LETTER TO THE EDITOR

The polymorphic deleted-form of the human α2B-adrenergic receptor and its wild-type counterpart display post-receptor signaling pathway differences in LLC-PK1 cells

A polymorphic variant of the human α2B-adrenergic receptor, which comprises the deletion of three glutamic acids (Del301-303) in the third intracellular loop, has been found to be common in Caucasians (31%) and less frequent in African-Americans (12%).1,2 The deletion occurs within a stretch of acidic residues that are essential for agonist-promoted receptor phosphorylation by G protein-coupled receptor kinases (GRKs), and its subsequent desensitization by the recruitment of β-arrestins at the plasma membrane.3 The Del301-303 variant of the α2B-adrenergic receptor consistently exhibits decreased GRK-mediated receptor phosphorylation and impaired desensitization in comparison to the wild-type receptor.2 Clinical genetic studies have previously revealed an association between the deleted variant receptor and cardiovascular pathologies, including acute coronary events, hypertension and sudden cardiac death, among others.1,4,5 In the present study, we employed clones of LLC-PK1 cells, stably expressing after transfection the wild-type α2B-AR or its deleted variant, and investigated the signaling pathways associated with the wild-type or deleted variant of human α2B-AR.

The expression vectors were derived from the pREP4 plasmid and contained hemagglutinin-tagged wild-type α2B-AR (pREP HA-α2B-WT) or hemagglutinin-tagged Del301-303 α2B-AR variant (pREP HA-α2B-Del), which were gifts from Dr. Mika Scheinin (University of Turku, Finland). Receptor expression and subcellular distribution were determined as depicted and described in Fig. 1. The receptor phosphorylation was measured using an anti-phospho-serine antibody on immuno-precipitated material, and the propensity to undergo desensitization was examined using the GTPγS binding assay. Soluble proteins were analyzed by Western blotting. Phospho-IKKα/β, β-arrestins, phospho-Erk, phospho-Akt and Erk were, respectively detected with their specific primary antibody and the corresponding horseradish peroxidase-conjugated secondary antisera. Anti-β-arrestin rabbit polyclonal antibody (A1CT) was a gift from Dr. Robert J. Lefkowitz (Duke University Medical Center, Durham, NC).

The receptor densities were comparable in the two clones: 2.4 ± 0.4 pmoles/mg of protein in LLC-α2B-WT4, and 2.1 ± 0.2 pmoles/mg of protein in LLC-α2B-Del4. The affinity of [3H]RX821002 for WT (Kd = 8.1 ± 1.2 nM) was not different from that for Del α2B-AR (Kd = 6.5 ± 0.7 nM), and the receptor density remained stable over at least 20 successive cell passages. Confocal microscopy examination of HA immunofluorescent staining indicated that WT and Del α2B-AR exhibited the same pattern of subcellular distribution (Fig. 1).

We found a significant decrease ~50% of the extent of receptor phosphorylation in LLC-α2B-Del4 compared with LLC-α2B-WT4, following short-term exposure of the cells to the α2-agonist UK14304 (not shown). [35S]GTPγS binding on membranes prepared from the two clones demonstrated a partial loss of desensitization in the LLC-α2B-Del4 clone (not shown), in agreement with previous observations in CHO cells.

Furthermore, immunoblotting with the β-arrestin antibody indicated that the Del α2B-AR exhibited slower kinetics of β-arrestin recruitment and more persistent coupling to its cognate G-proteins (data not shown). Moreover, a divergence in the type of β-arrestin recruited was observed...
between WT and Del α2B-adrenergic receptors: whereas β-arrestin 2 was preferentially recruited within the first minutes following exposure of LLC-a2BAR-WT4 to the UK14304 agonist, it was β-arrestin 1 that was finally engaged after a long period of stimulation of LLC-a2BAR-Del4. The functional consequences of this divergence are unknown; however, because β-arrestins 1 and 2 play discrete roles,3 they may result in a difference in the intensity and duration of receptor signaling.

proteins were separated on SDS-PAGE and immunoblotted using anti-phosphorylated IKKα/β. A representative blot from at least three sets of experiments is shown. C. Densitometric analysis was performed and the data are presented as the mean ± S.E.M. of three independent experiments.

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Fig. 1 Expression of WT and Del α2B-adrenergic receptors in LLC-PK1 cells. A. LLC-α2BAR-WT4 and LLC-α2BAR-Del4 clones were cultured on glass coverslips. The cells were fixed, permeabilized and immunolabeled with mouse anti-HA antibody/anti-mouse Cy3. The results were analyzed on a Zeiss laser confocal microscope. B. Membranes prepared from LLC-α2BAR-WT4 or LLC-α2BAR-Del4 cells were incubated in the presence of various concentrations of [3H]RX821002, and the amount of specifically bound radioligand was determined using 10 μM phentolamine to estimate the non-specific binding. The data presented are from a typical experiment. Computer-assisted analysis of the results from this specific experiment indicated that the Bmax and Kd values of [3H]RX821002 were, respectively 1.98 ± 0.26 pmol/mg of protein and 6.17 ± 0.73 nM for LLC-α2BAR-Del4 cells and 1.84 ± 0.12 pmol/mg of protein and 4.46 ± 0.51 nM for LLC-α2BAR-Del4 cells.

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Fig. 2 Activation of NF-κB and phosphorylation of IKKα/β. A. LLC-α2BAR-WT4 (open bars) or LLC-α2BAR-Del4 (dark bars) cells were transfected with the p-NF-κB Luc construct. Thirty-six hours post-transfection, the cells were placed in serum-free culture medium and subsequently treated or not (basal) with PMA (PMA) or with 10 μM UK14304 in the absence (UK) or the presence of 30 μM PD98059 (PD+UK) or 50 μM LY 240092 (LY+UK) or 10 μM PP1 (PP1+UK). The cells were collected and assayed for luciferase activity 6 hours after the beginning of the treatment. Luciferase activity is expressed as the fold-increase relative to basal. Data are the mean ± S.E.M. from three individual experiments. B. Serum-deprived LLC-α2BAR-WT4 (upper panel) or LLC-α2BAR-Del4 (lower panel) were treated or not (basal) with PMA or with 10 μM UK14304 in the absence (UK) or the presence of 30 μM PD98059 (PD+UK) or 50 μM LY 240092 (LY+UK) or 10 μM PP1 (PP1+UK). The cells were collected and assayed for phosphorylation of IKKα/β. C. Densitometric analysis was performed and the data are presented as the mean ± S.E.M. of three independent experiments.
Both receptors caused a rapid and long-lasting increase in the phosphorylation of Erk and Akt. Phosphorylation of Erk was partially inhibited by MMP inhibitors, heparin, and tyrocephalin, and persisted upon EGFR desensitization, indicating that it is triggered both by EGFR-dependent and EGFR-independent mechanisms, as previously described for other cell types. The effects of the α2-agonist are strongly attenuated by prior treatment of the cells with the inhibitor of MEK1/2 (PD98059) of PI3-K (LY240092) and of Src (PP1) (data not shown).

A variety of GPCRs have now been shown to regulate inflammation and cell survival processes by controlling the activation of NF-κB. The GPCRs include receptors for bradykinin, fMLP (N-formyl methionyl leucyl phenylalanine), lysophosphatidic acid, and dopamine, as well as the α2-adrenergic receptors. Therefore, the capacity of WT and Del α2-ARs to activate NF-κB was investigated by measuring luciferase activity and by following IKK phosphorylation. Luciferase activity was measured in cell extracts prepared from LLC-PK1 cells transiently transfected with pNF-κB-Luc plasmid, and then treated with regulatory substances in the presence or absence of inhibitors, using a Lumat LB 9501 luminometer (Berthold Technologies, Wildbad, Germany). As shown in Fig. 2A, long-term exposure of LLC-PK1 cells to UK14304 markedly enhanced the activity of the pNF-κB-Luc construct regardless of the type of α2-AR expressed. In LLC-α2B WT4, the effect of UK14304 was not affected by a prior treatment with the MEK1 inhibitor PD98059, but was completely abolished by prior treatment with the PI-3K inhibitor, LY294002 (Fig. 2A). The situation was somewhat different in LLC-α2B-Del4, where the induction of luciferase was inhibited to the same extent (55-60% reduction) in the presence of these inhibitors. Fairly similar findings were obtained with regard to the UK14304-induced IKKα/β phosphorylation (Fig. 2B). UK14304 caused IKKα/β phosphorylation and LY240092 inhibited IKKα/β phosphorylation and NF-κB activation by WT or Del α2-AR. In contrast, PD98059 was efficient in cells expressing the Del α2B-adrenergic receptor, but not the WT. The reasons for the differences in the sensitivity of the NF-κB pathway to inhibitors are difficult to understand, especially as we did not find any evidence for the difference in the kinetics and mechanisms of MAPK activation between the two receptors. Because β-arrestins 1 and 2 are both binding partners of β2α, the difference between WT and Del α2B-adrenergic receptor may be the consequence of their distinct capacity to recruit β-arrestins.

Overall, the present data have demonstrated great similarities but also certain major differences between the signaling pathways of the WT and Del α2-AR in LLC-PK1 cells. Given the aforementioned associations of this variant receptor with cardiovascular pathologies, the differences and their molecular basis found in the present study would merit confirmation in other cellular models relevant to cardiovascular pathologies. Furthermore, these post-receptor signaling differences add to the clinical relevance of the variant receptor in other domains, such as neurobiology and psychotherapy, based on the recently found association of the polymorphic variant α2B-adrenergic receptor with differential amygdala activation and emotional memory.

Abbreviations

| GPCR | G-protein-coupled receptor |
| MMP | matrix metalloproteinase |
| MEK | mitogen-activated protein kinase |
| PI-3K | phosphoinositide-3-kinase |
| Bmax | the maximal density of the receptor site in the membrane preparation |
| Kd | equilibrium dissociation constant |
| PMA | phorbol myristate acetate |
| IKKα/β | inhibitor of NF-κB kinase, subunits alpha & beta |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| PP1 | Src family tyrosine kinase inhibitor |

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