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Okadaic acid increases the phosphorylation state of α_{1A}-adrenoceptors and induces receptor desensitization

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Abstract

Okadaic acid, a protein phosphatase inhibitor, and phorbol myristate acetate, an activator of protein kinase C, increased the phosphorylation state of α_{1A}-adrenergic receptors. The effects of these agents were of similar magnitude but that of okadaic acid developed more slowly. Wortmannin (inhibitor of phosphoinositide 3-kinase), but not staurosporine (inhibitor of protein kinase C), abolished the effect of okadaic acid on the α_{1A}-adrenoceptor phosphorylation state. The effect of phorbol myristate acetate on this parameter was blocked by staurosporine and only partially inhibited by wortmannin. Okadaic acid markedly increased the co-immunoprecipitation of both the catalytic and regulatory subunits of phosphatidylinositol 3-kinase and of Akt/protein kinase B with the adrenoceptor and only marginally increases receptor association with protein kinase Cε. Okadaic acid induced desensitization of α_{1A}-adrenoceptors as evidenced by a decreased ability of noradrenaline to increase intracellular calcium. Such desensitization was fully reverted by wortmannin. Our data indicate that inhibition of serine/threonine protein phosphatases increases the phosphorylation state of α_{1A}-adrenergic receptor and alters the adrenoceptor function.

Keywords: α_1-adrenoceptor; Receptor phosphorylation; Protein kinase C; Protein phosphatase; Desensitization; Phosphatidylinositol 3-kinase

1. Introduction

Modulation of receptor function is a key event in the adaptation of cells to the changes in the internal milieu and to the overall homeostasis, finely tuning their sensitivity to different stimuli (desensitization–resensitization). Many molecular and cellular processes are involved in such modulation (receptor uncoupling to G proteins, internalization, receptor degradation and recycling and changes in the receptor gene expression, among others); receptor phosphorylation seems to be a very initial key event (Lefkowitz, 1998).

The phosphorylation state of a receptor results from the balance of the activities of two groups of enzymes: protein kinases and protein phosphatases. Three groups of protein kinases are the main modulators of G protein-coupled receptors: a) the G protein-coupled receptor kinases, b) second messenger activated protein kinases such as protein kinase A and protein kinase C, and c) receptors with tyrosine protein kinase activity (Vázquez-Prado et al., 2003). The identity of the specific phosphatases involved is largely unknown.

α_1-Adrenoceptors are a heterogeneous subfamily of G protein-coupled receptors comprised by three isoforms, the α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors (Hieble et al., 1995). There is evidence that the three α_1-adrenoceptor subtypes are subjected to phosphorylation and the roles of G protein-coupled receptor kinases, protein kinase C and other kinases has been studied in some detail (García-Sáinz et al., 2000; Vázquez-Prado et al., 2003). In contrast, the role(s) of protein phosphatases is very little known. Previously, we reported that okadaic acid, tautomycin and calyculin A, protein phosphatase 2A and 1 selective inhibitors, and cypermethrin, an specific protein phosphatase 2B inhibitor, are able to increase the α_{1B}-adrenoceptor phosphorylation state in living cells (Alcántara-Hernández et al., 2000); such effect seems to involve protein kinase C as evidenced by the ability of staurosporine and Ro 31-8220 to inhibit the effect okadaic acid (Alcántara-Hernández et al., 2000). Interestingly, in spite of inducing a marked increase in the receptor phosphorylation state, okadaic acid alters α_{1B}-adrenoceptor function only marginally, which is in marked contrast with the effect of direct activation of protein kinase C

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with phorbol myristate acetate that induces α_{1B}-adrenoceptor phosphorylation and an almost total desensitization (Alcántara-Hernández et al., 2000).

α_{1A}-Adrenoceptors mediate important actions of adrenaline and noradrenaline such as vasoconstriction, cardiac inotropy, genitourinary smooth muscle contraction and modulation of liver metabolism in some species (García-Sáinz et al., 1992; García-Sáinz et al., 1995); these receptors also participate in the development of prostatic hypertrophy (Piascik and Perez, 2001; Schulman et al., 1996). It is particularly in this latter action in which selective α_{1A}-adrenoceptor antagonists have shown important therapeutic value, since long-term therapy seems to be safe and well-tolerated, improving urinary flow and decreasing symptoms (Schulman et al., 1996). In spite of the clear physiological and pathophysiological importance of these receptors little is know on their regulation and even less on the role that protein kinases and phosphatases might play. We have previously shown that agonists and activation of protein kinase C by phorbol myristate acetate induce α_{1A}-adrenoceptor phosphorylation (Vázquez-Prado et al., 2000). Interestingly, the phosphorylation of α_{1A}-adrenoceptors and the desensitization observed were of much lesser magnitude than those observed with the α_{1B} subtype (Vázquez-Prado et al., 2000). It has been reported that okadaic acid markedly attenuated α_1-adrenoceptor (putatively α_{1A})-mediated actions in glia and that such effect is blocked by inhibitors of protein kinase C (Assari et al., 2003). In the present work we studied the effect of okadaic acid in rat-1 cells expressing α_{1A}-adrenoceptors, our results show that the protein phosphatase inhibitor is capable of increasing the receptor phosphorylation state associated to receptor desensitization and that this effect is associated to recruitment of protein kinase C, phosphoinositide 3-kinase and Akt/protein kinase B. Differences were observed with the actions of phorbol myristate acetate. Our results indicate that the actions of okadaic acid are more complex than previously anticipated and that differences in the sensitivity to this phosphatase inhibitor exist among α_1-adrenoceptor subtypes.

2. Materials and methods

2.1. Materials

(−)-Noradrenaline, phorbol myristate acetate, staurosporine, wortmannin, and protease inhibitors were obtained from Sigma. Dulbecco´s modified Eagle´s medium, fetal bovine serum, trypsin, antibiotics, and other reagents used for cell culture were from Life Technologies. [^{32}P]P_i (8500–9120 Ci/mmol) was from NEN Life Science Products. Sepharose-coupled protein A was from Upstate Biotechnology. Fura-2/AM was from Molecular Probes. Nitrocellulose membranes were from Bio-Rad and the chemiluminescence's kits were obtained form Pierce. Antibodies against protein kinase C-selective isoforms, p85αPI3K and p110αPI3K were from Santa Cruz Biotechnology, anti-Akt and anti-*phospho*-Akt antibodies were from BD Pharmigen, and secondary antibodies were from Zymed.

2.2. Cell line and culture

Rat-1 fibroblasts stably expressing the bovine α_{1A}-adrenoceptor, generously provided to us by Drs. R. J. Lefkowitz, M. G. Caron, and L. Allen (Duke University), were cultured in glutamine-containing high-glucose DMEM supplemented with 10% fetal bovine serum, 300 μg/ml neomycin analog G-418 sulfate, 100 μg/ml streptomycin, 100 units/ml penicillin, and 0.25 μg/ml amphotericin B at 37 °C under a 95% air, 5% CO_2 atmosphere as described previously (Vázquez-Prado and García-Sáinz, 1996; Vázquez-Prado et al., 2000).

2.3. Receptor phosphorylation

Rat-1 fibroblasts, expressing the bovine α_{1A}-adrenoceptors were cultured in culture dishes (10-cm diameter). Cells reaching confluence were incubated in phosphate-free DMEM over night in 3 ml that containing [^{32}P]P_i (50 μCi/ml) at 37 °C. Labeled cells were stimulated with as indicated, and then they were washed with ice-cold phosphate-buffered saline and solubilized with 1.0 ml of ice-cold buffer containing 1% Triton X-100, 0.05% sodium dodecyl sulfate, 50 mM NaF, 1 mM Na_3VO_4, 10 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM *p*-serine, 1 mM *p*-threonine, and 1 mM *p*-tyrosine. The plates were maintained on ice for 1 h. Then the extracts were centrifuged at 12,700 ×*g* for 15 min at 4 °C, and the supernatants were immunoprecipitated with a rabbit antiserum against GST-α_{1A}-adrenoceptor fusion protein (Vázquez-Prado and García-Sáinz, 1996; Vázquez-Prado et al., 2000). At least three independent experiments were performed for each treatment. Receptor phosphorylation was detected with a Molecular Dynamics PhosphorImager and quantified with ImageQuant software. Data were within the linear range of detection of the apparatus and were plotted using Prism 3.01, GraphPad software.

2.4. Intracellular calcium concentration

Confluent fibroblasts were incubated for 2 h in DMEM without serum and antibiotics. Cells were loaded with 5 μM Fura-2/AM in Krebs-Ringer Hepes containing 0.05% bovine serum albumin, pH 7.4, for 1 h at 37 °C as described (Vázquez-Prado and García-Sáinz, 1996; Vázquez-Prado et al., 2000). Cells were detached by gentle trypsinization. Experiments were performed with about 10^6 cells suspended in 3 ml of the above-mentioned buffer supplemented with 1.2 mM $CaCl_2$. Fluorescence measurements were carried out with an Aminco-Bowman Series 2 spectrometer with the excitation monochromator set at 340 and 380 nm, chopper interval of 0.5 s, and the emission monochromator set at 510 nm. $[Ca^{2+}]_i$ was calculated (Grynkiewicz et al., 1985) using the software provided by Aminco-Bowman; traces were directly exported to the graphs. When protein kinase inhibitors, okadaic acid or phorbol myristate acetate were used, the cells were in contact with each these agents for 15 min.