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European Journal of Pharmacology 372 (1999) 85–95

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Regulation of glomerular mesangial cell proliferation in culture by adrenomedullin

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Received 24 September 1998; received in revised form 1 December 1998; accepted 12 January 1999

Abstract

Adrenomedullin is a recently discovered vasodilatory peptide that has been shown to be a potent activator of adenylate cyclase in a variety of cell systems, including rat mesangial cells. The major aim of the present study was to determine the regulation of rat mesangial cell proliferation (using [^{3}H]thymidine incorporation as an index), apoptosis (using nucleosome-associated cytoplasmic DNA fragmentation as an index) and mitogen-activated protein kinase (MAPK) cascade, specifically extracellular signal-regulated kinase (ERK), *jun*-amino terminal kinase (JNK) and P38 mitogen-activated protein kinase (P38 MAPK) activities, by adrenomedullin-stimulated cyclicAMP-protein kinase-A pathway. Adrenomedullin increased cAMP levels significantly above basal and the response was inhibited by the adrenomedullin receptor antagonist, adrenomedullin-(22–52). Adrenomedullin also decreased [^{3}H]thymidine incorporation and increased nucleosome-associated cytoplasmic DNA fragmentation, in a concentration-dependent fashion. Both these responses were receptor mediated as, adrenomedullin-(22–52) inhibited these effects. The decrease in proliferation and increase in apoptosis were both mimicked by forskolin, a direct adenylate cyclase activator. Adrenomedullin-mediated decrease in proliferation and increase in apoptosis were inhibited by H89 [{*N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, hydrochloride}], a potent protein kinase-A inhibitor. Associated with the changes in proliferation and apoptosis, adrenomedullin decreased ERK2 activity, and increased JNK1 and P38 MAPK activities. All these kinase activities, except the increase in JNK1 activity could be simulated using forskolin. In addition, only adrenomedullin-mediated changes in ERK2 and P38 MAPK activities were inhibited by H89 while, adrenomedullin-stimulated JNK1 was not consistently inhibited by the protein kinase-A inhibitor. These results suggest that adrenomedullin might play an important role in mesangial cell turnover and that although adrenomedullin-mediated responses are primarily cAMP-dependent, it does not preclude the involvement of cAMP-independent pathways.

Keywords: Proliferation; Apoptosis; ERK (extracellular signal-regulated kinase); JNK (*jun*-amino terminal kinase); P38 MAPK (P38 mitogen-activated protein kinase); Adrenomedullin

1. Introduction

Adrenomedullin, a derivative of proadrenomedullin, is a potent vasodilator and natriuretic factor. Discovered in 1993, it is thought to belong to the calcitonin gene related peptide (CGRP) superfamily (Kitamura et al., 1993; Sakata et al., 1993). Since its initial discovery, a number of reports have appeared describing the actions of adrenomedullin, both in animal and cell culture models (Ebara et al., 1994; Gardiner et al., 1995; Haynes and Cooper, 1995; Jougasaki et al., 1995). Adrenomedullin has been shown to decrease proliferation or thymidine incorporation in mesangial cells (Chini et al., 1995; Segawa et al., 1996). In most systems including mesangial cells, adrenomedullin activates adenylate cyclase, with a subsequent increase in cyclicAMP accumulation and protein kinase-A activation (Chini et al., 1995; Kohno et al., 1995; Osajima et al., 1996). In endothelial cells, in addition to stimulation of cAMP, an increase in intracellular calcium release and phosphatidyl-inositol hydrolysis in response to adrenomedullin was reported (Shimekake et al., 1995). The mechanisms of adrenomedullin-mediated responses, however, have not been completely elucidated.

The major aim of the present study was to evaluate the effect of adrenomedullin on mesangial cell proliferation (using [^{3}H]thymidine incorporation as an index), apoptosis (using nucleosome-associated DNA fragmentation as an

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0014-2999/99/$ - see front matter
PII: S0014-2999(99)00022-9

index) and mitogen-activated protein kinase (MAPK) pathways, specifically that of extracellular signal-regulated kinase (ERK), *jun*-amino terminal kinase (JNK) and P38 mitogen-activated protein kinase (P38 MAPK) activities. In addition, to determine the regulation of proliferation, apoptosis and MAPKs by adrenomedullin-stimulated protein kinase-A pathway, we have used a potent protein kinase-A inhibitor, H89 [{*N*-[2-((*p*-bromocinnamyl)-amino)ethyl]-5-isoquinolinesulfonamide, hydrochloride}]. We have also compared adrenomedullin-mediated responses to that of forskolin, another cAMP elevating agent and a direct activator of adenylate cyclase. Our data indicate the presence of both cAMP-protein kinase-A-dependent and -independent pathways in rat mesangial cells, modulated by adrenomedullin.

2. Materials and methods

2.1. Materials

Adrenomedullin and adrenomedullin-(22–52) were purchased from Phoenix Pharmaceuticals (Belmont, CA), myelin basic protein (MBP), from Sigma (St. Louis). Polyclonal anti-ERK2, anti-P38 MAPK, and anti-JNK1 anti-bodies were purchased from Santa Cruz Laboratories (Santa Cruz, CA). Glutathione-*S*-transferase conjugated cJUN (GST-cJUN) was purchased from Alexis Biochemicals (San Deigo, CA). RPMI-1640, fetal bovine serum, penicillin and streptomycin were from Gibco (Grand Island, NY). All other reagents were of high quality available.

2.2. Cell culture

Rat mesangial cells were obtained from the glomeruli of kidney cortex isolated from Sprague–Dawley rats as described before (Albrightson et al., 1992), and were grown in RPMI-1640 with 15% fetal bovine serum. Passages between 15 and 30 were used for the experiments.

2.3. Cyclic nucleotides

cAMP measurements were performed as described before, with slight modifications (Haneda et al., 1996). Cells were plated in 24 well plates (50,000 cells/well) and grown for 2 days and serum starved overnight. Cells were preincubated with 0.5 mM isobutyl methyl xanthine (IBMX) for 10 min and then agonist solutions (prepared in phosphate buffered saline containing 0.2% bovine serum albumin, 0.2% magnesium chloride and 0.1% glucose) were added to the wells and incubated for an additional 5 min at 37°C. Reactions were stopped by adding 50 μl of 100% tri-chloro acetic acid. The cells in tri-chloro acetic acid were collected in separate tubes and centrifuged. The supernatants were collected after brief centrifugation and ether extracted 3 times with water-saturated ether. After overnight evaporation of the ether, a portion of the sample was used for measurement of cAMP levels, using a radioimmunoassay (RIA) kit from PerSeptive Biosystems (Framingham, MA). Each experiment was done in triplicates and repeated at least 3–5 times.

2.4. [^{3}H]thymidine incorporation

Cells were plated in 24 well plates (30,000 cells/well) and grown for 2 days, and then serum starved for 48 h. Cells were then treated with the compounds for a period of 16 h and pulsed with [^{3}H]thymidine (1 μCi/ml) for 4 h. The radioactivity was counted in Beckman LS counter, after washing the cells and stopping the reaction with 5% tri-chloro acetic acid and solubilising the cells in 0.5 ml of 0.25 N sodium hydroxide. Each experiment was done in quadruplicates and was repeated at least 3 times.

2.5. Kinase assays

Cells were plated in p100 plates and were serum starved overnight on reaching confluency. The agonist solutions were prepared in the growth media without serum. Cells were treated with the agonists for indicated time points. The cell lysates were prepared as described (Bogoyevitch et al., 1995; Li et al., 1995). In the meantime, specific anti-bodies (10 μg/reaction) were incubated with protein A agarose (Gibco) for 30 min at room temperature. After normalizing for protein concentration, the cell lysates were incubated with the specific anti-body agarose conjugate for 2 h at 4°C with constant shaking. The kinase assays were done after washing the immunoprecipitate 3 times with HNTG (20 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) buffer and 2 times with kinase buffer (50 mM Tris–HCl, 100 mM NaCl, 10 mM MnCl2 and 0.1 mM sodium ortho vanadate). The functional assay was done in the presence of 50 μM ATP, 5 μCi 32P-ATP, 10 μg of specific substrate (MBP for ERK2 and P38 MAPK, and GST-cJUN for JNK1), and the immunoprecipitate. The reactions were performed at 30°C for 15 min and then stopped with sodium dodecyl sulfate (SDS) buffer. The samples were electrophoresed in 12% polyacrylamide gel with proper molecular weight standards. The gels were dried and subjected to autoradiography or phosphoimager plates. The intensity of the bands in the autoradiogram was visualized using an ARCUS high-resolution optical scanner and quantitated using NIH image software or quantitated using imagequant program (for the gels exposed to phosphoimager plates). Results are expressed as percent change from the basal of the relative densitometric units or phosphoimager units.

2.6. Western blots

Western blot analysis was done as described before (Guo et al., 1998). Briefly, equal concentration of protein

samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) along with molecular weight standards and transferred to nitrocellulose membranes. The membranes were then blocked with 5% non-fat dry milk in tris-buffered saline containing 0.05% Tween-20 and incubated with primary anti-bodies followed by horse radish peroxidase-conjugated secondary anti-bodies according to manufacturer's instructions. The blots were then visualized by an enhanced chemiluminescence (ECL) kit obtained from Pierce.

2.7. ELISA for apoptosis

The ELISA kit was obtained from Boehringer Mannheim (Indianapolis, IN). The principle of the kit is based on the fact that on induction of apoptosis cells undergo DNA fragmentation, leading to oligonucleosomal DNA fragments in the cytoplasm of the cells. For the experiments, the cells were plated in 96 well plates for overnight, after which they were labeled with bromodeoxyuridine for 8 h and then treated with different agonists. At the end of the incubation period, the cells were lysed, centrifuged to separate the cytoplasmic DNA fragments from the nuclear DNA and then an ELISA was done to quantitate the cytoplasmic fragmented DNA, and the absorbance was read at 490 nm using a plate reader (Biorad, model550). Each experiment was done in quadruplicates and the experiment was repeated at least 3 times. The experiments were also repeated using a different ELISA kit (Boehringer Mannheim), which specifically detects the cytoplasmic nucleosome-associated DNA fragments. For that, cells were plated in 48 well plates and after 24 h were serum starved overnight. Different agonists (prepared in the media) were incubated for another 20 h. The cells were lysed with the lysis buffer and centrifuged to separate cytoplasmic and nuclear fractions. The cytoplasmic fraction was then tested for DNA still attached to nucleosomes using the ELISA protocol from Boehringer Mannheim. The assay was done in triplicates or quadruplicates and repeated at least 3–5 times. The results that are presented here are from the second ELISA method, which detects the nucleosome-associated DNA fragments in the cytoplasm. Similar results were also obtained with the first ELISA method.

2.8. Caspase activity assays

Cultured rat mesangial cells were treated with vehicle or the test compound for 18 h. Cell lysates were prepared as described (Yue et al., 1998). Briefly, the cells were washed with ice-cold phosphate buffered saline without calcium and magnesium, harvested and suspended in buffer containing 25 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 2 mM DDT, 5 mM EDTA, 1 mM PMSF, and 1 μM pepstatin A and allowed to swell for 20 min on ice. The suspension was forced through a 25 gauge needle to break the cells. The homogenate was centrifuged at 4°C for 1 h in a Beckman Airfuge at 90,000 rpm. The cleared lysates were stored at −70°C until used for assays.

2.9. Enzyme assays

The assay buffer and the substrates for caspase-3-like activity were used for the peptide-pNA hydrolysis assays as described before (Yue et al., 1998). The composition of the assay buffer was as follows: HEPES (pH 7.5), CHAPS (0.1%), DTT (1 mM), 50 mM KCl. Ac-DEVD-pNA was used as substrate. Cell extracts containing 20–30 μg of protein were diluted into the assay buffer and preincubated for 10 min for 30°C prior to the addition of the substrate. Levels of released pNA were measured with a plate reader colorimetrically at 405 nm for 30 min. The specificity of the assay was confirmed by using a specific caspase-3 inhibitor (DEVD-FMK).

2.10. Data analysis

Results are expressed as mean ± S.E. Analysis of variance (ANOVA) was used to compare three or more treat-

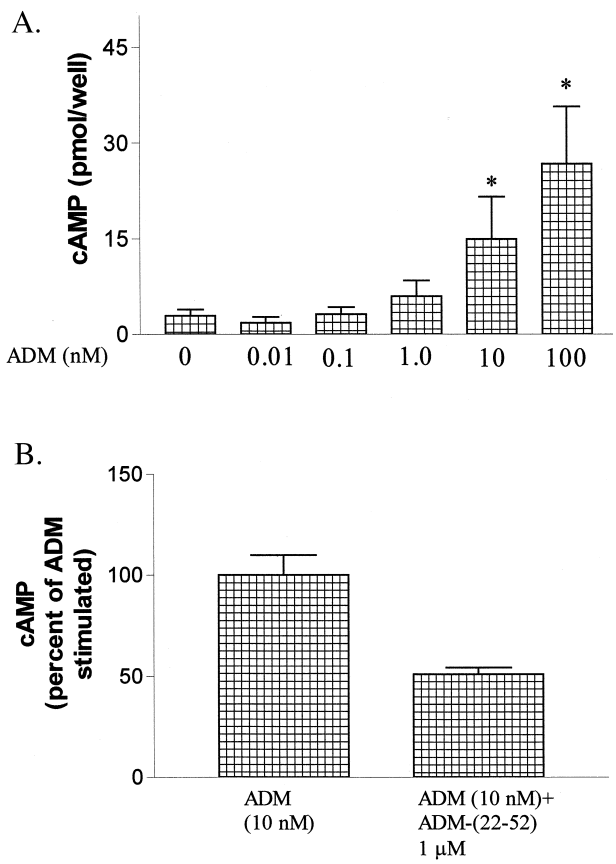

Fig. 1. (a) Effect of adrenomedullin (ADM) on cAMP levels in rat mesangial cells. Each experiment was done in triplicates and repeated at least 5 times. ANOVA P-value < 0.05 ($^*P < 0.05$ compared to basal). (b) Effect of adrenomedullin-(22–52), the adrenomedullin receptor antagonist on adrenomedullin-induced cAMP levels in rat mesangial cells ($n = 3$).

ments and Student's *t*-test for two treatment comparisons. A *P*-value of less than 0.05 was considered significant.

3. Results

3.1. cAMP

Adrenomedullin increased cAMP levels significantly above basal (Fig. 1a) and the adrenomedullin receptor antagonist, adrenomedullin-(22–52) inhibited adrenomedullin-stimulated cAMP response (Fig. 1b). Forskolin, a direct activator of adenylate cyclase also increased cAMP levels (2666 ± 578% change from basal).

3.2. [^{3}H]thymidine incorporation

Adrenomedullin decreased [^{3}H]thymidine incorporation in a concentration-dependent manner (Fig. 2a). Adrenomedullin-(22–52) almost completely reversed the inhibition (Fig. 2b), indicating the decrease in proliferation is mediated through adrenomedullin receptor. In addition, forskolin also decreased [^{3}H]thymidine incorporation in mesangial cells significantly below basal levels (Fig. 2c). Furthermore, H89, a potent protein kinase-A inhibitor completely reversed the adrenomedullin-mediated proliferation response at a concentration as low as 20 nM (Fig. 2d). At both 20 and 200 nM H89 by itself did not cause any statistically significant change in proliferation of rat mesangial cells. But at 2 μM, it caused approximately a 2-fold increase in mesangial cell proliferation.

3.3. Nucleosome-associated DNA fragmentation

Associated with a decrease in [^{3}H]thymidine incorporation, adrenomedullin also caused an increase in cytoplasmic nucleosome-associated DNA fragmentation (an index of apoptosis), in a concentration-dependent manner

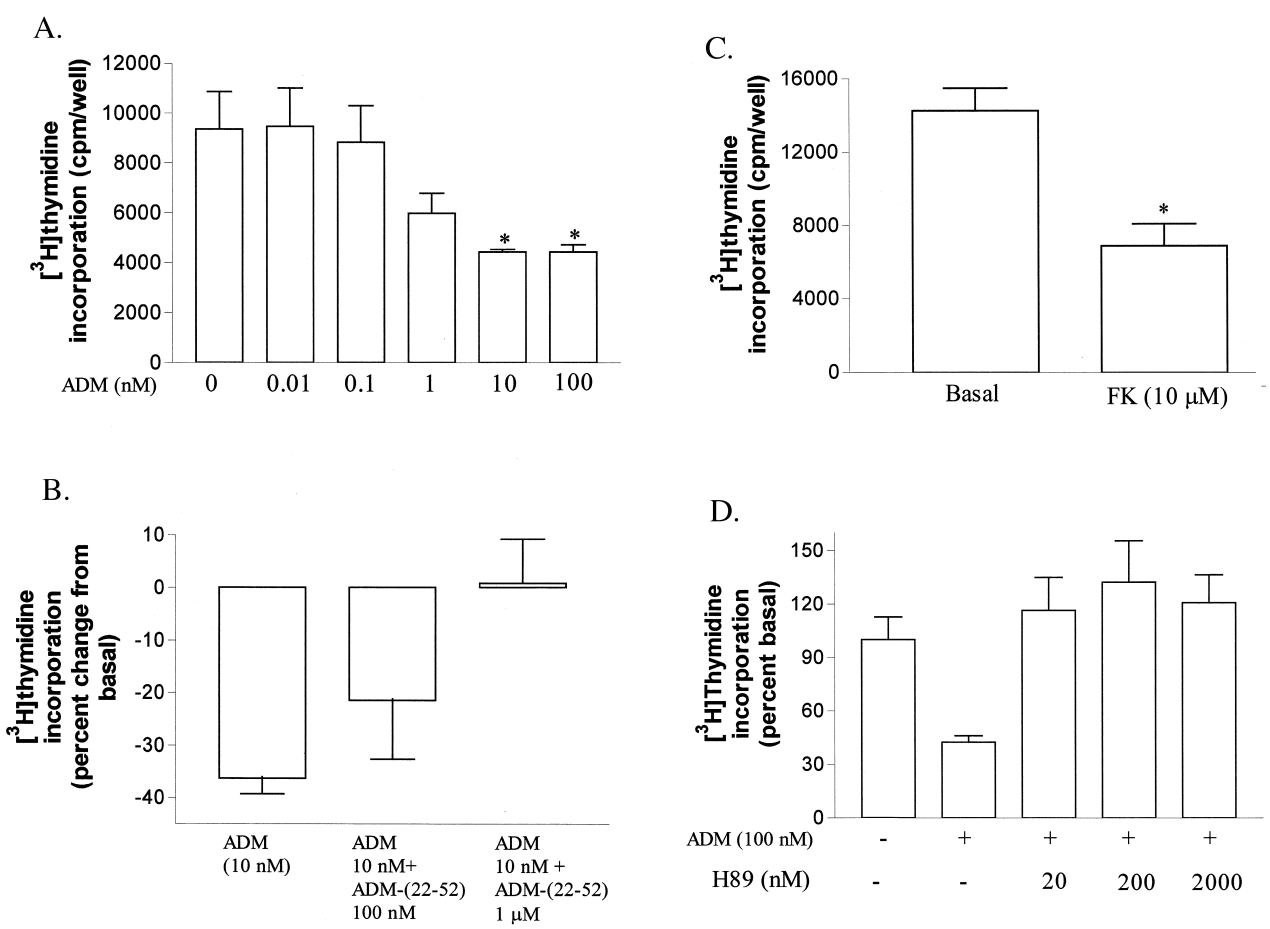

Fig. 2. (a) Effect of adrenomedullin (ADM) on [^{3}H]thymidine incorporation in rat mesangial cells. Each experiment was done in quadruplicates and repeated 4 times. ANOVA *P*-value < 0.05 (**P*-value < 0.05 compared to basal). (b) Effect of adrenomedullin receptor antagonist, adrenomedullin-(22–52) on adrenomedullin-mediated response on [^{3}H]thymidine incorporation in rat mesangial cells. Results are expressed as percent change from basal ($n = 3$). (c) Effect of Forskolin (FK) on [^{3}H]thymidine incorporation in rat mesangial cells. $n = 3$ ($P < 0.01$). (d) Effect of H89, protein kinase-A inhibitor on adrenomedullin (ADM)-mediated response on [^{3}H]thymidine incorporation in rat mesangial cells. Results are expressed as percent basal. $n = 3$ ($P < 0.05$). H89 by itself stimulated proliferation of mesangial cells significantly only at 2 μM.

(Fig. 3a) and the response was inhibited significantly by adrenomedullin-(22–52), the adrenomedullin receptor antagonist (Fig. 3b). Furthermore, forskolin also increased mesangial cell apoptosis (Fig. 3d). In addition, H89, a

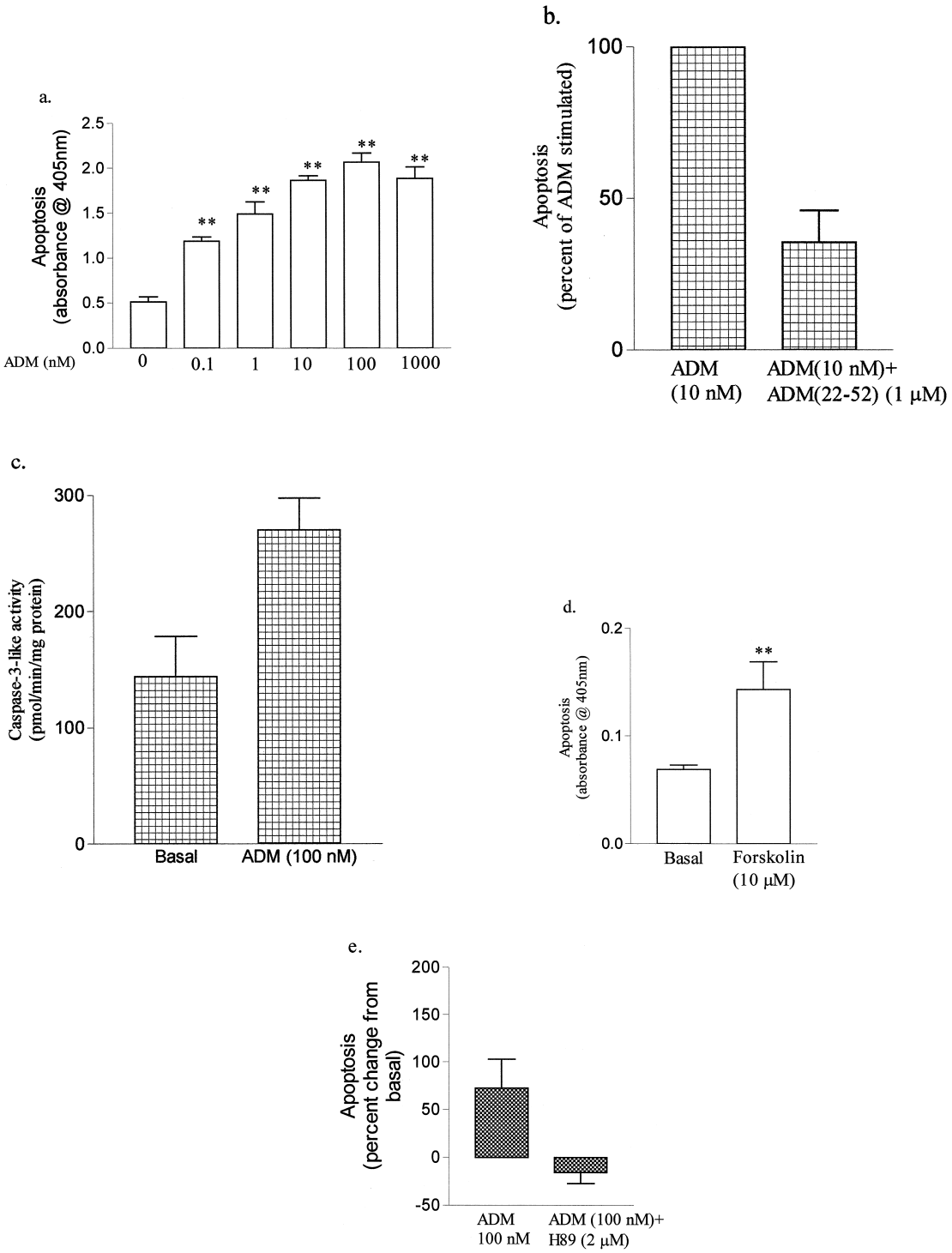

Fig. 3. (a) Effect of adrenomedullin (ADM) on nucleosome-associated DNA fragmentation (apoptosis), in rat mesangial cells. Each experiment was done in triplicates and repeated at least 3 times. ANOVA P-value < 0.01 ($^*P < 0.01$ compared to basal). (b) Effect of adrenomedullin receptor antagonist adrenomedullin-(22–52) on adrenomedullin-induced apoptosis in rat mesangial cells. $n = 3$. (c) Effect of adrenomedullin on caspase-3-like activity in rat mesangial cells ($P < 0.01$), $n = 3$. (d) Effect of forskolin (FK) on nucleosome-associated DNA fragmentation (apoptosis) in rat mesangial cells. $n = 3$ ($^*P < 0.01$). (e) Effect of inhibition of protein kinase-A, by H89, on adrenomedullin-induced nucleosome-associated DNA fragmentation (apoptosis) in rat mesangial cells. Analysis of raw values for stimulation of apoptosis by adrenomedullin indicated a significant increase in apoptosis ($P < 0.05$). Pretreatment with H89 completely blocked adrenomedullin-induced apoptosis. H89 by itself did not affect the basal DNA fragmentation ($n = 3$).

potent protein kinase-A inhibitor significantly inhibited adrenomedullin-stimulated apoptosis (Fig. 3e), although at that concentration of H89, there was no significant change in the basal DNA fragmentation/apoptosis.

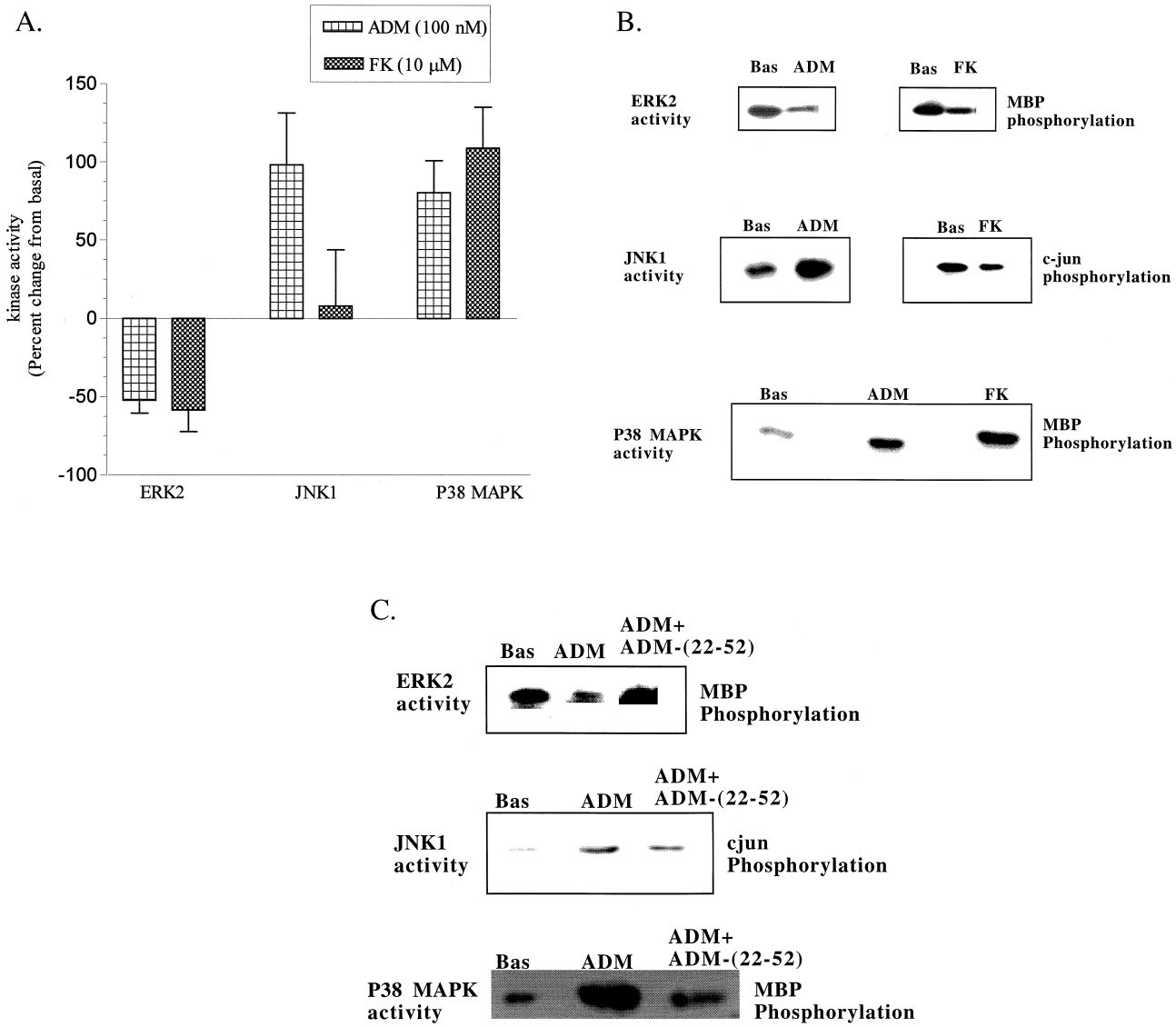

Fig. 4. (a) Effect of adrenomedullin (ADM) and forskolin (FK) treatment (for 30 min) on ERK2, JNK1, and P38 MAPK activities in rat mesangial cells ($n = 3–6$). (b) Representative autoradiograms showing the effect of adrenomedullin (ADM) and forskolin (FK) on ERK2, JNK1, and P38 MAPK activities. ERK2 activity was determined by specific immuno-complex assay with MBP as the substrate as described in Section 2. The degree of phosphorylation of MBP by the immunoprecipitated ERK2 indicates the activity of the enzyme. JNK1 activity was determined by specific immuno-complex assay with c-*jun* as the substrate. The degree of phosphorylation of c-*jun* by the immunoprecipitated JNK1 indicates the activity of the enzyme. P38 MAPK activity was determined by specific immuno-complex assay with MBP as the substrate. The degree of phosphorylation of MBP by the immunoprecipitated P38 MAPK indicates the activity of the enzyme. (c) Representative autoradiograms showing the effect of adrenomedullin receptor antagonist on ERK2, JNK1, and P38 MAPK activities modulated by adrenomedullin in rat mesangial cells. (d) Time course of modulation of ERK2 activity in rat mesangial cells by adrenomedullin. The top panel indicates the MBP phosphorylation by immunoprecipitated ERK2 and the bottom panel is the immunoblot showing ERK2, at the corresponding time points. There was no significant change in the expression of ERK2 between basal and treatments at different time points. The experiment was repeated at most of the time points at least two more times with similar results. (e) Time course of modulation of JNK1 activity in rat mesangial cells by adrenomedullin. The top panel indicates the c-*jun* phosphorylation by immunoprecipitated JNK1 and the bottom panel is the immunoblot showing JNK1, at the corresponding time points. There was no significant change in the expression of JNK1 between basal and treatments at different time points. The experiment was repeated at most of the time points at least two more times with similar results. (f) Time course of modulation of P38 MAPK activity in rat mesangial cells by adrenomedullin. The top panel indicates the MBP phosphorylation by immunoprecipitated P38 MAPK and the bottom panel is the immunoblot showing P38 MAPK, at the corresponding time points. There was no significant change in the expression of P38 MAPK between basal and treatments at different time points. The experiment was repeated at most of the time points at least two more times with similar results.

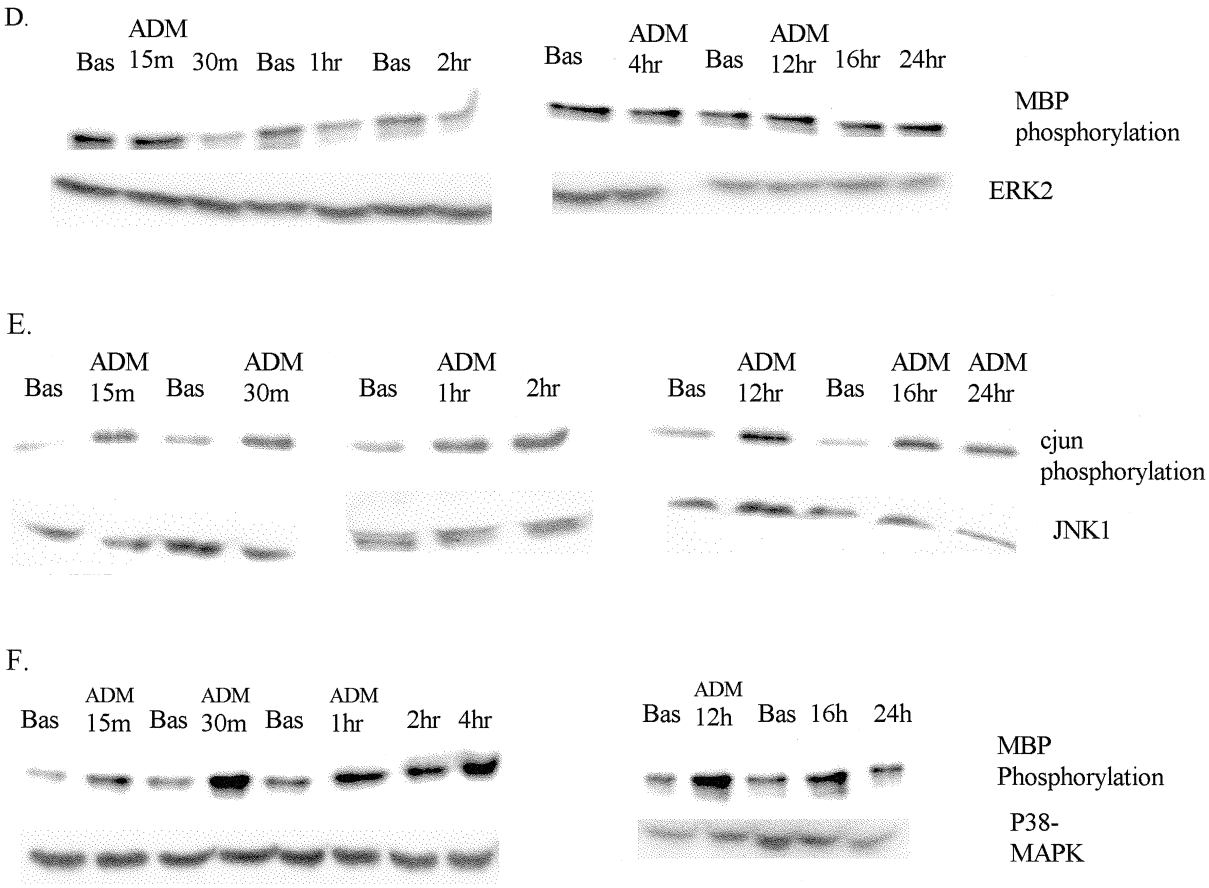

Fig. 4 (continued).

3.4. Caspase-3-like activity

To confirm the induction of apoptosis biochemically, we examined the effect of adrenomedullin on caspase-3-like (CPP 32 or apopain) activity in rat mesangial cells. Eighteen hour treatment with ADM caused a significant induction of caspase-3 activity confirming biochemically, the induction of apoptosis by ADM in rat mesangial cells (Fig. 3c).

3.5. MAPK activities

3.5.1. ERK

Associated with a decrease in proliferation and an increase in apoptosis, adrenomedullin and forskolin also caused a decrease in ERK2 activity (Fig. 4). ERK2 activity modulated by adrenomedullin was inhibited by adrenomedullin receptor antagonist (Fig. 4c). Adrenomedullin-mediated inhibition of ERK2 activity lasted only 4 h, without any change in the protein expression levels, indicating a possible dephosphorylation mechanism (Fig. 4d). Also, H89 a protein kinase-A inhibitor completely reversed the ERK inhibition caused by adrenomedullin (Fig. 5). By itself H89 did not affect the basal ERK activity.

3.5.2. JNK

Adrenomedullin increased JNK1 activity significantly above basal levels (Fig. 4). But forskolin, although a cAMP activator like adrenomedullin, did not cause any consistent change in JNK1 activity. JNK1 activity modulated by adrenomedullin was inhibited by adrenomedullin receptor antagonist (Fig. 4c). The effect of adrenomedullin on JNK1 activity remained for up to 16 h of treatment without any change in JNK1 expression levels, indicating a possible phosphorylation mechanism (Fig. 4e). Furthermore, H89 did not have any consistently significant effect on adrenomedullin-stimulated JNK1 activity (Fig. 5), indicating a possible protein kinase-A-independent activation. By itself H89 did not affect the basal JNK activity.

3.5.3. P38 MAPK

Adrenomedullin and forskolin significantly increased P38 activity (Fig. 4). P38 MAPK activity modulated by adrenomedullin was inhibited by adrenomedullin receptor antagonist (Fig. 4c). Adrenomedullin-stimulated P38 activ-

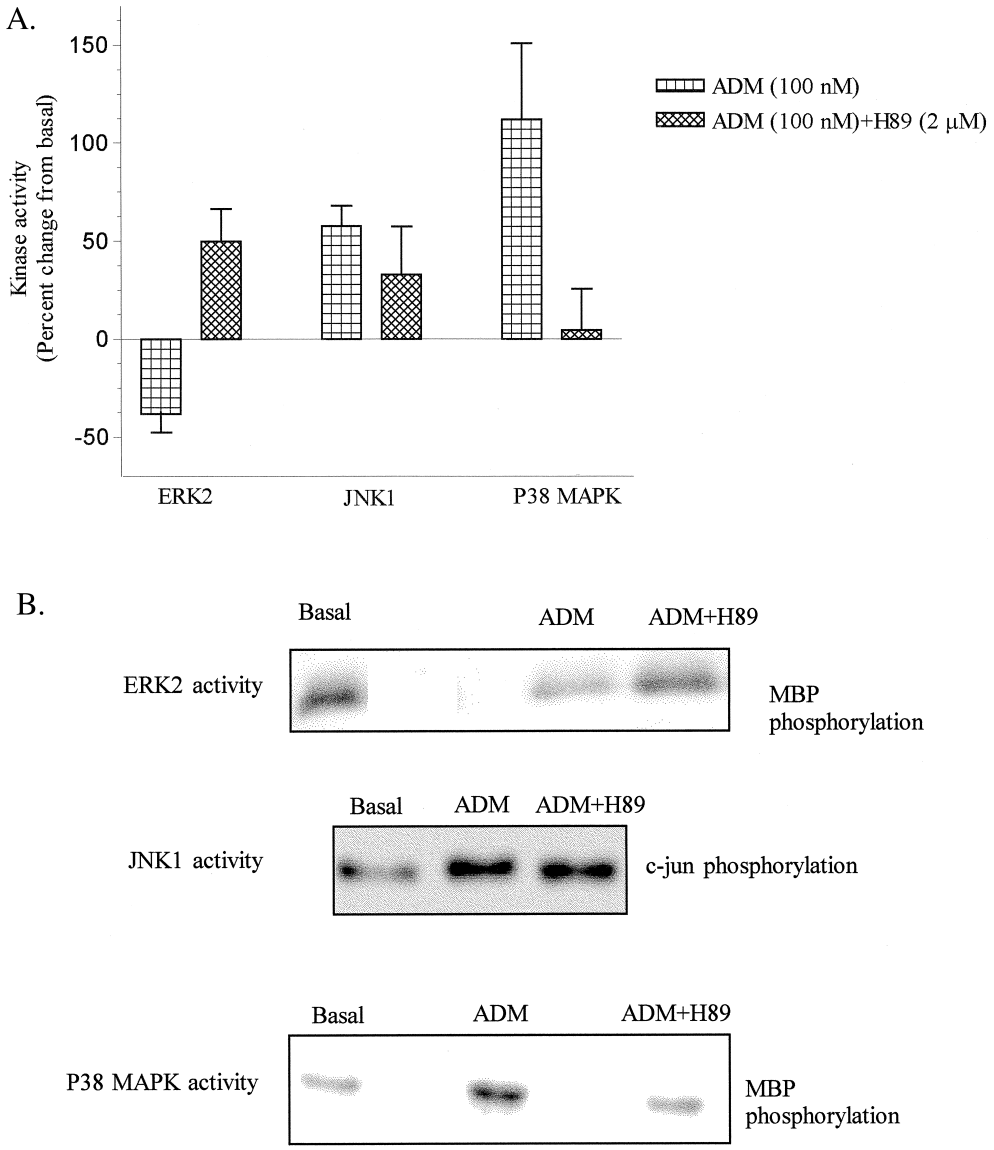

Fig. 5. (a) Effect of H89, on ERK2, JNK1 and P38 MAPK activities modulated by adrenomedullin treatment (for 30 min) in rat mesangial cells. H89 did not affect the basal kinase activities significantly ($n = 4$). (b) Representative autoradiograms showing the effect of adrenomedullin and H89 on ERK2, JNK1, and P38 MAPK activities.

ity also remained elevated for up to 16 h without any change in protein expression levels (Fig. 4f). In addition, H89 almost completely inhibited adrenomedullin-stimulated P38 MAPK activity (Fig. 5).

4. Discussion

An increase in cAMP resulting in the activation of protein kinase-A is followed by a multitude of changes in the signaling systems in a cell, such as changes in the MAPK pathways, leading to biological responses such as proliferation, apoptosis and matrix production. These responses have important implications in the initiation and progression of diseases like glomerulonephritis. A common finding in proliferative glomerulonephritis is the aberrant proliferation of mesangial cells. Several hormones, growth factors and inflammatory cytokines have been shown to modulate mesangial cell turnover in culture, indicating a possible role in nephropathies associated with aberrant proliferation (Klahr et al., 1988; Brenner and Stein, 1989; El Nahas et al., 1997).

Several studies have shown that adrenomedullin causes an increase in cAMP in mesangial cells, resulting in the activation of protein kinase-A (Chini et al., 1995; Chini et al., 1997). An increase in cAMP causes changes in proliferation depending on the cell type (Dumont et al., 1989; Withers et al., 1996). In mesangial cells, cAMP causes a decrease in proliferation (Floege et al., 1993). Our results using a protein kinase-A inhibitor indicate that adreno-

medullin causes a decrease in proliferation of rat mesangial cells through activation of protein kinase-A.

In addition to an aberrant proliferation, an altered apoptotic machinery may be an important mechanism in the progression of proliferative diseases. Although in some cases, resolution of renal disease or proliferative glomerulonephritis is seen, the mechanisms involved are not understood (Baker et al., 1994; Sugiyama et al., 1996). Mesangial cell apoptosis has recently been proposed to be one of the important mechanisms of resolution of hypercellularity in at least some of the experimental models of proliferative mesangiopathies (Baker et al., 1994). Accordingly, it is thought that alterations of the apoptotic machinery in the glomerulus which lead to the survival of excess mesangial cells, lead to further complications of the disease. We report here for the first time that adrenomedullin causes an increase in nucleosome-associated DNA fragmentation, an index of apoptosis (Martin et al., 1994; Kroemer et al., 1995). The increase in apoptosis is receptor-mediated since the adrenomedullin receptor antagonist inhibits it. Our findings confirm a recent report by Muhl et al. (1996) who showed that elevation of cAMP levels in mesangial cells was associated with an increase in apoptosis. We have also demonstrated that inhibition of protein kinase-A by H89 can inhibit adrenomedullin-induced apoptosis. These results are in contrast to the effect of adrenomedullin on endothelial cells, where it protects the cells from apoptosis through a cAMP-independent mechanism (Kato et al., 1997).

Recently, numerous reports have suggested a role for MAPK pathways, specifically of ERK, JNK, and P38 MAPK pathways in the regulation of proliferation and apoptosis (Denhardt, 1996; Neary, 1997; Robinson and Cobb, 1997). To understand the mechanism of adrenomedullin-induced apoptosis and adrenomedullin-mediated decrease in proliferation, we measured specific kinase activities of ERK2, JNK1, and P38 MAPK in response to adrenomedullin and forskolin. While both these agents caused a decrease in ERK2 activity and an increase in P38 MAPK, only adrenomedullin caused an increase in JNK1 activity. Previous reports have demonstrated that the activity of a MBP phosphorylating kinase decreases in response to adrenomedullin in the total cell lysate (Chini et al., 1995; Haneda et al., 1996; Chini et al., 1997). Our study shows for the first time that adrenomedullin causes specifically an increase in P38 MAPK and JNK1 and a decrease in ERK2 activities. Xia et al. (1995) recently showed that in PC12 cells, nerve growth factor withdrawal induces apoptosis through a mechanism that involves all of these three kinases; specifically, a decrease in ERK2 and a simultaneous increase in P38 MAPK and JNK1 was obligatory for the induction of apoptosis. It remains to be determined if the same prerequisite is necessary for adrenomedullin-induced apoptosis in mesangial cells. Forskolin-induced apoptosis, however, does not require JNK activity because it increases only P38 MAPK (in addition to a decrease in ERK2). The same could be true for adrenomedullin-induced apoptosis because inhibition of protein kinase-A with H89, inhibits adrenomedullin-stimulated apoptosis, although it does not consistently affect adrenomedullin-induced JNK activity. We have also found recently that SB203580, a P38 inhibitor completely inhibits both adrenomedullin-stimulated apoptosis and adrenomedullin-inhibited proliferation (manuscript submitted for publication, Eur. J. Pharmacol., 1998), suggesting there might be redundant pathways stimulated by adrenomedullin, that may or may not result in the same biological response. The role of adrenomedullin-stimulated JNK in mesangial cell function remains to be elucidated.

Based on our results using H89, adrenomedullin-mediated decrease in ERK2 and increase in P38MAPK activities are possibly mediated through protein kinase-A pathway, while JNK1 activity is probably protein kinase-A-independent. Several laboratories studying G-protein coupled receptors in the recent years have shown that the $\beta\gamma$ subunit of the G-protein, can activate several effectors including ERK, P38 MAPK, and JNK through a signaling cascade (Yamauchi et al., 1997; Gutkind, 1998; Lopez-Ilasaca et al., 1998). In view of the fact that the only second messenger system for adrenomedullin so far identified in mesangial cells is cAMP and that the adrenomedullin receptor is G-protein coupled, we postulate that the increase in JNK activity by adrenomedullin may be through $\beta\gamma$ subunit although experiments with specific inhibitor of $\beta\gamma$ subunit would be necessary to prove that.

It is quite surprising that even though forskolin elevated cAMP levels higher than adrenomedullin (about 3-fold), most of the other responses of forskolin are comparable to adrenomedullin or only slightly higher than that of adrenomedullin. In fact, adrenomedullin-stimulated apoptotic response is even slightly higher than that of forskolin. Lower concentrations of forskolin only gave lesser effects on proliferation and apoptosis (data not shown), suggesting that adrenomedullin might also act through cAMP-independent pathways. Moreover, forskolin and adrenomedullin cause differential caspase-substrate peptide hydrolysis (manuscript in preparation) again suggesting that although adrenomedullin stimulates cAMP-protein kinase-A pathway like forskolin, they also activate other pathways. Furthermore, in our studies on hyaluronic acid production in mesangial cells, forskolin-stimulated hyaluronic acid production is much higher than adrenomedullin and also, H89 does not inhibit adrenomedullin-stimulated hyaluronic acid secretion, while it inhibits forskolin-induced hyaluronic acid production (manuscript submitted for publication, Eur. J. Pharmacol., 1998).

Adrenomedullin, through its anti-proliferative and apoptotic effect on mesangial cells, may play a major role in the normal turnover of mesangial cells. It is known that the plasma levels of adrenomedullin are increased in hypertension and renal failure (Ishimitsu et al., 1994; Cheung and Leung, 1997). Tissue specific expression of adreno-

medullin under these pathophysiological conditions might provide insight into the role, adrenomedullin plays in the turnover of mesangial cells in such abnormal conditions.

In summary, adrenomedullin decreases proliferation and increases apoptosis possibly through a protein kinase-A pathway. In addition adrenomedullin decreases ERK and increases JNK and P38 MAPK activities in rat mesangial cells. Only adrenomedullin-modulated ERK and P38 are sensitive to H89, a protein kinase-A inhibitor, while JNK activity is not. These results suggest that while adrenomedullin-stimulated protein kinase-A pathway is critical for most responses, it does not preclude the involvement of adrenomedullin-stimulated protein kinase-A-independent pathways.

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