Suppression of Urokinase Expression and Invasiveness by Urinary Trypsin Inhibitor Is Mediated through Inhibition of Protein Kinase C- and MEK/ERK/c-Jun-dependent Signaling Pathways*

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Urinary trypsin inhibitor (UTI), a Kunitz-type protease inhibitor, interacts with cells as a negative modulator of the invasive cells. Human ovarian cancer cell line, HRA, was treated with phorbol ester (PMA) to evaluate the effect on expression of urokinase-type plasminogen activator (uPA), since the action of uPA has been implicated in matrix degradation and cell motility. Preincubation of the cells with UTI reduced the ability of PMA to trigger the uPA expression at the gene level and at the protein level. UTI-induced down-regulation of PMA-stimulated uPA expression is irreversible and is independent of a cytotoxic effect. Down-regulation of uPA by UTI is mediated by its binding to the cells. We next asked whether the mechanism of inhibition of uPA expression by UTI was due to interference with the protein kinase C second messenger system. An assay for PKC activity demonstrated that UTI does not directly inhibit the catalytic activity of PKC and that PMA translocation of PKC from cytosol to membrane was inhibited by UTI, indicating that UTI inhibits the activation cascade of PKC. PMA could also activate a signaling pathway involving MEK1/ERK2/c-Jun-dependent uPA expression. When cells were preincubated with UTI, we could detect suppression of phosphorylation of these proteins. Like several types of PKC inhibitor, UTI inhibited PMA-stimulated invasiveness. We conclude that UTI markedly suppresses the cell motility possibly through negative regulation of PKC- and MEK/ERK/c-Jun-dependent mechanisms, and that these changes in behavior are correlated with a coordinated down-regulation of uPA which is likely to contribute to the cell invasion processes.

A number of studies have indicated that exogenously applied UTI, also known as bikunin, to tumor cells could suppress their invasiveness and metastatic formation in an in vitro assay system and in an in vivo animal model (1–9). The UTI gene encodes a Kunitz-type protease inhibitor of molecular mass 40 kDa (9), which is composed of a ligand-binding domain (amino terminus) for cell-associated UTI-binding sites (10, 11) and protease inhibitor domain (carboxyl terminus) (9), which effectively inhibits trypsin, plasmin, and granulocyte elastase. In addition to its protease inhibiting effects, UTI plays a role in suppressing urokinase-type plasminogen activator (uPA) production responsible for the invasiveness of tumor cells (12), although UTI does not inhibit directly the catalytic activity of uPA. uPA converts plasminogen into plasmin, a serine protease with broad substrate specificity toward components of the basement membrane and the extracellular matrix including laminin, vitronectin, and fibronectin (13). These proteolytic functions facilitate the migration of tumor cells through the extracellular matrix and basement membrane barriers. Therefore, UTI apparently plays a key role in regulation of cell invasiveness and metastatic formation possibly through down-regulation of uPA expression.

Expression of uPA is controlled by a variety of extracellular signals such as phorbol ester, protein kinase C (PKC), and Fos/Jun-dependent signals, cAMP, cytoskeletal reorganization, tumor necrosis factor-α, interleukin-1β, interferon-γ, tumor growth factor-β, fibroblast growth factor-2, okadaic acid, retinoic acid, UV, and oncogene products v-Src and v-Ras (14). uPA activity of malignant cells is induced during the promotion stage of the carcinogenic process and phorbol myristate acetate (PMA) is one of the best characterized, tumor promoting agent. PMA is generally recognized to modulate cellular functions by activating a Cu2+-phospholipid-dependent PKC (15). Agents that modulate uPA have been shown to alter the rate of metastasis in in vitro experiments and in some animal models (16). Recent publication demonstrated that UTI with anti-inflammatory and anti-tumor promoting properties can influence the PKC-dependent signal pathway in uPA expression in cultured human umbilical vein endothelial cells and in the promyeloid leukemia cell line U937 (12); exogenous UTI inhibits a rapid increase in membrane-associated PKC activity, and a decrease in cytosolic PKC activity. However, the precise molecular mechanisms of the UTI-mediated changes occurring downstream of the PKC signal transduction have remained unclear.

In the present study, we have sought to define the UTI-dependent regulatory mechanisms involved in PMA-induced uPA expression and cell motility. First, we have determined the effect of UTI on PMA-induced uPA expression, as well as quantitating time- and dose-dependent alterations in the steady state levels of uPA mRNA and uPA activity. Second, we ask whether the inhibition was due to interference with the PKC second messenger system. For this, we have compared the effect of UTI and several types of PKC inhibitor on PMA-induced uPA expression, PKC translocation, and signal pathway involving a relay of phosphorylation of several proteins. Third, we have investigated the possibility that UTI binding to
tumor cells might be involved in the down-regulation of uPA expression. Finally, we have determined if there is a relation between UTI-dependent alterations in the uPA expression and cellular motility.

EXPERIMENTAL PROCEDURES

Materials—UTI was purified to homogeneity from human urine. A highly purified preparation of human UTI was kindly supplied by Mochida Pharmaceutical Co., Tokyo, Japan. The COOH-terminal fragment of UTI (HI-8) was purified as described previously (9). Polyclonal antibodies raised against MEK1, ERK2, and c-Jun were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies raised against human uPA and human high molecular weight uPA were supplied by Yoshitomi Pharmaceutical Co., Ltd. (Osaka, Japan). PMA, calcium ionophore A23187, H-7, calphostin C, staurosporin, aspirin, and 5,8,11,14-eicosatetraenoic acid were purchased from Sigma, and all other chemicals were of reagent grade or better and were purchased from major suppliers. Ethanol was used as the solvent for PMA, and the final concentration of ethanol was 0.1% in all experimental points.

Cell Line and Culture Conditions—Human ovarian cancer cell line HRA was obtained from Dr. Y. Kikuchi (17). The HRA was cultured in RPMI 1640 with 10% fetal calf serum (Life Technologies, Inc., Rockville, MD). Cells were disaggregated routinely with 0.1% trypsin/EDTA solution and replated at a split ratio of 1:10. The cells were harvested and aliquoted into tissue culture plates (0.5–1.0 × 106 cells/well) in RPMI 1640 supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal calf serum. On the next day, the cells were washed three times with phosphate-buffered saline to remove serum, and the medium was replaced with RPMI 1640 supplemented with antibiotics. Serum-free medium plus the test drugs were added and incubation was continued for different time lapses. The cells were incubated with various concentrations of UTI during three different periods, i.e. (a) during 30 min preceding the stimulation phase, (b) during the PMA-stimulation phase, and (c) 60 min after the stimulation, UTI being then added to the expression medium. Conditioned media were individually harvested, one of the remaining monolayers were trypsinized and hemocytometer cell counting or protein content determinations were performed. The protein concentration was determined by the method of Bradford (18) using bovine serum albumin as standard. 

Zymography was performed as described previously (20). Aliquots of both cytosolic and membrane extracts were assayed for PKC activity by a PKC enzyme assay system (PepTag™ Non-Radioactive Protein Kinase Assays; Promega, Madison, WI), according to the instructions of the manufacturer. The micrograms of PepTag™ C1 peptide were incubated as in the standard reaction with varying amounts of PKC in a final volume of 25 μl for 30 min at 23 °C. The reactions were stopped by heating to 95 °C for 10 min. The samples were loaded on a 0.8% agarose gel and run at 100 V for 15 min. Phosphorylated peptide migrated toward the cathode, while nonphosphorylated peptide migrated toward the anode. Using a razor blade, the negatively charged phosphorylated bands were excised from the gel and assayed for PKC activity according to the manufacturer’s instructions. Western blot analysis of PKC was performed using α-subtype-specific monoclonal antibodies. Immuneactivity was analyzed using ECL detection kit as described above.

Invasion Assays—Invasion assays were performed essentially as described previously (25). Assays were conducted using a 24-well Boyden chamber apparatus. For motility assays, 8-μm pore sized, polycarbonate filters were placed in the apparatus separating the upper from the lower wells. The lower wells contained 25 μl of fibroblast-conditioned media prepared by incubating confluent monolayers of NIH 3T3 fibroblasts for 24 h with Dulbecco’s modified Eagle’s medium containing 0.1% bovine serum albumin and 0.05 mg/ml ascorbic acid. HRA cells were harvested with trypsin/EDTA, washed twice with Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, resuspended in media containing the appropriate treatment, and added to the top well (5 × 105 cell/well). The filters were coated with 0.375 μg of Matrigel per filter. The apparatus was incubated in a humidified incubator at 37 °C in 5% CO2, 95% air for 18 h, after which the cells that had traversed the membrane and spread on the lower surface of the filter were stained with Diff-Quick and quantified electronically with the image analysis system. This system analyzes 32 independent fields for each filter. Cells that migrated on the basis of purely adhesive interactions to a confocal field is generated. The chemotactic assay was conducted as described previously (4). For chemotaxis assay, the upper filters were not coated with Matrigel.

Statistical Analysis—All experiments were performed using at least three different cell preparations. Data are presented as mean ± S.D. All statistical analysis was performed using StatView for Macintosh. The Mann-Whitney U test was used for the comparisons between two groups. In cases in which significant interactions were detected, Dun-can’s multiple range test was used for group comparisons. p less than 0.05 was considered significant.
UTI Modulates uPA Expression and Invasiveness

RESULTS

PMA Specifically Stimulates HRA Cell Expression of uPA Activity—It has been established that the cell-associated receptor-bound uPA activity is important for tumor cell invasiveness (26). The effect of various concentrations of PMA on the uPA expression was determined by a chromogenic assay. The HRA cells were treated with PMA, calcium ionophore A23187, or 8-Br-cAMP. A23187 and cAMP were included for comparison because they have been shown to induce the uPA in other cell types. Fig. 1 shows that PMA strongly induced PA activity on HRA cell surface. Plasminogen activator activity on the surface of the cells incubated with PMA (100 nM) was increased about 7-fold as compared with the control cells. In contrast, A23187 and cAMP showed a negligible effect by themselves or in combination with PMA (not shown). When cells were preincubated with anti-uPA IgG, PA activity was inhibited more than 85%, indicating that most of the PA activity expressed by HRA cells is uPA. As shown in Fig. 1, induction by PMA reached maximal and 50% values at the concentrations 100 and 1000 nM, respectively. Time course analyses showed that PMA induction reached a maximum at 9 h (see Fig. 6).

Suppression of PMA-induced uPA Expression and Secretion by UTI—We investigated whether UTI could inhibit PMA-induced stimulation of PA activity expressed on the cell surface. We initially reported (2) that UTI is able to reduce cell-associated protease activity directly via inactivation of plasmin and trypsin, while UTI fails to inhibit uPA activity. In the present study, to examine if UTI could be directly modifying pathways associated with UTI-induced down-regulation of PA activity, constitutive uPA expression were estimated utilizing several types of specific inhibitors (Fig. 5). The cells exposed to 100 nM PMA exhibited a 7.5-fold increase in PA activity on the cell surface. Preincubation of the cells with either aspirin (2 μg/ml) or eicosatetraenoic acid (0.1 mM) had no effect on the ability of PMA to stimulate PA activity. This experiment demonstrated that it is unlikely that cyclooxygenase or lipoygenase products of arachidonic acid are involved. We next compared the ability of UTI to reduce PMA-stimulated expression of PA activity by cells pretreated with each PKC inhibitor. When cells were incubated with 10 μM H-7, the ability of PMA to stimulate the expression of cell associated PA activity was inhibited about 90%. When cells were preincubated with 1.0 μM UTI, the ability of PMA to stimulate the expression of PA activity was inhibited about 60%. Higher concentrations of UTI (10 μM) gave similar results on inhibition of PMA-dependent stimulation of PA activity. Inhibition assays showed that UTI modulates uPA expression and secretion of enzymatically active uPA.

Results obtained after exposing the cells to UTI before, during, and after stimulation by PMA are presented in Fig. 4. Preincubation of the cells with UTI during 30 min before 100 nM PMA stimulation results in a concentration-dependent inhibition of the induction of cell associated PA activity. At concentrations of 100 and 1000 nM, PA activity is inhibited by 55 and 75%, respectively. This inhibition is irreversible since it cannot be reversed by washing the cells before PMA stimulation. The presence of UTI during stimulation by PMA (concurrent treatment) does not cause a dramatic reduction of PA activity. At a concentration of 1000 nM, PA activity is inhibited by ~25%. In contrast, no significant decrease of PA activity is observed when UTI is added to the medium 1 h after stimulation by PMA. More than 90% of the control value still remains at the highest UTI concentration (1000 nM) tested. Cell viability, monitored by lactate dehydrogenase leakage in the culture medium and trypan blue dye exclusion test, is not altered under the different exposure conditions (data not shown). These experiments demonstrated that a marked and a slight, but significant, decrease of PA activity are observed when UTI is added to the medium before and during stimulation by PMA.

The Effect of UTI and Several Types of PKC Inhibitor on PMA-stimulated Expression of PA Activity—The cell signaling pathways associated with UTI-induced down-regulation of PA expression were estimated utilizing several types of specific inhibitors (Fig. 5). The cells exposed to 100 nM PMA exhibited a 7.5-fold increase in PA activity on the cell surface. Preincubation of the cells with either aspirin (2 μg/ml) or eicosatetraenoic acid (0.1 mM) had no effect on the ability of PMA to stimulate PA activity. This experiment demonstrated that it is unlikely that cyclooxygenase or lipoygenase products of arachidonic acid are involved. We next compared the ability of UTI to reduce PMA-stimulated expression of PA activity by cells pretreated with each PKC inhibitor. When cells were incubated with 10 μM H-7, the ability of PMA to stimulate the expression of cell associated PA activity was inhibited about 90%. When cells were preincubated with 1.0 μM UTI, the ability of PMA to stimulate the expression of PA activity was inhibited about 60%. Higher concentrations of UTI (10 μM) gave similar results on inhibition of PMA-dependent stimulation of PA activity (see Fig. 2). Although UTI, like H-7, was effective to reduce PMA-stimulated PA activity, induction of PA activity was not synergistically reduced. This shows that component(s)
of UTI action are mediated dependently of PKC. Other PKC inhibitors such as calphostin C (250 nM), not shown, and staurosporin (50 nM) gave similar results. These data suggest that UTI may suppress PA activity in a manner analogous to PKC inhibitor.

The time-dependent accumulation of PA activity on the cells exposed to PMA is presented in Fig. 6. Cell associated PA activity did not increase until after 2 h of PMA stimulation and continued to increase over 9 h. As expected, PMA stimulated PA activity was significantly suppressed in a time-dependent manner when the cells were preincubated with 1 μM UTI or 10 μM H-7, respectively. Of note, both UTI and H-7 did not directly inhibit uPA activity.

The Effect of UTI on HRA cell uPA production during a 24-h treatment. uPA activity in the conditioned media is expressed in IU/10⁶ cells/24 h for control or treated groups. A, zymography; B, Western blot with anti-uPA antibody; and C, uPA activity in conditioned media. Lane 1, control high molecular weight uPA (0.1 μg/lane).

The Effect of UTI and Several Types of PKC Inhibitors on PMA-induced uPA mRNA Expression—The effect of UTI or PKC inhibitors on the PMA-induced expression of uPA mRNA was studied. RNA was prepared from cells treated with PMA and inhibitors and hybridized with probes derived from human cDNA clones of uPA. Fig. 7 shows the results from a blot probed for uPA mRNA. PMA produced a marked increase in uPA expression at the gene level. The expression of the uPA gene was increased by 12-fold at 100 nM PMA for 3 h. This stimulation was abrogated in cells pretreated with UTI or PKC inhibitors.

The time-dependent effect of UTI on PMA-stimulated expression of uPA mRNA was determined. As illustrated in Fig. 8, an increase in HRA cell uPA mRNA levels was observed after 1 h and peaked after 3 h. uPA mRNA levels dropped sharply over the next 6 h. The increase in levels of uPA mRNA observed in cells treated with PMA for 9 h was inhibited more than 50% (UTI) or 95% (H-7) when cells were preincubated with UTI or H-7, respectively.

Inhibition of PKC Translocation by UTI—We have investigated whether UTI can suppress PMA-mediated PKC translocation. HRA cells were grown to confluency and then maintained in serum-free conditions to keep the cells in a quiescent state. PKC enzymatic assay and immunoblot analysis showed that stimulation with 100 nM PMA for 30 min at 37 °C resulted in the translocation of PKC-α in the membrane fraction, with a concomitant decrease in the cytosolic pool,
while total PKC activity did not significantly change (Fig. 9). Abolition of PMA effect was achieved in the presence of UTI, but not of HI-8. UTI did not directly inhibit the catalytic activity of partially purified porcine brain PKC even at the concentration of 1 μM (data not shown).

UTI Suppresses PMA-triggered Phosphorylation of MEK1, ERK2, and c-Jun—The phosphorylation of proteins in tyrosine/threonine residues is a prerequisite for the activation of these enzymes. In some systems these events are PKC-dependent (27). Recent publication demonstrated that activation of a PMA-dependent signal pathway involves a relay of phosphorylation of several proteins making up the pathway (27). Therefore, we investigated the effect of UTI on the phosphorylation of MEK1, ERK2, and c-Jun in HRA cells stimulated with PMA. This may be detected by Western blot analysis as a mobility shift in SDS-polyacrylamide gel electrophoresis (Fig. 10). As expected, PMA could activate a signaling pathway involving MEK1/ERK2/c-Jun. In nonstimulated cells the expression of phosphorylated proteins was weak, whereas PMA significantly raised the levels of the phosphorylated form of these proteins. We found that phosphorylation of these proteins is modified within 30 min of induction by PMA and then returned to the unin-

The Effect of PMA, PKC Inhibitors, and UTI on Invasiveness—The effects of agents that alter the activity of PKC on the invasiveness of HRA cells were determined by measuring the ability of cells treated with these agents to pass through a layer of the extracellular matrix extract Matrigel coating a filter using chemoinvasion chambers as described under “Experimental Procedures.” Fig. 11 shows that treatment with PMA produced a significant stimulation of the invasiveness of the cells in a dose-dependent manner. The stimulation was maximal at 100 nM PMA (7-fold). This stimulation was reversed by concurrent treatment with either PKC inhibitor or UTI. Fig. 12 shows the effect of adding increasing concentrations of PKC inhibitors (staurosporin and H-7) or UTI on the invasiveness of cells stimulated with 100 nM PMA. Calphostin C was also able in our studies to inhibit the stimulatory effects of PMA on
invasiveness (not shown). Although concurrent treatment with UTI produced a dose-dependent reversal of the PMA-induced stimulation, the migration of cells treated with 100 nm PMA was reduced to about 60% by the addition of 1 µM UTI, indicating that UTI may exhibit less inhibitory to abrogate PMA-induced cell migration compared with the PKC inhibitors. The inhibition seen is not due to nonspecific cytotoxicity produced by these agents, as the concentrations used do not significantly inhibit the cell viability.

In addition, the cell chemotactic response was also tested to determine whether the inhibitory effect of UTI on cell invasion of basement membranes was due to an inhibition of chemotaxis. The cells tested showed good chemotactic migration in the presence of UTI (data not shown; see Ref. 4). In addition, we examined the effects of UTI on cell attachment. No inhibition of attachment to Matrigel (or fibronectin) was seen with UTI (data not shown; see Ref. 4). In addition, we confirmed that UTI has no ability to inhibit cell migration or chemotaxis, which is consistent with our previous data (4).

**DISCUSSION**

A variety of signal transduction pathways for uPA gene expression have been described for the action of cytokines and growth factors. Multiple signal transduction pathways, for example, PKC and cAMP protein kinase pathways, converge at the level of uPA gene regulation. The uPA promoter contains functional binding sites for transcription factors AP-1, PEA3, and NF-kB (28). The induction of uPA is regulated at least in part by a c-Jun-dependent MAP kinase pathway (28). Agents that regulate signaling pathways involved in uPA expression appear to be a useful probe to help dissect cell invasion process.

One may speculate that PMA can promote cell invasion possibly through an increase in cell-associated uPA expression. This by-passes the cell surface events involved in the phosphatidylinositol system. In HRA cells used in this study, PKC-dependent signal transduction needs to be activated to achieve uPA expression, whereas neither increase in intracellular calcium nor cAMP activation was able to stimulate uPA expression. It follows then that inhibition of PKC signaling pathway could result in the inhibition of uPA expression and cell invasion. Moreover, preincubation of cells with inhibitors of cyclooxygenase or lipoxygenase had no effect on PMA-induced uPA expression. These data suggest that the effect of PMA on uPA expression is not likely due to alterations in the permeability to calcium, adenylate cyclase activity, or arachidonic acid metabolism. Therefore we tested the effects of UTI on PKC-dependent signaling pathway only.

We have previously shown that in human umbilical vein endothelial cells and promyelocytic leukemia cell line U937 activation of PKC represents a critical event in tumor necrosis factor-triggered signaling cascade leading to uPA secretion (12). Furthermore, we had shown that induction of uPA expression by tumor necrosis factor-α was inhibited when these cells were incubated with UTI and that UTI may influence the PKC-dependent protein kinase pathway in uPA expression. However, the molecular mechanisms of UTI-mediated changes in signaling pathways regulating uPA gene expression is not fully understood. Therefore, we asked whether the inhibition was due to interference with the PKC signaling system which cooperates with the MAP kinase pathway.

We detected changes in the PKA-stimulated uPA expression by UTI. HRA cells normally only express low levels of this enzyme, and upon stimulation with PMA expression and secretion were each induced well. This induction was inhibited by pre- or concurrent treatment with UTI or PKC inhibitors. The UTI's action is relatively rapid and irreversible; it occurs after 30 min of contact and persists after UTI is removed and the cells are washed. One h after stimulation by PMA, however, UTI has no effect on uPA expression. We show that this reduction occurs at the level of gene transcription (or mRNA degradation) in that the mRNA levels for uPA were reduced in the presence of UTI in the Northern analysis. Present data do not indicate whether UTI is selectively inhibiting the uPA gene or also inhibits other gene transcription as well. Notwithstanding these limitations, we have also shown in HRA cells that the PKA-stimulated cell motility (invasiveness) can be suppressed by UTI. Increased uPA activity might be contributing to the increase in motility produced by PMA treatment, indicating that the level of uPA expression closely parallels the degree of cell motility. Thus, the reduction in uPA expression by UTI might appear to explain the degree of inhibition of cell motility.

Although UTI does not directly inhibit the catalytic activity of PKC, UTI inhibited the translocation of PKC from cytosol to membrane when stimulated with PMA. Thus UTI may prevent PKC from being up-regulated by PMA and PKC remained in an inactivated state. Therefore, the inhibition of PKA-stimulated uPA expression by UTI must be occurring at a point beyond the diacylglycerol activation event. Activated PKC also causes the phosphorylation of a wide variety of intracellular proteins (29). The MAP kinase pathway is activated via the stimulation of Ras → Raf → MAPKK (MEK1/2) → ERK1/2. It was suggested that PKC phosphorylates and activates Raf-1 and ERK1/2 (27). More recent data support the implication of different PKC isoforms in the phorbol ester-induced activation of the ERKs pathway in different cell types (30, 31). If phorbol ester leads phosphorylation and assembly of the MAP kinase pathway, it remains possible that UTI is inhibiting one or more of these phosphoprotein products. The present results show for the first time that UTI's function on PMA-induced production of uPA in tumor cells involves the PKC regulatory signaling pathway which may cooperate with the MEK/ERK/c-Jun pathway. We report that UTI blocks PMA-triggered phosphorylation of MAP kinases, when used at a concentration of 1 µM. Because this concentration of UTI affects cytosol-to-membrane translocation of PKC-α isoform, it is likely that at least in part PKC-α participates in the activation of MAP kinases in our system. These data allow us to conclude that the inhibitory effect of UTI for uPA expression involves an interfeference with the PKC
second messenger system, and appears to occur downstream of diacylglycerol activation and upstream of MEK phosphorylation, which are critical in the uPA expression and subsequently in cell motility. Transcription of human genes encoding uPA is a complex process that requires participation of several transcription factors. It is also well documented that NF-κB activity could be strongly induced by PMA (32). We cannot exclude the possibility that UTI may directly affect MAP kinase phosphorylation or that UTI may inhibit DNA binding activity of transcription factors. Also, there is no evidence whether UTI binds to PKC at a catalytic site to which PMA binds or a site other than the catalytic one.

Suppression by PKC inhibitors of uPA stimulation by PMA is complete. Contrary to several PKC inhibitors, inhibition by UTI of down-regulation of uPA expression is partial. PKC actually exists not as a single entity, but in a family of isoforms; their activation mechanisms are very complex and they may exhibit significant biochemical differences (33, 34). It is possible that the mechanism of action of both UTI and PKC inhibitors is partly different, although a PMA-sensitive subtype of PKC could be involved in UTI's function.

On the basis of our previous data obtained in our laboratories, we had shown that UTI binds to tumor cells via specific UTI-binding sites (10, 11). Cell-associated UTI-binding proteins (UTI-BPs) may be critical targets for UTI, since HI-8, which does not possess a ligand for UTI-BPs (11), failed to prevent the PMA-stimulated uPA expression. Membrane-associated UTI-BPs are believed to represent the rate-limiting step for UTI-dependent signal transduction or cellular uptake of the UTI. Taken together, we hypothesize the following mechanism in the UTI-dependent regulation: the UTI/UTI-BP interaction is followed by modulation of a PKC system, which inhibits translocation of certain PKC isoform(s) including PKC-α from the microsome to the plasma membrane compartment. UTI-dependent suppression of activation and redistribution of PKC-α results in the reduction of uPA expression possibly via inhibition of phosphorylation of the MEK/ERK/c-Jun proteins. Thus,

**Fig. 9.** Subcellular localization of PKC activity. A, the PKC activity is expressed as picomoles of phosphate transferred per minute per 10⁶ cells. B, cytosol; M, plasma membrane. PMA, 100 nM; UTI and HI-8, 1 μM. Data reported are the mean ± S.D. (n = 4–6). B, cells were grown in 10-cm dishes to confluence and then incubated for 30 min with 100 nM PMA. Cytosolic and membrane extracts were prepared, and 100 μg of each extract was separated by polyacrylamide gel electrophoresis, blotted, and subjected to Western analysis using PKC-α subtype-specific monoclonal antibodies. Experiments were performed twice with similar results. C, cytosol; M, membrane

**Fig. 10.** Western blot analysis of MEK1, ERK2, and c-Jun proteins. Modification of these proteins was shown. Whole cell extracts (20 μg of total protein) from cells treated for 30 min with 100 nM PMA in the absence or presence of 1 μM UTI were analyzed by Western blot. Western blots were first incubated with antibodies against MEK1, ERK2, and c-Jun and then with biotin-conjugated secondary antibodies and avidin-peroxidase, followed by enhanced chemiluminescence. The "m" shows the modified isoforms. Phosphorylation-dependent mobility shift of MEK1 and ERK2 proteins in response to PMA induction was detected. This is a representative experiment selected from two performed.

**Fig. 11.** Induction of HRA cell invasiveness by treatment with PMA. HRA cells were trypsinized and counted, and 5 × 10⁴ cells were placed in the top wells of a chemoinvasion chamber apparatus with the indicated concentration of PMA. After 18 h the cells that had passed through the Matrigel-coated filter toward the bottom wells containing fibroblast-conditioned medium as a chemoattractant were quantitated. Each point represents the mean and S.D. of measurements made on three independent wells. Asterisks indicate bars that are significantly different from the vehicle control (p < 0.05).
UTI Modulates uPA Expression and Invasiveness

In conclusion, we describe the in vitro regulation of the PMA-induced expression of uPA gene and cell motility by UTI in ovarian cancer cells and the signaling pathways involved in the UTI-dependent modulation of the PMA induction. We found that cytostol-to-membrane translocation of PKC-α isoenzyme may represent early steps in signaling cascades that lead to uPA production in human ovarian cancer cells and that UTI prevents translocation of PKC-α, and subsequently inhibits MEK1, ERK2, and c-Jun phosphorylation and inhibits cell motility at the concentrations relevant to reported IC50 values for uPA production. It is unlikely that UTI directly inhibits catalytic activity of PKC-α. At this point, it is not possible to implicate any UTI-specific targets (protease, adhesive molecule, or other determinant of motility). Nevertheless, as reported here, UTI exhibited no cytotoxic effects even at concentrations of 10 μM. The present effect of UTI occurs at concentrations in the micromolar range; it is thus likely that such effects may occur in vivo at therapeutically relevant concentrations.

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