

# Calcium Ionophore A23187 as a Regulator of Gene Expression in Mammalian Cells

Elpidio Resendez, Jr., Jerry Ting, Kyu Seong Kim, Scott K. Wooden, and Amy S. Lee

Department of Biochemistry and the Norris Cancer Research Institute, University of Southern California School of Medicine, Los Angeles, California 90033. Address correspondence to A. S. Lee.

**Abstract.** The calcium ionophore A23187 can reversibly induce the expression of two glucose-regulated genes, p3C5 and p4A3. This induction requires a continuous presence of the ionophore for over 2 h. Although extracellular  $\text{Ca}^{2+}$  is important for the optimal effect of A23187, it is not necessary for the induction, since a similar response with a lower magnitude can be triggered in cells cultured in low  $\text{Ca}^{2+}$  medium

buffered with EGTA. Both the basal and induced levels of p3C5 and p4A3 transcripts can be modulated by the calmodulin antagonist W-7, indicating the involvement of  $\text{Ca}^{2+}$ /calmodulin-associated pathways. In addition, the sensitivity of the A23187 induction to cycloheximide suggests that the induction process is dependent on de novo protein synthesis.

**I**NTRACELLULAR  $\text{Ca}^{2+}$  has profound effects on a wide variety of cellular activities (Campbell, 1983; Rasmussen and Barrett, 1984), and the  $\text{Ca}^{2+}$  ionophore A23187 is used extensively to mimic the effect of many physiological cell stimuli related to  $\text{Ca}^{2+}$ . For example, the ionophore has been used to induce cell movement (Devore and Nastuk, 1977), fusion (Rose and Loewenstein, 1976), transformation (Duffus and Patterson, 1974), differentiation (Maino et al., 1974), and hormonal responses (Keppens et al., 1977; Kaiser and Edelman, 1977).

Upon treating mammalian cells with A23187, the syntheses of two glucose-regulated proteins (GRPs)<sup>1</sup> with molecular masses of 94–100 kD and 78–80 kD were selectively enhanced (Martonosi et al., 1982; Welch et al., 1983; Lee et al., 1984). Using two cDNA clones, p4A3 and p3C5, encoding the hamster GRPs of 94 and 78 kD (GRP94 and GRP78), respectively (Lee et al., 1981; Lee et al., 1983), we demonstrated that A23187 enhanced the transcriptional rate of these two genes and that A23187, but not other ionophores, specifically stimulated this response (Resendez et al., 1985). We further showed that the 5' flanking sequence of the rat gene encoding GRP78 conferred A23187 regulation on heterologous fusion genes upon transfection into mammalian cells (Resendez et al., 1985). Within this promoter region, a 291-nucleotide fragment with enhancer properties has been identified and could compete for the positive regulatory factors mediating the A23187 induction (Lin et al., 1986).

In this report, we attempt to identify the regulatory components that affect the enhanced transcription of these two genes by A23187. Our results indicate that (a) the continuous presence of A23187 for 2–3 h is required for the increase in

the steady-state mRNA levels of p3C5 and p4A3; (b) ongoing protein synthesis is required for the A23187 induction; (c) extracellular  $\text{Ca}^{2+}$  can enhance the A23187 induction; however, low  $\text{Ca}^{2+}$ /EGTA medium by itself, or in combination with A23187, partially induces these mRNAs; and (d) W-7, an inhibitor of calmodulin-dependent activities, reduces both the basal and induced levels of these RNAs. We propose a possible model, based on these results, by which A23187 may regulate transcription of these genes.

## Materials and Methods

### Cell Lines and Culture Conditions

The Chinese hamster fibroblast cell lines Wg1A and K12 have been described previously (Lee, 1981; Roscoe et al., 1973). K12 is a temperature-sensitive mutant derived from the parental cell line Wg1A. The phenotypes of K12 and Wg1A are similar when the cells are incubated at the permissive temperature, 35°C. The cells are routinely maintained in DME containing 4.5 mg of glucose/ml and supplemented with 10% cadet calf serum.

### Media and Treatment Conditions

The regular DME contains 1.8 mM  $\text{Ca}^{2+}$  in the form of calcium chloride. For the preparation of low  $\text{Ca}^{2+}$  medium, calcium chloride was eliminated from the DME and 10% dialyzed cadet calf serum was used. The concentration of  $\text{Ca}^{2+}$  in the low  $\text{Ca}^{2+}$  medium is estimated to be  $\sim 30$   $\mu\text{M}$ , due to the trace presence of  $\text{Ca}^{2+}$  in the water used (Rasmussen and Barrett, 1984). When EGTA (1–5 mM) was added to the low  $\text{Ca}^{2+}$  medium, the free  $\text{Ca}^{2+}$  concentration was further reduced to below 2 nM (Ogawa, 1968; Drummond, I., personal communication). The concentration of A23187 used was 7  $\mu\text{M}$ , which is optimal for the induction (Resendez, E., unpublished results). W-7 (N-[6-aminohexyl]-5-chloro-1-naphthalenesulfonamide) and 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) were purchased from Sigma Chemical Co. To make stock solutions, W-7 was dissolved in distilled  $\text{H}_2\text{O}$  to a concentration of 5 mM, and TPA was dissolved in acetone to a concentration of 4 mM. They were stored at  $-20^\circ\text{C}$  and used after further dilution with distilled  $\text{H}_2\text{O}$ .

1. Abbreviation used in this paper: GRP(s), glucose-regulated protein(s).

## Plasmid DNA

The construction of the hamster cDNA plasmids p3C5 and p4A3, encoding hamster GRP78 and GRP94, respectively, have been described (Lee et al., 1981; Lee et al., 1983). The plasmid, pJ, is a cDNA clone encoding for mouse  $\alpha$ -skeletal actin (Artishevsky et al., 1984). Under our hybridization conditions, pJ cross-hybridizes with the beta forms. The human  $\alpha$ -tubulin cDNA plasmid was described (Cowan et al., 1983).

## Preparation of Cytoplasmic RNA

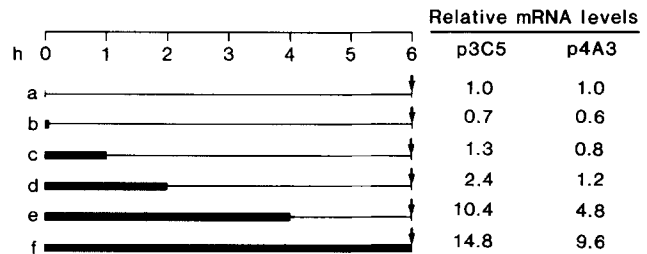
Extraction of cytoplasmic RNA is a modification of a previously described method (Lee et al., 1983). Briefly, ~80% confluent cell cultures from 10- or 15-cm diameter dishes were washed once with cold PBS. Cells were pelleted at 200 g in a centrifuge (Centra-7R; International Equipment Co., Needham Heights, MA) for 3 min at 4°C. The cell pellet was resuspended in 0.6 ml of cold Isohigh buffer (0.14 M NaCl, 10 mM Tris hydrochloride, pH 8.4, 1.5 mM MgCl<sub>2</sub>), lysed with 70  $\mu$ l of 5% Nonidet P-40, and centrifuged at 400 g in a Centra-7R centrifuge for 3 min. The supernatant was recovered and transferred into Eppendorf tubes containing 0.3 ml of NETS buffer (0.1 M NaCl, 10 mM Tris hydrochloride, pH 8.4, 1 mM EDTA, 1% SDS). This mixture was extracted twice with 0.5 ml of phenol/chloroform/isoamyl alcohol (25:24:1) pre-equilibrated with 10 mM Tris hydrochloride (pH 7.9), and 1 mM EDTA. The aqueous phase was extracted once with 0.5 ml of chloroform. The RNA in the aqueous phase was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.6) and 2 vol of 95% ethanol. After 16 h at -20°C, the RNA samples were centrifuged in a microfuge (Brinkmann Instruments Co., Westbury NY) for 15 min at 4°C. The RNA pellet was dried under vacuum and resuspended in 70  $\mu$ l of 10 mM Tris hydrochloride (pH 7.6), and 1 mM EDTA.

## RNA Blot Hybridization

10  $\mu$ g of cytoplasmic RNA from each sample were applied on denaturing formaldehyde-formamide agarose gels, subjected to electrophoresis, and transferred onto nitrocellulose filters as previously described (Lee et al., 1983). In the hybridization reactions, the filters were pretreated for 2-3 h at 42°C in a 10-ml mixture containing 10 $\times$  Denhardt (1 $\times$  Denhardt, 0.02% each of bovine serum albumin, polyvinylpyrrolidone, Ficoll), 5 $\times$  SET (1 $\times$  SET, 0.15 M NaCl, 30 mM Tris hydrochloride, pH 8.0, 2 mM EDTA), 50 mM sodium phosphate buffer (pH 6.8), and 0.3% SDS. Then the RNA blot was prehybridized for 1-2 h at 42°C in a 10-ml solution containing 50% deionized formamide, 5 $\times$  SET, 1 $\times$  Denhardt, 20 mM sodium phosphate buffer, 0.1% SDS, and 50  $\mu$ g/ml of denatured salmon sperm DNA (sonicated and phenol extracted; Sigma Chemical Co., St. Louis, MO). The hybridization of RNA blots was carried out with the same solution used during prehybridization, except that the labeled probe was denatured together with the salmon sperm DNA and added to the hybridization solution. Blots were hybridized with 10<sup>6</sup> cpm/ml of nick-translated cDNA plasmids with specific activities of 2-5  $\times$  10<sup>7</sup> cpm/ $\mu$ g of DNA. Hybridizations were carried out at 42°C for 16 h. Filters were washed once at room temperature for 40 min in 4 $\times$  SSC (1 $\times$  SSC, 150 mM NaCl, 15 mM sodium citrate), 0.1% SDS, 0.1% sodium pyrophosphate, and 25 mM sodium phosphate buffer (pH 6.8); three times at 50°C for 45 min each in 1 $\times$  SSC in the same buffer; and, finally, two times at room temperature for 30 min each in 0.3 $\times$  SSC in the same buffer. The dried filters were exposed to Kodak X-Omat AR films at -70°C for 1-2 d. Autoradiograms were scanned with a scanning densitometer (model GS300; Hoefer Scientific Instruments, San Francisco, CA), and peak areas were integrated with an integrator (model 3392A; Hewlett-Packard Co., Palo Alto, CA).

## Protein Labeling and Two-dimensional Gel Electrophoresis

Wg1A cells were seeded in 10-cm diameter dishes containing DME. At 90% confluency, the cells were changed to 10 ml of either DME supplemented with 10% dialyzed serum (control), DME supplemented with 10% dialyzed serum containing 7  $\mu$ M A23187, low Ca<sup>2+</sup> medium containing 1 mM EGTA, or low Ca<sup>2+</sup> medium containing 1 mM EGTA and 7  $\mu$ M A23187. After 3 h of incubation at 35°C, the cells were labeled for 2 h with 70  $\mu$ Ci of [<sup>35</sup>S]methionine (specific activity 1,131 ci/mmol; New England Nuclear, Boston, MA) in 5 ml of the same media (methionine omitted). At the end of the labeling period, the cells were scraped from the culture dishes, spun down, and the cell pellet rinsed twice with 10 ml of cold PBS. The cell pellets were each resuspended in 0.33 ml of TST buffer (0.01 M Tris hydrochloride, pH 7.4, 0.15 M NaCl, 0.5% Triton X-100) and frozen and



**Figure 1.** The effect of transient A23187 treatment on p3C5 and p4A3 transcript levels. Wg1A cells were grown in DME. At 80% confluency, A23187 (7  $\mu$ M) was added to the appropriate cell cultures (b-f). At the indicated time (b, 1 min; c, 1 h; d, 2 h; e, 4 h), the medium was removed, the cells were rinsed once with PBS, and fresh medium was added. The thick lines indicate incubation periods in DME supplemented with A23187. The thin lines indicate incubation periods in regular DME. At the end of 6 h, the incubation was terminated for all cultures by the extraction of cytoplasmic RNA. 10  $\mu$ g of cytoplasmic RNA from each sample were electrophoresed on a 1% agarose formaldehyde-denaturing gel. After electrophoresis, RNA was blotted onto nitrocellulose filter and hybridized to either radiolabeled p3C5 or p4A3 probes, as described in Materials and Methods. The autoradiograms were quantitated by densitometry to obtain relative mRNA levels of p3C5 and p4A3.

thawed three times. The cell debris was removed by centrifugation and the supernatant stored at -20°C. An aliquot of each supernatant was counted in a liquid scintillation counter for [<sup>35</sup>S]methionine incorporation and protein concentration was determined by protein assay (Bio-Rad Laboratories, Richmond, CA).

Two-dimensional isoelectric focussing/SDS PAGE was performed according to the method of O'Farrell (1975) as previously described (Lee, 1981). Analytical gels contained 8.5% acrylamide. For each sample, 20  $\mu$ g of protein (ranges from 0.6 to 1.5  $\times$  10<sup>6</sup> cpm) were applied on each gel. The gels were dried and exposed to Kodak X-Omat AR films at -70°C for 3-5 d.

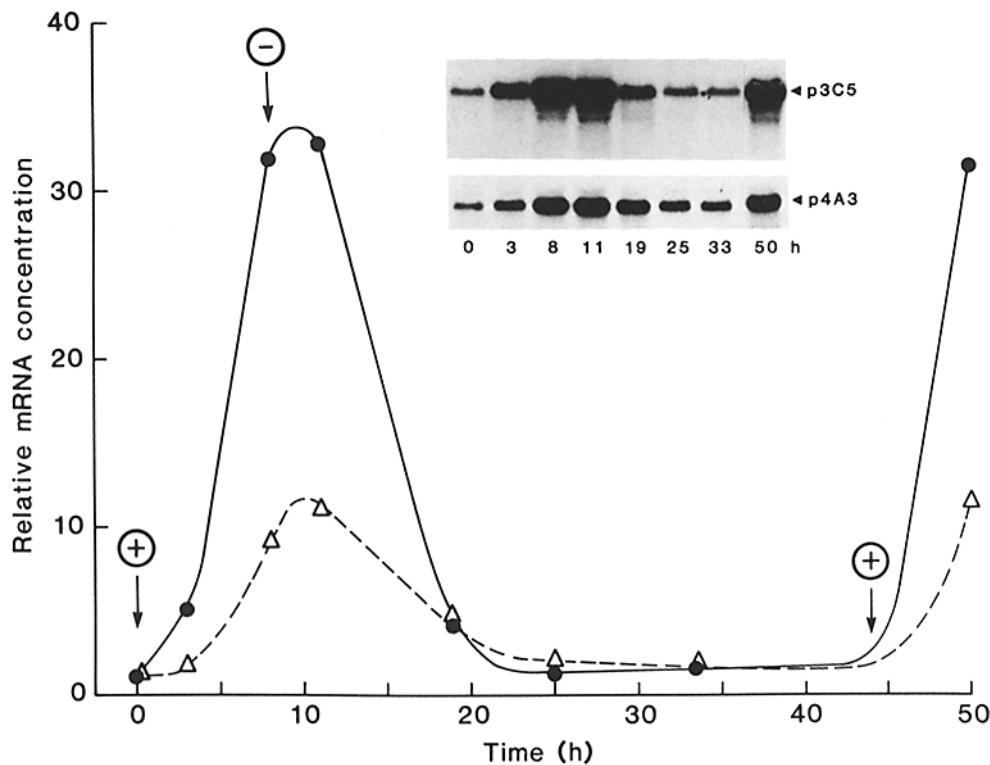
## Transcription Rate Measurements

The detailed procedures for labeling of nuclear RNA and the hybridization conditions of the labeled RNA with excess plasmid DNA baked on filters have been previously described (Resendez et al., 1985). The incorporation of radioactivity into nuclei in control and cycloheximide-treated samples ranged from 3.5 to 4.5  $\times$  10<sup>6</sup> cpm.

## Results

### Continuous Treatment of A23187 Is Necessary for Gene Expression

Using the cDNA plasmids p3C5 and p4A3 as hybridization probes, we previously demonstrated that on the addition of 7  $\mu$ M A23187 to the Wg1A hamster fibroblasts, there was a lag period of ~2 h, followed by a rapid increase in both the transcription rates and the steady-state concentrations of the p3C5 and p4A3 transcripts (Resendez et al., 1985). However, since A23187 has been shown to increase the intracellular concentration of Ca<sup>2+</sup> within a few seconds (Campbell and Dormer, 1978), it is possible that the signal for enhanced transcription of these two genes was triggered immediately upon the addition of A23187 and that the delayed expression did not require the continuous presence of A23187. To test this hypothesis, Wg1A cells were treated with A23187 for various lengths of time, and subsequently incubated in medium without the ionophore. Total cytoplasmic RNA was extracted from the cultures at the end of a 6-h incubation period, and



**Figure 2.** Reversibility of the A23187 induction. Wg1A cells were grown in DME until they reached 70% confluency. At the indicated time points, A23187 (7  $\mu$ M) was either added (+) or removed (-) from the cultured cells. A23187 was removed by a change to fresh medium. Cytoplasmic RNA was extracted from cells at the time points indicated and subjected to RNA blot hybridizations as described in Materials and Methods. The autoradiograms, as shown in the insert, were quantitated by densitometry to obtain the relative mRNA levels of p3C5 (solid circle) and p4A3 (open triangle) at different times of incubation.

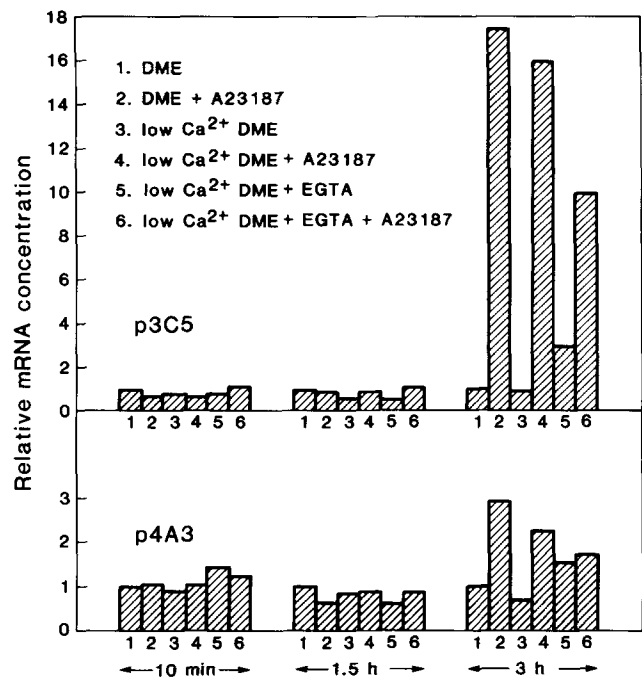
the levels of p3C5 and p4A3 transcripts were determined by RNA blot hybridization. As shown in Fig. 1, treatments of the cells with A23187 ranging from 1 min to 2 h followed by further incubation in regular medium failed to significantly elevate the mRNA levels of the two genes. Our results indicated that the continuous presence of A23187 for over 2 h was required for the increase in the steady-state levels of the specific mRNAs.

#### The A23187 Response Is Reversible and Reinducible

Next, we determined whether the increase in the p3C5 and p4A3 mRNA levels by A23187 was permanent once the activation event had been triggered. Wg1A cells were grown to 70% confluency and treated with A23187. As shown in Fig. 2, there was a rapid increase in both p3C5 and p4A3 transcripts when A23187 was added. After a change to regular medium, the levels of p3C5 and p4A3 transcripts remained high for  $\sim$ 3 h before decreasing. By 18 h, the transcripts were reduced to basal level. Upon readdition of A23187, both transcripts started to accumulate again. These observations suggested that the A23187 stimulation of the expression of the p3C5 and p4A3 genes was tightly and reversibly regulated within the cell.

#### EGTA Can Partially Inhibit the Induction by A23187

To test the involvement of extracellular  $Ca^{2+}$  in this induction process, we utilized EGTA, a chelator of divalent cations with high specificity for  $Ca^{2+}$ , to lower the extracellular  $Ca^{2+}$  concentration of the culture media. The rationale of the experiment was that if extracellular  $Ca^{2+}$  is absolutely required, the  $Ca^{2+}$  ionophore induction observed would be inhibited or abolished by decreasing the available free  $Ca^{2+}$  in the medium with the addition of EGTA. Therefore, the following media were prepared: (a) regular DME that con-



**Figure 3.** Effect of extracellular  $Ca^{2+}$  on p3C5 and p4A3 mRNA levels. Wg1A cells were grown to 80% confluency in regular DME (1.8 mM  $Ca^{2+}$ ). The medium was replaced with either DME (1.8 mM  $Ca^{2+}$ ), low  $Ca^{2+}$  medium (30  $\mu$ M  $Ca^{2+}$ ), or low  $Ca^{2+}$  medium containing 5 mM EGTA (2 nM free  $Ca^{2+}$ ), each supplemented with 10% dialyzed cadet calf serum. With the different media, the cells were incubated in the presence or absence of 7  $\mu$ M A23187. At the end of the incubation period, cells were harvested for cytoplasmic RNA isolation and subjected to RNA blot hybridizations as described in Materials and Methods. The autoradiograms were quantitated by densitometry to obtain relative mRNA levels of p3C5 and p4A3.

tained 1.8 mM of  $\text{Ca}^{2+}$  in the form of calcium chloride, (b) low  $\text{Ca}^{2+}$  medium that did not contain calcium chloride but was estimated to contain  $\sim 30 \mu\text{M}$  of  $\text{Ca}^{2+}$  due to the trace presence of  $\text{Ca}^{2+}$  in the water used, and (c) low  $\text{Ca}^{2+}$ /EGTA medium, the  $\text{Ca}^{2+}$  concentration of which was reduced to below 2 nM by the addition of EGTA. The levels of p3C5 and p4A3 transcripts were monitored for cells grown in these media in the presence or absence of the ionophore A23187. The results are shown in Fig. 3. For the first 1.5 h of incubation in these various media, the mRNA levels of p3C5 and p4A3 were unchanged. After 3 h of incubation, A23187 in DME caused a 17- and 3-fold induction of the p3C5 and p4A3 mRNAs, respectively (lanes 2). These levels of induction were slightly higher in this set of experiments where dialyzed serum was used. While low  $\text{Ca}^{2+}$  medium had no effect on the transcript levels of p3C5 and p4A3 (lanes 3), the addition of A23187 to the low  $\text{Ca}^{2+}$  medium increased the p3C5 and p4A3 mRNAs by 16- and 2.5-fold, respectively (lanes 4). The addition of EGTA to the low  $\text{Ca}^{2+}$  medium resulted in minor but detectable increases of both mRNAs (lanes 5). When A23187 was added to cells grown in low  $\text{Ca}^{2+}$ /EGTA medium, 10- and 1.8-fold increases in the p3C5 and p4A3 mRNA levels were observed (lanes 6). These results, taken together, indicated that extracellular  $\text{Ca}^{2+}$  is important for the optimal effect of A23187. Lowering the extracellular  $\text{Ca}^{2+}$  by EGTA results in a 40% decrease in the A23187 induction levels; however, A23187 is not absolutely required for the induction. In fact, a severe reduction of the extracellular free  $\text{Ca}^{2+}$  by addition of EGTA causes a detectable, but lower, increase of these transcript levels.

#### Fluctuations in Extracellular $\text{Ca}^{2+}$ Can Reversibly Regulate p3C5 Transcript Levels

To further investigate the effect of extracellular  $\text{Ca}^{2+}$  on the

induction of p3C5, Wg1A cells were subjected to  $\text{Ca}^{2+}$ -deficient conditions by the addition of 5 mM EGTA to low  $\text{Ca}^{2+}$  medium. As expected, we observed a 2.5-fold increase in p3C5 levels after 3 h (Fig. 4). When these cells were changed to regular medium containing the normal amounts of  $\text{Ca}^{2+}$ , there was a slight increase in the mRNA level of p3C5, followed by a gradual decrease. This process is reversible, as in the case of the A23187 induction in regular DME, but the magnitude is about 10-fold lower. The half-lives of the p3C5 transcripts were  $\sim 4$ –5 h when A23187 or EGTA was removed from DME. Similar kinetics were observed for the p4A3 gene, but with lower levels of mRNA as compared with p3C5 (data not shown).

#### Selective Induction of GRP Synthesis in $\text{Ca}^{2+}$ -free Medium Supplemented with A23187

To determine the profile of protein synthesis in cells deprived of  $\text{Ca}^{2+}$  in the culture medium and the effect of A23187 treatment in these cells, Wg1A cells were pulse-labeled with [ $^{35}\text{S}$ ]methionine after 3 h of incubation in various media supplemented with EGTA and/or A23187. The labeled proteins were analyzed by two-dimensional gel electrophoresis, and the autoradiograms are shown in Fig. 5. As expected, control cells grown in regular DME synthesized basal levels of GRP94 and GRP78 (Fig. 5A). Upon treatment with 7  $\mu\text{M}$  A23187, the syntheses of both GRPs were enhanced (Fig. 5B). When the cells were shifted to low  $\text{Ca}^{2+}$  medium buffered with EGTA, a low but detectable increase in the synthesis of GRP94 and GRP78 was observed (Fig. 5C). This increase was further enhanced for GRP78 in cells treated with A23187 in the low  $\text{Ca}^{2+}$ /EGTA medium (Fig. 5D). These results confirmed the analysis at the gene transcript levels (Fig. 4). In addition, they showed that the general protein and RNA metabolism were still intact in cells incubated

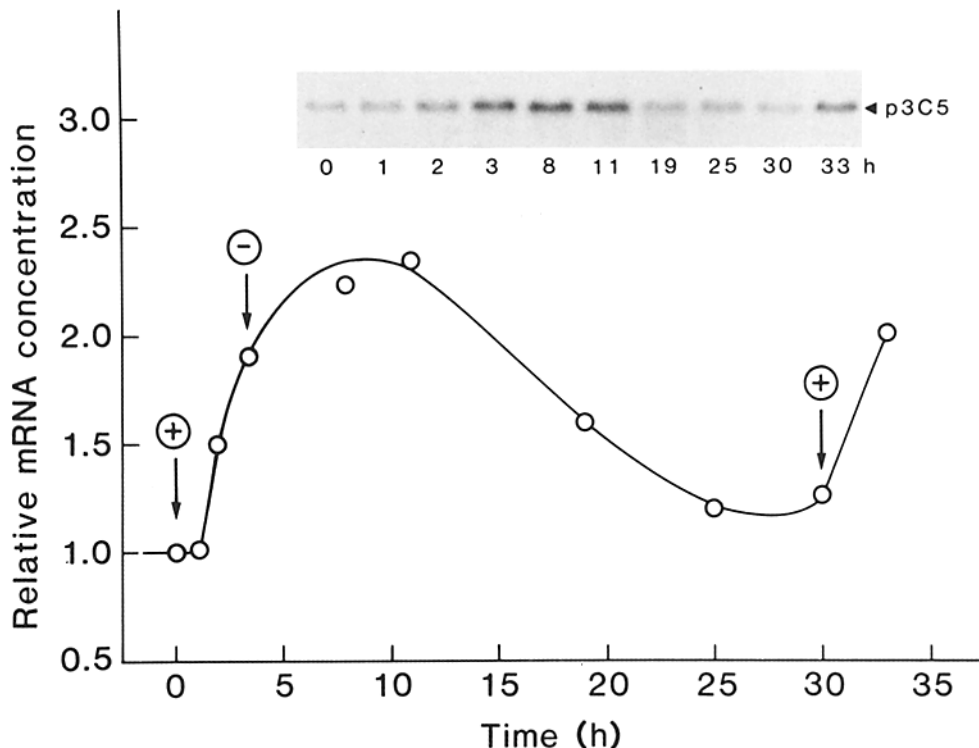
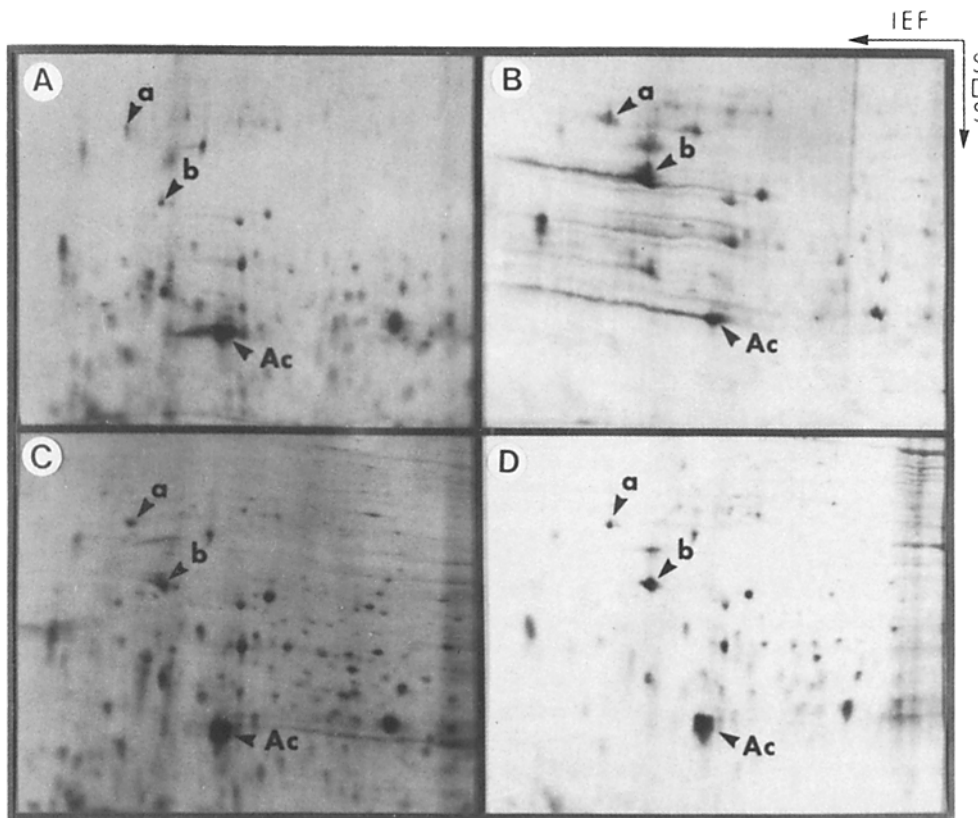


Figure 4. Reversibility of induction by fluctuations in extracellular  $\text{Ca}^{2+}$ . Wg1A cells were grown to 80% confluency. At indicated times, low  $\text{Ca}^{2+}$  medium containing 5 mM EGTA was added (+) or replaced with regular media (-) as denoted by the arrows. The autoradiogram of the RNA samples hybridized with radiolabeled p3C5 probe is shown in the insert. The relative mRNA levels were obtained from quantitation of the autoradiogram by densitometry.



**Figure 5.** Protein synthesis profile in  $\text{Ca}^{2+}$ -deficient cells. Subconfluent Wg1A cells were treated as follows: (A) Cells grown continuously in DME supplemented with 10% dialyzed serum; (B) same as A, except containing  $7 \mu\text{M}$  A23187; (C) in low  $\text{Ca}^{2+}$  medium containing 1 mM EGTA; (D) same as C, except containing  $7 \mu\text{M}$  A23187. After 3 h of incubation at  $35^\circ\text{C}$ , the cells were labeled for 2 h with [ $^{35}\text{S}$ ]methionine as described in Materials and Methods. About  $20 \mu\text{g}$  of protein from each sample were applied to the isoelectric-focussing/SDS polyacrylamide gels. The autoradiograms are shown. The isoelectric focussing was from right (basic) to left (acidic) in the gels presented. The positions of the GRP94 (a), GRP78 (b), and actin (Ac) are indicated.

in  $\text{Ca}^{2+}$ -deficient media for several hours, even though the cells began to round up and started to detach from the culture dish at the end of the pulsing period.

#### ***De Novo Protein Synthesis is Required for Transcriptional Activation***

The lag period observed for the A23187 induction of the p3C5 and p4A3 genes suggested that other events might be involved in the transcription activation process. To assess the role of de novo protein synthesis in the induction, Wg1A cells were pretreated with cycloheximide for 2 h before addition of the ionophore. After a further incubation period of 4 h, the cells were harvested for the extraction of cytoplasmic RNA and the determination of p3C5, p4A3, and actin mRNA levels.

The results, summarized in Table I, showed that under our experimental conditions, the cycloheximide treatment of the A23187-induced cells caused a two- to threefold reduction in the p3C5 and p4A3 mRNA levels, while treatment of the noninduced cells did not result in any substantial change of their steady-state levels. Similarly, cycloheximide treatment of glucose-starved cells also drastically reduced the levels of p3C5 and p4A3 transcripts (Grafsky, A., and A. Lee, unpublished results). In contrast, the actin mRNA levels were relatively unaffected by the A23187 and cycloheximide treatments.

To demonstrate that continuous protein synthesis was indeed required for the transcriptional activation as opposed to posttranscriptional regulation of the mRNA levels, the rates of transcription of the p3C5 gene stimulated by A23187 in the presence and absence of cycloheximide were measured directly. Nuclei extracted from the cells were used as templates for in vitro transcription assays. The results are shown

in Table II. Under these conditions, the relative increase in the rate of transcription of p3C5 by A23187 induction after 6 h was 17-fold. In the presence of cycloheximide, the transcriptional rate of p3C5 during A23187 induction was inhibited by 80%. In contrast, the rate of histone gene transcription was not affected (Wooden, S., and A. Lee, unpublished data). These results suggest that either a newly synthesized protein or the continuous synthesis of a pre-existing labile protein is required to mediate the transcriptional activation by A23187.

#### ***Expression of the Two Genes Can Be Modulated by a Calmodulin Inhibitor, but Is Insensitive to Phorbol Ester***

It has been suggested that the  $\text{Ca}^{2+}$  messenger system is primarily mediated by two branches, one involving calmodulin and the other involving C-kinase (Rasmussen and Barrett, 1984). From work with blood platelets, it has been concluded that A23187 increases the cytosolic  $\text{Ca}^{2+}$  concentration without activating the C-kinase branch, and the phorbol ester TPA activates the C-kinase branch (Castagna et al., 1982) without causing a rise in the cytosolic  $\text{Ca}^{2+}$  concentration (Sha'afi et al., 1983). A combination of A23187 and TPA induces a maximum response in the platelet system.

To test the involvement of the calmodulin activation pathway in the p3C5 and p4A3 gene induction, Wg1A cells were pretreated with W-7 before the addition of A23187. Unlike other calmodulin antagonists, W-7 can be used at a relatively low concentration that is nontoxic to the Wg1A cells and can inhibit  $\text{Ca}^{2+}$ /calmodulin-regulated enzyme activities (Hidaka et al., 1981). As shown in Fig. 6, upon treatment with the calmodulin inhibitor, the basal levels of both p3C5 and p4A3

**Table I. Effect of Cycloheximide on the A23187 Induction of p3C5, p4A3, and Actin Transcripts**

Conditions*	Relative mRNA levels‡		
	p3C5	p4A3	pJ
DME	1.0	1.0	1.0
DME + CHX	1.3	1.0	1.2
DME + A23187	6.4	3.2	0.9
DME + A23187 + CHX	3.0	1.2	1.3

\* Wg1A cells, grown to 80% confluency in DME, were treated as follows: sample 1, control cells grown continuously in DME; sample 2, cells treated with 0.1 mM cycloheximide (CHX) for 6 h; sample 3, cells treated with 7 μM A23187 for 4 h; and sample 4, cells pretreated with 0.1 mM CHX for 2 h before addition of 7 μM A23187 for another 4 h. Cytoplasmic RNA was extracted and subjected to RNA blot hybridization with nick-translated cDNA probes, p3C5, p4A3, and pJ, as described in Materials and Methods. The autoradiograms were quantitated by densitometry.

‡ The relative mRNA levels were determined by setting the peak area of sample 1 as unity. The numbers represent the ratios between the value obtained for the experimental and control samples.

mRNAs were reduced to 27 and 8% of the control levels, respectively. Under A23187-induced condition, increasing amounts of W-7 reduced the levels of p3C5 and p4A3 mRNAs by 64 and 63%. Similar inhibition was observed when W-7 was added at the same time as A23187 (data not shown). To rule out that W-7 might result in the rapid degradation of general cellular mRNAs, the same RNA blots were hybridized to pJ, a cDNA plasmid containing the actin gene. No significant changes of actin mRNA levels were observed with the addition of W-7, A23187, or both (our unpublished results). This demonstrated that W-7 inhibits both the basal and induced mRNA levels of p3C5 and p4A3, and that the inhibitory effect was specific to these glucose/calcium-regulated genes.

The involvement of the C-kinase pathway was tested by the phorbol ester treatment. In contrast to W-7, the addition of TPA did not have any major effect on the levels of p3C5 or p4A3 mRNAs (Fig. 7). This compound by itself was unable to elicit any response. In combination with A23187, it did not have any effect on the p3C5 mRNA and exhibited only a slight inhibitory effect on the p4A3 gene. As a control, measurement of phosphate incorporation into proteins using extracts from TPA-treated cells indicated that the C-kinase ac-

**Table II. Effect of Cycloheximide on the In Vitro Transcriptional Rate of the p3C5 Gene**

Conditions*	Input	cpm bound		p3C5 mRNA‡ hybridized	Relative§ rates of transcription
		pBR322	p3C5		
	<i>10<sup>6</sup> cpm</i>		<i>cpm</i>		
DME	3.3	66	92	26	1.0
DME + A23187	3.1	88	530	442	17.0
DME + A23187 + CHX	3.1	95	183	88	3.4

\* Wg1A cells were grown to 80% confluency in DME. An aliquot of cells (sample 3) was pretreated with 0.1 mM cycloheximide (CHX) for 4 h. Subsequently, A23187 (7 μM) was added to samples 2 and 3 and the cells were incubated for an additional 6 h. Control cells (sample 1) were incubated continuously in DME. At the end of 10 h, nuclei were isolated. The nuclear RNA was labeled in vitro and hybridized to plasmid DNA as described (Resendez et al., 1985).

‡ Calculated as counts subtracted from those of filters containing pBR322.

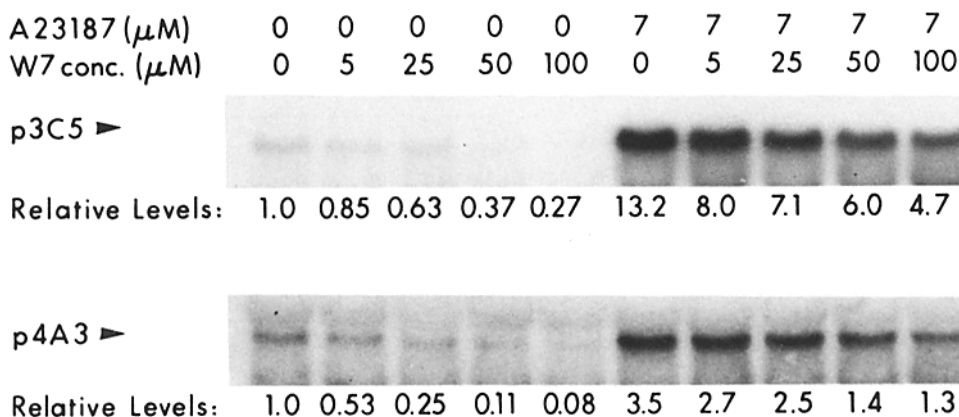
§ Calculated as ratio of the counts per minute between the experimental and the control samples.

tivities had been elevated by the TPA treatment (Ting, J., data not shown). Furthermore, for comparison, the effects of A23187 and TPA were tested on α-tubulin mRNA levels (Fig. 7). Interestingly, while the tubulin mRNA levels were relatively unchanged with the addition of A23187, there was a detectable increase with TPA treatment. This further demonstrated that the effects of A23187 on the p3C5 and p4A3 genes were specific, and that other genes, such as tubulin and actin (unpublished results), were not affected.

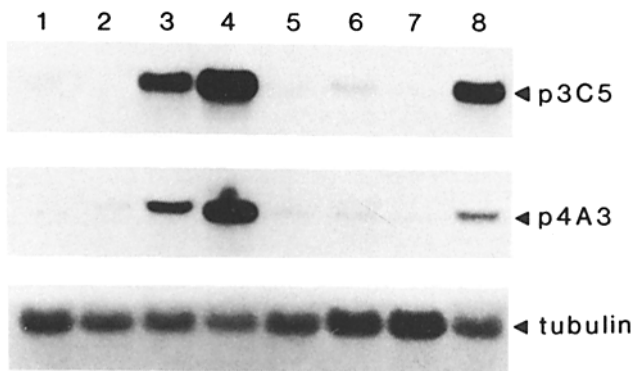
## Discussion

The calcium ionophore A23187 has been shown to affect the permeability of the cell membrane to Ca<sup>2+</sup> and is used extensively to study the role of Ca<sup>2+</sup> in cell regulation. Evidence is now accumulating to show that this ionophore can also selectively activate gene expression, and that DNA sequences flanking a glucose/calcium-regulated gene contain the *cis* elements required for its activation (Resendez et al., 1985; Lin et al., 1986).

While our studies with the A23187 ionophore strongly indicate that the activation of the p3C5 and p4A3 genes is coordinately regulated, the relatively long period (2 h) needed for



**Figure 6.** The effect of calmodulin antagonist W-7 on p3C5 and p4A3 mRNA levels. Wg1A cells were grown to 90% confluency in regular DME at 35°C. After a change to fresh medium, W-7 was added to a final concentration as indicated. In case of treatment with both A23187 and W-7, W-7 was added 1 h earlier than A23187 for the prior blockage of the calmodulin activity. After 5 h of incubation with W-7, cytoplasmic RNA was extracted from the cells and subjected to RNA blot analysis as described in Materials and Methods.



**Figure 7.** The effect of TPA on p3C5, p4A3, and  $\alpha$ -tubulin mRNA levels. Cytoplasmic RNA was extracted from K12 cells grown in DME (lane 1); in DME supplemented with 7  $\mu$ M A23187 for 1 h (lane 2), 4 h (lane 3), and 16 h (lane 4); in DME supplemented with 0.1  $\mu$ M TPA for 1 h (lane 5), 4 h (lane 6), and 16 h (lane 7); in 7  $\mu$ M A23187 and 0.1  $\mu$ M TPA for 4 h (lane 8).

transcriptional activation suggests that A23187 induction may not depend simply on the transient increase in cytoplasmic  $\text{Ca}^{2+}$  due to influx through the plasma membrane, as compared with other calcium messenger systems (Rasmussen and Barrett, 1984). The fact that the phorbol ester treatment does not result in the induction of these genes further indicates that the induction is unrelated to C-kinase activation and may result from  $\text{Ca}^{2+}$  perturbation, as suggested by the effectiveness of EGTA treatment. Since the endoplasmic reticulum and mitochondria are organelles that store  $\text{Ca}^{2+}$ , alterations in these  $\text{Ca}^{2+}$  stores or perturbations at these membrane sites may stimulate the process mediating the response. Taking into account the data obtained so far, we propose the following model for the activation of the p3C5 and p4A3 genes. When mammalian cells are cultured in regular medium, the cells are exposed to  $\sim 2$  mM of  $\text{Ca}^{2+}$ , which is several orders of magnitude higher than the cytosolic concentration of free  $\text{Ca}^{2+}$  measured to be 154 nM in Wg1A cells (Drummond et al., 1986). Since excess intracellular  $\text{Ca}^{2+}$  is toxic to cells, stringent regulation must exist to control the  $\text{Ca}^{2+}$  influx and efflux. When cells are grown in regular medium, the p3C5 and p4A3 genes are transcribed at low, basal levels (Lee et al., 1983; Lin et al., 1984; Resendez et al., 1985). These basal levels of mRNA are regulated at least partially by  $\text{Ca}^{2+}$ /calmodulin-dependent process(es), as they are sensitive to the inhibitor W-7. When A23187 is added to the culture medium (or when the cells are exposed to low  $\text{Ca}^{2+}$ /EGTA medium), this could result in the leaking of intracellular pools of stored  $\text{Ca}^{2+}$  into the cytosolic  $\text{Ca}^{2+}$  pool (Wu et al., 1981; Martonosi et al., 1982; James-Krache and Martonosi, 1983). It is plausible that both A23187 and low external  $\text{Ca}^{2+}$  could both have the effect of reducing the amount of  $\text{Ca}^{2+}$  sequestered within the lumen of the endoplasmic reticulum and within the mitochondria. A23187 would prevent the organelles from maintaining a  $\text{Ca}^{2+}$  gradient. With low extracellular  $\text{Ca}^{2+}$ , there would simply be no  $\text{Ca}^{2+}$  to be accumulated in the organelles. Both conditions may also result in the disruption of some membrane processes dependent on the integrity of  $\text{Ca}^{2+}$  concentration. Continuous treatment of the cells with the ionophore (or low  $\text{Ca}^{2+}$ /EGTA) elicits a secondary response resulting in the de

novo synthesis of one or more protein factor(s) that regulate the transcriptional activity of both genes. The transcript encoding for this factor may be pre-existing in the cells, or it could be newly synthesized. The factor triggers a transcriptional activation process that is also sensitive to W-7, and, as a consequence, the mRNA levels and the synthetic rates of the two GRPs are greatly enhanced. When the cells are restored to regular medium, the levels of p3C5 and p4A3 mRNAs remain high for a few hours, then decay to basal levels that are independent of A23187. When A23187 (or low  $\text{Ca}^{2+}$ /EGTA) is added again, the factor is resynthesized, and a second round of stimulation follows. Apparently, extracellular  $\text{Ca}^{2+}$  could enhance the effect of A23187, but the ionophore is effective even in  $\text{Ca}^{2+}$ -deficient medium. Our measurements at the mRNA levels confirmed previous GRP protein synthesis observations in cells shifted from low to normal  $\text{Ca}^{2+}$  media (Martonosi et al., 1982) and in cells grown in  $\text{Ca}^{2+}$ -deprived medium (Lamarche et al., 1985).

The primary stimulus generated by A23187 can be: (a) perturbations in the intracellular  $\text{Ca}^{2+}$  level; (b) physical disruption of the cell membrane, causing release of some integral components that serve as stimuli; or (c) a drop in ATP levels in the cells as the consequence of the  $\text{Ca}^{2+}$  ionophore treatment (Campbell and Siddle, 1976). Our observations concerning treatments with other ionophores suggest that the stimuli are more specific than the last two mechanisms would imply. First, only one other ionophore, ionomycin, which is also a  $\text{Ca}^{2+}$  ionophore, has been found to increase the levels of the glucose-regulated proteins (Martonosi et al., 1982). Second, when the cells were treated with ionophores such as valinomycin, gramicidin, or nigericin, the p3C5 and p4A3 genes were not affected to any major extent (Resendez et al., 1985). These ionophores, when added together with A23187, did not inhibit the inductive response. When nigericin and valinomycin were added together, a severe ATP shortage was created because of the drop in membrane potential as well as the proton gradient, resulting in the disruption of oxidative phosphorylation (Montal et al., 1970). Under these circumstances, A23187 was unable to stimulate p3C5 and p4A3 mRNA levels (unpublished results). Therefore, it appears that a drop in ATP level does not result in induction of the genes. On the contrary, energy is likely to be needed for the enhanced expression of these genes, including the synthesis of the required protein factor.

While the exact contribution of intracellular  $\text{Ca}^{2+}$  towards the induction process awaits direct measurements of the levels and distributions of intracellular  $\text{Ca}^{2+}$  during the time course of A23187 treatment, the localization of the 78-kD protein (GRP78) encoded by p3C5 in the endoplasmic reticulum (Zala et al., 1980) suggests that either the  $\text{Ca}^{2+}$  store inside the endoplasmic reticulum or the membrane site at the endoplasmic reticulum may be the major target site of A23187 in this response. Interestingly, the GRP78 has been shown to be phosphorylated and has the ability to bind ATP agarose (Welch et al., 1983; Lee et al., 1984). It is also found to be ADP-ribosylated (Carlsson and Lazarides, 1983) and is immunologically related to the 70-kD heat shock protein family (Chappell et al., 1986). Recent identification of the GRP78 as the immunoglobulin heavy chain binding protein that is constitutively expressed in many cell types indicates that this protein may be involved in the assembly of secreted and membrane-bound proteins (Munro and Pelham, 1986).

Therefore, it is possible that disruption of endoplasmic reticulum as caused by continuous A23187 treatment may trigger a signal leading to the transcription activation of this set of genes.

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## References

- Artishevsky, A., A. M. Delegeane, and A. S. Lee. 1984. Use of a cell cycle mutant to delineate the critical period for the control of histone mRNA levels in the mammalian cell cycle. *Mol. Cell. Biol.* 4:2364-2369.
- Campbell, A. K. 1983. Intracellular Calcium: its Universal Role as Regulator. John Wiley & Sons, Inc., New York.
- Campbell, A. K., and R. L. Dormer. 1978. Inhibition by calcium of cyclic AMP formation in sealed pigeon erythrocyte ghost: a study using the photoprotein obelin. *Biochem. J.* 176:53-66.
- Campbell, A. K., and K. Siddle. 1976. The effect of intracellular calcium ions on adrenaline-stimulated adenosine 3', 5'-cyclic monophosphate concentrations in pigeon erythrocytes: studied by using ionophore A23187. *Biochem. J.* 158:211-221.
- Carlsson, L., and E. Lazarides. 1983. ADP-ribosylation of the Mr 83,000 stress-inducible and glucose-regulated protein in avian and mammalian cells: modulation by heat shock and glucose starvation. *Proc. Natl. Acad. Sci. USA.* 80:4664-4668.
- Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, and Y. Nishizuka. 1982. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* 257:7847-7851.
- Chappell, T. G., W. J. Welch, D. M. Schlossman, K. B. Palter, M. J. Schlesinger, and J. E. Rothman. 1986. Uncoating ATPase is a member of the 70 kilodalton family of stress proteins. *Cell.* 45:3-13.
- Cowan, N. J., P. R. Bobner, E. V. Fuchs, and D. W. Cleveland. 1983. Expression of human  $\alpha$ -tubulin genes: interspecies conservation of 3' untranslated regions. *Mol. Cell. Biol.* 3:1738-1745.
- Devore, D. I., and W. L. Nastuk. 1977. Ionophore-mediated calcium influx effects on the post-synaptic muscle fibre membrane. *Nature (Lond.)*. 270:441-443.
- Drummond, I. A. S., S. A. McClure, M. Poenie, R. Y. Tsien, R. A. Steinhart. 1986. Large changes in intracellular pH and calcium observed during heat shock are not responsible for the induction of heat shock proteins in *Drosophila melanogaster*. *Mol. Cell. Biol.* 6:1767-1775.
- Duffus, J. H., and L. J. Patterson. 1974. Control of cell division in yeast using ionophore A23187 with calcium and magnesium. *Nature (Lond.)*. 251:626-627.
- Hidaka, H., Y. Susaki, T. Tanaka, T. Endo, S. Ohno, Y. Fujii, and T. Nagata. 1981. N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, a calmodulin antagonist, inhibits cell proliferation. *Proc. Natl. Acad. Sci. USA.* 78:4354-4357.
- James-Krache, M. R., and A. Martonosi. 1983. Incorporation of aequorin into developing chicken myoblasts. *Fed. Proc.* 42:996.
- Kaiser, N., and I. S. Edelman. 1977. Calcium dependence of glucocorticoid-induced lymphocytolysis. *Proc. Natl. Acad. Sci. USA.* 74:638-642.
- Keppens, S., J. R. Vandenhede, and H. DeWulf. 1977. On the role of calcium as a second messenger in liver for the hormonally induced activation of glycogen phosphorylase. *Biochim. Biophys. Acta.* 496:448-457.
- Lamarche, S., P. Chretien, and J. Landry. 1985. Inhibition of the heat shock response and synthesis of glucose-regulated proteins in  $Ca^{2+}$ -deprived rat hepatoma cells. *Biochem. Biophys. Res. Commun.* 131:868-876.
- Lee, A. S. 1981. The accumulation of three specific proteins related to glucose-regulated proteins in a temperature-sensitive mutant cell line K12. *J. Cell. Physiol.* 106:119-125.
- Lee, A. S., J. Bell, and J. Ting. 1984. Biochemical characterization of the 94- and 78-kilodalton glucose-regulated proteins in hamster fibroblasts. *J. Biol. Chem.* 259:4616-4621.
- Lee, A. S., A. Delegeane, and D. Scharff. 1981. Highly conserved glucose-regulated protein in hamster and chicken cells: preliminary characterization of its cDNA clone. *Proc. Natl. Acad. Sci. USA.* 78:4922-4925.
- Lee, A. S., A. M. Delegeane, V. Baker, and P. C. Chow. 1983. Transcriptional regulation of two genes specifically induced by glucose starvation in a hamster mutant fibroblast cell line. *J. Biol. Chem.* 258:597-603.
- Lin, A., and A. S. Lee. 1984. Induction of two genes by glucose starvation in hamster fibroblasts. *Proc. Natl. Acad. Sci. USA.* 81:988-992.
- Lin, A. Y., S. C. Chang, and A. S. Lee. 1986. A calcium ionophore-inducible cellular promoter is highly active and has enhancer-like properties. *Mol. Cell. Biol.* 6:1235-1243.
- Maino, V. C., N. W. Green, and M. J. Crumpton. 1974. The role of calcium ions in initiating transformation of lymphocytes. *Nature (Lond.)*. 251:324-327.
- Martonosi, A. N., L. Dux, R. L. Terjung, and D. Roufa. 1982. Regulation of membrane assembly during development of sarcoplasmic reticulum: the possible role of calcium. *Ann. N.Y. Acad. Sci.* 402:485-514.
- Montal, M., B. Chance, and C.-P. Lee. 1970. Ion transport and energy conservation in mitochondrial particles. *J. Membr. Biol.* 2:201-234.
- Munro, S., and H. R. B. Pelham. 1986. An hsp70-like protein in the endoplasmic reticulum: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell.* 46:291-300.
- O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007-4021.
- Ogawa, Y. 1968. The apparent binding constant of glycoetherdiamine tetraacetic acid for calcium of neutral pH. *J. Biochem.* 64:255-257.
- Rasmussen, H., and P. Q. Barrett. 1984. Calcium messenger system: an integrated view. *Physiol. Rev.* 64:938-984.
- Resendez, E., Jr., J. W. Antenello, A. Graftsky, C. S. Chang, and A. S. Lee. 1985. Calcium ionophore A23187 induces expression of glucose-regulated genes and their heterologous fusion genes. *Mol. Cell. Biol.* 4:1212-1219.
- Roscoe, D. H., M. Read, and H. Robinson. 1973. Isolation of temperature-sensitive mammalian cells by selective detachment. *J. Cell. Physiol.* 82:325-332.
- Rose, B., and W. R. Loewenstein. 1976. Permeability of a cell junction and the local cytoplasmic free ionized calcium concentration: a study with aequorin. *J. Membr. Biol.* 28:87-119.
- Sha'afi, R. I., J. R. White, T. F. Moshi, J. Shefeyk, M. Volip, P. H. Nacache, and M. B. F. Feinstein. 1983. Phorbol-12-myristate 13-acetate activates rabbit neutrophil without an apparent rise in level of intracellular free calcium. *Biochem. Biophys. Res. Commun.* 114:638-645.
- Welch, W. J., J. I. Garrels, G. P. Thomas, J. J. C. Lin, and J. R. Feramisco. 1983. Biochemical characterization of the mammalian stress proteins and identification of two stress proteins as glucose- and  $Ca^{2+}$ -ionophore-regulated proteins. *J. Biol. Chem.* 258:7102-7111.
- Wu, F. S., Y.-C. Park, D. Roufa, and A. Martonosi. 1981. Selective stimulation of the synthesis of an 80,000-dalton protein by calcium ionophore. *J. Biol. Chem.* 256:5309-5312.
- Zala, C. A., M. Salas-Prato, W. T. Yan, B. Banjo, and J. F. Perdue. 1980. In cultured chick embryo fibroblasts the hexose transport components are not the 75,000 and 95,000 dalton polypeptides synthesized following glucose deprivation. *Can J. Biochem.* 58:1179-1188.