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Angiotensin AT_2 Receptor Degradation Is Prevented by Ligand Occupation

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A substantial increase in [^{125}I]Sar^1,Ile^8-Angiotensin II binding activity can be observed 24 hours after treatment of R3T3 cells with AT_2 receptor agonists and antagonists. An increase in the radioligand binding activity, although less profound, can also be observed 6 hours after AT_2 receptor ligand treatment, on fetal human kidney cells expressing a recombinant human AT_2 receptor. However, the increase in radioligand binding activity cannot be detected unless the ligands are removed from the cell surface by an acid-glycine (pH 3) wash, just prior to the binding assay. Interestingly, an acid-glycine wash 24 hours prior to the binding assay causes a dramatic decrease in the radioligand binding activity on untreated R3T3 cells. This decrease, which was prevented by angiotensin II treatment, suggests the existence of an unknown endogenous factor which, like the AT_2 receptor ligands, seems to prevent AT_2 receptor degradation.

Angiotensin II (ANG II), the main effector peptide of the renin-angiotensin system, mediates its effects through angiotensin receptors. So far two angiotensin receptors have been cloned, namely the AT_1 and the AT_2 receptor (1). Most of the known effects of ANG II, in blood pressure control and body fluid homeostasis are mediated through the AT_1 receptor (2). Concerning the AT_2 receptor, several reports have demonstrated that it is involved in antiproliferative effects (3, 4), apoptosis (5-7) neurite outgrowth and differentiation (8-10), and in embryonic development (11).

A useful tool in AT_2 receptor research are R3T3 cells, which of the known angiotensin binding sites, exclusively express the AT_2 receptors. They are mouse embryonic fibroblasts derived from the Swiss 3T3 cell strain. Experimental results obtained from these cells might be representative of AT_2 receptor function in fetal development, since one of the sources of abundant AT_2 receptor expression in fetal mesenchymal tissue are undifferentiated fibroblasts (11).

The function of the AT_2 receptor concerning R3T3 cells is not clear. However, binding assays have shown that the number of cell surface AT_2 receptors increases dramatically when R3T3 cells are contact-inhibited and that stimulation with various mitogens causes a rapid decline of cell surface AT_2 receptors (12-15). In contrast, stimulation of cells with not only ANG II but also AT_2 receptor antagonists appears to markedly enhance the number of cell surface AT_2 receptors (12).

We present data showing that besides ANG II (ANG 1-8), also the AT_2 receptor ligands ANG I (ANG 1-10), ANG III (ANG 2-8) (13) and CGP 42112 (16, 17) are capable of increasing the number of cell surface AT_2 receptors. We also demonstrate that this increase 24 hours after treatment can only be observed when the ligands are removed from the cell surface by an acid-glycine wash, which indicates that ligands and the AT_2 receptor form a stable complex within the cell membrane. These experiments provide an explanation for the unexpected finding that both agonists and antagonists are capable of increasing the cell surface AT_2 receptors on R3T3 cells and on fetal human kidney cells (HEK293 cells) permanently expressing recombinant human AT_2 (hAT_2) receptors. Finally, we observed that an acid-glycine wash of R3T3 cells, 24 hours before the binding assay, led to a decrease in the level of cell surface AT_2 receptors. This raises the possibility that R3T3 cells produce an endogenous factor, which like the AT_2 receptor ligands, seems to prevent AT_2 receptor degradation.

MATERIALS AND METHODS

R3T3 cells were kindly provided by Dr. David Dudley (Department of Signal Transduction, Parke Davis Pharmaceutical Research Division, Warner-Lambert Company, Ann Arbor, MI USA). [^{125}I]Sar^1,Ile^8-ANG II (specific activity of 2000 Ci/mmol) and Sequenase version 2.0 sequencing kit was purchased from Amersham International plc (Buckinhamshire, UK). [^{125}I]Tyr^4-ANG II was purchased from NEN (Boston MA, USA). Dulbecco's modified essential medium (DMEM), minimum essential medium (MEM), essential amino acids, trypsin,

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and antibiotics were obtained from Gibco BRL (Eggenstein, Germany), whereas phosphate buffer saline (PBS) and fetal calf serum was purchased from Biochrom (Seromed) (Berlin, Germany). Cell culture dishes were obtained from Nunc (Wiesbaden, Germany). PD 123177 was a kind gift from Dr. David Taylor (Parke Davis Pharmaceutical Research Division, Warner-Lambert Company, Ann Arbor, MI USA), CGP 42112 was a kind gift from Dr. Marc de Gasparo (Novartis, Pharmaceutical Research Division). Hygromycin B was purchased from Boehringer Mannheim (Mannheim, Germany). ANG I, ANG II, ANG III, ANG 3-8, ANG 4-8, ANG 5-8 and ANG 1-7 were purchased from Sigma (Deisenhofen, Germany) or Bachem (Bubendorf, Switzerland). Cloning vectors were obtained from Invitrogen (San Diego, USA), whereas restriction enzymes were purchased from Gibco BRL (Pailsley, UK)

Cell culture. R3T3 cells were cultured as described previously (13), using DMEM supplemented with 10 % fetal calf serum. HEK293 cells were incubated in MEM containing 1 % essential amino acids and 10 % fetal calf serum and 200 μg/ml of Hygromycin B (to maintain selection of the cells hosting the pCEP4 plasmid containing the cDNA for the hAT_2 receptor). R3T3 cells were passaged using 1× trypsin whereas HEK293 cells were trypsinized using a 1× trypsin/EDTA solution. In all experiments, cells were usually grown as a monolayer in 24 well dishes (500 μl media/well) at 37 °C in a humidified atmosphere of air/CO_2 (19:1).

Establishment of a HEK293 cell line permanently expressing the recombinant hAT_2 receptor. RT-PCR was used to isolate the entire coding region of the hAT_2 receptor from human uterus mRNA. A 1.1 kb fragment was initially ligated into pCR3-Uni and both strands sequenced. For expression studies, the hAT_2 cDNA was subcloned into pCEP4 using HindIII and NOTI restriction cuts. A cell line stably expressing the hAT_2 receptor was established as previously described for the rat AT1a receptor (18).

Cell treatment. Cells were incubated in culture medium containing ligands (1 μM final concentration) for six hours. Subsequently, the cells were washed twice with PBS (0.5 ml), provided with fresh medium and a binding assay was performed 24-72 hours later (see figure legends). To specifically remove bound ligands from the AT_2 receptor, cells were washed twice with 0.5 ml PBS, incubated in an acid-glycine buffer (300 μl; 150 mM NaCl, 50 mM glycine, 1 % BSA, pH 3) at 4 °C for 3 minutes, followed by two consecutive washes with 0.5 ml PBS to remove traces of the acidic buffer.

[^{125}I]Sar^1,Ile^8-ANG II binding assay. Cells were either washed twice with 0.5 ml PBS or they underwent an acid-glycine wash. The radioligand [^{125}I]Sar^1,Ile^8-ANG II (40 pM/single well) was added to the media (500 μl/well, HEK293 cells in MEM, R3T3 in DMEM) for 2 hours, at 37 °C. The cells were washed twice with ice-cold PBS, and solubilized in 1 N NaOH for 20 minutes at 37 °C. The solubilized material was then counted for γ radioactivity. Non-specific binding was performed in parallel cultures, incubated with [^{125}I]Sar^1,Ile^8-ANG II in combination with 1 μM ANG II and was usually less than 8 % of total binding.

Data presentation. Results are representative of 3 separately performed experiments (except for the experiment described for figure 3b and 3c, which was repeated once). Single 24 well dishes contained different treatments to allow for direct data comparison. All data points presented per experiment correspond to an average of n=4 plus its standard deviation. Saturation analysis was performed with GraphPad PRISM℠ (GraphPad software, Inc. San Diego CA USA).

RESULTS

Effect of a PBS or an Acid-Glycine Wash Prior to the Binding Assay on Treated and Untreated Cells

R3T3 cells were washed twice with PBS and treated with ANG II or CGP 42112 (figure 1a and b). Non-treated cells served as controls. After treatment, the cells were washed twice with PBS and returned to culture for 24 hours or for 48 or 72 hours. Just prior to the binding assay, the cells underwent either a PBS wash or an acid-glycine wash as described in the methods section. Only the cells which had been treated with ANG II or CGP 42112 and were acid-glycine washed just prior to the binding assay, revealed an increase in binding activity. One day after treatment this increase was at least 2-fold compared to the PBS or acid-glycine washed controls and the treated PBS washed cells. The difference in binding activity was still observed 48 hours after treatment, but became much less pronounced 72 hours after treatment. Saturation analysis of [^{125}I]-ANG II binding to intact R3T3 cells revealed that the increase in binding activity observed after treatment, was due to an increase in the number of cell surface AT_2 receptors, rather than an increase in the receptors affinity (figures 1c and 1d: controls: Bmax = 42,3 fmol/well, Kd = 2.45 nM; ANG II treatment: Bmax = 95,2 fmol/well, Kd = 1.71 nM). R3T3 cells were also treated with ANG I, ANG III, PD 123177 and a number of ANG II derivatives to test their ability to increase the binding activity. Figure 1e shows that the increase in binding activity 24 hours after ANG I, ANG III, PD 123177 and CGP 42112 treatment is only revealed when the cells were acid-glycine washed prior to the binding assay. ANG 3-8, ANG 4-8, ANG 5-8, ANG 1-7 were without effect. These data are consistent with a mechanism involving ligand occupation of the AT_2 receptor.

Figure 2 shows the increase in binding activity after treatment of HEK 293 cells, permanently expressing recombinant hAT_2 receptors, with ANG II and PD 123177. Again, an increase in binding activity could only be observed when the treated cells were acid-glycine washed just prior to the binding assay. The interval between treatment and the binding assay was 6 hours. A 24 hour interval revealed no difference in the binding activity (data not shown).

Long Term Association of [^{125}I]-ANG II with the AT_2 Receptor on R3T3 Cells

Following addition of [^{125}I]-ANG II to R3T3 cells, a steady increase in specific AT_2 receptor binding was observed with a maximum around 90 minutes (figure 3a). At 90 minutes, unlabeled ANG II (1 μM) was added and the amount of radioligand that remained bound was determined over a further 2 hours. Figure 3a illustrates that following the binding of [^{125}I]-ANG II, unlabeled ANG II was not able to compete for binding to the occupied AT_2 receptors. In an additional experiment, we investigated the fate of [^{125}I]-ANG II bound to R3T3 cells over a 24 hour period. R3T3 cells were incubated with [^{125}I]-ANG II for 6 hours, then washed twice with PBS (to remove non-bound ligand) and then

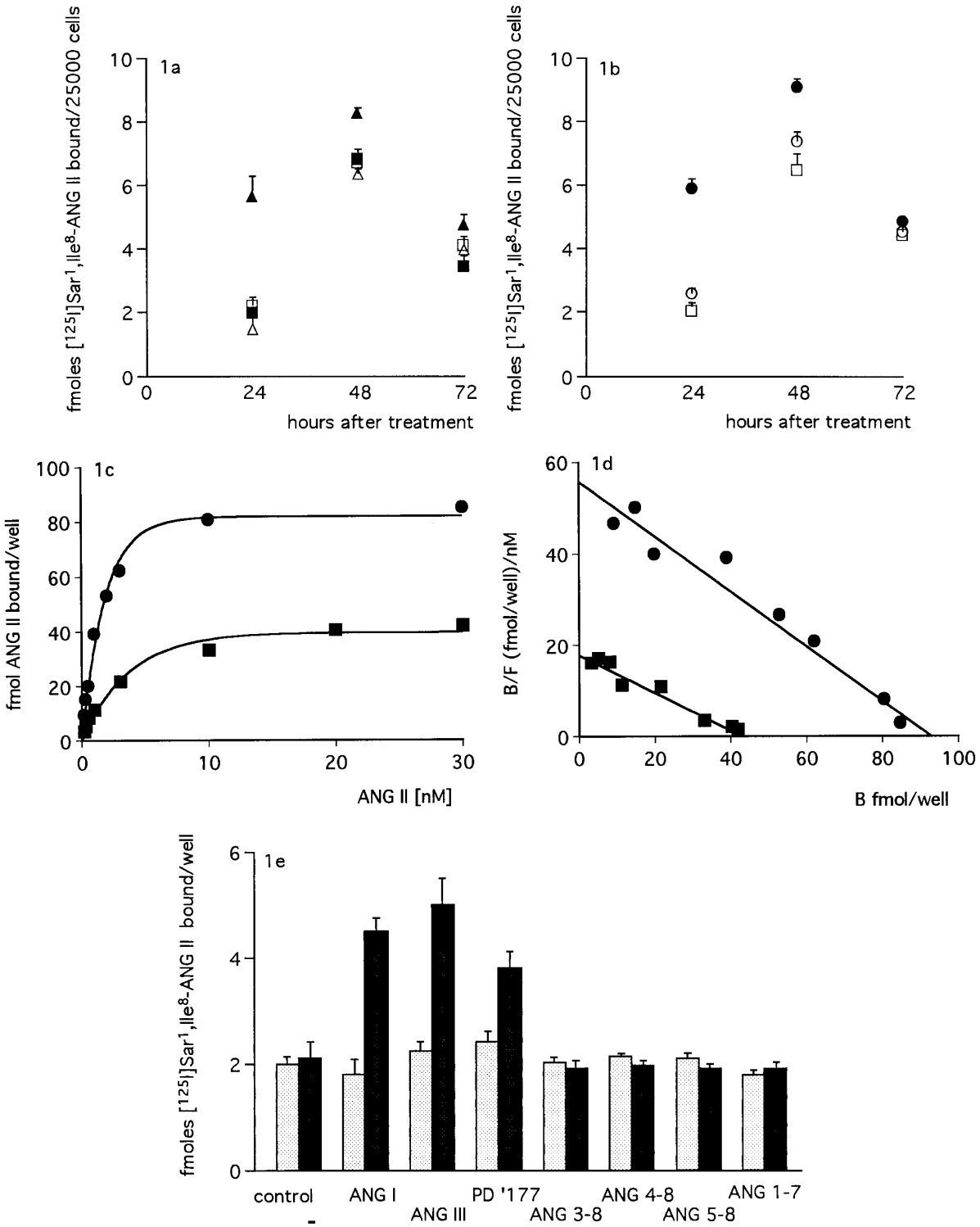

FIG. 1. (a) Radioligand binding activity after a PBS or acid-glycine wash on ANG II treated R3T3 cells. Near confluent R3T3 cells were treated for 6 hours with 1μM ANG II. Binding assays were performed 24, 48 and 72 hours after treatment. Cells underwent either a PBS or an acid glycine wash just prior to the binding assay. □: PBS washed (control), ■: acid-glycine washed, △: ANG II + PBS washed, ▲: ANG II + acid-glycine washed. Data is shown of a single experiment representative of three independent experiments. Data points correspond to an average of n = 4 plus standard deviation. (b) Radioligand binding activity after a PBS or acid-glycine wash on CGP 42112 treated R3T3 cells. Near confluent R3T3 cells were treated for 6 hours with 1μM CGP 42112. Binding assays were performed 24, 48 and 72 hours after treatment. Cells underwent either a PBS or an acid glycine just wash prior to the binding assay. □: PBS washed (control), ○: CGP 42112+ PBS washed, ●: CGP 42112 + acid-glycine washed. Data is shown of a single experiment representative of three independent experiments. Data points correspond to an average of n = 4 plus standard deviation. c. Saturation analysis of ANG II binding to intact

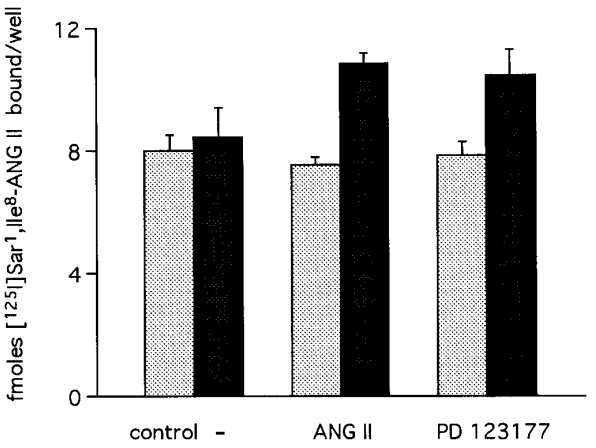

FIG. 2. Radioligand binding activity after a PBS or acid-glycine wash on HEK293 cells permanently transfected with the recombinant hAT2 receptor. HEK293 cells were treated with ANG II and PD 123177 (1 μM each). Binding assays were performed 6 hours after treatment. Cells underwent either a PBS or an acid glycine wash just prior to the binding assay. Control: no treatment + PBS washed. ▨: PBS washed, ■: acid-glycine washed. Data is shown of a single experiment representative of three independent experiments. Data points correspond to an average of n = 4 plus standard deviation.

either immediately, or following an additional 18 hours in fresh culture medium, subjected to an acid-glycine wash and cell solubilization. Figure 3b shows that following a 6 hour incubation with [^{125}I]-ANG II, the acid-glycine wash contained substantial radioactivity (which represents [^{125}I]-ANG II bound to cell surface AT_2 receptors) compared to the solubilized cell fraction. Following a further 18 hours in fresh medium, it appears from the acid-glycine wash data that approximately 30 % of the radioligand was still associated with AT_2 receptors present on the cell surface (figure 3c), suggesting a slow rate of ligand dissociation.

The Effect of an Acid-Glycine Wash 24 Hours Prior to the Binding Assay on R3T3 Cells

As shown in figure 1, no difference in binding activity was observed when untreated cells were either PBS- or acid-glycine washed just prior to the binding assay. However, an acid-glycine wash 24 hours prior to the binding assay resulted in a 5-fold decrease in the binding activity as compared to PBS washed cells (figure

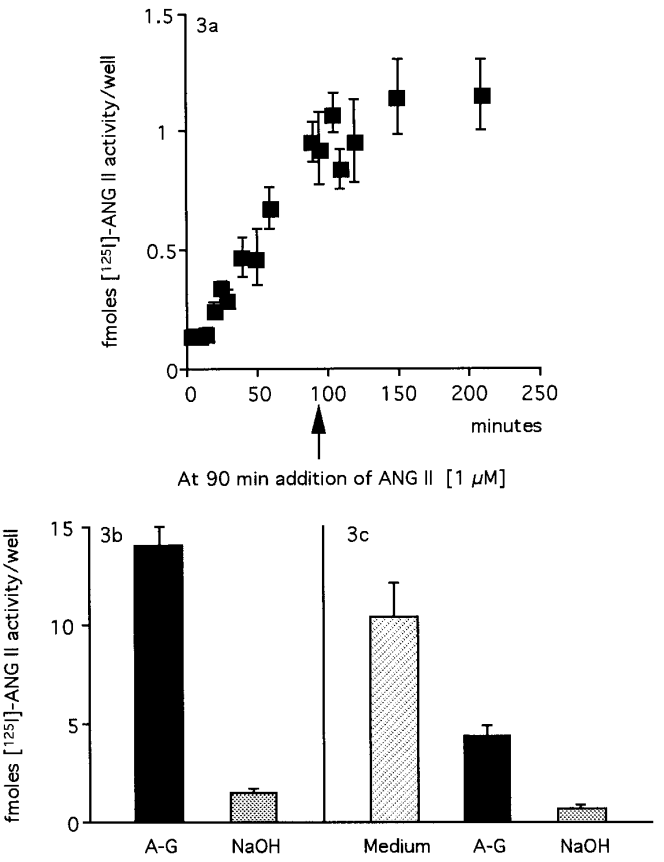

FIG. 3. (a) Lack of reversibility of [^{125}I]-ANG II binding on R3T3 cells. R3T3 cells were incubated for 6 hours with 40 pM [^{125}I]-ANG II and specific binding determined over time as described under methods. After 90 min, 1 μM ANG II was added to the wells and the amount of ligand remained bound was determined over the subsequent 2 hours. (b) and (c) Recovery of bound [^{125}I]-ANG II from R3T3 cells. The cells were incubated for 6 hours with 40 pM [^{125}I]-ANG II and then washed with PBS to remove unbound ligand. Cells were processed immediately (figure 3b) or incubated for a further 18 hours in fresh culture medium. The radioligand content of the medium (24 hour group only), acid-glycine washes (A-G) and solubilized cells (NaOH) were analysed using a γ-counter. Data are from single experiments representative of three (or two: figures 3b and 3c) independent experiments. Data points correspond to an average of n = 4 plus standard deviation.

4a). The decrease in binding activity was clearly prevented when the acid glycine wash was immediately followed by ANG II treatment. However, this binding activity was still significantly lower compared to ANG

R3T3 cells. Untreated and ANG II treated (1 μM ANG II for 6 hours, and left for 18 hours until saturation analysis) R3T3 cells were incubated with varying concentrations [^{125}I]-ANG II for 2 hours at 37 °C. Data represents a single experiment representative of three independent experiments. Each data point corresponds to the average of n = 4. (d) Scatchard transformation of the data, given in Fig. 1c. ■: Untreated, Kd = 2,45 nM; Bmax = 43,2 fmol/well, ●: ANG II treated, Kd = 1,71 nM; Bmax = 95,2 fmol/well. (e) Radioligand binding activity after a PBS or acid-glycine wash on R3T3 cells treated with ANG I, PD 123177 and ANG II derivatives. R3T3 cells were treated for 6 hours with compounds (1μM each). Binding assays were performed 24 hours after treatment. Cells underwent either a PBS or an acid glycine just wash prior to the binding assay. Control: no treatment + PBS washed. ▨: PBS washed, ■: acid-glycine washed. Data is shown of a single experiment representative of three independent experiments. Data points correspond to an average of n = 4 plus standard deviation.

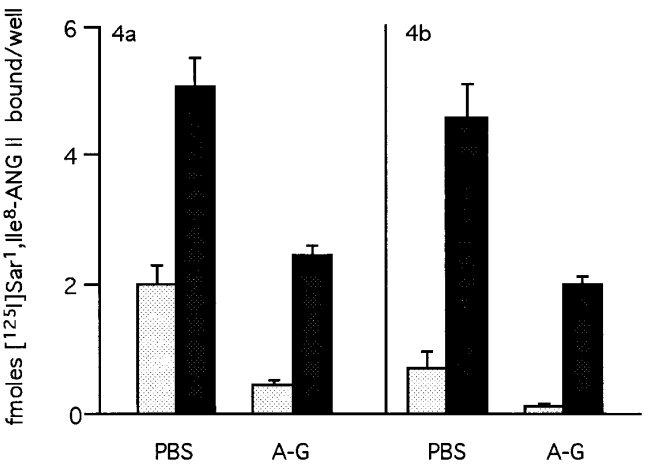

FIG. 4. (a) R3T3 cells were either PBS or acid-glycine washed and immediately treated with ANG II (1 μM) for 6 hours. The next day all cell groups were acid-glycine washed (A-G) prior to the binding assay. (b) Same protocol on parallel cultured cells one day later. Control: PBS wash, no treatment. ▨: no treatment, ■: ANG II treatment. Data are from single experiments representative of three independent experiments. Data points correspond to an average of n = 4 plus standard deviation.

II treated PBS washed cells (figure 4a). Exactly the same protocol was performed 24 hours later on cultures kept in parallel (figure 4b). This time an acid-glycine wash resulted in a more then 5-fold decrease in binding activity compared to untreated PBS washed cells. The decrease in binding activity was again prevented by the ANG II treatment resulting in a 2-fold higher binding activity as compared to untreated PBS washed cells.

To rule out the possibility that the dramatic decrease in binding activity is the result of cell death due to the acid-glycine wash, parallel treated cultures were trypan blue stained and counted. No difference in staining levels and cell numbers was observed 24 hours after an acid-glycine wash as compared to PBS washed cells (approximately $5 \cdot 10^4$ cells/ml for each wash group).

DISCUSSION

It has been previously established that treatment of R3T3 cells with either agonists or antagonists of the AT_2 receptor leads to an increase in the number of cell surface AT_2 receptors (12, 14). The present study, which includes saturation analysis using [^{125}I]-ANG II, firmly establishes that the increase in binding sites represents a change in AT_2 receptor number rather than affinity. Furthermore, a salient observation of the present study is that the increase in cell surface AT_2 receptors, associated with pretreatment of R3T3 cells with ligands, was not apparent when the standard acid-glycine wash of these cells was substituted with a PBS wash prior to the radioligand binding assay. Since there was no difference in the number of cell surface AT_2 receptors measured on untreated R3T3 cells, either PBS or acid-glycine washed (figure 1), it can be concluded that the acid-glycine wash itself was not responsible for the observed increase in cell surface AT_2 receptors. This led us to consider the possibility that the observed increase in cell surface AT_2 receptors, was related to a population of the cell surface AT_2 receptors still occupied by the ligand under investigation. The observation that the agonist [^{125}I]-ANG II could not be dissociated in the presence of 1 μM ANG II for at least 2 hours (figure 3a), and that 30 % of [^{125}I]-ANG II could be recovered from the cell surface by an acid-glycine wash (figure 3c) 18 hours after its removal from the media, suggests that ligands may dissociate very slowly from the AT_2 receptor.

Previous studies (12, 14, 15) have established that treatment of serum starved R3T3 cells with basic fibroblast growth factor (bFGF) leads to a rapid decrease in the number of cell surface AT_2 receptors, detectable within a few hours and maximal within 20 hours. The underlying mechanism appears to be an increase in the degradation rate of the AT_2 receptor mRNA. This suggests that in R3T3 cells the half life of AT_2 receptors present on the cell surface is extremely short, a matter of hours rather than days. Taking this fact into account and the observation that there was little difference between the number of cell surface AT_2 receptors measured on the untreated control cell groups and the PBS washed ligand pretreated group, suggests that this latter receptor population represents the steady state receptor turnover rate, composed mainly of receptors synthesized during the period under investigation. If this were so, then the additional receptors present on the ligand pretreated cells, represent receptors occupied with ligands which have not been removed from the cell surface during the period under investigation. Thus in a situation where there is continuing synthesis, lack of degradation (due to ligand occupancy) would result in accumulation of AT_2 receptors at the cell surface and an apparent increase in the number of AT_2 receptors in response to ligands, only observable if an acid-glycine wash was undertaken before measuring AT_2 receptors using a radioligand assay.

We have confirmed and extended the observation that the mechanism of ligand-induced increases in the number of cell surface AT_2 receptors relies on the occupation of the AT_2 receptor by ligands through expansion of a range of peptides used to pharmacologically characterize this system. Ligands that would be able to occupy the majority of AT_2 receptors present on R3T3 cells at 1 μM such as ANG I, ANG III and PD 123177 mimic the effect of ANG II, whilst those that could not occupy the AT_2 receptor (ANG 3-8, ANG 4-8, ANG 5-8 and ANG 1-7), do not (figure 1e).

Additional support for the observation that the increased number of cell surface AT_2 receptors is not the

result of ligand-induced activation of AT_2 receptor synthesis but due to prevention of receptor degradation comes from earlier observations that the increased binding activity is not accompanied by increased AT_2 mRNA levels in R3T3 cells (14) and stimulation of known signal transduction pathways in response to ANG II (13). Also, the lack of internalization of the AT_2 receptors upon ligand binding (a prerequisite for our model) seems to be a general characteristic for the AT_2 receptor. Indeed, earlier reports show a lack of internalization of the receptor upon binding in other cell systems (19, 20).

If ligand occupancy of the AT_2 receptor on R3T3 cells is responsible for reduced receptor degradation, then it should be observed for the AT_2 receptor in general. Similar data as with R3T3 cells could be obtained from HEK293 cells permanently expressing the recombinant hAT_2 receptor. However, the increase in binding activity was only observable with a 6 hour interval between treatment and radioligand binding assay (figure 2). The reason why a longer time interval did not reveal the increased binding activity is presently unknown. It could be due to the fact that transcription of the cDNA for the hAT_2 receptor in HEK293 cells is under the control of a CMV promoter, and thus the mechanisms regulating the steady state turnover of hAT_2 receptors in HEK cells are not comparable with the situation in R3T3 cells. Further work using other established cell culture models expressing endogenous AT_2 receptors would, therefore, be helpful.

The observation of a dramatic decrease in the number of cell surface AT_2 receptors one day after a single acid-glycine wash of R3T3 cells, raises the possibility of an endogenous ligand produced by R3T3 cells which prevents AT_2 receptor degradation (figure 4a). ANG II is able to rescue the binding activity. However, the ensuing activity is 2-fold less compared to PBS ANG II treated controls. The irreversible nature of the acid-glycine effect indicates that the factor is not membrane-bound, since it can be removed from the cell surface by a single wash, and that it probably is not continuously produced. The difference in the number of cell surface AT_2 receptors between acid-glycine washed and PBS washed cells prior to ANG II treatment indicates that the endogenous ligand and ANG II effects might be additive (figure 4a). The rescue of the binding activity by ANG II after removal of the putative endogenous factor by the acid-glycine wash suggests that this factor is part of the ligand-receptor complex, but seems not to be a prerequisite for the ligand induced stabilization of the receptor (figure 4b). The fact that there was no difference in the radioligand binding activity measured on untreated R3T3 cells, either PBS or acid-glycine washed (figure 1) shows that the endogenous ligand binds to a different site in the receptor, since it does not compete for the [^{125}I]Sar^1,Ile^8-ANG II binding site and is, therefore, probably not ANG II, or ANG II-related. Studies are in progress to determine whether this single acid-glycine wash-induced decrease in binding activity is accompanied by a decrease in AT_2 receptor mRNA. Such studies might answer the question as to whether this putative ligand is only an AT_2 receptor stabilizing factor or plays a role in a receptor mediated response, indicating the existence of a new, endogenous, non-ANG II-related, ligand for the AT_2 receptor.

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