Identification of subcellular compartments containing peptidylglycine α-amidating monooxygenase in rat anterior pituitary

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SUMMARY

Both soluble and integral membrane forms of peptidylglycine α-amidating monooxygenase (PAM) are expressed in the rat anterior pituitary, making it an ideal model system for studying the routing of proteins into secretory granules. To identify the subcellular compartments involved in the routing of integral membrane PAM, we used subcellular fractionation, metabolic labeling and immunoblot analysis. Mature secretory granules were found to contain full-length integral membrane PAM along with a significant amount of soluble PAM generated by endoproteolytic cleavage. PAM proteins were not co-distributed with tyrosylprotein sulfotransferase activity during sucrose gradient centrifugation, indicating that the trans-Golgi/TGN is not a major PAM-containing compartment at steady state. Fractionation of the 4,000 g and 10,000 g pellets obtained by differential centrifugation identified a significant amount of integral membrane PAM in a light fraction lacking soluble secretory granule proteins. Metabolic labeling experiments with primary anterior pituitary cells demonstrated that integral membrane PAM enters a light compartment with similar properties only after exit from the trans-Golgi/TGN compartment in organelles involved in the intracellular recycling of integral membrane PAM. Small amounts of full-length integral membrane PAM were also recovered in fractions containing internalized transferrin and may be in an endosomal compartment following retrieval from the cell surface.

Key words: secretory granule, amidation, anterior pituitary

INTRODUCTION

One of the steps in the synthesis of many bioactive peptides is the conversion of COOH-terminal glycine-extended peptide into α-amidated product (Bradbury and Smith, 1991; reviewed by Eipper et al., 1992a). The two-step α-amidation reaction is catalyzed by the bifunctional enzyme peptidylglycine α-amidating monooxygenase (PAM) (EC 1.14.17.3). The PAM precursor protein encodes both of the enzymatic activities involved in peptide amidation (Kato et al., 1990; Perkins et al., 1990). The initial step in the amidation reaction is the hydroxylation of the COOH-terminal glycine and is catalyzed by peptidylglycine α-hydroxylating monooxygenase (PHM). This step is rate limiting and requires copper, molecular oxygen and ascorbate (Eipper et al., 1992a). At physiological pH, peptidyl-α-hydroxyglycine α-amidating lyase (PAL) catalyzes the second step, converting the peptidyl-α-hydroxyglycine intermediate into an α-amidated product (Katopodis et al., 1990; Perkins et al., 1990; Takahashi et al., 1990).

Alternative splicing of the single copy PAM gene and post-translational processing of the PAM proteins both contribute to the generation of tissue-specific forms of PAM protein. The most prevalent forms of PAM mRNA found in the anterior pituitary are rPAM-2 and rPAM-3b, with less rPAM-3a (Fig. 1) (Eipper et al., 1992b). The PHM domain in rPAM-2 and -3b follows a typical signal sequence and is contained within the NH2-terminal third of the PAM precursor; it is followed by the PAL domain, a transmembrane domain and a COOH-terminal domain that is exposed to the cytosol (Yun et al., 1993); the PAM-3b protein lacks an 18 amino acid peptide in the cytoplasmic domain of rPAM-2. In contrast, rPAM-3a lacks a 68 amino acid peptide including the single transmembrane domain, and thus is a soluble protein. A soluble 75 kDa PAM protein predominates in anterior pituitary extracts and can be derived from rPAM-2, -3a or -3b by endoproteolytic cleavage.

Both soluble and integral membrane forms of peptidylglycine α-amidating monooxygenase (PAM) are expressed in the rat anterior pituitary, making it an ideal model system for studying the routing of proteins into secretory granules. To identify the subcellular compartments involved in the routing of integral membrane PAM, we used subcellular fractionation, metabolic labeling and immunoblot analysis. Mature secretory granules were found to contain full-length integral membrane PAM along with a significant amount of soluble PAM generated by endoproteolytic cleavage. PAM proteins were not co-distributed with tyrosylprotein sulfotransferase activity during sucrose gradient centrifugation, indicating that the trans-Golgi/TGN is not a major PAM-containing compartment at steady state. Fractionation of the 4,000 g and 10,000 g pellets obtained by differential centrifugation identified a significant amount of integral membrane PAM in a light fraction lacking soluble secretory granule proteins. Metabolic labeling experiments with primary anterior pituitary cells demonstrated that integral membrane PAM enters a light compartment with similar properties only after exit from the trans-Golgi/TGN compartment in organelles involved in the intracellular recycling of integral membrane PAM. Small amounts of full-length integral membrane PAM were also recovered in fractions containing internalized transferrin and may be in an endosomal compartment following retrieval from the cell surface.

Key words: secretory granule, amidation, anterior pituitary
secretory pathway in different ways (Milgram et al., 1992, 1994). A significant amount of the integral membrane PAM protein is localized in the vicinity of the Golgi complex and kinetic studies suggest that membrane PAM may be retrieved from immature granules without ever reaching the plasma membrane. Full-length integral membrane PAM is detectable on the surface of transfected AtT-20 cells, primary anterior pituitary cells and primary cardiomyocytes (Milgram et al., 1993; Maltese and Eipper, 1993); PAM protein that reaches the cell surface is rapidly internalized, and then accumulates in the vicinity of the Golgi apparatus. Mutant forms of PAM lacking most of the cytoplasmic domain were less extensively cleaved by the endoproteases characteristic of the regulated pathway, were localized on the plasma membrane, and failed to undergo internalization (Milgram et al., 1993; Tausk et al., 1992). These data suggest that the COOH-terminal domain of PAM contains information for its trafficking within the secretory pathway in addition to the information necessary for its retrieval from the plasma membrane.

Current data on the routing of integral membrane PAM suggest that PAM should be present in secretory granules and additional subcellular compartments. In an attempt to identify the subcellular compartments involved in routing of membrane-associated PAM, the distribution of PAM proteins in the rat anterior pituitary has been investigated. The anterior pituitary is a complex tissue composed of several different endocrine cell types, all of which contain PAM; highest levels of PAM occur in a subpopulation of gonadotropes, with moderate levels in corticotropes and lower levels in somatotropes and lactotropes (May et al., 1990). Anterior pituitary was selected for these studies because it is a rich source of PAM in which subcellular fractionation, metabolic labeling and immunoblot analyses can be carried out. Previous studies using subcellular fractionation techniques to examine the anterior pituitary indicated that, although the secretory granules of different cell types differ in size and density (Nansel et al., 1979), other organelles can be separated into well-defined fractions.

**MATERIALS AND METHODS**

**Differential and equilibrium centrifugation**

Anterior pituitaries from adult male Sprague-Dawley rats (150-200 g) (Harlan, Indianapolis, IN) were homogenized at 4°C in 10 volumes (w/v) of 0.32 M sucrose, 10 mM Tris-HCl, pH 7.4, containing protease inhibitors (30 µg/ml aprotinin, 30 µg/ml leupeptin, and 10 µg/ml leupeptin) using eight strokes of a motor-driven glass Potter-Elvehjem homogenizer with a teflon pestle. The anterior pituitaries of Sprague-Dawley rats purchased from Charles River (Wilmington, MA) consistently contained PAM-1-derived proteins not observed in rats from Harlan. The homogenate was subjected to differential centrifugation using a TLA100.3 rotor in a Beckman TL100 ultracentrifuge. The homogenate was centrifuged for 5 minutes at 120,000 g yielding a pellet (P1) containing nuclei and cell debris. The supernatant (S1) was centrifuged at 4,000 g for 15 minutes to obtain a P2 pellet. The resulting post-supernatant (S2) was separated into a P3 pellet and soluble fraction (S3) by centrifugation at 10,000 g for 15 minutes. A P4 pellet and a cytosolic fraction (S4) were obtained following centrifugation of S3 at 350,000 g for 15 minutes. Each pellet was resuspended in 350 µl of homogenization buffer and either centrifuged for 17 minutes to obtain a P2 pellet. The resulting post-supernatant (S2) was separated into a P3 pellet and soluble fraction (S3) by centrifugation at 10,000 g for 15 minutes. A P4 pellet and a cytosolic fraction (S4) were obtained following centrifugation of S3 at 350,000 g for 15 minutes. Each pellet was resuspended in 350 µl of homogenization buffer and either kept at 70°C for enzyme assays and western blot analysis or fractionated further on a discontinuous sucrose density gradient. All sucrose solutions were made in 10 mM Tris-HCl, pH 7.4, containing protease inhibitors and the gradients were prepared in 2-ml polycarbonate tubes 1 hour before use. The resuspended P2 pellet was layered onto a density gradient consisting of (from top to bottom): 200 µl of 0.4 M sucrose; 250 µl of 0.6 M sucrose; 350 µl of 0.8 M, 1.0 M, 1.2 M and 1.4 M sucrose; and 350 µl of 1.6 M sucrose. The density gradient used for P3 consisted of 200 µl of 0.4 M, 0.6 M, 0.8 M and 1.0 M sucrose; 350 µl of 1.2 M, 1.4 M and 1.6 M sucrose; and 200 µl of 2.0 M sucrose. The density gradient used for P4 consisted of 200 µl of 0.4 M, 0.6 M and 0.8 M sucrose; 350 µl of 1.0 M, 1.2 M and 1.4 M sucrose; and 250 µl of 1.6 M sucrose. The gradients were centrifuged in a swinging bucket TL855 rotor for 2 hours at 120,000 g; 150-µl fractions were collected from the top of the gradient and either analyzed further or stored at ~70°C. Density values were determined by measuring the refractive index of fractions of a parallel gradient.

**Characterization of subcellular fractions**

Fractions obtained after differential centrifugation and sucrose gradient centrifugation were assayed for subcellular marker enzymes.

**Tyrosylprotein sulfotransferase**

The activity of tyrosylprotein sulfotransferase, a marker for trans-Golgi/TGN, was determined by measuring the sulfation of the synthetic polymer EAY (Glu, Ala5, Tyr1) using 5'-[35S]phosphoadenosine 3'-phosphosulfate (Amersham, Corp.) essentially as described by Rens-Domiano and Roth (1989) except that 0.5% Triton X-100 was used instead of Lubrol. After incubation at 30°C for 30 minutes, the sulfated polymer was precipitated with 12% TCA and filtered onto 2.4 cm GF/A glass microfiber filters (Whatman Labsales, OR). Following two washes with 5% TCA, the filters were rinsed with...
cold acetone and dried. The radiolabeled product was measured in a Beckman LS1701 liquid scintillation counter.

5′-Nucleotidase
The activity of 5′-nucleotidase, a marker for plasma membrane, was determined by measuring the release of [3H]adenosine from [3H]AMP (Amersham, Corp.) as described by Newby et al. (1975). Reactions were terminated with 150 μl of 0.15 M ZnSO4, and subsequent addition of 150 μl of 0.15 M Ba(OH)2 to precipitate [3H]AMP; radiolabeled adenosine present in the supernatant was determined in a liquid scintillation counter.

Amidation assays
PHM activity was measured using 0.5 μM t-α-N-acetyl-Tyr-Val-Gly, 125I-t-α-N-acetyl-Tyr-Val-Gly (20,000 to 25,000 cpm) at pH 5.0 in the presence of 0.5 μM CuSO4, 0.5 mM ascorbate and 0.18 mg/ml catalase. Following incubation at 37°C for 1 hour, the α-hydroxylated product formed by PHM was converted into α-amidated product by the addition of base (Sakata et al., 1986; Eipper et al., 1991). PAL activity was determined as described (Eipper et al., 1991). For both assays, α-amidated product was separated from substrate by extraction with ethyl acetate (Perkins et al., 1990). Samples were assayed in duplicate at two or more concentrations of protein, and duplicates varied by less than 5%.

Western blot analysis
Samples of fractions obtained after differential centrifugation and sucrose density gradient centrifugation were fractionated on either 8 or 10% polyacrylamide gels. Western blot analysis was performed as described above. The radiolabeled transferrin in each sucrose fraction was depleated endogenous transferrin and then incubated for 30 minutes in 10% polyacrylamide gels and prepared for fluorography as described by Milgram et al. (1993).

RESULTS
Differential centrifugation
Anterior pituitaries from adult male rats were homogenized and fractionated by differential centrifugation. The fractions were characterized using enzyme assays and western blots. Significant amounts of PHM and PAL activity were recovered in all three particulate fractions (Fig. 2A). The forms of PAM protein in each fraction were not identical. While PAM proteins of the same medium containing 870 μCi [35S]methionine/cysteine labeling mix (5 μM methionine, Amersham Corp., Arlington Heights, IL) for 30 minutes. At the end of the incubation with [35S]methionine, the cells were harvested immediately (pulse) or further incubated for 4 hours in 500 μl CSFM (chase) at either 20°C or 37°C. Following removal of the medium, the cells were rinsed once with CSFM and then scraped into 0.32 M sucrose, 10 mM Tris-HCl, pH 7.4. Metabolically labeled cells were mixed with five adult male Sprague-Dawley rat (150-200 g) anterior pituitary lobes and then subjected to differential and equilibrium centrifugation analysis as described above. Samples (130 μl) of the sucrose density gradient fractions were diluted fivefold with 50 mM sodium phosphate, pH 7.4, 1% Triton X-100 containing protease inhibitors and PAM proteins were immunoprecipitated with a rabbit polyclonal antibody to PHM as described by Milgram et al. (1993). Samples were fractionated by SDS-PAGE on 10% polyacrylamide gels and prepared for fluorography as described by Milgram et al. (1993).

For the uptake of transferrin by primary anterior pituitary cells, human dfferfer transferrin (Sigma Chemical Co, St Louis, MO) was iodinated to a specific activity of 2×106 cpm/μg by incubating 100 μg transferrin with an iododeae (Pierce Chemical Co, IL) and 0.5 mCi Na125I in 100 mM sodium phosphate, pH 7.4, for 1 minute at room temperature; unincorporated iodine was removed by gel filtration in phosphate buffered saline (50 mM sodium phosphate, 150 mM NaCl, pH 7.4). Primary anterior pituitary cell cultures (2×105 cells/12 mm plate) were preincubated in serum-free DMEM for 30 minutes at 37°C to deplete endogenous transferrin and then incubated for 30 minutes in 400 μl of the same medium containing 125I-labeled transferrin (10 μg) (Schmid et al., 1988). After incubation, the cells were cooled to 4°C and surface-bound transferrin was removed by incubating the cells for 2 minutes in cold 25 mM acetic acid, 150 mM NaCl, pH 2.8. Following a rinse with cold phosphate buffered saline, the cells (two 12 mm wells) were scraped in 0.32 M sucrose, 10 mM Tris-HCl, pH 7.4, mixed with five adult anterior pituitary lobes and subjected to fractionation as described above. The radiolabeled transferrin in each sucrose fraction was determined in a Micromedic 4200 plus gamma counter.
Tyrosylprotein sulfotransferase is a marker for trans-Golgi/TGN (Lee and Huttner, 1985); since fluorescence microscopy indicates that transfected forms of integral membrane PAM localize to the Golgi/TGN region of the cell, the distribution of this marker was determined. Sulfotransferase activity was recovered primarily in the 10,000 g (P3) and 350,000 g (P4) fractions (Fig. 2D).

Antisera against markers for several subcellular organelles and proteins with which PAM might interact were used to characterize the fractions (Fig. 2E). Endoplasmic reticulum markers, GRP78, ERP72 (Lewis et al., 1985; Mazzarella et al., 1990) and protein disulfide isomerase (Akagi et al., 1988) were recovered in all subcellular fractions except S4 (data not shown). Based on biosynthetic labeling studies and resistance to digestion with endoglycosidase H, less than 10% of the PAM protein resides in the endoplasmic reticulum at steady state. Therefore, although endoplasmic reticulum is recovered in all the particulate fractions, very little of the PAM protein observed on immunoblots resides in the ER. Thy-1, a GPI-linked membrane protein that serves as a plasma membrane marker (Low and Kincade, 1985; Tse et al., 1985), was recovered primarily in P4. CI-M6PR, the protein that mediates the sorting of newly synthesized lysosomal enzymes to lysosomes and a late endosomal marker (Von Figura, 1991; Brown, 1990; Green and Kelly, 1992), was most prevalent in the P4 fraction. The distribution of synaptotagmin (p65), an integral membrane protein that binds acidic phospholipids and calcium (Brose et al., 1992) and is found in synaptic vesicles and large dense core vesicles in neurons (Walch-Solimena et al., 1993; Trifaro et al., 1989), resembles that of PAM. Synaptophysin, a marker for synaptic-like microvesicles (Nuvone et al., 1986), was found in the P3 and P4 fractions. In order to obtain a better understanding of the localization of PAM proteins, the pellets obtained by differential centrifugation were resuspended and subjected to sucrose density gradient fractionation.

**Sucrose density gradient fractionation-steady state distribution of PAM**

We were especially interested in identifying PAM proteins in the trans-Golgi/TGN or in secretory granules. Therefore the distributions of PAM, tyrosylprotein sulfotransferase activity and POMC were compared following sucrose density gradient
fractionation. The 4,000 g P2 fraction was separated into two regions containing the majority of the PAM protein and PAL activity. The lighter region (P2A) contained primarily intact PAM-2 while the denser region (P2C) contained intact and processed forms of PAM (Fig. 3). Intact and processed POMC were also present in the denser fractions, indicating that these fractions are enriched in peptide-containing secretory granules. Peak sulfotransferase activity (P2B) was clearly separated from any peaks of PAL activity or PAM protein, suggesting that the trans-Golgi/TGN is not a major PAM-containing compartment at steady state. The prevalence of intact PAM-2/3b in P2A suggests that it contains a subcellular compartment distinct from secretory granules. The small amount of soluble PAM and POMC at the top of the gradient comes from lysed organelles.

Antisera against several organelle markers were used to evaluate their distribution throughout the P2 gradient (Fig. 4).

Fig. 3. Sucrose density gradient fractionation of P2. The P2 pellet obtained by differential centrifugation was resuspended in 0.32 M sucrose and fractionated on a discontinuous sucrose density gradient. Fractions collected from the top of the gradient were analyzed for the steady state distribution of PAL activity, tyrosylprotein sulfotransferase activity and PAM and POMC protein. The densities corresponding to the peaks of PAL and sulfotransferase activities are indicated. PAM proteins in the gradient fractions were analyzed by western blot using an antiserum to PHM; POMC was identified using a γMSH antibody. Immunoblots were quantitated by densitometry to determine the percentage of total PAM protein (sum of all forms of PAM protein) recovered in each fraction. The presence of processed PAM proteins and POMC at the bottom of the gradient is consistent with the design of the P2 gradient to pellet secretory granules. Similar distributions of PAM, tyrosylprotein sulfotransferase and POMC were observed in five separate experiments.

Fig. 4. Distribution of markers for subcellular organelles following sucrose density gradient fractionation of P2. Fractions from gradients like that shown in Fig. 3 were subjected to western blot analysis; equal volumes of each fraction were separated by SDS-PAGE, transferred to Immobilon-P, and probed with the antibodies indicated. For comparison, the peaks of PAL (A and C) and tyrosylprotein sulfotransferase (B) activities are indicated. Similar distributions of organelle markers were obtained in three other analyses.

Synaptotagmin was prevalent in P2 and immunoblots showed a bimodal distribution of synaptotagmin quite similar to that of PAM proteins. The small amount of CI-M6PR in P2, was recovered in the denser region (B and C) of the gradient. Only very small amounts of synaptophysin were found in P2 and almost none co-distributed with the secretory granules. Instead, synaptophysin was found in the lighter fractions. The small amount of Thy-1 recovered in P2 was also found in light fractions enriched in intact PAM-2 and synaptophysin.

The 10,000 g pellet (P3) was analyzed on a sucrose density gradient designed to keep secretory granules from pelleting (Fig. 5). PAL activity and PAM protein were separated into two regions with most of the PAL activity and PAM protein in the denser fractions (P3C). The peaks of PAL activity and total PAM protein (sum of all forms) were again clearly resolved from the peak of sulfotransferase activity (P3B), suggesting that little PAM protein resides in the trans-Golgi/TGN at steady state. The denser region of the gradient (P3C) was enriched in secretory granules as evidenced by the presence of POMC and its 16 K fragment product peptide. The PAM proteins present in these denser fractions (P3C) were also enriched in processed forms. The lighter compartment (P3A) contained only intact PAM-2.

The P3 sucrose gradient fractions were assayed for marker proteins (Fig. 6). Of the modest amount of synaptotagmin found in P3, most co-distributed with proteolytically processed PAM and POMC proteins in the denser fractions (P3C) of the gradient. Smaller amounts of synaptotagmin were found in the lighter fractions (P3A) containing intact PAM-2. The CI-M6PR, found in significant amount in P3, was separated from
the PAM and POMC proteins resident in secretory granules and was recovered from lighter fractions. Synaptophysin was broadly distributed throughout the gradient, overlapping significantly with denser fractions containing PAM proteins and lighter fractions containing CI-M6PR. The Thy-1 present in P3 was recovered in two peaks overlapping, but not identical to, the peaks of PAL activity.

The 350,000 g pellet (P4), contained a lighter peak (P4A) and a denser peak (P4C) of PAL activity and PAM protein (Fig. 7). The peak of sulfotransferase activity (P4B) was again not coincident with either peak of PAL activity or total PAM protein. Intact PAM-2/3b predominated in the light region (P4A) and a significant amount of processed PAM protein was found in the denser region (P4C); the small amount of POMC and 16K fragment in P4 was recovered in P4C (data not shown). The distribution of synaptotagmin across the P4 gradient again paralleled that of PAM (Fig. 8). The CI-M6PR, synaptophysin and Thy-1 were all most prevalent in P4. All were found primarily in lighter fractions (A and B); none was prevalent in the P4 fraction containing secretory granules (P4C).

Identification of subcellular compartments containing PAM by biosynthetic labeling and temperature block analysis
To obtain a better identification of ER, trans-Golgi/TGN and post-TGN compartments, primary cultures of anterior pituitary cells were metabolically labeled, mixed with fresh anterior pituitary tissue and subjected to subcellular fractionation. Cultures maintained in serum-containing medium consist of endocrine cells and fibroblast-like cells and fractionate much like anterior pituitary tissue. Incubation times were selected on
the basis of detailed studies of soluble and integral membrane PAM proteins in stably transfected AtT-20 cells (Milgram et al., 1994). In AtT-20 cells labeled and chased at 37°C, ER to Golgi transport rates of approximately 60 minutes were observed for both soluble and integral membrane PAM proteins, and endoproteolytic cleavage of newly synthesized PAM proteins began approximately 2 hours after biosynthesis. Cleavage was completely blocked when chase incubations were carried out at 20°C to prevent protein export from the TGN (Saraste and Kuismanen, 1984; Griffiths et al., 1985), suggesting that proteolysis is initiated in immature secretory granules.

We labeled the primary cells for 15 minutes at 37°C (pulse) to identify PAM proteins in the ER compartment; PAM in the ER is a minor contributor to the PAM proteins visualized by immunoblotting. To enrich for radiolabeled PAM in the trans-Golgi/TGN, primary pituitary cells were labeled for 15 minutes at 37°C and then chased for 4 hours at 20°C. Secretory granules and other post-TGN compartments were identified by carrying out the chase at 37°C for 4 hours; results after shorter chase times (2 or 3 hours) were also examined and were similar (data not shown). PAM proteins were immunoprecipitated from gradient fractions with a PHM antibody, analyzed by SDS-PAGE and newly synthesized PAM proteins were detected by fluorography.

After the pulse incubation, most of the newly synthesized PAM proteins were recovered in P2 and P3. Intact radiolabeled PAM-2/3b (105 kDa) and PAM-3a (97 kDa) were recovered from the denser regions of both gradients and no difference was observed in the distribution of integral membrane and soluble PAM proteins (Fig. 9A, B, C); immunoblot analysis confirmed the presence of endoplasmic reticulum markers in these fractions (data not shown). The small percentage of PAM expected to reside in the ER at steady state makes this compartment difficult to visualize using immunoblot analysis (Milgram et al., 1994).

When cells were pulse labeled and chased at 20°C, no 75 kDa PAM protein was observed, radiolabeled PAM did not move into the light region of the gradient and radiolabeled soluble and integral membrane PAM proteins still exhibited identical distributions (except for the presence of some soluble PAM proteins at the top of the gradient). Furthermore, during the 4 hours chase at 20°C, more of the newly synthesized PAM protein moved into fractions enriched in the trans-Golgi/TGN marker, tyrosylprotein sulfotransferase (P2B, P3B, P4B). These data are consistent with accumulation of most of the radiolabeled PAM within the TGN under these conditions.

As expected, PAM proteins underwent endoproteolytic processing when the cells were chased at 37°C; cleavage of the
newly synthesized PAM proteins occurs only after the proteins exit the TGN (Milgram et al., 1993). The 75 kDa PAM protein was most prevalent in the region of the P2 and P3 gradients containing secretory granules. Following the 37°C chase, the distributions of integral membrane (105 kDa) and soluble (97 kDa and 75 kDa) PAM proteins were no longer identical. Intact radiolabeled PAM-2/3b was recovered in the lighter fractions of the P2 and P3 sucrose gradients when cells were chased at 37°C but not when cells were chased at 20°C. This result indicates that the intact integral membrane PAM proteins found in the lighter regions of the P2 and P3 gradients are located in a post-TGN, non-secretory granule compartment. In contrast, even after a 4 hour chase at 37°C, very little of the newly synthesized intact PAM-2/3b in the P4 gradient was recovered in the light fractions enriched in PAM-2/3b at steady state.

**DISCUSSION**

A model was developed to explain the trafficking of integral membrane and soluble PAM proteins in endocrine cells, based on data obtained by examining stably transfected AtT-20 cells, and suggests that PAM proteins that have passed through the endoplasmic reticulum and Golgi complex should be found in immature granules, a recycling compartment, constitutive-like vesicles, mature granules, endosomes and plasma membrane (Fig. 10) (Milgram et al., 1994). We used differential cen-
trifugation coupled with sucrose density gradient fractionation and immunoblot analysis to see if a similar model might apply to normal pituitary tissue. Metabolic labeling studies coupled with subcellular fractionation facilitated the identification of compartments not fully resolved on gradients.

PAM has been localized to secretory granules in several neural and endocrine tissues (Hu and Thorn, 1993; Oyarce and Eipper, 1993). As expected, sucrose density gradient fractionation of the P2, P3 and P4 fractions revealed the presence of PAM-containing organelles (P2C, P3C, P4C) with the buoyant density of mature granules (Figs 3, 5, 7) (Nansel et al., 1979). The anterior pituitary contains several cell types, most of which express PAM, and the secretory granules in the different cell types vary considerably in size and density (Nansel et al., 1979; May et al., 1990). The mature secretory granules characterized by immunoblot analysis contained intact PAM-2/3b, soluble PAM-3a and a 75 kDa PAM protein derived from the larger membrane and soluble PAM proteins by endoproteolytic processing. The presence of POMC and one of its processed products (16K fragment) (Figs 3, 5) as well as prohormone convertase 1 (PC1), one of the neuroendocrine-specific subtilisin-like endoproteases (data not shown), confirmed the localization of most of the mature secretory granules in the denser regions of the P2, P3 and P4 gradients. A significant amount of synaptotagmin is co-distributed with PAM in mature secretory granules, especially in P2C and P3C. Synaptotagmin has been identified in the membranes of chromaffin granules and large dense-core vesicles (LDCV) of the posterior pituitary, hypothalamus and sympathetic neurons (Fournier and Trifaro, 1988; Fournier et al., 1989; Schmidile et al., 1991; Walch-Solimena et al., 1993). Scanning densitometry of the PAM immunoblots indicates that at least 30% of the total PAM protein (sum of all forms) is in secretory granules at steady state.

Studies on stably transfected AtT-20 cells suggest that newly synthesized integral membrane PAM proteins, after removal from immature granules, are largely retained in the cell and perhaps repackaged into immature granules (Milgram et al., 1993; 1994). Little newly synthesized membrane PAM reaches the plasma membrane, making trafficking of PAM distinctly different from synaptic vesicle proteins. A major goal of these studies was identification of the compartments involved in recycling integral membrane PAM. At steady state, immunofluorescence studies indicate that a significant amount of the membrane PAM protein in AtT-20 cells is localized to the perinuclear region overlapping markers for the trans-Golgi/TGN (Milgram et al., 1992, 1993). Tyrosylprotein sulfotransferase was assayed as a biochemical marker for the trans-Golgi/TGN (Lee and Huttner, 1985; Baeuerle and Huttner, 1987; Huttner and Baeuerle, 1988; Leitinger et al., 1994). If a significant amount of integral membrane PAM resides in the trans-Golgi/TGN at steady state, we would expect to observe some regions of the sucrose density gradients with coincident peaks of PAM and tyrosylprotein sulfotransferase activity. In contrast, peaks of sulfotransferase activity and PAL activity or PAM protein were clearly separated in all three gradients (Figs 3, 5, 7), suggesting that integral membrane PAM accumulated in the perinuclear region is not located primarily in the trans-Golgi/TGN.

To identify biochemically a PAM-containing compartment that could correspond to the recycling compartment and be responsible for the perinuclear localization of integral membrane PAM, we made use of the fact that exit of proteins from the TGN is slowed substantially at 20°C. When primary anterior pituitary cells were pulse labeled at 37°C and chased at 20°C for 4 hours (Fig. 9), newly synthesized integral membrane and soluble PAM were enriched in fractions containing tyrosylprotein sulfotransferase (P2B, P3B, P4B). During a 2- to 4-hour chase at 37°C, soluble PAM proteins accumulated in fractions enriched in secretory granules while integral membrane PAM proteins accumulated in the lighter regions of the P2 and P3 gradients (P2A; P3A) as well as in secretory granules.

In light of our model for PAM trafficking (Fig. 10) (Milgram et al., 1994), this post-TGN compartment could include immature granules, constitutive-like vesicles and the recycling compartment. The buoyant density of even the lightest anterior pituitary secretory granules is considerably greater than that of P2A/P3A (Nansel et al., 1979). In addition, our inability to detect soluble forms of PAM or any forms of POMC or PC1 in this region of the gradient argues against its identification as immature granules or constitutive-like vesicles. Together these data strongly suggest that integral membrane PAM proteins in the lighter regions of the P2 and P3 sucrose density gradients are in organelles involved in the recycling of integral membrane PAM. Synaptotagmin is also recovered in P2A and P3A while very little cation-independent M6PR is recovered in membrane PAM. Synaptotagmin is also recovered in P2A and appears to be in organelles involved in the recycling of integral membrane PAM.

Fig. 10. PAM-containing subcellular compartments in the rat anterior pituitary. Subcellular fractionation, immunoblot analysis and metabolic labeling studies identified PAM in: endoplasmic reticulum (ER); Golgi; trans-Golgi/TGN (TGN); immature granules (IG); constitutive-like vesicles (CLV); recycling compartment (RC); mature granules (MG); endosomes (E) and plasma membrane (PM).
piritary. Our immunofluorescence and kinetic studies on AtT-20 cells are consistent with the idea that significant amounts of integral membrane PAM reside in this recycling compartment.

Immunoblot analysis of the P4 sucrose density gradient revealed the presence of lighter fractions (P4A) containing full-length PAM-2/3b but no soluble PAM or POMC (Fig. 7); metabolic labeling studies indicated that P4A occurred later in the biosynthetic pathway than P2A and P3A. Scanning densitometry of western blots indicates that P4A accounts for only about 4% of the total PAM protein. Markers for early and late endosomes, plasma membrane and synaptic-like microvesicles were recovered from this region of the gradient and our model suggests that each of these compartments could contain membrane PAM and would be accessible only with prolonged chase time. Further separation will be required to identify PAM in each of these compartments (Hopkins, 1983; Klausner et al., 1983; Wiedenmann et al., 1988; Navone et al., 1989; Regnier-Vigouroux et al., 1991; Cameron et al., 1991; Feany et al., 1993).

Our data on the subcellular distribution of PAM in the rat anterior pituitary have allowed us to identify biochemically a PAM-containing subcellular compartment that may correspond to the recycling compartment predicted from studies on transfected AtT-20 cells. Recycling of integral membrane PAM is not limited to neuroendocrine cells, suggesting that this recycling compartment is a specialization of the endosomal system (Tausk et al., 1992). Immunolocalization studies are underway to identify other proteins residing in this compartment.

We thank Drs W. Brown (Cornell University) and R. Scheller (Stanford University) for the kind gift of antibodies. We especially thank Drs Richard Mains and Sharon Milgram for help in the design, execution and interpretation of several experiments, Drs An Zhou and Luc Paquet for critically reading the manuscript and Marie Bell for general laboratory assistance. This work was supported by grants from the National Institute on Drug Abuse, DA-00266 and DA-00098.

REFERENCES


(Received 8 April 1994 - Accepted, in revised form, 29 August 1994)