α_{1B} -Adrenergic receptor phosphorylation and desensitization induced by transforming growth factor- β

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Transforming growth factor- β (TGF- β) induced α_{1B} -adrenergic receptor phosphorylation in Rat-1 fibroblasts stably expressing these adrenoceptors. This effect of TGF- β was rapid, reaching a maximum within 30 min and decreasing thereafter, and concentration-dependent (EC_{50} 0.3 pM). The phosphoinositide 3-kinase inhibitors wortmannin and LY294002, and the protein kinase C inhibitors staurosporine, Ro 318220 and bisindolylmaleimide, blocked the effect of this growth factor. α_{1B} -Adrenergic receptor phosphorylation was associated with desensitization, as indicated by a reduction in the adrenergic-mediated production of [³H] inositol phosphates. Phosphorylation of α_{1B} -adrenergic receptors

by TGF- β was also observed in Cos-1 cells transfected with the receptor. Co-transfection of the dominant-negative mutant of the regulatory subunit of phosphoinositide 3-kinase ($\Delta p85$) inhibited the phosphorylation of α_{1B} -adrenergic receptors induced by TGF- β . Our results indicate that activation of TGF- β receptors induces α_{1B} -adrenergic receptor phosphorylation and desensitization. The data suggest that phosphoinositide 3-kinase and protein kinase C play key roles in this effect of TGF- β .

Key words: adrenaline, cross-talk, phosphoinositide 3-kinase, protein kinase C.

INTRODUCTION

G-protein-coupled receptors constitute one of the major families in the mammalian genome [1]. They play primary roles in the perception by the organism of external stimuli such as light, odours and tastes, and in cell-to-cell communication mediating the response to many hormones and neurotransmitters. Control of receptor function is a cardinal event in the adaptation of whole organisms and individual cells to changes in the external and internal milieus, and to the overall maintenance of homeostasis.

α_1 -Adrenergic receptors (α_1 -ARs) mediate many of the actions of adrenaline and noradrenaline, such as the smooth muscle contraction that maintains vascular tone, and the regulation of hormone secretion and of intermediary metabolism [2]. They also participate in the pathogenesis of diseases, such as hypertension and benign prostatic hypertrophy [2]. Therefore knowledge about the molecular events that control their function is of major importance for the understanding of the physiological balance that we call health, and its perturbations during disease.

This family of receptors consists of three members, the α_{1A} -, α_{1B} - and α_{1D} -ARs, which mediate their actions mainly via the phosphoinositide-turnover/calcium-mobilization pathway [2–5]. The function of these ARs is modulated through phosphorylation via G-protein-coupled receptor kinases and second messenger-dependent kinases such as protein kinase C (PKC) ([6–13]; reviewed in [14]). Differences in sensitivity to phosphorylation and desensitization have been observed among these receptor subtypes [12,15].

The α_{1B} -AR (hamster) was the first receptor of the α_1 subfamily to be cloned [16], and it has been studied to a much greater extent than the other subtypes. G-protein-coupled receptor kinases 2 and 3 seem to be involved in the phosphorylation of agonist-

occupied α_{1B} -ARs during homologous desensitization [17,18]. The phosphorylation sites involved have been located at Ser⁴⁰⁴, Ser⁴⁰⁸ and Ser⁴¹⁰ in the receptor C-terminus [7].

In heterologous desensitization, activation of non-adrenergic receptors desensitizes α_{1B} -ARs, and this has also been associated with receptor phosphorylation (reviewed in [14]). Direct activation of PKC by active phorbol esters markedly desensitizes α_{1B} -ARs and leads to a pronounced increase in receptor phosphorylation [6–12,14]. The sites phosphorylated by PKC have been located at Ser³⁹⁴ and Ser⁴⁰⁰ of the α_{1B} -AR C-terminus [7]. A variety of receptors have also been observed to induce α_{1B} -AR phosphorylation. Thus we have shown that activation of seven-transmembrane-domain receptors coupled to G_{q/11} (such as endothelin ET_A receptors [8]) or G_i (e.g. lysophosphatidic acid receptors [10]) or of receptors with intrinsic tyrosine kinase activity (e.g. epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors [11]) can induce α_{1B} -AR phosphorylation/desensitization. PKC and phosphoinositide 3-kinase (PI3K) are key participants in these effects (reviewed in [14]).

In the present work, we studied the effect of activation of receptors of a different class, the transforming growth factor- β (TGF- β) receptors, on α_{1B} -AR phosphorylation/desensitization. TGF- β 1–3 constitute a family of growth factors involved in the control of cell differentiation, proliferation and apoptosis [19–21]. These growth factors signal through the activation of heteromeric type I and type II receptors that are serine/threonine kinases. In the presence of TGF- β , the constitutively active type II receptor phosphorylates and activates the type I receptor. The activated type I receptor phosphorylates receptor-associated Smads (Smad2 and Smad3), which then bind Smad4, and the complex translocates to the nucleus where it regulates the transcription of target genes [19–21]. Interestingly, there are some rapid actions of TGF- β that do not seem to involve gene transcription or *de*

Abbreviations used: α_1 -AR, α_1 -adrenergic receptor; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; SH, Src homology; TGF- β , transforming growth factor- β .

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novo protein synthesis [22–24]. PKC and PI3K have been reported to play a role in some of these actions and in other longer-term effects of this growth factor [23–27].

EXPERIMENTAL

Materials

Dulbecco's modified Eagle's medium (DMEM), G418, fetal bovine serum and other reagents used for cell culture were obtained from Gibco BRL. TGF- β 1 and TGF- β 2 were from RD Systems. (–)-Noradrenaline, staurosporine, wortmannin and protease inhibitors were from Sigma Chemical Co. Ro 318220, LY294002 and bisindolylmaleimide I were from Calbiochem. [32 P] P_i (8500–9120 Ci/mmol), [3 H]prazosin (74.4 Ci/mmol) and *myo*-[2,3- 3 H]inositol (22.9 Ci/mmol) were from New England Nuclear Life Science Products. Fura-2 acetoxymethyl ester was from Molecular Probes. Protein A–Sepharose beads were from Upstate Biotechnology. DNA purification kits were from Qiagen. The hamster α_{1B} -AR cDNA [16], kindly provided to us by Dr Robert J. Lefkowitz, was subcloned into pcDNA3 as described [12]. The SR α - Δ p85 plasmid containing the cDNA of the mutant p85 subunit of PI3K lacking the p110 binding site (Δ p85) was kindly provided to us by Dr Wataru Ogawa and Dr Masato Kasuga [28].

Cell culture

Rat-1 cells transfected with the hamster α_{1B} -AR [16] were generously provided by Dr R. J. Lefkowitz, Dr M. G. Caron and Dr L. Allen. Cells were grown at confluence in 10-cm culture dishes in DMEM supplemented with 10% (v/v) fetal bovine serum, G418 (300 μ g/ml), streptomycin (100 μ g/ml), penicillin (100 units/ml) and amphotericin B (0.25 μ g/ml) under a 5% CO $_2$ /95% air atmosphere at 37 °C. Cos-1 cells were cultured in the same medium but in the absence of G418. Transient transfection into Cos-1 cells was performed using DEAE-dextran [29]; experiments were performed 48–72 h after transfection, with an efficacy of 60–80%, as described in [12]. Total plasmid DNA was maintained at 3 μ g per 10 cm Petri dish using 1.5 μ g of plasmid containing the α_{1B} -AR cDNA and 1.5 μ g of plasmid containing the Δ p85 cDNA or the empty vector. For all the experiments, confluent cells were serum-deprived overnight in unsupplemented DMEM. α_{1B} -ARs were expressed at a density of $\sim$ 1 pmol/mg of membrane protein in Rat-1 cells, and $\sim$ 2 pmol/mg of membrane protein in Cos-1 cells, as determined by [3 H]prazosin binding, with K_D values in the range 0.15–0.3 nM [12].

Determination of α_{1B} -AR phosphorylation

Rat-1 cells expressing α_{1B} -ARs were incubated in phosphate-free DMEM for 1 h, and then labelled in the same medium containing [32 P] P_i (50 μ Ci/ml) for 3 h at 37 °C, as described previously [8–12]. In this procedure, a rabbit antiserum against the C-terminal decapeptide of the hamster α_{1B} -AR was used [8]. This antiserum immunoprecipitates α_{1B} -ARs with high efficacy ($\sim$ 80% of photolabelled receptors) [8]; the receptors were detected as a broad band of $\sim$ 80–85 kDa [8]. 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 μ M

Na $_3$ VO $_4$, 10 mM β -glycerophosphate, 10 mM sodium pyrophosphate, 1 mM phosphoserine, 1 mM phosphothreonine and protease inhibitors (20 μ g/ml leupeptin, 20 μ g/ml aprotinin, 100 μ g/ml PMSF, 500 μ g/ml bacitracin and 50 μ g/ml soybean trypsin inhibitor). Cell lysates were centrifuged at 12700 *g* for 15 min, and the supernatants were incubated with the anti- α_{1B} -AR antiserum and Protein A–Sepharose overnight at 4 °C. After five washes with buffer containing 50 mM Hepes, 50 mM NaH $_2$ PO $_4$, 100 mM NaCl, pH 7.2, 1% Triton X-100, 0.05% SDS and 100 mM NaF, followed by a final wash with 50 mM Tris/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 X-Omat X-ray films and intensifying screens. The level of receptor phosphorylation was assessed in the same gels with Molecular Dynamics PhosphorImager and Imagequant software.

Measurement of intracellular free Ca $^{2+}$ concentration

Confluent fibroblasts were incubated overnight in DMEM without serum and antibiotics. Cells were loaded with 5 μ M fura-2 acetoxymethyl ester in Krebs–Ringer/Hepes containing 0.05% BSA, pH 7.4, for 1 h at 37 °C. Cells were detached by gentle trypsinization, and fluorescence measurements were carried out as described previously [12] using an Aminco-Bowman Series 2 spectrometer with the excitation monochromator set at 340 and 380 nm, a chopper interval of 0.5 s, and the emission monochromator set at 510 nm. The intracellular free Ca $^{2+}$ concentration was calculated as described in [30] using the software provided by Aminco-Bowman; traces were exported directly to the graphs.

[3 H]inositol phosphate production

Cells were labelled with [3 H]inositol (5 μ Ci/ml) for 18–24 h in inositol-free DMEM containing 1% (v/v) fetal bovine serum. On the day of the experiment, cells were washed twice with Krebs–Ringer/Hepes buffer containing 1.3 mM CaCl $_2$ and preincubated for 20 min in 2 ml of the same buffer containing 20 mM LiCl at 37 °C in a 5% CO $_2$ atmosphere. Incubations were for 15 min and were terminated by the addition of 2 ml of chloroform/methanol (1:2, v/v); samples were mixed thoroughly and centrifuged in a clinical centrifuge. The aqueous phase was recovered and total [3 H]inositol phosphates were separated by Dowex AG1-X8 chromatography [31].

RESULTS

Incubation of Rat-1 cells stably expressing the α_{1B} -AR with 10 pM TGF- β 1 induced an increase in the phosphorylation of the AR that was evident at 5 min, reached its maximum within 15 min and had vanished by 60 min (Figure 1). A similar effect was observed in a few experiments performed using TGF- β 2 (results not shown). The effect of TGF- β was concentration-dependent, with an EC $_{50}$ of 0.3 pM, and reached its maximum at 10 pM (Figure 1).

This effect of 10 pM TGF- β was blocked by 100 nM staurosporine, 1 μ M bisindolylmaleimide I, 1 μ M Ro 318220 or 100 nM wortmannin (Figure 2). Wortmannin and LY294002, inhibitors of PI3K, blocked the receptor phosphorylation induced by 10 pM TGF- β in a concentration-dependent fashion; the EC $_{50}$ values were 1 nM and 50 nM respectively (Figure 3). Similarly, the PKC inhibitors staurosporine and bisindolylmaleimide I

Figure 1 Effect of TGF- β on α_{1B} -AR phosphorylation

Left panel: cells were incubated in the absence or presence of 10 pM TGF- β for the times indicated. Right panel: cells were incubated with the indicated concentrations of TGF- β for 15 min. Plotted are the means $\pm$ S.E.M. of triplicate determinations of 6–8 experiments using different cell cultures. Representative autoradiographs are shown.

Figure 2 Effect of protein kinase inhibitors on TGF- β -induced α_{1B} -AR phosphorylation

Left panel: cells were incubated in the absence of any agent (Basal) or in the presence of 10 pM TGF- β alone or with 1 μ M bisindolylmaleimide I (BIM), 100 nM staurosporine (STAU) or 1 μ M Ro 318220 (Ro). Plotted are the means $\pm$ S.E.M. of triplicate determinations of 6–7 experiments using different cell cultures; * P < 0.001 compared with control; ** P < 0.001 compared with TGF- β . A representative autoradiograph is shown. Right panel: cells were incubated in the absence of any agent (Basal) or in the presence of 10 pM TGF- β alone or with 1 μ M bisindolylmaleimide I (BIM) or 100 nM wortmannin (WT). Plotted are the means $\pm$ S.E.M. of triplicate determinations of 3–4 experiments using different cell cultures; * P < 0.001 compared with control; ** P < 0.001 compared with TGF- β . A representative autoradiograph is shown.

blocked the α_{1B} -AR phosphorylation induced by TGF- β with nearly identical EC_{50} values of ~ 15 nM (Figure 3). These inhibitors by themselves had no effect on basal receptor phosphorylation at the concentrations tested (results not shown; see [8–12]).

In order to determine whether the α_{1B} -AR phosphorylation induced by TGF- β has functional metabolic consequences, the effects of noradrenaline on intracellular cell calcium and [3 H] inositol phosphate production were tested. As shown in Figure 4

(upper left panel), incubation of the cells for 15 min with 100 pM TGF- β did not alter in a significant way the increase in intracellular calcium induced by 1 μ M noradrenaline. The concentration–response curve to the adrenergic agonist showed that TGF- β induced only a small shift to the right (EC_{50} values of 60 and 100 nM in cells preincubated in the absence and presence respectively of 100 pM TGF- β for 15 min). Nevertheless, when [3 H]inositol phosphate production was tested, a clear decrease in the maximal response to noradrenaline was observed in cells

Figure 3 Concentration–response curves for the effects of PI3K and PKC inhibitors on TGF- β -induced α_{1B} -AR phosphorylation

Left panel: cells were incubated in the absence of any agent (B) or in the presence of 10 pM TGF- β alone (■) or with different concentrations of wortmannin (WT; ●) or LY294002 (LY; ○). Right panel: cells were incubated in the absence of any agent (B) or in the presence of 10 pM TGF- β alone (■) or with different concentrations of staurosporine (STAU; ●) or bisindolylmaleimide I (BIM; ○). Plotted are the means $\pm$ S.E.M. of triplicate determinations of 3–4 experiments using different cell cultures. Representative autoradiographs are shown.

incubated with TGF- β ; no significant change was observed in the EC_{50} values, i.e. ~ 50 nM under both conditions (Figure 4, lower panel).

PI3K seems to play a key role in the TGF- β -mediated phosphorylation of α_{1B} -ARs. Therefore, in order to further document its participation, we used transfection of a dominant-negative mutant of the enzyme [28]. We attempted to perform these experiments with the Rat-1 cell line, but the efficacy of transfection was too low. Therefore we performed these experiments using Cos-1 cells co-transfected with the α_{1B} -AR and either the dominant-negative PI3K mutant or an identical amount of empty vector. As shown in Figure 5 (right panel), TGF- β was able to induce α_{1B} -AR phosphorylation in Cos-1 cells transfected with α_{1B} -ARs. This effect was blocked by the PI3K inhibitor wortmannin and by the PKC inhibitor bisindolylmaleimide I, which confirmed the data obtained with Rat-1 cells. The inhibitors by themselves were without effect (results not shown). When cells were co-transfected with the $\Delta p85$ mutant of PI3K, the ability of TGF- β to increase α_{1B} -AR phosphorylation was lost (Figure 5, left panel). The AR phosphorylation induced by noradrenaline was similar in cells transfected with or without the PI3K mutant (Figure 5, left panel).

DISCUSSION

The present data indicate that activation of TGF- β receptors increased α_{1B} -AR phosphorylation and thus induced receptor desensitization. Phosphorylation and desensitization of α_{1B} -ARs has been observed previously in response to activation of $G_{q/11}$ -coupled receptors such as the ET_A endothelin receptors [8], G_i -coupled receptors such as lysophosphatidic acid receptors [10], and receptors with intrinsic tyrosine kinase activity such as those for EGF and PDGF [11]. In the present study, receptors

belonging to a different family, i.e. those with intrinsic serine/threonine kinase activity, were studied. The data show clearly that activation of these receptors can also modulate the α_{1B} -AR sensitivity of cells.

There seems to be a relationship between α_{1B} -AR phosphorylation and desensitization, but as yet it is far from fully understood. Thus agents that induce a small increase in receptor phosphorylation, such as bradykinin, do not induce receptor desensitization [9]. This can be explained if only some site(s) or a fraction of the receptors are affected. However, okadaic acid, an inhibitor of protein phosphatases, or overexpression of G-protein-coupled receptor kinase 5 or 6 markedly increased receptor phosphorylation, but such covalent modifications do not seem to alter receptor function [17,32]. In the present study we observed that the TGF- β -mediated AR phosphorylation was associated with a decrease in the maximal adrenergic effect on [3 H]inositol phosphate production and a small shift to the right of the concentration–response curve to noradrenaline for intracellular calcium. This difference could be due to the time frames of agonist action (immediate for intracellular calcium and 15 min for [3 H]inositol phosphate production), and to the fact that a small increase in inositol trisphosphate could be sufficient to induce a near-maximal increase in intracellular calcium.

The effect of TGF- β was observed at low concentrations, and it was relatively rapid and transient in a short time frame. This time course is not consistent with the idea that it could be mediated through the classic TGF- β signalling pathway involving gene transcription and *de novo* protein synthesis. As mentioned in the Introduction, there is evidence that additional signalling events may participate in the action of this growth factor [22–27]. In agreement with these findings, we observed that inhibitors of PKC and PI3K effectively blocked the α_{1B} -AR phosphorylation induced by TGF- β ; the IC_{50} values were similar to what was

Figure 4 Effects of TGF- β on α_{1B} -AR-mediated functional responses

Upper left panel: fura-2-loaded cells were incubated in the absence (solid line) or presence (dashed line) of 10 pM TGF- β for 15 min and then challenged with 1 μ M noradrenaline (NA; arrow). Representative traces are shown. Upper right panel: concentration-response curves to noradrenaline for intracellular calcium. Cells were incubated for 15 min in the absence (○) or presence (●) of 10 pM TGF- β before being challenged with the indicated concentrations of noradrenaline (NA). Results are presented as a percentage of the maximum increase observed in response to noradrenaline in control cells. Plotted are the means $\pm$ S.E.M. of 4–5 experiments using different cell preparations. Lower panel: concentration-response curves to noradrenaline for [3 H]inositol phosphate production. Cells were incubated for 15 min in the absence (○) or presence (●) of 10 pM TGF- β before being challenged with the indicated concentrations of noradrenaline (NA). Plotted are the means $\pm$ S.E.M. of 4–5 experiments using different cell cultures.

Figure 5 Effects of protein kinase inhibitors on TGF- β -induced α_{1B} -AR phosphorylation

Left panel: Cos-1 cells transfected with the hamster α_{1B} -AR were incubated in the absence of any agent (Basal) or in the presence of 10 pM TGF- β alone or with 1 μ M bisindolylmaleimide I (BIM) or 100 nM wortmannin (WT). Plotted are the means $\pm$ S.E.M. of triplicate determinations from four experiments using different cell cultures; * P < 0.001 compared with basal; ** P < 0.001 compared with TGF- β alone. A representative autoradiograph is shown. Right panel: Cos-1 cells co-transfected with the hamster α_{1B} -AR and empty vector (□) or the Δ p85 PI3K dominant-negative kinase (■) were incubated in the absence of any agent (Basal) or in the presence of 10 μ M noradrenaline (NA) or 10 pM TGF- β . Plotted are the means $\pm$ S.E.M. of triplicate determinations of 7–9 experiments using different cell cultures; * P < 0.001, ** P < 0.01 compared with basal; *** P < 0.001 compared with TGF- β without Δ p85. A representative autoradiograph is shown.

anticipated and within the ranges observed previously by us [8–12]. As observed previously for α_{1B} -AR phosphorylation induced by lysophosphatidic acid and EGF [10,11], inhibitors of PKC and PI3K completely blocked the effect of TGF- β . This suggests that these kinases act in a sequence and not in independent pathways.

There is a large body of experimental evidence indicating that PKC phosphorylates α_{1B} -ARs [6–14]. PKC comprises a large family of enzymes with differences in sensitivity to activators and in substrate specificity [33,34]. There is no direct evidence with regard to the isoforms that participate in α_{1B} -AR phosphorylation. Nevertheless, we have observed that the α , δ and ϵ isoforms of PKC co-immunoprecipitate with α_{1B} -ARs under basal conditions, and this association is dynamically increased by cell treatment with phorbol esters or hormones that increase receptor phosphorylation [35]. These data indicate that several PKC isoforms participate in this process, and suggest the possibility that they may participate in different steps or that functional redundancy may exist. It should be mentioned that a recent publication presented experimental evidence that seriously questions the selectivity of many protein kinase inhibitors [36]. In the present work we used three different PKC inhibitors, and the EC₅₀ values were similar to what would be expected. However, in view of the above-mentioned finding [36], the possibility that other protein kinases may be involved cannot be ruled out.

PI3K is a family of enzymes with fundamental roles in signalling and the regulation of cellular activity [33,34]. These enzymes comprise catalytic and adaptor/regulatory subunits. In Class 1A, the adaptor p85 subunit has Src homology 2 (SH2) and SH3 motifs that bind phosphotyrosine residues and the p110 catalytic subunit [37,38]. Our present data using PI3K inhibitors or transfection of the Δ p85 dominant-negative mutant strongly indicate a role for PI3K in the phosphorylation and desensitization of α_{1B} -ARs induced by TGF- β . Wortmannin is a potent and selective non-competitive inhibitor of PI3K [39], whereas LY294002 is an inhibitor that competes at the binding site of ATP of this kinase [40]. The Δ p85 dominant-negative mutant of PI3K lacks the binding site for the catalytic subunit p110, and interferes with its interaction with tyrosine-phosphorylated residues [28]. These three inhibitors of PI3K clearly and consistently blocked α_{1B} -AR phosphorylation, underscoring the importance of this enzyme activity as a modulator of the AR phosphorylation state.

PI3K is a modulator of PKC activity through the synthesis of 3-phosphorylated phosphoinositides. These phospholipids are important modulators of phosphoinositide-dependent protein kinase 1, which phosphorylates the activation loop of some PKC isoforms [41,42]. 3-Phosphorylated phosphoinositides have also been reported to activate novel and atypical PKC isoforms [41,42].

The mechanism(s) through which TGF- β activates PI3K is not yet fully understood. Some authors have suggested that it takes place through indirect actions, not involving tyrosine phosphorylation [27]. Nevertheless, it is known that TGF- β stimulates Src kinase activity, increasing protein tyrosine phosphorylation [22]. On the other hand, it has been observed by several authors that PI3K p85 and PI3K activity co-immunoprecipitate with TGF- β receptors [25,26].

It has been elegantly demonstrated that activation of receptors for insulin or for insulin-like growth factor I leads to β_2 -AR desensitization associated with AR tyrosine phosphorylation ([43,44]; reviewed in [45]). A key hierarchical role for protein kinase B (Akt) in this effect has recently been described [46]. Similarly, we have shown that activation of other tyrosine kinase receptors, such as those for EGF or PDGF, leads to desensitiza-

tion of α_{1B} -ARs [11]. As shown here, activation of TGF- β receptors, which have endogenous serine/threonine kinase activity, modulates a G-protein-coupled receptor.

In summary, the present data show that TGF- β induces α_{1B} -AR phosphorylation and desensitization. The data suggest the following working model: activation of TGF- β receptors, directly or indirectly, stimulates PI3K activity; this leads to activation of PKC, which phosphorylates and desensitizes α_{1B} -ARs.

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