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Human urokinase receptor expression is inhibited by amiloride and induced by tumor necrosis factor and phorbol ester in colon cancer cells

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Abstract The modulation of urokinase plasminogen activator receptor (uPAR) gene expression by tumor necrosis factor alpha (TNFα), phorbol ester (PMA) and amiloride was studied in three colon cancer cell lines. uPAR mRNA and protein were induced by TNFα and by PMA but were inhibited by amiloride at concentrations of 0.1 to 1 mM in the presence or absence of TNFα and PMA. Nuclear run-on transcription assay indicated that the effects of amiloride and TNFα were mediated at least in part at the transcriptional level, whereas PMA may act in part via a post-transcriptional mechanism. These results suggested that uPAR gene expression is modulated by multiple signal transduction pathways.

Key words: Urokinase receptor; Tumor necrosis factor; Amiloride

1. Introduction

Activation of receptor-bound prourokinase (pro-uPA) on the cell surface appears to play an important role in some biological processes including cancer cell invasion and metastasis, monocyte migration, angiogenesis, wound healing and trophoblast implantation [1]. The urokinase plasminogen activator receptor (uPAR) specifically recognizes high molecular weight pro-uPA and active uPA by their epidermal growth factor (EGF)-like terminal domains. Receptor-bound uPA catalyzes the formation of plasmin on the cell surface to generate the proteolytic cascade that contributes to the breakdown of basement membranes and extracellular matrix [2]. uPAR is a 55–60 kDa glycoprotein that attaches to plasma membranes by glycosyl-phosphatidyl-inositol (GPI) linkage [3]. The 1.4 kb uPAR gene has been cloned [4] and mapped to chromosome 19q13.2 [5,6]. uPAR is expressed in human colon adenocarcinoma cells at the invasive front [7] and in tumor-associated macrophages in human breast carcinoma [8]. Blockade of uPAR on human PC3 prostate carcinoma cells by inactive uPA markedly inhibited metastatic activity in a nude mouse model [9]. There is increasing evidence that uPAR expression on the surface of adenocarcinoma cells is central to the invasion process and that receptor-bound uPA is the preferred site for mediating proteolysis. The regulation of the uPAR gene therefore may be a pivotal point in the control of the malignant behavior of a colon cancer cell.

The uPAR protein is inducible in monocytes by the tumor promoter, phorbol myristate acetate (PMA) [10] and by the cytokines, interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNFα) [11]. uPAR biosynthesis, the uPAR mRNA level and gene transcription are also increased by transforming growth factor beta (TGFβ) and dexamethasone in A549 lung carcinoma cells [12], but reports of transcriptional regulation of uPAR mRNA expression by TNFα in any cell type are lacking. The question of negative regulation of uPAR gene expression also remains unexplored.

Amiloride is a specific and an effective inhibitor of sodium channels in epithelial cell transport systems [13]. It inhibits tumor growth [14] and metastasis [15], and may represent a prototype for potential anticancer drugs because it also interferes with the regulation of intracellular pH [16]. Although amiloride competitively inhibits the catalytic activity of uPA [17], little is known concerning the potential role of amiloride in the regulation of uPA receptor mRNA and its protein. In this paper the positive modulation of uPAR mRNA and protein by TNFα, PMA and cycloheximide and the negative regulatory effects by amiloride on uPAR gene expression in human colon cancer cells are reported.

2. Materials and methods

2.1. Materials

Human urokinase (Actisolv, high M_r) was a gift from Dr. E. Schuler, Behringwerke, Marburg, Germany and recombinant human TNFα (activity: 1 unit ~0.0455 ng) was provided by the Asahi Chemical Company, Tokyo, Japan. PMA, CHX and amiloride were from Sigma Chem. Co., St. Louis, USA.

2.2. Cell culture

The human colon cancer cell lines, HCT116 [18], KM12SM [19] and LM1215 [20], have been described previously. The HCT116 cells were obtained from the American Type Tissue Collection. The KM12SM cells were a gift from Dr. I.J. Fidler, M.D. Anderson Cancer Center, University of Texas, Houston, USA and the LIM1215 cells were provided by Dr. R.T. Whitehead, Ludwig Institute for Cancer Research, Melbourne, Australia. All cell lines stained negative for *Mycoplasma* contamination using Hoechst stain 33258. Cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS). Before stimulation, cells were washed three times with PBS and incubated overnight or for 4 h in RPMI serum-free media. Stimulants were added to cells at a density of about 1×10^6 cells per ml. Immediately before harvest, cell viability was consistently found to be > 90%.

2.3. RNA preparation and Northern blot analysis

RNA was purified from stimulated or unstimulated cells using the guanidinium isothiocyanate method [21]. RNA samples were electrophoresed on 1% formaldehyde-containing agarose gels, transferred to a nitrocellulose membrane and further processed. Hybridization was carried out in 50% (vol/vol) formamide/6 × SSC/5 × Denhardt's solution/2% Sarcosyl/200 μg/ml salmon sperm DNA. The membranes were exposed to Kodak XAR film at −70°C using intensifying screens and

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Abbreviations: Am, amiloride; CHX, cycloheximide; TNF, tumor necrosis factor; PMA, phorbol 12-myristate 13-acetate; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor.

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multiple film exposure times were used to ensure linearity of band intensities. The intensities of mRNA bands in the autoradiographs were scanned and quantitated by a video densitometer (Model 620, Bio-Rad). mRNA intensities are calculated relative to the intensity of the 18 S rRNA internal control. The RNA molecular markers used were purchased from Promega corporation.

2.4. cDNA probes

The 1.144 kb human uPAR cDNA [4] used was a gift from Dr. E.K.O. Kruithof, Lausanne, Switzerland. Human 18S ribosomal DNA (18S rDNA) [22] was a gift from Dr. B.E.H. Maden, University of Liverpool, UK. Human β-actin cDNA [23] was a gift from Dr. H.D. Campbell, The Australian National University. Human ubiquitin cDNA [24] was a gift from Dr. R.T. Baker, The Australian National University. cDNA probes were labelled with [α-^{32}P]dCTP by the random priming method [21].

2.5. Nuclear run-on transcription assay

Nuclei were isolated from HCT116 cells treated with TNFα, PMA or amiloride, according to the method previously described [25,26]. In brief, about 5×10^7 cells were washed in buffer (10 mM Tris, pH 7.5/2 mM $MgCl_2$/3 mM $CaCl_2$/3 mM dithiothreitol/0.3 M sucrose). Nuclei were then pelleted after lysis of the cells in the same buffer containing 0.1% Triton X-100. For the transcription elongation reaction, freshly prepared nuclei were incubated at 30°C for 30 min with [α-^{32}P]UTP, and run-on analysis performed (26). In a given experiment, each filter was hybridized to DNA immobilized on nitrocellulose with the same amount of ^{32}P-labelled RNA. The filters were then exposed at −70°C to Kodak XAR film for 7–14 days using intensifying screens. The intensities of mRNA bands in the autoradiographs were scanned and quantitated by a video densitometer (Model 620, Bio-Rad). mRNA intensities are calculated relative to the intensity of the β-actin internal control.

2.6. uPAR protein assay

Cells were rinsed and maintained overnight in serum-free media in the presence or absence of stimulants. The cells were then rinsed with acidic glycine buffer (50 mM glycine-HCl, pH 3.0, 0.1 M NaCl), washed twice with binding buffer (RPMI containing 0.1% BSA and 20 mM HEPES pH 7.4), and incubated with a range of concentrations of [^{125}I]uPA in binding buffer for 45 min at room temperature [27]. To determine non-specific binding, 50-fold higher concentrations of unlabelled uPA were added to the incubate. Unbound uPA was removed and the radioactivity of the cell lysates measured by gamma counter. Scatchard analysis was used to calculate the dissociation constant value and the average number of the uPAR on the cell surface [28]. The statistical significance of the differences between uPAR protein assays was analyzed by a Student's *t*-test.

3. Results

3.1. Induction of uPAR mRNA accumulation by TNFα, PMA and cycloheximide (CHX)

To determine whether uPAR mRNA is induced by TNFα, PMA or CHX in colon cancer cells, RNA was isolated from cells treated with stimili and analyzed by Northern blotting. Unstimulated HCT116 cells expressed very low levels of 1.4 kb uPAR transcripts. After TNFα stimulation however, there were marked increases in the levels of uPAR mRNA which appeared at about 1 h, peaked at 2 h and declined at 6 h (Fig. 1A). uPAR mRNA was increased in a dose-dependent manner at TNFα concentrations of 2.2–2,200 units/ml (Fig. 1B). After PMA stimulation, uPAR mRNA was increased at 2 h and high levels were sustained for 24 h in HCT116 cells (data not shown). Similar TNFα or PMA effects on uPAR mRNA were found in the two other colon cancer lines, KM12SM and LIM1215 (Fig. 3).

To investigate whether induction of uPAR gene expression by TNFα or PMA was dependent on de novo protein synthesis,

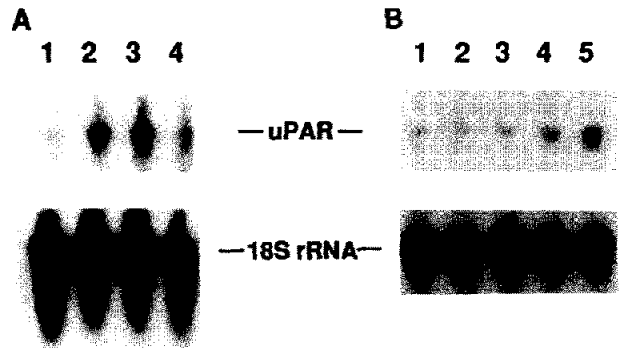

Fig. 1. Time- and dose-dependent induction of uPAR mRNA accumulation by TNFα in HCT116 cells. The same Northern blot was hybridized to ^{32}P-labelled uPAR cDNA and 18S rDNA probes as indicated. (A) Time course of uPAR mRNA accumulation induced by TNFα (2,000 units/ml). Autoradiographic exposure times were 12h (for uPAR as probe) and 8h (for 18S rDNA as probe). Lane 1, untreated; lanes 2–4, TNFα for 1, 2 and 6 h. (B) Dose-dependent induction of uPAR mRNA accumulation in cells induced by TNFα for 4h at the concentrations of 0, 2.2, 22, 220 and 2,200 units/ml (lanes 1, 2, 3, 4 and 5, respectively). Autoradiographic exposure times were 48h (for uPAR as probe) and 18 h (for 18S rDNA as probe). Each experiment was repeated at least two times and representative data shown in the figures.

CHX was used to inhibit protein synthesis. Cells were preincubated with CHX (20 μg/ml) for 30 min to ensure inhibition of protein synthesis at an early time point and then TNFα or PMA was added for 4 h. Northern blot analysis showed that in HCT116 cells, CHX alone induced uPA mRNA suggesting that the uPAR gene is regulated by a labile repressor protein (Fig. 2B, lane 4) [29]. uPAR mRNA was also induced by CHX in HCT116 cells stimulated by TNFα or PMA (data not shown), suggesting that stimulation of uPAR does not need de novo protein synthesis. As expected, synthesis of uPAR protein was totally inhibited by CHX addition (data not shown). No significant difference was observed in 18S rRNA, which served as a control.

3.2. Inhibition of uPAR mRNA by amiloride

As amiloride competitively inhibits the catalytic activity of uPA it was of interest to determine whether it also affects the synthesis of the uPA receptor. When concentrations of amiloride (0.001–1 mM) known to inhibit the Na^+/Ca^{2+} exchanger, the Na^+,K^+-ATPase and Na^+-coupled solute transport [30] were added in the presence of PMA to HCT116 cells, there was a dose-dependent inhibition of 55% and 80% of the uPAR mRNA level at concentrations of 0.1 mM and 1 mM, respectively, as determined by scanning densitometry (Fig. 2A, lanes 5,6). Inhibition was detected whether amiloride was added simultaneously with PMA (Fig. 2A, lanes 5,6), 2 h after PMA (lane 7) or 1 h before PMA (lane 8). Reductions of 41% of uPAR mRNA levels occurred in HCT116 cells treated with amiloride alone at a concentration of 1 mM (Fig. 2B, lane 3) but no inhibition of uPAR mRNA was detected at 0.1 mM (Fig. 2B, lane 2). Amiloride (1 mM) also markedly inhibited the uPAR mRNA expression induced by CHX (Fig. 2B, lanes 4,5) and TNFα (Fig. 2B, lanes 6,7). Similar effects of amiloride inhibition were found in the other colon cancer cell lines, KM12SM and LIM1215 (Fig. 3). Levels of 18S rRNA in these cell lines were not significantly affected by exposure to amiloride.

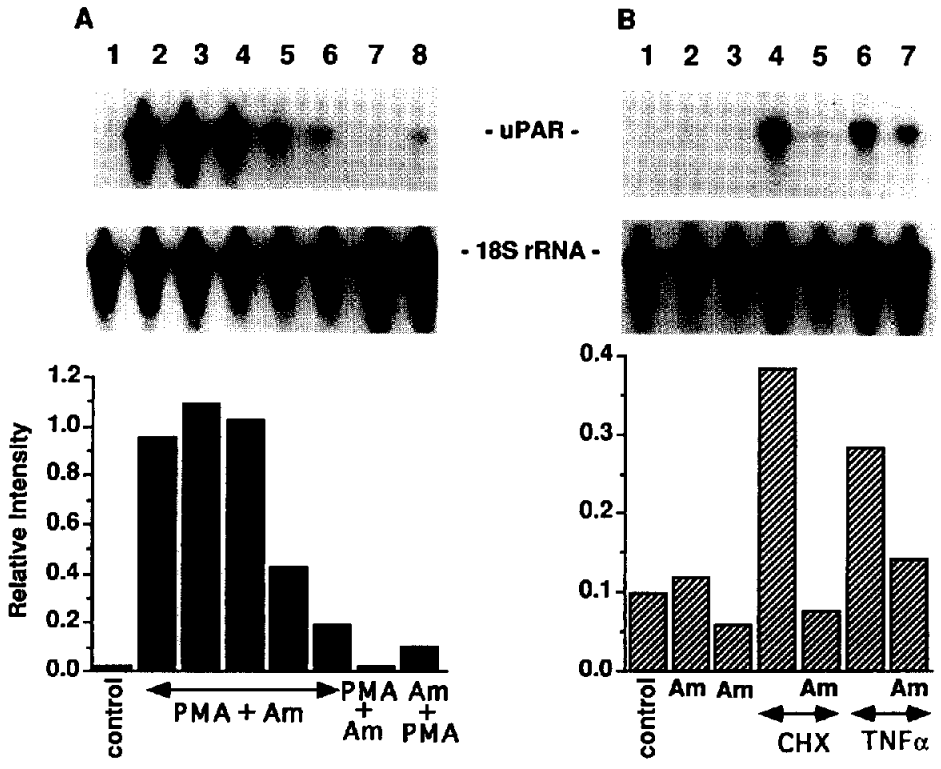

Fig. 2. Effect of amiloride on uPAR mRNA accumulation in HCT116 cells. Cells were treated for 4 h with PMA (50 ng/ml), CHX (20 μg/ml) or TNFα (220 units/ml) in the presence or absence of amiloride. The same Northern blot was hybridized to ^{32}P-labelled uPAR cDNA and 18S rDNA probes as indicated. (A) Dose-dependent inhibition of uPAR mRNA accumulation by amiloride. Autoradiographic exposure times were 24 h (for uPAR cDNA as probe) and 6 h (for 18S rDNA as probe). Lane 1, untreated; lanes 2–6, PMA plus amiloride at 0, 0.001, 0.01, 0.1, and 1 mM, respectively; lane 7, PMA-treated for 2 h then amiloride (1mM) added; lane 8, amiloride (1 mM)-treated for 1h then PMA added for 3 h. (B) Effect of amiloride on uPAR mRNA accumulation. Autoradiographic exposure times were 91 h (for uPAR cDNA as probe) and 5 h (for 18S rDNA as probe). Lane 1, untreated; lane 2, amiloride (0.1 mM); lane 3, amiloride (1 mM); lane 4, CHX; lane 5, CHX plus amiloride (1 mM); lane 6, TNFα; lane 7, TNFα plus amiloride (1 mM). The corresponding densitometry results are shown underneath. Each experiment was repeated at least three times and representative data shown in the figures.

3.3. *Effects of PMA, TNFα or amiloride on uPAR transcription in isolated nuclei*

Incorporation of label via elongation of nascent uPAR transcripts in isolated nuclei was used as a measure of the level of uPAR gene transcription in HCT116 cells. The radiolabelled transcripts were then hybridized with uPAR cDNA bound to nitrocellulose in order to detect uPAR mRNA. The results of these nuclear run-on transcription assays are shown in Fig. 4. uPAR transcripts were expressed in nuclei from unstimulated HCT116 cells but were markedly reduced in the cells that had been treated with amiloride (Fig. 4A). uPAR mRNA, however, was increased in the cells exposed to TNFα (Fig. 4B), but not PMA (Fig. 4C), as determined by scanning densitometry. These assays indicate that the effect of amiloride and TNFα, but not PMA, is mediated, at least in part, at the level of transcription.

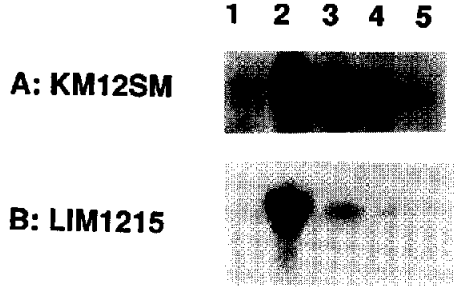

Fig. 3. Effect of amiloride (1 mM) on uPAR mRNA accumulation in KM12SM (A) and LIM1215 (B) cells. Cells were treated for 4 h with PMA (50 ng/ml) or TNFα (220 units/ml) in the presence or absence of amiloride (1 mM). Autoradiographic exposure times were 72 h (A) and 70 h (B). Lane 1, untreated; lane 2, PMA; lane 3, PMA plus amiloride; lane 4, TNFα; lane 5, TNFα plus amiloride. Each experiment was repeated at least three times and representative data shown in the figures.

3.4. *Effects of PMA, TNFα and amiloride on cell surface uPAR protein levels*

As shown in Fig. 5, the level of uPAR protein on the surface of unstimulated (control) HCT116 cells was 4.2 ± 0.13 ng per 10^6 cells (row 1). After stimulation by PMA, cell surface uPAR protein levels increased about 1.6-fold (row 3) while TNFα treatment induced less levels of uPAR protein production (row 5). After treatment with amiloride alone, a 52% reduction of uPAR protein levels was observed (rows 1 and 2). Amiloride also markedly decreased the levels of PMA-induced (rows 3 and 4) or TNFα-induced uPAR protein (rows 5 and 6). These effects were statistically significant ($P < 0.05$). Similar effects of PMA, TNFα and amiloride on cell surface uPAR protein levels were found in the other colon cancer cell lines, KM12SM and LIM1215 (data not shown). uPAR protein levels on the cell surface, therefore reflected PMA and TNFα induction and

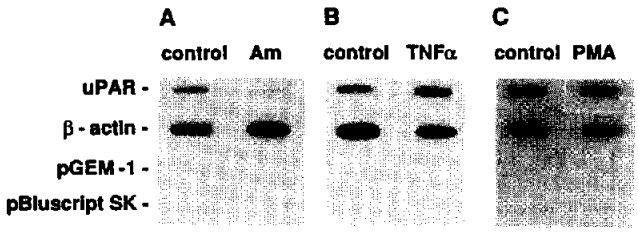

Fig. 4. Nuclear run-on transcription analysis of effects of TNFα, PMA and amiloride on uPAR gene transcription in HCT116 cells. Nuclei were isolated from HCT116 cells treated with amiloride (1 mM/ml) (A), TNFα (2,000 units/ml) (B), PMA (30 ng/ml) (C). Following in vitro transcription, RNA was purified from the nuclei and hybridized with uPAR cDNA, β-actin cDNA or vector DNA (pGEM-1 and pBluscript SK) immobilized on nitrocellulose filters. Hybridized ^{32}P-labeled nuclear RNA was detected by autoradiography. Autoradiographic exposure time was 6 days for experiments (A) and (B), and 10 days for experiments (C). Each experiment was repeated at least twice and representative data shown in the figures.

amiloride inhibition, of uPAR mRNA levels in colon cancer cells.

4. Discussion

This paper reports the negative effect of amiloride and the positive effects of TNFα and PMA on uPAR expression in colon cancer cell lines. Transcriptional elongation experiments suggested that the suppression effect of amiloride and the induction effect of TNFα were mediated, at least in part, at the level of transcription. The induction effect of PMA, however, may be mediated at the level of post-transcription.

TNFα has a wide range of biological activities affecting the growth, differentiation and functions of many cell types [31]. Amongst its protean biological functions, TNFα induces rapid expression of the uPA gene in pulmonary epithelial cells [32]. The results reported here indicate that treatment with TNFα also increases levels of uPAR mRNA and protein expression in colon carcinoma cells. In this context, it has been reported that a small proportion of infiltrating macrophages, but not colon cancer cells, expresses TNFα mRNA and protein in colon cancer tissue [33], suggesting that the tumor microenvironment may modulate uPA and uPAR expression in colonic cancers thereby influencing tumor invasiveness.

Amiloride is a specific inhibitor of sodium channels in many epithelial transport systems. It is also a competitive inhibitor of uPA [17] and its receptor, but not tissue plasminogen activator (tPA), plasmin, plasma kallikrein or thrombin, indicating that it does not exert a general inhibitory action on cell metabolism. This paper reports that amiloride markedly inhibits uPAR mRNA induction in colon cancer cell lines. We found that amiloride had no measurable effects on cell viability or on the yield of the total RNA. When the uPAR probe was eluted from the filters and the same RNA blot was then hybridized using ^{32}P-labelled human β-actin [23], ubiquitin cDNA [24] or 18S rRNA, no significant changes in the β-actin and ubiquitin mRNAs (data not shown) or 18S rRNA were detected regardless of whether the cells had been exposed to amiloride. This result shows that amiloride may be selective in inhibiting gene expression. There is evidence that amiloride given to rats in their drinking water completely prevented pulmonary metastasis when rat mammary adenocarcinoma cells were injected intravenously [15]. This observation combined with the inhibitory effects of amiloride on uPAR expression reported in this paper, encourage further research into the mechanisms of amiloride-induced inhibition of metastasis that may offer fresh insights into the prevention of invasion and metastasis by adenocarcinomas.

This paper suggests that uPAR gene expression may be modulated by multiple pathways of signal transduction in colon cancer cells. These signalling pathways may include the well-characterized protein kinase C/cAMP-dependent pathways, and other less-well understood pathways such as those involving the TNFα receptor and, as described here, the amiloride-sensitive Na^+ channel. PMA activates protein kinase C which can phosphorylate and modulate many proteins [34]. PMA has been shown to affect transcription of numerous genes, increasing transcription of many cytokine genes [35], and inhibiting phosphoenolpyruvate carboxykinase gene activity [36]. The results of the transcriptional elongation assays reported in this paper suggest that PMA stimulation may lead to stabilization of uPAR mRNA in colon cancer cells. This is also true in the case of GM-CSF gene expression in cells treated with PMA [37]. Human TNFα, however, is reported to regulate gene expression in eukaryotic cells by binding to the TNFα receptors [38]. The nuclear run-on transcription assay suggested that the effect of TNFα was mediated at the transcriptional level and may be mediated through the transcriptional factor NF-kB

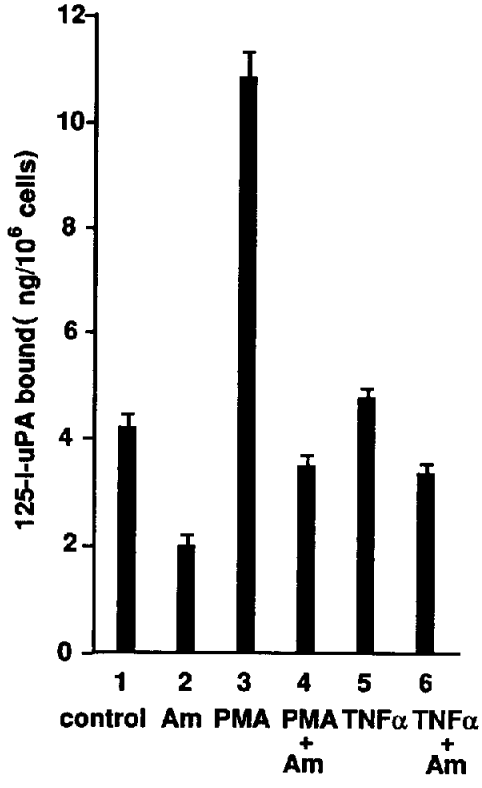

Fig. 5. Effects of PMA, TNFα and amiloride on uPAR protein production in HCT116 cells measured by the [^{125}I]uPA binding assay. Cells were treated with PMA (50 ng/ml) or TNFα (440 units/ml) in the presence or absence of amiloride (1 mM) for 12 h. Columns, means of three separate experiments; bars, S.D. Statistical evaluation showed that the effects of PMA, TNFα and amiloride in uPA binding in HCT116 cells were significant ($P < 0.05$).

which is activated by the sphingomyelin pathway in HL-60 cells [39]. The experiments reported here also show that amiloride downregulates uPAR mRNA at the level of transcription in colon cancer cells suggesting that the Na^+ channel may be involved in the regulation of the uPAR gene. Although the mechanism in the regulation of genes by amiloride is unclear, recent reports indicate that amiloride is able to bind to diamine oxidase, an amiloride binding protein, that is very similar to the Na^+ channel and to catalyze the degradation of compounds including putrescine and histamine [40]. To characterize the signal transduction pathways which regulate the uPAR gene, further experiments are currently in progress to study the signalling pathways and the regulatory sequence(s) and trans-acting factor(s) that contribute to regulation of uPAR gene expression by PMA, TNFα and amiloride.

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