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Research Article

Determinants Present in the Receptor Carboxy Tail Are Responsible for Differences in Subtype-Specific Coupling of β-Adrenergic Receptors to Phosphoinositide 3-Kinase

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An agonist-occupied β_2-adrenergic receptor (β_2-AR) recruits G protein receptor kinase-2 (GRK2) which is recruited to the membrane. Thus, the physical proximity of activated β_2-AR and PI-3K allows the activation of the latter. In contrast, it has been observed that the β_1-AR is unable to activate the PI-3K/Akt pathway. We hypothesized that the difference might be due to molecular determinants present in the carboxy termini of the two β-AR subtypes. Using transiently transfected HEK 293 cells expressing either β_1- or β_2-AR, we also observed that in presence of an agonist, β_2-AR, but not β_1-AR, is able to activate the PI-3K/Akt pathway. Switching the seventh transmembrane domain and the carboxy tail between the two receptors reverses this phenotype; that is, $\beta_1 \times \beta_2$-AR can activate the PI-3K/Akt pathway whereas $\beta_2 \times \beta_1$-AR cannot. Pretreatment with pertussis toxin abolished the activation of PI-3K by β_2- or $\beta_1 \times \beta_2$-AR stimulation. Ligand-mediated internalization of the β_2-AR induced by a 15-minute stimulation with agonist was abolished in the presence of a dominant negative of PI-3K or following pertussis toxin pretreatment. These results indicate that the subtype-specific differences in the coupling to PI-3K/Akt pathway are due to molecular determinants present in the carboxy tail of the receptor and further that β_2-AR activates PI-3K via a pertussis toxin-sensitive mechanism.

1. Introduction

β_2-AR activation induces antiapoptotic effects in cardiomyocytes mediated by stimulation of the PI-3K pathway [1]. The proposed mechanism by which this activation occurs is dependent on G protein-coupled receptor kinases (GRKs). Under basal conditions, GRK2 forms a complex with PI-3K in the cytosol [2]. When β_2-AR is occupied by an agonist, GRK2 is translocated to the membrane by a G$\beta\gamma$ subunit-dependent mechanism and subsequently recruits PI-3K from the cytosol to the membrane [2]. The proximity of the PI-3K and the stimulated receptor induces the activation of the enzyme. In contrast, β_1-AR has proapoptotic activity in cardiomyocytes due to stimulation of PKA or CAM KII [3]. Indeed, it has been observed that stimulation of β_1-AR with an agonist induces apoptosis and this can be prevented in presence of inhibitors of either PKA or CAM-KII [4].

Interestingly it has been shown that stimulated β_1-AR can also recruit GRK to the membrane. The interaction of either β-AR subtype with GRKs is mainly via the intracellular loops and carboxy tail of the receptor [5]. When activated, GRK induces the phosphorylation of certain serine/threonine residues in the carboxy tail of both β-AR subtypes resulting in functional uncoupling of the receptor from their primary signalling pathways. GRK2 phosphorylation also favours subsequent interaction with β-arrestin. This interaction with β-arrestin further desensitizes the receptor and is subsequently involved in receptor endocytosis [6, 7].

As GRK interacts with molecular determinants in carboxy tail of the receptor, we hypothesized that differences in the coupling of the β_1- and β_2-AR with PI-3K may be due to determinants located in this portion of the receptor. This study was designed to determine the respective efficiencies of

β_1-AR and β_2-AR to stimulate the PI-3K pathway and to test the above hypothesis.

2. Material and Methods

2.1. DNA Constructions, Cell Transfection, and Culture. Murine β_1-AR and human β_2-AR subcloned into pcDNA3 were used in this study. Two chimeric receptors consisting of the β_1-AR with the seventh transmembrane domain and the carboxyl-terminal tail of the β_2-AR and the reciprocal β_2-AR with the seventh transmembrane domain and the carboxyl-terminal tail of the β_1-AR were constructed as follows. A restriction site for Hpa I was created by polymerase chain reaction (PCR) at position 2070 in the β_1-AR. The new restriction site in the β_1-AR was created with three primers. Two primers were used for the hybridation with the receptor sequence. These primers contained 21 base pairs and had, respectively, CGCCTCAGAAGCCATAGAGCC and TCGTGTGCACAGTGTGGGCCA sequences. The third primer was utilized to introduce the restriction site. This primer which contained 24 base pairs had the following sequence: GGTGGAAAGCGTTAACCACGTTGG. The mutated β_1-AR and the β_2-AR wt were double digested with Hpa I and Xho I. The result of this digestion is two fragments of 6540 bp and 486 bp for the mutated β_1-AR and two fragments 5998 bp and 1362 bp for the β_2-AR wt. The appropriate restriction fragments (containing the seventh transmembrane domain and the carboxy-terminal portion of the receptor) were isolated, exchanged for their counterparts, and religated. Positive clones were selected by enzymatic digestion and confirmed by sequencing.

The β-AR wild type (wt), chimeric receptors, dominant-negative PI-3 kinase (p85ΔPI-3K), and/or carboxy-terminal domain of GRK2 (ct-GRK2) were transiently transfected in human embryonic kidney (HEK 293) cells using the calcium phosphate precipitation method. We performed all experiments at 48 hours posttransfection, that is, at maximal receptor expression determined by ligand binding. Cells were starved overnight 24 hours before the experiments in a medium without fetal bovine serum. HEK 293 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin and streptomycin, 1 mM glutamine, 0.25 μg/mL fungizone in an atmosphere of 95% air/5% CO_2 at 37°C. On the day of the experiment, cells were treated with 1 μM isoproterenol for the indicated times and fractionated for the cytosolic or membrane compartments. In some experiments, cells were pretreated with pertussis toxin (0.1 μg/mL; Sigma), 18 hours before stimulation with isoproterenol.

2.2. Preparation of Cytosolic and Membrane Fractions. Cells were washed three times with 10 mL of phosphate-buffered saline at 4°C and mechanically detached in 1 mL of ice-cold buffer containing 5 mM Tris-HCl, pH 7.4, 2 mM EDTA, 5 μg/mL leupeptin, 5 μg/mL soybean trypsin inhibitor, and 10 μg/mL benzamidine. Cells were then lysed with a sonicator (3 bursts of 10 seconds at max speed), and the lysates were centrifuged at 1000 × g for 5 minutes at 4°C. The supernatant was centrifuged at 45 000 × g for 20 minutes and was considered as the cytosolic preparation. Protein content was assessed using the Bradford method (Bio-Rad). The pelleted membranes were resuspended in 250 μL of a solubilization buffer (buffer A) containing 50 mM Tris pH 7.5, 20 mM β-glycerophosphate, 20 mM NaF, 5 mM EDTA, 10 mM EGTA, 1 mM Na_3VO_4, 10 mM benzamidine, 0.5 mM PMSF, 10 μg/mL leupeptin, 5 mM DTT, 1 μM microcystin LR, and 1% Triton X-100; and solubilized for 2 hours at 4°C. Then the membranes were centrifuged at 10 000 × g for 15 minutes. The protein content was assessed using the Lowry method (Bio-Rad).

2.3. Radioligand Binding Assay. Radioligand binding assays were conducted essentially as described previously [8] with ~5 μg of membrane proteins in a total volume of 0.5 mL containing 250 pM [^{125}I]CYP in the presence or absence of 10 μM alprenolol to define nonspecific binding. The binding reactions were incubated at room temperature for 90 minutes and terminated by rapid filtration with ice-cold 25 mM Tris-HCl, pH 7.4, over Whatman GF/C glass fiber filters preincubed for ≥30 minutes in a buffer containing 25 mM Tris-HCl, pH 7.4, 0.1% bovine serum albumin, and 0.3% polyethylenimine.

2.4. Western Blotting. Western blotting was conducted as described previously [9]. Briefly, aliquots of 50–75 μg of cytosolic or membrane protein preparations were subjected to 10% denaturing polyacrylamide gel electrophoresis as previously described. Transfer was performed with a Trans-Blot SD Semi-dry transfer cell (Bio-Rad) on Protran nitrocellulose membrane (Mandel, Montréal, QC, Canada). Protein transfer efficiency was assessed using Ponceau S staining. Membranes were blocked using 5% nonfat dry milk in TBS-T (10 mM Tris (pH 7.4), 150 mM NaCl, and 0.05% Tween 20) and membranes were incubated at 4°C overnight with primary antibody (anti-Phospho-Akt (Ser 473) and anti-Akt from Cell Signaling Technology, (Missisauga, Canada) or anti-PI-3K, anti-β_1- or anti-β_2-AR from Santa-Cruz (Calif, USA) diluted 1:1000 in 5% nonfat dry milk into TBS-T. Subsequently, membranes were washed and incubated for 45 minutes at room temperature with the secondary antibody (diluted 1:5000 in 5% nonfat dry milk into TBS-T) conjugated to horseradish peroxidase. Membranes were washed and exposed to scientific imaging film (Perkin Elmer Life Sciences, ON) or quantified using a Kodak ImageStation 440CF using enhanced chemiluminescence reagent (Perkin Elmer Life Sciences). Band intensities were analyzed using Kodak 1D v.3.5.5 Scientific Imaging Software.

2.5. Immunohistochemistry and Receptor Internalization. Sequestration of β-AR was observed by immunolocalisation. After agonist treatment, cells were washed with PBS and fixed with 3% paraformaldehyde for 15 minutes. After these washes, nonspecific sites were blocked with 0.2% BSA and 0.15% Triton x-100 (blocking solution) for 10 minutes. Primary antibody (β_1- and β_2-AR from Santa-Cruz), prepared in the blocking solution (1:200), was added for 30 minutes at room temperature. After another series

of washes, secondary antibody (antirabbit, Santa-Cruz,) also prepared in the blocking solution (1:500) was added for 30 minutes. After a final series of washes, slides were mounted and viewed using a Leica epi-illumination microscope.

2.6. PI-3K Activity. PI-3K activity was measured as previously described [10]. Briefly, 250–375 μg of cytosolic and membrane proteins were precipitated with anti-phosphotyrosine antibody conjuged to biotin (1:50, Santa-Cruz, Calif, USA) overnight at 4°C. The immune complex was pelleted (with streptavidin beads) and washed three times with lysis buffer and twice with phosphate-buffered saline buffer containing 0.1 mM Na_3VO_4. The immune pellet was then suspended in activation buffer (35 mM ATP, 0.2 mM adenosine, 30 mM $MgCl_2$, 10 mg/mL L-α-phosphatidylinositol, and 20 μCi [$\gamma^{32}P$]-ATP; (Amersham Pharmacia Biotech, Baie-d'Urfé, Canada) and incubated at room temperature for 20 minutes. The reaction was stopped with the addition of 100 μL HCl 1 M and 200 μL of chloroform:methanol (1:1). The aqueous phase was then discarded. Eighty μL of HCl:methanol (1:1) were then added before discarding the aqueous phase. Twenty μL of the organic phase containing ^{32}P-phosphatidylinositol were resolved by thin layer chromatography on K6 Silica Gel plates (Whatman, Clifton, NJ, USA) in a solvent system containing chloroform:methanol:ammonium hydroxide (45:35:10). Plates were exposed to film for three to five days (−80°C).

2.7. Statistical Analysis. Results are expressed as mean ± SEM and were evaluated using analysis of variance adapted for factorial experimental design. Orthogonalization was performed when necessary [11]. $P < .05$ was considered significant.

3. Results

3.1. Expression of β-AR Subtypes and p85ΔPI-3K in HEK 293 Cells. HEK 293 cells were transiently transfected with cDNAs encoding for β_1-AR Wt, β_2-AR Wt, $\beta_1 \times \beta_2$-AR, or $\beta_2 \times \beta_1$-AR and in some case, p85ΔPI-3K. Forty-eight hours after transfection β-AR expression levels were approximately 500 fmol/mg of proteins (compared to 10–20 fmol/mg in untransfected cells). Expression of p85ΔPI-3K determined by western blot was increased 3.24 times as compared to wild-type cells (data not shown, $n = 3$).

3.2. Stimulation of β_2-AR but not β_1-AR Induces Activation of PI-3K/Akt Pathway. PI-3K activation by the β_1- or β_2-AR was measured by in vitro phosphorylation of L-α-phosphatidylinositol. Transfected cells were stimulated with isoproterenol 1 μM for 0, 5, or 15 minutes at 37°C. Stimulation of β_2-AR- for 5 or 15 minutes induced a significant augmentation in PI-3K activity compared to basal conditions (nonstimulated; Figure 1). In contrast, β_1-AR stimulation had no effect on PI-3K activity. Thus, the stimulation of β_2-AR but not β_1-AR by isoproterenol induces activation of PI-3K in our model. We suspect that the apparent high basal level of PI-3K activation observed in our cell line may be due to the phosphotyrosine antibody used to immunoprecipitate the activated PI-3K. Using this antibody, we immunoprecipitate other PI-3K subtypes as well as phosphotyrosine proteins that although not activated by β-AR may still contribute to the basal level of activation.

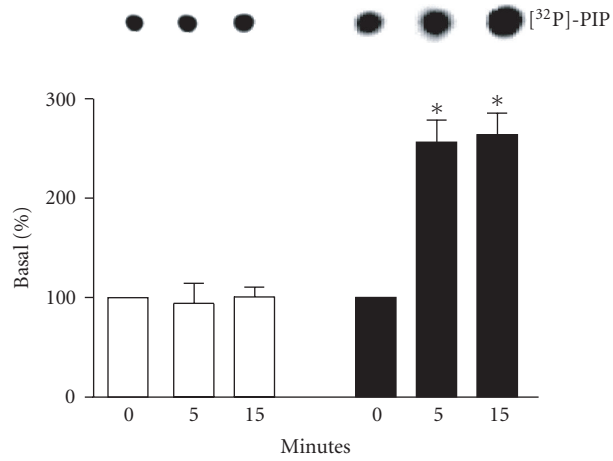

Figure 1: *PI-3K activity with β-AR stimulation.* HEK 293 cells were transfected with β_1-AR or β_2-AR. Forty-eight hours after transfection, cells were stimulated with 1 μM isoproterenol for the indicated times. PI-3K activity was determined by the level of [^{32}P]-PI produced by the stimulation of β_1-AR (□) or β_2-AR (■) expressing cells. Top panel is a representative autoradiograph of TLC separation (n = 4-5). Data from these experiments is quantitated in the bottom panel as described in Section 2. *$P < .05$ versus 0 minute.

To confirm differences between stimulation of the two β-AR subtypes on PI-3K activity, we used a measure of Akt (a downstream PI-3K effector) activation. Akt activation, as determined by the phosphorylation status of Serine 473, was significantly increased after β_2-AR-stimulation for 5 and 15 minutes as compared to the basal state (Figure 2). Stimulation of β_1-AR did not modify the phosphorylation status of Akt as compared to control, confirming that β_1-AR activation cannot stimulate PI3-kinase/Akt pathway activation. Stimulation of untransfected HEK293 with isoproterenol did not result in any significant activation of Akt (data not shown).

3.3. Stimulation of Either β_1- or β_2-AR Induces PI-3K Recruitment to the Plasma Membrane. To determine whether β_1-AR can recruit PI-3K to the plasma membrane, immunolocalisation of PI-3K was performed using Western blotting with anti-P110γ antibody. Transiently transfected cells with β_1- or β_2-AR were stimulated by isoproterenol 1 μM for 0, 5, or 15 minutes at 37°C. Both β_1- and β_2-AR stimulation results in recruitment of PI-3K to the particulate fraction (Figure 3). Compared with control (i.e., nonstimulated cells), the presence of PI-3K was significantly increased ($P < .05$) in membrane fractions by agonist stimulation of either β_1-AR or β_2-AR for 5 or 15 minutes. No significant difference was detected between 5 and 15 minutes of stimulation. Thus, either subtype of β-AR can recruit PI-3K to the membrane

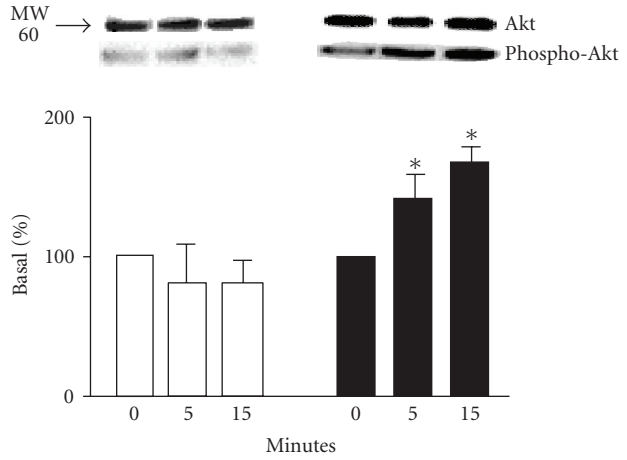

Figure 2: *Akt activation following β-AR stimulation.* HEK 293 cells were transfected with β_1-AR or β_2-AR. Forty-eight hours after the transfection, cells were stimulated with 1 μM isoproterenol for the indicated times. Akt activation status was determined by immunoblot for the phosphorylated form of Akt compared to the total amount of Akt after stimulation of β_1-AR (□) or β_2-AR (■) expressing cells. Top panel is a representative immunoblot of the experiments (n = 4-5). Data from these experiments is quantitated in the bottom panel as described in Section 2. $^*P < .05$ versus 0 minute.

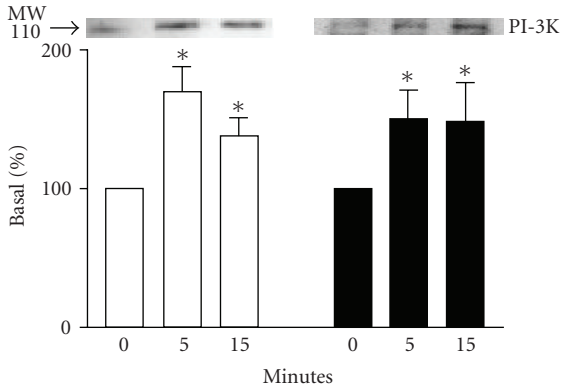

Figure 3: *PI-3K Partitioning of PI-3K to the membrane.* Forty-eight hours after transfection, cells were stimulated with 1 μM isoproterenol for the indicated times. Membranes were isolated, solubilized, and approximately 100 μg of membrane proteins were separated on a 10% SDS-PAGE gel. Membrane-associated PI-3K levels increased with cells expressing β_1-AR (□) or β_2-AR (■). Top panel is a representative immunoblot of the experiments (n = 3-4). Data from these experiments is quantitated in the bottom panel as described in Section 2. $^*P < .05$ versus 0 minute.

after agonist stimulation but only the β_2-AR results in PI-3K activation.

3.4. Recruitment of PI-3K to the Plasma Membrane is Gβγ Subunit-Dependent. It has been shown that PI-3K and GRK2 form a cytosolic complex [2]. To determine whether the mechanism of PI-3K recruitment to the plasma membrane was G$\beta\gamma$ subunit-dependent, β_1- or β_2-AR was transiently

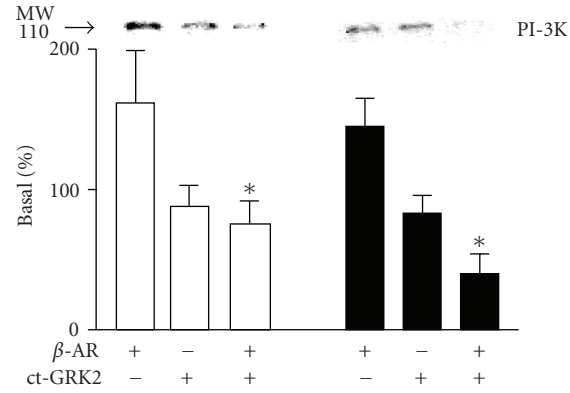

Figure 4: *GRK2 carboxy-terminal domain reduced PI-3K recruitment to membrane fractions in response to β-AR stimulation.* HEK 293 cells were cotransfected with ct-GRK-2 and β-AR subtypes. Forty-eight hours after the transfection, cells were stimulated with 1 μM isoproterenol for 5 minutes. PI3-kinase recruitment to particulate fractions decreased with cells expressing either β_1-AR (□) or β_2-AR (■) in presence of cotransfected ct-GRK2. Top panel is a representative immunoblot of the experiments (n = 4–6). Data from these experiments is quantitated in the bottom panel as described in Section 2. $^*P < .05$ versus 0 minute.

cotransfected with the carboxyl-terminal portion (ct) of GRK2 (a sequestering agent for G$\beta\gamma$) in HEK 293 cells. Cells cotransfected with β_1- or β_2-AR and ct-GRK2 were stimulated with 1 μM isoproterenol for 5 minutes. ct-GRK2 when transfected alone had no effect on PI-3K activity. Stimulation of the β_2-AR for 5 minutes with isoproterenol in cells cotransfected with ct-GRK2 resulted in a significantly decreased ($P < .001$) PI-3K recruitment to the particulate fraction (Figure 4) as compared to stimulation of the β_2-AR expressed alone. Similar results were obtained when cells expressing the β_1-AR were stimulated for 5 minutes with the agonist, that is, the presence of ct-GRK2 significantly decreased ($P < .001$) PI-3K recruitment to membranes (Figure 4). Thus, PI-3K recruitment to the plasma membrane by either β_1- or β_2-AR stimulation is G$\beta\gamma$ subunit-dependent although only β_2-AR-dependent recruitment results in subsequent PI-3K activation.

3.5. PI-3K/Akt Pathway Activation Following Stimulation of Chimeric $\beta_1 \times \beta_2$-AR and $\beta_2 \times \beta_1$-AR . To determine the importance of receptor-specific determinants in the β-AR carboxy tail for PI-3K activation, we constructed two chimeric receptors which consisted of the β_1-AR with the proximal seventh transmembrane domain and carboxy-terminal tail of the β_2-AR ($\beta_1 \times \beta_2$-AR) as well as the reciprocal receptor which consisted of β_2-AR with the carboxy-terminal tail of the β_1-AR ($\beta_2 \times \beta_1$-AR). When the $\beta_1 \times \beta_2$-AR was stimulated by 1 μM isoproterenol for 5 or 15 minutes, PI3-kinase activity was significantly increased (Figure 5; $P < .05$). On the other hand, agonist stimulation of the $\beta_2 \times \beta_1$-AR for 5 or 15 minutes did not result in increased PI3-kinase activity. These results suggest that the carboxy-terminal tail of the β_2-AR contains molecular determinants which are required for PI3-kinase activation.

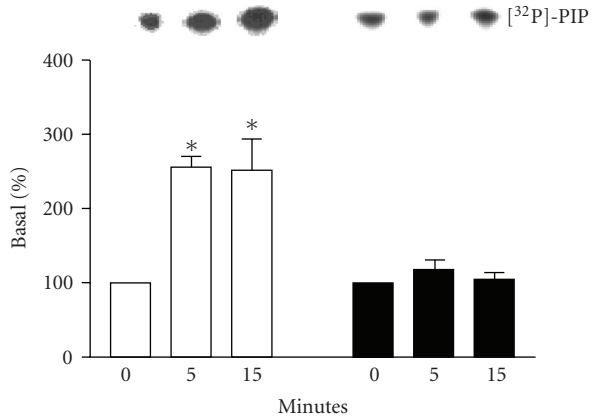

FIGURE 5: *PI-3K activity with chimeric β-AR stimulation*. HEK 293 cells were transfected with $\beta_1 \times \beta_2$-AR or $\beta_2 \times \beta_1$-AR. Forty-eight hours after transfection, cells were stimulated with 1 μM isoproterenol for the indicated times. PI-3K activity was determined by the level of [^{32}P]-PI produced by the stimulation of $\beta_1 \times \beta_2$-AR (□) or $\beta_2 \times \beta_1$-AR (■) expressing cells. Top panel is a representative autoradiograph of the TLC separation (n = 4-5). Data from these experiments is quantitated in the bottom panel as described in Section 2. $^*P < .05$ versus 0 minute.

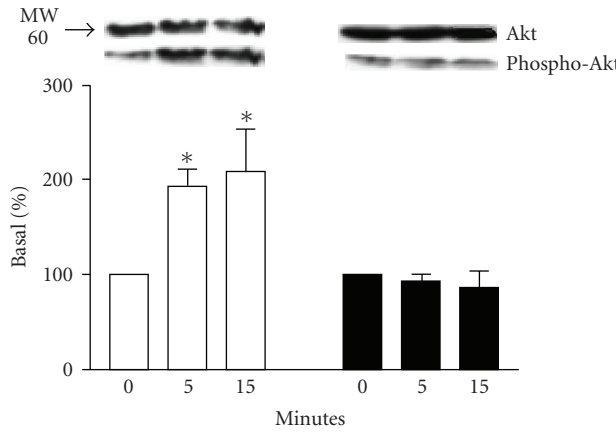

FIGURE 6: *Akt activity with chimeric β-AR stimulation*. HEK 293 cells were transfected with $\beta_1 \times \beta_2$-AR or $\beta_2 \times \beta_1$-AR. Forty-eight hours after the transfection, cells were stimulated with 1 μM isoproterenol for the indicated times. Akt activity was determined by measuring levels of phosphorylated Akt compared with total Akt produced by the stimulation of $\beta_1 \times \beta_2$-AR (□) or $\beta_2 \times \beta_1$-AR (■) expressing cells. Upper panel is a representative immunoblot of the experiments (n = 6-7). Data from these experiments is quantitated in the bottom panel as described in Section 2. $^*P < .05$ versus 0 minute.

To confirm results obtained for the PI-3K activation mediated by the two chimeric β-ARs, we again determined the activation status of Akt. Chimeric receptors were stimulated with 1 μM isoproterenol for 0, 5, or 15 minutes at 37°C. Akt phosphorylation was significantly increased ($P < .05$) in HEK 293 cells expressing $\beta_1 \times \beta_2$-AR (Figure 6). Stimulation of $\beta_2 \times \beta_1$-AR by isoproterenol had no effect on Akt phosphorylation as compared to basal conditions. Thus chimeric $\beta_1 \times \beta_2$-AR increased Akt phosphorylation and confirmed that the carboxy-terminal tail of the β_2-AR contained molecular determinants necessary and sufficient for PI-3K activation.

3.6. Effect of Pertussis Toxin Treatment on β-AR Stimulation of PI3-K Activity. It has been proposed that stimulation of PI-3K by β_2-AR is pertussis toxin sensitive [1, 12]. To determine the effect of pertussis toxin on β-AR induced stimulation of PI-3K, we incubated the cells with PTX for 18 hours followed by a stimulation with 1 μM isoproterenol for 15 minutes. Our results indicate that, in presence of PTX, no activation of PI-3K was observed with β_2-AR stimulation (Figure 7(b)). In presence of the chimeric receptors, PTX treatment abolished the activation of PI-3K observed with the chimeric $\beta_1 \times \beta_2$-AR (Figure 7(d)). Consistent with our earlier data, neither the β_1-AR (Figure 7(a)) nor the $\beta_2 \times \beta_1$-AR (Figure 7(c)) was sensitive to PTX pretreatment. These results indicate that stimulation of PI-3K by β_2-AR is pertussis toxin sensitive.

3.7. Involvement of PI-3K Activation on β-AR Sequestration. Cells expressing β_1-AR were treated with isoproterenol for 15 minutes. After stimulation, very little β_1-AR was observed in the interior compartments of treated cells and no significant difference was observed in presence of either cotransfected p85ΔPI-3K or PTX pretreatment (Figures 8(a) to 8(d)). In contrast, we observed a significant internalization of β_2-AR after a similar period of agonist stimulation as shown by clustered distribution of the receptor (Figure 8(f); outline of the cell is poorly defined in this particular condition). β_2-AR internalization was inhibited in the presence of either cotransfected p85ΔPI-3K or PTX pretreatment (Figures 8(g) and 8(h)). These results confimed that functional PI-3K is important for the sequestration of β_2-AR and attests to the effectiveness of our p85ΔPI-3K cotransfection or PTX pretreatment.

4. Discussion

The results of the current study demonstrate that stimulation of β_2-AR, but not β_1-AR, induces PI-3K activation by a pertussis toxin-sensitive mechanism. This subtype-dependent activation has been reinforced by the fact that Akt, a downstream PI-3K effector, was also selectively activated by β_2-AR stimulation. However, both receptor subtypes can recruit the PI-3K to the plasma membrane via a G$\beta\gamma$ subunit-dependent mechanism. Naga Prasad et al. [2] had previously demonstrated that PI-3K and GRK2 formed a cytosolic complex and the recruitment of the enzyme to the plasma membrane was facilitated by G$\beta\gamma$ subunits in an agonist-dependent manner. Our results confirmed that PI-3K is recruited to the plasma membrane via a G$\beta\gamma$ subunit-dependent mechanism. GRK-2 is important for PI-3K recruitment to the plasma membrane, thus we thought this might suggest that the carboxyl-terminal domain of the receptor is important for the PI-3K activation because the carboxyl-terminal domain of the β-AR contains sites phosphorylated by GRK2. Furthermore, Shiina et al. [13] had demonstrated that the carboxyl-terminal domain and

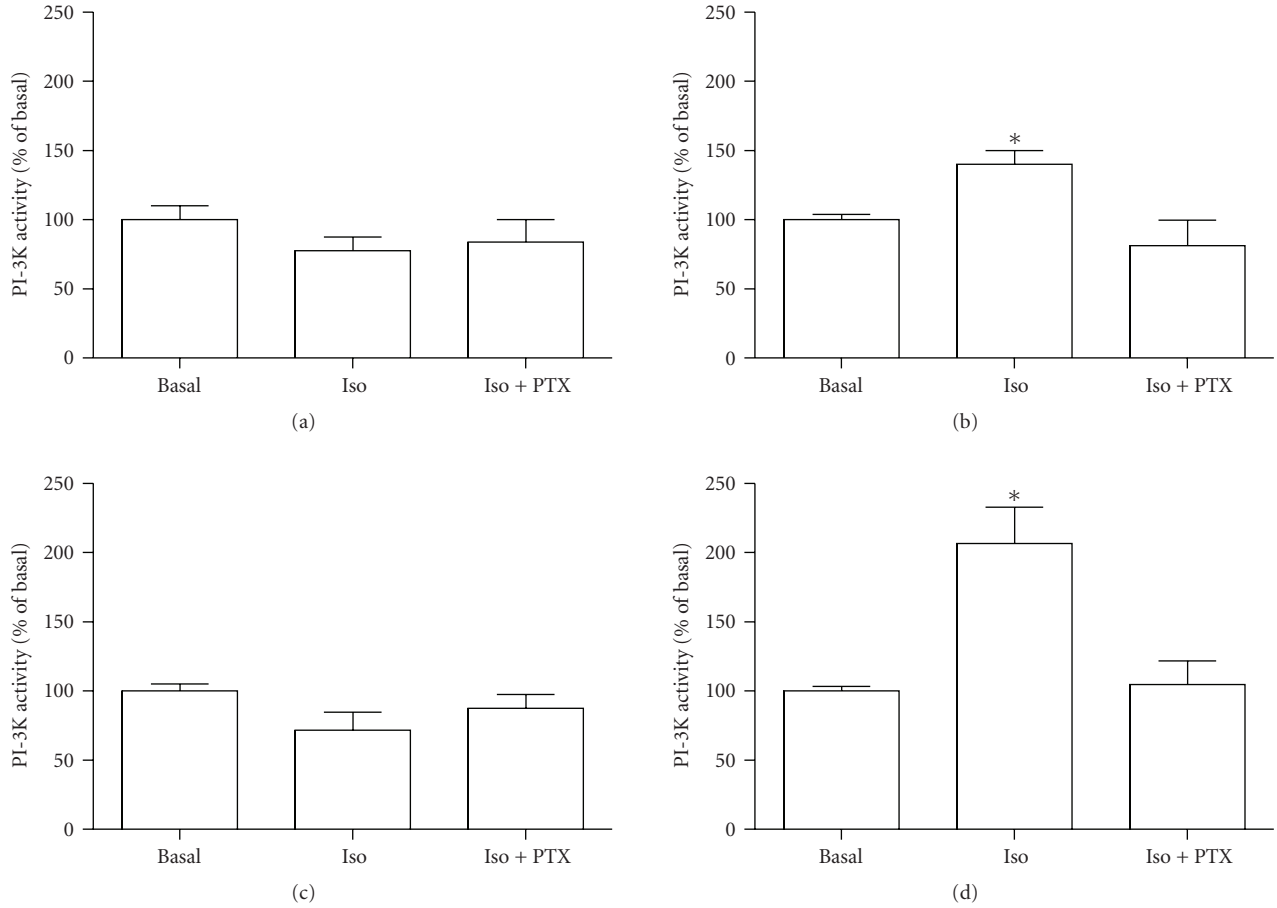

Figure 7: *Pertussis toxin effects on β-AR-mediated PI-3K activation*. Twenty-six hours after transfection, cells were incubated with 0.1 μg/mL pertussis toxin for 18 hours. Cells were stimulated for 5 minutes with 1 μM isoproterenol prior to processing for TLC as described in Figure 1 and Section 2. PI-3K activity was determined by the level of $[^{32}P]$-PI produced by the stimulation of β_1-AR (a), β_2-AR (b), $\beta_2 \times \beta_1$-AR (c), and $\beta_1 \times \beta_2$-AR expressing cells. (n = 3-4; $^*P < .05$ versus basal and PTX-Iso conditions).

the third cytosolic loop are the regions mostly responsible for the difference in internalization behavior between both β-AR subtypes.

To determine the potential contribution of the carboxyl-terminal domains of the β_1- and β_2-AR in the PI-3K activation, chimeric β_1- and β_2-AR in which the seventh transmembrane domain and the carboxyl-terminal domain have been exchanged were constructed. We observed that the chimeric $\beta_1 \times \beta_2$-AR could activate PI-3K in contrast to the wild type β_1-AR. Reciprocally, β_2-AR lost its ability to activate PI-3K when its carboxy-terminal domain was exchanged for that of the β_1-AR. This result demonstrated that the β_2-AR carboxyl-terminal domain contains important molecular determinants for PI-3K activation.

The present study confirms PI-3K activation by β_2-AR stimulation as observed in other studies [2, 12]. However the kinetics of PI-3K activation previously reported for β_2-AR stimulation was different to that observed in the present study. PI-3K was rapidly activated following β_2-AR stimulation and returned to basal levels after 10 minutes. In our study, we observed a significant PI-3K activation with a 5-minute β_2-AR stimulation that was maintained after 15 minutes. The difference in activation patterns may be due to the agonist concentration used. In the previous study [2], 10 μM isoproterenol was used which is 10 times higher than the concentration used here. Higher concentration of agonist may induce more rapid PI-3K recruitment to the membrane and thus activation of the enzyme but may also more rapidly stimulate mechanisms which terminate these signals as well.

Pretreatment with pertussis toxin results in a loss of PI-3K activation by β_2-AR stimulation confirming results obtained in other studies [1, 12]. The new information afforded by the present study is that the carboxy-terminal portion of the receptor is important for the interaction between the receptor, Gi heterotrimers (as stimulation depends on both the Gα and G$\beta\gamma$ subunits), and PI-3K. Indeed, the chimeric receptor $\beta_1 \times \beta_2$-AR can activate the PI-3K by a pertussis toxin sensitive mechanism whereas the chimeric receptor $\beta_2 \times \beta_1$-AR is unable to activate PI-3K. However, other regions of the receptor may also be important for full activation or regulation of the response by the desensitization machinery.

Our results demonstrate that isoproterenol-stimulated β_1-AR (for up to 15 minutes) cannot activate PI-3K. These

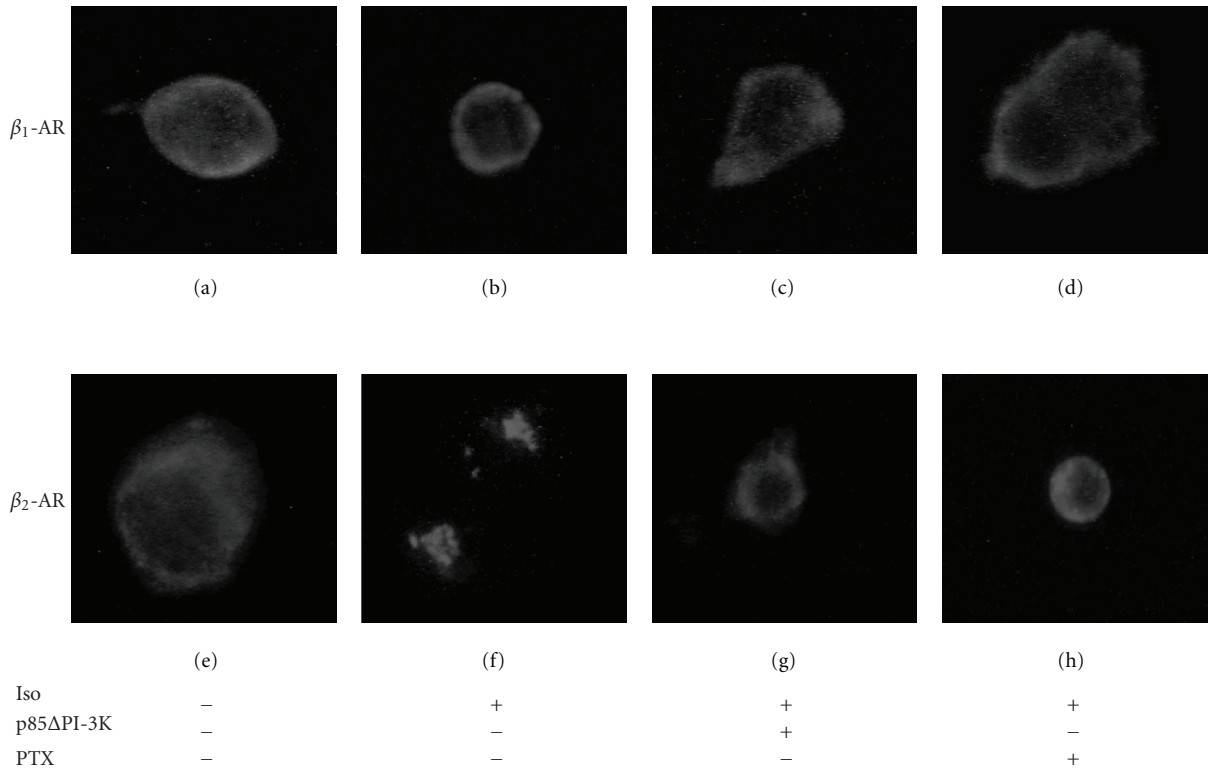

Figure 8: *p85ΔPI-3K reduced agonist-induced β_2-AR sequestration*. HEK 293 cells were transfected with p85ΔPI-3K and the two β-AR subtypes. Cells were stimulated with 1 μM isoproterenol for 15 minutes (b) and (f). Stimulation of β_2-AR-transfected cells induced a cluster distribution which is characteristic of receptor internalization (f). This sequestration was abolished in presence of either cotransfected p85ΔPI-3K (g) or PTX pretreatment (h). No significant β_1-AR sequestration was observed under the different conditions used in these experiments (a)–(d). Representative images of several experiments (n = 8–15) are shown.

results contrast with those reported in a previous study in which activation of PI-3K was observed after stimulation of β_1-AR transfected into HEK 293 cells [2]. Several possibilities can explain the discrepancy between both studies. First, 1 μM isoproterenol although sufficient to induce submaximal adenylyl cyclase activity [8] cannot induce PI-3K activation. Secondly, in our study we use transiently transfected cells that express approximately 500 fmol receptor/mg of membrane proteins, which is closer to physiological levels than seen with the study of Naga Prasad et al. [2]. Also, it is clear that the interplay between the signalling and internalization machinery is different for the two receptors even though they can interact with many of the same proteins. This study provides another such example that both β_1-AR and β_2-AR lead to PI-3K and GRK2 recruitment; both the signalling and desensitization outcomes for these events are markedly different. Some studies have also shown that β_1-AR, in contrast to the β_2-AR, cannot activate G_i [14] while others suggest that it can [15]. It is possible that other cell- and tissue-specific factors might regulate these events as well.

In this study, we observed that β_2-AR internalization is inhibited in the presence of a dominant negative PI-3K, p85ΔPI-3K, results that are similar to previous studies [2, 16]. We also observed that β_2-AR sequestration can be abolished with pertussis toxin pretreatment. In contrast to β_2-AR, we observed that the β_1-AR sequestration is minimal with 15 minutes of stimulation. This is similar to the 10% sequestration observed in β_1-AR expressing cells obtained by Suzuki et al. [17]. Since phosphorylated lipids generated by PI-3K are critical for the receptor internalization dynamics [18, 19], it is plausible that the weak sequestration observed for the β_1-AR might be due to the weak PI-3K activation induced by the stimulation of this receptor subtype. Results obtained with the pertussis pretreatment and p85ΔPI-3K also suggest that activated PI-3K is important for receptor sequestration.

In conclusion, we observed that PI-3K activation is β-AR subtype specific and the difference is due to molecular determinants present in the carboxy-terminal tail of the receptors. PI-3K activation is pertussis toxin sensitive and is necessary for the sequestration of the β_2-AR.

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