Induction of tissue plasminogen activator secretion from rat heart microvascular cells by fM 1,25(OH)₂D₃

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We investigated the effects of 1,25-dihydroxyvitamin D₃ [25(OH)₂D₃] on tissue plasminogen activator (tPA) secretion from primary cultures of rat heart microvascular cells. After an initial 5-day culture period, cells were treated for 24 h with 1,25(OH)₂D₃ and several of its analogs. The results showed that 1,25(OH)₂D₃ induced tPA secretion at 10⁻¹⁰ to 10⁻¹⁶ M. A less calcemic analog, Ro-25–8272, and an analog that binds the vitamin D receptor but is ineffective at perturbing Ca²⁺ channels, Ro-24–5531, were ~10% as active as 1,25(OH)₂D₃. An analog that binds the vitamin D receptor poorly but is an effective Ca²⁺ channel agonist, Ro-24–2287, required ~10⁻¹³ M to induce tPA secretion. Combinations of Ro-24–5531 and Ro-24–2287 were approximately as potent as 1,25(OH)₂D₃. Treatment of the cells with BAY K 8644 or thapsigargin also increased tPA secretion, suggesting that increased cytosolic calcium concentration ([Ca²⁺]c) induces tPA secretion. The results suggested that the sensitivity of the tPA secretory response of microvascular cells to 1,25(OH)₂D₃ was due in part to generation of a vitamin D-depleted state in vitro and in part to synergistic effects of 1,25(OH)₂D₃ on two different induction pathways of tPA release.

VITAMIN D₃ through its hormonally active metabolite 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] is an essential component of the systems that regulate bone calcification (36), intestinal Ca²⁺ absorption (29), kidney function, and parathyroid gland activity (10). 1,25(OH)₂D₃ may also be important to functions of the immune system, heart, pancreas, brain, pituitary, cancer cells, and vascular endothelium (32, 48). Most effects of 1,25(OH)₂D₃ are thought to result from its binding to a cytosolic/nuclear receptor, similar to receptors of steroids, thyroid hormone, and vitamin A (14), which has a dissociation constant (Kᵣ) of 10⁻¹⁰ to 10⁻¹¹ M (40). The ligand-activated vitamin D receptor is a heterodimer with the retinoid (RXR) receptor that binds to vitamin D response elements in responsive genes (13).

Recent studies suggest that actions of 1,25(OH)₂D₃ may involve both genomic and nongenomic pathways (37). Nongenomic actions of 1,25(OH)₂D₃ are rapid and include a prolonged open time of voltage-gated Ca²⁺ channels and a shift in the threshold of activation toward the resting cell potential (7). Several cellular signal transduction pathways may be involved in nongenomic actions of 1,25(OH)₂D₃ such as those involving cAMP, protein kinase C, and inositol phosphate (12, 41, 46).

The vascular endothelium plays an essential role in the fibrinolytic system by virtue of the secretion of tissue plasminogen activator (tPA) (11, 44). tPA converts inactive plasminogen to the active serine protease plasmin that degrades the fibrin component of thrombi. The regulation of tPA secretion involves second messengers, such as calcium (42), protein kinase C, cAMP, and diacylglycerol (20), and may involve increased transcription of the tPA gene (38). Among the agents known to increase tPA secretion from the endothelium are thrombin and histamine (21), butyrate (27), phorbol-12-myristate-13-acetate (47), and retinoids (26).

Vascular endothelial cells contain the vitamin D receptor (32), but the function(s) of 1,25(OH)₂D₃ in the vasculature have not been examined. We decided to test the hypothesis that 1,25(OH)₂D₃ could regulate the release of PA from cultured rat heart microvascular cells. This was based on our observation that 1,25(OH)₂D₃ induces PA secretion from bovine parathyroid cells (2) and the demonstration by Fukomoto et al. (18) that 1,25(OH)₂D₃ increases the release of PA activity from bone cells. In the present study, we show that 1,25(OH)₂D₃ and several of its synthetic analogs stimulate the secretion of tPA. Under the conditions of our experiments, 1,25(OH)₂D₃ increased tPA secretion within 24 h at concentrations of 10⁻¹⁰ M and above. By comparison, responses to 1,25(OH)₂D₃ in other cultured target cells have been observed at hormone concentrations of 10⁻¹³ to 10⁻⁹ M (1, 2, 25); the concentration of the free secoosteroid in the circulation in vivo is 1–2 × 10⁻¹³ M (45). In addition to the beneficial effects of preincubation in lipid-depleted medium, our experiments using synthetic analogs of 1,25(OH)₂D₃ suggest that the high level of sensitivity to 1,25(OH)₂D₃ may have resulted in part from synergy between two different mechanistic pathways of vitamin D action.

METHODS

Materials

Human 52 kDa urokinase, human 70 kDa tPA, goat anti-human tPA IgG, human Glu-type plasminogen, H-o-

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norleucyl-hexahydrotyrosyl-lysine-p-nitroanilide diacetate salt, human 2-thrombin, and plasminogen-free fibrinogen were purchased from American Diagnostica (Greenwich, CT). BAY K 8644 was purchased from Calbiochem (La Jolla, CA). Thapsigargin, amiloride, lipid-depleted controlled process serum replacement-1 (CPSR-1), glucose, MOPS, insulin, creatine, carnitine, SDS, and routine chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Acrylamide/bisacrylamide, ammonium persulfate, and glycerine were purchased from Bio-Rad (Richmond, CA). Fetal bovine serum was purchased from HyClone (Logan, UT). Purified collagenate was purchased from Sigma-Aldrich (St. Louis, MO). Polyacrylamide, tricine buffer (pH 8.3); they were stored at

**Cell Preparation and Treatment**

Female Sprague-Dawley rats (retired breeders) were anesthetized with xylazine and ketamine. Hearts were excised and briefly perfused with Ca- and Mg-free Hank’s balanced salt solution (HBSS) to remove red cells. Rat heart microvascular cells were isolated by the method of Nishida et al. (35), with minor modifications. Contamination by mesothelial and endothelial cells was minimized by devitalizing the endocardial and epicardial surfaces with 70% ethanol and dissecting away the atria, valves, right ventricle, and one-third to one-fourth of the left ventricular pericardial wall. The remaining tissue was minced. The tissue fragments from 2 or 3 hearts were digested in 30 ml of Leibovitz’s L15 medium, containing 250 U/ml of purified bacterial collagenase, 5 U/ml of papain (unactivated), and 0.8–1 U/ml (0.25 mg/ml) of elastase with continuous tumbling at 37°C. The tissue fragments were gently triturated with a 10-ml serological pipette every 20–30 min. After 2–3 h, the digest was filtered through a 70-µm Falcon cell strainer (Becton-Dickinson Labware, Franklin Lakes, NJ) and centrifuged at low speed for 10 min to sediment the cells. The pellet was suspended in HBSS and then washed by sedimentation for 5 min at the same speed. The wash was repeated twice more. The final pellet of microvascular segments and cells was suspended in modified Leibovitz’s L15 medium containing 11 mM glucose, 20 mM MOPS, 4 x 10^{-7}M insulin, 10 mM creatine, 5 mM taurine, 2 mM carnitine, and 20% fetal bovine serum, and was seeded into the wells of Falcon 48-well plates (Becton-Dickinson Labware). After 3 days, the medium was replaced with unmodified L15 medium containing 2% CPSR-1 that had been heated at 56°C for 30 min to inactivate inherent PA activity. Culture was continued for two additional days, and then media were removed and the cells were treated with test compounds in 250 µl of L15 containing 0.2% of the heat-treated CPSR-1. After 24-h test incubations, the media were removed for immediate PA assays. Cell extracts were prepared by lysing the cells with 0.5% Triton X-100 in 0.1 M tricine buffer (pH 8.3); they were stored at −20°C until further use.

**PA Activity Assay**

For assay of cellular PA, cell extracts were freeze-thawed three times before use; media samples were assayed directly. PA activity was assayed colorimetrically, essentially according to Campbell et al. (8) by use of human tPA as a standard. Briefly, 50 µl of media or diluted cell extracts were incubated at 37°C in 96-well plates with (final concentrations) plasminogen (10 µg/ml), alkali-denatured fibrinogen (170 µg/ml) (39), and the chromogenic peptide substrate of plasmin H-norleucyl-hexahydrotyrosyl-lysine-p-nitroanilide diacetate salt (170 µM). The samples were buffered with 0.1 M tricine buffer (pH 8.3). All assay mixtures contained Triton X-100 at a final concentration of 0.5%. The color development at 405 nm was read periodically on a plate reader. The anti-tPA antibody was used at concentrations by 10.220.33.1 on June 27, 2017 http://ajpendo.physiology.org/ Downloaded from

**Characterization of PA Activity**

Fibrin zymography. Single-step fibrin zymography for the media was carried out according to modifications of the method of Heussen and Dowdle (22). Fibrinogen (90 µg/ml), plasminogen (3.375 µg/ml), and thrombin (0.3 U/ml) were combined with acrylamide/bisacrylamide before gel formation or electrophoresis. By this means fibrinogen was converted to fibrin and copolymerized with the acrylamide in the electrophoretic gel. Samples were separated on 9% SDS-acrylamide-separating gels. After electrophoresis, gels were extracted by 2.5% Triton X-100 in water for 2 h with three changes to remove SDS. To express PA activity, the fibrin-containing gels were incubated in 0.1 M tricine buffer (pH 8.3) at 37°C for 15 h. The proteins were then fixed in 10% TCA and then extracted with 7% ethanol. The gels were stained for 1 h with Coomassie Brilliant Blue R250 and then destained with 7% acetic acid. They visualized PA activity as clear bands in the gels caused by plasmin-mediated digestion of the fibrin. Zymograms were scanned using a Hewlett-Packard Scanjet II CX/T scanner.

Inhibitor studies. The samples were incubated with a blocking antibody against human tPA or with amiloride. The mixtures were then assayed for PA activity or subjected to zymography to determine the nature of the PA(s) in the samples. The anti-tPA antibody was used at concentrations from 3 to 30 µg/ml. Amiloride was used at a concentration of 100 µM.

**Statistical Analysis**

The data were expressed as averages ± SE and were analyzed for significant differences between the means of experimental and control samples using Student’s t-test.

**RESULTS**

Rat heart microvascular preparations consisted of capillaries and some single cells. These cells were slow to migrate and flatten onto cultureware, but after 3 days in medium containing 20% fetal bovine serum, they formed semiconfluent monolayers. Two days after substitution of 2% lipid-depleted serum replacement for the fetal bovine serum, some cells retracted into cell clumps that remained attached to the plate. The freshly isolated microvascular cells contained high levels of PA activity. After 24 h in culture, however, 80 to 90% of the activity had disappeared (Fig. 1A). After 2 days of culture, the cellular levels of PA activity stabilized at 10% of the amounts in the freshly dispersed cells. In contrast to the rapid changes in cell content of PA, the secretory rates of PA under control conditions were stable throughout the periods of culture. After the cellular levels had stabilized on day 2, the cells secreted 1–2 times the cellular content each day, indicating that PA synthesis was an active process.
activity in media, but zymography studies showed that it inhibited rat tPA as well as urokinase and thus was not sufficiently selective to permit conclusions as to the contribution of urokinase to the PA activity of rat cell culture media (data not shown).

Zymography. Samples of culture media and cell extracts were subjected to electrophoresis in fibrin- and plasminogen-containing polyacrylamide gels followed by zymography. The zymography conditions were optimized for maximal sensitivity to PA activity but were not suitable for quantitative comparisons, although comparisons between the intensities of experimental zymography bands after scanning generally confirmed the results of activity assays. The appearance of all bands was dependent on the presence of plasminogen in the gels, indicating that the bands represent PA activity. On these gels three activity regions were consistently observed (Fig. 2) that were similar to patterns observed previously (1, 2). Both native human 70 kDa tPA and corresponding areas of lanes representing the PA of conditioned media displayed a zone of activity containing sub-bands. The band multiplicity probably reflected selective adsorption of fibrin-binding regions of the human and rat tPAs to the immobilized fibrin in the gels. This zone was shown by densitometry to become more intense after treatment of cells with 1,25(OH)2D3 or isoproterenol (e.g., Fig. 2, lanes 5 and 6 vs. lane 3). Second, media and cell extract samples contained a sharp band of activity that migrated more rapidly than did human 52 kDa urokinase; the intensity of this band was not observed to be affected by experimental treatments. Third, a sharp but not intense band of activity was observed that migrated more slowly than did the tPA standard.

With regard to the bands derived from culture media that migrated with authentic human tPA, their intensity was decreased in a dose-dependent manner by prior incubation of the media samples with an anti-human tPA antibody (Fig. 3). The antibody was the same as that used in the experiment shown in Table 1. At 3 µg/ml, the antibody when incubated with 4 mU of

Table 1. Effects of anti-human tPA on PA activity in media of microvascular cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>A405 NoAntibody</th>
<th>Anti-tPA</th>
<th>%Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.079 ± 0.005</td>
<td>0.039 ± 0.002*</td>
<td>51</td>
</tr>
<tr>
<td>1,25(OH)2D3</td>
<td>0.300 ± 0.013</td>
<td>0.071 ± 0.005*</td>
<td>76</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>0.303 ± 0.005</td>
<td>0.057 ± 0.005*</td>
<td>81</td>
</tr>
</tbody>
</table>

Cells were incubated for 24 h under control conditions or in the presence of 10−12 M 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] or 10−6 M isoproterenol, six wells/group. Media from each group were pooled, and triplicate samples from each pool were incubated with or without 30 µg/ml of anti-human tissue plasminogen activator (tPA) antibody for 1 h at 37°C and were assayed for PA activity. Values represent means ± SE of absorbances at 405 nm (A405). *P = 0.001 vs. respective “no antibody” group.

Fig. 2. Zymogram of PA standards and samples of media from control and 1,25-dihydroxyvitamin D3 [1,25(OH)2D3]-treated cardiac microvascular cells. Lane 1, 4 µl of human tissue PA (tPA); lane 2, 0.1 µl of human urokinase (h-uPA); lane 3, media from control cells; lanes 4–6, media from cells treated with 10−10, 10−11, or 10−12 M 1,25(OH)2D3. Samples of media were pools taken from replicate wells.
the human tPA standard completely prevented the formation of a zymographic band (not shown). It was less reactive with the putative rat tPA, and significant loss of intensity of the band in the 70-kDa region of the zymogram required incubations with 10 or 30 µg/ml of the antibody (Fig. 3, lanes 4 and 5). The antibody had no effect on the activity of human urokinase, nor did it diminish the intensity of the band of PA activity that appeared in the region of the gel characteristic of rat 35 kDa urokinase (1). On the basis of these results, the band migrating in the approximate position of authentic human tPA was identified as rat tPA. The slowest-moving band of activity, not characterized by us, migrated in a position typical of rat tPA-plasminogen activator inhibitor-1 (PAI-1) complexes (1, 43). The demonstration of activity of tPA-PAI-1 complexes during zymography is presumed to result from the overnight incubations of the gels in buffers containing Triton X-100, a detergent that converts PAI-1 into a latent form (19). The intensity of this band on the gels was decreased by incubation of media samples with anti-human tPA (Fig. 3, lanes 4 and 5) but was not affected by any experimental treatments. When culture media were subjected to reverse zymography (tPA is included during gel formation) to detect active PA inhibitor(s), no activity was observed (data not shown). This likely reflected the presence of excess tPA and urokinase in the media from rat cells, a characteristic that is not shared by cultured human endothelial cells.

Effects of 1,25(OH)₂D₃ and 1,25-dihydroxy-16-ene-24-oxo-vitamin D₃ on cellular PA activity and PA secretion. We tested the effects of different concentrations of 1,25(OH)₂D₃ and of a less calcemic analog, 1,25-dihydroxy-16-ene-24-oxo-vitamin D₃ (28). The amount of PA activity in cell lysates from the culture wells was unaffected by any of the treatments. The results with conditioned media (Fig. 4) showed that rat heart microvascular cells under the conditions of these experiments increased their PA secretory rates in response to very low concentrations of the test compounds. Significant increases in PA secretion were consistently observed at 10⁻¹⁶ M 1,25(OH)₂D₃. Maximal induction usually occurred between 10⁻¹¹ and 10⁻¹⁴ M secosteroid. The degree of increase of secretion resulting from 24-h treatment with maximally effective concentrations of either hormone varied between experiments from ~50% to a maximum of approximately fivefold. The maximal degree of secretory induction was often equivalent to that attained by treatment of the cells with 10⁻⁶ M isoproterenol (Table 1), which was used as a positive control for inducibility. In multiple experiments, there were parallels in magnitude between the effects of isoproterenol and 1,25(OH)₂D₃ suggesting that the interexperiment variability in degree of inducibility of PA secretion by 1,25(OH)₂D₃ did not reflect altered sensitivity to the secosteroid, but rather variations in the ability of the cells to increase their production and secretion of PA above their baseline rates.

Effects of two analogs of 1,25(OH)₂D₃ with different affinities for the vitamin D receptor. The great sensitivities of the microvascular cells to 1,25(OH)₂D₃ compared with the (Kᵣ) for its receptor of 10⁻¹⁰ to 10⁻¹¹ M suggested that the hormone might induce PA secretion from rat cardiac microvascular cells by more than one mechanism and that the mechanisms might interact to maximize hormone sensitivity. To examine this hypothesis, we tested the effects of two 1,25(OH)₂D₃ analogs, added either individually or mixed. The first, 1,25-dihydroxy-16-ene-23-yno-hexafluoro-vitamin D₃ (Ro 24–5531) interacts strongly with the vitamin D receptors of chick intestine and ROS 17/2.8 cells and is ~0.01% as active as 1,25(OH)₂D₃ in stimulating transmembrane Ca²⁺ influx into ROS 17/2.8 cells (15, 25). The second,
25-hydroxy-16,23E-diene-vitamin D₃ (Ro 24–2287) binds the vitamin D receptor only ~1/1,000 as well as does 1,25(OH)₂D₃ (9, 16) but is a stronger activator of Ca²⁺ channels than is 1,25(OH)₂D₃ itself (25). Experiments were performed in which cells were treated with different concentrations (from 10⁻¹³ to 10⁻¹⁶ M) of 1,25(OH)₂D₃ of each analog, individually or with equimolar mixtures of the analogs at the same concentrations.

Treatment of cells with 1,25(OH)₂D₃ at 10⁻¹⁶ to 10⁻¹³ M significantly increased PA secretion in all experiments, as illustrated in Fig. 4. 25-Hydroxy-16,23E-diene-vitamin D₃ (Ro 24–2287) was a weaker inducer than was 1,25(OH)₂D₃; it significantly induced PA secretion at 10⁻¹³ M, but at 10⁻¹⁵ or 10⁻¹⁴ M it did so in only one of three experiments (data not shown). 1,25-Dihydroxy-16-ene-23-yn-hexafluoro-vitamin D₃ (Ro 24–5531) induced PA secretion with potencies intermediate between 1,25(OH)₂D₃ and the other analog. At 10⁻¹⁴ M, it significantly increased PA secretion from control levels in three of three experiments, and at 10⁻¹⁵ M it significantly induced PA secretion in two of three experiments (data not shown).

In all three of the experiments performed, combinations of the two analogs, each at 10⁻¹⁵ M (two experiments) or at 10⁻¹⁶ M (one experiment), induced PA secretion similarly to the parent 1,25(OH)₂D₃ and the other analog. At 10⁻¹⁴ M, it significantly increased PA secretion from control levels in three of three experiments, and at 10⁻¹⁵ M it significantly induced PA secretion in two of three experiments (data not shown).

Effects of BAY K 8644 and thapsigargin. The demonstrated abilities of 1,25(OH)₂D₃ to activate Ca²⁺ channels (25) suggested that one mechanism by which 1,25(OH)₂D₃ might induce PA secretion is by the elevation of cytosolic [Ca²⁺]. To test this hypothesis, we determined the effects on PA secretion of several agents known to activate Ca²⁺ channels or to increase cytosolic [Ca²⁺]⁻¹. Table 3 shows that the calcium channel agonist BAY K 8644 (2.8 M) increased PA secretion by 43% above control levels (P < 0.001), similar to the effects of 1,25(OH)₂D₃ and the positive control isoproterenol. The effects of BAY K 8644 were not, however, additive to the effects of 1,25(OH)₂D₃ when the two agents were added to the same sample wells.

Thapsigargin increases cytosolic [Ca²⁺]⁻¹ by inhibiting the Ca²⁺ pump in the endoplasmic reticulum that removes Ca²⁺ from the cytosol (24). The effects of thapsigargin on PA secretion were examined at concentrations of 22–200 nM, alone or in combination with 1,25(OH)₂D₃. The results demonstrated that PA secretion was increased at all concentrations of thapsigargin, with maximal effects observed at 200 nM (data not shown). In the presence of 200 nM thapsigargin, neither 10⁻¹¹ M 1,25(OH)₂D₃ (Table 4) nor 1,25-dihydroxy-16-ene-24-yne-26,27-hexafluoro-vitamin D₃ (data not shown) was able to further increase PA secretion.

### Table 3. Effects of BAY K 8644 alone or in combination with 1,25(OH)₂D₃ on PA secretion from microvascular cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PA Secretion, mU/day</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>63 ± 7</td>
</tr>
<tr>
<td>1,25(OH)₂D₃</td>
<td>98 ± 2.5†</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>95 ± 6*</td>
</tr>
<tr>
<td>BAY K 8644</td>
<td>90 ± 3*</td>
</tr>
<tr>
<td>BAY K 8644 + 1,25(OH)₂D₃</td>
<td>85 ± 7*</td>
</tr>
</tbody>
</table>

Cells were incubated for 24 h under control conditions or in the presence of 10⁻¹¹ M 1,25(OH)₂D₃, 10⁻⁶ M isoproterenol, 3 µM BAY K 8644, or 1,25(OH)₂D₃ plus BAY K 8644. Values represent means ± SE for 6 wells/group. *P ≤ 0.01, †P ≤ 0.001 vs. control group.

### DISCUSSION

The results of this study showed that, in lipid-depleted rat heart microvascular cells, 1,25(OH)₂D₃ induced the secretion of tPA from adult rat heart microvascular cells at sub-fM concentrations. We showed further that the high sensitivity of the cells to 1,25(OH)₂D₃ may be due in part to the secosteroid acting through more than one cellular pathway. It is likely that one induction pathway involves binding to the vitamin D receptor, but the manner(s) in which the 1,25(OH)₂D₃-bound receptor might induce tPA production and release have not yet been determined. In addition, the results are consistent with the notion that cytosolic [Ca²⁺]⁻¹ may be a major factor in one pathway.

### Table 4. Effects of thapsigargin alone or in combination with 1,25(OH)₂D₃ on PA secretion from microvascular cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PA Secretion, mU/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>1,25(OH)₂D₃</td>
<td>40 ± 1.5*</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>72 ± 2*</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>58 ± 6*</td>
</tr>
<tr>
<td>Thapsigargin + 1,25(OH)₂D₃</td>
<td>66 ± 1*</td>
</tr>
</tbody>
</table>

Cells were incubated for 24 h under control conditions or in the presence of 10⁻¹¹ M 1,25(OH)₂D₃, 10⁻⁶ M isoproterenol, 200 nM thapsigargin, or thapsigargin plus 1,25(OH)₂D₃. Values represent means ± SE of 6 wells/group. *P ≤ 0.001 vs. control.
The Microvascular Cell Culture System

The experimental system used in these studies is adult rat heart microvascular cells in short-term primary culture. It is comprised primarily of microvascular endothelial cells, and smaller percentages of vascular smooth muscle cells, interstitial cells (fibrocytes), and (vascular) pericytes (6). Our initial decision to study this cell preparation was based on the hypothesis that PA secretion would originate from endothelial cells in the cultures, but that responses to 1,25(OH)\(_2\)D\(_3\) might require cooperative interactions between different cell types, because, for example, both endothelial cells and vascular smooth muscle cells contain vitamin D receptors (31). If pure cell populations or established endothelial cell lines were examined, therefore, physiologically relevant responses to 1,25(OH)\(_2\)D\(_3\) might not be generated or observed. We performed one preliminary study with a rat endothelial cell line (graciously supplied by Dr. Clement Diglio, Wayne State University School of Medicine, Detroit, MI) and observed that the cells did not increase their rate of tPA secretion in response to 1,25(OH)\(_2\)D\(_3\), isoproterenol, or thapsigargin. The results indicated that the ability to respond to 1,25(OH)\(_2\)D\(_3\) might be lost from endothelial cells as a function of increased numbers of passages in culture.

We performed additional preliminary studies (to be presented separately) that have supported the hypothesis that endothelial cells at low passages increase their rates of tPA secretion in response to 1,25(OH)\(_2\)D\(_3\). We isolated purified rat heart microvascular endothelial cells and examined the effects of 1,25(OH)\(_2\)D\(_3\) on PA secretion from cultures after three to four passages. The results showed that 1,25(OH)\(_2\)D\(_3\) induced secretion of PA from the cultured endothelial cells. Second, we examined the effects of 1,25(OH)\(_2\)D\(_3\) on fourth-passage newborn human dermal microvascular endothelial cells (purchased from BioWhittaker, San Diego, CA). PA secretion was increased by treatment with 1,25(OH)\(_2\)D\(_3\) in these cells also. In summary, studies with the cell culture models described above led us to conclude that 1,25(OH)\(_2\)D\(_3\) induced PA secretion from rat vascular endothelial cells and that no interactions with other cell types were required to generate the results.

Cell PA Content and Secretion

Freshly isolated microvascular cell preparations lost most of their cellular PA activity within 24 h of culture, but this phenomenon seemed unrelated to the rates of PA secretion that remained constant from the first time tested until the end of the culture and experimental periods (Fig. 1B). The initial rapid loss of PA activity, therefore, did not appear to result from secretion into the medium, and the fate of that enzyme is not known. It is possible that the cellular stores of PA in vascular endothelial cells in vivo represent the ability to secrete tPA rapidly in response to secretagogues such as histamine and thrombin. The loss of cellular PA in culture would be expected to correlate with a loss in the ability of the cells to secrete PA in response to acute stimuli. Our preliminary studies (data not shown) and the results of others (42) support the notion that acute secretory responses are compromised in cultured endothelial cells.

One of the problems encountered in the study of the responses of rat heart microvascular cells to 1,25(OH)\(_2\)D\(_3\) and other inducers of tPA secretion was that the degree of increase in induction of secretion varied from experiment to experiment. This variability can be observed in Tables 2–4 and Fig. 4. In these and many other experiments performed by us with microvascular cells, the greatest variability in secretory rates between experiments occurred not in the treated samples but in those representing the control or basal conditions. In virtually every case, moreover, the basal secretory rates were inversely related to the ability of a stimulatory agent like 1,25(OH)\(_2\)D\(_3\) to increase secretion of PA activity over the baseline levels. The highest basal secretory rates observed were those associated with an established endothelial cell line whose PA secretory rates were not increased by 1,25(OH)\(_2\)D\(_3\), isoproterenol, or thapsigargin. The reason(s) for the variability in basal secretory rates and degrees of its induction by stimulatory agents are not yet known. We speculate that unintended induction of tPA secretion may have occurred when the experimental period was initiated, or perhaps before. One possible variable was the amount of shearing stress that cells were subjected to during the media changes associated with initiation of the experiment. It has been reported, for example, that shear stress of endothelial cells causes Ca\(^{2+}\) entry into the cells by a mechanism involving myosin light-chain kinase (49). Variable degrees of shear stress between experiments could have caused variable degrees of Ca\(^{2+}\) entry that, in turn, may have induced release of PA activity from control and experimental groups of cells. If the results of Ca\(^{2+}\) entry and other experimental treatments were not additive (as seems the case), then the results would be observed as a reduced effect of test agents on the rates of release of PA activity into media. In the case of endothelial cells in particular, shear stress during media changes should probably be minimized as much as possible.

Effects of 1,25(OH)\(_2\)D\(_3\) and 1,25-Dihydroxy-16-ene-24-oxo-vitamin D\(_3\) (Ro 25–8272) on Secretion of PA Activity

Both 1,25(OH)\(_2\)D\(_3\) and a less calcemic analog strongly stimulated PA release; 1,25(OH)\(_2\)D\(_3\) was more potent than the analog. Most notable about their induction of PA secretion is the range of concentrations over which they occurred. In the experiment shown in Fig. 3, significant increases in PA secretion were observed at 10\(^{-10}\) M 1,25(OH)\(_2\)D\(_3\). At 10\(^{-14}\) M, PA secretion was increased almost threefold compared with controls and was one-half of the maximal rate attained. For both test compounds, the secretory rates were maximal at 10\(^{-14}\) M. These concentrations are much lower than the concentrations of 1,25(OH)\(_2\)D\(_3\) of 10\(^{-12}\) to 10\(^{-10}\) M reported previously to be required to generate effects of 1,25(OH)\(_2\)D\(_3\) in cultured cells (2, 18, 51). They are also well below the published values for the K\(_d\) of \(~10^{-10}\) M.
for the receptor for 1,25(OH)₂D₃ (40). One report of a low Kᵋ, however, is that of 5 × 10⁻¹³ M reported for binding of 1,25(OH)₂D₃ to crude receptor preparations of MCF-7 breast cancer cells (30).

One basis for the high potency of 1,25(OH)₂D₃ and its analogs in our experimental culture model rests on the treatment of the cells with a medium containing 2% of a lipid-depleted serum substitute before experimentation. We observed in early experiments that, without this treatment, the effects of 1,25(OH)₂D₃ on PA secretion were variable and sometimes insignificant; the maximal effects of the hormone were obtained at concentrations of 10⁻¹⁰ to 10⁻¹¹ M. The reason that the treatment with lipid-depleted serum was initiated, however, was not to deplete the cells of 1,25(OH)₂D₃ but to eliminate the high concentrations of 25-OH-D₃ normally present in serum. It has been reported that vascular endothelial cells can convert 25-OH-D₃ to 1,25(OH)₂D₃ (32). The lipid-depleted serum replacement presumably contains little 25-OH-D₃ or 1,25(OH)₂D₃, and so its inclusion in media for the final 72 h of culture may render the cells vitamin D deficient in vitro. In addition to the absence of vitamin D metabolites in the serum substitute, moreover, the treatment may additionally deplete the cells of bound 1,25(OH)₂D₃ because of its content (−100 nM) of lipid-stripped vitamin D-binding protein(s) that might extract receptor-bound hormone from the cells. Unfortunately, the concentrations of vitamin D metabolites in the serum replacement were not available.

In cells rendered vitamin D deficient, biochemical characteristics of the cells might change in ways that would increase the sensitivity of the cells to 1,25(OH)₂D₃. For example, 1,25(OH)₂D₃ reduces the levels of mRNA for the α₁C subunit of voltage-sensitive calcium channels in ROS 17/2.8 osteosarcoma cells (33). Depletion of 1,25(OH)₂D₃ in the rat heart microvascular cells might therefore increase the synthesis and thus the number per cell of such calcium channels. If part of the activity of 1,25(OH)₂D₃ in these cells is based on regulation of cytosolic [Ca²⁺], exposure to the hormone after a period of depletion would maximize the initial rate at which Ca²⁺ could enter the cells and might by that induce physiological responses at lower hormone concentrations.

In addition to the high potency of 1,25(OH)₂D₃ in rat heart microvascular cells, 1,25(OH)₂D₃ might be associated with cooperative effects on ligand binding to the receptors or with postreceptor binding effects such as increased metabolic stability of the receptor-ligand complex or high affinity for the DNA binding site (23). These possibilities have been discussed in connection with the biological activities of structural analogs of 1,25(OH)₂D₃, such as KH 1060, a 20-epi-analog of 1,25(OH)₂D₃ that does not bind to vitamin D-binding protein (51). KH 1060 has been shown to induce differentiation of U 937 cells at 10⁻¹⁴ M and to inhibit interleukin-1-induced proliferation of thymocytes at an IC₅₀ of 3 × 10⁻¹⁵ M, while displaying a Kᵋ for receptor binding of 10⁻¹¹ M (4). These concentrations are more in concert with the doses of 1,25(OH)₂D₃ shown here to increase the secretion of PA. This effect, therefore, may have resulted from the ability of low doses of 1,25(OH)₂D₃ to maintain or reinstate differentiated characteristics of rat heart microvascular cells, rather than a direct receptor-mediated effect of the hormone on transcription of the mRNA for tPA.

A third possibility to explain the high sensitivity to 1,25(OH)₂D₃ is that, in rat microvascular cells, 1,25(OH)₂D₃ may be acting by more than one signal transduction pathway and that the pathways used may interact synergistically to produce the high sensitivity to the hormone observed in our experiments. This hypothesis is based on recent demonstrations that 1,25(OH)₂D₃ is able to demonstrate effects by "nongenomic" as well as "genomic" pathways (37). For example, 1,25(OH)₂D₃ activates plasma membrane Ca²⁺ channels in target cells (3, 7, 34), thereby increasing the rate of Ca²⁺ entry and the cytosolic [Ca²⁺]. To examine the genomic and nongenomic pathways more deeply, numerous structural analogs of 1,25(OH)₂D₃ have been synthesized and tested for their abilities to bind to the vitamin D receptor and to influence the activity of Ca²⁺ channels (5, 25).

Two analogs of 1,25(OH)₂D₃ with different profiles of interaction with the vitamin D receptor or with plasma membrane Ca²⁺ channels were chosen to examine the hypothesis that the high sensitivity of rat vascular endothelial cells to 1,25(OH)₂D₃ may result from synergy between two interacting mechanisms. As described in the previous section, a genomic analog, 1,25-dihydroxy-16-ene-23-yn-26,27-hexafluoro-vitamin D₃, binds to the vitamin D receptor like 1,25(OH)₂D₃ and is a more powerful initiator of transcription (15), but it interacts only weakly with Ca²⁺ channels (15). A nongenomic analog, 25-hydroxy-16,23E-diene-vitamin D₃, on the other hand, binds poorly to the vitamin D receptor but is more powerful than 1,25(OH)₂D₃ at activating Ca²⁺ channels (25).

The results of the study with the analogs showed that they were both individually active at inducing PA secretion from rat microvascular cells. The genomic analog was the more powerful inducer and increased PA secretion consistently at 10⁻¹⁴ M. The nongenomic analog required 10⁻¹³ M or greater concentration to induce PA secretion. When added together at 10⁻¹⁵ M or even 10⁻¹⁶ M each, however, the combination was more potent than either compound alone, suggesting that synergistic effects may occur between the mechanisms used by each analog.

To determine whether the hypothesis was tenable that 1,25(OH)₂D₃ could increase PA secretion in part through increased cytosolic [Ca²⁺] resulting from increased Ca²⁺ channel activity, two drugs were tested that increase cytosolic [Ca²⁺]. BAY K 8644 is a Ca²⁺ (or cation) channel agonist (17), and thapsigargin is an inhibitor of the Ca²⁺ pump that transports Ca²⁺ from the cytosol into the lumen of the endoplasmic reticulum (24). Both agents stimulated PA secretion; thapsigargin did so very strongly. The results are in overall agreement with those of Tranquille and Emeis (42), who
concluded that Ca\textsuperscript{2+} influx was essential for the acute release of tPA. The effects of altering cytosolic [Ca\textsuperscript{2+}] on steady-state rates of tPA secretion have not yet been systematically investigated, however, and the effects of altering cytosolic [Ca\textsuperscript{2+}] under physiological conditions remain to be shown. The results obtained after thapsigargin treatment may be complicated by its demonstrated induction of apoptosis in several cell types (50, 52).

At maximally effective concentrations of either 1,25(OH)\textsubscript{2}D\textsubscript{3} or isoproterenol, no additive effects were observed when maximally effective doses of thapsigargin or BAY K 8644 were also present. Similar results were obtained when the effects of 1,25-dihydroxy-16-ene-23-yne-25,26-hexafluoro-vitamin D\textsubscript{3} combined with thapsigargin were examined. The results suggested that cytosolic [Ca\textsuperscript{2+}] may be an important component of the 1,25(OH)\textsubscript{2}D\textsubscript{3}-mediated mechanism for increasing PA secretion, but they do not infer that it comprises a separate pathway from those already in use by the hormone to increase PA secretion.

This work was supported by a grant from the American Heart Association, Kansas Affiliate (KS-97-GS-60). Address for reprint requests and other correspondence: R. R. MacGregor, Dept. of Anatomy and Cell Biology, Univ. of Kansas Medical Center, 39th St. and Rainbow Blvd., Kansas City, KS 66160–7400 (E-mail: rmacgreg@kumc.edu). Received 14 May 1999; accepted in final form 15 September 1999.

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