The Release of Parathyroid Hormone and the Exocytosis of a Proteoglycan Are Modulated by Extracellular Ca^{2+} in a Similar Manner

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Secretion of parathyroid hormone (PTH) is regulated in part by a classical “stimulus-secretion” pathway responsive to catecholamines. The primary physiological modulator of PTH exocytosis in parathyroid cells, however, is extracellular free Ca^{2+} . Ca^{2+} -modulated PTH release exhibits several characteristics suggestive of constitutive secretion. The aim of this work was to obtain further information about the possible intracellular origins of Ca^{2+} -modulated exocytosis in parathyroid cells. Freshly dissociated bovine parathyroid cells labeled with [^{35}S]sulfate synthesized a soluble chondroitin/dermatan sulfate proteoglycan ($M_r \sim 90\text{--}150\text{ K}$) that was secreted into the medium. The export of [^{35}S]sulfated proteoglycan satisfied several criteria that generally define constitutive release: 1) export is detected in the medium shortly (7–15 min) after a 5-min pulse, 2) there is minimal intracellular storage after equilibrium labeling (because of combined processes of rapid release and intracellular degradation), and 3) there is insensitivity to stimulation with isoproterenol, a known secretagogue in parathyroid cells. Nevertheless, the increase in extracellular Ca^{2+} from 0.5 to 2.0 mM reduced the export of the [^{35}S]sulfated proteoglycan from 60% of total labeled to 30%. In addition, a secreted pool of immunoreactive PTH and [^{35}S]sulfated proteoglycan was modulated by external Ca^{2+} to the same degree and sensitivity, although isoproterenol was more effective in stimulating the release of PTH than that of proteoglycan. Together, our experimental results show that in the parathyroid cell extracellular Ca^{2+} modulates negatively the export of both PTH and proteoglycan, a putative marker for constitutive secretion. We further suggest that a portion of newly synthesized PTH also enters this pathway, whereas another portion proceeds to an isoproterenol-releasable compartment from which the proteoglycan is largely excluded.

INTRODUCTION

The secretory products in exocrine and neuroendocrine cells travel from endoplasmic reticulum to the *trans*-Golgi network, where they are packed in constitutive secretory vesicles or immature secretory granules, to be transported outside the cell or to the storage compartment (Palade, 1975; Burgess and Kelly, 1987; Pfeffer and Rothman, 1987). The export of constitutive secretory proteins is continuous, occurs without intracellular storage, and is believed to be

insensitive to treatments with secretagogues. Regulated secretion, on the other hand, involves the concentration and storage of secretory products in storage granules, whose exocytosis is elicited by the action of specific extracellular stimuli (Kelly, 1985). It has been shown recently that the sorting process is not an exclusive function of *trans*-Golgi network and that sorting of stored proteins and other products may continue in the immature granules (von Zastrow and Castle, 1987; Grimes and Kelly, 1992a,b; Kuliawat and Arvan, 1992). Indeed, some proteins may be removed from immature granules and exocytosed in a process referred to as “constitutive-like” secretion (see Arvan and Castle, 1992 for review).

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In parathyroid cells, regulated secretion of parathyroid hormone (PTH)¹ is triggered by catecholamines that increase the level of intracellular cyclic AMP (Brown *et al.*, 1977; Chu *et al.*, 1983). Catecholamine-induced PTH release is transitory, being conditional to the presence of extracellular stimulus, and it emerges from the storage compartment, thus having the properties of regulated exocytosis (Brown *et al.*, 1985). The main physiological regulatory factor for PTH release from parathyroid cells, however, is the extracellular free Ca^{2+} . Although in long term adaptation lasting for hours to weeks Ca^{2+} regulates the rate of synthesis of PTH as well as the rate of cell growth (Brown, 1991), the immediate effect of changes in the level of external Ca^{2+} , i.e., seconds to minutes, consists in the inhibition of PTH release by 50–80%. The intracellular messenger responsible for modulation of PTH release by extracellular Ca^{2+} is thought to be a protein kinase C isoform (Brown *et al.*, 1984) activated via stimulation of the extracellular Ca^{2+} -sensing receptor (Brown *et al.*, 1993) and appears unrelated to the production of intracellular cAMP (Brown, 1991). These findings suggest that the pathways by which catecholamines and external Ca^{2+} regulate PTH release are independent. Moreover, in porcine parathyroid cells the exocytosis of labeled PTH becomes sensitive to stimulation with isoproterenol 60 min from pulse, after labeled PTH has already passed the Ca^{2+} -exportable compartment (Morrissey and Cohn, 1979b). These results indicate that parathyroid cells have two intracellular hormone pools. One, containing newly made PTH, is exported soon after synthesis in response to hypocalcemia, and another one, comprising the hormone in the storage compartment, is released in response to isoproterenol or other agents that increase intracellular cAMP (Morrissey and Cohn, 1979b; Cohn and MacGregor, 1981).

Ca^{2+} -modulated PTH export possesses a number of properties reminiscent of those described for the constitutive secretory pathway. First, PTH released under the control of external Ca^{2+} contains newly synthesized PTH, which reaches the external space 20–30 min after synthesis (Morrissey and Cohn, 1979a) and little, if any, "old" hormone from storage granules (MacGregor *et al.*, 1975; Morrissey and Cohn, 1979a). The short time elapsed between synthesis and release of secretory proteins, paralleled by the bypass of the storage compartment, are constitutive characteristics. In addition, whereas very high Ca^{2+} concentrations decrease PTH secretion rate to a minimum value, the inhibition is never complete (Mayer *et al.*, 1976). Indeed, at physiological concentration of extracellular Ca^{2+} , parathyroid cells secrete PTH at about one-fourth the maximum rate (Brent *et al.*, 1988). The continuous exocytotic activity is another characteristic suggestive of constitutive release. Other short term effects of external Ca^{2+} on the

parathyroid cell suggest a role of external Ca^{2+} in the modulation of the rate of transport along the constitutive secretory pathway. For example in a rat parathyroid cell line, transport to the cell surface of a plasma membrane-associated proteoglycan is negatively modulated by external Ca^{2+} (Takeuchi *et al.*, 1990). In addition, hypercalcemia, associated with reduced secretory activity, leads to a rapid regression of the Golgi complex (>15 min) and of the number of Golgi-associated small secretory vesicles (Emura *et al.*, 1992). These morphological changes together with modulated traffic of membrane components suggest the possibility that extracellular Ca^{2+} alters functions performed by the Golgi complex in the traffic of secretory products.

The aim of this work was to examine a possible relationship of Ca^{2+} -modulated export of secretory products in parathyroid cells with respect to a constitutive type of secretory route. Constitutive secretion from these cells has not been studied previously. We have characterized a sulfated proteoglycan from bovine parathyroid cells and found that its release satisfies several criteria of constitutive secretion. This form of secretion has potentially important implications for the Ca^{2+} modulation of PTH discharge.

MATERIALS AND METHODS

Chemicals

Collagenase from *Clostridium histolyticum* (type III) (Worthington Biochemical, Freehold, NJ) was purified by gel filtration on Sephadex G-100 (Schultz *et al.*, 1980). Collagenase CLSPA (Worthington Biochemical) was used as purchased. [³⁵S]Sulfuric acid (carrier free) was from New England Nuclear/Du Pont (Wilmington, DE). Tran³⁵S-label (1120 Ci/mmol), [³⁵S]methionine (514 Ci/mmol), and RPMI-1640, methionine-, cysteine-, cystine-, and glutamine-free were purchased from ICN Biomedicals (Irvine, CA). Isoproterenol was from Research Biochemicals (Natick, MA). Fast flow Q Sepharose anion exchanger and prepacked Sephadex G-50 columns were from Pharmacia Chemicals (Piscataway, NJ). Acrylamide, prestained molecular weight markers and all other chemicals used for polyacrylamide gel electrophoresis were from BioRad (Richmond, CA). Urea was from Fisher Scientific (Fairlawn, NJ); before use urea solution was filtered on a 4.2- μm Rainin filter. Pyridine was from Aldrich Chemical (Milwaukee, WI). 4-Methylumbelliferyl β -D-xyloside, chondroitin ABC lyase from *Proteus vulgaris* (chondroitinase ABC), sodium acetate, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 3-(N-morpholino)propanesulfonic acid, Triton X-100, Triton X-114, protease inhibitors (leupeptin, antipain, benzamidine, and phenylmethylsulfonyl fluoride [PMSF]), and all the other chemicals used in this study were purchased from Sigma Chemical (St. Louis, MO).

Preparation of Parathyroid Cells

Fresh bovine parathyroid glands have been used for obtaining dispersed cells by dissociation with collagenase (Brown *et al.*, 1976; MacGregor *et al.*, 1983) and elastase. Dispersed cells retained the biochemical and physiological features of parathyroid cells *in situ*, i.e., they synthesized PTH and secreted it with a rate sensitive to the concentration of extracellular Ca^{2+} .

Metabolic Labeling with [³⁵S]Methionine

Fresh parathyroid cells were depleted of methionine by 30-min preincubation in methionine-, cysteine-, and cystine-free RPMI-1640 me-

¹Abbreviation used: PTH, parathyroid hormone.

dium at 0.4 mM CaCl₂. To exclude any effect of Ca²⁺ on PTH synthesis, cells were treated in a single batch until the start of the chase incubation. Cells were labeled for 5 min in one batch with Tran³⁵S-label (2 mCi/ml) or [³⁵S]methionine (0.5 mCi/ml); labeling was terminated by 10- to 20-fold dilution of radioactivity with ice-cold medium containing methionine. Cells were separated into two equal batches that were pelleted by spinning 3 min at 250 × g. Each batch was resuspended and chase-incubated at 37°C in culture medium at the desired Ca²⁺ concentration. For changing media during the experiment, cells were pelleted by spinning at 250 × g, for 3 min, and supernatants were replaced with the desired medium. At the end of the chase incubations, the cells were pelleted by spinning at 10 000 × g for 1 min in a Beckman microfuge (Fullerton, CA). For gel electrophoretic analysis of released PTH, portions of supernatants were lyophilized and resuspended in one-fifth the original volume in sodium dodecyl sulfate (SDS) sample buffer (1×) (3% [wt/vol] SDS, 10% [wt/vol] glycerol, 3.3% [vol/vol] 2-mercaptoethanol, a trace of bromophenol blue, and 62.5 mM tris(hydroxymethyl)aminomethane [Tris]-HCl pH 6.8) (Huttner, 1984), or lyophilization was omitted and the required amount of 5× sample buffer was added.

Metabolic Labeling with [³⁵S]Sulfate

Before biosynthetic labeling with [³⁵S]sulfate, freshly dispersed parathyroid cells were depleted of sulfate by incubation for 30 min at 37°C in Waymouth MB752/1 medium containing 1.3 mM CaCl₂ and no antibiotics, in which 0.7 mM MgSO₄ was replaced with 0.63 mM MgCl₂ and 0.07 mM MgSO₄. This medium was referred to as "sulfate-depleted medium". For biosynthetic labeling, [³⁵S]sulfuric acid was added to sulfate-depleted medium at 200 µCi/ml or 2 mCi/ml for >1 h labeling or for a 5-min pulse, respectively. The labeling conditions described above were well tolerated by cells because the incorporation of [³⁵S]sulfate was linear for 20 h. To exclude any effect of Ca²⁺ on sulfation, the biosynthetic labeling was performed for all cells at 1.3 mM CaCl₂, and thereafter, as indicated, the cells were chased at either high (2 mM) or low (0.5 mM) CaCl₂. At the termination of chase incubations, samples were collected for analysis of cellular and released proteoglycan by pelleting cells in a microfuge (10 000 rpm, 30 s). A 400-µl aliquot of each conditioned culture medium was lyophilized and resuspended in 100 µl 1× electrophoresis SDS sample buffer (Huttner, 1984). The pelleted cells were lysed in the same buffer and boiled immediately for 3 min.

For biochemical analysis, proteoglycan was precipitated from the conditioned media by the addition of 5 vol -20°C cold acetone. The mixture was vortexed, kept for 2 h at -70°C, and centrifuged at 3000 × g for 15 min at -10°C.

Biochemical Characterization of Labeled Proteoglycan

Extraction. The extraction and the chromatographic separation of labeled proteoglycan were performed according to the procedures described by Hascall and Kimura (1982) and Yanagishita *et al.* (1987). Briefly, parathyroid cells were labeled with [³⁵S]sulfate for the indicated time. Cells or conditioned media were extracted with guanidine-HCl buffer (4 M guanidine-HCl, 2% [wt/vol] Triton X-100, 50 mM acetate buffer pH 6.0 containing protease inhibitors: 1 mM PMSF, 5 mM benzamidine). Free [³⁵S]sulfate was removed by gel filtration on 10-ml prepacked Sephadex G-50 columns equilibrated with urea buffer (8 M urea, 0.5% [wt/vol] Triton X-100, 0.15 M NaCl, 50 mM acetate buffer pH 6.0, containing 1 mM PMSF and 5 mM benzamidine).

Ion Exchange Chromatography. The radioactive fractions collected in the void volume after gel filtration were pooled and applied to fast flow Q Sepharose columns (0.7 × 4 cm), washed with 4 column volumes of urea buffer, and eluted with a linear gradient of increasing concentration of NaCl (0.15–1.5 M) in 50 mM Tris buffer pH 8.0. The fractions containing [³⁵S]sulfate-labeled macromolecules were combined, diluted in Tris buffer without NaCl, and then concentrated by a second ion exchange chromatography when [³⁵S]sulfate-labeled

material was eluted in one step with 1 M NaCl in 100 mM Tris-HCl buffer pH 8.0.

Metabolic Labeling with [³⁵S]Sulfate in the Presence of 4-Methylumbelliferyl β-D-Xyloside. The procedure was performed according to Matsuuchi and Kelly (1991). Briefly, freshly dispersed parathyroid cells were incubated in culture medium containing 0.5 mM 4-methylumbelliferyl β-D-xyloside (sample) or solvent only (0.3% pyridine) (control) for 2 h at 37°C, followed by 1 h in sulfate-depleted medium. Cells were then labeled with [³⁵S]sulfate (200 µCi/ml) for 1 h in the presence or absence of drug. Portions of the cells were lysed in SDS sample buffer (Huttner, 1984) immediately after labeling, whereas the rest were chased for 3 h in the absence of drug. Media were collected, and cells were lysed as before. Culture media contained 1.3 mM CaCl₂ throughout. The lysed cells and the collected medium were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 7.5% gels and autoradiography.

Digestion with Chondroitin ABC Lyase. [³⁵S]sulfated proteoglycan was treated for 1 h at room temperature with 1 U/ml chondroitin ABC lyase. The reaction mixture in 10 mM Tris buffer pH 8.0 contained 50 mM acetate, 1 mM PMSF, 10 mM benzamidine, and 1 mg/ml soybean trypsin inhibitor. Controls were treated with enzyme inactivated by 5 min boiling. The reaction was stopped by the addition of 5× electrophoresis sample buffer and boiling. Samples were analyzed by SDS-PAGE on 7.5% gels.

Deaminative Cleavage with Nitrous Acid. Pellets of parathyroid cells biosynthetically labeled with [³⁵S]sulfate or acetone precipitates of conditioned chase media were suspended in 3.5 M acetic acid (controls) or 0.2 M NaNO₂ in 3.5 M acetic acid (samples) (Cifonelli and King, 1972; Soroka and Farquhar, 1991). Deamination was carried out for 30 min at room temperature and was terminated by freezing and lyophilizing the samples. The dried residue was suspended in sample buffer and analyzed by SDS-PAGE and autoradiography. Quantitative measurements of the amount of radioactive proteoglycan in samples and controls were obtained with a gel scanner equipped with Ambis software (Ambis Systems, San Diego, CA).

Phase Separation of Proteoglycan in Triton X-114. Parathyroid cells labeled with [³⁵S]sulfate (3 h) were extracted for 3 h at 4°C with 1% Triton X-114 in 10 mM Tris buffer pH 7.4, 0.15 M NaCl, 1 mM EDTA, 20 µg/ml leupeptin, 10 µg/ml antipain, 5 mM benzamidine (Bordier, 1981). Phase separation was induced by incubating the extract at 37°C for 5 min; phases were separated by spinning the samples in a Beckman microfuge for 3 min at 10 000 × g. The aqueous and detergent phases were washed two times by reextraction and made up to equal volumes and similar detergent concentration before analysis by SDS-PAGE.

Electrophoretic Analysis, Quantitation, and Autoradiography

The [³⁵S]sulfate-labeled proteoglycan was analyzed by gel electrophoresis on 7.5% polyacrylamide minigels (BioRad Protean II) in Laemmli system (Laemmli, 1970). [³⁵S]Methionine-labeled PTH was assayed by separation on 13% polyacrylamide minigels in the same system. To increase the resolution for low molecular mass proteins, the anode buffer consisted of a sodium acetate solution: 0.025 M Tris, 0.192 M glycine, and 0.1 M sodium acetate (Christy *et al.*, 1989). After running, gels were fixed in 40% methanol, 10% acetic acid for ≥40 min (Huttner, 1984), then the alcohol-acid solution was washed out from gels by rinsing with several changes of distilled water. Gels used to separate samples containing PTH were fixed in 50% methanol supplemented with formaldehyde and processed as described above. Gels were vacuum dried on filter paper, and the radioactivity of the bands was counted with the gel scanner. The radioactive gels were recorded by autoradiography on Kodak XOMAT XAR-5 (Sigma Chemical) X-ray films.

Radioimmunoassay for PTH

Immunoreactive intact PTH was quantified using a goat anti-bovine PTH antibody, G-1811, at 1:70 000 dilution, as previously described (MacGregor *et al.*, 1983).

Figure 1. Export of [³⁵S]methionine-PTH by bovine parathyroid cells. Freshly dissociated cells were pulse-labeled (5 min) with [³⁵S]methionine in methionine-free RPMI-1640 at 0.4 mM CaCl₂; cells were chased in Waymouth culture medium at 0.5 or 2.0 mM CaCl₂, as indicated. (A) The figure shows the autoradiograms of SDS polyacrylamide gels loaded with equal amounts of incubation media collected for the indicated time periods from the end of pulse. The [³⁵S]methionine-PTH band is indicated by the label. Molecular size markers from top to bottom: 130, 75, 50, 39, 27, and 17 kDa. (B) Quantitative analysis of accumulation of [³⁵S]methionine-PTH in chase medium. The incubation media were collected and analyzed as described in A. The radioactivity in PTH bands was counted with a gel scanner equipped with a β -counter and an Ambis software and expressed as percentage of the total labeled hormone determined immediately after pulse. (C) Autoradiogram of SDS-PAGE of cell lysates obtained at the indicated time periods of chase incubations. The [³⁵S]methionine-PTH band is marked at the left of the autoradiograms; its intensity decreases as a result of its export and degradation. Other cell-associated proteins, for example those labeled with asterisks, retain the same intensity during the chase incubations, suggesting that uncontrolled cell

lysis during the experiment is minimal. Molecular size markers from top to bottom: 130, 75, 50, 39, 27, and 17 kDa. (D) The accumulation of immunoreactive PTH into the incubation medium is linear, a reflection of constant release of hormone by cells at either low or high concentrations of extracellular Ca²⁺. The amount of exported PTH is reduced by a high level of CaCl₂ in the incubation medium, an indication that cells are viable and functionally active. □, 0.5 mM CaCl₂; ■, 2.0 mM CaCl₂.

Statistical Analysis of the Data

When indicated, data are presented with the SE of the mean. Statistical significance was performed with the two-tailed Student's *t* test. Unless otherwise indicated, results are representative of at least two experiments performed in duplicate or quadruplicate with different cell preparations. In pulse-chase experiments, if not otherwise specified, the results are expressed as a percentage of total labeled secretory product as determined at the end of the labeling. The "Ca²⁺-exportable" secretory product is defined as the total amount of labeled product released when cells are chase-incubated in medium containing low concentration of Ca²⁺, i.e., 0.5 mM CaCl₂ and no isoproterenol.

RESULTS

PTH Release from Dispersed Bovine Parathyroid Cells

Previous observations have shown that slices of bovine parathyroid tissue release a fraction of [³⁵S]methionine-PTH shortly after synthesis, bypassing the intracellular hormone stores (MacGregor *et al.*, 1975). To establish if dissociated cells retain the secretory properties described for cells *in situ* (tissue slices), freshly dispersed bovine parathyroid cells were labeled 5 min with [³⁵S]methionine (at 0.4 mM CaCl₂) and chased in media containing 2.0 or 0.5 mM CaCl₂. Equal amounts of the incubation media were analyzed by SDS-PAGE and autoradiography. At a high level of external Ca²⁺, the secretory activity was reduced with regard to all ex-

ported proteins, the major ones being PTH and chromogranin A (*Mr* ~ 75 K) (Figure 1A). This is well documented in the parathyroid cell and reflects the physiological action of free external Ca²⁺ (Cohn and MacGregor, 1981). The quantitative analysis of accumulated [³⁵S]methionine-PTH in the extracellular medium generated curves with similar *t*_{1/2} when the chase was performed at 0.5 or 2.0 mM CaCl₂, but the amount of exported hormone was reduced 50% in the latter conditions (Figure 1B). [³⁵S]Methionine-PTH was first detected in the medium in the chase interval 30–60 min from the pulse and continued to accumulate steadily until 20–30% of the total labeled hormone was released (Figure 1, A and B). Importantly, ~80% of the Ca²⁺-dependent component was discharged in the first 90 min of chase (Figure 1, A and B).

To test for cell lysis during the experiment, SDS-PAGE and autoradiographic analysis of cell lysates obtained at various time periods after metabolic labeling with [³⁵S]methionine were carried out. As shown in the autoradiogram of SDS polyacrylamide gels (Figure 1C), the intensity of PTH band was gradually diminished during the chase incubation because of concurrent processes of release and degradation (Habener *et al.*, 1975). Numerous other proteins, such as those labeled with asterisks, however, retained the same intensity all over

the experiment. In addition, the rate of exocytosis of immunoreactive hormone was constant over the length of the experiment, leading to linear accumulation of PTH into the medium (Figure 1D). The rate of release of immunoreactive PTH was reduced by 50% when the level of external Ca²⁺ was increased from 0.5 to 2.0 mM (Figure 1D). These features are specific for the response of parathyroid cell to Ca²⁺ and indicate that the fresh dispersed cells are functional.

The release of [³⁵S]methionine-PTH shortly after synthesis indicates that a proportion of exported hormone is made of the newly synthesized PTH. To determine the contribution of newly synthesized hormone to total exocytosed PTH, we compared the specific radioactivity of that released to that remaining in the cell. Pulse-chase experiments were carried out in which noncumulative chase media were collected for 20 min at each indicated time after labeling. The specific activity of released [³⁵S]methionine-PTH was determined as the ratio between counts in the [³⁵S]methionine-PTH band as separated by SDS gel electrophoresis and the amount of immunoreactive hormone in the chase medium. The specific activity of [³⁵S]methionine-PTH increased rapidly during the first chase incubation periods, indicating immediate secretion of [³⁵S]methionine-PTH at both 0.5 and 2.0 mM CaCl₂ (Figure 2). The specific activity was similar, if not identical, at both Ca²⁺ concentrations (Figure 2). When the specific radioactivity of exported [³⁵S]methionine-PTH attained its maximum ~60 min after the synthesis, it was 20–30 times higher than that of intracellular hormone. Thus, newly synthesized PTH is released preferentially over the cellular hormone stores, in line with previous results obtained on slices of bovine parathyroid tissue (MacGregor *et al.*, 1975).

Together, these results indicate that fresh cells retain the characteristics of PTH release observed in tissue slices. In addition, external Ca²⁺ inversely modulates the exocytosis of newly synthesized PTH, at least some of which exhibits secretory kinetics consistent with a constitutive type of secretion.

Identification and Characterization of a Sulfated Bovine Parathyroid Cell Proteoglycan

Proteoglycans have been used as bona fide markers for constitutive secretion in various epithelial (Caplan *et al.*, 1987; Stow *et al.*, 1991) and neuroendocrine cell lines (Tooze and Huttner, 1990; Grimes and Kelly, 1992a,b). However, a proteoglycan from the AtT-20 cell line was packed with ACTH in the secretory granules and was released by treatment with cyclic AMP analogues (Burgess and Kelly, 1984). In search for an endogenous marker of the constitutive secretory pathway in parathyroid cells, we identified and biochemically characterized a proteoglycan and studied its release.

Freshly dispersed parathyroid cells biosynthetically labeled with [³⁵S]sulfate incorporated the radioactivity, principally into a highly anionic compound that bound

Figure 2. Specific activity of exported [³⁵S]methionine-PTH in noncumulative chase media and cells. Fresh parathyroid cells were pulse-labeled with Tran³⁵S-label for 5 min in methionine-free RPMI-1640 at 0.4 mM CaCl₂ and chased at 0.5 or 2.0 mM CaCl₂ for the indicated period of time, when cells were resuspended in fresh medium, and secretion was collected for 20 min. Equal amounts of these media were analyzed by SDS-PAGE in 13% polyacrylamide gels, and the radioactivity of [³⁵S]methionine-PTH band was determined. To calculate the specific activity of exported hormone, the number of cpm in the [³⁵S]methionine-PTH band was divided by the amount of immunoreactive PTH (ng) in the same samples. The specific activity of cellular [³⁵S]methionine-PTH was determined in a similar manner. Note that the labeled hormone was released as a wave with the peak in the chase intervals starting between 30 and 60 min after pulse and had similar values for samples collected at 0.5 or at 2.0 mM CaCl₂. □ or ♦, specific activity of released or cell-associated [³⁵S]methionine-PTH, respectively.

strongly to the positively charged matrix Q Sepharose being eluted at very high salt concentration (0.9 ± 0.1 M NaCl, n = 20) (Figure 3A). This is a property typical of proteoglycans (Yanagishita *et al.*, 1987). The sulfated material migrated in SDS-PAGE as a smear with Mr of 90–150 K (Figure 3B). The size heterogeneity and the ability to incorporate sulfate suggested that the labeled macromolecule was a proteoglycan. Biochemically, the presence of the core protein component was demonstrated by the protease sensitivity of the high molecular mass radioactive smear (Figure 3B). The formation of the high molecular mass sulfated compound was inhibited when cells were labeled with [³⁵S]sulfate in the presence of 4-methylumbelliferyl β-D-xyloside, an inhibitor of addition of glycosaminoglycan chains to core protein (Schwartz *et al.*, 1974) (Figure 3C). The labeled proteoglycan was resistant to deamination with nitrous acid (Figure 3D), an indication that it does not contain major amounts of heparan sulfate glycosaminoglycans (Cifonelli and King, 1972). The sensitivity of sulfated proteoglycan to digestion with chondroitinase ABC (Figure 3E) indicated that this proteoglycan fraction contains mainly chondroitin/dermatan sulfate chains.

An 88-kDa proteoglycan, possibly a posttranslational modification of chromogranin A has been found previously in bovine parathyroid slices (Gorr *et al.*, 1991). The differences in electrophoretic mobility of the proteoglycan labeled in our study and that of the heat stable

Figure 3. Biochemical characterization of the parathyroid cell proteoglycan. (A) Anion exchange chromatography of [^{35}S]sulfated proteoglycan. The elution profile of cell-derived [^{35}S]sulfate-labeled compound from fast flow Q Sepharose is shown. Freshly dissociated parathyroid cells were labeled biosynthetically with [^{35}S]sulfate for 20 h. Cells and media were extracted with guanidine-HCl buffer and filtered on Sephadex G-50 equilibrated with urea buffer. The radioactivity collected in the void volume was applied on a fast flow Q Sepharose (0.7×4 cm) column. The radioactive compound was eluted with increasing concentration of NaCl in urea buffer. Identical elution profiles were obtained with samples from cells or conditioned media. (B) Protease sensitivity of the [^{35}S]sulfated proteoglycan. The autoradiography of SDS polyacrylamide gel of the [^{35}S]sulfate-labeled compound eluted from the anion exchange column shows that it migrates as a smear (because of its mass and charge heterogeneity) with a $M_r \sim 90\text{--}150$ K. This radioactive smear was sensitive to digestion with trypsin, proteinase K, and papain. Molecular mass standards are shown on the left. (C) The synthesis of the sulfated high molecular mass compound was inhibited by 4-methylumbelliferyl β -D-xyloside. Parathyroid cells were metabolically labeled for 1 h with [^{35}S]sulfate in the presence or absence of 4-methylumbelliferyl β -D-xyloside (0.5 mM). A portion of cells was lysed immediately after labeling and analyzed by SDS-PAGE; the remaining cells were chased for 3 h, and the media were collected and analyzed by electrophoresis. The figure shows the autoradiograms of SDS gels of cell lysates and conditioned media of samples and controls. The xyloside analogue inhibited the formation of the high molecular mass sulfated compound; instead, low molecular mass sulfated entities were synthesized, probably free glycosaminoglycan chains that were released into medium. [^{35}S]sulfate was incorporated in the high molecular mass heterogeneous compound in the control samples. (D) The bovine parathyroid proteoglycan is resistant to deaminative cleavage with nitrous acid. Pellets of cells labeled with [^{35}S]sulfate and acetone precipitates of conditioned chase media containing the [^{35}S]sulfate-proteoglycan were suspended in 0.2 M NaNO_2 in 3.5 M acetic acid (sample) or 3.5 M acetic acid (control) and reacted for 30 min at 22°C . The autoradiograms show that most

fraction identified in the work of Gorr *et al.* (1991) indicates that the two sulfated species are not identical. Moreover, chondroitinase ABC digestion of proteoglycan labeled in tissue slices leads to the displacement of the proteoglycan smear and the appearance of a labeled band with $M_r \sim 75$ K (Gorr *et al.*, 1991). In our system, this band was revealed after chondroitinase ABC digestion of material obtained after labeling for extended time periods (20 h), but not after a 5-min pulse (Figure 3E). Together, these results indicate that the proteoglycan described by Gorr *et al.* (1991) is only a minor part of the sulfated compound characterized in this study. In addition, only 1% of chromogranin A is modified to a proteoglycan in bovine chromaffin cells (Gowda *et al.*, 1990) [$M_r \sim 92$ K and little heterogeneity (Rosa *et al.*, 1985)], indicating that bovine chromogranin A is not a good acceptor of glycosaminoglycan chains.

To determine if the cellular proteoglycan is soluble or membrane associated, cells were labeled with [^{35}S]sulfate and extracted with Triton X-114 (Bordier, 1981). The gel electrophoresis analysis of the detergent and aqueous phases of the Triton X-114 extract (Figure 3F) shows that the cellular proteoglycan partitions in the aqueous phase, an indication that it is not membrane bound, but soluble.

The Export of Sulfated Proteoglycan: Fast Release and Insensitivity to Stimulation with Isoproterenol

To test the utility of the bovine parathyroid cell sulfated proteoglycan as reporter for exocytotic secretion from other than the storage pool, cells were labeled with [^{35}S]sulfate for 5 min and chased for various time intervals, when the medium was collected and analyzed

of the [^{35}S]sulfate-proteoglycan is insensitive to deaminative treatment, i.e., it is not heparan sulfate proteoglycan. The mobility of molecular mass standards is indicated to the left. (E) Digestion of bovine parathyroid proteoglycan with chondroitinase ABC lyase. Samples of proteoglycan labeled biosynthetically with [^{35}S]sulfate for 20 h or 5 min, as indicated, were digested for 1 h with chondroitinase ABC (1 U/ml) (sample) or with inactivated chondroitinase (control). Before digestion, the 20 h-labeled proteoglycan, which has been collected from the labeling medium, was subjected to ion exchange chromatography and concentration; the proteoglycan labeled for 5 min was extracted from cells that were chased for 30 min after the pulse incubation. The autoradiograms of SDS polyacrylamide gels of samples and controls show that the digestion with chondroitinase ABC of the proteoglycan labeled for 20 h leads to a band with $M_r \sim 75$ K, probably the core protein. This band is not enhanced after enzyme treatment of the sulfated material labeled for 5 min, indicating that the 75-kDa protein does not make the core for the majority of the proteoglycan species. The molecular mass standards are indicated for each group of gels. (F) Phase partition of sulfated proteoglycan after extraction with Triton X-114. Parathyroid cells labeled with [^{35}S]sulfate for 3 h were extracted with Triton X-114 at 4°C ; the phase separation was induced by bringing the samples to 37°C . Detergent and aqueous phases were separated by centrifugation. The autoradiogram of the SDS gels loaded with equivalent amounts from the two phases shows that more than 95% of the sulfated proteoglycan partitioned in the aqueous phase.

for the amount of exocytosed proteoglycan. Noncumulative chase media collected every 30 min after the pulse indicated that the proteoglycan was released in the first 60 min after the termination of labeling, with little retained in the cells (Figure 4A). The fast release, paralleled by reduced retention in the cells, indicates that proteoglycan secretion did not derive primarily from the storage pool. To confirm this point, cells were labeled with [³⁵S]sulfate at equilibrium, (i.e., 20 h), then they were chased for 4 h to clear any constitutively secreted proteoglycan, and thereafter, cells were stimulated with 1 μ M isoproterenol for 30 min. Although isoproterenol stimulated the exocytosis of immunoreactive PTH with a factor of 1.5–1.8, stimulated release of labeled proteoglycan was negligible, i.e., 1.1-fold (Figure 4B). These results suggest that the chondroitin/dermatan sulfate proteoglycan is released from primary parathyroid cells via a constitutive type of secretory pathway.

The Export of Proteoglycan Is Modulated by Extracellular Ca²⁺

To test the effect of extracellular Ca²⁺ on the rate of release of proteoglycan, cells were pulsed for 5 min with [³⁵S]sulfate at 1.3 mM CaCl₂ and then chased at 0.5 or 2.0 mM CaCl₂. As shown in the autoradiography of SDS-polyacrylamide gels of chase incubation media (Figure 5A), at both Ca²⁺ concentrations the proteoglycan first appeared in the medium shortly after termination of the labeling, i.e., in the 7–15 min chase interval. The half-maximum release was attained at 20–30 min of chase (Figure 5B). However, at 2 mM CaCl₂, the amount of released proteoglycan was half of the corresponding values for chase at 0.5 mM CaCl₂,

Figure 4. Characteristics of exocytosis of parathyroid cell [³⁵S]sulfated-proteoglycan. (A) Cells were labeled biosynthetically with [³⁵S]sulfate for 5 min, and media were collected for 30 min at the indicated time periods after the end of pulse. The autoradiograms of SDS gel loaded with equal amounts of the incubation media or labeled cell lysates indicate that most of proteoglycan was released in the first 60 min after pulse. The molecular mass markers are indicated at the left of the gel. (B) Autoradiogram of SDS gel containing incubation media of controls and samples obtained by stimulation of cells for 20 min with 1 μ M isoproterenol. In this experiment, parathyroid cells were labeled with [³⁵S]sulfate for 20 h and chased for 4 h to clear the constitutively released proteoglycan. The numbers under the gel represent the stimulation factor of [³⁵S]sulfate-proteoglycan release relative to the control, to which a factor of 1.0 was arbitrarily attributed. The stimulation factor for immunoreactive PTH in this experiment was 1.5–1.8 $\times$.

Figure 5. The rate of exocytosis of bovine parathyroid cell proteoglycan is modulated by the level of extracellular Ca²⁺. Parathyroid cells were pulse-labeled (5 min) with [³⁵S]sulfate at 1.3 mM CaCl₂ and chased at 0.5 or 2.0 mM CaCl₂. Equal amounts of the incubation media were analyzed by SDS-PAGE. Gels were scanned with a β -counter to determine the amount of labeled proteoglycan in the sample and recorded by autoradiography. (A) Autoradiogram of SDS gel of cumulative chase media containing labeled proteoglycan. The chase was carried out at 0.5 or 2.0 mM CaCl₂, as indicated. In either case the [³⁵S]sulfate-proteoglycan was detected in the medium in the time interval of 7–15 min after pulse. (B) Quantitative analysis of accumulation of [³⁵S]sulfate-proteoglycan in the chase medium. A high concentration of Ca²⁺ in the chase medium inhibited the exocytosis of proteoglycan by 50%.

i.e., a fraction of 60 and 30% of the total labeled proteoglycan was released at 0.5 and 2.0 mM CaCl₂, respectively. (Figure 5, A and B). Ca²⁺-induced inhibition of release was a rapid process, because the amount of proteoglycan exported from cells chased at high Ca²⁺ was decreased as soon as this secretory product was detected in the medium (Figure 5A). Except for a reduced amplitude, the curve representing the accumulation of proteoglycan in the extracellular medium had the same $t_{1/2}$ at either 0.5 or 2.0 mM CaCl₂ (Figure 5B).

The decrease of cellular proteoglycan followed a double exponential decay, a rapid decline in the initial chase period (0–90 min) mainly because of exocytosis followed by a slower decrease at later times presumably because of intracellular degradation (Figure 6). For the decay in the initial period of chase, in six separate experiments performed in duplicate, we found a $t_{1/2}$ of 44 ± 17 min ($n = 12$) and 127 ± 25 min ($n = 12$) when cells were chased at 0.5 or 2.0 mM CaCl₂, respectively.

Ca²⁺-Modulated PTH and Proteoglycan Release Are Parallel

Increased concentration of extracellular Ca²⁺ resulted in decreased export of both PTH and of proteoglycan. To test if the exocytosis of the two secretory products has similar sensitivity to Ca²⁺, the dose-response relationships between Ca²⁺ concentration and the release of proteoglycan and PTH were investigated. This was carried out by labeling cells with [³⁵S]sulfate at 1.3 mM CaCl₂ (5 min) and chasing for 1 h at various concentrations of CaCl₂. The amount of [³⁵S]sulfated-proteoglycan in the incubation media was quantified by

Figure 6. Decay of [³⁵S]sulfate-proteoglycan in parathyroid cells. (A) Autoradiogram of SDS-polyacrylamide gel of cells labeled biosynthetically with [³⁵S]sulfate at 1.3 mM CaCl₂ (5 min) and chased at 0.5 or 2.0 mM CaCl₂. Equal amounts of cell lysates were analyzed by SDS-PAGE on 7.5% gels. The numbers of the lanes refer to the length of chase times as follows: 1, 30 min; 2, 60 min; 3, 90 min; 4, 120 min; 5, 200 min; and 6, 380 min. (B) Plot of logarithm of the amount of [³⁵S]sulfate-proteoglycan remaining in the cells. The amount of labeled proteoglycan present in cell lysates was determined by scanning the SDS gels (as shown in A) and counting the radioactivity in the smear associated with the proteoglycan. Lines are drawn by a best-fit program (correlation coefficients > 0.95) and used to calculate the times for 50% decay in the first 90 min after labeling (because of exocytosis) at 0.5 or 2.0 mM CaCl₂. The lines for time intervals >90 min reflect the decay of the amount of cellular [³⁵S]sulfate-proteoglycan most likely because of intracellular degradation. The $t_{1/2}$ values are the mean of six separate experiments performed in duplicate with different cell preparations.

measuring its radioactivity after SDS-PAGE (Figure 7, A and B). PTH released was quantified by radioimmunoassay (Figure 7C). Inverse sigmoidal curves, known to describe the secretory response of parathyroid cells to changes in level of extracellular Ca²⁺ (Cohn and MacGregor, 1981; Brown, 1991), were obtained in response to increasing CaCl₂ concentrations for both parameters tested (Figure 7, B and C). As a result, the ratio of exported proteoglycan/PTH was not changed in response to modifications in concentrations of extracellular Ca²⁺. Thus the effect of extracellular Ca²⁺ on the release of immunoreactive PTH and labeled proteoglycan was identical.

Effect of β -Adrenergic Agonists on the Release of Newly Synthesized Proteoglycan and PTH

In addition to controlled PTH release by extracellular Ca²⁺, parathyroid cells increase PTH exocytosis when stimulated with catecholamines (Brown *et al.*, 1977). It has been shown previously that in porcine parathyroid cells there are two intracellular hormone pools: one pool releasable by Ca²⁺, made up of newly synthesized hormone, and a second pool, isoproterenol-stimulatable, containing more mature hormone (60 min from synthesis) (Morrissey and Cohn, 1979a). Isoproterenol stimulation is thought to induce the exocytosis of the secretory granules, defining the regulated secretory route (Sharoni *et al.*, 1976; Burgess and Kelly, 1984). Because

sorting of secretory products can also occur in the immature granules (von Zastrow and Castle, 1989; Arvan and Castle, 1992; Grimes and Kelly, 1992a), we have used isoproterenol stimulation shortly after metabolic labeling to determine the fraction of proteoglycan or PTH contained in immature granules, which in other cell types are known to be responsive to secretagogue-induced exocytosis (Tooze *et al.*, 1991; Arvan *et al.*, 1991). Thus, isoproterenol stimulation of cells applied soon after labeling should induce the exocytosis of immature secretory granules, thereby identifying the secretory products emerging from the maturation compartment.

To determine the amount of proteoglycan contained within immature granules, cells were labeled for 5 min with [³⁵S]sulfate and stimulated for 30 min with 1.0 μ M isoproterenol at different time intervals after the pulse. Results were expressed as the fraction from the maximum amount of proteoglycan released by low Ca²⁺. Very little change in [³⁵S]sulfate-proteoglycan release was detected when isoproterenol was applied at 0.5 mM CaCl₂, although a small effect was observed at 2.0 mM CaCl₂ in the time intervals starting at 30 and 60 min after labeling (Figure 8). Only ~10% of total labeled proteoglycan was mobilized for exocytosis by isoproterenol over the first 6 h of chase incubation with a maximum of 1.6-fold (Figure 10A). It is important to observe that at each time point, when isoproterenol stimulus was applied to cells in high Ca²⁺, the amount of proteoglycan exocytosed was lower than that released by low Ca²⁺ (compare Figure 8, A and B versus C and D). This finding indicates that Ca²⁺-modulated proteoglycan release and isoproterenol-induced exocytosis originate from different intracellular pools. In addition, it shows that proteo-

Figure 7. Effect of external Ca²⁺ on the release of [³⁵S]sulfate-proteoglycan and immunoreactive PTH. (A) Parathyroid cells metabolically labeled with [³⁵S]sulfate (5 min at 1.3 mM CaCl₂) were chased for 1 h at the indicated CaCl₂ concentrations in the interval 0.5 to 3.0 mM. Equal volumes of chase media were analyzed by SDS-PAGE and autoradiography. (B) Plot of the amount of [³⁵S]sulfate-proteoglycan released when chased at various CaCl₂ concentrations. The gels obtained as described in A were scanned with a β counter to determine the radioactivity associated with the [³⁵S]sulfate-proteoglycan. (C) Plot of the immunoreactive PTH released, as measured in the same samples analyzed in A and B.

Figure 8. Effect of isoproterenol on the export of [³⁵S]sulfated proteoglycan at early time points after synthesis. Parathyroid cells were labeled biosynthetically with [³⁵S]sulfate (5 min at 1.3 mM CaCl₂) and then chased at 0.5 or 2.0 mM CaCl₂. At the indicated times after pulse, the media were changed and the cells were stimulated with 1 μ M isoproterenol for 30 min. Collected media were analyzed for [³⁵S]sulfate-proteoglycan by SDS-PAGE and autoradiography. (A and C) Autoradiograms of SDS gels. The radioactive smear corresponding to the proteoglycan is indicated. (B and D) Histograms representing the amount of [³⁵S]sulfate-proteoglycan in the media, expressed as a percentage of Ca²⁺-exportable proteoglycan, defined as the amount of labeled proteoglycan released by cells incubated at 0.5 mM CaCl₂. The radioactivity in the samples was quantified with a gel scanner from gels as those shown in A and C. ■, controls; ▨, samples stimulated with 1 μ M isoproterenol.

glycan release is fully modulated by external Ca²⁺, but less amount is sensitive to stimulation with isoproterenol. Thus, it would appear that only a small amount of proteoglycan passes through a compartment stimutable by isoproterenol.

In a similar set of experiments, to examine PTH release, freshly dispersed parathyroid cells were pulse-labeled with [³⁵S]methionine (5 min at 0.4 mM CaCl₂), chased at 0.5 or 2.0 mM CaCl₂, and stimulated with 1 μ M isoproterenol for 30 min at various times after labeling. When the stimulation with isoproterenol was carried out in medium containing 2.0 mM CaCl₂, but not at 0.5 mM CaCl₂, we observed a significant ($p < 0.05$) increase in the exocytosis of [³⁵S]methionine-PTH; the maximum stimulation (3.6-fold) was attained 60–90 min from the end of pulse (Figures 9 and 10B). The integration of [³⁵S]methionine-PTH exocytosed under stimulation with isoproterenol over the chase period (10–360 min) amounted to ~19% of total labeled PTH. Further, although stimulated proteoglycan secretion cannot be detected after the second hour of chase (Figure 8), isoproterenol-stimulated PTH secretion could still be detected at these later times (Figure 9). Thus, some PTH reaches more mature secretory granules, but the proteoglycan is largely excluded. However, even for PTH, the fold stimulation of isoproterenol-induced secretion fell as a function of time beyond 90 min of chase (Figure 10B). Therefore, prolonged storage of PTH in the cAMP-regulated secretory pathway is not a feature of these cells.

DISCUSSION

The Release of PTH from Bovine Parathyroid Cells

Because PTH export is continuous, one might believe that its secretion derives primarily from unstimulated exocytosis of storage granules (Matsuuchi and Kelly, 1991). However, although PTH secretion is modulated by extracellular Ca²⁺, it emanates from the newly synthesized hormone pool kinetically situated before the storage compartment. Indeed, freshly dispersed parathyroid cells metabolically labeled with [³⁵S]methionine released newly synthesized PTH as a wave that started 30 min after synthesis, peaked ~60 min after pulse, and decreased sharply afterward. These observations are in line with previous results obtained on slices of bovine parathyroid (MacGregor *et al.*, 1975) and on freshly dissociated porcine parathyroid cells (Morrissey and Cohn, 1979a,b). In agreement with these previous studies, the specific activity of released PTH, a measure of preferential export of the newly synthesized hormone, exceeded by at least one order of magnitude the specific activity of the hormone retained in the cell, suggesting that PTH export relies on the newly synthesized hormone. Such an outcome is possible because new PTH is synthesized fast enough to support this continuous exocytosis without mobilization of the hormone preexisting in the cell, i.e., the rate of synthesis exceeds by two- to five-fold the rate of exocytosis (Figure 1). However, the amount of cell-as-

Figure 9. Effect of isoproterenol on the export of [³⁵S]methionine-PTH at early time points after synthesis. Parathyroid cells were labeled for 5 min with [³⁵S]methionine-PTH in RPMI-1640 (methionine-free) at 0.4 mM CaCl₂ and chased at 0.5 or 2.0 mM CaCl₂ for the indicated time. This chase period was followed by isoproterenol stimulation (1 μM) at 0.5 or 2.0 mM CaCl₂ for 30 min when the secretion was collected in fresh media. Equal amounts of incubation media were analyzed by SDS-PAGE in 13% polyacrylamide gels. Gels were autoradiographed. (A and C) Autoradiograms of the SDS gels. The radioactive PTH band is indicated. (B and D) Quantitative analysis of the radioactivity of PTH band in SDS gels, as determined with a gel scanner equipped with a β-counter. Results are presented in histograms as percentages from Ca²⁺-exportable [³⁵S]methionine-PTH, defined as the amount of [³⁵S]methionine-PTH secreted by cells incubated at 0.5 mM CaCl₂. ■, controls; ▨, samples stimulated with 1 μM isoproterenol.

sociated hormone is regulated by an intense degradative process (Habener *et al.*, 1975; MacGregor *et al.*, 1986). An interesting observation of this study is that the specific activity of released [³⁵S]methionine-PTH at low or high external Ca²⁺ is similar, indicating that PTH release at low extracellular Ca²⁺ emanates from the same intracellular compartment as PTH exported at high external Ca²⁺.

Together, our results and others (MacGregor *et al.*, 1975; Morrissey and Cohn, 1979a,b) indicate that newly synthesized PTH moves rapidly along the vesicular secretory pathway from the endoplasmic reticulum to plasma membrane, being preferentially released with little mixing with the hormone preexisting in the cell. Thus Ca²⁺ modulates exocytosis of newly synthesized PTH, which nevertheless exhibits characteristics of a constitutive type of secretion.

The Bovine Parathyroid Cell Proteoglycan: Characterization and Exocytotic Properties

Metabolic labeling of bovine parathyroid cells with [³⁵S]sulfate revealed a chondroitin/dermatan sulfate proteoglycan with M_r ~ 90–150 K. A fraction of 30–60% of total labeled proteoglycan was released in the medium; its export started soon after sulfation, i.e., 7–15 min after the end of 5-min labeling. Only a nonsignificant amount of parathyroid cell proteoglycan was found in the storage compartment when tested with isoproterenol after equilibrium labeling and clearance

of constitutive release (Figure 4B). Instead of being stored, the proteoglycan that remained in the cells appeared to be degraded. In addition, a relatively little parathyroid cell proteoglycan was released by isoproterenol-stimulated exocytosis of immature granules (Figures 8 and 10). These features are different from those observed for a granule-associated proteoglycan from AtT-20 cells (Burgess and Kelly, 1984) but are not inconsistent with the secretory properties of the PC12 proteoglycan that describes the constitutive secretory route with a confidence of 70% (because 30% enters immature secretory granules) (Grimes and Kelly, 1992b). Because the parathyroid cell proteoglycan release exhibits the following characteristics: 1) rapid kinetics of release, 2) absence of intracellular storage, and 3) large insensitivity to stimulation with isoproterenol, this proteoglycan may serve as marker for the constitutive secretory route.

The role of this proteoglycan in bovine parathyroid cells is not known. Because a proteoglycan counterpart has not been found in porcine parathyroid (Gorr *et al.*, 1991), it appears that it is not essential for the endocrine activity of parathyroid glands. One possibility is that it may represent the contribution of endocrine cells, besides fibroblasts, to the renewal of their extracellular matrix. This is suggested by the observation that other endocrine cells continuously synthesize and secrete proteoglycans for the production and replacement of extracellular matrix components (Schubert *et al.*, 1988).

Figure 10. Isoproterenol-induced exocytosis of [³⁵S]sulfated-proteoglycan (A) and [³⁵S]methionine-PTH (B) tested shortly after pulse labeling. Data are obtained from experiments performed as described in Figures 8 and 9 and are expressed as fold-stimulation induced by secretagogue. The stimulation was carried out with 1 μ M isoproterenol for cells chased at either 0.5 mM CaCl₂ (□) or 2.0 mM CaCl₂ (■).

The Export of Proteoglycan Is Modulated by the Level of Extracellular Ca²⁺

Although the proteoglycan secretion exhibited certain constitutive characteristics, its exocytosis was strongly Ca²⁺ dependent, being reduced from 60% of total labeled to 30% by an increase in the concentration of external CaCl₂ from 0.5 to 2.0 mM CaCl₂ (Figure 5). In line with our observations, external Ca²⁺ decreased the amount of a membrane-associated heparan sulfate proteoglycan that is transported to the cell surface in a rat parathyroid cell line (Takeuchi *et al.*, 1990). Because the current view on the biogenesis of plasma membrane and of extracellular matrix is that they rely on the constitutive export of their components, these results suggest that in parathyroid cells external Ca²⁺ may play a role in the traffic along the constitutive secretory pathway.

Prior studies have shown that the sulfation of chondroitin sulfate chains occurs at the *trans*-Golgi network (Rosenzweig and Farquhar, 1980; Spiro *et al.*, 1991). Only a reduced amount of activated sulfate, 3'-phosphoadenosine 5'-phosphosulfate is transported by the membrane of immature secretory granules in parotid gland (Blair *et al.*, 1991), thus supporting a limited sulfation activity at this site. Therefore, the secretion of [³⁵S]sulfate-proteoglycan should in principal represent the trafficking from *trans*-Golgi network to plasma membrane. If so, the observed inhibition of proteoglycan release by high Ca²⁺ implies action on intracellular transport(s) of proteoglycan from the *trans*-Golgi network to plasma membrane.

Features of Parallel and Nonparallel Exocytosis of PTH and of the Proteoglycan

The release of PTH and proteoglycan were parallel regarding several aspects tested in this work. First, similar

kinetics of intracellular transport for proteoglycan and PTH were observed in this study. [³⁵S]Methionine-PTH was detectable in the culture medium 30–40 min after the pulse, whereas the proteoglycan appeared in the extracellular space in the time interval of 7–15 min after labeling with [³⁵S]sulfate in the Golgi system. Because in bovine parathyroid the time necessary for the transport of PTH from the endoplasmic reticulum to Golgi is ~20 min (Habener *et al.*, 1979), the kinetics of transport of PTH and proteoglycan from the Golgi to plasma membrane appear comparable. Second, our results show that external Ca²⁺ inhibits the release of [³⁵S]sulfated proteoglycan and immunoreactive PTH with a similar degree and in the same concentration range (Figure 7), suggesting the same mechanism of action on the secretion of these two products. A Ca²⁺ effect on the synthesis of PTH or proteoglycan was eliminated by our experimental setting where biosynthetic labeling was carried out for all cells under the same conditions. Because the rate of release of proteoglycan is modulated at a step between the Golgi and plasmalemma and because immunoreactive PTH export is inhibited within seconds after increases in the level of external Ca²⁺ (Cohn and MacGregor, 1981; Brown, 1991), it follows that in parathyroid cells external Ca²⁺ does not act primarily on the exit of secretory products from endoplasmic reticulum.

In spite of similar kinetics of release and parallel export of PTH and proteoglycan with respect to the level of extracellular Ca²⁺, isoproterenol, a secretagogue for regulated exocytosis, was more effective in stimulating the exocytosis of PTH than the export of proteoglycan (Figure 10). Thus, although PTH and proteoglycan are both present in a Ca²⁺-sensitive pool, part of PTH, found in the cAMP-regulated secretory pathway, must be segregated from the proteoglycan.

One explanation for the Ca²⁺-modulated secretion of proteoglycan would be that proteoglycan enters the immature granule together with PTH, and under continuous stimulation by low concentration of extracellular Ca²⁺, the whole immature granule would be exocytosed. Although controversial (Orci *et al.*, 1987; Tooze and Huttner, 1990), the origins of a constitutive-like secretory pathway from immature granules is supported by a large body of evidence obtained in exocrine (Arvan and Castle, 1987; von Zastrow and Castle, 1987) and neuroendocrine cells (Arvan *et al.*, 1991; Grimes and Kelly, 1992a; Kuliawat and Arvan, 1992), indicating that the process of sorting of secretory products is not confined to *trans*-Golgi network but can continue in the maturation compartment (Arvan and Castle, 1992 for review). The ability of immature granules to undergo exocytosis in response to extracellular stimuli (Arvan *et al.*, 1991; Tooze *et al.*, 1991) would thus result in regulated exocytosis of constitutive secretory products.

However, because a large fraction of proteoglycan is released in a Ca²⁺-dependent manner (similar to PTH release), but (in contrast to PTH) a disproportionately small fraction is secreted from immature granules in

response to isoproterenol stimulation, the exocytosis of immature granules seems unlikely to explain our results. Rather, our data suggest that in parathyroid cells extracellular free Ca^{2+} modulates a constitutive type of release. Two types of constitutive exocytosis have been described so far: the classical constitutive release via constitutive secretory vesicles formed at *trans*-Golgi network (Kelly, 1985) and the constitutive-like secretion carried by constitutive-like vesicles originating from immature granules (Arvan and Castle, 1992). This later type of release has been described to accompany the maturation of granules in pancreatic islet cells (Kuliawat and Arvan, 1992), and it was reconstituted *in vitro* for large immature regulated granules from permeabilized PC12 pheochromocytoma cells (Grimes and Kelly 1992a). Although it remains to be determined which pathway accounts for [^{35}S]sulfated proteoglycan release, alterations in the rate and efficiency of such transport could be regulated by heterotrimeric proteins (Barr *et al.*, 1991; Stow *et al.*, 1991; Leyte *et al.*, 1992; Pimplikar and Simons, 1993) or protein kinase C (Luini and De Matteis, 1993).

In summary, we characterized a releasable sulfated proteoglycan from bovine parathyroid cells, whose kinetics of export are consistent with its constitutive release. Isoproterenol augmented the release of PTH, and to a much lesser extent, the exocytosis of proteoglycan. However, the exports of this proteoglycan and of PTH were modulated by the level of external Ca^{2+} with a similar degree and sensitivity.

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REFERENCES

- Arvan, P., and Castle, D. (1987). Phasic release of newly synthesized secretory proteins in the unstimulated rat exocrine pancreas. *J. Cell Biol.* 104, 243–252.
- Arvan, P., and Castle, D. (1992). Protein sorting and secretion granule formation in regulated secretory cells. *Trends Cell Biol.* 2, 327–331.
- Arvan, P., Kuliawat, R., Prabakaran, D., Zavacki, A.-M., Elahi, D., Wang, S., and Pilkey, D. (1991). Protein discharge from immature secretory granules displays both regulated and constitutive characteristics. *J. Biol. Chem.* 266, 14171–14174.
- Barr, F.A., Leyte, A., Mollner, S., Pfeuffer, T., Tooze, S.A., and Huttner, W.B. (1991). Trimeric G-proteins of the *trans*-Golgi network are involved in the formation of constitutive secretory vesicles and immature secretory granules. *FEBS Lett.* 294, 239–243.
- Blair, E.A., Castle, A.M., and Castle, J.D. (1991). Proteoglycan sulfation and storage parallels storage of basic secretory proteins in exocrine cells. *Am. J. Physiol.* 261, C897–C905.
- Bordier, C. (1981). Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* 256, 1604–1607.
- Brent, G.A., LeBoff, M.S., Seely, E.W., Conlin, P.R., and Brown, E.M. (1988). Relationship between the concentration and rate of change of calcium and serum intact parathyroid hormone levels in normal humans. *Endocrinology* 67, 944–950.

- Brown, E.M. (1991). Extracellular Ca^{2+} sensing regulation of parathyroid cell function, and role of Ca^{2+} and other ions as extracellular (first) messengers. *Physiol. Rev.* 71, 371–411.
- Brown, E.M., Carroll, R.J., and Aurbach, G.D. (1977). Dopaminergic stimulation of cyclic AMP accumulation and parathyroid hormone release from dispersed bovine parathyroid cells. *Proc. Natl. Acad. Sci. USA* 74, 4210–4213.
- Brown, E.M., Gamba, G., Riccardi, D., Lombardi, M., Butters, R., Kifor, O., Sun, A., Hediger, M.A., Lytton, J., and Hebert, S.C. (1993). Cloning and characterization of an extracellular Ca^{2+} -sensing receptor from bovine parathyroid. *Nature* 366, 575–580.
- Brown, E.M., Hurwitz, S., and Aurbach, G.D. (1976). Preparation of viable isolated bovine parathyroid cells. *Endocrinology* 99, 1582–1588.
- Brown, E.M., Leombruno, R., Thatcher, J., and Burrowes, M. (1985). The acute secretory response to alterations in extracellular calcium concentration and dopamine in perfused bovine parathyroid cells. *Endocrinology* 116, 1123–1132.
- Brown, E.M., Redgrave, J., and Thatcher, J. (1984). Effect of phorbol esters TPA on PTH secretion. Evidence for a role for protein kinase C in the control of PTH release. *FEBS Lett.* 175, 72–75.
- Burgess, T.L., and Kelly, R.B. (1984). Sorting and secretion of adrenocorticotropin in a pituitary tumor cell line after perturbation of the level of a secretory granule-specific proteoglycan. *J. Cell Biol.* 99, 2223–2230.
- Burgess, T.L., and Kelly, R.B. (1987). Constitutive and regulated secretion of proteins. *Annu. Rev. Cell Biol.* 3, 243–293.
- Caplan, M.J., Stow, J.L., Newman, A.P., Madri, J., Anderson, H.C., Farquhar, M.G., Palade, G.E., and Jamieson, J.D. (1987). Dependence on pH of polarized sorting of secreted proteins. *Nature* 329, 632–635.
- Christy, K.G., Jr., LaTart, D.B., and Osterhoudt, H.W. (1989). Modifications for SDS-PAGE of proteins. *Biotechniques* 7, 692–693.
- Chu, L.L.H., MacGregor, R.R., and Hamilton, J.W. (1983). Effects of isoproterenol and cycloheximide on parathyroid secretion. *Mol. Cell. Endocrinol.* 33, 157–168.
- Cifonelli, J.A., and King, J. (1972). The distribution of 2-acetamido-2-deoxy-D-glucose residues in mammalian heparins. *Carbohydr. Res.* 21, 173–186.
- Cohn, D.V., and MacGregor, R.R. (1981). The biosynthesis, intracellular processing, and secretion of parathormone. *Endocrine Rev.* 2, 1–26.
- Emura, S., Shoumura, S., Utsumi, M., Hayakawa, D., Yamahira, T., Terasawa, K., Tamada, A., Arakawa, M., and Isono, H. (1992). Effects of short-term treatment with calcium on the parathyroid glands in golden hamsters of different ages, with special reference to large vacuolar bodies. *Acta Anat.* 143, 223–230.
- Gorr, S.-U., Hamilton, J.W., and Cohn, D.V. (1991). Sulfated secreted forms of bovine and porcine parathyroid chromogranin A (secretory protein-I). *J. Biol. Chem.* 266, 5780–5784.
- Gowda, D.C., Hogue-Angeletti, R., Margolis, R.K., and Margolis, R.U. (1990). Chromaffin granule and PC12 cell chondroitin sulfate proteoglycans and their relation to chromogranin A. *Arch. Biochem. Biophys.* 281, 219–224.
- Grimes, M., and Kelly, R.B. (1992a). Intermediates in the constitutive and regulated secretory pathways released *in vitro* from semi-intact cells. *J. Cell Biol.* 117, 539–549.
- Grimes, M., and Kelly, R.B. (1992b). Sorting of chromogranin B into immature secretory granules in pheochromocytoma (PC12) cells. *Ann. New York Acad. Sci.* 674, 38–52.
- Habener, J.F., Amherdt, M., Ravazzola, M., and Orci, L. (1979). Parathyroid hormone biosynthesis. Correlation of conversion of biosynthetic precursors with intracellular protein migration as determined by electron microscope autoradiography. *J. Cell Biol.* 80, 715–731.

- Habener, J.F., Kemper, B., and Potts, J.T., Jr. (1975). Calcium-dependent intracellular degradation of parathyroid hormone: a possible mechanism for the regulation of hormone stores. *Endocrinol.* 97, 431–441.
- Hascall, V.C., and Kimura, J.H. (1982). Proteoglycans: isolation and characterization. *Methods Enzymol.* 82, 769–800.
- Huttner, W.B. (1984). Determination and occurrence of tyrosine O-sulfate in proteins. *Methods Enzymol.* 107, 200–223.
- Kelly, R.B. (1985). Pathways of protein secretion in eukaryotes. *Science* 230, 25–32.
- Kuliawat, R., and Arvan, P. (1992). Protein targeting via the “constitutive-like” secretory pathway in isolated pancreatic islets: passive sorting in the immature granule compartment. *J. Cell Biol.* 118, 521–529.
- Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Leyte, A., Barr, F.A., Kehlenbach, R.H., and Huttner, W.B. (1992). Multiple trimeric G-proteins on the *trans*-Golgi network exert stimulatory and inhibitory effects on secretory vesicle formation. *EMBO J.* 11, 4795–4804.
- Luini, A., and De Matteis, M.A. (1993). Receptor-mediated regulation of constitutive secretion. *Trends in Cell Biol.* 3, 290–292.
- MacGregor, R.R., Hamilton, J.W., and Cohn, D.V. (1975). The bypass of tissue hormone stores during the secretion of newly synthesized parathyroid hormone. *Endocrinology* 97, 178–188.
- MacGregor, R.R., Jilka, R.L., and Hamilton, J.W. (1986). Formation and secretion of fragments of parathormone. *J. Biol. Chem.* 261, 1929–1934.
- MacGregor, R.R., Sarra, M.P., Jr., Houle, A., and Cohn, D.V. (1983). Primary monolayer cell culture of bovine parathyroids: effects of calcium, isoproterenol and growth factors. *Mol. Cell. Endocrinol.* 30, 313–328.
- Matsuuchi, L., and Kelly, R.B. (1991). Constitutive and basal secretion from the endocrine cell line. *J. Cell Biol.* 112, 843–852.
- Mayer, G.P., Habener, J.F., and Potts, J.T., Jr. (1976). Parathyroid hormone secretion in vivo: demonstration of a calcium independent, non-suppressible component of secretion. *J. Clin. Invest.* 57, 678–683.
- Morrissey, J.J., and Cohn, D.V. (1979a). Secretion and degradation of parathormone as a function of intracellular maturation of hormone pools. *J. Cell Biol.* 83, 521–528.
- Morrissey, J.J., and Cohn, D.V. (1979b). Regulation of secretion of parathormone and secretory protein-I from separate intracellular pools by calcium, dibutyryl cyclic AMP, and (1)-isoproterenol. *J. Cell Biol.* 82, 93–102.
- Orci, L., Ravazzola, M., Amherdt, M., Perrelet, A., Powell, S.K., Quinn, D.L., and Moore, H.-P.H. (1987). The *trans*-most cisternae of the Golgi complex: a compartment for sorting of secretory and plasma membrane proteins. *Cell* 51, 1039–1051.
- Palade, G.E. (1975). Intracellular aspects of the process of protein synthesis. *Science* 189, 347–358.
- Pfeffer, S.R., and Rothman, J.E. (1987). Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. *Annu. Rev. Biochem.* 56, 829–852.
- Pimplikar, S.W., and Simons, K. (1993). Regulation of apical transport in epithelial cells by a Gs class of heterotrimeric G protein. *Nature* 362, 456–458.
- Rosa, P., Hille, A., Lee, R.W.H., Zainini, A., DeCamilli, P., and Huttner, W. (1985). Secretogranins I and II: two tyrosine-sulfated secretory proteins common to a variety of cells secreting peptides by the regulated pathway. *J. Cell Biol.* 101, 1999–2011.
- Rosenzweig, L.J., and Farquhar, M.G. (1980). Sites of sulfate incorporation into mammothrophs and somatotrophs of the rat pituitary as determined by quantitative electron microscopic autoradiography. *Endocrinology* 107, 422–431.
- Schubert, D., Schroeder, R., LaCorbiere, M., Saitoh, T., and Cole, G. (1988). Amyloid β protein precursor is possibly a heparan sulfate proteoglycan core protein. *Science* 241, 223–226.
- Schultz, G.S., Sarra, M.P., Jr., Gunther, G.R., Hull, B.E., Alicea, H.A., Gorelick, F.S., and Jamieson, J.D. (1980). Guinea pig pancreatic acini prepared with purified collagenase. *Exper. Cell Res.* 130, 49–62.
- Schwartz, N.B., Galligani, L., Ho, P.-L., and Dorfman, A. (1974). Stimulation of synthesis of free chondroitin sulfate chains by β -D-xylosides in cultured cells. *Proc. Natl. Acad. Sci. USA* 71, 4047–4051.
- Sharoni, Y., Eimerl, S., and Schramm, M. (1976). Secretion of old versus new exportable protein in rat parotid slices. *J. Cell Biol.* 71, 107–122.
- Soroka, K.J., and Farquhar, M.G. (1991). Characterization of a novel heparan sulfate proteoglycan found in the extracellular matrix of liver sinusoids and basement membranes. *J. Cell Biol.* 113, 1231–1241.
- Spiro, R.C., Freeze, H.H., Sampath, D., and Garcia, J.A. (1991). Uncoupling of chondroitin sulfate glycosaminoglycan synthesis by Brefeldin A. *J. Cell Biol.* 115, 1463–1473.
- Stow, J.L., de Almeida, J.B., Narula, N., Holtzman, E.J., Ercolani, L., and Ausiello, D.A. (1991). A heterotrimeric G protein, Gai-3, on Golgi membranes regulates the secretion of a heparan sulfate proteoglycan in LLC-PK1 epithelial cells. *J. Cell Biol.* 114, 1113–1124.
- Takeuchi, Y., Sakaguchi, K., Yanagishita, M., Aurbach, G.D., and Hascall, V.C. (1990). Extracellular calcium regulates distribution and transport of heparan sulfate proteoglycans in a rat parathyroid cell line. *J. Biol. Chem.* 265, 13661–13668.
- Tooze, S.A., Flatmark, T., Tooze, J., and Huttner, W.B. (1991). Characterization of the immature secretory granule, an intermediate in granule biogenesis. *J. Cell Biol.* 115, 1491–1503.
- Tooze, S.A., and Huttner, W.B. (1990). Cell-free protein sorting to the regulated and constitutive secretory pathways. *Cell* 60, 837–847.
- Tooze, J., Kern, H.F., Fuller, S.D., and Howell, K.E. (1989). Condensation-sorting events in the rough endoplasmic reticulum of exocrine pancreatic cells. *J. Cell Biol.* 109, 35–50.
- von Zastrow, M., and Castle, J.D. (1987). Protein sorting among two distinct export pathways occurs from the content of maturing exocrine storage granules. *J. Cell Biol.* 105, 2675–2684.
- Yanagishita, M., Midura, R.J., and Hascall, V.C. (1987). Proteoglycans: isolation and purification from tissue cultures. *Methods Enzymol.* 138, 279–289.