Novel regulation of adrenomedullin receptor by PDGF: role of receptor activity modifying protein-3

WOJCIECH NOWAK, NARAYANAN PARAMESWARAN, CAROLYN S. HALL, NAMBI AIYAR, HARVEY V. SPARKS, AND WILLIAM S. SPIELMAN

Department of Physiology, Michigan State University, East Lansing, Michigan 48824

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The recent discovery of receptor activity modifying proteins (RAMPs) by McClatchie et al. (31) has significantly altered our understanding of mechanisms involved in the regulation of G protein-coupled receptors (GPCR). RAMP-1, RAMP-2, and RAMP-3 are distinct gene products and have been characterized as single-transmembrane domain proteins capable of direct interaction with two related members of GPCR: calcitonin receptor (CR) and CR-like receptor (CRLR) (4, 7, 20, 31). Although the exact nature of RAMP-CR and RAMP-CRLR interactions remains elusive, it has been clearly documented that RAMPs facilitate trafficking and determine the phenotype of these receptors (7, 28, 31). In particular, with regard to CRLR, RAMP-1 and CRLR coexpression renders the receptor a fully functional calcitonin gene-related peptide (CGRP) receptor. Cotransfection of CRLR with RAMP-2 or RAMP-3, on the other hand, confers on CRLR adrenomedullin (AM) receptor characteristics (31). Consequently, differential expression of RAMPs may prove to function as a regulatory step for CRLR activity and its ligand specificity toward CGRP and/or AM in physiological and pathophysiological states alike. In fact, RAMP gene expression has been observed to be nonconstitutive and reported to undergo dynamic alterations in several disease states including renal diseases (32, 33, 47, 48).

AM, a 52-amino acid peptide recently isolated from a pheochromocytoma (23), has been shown to activate CRLR causing an elevation in intracellular cAMP in several systems including rat mesangial cells (RMCs) (6, 26, 35). In particular, by activation of the cAMP-protein kinase A (PKA) pathway, AM exerts antiproliferative, proapoptotic, and antimigratory effects on RMCs (5, 37). Because disproportionate mesangial proliferation and matrix deposition are hallmark pathologic changes accompanying several glomerular diseases (10, 25), the antiproliferative effects of AM suggest an attractive, renoprotective role for this hormone. AM has also been shown to decrease platelet-derived growth factor (PDGF)-induced mesangial cell proliferation (6, 44). PDGF is a prime cytokine responsible for mediating both proliferative and migratory responses in the mesangium during glomerular injury (1, 29, 36). Elucidating the mechanisms of functional interplay between PDGF and AM receptor will provide novel information on regulation of proliferative/antiproliferative events in mesangial cells. Hence the present study was undertaken to examine the possible effect(s) of PDGF on AM receptor complex (CRLR-RAMP-3) expression in RMCs. Our results suggest that PDGF causes a significant increase in RAMP-3 mRNA and protein expression, with a corresponding elevation in AM-stimulated adenylate cyclase activity. Furthermore, the PDGF-mediated increase in RAMP-3 mRNA abundance is dependent on mRNA stability but not on transcription.
MATERIALS AND METHODS

Materials. AM, AM-(22-52) fragment, actinomycin D, α-amanitin, and PDGF-BB were purchased from Sigma RBI (St. Louis, MO). AG-1296, PD-98059, PD-153035, PD-168393, and SB-203580 were from Calbiochem (La Jolla, CA). RPMI 1640, fetal bovine serum, penicillin/streptomycin, and trypsin-EDTA were from GIBCO BRL (Grand Island, NY). All other reagents were of highest quality available.

Cell culture. Rat mesangial cell (RMC) cultures were established from glomeruli obtained from kidney cortex of 55–70 g male rats (Sprague-Dawley; Charles River). Glomeruli were isolated by sequential sieving, which removes tubules (300- to 150-μm sieves) and then retains glomeruli on the 65-μm sieve, as described by Wolthuis et al. (51). Isolated glomeruli were incubated for 10 min at 37°C in collagenase (750 U/ml) and then plated in flasks in RPMI 1640 medium supplemented with 0.6 U/ml insulin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 15% fetal bovine serum. Cells were grown at 37°C in 5% carbon dioxide with medium changed twice a week. At confluence for different experiments was determined by preliminary studies.

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Membrane preparation and adenylate cyclase assay. Cells were harvested from 6-well plates and homogenized in 10 mM Tris-HCl (pH 7.4)-10 mM EDTA buffer. Membranes were prepared by homogenization in a Dounce ground glass homogenizer, centrifuged for 20 min at 12,000 g at 4°C, and washed in 50 mM Tris-HCl (pH 7.4)-10 mM MgCl2 buffer. A final concentration of 40 μg protein/assay tube was obtained, and the membranes were immediately subjected to adenylate cyclase assay, as follows. Membrane-associated adenylate cyclase activity was measured as the rate of conversion of [α-32P]ATP to [32P]cAMP as described by Elshourbagy et al. (11). Accordingly, membranes were incubated for 20 min at 30°C with appropriate drugs and an assay mix containing an ATP regeneration system (50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 1.2 mM ATP, 0.1 mM CAMP, 2.8 mM phosphoenolpyruvate, and 5.2 μg/ml myokinase) and 1.0 μCi of [α-32P]ATP. Total reaction volume was 100 μl, and drug as well as AM concentrations were as described for particular experiments. Reactions were stopped with 1 ml of stop solution containing 0.28 mM cold cAMP, 0.33 mM ATP, and 22,000 dpm [3H]cAMP. The contents of the assay tubes were washed through a Dowex column, and subsequently through alumina columns, to separate the degradation products of ATP as previously described by Salomon et al. (41). Elution profiles were performed before experiments to determine the amount of water (for Dowex columns) and imidazole (for alumina columns) needed to wash and elute the products. Products eluted from the alumina column were counted for the presence of [3H]cAMP and [α-32P]cAMP. Each experiment was done in triplicate, repeated at least three times, and expressed as a percentage of AM-mediated adenylate cyclase activity compared with basal.

[3H]tytymidine incorporation. Cells were plated in 24-well plates (30,000 cells/well) and grown for 2 days with subsequent serum starvation for 48 h. They were then treated with the compounds for a period of 16 h and pulsed with [3H]thymidine for 4 h. The radioactivity was counted in a Beckman LS counter after the cells were washed, the reaction was stopped with 5% TCA, and the cells were solubilized in 0.5 ml of 0.25 N sodium hydroxide. Each experiment was performed in quadruplicates and repeated at least three times.

Northern blot analysis. Immediately after the cell culture medium was aspirated from the tissue culture plates, 2−4 ml of Trizol were added and dispersed uniformly, and plates were stored at −80°C until further use. After a quick thaw, cells were scraped with cell lifters and transferred to 15-ml centrifuge tubes. Total cellular RNA was isolated with Trizol according to the manufacturer’s specifications. RNA was pre-
cipated by adding 3 M sodium acetate and absolute ethanol and then washed with 75% ethanol, pelleted in microcentrifuge tubes, and dried before resuspension in RNAase-free water. The purity of the RNA was checked by measuring the ratio of absorbance at 260 nm to absorbance at 280 nm. All of the RNA used had a ratio ≥1.8. A standardized aliquot of RNA (30 μg) was separated by electrophoresis on a formaldehyde agarose denaturing gel and transferred to an Optitran membrane (Schleicher and Schuell, Keene, NH) by capillary transfer. Subsequently, RNA samples were immobilized to the membrane by ultraviolet cross-linking. Membranes were successively hybridized at 42°C for 16–24 h with four parts of a solution containing 15 ml of formamide, 0.6 ml of Denhardt's solution, 1.5 ml of 1 M phosphate buffer, 7.5 ml of 20× SSC, 1.5 ml of SDS, 2.4 ml of diethylpyrocarbonate water, and 1.5 ml of salmon sperm DNA per blot and one part of [32P]dCTP-labeled cDNA probes (specific for RAMP-1, -2, -3 or 18S ribosomal subunit). RAMP probes were obtained as RT-PCR products using RAMP-specific primers designed from published sequences. The cDNA was radiolabeled using a random prime labeling kit. After hybridization for 16–24 h, the membranes were washed and placed in an X-ray cassette for the requisite exposure time. Signals were quantitated by phosphorimager analyses and expressed relative to 18S levels.

**Analysis of RNA stability.** Rat mesangial cells were grown on P-150 tissue culture plates to ~80% confluence (as described above) and subsequently rendered quiescent by serum starving for 24 h. Next, cells were incubated with PDGF-BB (50 ng/ml) for 16 h, washed, and placed in serum-free medium containing actinomycin D, an inhibitor of gene transcription, at a final concentration of 10 μg/ml and in the presence or absence of PDGF-BB (50 ng/ml) for 0, 1, 2, 4, and 8 h. Total RNA was isolated and analyzed by Northern blotting as described above. RNA degradation curves were obtained by setting the 100% value to the amount of RAMP mRNA present immediately before actinomycin D exposure [maximum value at time 0 (t0)]. mRNA levels remaining at indicated times following t0 were compared as a percent of the maximum value. A one-phase exponential decay curve was fitted including the maximum value at t0 and decay rate constant K, calculated for each nonlinear regression curve. The half-life of the RAMP message was calculated as equal to ln (2/K).

**Western blot analysis.** Western blot analysis was done as described before (37). Briefly, equal concentrations of protein samples were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membranes were subsequently blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and incubated with RAMP-3 polyclonal primary antibodies at a final concentration of 400 ng/ml (Santa Cruz Biotechnology, Santa Cruz, CA) followed by horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG antibodies at a concentration of 1:10,000, Sigma), according to the manufacturer's instructions. An enhanced chemiluminescence (ECL) kit (Pierce, Rockford, IL) was used to visualize the blots.

**Statistical analysis.** Data are presented as means ± SE. Multiple group comparisons were made using a two-way ANOVA. Single group comparisons exercised the Student's t-test method. Statistical significance was set at P < 0.05.

**RESULTS**

**AM receptor components in RMCs.** We first characterized the expression of AM receptor components in quiescent RMCs by RT-PCR as described in MATERIALS AND METHODS. Under basal conditions, RMCs express CRLR as well as all three subtypes of RAMP (RAMP-1, -2, and -3; Fig. 1).

**Effects of RAMP overexpression on AM-induced adenylyl cyclase activity and [3H]thymidine incorporation in RMCs.** To investigate the effect of RAMP overexpression on mesangial cell responsiveness to AM, we transiently transfected RMCs with RAMP-1, -2, and -3 and subsequently examined AM-mediated adenylyl cyclase activity and [3H]thymidine incorporation. Western blot analysis revealed significantly higher levels of RAMP-1, -2, and -3 protein expression in the membrane fraction compared with vector transfected cells (data not shown). Transfection of RAMP-2 or RAMP-3 resulted in a 85.4 ± 3.02 and 54.5 ± 2.63% increase in AM-induced adenylyl cyclase activity, respectively (Fig. 2). These responses were further potentiated by cotransfection of CRLR with RAMP-2 or RAMP-3 (136.6 ± 7.9 and 90.71 ± 3.8% increase, respectively). AM-induced adenylyl cyclase activity in RAMP-2/RAMP-3-transfected cells was also inhibited by pretreatment with AM-(22–52), AM receptor antagonist, indicating that this effect is AM receptor mediated (results not shown). Transfection of RAMP-1, on the other hand, had no effect on AM-mediated responses (Fig. 2), which is consistent with previously reported data describing RAMP-1-CRLR as a receptor complex with low AM binding specificity (4, 15).

The antiproliferative effect of AM on mesangial cells has been previously demonstrated by our laboratory as well as others (6, 37, 44). Activation of PKA has been shown to induce an antimitogenic effect on mesangial cells. Because AM increases adenylyl cyclase activity and hence PKA, we hypothesized that overexpression of RAMP-2 and RAMP-3, in addition to increasing adenylyl cyclase activity, will also potentiate an AM-mediated decrease in [3H]thymidine incorporation. As predicted, transfection of RAMP-2 or RAMP-3 significantly potentiated AM-mediated inhibition of [3H]thy-
Involvement of signal transduction pathway(s) in PDGF-dependent RAMP-3 upregulation. PDGF has been shown to exert myriad biological effects principally by acting through its receptor, PDGFR, known to have an intrinsic tyrosine kinase activity (18). To investigate whether PDGF-induced RAMP-3 mRNA expression is PDGFR specific, we utilized several pharmacological tyrosine kinase inhibitors. AG-1296, a specific and selective inhibitor of PDGFR tyrosine kinase activity (27), abrogated the PDGF-induced response (Fig. 8). The effect was dose dependent for AG-1296 concentrations of 0.1–30 μM (data not shown). PD-153035 and PD-168393, selective inhibitors of epidermal growth factor receptor-associated tyrosine kinase (3, 17), did not, however, have any effect on PDGF-stimulated RAMP-3 mRNA expression (Fig. 8). Accordingly, the observed increase in RAMP-3 α-amanitin and PDGF resulted in a 58.49 ± 2.56% increase in RAMP-3 mRNA expression above basal compared with a 55.46 ± 3.47% increase in cells treated with 50 ng/ml PDGF alone (statistical difference between these results is not significant, with P = 0.665, n = 3). Next, a requirement for new protein synthesis was examined by pretreatment of PDGF-exposed cells to cycloheximide (CHX), a potent eukaryotic translational inhibitor. At 10 μg/ml, CHX significantly inhibited PDGF-induced RAMP-3 mRNA expression, identifying a requisite step of de novo protein synthesis (Fig. 6).

Because a PDGF-induced increase in RAMP-3 mRNA expression was not mediated through transcriptional events, we further hypothesized that PDGF enhances RAMP-3 mRNA stability, thus leading to increased abundance of the RAMP-3 message. Accordingly, we analyzed the effect of PDGF on the half-life of RAMP-3 mRNA as described in MATERIALS AND METHODS. PDGF (50 ng/ml) treatment increased the apparent half-life of RAMP-3 mRNA from 66.5 to 331.6 min (Fig. 7). This indicates that the mechanism of PDGF-induced RAMP-3 expression is mediated by a posttranscriptional event of mRNA stability enhancement.

Mechanism of PDGF-stimulated RAMP-3 expression. To determine whether PDGF affects transcriptional events leading to increased abundance of RAMP-3, we tested actinomycin D and α-amanitin (inhibitors of DNA-dependent RNA synthesis) for their ability to influence the PDGF-dependent effect. Preincubation of mesangial cells with actinomycin D (5 μg/ml) did not alter PDGF-induced RAMP-3 expression (Fig. 5A), suggesting the lack of regulation at a transcriptional level. To verify the effectiveness of transcriptional inhibition by actinomycin D, Northern blots were reprobed with mitogen-activated protein kinase (MAPK) kinase (MEK)-1 cDNA, and quantification of MEK-1 RNA was performed. As previously reported by Schramek et al. (43), PDGF induced MEK-1 mRNA, and this effect was inhibited by actinomycin D (Fig. 5B), indicating a good transcriptional inhibition by actinomycin D. Similarly, preincubation of the cells with α-amanitin (1 μg/ml) for 5–6 h did not have any effect on PDGF-induced RAMP-3 mRNA elevation. Coexposure of cells to

*Fig. 2. Effect of RAMP-1, RAMP-2, and RAMP-3 overexpression on adrenomedullin (AM)-stimulated adenylate cyclase activity in RMCs. RMCs were transfected with vector or vector + RAMP-1, RAMP-2, or RAMP-3 at 60% confluence. Cells were allowed to grow for another 24–36 h. Adenylate cyclase (AC) assay in response to 100 nM AM was performed as described in MATERIALS AND METHODS. Overexpression of RAMP-2 and RAMP-3 significantly enhanced AM-stimulated AC activity in RMCs. Overexpression of RAMP-1 had no significant effect on AC activity. *P < 0.01; n.s., statistically not significant; experiments performed in triplicates; n ≥ 3.

*Fig. 3. Effect of RAMP-2 and RAMP-3 overexpression on [3H]thymidine incorporation in RMCs. Cells were transiently transfected with RAMP-2 or RAMP-3 and allowed to grow for additional 24–36 h. Subsequently, labeled thymidine incorporation in response to 100 nM AM was followed per protocol described in MATERIALS AND METHODS. RAMP-2 and RAMP-3 overexpression significantly potentiated AM-mediated decrease of [3H]thymidine incorporation in RMCs; *P < 0.05 compared with vector; experiments performed in quadruplicates; n = 4.
mRNA abundance after exposure to PDGF is PDGFR-mediated and receptor-associated tyrosine kinase dependent.

Because initiation of intracellular tyrosine kinase activity often triggers a cascade of events mediated via MAPKs, we investigated the effects of PD-98059 (10 \( \mu \)M) and SB-203580 (10 \( \mu \)M) (selective inhibitors of MEK and p38 MAPK, respectively) on PDGF-induced RAMP-3 expression. Both inhibitors significantly attenuated the PDGF-induced response (Fig. 9), indicating that RAMP-3 expression in RMCs is, at least in part, regulated by the MAPK pathway.

**Effect of PDGF on RAMP protein expression and AM-mediated adenylate cyclase activity.** To determine whether the PDGF-induced increase in RAMP-3 mRNA corresponds to an elevated expression of RAMP-3 protein, we examined RAMP-3 protein levels in the membrane-associated fraction of cells cultured in the presence or absence of PDGF. Western blot data revealed a 3.3 ± 0.28-fold increase in the amount of RAMP-3 protein in cells exposed to PDGF compared with controls (Fig. 10). As predicted, PDGF-induced elevation of RAMP-3 correlated to a functional increase in cell responsiveness to various concentrations of AM as measured by AM-mediated adenylate cyclase activity (Fig. 11A). Also, membranes of cells treated with various concentrations of PDGF (1–100 ng/ml) exhibited a concentration-dependent increase in AM-stimulated adenylate cyclase activity (Fig. 11B). This effect was inhibited by pretreatment of membranes with AM-(22–52), suggesting a direct involvement of AM-specific receptors (Fig. 11C).

**DISCUSSION**

Initial reports characterizing glomerular mesangial cells emphasized their importance in providing structural integrity to the glomerulus as well as their likely involvement in glomerular filtration rate regulation...
Recent discoveries focus on the contribution of the mesangial cell to the genesis and progression of several glomerulopathies. Excessive mesangial cell proliferation and matrix deposition are considered to be the hallmark for several renal diseases (10, 12, 25). These alterations in mesangial cell biology instigate the maladaptive process of glomerular hypertrophy resulting in glomerulosclerosis, often leading to end-stage renal failure (13, 21, 46, 49, 52). Several agents have been proposed to influence the course and progression of renal disease (24). Of these, one of the most recently identified peptides, AM, has been shown to have several actions of glomerular mesangial cells, suggesting that it may serve to be an important autocrine/paracrine factor that regulates mesangial cell turnover. We have recently reviewed the current understanding of the actions of AM on glomerular mesangial cells (38). Restoration of normal kidney function and histology by AM has been suggested.

**Fig. 5.** Effect of actinomycin D (ActD; 5 μg/ml) on PDGF-stimulated RAMP-3 expression in RMCs. RMCs were serum starved overnight and treated with or without PDGF (50 ng/ml) in the presence of ActD for 24 h. Northern blot analysis was performed as described in MATERIALS AND METHODS, and the blot was probed for RAMP-3 or mitogen-activated protein kinase kinase (MEK)-1 mRNA expression. A: ActD did not inhibit PDGF-induced RAMP-3 expression, suggesting that RAMP-3 expression in mesangial cells may not be regulated by increase in transcription. Inset: representative Northern blot for this experiment; n ≥ 3. B: control experiment verifying that PDGF-increased transcription of MEK-1 is inhibited by ActD. This experiment is shown to eliminate the possibility that ActD is inactive. The representative Northern blot shown here for MEK-1 expression is the same one that was used for probing RAMP-3.

**Fig. 6.** Effect of cycloheximide (CHX; 10 μg/ml) on PDGF-induced RAMP-3 expression in RMCs. RMCs were serum starved overnight and treated with or without PDGF (50 ng/ml) in the presence of CHX for 24 h. RNA was extracted, and Northern blot analysis was performed as indicated in MATERIALS AND METHODS. Blots were first probed for RAMP-3 and then stripped and reprobed for 18S RNA. Raw values were converted to ratios of RAMP-3 mRNA to 18S and then expressed as percent change from basal. Inset: representative Northern blot of RAMP-3 expression in response to PDGF ± CHX. CHX significantly attenuated PDGF-induced RAMP-3 mRNA expression; **P ≤ 0.001; n = 3.

**Fig. 7.** Effect of PDGF on RAMP-3 mRNA rate of decay in RMCs. RNA stability assay was performed on RMCs as described in MATERIALS AND METHODS. RNA from each time point was analyzed by Northern blot hybridization, and relative RAMP-3 mRNA abundance was expressed as a percentage of that present at time 0 (percentage of maximum value). RAMP-3 mRNA half-lives were calculated by using the decay constants (K) obtained from one-phase exponential decay curves. Inset: representative Northern blot obtained following the RNA stability assay protocol. PDGF significantly increased RAMP-3 mRNA half-life from 1.1 to 5.5 h. Regression lines (half-lives) for RNA decay from PDGF-treated (●) and no PDGF (▲) RMCs were compared by analysis of covariance. Regressions were regarded significantly different with a value of P ≤ 0.01; n ≥ 3.

**Fig. 8.** Effects of AG-1296, PD-153035, and PD-168393 on PDGF-stimulated RAMP-3 mRNA expression in RMCs. Cells were pretreated with inhibitors and subsequently exposed to PDGF (50 ng/ml) for 24 h. Representative Northern blot showing the selective dependence of PDGF-induced response on the activation of PDGF receptor but not EGF receptor-associated tyrosine kinase activity.
gested in several in vivo studies of kidney disease models. Dobrzynski et al. (9) reported that renal damage in deoxycorticosterone acetate salt hypertensive rat models was significantly attenuated by AM gene delivery. Others also confirmed the beneficial effects of AM administration in humans as well as animal models of renal disease (22, 30, 50). Although the exact mechanism for the renoprotective effect of AM is unclear at this time, it is hypothesized that the antiproliferative (and proapoptotic) effects of AM on mesangial cells may in part serve to protect the kidney during development of glomerular disease. Accordingly, identification of mechanisms responsible for modulation of the activity of AM in mesangial cells will benefit our understanding of mesangial growth regulation and the associated progress of several renal pathologies.

![Fig. 9. Effects of SB-203580 (10 μM) and PD-98059 (10 μM) on PDGF-induced RAMP-3 expression in RMCs. Cells were pretreated with inhibitors and subsequently exposed to PDGF (50 ng/ml) for 24 h. RNA was collected as in previous experiments and analyzed by Northern blot hybridization with RAMP-3 probes. To correct for loading variability, blots were stripped and reprobed for 18S RNA. Raw values were converted to ratios of RAMP-3 mRNA to 18S and then expressed as percent change from basal. Inset: representative Northern blot of RAMP-3 expression in response to pretreatment of RMC with SB-203580 and PD-98059 followed by PDGF exposure. Both SB-203580 and PD-98059 significantly attenuated PDGF-induced RAMP-3 expression; *P < 0.001; n = 5.](image)

![Fig. 10. Effect of PDGF on membrane-associated RAMP-3 protein expression in RMCs. Cells were incubated with PDGF (50 ng/ml) for 24 h. Subsequently, membranes were extracted as per membrane preparation protocol for AC assay (see MATERIALS AND METHODS), and equal concentrations of protein were loaded onto a gradient polyacrylamide gel. Western blotting was performed as described in MATERIALS AND METHODS. Raw values were converted to ratios of RAMP-3 protein to actin and expressed as fold of basal (with basal expression arbitrarily set at 1). Right: an exemplary Western blot obtained in this set of experiments. PDGF significantly increased RAMP-3 protein expression in the membrane-associated fraction of RMC. *P < 0.001; n = 4.](image)

![Fig. 11. Effect of PDGF on AM-mediated AC activity in RMCs. Cells were incubated in the presence or absence of PDGF (50 ng/ml) for 24 h. Membranes were then extracted, and AC activity assay in response to indicated concentrations of AM was performed. A: AC activity increased with increasing doses of AM in PDGF-treated and control cells. PDGF-treated cells exhibited significantly higher AC activity compared with corresponding basals (*P < 0.01) and control-treated cells (**P < 0.01). Experiments were performed in triplicates; n = 5. B: cells were treated with different concentrations of PDGF, as indicated, for 24 h. Membranes were then extracted, and AC activity assay in response to 100 nM AM was performed. PDGF caused a concentration-dependent increase in AM-stimulated AC activity, which closely correlated with PDGF-stimulated RAMP-3 mRNA expression (Fig. 4A); experiments were done in triplicates; n = 3. C: effect of AM-(22–52), the AM receptor antagonist, on AM-mediated AC activity in RMCs exposed to PDGF. Cells were grown in the presence of PDGF (50 ng/ml) or its absence (control) for 24 h. Next, membranes were extracted and pretreated with AM-(22–52) at 100 nM (+) or 1 μM (++) for 10 min before AC activity assay in response to 100 nM AM. PDGF-dependent increase in AM-mediated AC activity was significantly inhibited by AM-(22–52). *P < 0.01 (compared with PDGF treatment); †P < 0.01 (compared with control); experiments were performed in triplicates; n = 3.](image)
Mesangial cells express a functional AM receptor complex (CRLR+RAMP-2/RAMP-3) in culture. In addition, as observed by others in different cell lines, overexpression of RAMP-2 or RAMP-3 also increased RMC responsiveness to AM as measured by AM-induced adenylate cyclase activity (15, 19). Fraser et al. (15) and Husmann et al. (19) have reported that co-transfection of HEK-293 and COS-7 cells with CRLR and RAMP-2/RAMP-3 exhibit increased $^{125}$I-labeled AM binding affinity and an augmented cAMP accumulation in response to various concentrations of AM. Clearly, modification of RAMP expression can serve as a major mechanism for effectively altering the responsiveness of cells to AM not only in other cell types, but also in mesangial cells. Here, we propose that factor(s) that influence the AM signaling system in mesangial cells may modulate this system through their action(s) on RAMP expression.

PDGF is a well-known, important autocrine/paracrine growth factor in mesangial cells and has been shown to interact with the AM antiproliferative pathway. Specifically, PDGF-induced mitogenesis is blocked by AM in RMCs in culture. Indeed, if PDGF enhances AM signaling in mesangial cells, it may serve as a negative feedback mechanism in regulating normal turnover of mesangial cells. Thus we investigated the effects of PDGF on the AM receptor system, including its effects on CRLR, RAMP-2, and RAMP-3 in RMCs. We report here that PDGF regulates AM receptor signaling through modulation of RAMP-3 mRNA expression. PDGF did not have any effect on CRLR or RAMP-2 mRNA expression. Others have found similar results in both animal and cell culture models. For example, Totsune et al. (47) presented findings from a rat model of 5/6 nephrectomy where RAMP-2 mRNA levels were unchanged whereas a significant decrease in RAMP-3 mRNA was found in the remnant kidney. In general, ample evidence exists for differential expression of RAMP mRNA in tissues from animal disease models, including those with renal pathology (32–34, 39, 48, 53). Frayon et al. (16) have reported that glucocorticoid treatment in vascular smooth muscle cells in culture causes a transient increase in RAMP-1 mRNA expression without any change in RAMP-2 expression. This glucocorticoid-directed RAMP expression results in a CGRP-responsive receptor (CRLR+RAMP-1). These results also suggest that, in vivo, glucocorticoid regulation of RAMP-1 mRNA expression may lead to a subsequent consequence on ligand recognition. Similarly, Robert-Nicoud et al. (40) reported vasopressin-induced RAMP-3 mRNA levels in the mouse clonal cortical collecting duct principal cell line. To date, it is not known if the well-established role for RAMP-3 in AM receptor pharmacology can be extended to other, unrelated systems such as that of vasopressin. However, ample evidence from related studies supports the hypothesis that PDGF-mediated changes in RAMP-3 expression in mesangial cells may have functional consequence in terms of AM signaling. In fact, our results suggest that PDGF-stimulated RAMP-3 expression leads to AM-stimulated adenylate cyclase activity. This effect, if present in vivo, may augment AM signaling and hence increase the antiproliferative effect of AM, opposing the proliferative effect of PDGF. This negative feedback mechanism may be present to keep the mesangial cell growth in check. Alternatively, this system of PDGF-induced RAMP-3 expression may be altered or absent under disease conditions, thus resulting in an aberrant negative feedback and, consequently, leading to uncontrolled mesangial growth. Though this hypothesis is attractive, clearly further studies are essential.

Although both Frayon et al. (16) and Robert-Nicoud et al. (40) demonstrated that exogenous addition of factors can alter RAMP expression, they did not address the underlying mechanisms, which may be responsible for these effects. Here we present evidence for PDGF-induced RAMP-3 expression to be transcriptionally independent, because it was not inhibited by actinomycin D or a-amanitin. Furthermore, our data indicate that PDGF acts via stabilizing RAMP-3 mRNA, consequently increasing the apparent half-life of the message by nearly fivefold. Though we established the requirement of de novo protein synthesis for this effect, future studies are necessary to further characterize the exact mechanism(s) by which PDGF influences the stability of the RAMP-3 message. It is worth noting that PDGF had no significant effect on RAMP-2 expression, suggesting a differential regulation between the closely related RAMP-2 and RAMP-3.

One of the aims of this study was to investigate the mechanism of PDGF-induced RAMP-3 expression at the signaling level (particularly the role of MAPKs). Cellular actions of PDGF have been shown to involve both its receptor tyrosine kinase activity and MAPK pathways. To examine the role of MAPKs, we utilized specific inhibitors to block these signaling molecules. The effect of AG-1296 (PDGFR tyrosine kinase blocker) suggests that the effects of PDGF on RAMP-3 expression are indeed mediated through PDGFR tyrosine kinase activity. Furthermore, inhibition of MEK (kinase upstream of extracellular signal-related kinase) by PD-98059, and p38 MAPK by SB-203580, suggests that activation of these kinases by PDGF may be important in the regulation of RAMP-3 expression. To our knowledge this is the first report of an involvement of MAPK pathways in regulating RAMP-3 expression in mesangial cells. Considering that MAPK pathways serve as a common denominator for a variety of divergent intracellular signals, this finding may bear an important implication for the complexity of RAMP gene expression regulation.

The discovery of the GPCR-associated family of RAMP proteins prompted a new look at the paradigm for receptor phenotype determination. It also provided a plethora of opportunities to recognize novel mechanisms responsible for modulation of receptor activity. RAMP-1, -2, and -3 have been shown to differentially couple to CR and CRLR, giving rise to distinct receptor phenotype characteristics (7, 31). Receptor trafficking,
glycosylation, and a direct RAMP-receptor interaction have all been proposed as possible mechanisms responsible for the critical role of RAMPs in CR and CRLR ligand specificity determination [for reviews see Foord et al. (14) and Sexton et al. (45)]. Accumulating data suggest that the dynamic alteration of RAMP expression levels may provide for yet another mode by which these proteins regulate the function of CRLR and possibly other receptors. The current study identified a model for RAMP mRNA regulation by a growth factor as a means for alteration of the functional responsiveness of a cell to a ligand.

In summary, we report that mesangial cells express CRLR, RAMP-1, RAMP-2, and RAMP-3 at basal conditions. PDGF, a pleiotropic cytokine well established to influence mesangial cell biology, increases RAMP-3 mRNA and membrane-associated RAMP-3 protein expression. This effect correlates with an elevation in cellular responsiveness to AM as measured by AM-stimulated adenylate cyclase activity. Moreover, our data show the PDGF-induced RAMP-3 mRNA elevation to be MEK and p38 MAPK dependent and post-transcriptionally regulated. PDGF augments the abundance of RAMP-3 mRNA via stabilization of the message and a consequent increase in RAMP-3 mRNA half-life. Taken together, these data suggest an important and novel mechanism for regulation of RAMP-3 in RMCs.

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