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Noradrenaline, oxymetazoline and phorbol myristate acetate induce distinct functional actions and phosphorylation patterns of α_{1A}-adrenergic receptors

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ABSTRACT

In LNCaP cells that stably express α_{1A}-adrenergic receptors, oxymetazoline increased intracellular calcium and receptor phosphorylation, however, this agonist was a weak partial agonist, as compared to noradrenaline, for calcium signaling. Interestingly, oxymetazoline-induced receptor internalization and desensitization displayed greater effects than those induced by noradrenaline. Phorbol myristate acetate induced modest receptor internalization and minimal desensitization. α_{1A}-Adrenergic receptor interaction with β-arrestins (colocalization/coimmunoprecipitation) was induced by noradrenaline and oxymetazoline and, to a lesser extent, by phorbol myristate acetate. Oxymetazoline was more potent and effective than noradrenaline in inducing ERK 1/2 phosphorylation.

Mass spectrometric analysis of immunopurified α_{1A}-adrenergic receptors from cells treated with adrenergic agonists and the phorbol ester clearly showed that phosphorylated residues were present both at the third intracellular loop and at the carboxyl tail. Distinct phosphorylation patterns were observed under the different conditions. The phosphorylated residues were: a) Baseline and all treatments: T233; b) noradrenaline: S220, S227, S229, S246, S250, S389; c) oxymetazoline: S227, S246, S381, T384, S389; and d) phorbol myristate acetate: S246, S250, S258, S351, S352, S401, S402, S407, T411, S413, T451. Our novel data, describing the α_{1A}-AR phosphorylation sites, suggest that the observed different phosphorylation patterns may participate in defining adrenoceptor localization and action, under the different conditions examined.

1. Introduction

The actions of adrenaline and noradrenaline (NA) are mediated through the adrenergic receptors (ARs), which is a family of G protein coupled receptors comprising three subfamilies (α_1-, α_2-, and β-ARs) with three members each (i. e., α_{1A}-, α_{1B}-, and α_{1D}-; α_{2A}-, α_{2B}-, and α_{2C}-; and β_1-, β_2-, and β_3-ARs) [1]. α_1-ARs are involved in a large variety of physiological responses and also in the pathogenesis of many diseases [1–3]. Agonist binding to α_1-ARs activates Gq and subsequently phospholipase C, which generates the second messengers inositol trisphosphate, which triggers calcium signaling, and 1–2-diacylglycerol. β-Arrestins have been shown to be key elements in both G protein-coupled receptor internalization, desensitization and plasma membrane-nucleus connection through the mitogen activated protein kinase pathway [4]. The functional regulation of these receptors is an area on great interest and receptor phosphorylation seems to be a crucial initial event [3].

Interestingly, marked differences exist in the function and regulation of the three α_1-AR subtypes. The α_{1B}-AR subtype is the best characterized, and it is desensitized and phosphorylated in response to agonist stimulation and also by activation of protein kinase C (PKC) ([5–7], see also [2,8] and references therein). The phosphorylation sites for α_{1B}-ARs have been reported and are located in the carboxyl terminus (CTail). These sites seem to be targeted by G protein-coupled receptors kinases (GRKs) and PKC [5,6]. However, the phosphorylation sites for the other two subtypes remain to be elucidated.

α_{1A}-ARs are phosphorylated during agonist stimulation and in response to PKC activation [9–12]. Basal α_{1A}-AR phosphorylation was much less than that observed for the α_{1B}-AR subtype [9]. Chimeric constructions in which the CTails of these receptors were exchanged indicated that the main phosphorylation substrate was the α_{1B}-AR CTail [9]. In addition, it was observed that activation of PKC was much less

Abbreviations: ARs, adrenergic receptors; PKC, protein kinase C; NA, noradrenaline; OXY, oxymetazoline; PMA, phorbol 12-myristate 13-acetate; eGFP, enhanced green fluorescent protein; PBS, phosphate-buffered saline; GRKs, G protein-coupled receptors kinases; IL3, intracellular loop 3; CTail, carboxyl terminus

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efficacious in desensitizing α_{1A}-ARs than α_{1B}-ARs [9,13]. The CTail-truncated α_{1A}-ARs are functional, phosphorylated in response to agonist stimulation and PKC activation, and also subjected to agonist-mediated desensitization and internalization. These data suggest that α_{1A}-AR function is primarily independent of the CTail and that phosphorylation sites could be present in other intracellular domains, such as the intracellular loop 3 (IL3) [12]. Using the chimeric CTail-exchanged α_{1A}-AR/α_{1B}-AR constructs [9], we observed that such exchanges changed the phosphorylation pattern of the core receptors but that there was no linear correlation with the functional consequences [11]. Interestingly, different α_{1A}-AR agonists induce receptor phosphorylation in distinct manners and no clear relationship was observed between receptor phosphorylation, internalization or desensitization, i.e., oxymetazoline (OXY), which was a partial agonist, as compared with NA for receptor phosphorylation, was more effective in inducing receptor desensitization and internalization than the catecholamine [14]. Such biased action has been observed for agonists of many receptors [15,16], and it has been suggested that the phosphorylation patterns or "bar codes"/"flute models" could be relevant for defining agonist pharmacodynamic behavior [17–19]. It was reported recently that in CHO cell, OXY is not only an α_{1A}-AR agonist but also activates 5-hydroxytriptamine (serotonin) receptors, and that this off-target action could explain its apparent biased agonism [20].

In the present work, we studied the effect of NA, OXY, and phorbol 12-myristate 13-acetate (PMA), on human α_{1A}-AR function (i.e., intracellular calcium concentration, receptor phosphorylation, internalization and association to β-arrestins and ERK 1/2 phosphorylation). Additionally, we purified α_{1A}-ARs and studied the phosphorylation sites observed under different conditions (agonist stimulation and PKC activation) through the use of mass spectrometry. Our data clearly indicate that these agents promote different phosphorylation patterns in human α_{1A}-ARs and indicate that OXY is a genuine biased agonist. In addition, to the best of our knowledge, this is the first report identifying phosphorylation sites of α_{1A}-ARs.

2. Materials and methods

2.1. Materials

(-) NA, OXY, PMA, propranolol, phentolamine, 5-hydroxytriptamine, Nonidet P40, Trizma base, and phosphatase and protease inhibitors were obtained from Sigma-Aldrich. 5-Methyl urapidil was purchased from Research Biochemicals, Inc. SB-216641 (N-[3-[3-(dimethylamino) ethoxy]-4-methoxyphenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)-[1,1′-biphenyl]-4-carboxamide hydrochloride) was purchased from Tocris Bioscience. RPMI medium 1640, Dulbecco's modified Eagle's medium, fetal bovine serum, trypsin and antibiotics used for cell culture were purchased from Life Technologies. The following reagents were acquired from the sources indicated: agarose-coupled protein A from Upstate Biotechnology; Fura 2/AM from Molecular Probes; and Lipofectamine 2000 from Invitrogen. [^{3}H] Tamsulosin (56.3 Ci/mmol) was a generous gift from Yamanouchi Europe (Leiderdorp, The Netherlands). [^{125}I] Arylazidoprazosin (2200 Ci/mmol), and [^{32}P]Pi (8500–9120 Ci/mmol) were from New England Nuclear Life Science Products. Rabbit polyclonal antibodies against extracellular signal-regulated kinases (ERK) 1/2 (4695S) and phospho-ERK 1/2 (9101S) were from Cell Signaling Technology. HRP-conjugated rabbit anti-mouse IgG and secondary antibodies were purchased from Zymed (Thermo Fisher Scientific) or Jackson ImmunoResearch, and chemiluminescence kits were from Merck-Millipore. β-Arrestin-1/2 (A-1) mouse monoclonal antibody was from Santa Cruz Biotechnology (Sc-74591). Tetramethyl-rhodamine-conjugated AffiniPure anti-donkey mouse IgG was from Jackson Immunology (715-025-150), and anti-eGFP monoclonal antibody (632381) was obtained from Clontech. Other reagents were from the sources indicated [9,11].

2.2. Cells

The LNCaP cell line, established from a metastatic lesion of human prostatic adenocarcinoma, was obtained from American Type Culture Collection (CRL-1470). Cells were cultured on poly-D-lysine (100 μg/ml) coated plates in RPMI medium 1640. The transfection was performed employing a previously described plasmid, containing the sequence for the α_{1A}-AR tagged, at the carboxyl terminus, with the enhanced-green fluorescent protein (eGFP) [11], using Lipofectamine 2000. The cells were cultured for stable expression in medium containing the neomycin analog, G-418 (600 μg/ml) and different clones were isolated and screened for robust responses and high density receptor expression. In all experiments using NA, 1 μM propranolol was also present, to avoid any β-AR action; the β-blocker did not alter baseline parameters by itself (data not shown). A clone was selected for all the experiments described here and unless otherwise indicated, it was cultured in medium containing G-418 (300 μg/ml).

2.3. Radioligand binding and receptor photoaffinity labeling

Membranes were prepared and radioligand binding and receptor photolabelling studies were performed as previously described [9,11]. In brief, the binding studies were carried out by incubating the radioligand (0.05–5 nM) with membranes for 60 min at 30 °C. The incubation was terminated by the addition of ice-cold buffer and filtration through GF/C filters using a Brandell harvester. Non-specific binding was determined in the presence of 10 μM phentolamine, and specific binding represented approximately 90% of total binding at the K_D. Photoaffinity labeling was performed by incubating membranes with 6 nM [^{125}I] arylazidoprazosin and exposing them to UV light. The samples were centrifuged, dissolved in Laemmli sample buffer [21] and subjected to 10% SDS- polyacrylamide gel electrophoresis containing 7 M urea under reducing conditions [9].

2.4. Intracellular calcium concentration

The cells were loaded with Fura 2/AM, detached by gentle trypsinization, washed, and fluorescence measurements were conducted using an Aminco-Bowman spectrometer with the excitation monochromator set at 340 and 380 nm (chopper interval 0.5 s) and the emission monochromator set at 510 nm [9,11,22]. The intracellular free calcium concentration was calculated according to Grynkiewicz et al. [23].

2.5. Receptor phosphorylation

Receptor phosphorylation was performed as previously described [7,11]. Briefly, cells were serum starved and incubated in phosphate-free Dulbecco's Modified Eagle's media for 1 h. After this, the media was aspirated, and the cells were incubated for 3 h in the same phosphate-free media supplemented with 100 μCi/ml [^{32}P]Pi. The cells were stimulated as indicated, washed with ice-cold phosphate-buffered saline (137.9 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4; pH 7.4) (PBS) and solubilized in buffer containing 100 mM NaCl, 10 mM Tris, 0.1% Nonident P 40, 0.05% SDS and phosphatase and protease inhibitors [11]. The extracts were centrifuged and supernatants were collected and incubated overnight with a rabbit anti-eGFP antiserum generated in our laboratory and validated to immunoprecipitate eGFP-tagged receptors [11,24–27].

2.6. Receptor internalization and colocalization with β-arrestins

The LNCaP cells were seeded at 60% confluence onto glass-bottomed Petri dishes coated with poly-D-lysine and cultured for 3 h at 37 °C in media containing 1% serum and subjected to the described treatments. After stimulation, the cells were washed with PBS and fixed