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Research Article

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Direct Regulation by Calcium of Cytoplasmic Messenger Ribonucleic Acid Coding for Pre-proparathyroid Hormone in Isolated Bovine Parathyroid Cells

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ABSTRACT DNA complementary to bovine pre-proparathyroid hormone mRNA was cloned and labeled by nick translation in order to measure mRNA by molecular hybridization. Bovine parathyroid cells were maintained in primary tissue culture for periods up to 96 h at 0.5 mM, 1.25 mM, and 2.5 mM calcium, which was followed by extraction of cellular RNA. Levels of mRNA showed no differences at 0.5 or 1.25 mM calcium, but at high calcium levels, there was a reversible decrease that began at 16 h to a plateau at 30% of control after 72 h. These studies suggest that the glandular capacity to synthesize hormone may be at or near maximal at normal calcium, but at high calcium, there is a decrease over time in steady state levels of mRNA.

INTRODUCTION

Regulation of parathyroid hormone (PTH)[1] secretion by calcium has been well documented, with secretion being stimulated by low and inhibited by high calcium (1). However, little information is available regarding the effects of calcium on PTH biosynthesis. Calcium has no effect on the processing of precursor, but intracellular degradation of hormone at high calcium is enhanced (2, 3). Moran et al. (4) showed that low calcium caused increased amino acid incorporation into proPTH, but Heinrich et al. (5) found this increase to be nonspecific. The latter also found no difference in hybridizable pre-proPTH mRNA when tissue slices were incubated at 0.5 or 5 mM calcium for 7 h.

To investigate regulation of synthesis, we measured steady state levels of cytoplasmic pre-proPTH mRNA with a cloned complementary DNA (cDNA) probe in isolated bovine parathyroid cells exposed for longer time periods to calcium. These studies showed a direct and reversible effect of high calcium on the levels of steady state mRNA.

METHODS

Preparation of cDNA probe. RNA was extracted from parathyroid glands (6) and purified by chromatography on oligodeoxythymidine-cellulose to yield a poly (A) fraction, which served as the template for cDNA synthesis, by using reverse transcriptase (7). Second-strand synthesis was performed with the Klenow fragment of DNA polymerase I and cleaved with S_1 nuclease. Double-stranded DNA was polydeoxycytidine-tailed with calf thymus terminal transferase, annealed to polydeoxyguanosine-tailed plasmid pBR322 that had been cleaved with Pst-1, and used to transform *Escherichia coli* strain RR1 (7). Plasmids were removed from tetracycline-resistant bacteria by alkaline lysis (8) and PTH cDNA inserts were identified by in situ hybridization and radioautography (7). The probe used for initial colony identification was a single-stranded cDNA copy labeled with ^{32}P and purified by rapid hybridization and hydroxylapatite chromatography (9). Positive inserts were removed by cleav-

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[1] *Abbreviations used in this paper:* cDNA, complementary DNA; pPTH10, plasmid containing pre-proPTH cDNA; PTH, parathyroid hormone.

age with Pst-1 and characterized for size on 5% polyacrylamide gel. The largest insert (650 base pairs) in a plasmid containing pre-proPTH cDNA (pPTH10) was tested by hybridization selection (10) and cleaved with restriction endonucleases Sst I, Sau 3A, Mbo II, and Taq I (11, 12).

Cell preparation. Fresh parathyroid glands were trimmed, minced, and digested with collagenase and DNAase for 3 h (12). About 5×10^6 cells were plated in Dulbecco's Modified Eagle's Medium, 5% fetal calf serum, 1% penicillin-streptomycin, and 1% fungazone. Cells were preincubated for 24–72 h; medium was removed and replaced with fresh medium containing 0.5 mM, 1.25 mM, or 2.5 mM calcium. At time intervals of 8–96 h, three plates at each condition were removed and cytoplasmic RNA was extracted by lysing cells in buffer containing 50 mM Tris (pH 7.4), 50 mM NaCl, 5 mM $MgCl_2$, 0.14 M sucrose, 1% sodium deoxycholate, and 0.5% Nonidet P-40. RNA was precipitated by 2 M LiCl at 4°C overnight. Resuspended RNA was quantitated at 260 nm.

Measurement of PTH mRNA. PTH mRNA was quantitated by two hybridization techniques using the insert in pPTH-10 labeled with ^{32}P by nick translation (14). Hybridization in solution (R_ot analysis) was carried out at 65°C in 0.3 M NaCl, 20 mM Tris (pH 7.4), 20 mM EDTA, and 0.1% sodium dodecyl sulfate with a large excess of RNA. Aliquots were taken at appropriate times and digested with S_1 nuclease. Percent hybridization was plotted against the log of RNA × time (R_ot). In control studies, in which no RNA was added, self-annealing accounted for only 3.5% hybridization during the experiment. The results were normalized to include only the hybridizing strand. For dot blot hybridization, RNA was blotted on nitro-cellulose filters and hybridized with ^{32}P labeled cDNA (1×10^6 cpm/ml) in 50% formamide, 5 × SSC, 1 × Denhardt's solution, and 100 μg/ml denatured salmon sperm DNA (15). Hybridization was carried out overnight at 42°C, filters were exposed to x-ray film (SB-5, Eastman Kodak Co., Rochester, NY) for 24 h, and the film was scanned with a densitometer.

Measurement of PTH secretion and RNA synthesis. PTH secreted into the medium was measured by radioimmunoassay (1). Total RNA synthesis after 72 h was determined by pulsing the cells with [^{3}H]uridine over 12 h (10 μCi/ml). Incorporation of [^{3}H]uridine into RNA was determined by counting aliquots of total RNA after extraction and reprecipitation with ethanol.

RESULTS

In the hybrid selection study, the poly(A)-rich RNA fraction translated multiple proteins in a wheat germ cell-free system including pre-proPTH (Fig. 1, left) (6). When pPTH10 was immobilized on nitrocellulose, hybridized to total RNA under stringent conditions, and boiled, only mRNA translating pre-proPTH was identified (Fig. 1, left) (10). The pPTH10 insert contained 650 base pairs and was characterized further by generating restriction fragments that were stained with silver (Bio-Rad Laboratories, Richmond, CA) (16) (Fig. 1, right). These fragments were compatible with the published DNA sequence (10, 11) and suggested that our clone included the entire coding region for pre-proPTH.

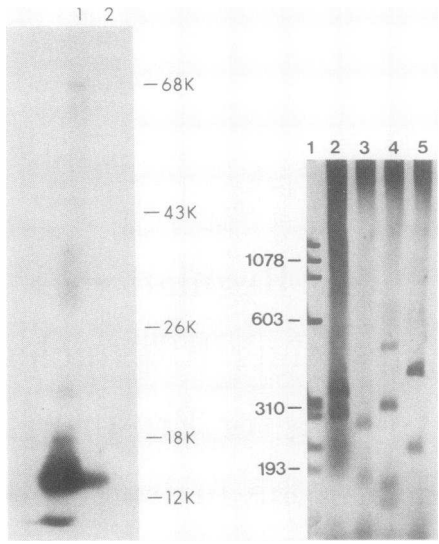

FIGURE 1 (*Left*) Results of hybridization selection (10) showing cell-free translation products of poly (A)-rich RNA in a wheat germ system (6) before (lane *1*) and after (lane *2*) incubation with pPTH-10 fixed to nitrocellulose. Immunoprecipitation (6) showed protein in lane 2 to be pre-proPTH. (*Right*) Polyacrylamide gel electrophoresis of fragments of pPTH10 insert (lanes 2–5) generated by Taq I (350 and 300 base pairs), Mbo II (290, 175, and 100), Sau 3A (310, 170, and 140), and Sst I (400, 210, and <100), respectively. Lane 1 contains Hae III marker fragments from ØX174 RF DNA used to estimate approximate size of insert fragments. Sau 3A digest contained one partly cleaved fragment (500 base pairs).

Using the cloned and nick-translated probe, we quantitated pre-proPTH mRNA in the experiments outlined above. The results of quantitation by R_ot analysis are shown in Fig. 2. At 8 h, there was a $\frac{1}{2}R_ot$ value of 0.72 at all three calcium concentrations, but at 16 (not shown) and 24 h, there was an increased value for cells grown in high calcium, with no change at low and normal calcium. The $\frac{1}{2}R_ot$ values for high calcium increased up to 72 h, indicating a decrease in mRNA of ~70%. The level then appeared to plateau as later time points revealed no further decrease. Fig. 3 shows the results of dot hybridization in which the density of purified pre-proPTH mRNA, which was hybridized, increased linearly over the range tested. When dispersed parathyroid cells were exposed to different levels of calcium and mRNA quantitated by dot hybridization, the results of the two methods were in excellent agreement (Fig. 4). There was a gradual decrease over time in cells exposed to high calcium levels, but no significant difference in mRNA content between cells grown in low or normal calcium levels. Other studies performed at 1.7 mM calcium (not shown) demonstrated partial suppression.

To show that this phenomenon was reversible, cells

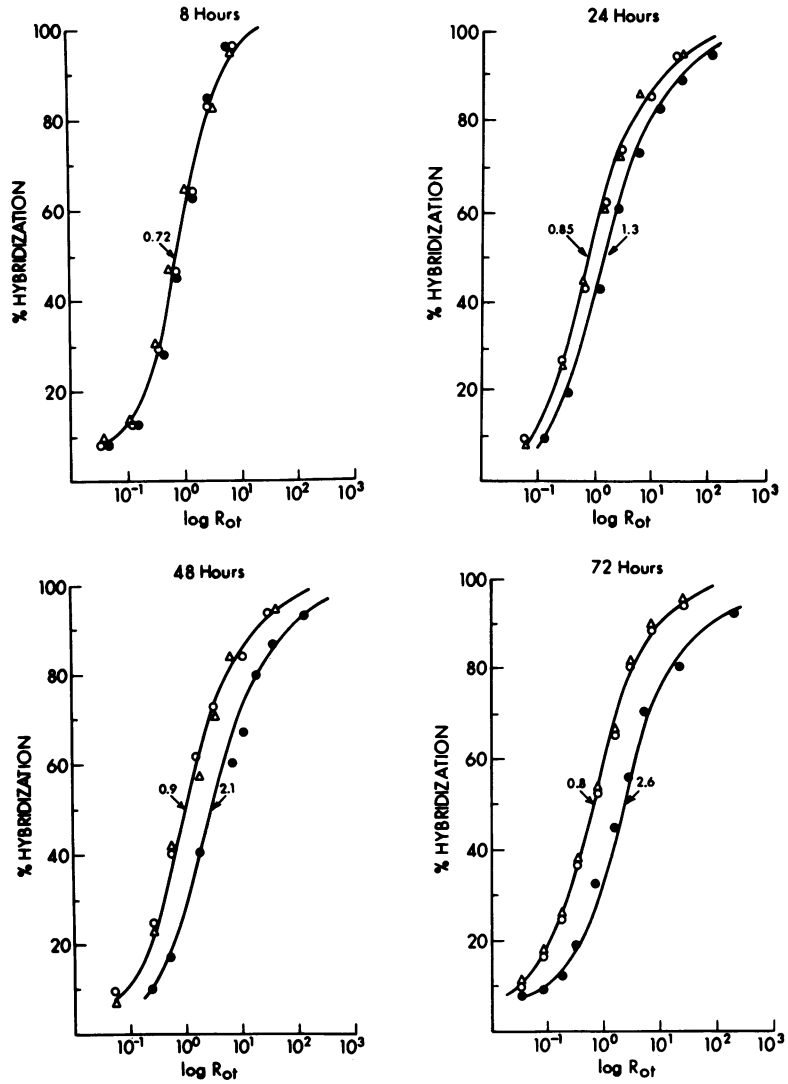

FIGURE 2 Quantitation of PTH mRNA from cells incubated in 0.5 mM (O), 1.25 mM (Δ), and 2.5 mM calcium (●). Arrows indicate points at which 50% hybridization occurred.

were incubated in high calcium concentrations for a period sufficient to suppress mRNA, and were then switched to low calcium medium. Fig. 5 shows the results of such an experiment (triplicate plates). Hybridizable mRNA in cells switched from high to low calcium was comparable to that of cells grown in low calcium alone. In contrast, cells grown only in high calcium showed a decrease of 30% at 36 h and 56% by 52 h.

Table I shows the effects of calcium on PTH secreted into the medium, on total cytoplasmic RNA, and on RNA synthesis at 72 h. PTH secretion was suppressed by 50% in cells exposed to high calcium, but there was no significant effect on total RNA extracted or RNA synthesis.

DISCUSSION

These experiments showed that chronic exposure of bovine parathyroid cells to high calcium leads to a

TABLE I
PTH Release, RNA, and RNA Synthesis at 72 H

Calcium	PTH Concentration	Total RNA	[³H]Uridine incorporation
			(cpm/μg RNA × 10⁻³)
0.5 mM Ca	2,860±460 ng/ml	27±6 μg	484±60
2.5 mM Ca	1,440±320 ng/ml*	33±7 μg‡	458±46‡

* $P < 0.05$.
‡ Not significant.

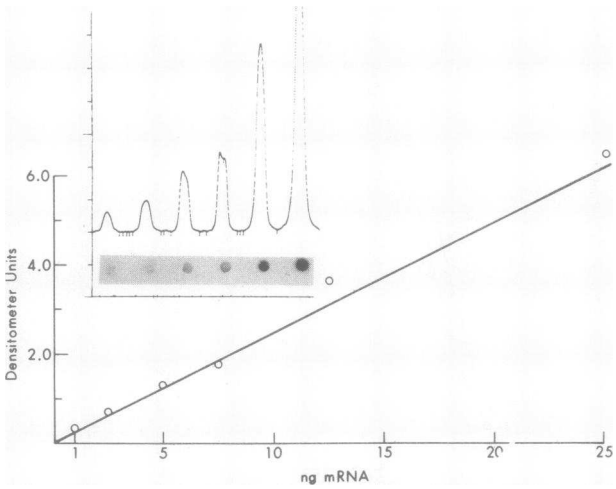

FIGURE 3 Dot hybridization using varying amounts of purified pre-proPTH mRNA. Graph shows linear relationship between mRNA blotted and the area under peak.

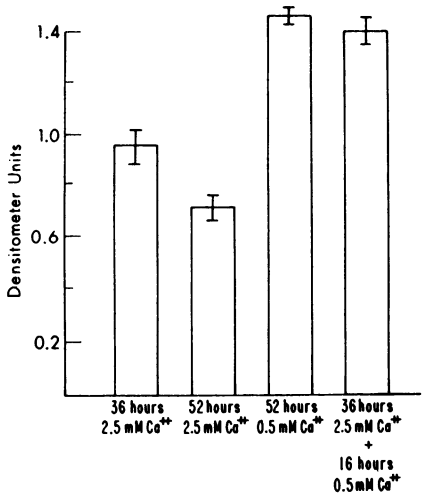

FIGURE 5 Effects on mRNA when cells grown in high calcium switched to low. Triplicate plates used for each point and results are mean±SEM. Lane 2 different from 3 and 4 by $P < 0.01$ and 3 not significantly different from 4.

gradual decrease in steady state levels of cytoplasmic pre-proPTH mRNA. This was never completely suppressed but reached a plateau at 30% of control. Since the phenomenon was fully reversible and RNA synthesis remained unchanged, the decrease in PTH mRNA was a calcium-regulated event. In contrast to cells grown in high calcium, there were no detectable differences in hybridizable mRNA between cells grown in low or normal calcium. There were also no differences at any calcium concentration when incubations < 12 h were examined, in agreement with the results of Heinrich et al. (5).

These data fit a theoretical model in which parathyroid cells at normal calcium concentrations synthesize PTH at or near maximal capacity. Varying calcium appears to regulate the events of PTH production at both posttranslational and pretranslational sites. The effect of low calcium is at the level of secretion and degradation; that is, it stimulates secretion and decreases intracellular degradation. High calcium, over short periods of time, decreases secretion and enhances degradation (2, 3), but chronic exposure causes a decrease in steady state levels of mRNA. Whether calcium exerts its effects by shortening mRNA half-life or by decreasing transcription remains to be determined.

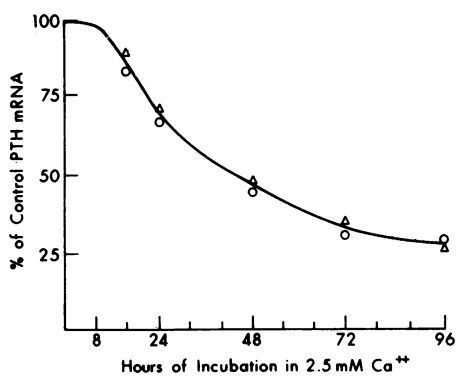

FIGURE 4 Decline in hybridizable PTH mRNA over time with exposure to 2.5 mM calcium (solution hybridization [Δ] and dot blot hybridization [O]).

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