Stimulation of Macrophage Urokinase Expression by Polyanions Is Protein Kinase C-dependent and Requires Protein and RNA Synthesis*

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Highly charged polyanionic ligands of the scavenger receptor trigger macrophage secretion of urokinase-type plasminogen activator (uPA). In experiments reported here, we have investigated the intracellular and extracellular regulation of polyanion-induced macrophage plasminogen activation. Exposure of a macrophage cell line (RAW264.7) to either fucoidan or phosphorethyl myristate acetate (PMA) stimulates the secretion of uPA, whereas calcium ionophore or dibutyryl cyclic AMP had no effect. Moreover, preincubation of macrophages with inhibitors of protein kinase C reduced (50–60%) the ability of both fucoidan and PMA to trigger the secretion of uPA, whereas aspirin and eicosatetraenoic acid had no effect. Both PMA and fucoidan treatment of RAW264.7 cells resulted in a rapid and transient increase in the steady state levels of uPA mRNA. However, in marked contrast to that observed with PMA, fucoidan-induced expression of RAW264.7 uPA activity was partially insensitive to cycloheximide and actinomycin D. In addition, fucoidan-induced uPA activity was detected in conditioned media in as little as 15 min, whereas PMA-induced uPA activity did not increase until 2 h. In addition to stimulating macrophage secretion of uPA, fucoidan bound uPA and had a small stimulatory affect on uPA activity. The binding does not interfere with the catalytic site on the B chain, or require the receptor binding or kringle domains on the A chain.

Macrophage generation of plasmin is tightly regulated by the secretion of a specific neutral protease, uPA, that converts inactive plasminogen to a serine protease possessing broad substrate specificities (1, 2). Cellular conversion of plasminogen to plasmin is an important regulator of migration (3–5), connective tissue remodeling (6), and the activities of other hydrolases (7) and growth factors (8, 9).

uPA has been demonstrated to exist in several molecular forms. It is secreted by a variety of cells as a single-chain uPA (10), which is converted to two-chain, heavy molecular weight uPA (HMW-uPA) by a single cleavage between amino acids 158 and 159 (11, 12). The catalytic site is on the B-chain, whereas the uPA receptor binding sequence is in the amino-terminal segment of the A-chain (13, 14). HMW-uPA can be modified further by limited cleavage resulting in a low molecular weight form (LMW-uPA) that is catalytically active (11) but does not express cell binding activity (15). In addition to uPA, macrophages secrete the serine protease inhibitor plasminogen activator inhibitor 2 (16, 17), which rapidly and irreversibly inactivates uPA (18). As expected, the proportions of plasminogen activator and inhibitor secreted by different macrophage populations varies and is effected by in vitro exposure to a diverse array of stimuli (16, 17, 19, 20).

In previous studies, we and others have demonstrated that acetyl-LDL and polyanionic inhibitors of acetyl-LDL binding to the scavenger receptor stimulated the secretion of plasminogen activator activity by activated peritoneal macrophages and a macrophage-like cell line (21–23). In later studies, we demonstrated that the secretion of plasminogen activator activity by the macrophage cell line, RAW264.7, was stimulated by heparin (24). Although heparin is not reported to be a ligand of the scavenger receptor, binding to RAW264.7 cells, and heparin partially inhibits both binding of acetyl-LDL to RAW264.7 cells and acetyl-LDL-induced cholesterol ester synthesis (24). The observation that a fraction of total heparin binding by RAW264.7 cells appeared to be via the scavenger receptor suggested that another cellular receptor/binding site may be responsible for polyanion induced-uPA secretion. However, neither heparin nor fucoidan were able to stimulate the release of uPA activity from U937 cells, a monocyte-like cell line devoid of scavenger receptor activity (25). These findings suggest that endocytosis of a diverse group of polyanions by the scavenger receptor may play a role in the regulation of macrophage-dependent plasminogen activation.

In experiments reported here, we have sought to define the regulatory mechanisms involved in polyanion-induced uPA expression by macrophages. First, we have identified the major cell signaling pathway operational in polyanion-induced expression of macrophage uPA activity. Second, we have determined the effect of inhibitors of protein and RNA synthesis on polyanion-induced uPA activity, as well as quantitating time-dependent alterations in the steady state levels of uPA mRNA. Third, we have determined whether polyan-
ions that stimulate the expression of macrophage uPA activity are able to bind uPA and affect its catalytic activity.

EXPERIMENTAL PROCEDURES

Materials—The murine macrophage-like line RAW264.7 was purchased from the American Type Culture Collection (Rockville, MD). RPMI 1640 medium, fetal calf serum, Dulbecco’s phosphate-buffered saline (DPBS), and penicillin/streptomycin sulfate were purchased from Flow Laboratories, Inc. (McLean, VA). The growth supplement ITS+ (insulin, transferrin, selenious acid, and albumin) was purchased from Collaborative Research, Inc. (Bedford, MA). Acetic anhydride (50 mCi/mmol), Na[35]I (17 Ci/mg), and [3H]-leucine (140.2 Ci/mmol) were obtained from Du Pont-New England Nuclear. Hydrofluor was obtained from National Diagnostics (Manville, NJ). Human plasminogen and HMW-uPA (50,000) were purchased from Calbiochem. Fucoidan (133 kDa), polyinosinic acid, polyadenylic acid, and calcium ionophore A23187 were purchased from Calbiochem. Fucoidan (133 kDa), polyinosinic acid, polyadenylic acid, and porcine heparin were purchased from Sigma. Acid-activated Sepharose 4B (column chromatography) was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Polyclonal goat anti-human uPA IgG, normal IgG, and LMW-uPA (33,000) were obtained from American Diagnostica Inc. (NY). The LMW-uPA was determined to be >98% pure by Western blot. Phorbol 12-myristate 13-acetate (PMA), Nω,Ο- dibutyryl cyclic adenosine-3,5'-monophosphate (db-cAMP), 5,8,11,14-eicosatetraenoic acid (ETYA), and calcium ionophore A23187 were purchased from Calbiochem. Fucoidan (133 kDa), polyinosinic acid, polyadenylic acid, polytetradecylidic acid, and porcine heparin were purchased from Sigma. The protein kinase C inhibitor H-7 (isooquinolinylsulfonfyl)pyr��anone and auranofin ([2(2,4-diaminophenyl)hexyl]-[4-methyl-3-phenyl](isoquinolinylsulfonylpiperazine) were obtained from Seikagaku America Inc. (Rockville, MD) and Dr. W. Borth (Mt. Sinai School of Medicine), respectively. Isogel was purchased from FMC Corp. (Rockland, ME).

Cell Culture and Isolation of Conditioned Media—RAW264.7 cells were harvested by scraping and aliquoted into 12-well tissue culture plates (0.5–1.0 × 10^6 cells/well) or 15 cm diameter dishes (1.0–1.5 × 10^6 cells/dish) in RPMI 1640 (without HEPES) supplemented with penicillin 100 IU/ml, streptomycin (100 μg/ml), and 10% fetal calf serum. On the next day, the cells were washed three times with DPBS to remove serum, and the medium was replaced with RPMI 1640 supplemented with antibiotics and ITS+ (1:100). Conditioned media derived from RAW264.7 cells were centrifuged to remove cellular elements and stored (−20 °C) until used for plasminogen activator activity. Following the recovery of conditioned media, monolayers were washed and dissolved in 0.2 N NaOH, and cellular protein was determined (26).

Determination of Plasminogen Activator Activity—Plasminogen activator (PA) activity in macrophage-conditioned media was quantitated utilizing a functional assay for plasmin (22). Briefly, the conversion of plasminogen to plasmin was monitored by quantitating the generation of acid-soluble [3H]acylpeptides in the presence and absence of plasminogen normalized for cellular protein. Conditioned media, monolayers were removed and assayed for the presence of labeled proteases secreted by the cells. Conditioned media (500 μl) were mixed with 70% ethanol containing 0.3 M sodium acetate, and stored at −20 °C. Following neutralization with HCl, conditioned media were preincubated with anti-uPA IgG, plasminogen, and aprotinin to stimulate macrophage uPA expression (22). The conditioned media were preincubated with H-7 or actinomycin D on control and polyanion- or PMA-induced uPA expression, individual comparisons were performed by t test. Statistical Analysis—The effect of preincubation of macrophages with either H-7 or actinomycin D on control and polyanion- or PMA-induced uPA expression was compared utilizing a two-factor analysis of variance. Following the determination of a significant overall effect of H-7 or actinomycin D on polyanion uPA expression, individual comparisons were performed by t test.

RESULTS

Ligands of the Scavenger Receptor Stimulate Macrophage Expression of uPA Activity—Plasminogen activator activity in the media of RAW264.7 cells incubated with the highly polyanionic ligands of the scavenger receptor fucoidan (a sulfated polymer of L-fucose derived from marine algae), dextran sulfate, or polyinosinic acid was increased 6–10-fold as compared with control cells (Fig. 1). When conditioned media were preincubated with anti-uPA IgG, plasminogen activator activity was inhibited >90%. Polyadenylic and polycytidylic acids, which are not recognized by the scavenger receptor, did not stimulate RAW264.7 expression of uPA (16). Treatment of cells with the above compounds did not affect the expression of macrophage uPA activity. Moreover, we report previously that chondroitin sulfate and dermatan sulfate, which are not recognized by the scavenger receptor, also failed to stimulate macrophage uPA expression (22).

Cell Signaling Pathway Responsible for Polyanion-induced UPA Secretion—We utilized two complementary approaches to determine the major cell signaling pathway responsible for the polyanion-induced expression of macrophage uPA activity. First, the cell signaling pathways associated with RAW264.7 uPA expression were identified utilizing specific
agonists. Second, the ability of specific inhibitors to block or stimulate the expression of uPA activity by cells pretreated with inhibitors of either cyclooxygenase (aspirin) or lipoxygenase (ETYA) was determined as described under "Experimental Procedures." Ctrl, control.

**TABLE I**

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<tr>
<th>Treatments</th>
<th>PA activity (dpm/mg x 10^-3)</th>
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<td>Ctrl</td>
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<td>Agonists</td>
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<td>Control</td>
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<td>Fucoidan (0.75 μM)</td>
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<td>PMA (0.1 μM)</td>
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<td>A23187 (5 μM)</td>
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<td>Dibutyryl cAMP (10 mM)</td>
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<td>Inhibitors</td>
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<td>Control</td>
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<td>Control + aspirin</td>
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<td>Control + ETYA (100 μM)</td>
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<td>Fucoidan + aspirin</td>
<td>1377</td>
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<td>Dibutyryl cAMP (10 mM)</td>
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FIG. 1. Anti-uPA IgG inhibits polyanion-induced expression of RAW264.7 PA activity. Aliquots of conditioned media (40 μl) derived from cells (0.5 x 10⁶/well) exposed for 6 h to 0.75 μM fucoidan, 100 μg/ml dextran sulfate (DxSO₄), or polyinosinic acid (Poly-I) were preincubated with anti-uPA IgG (50 μg) or normal IgG (nIgG) overnight at 4°C. The PA activity in the samples was determined as described under "Experimental Procedures." Ctrl, control.

FIG. 2. Dose-dependent inhibition of fucoidan-induced expression of RAW264.7 PA activity by auranofin. Cells (10⁶/well) were preincubated with 0-40 μM auranofin in 10% FCS for 1 h. The preincubation media were replaced with RPMI 1640-ITS+ containing auranofin and fucoidan (0.75 μM) (●) or auranofin alone (○). Media were collected after 6 h and assayed for PA activity.

FIG. 3. H-7 inhibits fucoidan and PMA-induced RAW264.7 PA activity. Cells (0.5 x 10⁶/well) were preincubated with H-7 (10 μM) for 30 min in the presence of 10% FCS. The preincubation media were replaced with RPMI 1640-ITS+ containing H-7 prior to the addition of fucoidan (0.75 μM) or PMA (0.1 μM). Media were collected 6 h later and assayed for PA activity. Data represent the mean ± S.D. of four separate wells. Ctrl, control.

It was recently demonstrated that auranofin, an antirheumatic drug, inhibits protein kinase C (32) and PMA-induced uPA secretion by macrophages (33). The dose-dependent ability of auranofin to inhibit polyanion-induced expression of uPA activity by macrophages is clearly demonstrated in Fig. 2. In contrast to polyanion-induced uPA expression, constitutive uPA expression was unaffected by auranofin. However, media derived from cells exposed to greater than 10 μM auranofin exhibited a significant increase in plasmin-independent proteolytic activity, which was subtracted from proteolytic activity observed in the presence of plasminogen in order to determine uPA activity (see "Experimental Procedures"). The release of these proteases from RAW264.7 cells reflects auranofin-induced cell injury. This is corroborated by the reduction in recoverable cellular protein from wells treated with the higher concentrations of auranofin and is consistent with the results of Lison et al. (33).

Since auranofin was injurious at higher concentrations, the putative role of protein kinase C in polyanion and PMA-induced uPA secretion was further examined utilizing H-7, another inhibitor of protein kinase C (34). As demonstrated in Fig. 3, exposure of cells to fucoidan or PMA enhanced their expression of uPA activity 4- and 10-fold, respectively. When cells were preincubated with 10 μM H-7, the ability of both fucoidan and PMA to stimulate the expression of uPA activity was inhibited 50% (p = 0.003) and 60% (p = 0.0001), respectively. Exposure of cells to H-7 did not lead to the release of plasminogen in extracellular fluids. These results are consistent with the demonstration that treatment of macrophages with H-7 inhibits fucoidan- and PMA-induced increases in plasmin activity (33).

Since both ionophore and dibutyryl cAMP were ineffective, the ability of both fucoidan and PMA to stimulate uPA secretion by auranofin was examined utilizing H-7. The dose-dependent inhibition of fucoidan-induced expression of RAW264.7 PA activity by auranofin was determined as described under "Experimental Procedures." Ctrl, control.
plasmin-independent proteases, as observed with auranofin, or affect recoverable protein per well. This is the first demonstration that the protein kinase inhibitor H-7 inhibits PMA or polyanion-induced macrophage uPA activity.

The Effect of Protein and RNA Synthesis Inhibitors on Polyanion- and PMA-induced uPA Secretion—Macrophage release of uPA following exposure to polyanions may reflect secretion of preformed uPA and/or the induction of uPA synthesis and its secretion. Therefore, we tested if an inhibitor of protein synthesis (cycloheximide) would affect polyanion-induced uPA secretion. The ability of cycloheximide to inhibit protein synthesis was tested by monitoring the incorporation of [3H]leucine into cellular protein by RAW264.7 cells over a 6-h period (Fig. 4). In the absence of cycloheximide, cellular incorporation of [3H]leucine increased linearly over the experimental period. In contrast, when cells were preincubated with cycloheximide (1 μg/ml) for 30 min, the uptake of [3H]leucine was markedly inhibited.

As seen in Fig. 4, uPA activity in the media of cells exposed to fucoidan rapidly increased over the first hour of incubation and then leveled off. In contrast, uPA activity in the media of cells preincubated with cycloheximide increased over the first 15–30 min of incubation and then leveled off at approximately 50% of that observed in untreated cells. In a similar experiment, preincubation with cycloheximide reduced fucoidan-induced uPA secretion 75% and essentially eliminated constitutive and PMA-induced uPA activity (data not shown). These data suggest that polyanions induce the secretion of a preformed pool of uPA, which is followed by the secretion of newly synthesized uPA.

The observed inhibition of polyanion- and PMA-induced macrophage uPA activity by cycloheximide demonstrates the requirement of protein synthesis for their effects. However, it is not clear from these studies if fucoidan or PMA are affecting translation of a stable uPA message or uPA gene activity. Therefore, we determined whether an inhibitor of RNA synthesis (actinomycin D) would affect polyanion and PMA-induced RAW264.7 uPA activity. Exposure of RAW264.7 cells to fucoidan and PMA stimulated their expression of uPA activity 4- and 10-fold, respectively (Fig. 5). Constitutive uPA activity expressed by control cells was unaffected by preincubation with actinomycin D. uPA activity in media derived from cells treated with actinomycin D and challenged with fucoidan, dextran sulfate, or polyinosinic acid were reduced 30% (p = 0.03). In marked contrast, actinomycin D inhibited the ability of PMA to trigger the release of uPA >90% (p = 0.0001). Consequently, in contrast to PMA-induced uPA expression, which is nearly totally inhibited by cycloheximide or actinomycin D, fucoidan-induced uPA expression is only partially sensitive to inhibitors of both protein and RNA synthesis.

PMA Induction of uPA Gene Transcription—The effect of fucoidan and PMA on RAW264.7 cell uPA mRNA was determined by Northern blot hybridization utilizing a murine uPA cDNA probe (27). As illustrated in Fig. 6, steady state levels of macrophage uPA mRNA increased rapidly following exposure to either fucoidan or PMA. In fucoidan-treated cells, a demonstrable increase in uPA mRNA was observed after 30 min of exposure, and after 2 h, uPA mRNA levels were >6-fold over that observed in control cells. However, the increase in RAW264.7 uPA mRNA following exposure to fucoidan was not sustained. uPA mRNA levels returned to control levels after 8 h (Fig. 6). In PMA-treated cells, an increase in the steady state level of uPA mRNA was observed after 1 h and peaked after 4 h. Steady state levels of uPA mRNA dropped sharply over the next 4 h, consistent with other reports (35, 36), but were still elevated over controls. The increase in steady state levels of uPA mRNA observed in cells treated with either fucoidan or PMA was inhibited >90% when cells were preincubated with the protein kinase C inhibitor H-7 (data not shown).

The time-dependent accumulation of uPA activity in the media of RAW264.7 cells exposed to fucoidan or PMA is presented in Fig. 7. uPA activity in the media of cells exposed to fucoidan is detected as early as 15 min following stimulation. Fucoidan-induced uPA expression peaks between 2 and 4 h and either remained steady or declined slightly by 6–8 h. In contrast, uPA activity in media of cells exposed to PMA did not increase until after 2 h of stimulation and continued to increase over the experimental period.

Fucoidan Affects uPA Activity—Polyanions have been demonstrated to enhance the conversion of plasminogen to plasmin by both uPA and tissue plasminogen activator (37–39). Since we have utilized a functional assay for uPA activity that depends on the ability of uPA to convert plasminogen to plasmin, we determined whether fucoidan directly affected
Regulation of Macrophage uPA Secretion

Fig. 6. Time-dependent effect of fucoidan and PMA on steady state levels of RAW264.7 uPA mRNA. Cells (15 × 10^5/flask) were exposed to fucoidan (0.75 μM) (A) or PMA (0.1 μM) (B) in RPMI 1640-ITS+ for 15 min to 8 h. Total RNA was isolated, and uPA mRNA levels were determined by Northern blot hybridization utilizing a murine uPA cDNA probe. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Fig. 7. Time-dependent effect of fucoidan and PMA on the expression of RAW264.7 urokinase activity. Cells (0.5 × 10^5/well) were exposed to 0.75 μM fucoidan (○) or 0.1 μM PMA (○) in RPMI 1640-ITS+ for 6 h. Media were recovered and assayed for PA activity.

The activity of uPA expressed by control cells without stimulating the cellular release of more uPA, uPA activity in media derived from cells incubated with 0.75 μM fucoidan for 6 h was 819 ± 81 dpm/mg × 10^-3 (±SD; n = 4). This was an 8-fold increase over uPA activity observed in media from control cells (111 ± 16). If fucoidan was added back to control cell media, there was a relatively small but significant (t = 3.64; p < 0.02) enhancement of the observed uPA activity (254 ± 76). As reported previously (24), media harvested from control cells between 15 min and 4 h contained low levels of uPA activity that was unaffected by the addition of fucoidan. Moreover, fucoidan did not affect plasmin activity or the activation of single chain uPA by plasmin (data not shown).

uPA Binds to Fucoidan—It has been demonstrated that heparin can bind to either tPA or uPA and enhances their catalytic activities (37–39). Therefore, we determined whether highly charged polyanions, capable of stimulating macrophage uPA expression, would also bind uPA. The ability of fucoidan to bind uPA was examined by monitoring the electrophoretic migration of ^125^I-HMW-uPA in an uncharged gel medium. As demonstrated in Fig. 8, labeled HMW-uPA remains at the origin following electrophoresis. When ^125^I-HMW-uPA was preincubated with fucoidan, the migration of uPA was dramatically shifted toward the anode. As we have demonstrated previously for fibronectin (39) and transforming growth factor β (30), polyanion-induced shifts in the electrophoretic migration of proteins to which they bind appear to be dependent on the concentration of the polyanion. As seen in Fig. 8, as little as 2 ng (~0.015 pmol) of fucoidan effectively altered the migration of 10 ng (0.182 pmol) of HMW-uPA. However, huge molar excesses of fucoidan continue to effect an anodic shift in the migration of ^125^I-HMW-uPA. These data suggest that fucoidan is functionally heterogeneous with respect to its ability to bind labeled uPA. Consequently, either the fraction of polyanion that binds HMW-uPA is very small, or HMW-uPA selectively binds the highest charged molecular species when a higher concentration of polyanion is available.

When HMW-uPA-fucoidan complexes were first electrophoresed in Isogel and then overlaid with an indicator gel containing casein and plasminogen, zones of lysis in the indicator gel became apparent (Fig. 9). The lytic zones in the indicator gel identify the position of uPA in the separating gel below. As seen in Fig. 9, free uPA remains at the origin following electrophoresis. When uPA is preincubated with fucoidan, the lytic zones are shifted toward the anode and spread over a greater area (hence appearing lighter). These
data corroborate the autoradiogram (Fig. 8) and demonstrate that fucoidan-uPA complexes are active. Consequently, we can conclude that fucoidan does not bind to the active site on the B-chain of HMW-uPA. Moreover, we determined that the binding of fucoidan to HMW-uPA did not involve the amino-terminal fragment of the A-chain, since the effect of fucoidan on the electrophoretic migration of a variant of uPA (LMW-uPA) devoid of this region was indistinguishable from that observed with HMW-uPA (data not shown).

**DISCUSSION**

A diverse group of highly charged polyanions have been demonstrated to trigger macrophage secretion of uPA (21, 22, 24). In experiments reported here, we have sought to define both intracellular and extracellular regulatory mechanisms operational in polyanion-induced macrophage plasminogen activation.

Secretion of uPA has been demonstrated to be regulated by protein kinase C and cyclic AMP dependent pathways (16, 35, 36, 40). We sought to determine if either of these pathways were involved in polyanion-induced uPA expression. When examined for their ability to stimulate macrophage uPA activity, both highly charged polyanions and PMA were very effective (Table I). The ability of PMA to induce macrophage release of uPA activity was first described by Vassalli et al. (20). Since then, it has become clear that PMA, in a manner similar to diacylglycerol, affects many cellular functions by binding to and activating protein kinase C (41, 42). In contrast to fucoidan or PMA, exposure of RAW264.7 cells to either calcium ionophore or dibutyryl cAMP had no effect on cellular expression of uPA activity (Table I). These data suggest that the effect of polyanions on uPA activity is not likely due to alterations in the permeability to calcium, adenyl cyclase activity, or arachidonic acid metabolism; rather it would appear that a disparate group of highly charged polyanions may affect protein kinase C-dependent pathways.

In order to determine if polyanion-induced uPA expression was dependent on protein kinase C, we utilized specific inhibitors. Froscio et al. (32) recently reported that the antirheumatic drug auranofin inhibits protein kinase C by interacting with thiols present in the catalytic portion of the enzyme. Moreover, auranofin inhibits macrophage response to a variety of agonists (43–46), including PMA-induced release of uPA activity (33).

As reported here, auranofin inhibited fucoidan-induced uPA secretion in a dose-dependent manner without affecting constitutive uPA activity expressed by control cells (Fig. 2). However these data, as well as previously reported data, must be interpreted with caution. As demonstrated here and reported by Lison et al. (33), auranofin exhibited cytotoxic effects at concentrations greater than 10 μM. Consequently, we examined the effect of H-7, another inhibitor of protein kinase C (47, 48), on both PMA and polyanion-induced uPA secretion. Preincubation of cells with H-7 significantly inhibited (50–60%) PMA and fucoidan-induced secretion of uPA (Fig. 3). As we observed with auranofin, H-7 did not appear to affect constitutive uPA expression. In contrast, preincubation of RAW264.7 cells with inhibitors of either cyclooxygenase or lipoxygenase had no effect on PMA or polyanion-induced uPA expression (Table I). We have concluded from these experiments that polyanions stimulate macrophage expression of uPA through a protein kinase C-dependent mechanism.

When macrophages are exposed to fucoidan, uPA activity in their media is observed to increase in as little as 15 min (Figs. 4 and 7). In contrast, induction of uPA expression with PMA required at least 2 h. The rapid response to fucoidan suggests that polyanions may stimulate the secretion of a storage pool of uPA. Therefore, we examined the effect of cycloheximide on polyanion-induced uPA expression. When fucoidan-induced uPA expression was examined over time, we observed that the initial release of uPA was unaffected by cycloheximide, despite the inability of these cells to incorporate [3H]leucine into protein (Fig. 4). In contrast, preincubation of macrophages with cycloheximide completely blocked the release of uPA by control cells and cells stimulated with PMA. Recently, Tranquille and Emens (49) demonstrated that protein synthesis was not necessary for platelet-activating factor or bradykinin-stimulated release of tPA from perfused rat hindlimb. Together, these results suggest that cells possess intracellular storage forms of plasminogen activator that can be released upon acute stimulation. However, these data also clearly demonstrate that fucoidan-induced uPA activity is partially inhibited by cycloheximide and therefore requires protein synthesis.

Since RAW264.7 constitutively express uPA activity, we determined whether polyanion-induced stimulation of uPA expression was due to enhanced translation of a stable message or if it required transcription. For this purpose, cells were preincubated with actinomycin D, an inhibitor of RNA synthesis, and then challenged with either fucoidan, dextran sulfate, polyinosinic acid, or PMA. Whereas actinomycin D inhibited PMA-induced uPA expression >90%, polyanion-induced uPA expression was inhibited 30% (Fig. 5). When the effect of polyanion and PMA on the uPA message was examined by Northern blot hybridization, uPA mRNA levels in cells exposed to fucoidan more than doubled after 30 min, peaked after 2 h (6.5-fold increase), and then declined (Fig. 6). PMA induced an order of magnitude greater increase in uPA mRNA levels (66.4-fold) than that observed with fucoidan. Furthermore, preincubation of cells with an inhibitor of protein kinase C effectively blocked the ability of either fucoidan or PMA to induce an increase in uPA mRNA.

Taken together, these data demonstrate that polyanion-induced uPA expression is partially dependent on transcription and protein synthesis, whereas the increased expression of uPA activity by cells exposed to PMA is completely dependent on transcription and protein synthesis. It appears that polyanions initially stimulate the release of a preformed pool of uPA. This interpretation is supported by the observation that fucoidan-induced uPA activity was detected in 15 min, whereas PMA-induced activity was not detected until 2 h (Fig. 7).

Polyanions capable of stimulating uPA secretion may affect macrophage plasminogen activation by other mechanisms. For example, it has been demonstrated that the ability of uPA and tPA to convert plasminogen to plasmin is enhanced by heparin (37–39). Recently the structural domains of tPA possessing heparin binding properties were detected, and located in the finger region and the second kringle (50). However, others have identified the catalytic portion of tPA as possessing heparin binding properties (51). In these studies, we have demonstrated that fucoidan had a small but significant stimulatory effect on uPA activity released by macrophage. In addition, fucoidan was shown to bind to uPA (Figs. 8 and 9). Binding was not mediated by either the receptor-binding domain or the kringle regions of the A-chain of HMW-uPA, since LMW-uPA bound fucoidan as well as intact HMW-uPA. These results corroborate our early finding demonstrating that fucoidan does not trigger the release of receptor-bound uPA by binding to its receptor-binding site (24). As expected, binding of polyanions was not mediated by
the active site of uPA located on the B-chain, since uPA-polyanion complexes were active (Fig. 9). Therefore, it is likely that polyanions capable of inducing uPA expression by macrophage will also affect uPA activity in the extracellular space.

REFERENCES