Ca\(^{2+}\) Potentiates cAMP-dependent Expression of Urokinase-type Plasminogen Activator Gene through a Calmodulin- and Protein Kinase C-independent Mechanism*

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In the porcine renal epithelial cell line, LLC-PK\(_1\), activation of the cAMP-dependent signal transduction pathway induces the urokinase-type plasminogen activator (uPA) gene. We show here that the cAMP response is enhanced when the intracellular calcium concentration is increased. When LLC-PK\(_1\) cells were treated with the calcium ionophore ionomycin alone, there was no uPA mRNA accumulation. However, in the presence of ionomycin the dose-response of 8-bromo-cAMP (Br-cAMP) with respect to uPA mRNA accumulation was shifted toward the lower concentrations of Br-cAMP. A Northern blot analysis after the inhibition of RNA synthesis and nuclear run-on assays showed that the synergistic effect of Ca\(^{2+}\) could be attributed to increases in uPA gene transcription and mRNA stability. In the presence of cycloheximide, a protein synthesis inhibitor, uPA mRNA was stabilized, but the effect of ionomycin on Br-cAMP-induced mRNA accumulation was still maintained. The result suggests that the Ca\(^{2+}\), at least on transcription, does not require new protein synthesis. Ionomycin treatment did not modify the activity of the cAMP-dependent protein kinase, suggesting that Ca\(^{2+}\) either affects a step in the pathway between the kinase and the uPA gene, or acts independently of the cAMP-dependent protein kinase pathway. The effect of ionomycin was not suppressed by protein kinase C down-regulation or by inhibitors of calmodulin. Synergism was also observed when Br-cAMP was replaced with calcitonin, a peptide hormone which is coupled to adenylate cyclase, and when ionomycin was replaced with another ionophore A23187, suggesting that the synergism is due to an interaction between cAMP-dependent and Ca\(^{2+}\)-dependent signal transduction pathways.

A variety of signal transduction pathways have been described for the action of hormones and growth factors. Well-known signal transduction pathways are those which utilize adenylate cyclase (Helmreich et al., 1976; Ross and Gilman, 1980) and phospholipase C (Smith et al., 1986; Taylor and Merritt, 1986). The activation of adenylate cyclase increases the intracellular concentration of cAMP, a second messenger which in turn activates cAMP-dependent protein kinase (cAMP-PK)\(^1\) (Walsh and Cooper, 1979; Beavo and Mummy, 1982; Cohen, 1982). Activated phospholipase C hydrolyzes phosphoinositides and consequently increases the concentration of two second messengers, diacylglycerol and inositol triphosphate (Berridge, 1987). The former activates protein kinase C (PK-C) (Nishizuka, 1984, 1986) and the latter increases the intracellular Ca\(^{2+}\) concentration, [Ca\(^{2+}\)], by mobilizing calcium storage from the endoplasmic reticulum (Hokin, 1985; Berridge, 1987).

Because individual cells are exposed to different extracellular stimuli, multiple signal transduction pathways may be activated simultaneously. Consequently, many investigations have focused on the interactions between different signal transduction pathways, especially those involving adenylate cyclase and phospholipase C. 12-O-tetradecanoylphorbol 13-acetate (TPA), which activates PK-C, enhances agonist-induced adenylate cyclase activities (Bell et al., 1985; Sibley et al., 1986; Yoshimasa et al., 1987), thus modulating the cAMP-dependent pathway. In turn, activation of cAMP-dependent protein kinase modulates PK-C activity (Narindrasorak et al., 1987). PK-C and cAMP-PK pathways also converge at the level of gene regulation. For example, IκB binds to the immunoglobulin gene-specific transcription factor, NFκ-B, resulting in a cytoplasmic localization and inhibition of its transcriptional activity. When IκB is phosphorylated by either PK-C or cAMP-PK, it releases NFκ-B, allowing it to enter the nucleus and activate transcription (Baeuerle and Baltimore, 1988a, 1988b; Shirakawa and Mizel, 1989). The AP1-binding sequence mediates the action of both CAMP-PK and PK-C on gene expression depending on the flanking sequences (Deutsch et al., 1988). Interactions are also observed between CAMP-PK and calcium pathways. For example, the regulation of phosphorylase kinase, a multisubunit enzyme involved in the glycolytic pathway, by cAMP-dependent phosphorylation is modulated by the binding of Ca\(^{2+}\) to one of its subunits, calmodulin (Cohen, 1978). In brain, Ca\(^{2+}\)-calmodulin affects the cAMP-dependent pathway by activating cyclic nucleotide phosphodiesterase (Cheung, 1970; Kakiuchi and Yamasaki, 1970) and adenylate cyclase (Wolf and Brostrom, 1979).

However, to our knowledge, synergism between cAMP and calcium resulting in the enhancement of specific mRNA production has not been reported.

In this report, we provide evidence that calcium interacts with the cAMP-dependent signal transduction pathway at the

\(^1\) The abbreviations used are: cAMP-PK, cAMP-dependent protein kinase; PK-C, protein kinase C; TPA, 12-O-tetradecanoylphorbol 13-acetate; uPA, urokinase-type plasminogen activator; Br-cAMP, 8-bromo-cAMP; DRB, 5,6-dichloro-1-β-ribofuransosyl-benimidazole; BAPTA/AM, [bis-(O-aminophenoxy-ethane-N,N',N'-tetraacetic acid, tetra(acetoxymethylester); FURA Z/AM, [bis-(O-carboxy-oxazol-2-yl)-6-aminobenzofuran-5-oxo]-2-[2'-amino-5'-methylphenoxo]ethane-N,N',N'-tetraacetic acid. 

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level of gene regulation in LLC-PK₁ cells. LLC-PK₁, is an epithelial cell line derived from porcine renal proximal tubules (Hull et al., 1976; Gstraunthaler, 1988). These cells have adenylyl cyclase-coupled receptors for the peptide hormone calcitonin (Goldring et al., 1978; Héron et al., 1981; Hemmings, 1986). Calcitonin is involved in the long term homeostasis of plasma calcium concentration in mammals by controlling bone resorption (Talmage et al., 1983) and calcium reabsorption in renal tubules (Ardaillou, 1975). In LLC-PK₁ cells, the activation of cAMP-PK by calcitonin leads to the induction of the urokinase-type plasminogen activator (uPA) gene in the absence of new protein synthesis (Dayer et al., 1981; Nagamine et al., 1983; Degen et al., 1985). Consequently, we were interested in the interaction between cAMP-dependent and Ca²⁺-dependent pathways with respect to uPA gene regulation. As the uPA gene is induced by TPA treatment in these cells (Degen et al., 1985), we also investigated the interaction between PK-C dependent and Ca²⁺-dependent pathways.

**EXPERIMENTAL PROCEDURES**

**Materials**—8-Bromo-cAMP (Br-cAMP) was purchased from Boehringer Mannheim, ionomicyn from Calbiochem, TPA from Pharmacia LKB Biotechnology Inc., cycloheximide and trifluoperazine from Sigma, 5,6-dichloro-1-b-D-ribofuranosylbenzimidazole (DRB) from Boehringer Mannheim, BAPTA/AM and FURA 2/AM were from Molecular Probes (Eugene, OR). Escherichia coli DNA polymerase I Klenow fragment was purchased from Biofines, and GeneScreen Plus nylon membrane from Du Pont-New England Nuclear. [α-³²P]dCTP (800 mCi/mmol) and [α-³²P]dGTP (400 mCi/mmol) were obtained from Amersham Corp. and Du Pont-New England Nuclear, respectively.

**Cells**—LLC-PK₁ cells (Hull et al., 1976) were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (GIBCO, Bethesda Research Laboratory) at 37 °C in a humidified 5% CO₂ (6%) incubator. Cells were plated at 10⁶ cells/35-mm (diameter) plastic dish with 2 ml of medium and 24 h later treated with reagents indicated.

**Plasmids**—cDNA clones for pig uPA, pYN15, and for pig β-actin have been described (Nagamine et al., 1984; Andrus et al., 1988). A full-length pig grp78 cDNA clone was obtained by screening a cDNA library of LLC-PK₁ cells using a pig grp78 cDNA clone, pPK7, which has historically been reported to code for grp78 (Andrus et al., 1988) and later confirmed to code for a member of the grp78 family, grp78.²

**Probes**—Radioactive probes were prepared by the random priming method (Feinberg and Vogelstein, 1983) using purified cDNA inserts. [α-³²P]dCTP (800 mCi/mmol) and [α-³²P]dGTP (400 mCi/mmol) were obtained from Amersham Corp. and Du Pont-New England Nuclear, respectively.

**Northern hybridization**—Total RNA was prepared by the acid guanidium thiocyanate method using guanidinium thiocyanate according to Chomczynski and Sacchi (1987). From 10⁶ cells, 20-30 μg of RNA was obtained. Total RNA was fractionated on agarose gel containing formaldehyde (5 μg/lane) and blotted onto a nylon membrane with UV (Khandjian, 1987) followed by heating at 80 °C in a vacuum oven for 2 h. Equal loading and blotting of each RNA sample were verified by staining the filter with methylene blue (Herrn and Schmidt, 1988). The filter was prehybridized and hybridized to radioactive probes as described (Nagamine et al., 1983), and then exposed to X-Omat AR film with an intensifying screen at -70 °C.

**Assay**—The levels of uPA mRNA by Br-cAMP and TPA—In LLC-PK₁ cells, uPA gene expression is induced by treatments that lead to the activation of cAMP-PK (Dayer et al., 1981; Nagamine et al., 1983) and PK-C (Degen et al., 1985). First, we used optimal concentrations of Br-cAMP (1 μm) and TPA (10 ng/ml) to test the effect of a calcium ionophore, ionomicyn, on uPA mRNA accumulation. Total RNA was prepared at several time points of incubation and analyzed by Northern blot hybridization. Equal loading of the RNA sample in each lane was verified by staining of the filter before hybridization and inclusion of an actin probe in hybridization. Fig. 1 shows that ionomicyn alone did not have any effect on uPA mRNA induction over the time course tested (lanes 5-7). Levels of uPA mRNA 2 h after induction by Br-cAMP were similar whether ionomicyn was absent or present (705 cpm in lane 8 and 745 cpm in lane 14 in Fig. 1a). However, while the concentration of uPA mRNA subsequently decreased in the absence of ionomicyn, 2.5 μl of conditioned medium was fractionated in sodium dodecyl sulfate-polyacrylamide (10%) gel. After electrophoresis, the gel was rinsed twice for 10 min at ambient temperature with 2.5% Triton X-100 and 0.1 M Tris-HCl, pH 7.5, and twice for 10 min with 0.1 M Tris-HCl, pH 7.5. The gel was then put on a 0.7% agarose layer (0.5 mm thick) containing 0.1 M Tris-HCl, pH 7.5, 40 μg/ml human plasminogen and 2% skim milk (Fluka), and kept at 37 °C with moisture for about 1 h. Region of uPA activity in the gel is revealed as a lytic zone due to the lysis of casein.

**Measurement of Intracellular Free Calcium Concentration**—Cells were loaded with 3 μm FURA 2/AM for 40 min at 37 °C, washed, and incubated for 30-60 min before initiating the experiments. A Zeiss microscope (Axiovert 35) equipped with a temperature-controlled stage, a Plan-Neofluar 40X objective, and a proximity focus-intensified camera (Hamamatsu C2400-09) were used. The ratio of the images generated at 350 and 380 nm were calculated using the Image/AT software purchased from Universal Imaging, West Chester, PA. The values obtained were calibrated by quenching with Mn²⁺ (Hesketh et al., 1983).

**CAMP-dependent Protein Kinase**—Cells (1 x 10⁶) were plated in a 35-mm plastic dish with 2 ml of Dulbecco's modified Eagles medium containing 10% fetal calf serum. After 24 h, the cells were treated for the times indicated in the figure, cell extracts were prepared, and protein kinase activities were measured in the absence or presence of 10 μM CAMP using the substrate Kemptide (Hemmings, 1986). The activity ratio (−cAMP)/(+cAMP) is a measure of the amount of inactive CAMP-dependent protein kinase.

**RESULTS**

**Calcium Ionophore Modifies the Accumulation of uPA mRNA by Br-cAMP and TPA**—In LLC-PK₁ cells, uPA gene expression is induced by treatments that lead to the activation of cAMP-PK (Dayer et al., 1981; Nagamine et al., 1983) and PK-C (Degen et al., 1985). First, we used optimal concentrations of Br-cAMP (1 μm) and TPA (10 ng/ml) to test the effect of a calcium ionophore, ionomicyn, on uPA mRNA accumulation. Total RNA was prepared at several time points of incubation and analyzed by Northern blot hybridization. Equal loading of the RNA sample in each lane was verified by staining of the filter before hybridization and inclusion of an actin probe in hybridization. Fig. 1 shows that ionomicyn alone did not have any effect on uPA mRNA induction over the time course tested (lanes 5-7). Levels of uPA mRNA 2 h after induction by Br-cAMP were similar whether ionomicyn was absent or present (705 cpm in lane 8 and 745 cpm in lane 14 in Fig. 1a). However, while the concentration of uPA mRNA subsequently decreased in the absence of ionomicyn,
it was maintained or slightly increased up to 8 h in the presence of ionomycin; the difference at 8 h was 2-fold (400 cpm in lane 10 versus 880 cpm in lane 16). When cells were treated with 10 ng/ml TPA, the concentration of uPA mRNA reached a maximum at 2 h and then declined (lanes 11–13). Ionomycin did not change the transient character of TPA-induced uPA mRNA accumulation, but retarded the rapid decline in uPA mRNA level (lanes 17–19). Because the influence of ionomycin was more pronounced with Br-cAMP-mediated induction than with TPA induction, we concentrated on the effect of ionomycin on CAMP-dependent uPA gene regulation.

When the optimal concentration of Br-cAMP was employed, the influence of ionomycin on uPA mRNA accumulation was not observed within 2 h of induction. To test whether ionomycin can modulate CAMP-dependent uPA gene activation at earlier stages of induction, we tested different concentrations of Br-cAMP in 2-h incubations. Fig. 2 shows that at suboptimal concentrations of Br-cAMP, uPA mRNA accumulation was significantly increased by ionomycin (panel a, lanes 9 and 10 versus lanes 3 and 4), while there was no change in either the maximal induction (lanes 11 and 12 versus lanes 5 and 6) or basal levels of uPA mRNA (lanes 7 and 8 versus lanes 1 and 2). Thus, the effect of ionomycin was seen to increase the sensitivity of cells to Br-cAMP with respect to uPA mRNA induction.

The enhancing effect of ionomycin on uPA mRNA induction was most notable with 0.1 mM Br-cAMP after 2 h of induction (Fig. 2 panel a, lane 9 versus lane 3). When incubation was continued for 24 h, the enhancement by ionomycin became more pronounced (Fig. 3). At this concentration, Br-cAMP alone induced uPA mRNA accumulation in a biphasic manner. The uPA mRNA reached a peak after 2 h, declined with time until 8 h, and then increased until the experiment ended at 24 h (Fig. 3 panel a, lanes 7–10); the second induction was always observed, but never exceeded the initial induction. In the presence of ionomycin, the same concentration of Br-cAMP induced a much higher level of uPA mRNA. The level of uPA reached a plateau at 4 h, instead of a peak, and the accumulation resumed again after 8 h (lane 14). Interestingly, two phases of uPA mRNA accumulation were observed regardless of the presence or absence of ionomycin: within 2 h of incubation a peak (in the absence of ionomycin, lane 7) or a plateau (in the presence of ionomycin, lane 11), and another increase after 8 h (lanes 10 and 14).

![FIG. 2. Effects of ionomycin on uPA mRNA induction with increasing concentration of Br-cAMP. Cells were treated with different concentrations of Br-cAMP in the presence or absence of 10 μM ionomycin for 2 h, then total RNA was prepared and 5 μg of RNA samples was analyzed by Northern blot hybridization.](http://www.jbc.org/)

**Fig. 2. Effects of ionomycin on uPA mRNA induction with increasing concentration of Br-cAMP.** Cells were treated with different concentrations of Br-cAMP in the presence or absence of 10 μM ionomycin for 2 h, then total RNA was prepared and 5 μg of RNA samples was analyzed by Northern blot hybridization. a, Northern blot hybridization. RNA was hybridized with uPA (10⁶ cpm/ml) and actin (10⁵ cpm/ml) probes. Concentrations of Br-cAMP are given on top of the gel. b, stained filter before hybridization.

Because we have used Br-cAMP to activate the CAMP-dependent signal transduction pathway leading to uPA gene activation, it may be argued that ionomycin synergizes only with a particular type of CAMP analogue. Rannels and Corbin (1980) have shown that the two CAMP-binding sites in each regulatory subunit of CAMP-PK have different affinities for CAMP analogues. Partial occupation of CAMP-binding sites by a CAMP analogue may confer a biological activity on the regulatory subunit different from its role as a modulator of the catalytic subunit of CAMP-PK. However, this is unlikely since ionomycin had a similar enhancing effect with suboptimal concentrations of salmon calcitonin, a peptide hormone which activates adenylate cyclase in LLC-PK1 cells (Goldring et al., 1978) (data not shown).

When a suboptimal concentration of TPA was used, the enhancing effect of ionomycin was also observed (data not shown). However, since PK-C is a Ca²⁺-dependent enzyme, it would be difficult to determine an effect of ionomycin other than that on PK-C. We, therefore, directed our attention on the effect of ionomycin on the Br-cAMP-dependent pathway.

**FIG. 3. Effects of ionomycin on Br-cAMP-induced uPA mRNA accumulation.** Cells were plated at 5 × 10⁶ cells/35-mm plastic dish, and 24 h later 0.1 mM Br-cAMP and 10 μM ionomycin were added and incubation was continued for 24 h. Conditioned medium (2.5 ml) was fractionated by 6% sodium dodecyl sulfate-polyacrylamide (nonreducing) gel electrophoresis and uPA activity in the gel was visualized by the casein underlay assay as described under "Experimental Procedures." Lanes: I, no treatment; 2, 0.1 mM Br-cAMP; 3, 10 μM ionomycin; 4, 0.1 mM Br-cAMP and 10 μM ionomycin. The enhancing effect of ionomycin was also observed (data not shown).

**Fig. 4. Effect of ionomycin on uPA expression.** Cells were plated at 5 × 10⁶ cells/35-mm plastic dish, and 24 h later Br-cAMP and ionomycin were added and incubation was continued for 24 h. Conditioned medium (2.5 ml) was fractionated by 6% sodium dodecyl sulfate-polyacrylamide (nonreducing) gel electrophoresis and uPA activity in the gel was visualized by the casein underlay assay as described under “Experimental Procedures.” Lanes: 1, no treatment; 2, 0.1 mM Br-cAMP; 3, 10 μM ionomycin; 4, 0.1 mM Br-cAMP and 10 μM ionomycin.
**Effect of Ionomycin on the Stability of uPA mRNA.** Cells were treated with 1 mM Br-cAMP (○) or 1 mM Br-cAMP plus 10 μM ionomycin (□), and chased 2 h later by adding 10 μg/ml DRB to the medium. Total RNA was collected at several time points over 6 h and analyzed for uPA mRNA by Northern blot hybridization. a, Northern blot hybridization. Treatment of cells and time after the start of chase are indicated on top of the gel. b, stained filter before hybridization. c, quantitation of uPA mRNA. Regions corresponding to uPA mRNA were cut, counted in a scintillation counter, and plotted.

**Effect of Ionomycin on the Stability of uPA mRNA in the Presence of Cycloheximide.** Cells were treated and analyzed as in Fig. 3, except that 10 μg/ml cycloheximide was added in every culture. a, Northern blot hybridization. RNA was hybridized with the uPA probe. Treatment and incubation time are given on top of the gel. b, stained filter before hybridization.

**Figure 5.** Effect of ionomycin on uPA gene transcription. Nuclei were prepared from untreated LLC-PK₁ cells (control) and cells from LLC-PK₁ cells that had been treated for 90 min with 10 μM ionomycin, 0.1 mM Br-cAMP, 0.1 mM Br-cAMP plus 10 μM ionomycin, 1 ng/ml TPA, or 1 ng/ml TPA plus 10 μM ionomycin. Transcription in the isolated nuclei was analyzed by hybridization of the 3²P transcripts (4 x 10 cpm) to 1 μg of pBR322, uPA or grp78 plasmid immobilized on nitrocellulose filters. Hybridization of each transcript was done in the same tube for three different filters.
Ca\(^{2+}\) Enhancement of cAMP-dependent uPA Gene Expression

Chase experiments increased from 150 min to \(\geq 20\) h in the presence of cycloheximide (Altus et al., 1987). Consequently, the effect of ionomycin on uPA mRNA stability was masked in the following experiment.

Without on-going protein synthesis the accumulation of Br-cAMP-induced uPA mRNA increased up to 8 h and then reached a plateau or declined in the presence or absence of ionomycin, respectively (Fig. 7). Still, the induction of uPA mRNA was higher in the presence of ionomycin than that in the absence of it; 3.5-fold at maximum points (2600 cpm in lane 13 versus 740 cpm in lane 9, Fig. 7a). As expected, the mRNA-stabilizing effect of cycloheximide, when DRB was added to inhibit new RNA synthesis at 2 h after induction, uPA mRNA did not decay in the following 6-h incubation with or without ionomycin (data not shown). Hence, the effect of ionomycin seen in the presence of cycloheximide was solely attributable to the enhancement of transcription. Therefore, the results shown in Fig. 7 indicate that the effect of ionomycin, at least on uPA gene transcription, was through preexisting factor(s).

In contrast to the time course of uPA mRNA accumulation in the absence of cycloheximide shown in Fig. 3, where a biphasic pattern of uPA mRNA accumulation was observed, the time course in the presence of cycloheximide was monophasic; the second increase in uPA mRNA accumulation did not occur (Fig. 7).

Effect of Ionomycin Is Due to Increased Intracellular Ca\(^{2+}\) Concentrations but Not Mediated by Calmodulin—Ionomycin is an ionophore highly specific for Ca\(^{2+}\) (Liu and Hermann, 1978), and therefore the expected immediate action of ionomycin in the cell is to increase the intracellular calcium ion concentration, [Ca\(^{2+}\)]. To confirm this, LLC-PK1 cells were loaded with the fluorescent indicator FURA 2/AM, and [Ca\(^{2+}\)], was monitored after ionomycin treatment as described under “Experimental Procedures.” The results show that the concentration of calcium before treatment with ionomycin was 0.1 \(\mu\)M and increases upon treatment to about 6 \(\mu\)M (Table I). The level of [Ca\(^{2+}\)], remained high at least for 2 h. In contrast, there was no change in [Ca\(^{2+}\)], by treatment with Br-cAMP or SCT.

To further verify that Ca\(^{2+}\) was responsible for the synergistic action of ionomycin on Br-cAMP-mediated uPA mRNA induction, we examined the effect of pretreatment of cells with BAPTA/AM, an intracellular Ca\(^{2+}\) chelator (Tsien, 1981). As seen in previous experiments, ionomycin enhanced the Br-cAMP-mediated uPA mRNA accumulation 3.2-fold (Fig. 8, lane 5 versus lane 4). But when cells were pretreated with BAPTA/AM, more than 70% of the enhancement by ionomycin was abrogated (lane 15 versus lane 14). Br-cAMP-mediated uPA mRNA induction was not affected at all by BAPTA/AM pretreatment (Fig. 8, lanes 4 and 14). The results, together with those shown in Table I, indicate that the enhancing effect of ionomycin on Br-cAMP-mediated uPA mRNA accumulation is through Ca\(^{2+}\). Another Ca\(^{2+}\) ionophore, A23187, could substitute for ionomycin (data not shown). The results also indicate that cAMP-dependent uPA mRNA induction does not require Ca\(^{2+}\).

Since many intracellular Ca\(^{2+}\) effects are mediated by the ubiquitous calcium-binding protein, calmodulin (Klee et al., 1980), we tested the calmodulin inhibitor, trifluoperazine (Weiss et al., 1980). Trifluoperazine did not affect Br-cAMP-mediated uPA mRNA induction (Fig. 8, lane 9) nor its enhancement by ionomycin (3.6-fold, lane 9 versus lane 10), suggesting that the synergistic effect of Ca\(^{2+}\) was not mediated by calmodulin. We also tested W-7, another calmodulin inhibitor (Hidaka et al., 1980), at concentrations up to 30 \(\mu\)M, but it also did not show any inhibitory effect (data not shown). As expected, ionomycin-induced increase in grp78 gene transcription shown in Fig. 5 was reflected in the level of uPA mRNA, and it was not affected by either Br-cAMP or by trifluoperazine, but it was strongly suppressed by BAPTA/AM (Fig. 8).

Ionomycin Does Not Act Through PK-C or cAMP-PK—Because PK-C is a Ca\(^{2+}\)-dependent enzyme and can modify many biological activities, we sought to exclude the possibility that PK-C mediates the enhancing effect of ionomycin. Cells were treated with 100 ng/ml of TPA for 24 h to down-regulate PK-C (Nishizuka, 1986). After the treatment PK-C was no longer detected in membrane fraction or in the cytoplasmic fraction of LLC-PK1 cells (data not shown). Down-regulated cells were treated with Br-cAMP in the presence or absence of ionomycin. Because PK-C down-regulation makes cells more sensitive to Br-cAMP in terms of uPA mRNA induction, we employed a lower concentration (0.03 mM) of Br-cAMP than that used in the preceding experiments. As shown in Fig. 9, when cells were pretreated with TPA for 24 h, uPA mRNA was no longer inducible by the addition of fresh TPA (lane 17 versus lane 2), indicating that PK-C was efficiently down-regulated. Under these conditions, the effect of ionomycin on Br-cAMP-induced uPA mRNA accumulation was

**TABLE I**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[Ca(^{2+})] (\mu)M</th>
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<tbody>
<tr>
<td>Exp. 1 ((n = 5))</td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>0.132 ± 0.095</td>
</tr>
<tr>
<td>Ionomycin 10 (\mu)M, 20 min</td>
<td>5.8 ± 0.36</td>
</tr>
<tr>
<td>Ionomycin 10 (\mu)M, 40 min</td>
<td>5.7 ± 0.33</td>
</tr>
<tr>
<td>Exp. 2 ((n = 5))</td>
<td></td>
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<tr>
<td>Untreated</td>
<td>0.122 ± 0.088</td>
</tr>
<tr>
<td>Br-cAMP 1 mM, 10 min</td>
<td>0.137 ± 0.126</td>
</tr>
<tr>
<td>Salmon calcitonin 100 ng/ml, 10 min</td>
<td>0.088 ± 0.099</td>
</tr>
</tbody>
</table>

2 A. Ziegler, unpublished data.

**Fig. 8. Effect of inhibitors of calcium metabolism.** Cells were pretreated with 10 \(\mu\)M trifluoperazine or 40 \(\mu\)M BAPTA/AM for 30 min and then treated with 10 \(\mu\)M ionomycin, 0.1 mM Br-cAMP or 10 \(\mu\)M ionomycin plus 0.1 mM Br-cAMP without changing the medium. After 4 h, total RNA was prepared and analyzed for uPA mRNA and grp78 mRNA by Northern blot hybridization. a, Northern blot hybridization. Types of pretreatment and types and lengths of treatments are indicated on top of the gel. Hybridization with uPA and grp78 probes were done with separate filters. Ig, ionomycin; Br, Br-cAMP; A, stained filter before hybridization. Only the filter hybridized with uPA probe is shown.
still clearly observed at all time points tested (lanes 12–15). Like the time course of uPA mRNA accumulation mediated by Br-cAMP in the absence of protein synthesis (see Fig. 7), the time course of PK-C down-regulation became monophasic.

To examine a reference RNA the blot was hybridized with the grp78 probe, and it was observed that grp78 mRNA was also induced by ionomycin after PK-C down-regulation and Br-cAMP did not affect the induction.

As Ca^{2+} may, directly or indirectly, change the characteristics of CAMP-PK we examined the effect of ionomycin on the activity of CAMP-PK after induction by Br-cAMP. The kinase activity in cell extracts was measured in vitro, using the synthetic CAMP-PK substrate Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide), and a ratio of the activity in the presence of CAMP over that in the presence of CAMP was obtained. Activity ratios, ranging from 0 to 1, denote the fraction of CAMP-PK in an active state under basal conditions. The value did not change significantly upon treatment with ionomycin for at least 6 h. As expected, Br-cAMP treatment increased the activity ratio to 0.17 within 1 h, and the value remained unchanged during the time course of experiment. Ionomycin did not further enhance the activity ratio attained with Br-cAMP but slightly suppressed it. Furthermore, the total enzyme activity measured in the presence of CAMP was not affected by ionomycin (data not shown). These data indicate that increased [Ca^{2+}] does not activate CAMP-PK nor modulate its activity induced by Br-cAMP.

DISCUSSION

In this work we have demonstrated that uPA mRNA induction by Br-cAMP was enhanced by the treatment of cells with the calcium ionophore, ionomycin. The effect of ionomycin was through increased intracellular Ca^{2+}. The concentration of Ca^{2+} increased upon ionomycin treatment and ionomycin action was suppressed by BAPTA/AM, a chelator of intracellular Ca^{2+} (Tsien, 1981). Furthermore, another calcium ionophore, A23187, had the same effect as ionomycin.

The induction of uPA mRNA by Br-cAMP does not require Ca^{2+}, suggesting that CAMP-dependent and Ca^{2+}-dependent signal transduction pathways are separate entities. Ionomycin alone does not induce uPA mRNA at least within 8 h of incubation, suggesting that Ca^{2+} modulates one of the steps of the CAMP-dependent pathway. At 24 h, we detected a low level of induction by ionomycin alone, but we do not know if this delayed Ca^{2+}-dependent induction was independent of other pathways, although it did not need new protein synthesis nor PK-C. In this context, it is noteworthy that the time course of Br-cAMP-dependent uPA mRNA accumulation, with or without ionomycin, was biphasic, and that it became monophasic when protein synthesis was suppressed or PK-C down-regulated. It is possible that the mechanisms underlying uPA gene regulation are different for primary and secondary induction. However, one should be cautious in interpreting the experiments measuring changes in the kinetics of uPA mRNA accumulation because inhibition of protein synthesis stabilizes uPA mRNA (Altus et al., 1987) and PK-C down-regulation makes cells more sensitive to CAMP in terms of uPA mRNA accumulation.

The effect of Ca^{2+} on Br-cAMP-induced uPA mRNA accumulation is mediated by two different mechanisms, an increase in the rate of transcriptional initiation and an increase in mRNA stability. These effects could be dissected by stabilizing uPA mRNA with cycloheximide. We also observed Ca^{2+}-induced uPA mRNA stabilization in the absence of uPA gene transcription.

Jans et al. (1987) have shown that in mutant LLC-PK1 cells with reduced levels of CAMP-PK, uPA induction by CAMP was decreased. We have shown that in a homologous cell-free transcription system the transcription of the uPA gene was enhanced by the addition of CAMP and that it was inhibited by a specific inhibitor of CAMP-PK (Nakagawa et al., 1988). In this system induction was also obtained by the addition of the purified catalytic subunit of CAMP-PK without the addition of CAMP (Nakagawa et al., 1988). These results suggest that CAMP-dependent uPA gene regulation in LLC-PK1 cells requires the activation of CAMP-PK. Therefore, in the CAMP-
dependent signal transduction pathway leading to uPA gene activation, cAMP-PK occupies a crucial place as a carrier of information from the cytoplasm to the nucleus. Upon cAMP binding to the regulatory subunit, the catalytic subunit of cAMP-PK is released from the inactive complex in the cytoplasm and is translocated to the nuclei (Nigg et al., 1985). Where does Ca"+ come into play in this pathway? As the characteristics of cAMP-PK were not altered by ionomycin treatment, an increased Ca"+ concentration should affect a step, most likely in the nucleus, between cAMP-PK and the uPA gene in the cAMP-dependent signal transduction pathway. This does not necessarily mean that a protein interacting with Ca"+ is present in nuclei; a chain of reactions ultimately directed to the nuclei might be initiated by Ca"+ in the cytoplasm. Ca"+ must interact with a preexisting protein, since the effect was observed in the absence of new protein synthesis.

Ca"+ by itself can activate some genes, e.g. grp78 (Resendez et al., 1985) and c-fos (Bravo et al., 1987). However, the mechanism underlying the regulation of uPA gene expression by Ca"+ is distinct from that of grp78 and c-fos induction in that uPA gene transcription is not induced by ionomycin alone. The difference cannot be attributed to cell specificity since grp78 mRNA was strongly induced by ionomycin alone in LLC-PK1 cells, and there was no synergism between Br-cAMP and ionomycin in grp78 mRNA induction. It seems, therefore, that there are at least two Ca"+-mediated gene regulatory systems in LLC-PK1 cells.

Different mRNAs have different half-lives (Brawerman, 1987). How the degradation of a particular mRNA is regulated is poorly understood, and so far, no mRNA degrading enzymes have been isolated. We have previously suggested that a labile protein was responsible for uPA mRNA degradation because grp78 mRNA was strongly induced by ionomycin alone in LLC-PK1 cells, and there was no synergism between Br-cAMP and ionomycin in grp78 mRNA induction. It seems, therefore, that there are at least two Ca"+-mediated gene regulatory systems in LLC-PK1 cells.

The characteristics of CAMP- and TPA-induced gene expression were closely related (Karin, 1989; Johnson and McKnight, 1989). Ca"+ regulation might have been evolutionarily maintained during the diversification of the mechanisms of gene regulation by cAMP and TPA. If so, the effect of Ca"+ will be found in many cAMP- and TPA-induced gene expression systems. However, it is also possible that the effects of Ca"+ on cAMP- and TPA-mediated uPA mRNA induction were realized by entirely different mechanisms.

Upon ionomycin treatment, [Ca"+]i, increased from 0.1 to 6 μM, which is within the range of physiological concentrations encountered in cell, suggesting that we can expect the effect of Ca"+ on uPA gene regulation in the context of normal cell physiology. An important role of renal epithelial cells is to regulate the flux of Ca"+, which is absorbed from the glomerular filtrate through the apical membrane by passive transport and secreted out through the basolateral membrane by a Ca"+/Mg"+·ATPase (Doucet and Katz, 1982) or a Na"+·Ca"+-antiporter (Friedman et al., 1981). Therefore, [Ca"+]i may fluctuate under physiological conditions and reach levels high enough to modulate the expression of uPA mRNA. As Ca"+ causes aggregation of some proteins and uPA is a key enzyme in the cascade of extracellular proteolytic process (Saksela and Rifkin, 1988), coordination between uPA gene regulation and Ca"+ metabolism may be physiologically important in preventing aggregation of proteins in the renal tubules.

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* M. S. Altus and Y. Nagamine, manuscript in press.
Ca\textsuperscript{2+} Enhancement of cAMP-dependent uPA Gene Expression


Ca2+ potentiates cAMP-dependent expression of urokinase-type plasminogen activator gene through a calmodulin- and protein kinase C-independent mechanism.
A Ziegler, J Hagmann, B Kiefer and Y Nagamine


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