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Phosphorylation, desensitization and internalization of human α_{1B}-adrenoceptors induced by insulin-like growth factor-I

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Abstract

The effect of insulin-like growth factor-I (IGF-I) on human α_{1B}-adrenoceptor function, phosphorylation state and cellular location was studied. Rat-1 fibroblasts were transfected with a plasmid construction containing enhanced green fluorescent protein joined to the carboxyl terminus of the human α_{1B}-adrenoceptor. Receptors were identified by radioligand binding and photoaffinity labeling, and were immunoprecipitated with an antiserum generated against the enhanced green fluorescent protein. The receptor was functional, as evidenced by noradrenaline action on intracellular calcium and inositol phosphate production. IGF-I had no significant effect by itself on these parameters but markedly reduced the effects of noradrenaline. IGF-I induced α_{1B}-adrenoceptor phosphorylation, which was markedly reduced by the following agents: pertussis toxin, a metalloproteinase inhibitor, diphtheria toxin mutant CRM 197, an epidermal growth factor (EGF) receptor intrinsic kinase activity inhibitor, and by phosphoinositide 3-kinase and protein kinase C inhibitors. IGF-I action appears to involve activation of a pertussis toxin-sensitive G protein, shedding of heparin-binding EGF and autocrine activation of EGF receptors. G protein subunits and phosphotyrosine residues stimulate phosphoinositide 3-kinase activity leading to activation of protein kinase C, which in turn phosphorylates α_{1B}-adrenoceptors. Confocal fluorescent microscopy showed that α_{1B}-adrenoceptors fussed to the green fluorescent protein were located in plasma membrane and intracellular vesicles in the basal state. IGF-I induced receptor redistribution favoring the intracellular location; this effect was blocked by hypertonic sucrose and concanavalin A. Our data show that IGF-I induces α_{1B}-adrenoceptor desensitization associated to receptor phosphorylation and internalization.

Keywords: α_{1B}-adrenoceptor; IGF-I; Receptor phosphorylation; Receptor internalization; Desensitization

1. Introduction

Cellular responses to a wide variety of stimuli such as light, odors, tastes, hormones, autacoids or neurotransmitters are mediated through G protein-coupled receptors. Cells modulate their ability to respond to these stimuli by desensitizing/resensitizing such receptors. These processes involve many cellular and molecular events with different time frames. However, at the molecular level, receptor phosphorylation is one of the earliest events that control receptor function (García-Sáinz et al., 2000; Pitcher et al., 1998).

Two major types of desensitization exist: homologous and heterologous desensitizations. Homologous desensitization, in which agonist-occupied receptors reduce their responsiveness, mainly involves receptor phosphorylation by G protein-coupled receptor kinases (Penn et al., 2000). In heterologous desensitization, the responsiveness of a given receptor decreases when another pharmacologically unrelated receptor or another signaling device (such as protein kinases) are activated; this desensitization principally involves phosphorylation of receptors and other signaling entities by second messenger-activated kinases, such as protein kinases A and C (Claing et al., 2002; Vázquez-Prado et al., 2003). It has been observed that phosphorylation of G protein-coupled receptors is associated with receptor internalization.

Our group has characterized the molecular processes and pathways involved in heterologous α_1-adrenoceptor desensitization. Thus, we have shown that activation of seven transmembrane domain receptors coupled to $G_{q/11}$ (such as endothelin ET_A

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(Vázquez-Prado et al., 1997) or bradykinin B_2 receptors (Medina et al., 1998)), or coupled to G_i (such as lysophosphatidic acid receptors (Casas-González and García-Sáinz, 2006; Casas-González et al., 2003; Casas-González et al., 2000)), intracellular receptor (estrogen receptor α (González-Arenas et al., 2006)) or receptors with intrinsic tyrosine kinase activity (such as those for insulin (García-Sáinz et al., 2004)), EGF (epidermal growth factor) (Medina et al., 2000) or platelet-derived growth factor (Medina et al., 2000) can induce α_{1B}-adrenoceptor phosphorylation/desensitization. Protein kinase C, phosphoinositide 3-kinase and EGF receptor transactivation are key participants in these effects (Casas-González and García-Sáinz, 2006; Casas-González et al., 2003; García-Sáinz et al., 2000; Medina et al., 2000; Molina-Muñoz et al., 2006; Vázquez-Prado et al., 2003).

Recently, we showed that insulin-like growth factor-I (IGF-I) induces adrenoceptor phosphorylation associated to desensitization of hamster α_{1B}-adrenoceptors (Molina-Muñoz et al., 2006). Such α_{1B}-adrenoceptor desensitization/phosphorylation takes place through a complex pathway that involves pertussis toxin-sensitive G proteins, EGF receptor transactivation, and phosphoinositide 3-kinase and protein kinase C activities (Molina-Muñoz et al., 2006).

In the present work, we further explore IGF-I effect by studying the following two aspects: a) whether this growth factor also affects the human α_{1B}-adrenoceptor, and b) whether its effect induces α_{1B}-adrenoceptor internalization. We have previously shown that human α_{1B}-adrenoceptors are subject to homologous and heterologous desensitization by adrenergic amines and phorbol esters, respectively, and that such effects are associated with receptor phosphorylation (García-Sáinz et al., 1999). We tagged the carboxyl terminus of the human α_{1B}-adrenoceptor with enhanced green fluorescent protein (eGFP) by molecular biological techniques and stably expressed the fused receptor in rat-1 fibroblasts. Our results indicate that IGF-I is able to induce α_{1B}-adrenoceptor desensitization and phosphorylation in these cells by means of a process similar to that observed with the hamster receptor and that such action is associated with adrenoceptor internalization.

2. Materials and methods

2.1. Materials

(−)-Noradrenaline, IGF-I, staurosporine, wortmannin, bis-indolyl-maleimide I, the diphtheria toxin mutant CRM 197, protease inhibitors, concanavalin A and chloroquine were obtained from Sigma Chemical Co. LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) and tyrphostin AG1478 (4-(3-chloroanillino)-6,7-dimethoxyquinazoline) were obtained from Calbiochem. Pertussis toxin was purified at our laboratory as described (García-Sáinz et al., 1992). Dulbecco's modified Eagle's medium, fetal bovine serum, trypsin, antibiotics, and other reagents used for cell culture were from Life Technologies. [^{32}P]Pi (8500−9120 Ci/mmol), [2,3-^{3}H]myo-inositol (22.9 Ci/mmol), [^{3}H]prazosin (74.4 Ci/mmol) and [^{125}I-aryl]-azidoprazosin (2200 Ci/mmol) were obtained from Perkin Elmer Life Sciences and sepharose-coupled protein A from Upstate Biotechnology. Dr. Mobashery (Wayne State University, Detroit, MI, USA) generously provided us with a sample of BB-94 (Batimastat) (N4-hydroxy-N1-(2-(methylamino)-2-oxo-1-(phenylmethyl) ethyl)-2-(2-methylpropyl)-3-((2-thienylthio) methyl)-,(2R-(1(S*),2R*,3S*))-butanediamide). DNA purification kits were obtained from Qiagen and chemiluminescence kits from Pierce. The pDrive cloning system was purchased from QIAGEN and the pEGFP-N1 vector from Clontech. FM-464 was obtained from Molecular Probes.

2.2. Plasmid construction

Dr. Gozoh Tsujimoto (Department of Genomic Drug Discovery Science, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan) generously provided us with the 1.8 kb-full length-coding region of the human α_{1B}-adrenoceptor cloned into pME-18S plasmid. The receptor gene sequence was amplified and its stop codon was removed by polymerase chain reaction, and the product was cloned into the pDrive cloning system with the following primers: forward 5′GGAAGATCTC-CACCATGAATCCCGACCTGGACACCG3′ and reverse 5′ CCGGAATTCCAAACTGCCCGGGCGCCAG3′. hα_{1B}Δstop

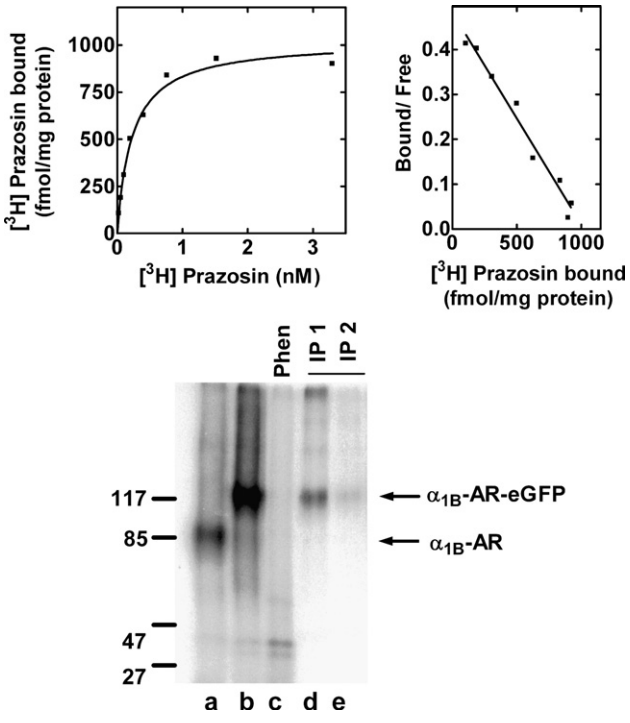

Fig. 1. Characterization of the α_{1B}-adrenoceptors fused to eGFP expressed in rat-1 fibroblasts. Upper panels: representative [^{3}H] prazosin saturation isotherm and Rosenthal transformation of the data. Bmax 990 fmol/mg of membrane protein, K_D 0.18 nM. Lower panel: Photoaffinity labeling (lanes a–c) and immunoprecipitation (lanes d and e) of α_{1B}-adrenoceptors. Photoaffinity labeling of membranes from cells expressing α_{1B}-adrenoceptors (lane a, α_{1B}-AR). Photoaffinity labeling of membranes from cells expressing α_{1B}-adrenoceptors fused to eGFP (α_{1B}-AR-eGFP) in the absence (lane b) or presence of 10 μM phentolamine (lane c, Phen). Immunoprecipitation of photolabeled α_{1B}-adrenoceptors fused to eGFP was performed using antiserum raised against eGFP (lane c, IP 1) or the carboxyl decapeptide of hamster α_{1B}-adrenoceptors (lane e, IP 2).

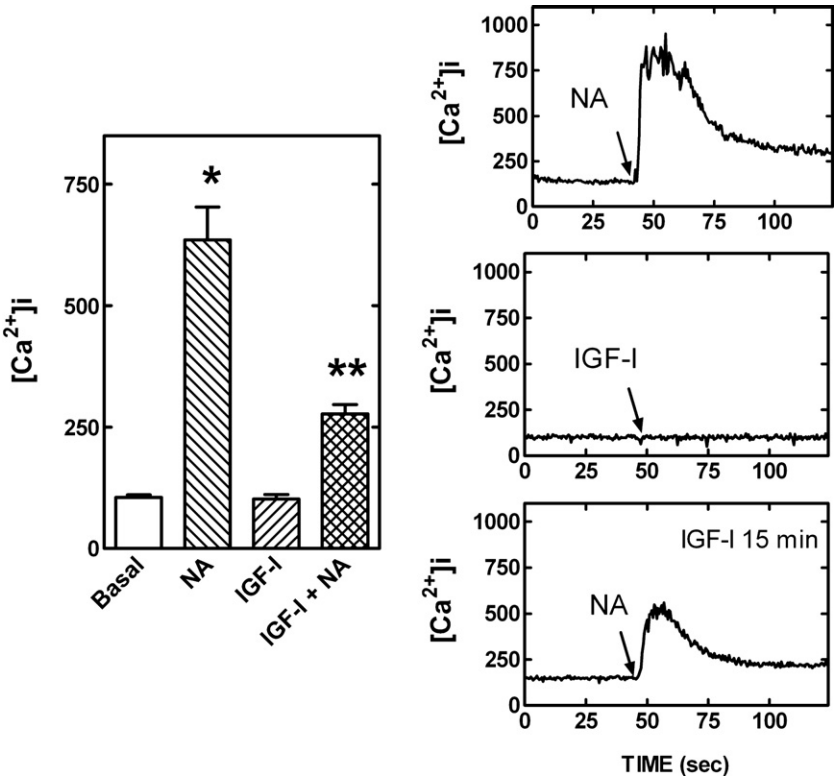

Fig. 2. Effect of IGF-I on α_{1B}-adrenergic-mediated increase in intracellular free calcium. Left panel: maximal intracellular calcium concentration observed after addition of vehicle (basal), 10 μM noradrenaline (NA), 100 ng/ml IGF-I or 10 μM noradrenaline in cells pre-incubated with 100 ng/ml IGF-I for 15 min (IGF-I+NA). Plotted are the means and vertical lines represent the S.E.M. of 7–8 determinations using different cell preparations. *$P<0.001$ vs. all other groups; **$P<0.01$ vs. basal and IGF-I. Right panels: representative tracings.

DNA was inserted into BglII/EcoRI sites of pEGFP-N1 vector (Clontech) to generate a receptor tagged at the C terminus with eGFP. Plasmid nucleotide sequences were verified by double-stranded DNA sequencing performed at our Institute's Molecular Biology Unit.

2.3. Cell line

Rat-1 fibroblasts obtained from the American Type Culture Collection, were used for this study because they have no detectable α_{1B}-adrenoceptors or responses to noradrenaline (data not shown). Cells were transfected with the plasmid containing the previously described human α_{1B}-adrenoceptor–eGFP sequence, with Lipofectamine according to the manufacturer's instructions, and were cultured in the following selection media: glutamine-containing high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 900 μg/ml of the neomycin analog, G-418 sulfate, 100 μg/ml streptomycin, 100 U/ml penicillin, and 0.25 μg/ml amphotericin B at 37 °C under a 95% air/5% CO_2 atmosphere. Several clones were isolated and were screened for α_{1B}-adrenergic responsiveness (noradrenaline-induced increase in intracellular calcium concentration). The clone with the best response was selected for this study and further cultured in the media described above, but with reduced G-418 (300 μg/ml). All experiments were performed utilizing this cell line that stably expresses α_{1B}-adrenoceptor–eGFP construction.

2.4. Receptor binding

Membranes were prepared as described (Vázquez-Prado et al., 1997; Vázquez-Prado et al., 2000). Binding studies were performed by incubating [^{3}H]Prazosin (0.025–8 nM) with

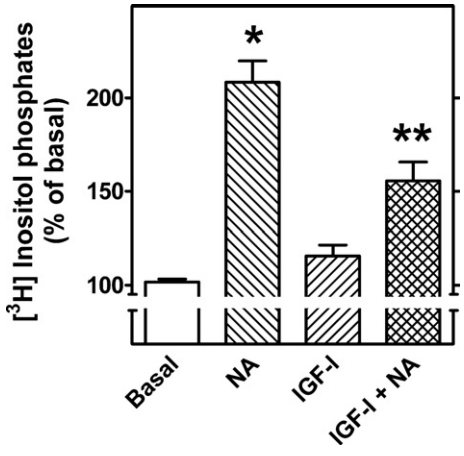

Fig. 3. Effect of IGF-I on α_{1B}-adrenergic-mediated [^{3}H] inositol phosphate production. [^{3}H] Inositol labeled cells were incubated in presence of vehicle (basal), 10 μM noradrenaline (NA), 100 ng/ml IGF-I or 100 ng/ml IGF-I+10 μM noradrenaline (IGF-I+NA) as indicated in Materials and methods. Plotted are the means, and vertical lines represent the S.E.M. of 5–6 experiments using different cell preparations. *$P<0.001$ vs. basal; **$P<0.001$ vs. basal or NA and $P<0.01$ vs. IGF-I.

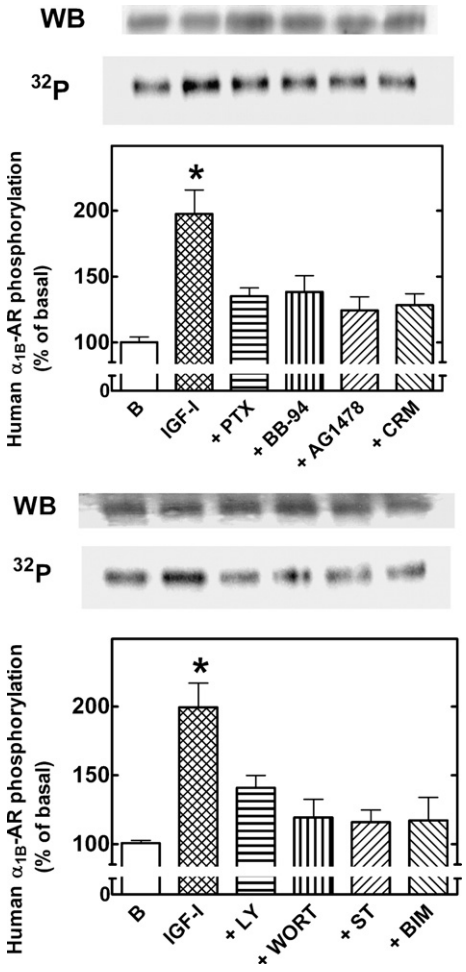

Fig. 4. Effect of IGF-I on α_{1B}-adrenoceptor phosphorylation. Cells were incubated with vehicle (B, basal) or challenged with 100 ng/ml IGF-I for 15 min (all remaining groups). Pre-incubation was overnight with 100 ng/ml of pertussis toxin (+PTX), for 2 h with diphtheria toxin mutant CRM 197 (200 ng/ml) (+CRM), or for 30 min with the remaining indicated agents: 10 μM BB-94 (+BB-94), 5 μM AG1478 (+AG1478), 1 μM LY 294002 (+LY), 100 nM wortmannin (+WORT), 1 μM staurosporine (+ST) or 1 μM bis-indolylmaleimide I (+BIM). Plotted are the means and vertical lines represent the S.E.M. of 6–8 experiments using different cell preparations. *$P<0.001$ vs. all remaining groups. Representative autoradiographs of phosphorylated receptor (^{32}P) and α_{1B}-adrenoceptor Western blots (WB) are presented.

membranes (15–20 μg of protein) in a final volume of 0.25 ml of binding buffer (Tris 50 mM, $MgCl_2$ 10 mM, pH 7.5) for 60 min at 25 °C in a water bath shaker. Incubation was ended by addition of 5 ml of ice-cold binding buffer and filtration through GF/C filters using a Brandel harvester. Filters were washed twice, and dried, and radioactivity was measured in a liquid scintillation counter. Non-specific binding was determined in presence of 10 μM phentolamine; specific binding was >90% of total binding at the K_D. The EBDA program (Biosoft-Elsevier) was used to analyze saturation curves.

2.5. Photoaffinity labeling

Membranes (250 μg protein), were incubated in the dark with 6 nM of [^{125}I-aryl]-azido-prazosin and exposed to ultraviolet light as described (Vázquez-Prado et al., 1997; Vázquez-Prado et al., 2000). After this treatment membranes were centrifuged, washed, solubilized and subjected to electrophoresis in 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions.

2.6. Intracellular free calcium ($[Ca^{2+}]_i$) measurements

Cells were incubated overnight in Dulbecco's modified Eagle's medium without serum and antibiotics and were loaded with 4 μM Fura-2 acetoxymethyl ester in Krebs–Ringer–HEPES containing 0.05% bovine serum albumin, pH 7.4 for 1 h at 37 °C. Cells were detached by gentle trypsinization and fluorescence measurements were carried out as described previously (Molina-Muñoz et al., 2006; Vázquez-Prado et al., 1997; Vázquez-Prado et al., 2000) with an Aminco-Bowman Series 2 Spectrometer with excitation monochromator set at 340 and 380 nm; a 0.5 s chopper interval was employed, and the emission monochromator was set at 510 nm. $[Ca^{2+}]_i$ was calculated as described (Grynkiewicz et al., 1985) using the software provided by Aminco-Bowman; traces were directly exported to the graphs.

2.7. [^{3}H]inositol phosphate production

Cells were labeled with [^{3}H]inositol (5 μCi/ml) for 18–24 h in inositol-free Dulbecco's modified Eagle's medium containing 1% 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 pre-incubated 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 stopped by addition of 2 ml of chloroform/methanol (1:2 v/v); samples were thoroughly mixed and centrifuged in a clinical centrifuge. Aqueous phase was recovered and total [^{3}H]inositol phosphates were separated by Dowex AG1-X8 chromatography (Berridge et al., 1983).

2.8. α_{1B}-Adrenoceptor phosphorylation

The procedure employed to study α_{1B}-adrenoceptor phosphorylation in rat-1 fibroblasts has been described previously in detail (Molina-Muñoz et al., 2006; Vázquez-Prado et al., 1997; Vázquez-Prado et al., 2000). In the present work the same procedure was used but a rabbit antiserum raised in our laboratory against eGFP was used. In brief, the day after transfection cells were collected by trypsinization and transferred to six-well dishes, cells were maintained overnight in phosphate-free Dulbecco's modified Eagle's medium without serum. The following day, cells were maintained in phosphate-free Dulbecco's modified Eagle's medium for 1 h and then incubated in 1 ml of the same medium containing [^{32}P]-orthophosphate (50 μCi/ml) for 3 h at 37 °C. Labeled cells were stimulated as indicated, washed with ice-cold phosphate-buffered saline, and solubilized with 0.5 ml of ice-cold solubilization buffer containing 50 mM Tris–HCl pH 8.0, 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 10 mM NaF, 1 mM Na_3VO_4, 10 mM β-glycerophosphate,

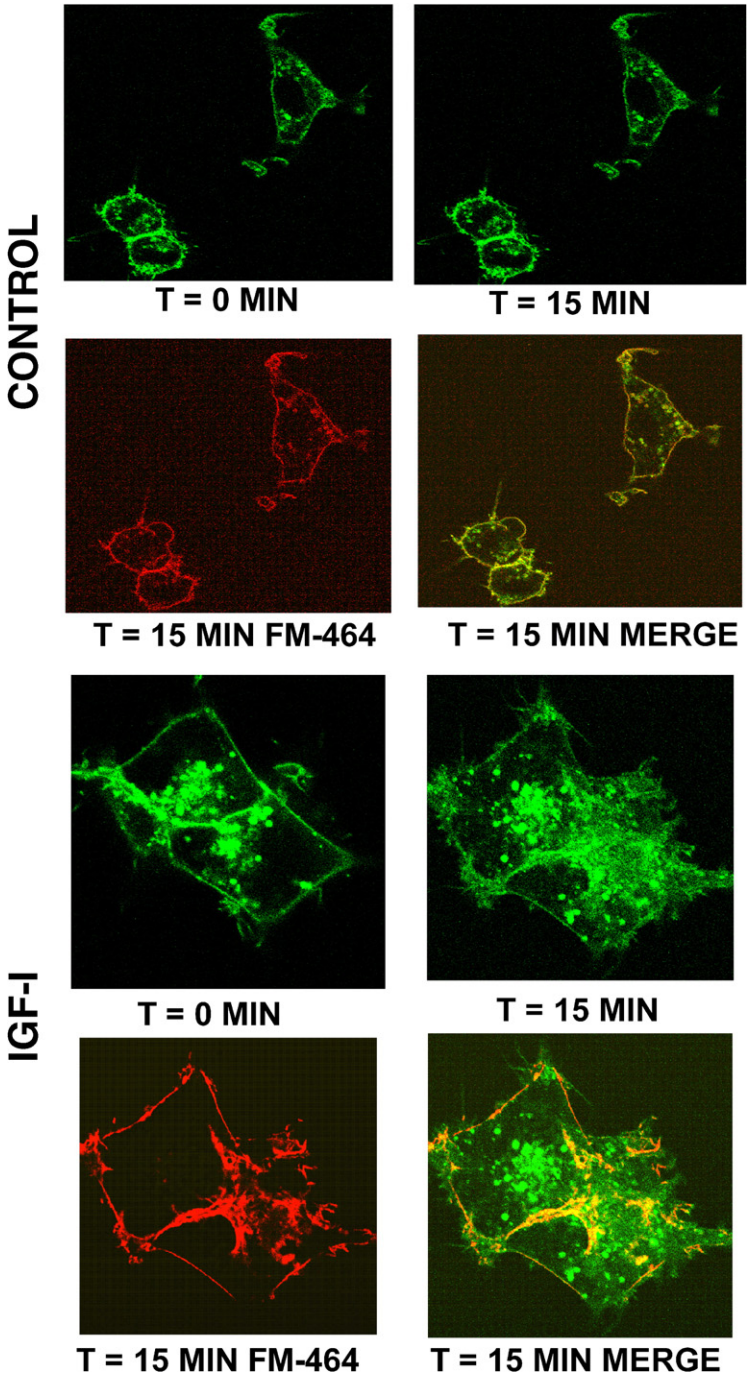

Fig. 5. Confocal images of rat-1 fibroblasts expressing α_{1B}-adrenoceptors fused to eGFP. Cells were incubated with vehicle (upper group of images) or with 100 ng/ml IGF-I (lower group of images) for the times indicated. FM-464 (red) was used as a plasma membrane marker.

10 mM sodium pyrophosphate, 1 mM p-serine, 1 mM p-threonine, 1 mM p-tyrosine, and protease inhibitors (Molina-Muñoz et al., 2006; Vázquez-Prado et al., 1997; Vázquez-Prado et al., 2000). Cell lysates were centrifuged at 12,700 ×*g* for 15 min at 4 °C and supernatants were incubated with the indicated antiserum and protein A-Sepharose overnight at 4 °C. After four washes with 50 mM HEPES, 50 mM NaH_2PO_4, 100 mM NaCl, pH 7.2, 1% Triton X-100, 0.1% SDS, and 100 mM NaF, the immune complexes were boiled for 5 min in SDS-sample buffer containing 5% β-mercaptoethanol, and subjected to SDS-polyacrylamide gel electrophoresis. Gels were dried and exposed for 18−24 h and receptor phosphorylation was assessed with a Molecular Dynamics PhosphorImager and Imagequant software (Amersham Biosciences). Data fell within the linear range of detection of the apparatus and were plotted using Prism 4 from GraphPad software. Recovery of the immunoprecipitated receptor was reproducible as evidenced by Western blotting and no correction for recovery was performed.

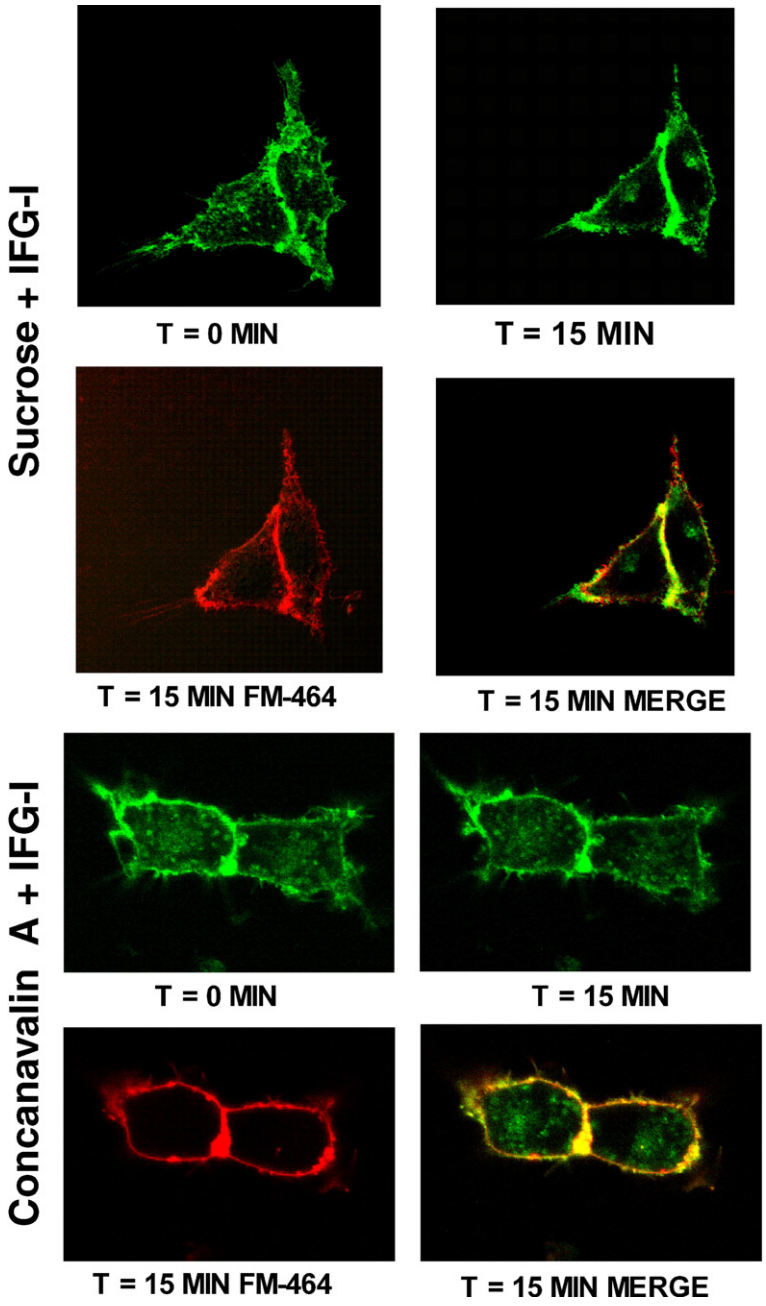

Fig. 6. Confocal images of rat-1 fibroblasts expressing α_{1B}-adrenoceptors fused to eGFP. Cells were pre-incubated with 0.4 M sucrose (upper group of images) or concanavalin A (0.25 μg/ml) for 30 min and then challenged with 100 ng/ml IGF-I for the times indicated. FM-464 (red) was used as a plasma membrane marker.

2.9. Confocal microscopy

Confocal images were obtained using a Flowview FV 1000 laser confocal system (Olympus) attached/interfaced to an Olympus IX81 inverted light microscope with a 40× glycerol-immersion objective. eGFP was excited using the 488 nm line of a krypton/argon laser, and the emitted fluorescence was detected with a 515–540 nm band pass filter. Operating the laser at a low power setting (97–99% attenuation) substantially reduced photobleaching and photodamage. Confocal images were viewed and processed using FV10-ASW 1.6 software (Olympus). FM-464 was used as a plasma membrane marker.

2.10. Statistical analysis

Statistical analysis between comparable groups was performed using ANOVA with Newman–Keuls analysis and was performed with the software included in the GraphPad Prism program.

3. Results

As indicated in Materials and methods, a cell line stably expressing α_{1B}-adrenoceptors tagged with eGFP at the carboxyl tail was isolated. These cells express α_{1B}-adrenoceptors at a density of 900–1600 fmol/mg membrane protein with high

affinity for [^{3}H]prazosin (Kd 0.2–0.3 nM) (Fig. 1). Membranes from cells expressing non-tagged α_{1B}-adrenoceptors (used as a control) or from cells expressing eGFP-tagged receptors were photolabeled with [^{125}I-aryl]-azido-prazosin, solubilized and subjected to polyacrylamide gel electrophoresis. Photolabeled non-tagged α_{1B}-adrenoceptors were identified as a band with relative molecular mass of ≈80–85 kDa, in agreement with previous observations (García-Sáinz et al., 1999; Vázquez-Prado et al., 1997; Vázquez-Prado et al., 2000). A major band with a relative molecular mass of ≈115–120 kDa was photolabeled in membranes from eGFP-tagged α_{1B}-adrenoceptor-expressing cells (Fig. 1). This is the expected molecular mass considering that the eGFP mass is ≈27 kDa. Labeling specificity was confirmed with the use of phentolamine. Photolabeled receptor immunoprecipitation was achieved using two antibodies, one generated against eGFP and another against the terminal carboxyl decapeptide of hamster α_1-adrenoceptors (Vázquez-Prado et al., 1997), which is capable of immunoprecipitating human α_1-adrenoceptors (García-Sáinz et al., 1999). As shown in Fig. 1, both antisera were able to immunoprecipitate the tagged receptor, but that generated against eGFP was more effective (15–20% of total photolabeled receptors) than the one against the receptor carboxyl tail (1–2%); therefore, in all subsequent experiments the former antiserum was utilized. The relative low efficacy of the antiserum generated against the hamster receptor carboxyl terminal decapeptide is not surprising, considering that it is less able to immunoprecipitate the human receptor (García-Sáinz et al., 1999) and the possible esteric restrictions imposed by the fused protein. No receptor immunoprecipitation is observed with non-immune serum (not shown) (see Vázquez-Prado et al., 1997).

We next examined the functional response of these cells and data are presented in Figs. 2 and 3. It can be observed that noradrenaline (10 μM) induced a nearly immediate increase in intracellular free calcium concentration and that IGF-I did not modify the resting intracellular concentration of this cation (Fig. 2). Nevertheless, when cells were pre-incubated with IGF-I for 15 min and then challenged with noradrenaline, response to the catecholamine was markedly decreased (Fig. 2). Similar results were obtained when production of [^{3}H] inositol phosphates from [^{3}H] inositol labeled cells was studied, i.e., noradrenaline markedly increased inositol phosphate production, and IGF-I induced a very small, statistically insignificant, increase but markedly decreased adrenergic action (Fig. 3). These data extend the observation that IGF-I induces functional desensitization of hamster α_{1B}-adrenoceptors to the human receptor.

Receptor phosphorylation was subsequently examined and the data are presented in Fig. 4. In agreement with our previous findings on studying hamster α_{1B}-adrenoceptors, IGF-I induced a clear increase in the human adrenoceptor phosphorylation state (Fig. 4). This IGF-I effect was markedly attenuated in pertussis toxin-incubated cells (100 ng/ml overnight or 300 ng/ml for 3 h). Similarly, this growth factor action was inhibited by BB-94 (a metalloproteinase inhibitor), AG1478 (an inhibitor of EGF receptor intrinsic tyrosine kinase), diphtheria toxin mutant CRM 197 and by inhibitors of phosphoinositide 3-kinase (LY294002 and wortmannin) and protein kinase C activities (staurosporine and bis-indoly-maleimide I) (Fig. 4). None of these agents modified basal α_{1B}-adrenoceptor phosphorylation by themselves (data not shown).

Because the enhanced fluorescent protein was attached to the receptor's carboxyl terminus, the possibility that it could be phosphorylated by IGF-I action was considered. However, this was not the case. No phosphorylation of free eGFP (i.e., not fused to the receptor) was detected in rat-1 cells that expressed it (expression was confirmed by confocal fluorescent microscopy and by immunoblotting) (data not shown).

Taking advantage of the fluorescent tag we studied α_{1B}-adrenoceptor location and tested the possibility that the action of IGF-I might lead to their internalization. Human α_{1B}-adrenoceptor-fused eGFP was localized at the plasma membrane level and also in relatively large vesicles (Fig. 5). Cell treatment with IGF-I induced a reduction of fluorescence at the plasma membrane level and accumulation into vesicles around the nucleus; this process was relatively fast, taking place within 10–15 min (Fig. 5 and also see Supplementary material "Movie"). Hypertonic sucrose markedly decreases clustering of surface receptors into endosomes (Daukas and Zigmond, 1985) and blocks receptor internalization (Walker et al., 1999). IGF-I-induced α_{1B}-adrenoceptor internalization was blocked by this treatment (Fig. 6). Similarly cell incubation with concanavalin A (0.25 μg/ml for 30 min), an inhibitor or receptor sequestration inhibited IGF-I-induced α_{1B}-adrenoceptor internalization (Fig. 6). In contrast, we were unable to detect any clear action of chloroquine (a lysosomal-proteolysis inhibitor) on this parameter (data not shown).

4. Discussion

Previously we showed that IGF-I induces desensitization and phosphorylation of hamster α_{1B}-adrenoceptors (Molina-Muñoz et al., 2006). This manuscript extends this observation to the human receptor and demonstrates that the action of IGF-I leads to α_{1B}-adrenoceptor internalization. Hamster and human α_{1B}-adrenoceptors are very similar but some differences exist (SWISS-PROT: P35368 and P18841). Cottechia and coworkers (Diviani et al., 1997; Lattion et al., 1994) have elegantly shown that the main phosphorylation sites for protein kinase C and G protein-coupled receptor kinases are located in the carboxyl terminus. This domain is present in both human and hamster α_{1B}-adrenoceptors but human adrenoceptor carboxyl terminus is four amino acids larger than that of the hamster and lacks a potential site for protein kinase C. Despite these differences, the results obtained with the human receptor are very similar to those using hamster α_{1B}-adrenoceptors.

The signaling cascade triggered by IGF-I that leads to α_{1B}-adrenoceptor phosphorylation and desensitization is complex and not yet completely defined. In agreement with our previous work, we observed that the increase in α_{1B}-adrenoceptor phosphorylation induced by IGF-I is pertussis toxin-sensitive. This indicates a role of G proteins (likely through their $\beta\gamma$ subunits (Molina-Muñoz et al., 2006)). Their role is clear but the manner in which this takes place is not. IGF-I exerts its actions through the type 1 IGF receptor a transmembrane

tyrosine kinase similar to the insulin receptor (Adams et al., 2000). G proteins are usually associated with seven transmembrane domain receptors; nonetheless there is evidence that certain receptors with a single transmembrane domain, including those with tyrosine kinase activity, can interact with G proteins, which might mediate some of their actions (reviewed in Patel, 2004). Therefore, the possibility of a direct G protein activation by IGF-I receptor remains open. On the other hand, there is growing evidence suggesting that many hormones and neurotransmitters induce sphingosine kinase activation, with generation of sphingosine-1-phosphate, which acts in an autocrine loop through G protein-coupled receptors (Hobson et al., 2001; Pyne and Pyne, 2000; 2002; Spiegel and Milstien, 2007; Sukocheva et al., 2006; Sukocheva et al., 2003). Therefore, indirect IGF-I receptor interaction with G proteins is also likely. These two possibilities are not mutually exclusive, and it is clear that further research is needed to clarify the pathway(s) through which the effect of IGF-I on α_{1B}-adrenoceptors takes place.

The effect of BB-94 (a general metalloproteinase inhibitor), the diphtheria toxin mutant CRM 197 (that binds with high affinity to HB-EGF and blocks its action) and of tyrphostin AG1478 (inhibitor of the EGF receptor tyrosine kinase activity) are consistent with the idea that HB-EGF shedding and EGF receptor transactivation play a role in this effect. Similarly, roles of phosphoinositide 3-kinase and protein kinase C were strongly indicated by the effects of these inhibitors.

The following sequence of events has been proposed (Molina-Muñoz et al., 2006) and is consistent with the present findings: a) IGF-I receptor activation stimulates, directly or indirectly, pertussis toxin-sensitive G proteins; b) these in turn activate metalloproteinases, which catalyze heparin-binding EGF shedding, and EGF receptor transactivation c) dissociated $G\beta\gamma$ subunits and phosphotyrosine residues appears to trigger phosphoinositide 3-kinase activity, which leads to protein kinase C activation, this resulting in α_{1B}-adrenoceptor phosphorylation and desensitization.

It has been frequently observed that G protein-coupled receptor phosphorylation is associated with internalization, and agonist-induced receptor internalization is a well-known phenomenon. Taking advantage of the possibility of visualizing receptor internalization in living cells by using fluorescent reagents such as eGFP, Tsujimoto and coworkers (Fukunaga et al., 2006) elegantly showed that the order of potency of agonists and antagonists for receptor internalization corresponded well with that observed in radioligand binding experiments. Such data suggest that monitoring agonist-induced receptor internalization could be considered as a novel pharmacological approach for characterizing receptors (Fukunaga et al., 2006).

Our present data indicate that heterologous desensitization of α_{1B}-adrenoceptors is associated with phosphorylation and internalization. This is consistent with previous observations indicating that protein kinase C activation induces α_{1B}-adrenoceptor internalization (Cowlen and Toews, 1988; Fonseca et al., 1995). It is interesting that differences have been observed in homologous- and heterologous desensitization-associated receptor internalization processes (Cowlen and Toews, 1988; Rapacciuolo et al., 2003). In DDT1 MF-2 cells, agonists and active phorbol esters induced α_{1B}-adrenoceptor internalization and the combination of both stimuli was additive (Cowlen and Toews, 1988; Rapacciuolo et al., 2003).

Differences in cellular location and internalization, among α_1-adrenoceptor subtypes have been reported (Chalothorn et al., 2002; McCune et al., 2000; Pediani et al., 2005; Rapacciuolo et al., 2003; Wang et al., 2007). The most prominent feature comprises that a large proportion of α_{1D}-adrenoceptors are located in intracellular vesicles, whereas α_{1A}- and α_{1B}-adrenoceptors are located in high density in plasma membrane and also in internal vesicles (Chalothorn et al., 2002; McCune et al., 2000; Morris et al., 2004; Pediani et al., 2005; Rapacciuolo et al., 2003; Wang et al., 2007). α_{1D}-Adrenoceptor internalization has been observed but is difficult to demonstrate (McCune et al., 2000; Wang et al., 2007). Agonists induce internalization of α_{1A}- and α_{1B}-adrenoceptors associated with β-arrestins and it has been shown that a dominant-negative mutant of this protein blocks this effect (Chalothorn et al., 2002; McCune et al., 2000). Interestingly, α_{1A}-adrenoceptor cellular traffic is very active, continuous and agonist-independent (Morris et al., 2004; Pediani et al., 2000); this constitutive internalization was not observed for α_{1B}-adrenoceptors (Morris et al., 2004; Pediani et al., 2000). Essentially, no information is available on α_1-adrenoceptor internalization in response to non-adrenergic agents (hormones, neurotransmitters or growth factors).

IGF-I is a small 70-amino acid peptide produced in liver and other organs in response to growth hormone, and it mediates the majority of its actions, playing a key role in fetal and post-natal growth and development (Adams et al., 2000; Laron, 2001). There are many cells that endogenously co-express the IGF-I receptors and adrenoceptors; however, their crosstalk has not been studied in many systems. The work of Malbon and coworkers has characterized in detail the molecular entities involved in IGF-I-mediated functional desensitization, phosphorylation, and internalization of β_1- and β_2-adrenoceptors (Fan et al., 2001; Gavi et al., 2006; Gavi et al., 2007; Hadcock et al., 1992). Our present and previous data (Molina-Muñoz et al., 2006) indicate that α_{1B}-adrenoceptor function, phosphorylation state and cellular location are influenced by IGF-I action. It is clear that for both β_2- and α_{1B}-adrenoceptors, the acute action of IGF-I attenuated their responsiveness. However, there are similarities and differences in the molecular mechanisms involved. In the case of the effect of IGF-I on β_2-adrenoceptors, tyrosine-residue phosphorylation appears to be of cardinal importance (Gavi et al., 2006; Karoor and Malbon, 1996) whereas the serine/threonine kinase, protein kinase C, plays such a key role for action of this growth factor on α_{1B}-adrenoceptors (Molina-Muñoz et al., 2006). A common player for these effects is the activity of phosphoinositide 3-kinase (Molina-Muñoz et al., 2006) and it is likely that other molecular entities (such as kinases or adaptors) might participate in these processes. It is worthwhile mentioning that phosphoinositide 3-kinase possesses dual activity as a phosphoinositide and as a protein kinase and it has been elegantly shown that both activities are important for G protein-coupled receptor endocytosis (Naga Prasad et al., 2005).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejphar.2007.08.047.

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