The Bradykinin B2 Receptor Is a Delayed Early Response Gene for Platelet-derived Growth Factor in Arterial Smooth Muscle Cells*

(Received for publication, January 24, 1996, and in revised form, March 20, 1996)

Bradley S. Dixon‡, Ram V. Sharma, and Michael J. Dennis
From the Departments of Medicine and Anatomy, University of Iowa College of Medicine and Veterans Affairs Medical Center, Iowa City, Iowa 52242

Bradykinin and platelet-derived growth factor (PDGF) are inflammatory mediators important in the response to vascular injury. Based upon the known effect of oncogenic Ras to increase bradykinin receptor expression and the ability of PDGF to stimulate Ras, we examined whether PDGF regulates bradykinin B2 receptor expression in cultured arterial smooth muscle cells. Treatment with PDGF (AB and BB, but not AA) produced a dose- and time-dependent increase in both mRNA (6–7-fold increase at 2–4 h) and cell surface receptors (2–4-fold at 6–12 h) for the B2 receptor. There was a 60-min delay between exposure to PDGF and the initial increase in B2 receptor mRNA. Transcriptional inhibitors, actinomycin D or 5,6-dichloro-1-O-ribofuranosylbenzimidazole, completely blocked the increase in B2 receptor mRNA when added up to 60 min after stimulation with PDGF. However, protein synthesis was not required, as treatment with the protein synthesis inhibitor, cycloheximide, did not block but rather superinduced the PDGF-induced increase in B2 receptor mRNA. Comparison with the immediate early response gene c-fos demonstrated that the increase in B2 receptor mRNA was similarly inhibited by the tyrosine kinase inhibitor, tyrphostin, as well as staurosporine. However, stimulation of c-fos was slightly more sensitive to genistein, while the B2 receptor mRNA was more sensitive to inhibition by the protein kinase C inhibitor, calphostin C. The increase in cell surface B2 receptors were functionally coupled to an increase in phosphoinositide-specific phospholipase C, and the effects of PDGF were selective as there was no increase in either angiotensin II- or arginine vasopressin-induced inositol phosphate formation or intracellular calcium release. Taken together, these results demonstrate that the B2 receptor is a delayed early response gene for PDGF in vascular smooth muscle cells.

Bradykinin is a potent vasoactive hormone released during activation of the intrinsic pathway of coagulation (1). In the presence of an intact endothelium, bradykinin acts on B2 receptors present on the endothelium to produce vasodilatation and an increase in vascular permeability (2, 3). However, when the endothelium is disrupted, bradykinin gains direct access to receptors present on the underlying vascular smooth muscle cells (4–6). The biological role of these smooth muscle B2 receptors is unclear. The available evidence suggests that these B2 receptors activate a phosphoinositide-specific phospholipase C leading to increases in intracellular calcium and activation of protein kinase C similar to that described for the vasoconstrictor hormones, angiotensin II and arginine vasopressin (7). This suggests that the direct effects mediated by the B2 receptor on arterial smooth muscle may antagonize the indirect endothelial-dependent vasoactive effects of bradykinin. Understanding the functional role and regulation of B2 receptor expression on vascular smooth muscle has important implications to elucidating the role of bradykinin in vascular injury and inflammation.

Previous studies have shown that cells transformed with the Ras oncogene have an increased number of cell surface bradykinin receptors and selectively enhanced bradykinin receptor coupling to phosphoinositide turnover (8–11). Although initial studies suggested that the effects were somewhat selective for the Ras oncogene, more recent studies have demonstrated that transformation with other oncoproteins also stimulates an increase in bradykinin receptor expression (10, 11). Of particular interest, the greatest increase in bradykinin receptor expression was reported in fibroblasts transformed by the constitutively activated receptor tyrosine kinase oncogene, trk. These observations suggest that activation of receptor tyrosine kinases may regulate bradykinin receptor expression possibly by a Ras-dependent mechanism. Despite these long-standing observations, there is no information as to whether activation of receptor tyrosine kinases known to stimulate Ras leads to an increase in bradykinin receptor expression in nontransformed cells. To approach this question, we recently surveyed several growth factors known to bind receptor tyrosine kinases and activate Ras in vascular smooth muscle cells (6). The studies demonstrated that platelet-derived growth factor (PDGF), and, to a lesser extent, epidermal growth factor increased bradykinin receptor expression in cultured arterial smooth muscle cells (6). PDGF is a mitogenic and chemotactic factor which is important in the response to vascular inflammation and injury (12, 13). When released from platelets, PDGF binds and dimerizes a receptor tyrosine kinase (reviewed in Refs. 14 and 15). Receptor dimerization and activation of the intrinsic tyrosine kinase promotes membrane association of a guanine nucleotide exchange factor (e.g. SOS) and activation of membrane-bound

* This research was supported by a merit grant from the Veterans Administration, Grant HL-42377 from the National Institutes of Health, a grant from the Iowa Affiliate of the American Heart Association, and by the University of Iowa Diabetes and Endocrinology Research Center. Preliminary data were presented at the American Society for Cell Biology Meetings, December 10–14, 1994, San Francisco, CA (Dixon, B. S., and Dennis, M. J. (1994) Mol. Biol. Cell. 5S, 10A). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Medicine, University of Iowa, E 300D GH, Iowa City, IA 52242. Tel.: 319-356-1626; Fax: 319-356-7893.

1 The abbreviations used are: PDGF, platelet-derived growth factor; BK, bradykinin; AII, angiotensin II; AVP, arginine vasopressin; IP, inositol phosphate; IP$_3$, inositol bisphosphate; IP$_7$, inositol trisphosphate; DRB, 5,6-dichloro-1-O-ribofuranosylbenzimidazole; bp, base pair(s); DMEM, Dulbecco's modified Eagle's medium.
Ras (16–19). Activation of Ras initiates several signaling cascades, the best characterized of which is the Raf-1/MEK/MEK pathway leading to regulation of gene transcription and cellular proliferation (20, 21 and reviewed in Refs. 22 and 23). Given the potential involvement of both bradykinin and PDGF in the response to vascular injury and inflammation, we further examined the effect of PDGF on bradykinin B₂ receptor expression in smooth muscle cells. The results demonstrated that the B₂ receptor gene is a delayed early response gene for PDGF in smooth muscle cells.

EXPERIMENTAL PROCEDURES

Materials—Chemicals and materials were from the following sources: ribonuclease protection assay kit (RPA II) and pTRI-β-actin-rat, Ambion (Austin, TX); bradykinin, angiotensin II, and arginine vasopressin, Bachem Inc. (Torrance, CA); Dowex 1-X8 (200-mesh) anion exchange resin, Bio-Rad; platelet-derived growth factor, genistein, staurosporine, and calphostin C, Calbiochem Corp.; tyrophosphitin B-46, LC Laboratories (Woburn, MA); Fura 2, Molecular Probes (Eugene, OR); [3H]myoinositol (15 Ci/mmol), DuPont NEN. 5,6-Dichloro-1-benzylselenite, 100 units/ml penicillin, and 100 μg/ml streptomycin, and 10% bovine calf serum. When the cultures attained 80% confluence, the medium was replaced with fresh medium and the cultures were allowed to equilibrate in binding buffer containing: 25 mM HEPES (pH 7.0), 140 mM NaCl, 2 mM MgCl₂, 1 mM phenanthroline, 30 μM aprotinin, 100 μM benzamidine, and 100 μM phenylmethylsulfonyl fluoride.

Cultured Arterial Smooth Muscle Cells—Arterial smooth muscle cells were isolated from the mesenteric artery of female Wistar rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN) and grown in culture as described previously (24). Cultures were grown in Dulbecco's modified Eagle's medium (DMEM) containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% bovine calf serum. When the cultures attained confluence (5–7 days), they were incubated in defined serum-free media (50:50 mixture of Ham's F-12 and DMEM supplemented with 5 mg/ml insulin, 5 mg/ml transferrin, 5 μM triiodothyronine, 10 mM sodium selenite, 100 units/ml penicillin, and 100 μg/ml streptomycin) for 24 h prior to study. Only cultures from the P₁ and P₂ passage were used for these studies.

Treatment with Tyrosine Kinase Inhibitors—In preliminary experiments, we observed that the activity of the tyrosine kinase inhibitors, genistein and tyrphostin B-46, decreased rapidly with storage in solution (−20 °C). Therefore, all experiments were performed using freshly dissolved kinase inhibitors. Immuno blotting with the PI-2 antiphosphotyrosine antibody (Calbiochem) demonstrated that PDGF-induced tyrosine phosphorylation was blocked by either genistein or tyrphostin B-46 (data not shown). Inhibition of PDGF-stimulated tyrosine kinase activity was also confirmed by performing paired control studies examining the effect of the inhibitors on the PDGF-stimulated immediate early response gene, c-fos (see below).

Bradykinin Receptor Binding Assay—Cell surface binding was measured as described previously (25). Confluent cultures of arterial smooth muscle cells grown in 12-well plates were deprived of serum for 24 h before treatment with PDGF at the concentrations and times indicated under “Results.” After treatment with PDGF, the cells were washed and allowed to equilibrate in binding buffer containing: 25 mM HEPES (pH 7.0), 140 mM NaCl, 2 mM MgCl₂, 1 mM phenanthroline, 30 μM aprotinin (gift from Squibb), and 100 μM benzamidine at 4 °C. To determine the effect of PDGF on bradykinin receptor binding, saturation binding experiments were performed using [3H]bradykinin in concentrations varying from ~10–15 nM (110 Ci/mmol) in 600 μl of binding buffer per well. For experiments measuring the time course and dose response of various PDGF ligands, total binding was determined using 0.5 nM [3H]bradykinin. Non-specific binding was determined in the presence of 2 μM unlabeled bradykinin. The binding was terminated after 16 h by aspirating the buffer and rapidly washing the cells with 3 ml of ice-cold binding buffer. The remaining cell-bound counts were solubilized with 1 ml of 1 N NaOH and quantitated by scintillation counting (Beckman LS 3801). The saturation binding data were analyzed by a nonlinear curve-fitting program ("LIGAND" developed by Peter Munson at the NIH) to determine the best model and model parameters (Kd, Bmax, and R₂) which fit the observed nontransformed data (26).

Preparation of Antisense 32P-Labeled Riboprobes—A plasmid (403-8) containing 1.9 kilobases of cDNA for the rat bradykinin B₂ receptor was graciously provided by Dr. Kurt Jarnagin (27). This plasmid was cut with Ncol and transcribed using T7 polymerase yielding a 515-base riboprobe (459-base protected length). To measure rat c-fos, a partial cDNA was cloned using RNA isolated from PDGF-treated vascular smooth muscle cells by reverse transcription followed by polymerase chain reaction with the primers 5'-CAGCCGACTCCTTCTCCAGCATG and 5'-TCCAGTTTTCCTCTTCTTCAGTAGTTG (28). The resulting 437-base product was cloned into pCR2 (Invitrogen), and the sequence was confirmed by the dideoxynucleotide chain termination method (29). The plasmid was linearized with SalI and transcribed using polynucleotide yielding a 334-base riboprobe (278-base protected length). The pTRI-β-actin-mouse template containing cDNA for the mouse β-actin (Ambion) was cut with HaellII and transcribed using T7 polymerase generating a 207-base length riboprobe (170-base protected length). In some experiments, the pTRI-β-actin-rat template containing cDNA for the rat β-actin (Ambion, 125-base protected length) was also transcribed. [3P]-labeled riboprobes were prepared by mixing 800 ng of cut plasmid, 20 units of RNasin, 0.5 mM each rATP, rUTP, and rGTP, 8 μM [α-32P]CTP (50 Ci/μl) for either the B₂ receptor or c-fos riboprobe and 15 μCi/μl for the β-actin riboprobe. Nonhybridized probe was digested with RNase A and T1 in Ambion's digestion buffer) for 30 min at 30 °C, 500 μl of lysis buffer (0.3 M NaCl, 1 mM EDTA, 0.2% SDS) overnight. A 2-μl sample of the probe was removed for quantitation by liquid scintillation counting, and the remainder was used immediately for the ribonuclease protection assay.

RNA Extraction—Total RNA was extracted from cultured cells using a modification of published procedures (29). Confluent cultures of arterial smooth muscle cells grown in 6-well panels (9 cm²/well) were deprived of serum for 24 h prior to treating with the indicated agents. After the indicated treatment, the buffer was aspirated, and the cells were scraped into 600 μl of ice-cold GIBCO buffer (5.8 mM guanidinium isothiocyanate, 38.6 mM sodium citrate, pH 7.0, 0.76% sarcosyl, and 0.7% β-mercaptoethanol). RNA was extracted by adding sequentially 500 μl of ice-cold sodium acetate (pH 5.2), 500 μl of isopropanol, 150 μl of chloroform/isoamyl alcohol with vortexing. After incubation on ice for 15 min, the sample was centrifuged at 10,000 × g for 20 min at 4 °C, and the upper aqueous phase was removed into a new 1.5-ml Eppendorf tube. For the ribonuclease protection assay, the [3P]-labeled riboprobes were added directly to the RNA at this time. The RNA was then precipitated by adding 750 μl of ice-cold isopropanol alcohol, incubating at −20°C for at least 30 min, and centrifuging at 10,000 × g for 20 min at 4 °C. The supernatant was discarded and the RNA pellet was used immediately for the ribonuclease protection assay.

RNA Protection Assay—The procedures are slightly modified from those provided with the RPA II kit (Ambion). Briefly, sample RNA and the [3P]-labeled riboprobes were combined as described above. The resulting pellet was resuspended in 20 μl of hybridization buffer (80% formamide, 10 mM sodium citrate, 300 mM sodium acetate, 1 mM EDTA, pH 6.4) with vortexing, heated at 90 °C for 4 min, and then allowed to hybridize overnight at 42 °C. Nonhybridized probe was hydrolyzed by incubating with 1:300 dilution of RNase (combination of RNase A and RNase T1 in Ambion's digestion buffer) for 30 min at 37 °C. RNase was inhibited, and the remaining protected RNA was precipitated with a proprietary buffer (300 μl, Ambion) and incubated at −20 °C. The supernatant was removed, and the pellet was resuspended in 8 μl of gel loading buffer (80% formamide, 0.1% xylene cyanol, 0.1% bromphenol blue, 2 μM EDTA) with vigorous vortexing and heated to 90 °C × 5 min. Gel electrophoresis was run on 5% acrylamide, 8 M urea gel. The bands were identified by autoradiography and quantitated by densitometry (PDI system). The results for the B₂ receptor and c-fos were normalized for the amount of β-actin measured simultaneously in each sample. Unless otherwise stated, the results are expressed as fractional increase over vehicle-treated control cells.

Calcium Imaging in Individual Cells—The effect of PDGF on bradykinin-stimulated calcium response was assessed by a video microscopic digital analysis system (Photon Technology International Inc., South Brunswick, N.J.) as described previously (7). Neurally confluent vascular smooth muscle cells grown on 25-mm coverslips were loaded with the calcium-specific dye Fura 2 by incubating with 5 μM Fura 2AM (Molecular Probes, Eugene, OR) for 40–45 min. After washing once with DMEM/bovine serum albumin, cells were reincubated in DMEM/bovine serum albumin for 30–45 min at 37 °C to allow complete hydrolysis of DNA.
B₂ Receptor Regulation by PDGF in Arterial Smooth Muscle

Characterization of PDGF Regulation of Bradykinin Receptor Binding—PDGF is a covalent dimer of two proteins, A and B, forming three possible PDGF ligands (AA, AB, and BB). When released from platelets, PDGF binds and dimerizes two B₂ receptors (7). Three PDGF ligands (AA, AB, and BB) were generated experimentally by adding PDGF (AB or BB) to cultured cells (data not shown). Association binding isotherms further demonstrated that treatment with PDGF (AB or BB) produced a 2-4-fold increase in cell surface bradykinin receptors (Fig. 1). The increase in receptor binding occurred within 4 h and peaked at 6-12 h after addition of PDGF (Fig. 1, upper panel). Subsequently, there was a gradual decrease in receptor binding which remained above control levels for over 48 h. Dose-response curves demonstrated that both the PDGF AB and BB isomers but not PDGF AA stimulated a dose-dependent increase in bradykinin receptor binding (Fig. 1, middle panel). This is consistent with the ability of PDGF AB and BB but not AA to stimulate mitogenesis in these cultured cells (data not shown). Association binding isotherms further demonstrated that treatment with PDGF (AB or BB) produced a 2-4-fold increase in cell surface bradykinin receptors (Fig. 1). The increase in receptor binding occurred within 4 h and peaked at 6-12 h after addition of PDGF (Fig. 1, upper panel). Subsequently, there was a gradual decrease in receptor binding which remained above control levels for over 48 h. Dose-response curves demonstrated that both the PDGF AB and BB isomers but not PDGF AA stimulated a dose-dependent increase in bradykinin receptor binding (Fig. 1, middle panel). This is consistent with the ability of PDGF AB and BB but not AA to stimulate mitogenesis in these cultured cells (data not shown). Association binding isotherms further demonstrated that treatment with PDGF (AB or BB) produced a 2-4-fold increase in cell surface bradykinin receptors (Fig. 1). The increase in receptor binding occurred within 4 h and peaked at 6-12 h after addition of PDGF (Fig. 1, upper panel). Subsequently, there was a gradual decrease in receptor binding which remained above control levels for over 48 h. Dose-response curves demonstrated that both the PDGF AB and BB isomers but not PDGF AA stimulated a dose-dependent increase in bradykinin receptor binding (Fig. 1, middle panel). This is consistent with the ability of PDGF AB and BB but not AA to stimulate mitogenesis in these cultured cells (data not shown). Association binding isotherms further demonstrated that treatment with PDGF (AB or BB) produced a 2-4-fold increase in cell surface bradykinin receptors (Fig. 1). The increase in receptor binding occurred within 4 h and peaked at 6-12 h after addition of PDGF (Fig. 1, upper panel). Subsequently, there was a gradual decrease in receptor binding which remained above control levels for over 48 h. Dose-response curves demonstrated that both the PDGF AB and BB isomers but not PDGF AA stimulated a dose-dependent increase in bradykinin receptor binding (Fig. 1, middle panel). This is consistent with the ability of PDGF AB and BB but not AA to stimulate mitogenesis in these cultured cells (data not shown). Association binding isotherms further demonstrated that treatment with PDGF (AB or BB) produced a 2-4-fold increase in cell surface bradykinin receptors (Fig. 1). The increase in receptor binding occurred within 4 h and peaked at 6-12 h after addition of PDGF (Fig. 1, upper panel). Subsequently, there was a gradual decrease in receptor binding which remained above control levels for over 48 h. Dose-response curves demonstrated that both the PDGF AB and BB isomers but not PDGF AA stimulated a dose-dependent increase in bradykinin receptor binding (Fig. 1, middle panel). This is consistent with the ability of PDGF AB and BB but not AA to stimulate mitogenesis in these cultured cells (data not shown). Association binding isotherms further demonstrated that treatment with PDGF (AB or BB) produced a 2-4-fold increase in cell surface bradykinin receptors (Fig. 1). The increase in receptor binding occurred within 4 h and peaked at 6-12 h after addition of PDGF (Fig. 1, upper panel). Subsequently, there was a gradual decrease in receptor binding which remained above control levels for over 48 h. Dose-response curves demonstrated that both the PDGF AB and BB isomers but not PDGF AA stimulated a dose-dependent increase in bradykinin receptor binding (Fig. 1, middle panel). This is consistent with the ability of PDGF AB and BB but not AA to stimulate mitogenesis in these cultured cells (data not shown). Association binding isotherms further demonstrated that treatment with PDGF (AB or BB) produced a 2-4-fold increase in cell surface bradykinin receptors (Fig. 1). The increase in receptor binding occurred within 4 h and peaked at 6-12 h after addition of PDGF (Fig. 1, upper panel). Subsequently, there was a gradual decrease in receptor binding which remained above control levels for over 48 h. Dose-response curves demonstrated that both the PDGF AB and BB isomers but not PDGF AA stimulated a dose-dependent increase in bradykinin receptor binding (Fig. 1, middle panel). This is consistent with the ability of PDGF AB and BB but not AA to stimulate mitogenesis in these cultured cells (data not shown). Association binding isotherms further demonstrated that treatment with PDGF (AB or BB) produced a 2-4-fold increase in cell surface bradykinin receptors (Fig. 1).
increase in the total number of cell surface receptors (Fig. 1, lower panel) associated with a small decrease in receptor affinity ($K_d = 0.46 \pm 0.12 \text{ nM}$ in control cells and 0.72 $\pm 0.09 \text{ nM}$ after PDGF). Cell surface bradykinin receptor binding from either control or PDGF-treated cells was completely displaced by incubation with the specific $B_2$ receptor antagonist, HOE140, indicating that the receptors are of the $B_2$ receptor subtype (data not shown). The increase in cell surface receptors required new protein synthesis since treatment with either cycloheximide or actinomycin D completely prevented the PDGF-induced increase in cell surface bradykinin receptor binding (Fig. 2). Hence, PDGF induced a rapid increase in the translation and expression of cell surface $B_2$ receptors in cultured arterial smooth muscle cells.

Effect of PDGF on mRNA for the $B_2$ Receptor—Messenger RNA for the $B_2$ receptor was quantitated using a ribonuclease protection assay. Treatment with PDGF induced an increase in $B_2$ receptor mRNA which peaked between 2 and 4 h and declined slowly thereafter (Fig. 3). In many experiments, a lag phase of approximately 60 min occurred before any significant increase in $B_2$ receptor mRNA was observed (Fig. 3, inset). Addition of the transcriptional inhibitors, actinomycin D (Fig. 4) or DRB (not shown), up to 60 min after addition of PDGF completely blocked synthesis of new $B_2$ receptor mRNA. To determine whether the lag phase in $B_2$ receptor mRNA induction was secondary to the synthesis of new proteins, we examined the effect of PDGF in the presence of cycloheximide. Treatment with cycloheximide, either 1 $\mu$g/ml as shown in Fig. 5 or 10 $\mu$g/ml (not shown), did not inhibit, but rather superinduced both basal and PDGF-stimulated $B_2$ receptor mRNA levels. At these concentrations of cycloheximide (1 and 10 $\mu$g/ml), protein synthesis was inhibited over 90% and 95%, respectively. These results demonstrate that the $B_2$ receptor gene is a delayed early response gene for PDGF and are compatible with an effect of PDGF to increase transcription of the $B_2$ receptor gene. The effects of PDGF on $B_2$ receptor mRNA are selective since no increase in mRNA for the angiotensin II AT$_1$ receptor was observed (data not shown, but see Figs. 7 and 8 below and Ref. 31).

Mechanism of the PDGF-induced Increase in $B_2$ Receptor mRNA—Most intracellular second messengers generated by PDGF involve activation of the tyrosine kinase on the cytosolic domain of the receptor (13–15). To test whether the PDGF-induced increase in $B_2$ receptor mRNA involved activation of tyrosine kinase, we examined the effect of the tyrosine kinase inhibitors genistein and tyrphostin B-46 (32). Since PDGF stimulation of the immediate early response gene, c-fos, is known to involve activation of tyrosine kinase (33), we compared the effects of these inhibitors on the PDGF-stimulated increase in mRNA for both the $B_2$ receptor and c-fos. Preliminary experiments demonstrated that exposure to PDGF stimulated an increase in c-fos mRNA which peaked at 15–30 min and decayed rapidly thereafter (data not shown). Therefore,
mRNA for c-fos was measured at 30 min while B2 receptor mRNA was measured after 3 h of exposure to PDGF. The results are shown in Table I and Fig. 6. Tyrosbin and genistein significantly inhibited the PDGF-stimulated increase in both c-fos and B2 receptor mRNA. Genistein inhibited the increase in c-fos more than the B2 receptor; however, interpretation of this observation was complicated by the fact that genistein also significantly increased basal levels of c-fos mRNA (Table I). These observations are consistent with the known signaling mechanisms for the PDGF receptor and indicate that activation of tyrosine kinases mediates the increase in both c-fos and the B2 receptor. To further determine whether activation of Ras was responsible for the increase in B2 receptor mRNA, we examined the effects of two reported Ras inhibitors, lovastatin and perillyl alcohol. These agents inhibit the activation of Ras by two different mechanisms (34). Pretreatment with either 50 μM lovastatin or 1 mM perillyl alcohol for 48 h potently inhibited PDGF-induced mitogenesis but did not significantly inhibit either farnesylation of Ras or the PDGF-induced increase in B2 receptor mRNA (data not shown). Higher doses of either inhibitor were associated with significant morphological evidence of cellular toxicity.

Further insight into the mechanism of the PDGF-induced increase in B2 receptor mRNA was sought by examining the effects of two additional protein kinase inhibitors, staurosporine and calphostin C (Table I and Fig. 6). The nonselective protein kinase inhibitor, staurosporine, significantly inhibited both the PDGF-stimulated c-fos and B2 receptor mRNA. In contrast, the more selective protein kinase C inhibitor, calphostin C, produced only a slight inhibition (24%) of PDGF-induced B2 receptor mRNA and had no effect on the PDGF-stimulated increase in c-fos. Overall, these results demonstrate a similar dependence on PDGF-stimulated kinase activation for both c-fos and the B2 receptor. However, stimulation of c-fos was more sensitive to genistein while the increase in the B2 receptor was more sensitive to inhibition by calphostin C.

Effect of PDGF on Bradykinin Activation of Phospholipase C—The B2 receptor is known to activate phosphoinositide-specific phospholipase C in arterial smooth muscle cells (7). To determine whether the increase in cell surface B2 receptors stimulated by PDGF are functionally coupled to activation of phosphoinositide-specific phospholipase C, we measured the effect of PDGF on bradykinin-stimulated inositol phosphate formation (Fig. 7) and intracellular calcium release (Fig. 8). As shown in Fig. 7 (upper panel), overnight treatment with PDGF produced at least a 2-fold increase in the maximal bradykinin-stimulated IP3 and IP2 formation. The EC50 for bradykinin-stimulated IP3 release appears to be shifted to the right which is consistent with the observed PDGF-stimulated decrease in receptor affinity (see above). However, analysis of the exact EC50 is complicated because the time to maximal IP2 formation varies with the concentration of bradykinin employed. Consistent with the effects on IP3 formation, overnight treatment with PDGF also produced a marked increase in bradykinin-stimulated calcium release (Fig. 8, upper panel). As shown in Fig. 8 (upper panel), PDGF significantly enhanced the kinetics of

![Figure 5](image_url)

**Fig. 5.** Effect of cycloheximide on the PDGF-stimulated increase in B2 receptor mRNA. Confluent serum-deprived cultures were treated with either vehicle or cycloheximide (1 μg/ml) for 60 min before adding either buffer or PDGF AB (12 ng/ml) for the indicated times. The upper panel shows representative autoradiographic data for vehicle-treated (A) and cycloheximide-treated (CHx, B) cells. The autoradiograms were exposed for 3 days with an intensifying screen to obtain the data shown for the B2 receptor and 16 h without the screen for β-actin. Note that the control cells shown on the left side of B were not exposed to cycloheximide. In the lower panel, mRNA for the B2 receptor was quantitated as described in Fig. 3, and the results are expressed as the fractional increase over the simultaneous vehicle-treated control cells. Each point represents the mean ± S.E. of 4-5 separate cultures from 2 different experiments.

**Table I**

| Inhibitor       | B2 receptor mRNA | c-fos mRNA |
|-----------------|------------------|------------|
|                 | Fractional increase | % control | Fractional increase | % control |
| Vehicle + PDGF |                   |           |                   |           |
| Inhibitor       | − PDGF + PDGF    |           | − PDGF + PDGF     |           |
| Tyrphostin (100 μM) | 15.8 ± 4.6       | 12 ± 4    | 1.6 ± 0.2         | 8 ± 4     |
| Genistein (100 μM)    | 6.4 ± 2.1        | 62 ± 13   | 26 ± 8.0          | 30 ± 8    |
| Stauroporine (50 nM)    | 7.3 ± 1.4        | 34 ± 6    | 3.2 ± 0.9         | 35 ± 13   |
| Calphostin C (500 nM)    | 3.7 ± 2.4        | 76 ± 13   | 2.1 ± 0.4         | 106 ± 12  |

Confluent serum-deprived cultures were treated with either vehicle (0.1% dimethylsulfoxide) or the indicated concentration of inhibitor for 30 min prior to adding additional media with or without PDGF AB (25 ng/ml). After incubation for an additional 30 min (c-fos) or 3 h (B2 receptor), the cultures were washed and mRNA for the B2 receptor, c-fos, and β-actin were measured as described under “Experimental Procedures.” The results for the B2 receptor and c-fos were normalized for differences in β-actin and expressed as fractional increase over vehicle-treated basal levels of mRNA (i.e. vehicle-treated basal mRNA for c-fos and B2 receptor = 1 ± 0.2). Data for % of control = (PDGF − basal) × 100/(PDGF − basal), where the subscripts i and c represent inhibitor-treated and vehicle-treated, respectively. All data represent mean ± S.E. of 8-12 independent determinations.
Bradykinin-stimulated calcium release and decreased the threshold concentration to less than 0.1 nM bradykinin. Overall, these results demonstrate that treatment with PDGF produced a similar increase in both the expression of cell surface B2 receptors and maximal bradykinin-stimulated inositol phosphate generation suggesting the new receptors are functionally coupled to activation of phospholipase C. In addition, prolonged exposure to PDGF also appeared to further enhance the kinetics of bradykinin-stimulated intracellular calcium accumulation.

To further examine whether the effects of PDGF were selective for bradykinin, we also examined the effect of PDGF on angiotensin II- and arginine vasopressin-stimulated inositol phosphate formation (Fig. 7) and calcium release (Fig. 8). As shown in the two lower panels of Figs. 7 and 8, angiotensin II produced only a small increase in inositol phosphate formation and calcium release while responses to vasopressin were similar to those produced by bradykinin in control cells. Chronic treatment with PDGF did modify the kinetics of hormone-stimulated calcium release leading to a more rapid peak follow by a discrete second increase in intracellular calcium concentration (Fig. 8, lower two panels). However, in contrast to the effects on bradykinin, pretreatment with PDGF produced essentially no increase in either vasopressin- or angiotensin II-stimulated inositol phosphate formation or the total time-averaged increase in intracellular calcium concentration (Figs. 7 and 8, lower two panels). These results suggest that chronic exposure to PDGF has a general effect on the kinetics of hormone-stimulated intracellular calcium concentration. However, these effects of PDGF are not mediated by a generalized increase in receptor coupling to inositol phosphate formation. These results further demonstrate that PDGF stimulates a selective increase in bradykinin receptors which are directly coupled to increased inositol phosphate formation.

**DISCUSSION**

Bradykinin and PDGF are local mediators of the response to vascular injury and inflammation. PDGF is released by degranulation of platelets while bradykinin is released during activation of the contact activation pathway of coagulation. The present study now further demonstrates that the bradykinin B2 receptor is also an early response gene for PDGF in cultured arterial smooth muscle cells. Similarly to other early response genes induced by PDGF (35–40), the increase in B2 receptor mRNA did not require new protein synthesis and was super-induced by pretreatment with cycloheximide. After a short delay, PDGF-induced a 6-10-fold increase in steady state mRNA which peaked between 2 and 4 h and lead to a 2-4-fold increase in cell surface B2 receptors at 6–12 h. Treatment with PDGF also increased bradykinin-stimulated inositol phosphate formation and markedly enhanced intracellular calcium release, suggesting that the increased expression of B2 receptors are functionally coupled to activation of phosphoinositidemr-specific phospholipase C. Finally, the effects of PDGF were selective for the bradykinin receptor since PDGF did not increase mRNA for the AT1a receptor (data not shown) or the activation of phosphoinositide-specific phospholipase C by either angiotensin II or arginine vasopressin. Consistent with these observations, previous investigators have also shown that PDGF decreased AT1 receptor binding and mRNA in vascular smooth muscle cells (31). Overall, these results demonstrate that PDGF exerts a selective effect to enhance vascular responsiveness to cellular signals generated by bradykinin via the B2 receptor on arterial smooth muscle cells.

The observation that the B2 receptor is an early response gene for PDGF has important implications both for the cellular
mechanisms which mediate the effect as well as the potential role of the B2 receptor in the biological response to PDGF. Studies in fibroblast cell lines using differential screening have shown that short-term exposure to PDGF or serum induces a large but restricted population (approximately 0.1–1%) of total cellular genes (35, 41, 42). Kinetic studies have suggested that these early response genes can generally be divided into two groups exhibiting either an immediate (=30 min) or delayed (>30 min) induction (42). Immediate early response genes include the transcription factors c-fos (37–39), c-jun (43), and the chemokine, KC (35, 44), while delayed responses are typical for the transcription factor c-myc (36, 39) and the chemokine, JE/MCP-1 (35, 45 and see Ref. 40 for review). The PDGF-induced increase in B2 receptor mRNA exhibited the slower kinetics observed for delayed early response genes. This was confirmed by the observation that addition of the transcriptional inhibitors, actinomycin D or DRB, 1 h after exposure to PDGF completely blocked the subsequent increase in B2 receptor mRNA.

For most early response genes, the growth factor-induced increase in mRNA involves a combination of both enhanced gene transcription and increased mRNA stability (42). Several elements, particularly when located in the 3′-untranslated region of the gene have been shown to regulate mRNA stability (46). The best characterized of these are the AU-rich regions which are associated with destabilizing mRNA and increasing mRNA degradation (47–49). Factors which bind to these regions are candidates for regulation by signal transduction pathways such as protein kinase C (50, 51). The rat B2 receptor gene has two ATTAA motifs in its 3′-untranslated region which might be associated with regulating mRNA stability (52). However, in preliminary studies, we have found that the half-life for B2 receptor mRNA is over 4 h and there was no increase in stability after treatment with PDGF. Further studies are required, but overall these results suggest that the PDGF-induced increase in B2 receptor mRNA is mediated primarily at the level of gene transcription.

Elucidation of the signaling pathways by which PDGF mediates gene transcription is still in its infancy. PDGF is a dimer of two proteins, A and B, forming three ligands (AA, AB, and BB) which bind and dimerize two PDGF receptors, α and β (13–15). PDGF-A ligand only binds the PDGF α receptor while the PDGF-B ligand can bind either receptor. The cytosolic domains of the two receptors are different, and differences in signal transduction pathways as well as gene transcription have also been observed (53–57). Nevertheless, both receptors have intrinsic tyrosine kinase activity and have been shown to activate Ras (19, 57). In our studies, treatment with PDGF-AA had no effect on B2 receptor expression, suggesting that the effects of PDGF are mediated via the PDGF β receptor. These results are compatible with the low level of expression of PDGF α receptors generally found on cultured rat vascular smooth muscle cells (55, 58). However, PDGF α has been shown to stimulate some early response genes even when expressed at low levels in vascular smooth muscle cells (53, 54). Hence, it will be important in future experiments to determine whether the inability of PDGF-AA to stimulate B2 receptor expression involves differences in postreceptor signaling mechanisms between the two PDGF receptors.

Consistent with our observations for the B2 receptor, most (but not all) PDGF-induced early response genes are blocked by inhibition of tyrosine kinase activation (33, 59). Activation of tyrosine kinase stimulates a number of second messenger pathways which may regulate gene transcription (see Refs. 22, 23, and 60 for reviews). For the prototypical immediate early response gene c-fos, two pathways have been shown to be important: the Ras-mediated activation of mitogen-activated protein kinases which increase transcription via the serum response element and the STAT pathway which directly activates transcription via the sis-inducible element (61–63 and reviewed in Ref. 60). A role for Ras was suggested by earlier studies demonstrating increased expression of cell surface B2 receptors in cells transformed with oncogenic Ras (8–11). Analysis of the recently reported promoter region (~1225 to +235) of the B2 receptor gene (52) does not reveal any consensus serum response element or CARG box to bind the SRF and no STAT
binding elements such as the sis-inducible element. However, there are several potential transcription factor binding sites which are known targets for Ras-activated signaling pathways. These include binding sites for Ets-related factors (i.e. Ets-1 and PEA) as well as several AP-1 sites. Moreover, the AP-1 sites may be functionally important, as we observed that activation of protein kinase C with phorbol 12-myristate 13-acetate increases bradykinin receptor binding (6). However, maximal and PEA) as well as several AP-1 sites. Moreover, the AP-1 sites may be functionally important, as we observed that activation of protein kinase C with phorbol 12-myristate 13-acetate increases bradykinin receptor binding (6). However, maximal kinetics of induction (65). We examined the 5'- and 3'-flanking regions that are both required for PDGF-induced transcription of this gene (64, 65). The 3' motif, called the 3' immediate response box (3'-IRB, TTGGTGA) was found to be present in the proximal 3'-untranslated region of all known immediate response genes (64). The immediate response box was found to be effective only when located in the 3'-flanking region of the gene; however, its activity was independent of its orientation as the sequence TACAAAAA functions equally well (64). This latter sequence is also present in the 3' region of the B2 receptor gene (+1988 to +1994). In addition, these investigators more recently identified two novel elements (sequences I and II) in the 5' region of the MCP-1 gene which bind a serinethreonine phosphoprotein and mediate the slow kinetics of induction (65). We examined the 5' region of the B2 receptor gene and have identified two elements having 80% homology to these two elements (−858 to −843 of the B2 receptor gene is homologous to sequence II and −803 to −799 of the B2 receptor gene is homologous to sequence III). Further studies will be required to determine whether these sequences are involved in the delayed response of the B2 receptor gene to PDGF.

The observation that the B2 receptor gene is an early response gene for PDGF suggests that it may have an important role in regulating vascular smooth muscle responses to PDGF during vascular injury. Since mitogenesis is an important biological response mediated by PDGF during tissue injury (12). Recent studies in fibroblasts have demonstrated that bradykinin can regulate the actin cytoskeleton via activation of the small G protein, Cdc42 (68). This may suggest a role for up-regulation of B2 receptors in modulating cell morphology and possibly the chemotactic response to PDGF.

Taken together, these observations suggest another interaction between platelets and the coagulation cascade during vascular injury. PDGF released from degranulating platelets may stimulate the rapid up-regulation of B2 receptors on the underlying vascular smooth muscle cells. Although the exact biological role for these B2 receptors is not currently known, based upon the similarities of the signal transduction pathways for the B2 receptor and the angiotensin AT1 receptor, we expect that the B2 receptor may be proinflammatory (7). This hypothesis would be consistent with the recent observations that in the absence of an angiotensin-converting enzyme inhibitor, treatment with a specific B2 receptor antagonist decreased neointimal formation after arterial injury (69). Further studies are in progress to better characterize the interactions between bradykinin and PDGF in the response to vascular injury.

Acknowledgments—We thank R. Hohl for his assistance in measuring farnesylated Ras (34).

REFERENCES

1. Bhola, K. D., Figueroa, C. D., and Worthy, K. (1992) Pharmacol. Rev. 44, 1–80
2. Hall, J. M. (1992) Pharmacol. Ther. 56, 131–190
3. Monrouxi, J.-V., and Vanhoute, P. M. (1995) Annu. Rev. Pharmacol. Toxicol. 35, 679–705
4. Förstmann, U., Hertting, G., and Neufang, B. (1986) Br. J. Pharmacol. 87, 521–532
5. Marceau, F. (1995) Immunopharmacology 30, 1–26
6. Dixon, B. S., and Dennis, M. J. (1996) Immunopharmacology, in press
7. Dixon, B. S., Sharma, V. R., Dickerson, T., and Fortune, J. (1994) Am. J. Physiol. 266, C1406–C1412
8. Parries, G., Hoebel, R., and Efraim, R. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 2648–2652
9. Downward, J., DeGuzman, J., Riehl, R., and Weinberg, R. A. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 5774–5777
10. Ruggiero, M., Srivastava, S. K., Fleming, T. P., Ron, D., and Eva, A. (1989) Oncogene 4, 767–771
11. Alonso, T., Srivastava, S., and Santos, E. (1990) Mol. Cell. Biol. 10, 3117–3124
12. Ross, R., Raines, E. W., and Bowen-Pope, D. F. (1986) Cell 46, 155–169
13. Ferns, G. A. A., Raines, E. W., Spruigel, K. H., Motani, A. S., Reidy, M. A., and Ross, R. (1993) Science 259, 1129–1132
14. Fanti, W. J., Johnson, D. E., and Williams, L. T. (1993) Annu. Rev. Biochem. 62, 453–481
15. Heldin, C.-H. (1993) Cell 80, 213–223
16. Satoh, T., Fanti, W. J., Escobedo, J. A., Williams, L. T., and Kaziro, Y. (1993) Mol. Cell. Biol. 13, 3706–3713
17. Zhang, K., Papageorge, A. G., and Lowy, D. R. (1992) Science 257, 671–674
18. Li, B.-Q., Subleski, M., Shalloway, D., Kung, H.-F., and Kamata, T. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 8504–8508
19. Nakenberg, E., and Westermark, B. (1993) J. Biol. Chem. 268, 18187–18194
20. Mulcahy, L. S., Smith, M. R., and Stacey, D. W. (1985) Nature 313, 241–243
21. de-Vries-Smits, A. M. M., Burgering, M. Th., Leevers, S. J., Marshall, C. J., and Tivol, E. A. (1991) Science 257, 671–674
22. Johnson, G. L., and Vaillancourt, R. R. (1994) Curr. Opin. Cell Biol. 6, 230–238
23. Segre, R., and Krende, E. G. (1995) FASEB J. 9, 726–735
24. Dixon, B. S., Brecken, R., Fortune, J., Vavrek, R. J., Stewart, J. M., Marzec-Carlet, R., and Linas, S. L. (1990) Ann. J. Physiol. 258, C299–C308
25. Dixon, R. S. (1984) Clin. Sci. Mol. Med. 66, 159–164
26. Munson, P. J., and Rodbard, D. (1984) in Sterosynthesis (Rodbard, D., and others, eds) Vol. 4, pp. 117–161, Raven Press, New York
27. McLaughlin, A. E., Shelton, E. R., Bishara, S., Obervoite, R., Bach, C., Zuapan, R., Fujisaki, J., Aldrich, R. W., and Jarnagin, K. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 7724–7728
28. Curran, T., Gordon, M. B., Rubino, K. L., and Sambucetti, L. C. (1987) Annu. Rev. Immunol. 5, 79–134
29. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
30. Berndige, M. J. (1983) Biochem. J. 212, 849–858
31. Nishida, G., and Murphy, T. J. (1994) Mol. Pharmacol. 46, 653–659
32. Levitzki, A., and Gazit, A. (1995) Science 267, 1782–1788
33. Zwiller, J., Sassone-Corsi, P., Kazakou, K., and Boynton, L. A. (1991) Oncogene 6, 219–221
34. Hohl, R. J., and Lewis, K. J. (1995) J. Biol. Chem. 270, 17580–17582
35. Cochran, B. H., Reiff, A. C., and Stiles, C. D. (1983) Cell 33, 939–947
36. Kelly, K., Cochran, B. H., Stiles, C. D., and Leder, P. (1983) Cell 35, 603–610
37. Greenberg, M. E., and Ziff, R. E. (1984) Nature 313, 433–438
38. Kruijer, W., Cooper, J. A., Hunter, T., and Verma, I. M. (1984) Nature 312, 711–716
39. Muller, R., Bravo, R., Burdickhardt, J., and Curran, T. (1984) Nature 312, 716–720

B2 Receptor Regulation by PDGF in Arterial Smooth Muscle
B₂ Receptor Regulation by PDGF in Arterial Smooth Muscle

40. Herschman, H. R. (1991) Annu. Rev. Biochem. 60, 281–319
41. Lau, F. L., and Nathans, D. (1985) EMBO J. 4, 3145–3151
42. Almendral, J. M., Sommer, D., MacDonald-Bravo, H., Burckhardt, J., Perera, J., and Bravo, R. (1988) Mol. Cell. Biol. 8, 2140–2148
43. Sukhatme, V. P., Kartha, S., Taub, R., Hoover, R. G., and Tsai-Morris, C. H. (1987) Oncogene Res. 1, 343–355
44. Ogunro, P., Albert, J., Wen, D., Gray, J. L., Derynck, R., and Stiles, C. D. (1989) J. Biol. Chem. 264, 4133–4137
45. Rollins, B. J., Stier, P., Ernst, T., and Wong, G. G. (1989) Mol. Cell. Biol. 9, 4687–4695
46. Decker, C. J., and Parker, R. (1995) Trends Biochem. Sci. 19, 336–340
47. Shaw, G., and Kamen, R. (1986) Cell 46, 659–667
48. Wilson, T., and Treisman, R. (1988) Nature 336, 396–399
49. Chen, C.-Y. A., and Shyu, A.-B. (1995) Trends Biochem. Sci. 20, 465–470
50. Lieberman, A. P., Pitha, P. M., and Shin, M. L. (1992) J. Biol. Chem. 267, 2123–2126
51. Perrone-Bizzozero, N. I., Cansino, V. V., and Kohn, D. T. (1993) J. Cell Biol. 120, 1263–1270
52. Pesquero, J. B., Lindsey, C. J., Zeh, K., Palva, A. C., Ganten, D., and Bader, M. (1994) J. Biol. Chem. 269, 26920–26925
53. Sahany, K. E., Robinson-Belton, C., Candia, A. F., Pledger, W. J., and Hidt, J. T. (1992) J. Cell Physiol. 150, 386–395
54. Sachinidis, A., Schulte, K., Ko, Y., Meyer zu Brinckwedde, M. K., Hoppe, V., Hoppe, J., and Vetter, H. (1993) FEBS Lett. 318, 221–224
55. Sachinidis, A., Locher, R., Vetter, W., Tatje, D., and Hoppe, J. (1990) J. Biol. Chem. 265, 10238–10243
56. Inui, H., Kitami, Y., Tani, M., Kondo, T., and Inagami, T. (1994) J. Biol. Chem. 269, 30546–30552
57. Bazzanet, C. E., and Kazlauskas, A. (1993) Oncogene 9, 517–525
58. Inui, H., Kitami, Y., Kondo, T., and Inagami, T. (1993) J. Biol. Chem. 268, 17045–17050
59. Mundschau, L. J., Forman, L. W., Weng, H., and Faller, D. V. (1994) J. Biol. Chem. 269, 16137–16142
60. Karin, M. (1994) Curr. Opin. Cell Biol. 6, 415–424
61. Wagner, B. J., Hayes, T. E., Hoban, C. J., and Cochran, B. H. (1990) EMBO J. 9, 4477–4484
62. Treisman, R. (1985) Cell 42, 899–902
63. Hill, C. S., and Treisman, R. (1995) EMBO J. 14, 5037–5047
64. Frater, R. R., Irminger, J. C., Porter, J. A., Jones, S. D., and Stiles, C. D. (1992) Mol. Cell. Biol. 12, 5288–5300
65. Frater, R. R., Albert, J. A., Lam, K. K., and Stiles, C. D. (1995) Mol. Cell. Biol. 15, 315–325
66. Rollins, B. J., Morrison, E. D., Usher, P., and Flier, J. S. (1988) J. Biol. Chem. 263, 16523–16526
67. Magistretti, J., Dettori, C., and Meldolesi, J. (1991) Exp. Cell Res. 192, 67–74
68. Kozma, R., Ahmed, S., Best, A., and Lim, L. (1995) Mol. Cell. Biol. 15, 1942–1952
69. Farhy, R. D., Carretero, O. A., Ho, K.-L., and Sdcli, A. G. (1993) Circ. Res. 72, 1202–1210