α2b-Adrenergic receptors activate MAPK and modulate proliferation of primary cultured proximal tubule cells

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Cussac, Daniel, Stéphane Schaan, Céline Gales, Christodoulos Flordellis, Colette Denis, and Hervé Paris. α2b-Adrenergic receptors activate MAPK and modulate proliferation of primary cultured proximal tubule cells. Am J Physiol Renal Physiol 282: F943–F952, 2002. —In the rat proximal tubule, the α2b-adrenergic receptor (α2b-AR) enhances Na+/H+ reabsorption by increasing the activity of Na+/H+ exchanger isoform NHE3. The mechanisms involved are unclear, and inhibition of cAMP production remains controversial. In this study, we reinvestigated α2b-AR signaling pathways using rat proximal tubule cells (PTC) in primary culture and LLC-PK1 cells permanently transfected with the RING gene (rat nonglycosylated α2b-AR). Binding experiments indicated that PTC express substantial amounts of α2b-AR (130 fmol/mg protein), and only RING transcripts were detected. In both cell types, the α2b-AR is coupled to G protein, and its stimulation by dexmedetomidine, but not by UK-14304, provoked a significant inhibition of the accumulation of cAMP induced by forskolin or parathyroid hormone. Exposure to α2-agonists increased arachidonic acid release and caused extracellular signal-regulated kinase (ERK)1/2 phosphorylation, which correlated with enhanced mitogen-activated protein kinase (MAPK) activity and nuclear translocation. MAPK phosphorylation was blunted by pertussis toxin but not by protein kinase C desensitization, and it coincided with transient phosphorylation of Shc. Finally, treatment with UK-14304 accelerated cell growth. Further studies will be necessary to clarify the precise mechanism of MAPK activation, but the present data suggest that α2b-AR may play a positive role during tubular regeneration.

FUNCTIONAL STUDIES, CARRIED OUT IN VARIOUS ANIMAL SPECIES, HAVE DEMONSTRATED THAT α2-adrenergic receptors (α2-ARs) ARE OF PREDOMINANT IMPORTANCE FOR THE MEDICATION OF THE REGULATORY EFFECTS OF CATECHOLAMINES ON SEVERAL RENAL FUNCTIONS, INCLUDING RENIN RELEASE, GLOMERULAR FILTRATION, AND Na+ AND WATER EXCRETION (8, 27, 38). PHARMACOLOGICAL CHARACTERIZATION AND MOLECULAR CLONING HAVE PROVIDED EVIDENCE THAT α2-ARs ARE A HETEROGENEOUS CLASS OF RECEPTORS COMPRISING THREE SUBTYPES (α2A, α2B, AND α2C) ENCODED BY DISTINCT GENES (33). All three subtypes have been detected as mRNA in the human and rat kidney (3, 15, 26). However, the respective abundance, precise anatomic location, and specific roles of the corresponding proteins remain somewhat controversial.

In the rat proximal tubule, the stimulation of α2-ARs induces an augmentation of Na+ and bicarbonate reabsorption by increasing the activity of the Na+/H+ exchanger (30). This increase is primarily due to the activation of the NHE3 isoform that is expressed in the brush-border membrane of proximal tubule cells (PTC). Additionally, α2-agonists were found to enhance the activity of the Na+/K+ATPase located in the basal membrane (2). Thus both mechanisms may coordinately contribute to the acceleration of Na+ reabsorption. According to binding and immunolocalization studies (17), it is clear that the effects of α2-agonists on the proximal tubule are primarily due to stimulation of α2b-AR, the expression of which is particularly high in this segment of the nephron. However, the molecular mechanisms responsible for the activation of NHE3 and Na+/K+ATPase are not fully understood.

In almost every known cell type, α2-ARs are coupled to Gs/Go proteins, and their stimulation causes inhibition of cAMP production. In agreement with this view, (−)-epinephrine was found to inhibit parathyroid hormone (PTH)-induced cAMP accumulation in microdissected proximal tubule (42) as well as in opossum kidney (OK) cells, a model for proximal tubule cells that express an α2-AR of the α2C-subtype (28). The search for alternative pathways of signal transduction demonstrated that α2b-AR stimulates protein kinase C (PKC) activity and inositol trisphosphate production in the distal convoluted tubule (14). On the other hand, studies in transfected Chinese hamster ovary (CHO) cells showed increased cystolic phospholipase A2 (cPLA2) activity (1). However, none of these effects has ever been demonstrated in the proximal tubule.

Originally identified as a signaling pathway activated by tyrosine kinase receptors, extracellular signal-regulated kinases (ERKs) are now well docu-
mented to be also stimulated by a large variety of receptors coupled to G proteins. As shown by experiments initially performed in transfected cells (21, 44), G, coupled receptors activate ERK via release of Bβ subunits and subsequent tyrosine phosphorylation of the adaptor protein Shc (25). Phosphorylation of Shc provokes its association with Grb2 and Son, which leads to increased GTP binding to ras and consecutive activation of raf, mitogen-activated protein kinase (MAPK)-ERK kinase (MEK), and MAPK. Recently, activation of ERK was reported after stimulation of the α2C-AR (LLC-PK1) gene (27). Brieﬂy, membranes were incubated for 30 min at 37°C in HBSS containing 0.3 mg/ml collagenase and 1 mg/ml BSA. The resulting cell suspension was centrifuged for 2 min at 500 g, and the particulate fraction was washed twice in HBSS containing 1 mg/ml BSA. The ﬁnal pellet was taken up in 30 ml of 42% Percoll made isotonic with 10× Krebs-Henseleit buffer. After centrifugation (20,000 g, 30 min at 4°C), the layer corresponding to proximal tubules was retrieved and washed twice in DMEM-Ham’s F-12 medium (1:1). The protein concentration was determined using BSA as a standard (5), and the isolated proximal tubules were plated (20 µg cellular protein/cm²) on collagen-coated plastic dishes in culture medium consisting of DMEM-Ham’s F-12 mixture containing 5% FCS, 10 µg/ml insulin, 10 ng/ml EGF, 5 ng/ml transferrin, 0.1 µM dexamethasone, and 5 µM triiodothyronine. The cells were maintained undisturbed under a 5% CO₂ atmosphere at 37°C for 48 h, after which the medium was exchanged for a serum-free medium.

RNA extraction and RNase protection assay. Cellular RNAs were extracted using the guanidinium isothiocyanate/phenol-chloroform method (6). The probe used in the RNase protection assay (RPA) was obtained by cloning the PstIUpdT1 fragment corresponding to nucleotides 628–897 of the ORF of the RING gene (47) into pKS+ (Stratagene, La Jolla, CA). Labeled antisense RNA was synthesized from the linearized matrix using T3 RNA polymerase (Promega) in the presence of [α-32P]UTP. Assays were performed as described previously (34); RNA were taken up in 30 µl of hybridization buffer (80% deionized formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM PIPES, pH 6.7) containing an excess of [α-32P]-labeled riboprobe. The samples were heated to 95°C for 5 min and then immediately placed at 55°C for 14 h. Nonhybridized probe was eliminated by the addition of 0.3 ml of RNase A (40 µg/ml) and RNase T1 (2 µg/ml) in 300 mM NaCl, 5 mM EDTA, and 10 mM Tris-HCl (pH 7.5). After 2 h at 37°C, digestion was stopped by addition of 5 µl of proteinase K (10 mg/ml), and the samples were further incubated for 15 min at 37°C. Carrier trDNA (10 µg) and 0.3 ml of 4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.1 M 2-mercaptoethanol, and 0.5% sarkosyl (solution D) were added to each tube, and protected hybrids were precipitated with isopropyl alcohol. Pellets were washed with 70% ethanol, air-dried, taken up in sample buffer (97% deionized formamide, 0.1% SDS, 10 mM Tris-HCl, pH 7.0) and run on a 5% polyacrylamide gel containing 7 M urea. The gels were ﬁxed, dried, and exposed for 48 h at –80°C to X-ray ﬁlm for autoradiography.

Membrane preparation and radioligand binding studies. Frozen cells were harvested in 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM EDTA and centrifuged at 27,000 g for 10 min (4°C). The pellet was taken up in 50 mM Tris-HCl and 0.5 mM MgCl₂, pH 7.5 (TM buffer) and centrifuged again. The ﬁnal pellet was suspended in the appropriate volume of TM buffer and immediately used for binding experiments as described previously (34). Brieﬂy, membranes were incubated at 25°C in a 400-µl ﬁnal volume of TM buffer containing the 3H-labeled antagonist. After a 45-min period of incubation, membrane-bound radioactivity was separated from free by rapid ﬁltration through a Whatman GFC ﬁlter. Retained radioactivity was counted by liquid scintillation spectrometry, and speciﬁc binding was calculated as the difference between total and nonspeciﬁc binding determined in the presence of 10⁻⁶ M phenolamine. For saturation experiments, the total radioligand concentration ranged from

Materials and Methods

Drugs and reagents. [3H]RX-821002 (59 Ci/mmol), [3H]arachidonic acid (202 Ci/mmol), and an enhanced chemiluminescence (ECL) Western blotting system were from Amersham Pharmacia Biotech (Courtabœuf, France). [3H]MK-912 (81 Ci/mmol) and Polyscreen polyvinylindene difluoride (PVDF) membranes were from NEN Life Science (Boston, MA). α-[32P]UTP (800 Ci/mmol) and γ-[32P]ATP (5,000 Ci/mmol) were purchased from ICN (Costa Mesa, CA). Phentolamine was donated by Ciba-Geigy (Basel, Switzerland), dexametomidine by Orion Pharma (Turku, Finland), and RX-821002 by Reckitt and Colman Laboratories (Kingston-upon-Hull, UK). UK-14304 and prazosin hydrochloride were generous gifts from Pfizer (Sandwich, UK). Collagenase, insulin, dexamethasone, transferrin, epidermal growth factor (EGF), triiodothyronine, PTH, oxytocin, forskolin, pertussis toxin, 5'-guanylylimidodiphosphate (GppNHp), phorbol 12-myristate 13-acetate (PMA), and all other chemicals were from Sigma (St. Louis, MO). Fetal calf serum (FCS) was from Calf Serum (Becton, Dickinson, Franklin Lakes, NJ). BSA at a concentration of 1 g/ml was used in all experiments. Cells were maintained undisturbed under a 5% CO₂ atmosphere at 37°C. Carrier trRNA (10 µg) and 0.3 ml of 4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.1 M 2-mercaptoethanol, and 0.5% sarkosyl (solution D) were added to each tube, and protected hybrids were precipitated with isopropyl alcohol. Pellets were washed with 70% ethanol, air-dried, taken up in sample buffer (97% deionized formamide, 0.1% SDS, 10 mM Tris-HCl, pH 7.0) and run on a 5% polyacrylamide gel containing 7 M urea. The gels were fixed, dried, and exposed for 48 h at –80°C to X-ray ﬁlm for autoradiography.

Culture of LLC-PK₁ cells and generation of α2AR transfectants. The renal tubular cell line LLC-PK₁ was routinely grown in DMEM containing 25 mM glucose, 100 µg/ml streptomycin, 100 IU/ml penicillin, and 5% FCS. LLC-PK₁ cells permanently expressing the rat α2B-AR (LLC-PK₁-α2B) or the human α2A-AR (LLC-PK₁-α2A) were respectively obtained by transfection with pcDNA3 vector containing the coding region of the RING or α2C10 genes. Cells were transfected using the calcium-phosphate method. Two days posttransfection, they were subcultured in the presence of G418-sulfate (1 mg/ml), and antibiotic resistant cells were selected.

Proximal tubule preparation and culture. PTC were isolated as previously described by Vinay et al. (43). Male Sprague-Dawley rats (4 wk of age, mean weight 40 g) were purchased from Harlan-France (Gannat, France). The animals were killed by intraperitoneal injection of pentobarbital sodium (50 mg/kg). The kidneys were rapidly removed, decapsulated, and placed in ice-cold Hanks’ balanced saline solution (HBSS) buffered with 10 mM HEPES (pH 7.4). Cortex slices were incubated for 30 min at 37°C in HBSS containing 0.3 mg/ml collagenase and 1 mg/ml BSA. The resulting cell suspension was centrifuged for 2 min at 500 g, and the particulate fraction was washed twice in HBSS containing 1 mg/ml BSA. The ﬁnal pellet was taken up in 30 ml of 42% Percoll made isotonic with 10× Krebs-Henseleit buffer. After centrifugation (20,000 g, 30 min at 4°C), the layer corresponding to proximal tubules was retrieved and washed twice in DMEM-Ham’s F-12 medium (1:1). The protein concentration was determined using BSA as a standard (5), and the isolated proximal tubules were plated (20 µg cellular protein/cm²) on collagen-coated plastic dishes in culture medium consisting of DMEM-Ham’s F-12 mixture containing 5% FCS, 10 µg/ml insulin, 10 ng/ml EGF, 5 ng/ml transferrin, 0.1 µM dexamethasone, and 5 µM triiodothyronine.
0.5 to 16 nM for [3H]RX-821002 and from 0.25 to 8 nM for [3H]MK-912. The values of maximal binding (B max), the dissociation constant (K d), and the inhibition constant (I K) were calculated from computer-assisted analysis of the data using GraphPad Prism (GraphPad Software, San Diego, CA).

Measurement of intracellular levels of cAMP. The measurement of intracellular levels of cAMP was carried out in cells at day 3 of culture. Cells were plated for 6 h in HEPES-buffered DMEM containing 0.2 mM 3-isobutyl-1-methylxanthine. The experiments were started by adding the appropriate concentration of hormone and/or drug to be tested. After a 20-min incubation period at 37°C, the medium was aspirated off and the reaction was terminated by adding 4 ml of methanol/acetic acid mixture (95:5). After 30 min of extraction, the cell layer was scraped off, sonicated, and centrifuged (2,500 g, 15 min). Aliquots of the supernatant were evaporated, and cAMP content was determined by RIA.

Arachidonic acid release. Cells were labeled with 1 μCi/ml [3H]arachidonic acid for 1 h at 37°C. They were carefully washed in DMEM 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, centrifuged (20,000 g, 10 min, 4°C), and the radioactivity was measured in the supernatant.

Detection of ERK1/2 and She. Three days postseeding, cells were placed for 24 h in culture medium free of serum and hormone. Cell layers were then exposed to the compound to be tested, rapidly rinsed with ice-cold PBS, and harvested in 1 ml of RIPA buffer (10 mM Tris·HCl, pH 7.4, 1% Triton X-100, 1% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM Na-orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 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 Polyvinylidene difluoride (PVDF) membrane (NEN-Life Sciences). Phosphorylated forms of MAPK were revealed by chemiluminescence (ECL Western blotting system, Amersham Pharmacia Biotech) using anti-active MAPK antibody (Promega). Equal protein loading was assessed by reprobing the membrane using a mixture of anti-ERK1 and anti-ERK2 antibodies (Santa Cruz Biotechnology). She phosphorylation was determined after immunoprecipitation. Briefly, 500 μl of cell lysate were incubated overnight at 4°C with 5 μg of rabbit polyclonal Shc-antibody (Upstate Biotechnology, Lake Placid, NY) and 50 μl of protein A-agarose beads. Immune complexes were extensively washed with ice-cold RIPA, dried, and denatured in Laemmli buffer. Samples were subjected to SDS-PAGE, transferred onto a Polyvinylidene difluoride membrane, and probed with an anti-phosphotyrosine-horseradish peroxidase-conjugated monoclonal antibody. Duplicates were subjected to SDS-PAGE, transferred onto aPVDF membrane, and probed with an anti-phosphotyrosine antibody to verify equal loading.

Measurement of MAPK activity. MAPK activity was measured as previously described (13). Briefly, the cells were rapidly rinsed in 10 ml of ice-cold PBS and harvested in 1 ml of 25 mM HEPES buffer (pH 7) containing 5 mM EDTA, 50 mM NaF, 0.1 mM Na-orthovanadate, 1 mM PMSF, and 10 μg/ml leupeptin. Lysates were further homogenized by passing repeatedly through a 25-gauge needle, and insoluble material was eliminated by ultracentrifugation (100,000 g, 20 min at 4°C). Aliquots of the cytosolic extracts were incubated for 10 min at 37°C with 25 μg of a synthetic peptide substrate corresponding to amino acids 95–98 of myelin basic protein (APRTPGRR) in 25 mM Tris·HCl buffer (pH 7.4) containing 10 mM MgCl2, 1 mM diethiothreitol, 40 μM ATP, 2 mM protein kinase inhibitor peptide, and 2 μCi of [γ-32P]ATP. The reaction was terminated by the addition of 125 mM cold ATP in 50% formic acid. An aliquot of the reaction mixture was spotted onto 4-cm2 Whatman P81 paper, extensively washed in 180 mM phosphoric acid, rinsed in ethanol, and counted.

Immunofluorescence microscopy. Cells plated on glass coverslips were grown, rendered quiescent, and exposed or not to the α2-agonist, as indicated above. The cells were fixed (15 min) in 4% paraformaldehyde and then treated (10 min) with 50 mM NH4Cl in PBS. They were permeabilized first in buffer consisting of PBS containing 0.05% saponin and 0.2% BSA (15 min) and then in methanol (10 min at −20°C). All subsequent steps were carried out in permeabilization buffer and were separated by several washes. Samples were incubated with ERK2 polyclonal antibody (1:40, Santa Cruz Biotechnology) and then with fluorescein-conjugated goat anti-rabbit IgG (1:400, Nordic Immunology). The coverslips were washed in PBS, mounted in fluorescent mounting medium (Dako, Carpinteria, CA), and examined under epifluorescence illumination. Digital images were captured using CoolSNAP software (Roper Scientific, Munich, Germany) and processed with Adobe Photoshop 4 (Adobe Systems, San Jose, CA).

Cell proliferation assay. PTC were seeded in six-well plates and grown for 2 days in DMEM-Ham’s F-12 medium (1:1) supplemented with 5% FCS. They were deprived of serum for 1 day and then treated or not with 1 μM UK14304. Culture plates were collected at the indicated time, and DNA content per well was measured by the fluorometric method using DAPI. LLC-PK1-α2B were seeded in six-well plates, grown for 2 days in DMEM supplemented with 5% FCS and then treated or not with 1 μM UK14304. At the indicated time, the cells were harvested by trypsin/EDTA treatment and counted.

Statistical analysis. Results are expressed as means ± SE for the number of experiments (n) indicated. The data were analyzed using Student’s t-test, and a P value <0.05 was considered statistically significant.

RESULTS

Cultured PTC having been never used for the study of α2-adrenergic receptivity, preliminary experiments were designed to assess the level of α2-AR expression under primary culture conditions. Results from binding experiments carried out with [3H]RX-821002 and [3H]MK-912 in membranes prepared from confluent PTC (5-day postseeding) are summarized in Table 1. Analysis of saturation isotherms revealed that these two specific α2-antagonists labeled a single class of sites with high affinity. With both radioligands, bind-

| Table 1. Measurement of α2-AR density in PTC and LLC-PK1-α2B cells |
|---------------------------------|------------------|-------------------|
|                                | [3H]RX-821002     | [3H]MK-912        |
|                                | Bmax            | Kd               | Bmax            | Kd               |
| PTC                            | 127 ± 11         | 4.76 ± 0.38      | 12              | 118 ± 16         | 1.65 ± 0.24      | 5                |
| LLC-PK1-α2B                    | 730 ± 51         | 5.05 ± 0.47      | 6               | ND               | ND               |

Values are means ± SE of n independent experiments. Maximal binding (Bmax; fmol of binding sites/mg protein) and the dissociation constant (Kd; nM) were calculated from computer-assisted analysis of the saturation. Membranes prepared from proximal tubule cells (PTC) and LLC-PK1-α2B cells were incubated in the presence of increasing concentrations of [3H]RX-821002 or [3H]MK-912. Non-specific binding was estimated in the presence of 10 μM phentolamine. ND, not determined.

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ing site number was fairly similar, B_max values being 127 ± 11 and 118 ± 16 fmol/mg protein for [3H]RX-821002 and [3H]MK-912, respectively. As also shown in Table 1, the level of receptor expression in LLC-PK1-α2B was about sixfold higher than in PTC (B_max for [3H]RX-821002 = 730 ± 51 fmol/mg protein). Prazosin inhibited [3H]RX-821002 binding with a slightly higher potency than oxymetazoline (data not shown), suggesting that the binding sites exhibited the pharmacological properties expected for an α2B-AR. This conclusion was confirmed by RPA with riboprobes specific for the different receptor subtypes. Indeed, RNG but not RG10 or RG20 transcripts were found in PTC (Fig. 1).

As a preliminary step in the study of the pathways of α2B-AR signal transduction, we examined the ability of the receptor to interact with G proteins by analyzing its propensity to exist under a high-affinity state for agonists (Fig. 2). In both PTC and LLC-PK1-α2B, inhibition of [3H]RX-821002 binding by the physiological amine (−)-epinephrine yielded shallow competition curves, and data were better fitted with a two-site model. From three independent experiments, the K_i values of (−)-epinephrine for the high- and low-affinity state receptor (K_H and K_L) were 4.1 ± 2.3 nM and 1.22 ± 0.55 μM, respectively, in PTC. The proportion of receptor under the high-affinity conformation for agonist represented 59 ± 8% of the whole population. As expected, this fraction was abolished in the presence of GppNHp/Na^+ or in membranes prepared from cells treated with pertussis toxin (data not shown). Fairly similar results were obtained with LLC-PK1-α2B cells, the K_H and K_L values of (−)-epinephrine being, respectively, 3.7 ± 1.7 nM and 0.63 ± 0.23 μM. The only difference is that the fraction of receptor under high-affinity conformation was lower (33 ± 5%). This is probably the consequence of higher receptor expression in LLC-PK1-α2B cells.

In isolated proximal tubules as well as in the OK cell line, α2-ARs are negatively coupled to adenylyl cyclase.

![Fig. 1. Characteristics of proximal tubule cells (PTC) in culture. A: phase-contrast microscopy of PTC monolayer. B: detection of RNG transcripts. An excess of labeled RNG probe was hybridized with either 100 μg of yeast tRNA (−), 5 μg of RNA from COS-7 cells transfected with pcDNA3-RNG (+), 100 μg of RNA from rat kidney (lane K), or 80 μg of RNA extracted from PTC in primary culture (lane PTC). Samples were digested with a mixture of RNases and resistant hybrids analyzed by electrophoresis. A typical autoradiogram is presented. P, undigested probe.](image)

### Table 2. Effect of UK-14304 on cAMP production induced by forskolin or PTH

<table>
<thead>
<tr>
<th></th>
<th>PTC (n = 4)</th>
<th>LLC-PK1-α2B (n = 4)</th>
<th>LLC-PK1-α2A (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>27 ± 2</td>
<td>42 ± 7</td>
<td>58 ± 10</td>
</tr>
<tr>
<td>FK</td>
<td>2,304 ± 80</td>
<td>2,352 ± 122</td>
<td>1,824 ± 57</td>
</tr>
<tr>
<td>FK + UK</td>
<td>2,012 ± 54</td>
<td>2,124 ± 91</td>
<td>1,280 ± 35*</td>
</tr>
<tr>
<td>FK + Dxm</td>
<td>1,774 ± 78*</td>
<td>1,961 ± 82*</td>
<td>1,348 ± 63*</td>
</tr>
<tr>
<td>PTH</td>
<td>111 ± 9</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PTH + UK</td>
<td>93 ± 12</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PTH + Dxm</td>
<td>75 ± 7</td>
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Values are means ± SE of n experiments expressed as pmol cAMP/mg cellular protein. Cells at 3 days postseeding were plated for 6 h in HEPES-buffered DMEM containing 0.2 mM 3-isobutyl-1-methylxanthine and then treated with vehicle (basal), 10 μM forskolin (FK), 10 μM forskolin plus 1 μM UK-14304 (FK + UK), 10 μM forskolin plus 1 μM dexametomidine (FK + Dxm), 0.1 μM parathyroid hormone (PTH), 0.1 μM PTH plus 1 μM UK-14304 (PTH + UK), or 0.1 μM PTH plus 1 μM dexametomidine (PTH + Dxm). After 20 min of incubation at 37°C, the reaction was stopped, and cAMP extracted and measured by RIA. *Significant effect of the α2-agonist (P < 0.05).

![Fig. 2. Analysis of receptor coupling to G proteins. Membranes prepared from PTC (A) and LLC-PK1-α2B cells (B) were incubated in the presence of [3H]RX-821002 (8 nM) and increasing concentrations of (−)-epinephrine. Inhibition experiments were carried out in binding buffer containing 0.1 mM 5′-guanylylimidodiphosphate (GppNHp) plus 100 mM NaCl (●) or not (○).](image)
The next series of experiments was designed to determine whether this mechanism of signal transduction is also effective in our models. As summarized in Table 2, PTC and LLC-PK₁-α₂B were similarly responsive to forskolin exposure. In both cell types, UK-14304 had a tendency to inhibit the forskolin-induced cAMP accumulation, but the effect was marginal and not statistically significant. By contrast, an inhibitory action was found with dexmedetomidine (20 and 17% decrease of cAMP level in PTC and LLC-PK₁-α₂B, respectively). The difference of efficacy between the two compounds was further confirmed in PTC treated with PTH. Indeed, dexmedetomidine caused a 43% inhibition of PTH-induced cAMP production, whereas UK-14304 did not. At this point, it is worth mentioning that the failure of UK-14304 to lower cAMP was likely due to a particularity of α₂B-AR subtype, because a significant reduction was observed when LLC-PK₁ cells expressing α₂A-AR (LLC-PK₁-α₂A) were assayed. It was previously reported that α₂-ARs can be linked to alternative effectors, in addition to adenylyl cyclase. In CHO cells transfected with α₂B-AR, agonist exposure stimulates cPLA₂ activity (1). In the distal segment of the rat nephron, an enhancement of PKC activity subsequent to an increase in diacylglycerol formation and to an elevation of intracellular Ca²⁺ concentration has been demonstrated (14). These observations raise the possibility that cAMP-independent mechanisms of signal transduction also exist in PTC. To investigate in this direction, PLA₂ activity was examined. As shown in Fig. 3, the exposure of PTC to UK-14304 resulted in an increase of [³H]arachidonic acid release. This effect was potentiated by the addition of the Ca²⁺ ionophore A-23187, and it was blocked in the presence of RX-821002 or quinacrine. Similar data were obtained in LLC-PK₁-α₂B cells, suggesting that cPLA₂ must be

Fig. 3. Effect of UK-14304 on arachidonic acid release. PTC (A) and LLC-PK₁-α₂B cells (B) were loaded with [³H]arachidonic acid (AA; 1 μCi/ml) for 1 h. They were extensively washed in HEPES-buffered DMEM containing 0.2% fatty acid-free BSA and treated with 1 μM A-23187 (A-23187), 1 μM UK-14304 (UK), 1 μM A-23187 plus 1 μM UK-14304 (A-23187+UK), 1 μM UK-14304 plus 10 μM RX-821002 (UK+RX), or 1 μM UK-14304 plus 100 μM quinacrine (UK+quin). Arachidonic acid release was measured as described in MATERIALS AND METHODS. Results are means ± SE from 4 independent experiments with triplicate determinations expressed as percentage of control. *P < 0.05 and **P < 0.02, values significantly different from control.

Fig. 4. Measurement of mitogen-activated protein kinase (MAPK) phosphorylation in PTC. PTC were grown, rendered quiescent, and treated as described in MATERIALS AND METHODS. Soluble proteins were extracted by centrifugation, separated by SDS-PAGE, and blotted onto a Polyscreen polyvinylidene difluoride (PVDF) membrane. ** Top: MAPK phosphorylation was revealed using anti-active MAPK antibody. Bottom: membranes were stripped of Ig and probed with anti-extracellular signal-regulated kinase (ERK) antibody to assess equal protein loading. Left: cells were treated with 1 μM UK-14304 for 2, 5, 10, 20, 30, and 60 min (UK) and 2 μM phorbol 12-myristate 13-acetate (PMA) for 5 min. Center: cells were treated with 1 μM (−)-epinephrine (Epi) for 2, 5, and 10 min. Right: cells were treated with 1 μM dexmedetomidine (Dxm) for 2, 5, and 10 min.
to assess equal protein loading. Membranes were stripped of Ig and probed with anti-ERK antibody. Bottom: phosphorylation was revealed using anti-active MAPK antibody. Top: proteins were extracted by centrifugation, separated by SDS-PAGE, and blotted onto a Polyscreen PVDF membrane. Proximal tubule (PTC) cells were grown and rendered quiescent as described in MATERIALS AND METHODS. Then, they were treated for 5 min with 1 μM UK-14304 (UK), 1 μM Dxm, 1 μM Epi, or 2 μM PMA. Soluble proteins were extracted by centrifugation, separated by SDS-PAGE, and blotted onto a Polyscreen PVDF membrane. Top: MAPK phosphorylation was revealed using anti-active MAPK antibody. Bottom: membranes were stripped of Ig and probed with anti-ERK antibody to assess equal protein loading.

Considered as an alternative effector in the mediation of the α2B-adrenergic signal in the proximal tubule.

The consequence of receptor stimulation on MAPK was investigated by estimating the extent of ERK phosphorylation as well as by measuring their enzymatic activity in cellular extracts and following their nuclear translocation in intact cells. In both PTC and LLC-PK1-α2B cells, the anti-active MAPK antibody preferentially recognized a protein that, when revealed with ERK1/2 antibody, corresponded to ERK2. As expected, exposure of PTC to PMA for 5 min resulted in a very marked increase in ERK2 phosphorylation (Fig. 4). A significant rise in the extent of ERK2 phosphorylation was also observed after treatment with UK-14304. The effect of the α2-agonist was clear as early as 5 min after the start of treatment. It was maximal at 10 min and persisted for at least 30 min. Similar kinetics were observed when PTC were exposed to (−)-epinephrine or dexmedetomidine (Fig. 4), and MAPK phosphorylation was also observed when the three agonists were assayed in LLC-PK1-α2B cells (Fig. 5). In agreement with the Western blot analysis data, a 5-min exposure to UK-14304 caused a threefold increase in MAPK enzymatic activity in PTC (Fig. 6) and resulted in massive translocation of ERK2 to the nucleus in LLC-PK1-α2B cells (Fig. 7). The change in MAPK activity matched the change in the extent of ERK2 phosphorylation. Indeed, both processes were blunted by the addition of the α2-antagonist RX-821002 and by prior treatment of the cells with pertussis toxin, indicating that the effects on MAPK are primarily due to activation of α2-AR and require Gi/Go integrity. Complementary experiments (Fig. 8) showed that the effect of UK-14304 was also totally abolished by the addition of genistein (25 μM), the inhibitor of protein tyrosine kinases, or the addition of PD-98059 (50 μM), the inhibitor of MEK1/2. The stimulation of α2-AR was previously reported to increase PKC activity in collecting ducts (14). As treatment of PTC with PMA causes a huge increase in MAPK phosphorylation (see Fig. 4), we wondered whether PKC activation is involved in the mediation of UK-14304 effects. The consequences of exposure to the α2-agonist were therefore studied after PKC desensitization induced by long-term treatment with PMA. As expected, such pretreatment totally abolished any further response to the phorbol ester (data not shown). By contrast, it did not affect the response to UK-14304, suggesting that α2B-AR triggers its effect through a PKC-independent pathway. Furthermore, because the activation of MAPK by several G protein-coupled receptors, including α2A-ARs, was previously shown to depend on Shc phosphorylation, we checked whether this holds true for α2B-AR in PTC (Fig. 9). The direct use of anti-Shc antibody on cellular
tion, this effect was transient and was totally abolished.

phorylation of both isoforms of Shc within the minutes of measuring the growth rate of PTC and LLC-PK1

rendered quiescent, and exposed (A) or not to UK-14304 (B) for 15 min as described in MATERIALS AND METHODS. The cells were treated. LLC-PK1 cells transfected with the gene encoding rat α2B-AR. According to RPA and to radioligand binding studies, cultured PTC express only the α2B-AR subtype at a density of ~130 fmol/mg protein. This value is fairly similar to that previously reported in freshly isolated proximal tubules using [3H]rauwolscine as radioligand (39). It is significantly higher than in cells cultured from inner medullary collecting duct (46), a difference that agrees with the fact that the proximal tubule is the major site of α2B-AR expression in the kidney. On the other hand, the level of receptor expression in LLC-PK1-α2B cells was ~sixfold that in PTC. In both models, a substantial proportion of the receptor was under the high-affinity state for an agonist, suggesting efficient coupling to G proteins. In support of this conclusion, dexmedetomidine attenuated cAMP production. The extent of inhibition was rather weak (~20%) in LLC-PK1-α2B cells or PTC stimulated with forskolin, but it was >40% in PTH-treated PTC. A similar amplitude of inhibition (35%) has been previously reported in microdissected tubule using (−)-epinephrine as an agonist (42). Unexpectedly, UK-14304 had a negligible action on cAMP level. The lack of effect in LLC-PK1-α2B is somewhat at variance with previous findings, demonstrating that this agonist is able to strongly inhibit forskolin-induced cAMP accumulation in another clone of LLC-PK1 cells transfected with α2B-AR (31). The reasons for this discrepancy are unclear. The clone used in that study expressed the receptor at a higher density (~2 pmol/mg of protein) than did our LLC-PK1-α2B cells (0.73 pmol/mg protein). In addition, the inhibitory effect was measured in polarized cell layers vs. nondifferentiated cells in the present study. As already found for other processes, it is possible that the lack of inhibition is due to nonoptimal assemblage of the α2B-AR signaling pathway in nonpolarized cells. A larger decrease in forskolin-induced cAMP accumulation and no difference between UK-14304 and dexmedetomidine efficiency was observed in LLC-PK1 cells expressing α2A-AR, suggesting that the data obtained under our experimental conditions also reflect intrinsic differences between the two subtypes. Regarding this point, it is noteworthy that experiments in CHO cells or on S115 mouse mammary tumor cells expressing the.

DISCUSSION

Although expression of α2B-AR in the rat proximal tubule has been known for a long time (37), the precise roles and the molecular mechanisms whereby this receptor exerts its actions on NHE3 are not completely understood. The purpose of the present study was to reinvestigate this using rat PTC in primary culture and a cell line of proximal tubule origin permanently transfected with the gene encoding rat α2B-AR. According to RPA and to radioligand binding studies, cultured PTC express only the α2B-AR subtype at a density of ~130 fmol/mg protein. This value is fairly similar to that previously reported in freshly isolated proximal tubules using [3H]rauwolscine as radioligand (39). It is significantly higher than in cells cultured from inner medullary collecting duct (46), a difference that agrees with the fact that the proximal tubule is the major site of α2B-AR expression in the kidney. On the other hand, the level of receptor expression in LLC-PK1-α2B cells was ~sixfold that in PTC. In both models, a substantial proportion of the receptor was under the high-affinity state for an agonist, suggesting efficient coupling to G proteins. In support of this conclusion, dexmedetomidine attenuated cAMP production. The extent of inhibition was rather weak (~20%) in LLC-PK1-α2B cells or PTC stimulated with forskolin, but it was >40% in PTH-treated PTC. A similar amplitude of inhibition (35%) has been previously reported in microdissected tubule using (−)-epinephrine as an agonist (42). Unexpectedly, UK-14304 had a negligible action on cAMP level. The lack of effect in LLC-PK1-α2B is somewhat at variance with previous findings, demonstrating that this agonist is able to strongly inhibit forskolin-induced cAMP accumulation in another clone of LLC-PK1 cells transfected with α2B-AR (31). The reasons for this discrepancy are unclear. The clone used in that study expressed the receptor at a higher density (~2 pmol/mg of protein) than did our LLC-PK1-α2B cells (0.73 pmol/mg protein). In addition, the inhibitory effect was measured in polarized cell layers vs. nondifferentiated cells in the present study. As already found for other processes, it is possible that the lack of inhibition is due to nonoptimal assemblage of the α2B-AR signaling pathway in nonpolarized cells. A larger decrease in forskolin-induced cAMP accumulation and no difference between UK-14304 and dexmedetomidine efficiency was observed in LLC-PK1 cells expressing α2A-AR, suggesting that the data obtained under our experimental conditions also reflect intrinsic differences between the two subtypes. Regarding this point, it is noteworthy that experiments in CHO cells or on S115 mouse mammary tumor cells expressing the.

Fig. 8. Effect of protein kinase C (PKC) desensitization, genistein, and PD-98059, PTC were grown and rendered quiescent as described in MATERIALS AND METHODS. They were treated for various periods of time with the indicated compound. Soluble proteins were separated by SDS-PAGE and blotted onto a Polyscreen PVDF membrane. MAPK phosphorylation was revealed using anti-active MAPK antibody. The cells were treated for 20 h with 2 μM PMA (PMA 20 h) or for 30 min with either 25 μM genistein (Genist) or 50 μM PD-98059 before exposure (+) or not (−) to 1 μM UK-14304 for 5 min.
different α2-AR subtypes at a similar density showed repeatedly that α2B-AR was less efficient than other subtypes in inhibiting adenyl cyclase (12, 18). Furthermore, measurement of pertussis toxin-sensitive GDPase in transfected CHO cells showed that UK-14304, but not dexmedetomidine, acts as a partial agonist at the α2B-AR (19).

Exposure of PTC or LLC-PK₁-α₂B cells to (−)-epinephrine or to α₂-agonists (UK-14304, dexmedetomidine) caused an increase in the phosphorylation state of p42/44 MAPK. As expected, this effect was blocked by the addition of RX-821002 (α₂-antagonist) and resulted in an enhancement of MAPK activity and translocation to the cell nucleus. The kinetics of MAPK phosphorylation were rapid and similar to those in other cell systems in which the α₂A-AR was found to exert mitogenic effects (4, 35). ERKs are expressed in the different segments of the nephron (41), and their pattern of expression changes with the stage of renal development (32). The activation of ERKs was demonstrated to play a crucial role in tubular cell regeneration after oxidative injury (9) and to be induced by various growth factors, cytokines, and vasoactive substances (16, 24). With regard to proximal tubule-derived cells, other G protein-coupled receptors already shown to activate ERKs include serotonin 1B receptor (5-HT1B), α₂C-AR, and lysophosphatidic acid receptor in OK cells (10, 22) as well as angiotensin II and PTH receptors in OK and rabbit proximal tubule cells (7, 20, 36, 40). It is now clearly established that the mechanisms responsible for the activation of ERKs by G protein-coupled receptors is highly dependent on the particular receptor or cell type examined. Phosphorylation of ERKs may be due to receptor interaction with Gq or Gs/Gi, and be triggered by direct activation of Raf by PKC or by indirect recruitment of p21-Ras via the formation of the Shc-Grb2-Sos complex. Because α₂-ARs were reported to stimulate PKC and to increase intracellular Ca²⁺ in renal epithelial cells (14), we investigated whether PKC is involved. As expected, short-term exposure of PTC or LLC-PK₁-α₂B cells to PMA caused a marked increase in the phosphorylation of ERK1/2, but the action of UK-14304 was unaffected by PKC desensitization. Conversely, the effect of the agonist was totally abolished by PTX treatment or by the prior addition of genistein or PD-98059, showing...
that it depends on G proteins and requires the activity of tyrosine kinases and MEK1/2. This view was also supported by measurement of Shc phosphorylation. Indeed, the two forms of Shc (46 and 52 kDa) found in PTC or LLC-PK1-α2B cells are transiently phosphorylated after agonist exposure. It is thus likely that the α2B-AR stimulates MAPK through a pathway comprising activation of G proteins, phosphorylation of Shc, recruitment of the Grb2-Sos complex, and subsequent activation of the p21-Ras-MAPK cascade. Measurement of cell growth in different culture conditions indicated that activation of MAPK resulted in accelerated proliferation of PTC and LLC-PK1-α2B cells.

In addition to MAPK activation, the treatment of PTC or LLC-PK1-α2B cells with α2-agonists induced a significant increase in arachidonic acid release that is potentiated by the Ca2+ ionophore A-23187. In this respect, our results match recent observations showing that, in transfected CHO cells, α2B-AR was much more efficient than other subtypes in stimulating cPLA2 (1). The relationship between MAPK and cPLA2 was not investigated in the present study. Although it is known that cPLA2 is a substrate for MAPK (29), in CHO cells, phosphorylation of cPLA2 does not correlate with its activation by α2B-AR, suggesting the involvement of another mechanism (1). Alternatively, activation of MAPK could be the consequence of arachidonic acid release. In this respect, it is noteworthy that arachidonic acid was recently demonstrated to mediate the effects of angiotensin II on MAPK in primary culture of proximal tubule cells from rabbit (11). The action of arachidonic acid was dependent on the production of epoxy derivative, and it involved activation of p21ras, subsequent to Shc phosphorylation and association with Grb2 (20). Preliminary results obtained in LLC-PK1-α2B cells indicate that MAPK activation by α2-agonists is ablated in the presence of quinacrine, suggesting that cPLA2 is involved in the mediation of the effect of α2B-AR. However, further studies will be necessary to determine whether other documented mechanisms (e.g., α2-AR internalization, EGFR polymerization, c-Src recruitment) should also be considered.

Depending on the cell type considered and on the duration of their activation, ERKs may have opposite effects on cell growth and result either in proliferation or in arrest of the cell cycle. For instance, activation of MAPK by angiotensin II has no mitogenic effect but induces hypertrophy in cultured murine PTC or in LLC-PK1 cells (45). According to recent reports, this effect depends on activation of NADPH-oxidase, generation of reactive oxygen species, and consecutive induction of p27kip, an inhibitor of cyclin-dependent kinases. Interestingly, our results demonstrate that α2B-AR stimulation enhances cell growth. Although the involvement of other factors cannot be ruled out, it is thus likely that activation of ERK is primarily responsible for the increased rate of proliferation.

In conclusion, the present work shows that the α2B-AR activates the ERK pathway and stimulates the proliferation of epithelial cells derived from the proximal tubule of rat and pig. Activation of ERK by α2B-AR is independent of adenyl cyclase inhibition or PKC stimulation, but it correlates with increased cPLA2 activity. Regardless of the precise mechanism responsible for ERK phosphorylation, it appears that the action of catecholamines on α2B-AR may play a role in the adaptive response to acute renal tissue injury in the rat.

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