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European Journal of Pharmacology 370 (1999) 313–318

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Mechanism of adrenomedullin-stimulated hyaluronic acid release in rat mesangial cells

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Received 9 November 1998; received in revised form 15 February 1999; accepted 19 February 1999

Abstract

Adrenomedullin is a potent vasodilatory peptide that increases cAMP in a number of different systems including rat mesangial cells. Since mesangial cells play a significant role in glomerular matrix production, we evaluated the effects and molecular mechanisms of adrenomedullin action on hyaluronic acid release, an important extracellular matrix component. Adrenomedullin increased hyaluronic acid release in mesangial cells in a concentration-dependent manner. Forskolin, an adenylate cyclase activator, and dibutyryl-cAMP, a cell permeable cAMP analog, also increased hyaluronic acid release significantly. Adrenomedullin-stimulated hyaluronic acid release was inhibited by the adrenomedullin receptor antagonist, adrenomedullin-(22-52). Inhibition of protein kinase A with H89 [{*N*-[2-((*p*-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, hydrochloride}], a potent protein kinase A inhibitor did not affect adrenomedullin-stimulated hyaluronic acid release; however, H89 [{*N*-[2-((*p*-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, hydrochloride}] inhibited forskolin- and dibutyryl-cAMP-induced hyaluronic acid production. In addition, SB203580 {[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole}, a P38 mitogen-activated protein kinase (P38 MAPK) inhibitor attenuated adrenomedullin-, forskolin-, and dibutyryl-cAMP-stimulated hyaluronic acid release. Hyaluronic acid release induced by adrenomedullin, forskolin and dbcAMP was also inhibited by wortmannin {[1*S*-(1α, 6bα, 9aβ, 11α, 11bβ)]-11-(Acetyloxy)-1, 6b, 7, 8, 9a, 10, 11, 11b-octahydro-1-(methoxymethyl)-9a, 11b-dimethyl-3*H*-furo[4, 3, 2-de]indeno[4, 5-h]-2-benzopyran-3, 6, 9-trione}. We conclude that adrenomedullin, forskolin and dbcAMP cause an increase in hyaluronic acid release in rat mesangial cells through a pathway that involves activation of wortmannin-sensitive kinase and P38 MAPK. Although cAMP stimulation and protein kinase A activation can induce hyaluronic acid release, adrenomedullin-stimulated hyaluronic acid release appears to be independent of protein kinase A activation. These data provide the first demonstration of the involvement of P38 MAPK- and wortmannin-sensitive kinase pathways in the stimulation of hyaluronic acid production by rat mesangial cells.

Keywords: Adrenomedullin; Hyaluronic acid; P38 Mitogen-activated protein kinase; SB203580; Wortmannin; Renal glomerular

1. Introduction

Hyaluronate, a non-sulfated glycosaminoglycan, is widely distributed in the extracellular space in animals. It is an important extracellular matrix component and also a potential mitogen secreted by mesangial cells (Couchman et al., 1994; Mahadevan et al., 1996). Serum hyaluronate levels are increased during renal insufficiency and chronic renal failure (Hallgren et al., 1987). Hyaluronic acid release by the glomerulus is also increased in experimental diabetes and postulated to be a significant factor in glomerular hypercellularity (Mahadevan et al., 1996). In addition, the expression of hyaluronic acid receptor is significantly increased in experimental proliferative glomerulonephritis (Nikolic-Paterson et al., 1996). Although certain factors have been shown to modulate hyaluronic acid release in different cell systems, there is little information on the molecular mechanisms of regulation of hyaluronic acid release, especially the role of mitogen-activated protein kinase (MAPK) pathways (Heldin et al., 1989; Heldin et al., 1992; Honda et al., 1993; Suzuki et al., 1995).

Adrenomedullin, discovered in 1993, increases cAMP in a variety of systems including rat mesangial cells (Chini et al., 1995; Kohno et al., 1995). Recently, we reported

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0014-2999/99/$ - see front matter
PII: S0014-2999(99)00117-X

that in rat glomerular mesangial cells adrenomedullin causes a decrease in extracellular signal-regulated kinase-2 (ERK2) activity and an increase in *jun*–amino terminal kinase-1 (JNK1) and P38 mitogen-activated protein kinase (P38 MAPK) activities with an associated induction of apoptosis and decrease in proliferation (Parameswaran et al., 1999). The major aim of this study was to identify the effects and molecular mechanisms of adrenomedullin, forskolin, and cAMP on hyaluronate release in rat mesangial cells. We demonstrate here for the first time that adrenomedullin, forskolin and dibutyryl-cAMP can stimulate hyaluronic acid release in mesangial cells, and that it is dependent on P38 MAPK as well as wortmannin-sensitive kinase pathways. These findings indicate a novel pathway of hyaluronic acid release in rat mesangial cells by adrenomedullin and cAMP elevating agents.

2. Materials and methods

2.1. Materials

Adrenomedullin was purchased from Phoenix Pharmaceuticals (Belmont, CA), RPMI-1640, fetal bovine serum, penicillin and streptomycin were from Gibco (Grand Island, NY). SB203580 {[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole} was a kind gift from Dr. John Lee (SmithKline Beecham pharmaceuticals, King of Prussia, PA). H89 [{*N*-[2-((*p*-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, hydrochloride}], forskolin, and wortmannin {[1*S*-(1α, 6bα, 9aβ, 11α, 11bβ)]-11-(Acetyloxy)-1, 6b, 7, 8, 9a, 10, 11, 11b-octahydro-1-(methoxymethyl)-9a, 11b-dimethyl-3H-furo[4, 3, 2-de]indeno[4, 5-h]-2-benzopyran-3, 6, 9-trione} were from Calbiochem. Dibutyryl cyclic AMP was from Sigma. All other reagents were of the highest quality available.

2.2. Cell culture

Mesangial cell cultures were established from glomeruli obtained from the kidney cortex of 55 to 70 g male rats (Sprague–Dawley, Charles River, MA). Glomeruli were isolated by sequential sieving which removes tubules (300 to 150 μ sieves), then retains glomeruli on the 63 μ sieve. Isolated glomeruli were incubated for 10 min at 37°C in collagenase (750 U/ml), then plated in flasks in RPMI 1640 medium supplemented with 0.6 U/ml of insulin, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 15% fetal bovine serum. Cells were grown at 37 C in 5% carbon dioxide and the medium was changed twice a

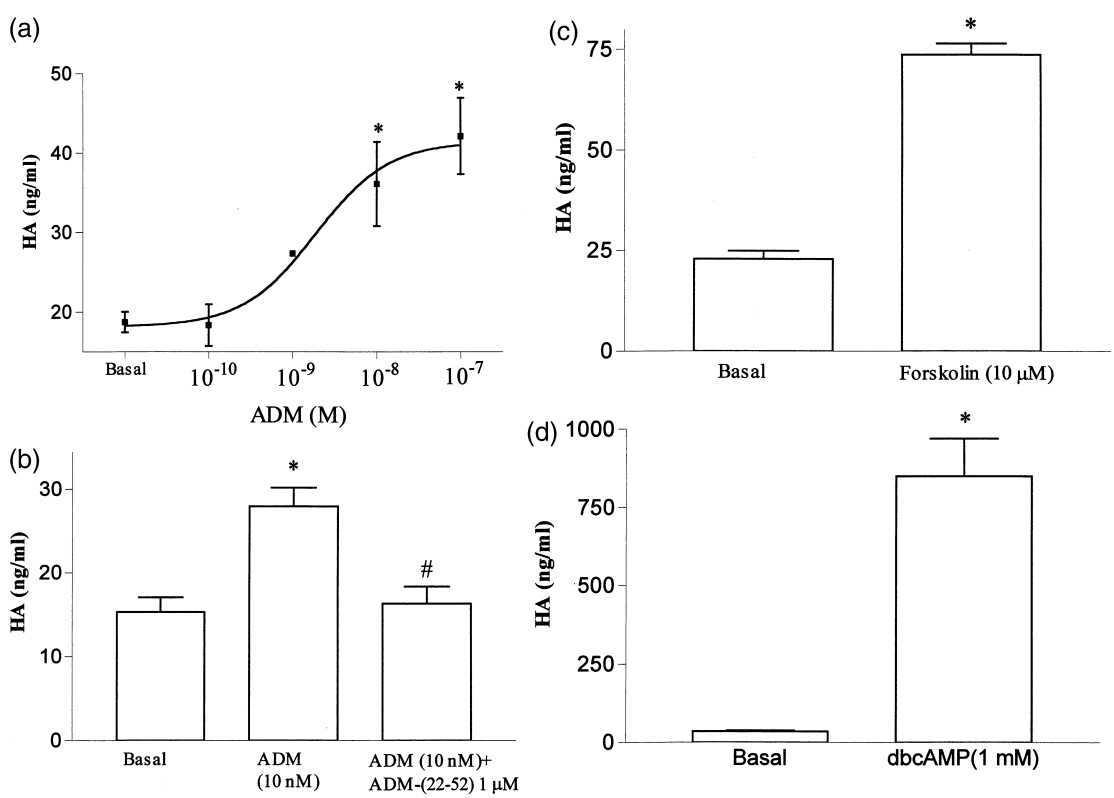

Fig. 1. Adrenomedullin (ADM) and cAMP activation of hyaluronate (HA) release from rat mesangial cells. (a) Adrenomedullin alone: Adrenomedullin caused a concentration-dependent increase in hyaluronate release. $^{*}P < 0.05$ compared basal. $n = 3$. (b) Adrenomedullin + adrenomedullin-(22-52): Adrenomedullin-(22-52), adrenomedullin receptor antagonist inhibited adrenomedullin-stimulated hyaluronate release. $n = 3$. $^{*}P < 0.05$ compared to basal, $\#P < 0.05$ compared to ADM. (c) Effect of forskolin (adenylate cyclase activator): Forskolin caused a significant increase in hyaluronate release. $^{*}P < 0.01$ compared to basal. (d) Effect of dibutyryl cAMP (cell permeable cAMP analog): Dibutyryl cAMP caused a significant increase in hyaluronate release. $^{*}P < 0.01$ compared to basal.

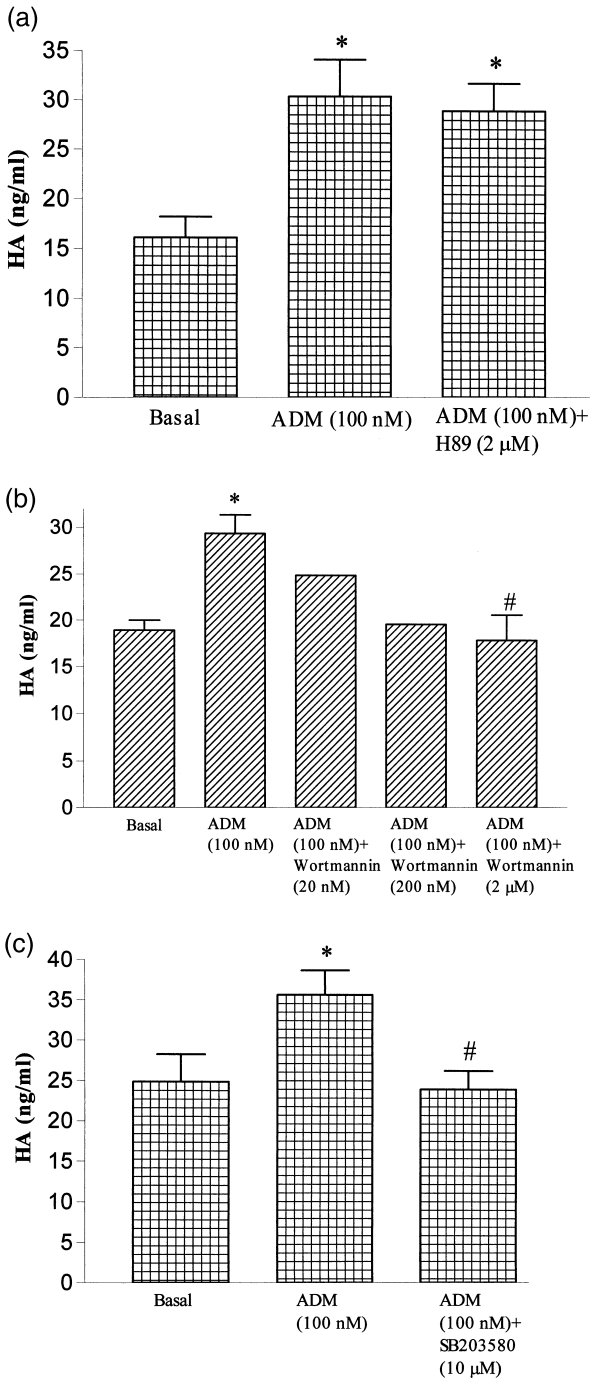

Fig. 2. Effect of adrenomedullin, H89 (protein kinase A inhibitor), wortmannin (phosphotidyl inositol 3-kinase inhibitor), SB203580 (P38 MAPK inhibitor) on hyaluronate release from rat mesangial cells. (a) Adrenomedullin + H89: H89 did not significantly affect adrenomedullin stimulated hyaluronate release. $n = 4$. $P = 0.7$ between ADM and ADM + H89. H89 by itself did not affect the basal hyaluronate release significantly (not shown). $^{*}P < 0.05$ compared to basal. (b) Adrenomedullin + wortmannin: Wortmannin significantly inhibited adrenomedullin-stimulated hyaluronate release. $^{*}P < 0.01$ compared to basal, $\#P < 0.05$ compared to ADM. $n = 3$. Wortmannin by itself did not affect basal hyaluronate release (not shown). (c) Adrenomedullin + SB203580: SB203580 significantly inhibited adrenomedullin-stimulated hyaluronate release. $^{*}P < 0.05$ compared to basal, $\#P < 0.05$ compared to ADM. $n = 4$. SB203580 by itself did not affect basal hyaluronate release (not shown).

week. At confluency, cells were subcultured by rinsing with Ca^{2+} and Mg^{2+} free phosphate buffered saline and then incubating with 0.05% trypsin supplemented with 20 mM EDTA (Albrightson et al., 1992). The cells were identified as mesangial cells by the following criteria: (1) a stellate morphology using phase contrast microscopy; (2) microfilaments and subplasmalemmal cytoplasmic densities using transmission electron microscopy; (3) insensitivity to puromycin aminonucleoside; and (4) positive immunofluorescence staining for actin and desmin but negative for keratin and factor VIII antigens. For the experiments, passages 15–30 were used and experiments were done in triplicates and repeated in different passages. All procedures were approved by the Institutional Animal Care and Use Committee and were in accordance with NIH Guidelines for the care and use of animals.

2.3. Radiometric assay for hyaluronic acid

Measurement of hyaluronic acid was done using a radiometric assay kit obtained from Pharmacia and Upjohn Diagnostics Division, (Kalamazoo, MI). The principle of the test is as follows.

The hyaluronic acid released in the cell culture media reacts with ^{125}I-HABP (Hyaluronic acid binding protein) in solution. The unbound ^{125}I-HABP is then quantitated by incubating with hyaluronic acid covalently coupled to sepharose particles of small size and low density. Separation is performed by centrifugation followed by decanting. The radioactivity bound to the particles is measured by a gamma counter and the counts are inversely proportional to the concentration of hyaluronic acid in the sample.

For our experiments, cells were plated in 24 well plates at 50,000 cells/well and serum starved overnight, after 48 h of plating. Cells were then treated with different agents

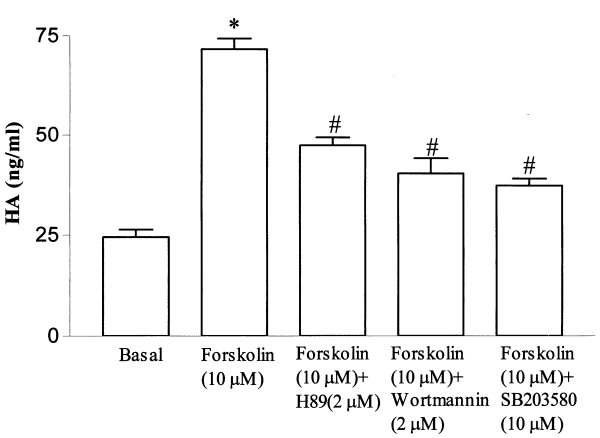

Fig. 3. Effect of forskolin (adenylate cyclase activator), H89 (protein kinase A inhibitor), wortmannin (phosphotidyl inositol 3-kinase inhibitor), and SB203580 (P38 MAPK inhibitor) on hyaluronate (HA) release from rat mesangial cells. All the inhibitors significantly inhibited forskolin-stimulated hyaluronic acid release from mesangial cells. $^{*}P < 0.01$ compared to basal, $\#P < 0.01$ compared to forskolin. $n = 3$.

in triplicates or quadruplicates for a period of 18 h and then the media were collected and assayed for hyaluronic acid released. Results are expressed as mean ± S.E. of hyaluronic acid in ng/ml of media.

2.4. Data analysis

Results are expressed as mean ± S.E. Analysis of variance (ANOVA) was used to compare 3 or more treatments and Student's *t*-test for 2 treatment comparisons. A *P* value of less than 0.05 was considered significant.

3. Results

Exposure of mesangial cells to adrenomedullin resulted in a concentration-dependent increase in hyaluronic acid release (Fig. 1a). Adrenomedullin-stimulated hyaluronic acid release was inhibited by adrenomedullin-(22-52), the adrenomedullin receptor antagonist (Fig. 1b). In addition to adrenomedullin, forskolin and db-cAMP also increased hyaluronic acid release significantly above basal levels (Fig. 1c,d).

Adrenomedullin-stimulated hyaluronic acid release was not significantly inhibited by H89 [{*N*-[2-((*p*-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, hydrochloride}], a potent protein kinase A inhibitor (Fig. 2a), whereas forskolin- and dbcAMP-mediated hyaluronic acid release was inhibited by H89 [{*N*-[2-((*p*-Bromocinnamyl)-amino)ethyl]-5-isoquinolinesulfonamide, hydrochloride}] (Figs. 3 and 4). Both wortmannin {[1*S*-(1α, 6bα, 9aβ, 11α, 11bβ)]-11-(Acetyloxy)-1, 6b, 7, 8, 9a, 10, 11, 11b-octahydro-1-(methoxymethyl)-9a, 11b-dimethyl-3*H*-furo[4, 3, 2-de]indeno[4, 5-h]-2-benzopyran-3, 6, 9-trione} (a phosphatidyl inositol-3 Kinase inhibitor) and SB203580 {[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole} (a P38-MAPK inhibitor) significantly blocked adrenomedullin-, forskolin-, and dbcAMP-stimulated hyaluronic acid release (Figs. 2–4).

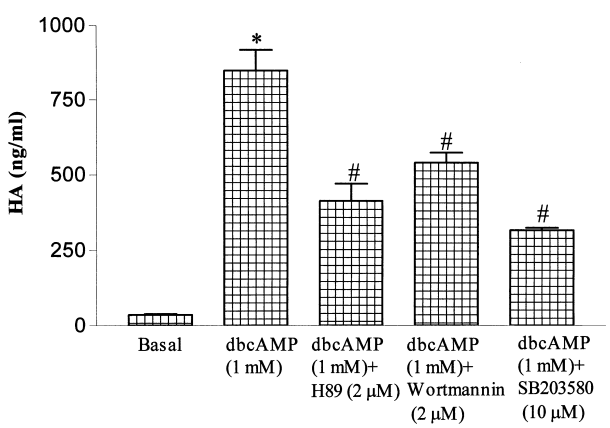

Fig. 4. Effect of dibutyryl cAMP (cell permeable cAMP analog), H89 (protein kinase A inhibitor), wortmannin (phosphotidyl inositol 3-kinase inhibitor), and SB203580 (P38 MAPK inhibitor) on hyaluronate (HA) release from rat mesangial cells. All the inhibitors significantly inhibited dbcAMP-stimulated hyaluronic acid release from mesangial cells. $^{*}P < 0.01$ compared to basal, $\#P < 0.01$ compared to dbcAMP. $n = 3$.

4. Discussion

The present study was initiated to evaluate the role of adrenomedullin and cAMP in mesangial cell matrix production, particularly that of hyaluronic acid. Because the glomerular extracellular matrix content plays a key role in the pathophysiology of glomerulonephritis, understanding the mechanisms of the extracellular matrix production is critical for therapeutic intervention. Moreover, understanding the regulation of hyaluronic acid release is important because of its possible role in mesangial cell proliferation in experimental diabetes (Mahadevan et al., 1996). The major aim of this study was to delineate the possible pathways especially, the role of MAPKs in adrenomedullin-, forskolin- and dbcAMP-induced hyaluronic acid release.

Mitogen-activated protein kinase (MAPK) pathway typically consists of a small G-protein like ras, activating a kinase cascade that ultimately leads to the activation of a MAPK. Three parallel MAPK pathways have been well characterized until now, namely the ERK, JNK and P38 MAPK pathways. In a previous study, we reported that adrenomedullin and forskolin produced a decrease in ERK and an increase in P38 activities, whereas, only adrenomedullin increased JNK activity. We also found that only adrenomedullin-modulated ERK and P38 MAPK activities could be inhibited by H89 [{*N*-[2-((*p*-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, hydrochloride}], a protein kinase A inhibitor. Forskolin, which stimulates cAMP through activation of adenylate cyclase did not stimulate JNK activity; also, H89 [{*N*-[2-((*p*-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, hydrochloride}] (protein kinase A inhibitor) did not consistently inhibit adrenomedullin-stimulated JNK activity indicating a cAMP-independent pathway regulating JNK activity (Parameswaran et al., 1999). In the present study, our results using forskolin and dbcAMP clearly show that an increase in cAMP levels can induce hyaluronic acid release in rat mesangial cells. Moreover, inhibition of forskolin- and dbcAMP-stimulated hyaluronic acid production by H89 [{*N*-[2-((*p*-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, hydrochloride}], a selective protein kinase A inhibitor, indicates that the elevation of cAMP levels and subsequent activation of protein kinase A can lead to an increase in hyaluronate production. Although adrenomedullin stimulates cAMP levels in rat mesangial cells and causes an increase in protein kinase A activation, H89 [{*N*-[2-((*p*-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, hydrochloride}], at the same

concentration that inhibited forskolin- and dbcAMP-stimulated hyaluronic acid release, did not have any effect on adrenomedullin-mediated increase in hyaluronic acid release. We have found that all the other responses (proliferation, apoptosis) mediated by adrenomedullin through cAMP can be completely inhibited by 2 μM H89 [{*N*-[2-((*p*-Bromocinnamyl)amino)ethyl]-5- isoquinolinesulfonamide, hydrochloride}] (Parameswaran et al., 1999). In a recent study in rat vascular smooth muscle cells, adrenomedullin was found to elevate MAPK activity and increase proliferation through a cAMP-independent mechanism, although cAMP is the only second messenger so far identified in vascular smooth muscle cells (Iwasaki et al., 1998). Our results indicate that the mechanism of adrenomedullin-stimulated hyaluronate release is likely to be predominantly dependent on a cAMP-independent pathway; for example, the pathway stimulated by βγ-subunit of the G protein or the JNK pathway. Since adrenomedullin receptor is G-protein coupled, activation of adrenomedullin receptor will lead to the release of αs and βγ-subunit of the Gs protein. Recent studies have shown that the release of βγ-subunit can lead to a variety of downstream effects like the activation of different MAPK pathways, such as ERK, JNK and P38 in different cell systems (Gutkind, 1998).

Stimulation by dbcAMP gives a dramatic effect on hyaluronate release but the other stimuli gives less than 10% of this stimulation. Although this might indicate that the stimulation by adrenomedullin compared to dbcAMP may not be that important physiologically, it should also be borne in mind that the physiological concentration of adrenomedullin in the immediate vicinity of mesangial cells is not known. Also, we do not know if a twofold increase in hyaluronate caused by adrenomedullin is pathophysiologically important. Moreover, it is hard to make a comparison between adrenomedullin and dbcAMP because of the fact that adrenomedullin-stimulated hyaluronate is not cAMP-dependent.

In rat mesangial cells activation of cAMP causes a decrease in proliferation and an increase in apoptosis (Li et al., 1995; Muhl et al., 1996). Because an increase in apoptosis may lead to an increase in detached cells in the media, the assay that we have used in the present study to quantitate hyaluronate released could have been influenced by an increase in dead cells. But in our preparation of mesangial cells, although adrenomedullin does induce apoptosis, the number of detached cells in the media between control and treatment is not significantly different with in the time frame tested. We do not know the exact amount of hyaluronate released from dead cells, but since we have found that there is no difference in the dead cells between control and treatment in the media, we are assuming that the hyaluronate from dead cells between treatments to be constant.

There appears to be a cell type specific regulation of hyaluronic acid release. In fibroblast cultures phorbol myritoyl acetate (PMA), platelet-derived growth factor-BB (PDGF-BB) and transforming growth factor-β1 (TGF-β1) stimulated hyaluronic acid production whereas forskolin did not have any effect on hyaluronic acid release (Suzuki et al., 1995). But in rabbit pericardial cells, prostaglandin E_2, forskolin and dbcAMP all stimulated hyaluronic acid release and hyaluronic acid synthase activity and also the prostaglandin E_2-mediated effects were shown to be mediated through the activation of protein kinase A (Honda et al., 1993).

SB203580 {[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole}, a selective inhibitor of P38 MAPK, has been shown to be powerful tool for evaluating the role of P38 MAPK in a number of systems (Lee et al., 1994). For example, P38 MAPK has been shown to play a key role in apoptosis and cardiac hypertrophy (Wang et al., 1998). Our results indicate that the adrenomedullin- and cAMP-stimulated hyaluronic acid release in mesangial cells are sensitive to SB203580 {[4-4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1--imidazole}. The concentration of SB203580 {[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole} we have used inhibits only P38 MAPK and does not inhibit JNK or ERK (Parameswaran et al., 1999, Manuscript submitted for publication, European Journal of pharmacology). To our knowledge this is the first demonstration on the role of P38 MAPK on hyaluronic acid release in rat mesangial cells.

In a previous study, we found that adrenomedullin-stimulated P38 MAPK activity can be inhibited by wortmannin {[1*S*-(1α, 6bα, 9aβ, 11α, 11bβ)]-11-(Acetyloxy)-1, 6b, 7, 8, 9a, 10, 11, 11b-octahydro-1-(methoxymethyl)-9a, 11b-dimethyl-3H-furo[4, 3, 2-de]indeno[4, 5-h]-2-benzopyran-3, 6, 9-trione}, a selective inhibitor of phosphatidyl inositol 3-kinase (Parameswaran et al., 1999; manuscript submitted for publication, European Journal of Pharmacology). It was a surprising finding that wortmannin {[1*S*-(1α, 6bα, 9aβ, 11α, 11bβ)]-11-(Acetyloxy)-1, 6b, 7, 8, 9a, 10, 11, 11b-octahydro-1-(methoxymethyl)-9a, 11b-dimethyl-3H-furo[4, 3, 2-de]indeno[4, 5-h]-2-benzopyran-3, 6, 9-trione}not only inhibits adrenomedullin-stimulated hyaluronic acid production, but also that of forskolin and dbcAMP. It remains to be determined if phosphatidyl inositol 3-kinase activity can be stimulated by protein kinase A either directly or indirectly in rat mesangial cells. It is tempting to hypothesise that the stimulation of a wortmannin-sensitive kinase in response to adrenomedullin, forskolin and dbcAMP causes downstream activation of P38 MAPK, which then leads to an increase in hyaluronic acid release. In fibroblast cultures, growth factor-stimulated hyaluronic acid release was not affected by wortmannin {[1*S*-(1α, 6bα, 9aβ, 11α, 11bβ)]-11-(Acetyloxy)-1, 6b, 7, 8, 9a, 10, 11, 11b-octahydro-1-(methoxymethyl)-9a, 11b-dimethyl-3*H*-furo[4, 3, 2-de]indeno[4, 5-h]-2-benzopyran-3, 6, 9-trione} (Heldin et al., 1992). This is again probably because of the differences

in cell system and hence cell-specific regulation of hyaluronate production.

It should also be noted that none of the reagents completely inhibited forskolin- or dbcAMP-stimulated hyaluronate release. The data likely indicates multiple signaling pathways that are activated by cAMP and forskolin, although further experiments (i.e., combination of blockers) will be necessary for a definitive answer. The focus of the present study was on adrenomedullin and the principal point is that although the only second messenger thus far identified for adrenomedullin in mesangial cells is cAMP and although cAMP does increase hyaluronate levels, adrenomedullin-stimulated hyaluronate release is not cAMP-dependent.

In glomerular cores, Dunlop et al. (1996) have found that fibronectin and PDGF can increase hyaluronic acid release from both diabetic and non-diabetic models. They also showed that both were protein kinase C-dependent mechanisms. While fibronectin-stimulated hyaluronic acid release was dependent on prostaglandin production, that of PDGF was not. Also in fibroblast cultures, stimulation of hyaluronic acid release by PDGF and TGFβ1 were dependent on protein kinase C pathway. Whether all the above findings were MAPK pathway-dependent or not, is not known.

In summary, adrenomedullin, forskolin and dbcAMP can stimulate hyaluronic acid release in mesangial cells, and that, the stimulation elicited by these three agents is dependent on P38 MAPK pathway and a wortmannin-sensitive kinase. These results have significant implications with regard to our understanding of the pathophysiology of mesangial cell proliferation and matrix production. Further studies are necessary to identify the role of JNK or G protein βγ subunit in adrenomedullin-mediated hyaluronic acid production. These can be performed when specific inhibitors are available.

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