The Effects of Tris(hydroxymethyl)aminomethane and Calcium Ionophores on the Biosynthesis of Proparathormone and the Formation and Degradation of Parathormone in Bovine Parathyroid Tissue

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A comparative study of ultrastructural and biochemical effects of Tris and ionophores X537A and A23187 on bovine parathyroid tissue is presented. When parathyroid slices were incubated with ^{3}H-leucine and ^{3}H-lysine for 10 minutes alone or with Tris (50 mM), A23187 (9.5–19 μM) or X537A (8.5–17 μM), the incorporation of the amino acids into radioactive proparathormone (proPTH) was similar, indicating that biosynthesis of the hormone was not affected. After 120 minutes of incubation, however, Tris inhibited the conversion of proPTH to parathormone (PTH), as judged by a decrease in cellular and secreted radioactive PTH and a corresponding increase in radioactive cellular proPTH. These changes were accompanied by marked dilatation of Golgi membranes. With both concentrations of A23187 and the low concentration of X537A there were no changes in amounts of radioactive proPTH, moderate decreases in cellular and secreted radioactive PTH, and little discernible distension of the Golgi membranes. At 17 μM X537A there was moderate increase in amount of radioactive proPTH, a marked decrease in amount of radioactive PTH, and swelling of the Golgi membranes. Taken together, these findings suggest that Tris inhibited conversion of proPTH to PTH by disrupting the Golgi zone—the site of conversion of proPTH to PTH; that A23187 and the low concentration of X537A decreased production of PTH by enhancing its degradation; and that X537A at the higher concentration acted both by inhibiting conversion of proPTH to PTH and by enhancing the degradation of PTH. (Am J Pathol 1981, 102:336–345)

IT WAS REPORTED previously that when bovine[1,2] and human[3] parathyroid tissue slices were incubated in physiologic buffers containing tris(hydroxymethyl) aminomethane (Tris) or other nonamphoteric amines such as glycinamide and diethylamine, there occurred a time-dependent and dose-dependent swelling of the Golgi vesicles of the parathyroid endocrine cells. The swelling was paralleled by inhibition of parathormone (PTH) formation and an accumulation of its precursor, proparathormone (proPTH). This action of Tris required the intact tissue, since the amine did not affect the conversion of proPTH to PTH in a cell homogenate.[1] Based on these and other data, it was postulated that Tris blocked the conversion of proPTH to PTH by mechanically disrupting the converting site within the Golgi complex.[1-3]

Recently the effects of calcium ionophores A23187 and X537A on parathyroid tissue were examined.[4] Ionophores are lipid soluble antibiotics that selectively transport or assist in the movement of cations across biologic membranes.[5-7] Since calcium plays a major role in regulating the secretion of PTH[8] and can modulate intracellular degradation[9-11] of the hor-

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mone, the action of these agents on parathyroid gland physiology was of interest. It was found that the chief cells of rat parathyroid glands, incubated with X537A, developed swelling and disorganization of the Golgi complex.[12] Then it was reported in bovine parathyroid tissue incubated with X537A or A23186[4] that at concentrations greater than 1 μM, these ionophores significantly inhibited the secretion of both radiolabeled and immunoreactive PTH. In addition, these agents appeared to block the cellular conversion of proPTH to PTH. Electron-microscopic evaluation of the X537A-treated tissues revealed striking ballooning and disruptive changes in the Golgi complex, but the ultrastructural features of A23187-treated tissue were not reported.

We have extended the previous studies by comparing in the present report biochemical and ultrastructural effects of Tris and ionophores X537A and A23187 on parathyroid tissue.

Materials and Methods

Preparation and Incubation of Tissue Slices

Fresh bovine parathyroid glands were obtained from a local abattoir. They were trimmed free of adipose and connective tissue and sliced with a Stadie-Riggs apparatus. Tissue (2 g per incubation) was incubated for 30–45 minutes in 10 ml of control medium (Krebs-Ringer buffer) or the same medium supplemented with 50 mM Tris, 9.5 or 19 μM A23187 or 8.5 or 17 μM X537A. 30 μCi each of ^{3}H-leucine and ^{3}H-lysine were added to the flasks and the incubations were continued for either 10 minutes or 2 hours. At the end of the 2-hour incubations about 0.1 g of tissue was removed from each flask for electron-microscopic examination. The remainder of the reaction mixtures were rapidly chilled on ice, followed by separation of medium from tissue and the addition of 10 ml of 8 M urea, 0.2 N HCl, and 0.1 M cysteine to the tissue slices. The medium was frozen for later analysis.

Isolation and Analysis of Incubation Products

The incubated tissue slices were homogenized in the 8 M urea, 0.2 M HCl, 0.1 M cysteine solution. To this homogenate were added 25 ml of a similar 10% homogenate of nonincubated frozen bovine glands, which served as carrier protein. The mixture was processed as described previously[13] through the carboxymethyl-cellulose (CM-cellulose) chromatography step in order to separate radioactive proPTH and PTH. A linear ammonium acetate gradient from 0.025 M, pH 5.3, to 0.2 M, pH 7.0, was employed for each column. Aliquots of each fraction were assayed for radioactivity. The recovery of the radioactive hormonal proteins was followed by inclusion in the initial homogenate of bovine ^{14}C-proPTH and ^{14}C-PTH as markers (prepared as described previously.[13] The recovery of the radioactive prohormone and hormone was adjusted for losses as determined by recovery of the markers. The incorporation of ^{3}H-amino acids into total proteins of the tissue was measured in a 10% trichloracetic acid precipitate derived from a sample of the original tissue homogenate. The precipitate was collected by centrifugation, washed 2 times with 10% trichloracetic acid, dissolved in 1% sodium dodecylsulfate, and assayed for radioactivity.

Radioactive assay was conducted by liquid scintillation spectroscopy as described earlier.[3] The efficiency of counting and degree of quenching for samples of interest were determined by means of ^{14}C-toluene and ^{3}H-toluene internal standards.

Electron Microscopy

Tissue samples were fixed in 4% glutaraldehyde, 0.1 M sodium phosphate, pH 7.2,[3] postfixed in 1.3% *s*-collidine-buffered osmium tetroxide (pH 7.4), dehydrated in ethanol, and embedded in an Epon-araldite mixture.[15] Thin sections were cut with diamond knives on the ultramicrotome and were stained with lead citrate[16] and uranyl acetate.[17]

Materials

Radioactive amino acids were purchased from New England Nuclear Corporation (Boston, Mass); Tris from Sigma Chemical Co. (St. Louis, Mo); X537A from Hoffmann La Roche (Nutley, NJ); A23187 from Eli Lilly and Company (Indianapolis, In); and carboxymethylcellulose (CM-52) from Reeve Angel (Clifton, NJ). All other chemicals were reagent grade and were purchased from various chemical suppliers.

Results

Electron Microscopy

Ultrastructural studies were performed on the bovine parathyroid tissue incubated in control medium and in medium containing either Tris, A23187, or X537A. The control tissue (Figure 1), incubated for 2 hours in Krebs-Ringer buffer only, appeared to be essentially normal morphologically. No abnormalities were noted in nucleus, plasma membrane, endoplasmic reticulum, Golgi complex, or secretory granules. There was occasional mildly increased density of mi-

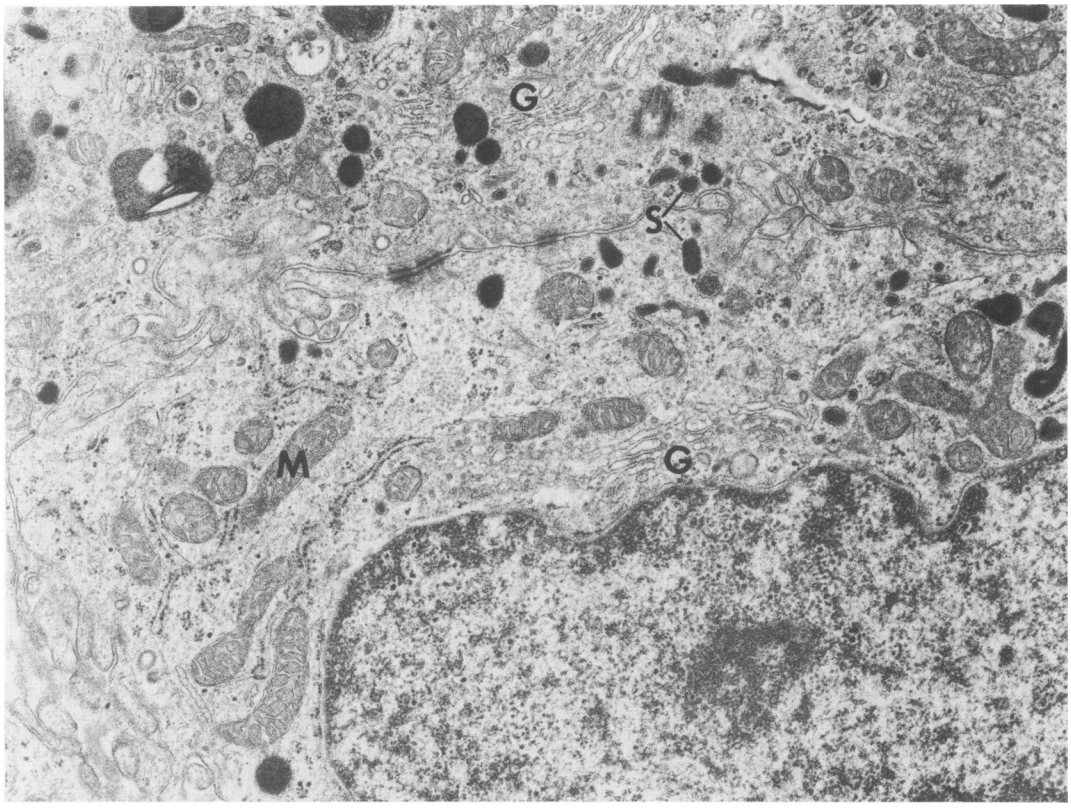


Figure 1—Parathyroid tissue incubated for 2 hours in Krebs-Ringer buffer. The chief cells appear essentially normal, except for focal mild elongation and increased matrix density of mitochondria (*M*), and no abnormalities are noted in organelles, including secretory granules (*S*) and Golgi complex (*G*). (Uranyl acetate and lead citrate, ×18,750)

tochondrial matrix. The tissue maintained this appearance up to at least 3 hours of incubation.

Tris-Treated Parathyroid Tissue

After incubation for 2 hours in Krebs-Ringer buffer containing 50 mM Tris (Figure 2), the cells contained many membrane-limited vacuoles that were approximately spherical and ranged from 0.2 to 2.0 μ in diameter. They frequently caused scalloping of the nucleus, and the membranes of the vacuoles were sometimes incomplete and coalesced with adjacent vacuoles. Most of the vacuoles were empty, but they occasionally contained distorted membranous material, secretory granulelike material, or lipoid material. The vacuoles appeared to be mostly concentrated in the apparent region of the Golgi complex; normal Golgi were not identified. The remainder of the cell organelles were normal in appearance, except for an apparent decrease in secretory granules, an increase in lysosomelike structures, and an increase in density of mitochondrial matrix. Some capillaries had mild distension and vacuolization of Golgi zones, but intravascular leukocytes had normal-appearing Golgi zones.

A23187-Treated Parathyroid Tissue

Figure 3 shows tissue after incubation for 2 hours in Krebs-Ringer buffer containing 9.5 μM of A23187. All cell organelles appeared normal, except for some increased density of mitochondrial matrix and a possible decrease in the number of secretory granules. The Golgi zones were not dilated. Figure 4 shows parathyroid tissue treated similarly, except that the buffer contained 19 μM of A23187. Ultrastructural changes were comparable to those seen in the tissue incubated in the lower concentration of A23187 (Figure 3), except that occasional degenerative changes were seen, including nuclear chromatin condensation, mild disruption of the Golgi zone, mitochondrial swelling, apparent increase in number of secretory granules, and focal mild distension of rough endoplasmic reticulum.

X537A-Treated Parathyroid Tissue

Figure 5 shows tissue after 2-hour incubation in

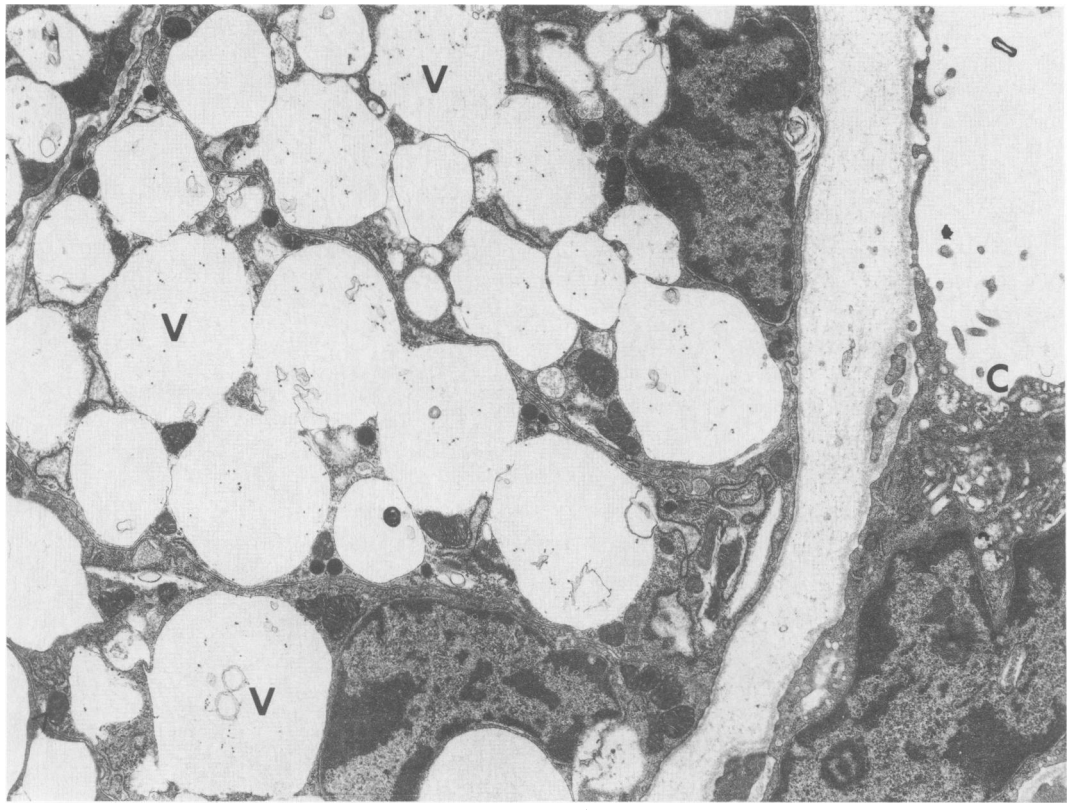
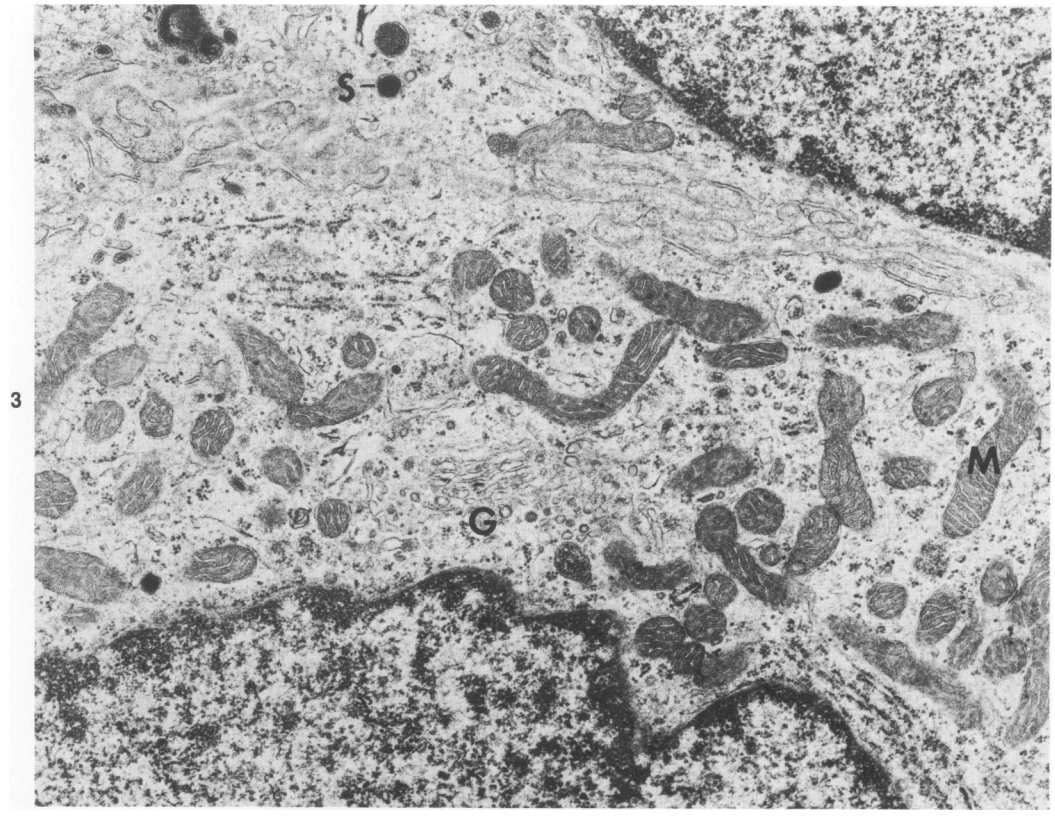


Figure 2—Parathyroid tissue incubated for 2 hours in Krebs-Ringer buffer containing 50 mM Tris. The chief cells contain many large membrane-limited vacuoles (*V*). Normal Golgi are not identified. A capillary endothelial cell (*C*) has mild distension of Golgi complex (Uranyl acetate and lead citrate, × 10,200)

Krebs-Ringer buffer containing 8.5 μM X537A. Most cells were normal in appearance except for mild distension of Golgi zones, along with some increased density of mitochondrial matrix, possible decrease in number of secretory granules, and increased number of lysosomes. Occasionally with this concentration of ionophore moderate distension of Golgi zones and moderate numbers of membrane-limited vacuoles were noted (Figure 6). These were similar to that observed when lesser concentrations of Tris were tested (about 5 mM; data not shown). Mild Golgi zone distension of capillary endothelial cells was also evident. Figure 7 shows parathyroid tissue treated similarly, except that the buffer contained 17 μM X537A. Most cells had moderate distension of Golgi zones and a moderate number of membrane-limited vacuoles. Only occasional cells, on the other hand, had mild distension of Golgi zones without vacuole formation.

Biochemical Studies

Neither Tris, X537A, nor A23187 altered the amount of radioactive proPTH found in the tissue during a 10-minute incubation (Table 1, Figure 8) demonstrating that the apparent biosynthetic rate of hormone precursor was not affected. After 120 minutes of incubation (Table 1, Figure 9), the tissue incubated in 50 mM Tris contained about five times more radioactive proPTH than control tissue and less than one-tenth the amount of radioactive PTH, such that the sum of proPTH and PTH remained the same. In contrast, treatment with the low or high concentrations of A23187 and the low concentration of X537A caused no change in radioactive proPTH and about a 50% decrease in radioactive PTH (Table 1, Figure 10). The high concentration of X537A, however, doubled the amount of radioactive proPTH and reduced the amount of radioactive PTH to one-fifth that of the control. In each situation the ionophores decreased the sum of radioactive proPTH and PTH. In no case did any of the agents affect the incorporation of amino acid into total protein of the tissue. In the experiment listed in Table 1, for example, the overall average radioactivity in the trichloroacetic acid precipitable protein of the tissue homogenate was 8.8 ×



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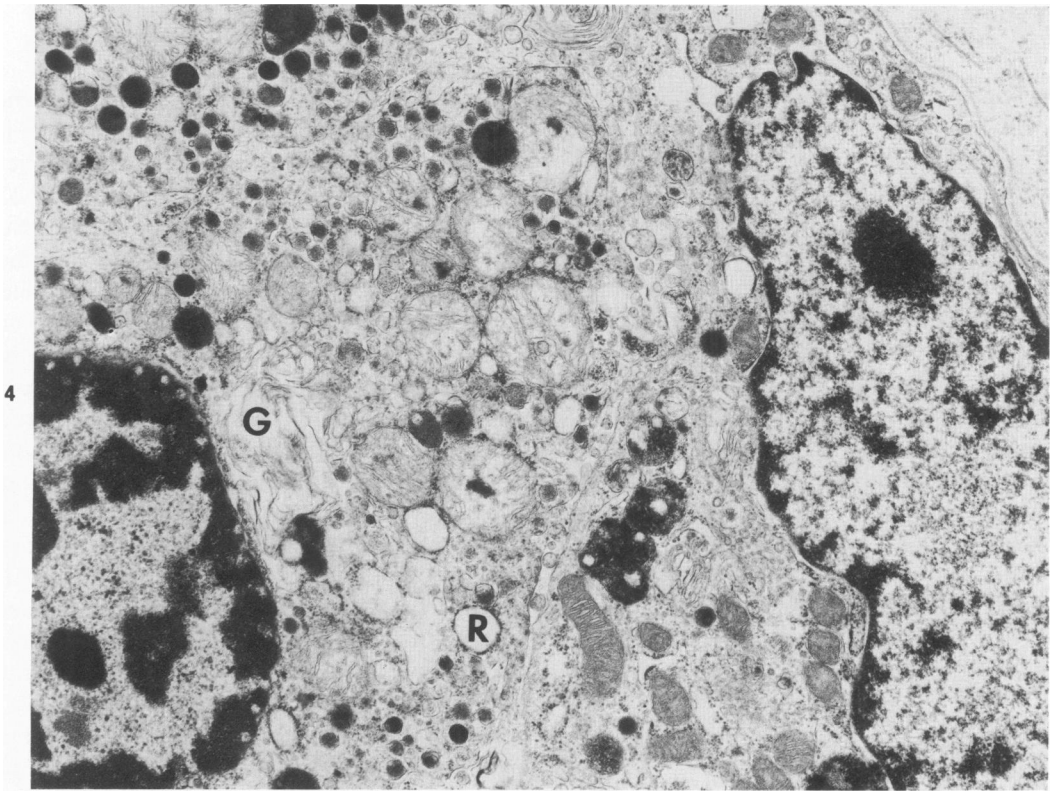

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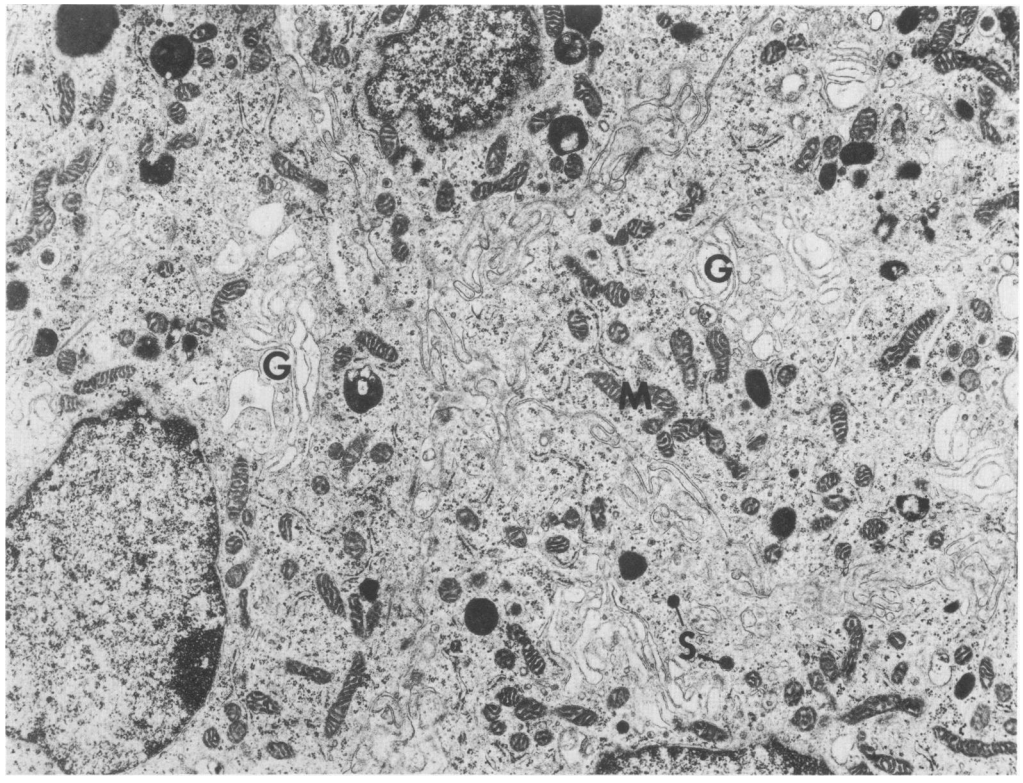


Figure 5—Parathyroid tissue incubated for 2 hours in Krebs-Ringer buffer containing 8.5 μM X537A. The chief cells appear normal except for mild distension of Golgi (*G*), along with some elongation and increased matrix density of mitochondria (*M*) and a possible decrease in the number of secretory granules (*S*). (Uranyl acetate and lead citrate, × 10,200)

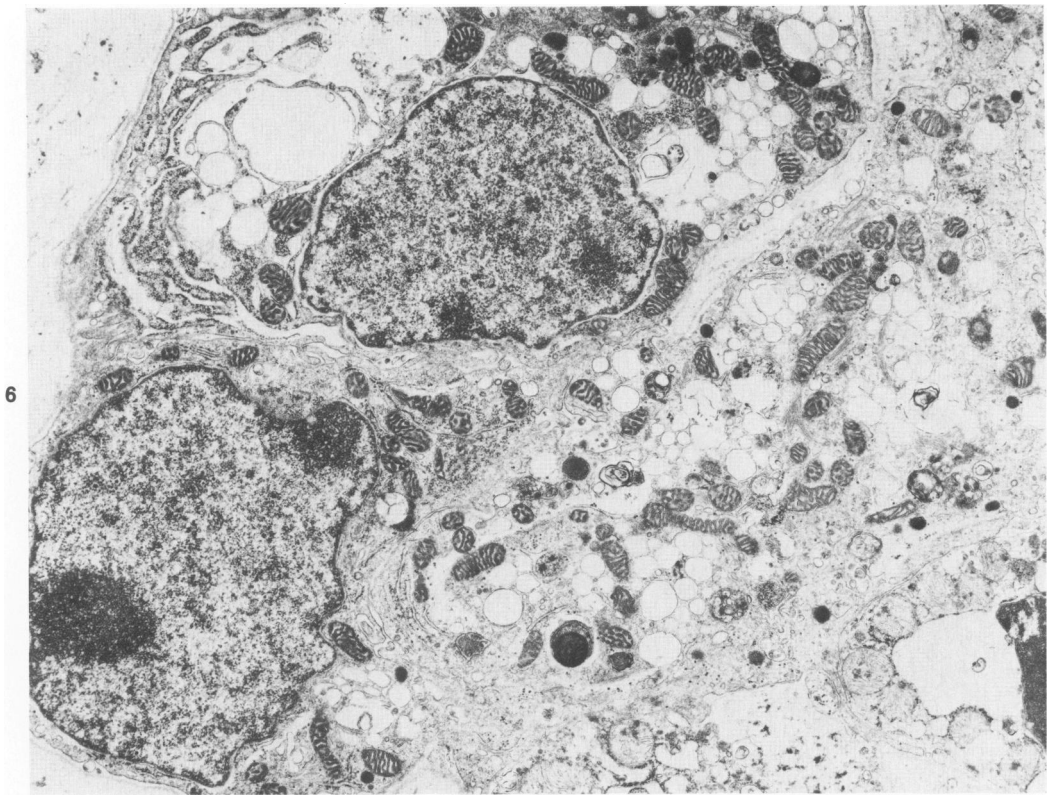
$10^6 \pm 0.1$ dpm (av ± SD) with the separate groups falling in the range $8.6 - 8.9 \times 10^6$ dpm.

Table 2 shows that Tris A23187 and X537A decreased the amount of radioactive PTH found in the incubation medium. Thus the decreases in cellular PTH listed in Table 1 caused by these agents did not result from enhanced secretion of PTH.

Discussion

Tris, A23187, and X537A each acted differently on the parathyroid tissue. Tris produced marked swelling of the Golgi, a major increase in radioactive proPTH, and a corresponding decrease in radioactive PTH (cell plus medium) such that the sum of proPTH and PTH remained constant. Thus this agent appeared to inhibit the conversion of proPTH to PTH—an interpretation that is in accord with our earlier reports.[1-3] A23187 lowered the amount of radioactive PTH in the tissue and in the incubation medium but did not cause appreciable Golgi swelling or change in amount of radioactive proPTH. This agent appeared to act by increasing the intracellular destruction of newly synthesized PTH—an event that under normal circumstances represents a major fate of unsecreted hormone.[9-11] This enhanced level of degradation could have been secondary to an increased intracellular level of calcium promoted by the ionophore, since it has been shown that the large amount of PTH normally degraded grows even greater when the cell is exposed to an elevated calcium concentration.[9-11] X537A seemed to exhibit dual effects. At the lower concentration, it acted like A23187, decreasing the amount of radioactive PTH in the cell and medium; at the higher concentration, it produced marked Golgi swelling, increased radioactive proPTH, and further decreased radioactive PTH in the cell. These findings extend our earlier studies with Tris and other amines.[1,3] They suggest that morphologic alteration of the Golgi complex by whatever chemical agent will inhibit for-

Figure 3—Parathyroid tissue incubated for 2 hours in Krebs-Ringer buffer containing 9.5 μM of A23187. Cell organelles of the chief cells appear normal except for focal mild elongation and increased matrix density of mitochondria (*M*) and a possible decrease in number of secretory granules (*S*). Golgi zones (*G*) are not dilated. (Uranyl acetate and lead citrate, × 18,750) **Figure 4**—Parathyroid tissue incubated for 2 hours in Krebs-Ringer buffer containing 19 μM of A23187. Cell organelles of the chief cells appear similar to those of Figure 3 except for focal degenerative changes, including nuclear chromatin condensation, occasional mild degenerative disruptions of Golgi zones (*G*), mitochondrial swelling, apparent increase in the number of secretory granules, and focal mild distension of rough endoplasmic reticulum (*R*). The chief cell on the right does not have these degenerative changes and is similar to the cells of Figure 3. (Uranyl acetate and lead citrate, × 14,960)



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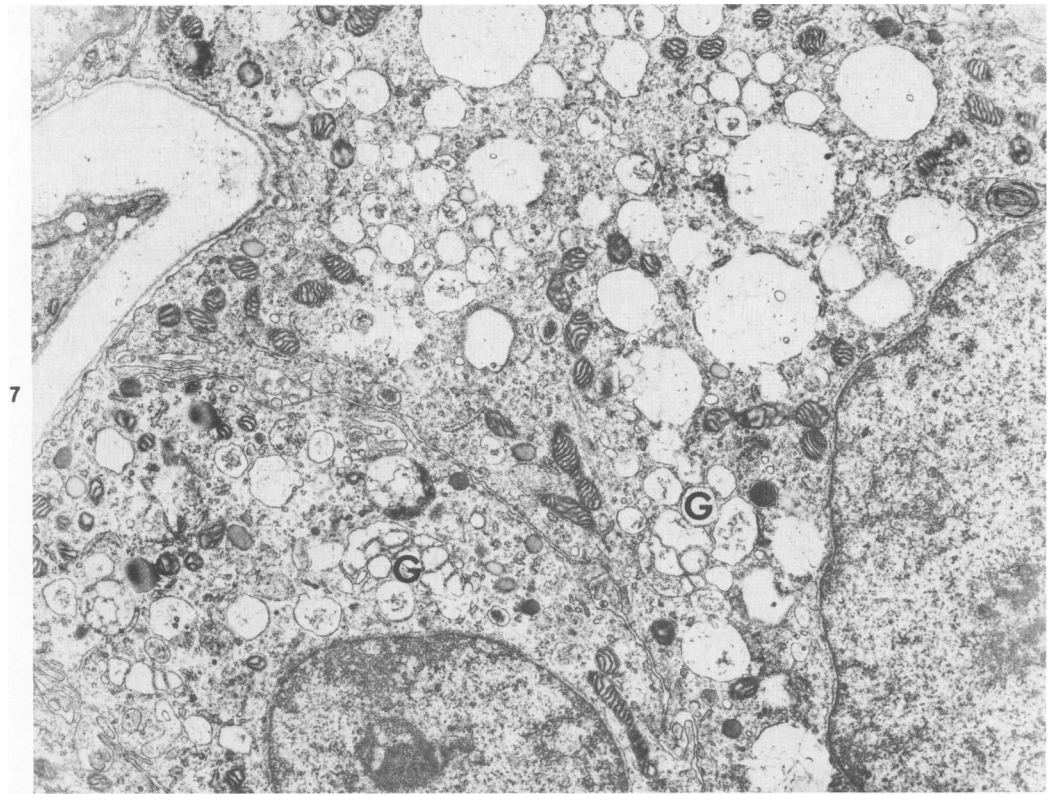

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Figure 6—Parathyroid tissue incubated for 2 hours in Krebs-Ringer buffer containing 8.5 μM X537A. Occasionally with this concentration of ionophore, moderate distension of Golgi zones and a moderate number of membrane-limited vacuoles are noted. (Uranyl acetate and lead citrate, × 10,200) **Figure 7**—Parathyroid tissue incubated for 2 hours in Krebs-Ringer buffer containing 17 μM X537A. Most cells have moderate distension of Golgi zones (*G*) and a moderate number of membrane-limited vacuoles. (Uranyl acetate and lead citrate, × 12,300)

Table 1—Effect of Tris and Calcium Ionophores on Cellular proPTH and PTH*

	Incubation period			
	10 minutes	120 minutes		
	proPTH	proPTH	PTH	proPTH + PTH
Control	9.0 ± 0.7	22.3 ± 1.8	96.1 ± 10.3	118.4 ± 12.1
Tris (50 mM)	9.5 ± 0.6	121.7 ± 20.8	8.6 ± 0.3	130.3 ± 20.7
A23187 (9.5 μM)	8.1 ± 0.3	23.9 ± 2.0	57.9 ± 6.5	81.8 ± 8.5
A23187 (19.0 μM)	8.5 ± 0.4	19.7 ± 2.5	51.4 ± 4.9	71.1 ± 7.4
X537A (8.5 μM)	9.1 ± 0.9	21.6 ± 1.8	54.6 ± 8.3	76.2 ± 10.1
X537A (17.0 μM)	9.4 ± 0.9	56.3 ± 5.8	16.9 ± 1.7	73.2 ± 7.5

* Data are presented as dpm × 10^{-3} (mean ± SD) for 3 determinations of each condition.

mation of PTH from its precursor. The most obvious reason for this would be that the site of the conversion reaction is the Golgi region itself.

The specific mechanism(s) by which Tris and X537A produce swelling is unknown. Presumably the severe distension of the Golgi vesicles is due to entry of fluid, since it is not likely that a morphologic change of this magnitude would be due to engorgement with prohormone or other unprocessed secretory proteins. The swelling agents might change the volume of fluid entering or leaving the Golgi by acting on an ion pump associated with this organelle. X537A, for example, can affect the transport of monovalent ions as well as calcium,[6] and monensin, which is a specific ionophore for Na^+ and K^+, has recently been shown to produce Golgi swelling in plasmacytoma cells.[18] In any event, the data rule out that the swelling is specifically due to the movement of calcium, since A23187 has a high selectivity for this ion and did not produce appreciable swelling in our studies.

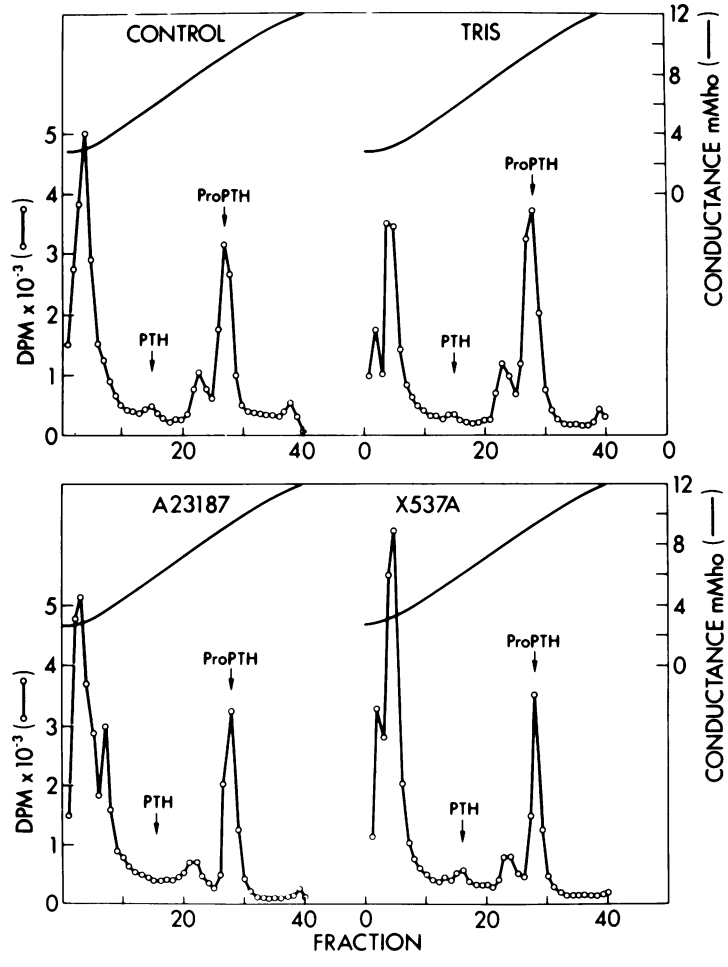

Figure 8—Separation of radioactive proPTH by carboxymethyl cellulose ion exchange chromatography derived from bovine parathyroid tissue after 30–45 minutes' incubation in control buffer or buffer containing 50 mM Tris, 9.5 μM A23187, or 8.5 μM X537A followed by 10 minutes' incubation in the same buffer containing ^{3}H-leucine and ^{3}H-lysine. Little or no radioactive PTH was detected (Fractions 13-17) because the incubation period was short. The radioactivity under the proPTH peak for this and repetitive experiments was determined and entered as data in Table 1.

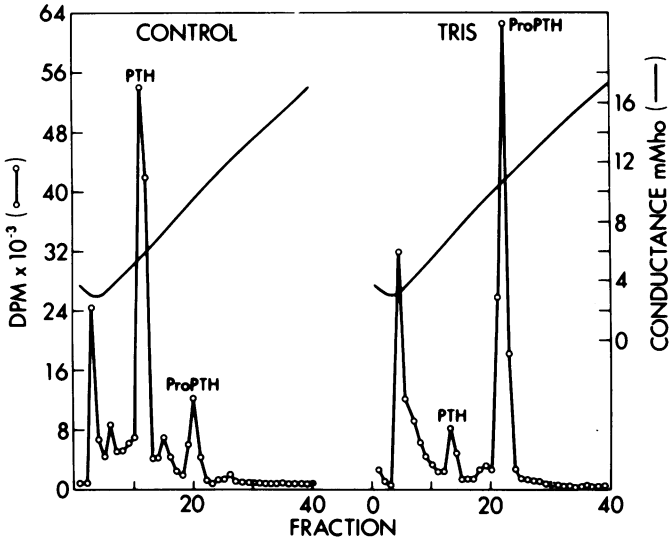

Figure 9—Separation of radioactive PTH and proPTH by carboxymethyl cellulose ion exchange chromatography derived from bovine parathyroid tissue after incubation in control or 50 mM Tris-containing buffer for 120 minutes. The data from this and repetitive incubations are entered in Table 1.

It was reported previously that X537A and A23187 strongly decreased the secretion of PTH.[4] It was suggested that this was due to an inhibition of secretion *per se* possibly as a consequence of an elevated level of calcium within the cell. Our results allow us to propose an alternative, but not mutually exclusive, possibility: since there was less new PTH in the cell as a consequence of inhibition of its formation, there was less available for secretion.

The Golgi action of Tris first reported for the parathyroid is not limited to this gland. Tris and other nonamphoteric amines have recently been found in short-term perfusion of isolated rat livers to cause swelling of Golgi complex, lysosomes, and secretory vesicles of this tissue.[19] Similar vacuolization was observed in Kupffer cells, pancreatic beta-cells, intestinal epithelial cells, and renal proximal tubule cells, but not in alpha-cells of the pancreas or in the kidney glomerular podocytes. Since protein synthesis in the liver was not affected, the inhibition by Tris of protein secretion into the perfusate indicated a block in protein processing at a postribosomal level, presumably in the Golgi region. X537A has been reported to induce vacuolation of Golgi complex of vascular and intestinal smooth muscle, epithelium, plasma cells, and cultured chick heart muscle cells.[20] Furthermore, rat anterior pituitaries incubated with X537A, but not A23187, contained vesicles apparently derived from the Golgi complex.[21]

Further biochemical correlations of the effect of amines and ionophores on endocrine and other organs have the potential of yielding valuable information

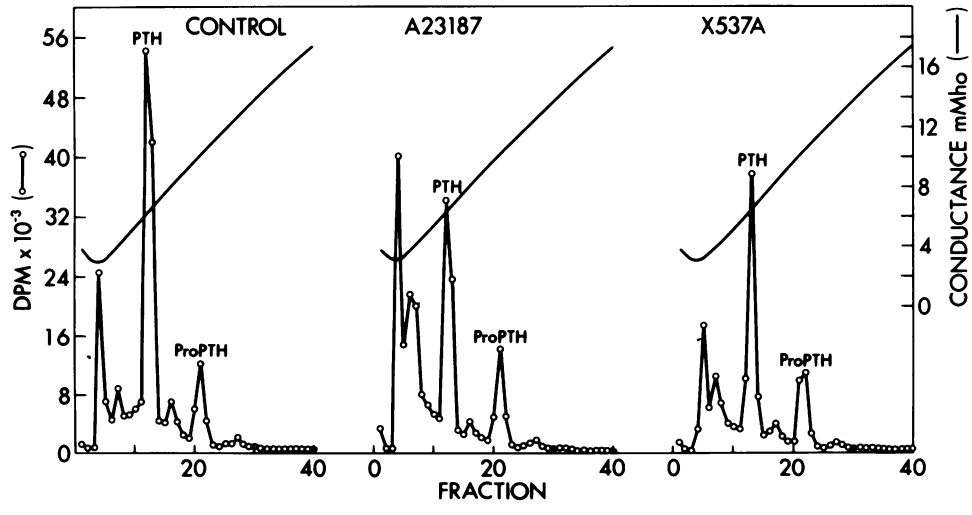

Figure 10—Separation of radioactive PTH and proPTH by carboxymethyl cellulose ion exchange chromatography derived from bovine parathyroid tissue after incubation in control buffer or buffer containing 19 μM A23187 or 17 μM X537A for 120 minutes. The curve for control is the same as that of Figure 9.

Table 2—Effect of Tris and Ionophores on the Secretion of Radioactive PTH by Parathyroid Tissue

Agent	PTH, dpm
Control	38,500 ± 4000
Tris, 50 μM	1,250 ± 200*
A23187, 9.5 μM	20,100 ± 5000†
X537A, 8.5 μM	17,800 ± 3900‡

Probability of difference from control.
* $P < .005$.
† $P < 0.05$.
‡ $P < 0.025$.

that will enhance our understanding of the processing and secretory mechanisms of hormones and other proteins. These agents, moreover, would seem to have the potential to serve as specific disrupters of various phases of the processing and secretion of exportable proteins.

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Acknowledgments

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