorylation sites (28), were also inhibited to the same extent as wild-type receptors. It is striking that phorbol ester-induced activation of PKC modulates $GABA_A$-Rs via phosphorylation of these sites and that the physiological activation of PLC-γ by growth factor receptors does not modulate $GABA_A$-R through a PKC-dependent mechanism under our recording conditions. Consequently, direct phosphorylation of $GABA_A$-R at known sites by PKC is not likely to play a role in this modulatory cascade. Moreover, PKC-dependent phosphorylation of either unknown sites in $GABA_A$-Rs or of an unidentified GABAergic modulatory protein does not appear to be important. These actions of PKC do not appear to be important because microinjection of PKCI into *Xenopus* oocytes and pretreatment of cerebellar microsacs with the PKC inhibitor GF109203X did not block the inhibitory actions of PDGFR.

There are other Ca^{2+}-dependent enzymes that could be directly activated by a PDGFR-mediated signal transduction cascade and that could modulate $GABA_A$-R function. Two of these enzymes are CAM kinase II and calcineurin (Ca^{2+}/calmodulin-dependent protein phosphatase 2B). Although an interaction between calcineurin and the $GABA_A$-R has not been demonstrated, CAM-kinase II phosphorylates $GABA_A$-Rs *in vitro* on both the β1 and γ2 subunits (40). However, CAM-kinase II does not appear to be important for the PDGFR-mediated inhibition of $GABA_A$-R function because (a) the removal of the γ2L subunit, which contains three known CAM-kinase II phosphorylation sites (S343, S348, and T350), and the removal of one of its known phosphorylation sites in the β1 subunit (S409) did not reduce the PDGFR inhibitory actions (40); and (b) microinjection of the specific inhibitor of this enzyme, KN-62, did not block the PDGFR-induced effects. In addition, calcineurin does not appear to play a role in the PDGFR-induced inhibition of $GABA_A$-Rs because the specific calcineurin inhibitor, deltamethrin, did not block the inhibition of $GABA_A$-R produced by PDGFR activation. Consequently, phosphorylation or dephosphorylation of $GABA_A$-Rs by CAM-kinase II or calcineurin does not appear to be involved in the PDGFR-induced inhibition of $GABA_A$ responses.

In addition to the above proteins, PKA, PKG, and protein tyrosine kinases can be indirectly activated by the PDGFR-mediated elevation in intracellular Ca^{2+} (41, 42). These kinases are known to interact with $GABA_A$-Rs (27, 40, 43, 44). However, our experiments with mutant $GABA_A$-Rs expressed in *Xenopus* oocytes suggest that PKA- or PKG-dependent phosphorylation of $GABA_A$-Rs at known sites does not play a role in mediating the inhibitory actions of PDGF. Ser^{409} on the β1 subunit is the only known phosphorylation site for both PKA and PKG on $GABA_A$-R (27, 40); however, $GABA_A$-Rs composed of $\alpha1\beta1_{(S490A)}$ were inhibited to the same extent as wild-type α1β1 $GABA_A$-R by PDGFR activation. Therefore, the effect of PDGFR on $GABA_A$-R does not appear to be mediated through direct PKA or PKG phosphorylation of the $GABA_A$-R at this site. In addition, it is unlikely that tyrosine phosphorylation or dephosphorylation of $GABA_A$-R is involved in this process because protein tyrosine kinases maintain or enhance $GABA_A$-R function (43, 44), and microinjection of sodium orthovanadate into *Xenopus* oocytes did not block the PDGFR-induced inhibition of $GABA_A$-Rs.

The results discussed above suggest that other Ca^{2+}-dependent enzymes or processes may be important mediators of the PDGFR-induced inhibition of $GABA_A$-R responses. We have screened inhibitors of other Ca^{2+}-dependent enzymes (i.e., $cPLA_2$, calmodulin, and calpain) that could play a role in this process. However, we have not found an inhibitor that significantly blocks the PDGFR-mediated inhibition of $GABA_A$-responses. Therefore, further study is required to determine whether the PDGFR-induced elevation of Ca^{2+} inhibits the $GABA_A$-R function directly or indirectly through other Ca^{2+}-dependent processes that are activated by the PDGFR-induced signal transduction pathway (Fig. 8).

In conclusion, this work demonstrates that a brief activation of PDGFRs produces a long-lasting inhibition of the function of native $GABA_A$-Rs in mouse brain microsacs and hippocampal CA1 pyramidal neurons and of cloned human $GABA_A$-Rs expressed in *Xenopus* oocytes. The mechanism of the PDGFR-induced inhibition of $GABA_A$-R involves a PLC-γ/IP_3-dependent rise in intracellular Ca^{2+} (Fig. 8). This work suggests that the mechanism of the PDGFR-induced inhibition of $GABA_A$-R is not mediated by Ca^{2+}-dependent enzymes such as PKC, CAM-kinase II, and calcineurin. These findings are significant because they establish both a novel action for PDGF in the mammalian CNS and a novel growth factor receptor-dependent modulatory mechanism for $GABA_A$-mediated synaptic transmission in the brain. This modulatory process could be important during disease states that are associated with decreased GABAergic function, such as epilepsy, anxiety, and alcohol withdrawal syndrome. Moreover, the inhibitory effects of growth factor receptors on $GABA_A$-R function could be important in situations where growth factor input might be elevated (i.e., neuronal injury) or during growth factor therapy for chronic neurological disorders where unwanted side effects can occur. An interesting task for future research will be to determine whether this long-lasting inhibitory effect of PDGFR activation plays a role in complex brain functions such as learning and memory.

Acknowledgments

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