Tumor-promoting phorbol esters and histamine induce tissue plasminogen activator (tPA) release from human endothelial cells in a dose- and time-dependent manner. Phorbol myristate acetate (PMA) and phorbol dibutyrate (PDBu) increased tPA concentration in the culture medium by eight to 12 times after 24 h with half-maximal stimulation at 1-3 nM, respectively. Maximum release by histamine was only half that of the phorbol esters and required 18 \mu M for half-maximal response. Kinetics of enhanced release was similar with both types of agonists: a 4-h lag period followed by a period of rapid release (4 h in PMA-treated and 10 h in histamine-treated cultures) followed by a decline toward pretreatment rates. The PMA and histamine effects were additive while histamine and thrombin, which also stimulates tPA release in human endothelial cells, were no more effective together than they were alone. Exposure of the cells to PMA, PDBu, or phorbol 12,13-didecanoate caused a loss of responsiveness to second treatment of the homologous agent that was time- and dose-dependent, sustained, and specific to active tumor promoters (half-maximal desensitization = 52 nM PDBu). A partial desensitized state was also established by histamine which resulted in a 60% lower response to a second challenge dose. Histamine-induced desensitization did not interfere with the PMA response. However, PMA-induced desensitization caused a 75% loss of the histamine and a 67% loss of the thrombin effects. These studies indicate that tumor promoters are potent agonists of tPA release from human endothelial cells and establish a desensitized state to further stimulation. Treatment of these cells with histamine has similar effects which may be mediated at least in part by pathways common to phorbol ester stimulation.

Tissue plasminogen activator (tPA) is produced by cultured cells from a variety of sources and its production can be regulated by hormones (1–3), tumor promoters (4–6), serine proteases (7), and mediators of inflammation (8). Among the cells producing tPA is the endothelial cell (9, 10). One of the proposed functions of the vascular endothelium is to maintain or to re-establish a homeostatic environment through a variety of pathways including the production and release of tPA. In vivo, tPA levels are elevated following exercise, venous occlusion, or infusion of DDAVP (11, 12). The appearance of tPA is rapid and transient with the initial increase occurring at or before 30 min and returning to base line by 5–6 h. This pattern of tPA increase and decrease in blood suggests that it results from a release of stored tPA. In contrast, stimulation of tPA release from cultured human endothelial cells by thrombin is much slower, requiring 4–8 h to begin (7). This is followed by 12–16 h of rapid release and then a decline to base-line levels. This response pattern is similar to that observed in granulosa cells following administration of luteinizing hormone (2).

The decline in the rate of tPA release 12–16 h after thrombin treatment is reminiscent of events which follow phorbol ester-mediated stimulation of various metabolic events. Treatment with these tumor promoters is followed by a refractory period during which the initial response is terminated and a second treatment has little, if any, additional effect (13–18). Establishing a desensitized state by and to phorbol esters may or may not lead to a loss of responsiveness to a second unrelated agonist, and these results have been used to define common pathways by which the response of interest is regulated (14, 15). Because protein kinase C appears to be the major cellular receptor for phorbol esters (19–22) and the loss of protein kinase C activity accompanies the loss of responsiveness to these tumor promoters (23–27), the role of this enzyme in the control of the synthesis and release of certain proteins can be assessed. Employing phorbol esters and histamine, we have investigated whether the loss of continued tPA release is due to desensitization of human endothelial cells and whether the response to the tumor promoters and the other agonists is mediated through similar pathways.

**EXPERIMENTAL PROCEDURES**

**Materials**

Tissue culture medium was purchased from MA Bioproducts, endothelial cell growth factor (ECGF) from Biotechnologies Incorporated, heparin, cinetidine, and diphenhydramine from Sigma, fetal calf serum from Reheis, and NuSerum from Collaborative Research. Phorbol esters were obtained from Behring Diagnostics and were dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide added to cultures never exceeded 0.1% and had no effect on tPA release. Human tissue plasminogen activator was a gift from Dr. Desire Collen (University of Leuven, Leuven, Belgium), and antiserum against tPA was raised in rabbits as previously described (28). The IgG fractions were isolated by ammonium sulfate precipitation and DEAES-cellulose chromatography, and affinity purified anti-tPA was prepared by passing 2 ml of the IgG fractions through a tPA-Sepharose 4B column containing 600 \mu g of tPA (7). Immunoaffinity-purified antibodies were labeled with \[^3^2^P\]I by the lactoperoxidase method (29). Human \(\alpha\)-thrombin was a gift from Dr. J. W. Fenton II (New York Department of Health) and had a specific activity of 3642 units/mg thrombin.
Desensitization of tPA Release

Methods

Cell Culture—Endothelial cells were isolated from human umbilical cord veins as described previously (7) and were cultured into 75-cm² tissue culture flasks or 12-well dishes coated with 20 mg/ml calf skin gelatin. Cells were grown to confluence in RPMI 1640 containing 10% fetal calf serum, 200 units/ml penicillin, 200 µg/ml streptomycin, 10 µg/ml ECGF, and 90 µg/ml heparin. Passaged cells were subcultured into 12-well dishes and allowed to grow to confluence under the same conditions as primary cultures except that 50 µg/ml ECGF was used. All experiments employed either primary or once passaged cultures. Cell density at confluence was 6 × 10⁵ cells/cm².

Studies were performed by washing confluent cultures twice with RPMI 1640 and incubating cultures at 37 °C in 0.5 ml of medium containing 5% NuSerum (final serum concentration = 1.25% newborn calf serum), 50 µg/ml ECGF, 90 µg/ml heparin, and the indicated agonist. The presence of ECGF and heparin had no effect on the stimulation of tPA release by any of the compounds employed. Desensitization experiments were performed by treating confluent cultures with a primary agonist for the time indicated followed by incubation in agonist-free medium containing 5% NuSerum (final serum concentration = 1.25% newborn calf serum), 50 µg/ml ECGF, 90 µg/ml heparin, and the indicated agonist. The presence of ECGF and heparin had no effect on the stimulation of tPA release by any of the compounds employed. Desensitization experiments were performed by treating confluent cultures with a primary agonist for the time indicated followed by washout of the medium and washing four times with RPMI 1640 maintained at 37 °C. The second agonist was added in fresh medium.

Control cultures in these experiments consisted of 1) treatment with the primary agonist followed by agonist-free medium, 2) treatment with medium alone followed by the second agonist, and 3) treatment with medium alone during both incubation periods. The conditions and times of incubation were identical to experimental cultures. The conditioned medium was centrifuged at 15,000 × g to remove all debris, made 0.01% Tween 80, and frozen at -70 °C until used. To ensure that the number of cells within different wells was consistent, cells were removed periodically by trypsinization and counted. The cell number of each well fell within 10% of the calculated mean at the times tested.

Radioimmunoassay—Rabbit anti-human tPA IgG at a final concentration of 10 µg/ml in 50 mM sodium borate buffer, pH 9.0, was added to microtiter wells in a total volume of 150 µl and allowed to incubate overnight at 4 °C. The contents of the wells were removed, and the wells were washed with PBS (0.05% Tween 20, 0.1% albumin) twice and stored wrapped at -20 °C. Samples and tPA standards (100 µl final, diluted in RPMI 1640, 5% NuSerum) were incubated in the antibody-covered wells for 5 h at room temperature on a tilting table. The samples were removed and the wells washed with wash buffer three times, cut from the plate, and counted. Standard curves were analyzed by log transformation and linear regression analysis of logit percent bound counts as a function of log tPA concentration. Best fitting lines were determined by computer. Standard curve values in the range of 0.2-25 ng/ml were employed. Samples not falling into the linear portion of the standard curve were diluted accordingly.

RESULTS

Dose Titration and Time Course of tPA Release in Response to Phorbol Esters and Histamine—To compare the effect of various tumor promoters on tPA release, dose titration experiments were performed with increasing concentrations of PMA, PDBu, PDD, and 4-α-PDD (Fig. 1). Both PMA and PDBu stimulated the largest increases in tPA concentration after 24-h incubation (8-12-fold), although the concentrations needed to achieve half-maximal levels were four times higher with PDBu than with PMA (55 versus 13 nM). With PDD, the highest concentration of tPA attained was 60% that of PMA-treated cultures (15 ng/ml) with half-maximum stimulation occurring at 18 nM, while 4-α-PDD, a phorbol ester with no tumor-promoting activity (19), had no effect at all. The concentration dependence of histamine-stimulated tPA release was also determined (Fig. 1). Cultures treated with increasing concentrations of histamine (0.1-100 µM/ml; 0.9-900 µM) were stimulated to release increasing levels of tPA with a maximum concentration of 11.0 ± 2.6 ng/ml (range 6-13 ng/ml) in 16 h. Half-maximal stimulation was achieved with 18 µM histamine, over 1000-fold greater than PMA while generating about one-half the tPA concentration.

Compounds that have been identified as specific H1 and H2 histamine receptor antagonists (30, 31) were employed to identify the endothelial cell receptor through which the histamine-mediated stimulation occurred (Table I). The H1 receptor antagonist diphenhydramine reduced the final level of tPA in a dose-dependent manner, while cimetidine, a H2 receptor antagonist, had only a small effect. Diphenhydramine at concentrations that abolished the response to histamine did not interfere with the stimulation by PMA.

Time course studies of tPA release showed similar kinetics of accumulation with either 10⁻⁶ or 10⁻⁵ M PMA (Fig. 2). The accumulation of tPA in the medium began several hours after agonist addition, rose rapidly, but began to decline shortly after. The average rate of release in 10⁻⁵ M PMA-treated cultures was 2.4 ng/ml/h from 4-8 h, 1.1 ng/ml/h from 8-18 h, and 0.4 ng/ml/h from 16-24 h. The pattern was identical with 10⁻⁶ M PMA-treated cells with corresponding values of 1.1 ng/ml, 0.7 ng/ml, and 0.2 ng/ml, respectively. PMA-treated cells continued to release tPA at an accelerated rate between 24 and 40 h (0.19-0.38 ng/ml/h). Untreated cultures released tPA at an average rate of 0.12 ng/ml/h over a 20 h period. PDBu-treated cells showed similar kinetics of release during the same time period (data not shown). Histamine on
we defined a fully desensitized culture as one in which the tPA concentration, after a second challenge, did not exceed the concentration of the primary agonist (Fig. 1). Therefore, desensitization was achieved with 1 μM PDBu, while cells treated with 100 and 10 nM PDBu released tPA after restimulation to about 45 and 75% of maximum, respectively. PDBu was as efficient a desensitizer as it was an inducer; half-maximal desensitization occurred at 52 nM as compared to the 55 nM needed for 50% induction. In addition to PMA and PDBu, treatment with 100 nM PDD also was followed by a refractory period while 4-α-PDD (1 μM) was ineffective at desensitizing the cells to 100 nM PMA (data not shown).

We took advantage of the reversibility of PDBu binding to cells (16, 32) to determine whether desensitization is established in a time-dependent manner (Fig. 4). [3H]PDBu could be removed from the cultures by four successive washes with RPMI 1640 maintained at 37 °C. Greater than 99% (99.5 ± 0.2%; four determinations) of 1 μM PDBu incubated with the cells for 2 h before washing was extracted by this procedure. PDBu (1 μM) was added to the cultures for 1, 2, 4, 6, or 8 h, removed by repeated washing at 37 °C, and replaced with desensitization was achieved with 1 μM PDBu, while cells treated with 100 and 10 nM PDBu released tPA after restimulation to about 45 and 75% of maximum, respectively. PDBu was as efficient a desensitizer as it was an inducer; half-maximal desensitization occurred at 52 nM as compared to the 55 nM needed for 50% induction. In addition to PMA and PDBu, treatment with 100 nM PDD also was followed by a refractory period while 4-α-PDD (1 μM) was ineffective at desensitizing the cells to 100 nM PMA (data not shown).

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Desensitization of tPA Release by Tumor Promoters and Histamine—The transient nature of the increase in tPA release is similar to drug-induced desensitization previously reported to occur following treatment with tumor-promoting agents (13, 15, 16). We examined whether these cells became desensitized to phorbol esters by exposing confluent cultures to several concentrations of PMA or PDBu for 20 h and re-exposing them to an optimal dose (100 nM PMA or 1 μM PDBu) of the homologous agonist for an additional 20 h (Fig. 3). The results are presented as a percent of the maximum tPA concentration released from fully induced cells. These studies were complicated by the tendency of phorbol ester-treated cultures to continue to release tPA at an accelerated rate during the second 20 h even after the initial dose was removed and replaced with medium free of the promoter. The amount of tPA released during this time was dependent on the concentration of the primary agonist (Fig. 3). Therefore, we defined a fully desensitized culture as one in which the tPA concentration, after a second challenge, did not exceed the level in cultures receiving no second treatment.

When cells pretreated with 100 nM PMA were rechallenged with an optimal dose of PMA, no response to the second addition was detected: the concentration of tPA at the end of the second incubation was no greater than that in cultures not re-exposed. This was observed whether the PMA was added in fresh medium or added directly to the original medium. In contrast, 10 nM PMA, which is capable of partially stimulating tPA release (to about 45% of maximum) (Fig. 1), did not desensitize the cells to the additional treatment. Close to 100% of the fully induced level of tPA was released into the medium at the end of the incubation period. Similar results were observed with PDBu. In this case, however, a much greater concentration range was involved.

the other hand stimulated a slightly different response than the phorbol esters (Fig. 2). While a lag period of the same length as PMA-treated cells was observed, the duration of accelerated tPA release was more prolonged, continuing until 14 h after histamine addition. At this time, tPA release returned to basal rates. The decline in tPA release was not due to limiting amounts of histamine since no additional tPA was released after 14 h when a concentration of histamine 10-fold greater than that employed in Fig. 2 was used.

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Complete desensitization was achieved with 1 μM PDBu, while cells treated with 100 and 10 nM PDBu released tPA after restimulation to about 45 and 75% of maximum, respectively. PDBu was as efficient a desensitizer as it was an inducer; half-maximal desensitization occurred at 52 nM as compared to the 55 nM needed for 50% induction. In addition to PMA and PDBu, treatment with 100 nM PDD also was followed by a refractory period while 4-α-PDD (1 μM) was ineffective at desensitizing the cells to 100 nM PMA (data not shown).

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fresh medium free of PDBu for 20 h and then challenged with
1 μM PDBu for an additional 20 h. The loss of responsiveness
to a challenge dose of PDBu was time-dependent and even
after 8 h complete desensitization was still not established
(half-maximal desensitization occurred after ~5 h; Fig. 4). It
is not likely that these results are due to small amounts of
PDBu that are retained after the washing procedure since the
remaining concentration predicted by our [3H]PDBu extrac-
tion experiments (less than 10 nM) would be too low to
desensitize the cultures to the levels observed (Fig. 3).
To determine whether loss of responsiveness was restricted
to tumor promoters or included non-phorbol ester agonists
such as histamine, cultures were treated with 0.1–100 μg/ml
histamine and then re-exposed to 10 μg/ml histamine for 20
h (Fig. 5). The cells did not continue to release tPA at elevated
levels after histamine was removed. Complete desensitization
was not achieved even at the highest concentration of hista-
mine employed. At 100 μg/ml, the cells were still capable of
responding to a second dose, although to only 35% of the
maximum. Decreasing the concentration of the first dose of
histamine resulted in a greater percent release upon restimu-
lation. Half-maximal desensitization occurred at 27 μM, 1.5
times the concentration inducing 50% maximal release.

**Heterologous Desensitization to PMA, Histamine, and Thrombin**—To determine how tPA release would be affected by histamine and PMA in combination, histamine (10 μg/ml) and PMA (10 and 100 nM) were added to the cultures simul-
taneously and the tPA concentration after 16 h compared to
samples from cultures treated with each agonist separately.
As shown in Table II, an additive effect was observed when the
cells were treated with either concentration of PMA and
histamine. An additive effect was also seen when PDBu was
used in place of PMA (data not shown).

Thrombin has been shown to induce enhanced release of
tPA from human endothelial cells: the maximum tPA levels
achieved and the time course of release are very similar to
that reported here for histamine (7). Thus, the response of
tPA release to two non-tumor-promoting agents whose inter-
action with the cell membrane is presumably mediated
through distinct sites could be examined. In these experi-
ments, histamine was slightly more stimulatory than throm-
bin (7.1 versus 5.4 ng/ml; 16 h). However, in contrast to the
additive effects of PMA and histamine, the final level of tPA
after histamine and thrombin treatment did not surpass that
of histamine alone (7.1 versus 7.2). This is in comparison to
an expected value of 12.5 ng/ml if the effect was additive.

The effect of desensitization on the stimulation of tPA
release by heterologous agonists was examined (Fig. 6). These
cultures were first treated with either 100 nM PMA or 10 μg/
ml histamine for 20 h and then re-exposed to a heterologous
agonist. Treatment of the cells with histamine had no effect
on the subsequent stimulation by the phorbol ester; greater
than 90% of the tPA released with PMA alone was present
after pretreatment with histamine. Histamine-induced desen-
sitization then has no effect upon the ability of PMA to
stimulate tPA release. The cells were also capable of respond-
ing to histamine or thrombin following PMA treatment. In
this case, however, the tPA levels generated were even higher
than the maximum levels induced by histamine or thrombin
alone (i.e. those treated with culture medium alone instead of

![Fig. 4. Desensitization of tPA release to PDBu following short-term exposure.](image)

![Fig. 5. Effect of histamine pretreatment on histamine-induced release of tPA.](image)

**TABLE II**

| Treatment               | tPA    | Additive concentration |
|-------------------------|--------|------------------------|
| PMA (100 nM)            | 17.4 ± 0.5 |
| PMA (10 nM)             | 11.3 ± 2.0 |
| Histamine               | 7.3 ± 0.3  |
| Thrombin                | 5.4 ± 0.2  |
| PMA (100 nM) + histamine| 25.2 ± 6.6 | 103 |
| PMA (10 nM) + histamine | 16.0 ± 1.8 | 90  |
| Thrombin + histamine    | 7.2 ± 0.8  | 57  |
Desensitization of tPA Release

We have demonstrated that stimulation of tPA release from human endothelial cells by either phorbol esters or histamine is transient and is succeeded by a phase during which the cells are refractile to additional stimulation by the homologous agonist. This series of events in response to phorbol esters is an example of drug-induced desensitization (14, 16, 27) and has also been described with non-tumor-promoting compounds such as vasopressin (14) and gonadotropin-releasing hormone (15).

In the studies presented here, the tumor promoters were active at nanomolar concentrations similar to the doses required to induce other biological responses in platelets, cultured cells, and in vivo. PMA treatment resulted in a lag period that lasted for 4 h followed by a rapid rise in tPA concentration. The maximum rate of release in PMA-treated cultures was short-lived (4-8 h) and declined over the next 8 h by 50% (average rate of release from 8 to 16 h) for both concentrations tested. This lag period and the eventual decline in tPA release is similar to that observed in rat granulosa cells treated with luteinizing hormone (2). Differences in this time course do occur under other conditions. For example, follicle-stimulating hormone stimulated tPA release in granulosa cells does not include a lag period but does show a decline after 8 h (2), while PMA-treated HeLa cells demonstrate the lag period but continue to release tPA at elevated levels indefinitely (5). Thus, neither the lag period nor the short-term response is a universal feature of tPA induction. Cycloheximide studies have suggested that this lag period allows for the synthesis of a protein(s) that is necessary for the increase in the steady-state levels of tPA mRNA leading to increased tPA synthesis and release (2, 5).

Stimulation of tPA release by histamine also followed a similar time course to that in response to PMA. However, several differences between histamine and PMA-mediated stimulation were observed. Most notable was the difference in the maximum concentration of tPA released after 16 h. tPA levels in PMA-treated cultures were consistently twice as high as those in histamine-treated cultures. In addition, the phorbol esters were much more efficient at generating these higher levels than histamine: half-maximal release occurred at concentrations 300-1000 times lower than that needed for histamine. Finally, the decline in tPA release during continued stimulus was more complete with histamine than the phorbol esters. Instead of the gradual decline in the rate of tPA release observed with PMA, histamine-treated cells returned to base-line rates more quickly. This was apparently not due to an inability of the cells to continue synthesizing tPA since exposure to PMA after histamine restimulated release (Fig. 5).

The effect of PMA on tPA release and the occurrence of a refractory period following treatment is most likely mediated through protein kinase C, which has been identified as the major cellular receptor for phorbol esters and through which these compounds exert their effects (19-22). Much evidence also exists to associate desensitization with the loss of protein kinase C activity and the level of immunoprecipitable material (15, 25). That protein kinase C is mediating the effect described here is supported by our observations that three of the tumor promoters tested, which are known to bind to the same high affinity binding sites (protein kinase C), were effective in stimulating tPA release and establishing desensitization while the phorbol ester which does not bind protein kinase C was not. In each case, the effect of pretreatment was time- and dose-dependent, sustained, and specific to the active tumor promoters.

The pathway by which histamine stimulates tPA release is less defined; however, it may also involve activation of protein kinase C. Treatment of endothelial cells with histamine leads to the breakdown of phosphoinositides leading to the formation of inositol triphosphate, an inducer of calcium mobilization, and diacylglycerol, an activator of protein kinase C (33, 34). While the effect of histamine treatment on thromboxane and prostacyclin synthesis, reduction in endothelial F-actin, and increased albumin diffusion across an endothelial cell barrier has been related to calcium mobilization (33, 35), we are not aware of any reports directly linking histamine to protein kinase C-mediated events, nor do the results presented here allow us to come to that conclusion. We have found that a loss of responsiveness to histamine has no effect on secondary stimulation by PMA, and simultaneous addition of optimum doses of both agonists leads to an additive increase in the level of tPA. These data alone can be interpreted as suggesting the response to phorbol esters and histamine are uncoupled and that desensitization of the histamine-stimulated pathway does not affect PMA activation of protein kinase C. However, this simple conclusion is compromised by our observation that the desensitized state established by PMA appears to reduce the secondary effect by histamine. Thus, cells refractory to phorbol esters also lose the ability to respond fully to histamine. These seemingly paradoxical results are not unique to these two agonists; phorbol esters...
desensitization of T3 cells to vasopressin-induced mitogenic stimulation while vasopressin, which desensitizes the cells to itself, has no effect on phorbol ester stimulation (14). Why maximum release of tPA by histamine (or thrombin) does not occur following desensitization to PMA is not clear. We have tested cell viability and it is not an issue, although the general metabolic capability of the cells may have been compromised during long-term incubation with PMA. Down-regulation of histamine receptors by PMA is a possibility and has been known to occur with other membrane receptors (36–40). Alternatively, the down-regulation in protein kinase C following PMA treatment may eliminate an effector pathway through which histamine transmits its effects. Since stimulation of endothelial cells by histamine involves pathways which include activation of protein kinase C (34), it could be affected by PMA-generated loss of protein kinase C activity leading to a partial abrogation of its effect. Histamine-induced desensitization (to itself) may occur through an entirely different pathway than the PMA-induced refractory period (41). It has been shown that the interaction of histamine with its H1 receptor on human endothelial cells results in its conversion to tele-methylhistamine which occurs upon contact. This biochemical modification eliminates histamine's biological activity at the H1 receptor, and without any additional metabolizing activity (diamine oxidase provided by an outside source) this intermediate metabolite is retained and accumulated by the cells. Thus, the onset of desensitization may occur at different levels.

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