Inhibition of protein synthesis in LLC-PK₁ cells increases calcitonin-induced plasminogen-activator gene transcription and mRNA stability

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The peptide hormone calcitonin induces the accumulation of urokinase-type plasminogen activator (uPA) mRNA in pig kidney LLC-PK₁ cells. By itself, inhibition of protein synthesis had a negligible effect on uPA mRNA accumulation. Inhibition of protein synthesis led to two superinductive effects: an increase in calcitonin-induced uPA mRNA accumulation over time, and a shift in the dose–response curve so that lower calcitonin doses became more potent. To explain these two superinductive effects of protein-synthesis inhibition on calcitonin treatment, we demonstrated that the inhibition of protein synthesis increased both calcitonin-induced uPA-gene transcription and uPA-mRNA stability. Different protein-synthesis inhibitors had similar actions, arguing against the possibility that the results were attributable to an anomalous action of a particular inhibitor. The superinductive effects of protein-synthesis inhibition could not be mimicked when a tumour promoter, 12-O-tetradecanoylphorbol 13-acetate (TPA), was used instead of calcitonin as an inducer. Calcitonin and TPA exert their effects through different pathways, suggesting a clue to the mechanism of superinduction. Although inhibition of protein synthesis has been reported to increase transcription and mRNA stability in a number of other systems, the one described here appeared unique in combining both effects in the context of hormonal regulation.

INTRODUCTION

Plasminogen activators are extracellular enzymes that convert the proenzyme plasminogen into the trypsin-like serine proteinase plasmin (Reich et al., 1975). A large body of data implicates the participation of plasminogen activators in many physiological regulatory processes, including tissue remodelling, cell migration and fibrinolysis [see Dana et al. (1985) and Reboud-Ravaux (1985)]. Depending on the type of cell or tissue, many cells produce either urokinase-type plasminogen activator (uPA) or tissue-type plasminogen activator. This often occurs under the influence of different extracellular stimuli, such as polypeptide hormones (Strickland & Beers, 1976; Dayer et al., 1981), steroid hormones (Mak et al., 1976; Vassalli et al., 1976), retinoids (Wilson & Reich, 1978), glucose (Virji et al., 1980), tumour promoters (Wigler & Weinstein, 1976; Ferraiuolo et al., 1984) and u.v. irradiation (Miskin & Reich, 1980). Thus the mechanisms underlying such versatile regulation are of general interest.

We have been investigating uPA expression in a pig kidney cell line, LLC-PK₁. In this cell line uPA-mRNA accumulation is induced by the peptide hormone calcitonin (Nagamine et al., 1983) and by the tumour promoter 12-O-tetradecanoylphorbol 13-acetate (TPA) (Degen et al., 1985). Previously we showed that pretreatment with cycloheximide, a protein-synthesis inhibitor, did not block calcitonin-induced uPA-mRNA synthesis, indicating that uPA gene activation was a primary transcriptional event resulting from calcitonin stimulation and did not require new protein synthesis (Nagamine et al., 1983). Here we show that the amounts of calcitonin-induced uPA mRNA were much higher when protein synthesis was inhibited (superinduction). This occurred because inhibition of protein synthesis increased uPA-gene transcription and uPA-mRNA stability. In contrast, the amounts of TPA-induced uPA mRNA were not much higher when protein synthesis was inhibited.

MATERIALS AND METHODS

Supplies and chemicals

Synthetic salmon calcitonin was given by Dr. S. Guttman (Sandoz A. G., Basel, Switzerland). Cycloheximide and puromycin (Sigma) were dissolved in water (final concn. 10 mg/ml) and stored frozen. Pectamycin, a gift from Infectious Diseases Research, The Upjohn Co., Kalamazoo, MI, U.S.A., was refrigerated until use, when it was dissolved in ethanol. Actinomycin D (Sigma) was dissolved in ethanol (final concn. 1 mg/ml) and stored at 4°C. α-Amanitin (Boehringer–Mannheim) was dissolved in water (final concn. 1 mg/ml) and stored frozen. TPA (Pharmacia) was dissolved in ethyl acetate (final concn. 2 mg/ml) and stored at −20°C until use, when a portion was air-dried and redissolved in dimethyl sulfoxide (final concn. 0.1 mg/ml). Plasmid pSP64 and SP6 polymerase were from Promega–Biotec. DNA polymerase I was obtained from BRL. Ribonuclease A (20 mg/ml; 100 Kunitz units/mg) was from Sigma (R-5250), and ribonuclease T₁ (100000 Egami units/ml; 350000 units/mg) was from Boehringer–Mannheim.

Abbreviations used: uPA, urokinase-type plasminogen activator; TPA, 12-O-tetradecanoylphorbol 13-acetate; PBS(+) phosphate-buffered saline containing Ca²⁺ and Mg²⁺.
Nitrocellulose membrane filters for mRNA quantification were from Millipore (HAWP02500), and for determination of mRNA stability from Schleicher & Schuell (401100). [*-32P]GTP and [*-32P]dCTP were from New England Nuclear, and [*H]uridine and [*H]UTP were from Amersham.

**Cell culture**

LLC-PK1 cells (Hull et al., 1976) were cultured as described by Nagamine et al. (1983). In most experiments about 5 × 10^5 cells were plated in serum-containing medium on 35 mm dishes, and then used 2 days later with additional reagents as described in Figure legends.

**Concentrations of protein-synthesis inhibitors**

We used concentrations of 10 µg of cycloheximide/ml and of 0.3 µg-pactamycin, which we had found to inhibit the incorporation of [*H]leucine by at least 90% into trichloroacetic acid-precipitable material after a 30 min treatment. We used a concentration of 10 µg of puromycin/ml, which we had found to inhibit calcitonin-induced uPA activity by 95%.

**Probes and plasmids**

A cDNA clone for uPA mRNA, pPK79 (Nagamine et al., 1984), was labelled with [*-32P]dCTP by nick translation (Rigby et al., 1977), and used as a probe in Northern hybridization and in the mRNA-stability experiment. Another cDNA clone, pYN15 (Nagamine et al., 1984), was used for construction of plasmids for use in the solution-hybridization assay (see section on RNA preparation and mRNA quantification). A plasmid, pYN1, which contains most of the uPA gene, was used for filter hybridization in the nuclear transcription experiment. It was constructed by excising 1.3 kb (kilobases) of the 5' flanking region, 6 kb of the transcribed region and 0.5 kb of the 3' flanking region of the pig uPA gene from the pig genomic clone AYN4 (Nagamine et al., 1984), and inserting them into the EcoRI and NruI (nearest to Thr1111 site) sites of plasmid pBR322.

**RNA preparation and mRNA quantification**

After cells were treated with drugs and hormones, RNA was extracted as described previously (Nagamine et al., 1983) and dissolved and stored in water.

In the experiment depicted in Fig. 2, amounts of uPA mRNA were assessed by Northern-blot hybridization analysis (Nagamine et al., 1983).

In the experiments depicted in Figs. 1 and 3, we sought to quantify uPA mRNA more accurately. We used the solution-hybridization assay of Durnam & Palmiter (1983), with several modifications. The 2 kb XbaI fragment of pYN1, which covers the entire coding region and part of the 3' non-coding region of the uPA cDNA, was inserted into the XbaI site in the poly linker of pSP64 in both orientations. The resulting plasmids were then linearized appropriately to enable transcription of the insert by SP6 polymerase (Melton et al., 1984). Transcription of one of the plasmids was performed in the presence of [*-32P]GTP to generate a complementary RNA probe of high specific radioactivity. Transcription of the other plasmid was performed in the presence of [*H]UTP to generate predominantly full-length mRNA-like molecules of low specific radioactivity. As the base composition of the XbaI fragment was known (Nagamine et al., 1984), the number of [*H]mRNA molecules was readily calculated from the incorporated radioactivity (c.p.m.). Then, various amounts of the [*H]mRNA standard were hybridized with fixed amounts (about 50 000 c.p.m.) of the [*P]-labelled complementary RNA probe. The standard and probe were hybridized in 50 µl of a buffer containing 50%, v/v formamide, 0.4 mM NaCl, 10 mM-Pipes (pH 6.4), 1 mM-EDTA, 80 µg of tRNA/ml plus a mineral overlay to prevent evaporation. The mixtures were heated at 90 °C for 1 min, and incubated overnight at 50 °C. Then the hybridization mixtures were treated with 450 µl of nuclease solution [20 mM-Tris/HC1 (pH 7.5), 0.1 mM-NaCl, 5 mM-MgCl2, 40 µg of ribonuclease A/ml, 1 µg of ribonuclease T1/ml], and incubated at 37°C for 1 h. Nuclease-resistant, presumably double-stranded, RNA was precipitated with an equal volume of 10% (w/v) trichloroacetic acid on to nitrocellulose filters, the filters were washed with 5% trichloroacetic acid and dried, and the acid-precipitable radioactivity was measured by scintillation counting. This enabled construction of a standard curve relating radioactivity (c.p.m.) to the number of mRNA molecules. Portions of test samples were hybridized with the probe in the same manner as for the [*H]mRNA standard, following by nuclease treatment, acid precipitation and radioactivity counting. The number of uPA molecules in test samples was deduced by referring the amount of acid-precipitable radioactivity from measurements on the samples to the standard curve. Assays were done in duplicate, the results of which agreed to within 10–15%.

**Nuclear transcription**

Nuclear preparations, transcription and filter hybridization were as described by McKnight & Palmiter (1979) except that [*H]UTP was used as the radioactive precursor instead of [*-32P]UTP, and that the genomic clone of uPA, pYN1, was immobilized to nitrocellulose filters, instead of a uPA cDNA.

**mRNA stability**

At the start of the experiment, cells were washed with phosphate-buffered saline containing Ca²⁺ and Mg²⁺ [PBS(+); contents per litre: 8 g of NaCl; 0.2 g of KCl; 1.44 g of Na₂HPO₄; 0.2 g of KH₂PO₄; 0.1 g of CaCl₂; 2H₂O; 0.175 g of MgCl₂·6H₂O; pH 7.3] and then provided with 1 ml of serum-free medium containing 10 ng of calcitonin/ml and 100 µCi of [*H]uridine. Then 4 h later, the medium was removed, cells were again washed, and a chase was started with serum-free medium containing calcitonin, 200 µg of unlabelled uridine/ml, and with or without 10 µg of cycloheximide/ml. At various times later, RNA was prepared. The amount of radioactivity remaining in the uPA mRNA was measured by filter hybridization as described by McKnight & Palmiter (1979).

**RESULTS**

**Time course of accumulation of uPA mRNA**

Analysis of the time course of uPA-mRNA accumulation revealed that protein synthesis was involved in the regulation of uPA-gene expression. uPA-mRNA contents in calcitonin-induced cells reached a peak at about 1500 molecules/cell at 4 h after the start of calcitonin
Superinduction of urokinase mRNA accumulation

Timing of addition of inhibitors

We observed superinduction of uPA mRNA regardless of the timing of addition of protein-synthesis inhibitors. Pretreatment with cycloheximide or with puromycin led to calcitonin induction of uPA mRNA amounts much higher than those noted without inhibitor pretreatment (Fig. 2, lanes 4 and 5 versus lane 2). When inhibitor treatment was postponed until 4 h after the start of calcitonin treatment, uPA mRNA continued to increase 4 h later, instead of decreasing (Fig. 2, lanes 9 and 10 versus lane 7).

We also confirmed our previous observation (Nagamine et al., 1983) that calcitonin induction of uPA-mRNA accumulation occurs as a result of transcription of the uPA gene. Pretreatment with actinomycin D or with α-amanitin, which inhibit transcription, blocked subsequent calcitonin induction (Fig. 2, lanes 3 and 6). When inhibitor treatment was postponed until 4 h after the start of calcitonin treatment, the amounts of uPA mRNA declined (Fig. 2, lanes 8 and 11).

Dose–response of calcitonin induction

Fig. 3(a) shows the dose–response of calcitonin induction of uPA-mRNA accumulation after 2 h in the presence of cycloheximide. Calcitonin concentrations of 0.1 ng/ml or less had negligible effects. When cells were treated with 0.1 ng of calcitonin/ml in the presence of cycloheximide, about 500 uPA-mRNA molecules/cell accumulated. Cycloheximide treatment also sharply increased the extent of accumulation.

Fig. 2. Effect of timing of addition of inhibitors of macromolecular synthesis on calcitonin-induced uPA-mRNA accumulation

At the start of the experiment, cells were washed with PBS(+), and then provided with 2 ml of serum-free medium. Additions were as follows: lane 1, no additions; lane 2, 10 ng of calcitonin/ml for 4 h; lane 3, 1 μg of actinomycin D/ml for 4 h, then also 10 ng of calcitonin/ml for 4 h; lane 4, 10 μg of cycloheximide/ml for 4 h, then also 10 ng of calcitonin/ml for 4 h; lane 5, 10 μg of puromycin/ml for 4 h, then also 10 ng of calcitonin/ml for 4 h; lane 6, 30 μg of α-amanitin/ml for 4 h, then also 10 ng of calcitonin/ml for 4 h; lane 7, 10 ng of calcitonin/ml for 8 h; lane 8, 10 ng of calcitonin/ml for 4 h, then also 10 ng of actinomycin D/ml for 4 h; lane 9, 10 ng of calcitonin/ml for 4 h, then also 10 μg of cycloheximide/ml for 4 h; lane 10, 10 ng of calcitonin/ml for 4 h, then also 10 μg of puromycin/ml for 4 h, lane 11, 10 ng of calcitonin/ml for 4 h, then also 30 μg/ml of α-amanitin/ml for 4 h. At the end of the incubation, RNA was prepared and 5 μg subjected to Northern-blot hybridization analysis.
The superinductive effect of protein-synthesis inhibition on uPA-mRNA accumulation appeared once again to be specific for calcitonin as an inducer. Fig. 3(b) shows the dose–response of TPA induction of uPA-mRNA accumulation in the presence and absence of cycloheximide. In contrast with the results noted with calcitonin (Fig. 3a), cycloheximide treatment did not shift the TPA dose–response curve to increased sensitivity to lower TPA concentrations. It also appeared that cycloheximide treatment decreased the inductiveness of higher TPA concentrations (10 and 100 ng/ml).

**Increase in uPA-gene transcription and in uPA-mRNA stability**

The results from Figs. 1, 2 and 3 suggested that, in the absence of protein synthesis, calcitonin-induced uPA-mRNA synthesis or stability, or both, increased. Accordingly, we performed experiments to look more directly at the effects on protein-synthesis inhibition on uPA-mRNA synthesis and stability.

Fig. 4 shows uPA-gene transcription in nuclei derived from cells treated in different ways. After treatment with a sub-optimal dose of calcitonin (0.1 ng/ml) in the presence or absence of cycloheximide, nuclei were induced by 1 ng of calcitonin/ml, which by itself was a sub-optimal dose.

Cycloheximide treatment thus shifted the dose–response curve of calcitonin, resulting in a greater sensitivity to the hormone. These results reflected another facet of superinduction; that is, low concentrations of calcitonin became more potent in the absence of protein synthesis. The results suggested that, in the absence of protein synthesis, calcitonin induction of uPA mRNA synthesis increased. An increase in stability would probably have been insufficient to explain the striking increase in uPA-mRNA accumulation on induction by 0.1 ng of calcitonin/ml. Similar results were obtained with other protein-synthesis inhibitors, puromycin and pactamycin (results not shown).

![Graph](image1)

**Fig. 3. Effect of cycloheximide on dose–response curves for induction by calcitonin and TPA of uPA-mRNA accumulation**

At the start of the experiment, cells were provided with (○) or without (●) 10 μg of cycloheximide/ml; 30 min later, the indicated concentrations of calcitonin (a) or of TPA (b) were added for 2 h. At the end of the incubation, RNA was prepared and the amount of uPA mRNA was measured by solution hybridization. The experiment was done twice, with similar results.

![Graph](image2)

**Fig. 4. Effect of cycloheximide on uPA-gene transcription**

Cells (1 x 10\(^6\)) were plated on 150 mm dishes and provided with serum-containing medium; 2 days later, cells were washed with PBS (+), and then provided with serum-free medium with (○, △) or without (●) 10 μg of cycloheximide/ml for 30 min. Then 0.1 ng of calcitonin/ml (○, △) was added. At the times indicated, nuclei fractions were prepared, transcription was continued for 90 min at 26 °C in the presence of [\(^3\)H]UTP, and the extent of uPA-gene transcription was measured by filter hybridization. The experiment was done twice, with similar results.
isolated and transcription was continued in the presence of [3H]UTP. Then RNA was extracted and the extent of radioactivity in uPA mRNA was determined by filter hybridization. By itself, cycloheximide caused measurable but, in our view, negligible increases in uPA-gene transcription. In the absence of cycloheximide, uPA-gene transcription was detected after a 20 min treatment with 0.1 ng of calcitonin/ml, but the rate of transcription fell to negligible values after a 2 h treatment. In the presence of cycloheximide, however, the rate of transcription induced by 0.1 ng of calcitonin/ml remained high through 2 h. These results showed that inhibition of protein synthesis led to an increase in calcitonin-induced uPA-gene transcription.

Fig. 5 shows the stability of uPA mRNA derived from cells treated in different ways. Cells were treated with a high dose (10 ng/ml) of calcitonin in the presence of [H]uridine for 4 h. Then the incubation was continued with calcitonin, with unlabelled uridine as a chase, and in the presence or absence of cycloheximide. At various times after the start of the chase, RNA was isolated and the radioactivity remaining in uPA mRNA was measured by filter hybridization. As shown in Fig. 5, the half-life of uPA mRNA was about 2.5 h in the absence of cycloheximide. In the presence of cycloheximide, the half-life increased to over 20 h.

We considered the possibility that cycloheximide treatment extended the half-life of uPA mRNA merely because cycloheximide, an inhibitor of elongation, could have caused polysomal aggregation, possibly rendering the mRNA less susceptible to ribonuclease action (Ernest, 1982; see also Pestka, 1971, for review). If this were the case, then an increase in stability noted with cycloheximide would not have been a consequence of the inhibition of protein synthesis itself. Accordingly, we performed a similar half-life experiment with pactamycin, an inhibitor of protein-synthesis initiation that disrupts polysomes. The results with pactamycin (not shown) were similar to those with cycloheximide (Fig. 5). Overall polysomal profiles of cells incubated for 30 min in the presence of 10 μg/ml of cycloheximide/mL, a relatively high dose that “freezes” polysomes (Shatkin et al., 1977), showed little change compared with untreated controls (results not shown). Polysomal profiles of cells incubated for 30 min in the presence of 0.3 μM-pactamycin showed decreases in polysomes and a corresponding increase in monosomes (results not shown), similar to that reported by Ernest (1982). We concluded that inhibition of protein synthesis led to an increase in calcitonin-induced uPA-mRNA stability.

DISCUSSION

We demonstrated that the inhibition of protein synthesis increased both calcitonin-induced uPA-gene transcription and uPA-mRNA stability. This explained the two sorts of superinductive effects of protein synthesis inhibition on calcitonin treatment: an increase in uPA-mRNA accumulation over time, and a shift in the dose–response curve so that lower calcitonin doses became more potent.

Our observations with other protein-synthesis inhibitors confirmed those with cycloheximide, arguing that our results were actually the consequence of inhibition of protein synthesis itself. This was of particular concern in the half-life study. Ernest (1982) noted that stabilization of tyrosine aminotransferase mRNA activity was observed with cycloheximide, but not with pactamycin. We found that pactamycin had the same effect as cycloheximide in stabilizing calcitonin-induced uPA mRNA.

The superinductive effects of cycloheximide could not be mimicked when TPA was used instead as the inducer. The superinductive effects that we observed were specific for calcitonin, which acts through the cyclic AMP-dependent protein kinase (Goldring et al., 1978). TPA acts through the Ca2+- and lipid-dependent protein kinase (Castagna et al., 1982). The different actions of calcitonin and TPA in LLC-PK1 cells might provide a clue to aid our further investigations into the mechanism of superinduction. In any case, the failure of TPA treatment to be superinduced by cycloheximide argued that superinduction in this system is not merely a general effect of protein-synthesis inhibition. If this were so, then we would have expected to observe superinduction with any inducer.

Interestingly, Stoppelli et al. (1986) reported superinduction by cycloheximide of TPA-induced uPA mRNA in human carcinoma cells. They did not elaborate by distinguishing between an increase in transcription, an increase in mRNA stability, or both. Nonetheless, their results taken with ours suggest that the effect of protein-synthesis inhibition on TPA induction depends on the system under study.

Inhibition of protein synthesis has been reported to increase transcription of various genes, including actin (Ringold et al., 1984; Elder et al., 1984), adenovirus early (Nevins, 1981; Cross & Darnell, 1983; Eggerding, 1985), cytochrome P450 (Israel et al., 1985), interferon (Ringold et al., 1984), and metallothionein (Mayo &
Palminteri, 1981) genes. Some have reported that inhibition of protein synthesis increases histone-gene transcription (Graves & Marzluff, 1984; Sive et al., 1984); others have reported no change (Stimac et al., 1984). On the other hand, enhanced transcription of the c-myc gene in some cells requires continuous protein synthesis, but not in others (Linial et al., 1985). Inhibition of protein synthesis has been reported to increase the stability of various mRNAs including histone (Stimac et al., 1984; Sive et al., 1984), interferon (discussed by Ringold et al., 1984), myc (Dani et al., 1984) and tubulin (Baker et al., 1986) mRNA.

A possible mechanism to explain these results and ours is that labile proteins decrease the synthesis and stability of various mRNAs, particularly those mRNAs that are inducible, short-lived, or both. As far as we know, our system is unique from the others in that we observed, in the context of hormonal induction, that inhibition of protein synthesis led both to increased transcription and increased mRNA stability. In studying the mechanism of superinduction, it will be interesting to find out which regions of the uPA gene and of the uPA mRNA are responsible.

We are grateful to Infectious Diseases Research, The Upjohn Co., Kalamazoo, MI, U.S.A., for the gift of pactamycin. We thank T. Hohn, D. Jans, A. Matus, D. Monard, C. Pearson and W.-D. Schleunig for critical reading of the manuscript.

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Received 1 August 1986/27 October 1986; accepted 4 November 1986

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