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Characterization of a fluorescent conjugate of the rabbit angiotensin AT_1 receptor

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1 The rabbit AT_1 receptor (AT_1R) for angiotensin II (A_{II}) has been conjugated to the yellow fluorescent protein (YFP) in order to establish the pharmacological profile of such a fusion protein and to facilitate the study of ligand-induced regulation.

2 A_{II} bound AT_1R-YFP (K_D 8.1 nM in transiently transfected cells) and stimulated HEK 293 cells expressing the fusion protein at concentration ranges similar to the ones that stimulate the contraction of the isolated rabbit aorta. Antagonists found to be insurmountable in the latter assay (candesartan and EXP-3174 being the most extreme cases) were also insurmountable in the phospholipase A_2 assay applied to cells expressing AT_1R-YFP, whereas losartan appeared to be surmountable in both assays.

3 Cells expressing AT_1R-YFP exhibited a membrane-associated fluorescence that was partly and reversibly translocated into intracellular structures upon A_{II} stimulation (confocal microscopy); the nonpeptide antagonists were not active in this respect, but prevented the effect of the agonist.

4 A_{II} treatment increased the quantity of the fusion protein in cells, and phorbol 12-myristate 13-acetate (PMA) treatment even more so (immunoblot, confocal microscopy) but, unlike the agonist, the latter drug did not induce receptor endocytosis. A protein kinase C (PKC) inhibitor prevented the effect of either A_{II} or PMA on AT_1R-YFP abundance.

5 The conjugate AT_1R-YFP retains the pharmacological properties of the parent rabbit AT_1R. Agonist-induced downregulation was not documented using this system; to the contrary, we have observed a PKC-mediated increased expression AT_1R-YFP likely to be the result of a decreased breakdown rate of the fusion protein.
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Abbreviations: A_I, angiotensin I; A_{II}, angiotensin II; AT_1R, AT_1 receptor; B_1R, B_1 receptor; CMV, cytomegalovirus; ERK1/2, extracellular signal regulated kinases 1/2; FAK, focal adhesion kinase; GFP, green fluorescent protein; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; YFP, yellow fluorescent protein

Introduction

Angiotensin II (A_{II}) is a cardiovascular hormone of prime importance with many effects mediated by the AT_1 receptor (AT_1R) subtype (contraction and trophic effect on vascular smooth muscle cells, aldosterone secretion, facilitation of sympathetic neurotransmission, etc., De Gasparo *et al*., 2001). Antagonists of the angiotensin AT_1Rs represent a clinically successful drug class for the treatment of hypertension and other cardiovascular conditions (Burnier, 2001). This drug class includes prominent examples of nonequilibrium interactions. Indeed, while losartan is a nonpeptide surmountable antagonist of A_{II} on the AT_1R, its major metabolite EXP 3174 is more active and insurmountable in experimental systems such as the rabbit aorta contractility (Wong *et al*., 1990; Wienen *et al*., 1992). Moreover, repeated washing of rabbit aortic tissue treated with EXP-3174 does not restore the maximum response to A_{II} (Panek *et al*., 1995), supporting the nonequilibrium variety of interaction. Radioligand binding results suggest that the interactions of EXP-3174 or that of the related tetrazole candesartan with the AT_1R results in a loss of B_{max}, without major change of the apparent affinity of the remaining receptors (Wienen *et al*., 1992; Chansel *et al*., 1994; Vanderheyden *et al*., 1999).

A number of fluorescent conjugates of G-protein-coupled receptors (GPCRs) have been produced, with a remarkable conservation of pharmacological properties (Milligan, 1999). They allow the visualization of ligand-induced translocation in live cells. Also, the green fluorescent protein (GFP) fused at the C-terminus of the receptors constitutes an excellent antigenic tag for immunoblotting. The present experiments aim at characterizing a fluorescent construction composed of the rabbit AT_1R fused to the yellow fluorescent protein (YFP; a variant of GFP). Specifically, the pharmacological profile of the construction has been compared to that of the wild-type receptor expressed in the rabbit aorta. AT_1R-YFP reaction to

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agonist and antagonist ligands (cellular translocation, possible downregulation) has been particularly studied.

Methods

Drugs

A_{II} was purchased from Sigma (St Louis, MO, U.S.A.). The AT_1R antagonists losartan sodium and EXP-3174 (2-*n*-butyl-4-chloro-1-[(2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole-5-carboxylic acid) were gifts from Merck Sharp & Dohme Research Laboratories (Rahway, NJ, U.S.A.). Irbesartan, valsartan and candesartan (CV 11974, active nonesterified form) were gifts from Bristol-Myers Squibb (Saint-Laurent, Canada), Novartis Pharma AG (Basel, Switzerland) and Astra Hässle AB (Mölndal, Sweden), respectively.

Contractility studies

Rings of rabbit aorta (New Zealand white, 1.5 – 2 kg, Charles River, St Constant, Canada) were suspended under a tension of 2 g in 5 ml tissue baths containing oxygenated (95% O_2 : 5% CO_2) and warmed (37°C) Krebs solution as described (Larrivée *et al*., 1998).

Contractility studies were based upon the construction of cumulative concentration – responses curves for A_{II} (a AT_1R agonist for this effect). These studies aimed to investigate the potency and surmountability of related tetrazole AT_1R antagonists in the vascular smooth muscle preparation. Two cumulative concentration – response curves were constructed at times 1 and 3 h from the beginning of the incubation of the preparation. One of the five antagonists at a given concentration or its dimethylsulfoxide (DMSO) vehicle (0.04% v/v) was introduced at time 2 h, and maintained in the bathing fluid during the construction of the second curve (effect of the antagonist at 3 h). Contractility results were expressed as a percent of the maximal response recorded when constructing the control curve (1 h).

All statistics were expressed as means ± s.e.m. and were calculated using the InStat 2.0 computer program (GraphPad Software, San Diego, CA, U.S.A.).

Construction and expression of the rabbit AT_1R – YFP

Using rabbit DNA as a template, the entire coding region of the AT_1R gene (Burns *et al*., 1993; excluding the stop codon) was amplified by PCR. 5′-AAATAAGCTTAATGATGCTC-AACTCTTCTACCG-3′ and 5′-TATTGGATCCTCAACCT-CAAAGCAGGGCAC-3′ were used as PCR sense and antisense primers, respectively. These primers contain additional *Hin*dIII and *Bam*HI sites (underlined) for the directional cloning of the rabbit AT_1R coding region in the eucaryotic expression vector pEYFP-N1 (Clontech Laboratories, Inc., Palo Alto, CA, U.S.A.), encoding YFP, a variant of GFP. Both the PCR fragment and the pEYFP-N1 vector were digested with *Hin*dIII and *Bam*HI and ligated at 12°C overnight. The resultant vector (AT_1R – YFP) contained the rabbit AT_1R coding sequence fused in frame at its carboxyl terminus with the YFP, expressed under the control of the cytomegalovirus (CMV) promoter. The AT_1R – YFP vector was transfected in HEK 293 cells using the X-Gen 500 transfection reagent (MBI Fermentas Inc., Flamborough, ON, Canada) as directed. Some cells were used 24 h after transfection (transient transfection); stable transfectants were selected after growing the cells for 1 month in α-MEM medium supplemented with fetal bovine serum (5%), horse serum (5%), penicillin – streptomycin (1%) and geneticin (500 μg ml^{-1}; Invitrogen, Burlington, ON, Canada).

Binding assays

The binding of [^{3}H]A_{II} (Perkin-Elmer Life Sciences, Boston, MA, U.S.A.; 40 Ci/mmol) to adherent intact HEK 293 cells was evaluated using confluent 24-well plates of cells. Cell wells were washed with ice-cold binding medium (phosphate-buffered saline, pH 7.4, supplemented with 0.02% sodium azide, 0.1% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride and 1 μM captopril), and 500 μl of the same medium was added to each well. To construct saturation curves, the total binding of the ligand (0.25 – 16 nM) to cells transiently or stably expressing AT_1R – YFP construction or nontransfected cells was determined in duplicate wells, and 1 μM of unlabelled A_{II} was added to matched wells to determine the nonspecific binding. Upon 90 min incubation, the wells were rinsed (× 3) with ice-cold phosphate-buffered saline, the supernatant removed and the cells dissolved in 1 ml of 0.1 M NaOH. The resulting suspension was then counted by scintillation. Binding experiments involving 4 nM [^{3}H]A_{II} and the same general conditions as outlined above were also performed on the HEK 293 stable transfectant cell line to verify the pharmacological profile of the fusion protein: plates of cells were concomitantly treated at 0°C with the unlabelled competitors (A_{II}, A_I, saralasin or the set of five nonpeptide antagonists) at various concentrations and the radioligand. Variants of the binding assay were applied to cells treated with phorbol 12-myristate 13-acetate (PMA) to characterize the drug effect of receptor abundance at the cell surface. The effect of PMA on the binding of [^{3}H]Lys-des-Arg^9-bradykinin to HEK 293 cells stably expressing B_1 receptor-yellow fluorescent protein (B_1R – YFP) was established in 24-well plates, precisely as described (Sabourin *et al*., 2002).

Effect of A_{II}, antagonists or PMA on the subcellular distribution of AT_1R – YFP

The drugs studied were added to the culture medium of the stably transfected HEK 293 cells, and the subcellular fluorescence distribution observed without fixation or drug washout using a BioRad 1024 confocal microscope as a function of treatment duration (× 60 objective with oil immersion, emission 488 nm, detection above 510 nM).

Phospholipase A_2 assays

An arachidonic acid release assay was performed to evaluate the function of AT_1R – YFP stably transfected in HEK 293 cells. Cells 2.5×10^5 stably expressing the receptor or untransfected cells were seeded in 2 cm^2 wells (24-well plates) containing 1 ml of the complete culture medium (see above). After 24 h, as the cells were 50 – 60% confluent, 0.1 μCi of [^{3}H]arachidonic acid (Perkin-Elmer Life Sciences; specific activity 185 Ci mmol^{-1}) was added to each well. The cells were further incubated for 18 h, then washed three times with

Earle's balanced salt solution containing 2 mg ml^{-1} of bovine serum albumin. A measure of 1 ml of this medium was left in each well. Upon adding of the agonist A_{II}, the plates were further incubated at 37°C for 30 min, at which point 500 μl of the medium from each well was recovered in 1.5 ml conical tubes and centrifuged for 5 min at 15,000 × *g*. A volume of 40 μl of the supernatants were transferred in vials for scintillation counting of the released arachidonate. A variation of this protocol involved the 60 min preincubation of AT_1R – YFP-expressing cells with antagonists after washing with Earle's balanced salt solution and before the 30 min treatment with A_{II}.

Immunoblot

Two different monoclonal antibodies to GFP were purchased from Clontech and Zymed (San Francisco, CA, U.S.A.). For the analysis of AT_1R – YFP, most experiments were based on total cell extracts. Transfectant HEK 293 cells (confluent 75 cm^2 flasks) were put in boiling lysis buffer containing 10 mM Tris pH 7.4, 1.0 mM Na_3VO_4 and 1.0% SDS. The cell lysates were incubated for 5 min at 100°C and then centrifuged at 15,000 × *g* for 5 min. Total protein concentrations were then determined using the BCA Protein Assay (Pierce, Rockford, IL, U.S.A.). Total proteins of 25 μg were run on a 9% SDS-PAGE and transferred to a PVDF membrane. The membranes were then incubated for 1 h at room temperature in blocking buffer (washing buffer (10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween-20) containing 5% skimmed milk). The primary antibodies (monoclonal anti-GFP; dilution 1 : 1000 for either ones from Clontech or Zymed) were added for 2 h at room temperature in fresh blocking buffer. The membranes were washed for 30 min in washing buffer at room temperature before adding the secondary antibody (horseradish peroxidase-conjugated, preadsorbed goat anti-mouse IgG; Santa Cruz Biotechnologies, Santa Cruz, CA, U.S.A., dilution 1 : 16 000) for 1 h at room temperature in blocking buffer. The membranes were washed in washing buffer for another 30 min and then the presence of immunreactive proteins was revealed using the Western Blot Chemoluminescence Reagent Plus (Perkin – Elmer Life Sciences), as directed.

Kinase assays (ERK and FAK)

In order to extend the investigation of cellular responses mediated by AT_1R – YFP, we tested the presence of phospho-extracellular signal regulated kinases 1/2 (ERK1/2) and phospho-focal adhesion kinase (FAK) in resting or drug-treated cells (either untransfected HEK 293 cells or cells stably expressing AT_1R – YFP). These kinases are known to be activated by A_{II} via AT_1Rs in cellular systems (Touyz & Schiffrin, 2000). Confluent 75 cm^2 flasks were cultured overnight with medium containing a reduced fetal bovine serum concentration (0.5%) to minimize the background phosphorylation of both tested kinases. The cells were treated with the stimulants agonist A_{II} (10 nM) or PMA (1 μM, both applied 10 min before extraction), inhibitory drugs (candesartan or GF 109,203X, 1 or 10 μM, respectively, both 15 min before extraction) or both. Total cell extracts applicable to immunoblots were then prepared as outlined above. Transferred proteins were revealed using three types of antibodies for each sample: phospho-ERK1/2 (monoclonal, New England Biolabs, dilution 1/1000), total ERK1/2 (to show comparable loading; polyclonal, New England Biolabs, dilution 1/1000) and phospho-FAK (polyclonal, Upstate Biotechnology, dilution 1/1000). Staining was revealed using the appropriate peroxidase-conjugated secondary antibody, as described above.

Results

Vascular contractility mediated by AT_1Rs in the rabbit isolated aorta

The rabbit aortic rings were stimulated twice with A_{II} at times 1 and 3 h; the first response was a control sigmoidal curve with an EC_{50} of 4.8 ± 0.4 nM (n = 115). The curve recorded at time 3 h in the presence of the DMSO vehicle of the antagonists was very similar to the control curve (pooled values from control curves shown in Figure 1: EC_{50} of 5.7 ± 1.0 nM, E_{max} of 104 ± 4% of the control maximal effect, n = 30, no significant difference by Mann – Whitney test).

Losartan shifted the 3 h A_{II} concentration – effect curve to the right at 10 – 100 nM without depression of the maximal effect (Figure 1a). A pA_2 value of 8.46 ± 0.10 was calculated for losartan using the Schild regression based on data in Figure 1a. Irbesartan and valsartan did the same, but were more potent (Figure 1b, c). A minor depression of the maximal effect of A_{II} may have occurred in the presence of the highest tested concentrations of irbesartan or valsartan (Figure 1). The 0.1 nM concentration level of the antagonist EXP 3174 significantly increased the A_{II} EC_{50}, but did not change the maximal effect, relative to the control recordings (Figure 1d). However, higher concentrations of EXP 3174 depressed the maximal response to A_{II} (P < 0.01 for either 1 or 10 nM, Figure 1), while further shifting the curves to the right. Candesartan also exerted a profound inhibitory effect on the agonist E_{max} (Figure 1e).

Properties of AT_1R – YFP: radioligand binding

Untransfected HEK 293 cells failed to bind [^{3}H]A_{II} (Figure 2a). Cells transiently transfected with the AT_1R – YFP coding vector exhibited one major saturable binding site (Scatchard plot parameters K_D 8.1 nM, B_{max} 124 fmol well^{-1}). HEK 293 cells stably expressing AT_1R – YFP exhibited a much higher specific binding when treated with [^{3}H]A_{II} in the 0.25 – 16 nM concentration range (Figure 2a). However, reminiscent of the behavior of the related construction bradykinin B_2 receptor-GFP in the same type of cells (Houle *et al*., 2000), the saturation curve exhibited an irregular shape suggestive of more than one affinity state for AT_1R – YFP. This shape was not more regular if the nonpeptide antagonist losartan (1 μM) was used to determine the nonspecific binding (data not shown). A series of unlabelled drugs were coincubated with the radioligand (4 nM) in order to verify the pharmacological profile of the receptor fusion protein in stably transfected cells (Figure 2b). A_I was about 100-fold less potent than unlabelled A_{II} to displace the radioligand, and the peptide antagonist saralasin was somewhat less potent than A_{II}. No large difference of potency was noted between the five nonpeptide antagonists, the IC_{50} values falling between 2 and 25 nM (Figure 2b).

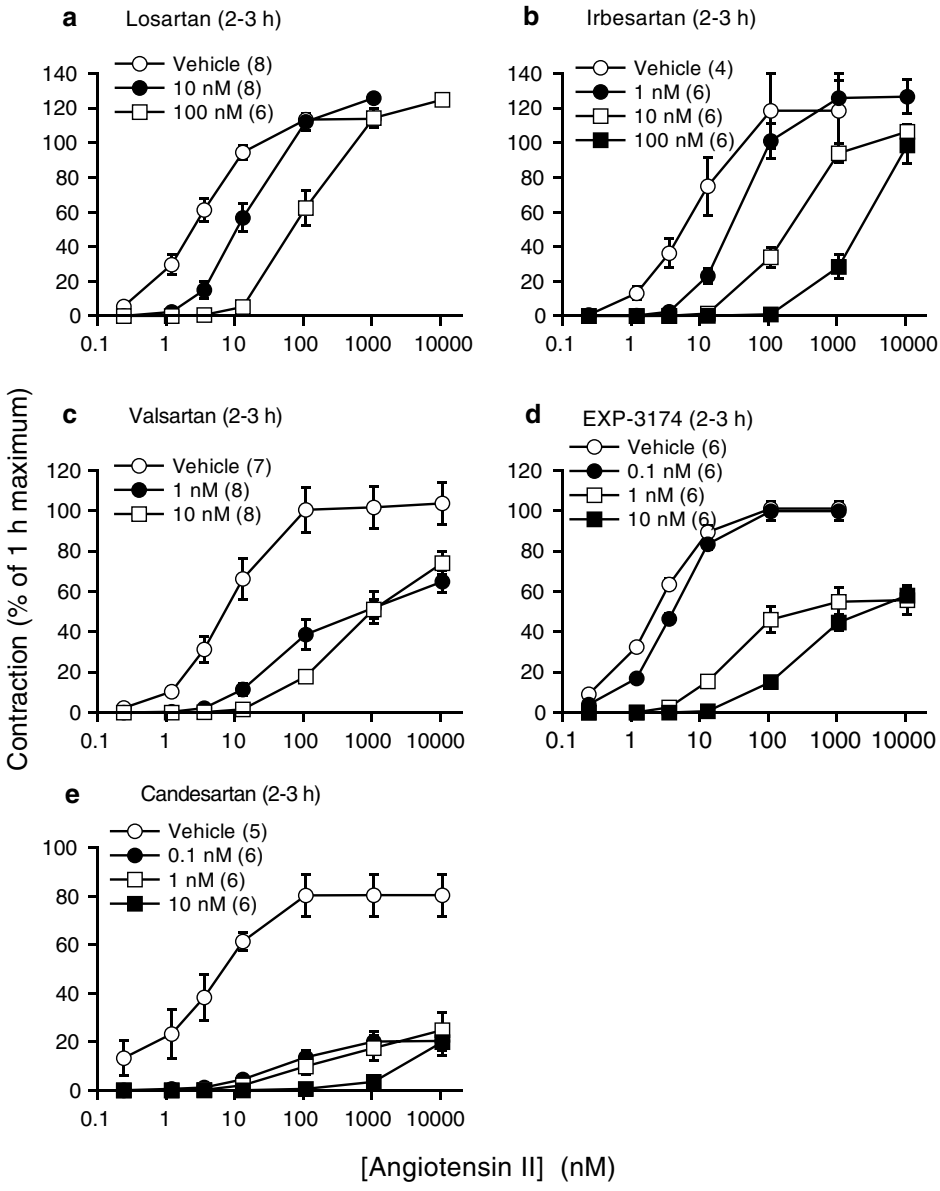

Figure 1 Effect of AT_1R antagonists (given 1 h before the 3 h concentration – effect curves construction) or their DMSO vehicle on A_{II}-induced contraction in the rabbit isolated aorta. Responses are expressed as a percent of the maximal effect of A_{II} in a control recording established at time 1 h in each tissue. Values are means ± s.e.m. (number of determinations between parentheses). See text for statistical analysis.

Stimulation of effectors mediated by AT_1R – YFP

A phospholipase A_2 assay was performed to verify that AT_1R – YFP is a functional receptor (Figure 3). A_{II} released [^{3}H]arachidonate from HEK 293 cells stably expressing the receptor (EC_{50} 6.3 nM), but untransfected cells did not respond to A_{II} (Figure 3a). Pretreatment for 1 h with the set of five nonpeptide antagonists or their DMSO vehicle was performed to document the conservation of the surmountable or insurmountable behavior of each drug (Figure 3b). The concentration of 10 nM for each of the five drugs was used as a large significant receptor occupancy occurs at this concentration (Figure 2b). In the phospholipase A_2 assay, it was shown that losartan is a surmountable antagonist, as the maximal effect of the agonist was recovered by increasing its concentration (Figure 3b). As for the contractility assay, irbesartan, valsartan, EXP-3174 and candesartan appeared to be insurmountable.

In cells stably expressing AT_1R – YFP, stimulation with A_{II} promoted the phosphorylation of ERK1/2 (1 and 10 nM of agonist) and of FAK (0.1 – 10 nM; Figure 4) as reported with wild-type receptors (Touyz & Schiffrin, 2000). Untransfected cells were not responsive to A_{II} in these assays. Candesartan (1 μM) had no direct effect on the systems, but prevented the effect of A_{II} (10 nM) in AT_1R – YFP-expressing cells (Figure 4). Comparable loading of extract samples was achieved in blots submitted to these assays, as shown by total ERK1/2 content.

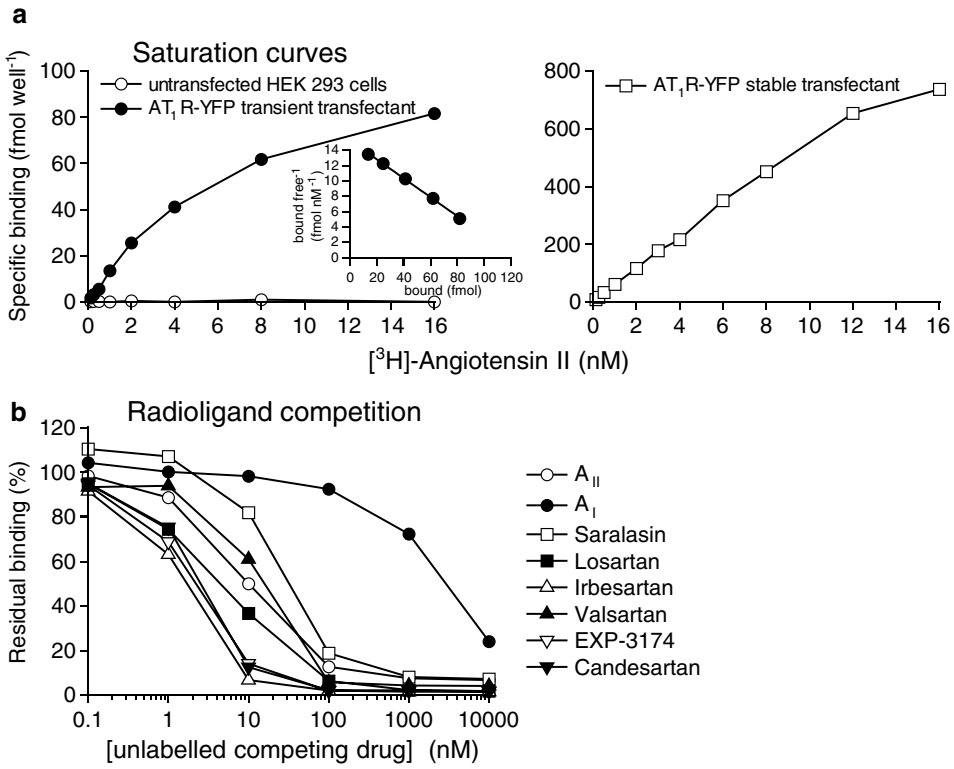

Figure 2 Radioligand binding to AT_1R-YFP. (a) Specific binding of $[^3H]A_{II}$ to HEK 293 cells stably or transiently expressing AT_1R-YFP or to untransfected cells. Nonspecific binding, established in the presence of 1 μM unlabelled A_{II}, typicaly 15% of the total binding, was subtracted. Values are the means of duplicate determinations in each experiment. (a) Representative experiment for each condition is shown out of three for the stable transfectant cells, two for untransfected cells and two for transiently transfected cells. Inset: Scatchard plot derived from the five data points with most intense binding in transiently transfected cells. (b) Pharmacological profile of AT_1R-YFP established using competition of the binding of $[^3H]A_{II}$ (4 nM) by a panel of unlabelled peptides and nonpeptide drugs in stably transfected HEK 293 cells. The unlabelled cold drugs were concomitantly present with the radioligand during the 90 min equilibration period at 0°C. Values are the means of two experiments with duplicate determinations in each experiment.

Subcellular distribution of AT_1R-YFP as a function of treatments with drugs

Most of the fluorescent labelling of resting HEK 293 cells stably expressing AT_1R-YFP is located at the level of plasma membranes (Figure 5). The imaging experiments were performed in the serum-containing culture medium and in the presence of cycloheximide (71 μM) to prevent the replacement of receptors by newly synthesized ones. The addition of the agonist A_{II} (10 nM, Figure 5a, or 1 – 100 nM, data not shown) was rapidly followed by a translocation of the fluorescence into the cells (apparently vesicular structures of various sizes). There was a variable loss of membrane fluorescence, with apparent loss of membrane labelling continuity in some cells (Figure 5a). These changes were reversible over 3 h upon agonist washout (data not shown). No global loss of cell fluorescence was noted following any form of treatment with A_{II}.

Losartan pretreatment (1 μM, 15 min) reduced membrane fluorescence translocation induced by A_{II} (10 nM, 30 min) in stably transfected cells (Figure 5b). The surmountable antagonist losartan did not exert a direct effect on the system (Figure 5b). The insurmountable antagonists failed to unequivocally translocate the membrane fluorescent receptors (the effect of 0.5 or 3 h treatments with candesartan is illustrated in Figure 5a, but this also applies to the three other insurmountable antagonists, not shown).

AT_1R-YFP immunoblotting

Two different monoclonal antibodies to GFP were tested to reveal the fusion protein. The antibody from Zymed reacted nonspecifically with several protein bands in the cell extract from untransfected HEK 293 cells (Figure 6, top). The Clontech antibody exhibited less background reactivity. Either could reveal two major classes of bands in HEK 293 cells stably expressing AT_1R-YFP (arrows at the right of Figure 6, top part): faint bands close to $\sim$120 kDa, presumably representing the fusion protein, and a stronger band corresponding to a protein resembling YFP. The similarity of the YFP-like band to YFP is further shown by the extract of HEK 293 cells transiently expressing authentic YFP (Figure 6, top). Intermediate proteins with specific reactivity are also seen in some immunoblots. The intense YFP-like band in the cells expressing AT_1R-YFP may arise either from the spontaneous ligand-independant degradation of the fusion protein or as an artifact during sample preparation, as an enriched plasma membrane fraction from AT_1R-YFP-expressing cells also exhibits some free YFP (data not shown).

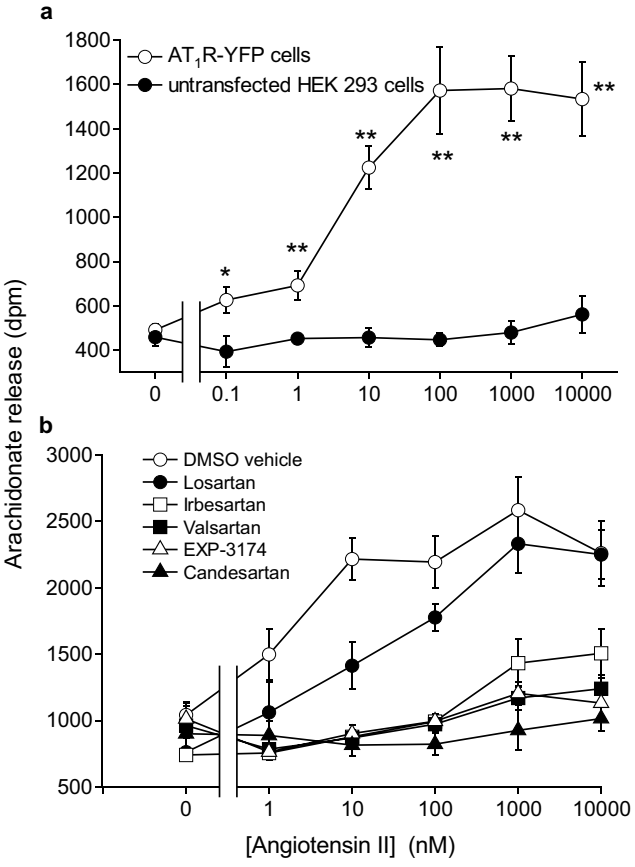

Figure 3 (a) [^{3}H]Arachidonate released by HEK 293 cells stably expressing AT_1R-YFP or untransfected cells and exposed to A_{II} for 30 min. Results are expressed as means ± s.e.m. ($n=8$ for most points, except for controls without agonist: $n=16$). The values from AT_1R-YFP stimulated cells only were statistically heterogeneous (Kruskall–Wallis test $P<10^{-4}$). Mann–Whitney test was applied to compare agonist-stimulated cells with their appropriate controls (*$P<0.01$; **$P<0.001$). (b) Effect of five nonpeptide antagonists on A_{II}-induced arachidonate release by the same type of cells. The drugs were incubated with the cells at 37°C for 60 min before introducing the agonist, which was further maintained for 30 min with the cells. Results are the means ± s.e.m. ($n=4-6$).

Signal transduction and the stability of AT_1R-YFP conjugate

Total HEK 293 cell extracts were immunoblotted using the Clontech monoclonal anti-GFP antibody to study the influence of ligands on the stability of the ~120 kDa fusion protein AT_1R-YFP (Figure 6, bottom; film less exposed than the ones shown at the top of Figure 6). Specifically, agonist-induced downregulation was investigated. Untransfected cells were included as control; the band presumably corresponding to AT_1R-YFP (arrow at the right of bottom part of Figure 6) was discernible in cells stably expressing this protein. Unexpectedly, pretreating these cells with the agonist A_{II} (10 nM for 3 h, Figure 6, or 1 μM for 3 h, data not shown) consistently reinforced the fusion protein band, relative to untreated cells. Antagonist treatment had no effect (candesartan or losartan, 1 μM of each for 3 h, Figure 6 and data not shown). Thus, A_{II}, while promoting receptor endocytosis (Figure 5), did not produce agonist-mediated downregulation; on the contrary, it may increase the receptor population by a mechanism related to AT_1R signalling. As protein kinase C (PKC) is part of this signalling (Touyz & Schiffrin, 2000), we tested the effect of a direct PKC stimulant, PMA (1 μM for 3 h). This drug increased the AT_1R-YFP content of the total cell extract more than A_{II} (Figure 6, bottom). Other drug treatments related to different signal transduction systems (forskolin 1 μM, dexamethasone 100 nM, interleukin-1β 5 ng ml^{-1} for 3 h) failed to influence AT_1R-YFP abundance in cells (data not shown), supporting the specificity of the PMA effect. The stimulatory effect of either A_{II} or PMA on AT_1R-YFP expression was prevented by concurrent treatment with the PKC inhibitor GF 109203 X (10 μM; Figure 6, bottom). The latter drug had no direct effect.

Confocal microscopy evidenced a striking reinforcement of membrane-associated fluorescence following PMA treatment in HEK 293 cells expressing AT_1R-YFP, but no fluorescent receptor endocytosis was observed (Figure 7 shows the effect 18 h after PMA application, as the optimal time window is 16–24 h. A 30 min PMA treatment does not promote significant endocytosis (Figure 5c); by contrast, A_{II} induces massive internalization of fluorescent receptors in 30 min (Figure 5a). The effect of PMA on membrane-associated fluorescence was prevented by a 10-fold higher concentration of the PKC inhibitor GF 109203 X (Figure 7).

The radioligand binding assay was used to verify if the 18 h PMA treatment increases [^{3}H]A_{II} binding to intact cells (Figure 8a). As compared to a control run in the same experiment, the PMA pretreatment approximately induced a ~three-fold increase of specific binding, the saturation curve keeping the irregular shape typical of stably transfected cells. A time course study showed that the binding of radiolabelled A_{II} increased progressively from the control level in cells treated with PMA (Figure 8b).

To further test whether PMA may influence the synthesis rate of AT_1R-YFP, we have conducted comparative experiments using a similar construction expressed in the same cell type and the same expression vector: B_1R-YFP. This fusion protein consists of the rabbit kinin B_1R fused to YFP and was also cloned into pEYFP-N1 (Sabourin *et al*., 2002). These receptors, expressed in stably transfected HEK 293 cells, were visualized as a membrane-associated fluorescence that was not reinforced by PMA pretreatment (Figure 7, bottom). Futher, the PMA pretreatment only slightly stimulates (×1.2) the abundance of the binding site for the cognate radioligand, [^{3}H]Lys-des-Arg^9-bradykinin (Figure 8c).

Discussion

As observed with other GPCRs (Milligan, 1999; Houle *et al*., 2000; Sabourin *et al*., 2002), conjugation of the rabbit AT_1R with a GFP-related molecule was not associated with noticeable pharmacological changes. Agonist potencies recorded in biological activities mediated by the naturally expressed receptor (Figure 1) or recombinant conjugate (PLA_2 or kinase phosphorylation assays, Figures 3a and 4) were consistent with the binding K_D measured in cells transiently transfected with AT_1R-YFP (8.1 nM). This figure is somewhat higher than, but close to the value reported in a binding assay to the wild-type rabbit AT_1R applied to intact cells (4.8 nM; Park & Han, 2002). The irregular shape of the radioligand saturation curve

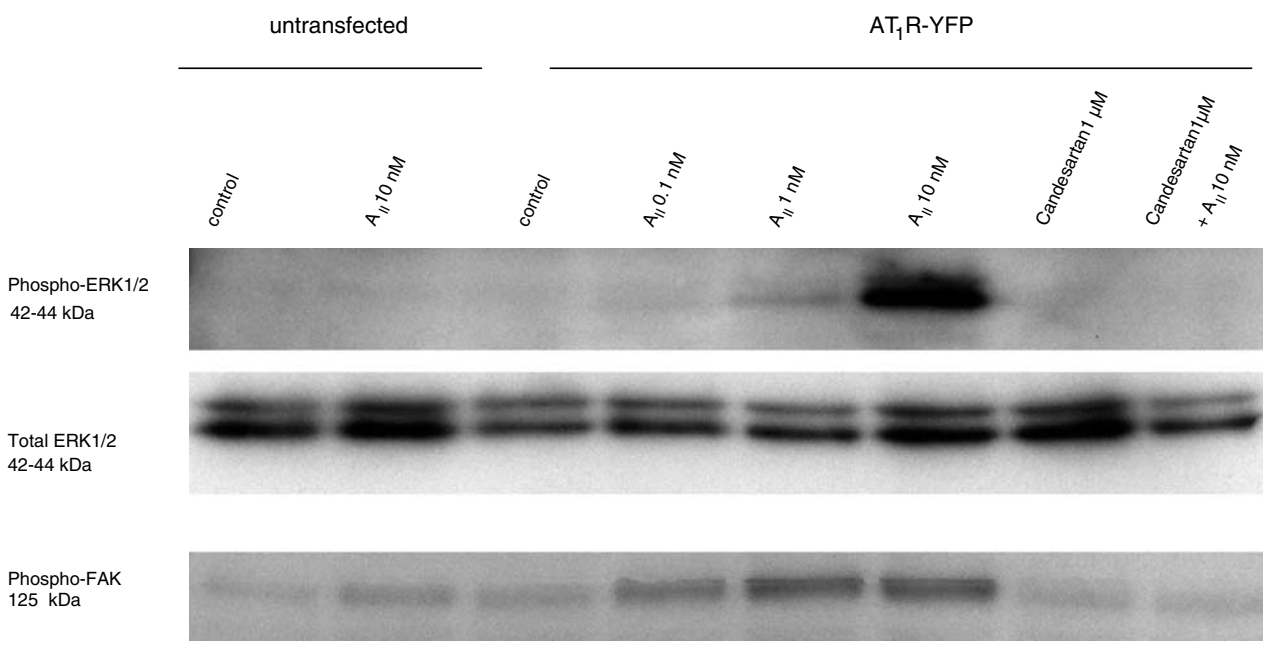

Figure 4 Kinase phosphorylation mediated by AT_1R – YFP. Untransfected HEK 293 cells or cells stably expressing AT_1R – YFP were treated with A_{II} (0.1 – 10 nM, 10 min), candesartan (1 μM, 15 min), or a combination of both. The same total cell extracts were immunoblotted for phospho-ERK1/2, total ERK1/2 (to assess comparable loading of tracks) and phospho-FAK. Representative results of two experiments.

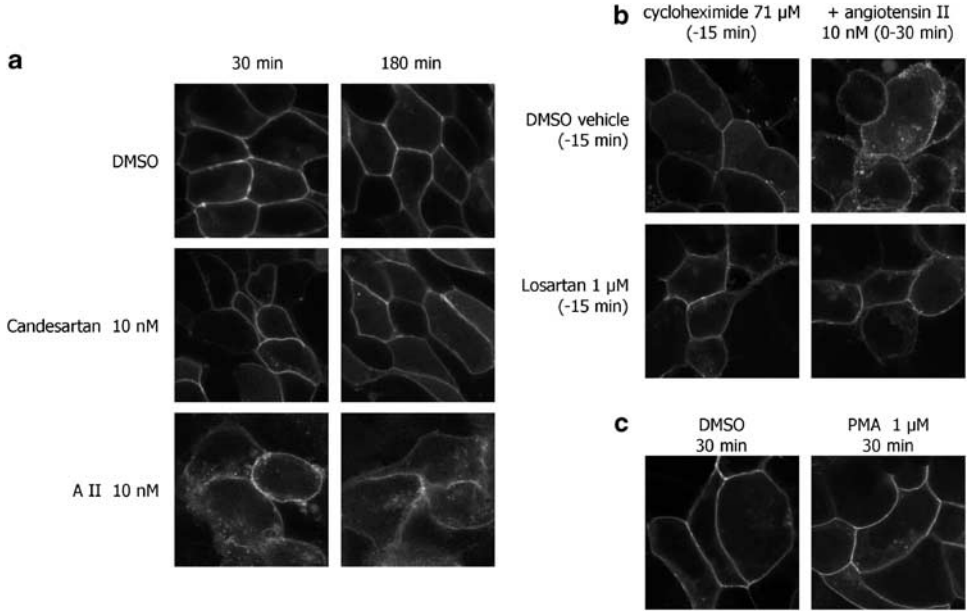

Figure 5 Subcellular localization of the AT_1R – YFP fusion protein in stably transfected HEK-293 cells maintained in the complete culture medium and treated with cycloheximide (71 μM). (a) Cells were treated with the indicated concentration of the agonist A_{II} or the antagonist candesartan for definite time periods. (b) Losartan treatments reduces A_{II}-induced receptor endocytosis. (c) Effect of a short treatment (30 min) with PMA. The selected confocal planes are halfway to the thickness of most cells. All photomicrographs represent square fields with 40 μm sides. In all panels, control cells were treated with the DMSO vehicle of candesartan, losartan or PMA.

in stably transfected cells may reflect multiple affinity states because of the high expression of the recombinant receptors in excess of available G proteins; the less intense transient transfection was more convincingly saturable (Figure 2). However, all components of $[^3H]A_{II}$ binding were determined by the expression of AT_1R – YFP, as shown by the absence of specific binding to untransfected cells (Figure 2). It can at least be said that there is a sizeable specific binding in stably transfected cells at A_{II} concentrations that functionally activate those cells (1 – 10 nM). The low potency of A_I in the

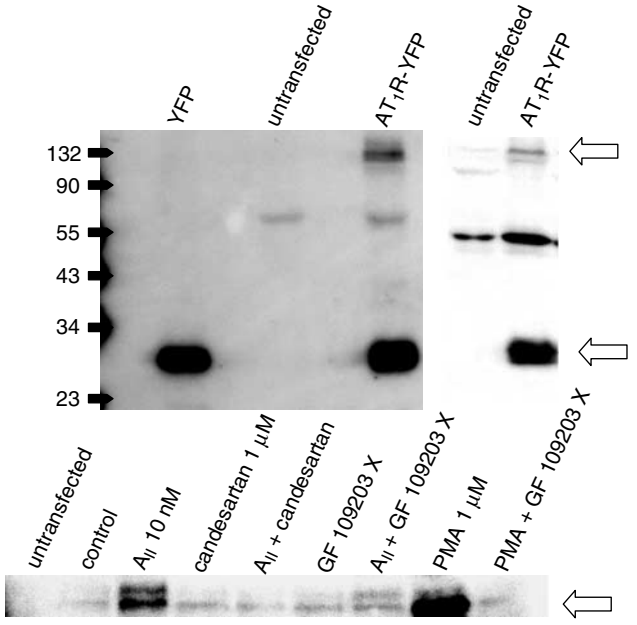

Figure 6 Immunoblots of total HEK 293 cell extracts based on anti-GFP monoclonal anti-GFP antibodies. Top: Total cell extracts from cells transiently transfected with YFP, from untransfected cells or from cells stably expressing AT_1R – YFP were revealed using the Clontech monoclonal (three tracks at the left). Total cell extracts from untransfected cells or cells stably expressing AT_1R – YFP were also submitted to reaction with the Zymed monoclonal (two tracks at the right). Bottom: Immunoblots of total HEK 293 cell extracts based on the Clontech anti-GFP antibodies. Cells were pretreated as indicated before extraction. Results are representative of two to three experiments for each condition.

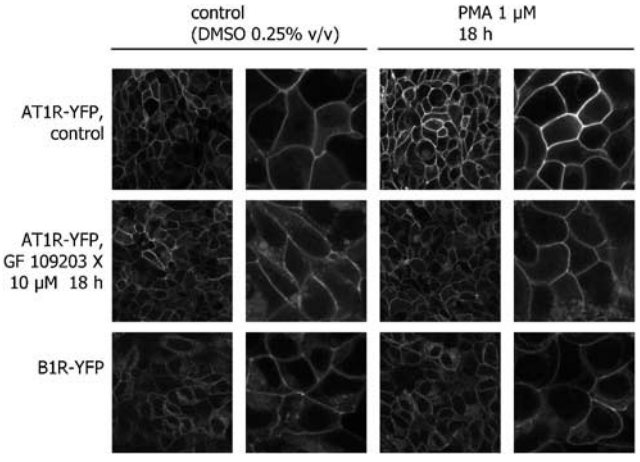

Figure 7 Effect of the PKC activator PMA, alone or in combination to the PKC inhibitor GF 109203 X, on the expression of AT_1R – YFP in stably transfected HEK 293 cells. Presentation as in Figure 5. Each frame at the left is a lower magnification (sides of the square fields 120 μm).

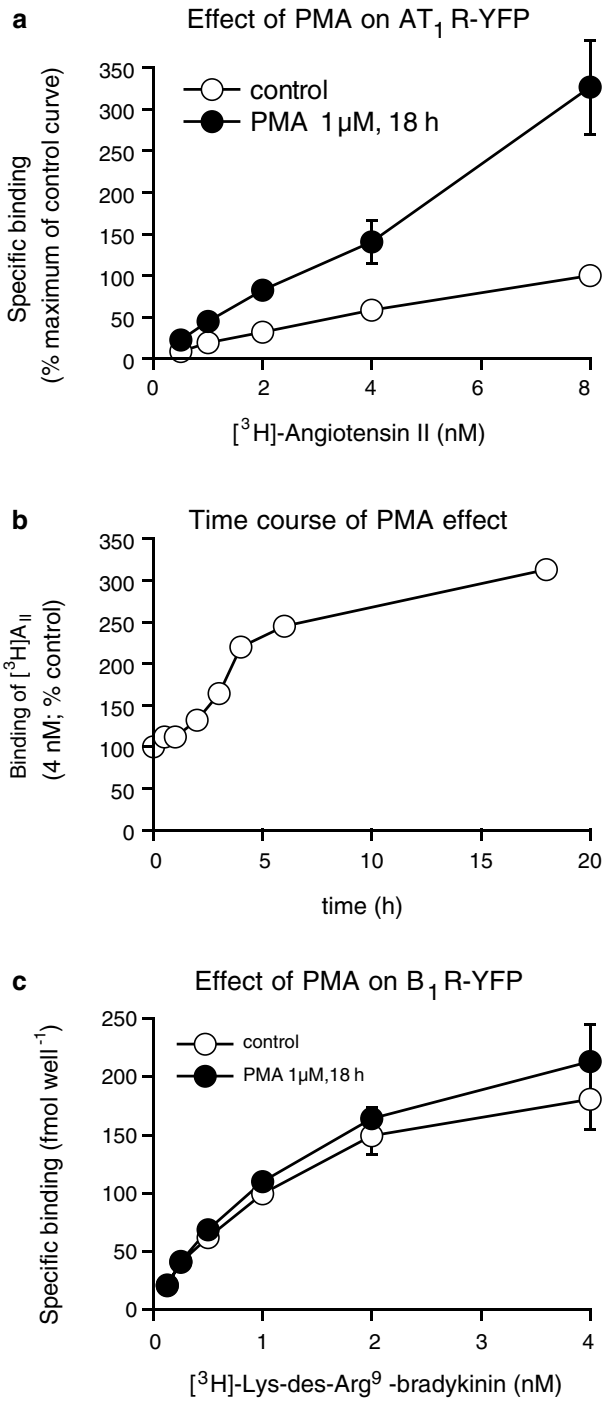

Figure 8 Investigation of the effect of PMA on HEK 293 cells using radioligands. (a) Effect of PMA on $[^3H]A_{II}$ binding to HEK 293 cells stably expressing AT_1R – YFP. Results are expressed as percent of the maximal specific binding recorded in the control curve (8 nM of radioligand). (b) Time course of PMA effect on $[^3H]A_{II}$ (4 nM) binding to HEK 293 cells stably expressing AT_1R – YFP. (c) Effect of PMA on [^{3}H]Lys-des-Arg^9-bradykinin binding to HEK 293 cells stably expressing B_1R – YFP. In panels a and c, values are means ± s.e.m. of five or three separate experiments, respectively.

aorta (Regoli *et al*., 1974) was also observed in the radioligand binding competition assay (Figure 2b). As established for the human AT_1R (Vanderheyden *et al*., 1999), all tetrazole antagonists tested could displace completely $[^3H]A_{II}$ binding from AT_1R – YFP, although some of these drugs were insurmountable in both the contractility assay (WT receptor, Figure 1) and PLA_2 assay (Figure 3b). Candesartan and EXP-3174 were the most consistently insurmountable antagonists in the contractility and PLA_2 assays, while losartan appeared

fully surmountable. This difference in behavior has been recently attributed to a slow dissociation kinetics for the insurmountable drugs (Vauquelin *et al*., 2001). Our functional data are consistent with this idea, as long drug washout is not sufficient for recovery of a full A_{II} maximal effect in the rabbit aorta for candesartan, EXP-3174 and irbesartan (data not shown). Also, tetrazole antagonists do not promote fluorescent receptor sequestration into the cells (Figure 5), a finding opposed to other systems (some insurmountable antagonists promote the internalization of the rabbit bradykinin B_2R, Houle *et al*., 2000; or cholecystokinin receptor, Roettger *et al*., 1997).

The fluorescent form of the AT_1R retains the capacity of the wild-type receptor to recruit PLA_2, ERK1/2 and FAK (Figures 3 and 4; Touyz & Schiffrin, 2000). Miserey-Lenkei *et al*. (2001) have shown that a similar fusion protein composed of the human AT_1R conjugated to GFP is coupled to the $G\alpha_{q/11}$ protein in a dynamic manner, further supporting that such a fluorescent receptor is fully functional. Another signalling-dependent response, receptor endocytosis, was documented using confocal microscopy in agonist-stimulated cells that express AT_1R-YFP (Figure 5). The present evidence for this response, obtained in live cells, is complementary to that obtained using antigenically flagged AT_1R in permeabilized cells (Hein *et al*., 1997). Previous studies support that AT_1R endocytosis is preceded by the phosphorylation of a Ser–Thr-rich domain in the C-terminal tail (a GRK phosphorylation domain; Smith *et al*., 1998), and is followed by extensive recycling at the plasma membrane level without important agonist-induced downregulation, at least in some cell types (Hein *et al*., 1997; Richard *et al*., 1997). The recycling mechanism of the AT_1R has been recently addressed, and part of the presented evidence was based on a receptor–GFP conjugate (Hunyady *et al*., 2002).

The fusion protein AT_1R-YFP is apparently a $\sim$120 kDa band when immunoblotted using anti-GFP (Figure 6). Subtracting the molecular weight of YFP (27 kDa) from that figure would yield an estimate of $\sim$95 kDa for the rabbit receptor. This value is similar to the one found for the fully glycosylated human AT_1R (Lanctot *et al*., 1999). Adding A_{II} to cells stably expressing AT_1R-YFP does not result in a downregulation of the fusion protein, but rather on an increased expression (immunoblot, Figure 6). The use of PMA and of a PKC inhibitor suggests that the PKC activation is the AT_1R signalling event responsible for this effect. PMA treatment increases both the surface binding sites for A_{II} (Figure 8a) and the intensity of the fluorescent protein at the membrane level (Figure 7), but the drug does not promote receptor endocytosis as does A_{II}. The increased AT_1R expression in vascular smooth muscle cells treated with PMA, attributed to increased gene transcription (Holzmeister *et al*., 1997), is irrelevant to explain PMA effect on AT_1R-YFP abundance, because the fusion protein is not expressed under the control of the endogenous promoter. Rather, a constitutive CMV promoter is involved in the expression vector utilized and PMA may not increase CMV promoter activity unless phorbol ester responsive elements are deliberately inserted into to the promoter structure (Kotarski *et al*., 2001). Further, the cell surface content of B_1R-YFP, as based on the same expression vector, is only modestly stimulated by PMA (Figures 7 and 8b), supporting that PMA-activated system(s) selectively interact with the AT_1R protein. Thus, a PKC-dependent mechanism may reduce agonist-independent AT_1R-YFP breakdown. Partly different sets of Ser/Thr residues located in the AT_1R C-terminal tail are involved in GRK- and PKC-mediated receptor phosphorylation (Tang *et al*., 1998). Different molecular PKC substrate(s) may also be involved in the decrease of an agonist-independent clearance mechanism that remains to be clarified. For comparison, the B_1R is not phosphorylated upon agonist stimulation (Blaukat *et al*., 1999), perhaps consistent with the minimal effect of PMA on B_1R-YFP abundance in HEK 293 cells.

In summary, the conjugate AT_1R-YFP retains the pharmacological properties of the parent rabbit AT_1R; tetrazole receptor antagonists do not promote receptor translocation; PKC activation increases the abundance of AT_1R-YFP possibly by attenuating a form of agonist-independent receptor decay.

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