α2B-Adrenergic Receptor Activates MAPK via a Pathway Involving Arachidonic Acid Metabolism, Matrix Metalloproteinases, and Epidermal Growth Factor Receptor Transactivation*

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Received for publication, October 22, 2001, and in revised form, February 20, 2002 Published, JBC Papers in Press, March 12, 2002, DOI 10.1074/jbc.M110142200

We have investigated the mechanisms whereby α2B-adrenergic receptor (α2B-AR) promotes MAPK activation in a clone of the renal tubular cell line, LLC-PK1, transfected with the rat nonglycosylated α2B-AR gene. Treatment of LLC-PK1-α2B with UK14304 or dexamethasone caused arachidonic acid (AA) release and ERK2 phosphorylation. AA release was abolished by prior treatment of the cells with pertussis toxin, quinacrine, or methyl arachidonyl fluorophosphonate but not by the addition of the MEK inhibitor U0126. The effects of α2-agonists on MAPK phosphorylation were mimicked by cell exposure to exogenous AA. On the other hand, quinacrine abolished the effects of UK14304, but not of AA, suggesting that AA released through PLA2 is responsible for MAPK activation by α2B-AR. The effects of α2-agonists or AA were PKC-independent and were attenuated by indomethacin and nordihydroguaiaretic acid. Agonists or AA were PKC-independent and were attributable for MAPK activation by α2B-AR. The effects of α2-agonists or AA were PKC-independent and were attenuated by indomethacin and nordihydroguaiaretic acid.

Like for other ARs (9), it is probable that the mechanisms whereby α2-ARs promote MAPK activation are highly dependent upon the subtype considered and the particular cell type it is expressed in. So far, the precise pathways of the mitogenic signal transmission were exclusively examined for α2A-AR. In HEK 293 cells (10), activation of ERK1/2 by α2A-AR is primarily triggered through release of βγ subunits from pertussis toxin-sensitive G proteins, stimulation of phospholipase Cβ, phosphoinositide hydrolysis, increase of intracellular Ca2+, and successive activation of Pyk2 and Src. Activation of Src causes the formation of the Shc-Grb2-Sos complex, which leads to ERK phosphorylation via the Ras/Raf/MEK cascade. In COS cells (11), α2A-AR-induced phosphorylation of ERK2 proceeds via two distinct pathways, which are dependent (“transactivation pathway”) or not (“direct pathway”) on the tyrosine kinase activity of the EGF receptor (EGF-R).

Recent experiments carried out on rat proximal tubule cells in primary culture and on LLC-PK1 cells transfected with the rat nonglycosylated α2-B-AR (RNG) gene (LLC-PK1-α2B) have shown that α2B-ARs promote MAPK activation and arachidonic acid (AA) release (13). The sequential relationship between PLA2 and MAPK activation was not investigated. As demonstrated in eosiophil cells during the process of adhesion to fibronectin (14), PLA2 activation may result from its phosphorylation by MAPKs. Conversely, as shown in rabbit renal epithelial cells for angiotensin II receptor, the activation of MAPK could be the consequence of AA release (15). Based on

* This work was supported by the BIOMED 2 Program PL963373 (European Commission, Brussels, Belgium) and by a grant from the Fondation pour la Recherche Médicale (Paris, France). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The α2-adenoreceptors (α2-ARs) are members of the G-protein-coupled receptor superfamily that mediate physiological responses to the endogenous catecholamines, such as reduction of blood pressure, sedation, platelet aggregation, and inhibition of renin release or insulin secretion. Three subtypes of α2-ARs (namely α2A, α2B, and α2C) have been identified (1). Although recent studies, conducted on mice with genetic alterations of α2-AR expression, have clarified the respective roles of α2A-, α2B-, and α2C-ARs in the mediation of the cardiovascular and sedative effects of α2-agonists, the precise functions of each subtype are far from being definitively elucidated (2). Until recently, the effects of α2-ARs were generally considered as exclusively due to the modulation of effectors such as adenyl cyclase and phospholipase Cβ. There is now accumulating evidence that, in addition to these pathways, α2-ARs are also involved in the regulation of cell growth via stimulation of mitogen-activated protein kinases (MAPKs). The phosphorylation of MAPKs has been observed in transfected cells (3, 4) as well as in various types of cells spontaneously expressing α2-ARs (5, 6). The three receptor subtypes promoted phosphorylation of ERK1 and ERK2 in Chinese hamster ovary cells (3). According to results obtained in HEK 293 and COS cells (7, 8), this effect is independent of receptor internalization via clathrin-coated pits.

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Mechanism of ERK Activation by α2B-Adrenergic Receptor

The use of different inhibitors, the present work demonstrates that activation of MAPK by α2B-AR is, in LLC-PK1-α2B, primarily mediated by a pathway involving stimulation of PLA2, generation of AA derivatives, activation of matrix metalloproteinases, release of HB-EGF, and transactivation of EGF-R.

EXPERIMENTAL PROCEDURES

Drugs and Reagents—UK14304, dexmedetomidine, and RX821002 were respectively donated by Pfizer (Sandwich, UK), Orion Pharma (Turku, Finland), and Reckitt and Colman Laboratories (Kingston-upon-Hull, UK). [3H]RX21002 (59 Ci/mmol), [3H]AA (202 Ci/mmol), nitroblue tetrazolium, and A-agarose beads. Immune complexes were extensively washed with Amersham Biosciences (Courtaboeuf, France). Arachidonic acid, quinacrine, methyl arachidonyl fluorophosphonate, [Glu52]diphtheria toxin (CRM 197), and U0126 were obtained from Calbiochem. Indo- methacin, ketokonazole, nordsydroguaeric acid (NDGA), phorbol 12-myristate 13-acetate (PMA), tyrphostin AG1478, EGF, staurosporine, 1,10-phenanthroline, and all other chemicals were from Sigma. Petal calf serum was purchased from Invitrogen. Anti-ERK1 and anti-ERK2 polyclonal Ab were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-active MAPK was from Promega (Madison, WI). Anti-Shc polyclonal Ab and fluorescein-conjugated goat anti-rabbit IgG were respectively purchased from Upstate Biotechnology, Inc. (Lake Placid, NY) and Nordic Immunological Laboratories (Tilburg, The Netherlands).

Culture of LLC-PK1-α2B Cells—The clone of the renal tubular cell line, LLC-PK1, permanently expressing the rat α2B-AR was obtained by transfection with a pcDNA3 vector containing the coding region of the R Ng gene. LLC-PK1-α2B cells were routinely grown in Dulbecco’s modified Eagle’s medium containing 25 mM glucose, 100 μg/ml streptomycin, 100 μg/ml penicillin, and 5% fetal calf serum. Binding experiments with [3H]RX21002 showed that the level of receptor expression was 73 ± 5 fmol/mg of protein.

Detection of ERK1/2 and Shc—Three days postseeding, cells were placed for 24 h in culture medium free of serum. They were then exposed to the compound to be tested, rapidly rinsed with ice-cold phosphate-buffered saline, and harvested in 1 ml of radioimmune precipitation buffer (10 mM Tris-HCl, pH 7.4, 1% Triton-X100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM aprotinin). Soluble proteins were extracted by centrifugation (15,000 × g, 15 min at 4 °C), separated by SDS-PAGE, and blotted onto a nitrocellulose membrane. Phosphorylated forms of MAPKs were revealed by chemiluminescence using an anti-active MAPK Ab. Shc phosphoproteins were detected by immunoprecipitation. Briefly, 500 μl of cell lysate were incubated over-night at 4 °C with 5 μg of rabbit polyclonal Shc-Ab and 50 μl of protein A-agarose beads. Immune complexes were extensively washed with ice-cold radioimmunoprecipitation buffer, dried, and denatured in Laemmli buffer. Samples were subjected to SDS-PAGE, transferred onto an nitrocellulose membrane, and probed with horseradish peroxidase-conjugated anti-phosphotyrosine Ab. In all experiments, the membranes were stripped of Ig and reprobed using either a mixture of anti-ERK1 Ab and anti-ERK2 Ab or anti-Shc Ab. Films were analyzed by densitometry, and the extent of phosphorylation was normalized to protein loading.

Measurement of AA Release—Cells rendered quiescent by a 24-h period of serum deprivation were labeled for 10 h with 1 μCi/ml [3H]AA. They were carefully washed in Dulbecco’s modified Eagle’s medium containing 10 mM Hepes and 0.2% fatty acid-free bovine serum albumin and then exposed to the drug to be tested. Aliquots of the incubation medium were collected every 10 min over a period of 30 min and centrifuged (20,000 × g, 10 min, 4 °C), and the radioactivity was measured in the supernatant.

Immunofluorescence Microscopy—Cells plated on glass coverslips were grown, rendered quiescent as indicated above, and exposed or not to the compound to be tested. They were fixed in 4% paraformaldehyde (15 min) and treated with 50 mM NH4Cl in phosphate-buffered saline (10 min). The cells were permeabilized first in phosphate-buffered saline buffer containing 0.05% saponin and 0.2% bovine serum albumin (15 min) and then in methanol (10 min at −20 °C). All subsequent steps were carried out in a permeabilization buffer and were separated by several washes. The cells were incubated with ERK2 polyclonal Ab (1:400) and then with fluorescein-conjugated goat anti-rabbit IgG (1:400). The coverslips were finally washed in phosphate-buffered saline, mounted in fluorescent mounting medium (Dako Corp., Carpinteria, CA), and examined under epifluorescence illumination. Digital images were captured using the software CoolSNAP (Roper Scientific GmbH, Munich, Germany) and processed with Adobe Photoshop 4 (Adobe Systems Inc., San Jose, CA).

Statistical Analysis—Results are expressed as mean ± S.E. for the number of experiments indicated (n). The data were analyzed using Student’s t test, and a p value <0.05 was considered statistically significant.

RESULTS

AA Release Is Involved in α2B-AR-induced MAPK Phosphorylation—A previous study from our group has shown that exposure of proximal tubule cells to α2B-agonists resulted in activation of MAPK and in an increase of AA release (13). As depicted in Fig. 1A, the treatment of LLC-PK1-α2B with 1 μM UK14304 induced a time-dependent increase of the tyrosine phosphorylation of p42 MAPK. The effect is maximal between 10 and 20 min and persists for at least 40 min. In addition, cell exposure to UK14304 caused an acceleration of AA release, which was abolished by 20 μM quinacrine or 50 μM methyl arachidonyl fluorophosphonate (not shown). Effects of the α2B-agonist on MAPK phosphorylation and AA release were abolished by pretreatment of the cells with pertussis toxin (Fig. 1, B and C). On the other hand, the addition of the MEK inhibitor, U0126, blunted phosphorylation of MAPK but did not affect the augmentation of AA release induced by UK14304. Similar results were obtained using dexmedetomidine, suggesting that stimulation of PLA2 activity by α2B-agonists is not the consequence of MAPK activation.

In primary culture of rabbit proximal tubule cells, activation of MAPK by angiotensin II receptor is the consequence of AA release (16). In a first step to evaluate the putative role of AA as an intermediary between activated α2B-AR and MAPK, LLC-PK1-α2B cells were treated with AA. As shown in Fig. 2A, AA induced a dose-dependent increase in MAPK phosphorylation. The effect of AA was detectable at 200 nM and reached a maximum at 20 μM. Such concentrations are in the physiological range, since the level of free AA was estimated to be 5 μM in the rat kidney (17). Exposure to AA also resulted in the redistribution of ERK2 from the cytoplasm to the nucleus (Fig. 2B), showing that phosphorylation of MAPK could be correlated with the translocation of ERK2. However, in contrast to that for UK14304, the effect of AA was not affected by pretreatment of the cell with pertussis toxin (Fig. 2C). In a second step, the role of endogenous AA release in the activation of MAPK by α2B-agonist was evaluated using PLA2 inhibitors. Of the compounds tested, quinacrine was the only one with no side effect; all others, including methyl arachidonyl fluorophosphonate and AACOCF3, caused by themselves a significant increase in ERK2 phosphorylation. Such an undesirable effect was previously reported for methyl arachidonyl fluorophosphonate in macrophage (18). Preincubation of LLC-PK1-α2B for 5 min with 20 μM quinacrine totally abolished MAPK phosphorylation induced by UK14304 but not by AA (Fig. 3A). Again, results from Western blotting were confirmed by examination of the subcellular distribution of ERK2. Indeed, translocation to the nucleus following exposure to UK14304 is abrogated by quinacrine pretreatment (Fig. 3B). Altogether, these results are therefore consistent with the implication of PLA2 and AA generation in MAPK activation induced by α2B-AR.

MAPK Phosphorylation Depends on Generation of AA Derivatives—In rabbit proximal tubule, the effect of AA on MAPK depends on the generation of an epoxy metabolite (16). The production of AA derivatives results in, most mammalian cells, from the activation by distinct enzymatic systems, namely the lipoxygenase, cyclooxygenase, and cytochrome P450-dependent epoxygenase. To evaluate the respective contribution of these pathways, MAPK activation was examined in LLC-PK1-α2B treated with inhibitors prior to stimulation with UK14304. As shown in Fig. 4, the lipoxygenase inhibitor NDGA...
significantly diminished the MAPK phosphorylation induced by UK14304. A decrease was also observed with the cyclooxygenase inhibitor indomethacin (50 \( \mu \)M) but not with the epoxygenase inhibitor ketoconazole (30 \( \mu \)M). Of note, the combined pretreatment with NDGA and indomethacin completely abolished the phosphorylation of ERK induced by UK14304 or exogenous AA. These findings strongly suggest that cyclooxygenase and/or lipooxygenase activities are essential in the mediation of the effects of \( \alpha_2 \)-AR.

Although metabolic products are responsible for many of the indirect effects of AA, some are the direct consequence of PKC activation. To determine whether PKC participated in MAPK activation by AA, experiments were carried out in the presence of staurosporine (Fig. 5). Treatment of the cells with 200 nM staurosporine (Fig. 5).

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Fig. 3. Quinacrine abolishes MAPK activation by UK14304. A, effect of quinacrine on MAPK phosphorylation induced by UK14304 or AA. Serum-deprived LLC-PK1-α2B cells were incubated for 5 min in the presence of vehicle or 20 μM quinacrine and then exposed or not for 10 min to 1 μM UK14304 or 20 μM AA. Phosphorylated and total MAPKs were respectively revealed using anti-active MAPK Ab (upper panels) and a mixture of anti-ERK1 Ab and anti-ERK2 Ab (lower panels). B, effect of quinacrine on UK14304-induced translocation of ERK2. LLC-PK-α2B cells grown on glass coverslips were rendered quiescent by serum deprivation and treated (Qui) or not (Control) for 5 min with quinacrine prior to 15-min exposure to 1 μM UK14304 (UK). Cells were fixed and permeabilized, and the localization of ERK2 was assessed by immunofluorescence using anti-ERK2 polyclonal Ab and fluorescein-conjugated goat anti-rabbit IgG. Images shown are representative of three independent experiments (scale bar = 10 μm).

Fig. 4. Effects of inhibitors of AA metabolism on MAPK phosphorylation. Serum-deprived LLC-PK1-α2B cells were incubated for 5 min in the presence of 10 μM NDGA (NDGA), 50 μM indomethacin (INDO), 30 μM ketoconazole (KETO), or 10 μM NDGA plus 50 μM indomethacin (NDGA/INDO) and then exposed for 10 min to 1 μM UK14304 (UK) or 20 μM AA (AA). MAPK phosphorylation was revealed using anti-active MAPK Ab (upper panel), and protein loading was assessed by reprobing the blot with a mixture of anti-ERK1 Ab and anti-ERK2 Ab (middle panel). Band intensity was semiquantified by densitometric scanning of the film (lower panel). Reported results are mean ± S.E. (n = 3). *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus respective control values.

staurosporine totally abolished the phosphorylation of MAPK induced by PMA, proving that the different isoforms of PKC were truly inhibited. By contrast, it did not prevent AA-induced MAPK phosphorylation. Thus, as previously found for α2-agonists (13), the effect of AA is independent of PKC.

MAPK Phosphorylation Requires Metalloproteinase Activity and EGF-R Transactivation—Previous studies carried out on rabbit proximal tubule have shown that cell treatment with AA resulted in a significant increase of EGF-R phosphorylation and its subsequent association with Shc (19). In our model, the role of EGF-R activation was first investigated using the specific inhibitor of EGF-R tyrosine kinase activity, tyrphostin AG1478. As shown in Fig. 6A, the preincubation of LLC-PK1-α2B cells in culture medium containing 100 nM tyrphostin AG1478 prevented the phosphorylation of MAPK caused by UK14304, dexametomidine, or AA. Thus, the transactivation of EGF-R plays a critical role in the mediation of the effect of α2B-AR on MAPK. According to recent evidence, EGF-R transactivation by G-protein-coupled receptor requires the cleavage of pro-HB-EGF by matrix metalloproteinases. Therefore, we next investigated whether MAPK phosphorylation was sensitive to the inhibitor of the matrix metalloproteinases, batimastat (Fig. 6B). Pretreatment of the cells with 5 μM batimastat neither affected the basal level of MAPK phosphorylation nor inhibited the response to EGF but resulted in a blockade of the effect of UK14304 or AA. Similar results were obtained with 1,10-phenanthroline (not shown). The implication of HB-EGF release was examined using the diphtheria toxin mutant, CRM 197. Pretreatment of LLC-PK1-α2B cells with CRM 197 (200 ng/ml) had no effect on the ability of exogenous EGF to activate MAPK (not shown) but strongly inhibited the effect UK14304 or AA (Fig. 6C). The release of a factor, with EGF activity and acting in an autocrine/paracrine mode, was confirmed by experiments in which the effect of conditioned medium from LLC-PK1-α2B was assayed on wild-type LLC-PK1 (Fig. 7). Incubation of wild-type cells in medium collected from nonstimulated LLC-PK1-α2B or their direct treatment with UK14304 did not cause any change in the extent of MAPK phosphorylation (not shown). In contrast, a clear increase was observed when medium came from LLC-PK1-α2B treated with UK14304. As expected, this response was blocked by tyrphostin AG1478 but was unaffected by the addition of quinacrine or batimastat or by the prior treatment of wild-type LLC-PK1 with pertussis toxin. It is well established that activation of MAPK by EGF-R occurs via the tyrosine phosphorylation of adapter proteins such as Shc and the recruitment of Grb2-Sos complexes. Previous experiments on rat proximal tubule cells in primary
Therefore, we examined whether AA induces phosphorylation of the p46 and p52 isoforms of Shc (13). They were then exposed for 10 min to either 1 μM UK14304 (UK), 20 μM AA (AA), or 5 ng/ml EGF (EGF). Phosphorylated and total MAPKs were respectively revealed using anti-active MAPK Ab (upper panel) and a mixture of anti-ERK1 Ab and anti-ERK2 Ab (lower panel). A, effect of tyrphostin AG1478 on MAPK phosphorylation induced by G-protein-coupled receptors. As depicted in Fig. 8, treatment of the cells with the inhibitor of EGF-R tyrosine kinase was stripped of Ig and reprobed using anti-Shc Ab to assess protein expression. B, effect of batimastat on MAPK phosphorylation induced by UK14304 or AA. Serum-deprived LLC-PK1-α2B cells were incubated or not for 30 min in the presence of 5 μM batimastat (BAT). C, effect of CRM 197 on MAPK phosphorylation induced by UK14304 or AA. Serum-deprived LLC-PK1-α2B cells were incubated or not for 1 h in the presence of 200 ng/ml CRM 197. The mechanisms whereby G-protein-coupled receptors activate the MAPK cascade are highly dependent upon the receptor considered and the cell type it is expressed in. Although previous studies have shown that the action of the α2-AR is independent of receptor internalization (7, 8), the signaling pathway(s) accounting for the phosphorylation of MAPK by this receptor subtype remains poorly defined. The results obtained in this study provide substantial evidence that, in LLC-PK1-α2B, the activation of ERK by α2-agonists is triggered via a mechanism comprising the activation of matrix metalloproteinases, the release of HB-EGF, and the subsequent activation of the EGF-R. This cascade was demonstrated by the following observations. First, UK14304-induced phosphorylation of MAPK is totally abrogated in the presence of the matrix metalloproteinase inhibitors (batimastat or 1,10-phenanthroline) and by prior treatment of the cells with CRM 197. Second, conditioned medium from LLC-PK1-α2B cells treated with UK14304 causes activation of MAPK in wild-type LLC-PK1, even in the presence of batimastat. Third, the consequences of LLC-PK1-α2B exposure to α2-agonists or of wild-type LLC-PK1 exposure to conditioned medium are abolished by prior treatment of the cells with the inhibitor of EGF-R tyrosine kinase activity, tyrphostin AG1478. Previous studies of lysophosphatidic acid receptor or α2AR have demonstrated that the con-
tetroentaonic acid and on PKC activation (25). Dependence on lipoxygenase and PKC activity was also found in human neutrophils (26). In this cell type, the effects of AA engaged a membrane receptor linked to G_{13} proteins (27). This is not the case in LLC-PK1-α_{2B}, since neither staurosporine nor pertussis toxin treatment abolished ERK phosphorylation caused by AA. Regarding these points, LLC-PK1-α_{2B} resembles rabbit renal epithelial cells. However, it is epoxy derivatives that mediate the effects of AA on MAPK phosphorylation in these cells (16). Whereas involvement of the cytochrome P450 pathway can be excluded in our model, the respective contribution of COX and LOX is still unclear, since it is difficult to reconcile why products from either pathway could function similarly. The implication of COX activity is beyond doubt, because the effects of α_{2B}-agonists and AA were also blocked by aspirin (not shown). By contrast, that of LOX is more questionable, since NDGA can also interfere with COX activity and act as an antioxidant. Alternatively, the possibility that prostaglandins and leukotrienes act in concert cannot be definitively ruled out. Indeed, the combined action of COX and LOX was already demonstrated to be necessary for some of the effects of angiotensin II in rat kidney and bovine bronchi (28, 29). It is therefore clear that the identification of the AA metabolites responsible for MAPK activation in LLC-PK1-α_{2B} will require future study. In addition, the mechanism whereby these products may affect matrix metalloproteinase activity has yet to be defined. In line with the existence of a relationship between the two phenomena, constitutive expression of cyclooxygenase-2 in human colon cancer cells results in increased activation of MMP-2 (30), whereas inhibitors of PLA2 and cyclooxygenase-2 reduce the release of matrix metalloproteinases in prostate tumor cells (31).

In conclusion, our results provide evidence for a pathway by which α_{2B}-AR activates MAPK through stimulation of PLA2, generation of AA metabolites by cyclooxygenase and/or lipoxygenase, stimulation of matrix metalloproteinases, release of HB-EGF, and transactivation of the EGF-R (Fig. 9). Whether this scenario is particular to α_{2B}-AR in LLC-PK1 or whether it can be extended to other cell types and/or other G-protein-coupled receptors remains to be established.

**Acknowledgments**—We thank Dr. C. Flordellis for valuable discussion and F. Quincho for excellent technical assistance.
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J. Biol. Chem. 2002, 277:19882-19888.
doi: 10.1074/jbc.M110142200 originally published online March 12, 2002

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