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Internalization of the rat AT_{1a} and AT_{1b} receptors: pharmacological and functional requirements

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Abstract

The capacity of the angiotensin II (AngII) agonist [Sar1]AngII, the antagonist [Sar1-Ile8]AngII and the non-peptidic antagonist DuP753 to undergo receptor internalization were studied in Chinese hamster ovary cells expressing rat AngII type 1a or 1b receptors (AT_{1a} or AT_{1b}) or a mutant of AT_{1a} (Asn^{74}) unable to couple G-protein. In this expression system, the ligand-induced internalization of rat AT_{1a} and AT_{1b} are similar. Moreover, peptidic ligands, either the agonist or antagonist, induce a significant internalization of AT_1 receptors, but the non-peptidic antagonist DuP753 is far less potent. Finally, the normal internalization of the mutant Asn^{74} demonstrates that receptor activation and G-protein coupling are not required for AT_{1a} internalization.

Key words: Angiotensin II; G-protein coupled receptor; Internalization

1. Introduction

Two pharmacologically distinct classes of receptors have been identified for the vasoactive peptide angiotensin II (AngII): AT_1 and AT_2 [1,2]. Both of them belong to the seven transmembrane domain receptor family [3–6]. The precise functions and intracellular signalling pathways of AT_2 have not yet been well defined. AT_1 receptors are responsible for most of the classical physiological actions of AngII by interacting with a G-protein, which induces phospholipase C (PLC) activation and the subsequent cascade of intracellular events (phosphoinositide hydrolysis, calcium signalling, protein kinase C activation) [7]. Two AT_1 isoforms (AT_{1a} and AT_{1b}) that are encoded by different genes have been identified in rodents [8–10]. AT_{1a} and AT_{1b} have different patterns of expression, in terms of tissue specificity [11,12] and expression during development (J.-M. Gasc, personnal communication), but no major difference in their pharmacology and signalling pathways has been identified up to now [11,13].

Agonist-induced receptor internalization occurs during activation of a large number of cell surface receptors, including G-protein coupled receptors [14]. There is increasing evidence that the binding of the ligand to G-protein coupled receptors induces a lateral movement of ligand–receptor complexes followed by their association with coated pit structures and their subsequent internalization [15,16]. This process has already been clearly demonstrated for other membrane-bound receptors, such as those for transferrin or EGF [17,18]. Internalization of the AngII–AT_1 complexes has been described in adrenal glomerulosa cells [19,20] and vascular smooth muscle cells [21,22] using radioiodinated ligands. However, the nature of the AT_1 subtype involved in this process and the molecular mechanisms underlying this process, such as receptor activation and G-protein coupling, are not known. Mutational analysis of different G-protein coupled receptors has shown that coupling to a G-protein may or not be a prerequisite for internalization, depending on the nature of the receptor and its cognate G-protein [23,24].

Several questions are addressed in the present study: (i) is there any difference in internalization of AT_{1a} and AT_{1b} receptors?; (ii) are the peptidic or non-peptidic analogs of AngII similarly internalized?; and (iii) is a functional G_q coupled AT_1 receptor required for internalization?

For this purpose, three different recombinant forms of AT_1 were separately expressed in CHO cells and analyzed for their capacity to internalize the AngII peptidic agonist [Sar1]AngII, the peptidic antagonist [Sar1-Ile8]AngII and the non-peptidic antagonist DuP753 (or losartan). The three recombinant receptors used were wild-type rat AT_{1a} and AT_{1b} and a rat AT_{1a}, shown to be incapable of coupling to the G-protein [25], in which a conserved charged residue of the second transmembrane domain had been mutated ($Asp^{74}\rightarrow Asn$).

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Abbreviations: AngII, angiotensin II; [Sar1]AngII, [sarcosine-1] angiotensin II; [Sar1-Ile8]AngII, [sarcosine-1,isoleucine-8]angiotensin II; PLC, phospholipase C; PKC, protein kinase C; IP, inositol phosphate; CHO, Chinese hamster ovary.

2. Materials and methods

2.1. Transfection and expression

CHO AT_{1a} and CHO Asn^{74} clones were described previously [25,26]. In order to establish CHO AT_{1b} clones, the plasmid $pEAT_{1b}$ was constructed by inserting the AT_{1b} cDNA insert of the plasmid pCDNAI-

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IAT_{1b} (a generous gift from K. Sandberg) into the *Not*1 and *Eco*R1 sites of the expression vector pECE [27]. Cells were co-transfected with 10 μg $pEAT_{1b}$ and 2 μg of the selection marker pSV2neo by the calcium phosphate precipitation method [28]. Transfected cells were selected by their resistance to 750 μg/ml G418 (Gibco). Individual resistant colonies expressing high levels of AT_{1b} were cloned by limiting dilution.

2.2. Cell culture

CHO K1-transfected cells were maintained in Ham's F12 medium supplemented with 10% fetal calf serum plus 0.5 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Boehringer-Mannheim).

2.3. Functional characterization

[Sarcosine-1]AngII and [Sarcosine-1, Isoleucine-8]AngII ([Sar1]AngII and [Sar1-Ile8]AngII, Sigma) were labelled by the chloramine T method [29] and monoiodinated products were purified by HPLC.

For saturation binding assays, cells were subcultured into 24-well culture trays and incubated for 45 min at 22°C with various ^{125}I-labelled [Sar1]AngII concentrations in 50 mM Tris-HCl, 6.5 mM $MgCl_2$, 125 mM NaCl, 1 mM EDTA and 1 mg/ml BSA, pH 7.6. For competitive binding assays, cells were incubated in the same buffer with 0.5 nM [^{125}I][Sar1]AngII and various concentrations of competing ligands. Non-specific binding was determined in the presence of 1 μM AngII. Each experiment was carried out in duplicate. Binding data were analysed with a non-linear least-squares curve fitting procedure, Ebda-Ligand (Elsevier-Biosoft, Cambridge, UK) [30].

[^{3}H]Inositol phosphate (IP) production in response to increasing concentrations of AngII or [Sar1-Ile8]AngII was determined in CHO AT_{1a} and CHO AT_{1b} cells, essentially as previously described [31]. Cells were labelled with 2 μCi/ml [^{3}H]*myo*inositol for 24 h and then incubated with AngII or [Sar1-Ile8]AngII for 30 min in the presence of 10 mM LiCl. After purification on a Dowex 1 × 8 anion exchange resin (Bio-Rad), the total IP fraction was measured.

2.4. Internalization assay

Two procedures were used depending on the ligand. For radioactive agonist or antagonist peptides, cells in 24-well culture trays were placed at 4°C, washed with PBS and incubated for 180 min in binding buffer containing [^{125}I][Sar1]AngII or [^{125}I][Sar1-Ile8]AngII, in the absence (total binding) or presence (non-specific binding) of 1 μM AngII. The concentrations of radioligands used corresponded to the previously determined dissociation constant (K_d) so that approximately 50% of the receptor were occupied. Cells were then washed twice with 0.5 ml ice-cold binding buffer and incubated at 37°C for various times to permit internalization. Finally, the cells were placed at 4°C and washed once in binding buffer. Half of the replicate wells were immediately incubated with 0.5 ml of 1 N NaOH and the solution counted to determine total and non-specific binding at each time. In the other wells, the radioactivity bound to the surface of the cells was eluted by a 5 min incubation in 0.5 ml of ice-cold buffer containing 50 mM glycine and 125 mM NaCl, pH 3. Cells were washed with an additional 0.5 ml of this buffer, and radioactivity in the combined acid washes was counted and considered to represent the total and non-specific surface-associated radioactivity. Radioactivity remaining within the cells after acid treatment was solubilized with 0.5 ml of 1 N NaOH and was considered to represent total and non-specific internalized radioactivity. The sum of the specific surface-bound and internalized fractions was comparable to the total specific binding measured, thus verifying the validity of this procedure. When the radiolabelled ligand was [^{3}H]DuP753 (NEN), the procedure for measuring receptor internalization was modified because the binding steady state was only obtained after incubation at 4°C for 24 h. Since this resulted in significant cell loss, the binding and internalization were realized together at 37°C for varying times. After these incubations the cells were placed at 4°C, washed twice in ice-cold buffer and processed as above. To confirm that acid-resistant radioactivity corresponds to internalization of the ligand–receptor complexes, cells were incubated in binding buffer with or without 0.4 M sucrose for 30 min at 37°C prior to a 15 min exposure to [^{3}H]DuP753 under the same conditions. For [^{125}I][Sar1]AngII or [^{125}I][Sar1-Ile8]AngII the procedure was as described above except that 0.4 M sucrose was present during the binding and internalization (20 min at 37°C) steps. Cells were then processed normally.

3. Results

The pharmacological and signalling properties of AT_{1a} and AT_{1b} receptors expressed separately in CHO cells were first evaluated. As indicated in Table 1, CHO AT_{1a} and CHO AT_{1b} exhibit a similar unique high affinity binding site (K_d = 1.07 ± 0.03 nM and 1.23 ± 0.14 nM, respectively). The density of receptors in CHO AT_{1b} (B_{max} = 3.54 ± 0.84 × 10^5 sites/cell) is twofold higher than in CHO AT_{1a} (B_{max} = 1.65 ± 0.39 × 10^5 sites/cell), reflecting clonal variations. Competition assays with [Sar1-Ile8]AngII and DuP753 show no significant difference in the K_i of these two antagonists for AT_{1a} and AT_{1b} receptors.

The production of IP in response to increasing concentrations of AngII was then compared in CHO AT_{1a} and CHO AT_{1b} (Fig. 1). The half maximal response was obtained with similar doses of AngII in CHO AT_{1a} (EC_{50} = 0.49 ± 0.07 nM) and CHO AT_{1b} (EC_{50} = 0.37 ± 0.04 nM) (non significant difference). However, the maximal response obtained for CHO AT_{1b} was 60% higher than that of CHO AT_{1a}. Analysis of the maximal IP response in several clones of CHO AT_{1a} expressing this recombinant receptor at different densities (data not shown) clearly indicates that the maximal IP response correlates with the B_{max}. Therefore the most likely explanation for the increased maximal IP response in the CHO AT_{1b} cell line is the higher level of expression of AT_{1b} receptors. The production of IP in response to increasing concentrations of [Sar1-Ile8]AngII was assayed in CHO AT_{1a} and CHO AT_{1b} cells. The partial agonist activity of this compound observed in some cellular models [32] could not be detected in these cells since the intracellular IP level remained similar to the basal level with up to 10^{-6} M [Sar1-Ile8]AngII.

Ligand-mediated internalization of the AT_{1a} and AT_{1b} was analysed for three different ligands. Fig. 2a shows the time–course of internalization for these two receptors when it was induced by [Sar1]AngII, a peptidic agonist of AngII. Maximal internalization was obtained after

Table 1
Pharmacological characterization of rat AT_{1a} and AT_{1b} receptors expressed in CHO cells

	AT_{1a}	AT_{1b}	n
(a) K_d (nM)	1.07 ± 0.03	1.23 ± 0.14	3
B_{max} (10^5 sites/cell)	1.65 ± 0.39	3.54 ± 0.84	3
(b) K_i (nM)			
[Sar1-Ile8]AngII	0.83 ± 0.18	0.67 ± 0.23	3
DuP753	2.81 ± 0.25	2.53 ± 0.46	3

(a) Binding parameters of [^{125}I][Sar1]AngII
(b) Affinities of both receptor subtypes for a peptidic and a non-peptide antagonist.
Data represent the mean ± S.E.M. obtained from the indicated number of experiments (n) with each point being performed in duplicate.

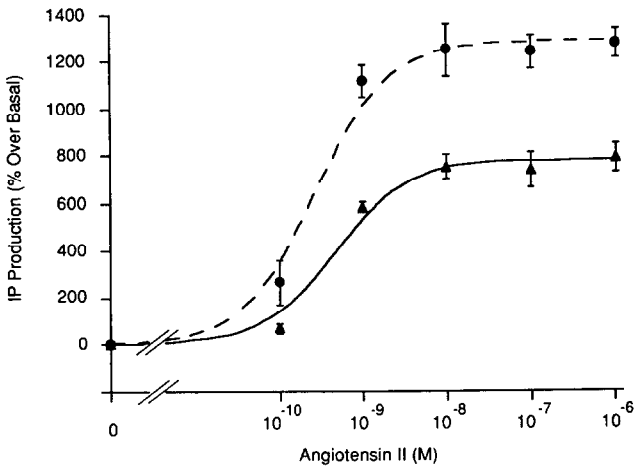

Fig. 1. AngII-induced stimulation of inositol phosphate production. Total inositol phosphates were measured in CHO AT_{1a} (▲) and CHO AT_{1b} (●) cloned cell lines, in the presence or absence of increasing amounts of AngII. Results are expressed as the ratio of the cpm in stimulated vs. unstimulated cells and represent the mean ± S.E.M. of three independent experiments performed in triplicate.

20 min at 37°C and was 84.28 ± 1.54% and 78.68 ± 1.38% of the total specific binding for AT_{1a} and AT_{1b}, respectively. The time necessary to internalize 50% of the ligand–receptor complex ($t_{1/2}$) was 5 min for both receptors. Receptor internalization mediated by a peptidic antagonist ([Sar1-Ile8]AngII) was then analyzed (Fig. 2b). Internalized radioactivity increased to a maximum (77.26 ± 3.86% for AT_{1a} and 75.60 ± 3.25% for AT_{1b}) after 20 min at 37°C. In the presence of sucrose, only 5% of this radioactivity was detected, indicating that 95% of the acid-resistant radioactivity corresponded to internalization via clathrin-coated pits (data not shown). The $t_{1/2}$ was also of approximately 5 min for the two isoforms. Finally, the internalization induced by a non-peptidic antagonist (DuP753) is shown in Fig. 3a. The acid-resistant [^{3}H]DuP753 fraction reached a maximum of 29.58 ± 3.34% after 10 min at 37°C for AT_{1b} and 28.09 ± 2.09% after 15 min at 37°C for AT_{1a}. To verify that the acid-resistant fractions did not represent incomplete dissociation of the bound DuP753 during the acid wash procedure rather than a specific internalization process, the effect of sucrose, which is known to abolish receptor internalization via clathrin-coated pits was studied [33]. As shown in Fig. 3b, approximately 60% of the acid-resistant radioactivity associated with either AT_{1a} or AT_{1b} is inhibited by sucrose. Therefore, approximately 17% of the bound [^{3}H]DuP753 is internalized.

To determine whether G-protein coupling is required for AT_1 internalization, we have analysed the internalization of an AT_{1a} receptor which contains a point mutation (Asp^{74}→Asn) that has been shown to abolish G-protein coupling of the receptor. As can be seen in Fig. 2a and b, the uncoupled AT_{1a} receptor is internalized to the same extent as the wild-type, after binding of [^{125}I][Sar1]AngII or [^{125}I][Sar1-Ile8]AngII at 4°C. [^{3}H]DuP753-mediated internalization of this mutant could not be assayed because of the low affinity of the mutated receptor for this compound (K_i = 17 nM).

4. Discussion

AT_1 receptors belong to the large family of seven transmembrane domain receptors of which the more familiar examples include the adrenergic, dopaminergic, serotoninergic, muscarinic and pituitary hormone receptors [34]. Several of these classes of receptors are composed of multiple isoforms, all of which bind a specific agonist but differ by their pharmacology and/or by their signal transduction mechanisms. In the case of AT_1 receptors, two subtypes have been clearly identified in rodents through cDNA cloning studies, but their potential

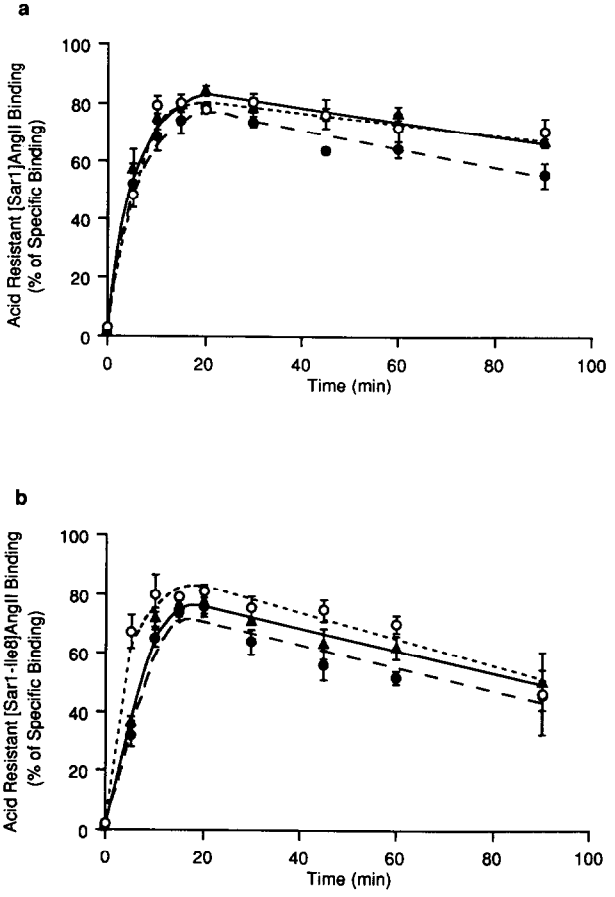

Fig. 2. [Sar1]AngII- and [Sar1-Ile8]AngII-induced receptor internalization. CHO cells expressing AT_{1a} (▲), AT_{1b} (●) or Asn^{74} (○) were prelabelled with [^{125}I][Sar1]AngII (a) or [^{125}I][Sar1-Ile8]AngII (b) on ice for approximately 3 h. The cells were then washed and incubated at 37°C for various time periods, to allow internalization. Non-internalized tracer was removed by acid washing, and internalized tracer levels were determined after NaOH treatment. Results are expressed as percent of total specific binding and represent the mean ± S.E.M. of three independent experiments performed in duplicate.

functional differences are still a matter of debate. It was therefore important to first compare the pharmacological properties and signalling mechanisms of AT_{1a} and AT_{1b}, before investigating potential internalization differences.

The most extensive comparison of rat AT_{1a} and AT_{1b} pharmacologies was recently described by Chiu et al. [13] in CHO cells and did not show any significant differences in the pharmacological profiles of the two receptors. However, Sandberg et al. [9] have reported that AT_{1b} transiently expressed in COS cells has a slightly higher affinity for [desAsp1]AngII as compared to that of AT_{1a} reported in the literature. The preliminary results presented here indicate no major difference in the pharmacological profiles of these two receptors, confirming the results of Chiu et al.

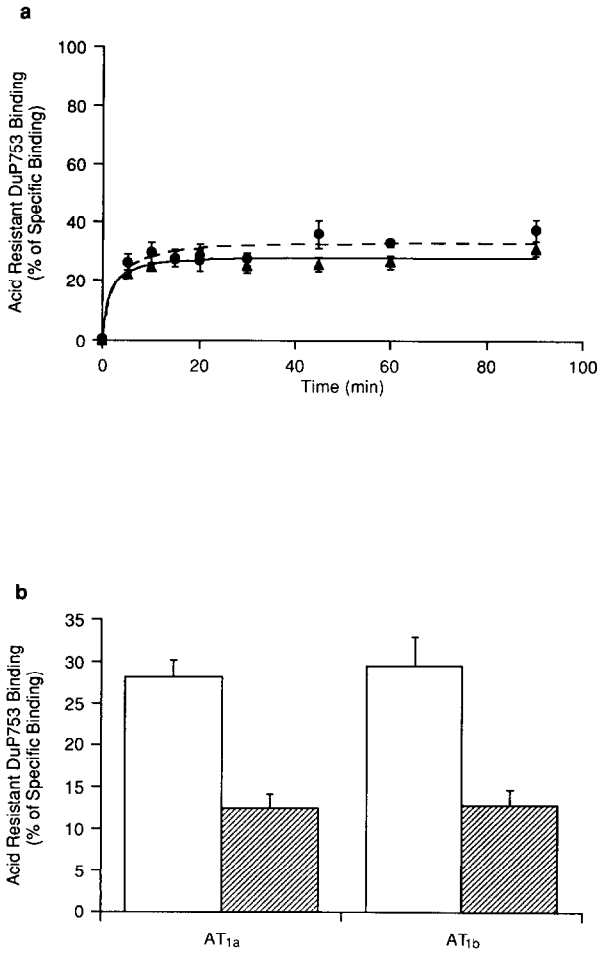

Fig. 3. DuP753-induced receptor internalization. a: CHO cells expressing AT_{1a} (▲) or AT_{1b} (●) were incubated with [^{3}H]DuP753 for various time periods at 37°C. b: Cells were preincubated for 30 minutes at 37°C with (hatched bars) or without (open bars) 0.4 M sucrose, prior to a 15 minute incubation with [^{3}H]DuP753 in the same buffer at 37°C. In both cases, cells were then placed on ice and after extensive acid washing, the remaining cell-associated radiactivity was determined after NaOH treatment. Results are expressed as percent of total specific binding and represent the mean ± SEM of three independent experiments performed in duplicate.

Both AT_{1a} and AT_{1b} activate PLC, which results in an increase in intracellular inositol-(1,4,5)-trisphosphate and diacylglycerol concentrations leading to intracellular calcium mobilization and PKC activation. No major difference in the signalling pathways of rat AT_{1a} and AT_{1b} has been reported so far [11]. However, a difference has been reported in the shape of the dose–response curve for Ca^{2+} mobilization at high concentrations of AngII ($>10^{-7}$ M), when rat AT_{1a} or AT_{1b} mRNAs were injected into *Xenopus* oocytes. The maximal response mediated by the rat AT_{1b} was lower at the higher ligand concentrations, whereas the maximal response was maintained for the rat AT_{1a} [9]. In the present study, where the AT_1 receptors were expressed in CHO cells, these differences were not observed for another second messenger (IP), therefore suggesting a cell- or species-specific phenomenon.

In conclusion, the preliminary comparative characterization of AT_{1a} and AT_{1b} receptors did not identify functional differences and the study was therefore extended to the internalization process.

Internalization of natural AngII receptors was previously studied in two main cellular models: rat vascular smooth muscle cells which express mainly the AT_{1a} subtype, and bovine adrenocortical cells which express a single AT_1 receptor species. When AngII agonist ligands were used, as much as 80% of the bound analog was internalized and the $t_{1/2}$ ranged from 1.5 to 10 min in these cells [19–22]. Interestingly, differences were observed in the internalization of AngII peptidic antagonist as rat vascular smooth muscle cells were found to internalize AngII peptidic agonists and antagonists to the same extent [35], whereas bovine adrenocortical cells only internalize 10–25% of the peptidic antagonist [19].

The present data show that the peptidic agonist [Sar1]AngII and antagonist [Sar1-Ile8]AngII are internalized to the same extent (80%) by both AT_{1a} and AT_{1b} and have similar kinetics of internalization ($t_{1/2}$ = 5min and t_{max} = 20 min). As similar profile of internalization are observed for the peptidic antagonist with both rat AT_{1a} and AT_{1b}, it is apparent that the species-specific differences in response to AngII peptidic antagonist are not displayed by the two rat AT_1 isoforms.

Another interesting observation is the low level (17%) of receptor internalization induced by the non-peptidic AngII antagonist DuP753, via clathrin coated pits. A low rate of internalization, via this pathway, has also been reported for the insulin receptor, using specific antibodies as ligand. This observation led to the identification of the constitutive pathway of internalization as opposed to the ligand-induced pathway [36]. There is no clear demonstration of constitutive internalization of G-protein coupled receptors, but it can be postulated that DuP753 internalizes via a constitutive and not a ligand-dependent pathway. Such an hypothesis merits further study.

Finally, a previously described mutant of the rat AT_{1a} receptor was used to analyse the relationship between G-protein coupling and receptor internalization. This mutant was selected because the single mutation of Asp^{74}-to-Asn does not alter the affinity of the receptor for peptidic agonists, but results in a receptor unable to transduce any measurable signal into CHO cells after agonist binding [25]. This receptor was found to internalize normally with either peptidic agonist or antagonist, thus excluding a role for G-protein coupling and/or effector activation in internalization of the AT_{1a} receptor subtype. This conclusion is also sustained by the normal internalization of peptidic antagonists, which do not activate the signalling pathway.

This result seems to be unique among the G-protein coupled receptors which activate PLC. Accumulated data indicate that, in this subfamily, G-protein coupling is needed for internalization. For example the mutation of Asp^{71} in the second transmembrane domain of the TRH receptor results in an inactive receptor which internalizes very poorly (15% vs. 60% for the wild-type) [23]. The homologous mutation of the M1 muscarinic receptor expressed in U293 human kidney cells produces a similar result [37]. Moreover compounds such as the aminosteroid U73122, which directly inactivate the G-protein that couples to the M3 muscarinic receptor, inhibit the internalization of this receptor [38].

In the subfamily of receptors coupled to G_s-protein and adenylate cyclase, there is no apparent link between G-protein coupling and internalization. Several mutations of the third intracellular loop and proximal carboxyl-terminal domain of the β_2-adrenergic receptor impair G-protein coupling but not internalization [24]. Moreover, while another deletion of the amino-terminal part of the third intracellular loop of the hamster β_2-adrenergic receptor resulted in the destruction of its ability to both couple to G_s-protein and undergo ligand-mediated internalization, replacement of the deleted region by the corresponding region of the M1 muscarinic receptor restored internalization but not G-protein coupling [39,40].

The nature of the mutations introduced in each of these receptors may explain the variable implication of G-protein coupling in internalization. However, it is unlikely since the same mutation in the second transmembrane domain of either AT_1 or TRH and M1 muscarinic receptors results in similar G-protein uncoupling but different internalization profiles. Our results suggest that, while normal G-protein coupling is required for internalization of some of these receptors, a conformational change of the specific structural motif that mediates internalization can occur independently of G-protein coupling for other receptors (such as AT_1). Thus, this internalization process depends more on the nature of the receptor than on the nature of its signalling pathway. We can postulate that AT_1 receptor internalization, like that of the β-adrenergic receptors, has conformational rather than functional requirements.

In conclusion, the present study provides the first evidence that both AT_{1a} and AT_{1b} are able to undergo internalization upon interaction with either AngII peptidic agonist or antagonist, but that non-peptidic antagonists are poorly internalized. Furthermore, it demonstrates that AT_1 receptors are unique among the G-protein coupled receptors which activate PLC as there is no link between activation of the signalling pathway and receptor-ligand internalization.

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