Calcitonin Stimulates Plasminogen Activator in Porcine Renal Tubular Cells: LLC-PK₁

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ABSTRACT Plasminogen activators are highly selective proteases that activate the proenzyme plasminogen to the general protease, plasmin. We studied a porcine kidney cell line, originally isolated as a high producer of plasminogen activator, in which activities of cellular adenylate cyclase and cAMP-dependent protein kinase are increased in response to calcitonin. We found that salmon calcitonin, in the concentration range 0.03-300 nM, increased plasminogen activator production up to ~1,000-fold and concurrently inhibited cell multiplication; both of these effects were reversible. Human calcitonin was ~0.01 times as potent as salmon calcitonin, corresponding to potency differences observed in other biological systems. Plasminogen activator production was also stimulated by other agents that raise cellular CAMP levels such as cholera toxin, phosphodiesterase inhibitors, and vasopressin, but not to the same extent as by calcitonins. The rapidity and sensitivity of the plasminogen activator determination and other cellular responses may make it possible in the future to use this cell strain in a convenient bioassay for calcitonins and their analogues.

Calcitonins stimulate adenylate cyclase activity in bone and kidney. These hormones act to decrease bone resorption and renal transport of calcium, sodium, magnesium, and inorganic phosphate (5, 16). We have found that a cultured cell line derived from porcine kidney (LLC-PK₁) responds to calcitonins and vasopressin with an increase in cellular cAMP content (8, 11) and activation of cAMP-dependent protein kinase (1). These cells had originally been isolated by Hull et al. (12) as a potential source for plasminogen activator, an enzyme whose secretion in other cell cultures is modulated by several hormones, including some whose effects are normally mediated by cAMP. It therefore appeared interesting to determine whether calcitonins and vasopressin could affect enzyme production in the porcine renal tubular cells as well.

In this report we show that calcitonins can markedly stimulate plasminogen activator production by LLC-PK₁ cells and, in addition, inhibit their replication. Other agents that increase cAMP levels in these cells also increase release of plasminogen activator, although a direct correlation of cAMP with plasminogen activator levels has not been established.

MATERIALS AND METHODS

Hormone Solutions and Other Reagents

Test solutions of salmon calcitonin were prepared from lyophilized samples of synthetic salmon calcitonin that were supplied by Dr. H. T. Keutmann (Endocrine Unit, Massachusetts General Hospital, Boston, Mass.). Salmon calcitonin was dissolved in 0.001 N acetic acid and used after dilution without sterilization. For some experiments, salmon calcitonin was obtained from commercial sources (Calcimar, Armour Pharmaceutical Co., Phoenix, Ariz.; 5,000 MRC U/mg). Synthetic human calcitonin (Cibacalcin) was a gift of Ciba-Geigy A. G., Basel, Switzerland. The antidiuretic hormone was a preparation of arginine vasopressin grade VI (Sigma Chemical Co., St. Louis, Mo. 367 IU/mg). Cholera enterotoxin was obtained from Becton, Dickinson, and Co., Orangeburg, N. Y. Phorbol myristate acetate (Consolidated Midland, Brewster, N. Y.) was diluted from a stock solution in absolute ethanol. 3-isobutyl-1-methylxanthine (IBMX) was obtained from Becton, Dickinson, and Co., Orangeburg, N. Y. Benzylsalicylate (Benzylsalicylic acid, Sigma) was dissolved in absolute ethanol.

Cell Culture

The LLC-PK₁ cell strain was originally isolated in 1958 and had been maintained in culture through at least 400 passages (12). During this work, cells
were carried in 10-cm Diam Falcon tissue culture dishes (Falcon Plastics, Oxnard, Calif.) using Dulbecco's modification of Eagle's medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum (FCS) from Microbiological Associates, Walkersville, Md., plus 100 U of penicillin and 100 μg of streptomycin/ml (Gibco Laboratories) (DMEM, calf serum (FCS) from Microbiological Associates, Walkersville, Md., plus 100 

Experimental Incubations

After the cells were washed, 1 ml of serum-free medium (DMEM containing penicillin and streptomycin and 10 mM HEPES, pH 7.4) and the compounds being tested were added to each dish and the cultures incubated at 37°C, 95% air, 5% CO₂. At the times indicated for each experiment, the media were removed and frozen at -20°C for subsequent assay. Cells were trypsinized and counted using a hemocytometer or a Coulter counter model Z (Coulter Electronics, Inc., Hialeah, Fla.).

Plasminogen Activator Determination

Plasminogen activator activity in samples of conditioned media or cell lysates was measured as the plasminogen-dependent rate of fibrinolysis using 125I-fibrin-coated multwell plates (20). Plasminogen was purified from human plasma by affinity chromatography using L-lysine-substituted Sepharose (6). Dilutions were made to contain the equivalent of 0.001-5 μl of the undiluted sample which were then added to 250 μl of 0.1 M Tris-HCl, pH 8.1, containing 8 μg/ml of plasminogen. The assay mixtures were incubated for 1 and 3 h at 37°C and the radioactivity of the solubilized 125I-fibrin peptides determined. Results are expressed in Ploug units, as determined with reference to a standard preparation of urokinase (Urokinase Reference Standard, Leo Pharmaceutical Products, Bella-rup, Denmark). The molecular weight of the enzyme was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (10), except that casein (21) was used instead of fibrin in the indicator gel. All experiments included appropriate controls for reagents and media; the modulators of plasminogen activator production tested did not affect activity in the assays at the concentrations used. Assays performed in the absence of plasminogen did not reveal plasminogen-independent proteolytic activity, under any of the experimental conditions.

RESULTS

Dose-dependent Stimulation of Plasminogen Activator Secretion by Calcitonins and by Vasopressin

LLC-PK₁ cells produce substantial levels of plasminogen activator (12). When cultures of those cells were exposed to synthetic salmon or human calcitonin, or vasopressin, the level of plasminogen activator activity in the conditioned medium rose markedly (Fig. 1). The response was dose dependent: the threshold for the effect of salmon calcitonin was below 0.29 nM; maximum stimulation occurred at ~290 nm, resulting in a 500- to 1,000-fold increase in secreted enzyme activity. The human hormone was at least two orders of magnitude less potent and the maximum effect achieved at 1.45 μM was less than that of salmon calcitonin at 290 nM. Plasminogen activator activity was also increased in cultures exposed to vasopressin, but 10,000-fold higher concentrations than salmon calcitonin and 100-fold higher concentrations than human calcitonin were needed to produce comparable effects.

![Figure 1](image1.png)

**FIGURE 1** Effects of calcitonins and vasopressin on LLC-PK₁ cells. Stimulation of plasminogen activator activity as a function of hormone concentration. LLC-PK₁ cells were plated in 3-cm diam dishes at 0.5 x 10⁵ cells/dish in DMEM, 10% FCS, 5 d before the experiment. After 2 d, media were removed, cells were washed once with PBS, and then incubated for 72 h under conditions indicated: DMEM without serum (○); salmon calcitonin (■); human calcitonin (▲); vasopressin (○). At the end of the incubation, media were removed and assayed for plasminogen activator activity.

![Figure 2](image2.png)

**FIGURE 2** Effects of calcitonin on stimulation of plasminogen activator production by LLC-PK₁ cells. LLC-PK₁ cells were plated in 3-cm diam dishes at 0.5 x 10⁵ cells/dish in DMEM, 10% FCS, 2 d before the experiment. Media were then removed, cells washed once with PBS, and then incubated for various periods in the absence (○) or presence (■) of 290-nM salmon calcitonin. At the times indicated, media were removed and assayed for plasminogen activator activity. Values are means ± SEM for three dishes.
Time-Course of Calcitonin Effects

The time-course of the effects of maximally stimulatory concentrations (290 nM) of salmon calcitonin on plasminogen activator in culture media is shown in Fig. 2. A substantial increase of ~10-fold in extracellular plasminogen activator was found at 4 h after addition of hormone (not readily appreciated because of the scale of Fig. 2). Although increases in plasminogen activator in this experiment were not strictly linear, enzyme accumulation persisted for at least 48 h in the same medium, although at progressively slower rates. Other experiments were performed in which cultures were exposed to salmon calcitonin (290 nM) for only 1 h, washed, incubated further and the conditioned media was analyzed for enzyme activity. Under these conditions, significant increases in extracellular plasminogen activator were observed in media sampled 20 min after removal of calcitonin (0.155 ± 0.020 U/ml after calcitonin preincubation, compared with 0.075 ± 0.005 U/ml in cells not preincubated with calcitonin). In other similar experiments such as that illustrated in Fig. 3, the persistent stimulatory effects of calcitonin were demonstrated in media assayed from 2 to 28 h after removal of the hormone. An increased rate of enzyme secretion was observed up to 8 h after removal of the hormone and then appeared to reach a plateau. Hence the effects of salmon calcitonin, although slowly reversible, were long-lasting, and once initiated, did not dissipate immediately on removal of hormone from the medium.

The increase in medium plasminogen activator activity induced by calcitonin required genetic transcription and translation as shown by the inhibitory effects of cycloheximide and actinomycin (Table I). At the concentration of the inhibitors utilized in these experiments, the stimulatory effect of calcitonin was markedly suppressed (by 97% with cycloheximide and by 93% with actinomycin D) but nevertheless still apparent. Furthermore, in the absence of inhibitors, total plasminogen

### Table I

| Inhibitors | Conditions | Plasminogen activator (U/culture) |
|------------|------------|----------------------------------|
| No inhibitor | Control cultures | 2.2 ± 0.3 |
| | Salmon calcitonin-treated cultures | 100 ± 8.6 |
| Cycloheximide (5 μg/ml) | Control cultures | 2.0 ± 0.2 |
| | Salmon calcitonin-treated cultures | 2.2 ± 0.2 |
| Actinomycin D (5 μg/ml) | Control cultures | 2.2 ± 0.2 |
| | Salmon calcitonin-treated cultures | 2.2 ± 0.2 |

Cells were plated in 3-cm Diam dishes at 0.5 × 10⁶ cells/dish, 3 d before the experiment, in DMEM, 10% FCS. After 3 d cells were washed once with PBS and preincubated for 1 h in 1 ml DMEM (without serum) alone, with 5 μg/ml cycloheximide, or 5 μg/ml actinomycin D. After 1 h, media were removed and the cells washed twice with PBS. Each of the three sets of cell culture dishes were then distributed into two subgroups with or without salmon calcitonin plus the corresponding inhibitors for 24 h (1 ml/dish). Triplicate dishes were used for cell number and plasminogen activator activity. Values for cell number are means ± SEM for three dishes.

### Table II

| Effect of Salmon Calcitonin on Cell-associated and Cell-secreted Plasminogen Activator |
|----------------------------------------|----------------------------------|
| | Plasminogen Activator (U/culture) |
| Control cultures | 2.2 |
| Salmon calcitonin-treated cultures | 100 |

Confluent cultures were washed and placed in DMEM without serum in the absence or presence of 290 nM salmon calcitonin. After 24 h the media was collected, and the cells were washed four times with PBS, before scraping into 0.2% Triton X-100 for plasminogen activator assay, and the total plasminogen activator in the cells and the media calculated.
Activator activity in the cultures was increased in the presence of salmon calcitonin (Table II); both the cell-associated and the secreted enzyme levels were markedly higher in the hormone-treated cultures. Hence, the stimulatory effects of the hormone on increasing plasminogen activator in culture media are dependent upon formation of active enzyme; probably through increased synthesis. In this respect, this cell system resembles others in all of which hormonal modulation of plasminogen activator production is dependent upon the maintenance of RNA and protein synthesis (19, 20).

**Effects of Other Agents on Induction of Plasminogen Activator Synthesis**

Because calcitonin and vasopressin are known to stimulate adenylate cyclase in these and in other cells, it was important to test other agents that influence cAMP metabolism for their ability to increase medium plasminogen activator activity. As seen in Table III, although added cAMP itself was ineffective, the cAMP-phosphodiesterase inhibitor IBMX stimulated plasminogen activator synthesis, and the effects of cAMP and IBMX were synergistic. Levels of enzyme activity in the media from cells incubated with both cAMP and IBMX were only slightly lower than the maximum achieved in this experiment with salmon calcitonin. Cholera toxin, which activates adenylate cyclase in most cells, also stimulated plasminogen activator production but only to the extent produced by exposure to IBMX alone.

Glucocorticoids and the tumor promoter phorbol myristate acetate (PMA) modulate plasminogen activator production in a variety of cell types; the former usually inhibits whereas the latter usually stimulates enzyme synthesis (21). In LLC-PK1 cells, the glucocorticoid, dexamethasone, decreased both spontaneous and calcitonin-stimulated plasminogen activator production (Table III). In five separate experiments the magnitude of this inhibitory effect tended to be variable, ranging from 20% to 90%, although we have, so far, not succeeded in identifying the basis of this variability.

Similar variability was observed in the effects of PMA. At concentrations ranging from 1 ng to 1 μg/ml, PMA always stimulated plasminogen activator production. Although the degree of stimulation varied from 5- to 50-fold, it was always below the maximum produced by stimulation of the cells with salmon calcitonin.

**Characterization of Plasminogen Activator Produced by LLC-PK1 Cells**

To identify the species of plasminogen activator produced by LLC-PK1 cells, aliquots of conditioned medium and cell extracts from basal and calcitonin-stimulated cultures were analyzed by a zymographic method based on SDS-PAGE (109). As seen in Fig. 4, there were two major bands of plasminogen activator activity that migrated with apparent molecular weights of ~50,000 and ~29,000, respectively. These results are consistent with the values for molecular weight of plasminogen activator species previously purified from these cells (3). The major band (~50,000 mol wt) coincided in its migration with the major cell culture and urinary plasminogen activator of rats, mice, and hamsters, whose apparent molecular weights are somewhat lower than that of human urokinase. The enzyme species in media from unstimulated cultures were indistinguishable with respect to their electrophoretic mobility from those from calcitonin-treated cultures (data not shown).

**Inhibition of Cell Multiplication by Calcitonin**

In addition to the effects of calcitonin on plasminogen activator production, we also observed that calcitonin inhibited proliferation of the LLC-PK1 cells (Tables I and III). These effects were further studied in experiments such as those illustrated in Fig. 5, where it can be seen that the concentration of salmon calcitonin that produced 50% inhibition of cell replication was ~2.9 nM; complete inhibition of cell replication was observed at concentrations of 29 nM and greater. The lower

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**Table III**

| Culture conditions | Plasminogen activator | Number of cells x 10^-6 |
|-------------------|----------------------|-------------------------|
| No addition       | 6                    | 2.29 ± 0.04             |
| IBMX (1 mM)       | 40                   | 2.35 ± 0.07             |
| cAMP (1 mM)       | 2                    | 2.59 ± 0.02             |
| cAMP (1 mM) + IBMX (1 mM) | 226          | 2.20 ± 0.02             |
| Cholera toxin (10 ng/ml) | 39             | 2.15 ± 0.01             |
| Salmon calcitonin (290 nM) | 375        | 1.18 ± 0.03             |

**Notes:** Cells were plated in 3-cm dishes each containing 0.8 x 10^6 cells (A) or 0.4 x 10^6 cells (B) in DMEM, 10% FCS, 1 d before each experiment. After 1 d, media were removed, cells washed once with PBS, and then incubated in 1 ml DMEM without serum under indicated conditions for an additional 24 h. At the end of the incubation, media were removed and assayed for plasminogen activator activity. Values for cell number are means ± SEM for three dishes.
The specificity of the calcitonin effects in the LLC-PK₁ cells has not been investigated sufficiently.

Of several other hormones tested, only vasopressin produced a measureable increase in plasminogen activator production as well as an increase in cellular content of cAMP in these cells. The concentration of vasopressin required for threshold effects on plasminogen activator was ~30 nM (~100- to 1000-fold that of salmon calcitonin) and even at 2 μM, vasopressin stimulation was only ~2-4% that of salmon calcitonin. However, vasopressin, at concentrations equal to those of calcitonin, increased cAMP content acutely in the LLC-PK₁ cells to an extent similar to that of salmon calcitonin (8, 11). We do not know why the observed potency differences between salmon calcitonin and vasopressin are so large if the mechanisms of their effects on plasminogen activator production are mediated entirely through changes in cellular cAMP content.

In comparing the effects of vasopressin and calcitonin on plasminogen activator in the renal tubular cells and relating them to changes in concentrations of cAMP, one must take into account the kinetics of these changes. For example, in our initial report (8) we showed that in the absence of phosphodiesterase inhibitors, equivalent concentrations (2.9 nM) of salmon calcitonin and arginine vasopressin produced a similar increase (fourfold) in the cAMP content after 10 min. These levels were markedly increased in the presence of IBMX. We also found that the content of cAMP in the cells exposed to either hormone in the absence of IBMX did not remain elevated but returned to base line by 24 h. Despite the fact that the cAMP contents were similar in cells exposed briefly to either hormone, activation of adenylate cyclase persists in cells exposed to calcitonin but not in cells exposed to vasopressin (11). The persistence of adenylate cyclase activation in cells preincubated with salmon calcitonin could be measured by exposing the cells to IBMX alone.

The long-term effects of cAMP in the presence of IBMX or cholera toxin alone, described here, of stimulation of plasminogen activator synthesis, nevertheless suggest that enzyme induction by salmon calcitonin could be mediated at least in part by hormonal activation of adenylate cyclase. In several other experiments (not shown) attempts to incubate cells for long periods, in the absence of serum, with IBMX in addition to hormones or cholera toxin, were associated with cell toxicity, and were therefore difficult to interpret. It is also possible that effects of calcitonins other than those responsible for changes in cAMP content could explain the marked effects on plasminogen activator. For example, because calcitonins in vivo alter renal transport of several ions such as calcium and sodium (5, 16), it is conceivable that altered ion fluxes in these cells could influence specific protein synthesis. To begin to explore this possibility, in preliminary studies, we have observed significant inhibition of calcitonin-stimulated plasminogen activator production in LLC-PK₁ cells by 10 μM ouabain. [³H]Ouabain binds to sites on the plasmalemma of these cells which corresponds to basolateral membranes of other cells (15). This binding is presumably to the Na⁺-K⁺-ATPase, an accepted marker for, if not the actual “sodium pump.”

Inhibition of cell replication was a second facet of calcitonin action observed in these cultures. The extent of growth inhibition as a function of salmon calcitonin concentration was generally parallel with the dose-response curve for plasminogen activator induction, whereas the low potency of human calcitonin and vasopressin as inducers of enzyme synthesis was associated with relatively feeble or negligible effects on growth.
A role for cAMP in mediating effects of hormones and other substances on cell growth has been proposed (7, 9, 17, 22). The observations that vasopressin or agents that increase cellular cAMP such as cholera toxin and exogenous cAMP in the presence of IBMX did not inhibit replication of the LLC-PK1 cells, cannot be offered as proof that salmon calcitonin inhibition is not mediated by cAMP, because experiments have not yet been performed to compare the kinetics or duration of action of the different compounds.

It is of interest to consider which physiologic or pharmacologic aspects of the action of these hormones in vivo, if any, may be correlated with the induction of plasminogen activator synthesis. For example, it is established that injections of large amounts of vasopressin shorten human euglobulin lysis time (4), an effect subject to tachyphylaxis. This suggests the possibility that our observations of the responses in cell culture could reflect some action of these hormones in vivo, at least when administered in pharmacologic doses. In support of this possibility are observations that salmon calcitonin administered to humans in amounts used to treat Paget’s disease of bone acutely increase urinary plasminogen activity (14). An additional consideration is that effects of hormones such as calcitonin could be modulated in a target tissue (kidney) by secretion of a protease such as plasminogen activator and by activation of extracellular plasminogen. The protease generated could be involved in the processing or degradation of that hormone, or other protein substrates. Investigations of such possibilities are targets for future efforts.

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