Expression of dopamine β-monooxygenase (DBM), the enzyme that converts dopamine into norepinephrine, is limited to adrenal chromaffin cells and a small population of neurons. We studied DBM trafficking to regulated granules by stably expressing rat DBM in AtT-20 corticotrope tumor cells, which contain regulated granules, and in Chinese hamster ovary (CHO) cells, which lack regulated granules. The behavior of exogenous DBM in both cell lines was compared with endogenous DBM in adrenal chromaffin cells. CHO cells secreted active DBM, indicating that production of active enzyme does not require features unique to neuroendocrine cells. Pulse-chase experiments indicated that early steps in DBM maturation followed a similar time course in AtT-20, CHO, and adrenal chromaffin cells. Use of a conformation-sensitive DBM antiserum indicated that acquisition of a folded structure occurred with a similar time course in all three cell types. Cell type-specific differences in DBM trafficking became apparent only when storage in granules was examined. As expected, DBM was stored in secretory granules in chromaffin cells; CHO cells failed to store DBM. Despite the fact that AtT-20 cells have regulated granules, exogenous DBM was not stored in these granules. Thus storage of DBM in secretory granules requires cell type specific factors.

Although catecholamines and peptides are often stored in the same granules, their biosynthetic pathways are distinctly different. Catecholamines are synthesized from tyrosine by the action of several cytosolic enzymes plus dopamine β-monooxygenase (DBM1, EC 1.14.17.1), the only enzyme in this biosynthetic pathway that is located in the secretory granule lumen (1, 2). Expression of DBM is restricted to adrenergic neurons and adrenal medullary cells (3, 4). The hydroxylation reaction catalyzed by DBM requires copper, reduced ascorbate, and molecular oxygen (Fig. 1A) (5–7). Neuropeptides are synthesized from larger inactive precursors that undergo a series of post-translational modifications, all of which occur within the secretory pathway lumen (8, 9). Amidation, essential for the bioactivity of many neuropeptides, is catalyzed by the bifunctional peptidylglycine α-amidating monooxygenase (PAM) enzyme in a two-step reaction (Fig. 1A) (9–11). Peptidylglycine α-hydroxylating monooxygenase (PHM) catalyzes the first step of the reaction and, like DBM, requires copper, reduced ascorbate, and molecular oxygen. PAM is expressed at varying levels in a wide variety of tissues (12). Cells expressing DBM also express PAM, and catecholamines and neuropeptides are stored together in regulated granules (13).

The catalytic core of PHM is 32% identical to a 296-amino acid region of DBM (Fig. 1B) (6, 14). Interestingly, this region contains four conserved disulfide bridges and six conserved copper ligands (15, 16). Despite these similarities, the topologies of DBM and PAM are very different (Fig. 1B). Each DBM monomer contains six additional Cys residues and multiple sites for N-glycosylation (17–19); DBM monomers form disulfide-linked dimers that associate noncovalently to form a tetrameric glycoprotein of 290 kDa (20, 21). In contrast, PHM is not glycosylated and is followed by a noncatalytic region (exon A), the second catalytic domain, and a single transmembrane domain that attaches to a cytosolic COOH-terminal domain responsible for localizing PAM in cells (5). Based on studies on Cnidarians, the most primitive organisms with an organized nervous system, the use of amidated peptides for intercellular communication preceded the use of catecholamines (22).

DBM and PHM are both found in soluble and membrane-bound forms, but have adopted different mechanisms for membrane association. Membrane and soluble forms of DBM derive from one translation product (23). Phospholipids as well as an uncleaved NH2-terminal signal peptide play a role in the association of DBM with membranes (24–27). In contrast, tissue-specific alternative splicing and endoproteolysis generate integral membrane and soluble forms of PHM (28, 29). Despite their different topologies, DBM and PAM are both stored in secretory granules and released along with stored peptides upon stimulation (30–32). Following fusion of the secretory granules with the plasma membrane, soluble DBM is released along with catecholamines, whereas the membrane form of DBM undergoes endocytosis (33, 34). Although the relationship between membrane and soluble forms of DBM is not completely understood, a precursor-product relationship has been proposed (20). The domains of DBM important for its trafficking have not been clearly defined.

With the goal of understanding its routing, we expressed rat DBM in AtT-20 corticotrope tumor cells and Chinese hamster ovary (CHO) cells. These cell lines were chosen because AtT-20 cells contain regulated granules, whereas CHO cells do not. DBM expressed in these cell lines was compared with endogenous DBM in primary rat chromaffin cells. We used metabolic labeling and immunoprecipitation to analyze the maturation
and secretion of DBM. In addition, we used immunofluorescence microscopy and Western blot analysis to determine the subcellular localization of DBM. Our studies show that newly synthesized DBM undergoes a conformational change with similarly slow kinetics in all three cell types and that secretion of active DBM does not require the presence of regulated granules. However, the subcellular localization of DBM is cell type-specific; despite the presence of granules, AtT-20 cells fail to store DBM in regulated granules.

MATERIALS AND METHODS

Construction of DBM Expression Vectors—Two DBM expression vectors were constructed from a pBluescript plasmid carrying the cDNA for DBM (rDBM [nucleotides 1–2445]) (35) that was kindly provided by Dr. E. Sabban (New York Medical College, Valhalla, NY). For construction of pCIS.DBM, the XhoI to EcoRI fragment was inserted into the pCIS.2CXXNH vector (29). DBM was also expressed with a rhodopsin epitope tag (Thr-Glu-Thr-Ser-Gln-Val-Ala-Pro-Ala, mouse rhodopsin (293–301)) (36) appended to its COOH terminus. Sense and antisense oligonucleotides encoding the rhodopsin epitope tag (nucleotides 4761–4788) were annealed, and EcoRI and NarI sites were used to ligate the rhodopsin tag oligonucleotides to the 3′-end of DBM cDNA in the pBluescript plasmid. The NarI site was created as a linker between the DBM cDNA and rhodopsin oligonucleotides. The cDNA fragment encoding the DBM-rhodopsin tag was inserted into pCIS.2CXXNH as above. The plasmid region containing the rhodopsin tag was verified by sequencing.

Tissue Culture and Stable Transfection—AtT-20 and CHO cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 medium (DMEM:F12 medium) containing 20 mM HEPES, pH 7.4, instead of NaHCO3. The adult male rats were diced with scissors and washed with DMEM:F12 cell medium containing 5% fetal calf serum, dispersed by pipetting up and down with a flame Pasture Pipette and filtered through a 70-µm cell strainer. The cells were resuspended in DMEM:F12 containing 5% fetal calf serum and plated at a density of 1 × 10⁶ cells/well in a 12-mm culture dish. The dishes were coated with 0.1 mg/ml polylysine for 10 min, following by a 5-min incubation with Nu-Serum. Adrenal cells were cultured for 2–4 days before use.

Purification and Assay of DBM Expressed in CHO Cells—DBM was precipitated from 50 ml of spent serum-free medium harvested from CHO-DBM cells by adding (NH₄)₂SO₄ to 80% saturation. The pellet was resuspended in 1 ml of 50 mM NaOAc, pH 5.5, centrifuged to eliminate insoluble material, and 0.5 ml of the supernatant (equivalent to 25 ml of spent medium) was loaded onto a BioGel A15 m column eluted with 50 mM sodium phosphate, 0.15 mM NaCl, pH 6.5. Fractions of 0.85 ml were collected and the peak of DBM protein was located by Western blot analysis.

DBM activity in the column fractions was determined using the method of Wimalasena and Wimalasena (37). 2.5 µl of catalase (20 mg/ml), 1 µl of 0.5 mM CuSO₄, 10 µl of 250 mM tyramine, and 10 µl of 250 mM N,N-dimethyl-1,4-phenylenediamine (freshly dissolved in water) were added to 0.5 µl of buffer (10 mM fumarate, 125 mM NaOAc, pH 5.2). The reaction mixture was equilibrated in a water-jacketed cuvette at 37 °C, and after adding the sample the absorption at 515 nm was recorded at 5 s intervals over a 1-min time period. Peak fractions were assayed at a 5-, 10-, and 20-µl volume. The concentration of DBM protein was estimated from a Coomasie Blue-stained membrane, and an extinction coefficient of 5200 M⁻¹ cm⁻¹ was used for N,N-dimethyl-1,4-phenylenediamine, yielding a turnover number of recombinant DBM of approximately 5/s. Using the same assay, purified DBM has a turnover number of 7.3/s (37).

Biosynthetic Labeling and Immunoprecipitation—Pulse-chase experiments were performed as described previously (29). Cells were pulse-labeled using 300 µCi of [35S]methionine (1 mCi/ml, 1000 Ci/mmol; Amersham Pharmacia Biotech) in 300 µl of methionine-free CSFM-air. Primary adrenal cells, AtT-20 cells, and CHO cells can be grown in CSFM for several days without compromising their ability to synthesize and secrete endogenous and exogenous proteins. Following the chase time, cellular proteins were extracted in 20 mM NaTES, pH 7.0, 10 mM mannitol, 1% TX-100 (Pierce) containing protease inhibitors (30 µg/ml phenylmethylsulfonyl fluoride, 16 µg/ml benzamidine, 2 µg/ml leupeptin, and 10 µg/ml leupeptin trypsin inhibitor). Immunoprecipitation of cell extract and medium was performed using rabbit polyclonal antibody (Ab2047 directed against the putative catalytic domain of DBM (rDBM-[217–257]))). Unless indicated otherwise, samples for immunoprecipitation were denatured by boiling for 5 min in 1% SDS and then diluted with a 7-fold weight excess of 15% Nonidet P-40. The immune complexes were isolated and analyzed as described (38). DBM immunoprecipitated from the culture medium, and cell extract was analyzed by SDS-polyacrylamide gel electrophoresis or subjected to endoglycosidase H treatment prior to electrophoresis.
FIG. 2. Expression of DBM in CHO cells. A, Western blot analysis. Aliquots of culture medium (Sec), soluble (Sol), and membrane (Mb) fractions prepared from CHO-DBM cells were fractionated by SDS-polyacrylamide gel electrophoresis and analyzed using an antiserum to DBM (Ab2047). Molecular masses are indicated in kDa. B, secretion of active DBM. DBM was partially purified from the spent medium by (NH₄)₂SO₄ precipitation and gel filtration. Fractions resolved by SDS-PAGE were visualized using Coomassie Blue (inset, lower panel) or the same DBM antibody (inset, upper panel). DBM activity was quantified as described under “Materials and Methods.” C, CHO cells expressing DBM or DBM-rhodopsin were immunostained with antibody to DBM or rhodopsin. Detection of DBM and DBM-rhodopsin in extracts of CHO and AtT-20 cells. Extracts of CHO cells expressing DBM (1) or DBM-rhodopsin (2) and AtT-20 cells expressing DBM-rhodopsin (3) were fractionated by SDS-PAGE and visualized using the indicated antiserum.

Stimulation of Secretion—To stimulate secretion, AtT-20 DBM-rhodopsin cells were labeled and chased for 2 h as described above. Following the chase, cells were incubated for 1 h in control medium (CSFM-air) or in CSFM-air containing either 1 μM phorbol 12-myristate 13-acetate (PMA) or 1 mM BaCl₂. DBM was immunoprecipitated from the cell extracts and culture media as described above. Steady-state secretion of DBM and PC1 by AtT-20 cells was quantified by incubating the cells in CSFM-air containing 0.2 mg/ml bovine serum albumin for two sequential 1-h periods (basal secretion); cells were then incubated in control medium or medium containing PMA or BaCl₂ and analyzed by Western blot as described below.

Treatment of Immunoprecipitated DBM with Endoglycosidase H or N-Glycanase F—Following immunoprecipitation, the DBM-antibody complex was eluted from the protein A-Sepharose by boiling for 5 min in 0.1 M sodium phosphate buffer, pH 5.5, 0.5% SDS, 2 mM β-mercaptoethanol. Following centrifugation, the supernatant was diluted 2-fold in 0.1 M sodium phosphate, pH 5.5, containing 30 μg/ml ovalbumin, 5 mM β-mercaptoethanol, protease inhibitors, and 2 milliunits of endoglycosidase H (Roche Molecular Biochemicals). For incubation with N-glycanase F, the supernatant was diluted in 0.1 M sodium phosphate, pH 7.5, 0.5% Nonidet P-40, 10 mM β-mercaptoethanol, 300 μg/ml phenylmethylsulfonyl fluoride, and 2 units of N-glycanase F (Roche Molecular Biochemicals). After 16 h at 37 °C, the DBM was analyzed by SDS-polyacrylamide gel electrophoresis and fluorography.

Western Blot Analysis—Samples were fractionated on 10% polyacrylamide, 0.25% N,N'-methylenebisacrylamide/SDS gels (39), transferred to polyvinyldene difluoride membranes (NEN Life Science Products), and visualized as described (40). The antisera used in these studies were: a rabbit polyclonal antibody (Ab2047) generated against a fragment of rat DBM (Fig. 2 inset, upper panel), a mouse monoclonal antibody against the rhodopsin tag (42) and a rabbit polyclonal antiserum against β-endorphin (JH2, β-endorphin(1–31)) (46), TGN38, BiP, chromogranin B (Santa Cruz Biotechnology, Santa Cruz, CA), or a rat monoclonal antiserum (1D4B) directed against lysosome-associated membrane protein (LAMP-1) (45) (Developmental Studies Hybridoma Bank). Secondary antisera included fluorescein-conjugated goat anti-rabbit immunoglobulin G (green) (1:1000); goat anti-rabbit immunoglobulin G coupled to Cy-3 (Jackson ImmunoResearch) (red) (1:500), donkey anti-mouse immunoglobulin G coupled to Cy-3 (1:1000), and fluorescein-conjugated goat anti-rat immunoglobulin G (1:1000) (CalTag Laboratories).

RESULTS

Active DBM Is Secreted by CHO Cells—DBM was expressed in CHO cells, which lack regulated secretory granules, to determine whether the production of active enzyme required features unique to neuroendocrine cells. Stably transfected CHO cells expressing DBM were separated into soluble and membrane fractions and analyzed by Western blot using an antibody generated against a fragment of rat DBM (Fig. 2A). Similar amounts of DBM were recovered in both the soluble and membrane fractions; DBM from both fractions has a molecular mass of 69 kDa. In contrast, the DBM secreted into the culture medium has a molecular mass of 72 kDa. Based on N-glycanase treatment of DBM immunoprecipitated from transfected CHO cells (see Fig. 4B) the difference in the mass of cellular and secreted DBM is a result of N-glycosylation.

To determine if the expressed DBM were active, spent medium was assayed following partial purification of DBM by gel filtration (Fig. 2B). A peak containing DBM protein was located by Coomassie Blue staining and Western blot analysis following SDS-PAGE. When the corresponding column fractions were assayed for DBM activity, a peak of activity was observed in the same fractions where the DBM protein was located. The specific activity of DBM expressed in CHO cells indicated that it is as active as DBM purified from adrenal medulla (37). Interestingly, the secretion of active DBM by CHO cells indicates that neither the presence of secretory granules nor the ability to produce endogenous DBM is a requirement for generation of a fully active enzyme.

The subcellular localization of DBM with and without an epitope tag was analyzed by immunofluorescence microscopy (Fig. 2C). Diffuse, reticular staining for both proteins was observed throughout the cell; staining was excluded from the nucleus. The distribution of DBM and DBM-rhodopsin closely resembled that of BiP, indicating that a significant amount of
DBM with or without the epitope tag is localized to the ER of CHO cells at steady state. Although many secretory products produced in CHO cells, including PHM, exhibit some concentration of protein in the trans-Golgi network region, no accumulation of DBM in the TGN region was observed. Both DBM and DBM-rhodopsin could be visualized with antisera to DBM; the rhodopsin antiserum visualized the epitope-tagged DBM (Fig. 2D). Appending the rhodopsin tag to the COOH terminus of DBM does not appear to affect the properties of the protein.

DBM Produced in AtT-20 Cells, CHO Cells, and Primary Adrenal Cells Is Secreted Slowly—Pulse-chase metabolic labeling experiments were performed to compare the biosynthesis and secretion of exogenous DBM expressed in AtT-20 and CHO cells to that of the endogenous DBM in primary adrenal chromaffin cells. The cells were labeled with [35S]methionine for 20 min and then chased for up to 6 h. DBM was immunoprecipitated from cell extracts and culture medium, fractionated by SDS-PAGE, and detected by fluorography. Endogenous DBM in primary adrenal cells was synthesized as a single species of 72 kDa, whereas DBM expressed in AtT-20 or CHO cells had a mass of 69 kDa (Fig. 3). For all three cell types there was a lag time of 3 h before a significant amount of radiolabeled DBM appeared in the culture medium. The DBM secreted by AtT-20 cells and CHO cells had a mass of 72 kDa. Although the amount of DBM secreted into the medium increased with time, a significant amount of DBM remained in all three cell types after 6 h of chase. With prolonged (16 h) chase times, more DBM was secreted by both AtT-20 cells and CHO cells (Fig. 3, insets), with almost all of the newly synthesized DBM released from AtT-20 cells after the long chase.

DBM Produced in AtT-20, CHO, and Primary Adrenal Cells Acquires Mature Oligosaccharides Slowly—Other soluble secretory proteins expressed in CHO and AtT-20 cells are secreted much more quickly than DBM (29, 44, 45). The fact that DBM showed a long lag time between synthesis and secretion raised the possibility that it spends a significant amount of time in the ER, Golgi complex, or in a post-Golgi compartment. Because all three cell types secreted fully or partially Endo H-resistant DBM (Fig. 4A), we used acquisition of resistance to digestion with Endo H as diagnostic of the presence of complex oligosaccharides and passage of the newly synthesized protein through the medial Golgi (19). As expected, DBM analyzed after the 20-min pulse is completely sensitive to digestion with Endo H in all three cell types (Fig. 4A). After the 3-h chase at 37 °C, primary adrenal cells contain some DBM that is resistant to Endo H (Fig. 4A, asterisk). In contrast, all of the cellular DBM in AtT-20 and CHO cells is still sensitive to Endo H after a 3-h chase at 37 °C. In all three cell types, newly synthesized DBM acquires resistance to Endo H very slowly. For comparison, PAM expressed endogenously in atrial myocytes or exogenously in AtT-20 corticotropes acquired Endo H resistance with a half-time of 1–1.5 h (46, 47).

Adrenal chromaffin cells, unlike AtT-20 cells and CHO cells, are able to store DBM with complex N-linked oligosaccharides. Although DBM secreted by AtT-20 and CHO cells has a larger
molecular mass than the cellular enzyme, little high molecular mass product was detectable in the cell extracts. To determine if the size difference between the secreted and cellular DBM was because of glycosylation, immunoprecipitated DBM was subjected to treatment with N-glycanase. Following deglycosylation, cellular and secreted DBM both exhibited a molecular mass of 65 kDa (Fig. 4B). Rat DBM lacking its signal sequence has a predicted molecular mass of 65 kDa (20). Although cellular and secreted DBM do not differ in apparent molecular mass in primary adrenal cells, deglycosylation converts both to a 65-kDa protein identical in size to rat DBM produced exogenously in AtT-20 and CHO cells. These data indicate that DBM in primary adrenal chromaffin cells has a different pattern of glycosylation than DBM expressed in AtT-20 and CHO cells.

DBM Produced in AtT-20, CHO, and Primary Adrenal Cells Acquires a Folded Conformation Slowly—The Endo H sensitivity experiments suggested that newly synthesized DBM exited the ER slowly, delaying its acquisition of Endo H-resistant oligosaccharides. To investigate earlier stages in the maturation of DBM, we took advantage of the fact that one of our DBM antibodies detects the mature DBM secreted by adrenal chromaffin cells, AtT-20/DBM cells, and CHO/DBM cells only after the medium has been denatured with SDS (Fig. 5, Secreted DBM). This antiserum was raised to a fragment of DBM that contains four Cys residues that form two disulfide bridges in the native protein (17); the recombinant protein was reduced or alkylated before use as an immunogen.

A pulse-chase experiment was carried out as described in Fig. 3; radiolabeled proteins were extracted in a nondenaturing buffer containing 1% Triton X-100. Equal aliquots of extract were then incubated directly with DBM antibody (native) or following denaturation with SDS and then incubated with DBM antibody (denatured) (Fig. 5, Cellular DBM). After the pulse or the 30-min chase, similar amounts of DBM were immunoprecipitated with or without SDS denaturation. In contrast, after the 1-h chase, significantly less DBM was immunoprecipitated unless the sample was first denatured with SDS. After 3 h, very little DBM could be recognized by this antibody unless the protein was first denatured. Following the 6-h chase, no DBM was recognized in extracts of adrenal chromaffin cells or AtT-20/DBM cells unless the sample was first denatured with SDS; CHO cells contained a small amount of DBM that was precipitated without denaturation. These results indicate that the folding step that eliminates the ability of this antiserum to detect DBM does not begin to occur until 1 h after synthesis and is completed between 3 and 6 h of chase in AtT-20 and chromaffin cells. The occurrence of a slow step early in the maturation of DBM is consistent with the steady-state localization of a significant amount of DBM in the ER.

DBM Expressed in AtT-20 Cells Is Not Localized to Secretory Granules—Like adrenal chromaffin cells, AtT-20 cells contain secretory granules that are responsive to the addition of secretagogue (47). The granules in AtT-20 cells have been shown to store a variety of exogenous proteins including NPY (48), insulin (49), and trypsin (50). We compared the localization of endogenous DBM in adrenal chromaffin cells and neuroblastoma cells to the localization of exogenous DBM-rhodopsin in AtT-20 cells using immunofluorescence microscopy (Fig. 6). Adrenal chromaffin cells and SH-SY5Y cells have a regulated secretory pathway and have the ability to produce catecholamines (51, 52). Punctate DBM staining was observed throughout each chromaffin cell and was excluded from the nucleus (Fig. 6A). DBM staining in chromaffin cells resembles staining for chromogranins A and B, which are located in secretory granules dispersed throughout the cell (data not shown) (53). Endogenous DBM in SH-SY5Y cells was localized to the perinuclear region with a significant amount of DBM also observed in cellular processes (Fig. 6B).

In contrast, diffuse reticular staining for DBM-rhodopsin was observed in the stably transfected AtT-20 cells; the DBM staining extended close to the margins of the cells and was excluded from the nucleus (Fig. 6C). The staining pattern observed for DBM-rhodopsin is distinctly different from the staining for a soluble PHM protein expressed in AtT-20 cells (Fig. 6D). The soluble PHM protein was localized to the perinuclear, TGN region as well as to vesicular structures concentrated at the tips of the cellular processes where secretory granules are

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**Fig. 5. Time course of folding of DBM.** Adrenal cells, AtT-20/DBM cells, and CHO/DBM cells were labeled with [35S]methionine and harvested immediately (Pulse) or chased in CSFM-air for the indicated amount of time. DBM was immunoprecipitated from cell extracts and culture medium without denaturation (N, native) or following denaturation with SDS (D, denatured). Apparent molecular masses are in kDa. Similar results were obtained in four additional experiments.
located. No DBM-rhodopsin staining was observed at the tips of the cells. Despite similarities in the early compartments of the secretory pathway, the trafficking of DBM in AtT-20 cells is distinctly different from the trafficking of DBM in chromaffin cells.

With the goal of further identifying the subcellular compartment containing the majority of the DBM in AtT-20 cells, confocal microscopy was used to compare the distribution of DBM-rhodopsin and selected marker proteins. AtT-20 cells produce proopiomelanocortin, the precursor to several neuropeptides including ACTH, β-endorphin, and α-melanotropin; the proopiomelanocortin products are stored in secretory granules in AtT-20 cells (54). AtT-20/DBM cells visualized simultaneously with the rhodopsin monoclonal antibody and a rabbit polyclonal antibody to PC1, a marker for the trans-Golgi network (55) (Fig. 7A). The immunostaining results, in addition to metabolic labeling and subcellular fractionation experiments (not shown), indicate that DBM-rhodopsin expressed in AtT-20 cells is localized primarily in the endoplasmic reticulum and is not stored in secretory granules.

**Secretion of DBM from AtT-20 Cells Is Not Stimulated by Secretagogue**—The DBM staining indicated that the majority of the protein is not localized to secretory granules in AtT-20 cells. To determine whether a small fraction of the DBM is stored in granules, we asked whether DBM reaches a stimulusable compartment in these cells. AtT-20 cells expressing DBM-rhodopsin were labeled with [35S]methionine for 20 min and chased at 37 °C for 2 h. After the chase, the cells were further incubated for 1 h with control medium or medium containing secretagogue (either 1 μM PMA or 1 mM BaCl₂), and DBM-rhodopsin was immunoprecipitated from the culture media (Fig. 8A) and cell extracts (not shown). Because basal DBM-rhodopsin secretion is detectable at this time, any diversion of newly synthesized DBM into secretory granules should be detectable. No increase in DBM secretion occurred following incubation with either secretagogue. To ensure that the AtT-20 cells were responsive to stimulation, the secretion of PC1, which is stored in secretory granules, was examined. An approximately 4-fold stimulation of secretion of the cleaved form of PC1, PC1ΔC, was observed (Fig. 8A) (58, 59).

As an additional way to detect a small amount of DBM stored in the secretory granules of AtT-20 cells, basal and stimulated secretion were evaluated by Western blot analysis (Fig. 8B). Secretion of endogenous PC1 was evaluated for comparison. Secretion under basal conditions was evaluated over two 1-h periods and secretagogue was then added for the final 1-h collection (Fig. 8B). As expected, similar amounts of DBM were secreted during the two basal collection periods (B1 and B2). The amount of DBM secreted did not increase after stimulation with PMA or BaCl₂. In contrast, secretion of PC1ΔC was stimulated by each secretagogue. The fact that secretion of DBM from AtT-20 cells was not stimulated in response to secretagogues indicates that DBM is not stored in secretory granules in AtT-20 cells.

**DISCUSSION**

The expression of DBM is limited to chromaffin cells in the adrenal medulla and to the subset of catecholamine producing neurons in the central and peripheral nervous systems (1–3). Nevertheless, production of active enzyme does not require cell type-specific factors. CHO cells, an epithelial line derived from Chinese hamster ovary and lacking regulated secretory granules, produce and secrete fully active DBM (Fig. 2B). This finding is consistent with secretion of active DBM by RK13 cells (26) and Schneider 2 cells, epithelial lines derived from normal rabbit kidney and Drosophila embryos, respectively (25, 60). In contrast, production of active prohormone converted 2 is limited to cells that also express 7B2, a helper protein that binds to proPC2 (61). Storage of DBM in regulated granules is, however, cell type-specific.

Many features of DBM maturation are indistinguishable in
stably transfected CHO and AtT-20 cells and in primary adrenal cells. N-Glycanase treatment of secreted and cellular DBM from all three cell types eliminates all size differences, yielding a single protein of 65 kDa (Fig. 4B). As observed here for rat DBM, bovine adrenal DBM (62, 63), human SH-SY5Y neuroblastoma cell DBM (64), and rat PC12 cell DBM (65, 66) all yielded core proteins of a single mass following deglycosylation. In contrast, human DBM expressed in AtT-20 cells using vaccinia virus yielded two proteins that still differed in mass following deglycosylation (26).

Fig. 7. Confocal immunofluorescence microscopy of DBM-rhodopsin in AtT-20 cells. The distribution of DBM-rhodopsin was compared with that of β-endorphin (A), which is present in secretory granules, the TGN marker TGN38 (B), the lysosomal marker LAMP-1 (C), and the ER protein BiP (D). Cells were fixed and incubated simultaneously with a monoclonal antibody to rhodopsin and rabbit anti-BiP, rabbit anti-β-TGN38, or rat anti-LAMP-1. All samples were analyzed by confocal microscopy using 1.5-μm optical sections.

Fig. 8. Lack of stimulation of DBM secretion from AtT-20 cells. A, duplicate wells of AtT-20 DBM-rhodopsin cells were labeled with [35S]methionine and chased in CSFM-air at 37 °C for 2 h. After the chase, one well of cells was incubated for 1 h in control medium (Con), whereas the other was incubated in medium containing secretagogue (1 μM PMA or 1 mM BaCl2). DBM (denatured) and PC1 were immunoprecipitated from the culture medium, analyzed by SDS-PAGE, and detected by fluorography. B, AtT-20 DBM-rhodopsin cells were incubated in CSFM-air twice for 1 h each time (B1 and B2) to evaluate basal secretion; the subsequent 1-h incubation included either 1 μM PMA or 1 mM BaCl2. Secreted DBM and PC1 were visualized by Western blot analysis of aliquots of medium. PC1αC is the major form of PC1 stored in granules. The bovine serum albumin (0.2 μg/μl) added to the serum-free medium creates the blurry pattern observed in some samples. Similar results were obtained in three additional experiments.
Dopamine β-Monoxygenase Expression

of DBM. Mature, secreted DBM is not recognized by this antibody until it has been denatured with SDS. DBM synthesized in chromaffin cells or AtT-20 cells first begins to acquire enough structure to limit antibody cross-reactivity after an hour of chase, and the process is largely complete within 3 h of synthesis. Maturation of DBM requires the formation of tetramers from disulfi de-linked dimers (67, 68); each monomer contains 14 Cys residues that form 5 intramolecular and 2 intermolecular disulfi de bonds (17). Conversion of mature DBM into a form that is recognized by the antibody requires SDS but no reducing agent, so it is not clear whether disulfi de bond formation precedes or follows the conformational change detected by this antiserum.

Cell type speciﬁcity is observed in the N-glycosylation of secreted DBM. The detailed oligosaccharide structure of rat DBM has not been determined. Three of the six potential N-glycosylation sites in rat DBM are conserved in the human enzyme (65). Based on its susceptibility to Endo H (66), rat adrenal DBM contains biantennary complex oligosaccharides (Endo H resistant) as well as high mannose oligosaccharides (Endo H sensitive). Our data showing that DBM secreted by primary adrenal cells is partially resistant to Endo H are consistent with this observation (Fig. 4A). In contrast, DBM secreted by CHO cells and AtT-20 cells is totally resistant to digestion with Endo H, indicating a different pattern of glyco-
sylation in these cell types. It is not clear whether these differences in N-glycosylation could play a role in cell type-speciﬁc trafﬁcking.

Unexpectedly, the ability to store DBM in secretory granules is cell type-speciﬁc. DBM in primary adrenal chromafﬁn cells is stored in secretory granules (Fig. 6A) (53). DBM in SH-SY5Y cells is localized to the perinuclear region as well as to punctate structures in the cell body and neuritic processes (Fig. 6B); endogenous peptide Y exhibits a similar staining pattern in neurites (data not shown). In contrast, DBM expressed in AtT-20 cells (Fig. 6C) is localized to the ER. No DBM could be detected in AtT-20 secretory granules by immunofluorescence or by examining proteins released upon secretagogue treatment. Immunofluorescence was not used to evaluate the subcellular localization of human DBM expressed in AtT-20 cells, and the inability of DBM to localize to secretory granules was not apparent (26). Thus DBM trafﬁcking in primary adrenal chromafﬁn cells, neuroblastoma SH-SY5Y cells, and AtT-20 cells exhibits distinct differences.

The secretory granules of AtT-20 cells accommodate many exogenous proteins including trypsinogen (49), PHM and pep-
tidyl-o-hydroxyglycine a-ami
dizing lyase (29), insulin (50), proline-rich protein (69), egg-laying hormone (70), PC1 and PC2 (44), and ﬁbronectin (71). The fact that DBM is not stored in AtT-20 secretory granules raises the possibility that cells which normally produce DBM contain speciﬁc proteins, lipids, or oligosaccharide modiﬁcations essential for targeting DBM to granules. Another possibility to consider is that the milieu in AtT-20 granules is different enough to exclude DBM. Consistent with this, the conditions required to demonstrate pH-de-pendent aggregation of chromafﬁn granule proteins and ante-
rior pituitary granule proteins were different (72). If the DBM signal sequence is retained (27, 35), a cell type-speciﬁc cytosolic factor could be necessary for the sorting of DBM to secretory granules. Proteins that interact with the cytosolic domains of PAM (73) and furin (74, 75) have recently been identiﬁed.

Cell type-speciﬁc trafﬁcking of proteins is being observed more commonly (76, 77). For example, amylase and GP2 are localized to secretory granules in exocrine cells but not in endocrine AtT-20 cells (78). In addition, the glucose transporter GLUT-4 is found in secretory vesicles in cardiomyocytes (79) but is primarily sorted to smaller vesicles in PC12 cells (80). Although mislocated, expression of DBM in AtT-20 cells is without effect on the localization of endogenous proteins such as TGN38 and β-endorphin. This is in sharp contrast to the effect of expression of syntaxin 1A on the Golgi complex and endoplas
dermal reticulum of cells that do not normally express it (81); co-expression with rbSec1 allows syntaxin 1A to localize to the plasma membrane. Similarly, accessory proteins that bind to cellubrevin in rat liver (82), Factor VIII in AtT-20 cells (83), and thyroglobulin in CHO cells (84) have been identiﬁed. Further studies will be required to determine the mechanism underly

REFERENCES
