Selective regulation of trypsin gene expression by calcium and by glucose starvation in a rat exocrine pancreas cell line

(A4R-2J cells/A23187/phorbol 12-myristate 13-acetate/chloramphenicol acetyltransferase assay)

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ABSTRACT Treatment of the rat pancreatic acinar cell line A4R-2J with the calcium ionophore A23187 selectively increases, within a few hours, the steady-state level of trypsin mRNA. Addition of the tumor-promoting phorbol ester phorbol 12-myristate 13-acetate potentiates the calcium-induced increase. The mRNA level of the other tested exocrine pancreatic genes decreases. These results were confirmed by DNA transfection experiments, using the 5' flanking region of the trypsin and chymotrypsin genes linked to the coding sequence of the chloramphenicol acetyltransferase (CAT) gene. In calcium-induced cells transfected with the trypsin constructs, an increase in CAT activity was observed, whereas the chymotrypsin constructs revealed a decreased CAT activity. Glucose starvation of A4R-2J cells similarly elicited a selective increase in trypsin mRNA. This selective regulation of trypsin may reflect its role as the key activator of the other zymogen species.

Calcium is a major intracellular messenger stimulating enzyme release from pancreatic acinar cells (for review, see ref. 1). It is unknown whether calcium is also involved in regulation of the expression of the exocrine pancreatic genes. Could there be a correlation between Ca-induced enzyme secretion and Ca-regulated expression of the genes for those pancreatic enzymes whose secretion is triggered by calcium?

Early studies revealed a secretagogue-induced efflux of Ca2+ in pancreatic fragments. More compelling evidence for the importance of Ca2+ in secretion was provided by studies in which an increase in intracellular free Ca2+ was produced by calcium ionophores, such as A23187 (1). The Ca ionophores stimulate amylase secretion from pancreatic acini (2, 3). Furthermore, the tumor-promoting phorbol ester phorbol 12-myristate 13-acetate (PMA) has a synergistic effect on amylase release (4). Similar experiments showed that A23187 and PMA in combination simulate the biphasic pattern of glucose-induced insulin secretion from pancreatic islets (5). These experiments suggest that the calcium-activated phospholipid-dependent protein kinase C (6) is also involved in the activation of secretion.

We have used the rat pancreatic acinar cell line A4R-2J, which displays a relatively high level of expression for several exocrine gene products (7), to study the influence of calcium on the expression of these genes. Our studies revealed a selective induction of trypsin by A23187 treatment, which is further potentiated by PMA. Using gene chimeras composed of the 5' flanking regions of the trypsin or chymotrypsin genes linked to the coding sequence of the bacterial chloramphenicol acetyltransferase (CAT) gene (8), we demonstrated that sequences upstream of these genes are involved in the effects of A23187. In addition, glucose starvation produces a selective increase in the level of trypsin mRNA.

EXPERIMENTAL PROCEDURES

Materials. A23187, PMA, dibutyryl cAMP (Bt2-cAMP), dibutyryl cGMP (Bt2-cGMP), and 3-isobutyl-1-methylxanthine were purchased from Sigma. Forskolin was obtained from Calbiochem–Behring.

Cell Culture. A4R-2J is a rat pancreatic tumor cell line that was derived from an azaserine-induced tumor of the rat exocrine pancreas (9). Cultures of A4R-2J cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine and 4.5 mg of glucose per ml and supplemented with 10% fetal calf serum/penicillin (100 units/ml)/streptomycin (50 μg/ml). Cells were grown at 37°C under 5% CO2/95% air. Under these conditions, A4R-2J cells have a doubling time of ~27 hr. They grow in colonies that form clumps rather than monolayers; therefore, they never reach confluency. To establish defined cell culture conditions, DMEM/10% fetal calf serum was replaced by DMEM containing 10% serum substitute (10) but without insulin before carrying out the different experiments.

Conditions for Ionophore Treatment. Cells were seeded in 100-mm Petri dishes at a density of 4 × 10⁶ cells per dish. Care was taken to ensure that for a defined experiment each plate contained the same number of cells. When cells reached a density of ~2 × 10⁷ cells per dish, medium was replaced by complete DMEM supplemented with 10% serum substitute. After 12 hr, fresh DMEM/10% serum substitute was added to ensure that the cells were not starved of glucose at the time of ionophore treatment. Different concentrations of A23187 and/or PMA, forskolin, Bt2-cAMP, Bt2-cGMP were used. At various times after addition of different compounds, RNA samples were prepared from treated and untreated cells.

Glucose Starvation. A4R-2J cells were seeded at a density of 4 × 10⁶ cells per 100-mm Petri dish in complete DMEM/10% fetal calf serum. Again, cells were seeded so that each plate contained the same number of cells. Cells were fed every 2 days, until they reached a density of ~2 × 10⁷ cells per dish. At that point (time 0), regular medium was removed and cells were washed twice with glucose-free medium and then incubated in glucose-free DMEM supplemented with 10% serum substitute. In parallel, cells were kept in complete DMEM/10% serum substitute as control. At various times after removal of glucose from the medium, total cytoplasmic RNA was extracted from glucose-starved cells as well as from control cells as described below.

Isolation of Cytoplasmic RNA. For preparation of RNA, cells were fractionated by lysis with 0.5% Nonidet P-40 in isotonic buffer (0.15 M NaCl/0.01 M Tris·HCl, pH 7.5). Total cytoplasmic RNA was extracted from the Nonidet P-40 wash.

Abbreviations: PMA, phorbol 12-myristate 13-acetate; Bt2-cAMP and Bt2-cGMP, dibutyryl cAMP and cGMP; CAT, chloramphenicol acetyltransferase; bp, base pair(s).
supernatants (11). After adding NaDodSO₄ to give a final concentration of 0.5%, the cell supernatant was extracted two times with phenol/chloroform (1:1) and once with chloroform/isoamyl alcohol (24:1). After ethanol precipitation, RNA was taken up in H₂O and stored at -70°C.

**RNA Dot Blot Analysis.** RNA samples (2 µg and 4 µg of each sample) were denatured in 50% formamide/2.2 M formaldehyde/10 mM phosphate buffer, pH 7.0, by heating at 60°C for 10 min. After dilution in 100 µl of ice-cold 20× SSC (1× SSC = 0.15 M NaCl/0.015 M Na citrate), samples were passed through a nitrocellulose filter equilibrated with 20× SSC by using a manifold apparatus (Schleicher & Schuell) to aid suction (12). Filters were baked at 80°C in a vacuum oven for 2 hr.

For hybridization, the following exocrine pancreatic cDNAs were used: amylase (13), trypsin I (14), chymotrypsin B (15), and carboxypeptidase A1 (16). Labeled probes were prepared by nick-translation as described (17). Using each of four [α-³²P]dNTPs (Amersham; 3000 Ci/mmol; 1 Ci = 37 GBq), the specific activity of each probe was 2.5 × 10⁶ cpm/µg.

Hybridization and washing conditions were as described (12). Autoradiograms were scanned with a Zeinhe soft laser scanning densitometer equipped with an area integrator (LKB).

**Description of Hybrid CAT Plasmids.** All plasmids contain 5′ flanking regions of different genes linked to the CAT coding region.

Plasmid Trp.CAT consists of a 1162-base-pair (bp) HgiAI fragment (−10 to −1172) from the 5′ flanking region of the trypsin I gene (18), inserted into the HindIII site of pBR.CAT by using HindIII linkers (7, 19). Plasmid TrpΔ500.CAT is a 500-bp Aeu I/Rsa I subfragment (−139 to −639) of above HgiAI fragment inserted into the HindIII site of pTE1 by using HindIII linkers. Plasmid pTE1 contains a polylinker located 600 bp upstream of the thymidine kinase promoter fused to the CAT coding region (20).

The construction of pCh₄.CAT and pCh₄Δ275.CAT have been described (7). Plasmid Ch₄.CAT contains the chymotrypsin B 5′ flanking region fragment (−3 to −711) linked to the CAT gene, whereas pCh₄Δ275.CAT consists of a 182-bp Sac I fragment (−93 to −275) from the chymotrypsin upstream region (15) inserted into the Bgl II site of pTE1 by using BamHI linkers.

For control experiments, plasmids pTE1 and pRSV.CAT were used. In pRSV.CAT the Rous sarcoma virus promoter was used to activate the expression of CAT sequences (21).

**Assay for CAT Activity.** One day after seeding AR4-2J cells at a density of 5 × 10⁴ cells per 100-mm Petri dish, a calcium phosphate coprecipitate (22) containing 20 µg of plasmid DNA was added to the cells. Four hours after the addition of DNA, cells were subjected to 20% (vol/vol) glycerol for 2 min, and 40 hr after transfection fresh DMEM containing 10% serum substitute was added to the cells and incubation continued for 8 hr with or without A23187 plus PMA added. Cells were disrupted by three freeze–thaw cycles, and the supernatant was assayed for the amount of protein by Lowry protein assay. Cell extract containing 60 µg of protein was assayed for CAT activity by the procedure of Gorman et al. (8). The cell extract was assayed in a final vol of 150 µl containing 0.14 M Tris-HCl, pH 7.8/4.4 mM acetyl coenzyme A/0.2 µCi of [1⁴C]chloramphenicol (40–60 mCi/mmol; New England Nuclear). After 12 hr of incubation at 37°C, samples were extracted with ethyl acetate, dried, and spotted on silica gel thin-layer chromatography (TLC) plates. After developing the TLC plates in chloroform/methanol (95:5), they were exposed to Kodak XAR-2 film for 24 hr. For quantitation of the acetylation reaction, spots corresponding to the unacetylated and acetylated forms were cut out and counted in a liquid scintillation spectrometer. The percentage of conversion was calculated by dividing the cpm for the two acetylated forms by the total cpm (acetylated and nonacetylated forms).

**RESULTS**

**Induction of Trypsin Expression by Calcium.** To determine whether Ca²⁺ is involved in the regulation of the exocrine pancreatic genes, we measured the effects of the calcium ionophore A23187 plus PMA on mRNA accumulation in the rat acinar cell line AR4-2J. As shown in Fig. 1, the level of trypsin mRNA increases within 2 hr of treatment with A23187 and PMA; by 8 hr, an 11-fold increase in the trypsin mRNA level was observed. In contrast, the levels of chymotrypsin and amylase transcripts decrease to 40% of the initial mRNA levels, and carboxypeptidase A1 mRNA decreases to 20% of the basal level.

To confirm the role of Ca²⁺ in obtaining these effects, we carried out a competition experiment with CoCl₂, a potent calcium antagonist. Addition of 2 mM CoCl₂, together with 1 µM A23187, 0.5 µM PMA, and 2 mM CaCl₂ to AR4-2J cells suppresses the effect of the latter compounds on the level of trypsin mRNA to a value 72% of the basal level. (Addition of 2 mM CoCl₂ alone causes a decrease by a factor of 3.2 in trypsin mRNA.)

To show that the results of Fig. 1 obtained with dot blot hybridization are specific for each gene, RNA blot analysis was carried out (Fig. 2). Although the trypsin mRNA level in AR4-2J cells is very low [only 0.08% of total poly(A)⁺ RNA], the selective induction of trypsin by A23187 can be clearly seen.

**Synergistic Effects of A23187 and PMA on Trypsin Induction.** Because of the synergistic effects of A23187 and PMA on enzyme secretion in the pancreas (4, 5), we examined the possibility that A23187 and PMA also have a combined effect...
on induction of trypsin mRNA. The results of such an experiment with various concentrations of A23187 are shown in Fig. 3. Although PMA alone in a concentration range of 0.1 μM to 5 μM does not show any effect, it increases the effect of 1 μM A23187 on trypsin mRNA accumulation by 1.6-fold. Also, in the presence of PMA the effect of A23187 on chymotrypsin and amylase decrease is less dramatic.

**Cyclic Nucleotides Do Not Induce Trypsin Expression.** We not only examined the effects of A23187 and PMA on trypsin mRNA levels, which influence calcium and protein kinase C as second messengers, but also the effects of cyclic nucleotides. Neither addition of 0.5 mM Bt2-cAMP, a lipid soluble derivative of cAMP, nor of 25 μM forskolin, a cardiotonic diterpene that activates the adenylate cyclase directly, show a remarkable effect on trypsin, chymotrypsin, amylase, or carboxypeptidase A1 mRNA levels. Treatment of AR4-2J cells with Bt2-cAMP or forskolin results in a 1.5-fold or a 1.8-fold increase in trypsin mRNA, respectively. To enhance the effect of Bt2-cAMP and forskolin on intracellular cAMP levels, 0.1 mM 3-isobutyl-1-methylxanthine, an inhibitor of cAMP phosphodiesterase was also used in these experiments.

Another possible intracellular messenger is cGMP. However, increase of intracellular cGMP by addition of the derivative Bt2-cGMP or by activation of endogenous guanylate cyclase by nitroprusside had no effect on mRNA levels. Furthermore, neither cAMP nor cGMP derivatives showed a potentiative effect when applied to the cells together with A23187 and PMA (data not shown).

**Regulatory Sequences Are Contained Within the 5′ Flanking Region.** To test whether the regulatory sequences for the Ca2+-induced response reside at the 5′ flanking region of the trypsin and chymotrypsin genes, we used recombinants containing 5′ upstream fragments of these genes linked to the CAT coding sequence to transfet AR4-2J cells. After preparation of cell extracts, their CAT activities were determined (Fig. 4).

The CAT activities directed by the Herpes simplex thymidine kinase (TK) promoter–CAT fusion (pTE1) and the Rous sarcoma virus promoter–CAT fusion (pRSV.CAT), used as negative and positive controls, respectively, are almost unaffected by Ca-ionophore treatment of the cells. In marked contrast, when recombinants containing the 5′ flanking sequences of the trypsin and chymotrypsin genes fused to the CAT coding region were transfected into AR4-2J cells, a Ca2+-dependent effect on CAT expression was observed. The 1.16-kilobase trypsin 5′ flanking sequence of pTrp.l.CAT, which contains a functional promoter and the putative enhancer region (7), elicited a 4-fold higher level of CAT activity in response to A23187. When a 500-bp subfragment containing only the cell-specific enhancer sequence was used in conjunction with the TK promoter, CAT activity increased 6-fold in A23187-treated cells. In chymotrypsin CAT constructs, CAT activity decreased, as expected, when

![Fig. 2. RNA blot analysis. Ten-microgram samples of cytoplasmic RNA isolated from AR4-2J cells, either untreated (−) or treated with 1 μM A23187/0.5 μM PMA/2 mM CaCl2 (+), were analyzed on a 1.5% agarose gel containing 2.2 M formaldehyde, transferred to a nitrocellulose filter, and hybridized with different nick-translated cDNAs. The positions of 28S and 18S rRNAs are shown. Trp, trypsin; Chtr, chymotrypsin; CPA1, carboxypeptidase A1; Amy, amylase.](image)

![Fig. 3. Combined effects of A23187 and PMA on mRNA levels. After adapting AR4-2J cells to DMEM supplemented with 10% serum substitute, fresh medium was added and the cells were treated with different concentrations of A23187 together with 2 mM CaCl2 with or without 0.5 μM PMA. Control cells remained untreated. Equal amounts of cytoplasmic RNA isolated 8 hr later from treated and untreated cells were subjected to dot blot analysis. Quantitation was done by scanning the autoradiograms as described. Closed symbols represent the values for A23187 and PMA; open symbols indicate treatment with A23187 alone. Trp, trypsin; Amy, amylase; Chtr, chymotrypsin; CPA1, carboxypeptidase A1.](image)

![Fig. 4. CAT activity directed by 5′ flanking sequences of the trypsin and chymotrypsin genes. AR4-2J cells were transfected with 20 μg of plasmid DNA per 100-mm dish. After 40 hr, fresh DMEM/10% serum substitute (−) or fresh DMEM/10% serum substitute containing 1 μM A23187, 0.5 μM PMA, and 2 mM CaCl2 (+) was added to identical sets of transfected cells. Eight hours later, cell extracts were prepared, protein content was determined, and 60 μg of protein was assayed for CAT activity. Lanes: a, pTE1; b, pRSV.CAT; c, pTrp.l.CAT; d, pTrp.D500.CAT; e, pChtr.CAT; f, pChtr.D275.CAT. The percentage of chloramphenicol (CM) converted to its 1Ac, 3Ac, and 1,3Ac acetylated forms is indicated.](image)
AR4-2J cells were treated with the Ca ionophore. As before, the Ca\(^{2+}\)-induced response was smaller when plasmid Ch트.CAT containing the chymotrypsin promoter was used than when CAT activity was driven by the TK promoter to which the chymotrypsin enhancer region was attached (a decrease by a factor of \(-1.6\) versus \(3.6\) in CAT activity).

From these data, it is evident that the observed increase in trypsin mRNA after treatment of AR4-2J cells with A23187 is at least partly due to a regulatory sequence at the 5' end of the trypsin gene.

**Accumulation of Trypsin Transcripts During Glucose Starvation.** Recently, Resendez et al. (23) have reported that the transcription rates of the glucose-regulated proteins, which are activated in Chinese hamster fibroblasts by glucose starvation (24, 25), are also increased by treatment of the cells with A23187. We therefore examined the influence of glucose starvation of AR4-2J cells on the mRNA levels of the different exocrine genes. As shown in Fig. 5, after a lag period of \(\sim 13\) hr, there is a gradual increase in trypsin mRNA, which reaches a plateau after 30 hr of 6-fold over the basal level.

In contrast to trypsin, the kinetics for chymotrypsin, amylase, and carboxypeptidase A1 transcripts is reversed. At 20 hr after glucose removal, a dramatic decrease in the mRNA content of these genes can be observed. Only \(-20\%\) of the initial mRNA levels can be detected.

**DISCUSSION**

Few studies of the role of calcium in the control of gene expression have been reported. Ca\(^{2+}\) interacts synergistically with growth factors and hormones to increase the transcription of the prolactin gene (26). Recently, Resendez et al. (23) showed that the glucose-regulated proteins, which belong to the class of mammalian stress proteins (27), are regulated at the transcriptional level by calcium. In this paper, we report the involvement of calcium in the regulation of the trypsin gene.

Previous studies have demonstrated the usefulness of the rat acinar cell line AR4-2J for studying exocrine gene expression (7, 19). It was shown that the mRNAs for trypsin, chymotrypsin, amylase, and carboxypeptidase A1 account for \(\approx 0.08\%\), \(\approx 1.5\%\), \(\approx 10.5\%\), and \(\approx 6\%\) of total poly(A)+ RNA in AR4-2J cells, representing 0.9%, 18%, 42%, and 34% of the amounts present in adult rat pancreas (J. H. Han, C. R. Erwin, and W.J.R., unpublished observations). Furthermore, these cells secrete measurable quantities of amylase in response to the secretagogue cholecystokinin (28), which is known to act via Ca\(^{2+}\) as an intracellular second messenger (1).

Our results demonstrate that trypsin mRNA is selectively increased 7-fold in response to A23187 treatment. In contrast, the mRNA levels of the other exocrine genes measured is decreased (amylase, chymotrypsin, carboxypeptidase A1). This decrease is in agreement with earlier observations that A23187 at concentrations that stimulate amylase secretion profoundly inhibits protein synthesis (29). PMA alone does not exert any effect, but it potentiates the A23187-induced increase in trypsin mRNA (11-fold induction). In contrast, neither cAMP derivatives nor forskolin, which are also known to stimulate amylase secretion (30), were able to activate Ca\(^{2+}\)-induced amylase release (31). Similarly, our experiments with Bt-cGMP or nitroprusside show no effect on exocrine mRNA levels. From these experiments, it is evident that calcium is the main intracellular messenger responsible for trypsin induction. From the two branches of the calcium messenger system, as discovered by Nishizuka and coworkers (34–36), the calmodulin branch activated by the divalent ionophore A23187 (37) seems to be more important in trypsin induction than the C kinase branch activated by the phorbol ester PMA (6). Nevertheless, the direct involvement of calmodulin and protein kinase C remains to be established.

Recent experiments from our laboratory have demonstrated that sequences in the 5' region of the amylase, trypsin, and chymotrypsin genes control the cell-specific expression of a linked reporter function (CAT) in the pancreatic cell line AR4-2J (7, 19). Mapping the control region of the amylase and chymotrypsin genes has revealed a cell-specific enhancer sequence, and related sequences have been identified in the 5' flanking region of the other exocrine genes, including the trypsin gene, suggesting an exocrine pancreas-specific enhancer element (7). Thus, we decided to transfect AR4-2J cells with recombinant plasmids, where part of the 5' flanking region of the trypsin and chymotrypsin genes containing the putative enhancer sequence is linked to the CAT coding sequences. In parallel transfection experiments with or without A23187 and PMA, we found that the CAT activity of cell extracts measured after transfection with trypsin recombinants increased \(-6\)-fold in response to calcium induction, whereas the CAT activity for chymotrypsin recombinants decreased by a factor of 2–4. This effect is less dramatic than for the native trypsin gene and suggests that the putative enhancer region is at least partly involved in the selective increase in trypsin mRNA.

Elsewhere, it was hypothesized that a set of trans-acting factors, called differentiators, may interact with the pancreas-specific genes to control transcription (7, 12, 19). From our observations, it seems plausible that the set of postulated differentiators might be regulated partly by phosphorylation/dephosphorylation mechanisms, which may also account for the highly complex developmental profiles of the exocrine mRNAs observed in pancreatic acinar development (12). These developmental data have already revealed a unique pattern of activation of the trypsin gene, which is expressed...
at very low levels in the embryonic differentiated state but shows a dramatic increase in mRNA levels after birth (12, 38). Our results demonstrate at another level that the trypsin gene is regulated differently from other pancreatic genes. Although the physiological meaning of the selective induction of trypsin by calcium is not established, it may be related to the unique role of trypsin in activating pancreaticzymogens. In this sense, trypsin can be considered a regulatory enzyme and its independent control is not surprising. Since cholecystokinin and other secretagogues are known to stimulate exocrine secretion via the Ca**2+**-protein kinase C second messenger system, it is possible that one or more of these regulators selectively activates the expression of the trypsin gene.

The selective regulation of trypsin among the pancreatic genes by glucose depletion emphasizes further a possible independent regulatory role of this enzyme. The other glucose-regulated genes belong to a set of mammalian stress genes (27). Does trypsin play a role in stress situations?

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