Peptidylglycine α-amidating monoxygenase (PAM) is a bifunctional enzyme that catalyzes the COOH-terminal α-amidation of neural and endocrine peptides through a two-step reaction carried out sequentially by its monoxygenase and lyase domains. PAM occurs in soluble and integral membrane forms. Metabolic labeling of stably transfected hEK-293 and AtT-20 cells showed that [32P]PO₄ was efficiently incorporated into Ser and Thr residues of membrane PAM but not into soluble PAM. Truncation of integral membrane PAM proteins (which terminate with Ser976) at Tyr936 eliminated their phosphorylation, suggesting that the COOH-terminal region of the protein was the site of phosphorylation. Recombinant PAM COOH-terminal domain was phosphorylated on Ser937 and Ser937 by protein kinase C (PKC). PAM-1 protein recovered from different subcellular fractions of stably transfected AtT-20 cells was differentially susceptible to calcium-dependent staurosporine-inhibitable phosphorylation catalyzed by endogenous cytosolic protein kinase(s). Although phorbol ester treatment of hEK-293 cells expressing PAM-1 stimulated the cleavage/release of a bifunctional 105-kDa PAM protein, the effect was an indirect one since it was also observed in hEK-293 cells expressing a truncated PAM-1 protein that was not phosphorylated. AtT-20 cells expressing PAM-1 lacking one of the PKC sites (PAM-1/Ser937 → Ala) exhibited an altered pattern of PAM-PAM antibody internalization, with the mutant protein targeted to lysosomes upon internalization. Thus, phosphorylation of Ser937 in the COOH-terminal cytosolic domain of membrane PAM plays a role in a specific step in the targeting of this protein.

Maturation of bioactive peptides in the nervous and endocrine systems involves a series of post-translational processing steps that occur as the precursor proteins and their products pass through the secretory pathway. Peptide α-amidation, which occurs late in the pathway and is often essential for biological activity, is a two-step reaction catalyzed in sequence by two enzymes contained within the bifunctional enzyme, peptidylglycine α-amidating monoxygenase (PAM; EC 1.14.17.3) (1, 2). The first reaction is catalyzed by peptidylglycine α-hydroxylating monoxygenase (PHM) and requires copper, ascorbate, and molecular oxygen, and the second reaction is catalyzed by peptidyl-α-hydroxyglycine α-amidating lyase (PAL) and generates α-amidated peptide and glyoxylate (3–7).

Alternative splicing of the single-copy PAM gene generates mRNAs encoding integral membrane and soluble PAM proteins in a tissue-specific and developmentally regulated manner (8–10). PAM-1 includes Exon 16, a noncatalytic region separating PHM and PAL, a transmembrane domain and a COOH-terminal domain (Fig. 1); Exon 16 is absent from PAM-2. Exon 25 encodes a transmembrane domain and is absent from PAM-3; the splicing of Exon 25 determines the intracellular fate of the COOH-terminal domain. The COOH-terminal domain of integral membrane PAM is exposed to the cytoplasm, whereas the same domain in soluble bifunctional PAM resides in the lumen of the secretory compartment (12). The COOH-terminal domain of PAM is essential for retrieval of integral membrane PAM from the cell surface in neuroendocrine and non-neuroendocrine cells (13, 14) and plays a modular role in the in vitro PHM activity of soluble bifunctional PAM-3 (15).

PAM proteins undergo various post-translational processing events while moving through the secretory pathway. The NH₂-terminal signal peptide is cleaved and Asn765 is glycosylated in the endoplasmic reticulum (12). In cultured cardiomyocytes and transfected hEK-293 cells, PAM-1, but not PAM-2, is sialylated (13, 16). Multiple sialylated and/or sulfated O-linked oligosaccharides are present when Exon 16 is included (17). Endoproteolytic processing gives rise to smaller soluble and membrane-associated PAM proteins in a tissue- and cell type-specific manner (18–20). Tyr936 in the COOH-terminal domain is sulfated only in PAM-3 (17).

The COOH-terminal domain of PAM is highly conserved (63% amino acid identity for man and Xenopus laevis) and includes several conserved potential phosphorylation sites (8). We set out to determine whether PAM was phosphorylated, and, if so, whether phosphorylation might play a role in regulating its routing. Metabolic labeling and immunoprecipitation of stably transfected hEK-293 and AtT-20 cells demonstrated that integral membrane PAM proteins were phosphorylated on Ser and Thr residues in the COOH-terminal domain. PAM proteins in different subcellular compartments were differential...
Phosphorylation of PAM

tially susceptible to phosphorylation by endogenous protein kinases. A membrane PAM protein lacking one of the two PKC sites in recombinant PAM COOH-terminal domain was misrouted after internalization from the plasma membrane.

MATERIALS AND METHODS

Cell Culture/ Metabolic Labeling—HEK-293 or AIT-20 cells expressing individual PAM proteins (13, 20) were maintained in Dulbecco’s modified Eagle’s medium-nutrient mixture F-12 mixed in equal volumes (DMEM/F-12, LifeTechnologies, Inc.) containing 10% Fetal Clone serum (HyClone) (HEK-293 cells) or 10% Fetal Clone serum and 10% NuSerum (AIT-20 cells), antibiotics and 0.5 mg/ml G418 at 37°C in 5% CO2 and 100% humidity. Cells plated on 12-mm culture dishes coated with 25 μg/ml fibronectin (HEK-293 cells) or 0.1 mg/ml poly-lysine (AIT-20 cells) were grown a minimum of 36 h and were 50 to 75% confluent before experiments were begun. Biosynthetic labeling with [35S]methionine/cysteine was performed as described (14, 21). For [32P]PO4 labeling, cells were incubated for 1 h in PO4-free complete serum medium (CSFM) and then incubated in 300 μCi of [32P]PO4 (850 Ci/mm; Amersham) for 6 h. At the end of the incubation with [35S]methionine/cysteine or [32P]PO4, the cells were rinsed once with CSFM and extracted. Whole cell extracts were prepared by scraping cells into ice-cold 20 mM sodium TES, 10 mM mannitol, pH 7.4, 1% Triton X-100, plus protease inhibitors (30 mM sodium fluoride, 0.5 mM benzamidine, 0.25 mM EDTA, 0.25 mM 1,10-phenanthroline, 0.5 mg/ml leupeptin, and 10 μg/ml pyrophosphatase, and 50 mM NaF) (22).

Pulse-chase experiments were carried out as described (20). Phorbol 12-myristate 13-acetate (PMA, 1 μM final concentration) (Calbiochem) was diluted into CSFM from a 1 mg/ml (1.62 mw) stock in dimethyl sulfoxide, and staurosporine (1 μM final concentration) (Calbiochem) was diluted into CSFM from a 1 mg/ml stock in dimethyl sulfoxide. Aliquots of cell extracts or media (50–100 μl) were diluted 5-fold in 50 mM sodium phosphate, pH 7.4, 1% Triton X-100 (Super E) containing protease inhibitors and protein phosphatase inhibitors and incubated with 10 μg/ml monoclonal antibody against PHM, PAL, CD, PAM COOH-terminal domain peptide (40 Ci of [32P]PO4) at 37°C for 2 h (12, 14). For immunoprecipitation with PAL antibody, extracts or media were boiled in 1% SDS for 5 min after addition of protease inhibitors and protein phosphatase inhibitors and then diluted 4-fold with Super E containing 0.5% Nonidet P-40 before addition of antisera. Immune complexes were isolated using protein A-Sepharose (Sigma), and immunoprecipitates were fractionated by SDS-PAGE and analyzed as described (14). Films were densitometrically using an Abaton Scan 300/GS and NIH Image 1.3 software (14).

Phosphoinoide Acid Analysis—PAM proteins immunoprecipitated from cells metabolically labeled with [32P]PO4 were fractionated by SDS-PAGE, visualized by autoradiography, extracted (23), and digested with 50 μg/ml trypsin (tosylphenylalanyl phenylmethyl ketone, 150,000 u/g, Fluka Chemical Corp., Ronkonkoma, NY) at 37°C for 16 h; a subsequent 4-h incubation was carried out with an additional 2 ml of 25 mM MgCl2, 0.5 mM CaCl2, 1 μl of [γ-32P]ATP (6.7 nM ATP), and 0.1 μg of PKC purified from rat brain (kindly provided by Dr. Richard Huganir, Johns Hopkins University School of Medicine) (24). Reactions were terminated by adding 20 μl of 0.5 M EDTA (pH 8.0). Samples were desalted by adsorption to a C18 Sep-Pak cartridge (Waters, Milford, MA) equilibrated with 0.1% trifluoroacetic acid, the cartridge was washed with the same buffer, and the peptide was eluted with 0.1% trifluoroacetic acid, 80% acetonitrile (25), dried by vacuum centrifugation, and analyzed by SDS-PAGE on gels containing 16.4% acrylamide (0.6% N,N’-methylenebisacrylamide) and autoradiography.

Phosphopeptide Mapping by Reverse Phase HPLC—Desalted in vitro phosphorylated PAM COOH-terminal domain protein was incubated with 50 μl of 25 mM ammonium bicarbonate (pH 8.0) containing trypsin (5% by weight of sample protein) at 37°C for 16 h. Samples were diluted in 500 μl of 0.1% trifluoroacetic acid (buffer A) and passed through a HPLC nylon filter (3-mm membrane, 0.45-μm pore size, MSI Micron Separations Inc., Honeyeye Falls, NY) to remove particulate material. Samples were fractionated on a reverse phase C8 bonded to HPLC column (Waters) using a linear gradient of 0–40% acetonitrile in buffer A over 60 min followed by a linear gradient to 76% acetonitrile in buffer A over 15 min. The flow rate was 1 ml/min, and the elution pattern was monitored at 220 nm; an internal standard of phenol red or 5-Tyr-Trp-Dansyl (Roth Biochemicals, West Grove, PA) was included to facilitate comparisons of HPLC runs. Aliquots of fractions were assayed for 32P radioactivity using a Bedman liquid scintillation counter.

Purification and Analysis of Phosphoproteptides—Purified recombinant PAM COOH-terminal domain peptide (40 μg) was phosphorylated in vitro by PKC with 1 mM ATP plus 1 μCi of [γ-32P]ATP. Reactions were carried out at 30°C for 1 h. Reaction mixtures were desalted using a C18 Sep-Pak, dried, and treated with trypsin as described. Samples were chromatographed on a C8-Bondapak HPLC column (Waters) equilibrated with 20 mM sodium phosphate (pH 7.0) containing 10 mM sodium perchlorate and eluted with a linear gradient of 0–45% acetonitrile in 68 min (26). Fractions containing 32P radioactivity were desalted and subjected to amino acid sequence analysis using an Applied Biosystems Model 477A pulsed-liquid instrument with on-line HPLC identification of phenylthiohydantoin derivatives.

Subcellular Fractionation—AIT-20 cells grown to 60% confluence in 15-cm dishes were harvested with 20 ml of 0.5 M sucrose, and staurosporine (1 μM final concentration) (Calbiochem) was added to the cells for 1 h. For immunoprecipitation with PAL antibody, the cells were fixed by adding 20 ml of 0.5 M EDTA. Cells were then immunoprecipitated using rabbit polyclonal Ab571 (12, 15) and analyzed by SDS-PAGE and Western blot using PHM antibody 475 and ECL (Amersham).

In vitro phosphorylation of the resuspended pellets was carried out at 30°C for 1 h in a reaction volume of 50 μl containing 20 μl of resuspended differential centrifugation pellet and 6 μl of [γ-32P]ATP in 20 mM HEPES (pH 7.0), 10 mM MgCl2, and 0.25 mM succrose in the absence or presence of 0.5 mM Ca2+ or cyclosporine (1 μM). Reactions were terminated by adding 20 μl of 0.5 M EDTA. Samples were then immunoprecipitated using rabbit polyclonal Ab571 (12, 15) and analyzed by SDS-PAGE and fluorography.

Site-directed Mutagenesis and Generation of Stable Cell Lines—The plasmid pBluescript plasmid encoding PAM-1 with Ser937 mutated to Ala937 (pBS.KrPAM-1/S937A) was created using the method of Horton et al. (28). pBS.KrPAM-1 was amplified twice once with an upstream primers (rPAM-1-(2791–2809)) and a mutagenic antisense primer with a codon for Ala937 (underlined): [5-TTT TCT GGC GTA GCC TTT CCG]-3; once with a mutagenic sense primer [5-CGA AAA GGC TAC GCC AGA AAA-3] and the T7 antisense primer. The two polymerase chain reaction products were combined and amplified using rPAM-1-(2791–2809) and the T7 primer. The amplified fragment was inserted into pBS.KrPAM-1 using Smal (nucleotide 2950) and XbaI, creating pBS.KrPAM-1/S937A. The vector generated by polymerase chain reaction was verified by sequencing. The pCIS.KrPAM-1/S937A expression vector was constructed using Bsp961 and XbaI. AIT-20 cells were transfected using Lipofectin and screened as described (20).

Antibody internalization experiments were carried out using a rabbit polyclonal antibody to PHM (Ab479) as described previously (13, 14); primarily the antibody was visualized using Cy3-AffiniPure F(ab)2 donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Lysosomes were visualized simultaneously using rat monoclonal antibody 1D4B (Ab479), which is directed against the monomeric low molecular weight 100,000 Da subunit of rat lysosomal protein 1 (LAMP-1) (29) (Developmental Studies Hybridoma Bank, Dept. of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine) and FITC-goat F(ab)2 (Jackson Immunoresearch Laboratories, Inc., San Francisco, CA). Confocal microscopy was performed on a 60× oil-immersion objective with 1.4 NA. Images were visualized by the simultaneous collection of the two fluorescent signals; optical sections were 1 μm thick, and each image was a composite of eight frames.
RESULTS

Phosphorylation of PAM Proteins Expressed in hEK-293 and AtT-20 Cells—Stably transfected hEK-293 cell lines expressing three alternatively spliced forms of PAM or a mutant integral membrane PAM protein with a truncated COOH-terminal domain (PAM-2/899) (13, 14) (Fig. 1) were metabolically labeled with [35S]methionine/cysteine or [32P]PO4 for 6 h, and PAM proteins were immunoprecipitated from cell extracts (Fig. 2A). As expected, [35S]methionine/cysteine-labeled 120-kDa PAM-1, 105-kDa PAM-2, 95-kDa PAM-3, and 95-kDa PAM-2/899 proteins were identified (13). [32P]PO4 was efficiently incorporated into PAM-1 and PAM-2 proteins, but not into PAM-3 or PAM-2/899 proteins. PAM-1 and PAM-2 proteins appeared to be phosphorylated with similar efficiency, suggesting that the exon 16 region was not a major phosphorylation site. Deletion of the transmembrane domain (PAM-3) or truncation of the COOH-terminal 77 amino acids (PAM-2/899) abolished phosphorylation. These data are consistent with phosphorylation of the COOH-terminal domain of PAM proteins only when this domain is exposed to cytosol. Phosphoamino acid analysis of acid-hydrolyzed PAM-1 labeled with [32P]PO4 revealed phosphoserine with lesser amounts of phosphothreonine and no phosphotyrosine (Fig. 3B).

In Vivo Phosphorylation of Purified Recombinant PAM COOH-terminal Domain—The COOH-terminal domain of PAM contains several potential PKC phosphorylation sites (11) (Fig. 1B). To determine whether PKC could phosphorylate the COOH-terminal domain of PAM, purified recombinant PAM COOH-terminal domain was incubated with PKC and [γ-32P]ATP. Fractionation of the 32P-labeled proteins by SDS-PAGE followed by autoradiography indicated that PKC efficiently phosphorylated recombinant PAM COOH-terminal domain (Fig. 4A). Recombinant PAM COOH-terminal domain protein phosphorylated in vitro by PKC with [γ-32P]ATP was subjected to tryptic digestion and fractionation by reverse phase HPLC (Fig. 4B). Major and minor 32P-labeled tryptic peptides (Peaks I and II) were detected. In order to identify these tryptic phosphopeptides, phosphorylation of recombinant PAM COOH-terminal domain by PKC was driven to completion by using a higher concentration of ATP. Tryptic peptides were fractionated by reverse phase HPLC using a phosphate-perchlorate system (pH 7.0) to resolve phosphorylated and nonphosphorylated peptides (26). Under these conditions, approximately equal amounts of phosphopeptides I and II were produced (data not shown); both phosphopeptides were subjected to Edman degradation.

Peak I yielded two overlapping sequences Lys934-Gly-Tyr-Ser-Arg and Gly935-Tyr-Ser-Arg (Fig. 5, A and B); Ser937 was identified as the phosphorylation site since it was the only Ser or Thr residue in the peptide, and recovery of Ser at cycle 4 of the Edman degradation was low, consistent with the low recovery of phenylthiodyantoin-phosphoserine (30). Edman degradation of peak II (Fig. 5C) provided the sequence Gly920-Ser-Gly-Gly-Leu-Asn-Leu-Gly-Asn-Phe-Phe-Ala-Ser-Arg; Ser was recovered in good yield at the 2nd cycle, but little Ser was recovered at the 13th cycle (Ser932; underlined) (Fig. 5C). Thus, phosphorylated PAM protein (Fig. 3A). Phosphoamino acid analysis of acid-hydrolyzed PAM-1 labeled with [32P]PO4 revealed phosphoserine with lesser amounts of phosphothreonine and no phosphotyrosine (Fig. 3B).
PKC first catalyzed the phosphorylation of Ser937; more extensive phosphorylation included Ser932.

PAM-1 Located in Different Subcellular Fractions Is Phosphorylated to Different Extents—

If phosphorylation of the COOH-terminal domain plays a role in the targeting of membrane PAM to different subcellular sites, we reasoned that PAM proteins enriched in different subcellular fractions might be differentially susceptible to in vitro phosphorylation. AtT-20 cells expressing PAM-1 were dispersed with a ball-bearing homogenizer and subjected to differential centrifugation; the PAM proteins in each fraction were then visualized with an antiserum to Exon 16 (Fig. 1) that recognizes PHM and PAL (Fig. 6A). Monofunctional PHM (45 kDa) is a secretory granule-associated product (20, 21) and is enriched in the 20,000 rpm pellet; the 40,000 rpm pellet contained very little total PAM and almost no 45-kDa PHM. The integrity of the organelles in the resuspended pellets was evaluated by assaying PHM activity in the absence or presence of 1% Triton X-100. The peptidylglycine substrate should fail to gain access to luminal PHM if the organelles are intact, and PHM activity assayed in the absence of detergent was less than 5% of the PHM activity assayed following addition of detergent (data not shown).

Each pellet was resuspended and incubated with \([\gamma-32P]\)ATP under 4 conditions: no additions, Ca\(^2+\) only, AtT-20 cytosol without or with added Ca\(^2+\) (Fig. 6B). PAM-1 and the 70-kDa PAL protein (PALm) generated when 45-kDa PHM is produced were phosphorylated in all subcellular fractions under all conditions examined. In the absence of added Ca\(^2+\) or cytosol, the most extensive phosphorylation of PAM-1 and PALm was observed in the fraction containing the highest concentration of PAM, the 20,000 rpm pellet (Fig. 6B). Addition of Ca\(^2+\) in the absence of cytosol had little effect on the phosphorylation of PAM in any of the fractions. Addition of cytosol to the 20,000 rpm fraction resulted in a substantial inhibition of the phosphorylation of PAM-1 and PALm; no effect of cytosol alone was observed with the other fractions. When cytosol was present...
along with Ca$^{2+}$, phosphorylation of PAM-1 and PALm in the 30,000 rpm and 40,000 rpm pellets was stimulated substantially. In the presence of cytosol and Ca$^{2+}$, the relatively small amount of PAM protein in the 40,000 rpm pellet was phosphorylated much more efficiently than PAM proteins in other subcellular fractions.

The Ca$^{2+}$ requirement for cytosol-dependent phosphorylation of PAM proteins in the 30,000 rpm and 40,000 rpm pellets suggested a role for Ca$^{2+}$-stimulated protein kinase(s). Since PKC phosphorylated purified PAM COOH-terminal domain in vitro (Fig. 4A), a role for endogenous PKC was examined using staurosporine, a PKC inhibitor (Fig. 6C). Inclusion of $1 \mu M$ staurosporine during the incubation with $[\gamma-^{32}P]ATP$ completely blocked the effects of Ca$^{2+}$ and cytosol on PAM phosphorylation in both the 30,000 and 40,000 rpm pellets and even reduced phosphorylation below the levels observed in the absence of cytosol. Thus, a staurosporine-inhibitable PKC-like enzyme could be the Ca$^{2+}$-dependent cytosolic factor that phosphorylates PAM in the 30,000 and 40,000 rpm pellets.

Effect of PKC Activator and Inhibitor on Phosphorylation of PAM-1 in hEK-293 Cells—We next sought to determine whether activation or inhibition of PKC could affect the phosphorylation of PAM in intact cells. We selected hEK-293 cells for these initial studies because they have a less complex pattern of PAM proteolytic processing than AtT-20 cells (13, 14). Although treatment of hEK-293 cells with PMA for 1 h did not significantly increase $[^{32}P]PO_4$ labeling of 120-kDa PAM-1, the amount of $[^{32}P]PO_4$-labeled 22-kDa protein immunoprecipitated by antibody to the COOH-terminal domain of PAM was increased approximately 5-fold over control (Fig. 7A). Treatment of PAM-1 hEK-293 cells with staurosporine yielded a slight but significant decrease in $[^{32}P]PO_4$ labeling of 120-kDa PAM-1 and the 22-kDa PAM COOH-terminal fragment (Fig. 7B). The addition of PMA in the presence of staurosporine failed to produce an increased amount of $[^{32}P]PO_4$-labeled 22-kDa protein. Little effect on $[^{32}P]PO_4$ labeling of 120-kDa PAM-1 or the 22-kDa PAM COOH-terminal fragment was observed following long term treatment with PMA (24-h PMA) to down-regulate PKC activity (31).

Under basal conditions, a small fraction of the PAM-1 produced in hEK-293 cells is cleaved at or near the cell surface to release a soluble 105-kDa bifunctional PAM protein and generate an integral membrane COOH-terminal fragment (Fig. 1) (13). The 105-kDa PAM protein is not detected in the cells.
Based on its apparent molecular mass and recognition by an
tiserato the COOH-terminal domain of PAM, the 22-kDa phos-
phoprotein observed here consists of a small portion of the
luminal domain, the transmembrane domain, and the COOH-
terminal domain of PAM. The increased level of phosphoryl-
ated 22-kDa PAM COOH-terminal protein upon PMA treat-
ment may reflect increased cleavage producing the 22-kDa
PAM COOH-terminal protein and/or preferential phosphoryl-
ation of the 22-kDa PAM COOH-terminal protein by activated
PKC.

Effect of PKC Activator on Cleavage/Release of PAM proteins from hEK-293 cells. Duplicate wells of hEK-293 cells
expressing PAM-1, PAM-3, or PAM-2/899 were labeled with (35)S)methionine/cysteine for 15 min and chased for
the indicated times with (+) or without (−) PMA (1 µM). Chase times were selected based on the experi-
mentally determined endoplasmic reticulum to Golgi transit time (t1/2; 1 h) and secretion rate of PAM proteins
from hEK-293 cells (<5% of cellular content of PAM-1/h; <50% of cellular content of PAM-3/h;
<25% of cellular content of PAM-2/899/h) (13). Chase media and final extracts were immunoprecipitated
with PHM antibody and analyzed by SDS-PAGE and fluorography; films were densitized to quantify the
effects of PMA. The same type of experiment was repeated twice with similar results. The doublet observed in the
medium of PAM-2/899 cells presumably reflects endoproteolytic cleavage at different sites near the
transmembrane domain.
expressing PAM-3 were pulse-labeled with \(^{35}\text{S}\)methionine/cysteine for 15 min and chased for 90 min with or without PMA. A shorter chase time was chosen to evaluate the effect of PMA on PAM-3 secretion because little PAM-3 remains in the hEK-293 cells after a 2-h chase (13). PMA had no effect on the secretion of PAM-3 from hEK-293 cells (Fig. 8B).

The effect of PMA on release of PAM proteins is limited to membrane forms of PAM. PMA could act directly by stimulating phosphorylation of integral membrane PAM or indirectly by affecting membrane flux or proteolytic cleavage. To determine whether the effect was direct or indirect, hEK-293 cells expressing integral membrane PAM lacking all of the identified phosphorylation sites (PAM-2/899) and shown not to undergo phosphorylation (Fig. 2A) were treated with PMA (Fig. 8C). PMA treatment significantly (−5-fold; \(n = 2\)) increased the cleavage/release of soluble PAM derived from PAM-2/899. Thus, the PMA effect on cleavage/release of soluble PAM derived from integral membrane PAM was not mediated directly by phosphorylation of the PAM protein.

Analysis of PAM-1/ Ser\(^{937}\) → Ala expressed in AtT-20 Cells—
Since both our biochemical studies and in vitro studies indicated that phosphorylation of membrane PAM by PKC-like enzyme might be of functional importance, a vector encoding PAM-1 in which the major PKC site was mutated to Ala was constructed. Stably transfected AtT-20 cells expressing PAM-1/S937A were then compared to AtT-20 cells expressing PAM-1. The steady state distributions of PAM-1 and PAM-1/S937A were quite similar, with intense perinuclear staining in the region of the trans-Golgi network and punctate, vesicular staining at the tips of processes (Fig. 9A).

At steady state, a small fraction of the membrane PAM in AtT-20 cells is on the plasma membrane; the PAM proteins on the cell surface are rapidly internalized and enter the endocytic pathway (13, 14, 21, 34). Antibody internalization experiments were carried out to compare the manner in which these two PAM proteins traverse the endocytic pathway (Fig. 9B). AtT-20 cells expressing PAM-1 or PAM-1/S937A were incubated with antibody to PHM; the antibody bound to the small fraction of the PAM protein accessible on the plasma membrane. The cells were then washed and allowed to internalize the PAM-PAM antibody complex for different amounts of time. After 10 min of chase, antibody internalization by cells expressing PAM-1 and PAM-1/S937A appeared quite similar; the PAM-PAM antibody complex accumulated in the perinuclear region. After a longer chase, the PAM-PAM antibody complex remained localized in the perinuclear region in AtT-20 cells expressing PAM-1. In contrast, the PAM-PAM antibody complex in AtT-20 cells expressing PAM-1/S937A was targeted to vesicular structures distributed more widely throughout the cytoplasm.

The PAM-PAM antibody complex internalized by AtT-20 cells expressing PAM-1/S937A assumed a distribution resembling that of lysosomes. To evaluate this possibility, AtT-20 cells expressing both PAM proteins were allowed to internalize antibody for 30 min, and the internalized antibody and lysosomes were then visualized simultaneously by confocal microscopy (Fig. 10). In AtT-20 cells expressing PAM-1, the internalized PAM antibody was localized primarily in structures distinct from lysosomes. In AtT-20 cells expressing PAM-1/S937A, much of the internalized PHM antibody was localized in structures recognized by the LAMP antibody. These results suggest that phosphorylation at Ser\(^{937}\) plays a crucial role in trafficking of membrane PAM after internalization from the plasma membrane.

**DISCUSSION**

In this study we demonstrated that PAM proteins were phosphorylated on Ser and Thr residues within their COOH-terminal domain only when this domain was exposed to the cytosol because of the presence of a transmembrane domain. Phosphorylation of membrane PAM was eliminated when the protein was truncated at Tyr\(^{936}\). Since recombinant PAM COOH-terminal domain was phosphorylated in vitro on Ser\(^{932}\) and Ser\(^{937}\) by PKC, we decided to investigate a role for PKC and phosphorylation in the trafficking of membrane PAM.

Our in vitro phosphorylation studies using subcellular fractions of AtT-20 cells indicated that the PAM proteins localized to different subcellular compartments at steady state were differentially susceptible to phosphorylation. Phosphorylation of 120-kDa PAM-1 and PALm in the particulate fractions occurred in the absence of cytosol. Phosphorylation of PAM proteins in the secretory granule-enriched 20,000 rpm fraction was inhibited by the addition of cytosol. In contrast, PAM proteins in the 30,000 and 40,000 rpm fractions were phosphorylated in a Ca\(^{2+}\)-dependent manner by protein kinases(s) in the cytosol. Although the identity of the kinase(s) responsible for the phosphorylation of PAM was not investigated, kinase activity was...
dependent on the addition of Ca\(^{2+}\) and was inhibited in the presence of staurosporine. These results indicate that PAM proteins in this subcellular compartment differ in their ability to undergo additional phosphorylation. This could reflect prior phosphorylation of the relevant sites or the presence of inhibitors of phosphorylation. In any case, these results suggest that phosphorylation of PAM could differentially affect its function depending upon its subcellular localization.

PMA treatment of hEK-293 cells expressing PAM-1 failed to increase the level of phosphorylation of PAM-1. However, it increased the cleavage/release of a soluble bifunctional PAM protein derived from PAM-1. Since truncation of the COOH-terminal domain of integral membrane PAM eliminated its phosphorylation without abolishing the PMA effect on cleavage/release of soluble bifunctional PAM, this effect of PKC must be an indirect one not involving phosphorylation of PAM. Lack of a PMA effect on the release of soluble PAM-3 from hEK-293 cells indicated that the effect was confined to integral membrane proteins.

The proteolytic release of soluble fragments derived from the \(\beta\)-amyloid protein precursor (35), tumor necrosis factor receptor (36), pro-TGF-\(\alpha\) (37, 38), and CSF-1 receptor (39) is also promoted by activators of PKC. As observed for PAM-1, this effect does not involve the conversion of \(\beta\)-amyloid protein precursor (40), pro-TGF-\(\alpha\) (38), and CSF-1 receptor (39) into better substrates for proteolysis by direct phosphorylation. The proteolytic enzyme(s) mediating these cleavages have not been identified; mutant CHO cells selected for an inability to cleave pro-TGF-\(\alpha\) also fail to cleave many other surface proteins, suggesting the involvement of a common factor (37, 41). PMA treatment may activate a proteolytic enzyme(s) or increase the accessibility of membrane proteins to the compartment containing the proteolytic enzyme(s) (41). Participation of PKC in regulating the cleavage/release of PAM raises the intriguing possibility that there is a physiological significance to the regulated release of PAM from integral membrane PAM. In primary atrial myocyte cultures, phorbol ester treatment also stimulates the cleavage/release of PAM proteins derived from PAM-1 and PAM-2 (42).

The COOH-terminal domain of integral membrane PAM contains information critical for the intracellular trafficking of PAM in endocrine cells (14, 21, 34) and in fibroblast-like cells (13). Truncation of most of the COOH-terminal domain resulted in mistargeting of integral membrane PAM to the cell surface and loss of the ability of cells to internalize PAM that has reached the cell surface. More detailed mutagenesis studies indicated that signals mediating the internalization of PAM from the plasma membrane are somewhat distinct from signals mediating the localization of PAM in the trans-Golgi network region of the cell (34). Mutation of the PKC site at Ser\(^{937}\) to Ala in the COOH-terminal domain of PAM-1 resulted in a protein whose steady state localization in AtT-20 cells was very similar to that of wild type PAM-1. Nevertheless, when an antibody internalization paradigm was used to look specifically at the small fraction of PAM-1 undergoing internalization, mutation of this site was found to have a profound effect. While the PAM-PAM antibody complex internalized by AtT-20 cells expressing PAM-1 was rapidly accumulated in the perinuclear region of the cells, the PAM-PAM antibody complex internalized by AtT-20 cells expressing PAM-1/S937A was first accumulated in the perinuclear region and then dispersed to structures that appear to be lysosomes.

In endocrine cells, integral membrane PAM proteins are thought to be retrieved from immature secretory granules via constitutive-like vesicles with very little membrane PAM actually reaching the cell surface under basal conditions. Membrane PAM protein that reaches the plasma membrane is thought to enter the endocytic pathway (14, 34). We propose that phosphorylation of Ser\(^{937}\) is crucial for a step in the endocytic pathway that directs PAM proteins in the recycling pathway; in the absence of phosphorylation at this site, the protein appears to enter lysosomes. If the Ca\(^{2+}\)-stimulated, staurosporine-inhibitable process that phosphorylates PAM proteins in subcellular fractions functions in cells, recycling of PAM proteins may be regulated in concert with Ca\(^{2+}\)-stimulated exocytosis. Phosphorylation has been implicated in the trafficking of integral membrane proteins such as the mannose 6-phosphate (43, 44), epidermal growth factor (45), and polymeric immunoglobulin (26) receptors. Phosphorylation of the mannose 6-phosphate receptor is crucial for its interaction with AP-1 Golgi adaptor proteins (46). Interaction of the COOH-terminal domain of membrane PAM with cytosolic factors involved in the trafficking of membrane proteins may involve phosphorylation of the COOH-terminal region of PAM. Additional studies...
on cytosolic protein kinases that phosphorylate PAM and proteins that interact with the COOH-terminal domain of PAM will provide insight into the role of phosphorylation in the trafficking of membrane PAM.

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Phosphorylation of PAM

30083
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