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The three α_1-adrenoceptor subtypes show different spatio-temporal mechanisms of internalization and ERK1/2 phosphorylation

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ABSTRACT

We analyzed the kinetic and spatial patterns characterizing activation of the MAP kinases ERK 1 and 2 (ERK1/2) by the three α_1-adrenoceptor (α_1-AR) subtypes in HEK293 cells and the contribution of two different pathways to ERK1/2 phosphorylation: protein kinase C (PKC)-dependent ERK1/2 activation and internalization-dependent ERK1/2 activation. The different pathways of phenylephrine induced ERK phosphorylation were determined by western blot, using the PKC inhibitor Ro 31-8425, the receptor internalization inhibitor concanavalin A and the siRNA targeting β-arrestin 2. Receptor internalization properties were studied using CypHer5 technology and VSV-G epitope-tagged receptors. Activation of α_{1A}- and α_{1B}-ARs by phenylephrine elicited rapid ERK1/2 phosphorylation that was directed to the nucleus and inhibited by Ro 31-8425. Concomitant with phenylephrine induced receptor internalization α_{1A}-AR, but not α_{1B}-AR, produced a maintained and PKC-independent ERK phosphorylation, which was restricted to the cytosol and inhibited by β-arrestin 2 knockdown or concanavalin A treatment. α_{1D}-AR displayed constitutive ERK phosphorylation, which was reduced by incubation with prazosin or the selective α_{1D} antagonist BMY7378. Following activation by phenylephrine, α_{1D}-AR elicited rapid, transient ERK1/2 phosphorylation that was restricted to the cytosol and not inhibited by Ro 31-8425. Internalization of the α_{1D}-AR subtype was not observed via CypHer5 technology. The three α_1-AR subtypes present different spatio-temporal patterns of receptor internalization, and only α_{1A}-AR stimulation translates to a late, sustained ERK1/2 phosphorylation that is restricted to the cytosol and dependent on β-arrestin 2 mediated internalization.

1. Introduction

The α_1-adrenoceptors (α_1-ARs) have been extensively investigated with regard to their pharmacology and structure–function relationship. In contrast, less is known about their regulatory properties, and the trafficking and protein interactions involved in these processes. They are heptahelical transmembrane proteins that belong to the G protein-coupled receptor (GPCR) superfamily. Upon agonist binding, α_1-ARs stimulate activation and dissociation of the α and βγ subunits of $G_{q/11}$ proteins [1], and promote PKC activation [2], as well as an increase of intracellular IP3 and calcium content [3,4]. Extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) activation promoted by the different α_1-AR subtypes has also been detected [5,6]. Termination of GPCR signaling is triggered by the phosphorylation of the agonist-occupied receptor by G-protein-coupled receptor-kinases (GRKs) and the second-messenger-dependent protein kinases PKA and PKC [5,7], promoting high affinity binding of cytoplasmatic β-arrestins to the receptor, resulting in its desensitization and endocytosis [8].

Three different α_1-AR subtypes (α_{1A}, α_{1B} and α_{1D}) have been cloned and exhibit differences in amino acid sequence and antagonist binding affinities [9,10]. Different cellular localization is also exhibited by the three subtypes: α_{1B}-AR is primarily expressed on the cell surface [11], α_{1A}-AR is detected not only on the cell surface, but also intracellularly [12]. In contrast, α_{1D}-AR appears to be localized perinuclearly and produces ERK1/2 activation in the absence of agonists [11]. This α_{1D}-AR constitutive activity has also been measured by a spontaneous increase in the resting tone [13–16] and calcium and IP3 accumulation [17,18] in rat aorta, where the main α_1-AR subtype is α_{1D}.

An increasing number of studies indicate that the α_1-AR subtypes display striking differences in their internalization properties and, although controversial, it has been suggested that while α_{1B}-AR undergoes

Abbreviations: α_1-AR, α_1 adrenoceptor; β_2-AR, β_2 adrenoceptor; $AT_{1A}R$, angiotensin receptor type 1A; Bmax, maximum number of binding sites; BSA, bovine serum albumin; ConA, concanavalin A; DMSO, dimethyl sulfoxide; EGFR, epidermal growth factor receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPCR, G-protein coupled receptor; GRK, G-protein-coupled receptor kinase; HEK, human embryonic kidney; KRH, Krebs Ringer Hepes; MAPK, mitogen-activated protein kinase; PCR, polymerase chain reaction; PBST, phosphate-buffered saline with 0.1% Tween 20; PE, phenylephrine; PKA, protein kinase A; PKC, protein kinase C; PTHR, parathyroid hormone receptor type 1; PZ, prazosin

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robust agonist-induced endocytosis, α_{1A}-AR does not [19]. Other reports disagree and suggest that α_{1A}-AR also internalizes upon agonist activation, although to a lesser extent than the α_{1B}-AR, and that α_{1D}-AR is constitutively internalized [12,20]. It has also been suggested that α_{1A}-AR is located in lipid rafts under basal conditions, but exits from these rafts following stimulation, therefore allowing endocytosis [21].

Recently, distinct models of regulation of ERK1/2 activation over time after GPCR activation have been proposed and for many GPCRs, it has been shown that β-arrestin recruitment and receptor-endocytosis are implicated in ERK1/2 activation [22]. For example, angiotensin II activation of the AT_{1A} receptor elicits both, a rapid (peak 2 min), G protein-dependent, ERK1/2 activation and a delayed (peak 5–10 min) and persistent ERK1/2 activation, that is β-arrestin2-dependent [23]. Similar patterns were found for the β_2-AR [24] and the parathyroid hormone receptor [25].

To our knowledge, no coherent analysis of the temporal profile of internalization and ERK1/2 MAPK activation by the three α_1-AR subtypes is available. In the present work, we performed a study of the temporal and spatial patterns of ERK1/2 activation after stimulation of each human α_1-AR stably expressed in HEK293 cells. We explored whether such signals were modified by β-arrestin 2 transcriptional silencing, by blocking receptor endocytosis with concanavalin A (ConA), and by inhibiting PKC with a selective inhibitor. We also studied the different properties of receptor internalization into acidic endosomes by using CypHer5 technology [26,27] and VSV-G epitope tagged receptors to shed light into potential differences between the α_1-AR subtypes.

2. Materials and methods

2.1. Antibodies and reagents

The antibodies used included phospho-p42/44 ERK MAPK (Thr202/ Thr204) and p42/44 ERK MAPK (Cell Signaling Technology, Beverly, MA). CypHer5-anti-VSV-G tag antibody (PA45407), horseradish peroxidase-labeled secondary antibodies and chemiluminescent reagents were from GE Healthcare (Amersham, Buckinghamshire, UK). 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) was purchased from Panreac (Barcelona, Spain). Anti-actin antibody (A2066), concanavalin A (ConA), phenylephrine, Ro 31-8425, prazosin, antibiotic G418, 5-methylurapidil, BMY7378, phentolamine, ethylenediaminetetraacetic acid (EDTA), Nonidet™ P-40, phenylmethanesulfonyl fluoride (PMSF), sodium vanadate, sodium fluoride, ditiothreitol and poly-L-lysine (molecular weight 70,000–150,000) were purchased from Sigma (Sigma, St Louis, MO).

2.2. Construction of the VSV-G-Tagged α_1-AR subtypes

The VSV-G (YTDIEMNRLGK) epitope tag was introduced immediately upstream of each human α_{1A}-AR, α_{1B}-AR and α_{1D}-AR. The amino-terminal primer (5′-AAAAAAAAGGATCCGCCACCATGTACACCGATATAG AGATGAACAGGCTGGGAAAGGTGTTTCTCTCGGGAAATGC-3′) or (5′-AAA AAAAGCTTCCACCATGTACACTGATATCGAAATGAACCGCCTGGGTAAGAA TCCCGACCTGGACACCG-3′) or (5′-AAAAAAAGCTTCCACCATGTACACTG ATATCGAAATGAACCGCCTGGGTAAGACTTTCCGCGATCTCCTGAGCG-3′) was used to incorporate a VSV-G tag, a consensus Kozak sequence and BamHI, HindIII and HindIII sites for α_{1A}-, α_{1B}- and α_{1D}-AR, respectively. Depending on the subtype used as a template, the following carboxyl terminal reverse primer was hybridized: α_{1A}-AR, 5′-AAAAAAAACTCG AGCTAGACTTCCTCCCCGTTC-3′; α_{1B}-AR, 5′AAAAAGAATTCCTAAAACTG CCCGGGCGC-3′; and α_{1D}-AR, 5′-AAAAAGAATTCTTAAATATCGGTCTCCC GTAGGTTGC-3′ respectively, incorporating a XhoI site, EcoRI and EcoRI sites downstream of the coding sequence. All the PCR fragments were subsequently cloned into the plasmid pcDNA3 (Invitrogen, Paisley, Renfrewshire, UK) by in-frame ligation. Construct sequences were confirmed by nucleotide sequencing.

2.3. Cell culture and transfection

HEK293 cells were grown in Dulbecco's modified Eagle's minimum essential medium (Sigma) supplemented with 10% newborn calf serum (Gibco BRL, Gaithersburg, MD), L-glutamine 2 mM (Gibco BRL), 100 μg/ml streptomycin and 100 units/ml penicillin at 37 °C in a humidified atmosphere of 5% CO_2. Cells were transfected by electroporation (300 V, 50 μs, 2 mm gap) using a Multiporator (Eppendorf AG, Hamburg, Germany). After 48 h, cells were selected with G418 400 μg/ml to identify stably expressing clones. All the experiments were performed at around 75% cell confluence.

2.4. RNA extraction and quantitative reverse-transcription polymerase chain reaction

RNA extraction and quantitative reverse-transcription polymerase chain reaction was performed as previously described [28,29]. The threshold cycle (Ct) values obtained for each gene were referenced to GAPDH and converted into the linear form using the term $2^{-\Delta Ct}$ as a value directly proportional to the copy number of mRNA.

2.5. Saturation binding experiments

Intact HEK293 cells (1.25×10^5, 5×10^5 and 1×10^6) stably transfected with each α_1-AR were incubated in duplicate for 45 min at 25 °C with [^{3}H]prazosin in 50 mM Tris HCl (pH 7.5) in a final volume of 1 ml. Samples were incubated with [^{3}H]prazosin concentrations ranging from 0.01 to 6 nM. The experiments were terminated by rapid filtration through fiberglass filters (Schleicher and Schuell, GF 52) pre-soaked in 0.3% polyethyleneimine using a Brandel cell harvester (M24R). The filters were then washed three times with 4 ml of ice cold 50 mM Tris–HCl buffer (pH 7.5), and filter bound radioactivity was determined by liquid scintillation counting. Nonspecific binding was measured in the presence of 10 μM phentolamine. The binding data were analyzed by non-linear regression using Prism, version 4.0 (Graph Pad Software; San Diego, California, USA) to determine the dissociation constant (K_d), and the maximum number of binding sites (B_{max}) for the saturation data. The saturation data were fitted to hyperbolic functions (one or two sites). The best fit to the one-site or two-sites was evaluated using the F-test ($P < 0.05$).

2.6. β-Arrestin 2 siRNA knockdown

The expression of β-arrestin 2 was silenced using HP validated siRNA duplexes targeting human ARRB2 (Hs_ARRB2_10 siRNA; Catalog no. SI02776928, Qiagen). AllStars non-targeting siRNA (Catalog no. 1027284, Qiagen) was used as negative control. The day before transfection, cells were seeded in 30 mm dishes in regular growth medium without antibiotics and grown overnight. The day of transfection, Lipofectamine™2000 (Invitrogen)-siRNA complexes were prepared in Opti-MEM I reduced serum medium according to manufacturer's protocol. Cells were transfected with either 50 nM β-arrestin 2 siRNAs or non-targeting control siRNA complexes for 6 h followed by replacement with fresh growth medium including antibiotics. Transfected cells were assayed 48 h post-transfection. Suppression of the target gene was confirmed by western blot.

2.7. Real-time imaging of the internalization of the α_1-AR subtypes to acidified endosomes in HEK293 cells

HEK293 cells stably expressing the N-terminal VSV-G tagged human α_{1A}-, α_{1B}- and α_{1D}-AR subtypes were plated onto poly-L-lysine coated sterile coverslips 48 h before experimentation. Live cells were washed three times with cold Krebs-Ringer-Hepes buffer (KRH, 120 mM NaCl, 25 mM HEPES, 4.8 mM KCl, 1.2 mM $MgSO_4$ and 1.3 mM $CaCl_2$ at pH 7.4) at 4 °C and were then incubated with CypHer5E linked