Extracellular Calcium Regulates Distribution and Transport of Heparan Sulfate Proteoglycans in a Rat Parathyroid Cell Line*

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The regulation of the cellular distribution of proteoglycans in a clonal rat parathyroid cell line by extracellular Ca²⁺ concentrations ([Ca²⁺]₀) was studied. Proteoglycans synthesized by the cells metabolically labeled with [³⁵S]sulfate have been shown to be almost exclusively heparan sulfate (HS) proteoglycans (Yanagishita, M., Brandi, M. L., and Sakaguchi, K. (1989) J. Biol. Chem. 264, 15714–15720), which are generally associated with the plasma membrane. The proportion of HS proteoglycans on the cell surface was ~20% in 2.1 mM (high) [Ca²⁺]₀, whereas it increased to 50–60% in 0.05 mM (low) [Ca²⁺]₀. Cell-associated HS proteoglycans redistribute in response to changing [Ca²⁺]₀, with a t½ < 4 min; HS proteoglycans appear on the cell surface as [Ca²⁺]₀ decreases and disappear from the cell surface as [Ca²⁺]₀ increases. Further, HS proteoglycans on the cell surface recycle to and from an intracellular compartment ~10 times before their degradation in low [Ca²⁺]₀, but do not recycle in high [Ca²⁺]₀. The distribution of newly synthesized HS proteoglycans is regulated by [Ca²⁺]₀, but is independent of [Ca²⁺]₀ during biosynthesis. In low [Ca²⁺]₀, at least 50% of the HS proteoglycans pulse-labeled for 10 min are transported from the Golgi complex to the cell surface or to the recycling compartment with a t½ of ~20 min. Another ~10% appear on the cell surface in either low or high [Ca²⁺]₀, in a compartment with a long half-life. Addition of Mg²⁺ or Ba²⁺ to the low [Ca²⁺]₀ cultures had little effect on the distribution of HS proteoglycans. These observations suggest that [Ca²⁺]₀ specifically regulates the distribution and recycling of cell-associated HS proteoglycans in the parathyroid cells.

Parathyroid glands uniquely regulate the secretion of parathyroid hormone in response to extracellular free Ca²⁺ concentrations ([Ca²⁺]₀); an increase in [Ca²⁺]₀ elicits increasing hormone secretion in response to extracellular free Ca²⁺ concentrations ([Ca²⁺]₀); an increase in [Ca²⁺]₀ elicits increasing hormone secretion in response to extracellular free Ca²⁺ concentrations ([Ca²⁺]₀). However, [Ca²⁺]₀ is usually required for the optimal response to stimulators (4, 5). Although several mechanisms are involved in controlling parathyroid hormone secretion (6–10), precise mechanisms coupling the signal ([Ca²⁺]₀) and the cell secretory machinery are still far from being understood.

A rat parathyroid cell line established by Sakaguchi et al. (11) retains certain characteristics of normal parathyroid cells. Secretory processes and growth of the cells are suppressed by an increase of [Ca²⁺]₀ (11, 12). We have reported that the proteoglycans synthesized by this cell line are almost exclusively (>95%) heparan sulfate-proteoglycans (HS-PGs) of two distinct types; HS-PG1 has a mass of ~160 kDa with a single HS chain (~12 kDa) and a core protein of ~150 kDa including oligosaccharides, and HS-PG2 has a mass of ~170 kDa with three to four HS chains (~30 kDa) and a core protein of 70–80 kDa including oligosaccharides (13). Interestingly, the distribution of the cell-associated HS-PGs is influenced by [Ca²⁺]₀; the proportion of HS-PGs on the cell surface increases as [Ca²⁺]₀ is reduced (13). This observation may lead to further understanding how Ca²⁺ regulates functions of parathyroid cells.

This report describes experiments demonstrating that these cells can sense and rapidly respond to changes of [Ca²⁺]₀ and that [Ca²⁺]₀ specifically regulates both the distribution and a rapid cycling of a large proportion of the HS-PGs between the cell surface and an intracellular compartment.

EXPERIMENTAL PROCEDURES

Materials—Guandine HCl and urea were purchased from Life Technologies/Bethesda Research Laboratories; [³⁵S]sulfate (~43 Ci/mg) from Du Pont-New England Nuclear; Sephadex G-50 (fine) and Q-Sepharose from Pharmacia LKB Biotechnology Inc.; heparitinase (Flavobacterium heparinum) from Seikagaku Kogyo, Tokyo through ICN Biochemicals, and diphenylcarbamyl chloride-treated trypsin from Sigma. The culture medium used was a mixture (1:1) of Coon's modified Ham's F-12 and Dulbecco's modified Eagle's minimal essential medium (both without Ca²⁺ and Mg²⁺) and obtained from the NIH media unit supplemented with 5% calf serum (Biofluids), 1% Nutridoma-SP (Boehringer Mannheim), 10 mM HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate (11). CaCl₂ and MgSO₄ or MgCl₂ were added to the indicated final concentrations.

Free Ca²⁺ concentrations in the final media were determined with a calcium electrode (Orion). [Ca²⁺]₀ in medium for cell growth and passage was 0.7 mM. In this study, medium containing 2.1 mM [Ca²⁺]₀ was utilized as high Ca²⁺ medium and 0.05 mM [Ca²⁺]₀, as low Ca²⁺ medium. Medium without CaCl₂ supplemented with 0.5 mM EGTA was used as Ca²⁺-free medium. All studies were done in medium containing 0.5 mM MgSO₄ except for the experiment to examine the effects of Mg²⁺ and Ba²⁺. MgSO₄ was replaced with MgCl₂ in the medium used for labeling cells with [³⁵S]sulfate. Other reagents used were obtained at their purest grade commercially available.

Overnight Labeling of Cell Cultures and Chase Protocols—Cells were cultured in 6-well plastic culture plates (Falcon) with 2 ml of growth medium as described previously (11) and labeled at confluence...
with 10 mM CaCl₂/[^35]S-sulfate in medium with the indicated [Ca²⁺], and 5% calf serum for 20 h. After labeling, cells were washed three times with serum-free medium containing the same [Ca²⁺], as for labeling and chased for 30 min in the radioisotope-free medium. Some cultures in low [Ca²⁺], were then washed three times with and chased in the high Ca²⁺ medium. Some cultures in high [Ca²⁺], were washed twice with the Ca²⁺-free medium with 0.5 mM EGTA and once with the low Ca²⁺-medium followed by a chase in low [Ca²⁺],. Other cultures in high [Ca²⁺], were depleted of extracellular Ca²⁺ rapidly by washing with and chased in Ca²⁺-free medium containing 0.5 mM EGTA. For the determination of trypsin-accessible proteoglycans, cells were treated with 200 μg/ml trypsin at 37 °C for 2 min in serum-free medium containing the same [Ca²⁺], as for the prelabeling period. Cultures were then chased in serum-free medium with appropriate [Ca²⁺], for the times indicated followed by the treatment with trypsin to determine cellular localization of proteoglycans.

For alteration of [Ca²⁺], in medium immediately after labeling, cells labeled in high [Ca²⁺], were washed four times with Ca²⁺-free medium containing 0.5 mM EGTA and once with the low Ca²⁺-medium and followed by chase in low [Ca²⁺],. Cells labeled in low [Ca²⁺], were washed five times with the high Ca²⁺-medium and followed by chase in high [Ca²⁺],. For change of [Ca²⁺], in medium during the chase period, small aliquots of 1 M CaCl₂ or 200 mM EGTA were added to cultures in low or high Ca²⁺ medium, respectively. The final concentration of EGTA was 4.0 mM. The volume of the aliquots added was less than 2% of the final volume. Final concentrations of Ca²⁺ were ∼2.1 mM or <0.05 mM after addition of aliquots of CaCl₂ or EGTA, respectively. Treatment with trypsin for 2 min in the presence of EGTA detached a small proportion of cells. Detached cells were pelleted by microfuge centrifugation to separate them from solubilized materials.

In some experiments, pulse-labeled cells in low [Ca²⁺], were maintained in low [Ca²⁺], in the presence of 4 mM MgCl₂ or 4 mM BaCl₂ followed by trypsin digestion protocols at 90 min of chase time.

Isolation of Proteoglycans—Cell layer extracts, media, and trypsin-solubilized samples in 4 M guanidine HCl solutions were chromatographed on Sephadex G-50 columns equilibrated with 6 M urea, 50 mM sodium acetate, 0.15 M NaCl, 0.5% (v/v) Triton X-100, pH 6.0 (8 M urea buffer) (13). Excluded fractions were applied to Q-Sepharose chromatography (Fig. 1). The two different concentrations of trypsin, 200 μg/ml and 10 mg/ml trypsin for 2 or 15 min as indicated (Table I). The two different concentrations of trypsin, 200 μg/ml and 10 mg/ml, released essentially the same amounts of[^35]S-HS-PGs comprised two species, HS-PG1 and HS-PG2, and the latter contained more than 90% of the radioactivity incorporated into proteoglycans (13). Thus, the results of this study reflect primarily, if not exclusively, the metabolism of the HS-PG2 molecules.

RESULTS

Trypsin Accessibility of Heparan Sulfate Proteoglycans—Trypsin treatment of metabolically radioabeled cell cultures has been shown to be a convenient method to classify operationally cell-associated HS-PGs into cell surface (trypsin-accessible) and intracellular (trypsin-inaccessible) forms (14). For the critical assessment of the amounts and the temporal profiles of HS-PGs in these two forms, it is necessary to determine concentrations of trypsin and incubation times required for the maximum removal of accessible HS-PGs from the cell cultures.

Rat parathyroid cells labeled with[^35]S-sulfate for 20 h in low [Ca²⁺], were treated with 200 μg/ml trypsin at 37 °C for 2 min, and trypsin-accessible and -inaccessible materials were extracted separately. In other cell culture systems, such as rat ovarian granulosa cell cultures (14), 100 μg/ml trypsin is sufficient to cleave all trypsin-accessible proteoglycans in 2 min at 37 °C. Macromolecules metabolically labeled in parathyroid cells with[^35]S-sulfate contained[^35]S-glycoproteins, glycopeptides, and[^35]S-HS-PGs, which were clearly separated into two peaks by Q-Sepharose chromatography (Fig. 1). In subsequent experiments, the two peaks were separately pooled by step elution with 0.3 M NaCl, 8 M urea, and 4 M guanidine HCl buffers and quantitated.[^35]S-HS-PGs comprised two species, HS-PG1 and HS-PG2, and the latter contained more than 90% of the radioactivity incorporated into proteoglycans (13). Thus, the results of this study reflect primarily, if not exclusively, the metabolism of the HS-PG2 molecules.

The incorporation of[^35]S into proteoglycans was ∼70% of the total radioactivity in macromolecules in cultures labeled for 5 min at 37 °C. Treatment with 20 μg/ml trypsin for 2 min failed to release accessible[^35]S-HS-PGs completely, while that for 15 min in low [Ca²⁺], gave essentially the same result (58%) as did 200 μg/ml trypsin, indicating that the 15-min digestion is sufficient for releasing all accessible molecules with 20 μg/ml trypsin. In contrast, release of[^35]S-glycoproteins from the same cell cultures was essentially the same for all concentrations of trypsin and both incubation times (Table I). In high [Ca²⁺], every condition tested (20 μg/ml for 15 min, 200 μg/ml for 2 min) failed to release accessible[^35]S-HS-PGs completely, while that for 15 min in low [Ca²⁺], gave essentially the same result (58%) as did 200 μg/ml trypsin, indicating that the 15-min digestion is sufficient for releasing all accessible molecules with 20 μg/ml trypsin.
portion of HS-PGs present on the cell surface at any instant in accessible become accessible to the enzyme during the incubation. The translocation of HS-PGs from the trypsin-inaccessible indicates that some HS-PGs (-34%) that are initially inaccessible (26-29%) and were used for determining the localization of the cell-associated HS-PGs in subsequent experiments.

Digestion times for cultures in low [Ca2+] were quantitated in more detail (Fig. 2). The extrapolated value for time 0 of digestion (48 ± 3%, mean ± S.E.) indicates the actual proportion of HS-PGs released by increasing trypsin. Trypsin-accessible and -inaccessible molecules were separated into two fractions in the same manner.

Fig. 1. Q-Sepharose anion-exchange chromatograms of macromolecules labeled with [35S]sulfate for 20 h. The parathyroid cells labeled with [35S]sulfate for 20 h in 0.05 mM [Ca2+], medium were treated with trypsin. Trypsin-accessible and -inaccessible 35S-macromolecules excluded from Sephadex G-50 columns were applied to Q-Sepharose chromatography and eluted with a NaCl gradient. A cell extract without trypsin shows three peaks (GP1, GP2, and PGs) in the 0.15-1.0 M NaCl gradient (A). The two early peaks contained 35S-labeled glycoproteins, and the third peak contained proteoglycans (13). The same sample as for A eluted with a 0.3-1.2 M NaCl gradient shows base-line separation into the two peaks, unbound and bound to the column (GPs and PGs), containing 35S-glycoproteins and proteoglycans, respectively (B). Both trypsin-inaccessible (C) and -accessible (D) molecules were separated into two fractions in the same manner.

ml for 2 min, and 10 mg/ml for 2 min) gave essentially the same values for trypsin-accessible 35S-HS-PGs (26-29%) and of 35S-glycoproteins (28-34%), values which are significantly less than those in low [Ca2+], (Table I). Since treatment with 200 µg/ml trypsin for 2 min or 20 µg/ml for 15 min at 37°C was sufficient to release all accessible 35S-HS-PGs and 35S-glycoproteins and proteoglycans (13). The same sample as for A eluted with a 0.3-1.2 M NaCl gradient shows base-line separation into the two peaks, unbound and bound to the column (GPs and PGs), containing 35S-glycoproteins and proteoglycans, respectively (B). Both trypsin-inaccessible (C) and -accessible (D) molecules were separated into two fractions in the same manner.

The amounts of HS-PGs removed by increasing trypsin digestion times for cultures in low [Ca2+] were quantitated in more detail (Fig. 2). The extrapolated value for time 0 of digestion (48 ± 3%, mean ± S.E.) indicates the actual proportion of HS-PGs present on the cell surface at any instant in accessible become accessible to the enzyme during the incubation.

The translocation of HS-PGs from the trypsin-inaccessible to the trypsin accessible compartment can be approximated by an exponential process with a $t_1/2$ of 3.8 min (S.E. of 5%). The parathyroid cells labeled with [35S]sulfate for 20 h in 0.05 mM [Ca2+], medium were treated with 200 µg/ml trypsin at 37°C for the indicated times. Released 35S-macromolecules containing sulfated glycosaminoglycans were separated with Q-Sepharose chromatography from sulfated glycoproteins or glycopeptides (see "Experimental Procedures" for details). Total radioactivity in 35S-proteoglycans is 30,500 ± 500 (low [Ca2+]) and 27,000 ± 740 (high [Ca2+]). cpm/well and in 35S-glycoproteins is 6,900 ± 110 (low [Ca2+]) and 5,400 ± 130 (high [Ca2+]). cpm/well. Each experiment in low or high [Ca2+] was done in different sets of cultures. Data are expressed as mean ± S.D. of four cultures unless otherwise indicated.

| Trypsin | 35S-Proteoglycans released | 35S-Glycoproteins released |
|---------|---------------------------|---------------------------|
| Low     | High                      | Low                       |
| 20 µg/ml| 2 min                     | ND                        | ND                        |
|         | 15 min                    | 29 ± 3                    | 46 ± 5                    |
| 200 µg/ml| 2 min                     | 26 ± 2                    | 42 ± 5                    |
|         | 15 min                    | ND                        | 43 ± 5                    |
| 10 mg/ml| 2 min                     | 28 ± 5                    | 40 ± 2                    |

* ND, not determined.

** Significantly different from values obtained with the 2-min digestion ($p < 0.005$).

† Significantly different from values obtained in low [Ca2+] cultures ($p < 0.005$).

‡ Significantly different from values obtained in low [Ca2+] cultures ($p < 0.05$).

Fig. 2. Effect of digestion time on the amount of trypsin-accessible proteoglycans in 0.05 mM [Ca2+]. The parathyroid cells labeled with [35S]sulfate for 20 h in 0.05 mM [Ca2+], medium were treated with 200 µg/ml trypsin at 37°C for the indicated times. Trypsin-accessible and -inaccessible proteoglycans were separately quantitated by Q-Sepharose column chromatography. Percentages of 35S proteoglycans released are shown. Data represent averages of duplicate cultures in a representative experiment among four independent experiments. Data were approximated by an exponential process with a curve-fitting program. A $t_1/2$ value of 3.8 min (27% S.E.) was obtained, the time 0 and plateau values are 48 ± 3 and 82 ± 3% (mean ± S.E.), respectively.

27% (Fig. 2, solid line). In several independent experiments, the actual proportions of trypsin-accessible IHS-PGs varied (40-60% in 2-min digests and 60-80% in 15-min digests). However, the ratio of trypsin-accessible HS-PGs for the 2-min digest to that for the 15-min digest was constant in every experiment (0.75 ± 0.01, mean ± S.E. in five independent experiments).

A proportion of HS-PGs on the cell surface determined by
the 2-min trypsin digestion was essentially the same at any time (Fig. 3A, see below), while significantly larger proportions appeared on the cell surface during the 15-min trypsin digestion. This observation suggests the presence of internalization of HS-PGs from the cell surface in conjunction with their translocation from the intracellular compartment to the cell surface to maintain cell-associated HS-PGs in a steady state in low [Ca\(^{2+}\)]. In contrast, any movement of HS-PGs between the cell surface and the intracellular compartment was not obvious in high [Ca\(^{2+}\)].

Treatment of cultures with heparitinase for 30 min at 37 °C released essentially the same amount of \(^{35}S\) activity as did the 15-min trypsin treatment, both in low and high [Ca\(^{2+}\)]. (data not shown). The ratio of heparitinase-inaccessible HS-PGs to trypsin-inaccessible HS-PGs was 1.00 ± 0.02 in low [Ca\(^{2+}\)], and 0.98 ± 0.03 in high [Ca\(^{2+}\)], experiments (mean ± S.E., n = 4). This provides evidence that the enzymes were accessible to the HS-PGs without proteolytic degradation of pericellular molecules and that the trypsin-accessible HS-PGs did not contain any intracellular HS-PGs leaking through plasma membrane damaged by the trypsin digestion procedure.

Change of [Ca\(^{2+}\)] in Medium Elicits Redistribution of HS-PGs—Parathyroid cells were maintained in high or low [Ca\(^{2+}\)], and labeled for 20 h with [\(^{35}S\)]sulfate. Treatment with trypsin for 2 min at different times during the chase released ~20 or ~60% of the total cell-associated HS-PGs when the cells were maintained throughout in high or low [Ca\(^{2+}\)], respectively (Fig. 3A). When cells labeled in high [Ca\(^{2+}\)], were washed and chased in low [Ca\(^{2+}\)], the trypsin-accessible HS-PGs increased from ~20 to ~60% by 60 min (t\(_{1/2} \sim 30\) min), nearly the level observed in constant low [Ca\(^{2+}\)], (Fig. 3A). Conversely, when cells labeled in low [Ca\(^{2+}\)], were washed and chased in high [Ca\(^{2+}\)], the trypsin-accessible HS-PGs rapidly decreased (t\(_{1/2} \sim 2-4\) min) from ~60 to ~25%, nearly to the level observed in constant high [Ca\(^{2+}\)], cultures (Fig. 3A).

The time courses for the redistribution of HS-PGs were significantly different for the two changes, from high to low or from low to high [Ca\(^{2+}\)]. This difference may reflect the time required for intracellular Ca\(^{2+}\) to re-equilibrate when medium is changed from high to low [Ca\(^{2+}\)]. For this reason, a second protocol was tested in which cells labeled in high [Ca\(^{2+}\)], were chased in Ca\(^{2+}\)-free medium containing 0.5 mM EGTA to chelate Ca\(^{2+}\) rapidly; as a control the same medium was added to cells labeled in low [Ca\(^{2+}\)], as well (Fig. 3B). Addition of Ca\(^{2+}\)-free medium with EGTA induced a rapid (t\(_{1/2} \sim 1-2\) min) increase of trypsin-accessible HS-PGs, a time course of redistribution close to that observed in the first experiment when cultures were changed from low to high [Ca\(^{2+}\)], (Fig. 3A). Addition of EGTA to cells in low [Ca\(^{2+}\)], only slightly increased the proportion of trypsin-accessible HS-PGs during the chase (shaded square). These observations suggest that the distribution of the HS-PGs between cell surface and intracellular compartments is determined by [Ca\(^{2+}\)], and that the cells rapidly redistribute HS-PGs (t\(_{1/2} < 4\) min) in response to a rapid change of [Ca\(^{2+}\)].

The net incorporation of [\(^{35}S\)]sulfate into HS-PGs that remain in the cell layer during the 20-h labeling period was essentially the same for cultures labeled in either high or low Ca\(^{2+}\) media (data not shown).

**Distribution and Metabolism of Newly Synthesized HS-PGs—** Parathyroid cells were labeled for 10 min with [\(^{35}S\)]sulfate in the low or high Ca\(^{2+}\) medium, washed, and chased for up to 480 min. The synthetic rate of \(^{35}S\) proteoglycans was not different between low and high [Ca\(^{2+}\)], experiments (15,000 ± 600 and 15,500 ± 600 cpm/culture/10 min, mean ± S.E., n = 12 in low and high [Ca\(^{2+}\)], experiments, respectively. In low [Ca\(^{2+}\)], the proportion of trypsin-accessible (2-min digestion) \(^{35}S\)-HS-PGs increased rapidly after a 10-min lag time and reached a plateau of ~40% of the total between 45–60 min, a value which was maintained until 120 min (Fig. 4A). Between 120–240 min most of the \(^{35}S\)-HS-PGs in this compartment disappeared. Approximately 20% of the \(^{35}S\)-HS-PGs was secreted into the medium after a lag time of 10–30 min, reaching a plateau by 120 min. In contrast only ~10% of the total \(^{35}S\)-HS-PGs reached the trypsin-accessible compartment in high [Ca\(^{2+}\)], and approximately 10% was secreted into the medium during the chase (Fig. 4B).

The proportion of \(^{35}S\)-HS-PGs in the trypsin-inaccessible
(intracellular) compartment showed complex changes during the chase in both [Ca\(^{2+}\)] conditions. In low [Ca\(^{2+}\)], the amount decreased from ~100 to ~65% with the same kinetics as for the appearance of \(^{35}\)S-HS-PGs in the trypsin-accessible (cell surface) compartment, reflecting the transport of HS-PGs from the site of sulfation in the Golgi complex to the cell surface. Similar kinetic analyses have also been observed in the rat ovarian granulosa cell system (14). There was an additional, slower decrease between 30–120 min of chase which coincided with the secretion of \(^{35}\)S-HS-PGs into the medium. Until 120 min of chase there is little net loss of \(^{35}\)S-HS-PGs from the cultures (inset, Fig. 4) whereas between 120–240 min chase ~65% are lost, reflecting depolymerization in lysosomes. Both the trypsin-accessible and -inaccessible compartments contributed to this loss. In high [Ca\(^{2+}\)], the trypsin-inaccessible compartment showed only a small decrease (~10%) between 5–60 min, reflecting the appearance of only ~10% of the \(^{35}\)S-HS-PGs in the cell surface compartment. A further decrease (~10%) was observed between 60–120 min reflecting primarily secretion into the medium. This was followed by a large decrease between 120–240 min reflecting lysosomal depolymerization. Net loss of \(^{35}\)S-HS-PGs from the high and low [Ca\(^{2+}\)] cultures between 60–240 min was essentially the same.

The translocation of HS-PGs from the trypsin-inaccessible to the trypsin-accessible compartment and their simultaneous internalization in a steady-state experiment in low [Ca\(^{2+}\)], was demonstrated in a previous section. This observation was further studied by following the movement of \(^{35}\)S-HS-PGs pulse labeled for 10 min in low and high [Ca\(^{2+}\)]. Cultures were treated with trypsin for either 2 or 15 min after the indicated chase periods. In low [Ca\(^{2+}\)], the 15-min trypsin digestion released more \(^{35}\)S-HS-PGs than the 2-min digestion at all times after 30 min of chase (Fig. 5A). The difference reached a maximum (~24% of the total) at 60-min chase and gradually decreased thereafter (Fig. 5, inset). By 120 min, about 20% of the total was secreted into the medium. In high [Ca\(^{2+}\)], in contrast, 15-min trypsin digestions failed to release more \(^{35}\)S-HS-PGs than the 2-min digestions, and ~80% remained in the trypsin-inaccessible compartment throughout the 120-min chase (Fig. 5B). This indicates that most HS-PGs labeled during the 10-min pulse did not reach the cell surface in high [Ca\(^{2+}\)]. These results from the low [Ca\(^{2+}\)] experiments provide additional support for the translocation of HS-PGs from the intracellular compartment to the cell surface and also provide evidence for translocation from the cell surface to the intracellular compartment. Between 30–120 min of chase \(^{35}\)S-HS-PGs are in a relatively steady-state distribution with each 2 or 15 min trypsin digestion releasing the same proportion of the total labeled HS-PGs. Thus, if the HS-PGs are appearing on the cell surface during the time of digestion, steady state requires internalization of the same proportion of HS-PGs when trypsin is not present. Further, the cumulative amount of HS-PGs translocated between 30–120 min (as indicated by the sum of the differences between the amount of HS-PGs released by the 2- and 15-min trypsin digestions, Fig. 5, inset) far exceeds the amount of intracellular HS-PGs at any moment. Thus, HS-PGs must recycle between these two compartments in low [Ca\(^{2+}\)]. The results for the high [Ca\(^{2+}\)] experiments indicate that HS-PGs recycle very little in this condition.

\[\text{[Ca}^{2+}]\text{, in Medium Regulates the Transport and Distribution of Newly Synthesized HS-PGs—Parathyroid cells maintained in various [Ca\(^{2+}\)] were labeled for 10 min and chased for 60 min before trypsin treatment for 2 min. The proportion of trypsin-accessible \(^{35}\)S-HS-PGs decreased with increasing}

\[\text{Refer to the figure for graphical representation of these data.}

\[\text{Fig. 5. Determination of trypsin-accessible \(^{35}\)S-proteoglycans obtained with 2- or 15-min trypsin treatments in cultures pulsed for 10 min and chased in 0.05 or 2.1 mM [Ca\(^{2+}\)]. Cells labeled in low (A) or high (B) for 10 min were chased for the indicated times before determining the trypsin-accessible \(^{35}\)S-proteoglycans by trypsin treatment at 37 °C for 2 min (□) or 15 min (△). \(^{35}\)S-Proteoglycans secreted into medium (Δ) are also indicated. Inset, the difference in trypsin-accessible \(^{35}\)S-proteoglycans is shown for the 15 min versus the 2-min treatments in low [Ca\(^{2+}\)]. (●) or in high [Ca\(^{2+}\)]. (○). Data represent averages of duplicate cultures. Total radioactivity incorporated into proteoglycans is 17,600 ± 440 cpm/well in low [Ca\(^{2+}\)] experiments and 11,700 ± 660 cpm/well in high [Ca\(^{2+}\)]. (mean ± S.E.) Each experiment was done in different sets of cultures.}
[Ca\(^{2+}\)], in media in a dose-dependent manner with \(\sim 0.3\ \text{mM}\) [Ca\(^{2+}\)], for the ED\(_{50}\) (Fig. 6).

We next examined at which times [Ca\(^{2+}\)], affects the transport and distribution of HS-PGs. Cultures were incubated in low or high [Ca\(^{2+}\)], for 20 h and pulse labeled for 10 min in the same [Ca\(^{2+}\)], medium. The cultures were then washed and chased either with the medium containing the same [Ca\(^{2+}\)], or with the medium containing reciprocal [Ca\(^{2+}\)]. The trypsin accessibilities of \(^{35}\text{S}-\text{HS-PGs}\) observed after changing [Ca\(^{2+}\)], from low to high are virtually identical to those for cultures maintained in high [Ca\(^{2+}\)], throughout (Fig. 7A). Changing [Ca\(^{2+}\)], from high to low gave nearly identical results as for cultures maintained in low [Ca\(^{2+}\)], throughout (Fig. 7B). Thus, trypsin accessibility of \(^{35}\text{S}-\text{HS-PGs}\) was determined entirely by [Ca\(^{2+}\)], in the chase media but not by [Ca\(^{2+}\)], during the biosynthesis period.

This observation was further verified by switching [Ca\(^{2+}\)], at the indicated times during the chase following a 10-min pulse (Fig. 8). The parathyroid cells responded rapidly (\(t_{\text{chase}} < 5\ \text{min}\)) to a change of [Ca\(^{2+}\)], throughout the 120-min chase (Fig. 8, dashed lines). As calcium ions in high Ca\(^{2+}\) medium were chelated by EGTA, 2 min trypsin accessible \(^{35}\text{S}-\text{HS-PGs}\) rapidly increased to 40–45% at any time between 30- and 90-min chase (Fig. 8B). Amounts of secreted \(^{35}\text{S}-\text{HS-PGs}\) were also stimulated to \(\sim 20\%\) in response to lowering [Ca\(^{2+}\)]. Changing from low to high [Ca\(^{2+}\)], by adding an aliquot of CaCl\(_2\) at a 30-min chase rapidly decreased the trypsin-accessible \(^{35}\text{S}-\text{HS-PGs}\) to the level obtained in the constant high [Ca\(^{2+}\)], treatment (Fig. 8A). Thus, HS-PGs labeled and chased in high [Ca\(^{2+}\)], for up to at least 90 min were fully capable of reaching the cell surface when [Ca\(^{2+}\)], was lowered.

Effects of Other Divalent Cations on the Distribution of HS-PGs—Parathyroid cells were labeled with \(^{35}\text{S}\)-sulfate for 10 min in low [Ca\(^{2+}\)], and then chased in the low Ca\(^{2+}\) medium without or with either 4 mM MgCl\(_2\) or 4 mM BaCl\(_2\) for 90 min followed by trypsin treatment for 2 or 15 min (Table II). The proportions of trypsin-accessible HS-PGs were not significantly altered by either Mg\(^{2+}\) or Ba\(^{2+}\), and the 15-min trypsin digestion in the presence of 4 mM MgCl\(_2\) or BaCl\(_2\) released more \(^{35}\text{S}-\text{HS-PGs}\) than the 2-min treatment. Increasing concentrations of MgCl\(_2\) up to 8 mM did not significantly alter the distribution of HS-PGs (data not shown). A control maintained in high [Ca\(^{2+}\)], yielded the characteristic low proportion of trypsin-accessible \(^{35}\text{S}-\text{HS-PGs}\) for both digestion times. These results indicate that neither Mg\(^{2+}\) nor Ba\(^{2+}\)
Effects of Mg2+ and Ba2+ on the trypsin accessibility of labeled proteoglycans in parathyroid cells

Cells labeled with [35S]sulfate for 10 min in 0.05 mM [Ca2+], were maintained in medium containing 4 mM MgCl2 or 4 mM BaCl2 in addition to 0.05 mM [Ca2+]. After 90 min, cells were treated with 200 µg/ml trypsin for 2 or 15 min at 37 °C. Data represent averages of duplicate cultures.

| Tryptsin-accessible 35S-proteoglycans | 2-Min treatment | 15-Min treatment |
|--------------------------------------|-----------------|-----------------|
| % cell-associated 35S-proteoglycans   |                 |                 |
| **Experiment 1**                     |                 |                 |
| 2.1 mM Ca2+                         | 10              | 13              |
| 0.05 mM Ca2+                        | 56              | 72              |
| + 4 mM Mg2+                         | 52              | 70              |
| + 4 mM Ba2+                         | 45              | 67              |
| **Experiment 2**                     |                 |                 |
| 0.05 mM Ca2+                        | 43              | 43              |
| + 4 mM Mg2+                         | 43              | 43              |
| + 4 mM Ba2+                         | 43              | 43              |

substituted for Ca2+ in terms of the distribution or recycling of 35S-HS-PGs regulated by [Ca2+].

DISCUSSION

Cell-associated HS-PGs are widespread, and a large proportion appears to be intercalated in the plasma membrane (15, 16) with a smaller proportion in some cells anchored by phosphatidylinositol (17-19). Although precise functions of cell surface HS-PGs are uncertain, they appear to be involved in cell-matrix interactions (20-22), cytoskeletal organization (23), and as receptors for molecules, such as transforming growth factor-β (24-26). The rat parathyroid cell line used in this study synthesizes proteoglycans which are almost exclusively HS-PGs (15). The predominant form, HS-PG2, has a structure similar to that described for many other cell surface HS-PGs; a core glycoprotein of ~70 kDa with three to four HS chains attached4 (15). HS-PG2 contains more than 90% of the radioactivity incorporated into proteoglycans in all cellular compartments studied in this paper when [35S]sulfate is used as a metabolic precursor (13). Thus, the results of this study reflect primarily, if not exclusively the metabolism of the IIS-PG2 molecules in both high and low [Ca2+] conditions.

We described several pulse label and chase experiments of parathyroid cells followed by treatment of cell layers with trypsin to demonstrate effects of [Ca2+], on the cellular distribution of HS-PGs and how the cells respond to changes of [Ca2+]. The results in steady state (Table I and Figs. 2 and 3) indicate that the distribution of HS-PGs is reversibly regulated by [Ca2+], and that a portion of HS-PGs recycles between the cell surface and intracellular compartment in low [Ca2+], but not in high [Ca2+].

The 10-min pulse-chase experiments (Figs. 4 and 5) define the following three distinct proteoglycan populations: population 1 with a long half-life on the cell surface or in a pericellular matrix; population 2 which can cycle between the cell surface and an intracellular compartment; and population 3 which is secreted. Population 1 is most readily apparent in the cultures maintained in high [Ca2+],. In these cultures ~10% of the total HS-PGs appear in the trypsin-accessible compartment after a lag of ~10 min reflecting the transit time from completion in the Golgi complex to appearance at the cell surface. This population remains nearly constant through 8 h of chase indicating that it has a long half-life in the cultures. It is also present in cultures maintained in low [Ca2+], as indicated by the 10-15% of the labeled HS-PGs remaining in the trypsin-accessible compartment after 4 h of chase in which time population 2 has been degraded (see below). HS-PGs in population 1, then, may be involved in structural functions such as cytoskeletal organization (23) or cell attachment (20-22).

Population 2, at least 50% of the total HS-PG, is most apparent in the cultures maintained in low [Ca2+],. HS-PGs in this population appear in the trypsin-accessible compartment after a lag of ~10 min as in the case of population 1 HS-PGs. In low [Ca2+],, molecules in this population cycle between the cell surface and an intracellular compartment (see below). Population 2 has a functional life time of at least 90 min (30-120 min of chase) before depolymerization in lysosomes, a process which occurs sometime between 120-240 min of chase. In high [Ca2+],, this population is not identified by the procedure of trypsin digestion. When [Ca2+], is switched, however, a similar proportion of HS-PGs to that of population 2 rapidly (ts ~ 4 min) changes their distribution to reflect the final [Ca2+]; either redistributing to the cell surface when [Ca2+] is increased or becoming sequestered in the intracellular compartment when [Ca2+] is decreased. Thus, it is suggested that the distribution of population 2 is regulated by the changes of [Ca2+] or [Ca2+]4.

The experiment depicted in Fig. 2 showed that the proportion of population 2 HS-PGs in the trypsin-accessible compartment for cultures in low [Ca2+], changed with time of digestion, demonstrating that the molecules in this compartment recycle. Extrapolation of the curve to zero digestion time revealed that ~48% of the total labeled HS-PGs are on the cell surface at any time; these include the population 1 HS-PGs plus the proportion of population 2 HS-PGs which are on the surface. The plateau value, ~82%, includes the additional amount of population 2 HS-PGs which cycled to the cell surface during the longer digestion times. When these cultures were labeled overnight, the proportion of the labeled HS-PGs in population 1, ~25% (see Table I and Fig. 3A), was greater than for the 10-min pulse-chase experiment, as would be expected because of the longer half-life of population 1 molecules. When this 25% is subtracted from the plateau

| Experiment | 2-Min treatment | 15-Min treatment |
|------------|-----------------|-----------------|
| 2.1 mM Ca2+  | 10              | 13              |
| 0.05 mM Ca2+  | 56              | 72              |
| + 4 mM Mg2+   | 52              | 70              |
| + 4 mM Ba2+   | 45              | 67              |

4 Phospholipase C (Bacillus thuringiensis) specific for phosphatidylinositol involved in membrane anchorage (a generous gift from Dr. M. G. Low, Columbia University) did not release a significant amount of 35S-HS-PGs from the rat parathyroid cell cultures (Y. Takeuchi, M. Yanagishita, and V. C. Hascall, unpublished observation).
value it is evident that ~57% of the labeled HS-PGs in this experiment were in population 2, of which ~23% were on the cell surface and ~54% in the intracellular compartment. The time of diastere was required to reach halfway between the initial value (time 0) and the plateau value is ~3.8 min and corresponds to the t_r for exocytosis from the intracellular compartment to the cell surface. Conversely, the t_r for endocytosis from the cell surface to the intracellular compartment is ~2.6 min. Assuming that the functional life time of population 2 HS-PGs is 90 min (30-120 min of chase) (Fig. 4A), these molecules would recycle ~10 times between these two compartments.

The kinetics exhibited by these HS-PGs are similar to those observed for lysosomal acid phosphatase, most of which migrates from the Golgi complex to the cell surface where it recycles with the endosome compartment for ~5 h before being transferred to lysosomes (27). Parameters for recycling of the HS-PGs are also similar to those reported for cell surface receptors which recycle with the endosome compartment (28), such as receptors for transferrin, low density lipoprotein, and mannose 6-phosphate/insulin-like growth factor II (29). Thus, it is likely that population 2 HS-PGs are being transferred to lysosomes (27). Parameters for recycling of the HS-PGs molecules have the same probabilities for (a) exocytosis from the intracellular pool and (b) endocytosis from the cell surface. In this case, t_r (endocytosis)/t_r (exocytosis) = [HS-PGs (cell surface)]/[HS-PGs (intracellular compartment)]. This gives the t_r (endocytosis) = ~3.8 min × 23%/34% = ~2.6 min.

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