Biosynthesis and Secretion of Parathyroid Hormone Are Sensitive to Proteasome Inhibitors in Dispersed Bovine Parathyroid Cells* 

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Preproparathyroid hormone (prepro-PTH) is one of the proteins abundantly synthesized by parathyroid chief cells; yet under normal growth conditions, little or no prepro-PTH can be detected in these cells. Although this may be attributed to effective cotranslational translocation and proteolytic processing, proteasome-mediated degradation of PTH precursors may be important in the regulation of the levels of these precursors and hence PTH secretion. The effects of N-acetyl-Leu-Leu-norleucinal, N-acetyl-Leu-Leu-methioninal, carbobenzoxoxy-Leu-Leu-leucinal (MG132), benzoylcarboxyl-Ala-Glu(-butyl)-Ala-leucinal (proteasome inhibitor I), and lactacystin on the biosynthesis and secretion of PTH were examined in dispersed bovine parathyroid cells. We demonstrate that treatment of these cells with proteasome inhibitors caused the accumulation of prepro-PTH and pro-PTH. Compared with mock-treated cells, the processing of pro-PTH to PTH was delayed, and the secretion of intact PTH decreased in proteasome inhibitor-treated cells. Relieving the inhibition of the proteasome by chasing MG132-treated cells in medium without the inhibitor led to the rapid disappearance of the accumulated prepro-PTH, and the rate of PTH secretion was restored to levels comparable to those in mock-treated cells. Furthermore, overexpression of the Hsp70 family of molecular chaperones was observed in proteasome inhibitor-treated cells, and we show that PTH/PTH precursors interact with these molecular chaperones. These data suggest the involvement of parathyroid cell proteasomes in the quality control of PTH biosynthesis.

The calcium concentration in mammalian body fluids is tightly regulated predominantly by the actions of parathyroid hormone (PTH) on bone, kidney, and intestine (1–4). The release of PTH by parathyroid chief cells is in turn strictly regulated predominantly by the actions of parathyroid hormone (PTH) (1, 2). The release of PTH by parathyroid chief cells is in turn strictly calcium-dependent. Slight changes in the extracellular calcium concentration ([Ca²⁺]o) perceived by the calcium-sensing receptor (5) are translated intracellularly to elicit stimulated (low [Ca²⁺]o) or repressed (high [Ca²⁺]o) secretion of PTH. However, the signaling pathway(s) leading to the rapid and specific changes in PTH secretion remains largely unknown.

The primary precursor prepro-PTH is translocated into the endoplasmic reticulum (ER) and cleaved to pro-PTH within 1 min. Pro-PTH then transits the ER and attains the trans-Golgi network within 20 min, where it is cleaved to mature PTH. Depending on the needs and predominantly on [Ca²⁺]o, mature PTH is packaged into either secretory granules for exocytosis or storage granules. Secretion of de novo synthesized PTH is believed to occur within 30 min of prepro-PTH formation (6) and constitutes the bulk of secreted PTH (7–9). At the post-transcriptional level, acute changes in [Ca²⁺]o (<24 h) do not significantly affect the rate of PTH biosynthesis. However, sustained or chronic hypocalcemia and hypercalcemia affect the stability and hence the levels of PTH mRNA (10–14, 44).

Intracellular proteolysis of mature bioactive PTH has been reported to be one of the mechanisms by which parathyroid cells regulate the amount of hormone available for secretion in response to changes in [Ca²⁺]o (15, 16). This PTH metabolism is now known to be mediated by calpains (17) and cathepsins B and D (18–21). Inhibition of these PTH-degrading activities potentiates PTH secretion, thus providing unequivocal evidence that PTH secretion is partly regulated by intracellular degradation of the mature bioactive hormone at the distal portion of the secretory pathway. However, this does not exclude the possibility that regulated processing and/or degradation of PTH precursors occurring early in the secretory pathway, e.g. by proteasome-mediated ER-associated degradation, might influence the overall secretion of PTH.

In most mammalian cells, the proteasome, a multicatalytic protease complex, accounts for the degradation of short-lived and most regulatory proteins. An increasing number of its physiological targets as well as its mechanism of action have been uncovered in recent years either through the use of proteasome inhibitors (22) or by genetic mutant studies (23). Using these strategies, the proteasome has also been implicated in the turnover of proteins that transit the secretory pathway (24–26). Among these proteins are the PTH-related protein (27), apolipoprotein B100 (28), the amyloid precursor protein (29), thyroglobulin (30), and macrophage inhibitory cytokine-1 (31). In these few examples, the proteasome has been demonstrated to directly or indirectly regulate the intracellular levels of the precursor proteins.

In this study, the effects of peptide aldehyde proteasome inhibitors and lactacystin on the fate of intracellular and secreted PTH and/or its precursors were investigated. We demonstrate that treatment of dispersed bovine parathyroid cells with proteasome inhibitors caused the accumulation of PTH precursors, a delay in the processing of pro-PTH, and decreased secretion of intact PTH. This was accompanied by overexpres-
sion of the Hsp70 family of molecular chaperones in proteasome-inhibitor-treated cells, which may lead to enhanced interactions with the accumulating PTH precursors and retard their transit into or through the secretory pathway. These data suggest the involvement of parathyroid cell proteasomes in the quality control of PTH biosynthesis.

**EXPERIMENTAL PROCEDURES**

**Materials—**N-Acetyl-Leu-Leu-norleucinal (ALLN), N-acetyl-Leu-Leu-methioninal (ALLM), lactacystin, benzoylcarbonyl-Leu-Leu-leucinal (MG132), benzoylcarbonyl-Arg(4-butoxy)-Ala-leucinal (proteasome inhibitor I (PSI)) and phorbol 12-myristate 13-acetate (PMA) were purchased from Calbiochem. The fluorogenic proteasome substrates succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (AMC) and benzoylcarbonyl-Leu-Leu-Glu-AMC were also purchased from Calbiochem. Chloroquine diphosphate, cycloheximide, collagenase type V, DNase I, and brefeldin A were from Sigma. Protein G-agarose; monoclonal antibody to ubiquitin (P4D1); goat polyclonal antibodies to GRP78 (BiP), Hsp70, and chromogranin A; and peroxidase-conjugated antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Rabbit polyclonal antiserum to bovine PTH was purchased from Biogenesis Ltd. (Poole, UK). The rat intact PTH IRMA kit was from Immutopics Inc. (San Clemente, CA). Heat-inactivated fetal bovine serum was purchased from Invitrogen. Modified Eagle’s medium (MEM) was freeze-thawed and centrifuged at 200,000 g before use in experiments. The viability of the recovered cells was routinely observed to be >96% by trypan blue exclusion.

Except as otherwise indicated, equal numbers of acutely dispersed cells (2–5 × 10^6 assay) and MCF medium supplemented as described above with the Ca^2+ adjusted to the indicated final concentrations (herein referred to as complete MCF medium) and used for all treatments. All incubations were carried out at 37 °C in 5% CO_2 for the indicated times. To determine the effects of proteasome inhibitors on the fate of intracellular PTH/PTH precursors, cells were incubated in complete MCF medium containing 0.5, 1.25, or 2.0 mM Ca^2+; the inhibitors at the indicated concentrations, or 0.2% dimethyl sulfoxide vehicle. At the end of the incubation, the cells were washed twice with ice-cold phosphate-buffered saline (pH 7.4), harvested, and stored at −70 °C until required for use. Where necessary, the culture supernatants were collected and centrifuged at 10,000 × g for 5 min, and the cleared supernatants were preserved in aliquots at −70 °C.

**Metabolic Labeling—**Acutely dispersed cells were preincubated for 2 h in complete MCF medium containing either 1.25 or 2.0 mM Ca^2+. To observe the steady-state effects of proteasome inhibitors, the cells were rinsed twice with the same medium and resuspended in the same medium containing 50 μCi/ml Tran-35S-label, inhibitors at the indicated concentrations, and Ca^2+ at a final concentration of either 1.25 or 2.0 mM for 5 or 20 h. At the end of the labeling, the cells and culture supernatants were harvested as described above and stored at −70 °C until required for use.

In pulse-chase labeling, cells were pretreated with MeSO vehicle (mock-treated) or proteasome inhibitors at the indicated concentrations for 60 min during methionine deprivation and pulse-labeled in complete MCF medium containing the inhibitors or vehicle, 50 μCi/ml Tran-35S-label, and Ca^2+ at 1.25 mM for 30 min. Following the labeling, the cells were washed twice with the same medium supplemented with 0.1 mM cycloheximide in which the radiolabel was replaced with 0.2 mM nonradioactive methionine and incubated in this medium for the indicated times. At the end of each chase period, the cells and culture supernatants were harvested as described above and stored at −70 °C until required for use.

**N-terminal Amino Acid Sequencing—**Cells were double-labeled with Tran-35S-label and a mixture of [3H]valine/leucine in the presence of MG132 (50 μM) in complete MCF medium containing 2.0 mM Ca^2+ for 5 h as described under “Metabolic Labeling.” The cell lysates were subjected to immunoprecipitation as described below using rabbit anti-PTH or control serum. The immune complexes were separated on 16.5% Tris/Tricine/SDS-polyacrylamide gels (34) and blotted onto Hybond ECL-P membrane (polyvinylidene difluoride, Amersham Biosciences). After autoradiography of the polyvinylidene difluoride blot, the autoradiograph was aligned with the polyvinylidene difluoride filter, and the two slowly migrating anti-PTH reactive bands in the MG132-treated cell lysates were excised for N-terminal protein sequencing using an Applied Biosystems 477A protein sequencer. The released phenylthiohydantoin-amino acid derivatives were subjected to scintillation counting.

**Measurement of Secreted Intact PTH—**Treatment of cells for the assay of secreted intact PTH was performed as previously described (35). Secreted intact PTH in the culture supernatants was assayed using a two-site immunoradiometric assay developed for the measurement of rat intact PTH. Routinely, the samples were assayed in duplicate according to the manufacturer’s instructions (Immutopics Inc.) with the appropriate controls and standards.

**In Vitro Proteasome Activity Assays—**Cells were either exposed to different concentrations of Ca^2+ for 10 or 90 min (acute treatment) or for 18–20 h (chronic treatment) or treated with proteasome or cysteine protease inhibitors or mock-treated with MeSO for 90 min (as described above). Crude cell lysates as the source for parathyroid proteasome proteolysis were freeze-thawed (10 times) and the resulting supernatants were harvested as described (38) in lysis buffer containing 25 mM Tris·HCl (pH 7.5), 250 mM sucrose, 5 mM MgCl_2, and 1 mM dithiothreitol. The ATP-dependent proteasome activities were assayed in a 96-well microtiter plate with the fluorogenic proteasome substrates succinyl-Leu-Leu-Val-Tyr-AMC and benzoylcarbonyl-Leu-Leu-Glu-AMC at 100 μM essentially as described (36, 37). The fluorescence of the released AMC moiety was determined using an Amersham Biosciences Fluorocount-96 set at 365-nm excitation and 450-nm emission wavelengths.

**Subcellular Fractionation of Proteasome Inhibitor- and Mock-treated Parathyroid Cells—**Fractionation of subcellular components was carried out by differential velocity centrifugation as described previously for parathyroid cells (39). Briefly, cells were metabolically labeled with [35S]methionine in the presence of 25 μM MG132, 10 μM brefeldin A, or 0.2% MeSO vehicle for 20 h. The cells were lysed in equal volumes of 25 mM Tris·HCl (pH 7.5), 250 mM sucrose, 5 mM MgCl_2, and 1 mM dithiothreitol by 50 strokes of a Dounce homogenizer. Nuclei and broken cells were harvested by centrifugation at 1000 × g for 10 min, and the post-nuclear fraction was sequentially centrifuged at 10,000 × g for 30 min and 105,000 × g for 60 min. The pellets obtained from these centrifugations were re-suspended in 400 volumes of the same buffer and fractionated by 20% sucrose. Cell lysates were extracted in equal volumes of the immunoprecipitation lysis buffer (incubation with end-over-end mixing for 60 min at 4 °C, followed by centrifugation at 10,000 × g for 10 min). The resulting supernatants and the cytosolic fractions (S100) were analyzed by immunoprecipitation with anti-PTH antiserum, followed by electrophoresis on 15% SDS-polyacrylamide gels, blotting onto Hybond C-Extra membranes (Amersham Biosciences), and autoradiography or Western blotting as described below.

**Protease Protection—**Cells were pretreated with MeSO vehicle or MG132 at 10, 25, or 50 μM for 60 min and then labeled as described under “Metabolic Labeling” for 30 min. Cells were washed twice and permeabilized with 10 μg/ml saponin in cytoskeletal buffer (300 mM sucrose, 100 mM KCl, 2.5 mM MgCl_2, 1 mM EDTA, and 10 mM PIPES (pH 6.8)) for 30 min at 4 °C as previously described (40). Saponin-permeabilized cells were then washed twice with cytoskeletal buffer and incubated on ice for 60 min with 150 μg/ml protease K in cytoskeletal buffer with or without 0.5% Triton X-100. Cell lysates were prepared and analyzed by immunoprecipitation with anti-PTH antiserum, followed by electrophoresis on 15% SDS-polyacrylamide gels, blotting onto Hybond C-Extra membranes (Amersham Biosciences), and autoradiography or Western blotting as described below.

**In Vitro Signal Peptidase Activity Assays—**Total RNA was isolated from dispersed parathyroid cells using the Promega SV total RNA isolation procedure, and mRNA was isolated from the total RNA using the Amersham Biosciences mRNA isolation procedure. In vitro translation reactions were programmed with either parathyroid cell mRNA or pre-β-lactamase RNA (Promega) using rabbit reticulocyte lysate in the presence of [35S]methionine or [35S]cysteine in the presence of MeSO or MG132 at concentrations up to 50 μM. The translated proteins were analyzed by immunoprecipitation, electrophoresis on 15% SDS-polyacrylamide gels, blotting onto Hybond C-Extra membranes, and autoradiography.

**Immunoprecipitation and Immunoblotting—**Frozen cell pellets were resuspended in ice-cold lysis buffer (50 mM Tris·HCl (pH 7.4), 5 mM
**Inhibition of Parathyroid Proteasomes Leads to Accumulation of Precursors of PTH in Acutely Dispersed Bovine Parathyroid Cells**—The biosynthesis of PTH from its primary precursor, prepro-PTH, comprises two successive proteolytic cleavages, which occur at distinct compartments of the secretory pathway. To examine the effects of proteasome inhibitors on its biosynthesis, acutely dispersed bovine parathyroid cells were treated with MG132, PSI, lactacystin, or Me2SO vehicle in complete MCF medium containing Ca2+ at a final concentration of 0.5, 1.25, or 3.0 mM. Analysis of cell lysates to optimize the incubation time and concentration of inhibitors was performed by immunoprecipitation with rabbit polyclonal antiserum to bovine PTH, followed by Western blotting with the same antiserum. Treatment of cells with these proteasome inhibitors provoked the accumulation of slowly migrating anti-PTH reactive molecules. These effects could be detected following 5–20 h treatment of cells, during which the viability of the cells remained high (>95%) when incubated with the peptide aldehyde inhibitors at concentrations up to 50 μM (data not shown). Typical results from a 5-h treatment are shown in Fig. 1A. Compared with mock-treated cells (Me2SO), the three proteasome inhibitors caused the accumulation of anti-PTH reactive molecules (arrows) to different extents. Thus, MG132 was more potent than PSI, which in turn was more potent than lactacystin; and the effects of these compounds were comparable when cells were treated in medium containing 0.5, 1.25, or 3.0 mM Ca2+.

Previous studies have shown that the bulk of secreted PTH is synthesized de novo. It was thus necessary to examine whether the accumulating anti-PTH reactive molecules were nascent precursors. Cell lysates from [35S]methionine-labeled bovine parathyroid cells were immunoprecipitated with anti-PTH antiserum and analyzed by SDS-PAGE and autoradiography. Fig. 1B confirms that prior treatment of parathyroid cells with MG132 or PSI (Fig. 1B, lanes 2 and 3, respectively) led to the accumulation of newly synthesized anti-PTH reactive molecules migrating with apparent molecular masses of 16 and 13 kDa. Compared with the mock-treated cells (Fig. 1B, lane 1), the accumulation of the 13-kDa molecule predominated in the proteasome inhibitor-treated cells. Similar results were obtained when cells were pretreated with these proteasome inhibitors for 60 min and pulse-labeled in the presence of the inhibitors for shorter periods (data not shown). As shown in Fig. 1 (A and B), molecules migrating with apparent molecular masses of 23 and 30 kDa (asterisks) were also enhanced in the lysates from proteasome inhibitor-treated cells. Likewise, in the metabolically labeled cells (Fig. 1B), a doublet corresponding to apparent masses of 70–80 kDa was consistently detected in the anti-PTH antiserum immune complexes, but these molecules also reacted with preimmune rabbit serum.

To identify the major anti-PTH reactive proteins, parathyroid cells were labeled with [35S]methionine and H[3]leucine/cysteine in the presence of MeSO (0.2%) or MG132 at concentrations ranging from 0.1 to 50 μM for 20 h at 37°C. Cell lysates were immunoprecipitated with anti-PTH antiserum (lanes 1–7) or with normal rabbit serum (lane 8) and analyzed as described for B. The control immunoprecipitation with normal rabbit serum was performed with cell lysate from cells treated with 50 μM MG132.

**RESULTS**

**FIG. 1.** Peptide aldehyde proteasome inhibitors and lactacystin cause the accumulation of PTH precursors in dispersed parathyroid cells. A, acutely dispersed bovine parathyroid cells were treated for 5 h at 37°C with 0.2% MeSO vehicle (DMSO), 25 μM MG132 (MG), 20 μM lactacystin (LAC), or 50 μM PSI. Incubations were done in complete MCF medium containing Ca2+ at a final concentration of 0.5 mM (lanes 1), 1.25 mM (lanes 2), or 3.0 mM (lanes 3). Cell lysates were analyzed by immunoprecipitation with anti-PTH antiserum, electrophoresis on 15% SDS-polyacrylamide gels, and Western blotting using the same antiserum. Asterisks in A and B indicate other molecules (23 and 30 kDa) accumulating in proteasome inhibitor-treated cells. Arrows indicate (from the top) the positions of prepro-PTH, pro-PTH, and PTH. Molecular masses (in kilodaltons) are indicated to the left. B, dispersed parathyroid cells were labeled with [35S]methionine/cysteine in complete MCF medium containing Ca2+ at a final concentration of 1.25 mM in the presence of 0.2% MeSO vehicle (lane 1), 25 μM MG132 (lane 2), or 25 μM PSI (lane 3) for 5 h at 37°C. Cell lysates were subjected to immunoprecipitation with anti-PTH antibodies, and the immune complexes separated on 15% SDS-polyacrylamide gel. Separated proteins were blotted onto Hybond C-Extra membranes, and radioactive proteins were visualized by autoradiography. The positions of prepro-PTH, pro-PTH, and PTH are indicated. C, parathyroid cells were labeled with [35S]methionine/cysteine in the presence of MeSO (0.2%) or MG132 at concentrations ranging from 0.1 to 50 μM for 20 h at 37°C. Cell lysates were immunoprecipitated with anti-PTH antiserum (lanes 1–7) or with normal rabbit serum (lane 8) and analyzed as described for B. The control immunoprecipitation with normal rabbit serum was performed with cell lysate from cells treated with 50 μM MG132.

EDTA, 150 mM NaCl, 50 mM β-glycerophosphate, 1 mM sodium orthovandate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each aprotinin and leupeptin, 1% Nonidet P-40, and 0.26% sodium deoxycholate) and incubated by end-over-end mixing overnight at 4°C, followed by centrifugation at 10,000 × g for 10 min at 4°C. The supernatants were transferred to new tubes and precleared with protein G-agarose for 1 h at 4°C. For immunoprecipitation, equal volumes of the cleared lysates were mixed with 2.5–5.0 μl of rabbit polyclonal antiserum to bovine PTH or other primary antibodies where necessary and incubated by end-over-end mixing overnight at 4°C. Protein G-agarose was then added, and incubation was continued under the same conditions for 2 h. The immune complexes were harvested, washed four times with the same buffer, and dissociated by heating for 5 min in 2 × SDS-PAGE loading buffer (125 mM Tris-HCl (pH 6.8), 40% glycerol, 4% SDS, 5% 2-mercaptoethanol, and 0.001% bromphenol blue). Cell lysates or immune complexes prepared as described above were separated on denaturing gels and either processed for fluorography and autoradiography or blotted onto Hybond C-Extra membranes. For Western blotting, the membranes were probed with primary antibodies as indicated, followed by the corresponding peroxidase-conjugated secondary antibodies according to standard procedures. The blots were developed using the ECL system (Amersham Biosciences). Where necessary, densitometric quantitation was carried out using a Fuji BAS-2500 phosphorimager to compare the intensities of the protein bands.
cultured at 37 °C described in the legend to Fig. 1. The presence of either Me2SO vehicle or the inhibitors for 5 h. (Fig. 1, lanes 6, 7), but also pro-PTH compared with mock-treated cells. This suggests that based on its mechanism of action, chloroquine might affect more than one cellular compartment. On the other hand, brefeldin A blocks the ER-to-Golgi transport and therefore interferes with the processing of proforms of exported proteins. To compare this effect with those of proteasome inhibitors, parathyroid cells were labeled with [35S]methionine in the presence of 10 μg/ml brefeldin A alone (lane 1) or in combination with either 10 μg MG132 (lane 2) or 50 μg chloroquine (lane 3). The cells were lysed, immunoprecipitated with anti-PTH antiserum, and analyzed as described in the legend to Fig. 1B. The positions of pro-pre-PTH, PTH, and PTH are indicated. Molecular masses (in kilodaltons) are indicated to the left.

Because of its potency as well as the reversibility of its effects on the proteasome, MG132 was used in most of the following experiments. To examine whether the accumulation of pro-PTH and pro-PTH was concentration-dependent with regard to MG132, parathyroid cells were labeled with [35S]methionine in the presence of either Me2SO vehicle or MG132 at concentrations ranging from 0.1 to 50 μM for 20 h. Cell lysates were analyzed by immunoprecipitation with anti-PTH antibodies, followed by electrophoresis and autoradiography. As depicted in Fig. 1C, the accumulation of pro-PTH could be detected at concentrations of MG132 as low as 1 μM (lane 3). Higher concentrations led to a dose-dependent accumulation of both pro-PTH and pro-pre-PTH. Thus, treatment of these cells with MG132 at concentrations ≥25 μM caused a >2-fold accumulation of pro-PTH and a ≥5-fold accumulation of pro-pre-PTH (Fig. 1C, lanes 6 and 7). Interestingly, newly synthesized PTH was present at all inhibitor concentrations, but the relative amounts decreased with increasing concentrations of MG132. Under similar conditions, treatment of parathyroid cells with up to 50 μM MG132 had little or no effect on the processing and/or post-translational modification (glycosylation) of chromogranin A as far as can be judged from the size and intensity of the anti-chromogranin A reactive molecule (data not shown). This suggests that treatment of parathyroid cells with MG132 or related compounds led to a dose-dependent accumulation of PTH precursors with a corresponding decrease in de novo synthesized mature PTH.

The related cysteine proteinase inhibitors ALLN and ALLM efficiently inhibit calpains, which are partly responsible for limited proteolysis of mature PTH. ALLN has been shown to also inhibit proteasome activity, whereas ALLM has a relatively weak effect on proteasomes (41). These inhibitors were therefore used to further confirm that the accumulation of PTH precursors following treatment of cells with PSI, MG132, or lactacystin was due to inhibition of proteasomes. Thus, dispersed parathyroid cells were labeled with [35S]methionine in the presence of either Me2SO vehicle or the inhibitors for 5 h. Fig. 2A demonstrates that treatment of cells with the peptide aldehyde protease inhibitors ALLN, MG132, and PSI (25 μM each) as well as lactacystin (20 μM) caused the accumulation of predominantly pro-PTH. MG132 at this concentration also provoked the accumulation of pre-pro-PTH, whereas the effects of ALLM were the weakest, in agreement with its mild effects on proteasome activity. Furthermore, assay of the ATP-dependent chymotrypsin-like and peptidylglutamyl-peptide hydrolase proteasome activities in crude lysates from cells treated with the inhibitors used in this study confirmed the potency of MG132 (data not shown) and previous reports describing the differential potency of the cysteine proteinase inhibitors on the proteasome in other cells (42, 43).

Meanwhile, treatment of cells with chloroquine (50 μM) caused the accumulation of predominantly PTH (Fig. 2A, lane 7), but also pro-PTH compared with mock-treated cells. This suggests that based on its mechanism of action, chloroquine might affect more than one cellular compartment. On the other hand, brefeldin A blocks the ER-to-Golgi transport and therefore interferes with the processing of proforms of exported proteins. To compare this effect with those of proteasome inhibitors, parathyroid cells were labeled with [35S]methionine in the presence of 10 μg/ml brefeldin A alone or in combination with 10 μg MG132 or the indirect inhibitor of lysosomal proteolysis, chloroquine (50 μM). As expected, treatment of cells with brefeldin A led to the accumulation of pro-PTH with little or no de novo synthesized PTH. Of particular interest is the fact that the effects of MG132 or chloroquine on pro-PTH accumulation at the concentration used were additive to that of brefeldin A (Fig. 2B). This suggests that the accumulating pro-PTH in proteasome inhibitor-treated cells could be predominantly in the pre-Golgi compartment.

**PTH Secretion Is Inhibited in Proteasome Inhibitor-treated Parathyroid Cells**—To examine the effects of accumulating PTH precursors in proteasome inhibitor-treated cells on the secretion of intact PTH, parathyroid cells were treated with Me2SO or 10 μM proteasome inhibitors in medium containing 0.5, 1.25, or 3.0 mM Ca2+. Bovine secreted intact PTH in the culture supernatants was assayed using an immunoradiometric assay developed to measure rat intact PTH. In these experiments, the stimulation of PTH secretion by phorbol 12-myristate 13-acetate at high [Ca2+]o was used as an internal control in addition to rat PTH standards (data not shown). Fig. 3A demonstrates that MG132, PSI, and lactacystin inhibited PTH secretion at low [Ca2+]o, whereas the cysteine proteinase inhibitors ALLN and ALLM had little or no effect. MG132 and PSI also inhibited the non-regulated PTH secretion at physiological [Ca2+]o (p < 0.05). Moreover, treatment of parathyroid cells with MG132 for 90 min (data not shown) or for relatively long periods (18–20 h) led to a dose-dependent inhibition of PTH secretion (Fig. 3B). These data indicate that treatment of parathyroid cells with potent peptide aldehyde protease inhibitors or lactacystin affected the secretion of PTH, but the regulation of PTH secretion by [Ca2+]o per se was not perturbed, thus suggesting that changes in proteasome activities may lead to unprecedented consequences on the biosynthesis and hence the stimulated secretion of the mature hormone.

Concentrations of MG132 ≥25 μM were used to examine the fate of the accumulated PTH precursors following relief of inhibition of the proteasome. Parathyroid cells were pretreated with MG132 or Me2SO for at least 2 h, pulse-labeled with [35S]methionine for 30 min, and then chased for up to 4 h in the presence or absence (Me2SO) of MG132. Analysis of cell lysates by immunoprecipitation with anti-PTH antiserum, electrophoresis on SDS-polyacrylamide gels, and autoradiography confirmed that compared with the mock-treated cells, treatment of parathyroid cells with 25 μM MG132 led to the accumulation of PTH precursors (Fig. 4, A and B, lanes 1). When chased in the presence of this inhibitor (Fig. 4B), the levels of pro-pre-PTH gradually decreased, whereas the processing of
two independent measurements. Supernatants as described for the presence of Me₂SO vehicle or MG132 at concentrations ranging from 0.1 to 50 μM (black bars), 1.25 mM (white bars), or 3.0 mM (hatched bars) for 90 min. Secreted intact PTH in the culture supernatants was measured in duplicate by a two-site immunoradiometric assay. Results are expressed as picograms/ml secreted PTH. Each bar represents the mean ± S.D. of a total of four independent determinations from two separate experiments. Statistical analysis was determined by unpaired, two-tailed Student’s t test; p < 0.05 was considered statistically significant. *, p < 0.05 compared with cells in Me₂SO vehicle. MG, MG132; Lac, lactacystin. ALLN and ALLM are cysteine proteinase inhibitors. B, parathyroid cells were cultivated as described in A, for 20 h at 37 °C in medium supplemented with Ca²⁺ at a final concentration of 1.25 mM in the presence of Me₂SO vehicle or MG132 at concentrations ranging from 0.1 to 50 μM. Secreted intact PTH was measured in the culture supernatants as described for A. Each bar represents the mean from two independent measurements.

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Pro-PTH in proteasome inhibitor-treated cells occurred over a longer time (≥60 min) relative to mock-treated cells (≥30 min).

Relieving the inhibition of the proteasome by chasing in the absence of MG132 (Fig. 4C) led to the instantaneous disappearance of the accumulated prepro-PTH as well as decreased levels of pro-PTH and de novo synthesized PTH, but the delay in the processing of pro-PTH remained apparent. When cells were treated and chased in the presence of 50 μM MG132, ~50% of the newly synthesized prepro-PTH persisted, but decreased to undetectable levels in cells chased in the absence of the inhibitor. Moreover, the amount of de novo synthesized mature PTH was severely decreased in cells treated with 50 μM MG132 (data not shown). This suggests that the disappearance of prepro-PTH and the decreased levels of pro-PTH in cells chased in the absence of MG132 were due to proteasome-mediated degradation.

Because the bulk of secreted PTH originates from the newly synthesized pool (7–9), an easily measurable consequence of the accumulation of PTH precursors is the secretion of intact PTH (Fig. 3). To verify that the delay in pro-PTH processing in proteasome inhibitor-treated cells influenced the secretion of PTH, secreted intact PTH was assayed in the culture supernatants collected during the pulse-chase experiments. Fig. 4D reveals that relieving the inhibition of the proteasome increased the rate of intact PTH secretion to levels almost comparable to those in control cells. Thus, in cells treated with 25 μM MG132 and chased in the presence or absence of the same concentration of the inhibitor, secretion of intact PTH decreased to ~60 and 70% of that in control cells, respectively, during the 4-h chase period. Similarly, secreted intact PTH in
cells treated with 50 μM MG132 and chased in the presence or absence of the same concentration of the inhibitor decreased to ~30% and 70% of that in control cells, respectively, during the 4-h chase period. This indicates that decreased transit of proPTH through the secretory pathway compromised the rate of intact PTH secretion. It should be noted that during the chase period, protein synthesis was inhibited with 0.1 mM cycloheximide to limit the observations to the newly synthesized pool of PTH/PTH precursors in the secretory pathway. Despite the inhibition of protein synthesis, a linear trend in PTH secretion was observed even in the later periods of the chase (~4 h), during which newly synthesized PTH was almost undetectable in the autoradiographs (Fig. 4, A–C). This suggests that the deficiency in nascent PTH was compensated with PTH in storage granules.

Mechanism of Proteasome Inhibitor Effects on Accumulation of PTH Precursors and Secretion of PTH—The results so far indicate that treatment of the cells with proteasome inhibitors did not affect their responsiveness to [Ca2+]i, with regard to PTH secretion (Fig. 3A). Moreover, no direct relationship appeared to exist between [Ca2+]i and the accumulation of PTH precursors in proteasome inhibitor-treated parathyroid cells. Assay of the ATP-dependent chymotrypsin-like activity in crude lysates from parathyroid cells cultivated in medium containing various [Ca2+]i for 18 h or for short time intervals of 10–90 min revealed that proteasome activity tended to decrease with increasing Ca2+ concentrations following prolonged exposure of cells to Ca2+ (data not shown). However, the accumulation of prepro-PTH and its stability in pulse-chase experiments in proteasome inhibitor-treated parathyroid cells (Fig. 4) might indicate that a pool of abnormal precursor is formed under such conditions. To exclude the possibility that this was due to unspecified inhibition of signal peptidases, we investigated the translocation and processing of [35S]methionine-labeled in vitro translated prepro-PTH and pro-β-lactamase into canine microsomal membranes in the absence and presence of MG132 at concentrations up to 50 μM. The translocation and signal peptide cleavage of either prepro-PTH or pro-β-lactamase were unaffected by this proteasome inhibitor (data not shown), making it unlikely that the accumulation of prepro-PTH in the inhibitor-treated parathyroid cells was the result of decreased signal peptidase activity.

Following translocation and signal peptide cleavage of prepro-PTH, the nascent pro-PTH transits to the distal portion of the secretory pathway, where it is cleaved to mature PTH. To examine the intracellular localization of the accumulating PTH precursors, cells were labeled with [35S]methionine in the presence of MeSO vehicle, MG132, or brefeldin A; homogenized; and fractionated by differential velocity centrifugation. Analysis of the detergent-solubilized particulate fractions and the final supernatant by immunoprecipitation with anti-PTH antiseraum, SDS-polyacrylamide gel electrophoresis, and autoradiography revealed that brefeldin A treatment provoked the accumulation of pro-PTH with little or no processing to PTH (Fig. 5A). The bulk of pro-PTH was associated with membrane/particulate fractions (P10 and P100), with only a relatively low amount in the cytosolic fraction (S100). Treatment of cells with MG132 caused the accumulation of both prepro-PTH and pro-PTH. Prepro-PTH almost exclusively resided in the membrane fractions (P10), whereas most of the pro-PTH was associated with both the membranes and microsomes (P10 and P100). Moreover, a portion of the pro-PTH was found in the cytosolic fraction in these cells (cf., relative distribution of pro-PTH in the P100 and S100 fractions in the MG132- and brefeldin A-treated cells). In the control cells, little or no pro-PTH was found in these fractions (Fig. 5A).

The subcellular localization of prepro-PTH and pro-PTH was further studied by protease sensitivity assay in saponin-permeabilized MG132-treated parathyroid cells. Fig. 5B depicts a typical experiment and demonstrates that in the mock-treated cells (MeSO), >90% of pro-PTH was protected against proteolytic degradation in the permeabilized cells. In contrast, in the MG132-treated cells, approximately one-third of the pro-PTH disappeared after the protease K treatment (cf. Fig. 5B, lanes 2 and 3), whereas prepro-PTH was completely accessible to the protease. Together, these data suggest that most of the accumulating prepro-PTH was accessible from the cytosol, whereas only a portion of the pro-PTH was cytosolic and hence must have attained this cellular compartment by retrotranslocation. These results are consistent with the rapid disappearance of prepro-PTH in MG132-treated cells upon removal of the inhibitor (Fig. 4, B and C).

Molecular chaperones of the Hsp70 family are known to be involved in the translocation and folding of secreted and other proteins in the secretory pathway (45–47). They also promote the degradation of some proteins by proteasomes (48–51). To assess whether treatment of parathyroid cells with proteasome
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Fig. 6. Prolonged MG132 treatment of parathyroid cells leads to overexpression of the Hsp70 family of chaperones. A, parathyroid cells were treated with 0.2% DMSO vehicle (DMSO) or 25 µM MG132 for 2 or 20 h in MCF medium containing either 0.5 or 2.0 mM CaCl₂ at 37 °C in 5% CO₂. Cells were disrupted by Dounce homogenization, and the post-nuclear supernatants were separated on a 10% SDS-polyacrylamide gel. The separated proteins were transferred to Hybond C-Extra membranes and probed with goat polyclonal antibodies to human BiP. Blots were developed by ECL. Arrows indicate the positions of the anti-BiP reactive proteins. Molecular masses (in kilodaltons) are indicated to the left. B, parathyroid cells were treated with DMSO vehicle or 25 µM MG132 for 20 h. The cells were lysed by Dounce homogenization, and the post-nuclear cell lysates were centrifuged at 100,000 × g for 1 h. The lysate (lane 1) and the resulting pellet (lane 2) and supernatant (lane 3) were analyzed by Western blotting with goat polyclonal antibodies to human BiP after separation on a 10% SDS-polyacrylamide gel as described for A.

Inhibitors influenced the levels of the ER resident molecular chaperone BiP, cells treated with or without 25 µM MG132 for 2 or 20 h in complete MCF medium containing 0.5 or 2.0 mM CaCl₂ were lysed by Dounce homogenization. The post-nuclear supernatants were separated on a 10% SDS-polyacrylamide gel and analyzed by Western blotting with anti-BiP serum against human BiP. Fig. 6A demonstrates that two anti-BiP reactive molecules with apparent molecular masses of 70 and 78 kDa were detected. In cells treated with MG132 for 20 h, the amounts of both molecules increased at least 4-fold at both low and high [Ca²⁺]ᵣ, relative to the mock-treated cells (Me₂SO). In a similar experiment, the post-nuclear cell lysates were fractionated by differential velocity centrifugation. Western blotting with polyclonal antiserum to BiP revealed that the 78-kDa protein was mainly membrane-bound, whereas the 70-kDa protein was predominantly cytosolic, consistent with the former being BiP and the latter presumably its cytosolic homolog Hsp70 (Fig. 6B). The intracellular levels of BiP as well as Hsp70 as assessed by Western blotting of cell lysates from mock- and MG132-treated cells with goat polyclonal antibodies to BiP or Hsp70 confirmed that the relative amounts of these molecular chaperones also increased with increasing concentrations of the drug (data not shown).

To examine whether PTH/PTH precursors interact with BiP or its cytosolic homolog Hsp70, cell lysates from parathyroid cells treated with 25 µM MG132 were immunoprecipitated with anti-PTH antiserum, anti-BiP antibodies, or preimmune rabbit immunoglobulin G heavy chain. The immune complexes were separated on 10% SDS-polyacrylamide gels and analyzed by Western blotting with goat polyclonal antibody against BiP. Fig. 7 (representing a typical blot) reveals that anti-BiP antiserum (lane 3) as well as anti-PTH antiserum (lane 2) immunoprecipitated BiP, in contrast to preimmune rabbit serum (lane 1). Similar results were observed with recombinant pro-PTH and prepro-PTH expressed in Sf9 insect cells (data not shown). This demonstrates that BiP and PTH precursors effectively interact in bovine parathyroid cells.

**DISCUSSION**

Parathyroid hormone is one of the dominant proteins synthesized by parathyroid chief cells, yet little or no prepro-PTH is normally detectable in these cells. This may be attributed to efficient cotranslational translocation and/or processing to pro-PTH. So far, most studies on the biosynthesis of PTH and mechanisms of PTH secretion using primary parathyroid cells or parathyroid gland slices have focused on the events at the distal portion of the secretory pathway, where mature PTH predominates. However, events early in the secretory pathway may be significant in the maintenance of intracellular levels of the newly synthesized mature hormone and hence the secretion of the hormone. In this study, we have demonstrated that nascent PTH precursors specifically accumulated in proteasome inhibitor-treated cells, representing untranslocated prepro-PTH and retrotranslocated pro-PTH in the cytosol as well as pro-PTH in the secretory pathway, available for processing to mature PTH. The processing of pro-PTH to PTH was delayed, and the secretion of intact PTH decreased in proteasome inhibitor-treated cells relative to mock-treated cells. This suggests the involvement of parathyroid cell proteasomes in the quality control of PTH biosynthesis.

Primary parathyroid cells cannot be easily transformed in vitro, making the use of drugs such as proteasome inhibitors the only possibility for studying specific intracellular processes. Several studies have demonstrated that most of the proteasome inhibitors currently used also inhibit other intracellular proteases (22, 52, 53). Consequently, three peptide aldehyde proteasome inhibitors (MG132, PSI, and ALLN) and lactacystin were used in this study. In addition to this, in vitro assays of the peptidylglutamyl-peptide hydrolase and chymotrypsin-like proteasome activities in proteasome inhibitor-treated cells confirmed the inhibition of these proteasome activities, as in other cells (42, 43). Furthermore, the cysteine proteinase in-
proteasome inhibitors (Fig. 2A). We were unable, however, to demonstrate consistently the ubiquitylation of the PTH precursors destined for degradation. However, ubiquitin-independent proteasome-mediated degradation is not uncommon (54), and Meerovitch et al. (27) were also unable to demonstrate the ubiquitylation of human prepro-PTH in vitro.

The accumulation of PTH precursors in proteasome inhibitor-treated parathyroid cells might theoretically be interpreted as due to inhibition of the proteases (signal peptidases and prohormone convertases) that process these precursors to mature PTH. However, with the exception of certain β-lactam compounds (56), most signal peptidases are insensitive to the commonly used protease inhibitors (57). Translocation into canine microsomes and processing of in vitro translated bovine prepro-PTH to pro-PTH were not inhibited by MG132 (data not shown). In dispersed parathyroid cells, brefeldin A caused the accumulation of predominantly pre-pro-PTH due to inhibition of its transfer from the ER to the Golgi, whereas chloroquine caused a remarkable increase in intracellular mature PTH, consistent with lyosomal degradation of mature PTH (18, 20, 21, 55). Chloroquine treatment most likely also abolished the pH gradient between the cis-Golgi (high) and the trans-Golgi (low), making it possible that this gradient is necessary for proper transfer, processing, and post-translational modification of proteins in the Golgi, hence the accumulation of pre-PTH relative to mock-treated cells (Fig. 2A). The effects of either brefeldin A or chloroquine were observed to be distinct from those in proteasome inhibitor-treated cells and suggest that proteasome inhibitors did not directly affect the ER-to-Golgi transport or the processing of pre-PTH within the Golgi most likely by the prohormone convertase furin (58, 59). This was demonstrated by pulse-chase metabolic labeling (Fig. 4) in that the processing of pro-PTH as well as PTH secretion in proteasome inhibitor-treated parathyroid cells occurred normally, although at reduced rates.

The data presented in this study is consistent with a quality control function of proteasomes in the biosynthesis of PTH (24–26). In addition to the accumulation of PTH precursors, treatment of parathyroid cells with proteasome inhibitors led to a delay in the processing of pro-PTH and decreased amounts of both de novo synthesized PTH and secreted intact PTH. The degradation of prepro-PTH and a portion of pro-PTH by the proteasome appears to be important in the overall secretory response. This is justified by the observation that relieving the inhibition of the proteasome led to the rapid disappearance of the accumulated prepro-PTH, decreased levels of pro-PTH, and restoration of the rate of PTH secretion to levels almost comparable to those in control cells. Despite the instantaneous disappearance of prepro-PTH and a portion of pro-PTH upon release of proteasome inhibition, the pool of accumulated PTH precursors could include normal and translation-competent prepro-PTH rendered translocation-incompetent by interaction with cytosolic molecular chaperones. However, given the small difference in intact PTH secretion between proteasome inhibitor-treated cells upon release of proteasome inhibition and mock-treated cells, it is unlikely that a significant amount of the secreted PTH resulted from the processing of the accumulated prepro-PTH.

The observed differences in PTH biosynthesis and secretion between proteasome inhibitor- and mock-treated cells may be attributed to the extent of retention of pro-PTH by molecular chaperones and/or its rate of transit through the secretory pathway. The accumulating PTH precursors and other proteasome substrates in proteasome inhibitor-treated parathyroid cells would provoke cellular stress, with a major consequence being the overexpression of molecular chaperones (60–64). In agreement with this hypothesis, we observed a concentration- and time-dependent overexpression of the ER resident chaperone BiP in response to treatment of cells with MG132 and also the interaction of BiP with PTH/PTH precursors in parathyroid cells (Fig. 7). Thus, overexpression of BiP or its cytosolic homolog Hsp70 would lead to enhanced interaction with PTH precursors and may in part account for the delay in pro-PTH processing as well as the decrease in the rate of both de novo PTH synthesis and PTH secretion in proteasome inhibitor-treated cells. Thus, the persistence of the delay in the processing of pro-PTH in proteasome inhibitor-treated cells upon relief of inhibition of the proteasome reflects the slow release of pro-PTH from ER resident molecular chaperones. This is consistent with previous reports that overexpression and increased binding of BiP or GRP94 delay the transport of thyroglobulin in the secretory pathway of Chinese hamster ovary cells (30). This also explains why treatment of cells with proteasome inhibitors repressed PTH secretion, as opposed to inhibitors of proteases that degrade mature PTH such as 3-methyladenine, chloroquine, and 1-deoxynojirimycin (21).

REFERENCES
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