Cross-talk between receptors with intrinsic tyrosine kinase activity and α_{1b}-adrenoceptors

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The effect of epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) on the phosphorylation and function of α_{1b}-adrenoceptors transfected into Rat-1 fibroblasts was studied. EGF and PDGF increased the phosphorylation of these adrenoceptors. The effect of EGF was blocked by tyrphostin AG1478 and that of PDGF was blocked by tyrphostin AG1296, inhibitors of the intrinsic tyrosine kinase activities of the receptors for these growth factors. Wortmannin, an inhibitor of phosphoinositide 3-kinase, blocked the α_{1b}-adrenoceptor phosphorylation induced by EGF but not that induced by PDGF. Inhibition of protein kinase C blocked the adrenoceptor phosphorylation induced by EGF and PDGF. The ability of noradrenaline to increase [^{35}S]guanosine 5′-[γ-thio]triphosphate ([^{35}S]GTP[S]) binding in membrane preparations was used as an index of the functional coupling of the α_{1b}-adrenoceptors and G-proteins. Noradrenaline-stimulated [^{35}S]GTP[S] binding was markedly decreased in membranes from cells pretreated with EGF or PDGF. Our data indicate that: (i) activation of EGF and PDGF receptors induces phosphorylation of α_{1b}-adrenoceptors, (ii) phosphatidylinositol 3-kinase is involved in the EGF response, but does not seem to play a major role in the action of PDGF, (iii) protein kinase C mediates this action of both growth factors and (iv) the phosphorylation of α_{1b}-adrenoceptors induced by EGF and PDGF is associated with adrenoceptor desensitization.

Key words: α_1-adrenoceptor, desensitization, epidermal growth factor, phosphoinositide 3-kinase, receptor phosphorylation.

INTRODUCTION

α_{1b}-Adrenoceptors (α_{1b}-ARs) are members of the superfamily of seven-transmembrane-domain G-protein-coupled receptors. These receptors couple to $G_{q/11}$ protein to stimulate phospholipase C activity, which induces hydrolysis of phosphatidylinositol 4,5-bisphosphate and the subsequent formation of inositol 1,4,5-trisphosphate and diacylglycerol; these second messengers mediate intracellular Ca^{2+} release and activation of protein kinase C (PKC), respectively [1,2].

Similar to most receptors of this superfamily, the activity of α_{1b}-ARs is tightly regulated. Stimulation by an agonist results in rapid attenuation of receptor responsiveness (desensitization), and receptor phosphorylation seems to be an essential event in this process [3,4]. Homologous desensitization, in which stimulation by a particular agonist reduces the receptor's responsiveness to that agonist, involves phosphorylation of the receptor by G-protein-coupled receptor kinases [3,4]. Receptor phosphorylation by G-protein-coupled receptor kinases promotes the binding of β-arrestin proteins, which in turn uncouple the receptor from the G-protein and enhance their sequestration [3,4]. Heterologous desensitization, in which the stimulation by an agonist attenuates the response to an unrelated agent, mainly involves phosphorylation of receptors and other signalling entities by second-messenger-activated kinases, such as protein kinase A and PKC [3,4]. Agonist-induced phosphorylation of α_{1b}-ARs seems to be mediated by G-protein-coupled receptor kinase 2 and G-protein-coupled receptor kinase 3 isoenzymes [5], whereas the phosphorylation of these adrenoceptors by PKC mainly participates in heterologous desensitization [6,7].

Growth-factor receptors, such as the epidermal growth factor (EGF) receptor and the platelet-derived growth factor (PDGF) receptor have intrinsic tyrosine kinase activity. Agonist binding to these receptors induces their dimerization and autophosphorylation on multiple tyrosine residues within their intracellular domains. The autophosphorylated receptors provide specific binding sites for SH2-domain-containing adaptor proteins, such as Shc and Grb2. Formation of the receptor–Shc–Grb2 complexes leads to activation of the guanine nucleotide-exchange factor, Sos, which in turn activates the Ras/Raf/mitogen-activated protein kinase (MAP kinase) cascade. Another class of receptor-associated proteins consists of enzymes, including Src family members, the protein tyrosine phosphatases SHP-2 and SHP-1, phospholipase Cγ and phosphoinositide 3-kinase (PI3K) [8,9].

Numerous pieces of evidence have suggested that transmodulation of growth-factor receptors represent an important event in specific responses mediated by G-protein-coupled receptors in different cell types. Thus stimulation of Rat-1 cells with endothelin-1, lysophosphatidic acid or thrombin induces a rapid increase in tyrosine phosphorylation of the EGF receptor and p185^{neu}, which results in activation of MAP kinase. Tyrphostin AG1478, a selective EGF-receptor kinase inhibitor, and expression of dominant-negative EGF-receptor mutants decrease the ability of these G-protein-coupled receptors to induce mitogenic responses [10,11]. In COS-7 cells the activation of G_i- or G_q-coupled receptors induces phosphorylation of the EGF receptor associated with assembly of Shc and Grb2 [12,13]. Angiotensin II elicits rapid phosphorylation of PDGF receptors and EGF receptors and the subsequent formation of Shc–Grb2

Abbreviations used: α_{1b}-AR, α_{1b}-adrenoceptor; PKC, protein kinase C; PI3K, phosphoinositide 3-kinase; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; GTP[S], guanosine 5′-[γ-thio]triphosphate; MAP kinase, mitogen-activated protein kinase.

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complex and MAP kinase activation in vascular smooth-muscle cells [14,15]. Furthermore, Ca^{2+}-dependent EGF-receptor activation following membrane depolarization or stimulation of bradykinin receptors has been observed in PC12 cells, suggesting that the EGF receptor can function as an integrator of different Ca^{2+}-mediated signals in neuronal cells [16,17].

Although much attention has been focused on how activation of G-protein-coupled receptors transmodulates receptors with endogenous tyrosine kinase activity, the information that exists about cross-talk from growth-factor receptors to G-protein-coupled receptors is limited. Nevertheless, Malbon and co-workers [18–20] have provided ample evidence that the β_2-adrenoceptor is cross-regulated by activation of the insulin receptor. Stimulation of DDT1 MF-2 cells with insulin attenuates β_2-adrenoceptor-mediated activation of adenylate cyclase and increases β_2-adrenoceptor phosphorylation [18]. Insulin promotes the phosphorylation of the β_2-adrenoceptor both *in vivo* and *in vitro*, and the main residues for insulin-induced phosphorylation were identified as Tyr^{350}, Tyr^{354} and Tyr^{364} [19,20], indicating that β_2-adrenoceptors are substrates of the insulin-receptor kinase activity.

The present study was designed to investigate the possible cross-talk from EGF and PDGF receptors to α_{1b}-ARs expressed in Rat-1 fibroblasts. We provide evidence that the activation of EGF and PDGF receptors leads to phosphorylation and desensitization of α_{1b}-ARs.

EXPERIMENTAL

Materials

Dulbecco's modified Eagle's medium, G418, PDGF-BB, EGF, fetal bovine serum and others reagents used for cell culture were obtained from Gibco-BRL. (−)-Noradrenaline, lysophosphatidic acid, staurosporine, wortmannin, GDP, guanosine 5′-[γ-thio]triphosphate (GTP[S]) and protease inhibitors were from Sigma. Ro 31-8220, and tyrphostin AG1478 and AG1296 were from Calbiochem. [^{35}S]GTP[S] (1250 Ci/mmol) and [^{32}P]P_i (8500–9120 Ci/mmol) were from New England Nuclear Life Science Products. Protein A–Sepharose beads were from Upstate Biotechnology. Pertussis toxin was purified from vaccine concentrates [7].

Cell culture

Rat-1 cells transfected with the hamster α_{1b}-AR [21] were a generous gift from Dr R. J. Lefkowitz, Dr M. G. Caron and Dr L. Allen (Duke University, Durham, NC, U.S.A.). Cells were grown at confluence in 10-cm culture dishes in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum, G418 (300 μg/ml), streptomycin (100 μg/ml), penicillin (100 units/ml) and amphotericin B (0.25 μg/ml) under a 5 % CO_2/95 % air atmosphere at 37 °C. Cells were serum-starved overnight in all experiments. Treatment with pertussis toxin was as described in [7].

Determination of α_{1b}-AR phosphorylation

Rat-1 cells expressing the α_{1b}-ARs were incubated in phosphate-free Dulbecco's modified Eagle's medium for 1 h, and then labelled in the same medium containing [^{32}P]P_i (50 μCi/ml) for 3 h at 37 °C, as described previously [7]. In this procedure, a rabbit antiserum against the decapeptide of the C-terminal sequence of the hamster α_{1b}-AR was used [7]. This antiserum immunoprecipitates α_{1b}-ARs with high efficacy (≈ 80 % of photolabelled receptors) [7]; the receptors were detected as a single broad band of ≈ 80–85 kDa [7]. In brief, following treatment with inhibitors and/or agonists, cells were washed with ice-cold PBS and lysed for 1 h on ice in lysis buffer containing 10 mM Tris/HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, 1 % Triton X-100, 0.05 % SDS, 50 mM NaF, 100 μM Na_3VO_4, 10 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM phosphoserine, 1 mM phosphothreonine and protease inhibitors (20 μg/ml leupeptin, 20 μg/ml aprotinin, 100 μg/ml PMSF, 500 μg/ml bacitracin and 50 μg/ml soya-bean trypsin inhibitor). Cell lysates were centrifuged at 12700 ***g*** for 15 min and the supernatants were incubated with the anti-α_{1b}-AR serum and Protein A–Sepharose overnight at 4 °C. After five washes with 50 mM Hepes, 50 mM NaH_2PO_4, 100 mM NaCl, pH 7.4, 1 % Triton X-100, 0.05 % SDS and 100 mM NaF followed by a final wash with 50 mM Tris and 150 mM NaCl, pH 7.4, the immune complexes were denatured by boiling in SDS sample buffer containing 5 % β-mercaptoethanol, and subjected to SDS/PAGE. The gels were dried and exposed for 3–24 h at −70 °C using Kodak X-Omat X-ray films and intensifying screens. The level of receptor phosphorylation was assessed in the same gels with a Molecular Dynamics PhosphorImager and ImageQuant software.

Membrane preparation and [^{35}S]GTP[S] binding

Confluent cells were stimulated in the absence (control) or presence of EGF (100 ng/ml) or PDGF (50 ng/ml) for 15 min at 37 °C. Washing with ice-cold PBS terminated the reaction and cells were scraped with 1 ml of ice-cold buffer (50 mM Tris, 150 mM NaCl, pH 7.5, 5 mM EDTA, 100 μM Na_3VO_4, 10 mM β-glycerophosphate and 10 mM sodium pyrophosphate plus protease inhibitors). Membranes were prepared according to the method of Mattingly et al. [22]. Freshly isolated membranes were used, since it was observed that noradrenaline-stimulated [^{35}S]GTP[S] binding was greater than in membranes that had been frozen. [^{35}S]GTP[S] binding was performed as described by Wieland and Jakobs [23] with minor modifications. Briefly, membranes were resuspended in binding buffer (50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, 1 mM dithiothreitol, 1 μM GDP and 0.1 % BSA). Binding was performed at 25 °C for 30 min in a volume of 250 μl of binding buffer containing 0.2 nM [^{35}S]GTP[S]. The reaction was initiated by the addition of membranes (25 μg of protein/tube) and terminated by rapid filtration through Whatman GF/C filters followed by three washes of the filters with ice-cold buffer (50 mM Tris and 10 mM $MgCl_2$, pH 7.5). The filters were dried and the radioactivity was measured by liquid scintillation. Non-specific binding was determined in the presence of unlabelled GTP[S] (30 μM) and represented 10 % of total binding. Statistical analysis between comparable groups was performed using analysis of variance with the Newman–Keuls post test.

RESULTS

EGF-induced phosphorylation of α_{1b}-AR

Rat-1 cells were exposed for 5 min to vehicle (basal) or different concentrations of EGF (0.01–100 ng/ml). EGF induced the phosphorylation of α_{1b}-ARs in a dose-dependent manner (EC_{50} ≈ 1 ng/ml) with a maximal increase of 60–70 % over basal (Figure 1, upper panels). The response to EGF was relatively rapid, reaching a maximum at 15–30 min and decreasing towards 60 min of stimulation (Figure 1, lower panels).

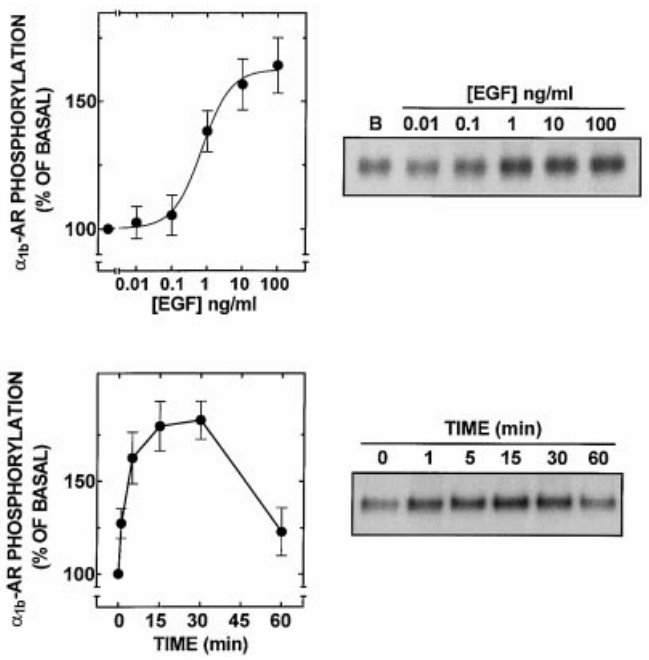

Figure 1 Effect of EGF on α_{1b}-AR phosphorylation

Upper panels: cells were incubated in the absence (B) or presence of different concentrations of EGF. Lower panels: cells were incubated with 10 ng/ml EGF for the times indicated. Plotted are the means ± S.E.M. of triplicate determinations of 4–5 experiments using different cell cultures. Representative autoradiographs are shown.

The role of the EGF-receptor tyrosine kinase activity in this effect was studied by using tyrphostin AG1478, a highly specific inhibitor of the EGF-receptor kinase [24]. Treatment of cells with AG1478 for 30 min inhibited EGF-induced phosphorylation of α_{1b}-AR in a concentration-dependent manner, with an IC_{50} of $\approx$ 300 nM (Figure 2, left-hand panels). The response to EGF was blocked in the presence of AG1478 (5 μM; $P < 0.01$ versus EGF alone) but remained unaffected by the same concentration of tyrphostin AG1296, a specific inhibitor of the PDGF-receptor tyrosine kinase activity (Figure 2, right-hand panels). The inhibitors by themselves were without effect on basal receptor phosphorylation (results not shown).

EGF-mediated α_{1b}-AR phosphorylation occurs through activation of PI3K

PI3K is an important mediator of cellular processes modulated by EGF. Therefore, we examined the role of this kinase on the phosphorylation of α_{1b}-ARs stimulated by EGF. Rat-1 cells were preincubated for 30 min with increasing concentrations of wortmannin (0.1–100 nM), an inhibitor of PI3K [25], followed by EGF treatment (10 ng/ml) for 5 min. Figure 3 (upper panels) shows that wortmannin inhibited EGF-induced α_{1b}-AR phosphorylation in a dose-dependent manner (IC_{50} $\approx$ 5 nM). An almost complete blockade was observed at 100 nM wortmannin, which is within the range of concentrations required for inhibition of PI3K activity [25]. Wortmannin (100 nM) was without effect on basal receptor phosphorylation (results not shown). These results indicate that the phosphorylation of α_{1b}-ARs induced by EGF was mediated through PI3K.

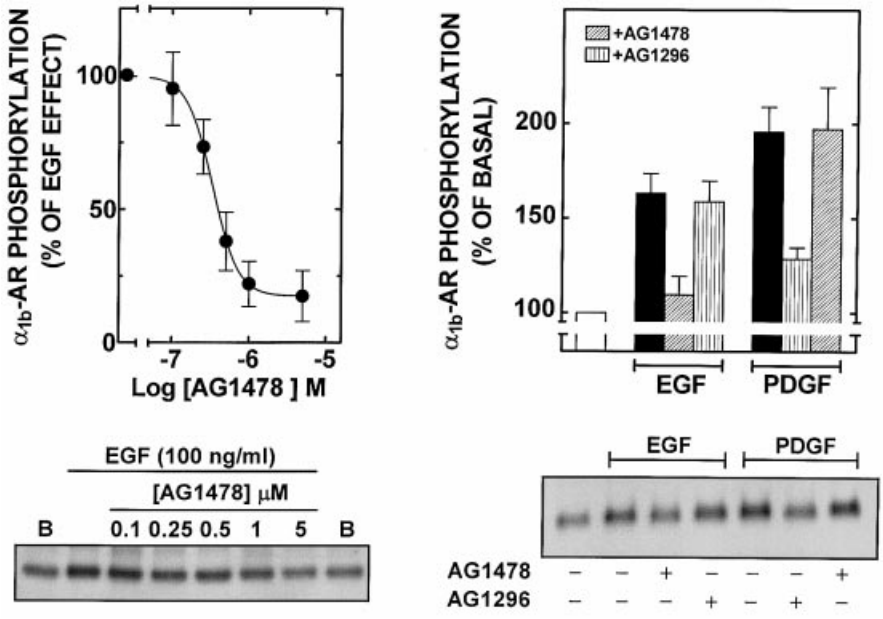

Figure 2 Effect of AG1478 and AG1296 on EGF- and PDGF-induced α_{1b}-AR phosphorylation

Left panels: cells were incubated in the absence of any agent (B) or with 100 ng/ml EGF and different concentrations of AG1478. Plotted are the means ± S.E.M. of triplicate determinations of four experiments using different cell cultures. A representative autoradiograph is shown. Right panels: cells were incubated in the absence of any agent (white bar), or with 100 ng/ml EGF or 50 ng/ml PDGF alone (black bars) or with 5 μM AG1478 or 5 μM AG1296. Plotted are the means ± S.E.M. of six independent determinations using two different cell cultures. A representative autoradiograph is shown.

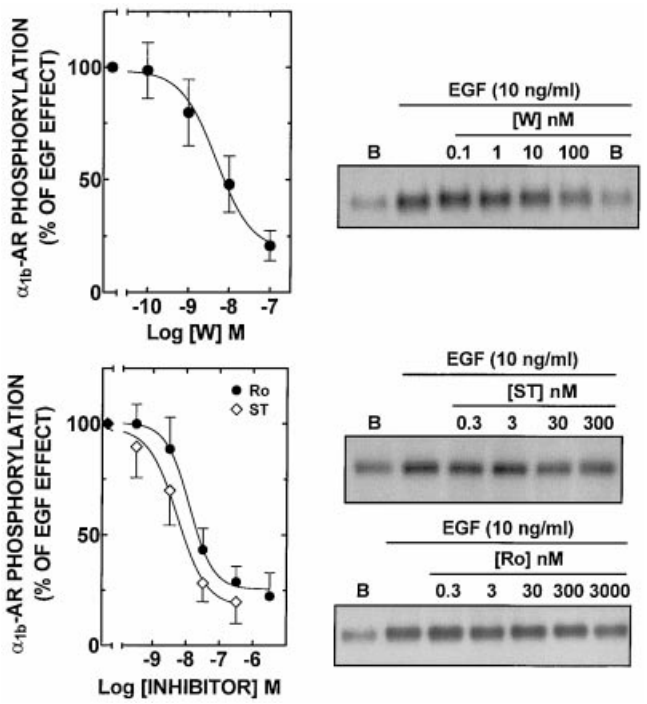

Figure 3 Effect of wortmannin, staurosporine and Ro 31-8220 on EGF-induced α_{1b}-AR phosphorylation

Upper panels: cells were incubated in the absence of any agent (B) or with 10 ng/ml EGF alone or with different concentrations of wortmannin (W). Plotted are the means ± S.E.M. of triplicate determinations of five experiments using different cell cultures. A representative autoradiograph is shown. Lower panels: cells were incubated in the absence of any agent (B) or with 10 ng/ml EGF alone or with different concentrations of staurosporine (ST) or Ro 31-8220 (Ro). Plotted are the means ± S.E.M. of triplicate determinations of three (ST) or four (Ro) experiments using different cell cultures. Representative autoradiographs are shown.

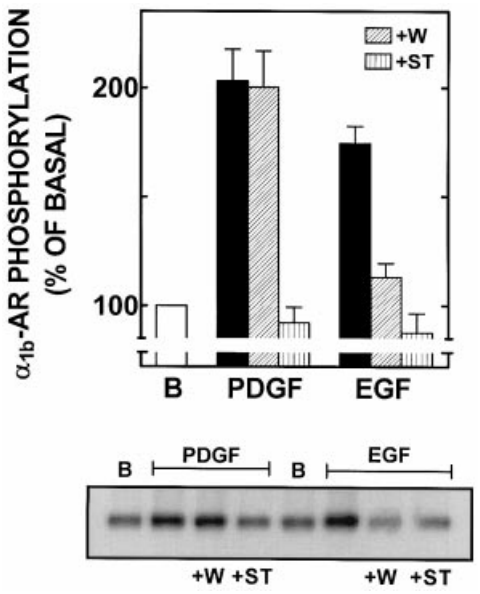

Figure 4 Comparative effect of wortmannin and staurosporine on EGF- and PDGF-induced α_{1b}-AR phosphorylation

Cells were incubated in the absence of any agent (B), with 100 ng/ml EGF or 50 ng/ml PDGF alone (black bars) or with 100 nM wortmannin (W) or 300 nM staurosporine (ST). Plotted are the means ± S.E.M. of six determinations using two different cell cultures. A representative autoradiograph is shown.

EGF-induced α_{1b}-AR phosphorylation requires activation of PKC

Previous studies have shown PI3K-dependent activation of PKC [26,27]. Therefore, we examined the contribution of PKC in the EGF signalling pathway that leads to phosphorylation of α_{1b}-ARs. Treatment of Rat-1 cells with the specific inhibitors of PKC, staurosporine and Ro 31-8220, for 30 min, reduced the EGF-stimulated phosphorylation of α_{1b}-ARs in a concentration-dependent manner. The IC_{50} values were ≈ 5 and ≈ 12 nM for staurosporine and Ro 31-8220, respectively (Figure 3, lower panels). These data indicate that, besides PI3K, PKC participates in mediating the EGF-induced phosphorylation of α_{1b}-ARs.

PDGF-induced phosphorylation of α_{1b}-AR in Rat-1 cells

To determine whether the induction of α_{1b}-AR phosphorylation was limited to the EGF receptor or common to other receptors with intrinsic tyrosine kinase activity, similar studies were performed using PDGF. Rat-1 cells were exposed to PDGF (50 ng/ml) for 5 min. Similar to EGF, PDGF markedly increased α_{1b}-AR phosphorylation. In fact, the increase in adrenoceptor phosphorylation induced by PDGF was 90–100 % above basal (Figure 2, right-hand panels), consistently greater than that induced by EGF. Treatment of the cells for 30 min with AG1296 (5 μM), a specific inhibitor of PDGF-receptor tyrosine kinase activity, inhibited α_{1b}-AR phosphorylation induced by subsequent exposure to PDGF (50 ng/ml) for 5 min (Figure 2, right-hand panels; $P < 0.01$ versus PDGF alone); the response of PDGF was not altered by the same concentration of AG1478 (Figure 2, right-hand panels).

Roles of PKC and PI3K in PDGF-induced phosphorylation of α_{1b}-ARs

To examine the role of PI3K in the PDGF-induced α_{1b}-AR phosphorylation, we assessed the effect of PI3K inhibition by wortmannin. As shown in Figure 4, treatment of Rat-1 cells for 30 min with 100 nM wortmannin prevented receptor phosphorylation induced by EGF ($P < 0.005$ versus EGF alone), but surprisingly it did not affect the phosphorylation of α_{1b}-ARs in response to PDGF.

We next studied the involvement of PKC in the effect of PDGF. Rat-1 cells were incubated for 30 min with staurosporine (300 nM), followed by PDGF stimulation (50 ng/ml, 5 min). Treatment with the inhibitor completely blocked α_{1b}-AR phosphorylation induced by PDGF ($P < 0.001$ versus PDGF alone; Figure 4), indicating that PKC activation is a critical step in the PDGF signalling pathway that leads to phosphorylation of α_{1b}-ARs.

Functional significance of the phosphorylation of α_{1b}-ARs induced by EGF and PDGF

The effects of EGF and PDGF on α_{1b}-AR coupling to G-proteins were studied by measuring receptor-mediated stimulation of [^{35}S]GTP[S] binding to membranes. Membrane preparations were obtained from Rat-1 cells preincubated for 15 min in the absence (control) or presence of noradrenaline (10 μM), EGF (100 ng/ml) or PDGF (50 ng/ml). As shown in Figure 5,

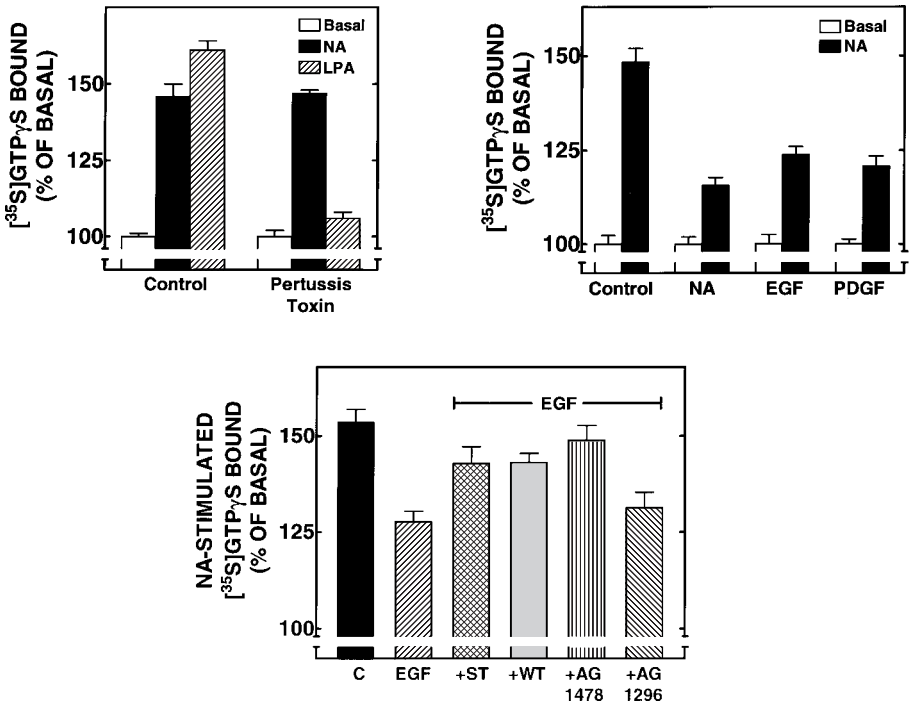

Figure 5 [^{35}S]GTP[S] binding to membranes

Upper-left panel: effect of pertussis toxin. Cells were incubated overnight in the absence or presence of pertussis toxin (100 ng/ml), membranes were isolated and *in vitro*-stimulated with vehicle (Basal), 10 μM noradrenaline (NA) or 1 μM lysophosphatidic acid (LPA). Basal [^{35}S]GTP[S] ([^{35}S]GTPγS) binding was 3053 $\pm$ 100 d.p.m./25 μg of protein (mean $\pm$ S.E.M., $n = 10$). Plotted are the means $\pm$ S.E.M. of 6–9 determinations using three different membrane preparations. Upper-right panel: effects of cell treatment with noradrenaline, EGF and PDGF. Cells were incubated for 15 min in the absence of any agent (Control), or with 10 μM noradrenaline, 100 ng/ml EGF or 50 ng/ml PDGF, membranes were obtained and basal (white bars) and noradrenaline-stimulated (black bars) [^{35}S]GTP[S] binding was determined. Basal [^{35}S]GTP[S] binding was 2810 $\pm$ 100 d.p.m./25 μg of protein ($n = 20$). Plotted are the means $\pm$ S.E.M. of 16 determinations using four different membrane preparations. Lower panel: effect of protein kinase inhibitors on the action of EGF on noradrenaline-stimulated [^{35}S]GTP[S] binding. Cells were incubated in the absence of any agent or the presence of 100 nM wortmannin (+WT), 300 nM staurosporine (+ST), 5 μM AG1478 or 5 μM AG1297. After 30 min with the inhibitors cells were incubated in the absence or presence of 100 ng/ml EGF for 15 min, membranes were obtained and basal and noradrenaline-stimulated [^{35}S]GTP[S] binding was assayed. Basal [^{35}S]GTP[S] binding was 3280 $\pm$ 90 d.p.m./25 μg of protein ($n = 20$); basal binding was not altered by the inhibitors. Plotted are the means $\pm$ S.E.M. of 15 determinations using three different membrane preparations.

noradrenaline significantly stimulated [^{35}S]GTP[S] binding in membranes from control cells ($P < 0001$ versus basal binding). Noradrenaline-stimulated [^{35}S]GTP[S] binding was pertussis toxin-insensitive (Figure 5, upper-left panel). As a control the effect of lysophosphatidic acid was tested; as shown, lysophosphatidic acid-stimulated [^{35}S]GTP[S] binding was sensitive to pertussis toxin (Figure 5, upper-left panel; $P < 0.001$ versus absence of pertussis toxin). Noradrenaline-stimulated [^{35}S]GTP[S] binding was decreased markedly in the membranes from cells pretreated with the adrenergic agonist ($P < 0.001$ versus control). Similarly, in membranes from cells pretreated with EGF or PDGF the increase in [^{35}S]GTP[S] binding induced by noradrenaline was significantly decreased ($P < 0.001$ versus control in both cases; Figure 5, upper-right panel).

The actions of the different protein kinase inhibitors on the effects of EGF on noradrenaline-stimulated [^{35}S]GTP[S] binding were studied. The EGF-receptor tyrosine kinase inhibitor, AG1478, blocked the effect of EGF ($P < 0.001$; Figure 5, lower panel), but it was not inhibited by AG1296. Staurosporine and wortmannin also inhibited the effect of EGF on noradrenaline-stimulated [^{35}S]GTP[S] binding (Figure 5, lower panel; $P < 0.05$ versus EGF alone in both cases). Similar experiments were carried out using PDGF, but the results were less clear. The effect of PDGF was inhibited by AG1296 but not by AG1478 or wortmannin, and staurosporine decreased the effect of the growth factor in a marginally significant way (results not shown).

DISCUSSION

Our present data show that EGF and PDGF induce phosphorylation of α_{1b}-ARs. The adrenoceptor phosphorylation induced by these growth factors seems to involve the tyrosine kinase activity of their receptors, as indicated by the ability of tyrphostin AG1478 and AG1296 to inhibit the actions. The selectivity of these inhibitors was shown by the absence of cross-inhibition.

It is known that Rat-1 cells endogenously express functional EGF receptors and PDGF receptors [10,11]. Stimulation of these receptors leads to activation of their intrinsic tyrosine kinase activity and subsequently to autophosphorylation of specific residues in their intracellular domains. PI3K and other signalling and adaptor proteins associate with phosphotyrosine residues on the receptors initiating the signalling cascades [26–28].

Of particular interest in our study were the roles of PI3K and PKC. The PI3K family of enzymes has been grouped into several classes. In Class-IA PI3K isoforms the adaptor p85 subunit interacts with phosphorylated tyrosine motifs of receptors with intrinsic tyrosine kinase activity, whereas PI3Kγ (Class-IB isoform) interacts with heterotrimeric G-proteins via the p101 protein; such interactions seem to control PI3K activity [26].

The mechanism through which PI3K stimulates PKC activity probably involves a direct interaction with the phosphoinositides generated by PI3K. Phosphatidylinositol 3,4-bisphosphate and

phosphatidylinositol 3,4,5-trisphosphate have been reported to activate novel PKC (δ, ϵ and η) and atypical PKC (ζ and λ) isoforms [26,27,29]. An intermediary kinase, such as the recently identified phosphoinositide-dependent protein kinase-1 [30,31] may also participate in the control of PKC. Phosphoinositide-dependent protein kinase-1, which binds with high affinity to phosphatidylinositol 3,4,5-trisphosphate, phosphorylates the activation-loop sites of PKCζ and PKCδ *in vitro* and in a PI3K-dependent manner *in vivo* [32,33]. Interestingly, classical PKCα, βI and βII bind directly to phosphoinositide-dependent protein kinase-1 co-expressed in HEK-293 cells [33], raising the possibility of general control of the PKC family by phosphoinositide-dependent protein kinase-1.

Our data show that EGF induces α_{1b}-AR phosphorylation through a PI3K- and PKC-dependent pathway. The almost complete inhibition of receptor phosphorylation observed in the presence of either a PI3K inhibitor (wortmannin) or PKC inhibitors (staurosporine and Ro 31-8220) suggests that these enzymes act sequentially in the same signalling pathway. In accord with current ideas our data suggest that: (i) EGF activates its receptor, which autophosphorylates in tyrosine residues, and (ii) this allows the association and activation of PI3K, which (iii) leads to activation of PKC, either via interaction with 3-phosphoinositides or phosphorylation by phosphoinositide-dependent protein kinase-1, and (iv) PKC catalyses the phosphorylation of α_{1b}-ARs.

We have shown previously that PKC plays an important role in the phosphorylation of α_{1b}-ARs induced by activation of endothelin ET_A receptors in these cells [7]. No effect of wortmannin was observed on this action of endothelin, which is consistent with our suggestion that EGF-receptor transmodulation did not play a major role in this effect. However, it is clear from our data that multiple pathways may contribute to α_{1b}-AR phosphorylation and that several of them may contribute marginally to the overall effect.

Lysophosphatidic acid stimulated α_{1b}-AR phosphorylation [34]. The effect of this phospholipid is mediated through G_i-coupled receptors and, interestingly, also involves PI3K and PKC [34]. It is not presently known if the same or different PI3K isoforms are involved in the actions of EGF and lysophosphatidic acid [26]. It was surprising that the α_{1b}-AR phosphorylation induced by EGF was very potently inhibited by staurosporine and Ro 31-8220; the EC_{50} values were 10–20 times lower than those observed for lysophosphatidic acid-stimulated α_{1b}-AR phosphorylation [34]. We cannot rule out the possibility that different PKC isoenzymes could participate in these effects but the potent effect further emphasizes the role of this protein kinase in the action of EGF.

The activation of PDGF receptors also significantly stimulated α_{1b}-AR phosphorylation. The PDGF response was strongly attenuated by AG1296, but it was not affected by AG1478. The PI3K inhibitor wortmannin did not block the effect of PDGF, suggesting that PI3K plays, if any, a minor role in this effect and that one or more other pathways are probably involved. In contrast, treatment of cells with staurosporine before PDGF stimulation prevented the adrenoceptor phosphorylation. Thus the α_{1b}-AR phosphorylation induced by PDGF clearly involved a PKC-mediated pathway. It is possible that this action of PDGF may involve activation of phospholipase Cγ. Several cellular responses induced by PDGF, such as growth and development [35], activation of phospholipase D [36] and chemotaxis [37], occur through phospholipase Cγ-mediated pathways. In Rat-1 fibroblasts, it has been reported that the serine/threonine kinase protein kinase D acts downstream of PKC in the PDGF signalling mediated by phospholipase Cγ [38]. It is, therefore, possible that protein kinase D may also participate in the α_{1b}-AR phosphorylation, a view that needs further exploration.

Our data provide evidence of differences in the growth-factor signalling pathways that lead to α_{1b}-AR phosphorylation. This is not completely unexpected since studies in Rat-1 cells and other cell types have shown differences in the signal-transduction pathways for EGF and PDGF receptors. For example, in Rat-1 cells PDGF activates phospholipase D and phosphatidylcholine-specific phospholipase C but EGF has no effect on this phospholipase C activity and stimulates phospholipase D only weakly; similarly, the activation of phospholipase Cγ induced by PDGF is much bigger than that induced by EGF [39,40].

The phosphorylation of α_{1b}-AR promoted by EGF and PDGF was associated with receptor desensitization, as illustrated by the [^{35}S]GTP[S]-binding assays. The data indicate that pretreatment of the cells with EGF and PDGF induced substantial reduction in the coupling efficiency of α_{1b}-ARs and G-proteins. The use of the protein kinase inhibitors confirmed the roles of the intrinsic tyrosine kinase activities of these receptors. In the case of the effects induced by EGF the inhibitors of PKC and PI3K clearly showed a negative correlation between α_{1b}-AR phosphorylation and receptor coupling, as evidenced by noradrenaline-stimulated [^{35}S]GTP[S] binding. With PDGF both the receptor phosphorylation and the [^{35}S]GTP[S]-binding assays indicated that additional factors were involved.

In conclusion, our data indicate that: (i) activation of EGF and PDGF receptors promotes phosphorylation of α_{1b}-ARs, (ii) PI3K is involved in the EGF response, but does not seem to play a definitive role in the action of PDGF, (iii) PKC plays an important role in mediating the α_{1b}-AR phosphorylation induced by both growth factors, and (iv) the phosphorylation of α_{1b}-AR induced by EGF and PDGF is associated with adrenoceptor desensitization. These results indicate that a cross-talk exists between the pathways activated by EGF receptors and PDGF receptors and α_{1b}-ARs in Rat-1 fibroblasts. This process could be of physiological relevance in the regulation of cell responsiveness to adrenergic agonists.

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