Hellenic Journal of Cardiology (2016) 57, 292–295

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: http://www.journals.elsevier.com/hellenic-journal-of-cardiology/

LETTER TO THE EDITOR

The polymorphic deleted-form of the human α_{2B}-adrenergic receptor and its wild-type counterpart display post-receptor signaling pathway differences in LLC-PK1 cells

KEYWORDS
α_{2B}-adrenergic receptor;
β-arrestin;
MAPK;
Akt;
NF-κB

A polymorphic variant of the human α_{2B}-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 α_{2B}-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 α_{2B}-AR or its deleted variant, and investigated the signaling pathways associated with the wild-type or deleted variant of human α_{2B}-AR.

The expression vectors were derived from the pREP4 plasmid and contained hemagglutinin-tagged wild-type α_{2B}-AR (pREP HA-α_{2B}-WT) or hemagglutinin-tagged Del301-303 α_{2B}-AR variant (pREP HA-α_{2B}-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-arrestins 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-α_{2B}-WT4, and 2.1 ± 0.2 pmoles/mg of protein in LLC-α_{2B}-Del4. The affinity of [^{3}H]RX821002 for WT (Kd = 8.1 ± 1.2 nM) was not different from that for Del α_{2B}-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 α_{2B}-AR exhibited the same pattern of subcellular distribution (Fig. 1).

We found a significant decrease ~50% of the extent of receptor phosphorylation in LLC-α_{2B}-Del4 compared with LLC-α_{2B}-WT4, following short-term exposure of the cells to the α_2-agonist UK14304 (not shown). [^{35}S]GTPγS binding on membranes prepared from the two clones demonstrated a partial loss of desensitization in the LLC-α_{2B}-Del4 clone (not shown), in agreement with previous observations in CHO cells.

Furthermore, immunoblotting with the β-arrestin antibody indicated that the Del α_{2B}-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

Peer review under responsibility of Hellenic Cardiological Society.

http://dx.doi.org/10.1016/j.hjc.2016.05.009

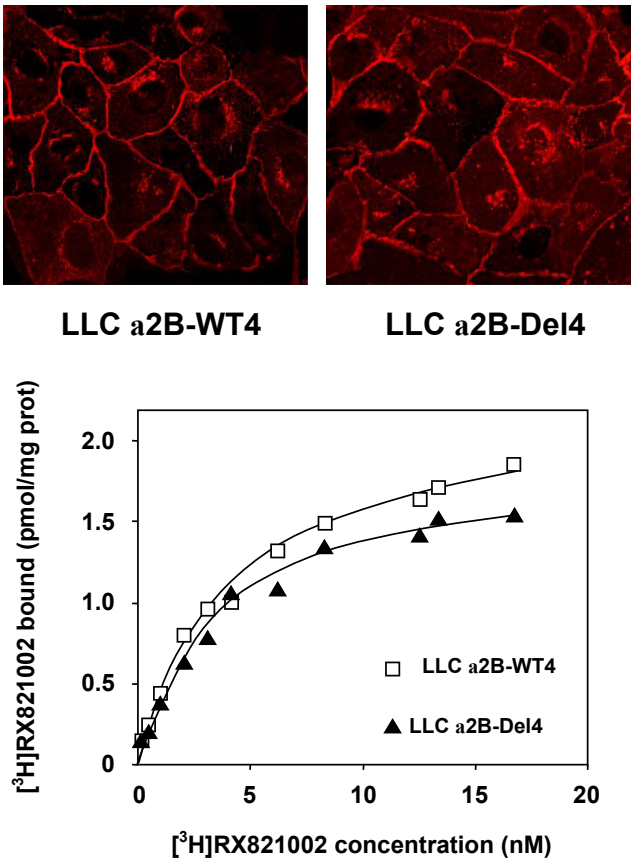

Fig. 1 Expression of WT and Del α_{2B}- adrenergic receptors in LLC-PK1 cells. A. LLC-α_{2B}AR-WT4 and LLC-α_{2B}AR-Del4 clones were cultured on glass coverslips. The cells were fixed, permeabilized and immunolabeled with mouse anti-HA antibody/anti-mouse Cy3. The results were analyzed on a Zeiss laser confocal microscope. B. Membranes prepared from LLC-α_{2B}AR-WT4 or LLC-α_{2B}AR-Del4 cells were incubated in the presence of various concentrations of [^{3}H]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 [^{3}H]RX821002 were, respectively 1.98 ± 0.26 pmol/mg of protein and 6.17 ± 0.73 nM for LLC-α_{2B}AR-Del4 cells and 1.84 ± 0.12 pmol/mg of protein and 4.46 ± 0.51 nM for LLC-α_{2B}AR-Del4 cells.

between WT and Del α_{2B}-adrenergic receptors: whereas β-arrestin 2 was preferentially recruited within the first minutes following exposure of LLC-α_{2B}-WT4 to the UK14304 agonist, it was β-arrestin 1 that was finally engaged after a long period of stimulation of LLC-α_{2B}-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.

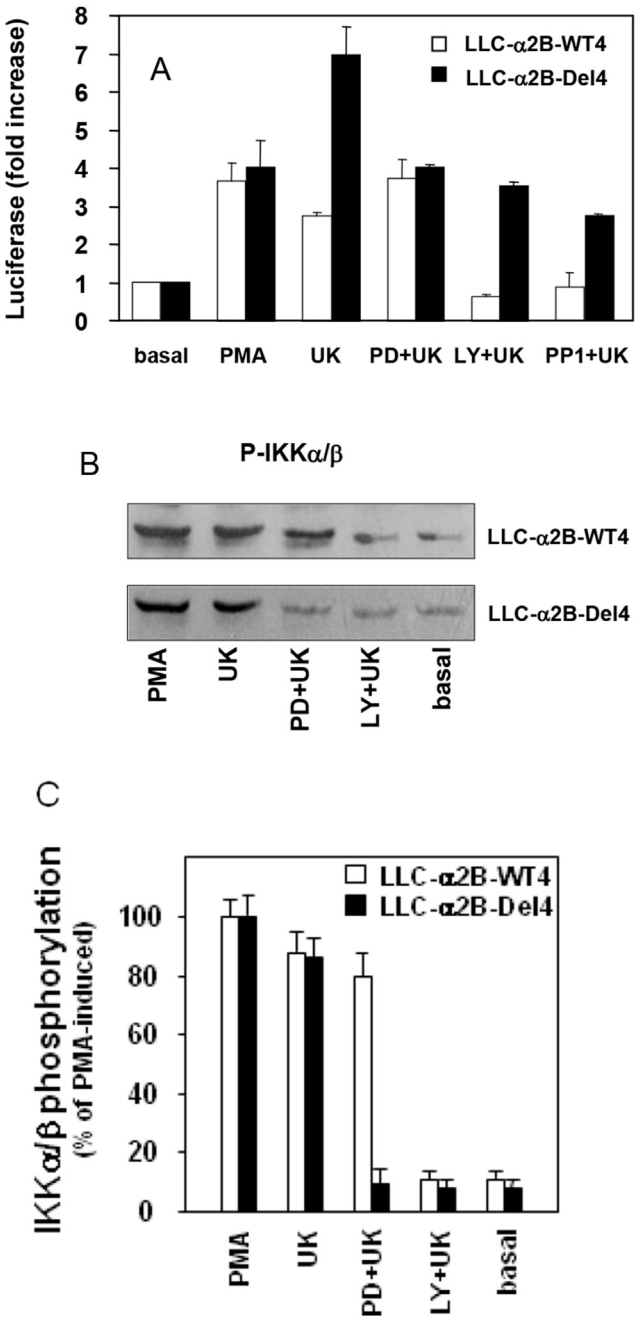

Fig. 2 Activation of NF-κB and phosphorylation of IKKα/β. A. LLC-α_{2B}AR-WT4 (open bars) or LLC-α_{2B}AR-Del4 (dark bars) cells were transfected with the p-NF-κB Luc construct. Thirty-six hours post-transfection, the cells were placed in serum-free culture medium and subsequently treated or not (basal) 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-α_{2B}AR-WT4 (upper panel) or LLC-α_{2B}AP−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). Cell lysates were prepared in RIPA, 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.

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 tyrphostin, 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.[6,7] The effects of the α_2-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.[8] The GPCRs include receptors for bradykinin, fMLP (N-formyl methionyl leucyl phenylalanine), lysophosphatidic acid, and dopamine, as well as the α_2-adrenergic receptors.[9] Therefore, the capacity of WT and Del α_{2B}-ARs to activate NF-κB was investigated by measuring luciferase activity and by following IKKα/β phosphorylation. Luciferase activity was measured in cell extracts prepared from LLC-PK1 cells transiently transfected with pNF-κB Luc plasmid,[9] and then treated with regulator substances in the presence or absence of inhibitors, using a Lumat LB 9501 luminometer (Berthold Technologies, Bad 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 α_{2B}-AR expressed. In LLC-α_{2B}-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-α_{2B}-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 α_{2B}-AR. In contrast, PD98059 was efficient in cells expressing the Del α_{2B}-adrenergic receptor, but not the WT. The reasons for the differences in the sensitivity of the NF-kB 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 IκBα, the difference between WT and Del α_{2B}-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 α_{2B}-AR in LLC-PK1 cells. Given the aforementioned associations of this variant receptor with cardiovascular pathologies,[1,4,5] 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.[10]

Abbreviations

GPCR	G-protein-coupled receptor
MMP	matrix metalloproteinase
MEK	mitogen-activated protein kinase kinase
PI-3K	phosphoinisitide3-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

References

1. Heinonen P, Koulu M, Pesonen U, et al. Identification of a three-amino acid deletion in the alpha2B-adrenergic receptor that is associated with reduced basal metabolic rate in obese subjects. *J Clin Endocrinol Metab*. 1999;84:2429—2433.
2. Small KM, Brown KM, Forbes SL, Liggett SB. Polymorphic deletion of three intracellular acidic residues of the alpha 2B-adrenergic receptor decreases G protein-coupled receptor kinase-mediated phosphorylation and desensitization. *J Biol Chem*. 2001;276:4917—4922.
3. DeGraff JL, Gagnon AW, Benovic JL, Orsini MJ. Role of arrestins in endocytosis and signaling of alpha2-adrenergic receptor subtypes. *J Biol Chem*. 1999;274:11253—11259.
4. Snapir A, Heinonen P, Tuomainen TP, et al. An insertion/deletion polymorphism in the alpha2B-adrenergic receptor gene is a novel genetic risk factor for acute coronary events. *J Am Coll Cardiol*. 2001;37:1516—1522.
5. Snapir A, Mikkelsson J, Perola M, Penttilä A, Scheinin M, Karhunen PJ. Variation in the alpha2B-adrenoceptor gene as a risk factor for prehospital fatal myocardial infarction and sudden cardiac death. *J Am Coll Cardiol*. 2003;41:190—194.
6. Cussac D, Schaak S, Denis C, Paris H. alpha 2B-adrenergic receptor activates MAPK via a pathway involving arachidonic acid metabolism, matrix metalloproteinases, and epidermal growth factor receptor transactivation. *J Biol Chem*. 2002;277:19882—19888.
7. Karkoulias G, Mastrogianni O, Lymperopoulos A, Paris H, Flordellis C. Alpha(2)-Adrenergic receptors activate MAPK and Akt through a pathway involving arachidonic acid metabolism by cytochrome P450-dependent epoxygenase, matrix metalloproteinase activation and subtype-specific transactivation of EGFR. *Cell Signal*. 2006 May;18(5):729—739.
8. Ye RD. Regulation of nuclear factor kappaB activation by G-protein-coupled receptors. *J Leukoc Biol*. 2001;70:839—848.
9. Lymperopoulos A, Karkoulias G, Koch WJ, Flordellis CS. Alpha2-adrenergic receptor subtype-specific activation of NF-kappaB in PC12 cells. *Neurosci Lett*. 2006;402:210—215.
10. Rasch B, Spalek K, Buholzer S, et al. A genetic variation of the noradrenergic system is related to differential amygdala activation during encoding of emotional memories. *Proc Natl Acad Sci U S A*. 2009;106:19191—19196.

Orthodoxia Mastrogianni[a]
Department of Pharmacology, School of Medicine, University of Patras, 26504 Rio Patras, Greece

Pierre-Antoine Crassous[a]
INSERM Unit 388, Institut Louis Bugnard, IFR31, CHU Rangueil, 31400 Toulouse, France

Georgios Karkoulias
Dimosthenis Lykouras
Department of Pharmacology, School of Medicine, University of Patras, 26504 Rio Patras, Greece

Stéphane Schaak
INSERM Unit 388, Institut Louis Bugnard, IFR31, CHU Rangueil, 31400 Toulouse, France

Nicholas Patsouras
George Panayiotakopoulos
Department of Pharmacology, School of Medicine, University of Patras, 26504 Rio Patras, Greece

Gregory Sivolapenko
Laboratory of Pharmacokinetics, Department of Pharmacy, University of Patras, Patras, Greece

Hervé Paris
INSERM Unit 388, Institut Louis Bugnard, IFR31, CHU Rangueil, 31400 Toulouse, France

Antonis S. Manolis*
Third Department of Cardiology, Athens University School of Medicine, Athens, Greece

Christodoulos Flordellis**
Department of Pharmacology, School of Medicine, University of Patras, 26504 Rio Patras, Greece

*Corresponding author. Professor Antonis S. Manolis, Third Department of Cardiology, Athens University School of Medicine, Athens, Greece.
E-mail address: asm@otenet.gr (A.S. Manolis)

**Corresponding author. Professor Christodoulos Flordellis, Department of Pharmacology, Patras University School of Medicine, Patras, Greece.
E-mail address: flordell@med.upatras.gr (C. Flordellis)

12 August 2015
Available online 20 August 2016

[a] These authors contributed equally to this study.