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Relationship between Internalization and mRNA Decay in Down-Regulation of Recombinant Type 1 Angiotensin II Receptor (AT_1) Expression in Smooth Muscle Cells

BRIAN ADAMS, TRACY S. OBERTONE, XIAOFEI WANG, and T. J. MURPHY

Department of Pharmacology (B.A., T.S.O., X.W., T.J.M.) and Graduate Program in Molecular Therapeutics and Toxicology (B.A., X.W., T.J.M.), Graduate Division of Biological and Biomedical Sciences, Emory University School of Medicine, Atlanta, Georgia

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ABSTRACT

In vascular smooth muscle cells, the hormone angiotensin II is thought to cause internalization of the seven-transmembrane domain type 1 angiotensin II receptor (AT_1-R) but it also suppresses expression of the receptor mRNA. As for similarly regulated members of this gene superfamily, the relative roles of these processes in receptor down-regulation are not well understood. In this study a recombinant AT_1-R mRNA was synthesized in A7r5 vascular smooth muscle cells from a tetracycline-suppressible promoter using a retroviral vector system. Angiotensin II induces a profound internalization of the cell surface AT_1-R protein but has no effect on steady-state AT_1-R mRNA levels. Shortly after either bolus or prolonged dosing with angiotensin II, cell surface AT_1-R expression recovers, indicating the existence of a significant restorative externalization pathway. The extent of this recovery is attenuated markedly when transcription of the recombinant AT_1-R gene is suppressed by cotreatment of the cells with anhydrotetracycline. Although agonist-stimulated internalization appears to contribute directly to a loss of AT_1-R protein, these observations provide direct evidence that a reduction in AT_1-R mRNA content plays a significant role in sustained AT_1-R down-regulation.

Several types of G protein- coupled receptors are regulated through persistent or skewed exposure to their natural agonists (Hausdorff et al., 1990). Several general mechanisms that change receptor function are thought to account for the ability of cells to adapt to changing extracellular conditions (Bohm et al., 1997). Desensitization defines a rapid uncoupling of the receptors from signaling systems (Lefkowitz et al., 1998), whereas receptor internalization, which can involve either sequestration of the receptor protein at the plasma membrane or vesicular endocytosis, removes the receptor from exposure to agonist (Koenig and Edwardson, 1997; J. Wang et al., 1997). Down-regulation of G protein-coupled receptors defines a prolonged period of attenuated expression that often follows exposure to agonist. The mechanisms associated with receptor down-regulation include a collection of cellular processes that are stimulated by agonists, which disrupt the normal balance between receptor protein synthesis and degradation (Bohm et al., 1997). In many specific instances of native systems, all of these processes occur simultaneously so that the relative contributions to down-regulation made by desensitization, receptor internalization, and mechanisms disrupting the balance of gene expression have not been clarified.

The octapeptide hormone angiotensin II (AngII) plays a central role in cardiovascular homeostasis by regulating blood volume and vascular tone (Peach, 1977). The direct vascular effects of AngII are mediated by activation of Gαq protein-coupled type 1 AngII receptor (AT_1-R) expressed on the surface of vascular smooth muscle cells (VSMC) (Griendling et al., 1994). Target cell sensitivity to AngII is important for normal cardiovascular homeostasis, and may participate in the development of hypertension and other cardiovascular diseases. For these reasons, the mechanisms involved in AT_1-R regulation are an area that receives significant interest.

Numerous studies indicate that AT_1-R expression both in VSMC and in other cell types is controlled dynamically (Griendling et al., 1987; Anderson et al., 1993; Regitz-Zagrosek et al., 1994; Ouali et al., 1997). In VSMCs, AngII down-regulates AT_1-R expression, which appears to involve several mechanisms. A disruption in steady-state AT_1-R mRNA levels occurs as a result of suppressing AT_1-R gene transcription and stimulating a protein kinase A-regulated mRNA destabilization process (Lassegue et al., 1995; Nick-

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ABBREVIATIONS: AT_1-R, type 1 angiotensin II receptor; VSMC, vascular smooth muscle cells; AnTet, anhydrotetracycline; AngII, angiotensin II; Sarile, Sar^1, Ile^8-angiotensin II; RNase, ribonuclease; tTA, tetracycline transactivator.

enig and Murphy, 1996; X. Wang et al., 1997; Wang and Murphy, 1998). AngII also induces AT_1-R internalization in these cells (Griendling et al., 1987), as well as in surrogate cell lines expressing a recombinant receptor (Hunyady et al., 1995; Thomas et al., 1995; Hein et al., 1997). Many of these latter studies have led to insights about regions of the AT_1-R protein that are necessary for desensitization and/or internalization or the cellular cofactors associated with these processes (Oppermann et al., 1996; Zhang et al., 1996; Tang et al., 1998). But few studies have been designed to clarify the relationship between receptor internalization, reductions in AT_1-R mRNA levels, and how either alone contributes to down-regulation of AT_1-R expression.

The present objectives were to gain selective and nontoxic control over recombinant AT_1-R mRNA production in a VSMC phenotype, but in a manner that is independent of effects evoked by AngII. The embryonic rat A7r5 VSMC cell line was used for this study because it expresses several markers associated with the VSMC lineage (Firulli et al., 1998) but does not express the AT_1-R endogenously. To perform the present studies in this nonstandard surrogate expression system, we engineered the tetracycline-regulated expression system (Gossen and Bujard, 1992) into retroviral vectors. By inhibiting synthesis of the AT_1-R mRNA selectively using anhydrotetracycline (AnTet) (Gossen and Bujard, 1993), we show substantial effects on cell surface AT_1-R expression. These data demonstrate an important role for AT_1-R mRNA modulation in down-regulation of cell surface AT_1-R protein expression, even in a system wherein 90% of the cell surface receptors are internalized by the agonist. The findings also provide new insights into the dynamics of basal and agonist-regulated AT_1-R turnover in the plasma membrane of smooth muscle cells.

Experimental Procedures

Materials. A7r5 VSMC, a cell line derived from the embryonic rat thoracic aorta (Kimes and Brandt, 1976), were purchased from the American Type Culture Collection (Rockville, MD). Antibiotics and cell culture media were purchased from Life Technologies, Inc. (Grand Island, NY). Fetal bovine serum was purchased from Atlanta Biologicals Inc. (Norcross, GA). α-[^{32}P]UTP (800Ci/mmol), Na^{125}I (2000 Ci/mmol) and the Tyramide Signal Amplification Direct-Green immunodetection kit were obtained from New England Nuclear Inc. (Boston, MA). The Maxiscript T7 in vitro transcription and RPAII kits were purchased from Ambion, Inc. (Austin, TX). AngII, cycloheximide, salts, and buffers were purchased from Sigma Chemical Co. (St. Louis, MO), whereas AnTet was purchased from Acros Organics, Inc. (Pittsburg, PA). Radiolabeled Sar^1, Ile^8-AngII ([^{125}I]-Sarile) was iodinated and purified to homogeneity by HPLC as described previously (Murphy et al., 1993). The monoclonal antibody HA.11 was purchased from Babco, Inc. (Richmond, CA). Biotin-SP-conjugated goat anti-mouse antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Cell culture plates and flasks were purchased from Corning Glass Works (Corning, NY) or Nunc Inc. (Naperville, IL). The retroviral plasmids pLNCX and pLXSH (Miller and Rosman, 1989) were gifts from Dr. A. D. Miller (Seattle, WA), whereas components of the tetracycline-regulated expression system were derived from plasmids obtained from Dr. H. Gossen (Heidelberg, Germany).

Plasmid Construction. Standard molecular protocols were used to create the plasmids used in this study, and all modifications were confirmed by restriction mapping or sequencing. The retroviral vector termed pTSO31 is a derivative of pLNCX (GenBank accession number M28247) in which the internal cytomegalovirus (CMV) promoter has been discarded and replaced with a transcriptional cassette directing tetracylcine-regulated expression of an AT_1-R mRNA (Fig. 1). The tetracycline- regulated promoter in pTSO31 was derived from the vector pUHD 10-3 (Gossen and Bujard, 1992), and the Simian virus 40 (SV40) polyadenylation signals were derived by the polymerase chain reaction from the vector pCDNA-1 (Invitrogen, San Diego, CA). The AT_1-R sequence was derived from the vascular AT_1-R expression vector pCa18b (Murphy et al., 1991). The most 5′ end of the AT_1-R mRNA expressed from pTSO31 incorporates pCDM8 vector remnant sequence between the *Hin*dIII and *Bst*XI

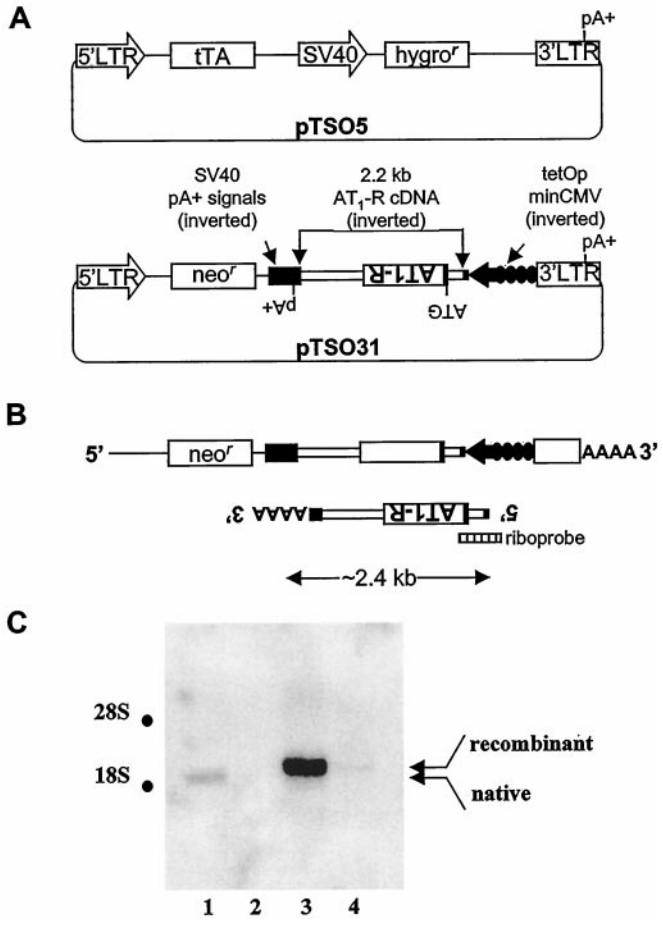

Fig. 1. Retroviral, tetracycline-regulated AT_1-R expression vectors and mRNAs. A, vectors: in pTSO5, the 5′ LTR promotes tTA expression, whereas a SV40 promoter drives expression of the hygromycin (hyrgro^r) resistance gene. The 3′ LTR pA+ signal serves as terminator for both of these promoters. In pTSO31 the neomycin resistance (neo^r) mRNA also terminates at the 3′ LTR pA+ signals, so that its 3′ untranslated region contains antisense sequences encoding the SV40 pA+ signals, ~2.2 kb of AT_1-R mRNA, and a tetracycline-operated minimal CMV promoter complex (tetOp minCMV). This promoter functions on bottom strand to direct transcription of a predicted ~2.4 kb AT_1-R mRNA that terminates at the SV40 polyadenylation signals. B, transcripts produced from TSO31: shaded regions at 5′ and 3′ ends of AT_1-R mRNA and in beginning of open reading frame (box) represent, respectively, a total of ~200 bases of heterologous CMV, SV40, and HA epitope sequence in mRNA. Slashed box depicts AT_1-R antisense riboprobe used for RNase protection assays. C, Northern blot: each lane contains 5 μg of total RNA fractionated on a formaldehyde agarose gel that was blotted and hybridized with a random primed, double-stranded [^{32}P]dCTP-labeled AT_1-R cDNA probe as described previously (Nickenig and Murphy, 1994). Lane 1, primary rat thoracic VSMC that express a native 2.2 kb AT_1-R transcript endogenously; lane 2, nonrecombinant A7r5 cells; lane 3; A7r5 cells infected with TSO31 virus grown in the absence of AnTet; lane 4, same cells as lane 3 grown for 5 days in the presence of 1.0 μg/ml AnTet. Results are representative of two experiments.

restriction sites in addition to sequence immediately 3′ of the CMV promoter transcription start site. The recombinant AT_1-R protein begins with the sequence MALKYPYDVPDYAVKSS, where the underlined sequence represents the HA immunogenic epitope that was formed by ligation of an oligonucleotide pair into a *Afl*III restriction site created in an AT_1-R cDNA by mutagenesis. This process converts the fourth amino acid in the native AT_1-R protein from asparigine to lysine, destroying a putative glycosylation site. The most distal 3′ end of the AT_1-R sequence in pTSO31 is modified by removal the AT_1-R cDNA sequence in pCa18b from the upstream polyadenylation signal (AAUAAA) through the polyadenylate tract and its replacement with a NsiI site using site- directed mutagenesis. SV40 polyadenylation signals were then inserted onto the 3′ end of the modified AT_1-R cDNA sequence using this NsiI site. The retroviral plasmid pTSO5 was created by ligation of the tetracycline transactivator (tTA) cDNA derived from pUHD 15-1 (Gossen and Bujard, 1992) into the vector pLXSH (GenBank accession number M77239). The complete sequences of these circular plasmids are available on request.

Cell Culture. A7r5 cells and derivatives were grown in bicarbonate-buffered high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a 5% CO_2 incubator. After reaching confluence between 5 to 6 days after plating, the cells received fresh growth media 24 h before experimental treatments.

Retroviral Infection. The protocols used for retroviral production and smooth muscle cell infection have been described previously (Boss et al., 1998). After infecting A7r5 cells with retrovirus prepared from pTSO5, a population of recombinant cells was selected over 7 to 10 days using hygromycin (100 μg/ml). After expansion and subsequent infection with retrovirus prepared from the pTSO31 vector, the cells were treated with geneticin (100 μg/ml). After establishing dually infected cell lines, the antibiotics were no longer used. Recombinant AT_1-R expression remained stable for up to 3 months.

Ribonuclease (RNase) Protection Assays. Cells were grown in 6 × 35-mm plates in 3.0 ml of cell culture media for 4 to 6 days until confluence before replenishment with fresh media 24 h before experimental treatments. Following the given treatments for the indicated times, the media was aspirated and the cells were lysed in 1 ml of the Trizol Reagent (Life Technologies) before isolating and quantifying total RNA according to directions supplied by the manufacturer. Total RNA (5–15 μg) was hybridized with a mixture of polyacrylamide gel-purified α-[^{32}P]UTP-labeled riboprobes prepared from rat AT_1-R and cyclophilin cDNA templates synthesized using T7 RNA polymerase and the Maxiscript Kit and using the RPAII kit, according to their directions. The cyclophilin probe template (pTRI-Cyp) was purchased from Ambion, Inc. The AT_1-R riboprobe is a 593-base runoff from a vector termed pRPA-1, which was linearized with EcoO109I. The insert in pRPA-1 is a 532 bp *Hin*dIII–MluNI fragment derived from the most 5′ end of the AT_1-R sequence encoded in pTSO31 that was subcloned into pBluescript(KS+) (Stratagene, Inc., LaJolla, CA).

Binding Assays. For intact cell assays, cells were grown in 12 × 2-cm tissue culture plates to confluence before aspiration and replenishment of the growth media. Within 24 h, all treatments were performed in duplicate for the indicated times by adding drugs from 100× to 1000× concentrated stocks. Following this, the bottom of the plates were placed in contact with ice, the media aspirated, and each well was washed rapidly twice with 0.5 ml of ice-cold DMEM, buffered with 10 mM HEPES, pH 7.4, (DMEM-HEPES) containing 0.1% BSA. [^{125}I]-Sarile (~2000 Ci/mmol) prepared in this same media was added to a final concentration of 1.2 to 1.5 nM (a concentration that is approximately 6- to 7-fold greater than the K_D value) in a 0.5 ml volume. Nonspecific binding was determined in duplicate on untreated wells in the presence of 10 μM losartan. After an overnight incubation at 4°C, the wells were washed twice with 0.5 ml of ice-cold DMEM-HEPES. The cells were then solublized and collected in 0.5 ml of 0.2 N NaOH before measuring bound radioactivity on a Beckman 4000 gamma counter at 72% efficiency. Membrane binding assays were performed on crude particulate fractions of cells homogenized in 50 mM Tris-HCl as described previously using 10 μM losartan to define nonspecific binding (Murphy et al., 1993).

Immunodetection of HA-Tagged AT1-R. Eight-well glass slide culture chambers (Nunc, Inc.) were treated for 20 min with 0.3 ml of 0.01 mg/ml poly-*d*-lysine prepared in sterile deionized H_2O before aspirating, drying to air, and seeding with 10,000 cells per well. The cells were allowed to grow for 48 h before treatment with vehicle or 2 μM AngII for the indicated times. After aspiration, 0.3 ml of 2% formaldehyde in PBS was added for 15 min before washing with PBS. A block solution containing 2% horse serum and 0.5% Triton X-100 in PBS was placed into each well. Following aspiration, 0.1 ml of block solution containing 0.4 μg/ml of the monoclonal antibody HA.11 or 0.4 μg/ml mouse ascites fluid as control, was added to each well. After a 60-min incubation at 22°C, the solutions were aspirated before washing the wells with three changes of 0.5 ml 0.2% Tween-80 in PBS over 30 min. After incubation for 60 min at 22°C with 0.4 μg/ml Biotin-SP-conjugated goat anti-mouse antibody in 0.1 ml of block solution, the cells were washed as above. Signal development was performed according to the protocol suggested by the manufacturer in the Tyramide Signal Amplification Direct-Green kit.

Results

Tetracycline-Regulated Retroviral Vectors. The relationship between AT_1-R mRNA levels and cell surface AT_1-R protein expression was studied by tetracycline-regulated expression from retroviral vectors (Fig. 1). Cells expressing the tTA from pTSO5 were first stabilized after retroviral infection using hygromycin. These were infected with a second virus prepared from pTSO31 and subsequently stabilized with geneticin. The pTSO31 vector produces a recombinant rat vascular AT_1-R mRNA from an opposite-strand, tTA-responsive promoter that can be suppressed with Antet. The opposite-strand strategy was designed so that the composition of the AT_1-R mRNA could be better specified than if production occurred from the top strand of the vector. This surmounts a retroviral packaging requirement wherein the polyadenylation signal in the 3′ long terminal repeat (LTR) must serve as the sole transcriptional terminator on the sense, or upper, strand of this vector (Boris-Lawrie and Temin, 1994). In concept, two transcripts are produced from TSO31 (Fig. 1B). The predicted size of the 5′ LTR transcript is ~4.6 kilobases (kb) because it terminates at the polyadenylation signal in the 3′ LTR. This is a chimera mRNA that produces a functional neomycin resistance protein but has a 3′ untranslated region that terminates at the 3′ LTR polyadenylation signals (pA+) encoding approximately 2.2 kb of antisense AT_1-R mRNA. The sense AT_1-R mRNA is produced from the bottom strand of this vector off of the tetracycline-regulated promoter, but it terminates at pA+ signals derived from the SV40 genome. The predicted size of this latter transcript (~2.4 kb) is approximately 200 bases larger then the native transcript due to a heterologous sequence derived from the CMV promoter and the SV40 polyA+ signals at its 5′ and 3′ ends, respectively, as well as an HA-epitope coding sequence.

Using a double-stranded, α-[^{32}P]dCTP-labeled AT_1-R cDNA probe, Northern hybridization analysis indicated that this latter mRNA is the dominant hybridizing AT_1-R transcript produced in A7r5 cells infected with the expression vectors (Fig. 1C, lane 3). This transcript was slightly larger, as predicted, than the 2.2-kb AT_1-R mRNA expressed in a

VSMC line that natively express the AT_1-R gene (Fig. 1C, lane 1). The 2.4-kb transcript was also absent in uninfected A7r5 cells (Fig. 1C, lane 2), and was suppressed in cells that are grown in AnTet (Fig. 1C, lane 4). Notably, only trace amounts of the predicted 4.6-kb transcripts were seen in the recombinant A7r5 cells. It is of interest to note that suppression of the smaller transcript with AnTet did not lead to enhanced expression of the larger transcript, as might be expected if there were any reciprocal antisense interactions between these two recombinant mRNAs in these cells. These Northern hybridization data indicate that the recombinant AT_1-R produced in these cells is translated from a precisely specified mRNA, albeit with some heterologous sequences. In subsequent experiments, levels of this mRNA were measured by RNase protection assay using an antisense AT_1-R mRNA probe.

Internalization of Cell Surface AT_1-R by AngII. Cell surface AT_1-R expression was measured following AngII treatment using an intact cell binding assay, which was performed at 4°C with a saturating concentration of [^{125}I]-Sarile. A 1-h treatment induces a dose-dependent reduction of cell surface AT_1-R expression and occurs with an EC_{50} of 5 ± 1 nM (mean ± S.E.M., n = 3) (Fig. 2A). The maximal effect represents a 90% reduction in cell surface AT_1-R expression, which occurs at 100 nM AngII and higher concentrations. This reduction occurs rapidly, and is evident as early as 5 min after a single bolus dose of AngII (Fig. 2B). Because the AngII peptide is unstable in cell culture media (see below), a high bolus dose (2 μM) was used to determine whether the agonist affects recombinant AT_1-R mRNA levels. As shown in Fig. 2C, however, AT_1-R mRNA levels in VSMC are not affected by this treatment. Although AngII signaling suppresses AT_1-R mRNA expression in VSMC that natively express the AT_1-R gene (Nickenig and Murphy, 1996; Wang and Murphy, 1998), this does not occur in the A7r5 cell line. This observation indicates that A7r5 cells provide a suitable background to study how AT_1-R protein levels are modulated by agonist without collateral effects of the agonist on AT_1-R mRNA levels.

AT_1 R mRNA Decay Kinetics Following AnTet. Figure 3A shows AT_1-R mRNA levels as a function of time after selective transcriptional inhibition with AnTet, in the absence or presence of 2 μM AngII. The mRNA levels are reduced to 5% of control levels by 24 h after AnTet addition (Fig. 3B). This low level appears to represent the transcriptional leak in the system. AngII addition does not accelerate the rate of AT_1-R mRNA decay induced by AnTet in this preparation (Fig. 3B), again indicating that mRNA levels are selectively and strictly controlled by AnTet but not by the agonist. The combined data from three experiments are best fit by a one-phase exponential decay equation yielding AT_1-R mRNA decay rate constants (mean ± S.E.M) of 0.102 ± 0.028 h^{-1} ($T_{1/2}$ = 6.8 h) and 0.116 ± 0.020 h^{-1} ($T_{1/2}$ = 6.0 h), respectively, in the absence and presence of AngII.

Cell Surface AT_1-R Decay Following AnTet. The response of the cell surface AT_1-R to AngII was measured over more prolonged periods of time than are shown in Fig. 2. The data in Fig. 4A shows that cell surface AT_1-R expression remains suppressed for 2 h after the addition of 2 μM AngII, after which a recovery of cell surface expression becomes evident. Cell surface AT_1-R levels return fully to control levels of expression within 10 h. This initial recovery is delayed compared with what is seen after treatment with a bolus of 100 nM angiotensin instead (see Fig. 2B), and likely reflects a more sustained concentration of the peptide under the higher dose condition. The addition of AnTet alone leads to a gradual reduction of cell surface AT_1-R expression (Fig. 4A). Within 48 h after starting this treatment, AT_1-R expression is reduced to less than 3% of maximal expression by AnTet alone, which represents the lowest possible steady-

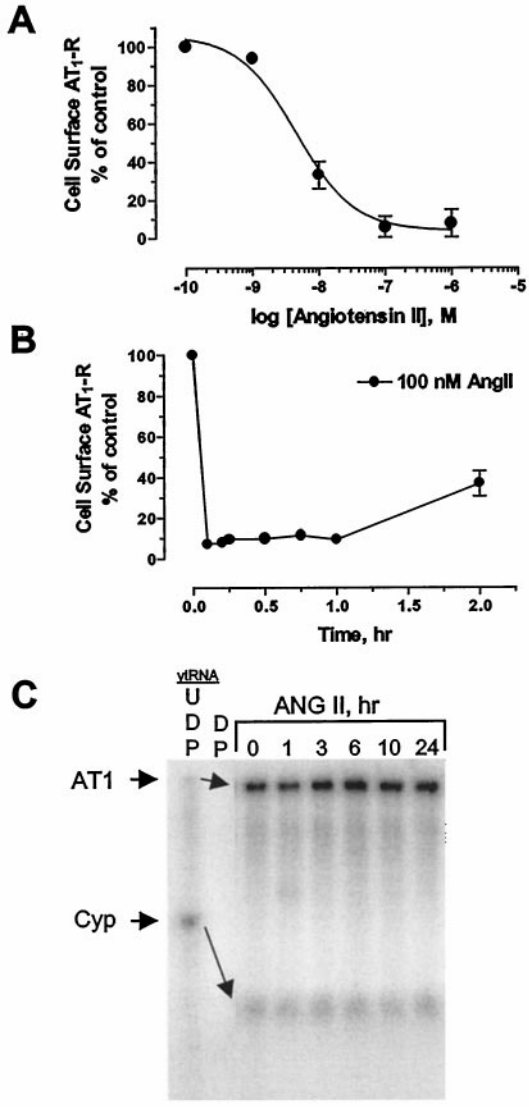

Fig. 2. AngII induces internalization of cell surface AT_1-R expression but does not affect steady-state AT_1-R mRNA levels. A, dose-effect relationship: A7r5 cells infected with retroviruses prepared from pTSO5 and pTSO31 were treated with indicated doses of AngII for 60 min before washing and measuring cell surface AT_1-R by intact cell radioligand binding assay. B, early response time course: the cells were treated with a single dose of 100 nM AngII and incubated for indicated times before washing and measuring cell surface binding. A and B, control represents levels of expression in vehicle-treated cells. Each data point represents mean ± S.E.M. of three to six experiments performed in duplicate. In these and subsequent binding experiments, total binding in control wells ranged from 10,000 to 30,000 cpm and nonspecific binding ranged from 500 to 1500 cpm. C, lack of effect of AngII on AT_1-R mRNA levels: cells were treated with a single dose of 2 μM AngII and incubated for indicated times before extracting RNA and performing RNase protection assay. Each lane was loaded with 15 μg of total RNA. AT1, AT_1-R riboprobe; Cyp, rat cyclophilin riboprobe; UDP, undigested probe; DP, digested probe; ytRNA; yeast transfer RNA. Phosphorimage is representative of two experiments.

state expression in this system. This reduction is best fit by a one-phase exponential decay equation with a rate constant of 0.039 ± 0.006 h^{-1} (mean ± S.E.M., $n = 6$; $T_{1/2} = 17.8$ h) (Fig. 4B). When AngII and AnTet are administered simultaneously, the profound early reduction in cell surface AT_1-R expression is followed by a period of recovery, similar to that seen with AngII treatment alone (Fig. 4A). However, the maximal level of AT_1-R recovery is suppressed under this permissive condition for AT_1-R mRNA decay. The peak of this recovery occurs 6 h after initiating treatment and represents approximately 60% of control levels of cell surface AT_1-R. These observations indicate that although a bolus of AngII induces a profound internalization of the AT_1-R, *sustained* down-regulation of cell surface expression occurs only if AT_1-R mRNA levels are also reduced. Figure 4B compares the best-fit exponential decay curves showing the temporal relationship between AT_1-R mRNA decay and cell surface protein decay initiated by the addition of AnTet alone and illustrates the lag period between decay of the mRNA and loss of cell surface protein expression.

As shown in Fig. 4C, complete inhibition of protein synthesis by treatment of the cells with cycloheximide results in a 40% decline of cell surface AT_1-R expression within 8 h, which stabilizes after this point. (The dose of cycloheximide

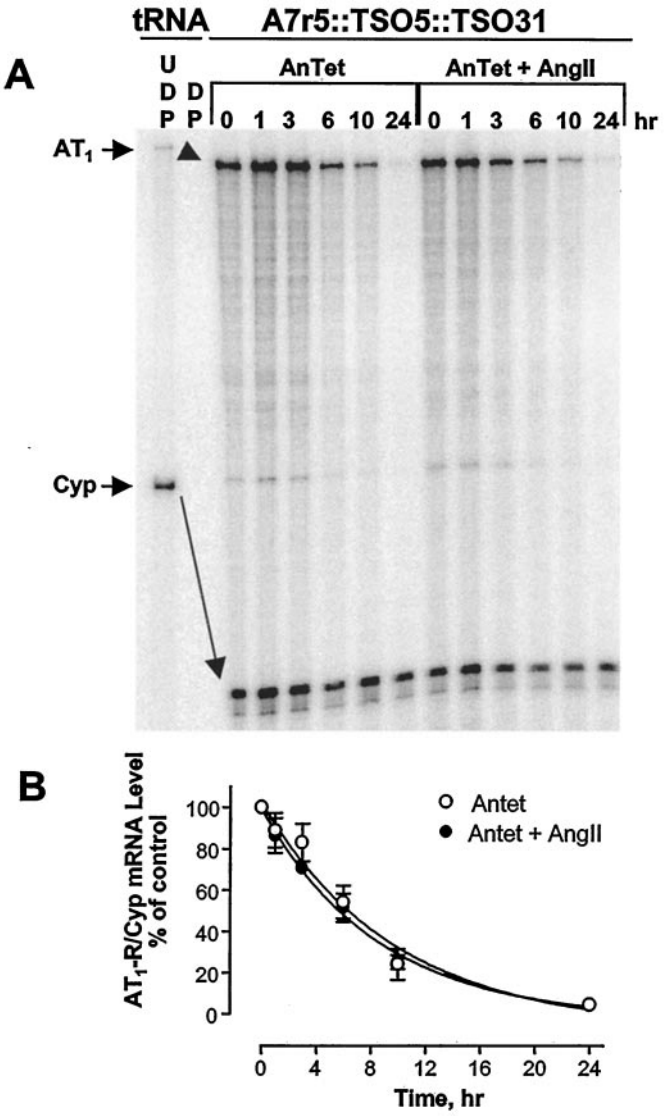

Fig. 3. AnTet-induced AT_1-R mRNA decay. VSMC were treated for indicated times with AnTet (0.3 μg/ml) in the absence or presence of 100 nM AngII before extracting RNA and performing RNase protection assays. A, representative RNase protection assay results: yeast tRNA (tRNA) or total RNA (15 μg) prepared from A7r5 cells infected with retrovirus prepared from pTSO5 and pTSO31 was hybridized with riboprobes directed against the AT_1-R (AT1) and cyclophilin (Cyp) sequences before digestion with RNases and resolution on a sequencing gel. UDP, undigested riboprobes; DP, digested riboprobes. B, quantitative results: each point represents mean ± S.E.M. ($n = 3$) ratio of AT_1-R to Cyp hybridization signals normalized to levels in untreated cells (control).

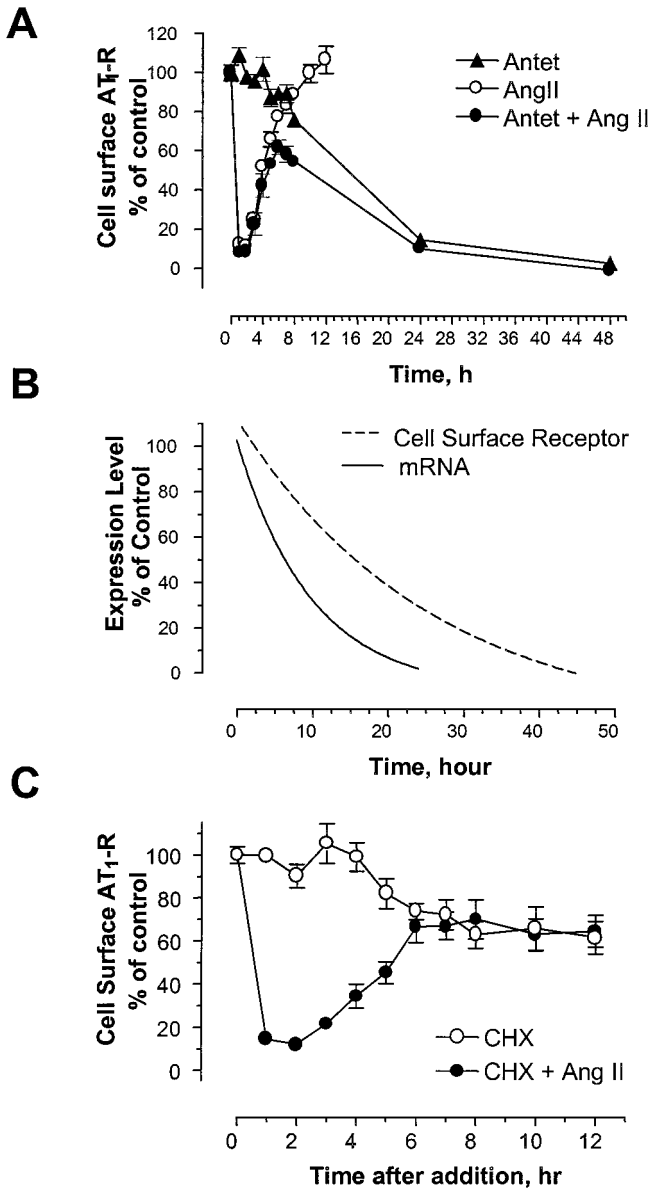

Fig. 4. Decay of cell surface AT_1-R expression. A, prolonged effect of AnTet, AngII, or the two drugs combined. Cells were given a bolus of AnTet (0.3 μg/ml), AngII (2 μM), or both and incubated for indicated times before measuring cell surface AT_1-R expression with an intact cell binding assay. B, comparison of mRNA and cell surface expression decay rates. Each curve represents a one-phase exponential decay best-fit for receptor binding or mRNA level as a function of time after AnTet administration. C, effect of cycloheximide (CHX) in the absence or presence of AngII. Cells were treated for indicated times following a bolus of 30 μg/ml CHX in the absence or presence of 2 μM AngII before measuring cell surface AT_1-R expression. Each data point represents mean ± S.E.M. of three to five experiments performed in duplicate.

used in these experiments completely abolished subsequent incorporation of [^{35}S]-methionine/cysteine into trichloroacetic acid precipitates of metabolically labeled A7r5 cells.) This steady state presumably represents nonspecific effects of the drug, perhaps through interference with the normal processes controlling AT_1-R decay. However, cycloheximide treatment does not inhibit AngII-induced AT_1-R internalization. Strikingly, a significant recovery of cell surface AT_1-R expression occurs within 6 h in the presence of cycloheximide. This observation indicates that some 60% of the cell surface AT_1-R that recovers within 6 h of the bolus internalization stimulus is pre-existing protein.

Effects of Sustained versus Bolus AngII. Compared with the higher bolus dose condition, the recovery of cell surface AT_1-R expression occurs earlier after a lower AngII dose. The simplest explanation for this is that recovery is only apparent and reflects of the rate of AngII degradation in the culture media rather than the rate of externalization. To examine this, AT_1-R levels were measured during and after a period of sustained agonist administration and compared with that following a single bolus dose (100 nM) of AngII. Sustained administration was achieved by repetitive addition of 100 nM AngII every hour over a 7-h period. The recovery of cell surface AT_1 R expression was then assessed at various times up to 7 h after the last dose of AngII in each of these two protocols. As shown in Fig. 5, cell surface AT_1-R expression remains repressed during the course of a sustained dosing regimen, indicating that internalization will persist so long as an appropriate concentration of agonist is present. However, following the last dose of the repetitive series, a marked recovery is evident beginning 1 h later. The maximal extent of recovery after the bolus and repetitive dosing conditions, respectively, is 95 ± 6 and 78 ± 3% of the level of cell surface AT_1-R in untreated cells (mean ± S.E.M.; $n = 3$). Thus, sustained treatment with AngII continuously suppresses cell surface AT_1-R expression and impacts the level by which it can recover.

This persistent internalization and its following recovery, along with the recovery following bolus dosing, nevertheless suggests that a fairly robust externalization process likely occurs continuously. Likely, the rate of recovery of cell surface AT_1-R expression after internalization is more rapid then the previous curves indicate. To test this, the cells were stimulated for either 10 or 30 min with 100 nM AngII, after which 10 μM of the antagonist losartan was added to block the effects of residual agonist. As shown in Fig. 6C, after 1 h in a losartan block, cell surface AT_1-R expression recovers to 69 ± 7% of control, whereas without the block the recovery is only 19 ± 5% of control. This observation indicates that the cells are continuously exchanging cell surface AT_1-R at a fairly rapid rate during a period in which agonist is present.

Binding assays on crude particulate fractions, rather than measurements of cell surface AT_1-R on intact cells, assess the total cellular content of AT_1-R protein following a treatment. These fractions were collected 1 h after stimulating with 100 nM AngII either once or repetitively over a 7-h period to compare agonist effects on total cellular AT_1-R protein levels. After the shorter treatment, total cellular AT_1-R protein was 73 ± 4% of control, whereas it was 55 ± 3% (mean ± S.E.M., $n = 3$) of control after the prolonged treatment (Fig. 6B). Thus, approximately 25% and 45% of the total cellular receptor protein is destroyed following the short and prolonged stimulation conditions, respectively. Thus, internalization is associated with a gradual loss of the total cellular pool of receptor.

Immunolocalization Studies. Immunohistochemical staining experiments were performed using antibodies directed against the HA-epitope encoded in the recombinant receptor. No staining is seen in A7r5 control cells that do not express the recombinant receptor (Fig. 6F), or in recombinant A7r5 cells treated with mouse ascites control antibody (data not shown), demonstrating the specificity of the antibody. As

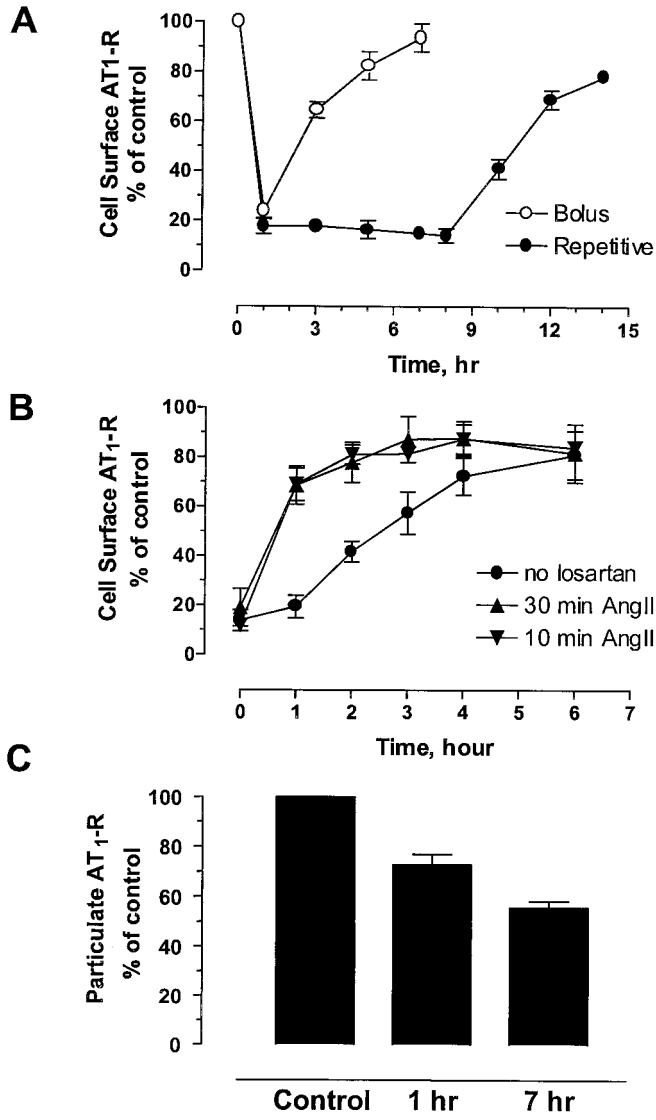

Fig. 5. Cell surface and total cellular AT_1-R levels. A, comparison of responses to bolus and repetitive AngII administration. Cells were treated with a single 100 nM AngII dose and incubated for indicated times (○) or with repetitive doses of 100 nM AngII for each of 7 h and then incubated for indicated times thereafter (●) before measuring intact cell surface binding. B, recovery after early blockade with losartan. Cells were treated with 100 nM AngII for 10 min (▼) or 30 min (▲) and then given 10 μM losartan or antagonist vehicle (●). Abscissa represents incubation time after losartan addition, after which cell surface receptor expression was assessed. C, effect of bolus and repetitive agonist on total cellular AT_1-R protein content. Cells in 6-cm diameter dishes were treated with vehicle, a single dose of 100 nM AngII for 1 h, or with repetitive 100-nM AngII doses every 60 min over 7 h. After these treatments, cells were scraped, homogenized, and subjected to membrane binding assays. Each point or bar in these panels represents mean ± S.E.M. of three or four experiments with duplicate determinations in each.

early as 5 min after AngII treatment (Fig. 6B), expression is reduced markedly from the peripheral cellular structures compared with that seen in vehicle- treated cells (Fig. 6A) and expression at the cell borders is largely lost. This peripheral and cell border staining likely represents expression of the receptor in the plasma membrane. Re-expression in these structures is evident within 4 h after AngII stimulation (Fig. 6D), and within 10 h the level of AT_1-R expression is similar to that in untreated cells (Fig. 6E). These observations are consistent with the loss of cell surface expression observed by the intact cell radioligand binding. The levels of perinuclear staining that are observed in untreated cells are not clearly affected by agonist treatment. Coimmunolocalization experiments that were conducted to determine whether this perinuclear AT_1-R staining represents lysosomal pools were inconclusive (data not shown). Although this remains a possibility, the staining in this location may represent a pool of newly translated protein associated with polyribosomes or in early sorting pathways.

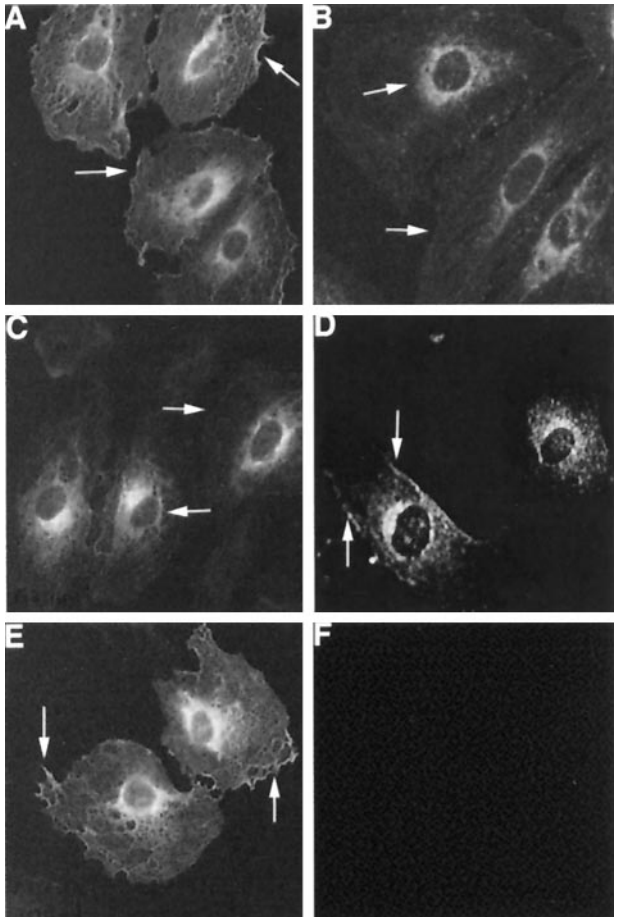

Fig. 6. AngII-evoked internalization of HA-tagged AT_1-R in A7r5 VSMC. Cells were treated with 2 μM AngII for indicated times before washing and detecting HA epitope-tagged AT_1-R immunohistochemically. A, expression after treatment with vehicle. Staining evident on cell edges (arrows) is quite clear and represents receptors at plasma membrane. B, expression after 2 μM AngII treatment for 5 min. Staining around cell surface (lower arrow) is reduced noticeably compared with vehicle-treated cells, indicating that receptors have internalized. However, perinuclear staining intensity (upper arrow) does not differ from that in vehicle treated cells. C, expression after 60-min treatment with AngII. Almost all staining is located around nucleus (lower arrow), with very little at cell surface (upper arrow). D, expression after 4-h treatment with AngII. Although perinuclear staining persists, staining can be seen now at cell edges (arrows), indicating re-expression of plasma membrane receptors. E, expression after 10 h treatment with AngII. Pattern of staining in these cells is indistinguishable from that seen in vehicle-treated cells as shown in A. F, absence of staining in nonrecombinant A7r5 cells. No signal is generated when nonrecombinant A7r5 cells are incubated with anti-HA epitope antibody and developed with fluorescent stain as for recombinant cells. Similar results were obtained in recombinant cells incubated with control mouse ascites IgG rather than anti-HA antibody. Data are representative of three experiments.

Discussion

A principal goal of these experiments was to understand the relative contributions to down-regulation that are made by processes affecting AT_1-R mRNA content and AT_1-R internalization. A major conclusion is that the regulation of AT_1-R mRNA levels in VSMC plays a crucial role in dictating the degree to which down-regulation will occur. We show that AngII stimulates a rapid and profound AT_1-R internalization. Certainly, a status of net internalization will persist for as long as the agonist is present in the extracellular space. However, experiments herein also show that there is also a persistent recovery from internalization during this period, which although masked by the continued presence of agonist, at some point must provide the rate limitation on the actual amount of receptor that can be internalized.

Presumably, if a stimulus does not affect metabolism of the mRNA, the mRNA provides a continuous source of new protein available for externalization in the membrane and if agonist is present, this receptor protein is rapidly internalized. At some point, the steady state in such a system will reflect a balance between the rate of protein synthesis and the rate that internalized receptor is destroyed. If instead the AT_1-R mRNA steady state is disrupted, the pool of receptor protein available for internalization will decrease over time at a rate dominated by the mRNA decay rate. The data shown in Fig. 4A dramatically illustrates this point by showing that a recovery of cell surface AT_1-R protein levels are blunted when a loss of AT_1-R mRNA is triggered. Indeed, this blunting effect becomes quite evident at a time representing a single half-life of the mRNA, after which there is a dramatic decline in the ability of the cells to maintain control levels of surface AT_1-R expression. These observations do not discount the role of internalization in down-regulation and in particular the destruction of receptor protein that appears to accompany this process. However, they do provide direct and compelling evidence for the importance of the mechanisms controlling AT_1-R mRNA levels in determining the degree to which AT_1-R down-regulation will occur.

To a degree, the present findings are intuitively obvious, and support long-held notions about what processes contribute to sustained down-regulation of receptors. However, in studies of natively expressed genes and their receptors, most evidence is correlative because it is not possible to know with any certainty how loss of an mRNA contributes to loss of its receptor protein when both responses are triggered by a common stimulus. Delineation of these relative roles is difficult to obtain in native systems principally because there is no means for selectively controlling for the effects of signaling on the mRNA steady state. Thus, the present studies were conducted in A7r5 cells in the manner described because it affords better experimental control over mRNA production.

Even though AngII signaling does not appear to modulate

AT_1-R mRNA metabolism in A7r5, it is reasonable to infer from the present data that a similar relationship between mRNA content and receptor protein content exists in cells that natively express the AT_1-R gene. Consistent with this are observations in native VSMC that indicate that mRNA down-regulation is more tightly coupled to AT_1-R activation than is degradation of the receptor protein. AngII is approximately 10-fold more potent at reducing AT_1-R mRNA levels in VSMC than it is at reducing the total cellular content of the AT_1-R protein (Lassegue et al., 1995). Other studies have shown that down-regulation of the native AT_1-R mRNA in VSMC can be highly sensitive to modest changes in the extracellular environment (X. Wang et al., 1997). Thus, in terms of dose relationships, levels of the AT_1-R mRNA are clearly more sensitive to perturbations in the concentrations of AngII and other agonists than are levels of the cell surface protein. However, this does not necessarily imply that internalization of the receptor is only a pharmacological effect. Because the EC_{50} for internalization is indistinguishable from the apparent affinity of the receptor for AngII, it is quite likely that internalization is the fate of any cell surface AT_1-R that is occupied by agonist.

An interesting facet of the present data is the demonstration that internalized receptors are replaced rather rapidly at the cell surface with additional receptors (see Fig. 5B). This externalization pathway seems to represent a substantial force against the contribution that receptor internalization alone can make in down- regulating responsiveness. The data shown in Fig. 5A emphasize this point, wherein even after a period of persistent stimulation and internalization over several hours, substantial recovery of cell surface expression can occur. Notably, this follows a period after which the total cellular AT_1-R receptor content has been reduced by some 45% (see Fig. 5C). It appears that so long as sufficient concentrations of extracellular AngII are present, our data show that these replacement receptors are quickly internalized. But it is reasonable to expect that externalized receptor likely contributes to signaling responses before this occurs. Taken together, the cells possess a capability for a substantial recovery of cell surface AT_1-R following a wave of internalization, and even following persistent internalization, so long as AT_1-R mRNA levels have not been disrupted.

Our experiments do not directly address precisely which receptors become expressed at the cell surface following internalization. They may be recycled internalized receptors, or they could reflect a pool of protein that has not before been expressed on the cell surface. Because cycloheximide fails to block a substantial component of the recovery, new synthesis is not required for a large fraction of the receptor that is expressed on the cell surface in the first hours following AngII-evoked internalization. Current general paradigms of G protein-coupled receptor regulation suggest that internalization and recycling of the protein back to the cell surface provides a mechanism to reactivate a desensitized receptor (Bohm et al., 1997). Results from previous reports are consistent with the notion that some degree of AT_1-R recycling occurs in VSMC plasma membranes (Griendling et al., 1987; Ullian and Linas, 1989) and recent immunohistochemical studies suggest this occurs in HEK293 cells as well (Hein et al., 1997). Although it is possible recycling contributes to this recovery, two observations argue against this. First, we find no obvious evidence in our immunohistochemical data for vesicular clustering or retention of recombinant AT_1-R near the cell surface shortly after treatment with AngII. Rather, AT_1-R expression appears largely lost from peripheral cell structures. If recycling explains the recovery, it would seem the receptor has trafficked some distance from the plasma membrane before it returns. Second, total cellular AT_1-R content is reduced by ~25% after 1 h of AngII treatment. We postulate that this loss reflects the fraction of receptors that are expressed on the cell surface during this period of stimulation and that their internalization is coupled to rapid degradation of the receptor protein. In the future, it will be important obtain better measurements of the time necessary for a newly synthesized receptor to traffic out to the plasma membrane, and to know how long lived it is once there. These are dynamic parameters the present system is poorly equipped to assess directly but should give better quantitative insight into how internalization contributes to down-regulation.

The tetracycline-regulated expression system shown here has a more general utility for understanding factors involved in the dynamic regulation of cellular mRNAs and proteins. Although a previous report demonstrated that tetracycline-regulated promoters can function when placed within a self-inactivating retroviral LTR (Hofmann et al., 1996), that system uses an internal ribosome entry site to express two proteins, one of which is the tTA, from a single mRNA. The system developed for the present study differs fundamentally from that system because it allows for production of a precisely specified experimental mRNA from within a retroviral LTR. This is an important distinction, because the composition of a mRNA can impact its translational efficiency and its rate of degradation (Sachs et al., 1997). In this regard, further refinements of these vectors are possible. In some instances, the heterologous minimal CMV promoter and the SV40 pA+ signals used here for entry and termination of transcription may affect normal control processes associated with regulation of the recombinant mRNA. For example, it should be possible to fuse the tTA enhancer elements to the minimal promoters of specific genes, to link this to their homologous mRNA coding sequence, and even to use their downstream pA+ signals rather than heterologous pA+ signals. This type of strategy would result in the expression of a precise mimic of a native transcript that is devoid of any heterologous sequences. Combined with the high efficiency associated with retroviral- mediated gene transfer, such designs have great promise for understanding the mechanisms associated with post-transcriptional control of gene expression in a diverse cell phenotypes.

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Send reprint requests to: Dr. T. J. Murphy, Department of Pharmacology, Emory University School of Medicine, Room 5031, O. W. Rollins Research Center, 1510 Clifton Rd., Atlanta, GA 30322. E-mail: tmurphy@pharm.emory.edu