

Differential Storage of Prolactin, Granins (Chromogranin B and Secretogranin II), and Constitutive Secretory Markers in Rat Pituitary GH₄C₁ Cells*

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The rat pituitary cell line GH₄C₁ secretes granins (chromogranin B and secretogranin II) and prolactin by the regulated secretory pathway. The intracellular storage of prolactin is preferentially induced by hormone treatment with estradiol, insulin, and epidermal growth factor. The goal of this study was to determine the effect of hormone treatment on storage of granins and constitutive secretory markers. The granins were efficiently stored in both hormone-treated and -untreated cells (17% of total secreted in 4 h). Secreted alkaline phosphatase (SEAP), a truncated membrane protein that would not be expected to enter secretory granules, and glycosaminoglycan, a marker for the constitutive secretory pathway, exhibited 70–80% secretion under both conditions. In comparison, the relative prolactin secretion was 31 and 68% from hormone-treated and -untreated cells, respectively. Phorbol ester and KCl stimulated prolactin secretion 2.3-fold from untreated cells and 5.5-fold from hormone-treated cells. In contrast, SEAP secretion was stimulated 1.5-fold from both treated and untreated cells, consistent with secretion by the constitutive secretory pathway. Stimulated secretion of granins was detected from both hormone-treated and -untreated cells. These results suggest that granin and prolactin storage are differentially regulated and that the constitutive secretory pathway is not affected by hormone treatment.

Endocrine cells exhibit a constitutive secretory pathway, common to all eukaryotic cells, as well as a regulated secretory pathway (Kelly, 1985). The latter is characterized by intracellular storage of secretory proteins, slow basal release, and stimulated secretion in response to secretagogues. Granins (chromogranin A, chromogranin B, secretogranin II) are a family of sulfated, calcium-binding proteins that are co-stored with peptide and amine hormones in secretory granules of endocrine cells. Thus, granins can serve as general markers for the regulated secretory pathway in endocrine cells (Scammell, 1993). It has been suggested that granins play a direct role in the sorting and packaging of peptide hormones in secretory granules (Rosa *et al.*, 1985; Gorr *et al.*, 1987a; Huttner *et al.*, 1991; Scammell, 1993). In support of this hypothesis, granins exhibit

calcium-induced aggregation at low pH, *i.e.* the conditions found in the trans-Golgi network and secretory granules of endocrine cells (Gorr *et al.*, 1987b, 1988, 1989; Gerdes *et al.*, 1989; Chanaat and Huttner, 1991; Thompson *et al.*, 1992). These aggregates can include other regulated secretory proteins but exclude constitutive secretory proteins (Gorr *et al.*, 1989; Huttner *et al.*, 1991). Specific sorting receptors have also been proposed to act in the segregation of regulated secretory proteins (Kelly, 1985). However, such sorting receptors have not been conclusively identified (Gorr *et al.*, 1992).

The rat pituitary cell line GH₄C₁, a subclone of the GH3 cell line, stores prolactin, chromogranin B, and secretogranin II in secretory granules (*e.g.* Scammell *et al.* (1990a)) and secretes all three proteins in response to extracellular stimulation (Hinkle *et al.*, 1992). The expression of prolactin and granins is induced by treating the cells with a combination of estradiol, insulin, and epidermal growth factor (EGF)¹ (Scammell *et al.*, 1986, 1990a; Thompson *et al.*, 1992). In addition, this hormone treatment preferentially induces prolactin granulogenesis and the intracellular storage of prolactin (Scammell *et al.*, 1986; Reaves *et al.*, 1990). However, the expression or storage of growth hormone, which is present in small amounts in these cells, is not induced by hormone treatment (Scammell *et al.*, 1986). Also, when insulin is expressed in transfected GH₄C₁ cells, the peptide is sorted to the regulated secretory pathway, but insulin does not exhibit a preferential increase in storage in hormone-treated cells (Reaves *et al.*, 1990). To determine if induction of storage and granulogenesis is specific for prolactin, the storage of the endogenous-regulated secretory proteins secretogranin II and chromogranin B, as well as constitutive secretory markers were compared. Hormone treatment induced granin synthesis, but it did not preferentially stimulate granin storage. The secretion and storage of secreted placental alkaline phosphatase (SEAP) and glycosaminoglycans was not affected by hormone-treatment.

EXPERIMENTAL PROCEDURES

Materials—Gelding horse serum was from Hyclone Laboratories, Logan, UT; Lipofectamine, geneticin (G418), and mouse EGF were obtained from Life Technologies, Inc.; Na₂³⁵SO₄ (carrier-free) was purchased from ICN Radiochemicals, Irvine, CA. Phorbol 12-myristate 13-acetate, 4-methylumbelliferyl β -D-xyloside, β -estradiol, and bovine insulin were from Sigma; 4 α -phorbol 12,13-didecanoate was purchased from Calbiochem, La Jolla, CA, and bovine serum albumin, fraction V (protease free), was from Boehringer Mannheim. The plasmid pcDNA3 was obtained from Invitrogen Corp., San Diego, CA, while pSEAP-control and the Great Escape SEAP detection kit were purchased from Clontech, Palo Alto, CA. Horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin was from Amersham Corp.; horseradish-conju-

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¹ The abbreviations used are: EGF, epidermal growth factor; SEAP, secreted human placental alkaline phosphatase; EIE, estradiol/insulin/EGF; PAGE, polyacrylamide gel electrophoresis.

gated goat anti-rabbit immunoglobulin was from Bio-Rad. Nitrocellulose membrane (Protran BA83) was obtained from Schleicher and Schuell, Keene, NH. Other reagents were analytical grade and were obtained from various suppliers.

Cell Culture—GH₄C₁ cells were cultured in Gelding medium (Dulbecco's modified Eagle's medium (42.5%), Ham's F-10 medium (42.5%), and gelding horse serum (15%) supplemented with penicillin (100 µg/ml) and streptomycin (100 units/ml)) at 37 °C, in a humidified atmosphere with 5% CO₂. For each experiment, the cells were detached with phosphate-buffered saline, pH 7.2, containing 0.2 g/liter of EDTA and then plated in gelding medium at 10⁵ cells/well in 6-well cell culture plates (10⁴ cells/cm²). Hormone-treated cells were cultured for 4 days in gelding medium supplemented with 1 nM estradiol, 300 nM insulin, and 10 nM EGF (+EIE). Untreated cells were cultured in parallel in gelding medium (−EIE).

Transfection and SEAP Assays—The plasmids pSEAP-control and pcDNA3 were co-transfected at a ratio of 10:1 into GH₄C₁ cells using Lipofectamine, as recommended by the manufacturer. Control cells were only transfected with pcDNA3, which contains the neomycin resistance gene. Transfected cells were selected with Geneticin (320 µg/ml). Colonies of resistant cells were directly collected with a pipette, expanded, and tested for SEAP secretion. Two transfected GH₄C₁ cell lines, S17 (SEAP positive) and S2 (negative control) were used for the experiments reported. Both transfected cell lines expressed and secreted prolactin. SEAP activity in secretion media and cell extracts were quantitated with the Great Escape chemiluminescent assay in a Berthold LB 9501 luminometer (Wallac, Inc., Gaithersburg, MD).

Metabolic Labeling—This was conducted at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were preincubated for 30–45 min in Labeling Buffer (15 mM Hepes, pH 7.4, 0.3 mM CaCl₂, 3.82 mM KCl, 0.61 mM KH₂PO₄, 0.62 mM MgCl₂, 127 mM NaCl, 1.08 mM Na₂HPO₄, 6.1 mM D-glucose, 1.0 mM sodium pyruvate, 2.05 mM glutamine, 1 mg/ml bovine serum albumin) and then incubated for 4 h in Labeling Buffer containing 200 µCi/ml of Na₂³⁵SO₄. The medium was collected and centrifuged for 2 min at 16,000 × *g*, and the supernatant fraction (Labeling Medium) was stored at −20 °C until use. The cells were rinsed with Labeling Buffer containing 5 mM Na₂SO₄ and then chase incubated for 15 min in the same buffer supplemented with 50 mM NaCl or 50 mM KCl. At the end of the chase period, the medium was centrifuged and stored frozen (Chase Medium). The cells were stored frozen until extraction. The cells were either directly solubilized in SDS-sample buffer or extracted in 1% Triton X-100. In both protocols, the cell extracts were centrifuged for 2 min at 16,000 × *g* and the supernatant fractions were analyzed by SDS-PAGE. The two extraction procedures produced similar results.

In some experiments, 4-methylumbelliferyl β-D-xyloside was added from a 100 mM stock solution in Me₂SO₄ to a final concentration of 1 mM. The xyloside was added to the preincubation and labeling buffers. Control incubations contained 1% Me₂SO₄.

Secretion Experiments with Unlabeled Proteins—Cells cultured in 6-well plates were rinsed with Labeling Buffer or, for experiments with SEAP containing samples, Krebs-Ringer-Hepes buffer (129 mM NaCl, 5 mM NaHCO₃, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1 mM CaCl₂, 1.2 mM MgCl₂, 2.8 mM glucose, and 10 mM HEPES, pH 7.4) and preincubated in this buffer for 1 h. Fresh buffer was added to the wells, and incubation continued at 37 or 16 °C for 4 h. Stimulated secretion was measured in buffer containing 50 mM KCl and 100 nM phorbol 12-myristate 13-acetate. Control samples contained 50 mM NaCl and 100 nM phorbol didecanoate. Medium samples were centrifuged for 2 min at 16,000 × *g* to remove cell debris before use. Cells were resuspended in 50 mM HEPES, 12.5 mM EDTA, pH 7.2, frozen and thawed, and homogenized through a 26-gauge needle. SEAP containing samples were homogenized in Krebs-Ringer-Hepes buffer.

Immunoblotting—Total cellular and secreted prolactin and secretogranin II were analyzed by dot-immunoblotting using antibodies to ovine prolactin and rat secretogranin II, respectively. The antibodies were a kind gift from Dr. Jonathan G. Scammell, University of South Alabama. The specificity of the antibodies has previously been established in GH₄C₁ cells (Scammell *et al.*, 1990b; Hinkle *et al.*, 1992). Medium and cell extracts were applied to a nitrocellulose membrane in a 96-well dot-blotting apparatus (Bio-Rad). The wells were washed with 100 µl of TTBS (20 mM Tris-HCl, 500 mM NaCl, 0.05% Tween-20, pH 7.5), and then the membranes were blocked for 30 min with 5% nonfat dry milk in TTBS. The membranes were incubated overnight with the monoclonal anti-prolactin antibody diluted 1:2000 or the polyclonal anti-secretogranin II antiserum diluted 1:100–1:500 in TTBS containing 1 mg/ml of bovine serum albumin. The blots were incubated with horseradish peroxidase-conjugated sheep anti-mouse or goat anti-rab-

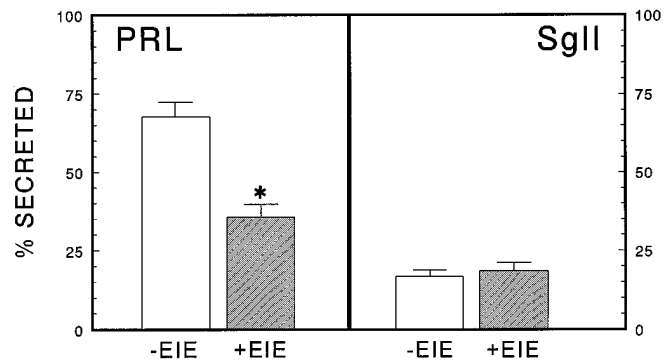


FIG. 1. Relative secretion of prolactin (PRL) and secretogranin II (SgII) from GH₄C₁ cells. The secreted and cellular amounts of PRL and SgII from untreated (−EIE) and hormone-treated cells (+EIE) were quantitated by dot-immunoblotting after 4 h of incubation. % Secreted = secreted amount/total (secreted + cellular) amount × 100%. Results from seven (PRL) and four (SgII; *n* = 14) independent experiments were analyzed and presented as mean ± S.E. Asterisk, different from untreated PRL samples (*p* < 0.0001; *n* = 21–23).

bit immunoglobulin (1:2000 in TTBS with 1 mg/ml of bovine serum albumin), respectively, for 2 h and developed with chemiluminescent substrate and recorded on x-ray film. The films were quantitated by densitometric scanning, as described below. Incubation with the second antibody alone produced a negligible signal.

SDS-PAGE—Aliquots of media samples and cell extracts were analyzed by SDS-PAGE on 10 or 15% gels (Laemmli, 1970). The latter were used for analysis of glycosaminoglycans. The gels were fixed and stained in 10% methanol, 15% acetic acid containing Coomassie Blue R-250 followed by destaining in methanol/acetic acid. The gels were rinsed for 30 min in distilled water followed by 30 min in 1 M salicylate (Chamberlain, 1979). The gels were then dried and exposed to Kodak X-Omat film at −80 °C.

Fluorographs and dot-blots were quantitated by densitometric scanning using a Bio Image Visage 60 image scanner (Bio Image, Ann Arbor, MI). The film exposure times were adjusted to ensure that the bands were in the linear range of the film. In general, the samples from chase incubations were exposed longer than the cell extracts and labeling medium samples and are, therefore, not directly comparable. Due to some interexperimental variation, the data on stimulated secretion of prolactin and SEAP were normalized by dividing all values in an experimental group (± stimulation) by the lowest value from the unstimulated samples. The data were analyzed by two-tailed Student's *t* test or analysis of variance with Student-Newman-Keuls multiple comparison post test. *p* < 0.05 was considered statistically significant.

RESULTS

Rat pituitary GH₄C₁ cells were cultured for 4 days with or without hormone treatment (EIE: 1 nM estradiol, 300 nM insulin, 10 nM EGF). Immunofluorescence microscopy of untreated cells indicated that prolactin was predominantly found in a perinuclear region consistent with localization in the Golgi complex. Only few cytoplasmic granules were detected in these cells. Hormone-treated cells, on the other hand, exhibited a bright punctuate pattern throughout the cytoplasm, consistent with storage of prolactin in secretory granules (not shown). Prolactin was quantitated by dot-immunoblotting of cell extracts and media samples. Densitometric scanning of the immunoblots indicated that hormone treatment induced the cellular and extracellular prolactin levels by 20- and 3-fold, respectively, in agreement with earlier reports (Scammell *et al.*, 1986; Reaves *et al.*, 1990; Hinkle *et al.*, 1992).

To determine if the storage of secretogranin II was similar to that of prolactin, the relative secretion of both proteins in 4 h was quantitated (Fig. 1). The relative prolactin secretion was 68% from untreated cells and 36% from hormone-treated cells, consistent with increased prolactin storage in hormone-treated cells. In contrast, secretogranin II exhibited 15–20% secretion under both conditions, suggesting that the protein is predominantly stored in both untreated and hormone-treated cells.

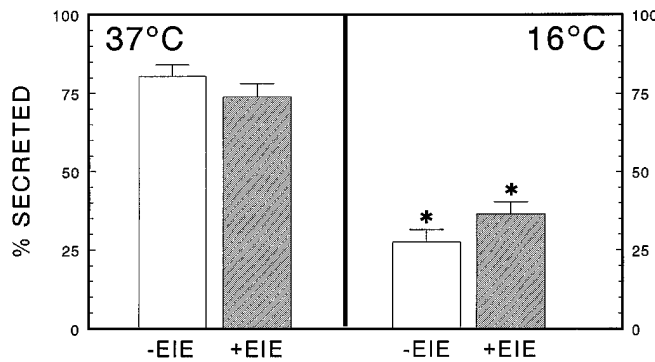


FIG. 2. **Relative secretion of SEAP.** The secreted and cellular amounts of SEAP from untreated (–EIE) and hormone-treated cells (+EIE) were quantitated enzymatically after 4 h of incubation at 37 or 16 °C. % Secreted = secreted amount/total (secreted + cellular) amount \times 100%. Results from two independent experiments were analyzed and presented as mean \pm S.E. asterisk, different from 37 °C samples ($p < 0.001$; $n = 6-11$).

Since an endogenous marker protein for the constitutive secretory pathway has not been identified in GH₄C₁ cells, a truncated form of human placental alkaline phosphatase (SEAP) was expressed in these cells and evaluated as a potential marker for constitutive secretion. SEAP was released linearly from transfected cells, and about 80% of total SEAP activity was found in the medium of untreated cells after 4 h (Fig. 2). Hormone treatment had no significant effect on the relative secretion of SEAP. When the cells were incubated at 16 °C, to block protein export, only about 30% of total SEAP activity was secreted in 4 h (Fig. 2). Incubation at 16 °C did not lead to a build-up in intracellular SEAP, suggesting that synthesis or degradation of SEAP was affected in addition to secretion.

The rapid basal secretion of SEAP suggested that the enzyme was secreted constitutively from both untreated and hormone-treated GH₄C₁ cells. To further test this, secretion was stimulated with phorbol ester and KCl and compared with unstimulated secretion. SEAP secretion was stimulated 1.5-fold from both untreated and hormone-treated cells (Fig. 3A), consistent with secretion by the constitutive secretory pathway. Basal SEAP secretion resumed when cells that had been incubated at 16 °C were warmed to 37 °C, but stimulated secretion did not increase (not shown). Phorbol ester and KCl stimulated prolactin secretion 2.3-fold from untreated cells and 5.5-fold from hormone-treated cell, respectively (Fig. 3B). The stronger stimulation of hormone-treated cells was in agreement with an earlier report (Reaves *et al.*, 1990).

Sulfate-labeled GH₄C₁ cells were used to further analyze the secretion of granins from untreated and hormone-treated cells. Since tyrosine-sulfation of secretogranin II and chromogranin B occurs in the trans-Golgi network, this modification is a specific marker for the late stages of the secretory pathways (Huttner, 1988). GH₄C₁ cells were radiolabeled with [³⁵S]sulfate for 4 h to allow sufficient time for the sulfated granins to reach the storage compartment. Medium and extracts of hormone-treated cells contained a high molecular weight sulfated molecule, presumably representing a heparansulfate proteoglycan (Hinkle *et al.*, 1992), chromogranin B, and secretogranin II (Fig. 4, lanes 1 and 3). Similar results have been reported for untreated cells (Hinkle *et al.*, 1992). The cellular and secreted amounts of secretogranin II each increased about 6-fold upon hormone treatment, while chromogranin B synthesis was increased about 2-fold (Table I). The higher stimulation of secretogranin II synthesis compared with chromogranin B synthesis reflects similar differences in mRNA levels after hormone-treatment (Thompson *et al.*, 1992). Hormone treatment did not

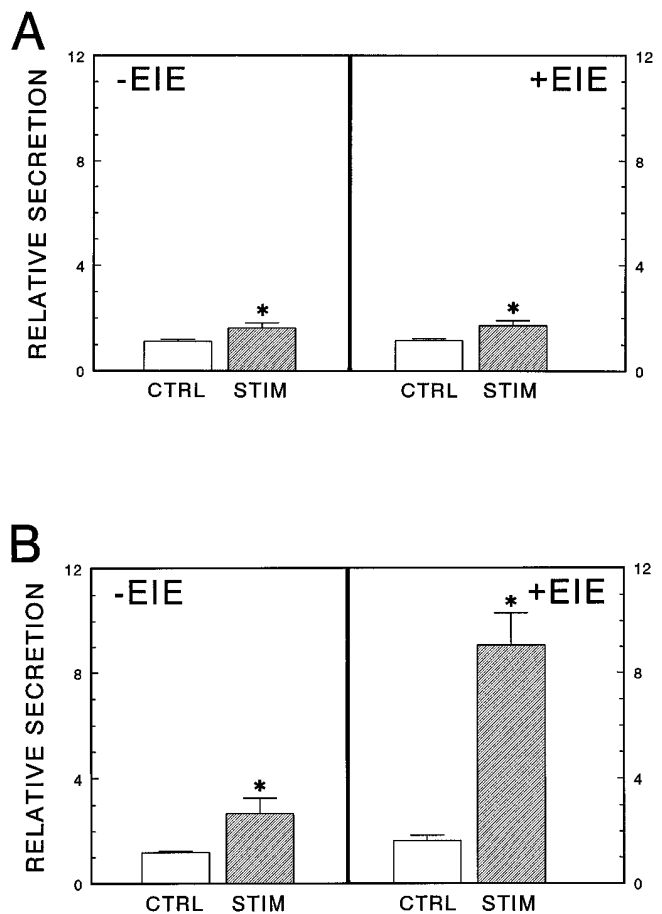


FIG. 3. **Stimulated secretion of SEAP (A) and prolactin (B) from untreated (–EIE) and hormone-treated cells.** The cells were incubated with 50 mM NaCl + 100 nM 4 α -phorbol 12,13-didecanoate (CTRL) or 50 mM KCl + 100 nM phorbol 12-myristate 13-acetate (STIM). Data from two to six independent experiments, each performed in triplicate, were normalized as described under “Experimental Procedures,” and expressed as mean \pm S.E. Asterisk, different from control (SEAP, –EIE, $p < 0.03$, $n = 6$; SEAP, +EIE, $p < 0.02$, $n = 6$; prolactin, –EIE, $p < 0.02$, $n = 9$; prolactin, +EIE: $p < 0.0001$, $n = 17-18$).

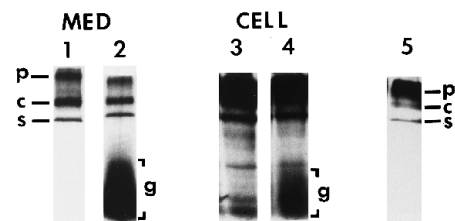


FIG. 4. **Metabolic labeling of GH₄C₁ cells with Na₂³⁵SO₄.** Hormone-treated cells were labeled with [³⁵S]sulfate in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of methylumbelliferyl xyloside. Aliquots of labeling medium (MED) and cell extracts (CELL) were analyzed by SDS-PAGE and fluorography. The positions of sulfated proteoglycan (p), chromogranin B (c), secretogranin II (s), and glycosaminoglycans (g) are indicated. Lane 5 is a shorter exposure of lane 3 to better visualize the chromogranin B band.

appear to selectively increase the cellular amounts of sulfated granins (Table I). To further determine if hormone-treatment affected granin storage, the relative secretion of sulfated granins was calculated for hormone-treated and untreated cells. Approximately 33% of each granin was secreted from both hormone-treated and untreated cells, confirming that hormone-treatment did not increase the fraction of stored granins (Fig. 5).

To test if the 30% secretion rate of sulfated granins represented the regulated (slow) or constitutive (fast) secretory

TABLE I

Stimulation of cellular and secreted granins by hormone treatment

Hormone-treated and -untreated GH_4C_1 cells were sulfate-labeled for 4 h. The cellular (cell) and secreted (medium) amounts of sulfated chromogranin B (CgB) and secretogranin II (SgII) were quantitated by SDS-PAGE and fluorography. The mean ratio of hormone-treated/untreated is shown for each experiment.

	CgB	SgII	n
Experiment I			
Cell	1.7	6.0	(3)
Medium	2.6	7.4	(3)
Experiment II			
Cell	1.5	5.1	(2)
Medium	2.8	5.8	(2)

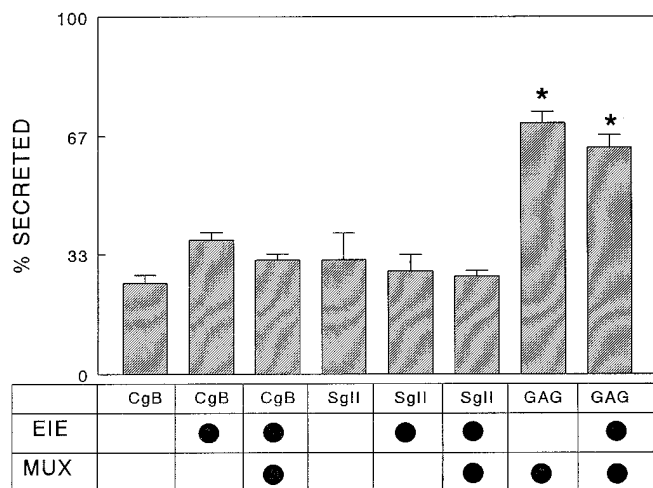


FIG. 5. Relative secretion of granins and glycosaminoglycan from $[^{35}S]$ sulfate-labeled GH_4C_1 cells. Hormone-treated (EIE) or untreated (blank) cells were radiolabeled in the presence (MUX) or absence (blank) of methylumbelliferyl xyloside. Aliquots of labeling medium and cell extracts were analyzed by SDS-PAGE and fluorography. Radioactive chromogranin B (CgB), secretogranin II (SgII), and glycosaminoglycans (GAG) were quantitated by densitometric scanning of the radiographic film. % Secreted = secreted amount/total (secreted + cellular) amount $\times 100\%$. Results from three independent experiments were analyzed and presented as mean \pm S.E. Asterisk, different from granin samples ($p < 0.001$, $n = 3-11$).

pathway, the cells were labeled in the presence of methylumbelliferyl xyloside to stimulate synthesis of sulfated glycosaminoglycans, a marker for the constitutive secretory pathway (Fig. 4; lanes 2 and 4). The xyloside did not affect the proportion of sulfated granins detected in the secretion medium (Fig. 5). Glycosaminoglycan synthesis was induced approximately 11-fold by xyloside treatment. In contrast to the granins, the glycosaminoglycans were predominantly (67% of total) found in the secretion medium (Figs. 4 and 5). These results are consistent with sorting and storage of granins in the regulated secretory pathway in both hormone-treated and untreated cells, while glycosaminoglycans are rapidly secreted by the constitutive secretory pathway. Stimulated secretion of sulfated granins was tested by incubating the cells for 15 min in the presence of 50 mM KCl. In two experiments, secretion of chromogranin B was stimulated about 2.3-fold from both hormone-treated and untreated cells (Fig. 6A), while secretion of secretogranin II was stimulated 2-fold from hormone-treated cells and 1.6-fold from untreated cells (Fig. 6B). Due to the small amounts of the diffuse sulfated glycosaminoglycan band present in the chase media, the constitutive marker could not be reliably quantitated in the stimulation experiments. However, in a control experiment, SEAP secretion was stimulated 1.2-fold ($p < 0.06$) in 1 h by 50 mM KCl (not shown).

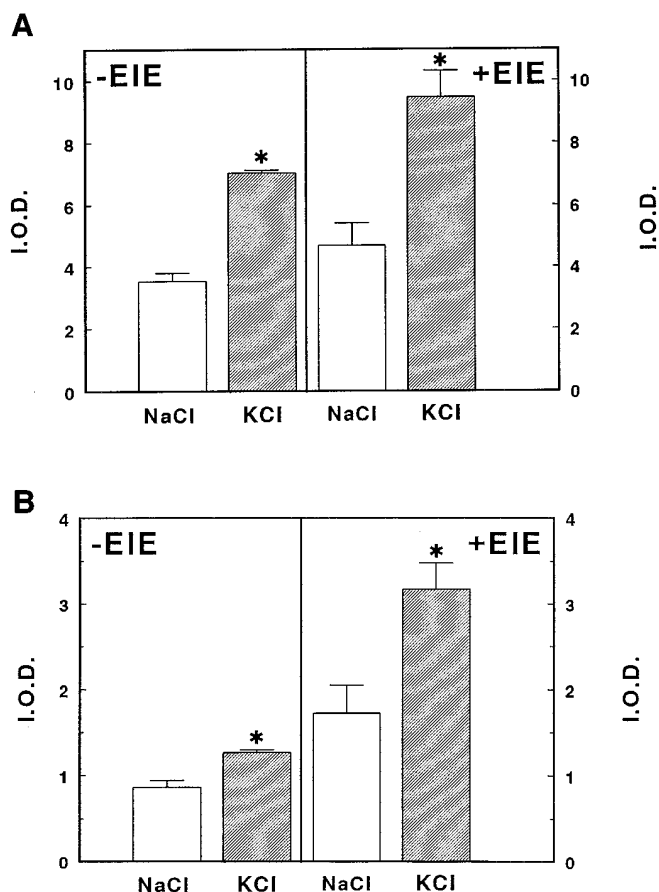


FIG. 6. Stimulated secretion of granins from hormone-treated (+EIE) and untreated (-EIE) cells. $[^{35}S]$ sulfate-labeled GH_4C_1 cells were chase incubated for 15 min in the presence of 50 mM NaCl (control) or 50 mM KCl (stimulated). Secreted chromogranin B (top) and secretogranin II (bottom) were quantitated by SDS-PAGE and fluorography and presented as mean \pm S.D. Asterisk, different from control; CgB, -EIE, $p < 0.0002$, $n = 2-3$; CgB, +EIE, $p < 0.002$, $n = 3$; SgII, -EIE, $p < 0.004$, $n = 2-3$; SgII, +EIE, $p < 0.005$, $n = 3$). IOD, integrated optical density.

GH_4C_1 cells express a cell-associated heparansulfate proteoglycan (Hinkle *et al.*, 1992). Methylumbelliferyl xyloside reduced the amount of sulfated proteoglycan in the cells about 30% (Fig. 4), but the xyloside did not prevent stimulated secretion of sulfated granins (Fig. 7). Thus, neither the reduction of proteoglycan synthesis nor the increased constitutive secretion of glycosaminoglycans prevented sorting of granins to the regulated secretory pathway.

DISCUSSION

Chromogranin B and secretogranin II, which serve as markers for the regulated secretory pathway in most endocrine cell types (Scammell, 1993), are expressed in GH_4C_1 cells, and their expression is induced by hormone treatment with estradiol, insulin, and EGF (Scammell *et al.*, 1990a; Thompson *et al.*, 1992). In contrast to prolactin (Scammell *et al.*, 1986; Reeves *et al.*, 1990), the storage of granins is not preferentially induced by hormone treatment, although the relative secretion remained constant, indicating that granin storage was increased in parallel with granin expression in hormone-treated cells. Thus, both secretogranin II and chromogranin B are efficiently stored in untreated cells, while prolactin is rapidly secreted from these cells, as measured by the relative secretion in the absence of stimulation. In hormone-treated cells, on the other hand, both granins and prolactin are efficiently stored, *i.e.* they exhibit low relative secretion (Table II). The relative secretion

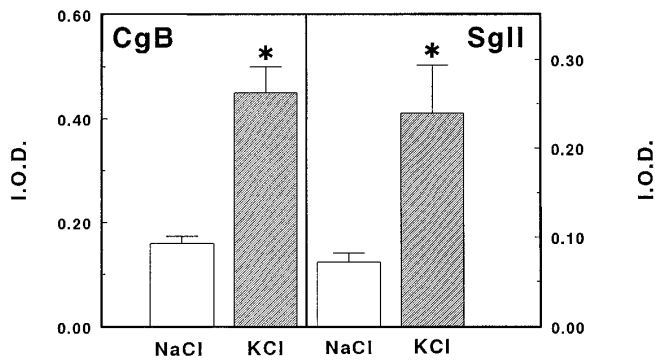


FIG. 7. **Stimulated secretion of granins in the presence of methylumbelliferyl xyloside.** GH₄C₁ cells were labeled with [³⁵S]sulfate in the presence of 1 mM methylumbelliferyl xyloside and then chase incubated for 15 min in the presence of 50 mM NaCl (control) or 50 mM KCl (stimulated). Secreted granins were quantitated by SDS-PAGE and fluorography and presented as mean \pm S.D. Asterisk, different from control (CgB, $p < 0.0007$; SgII, $p < 0.007$; $n = 3$). IOD, integrated optical density.

TABLE II

Relative secretion of regulated and constitutive secretory proteins from GH₄C₁ cells

The relative secretion in 4 h of prolactin (published) was calculated from published 24 h secretion data (Scammell *et al.*, 1986; Reaves *et al.*, 1990; Hinkle *et al.*, 1992), assuming constant cellular amounts and linear secretion rates. The relative secretion rates described in the present report for prolactin, secretogranin II (SgII), sulfated secretogranin II, chromogranin B (CgB) and glycosaminoglycans (GAG), and SEAP have been tabulated for comparison.

Sample	-EIE	+EIE
	%	%
Prolactin (published)	67	31
Prolactin (this study)	68	36
SgII	17	18
35SO4-SgII	32	29
35SO4-CgB	25	37
35SO4-GAG	70	63
SEAP (37 °C)	81	75
SEAP (16 °C)	28	36

of prolactin in 4 h reported here is similar to that previously reported (Scammell *et al.*, 1986; Reaves *et al.*, 1990; Hinkle *et al.*, 1992) (Table II), assuming constant cellular amounts and linear secretion in 24 h (see Reaves *et al.* (1990)). These results indicate that prolactin and granin storage are differentially regulated in GH₄C₁ cells.

Granins have been suggested to play a role in the sorting and packaging of peptide hormones in the regulated secretory pathway (*e.g.* Gorr *et al.* (1987a) and Huttner *et al.* (1991)). This is supported by the co-aggregation *in vitro* of parathyroid hormone and chromogranin A and B under the conditions of low pH and millimolar concentrations of calcium that are found in the trans-Golgi network and secretory granules (Gorr *et al.*, 1989). Similarly, prolactin exhibits low pH-induced aggregation (Thompson *et al.*, 1992). Recent evidence, however, suggests that calcium-induced aggregation at low pH is neither necessary (Schmidt and Moore, 1994) nor sufficient (Chanat *et al.*, 1994) for sorting of regulated secretory proteins. The present findings that granins, but not prolactin, are efficiently stored in untreated GH₄C₁ cells indicates that granin storage is not sufficient for the efficient storage of prolactin. This finding suggests that the granins do not directly interact with prolactin in the formation of a storage complex. Indeed, it appears that the prolactin storage complex is highly specific since it is disrupted by the expression of small amounts of human prolactin

in the rat cell line (Arrandale and Dannies, 1994).

One possible explanation for the differential storage of prolactin and granins is that the proteins are stored in separate granule populations. This is directly supported by the finding that prolactin and secretogranin II are stored in distinct secretory granule populations in bovine pituitary somatomotrophs (Hashimoto *et al.*, 1987). Similarly, peptide hormones derived from a common prohormone are sorted to, and stored in, separate granule populations in *Aplysia* neuronal cells (Jung and Scheller, 1991). Thus, individual cell types can contain separate regulated secretory pathways including separate granule populations. Differential storage in separate granule populations may be mediated by distinct sorting signals (Gorr and Darling, 1995), presumably including Asn³¹ and Ser³⁴ in human prolactin (Arrandale and Dannies, 1994) or differential retention during granule maturation (Kuliawat and Arvan, 1994). A second, but not mutually exclusive, explanation for differential storage is that prolactin and granin storage involves different subpopulations of GH₄C₁ cells. Thus, this cell line is heterogeneous and prolactin and granin expression are not uniformly induced in hormone-treated cells, although most prolactin expressing cells also express at least one of the two granins (Scammell *et al.*, 1990a). Scammell and co-workers (Scammell *et al.*, 1990a; Thompson *et al.*, 1992) also noted that granin expression is less strongly induced than that of prolactin upon hormone treatment, although granins exhibited a redistribution to secretory granules, similar to that of prolactin, as judged by immunofluorescence (Scammell *et al.*, 1990a). It is possible that the preferential storage of prolactin correlated with the large increase in expression levels in hormone-treated cells. However, prolactin storage is not directly induced by high prolactin levels, since cells treated with EGF alone exhibit increased prolactin expression with no preferential increase in prolactin storage (Reaves *et al.*, 1990).

A truncated, soluble form of vesicular stomatitis virus G-protein has been used as a marker for constitutive secretion from endocrine cells (*e.g.* Moore and Kelly (1986)). Here it is shown that a truncated form of human placental alkaline phosphatase (SEAP) is secreted constitutively from GH₄C₁ cells. Since alkaline phosphatase activity is readily quantitated, SEAP is a convenient marker for the constitutive secretory pathway. SEAP and the constitutive secretory marker glycosaminoglycan (Burgess and Kelly, 1984) were rapidly secreted from both untreated and hormone-treated cells. Thus, it appears that the constitutive secretory pathway is not affected by hormone treatment. Furthermore, the rapid secretion of SEAP and glycosaminoglycans from hormone-treated cells, demonstrates that the slow release of prolactin and granins is not due to a general block of the secretory pathways due to hormone-treatment. The relative secretion of SEAP was blocked at 16 °C, but without an increase in cellular storage of SEAP, suggesting that SEAP is not shifted to a storage compartment at the lower temperature. Similarly, the absence of increased stimulated SEAP secretion after return to 37 °C suggests that reducing its rate of secretion is not sufficient to target SEAP to the regulated secretory pathway.

Stimulated secretion of prolactin has been detected in both untreated and hormone-treated cells (this study and Reaves *et al.* (1990)). The stronger stimulation of prolactin secretion from hormone-treated cells is presumably accounted for by the increased intracellular storage of the hormone. In untreated cells, the stimulation of prolactin secretion is somewhat stronger than that of SEAP. However, taken together with the Golgi localization of prolactin and the relative secretion data, it appears that prolactin is mainly secreted by the constitutive secretory pathway in untreated cells. Since untreated and hor-

mone-treated cells exhibit both regulated and constitutive secretory pathways, as evidenced by granin and SEAP secretion, respectively, these results suggest that a regulated pathway for prolactin secretion is up-regulated by hormone treatment.

Chromogranin B and secretogranin II are tyrosine-sulfated in the trans-Golgi network (Rosa *et al.*, 1985; Huttner, 1988); thus, analysis of granins in untreated and hormone-treated cells allowed the use of sulfation as a marker for the late stages of the secretory pathway, thereby eliminating differences due to transit through the endoplasmic reticulum and Golgi apparatus. Stimulation of sulfated chromogranin B secretion was similar in both untreated and hormone-treated cells, while a consistent but small difference was detected for secretogranin II secretion. These results suggest that the two granins are sorted to separate granule populations that are exocytosed at different rates upon stimulation. These granules may exist in the same cells or in different subpopulations of GH_4C_1 cells, in agreement with fluorescent localization of granins in different cells (Scammell *et al.*, 1990a), as discussed above. If chromogranin B and secretogranin II are differentially sorted in individual cells, this would imply the presence of functionally distinct sorting signals on chromogranin B and secretogranin II. Recent reports indicate that this is indeed the case. Thus, chromogranin B, but not secretogranin II, is rerouted to the constitutive pathway in dithiothreitol-treated PC12 cells (Chanat *et al.*, 1993). In addition, our recent proposal that an amino-terminal hydrophobic peak serves as the sorting signal for chromogranin B, but not secretogranin II (Gorr and Darling, 1995), is consistent with this possibility.

Methylumbelliferyl xyloside inhibits proteoglycan synthesis but stimulates synthesis of free glycosaminoglycan chains (Schwartz, 1977; Burgess and Kelly, 1984). GH_4C_1 cells express a heparan sulfate proteoglycan that is cellularly located (Hinkle *et al.*, 1992). Xyloside treatment reduced sulfate incorporation into this proteoglycan about 30% and reduced sulfation of granins while strongly stimulating glycosaminoglycan synthesis. The decrease in granin sulfation does not prevent their sorting to the regulated secretory pathway in hormone-treated cells. Similarly, inhibition of sulfation with sodium chlorate does not prevent sorting of granins in untreated cells (Hinkle *et al.*, 1992). Based on these and earlier data (Burgess and Kelly, 1984; Gorr and Cohn, 1990; Gorr *et al.*, 1991), it is concluded that tyrosine-sulfation, oligosaccharide-sulfation, or proteoglycan synthesis are not required for sorting or storage of

secretory proteins in the regulated secretory pathway in endocrine cells.

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