Proteases within secretory vesicles are required for conversion of neuropeptide precursors into active peptide neurotransmitters and hormones. This study demonstrates the novel cellular role of the cysteine protease cathepsin L for producing the (Met)enkephalin peptide neurotransmitter from proenkephalin (PE), in the regulated secretory pathway of neuroendocrine PC12 cells. These findings were achieved by coexpression of PE and cathepsin L cDNAs in PC12 cells with analyses of PE-derived peptide products. Expression of cathepsin L resulted in highly increased cellular levels of (Met)enkephalin, resulting from the conversion of PE to enkephalin-containing intermediates of 23, 18-19, 8-9, and 4.5 kDa that were similar to those present in vivo. Furthermore, expression of cathepsin L with PE resulted in increased amounts of nicotine-induced secretion of (Met)enkephalin. These results indicate increased levels of (Met)enkephalin within secretory vesicles of the regulated secretory pathway. Importantly, cathepsin L expression was directed to secretory vesicles, demonstrated by colocalization of cathepsin L/DsRed fusion protein with enkephalin and chromogranin A neuropeptides that are present in secretory vesicles. In vivo studies also showed that cathepsin L in vivo was colocalized with enkephalin. The newly defined secretory vesicle function of cathepsin L for biosynthesis of active enkephalin opioid peptide contrasts with its function in lysosomes for protein degradation. These findings demonstrate cathepsin L as a distinct cysteine protease pathway for producing the enkephalin member of neuropeptides.

The biosynthesis of enkephalin opioid neuropeptides requires proteolytic processing of protein precursors within regulated secretory vesicles (1–3). Active enkephalin and related neuropeptides are stored in such secretory vesicles for regulated secretion that is induced by receptor-mediated mechanisms. Secreted enkephalin functions as an active peptide neurotransmitter in the control of analgesia for pain relief, behavioral responses, and related brain and physiological functions (4–6).

Secretory vesicles represent the primary subcellular site for proteolytic processing of proenkephalin and other proneuropeptides (1, 3). Secretory vesicles isolated from adrenal medullary chromaffin cells (also known as chromaffin granules) have provided a model system for identification of proteases for proneuropeptide processing which include PC1/3 and PC2 endopeptidases (7, 8) and the exopeptidase carboxypeptidase E/H (9, 10). These secretory vesicles contain (Met)enkephalin and its proenkephalin precursor (11, 12), and, therefore, contain the appropriate processing proteases.

In vitro proneuropeptide processing assays have identified cathepsin L as a major proenkephalin-cleaving activity purified from neuropeptide-containing chromaffin granules (13, 14). Cathepsin L generates (Met)enkephalin in vitro from enkephalin-containing peptide substrates, resulting from cleavage at paired basic
residue processing sites, as well as monobasic residue sites.

It is notable that these findings for a potential secretory vesicle function of cathepsin L contrast with the well known lysosomal function of cathepsin L for protein degradation. Therefore, the important question of whether cathepsin L expression is directed to secretory vesicles for proteolytic processing of proenkephalin must be answered by direct cellular gene expression experiments. Therefore, this study evaluated (Met)enkephalin production during expression of cathepsin L in the regulated secretory pathway of neuroendocrine PC12 cells. PC12 cells have been extensively utilized as a model for regulated secretion (15-24).

Results showed that expression of cathepsin L in PC12 cells resulted in increased production of (Met)enkephalin, accompanied by the production of proenkephalin-derived intermediates that resemble those in vivo. Importantly, expression of cathepsin L resulted in elevated amounts of nicotine-induced secretion of (Met)enkephalin. Colocalization of cathepsin L with enkephalin in regulated secretory vesicles was demonstrated by immunofluorescent microscopy. While expression of the cathepsin L/DsRed fusion protein was directed to neuroendocrine-containing secretory vesicles, expression of the DsRed protein alone resulted in its localization throughout the cell. These novel findings demonstrate that the cysteine protease cathepsin L participates in the biosynthesis of the enkephalin peptide neurotransmitter in the regulated secretory pathway of neuroendocrine PC12 cells. These findings provide evidence for cathepsin L as a cysteine protease pathway for the biosynthesis of enkephalins and possibly other neuropeptides.

Experimental Procedures

Construction of prepro-cathepsin L cDNA in the pcDNA3.1 vector—The pcDNA3.1 plasmid vector was used for expression of cathepsin L in PC12 cells. The bovine cDNA encoding prepro-cathepsin L was obtained by RT-PCR from total bovine pituitary RNA isolated by the Trizol reagent (Invitrogen, Carlsbad, CA). The first strand cDNA was generated by SuperScriptII reverse transcriptase with oligo (dT) 12-18 (Invitrogen) using conditions recommended by the manufacturer (Invitrogen, Carlsbad, CA). PCR with the first strand cDNA as template and Taq polymerase (Qiagen, Valencia, CA) utilized primers (0.4 µM) consisting of 5'-AAAAAGCTAGCATCCACCATGAATCTTTCAT TCTTCTGT ACTGT-3' (with NheI site, underlined) and 5'-AAAAAGGATCCCTCAAAAAGTGGATAGCT GGCTGCTGT-3' (with BamHI site, underlined) for the 3'-primer, under PCR cycle conditions of 94°C for 60 seconds, 44°C for 60 seconds, and 72°C for 80 seconds, for a total of 30 cycles. The amplified prepro-cathepsin L cDNA fragment (size of about 1.0 kb) was double-digested with NheI/BamHI and ligated to NheI/BamHI digested pcDNA3.1 plasmid expression vector (Invitrogen, Carlsbad, CA). This construct was subjected to DNA sequencing (Davis DNA sequencing Inc., Davis, CA) to verify the nucleotide sequence, and deduced primary amino acid sequence, of the bovine prepro-cathepsin L cDNA.

Preproenkephalin cDNA in the pcDNA3.1 vector—The preproenkephalin cDNA (bovine) was obtained by RT-PCR as described in the previous paragraph, using polyA(+) RNA isolated from bovine adrenal medulla, as described previously (25). PCR utilized the primers 5'-AAAAAGCTAGCCACCATGGCG GTTCCCTGGGACT-3' (with NheI site, underlined) and 5'-AAAAAGGATCCACTGATGGGAAAGGATATTAATCTCAT-3' (with BamHI site, underlined) to generate the preproenkephalin cDNA, verified by DNA sequencing (by Davis DNA Sequencing Inc., Davis, CA).

Prepro-cathepsin L/DsRed fusion construct for expression—A fusion construct of prepro-cathepsin L (bovine) with DsRed fused to the COOH-terminus of cathepsin L was prepared in the pDsRed1-N1 vector (BD Biosciences, Palo Alto, CA). The prepro-cathepsin L without termination codon was obtained by PCR of the bovine prepro-cathepsin L cDNA with Pfx DNA polymerase (Invitrogen, Carlsbad, CA) with primers consisting of 5'-AAAATGATGGCTCACCACATGAATCTTTCAT TCTTCTGTACTGT-3' (with NheI site, underlined) and 5'-AAAAAGGATCCCTCAAAAAGTGGATAGCTGGCTGCTGT-3' (with BamHI site, underlined), using PCR cycling conditions (30 cycles total) with each cycle consisting of 94°C for 60 seconds, 44°C for
60 seconds, and 68°C for 80 seconds. The amplified DNA fragment was digested with BamHI/NheI and ligated to BamHI/NheI digested pDsRed2-N1 vector (BD Biosciences, Palo Alto, CA) to generate the prepro-cathepsin L/DsRed construct. DNA sequencing verified that the cathepsin L-DsRed sequence was obtained by PCR and subcloning. Coexpression of PE and cathepsin L in PC12 cells—The rat adrenomedullary PC12 neuroendocrine cell line (obtained as a gift from Dr. Daniel O’Connor, Univ. of Calif., San Diego) was grown at 37°C with 6% CO2 in DMEM high glucose medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum, 10% horse serum, 100 U/ml of penicillin and 100 µg streptomycin. Five microgram preproenkephalin cDNA/pcDNA3.1 (PPE/pcDNA 3.1) was transfected into PC12 cells, plated the previous day at 1.5 x 10^6 cell/well (70% confluency) in 6 well plates, with the GenePorter-2 transfection reagent using the protocol recommended by manufacturer (Gene Therapy Systems, San Diego, CA). Three days after the PPE/pcDNA3.1 was transfected, the prepro-cathepsin L/pcDNA3.1 construct was then transfected into the proenkephalin expressing PC12 cells using the GenePorter-2 reagent. Control experiments were performed with PC12 cells transfected with pcDNA3.1 vector alone, PPE/pcDNA3.1 alone, and prepro-cathepsin L/pcDNA3.1 alone. Cells were collected three days after transfection with prepro-cathepsin L/pcDNA3.1. Evaluation of PE processing products by western blotting and radioimmunoassay (RIA)—Transfected cells were collected by centrifugation at 12,000 x g for 15 min. and pelleted cells were subjected to homogenization in buffer containing 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 10 µM E64c, 10 µM pepstatin A, 10 µM chymostatin, 0.1 mM AEBSF, 10 µM leupeptin, 0.2% Tween-20, 0.2% Triton X-100, and 3 mM CHAPS. Cells were then disrupted by freeze-thawing (three times). Solubilized proteins were collected by centrifugation at 12,000 x g for 15 minutes at 4°C. Protein concentration was determined by the Bradford reagent from Biorad (Hercules, CA) according to the protocol provided by the manufacturer. High molecular weight proenkephalin (PE) derived products were analyzed by western blots with anti-(Met)enkephalin and anti-synenkephalin (recognizing the NH2-terminal domain of PE that lacks enkephalin sequences). Twenty microgram protein from transfected cells was subjected to SDS-PAGE on 12% NuPAGE gels (Invitrogen, Carlsbad, CA), and western blots were performed using anti-(Met)enkephalin (from Chemicon Inc., Temecula, CA) or anti-synenkephalin, as we have described previously (25). Processed (Met)enkephalin was measured by radioimmunoassay (RIA) as we have described previously (14). This RIA does not crossreact with PE, and therefore, measures processed (Met)enkephalin. Regulated secretion of (Met)enkephalin induced by nicotine—Subsequent to cotransfection of PC12 cells with both PE and cathepsin L cDNAs, as described above, evaluation of regulated secretion of (Met)enkephalin was conducted. Regulated secretion was induced by nicotine (10 µM) by incubation of transfected cells with nicotine for 15 minutes, as described previously (26). Culture media was collected and levels of (Met)enkephalin were measured by RIA. Colocalization of cathepsin L with enkephalin in secretory vesicles of PC12 cells by confocal immunofluorescent microscopy—PC12 cells were transfected with the prepro-cathepsin L/DsRed construct as described above for transfection of prepro-cathepsin L/pcDNA3.1, and cathepsin L/DsRed immunofluorescence was examined 3-4 days post-transfection. For cellular fixation, cells were harvested and re-seeded at 100,000 to 500,000 cells/well in poly-D-lysine coated 2-well chamber slides for 4 hours in culture media. Culture media was then aspirated and cells were rinsed in PBS 3 times. Cells were then fixed in 3.7% paraformaldehyde at room temperature for 15 min., rinsed with PBS 4 times, and permeabilized with 0.3% Triton X-100. Colocalization of cathepsin L/DsRed with (Met)enkephalin was performed by immunostaining of cells with anti-(Met)-enkephalin (1:100 dilution, Chemicon Inc., Temecula, CA) in PBS containing 1% BSA overnight at 4°C. Cells were washed and then incubated with Alexa Fluor 488 goat anti-mouse (Molecular Probes/Invitrogen, Carlsbad, CA) in PBS containing 1% BSA for 1 hr at room temperature. Finally, slides were mounted with VectaShield Mounting medium containing 4',6-
diamidino-2-phenylindole staining (DAPI) and analyzed for immunofluorescence of cathepsin L-DsRed (red fluorescence) and enkephalin immunofluorescence (green fluorescence) using a Nikon Eclipse 800 microscope coupled to a PCM-2000 confocal system with image analyses by SimplePCI software. Alternatively, cathepsin L-DsRed colocalization was also examined with the secretory vesicle marker chromogranin A (1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), visualized with anti-rabbit AgG/Alexa 488 (green fluorescence).

*In vivo colocalization of cathepsin L with enkephalin in adrenal medulla (bovine)—* Fresh adrenal medullas (bovine) were obtained and tissue slices were fixed as we have described previously (27). Cathepsin L localization with (Met)enkephalin was performed as described previously (27), with cathepsin L visualized by rabbit anti-cathepsin L (Athens Research & Technology) and mouse anti-(Met)enkephalin (Chemicon, Temecula, CA). Cathepsin L was detected with anti-rabbit IgG-Alexa Fluor 488 (green fluorescence, Molecular Probes/Invitrogen, Carlsbad, CA), and (Met)enkephalin was detected with anti-mouse IgG-Alexa Fluor 594 (red fluorescence). Confocal immunofluorescence microscopy was conducted as described above for PC12 cells.

**RESULTS**

*Cellular cathepsin L expression generates (Met)enkephalin in the regulated secretory pathway of PC12 cells—* Neuroendocrine PC12 cells were utilized in this study because they contain the regulated secretory pathway that is utilized for proteolytic processing of proneuropeptides and prohormones (15-24, 28-30). In addition, PC12 cells lack substantial amounts of prohormone processing enzymes, demonstrated by expression of intact POMC in these cells (29). For these reasons, PC12 cells were an ideal choice for studies of proenkephalin expression and processing during expression of cathepsin L in the regulated secretory pathway.

PC12 cells were transfected with the preproenkephalin (PPE) and preprocathepsin L cDNAs for analyses of (Met)enkephalin production. Production of (Met)enkephalin was quantitated by a specific radioimmunoassay (RIA) that specifically detects (Met)enkephalin, but not its proenkephalin (PE) precursor.

Cellular (Met)enkephalin was elevated 8-fold in cells expressing cathepsin L and PE compared to cells expressing PE alone (fig. 1). Cathepsin L expression in PE-containing cells generated approximately 80-fold greater levels of (Met)enkephalin compared to vector control. Expression of cathepsin L alone produced a small increase in (Met)enkephalin, suggesting that PC12 cells endogenously express low levels of PE. In addition, expression of PE alone resulted in a small increase in (Met)enkephalin levels, suggesting the presence of low amounts of endogenous PE processing enzyme(s); endogenous enzymes may include low levels of prohormone convertases or cathepsin L itself. Notably, (Met)enkephalin production was increased by nearly 10-fold in cells coexpressing cathepsin L and PE, compared to cells expressing only PE or only cathepsin L. These results indicate participation of cathepsin L in the cellular production of (Met)enkephalin.

To assess cathepsin L-mediated production of cathepsin L with enkephalin in the regulated secretory pathway, nicotine-stimulated secretion of (Met)enkephalin was tested. Amounts of (Met)enkephalin secreted during nicotine stimulation of cells were increased by 5-fold in cells expressing cathepsin L, compared to control cells without expression (fig. 2). These results demonstrate a functional role for cathepsin L in the regulated secretory pathway for production of (Met)enkephalin.

*Cathepsin L expression in PC12 cells generates PE-derived intermediates that resemble those in vivo in chromaffin granules—* Cathepsin L processing of PE in PC12 cells was analyzed by epitope-specific antisera to proenkephalin domains in western blots. Cells transfected with the preproenkephalin (PPE) cDNA showed the presence of PPE and PE as 33 kDa and 31 kDa bands, respectively, detected by western blots with anti-enkephalin serum (fig. 3a). These findings are consistent with production of PPE with a signal peptide that is removed at the RER (rough endoplasmic reticulum) to generate the slightly smaller PE, which is routed to secretory vesicles for proteolytic processing (1).

Coexpression of cathepsin L with PE resulted in the production of PE-derived intermediates of
23 kDa and 18-19 kDa detected by anti-(Met)enkephalin western blots (fig. 3a). Western blots with anti-sytenkephalin serum, which recognizes the NH₂-domain of bovine PE (12), detected PE-derived intermediates of 26, 23, 18-19, and 8-9 kDa (fig. 3b). Expression of 27 kDa cathepsin L, corresponding to the single chain form of this protease, was confirmed by anti-cathepsin L immunoblotting (fig. 3c).

Importantly, cathepsin L generated PE-derived peptides in transfected PC12 cells that resemble in vivo PE-derived intermediates (fig. 4). In vivo, the primary PE-derived products in adrenomedullary chromaffin cells consisted of 23, 18-19, 8-9, and 4.5 kDa enkephalin-containing peptides (fig. 4a). PC12 cells expressing PE and cathepsin L showed the same intermediates of 23, 18-19, 8-9, and 4.5 kDa (fig. 4b). These results demonstrate that cathepsin L expression in PC12 cells produces PE-derived intermediates that resemble those in vivo in adrenal medulla. These results support a role for cellular cathepsin L processing of PE.

**Secretory vesicle localization of cathepsin L with enkephalin in vitro and in vivo**—Support for cathepsin L production of enkephalin in secretory vesicles was indicated by their cellular colocalization, examined by immunofluorescence confocal microscopy. During coexpression of cathepsin L and PPE cDNAs, cathepsin L (red immunofluorescence) and (Met)enkephalin (green immunofluorescence) were colocalized as demonstrated by the merged images indicating their colocalization (indicated by yellow immunofluorescence) (fig. 5). Cathepsin L and enkephalin showed punctate patterns of immunostaining that are consistent with localization to secretory vesicles. Furthermore, in vivo adrenal medulla chromaffin cells showed a discrete pattern of cathepsin L immunofluorescence that was colocalized with (Met)enkephalin (fig. 6). These findings illustrate the secretory vesicle localization of cathepsin L in neuronal PC12 cells in vitro and in vivo in adrenal medullary chromaffin cells.

**Trafficking of cathepsin L/DsRed fusion protein to secretory vesicles in PC12 cells**—To assess cathepsin L expression and protein trafficking to secretory vesicles, the cathepsin L/DsRed fusion protein construct was utilized to allow direct fluorescence visualization of the subcellular localization of DsRed fused with cathepsin L, compared to expression of DsRed alone. Expression of cathepsin L/DsRed resulted in its localization with the endogenous secretory vesicle marker chromogranin A (CgA) (fig. 7a). Notably, nearly all the cathepsin L/DsRed was colocalized with CgA in a punctate pattern of staining. In contrast, expression of DsRed alone resulted in its presence throughout the cell in nuclei and cytoplasm (fig. 7b). These results demonstrate that cathepsin L possesses the ability to direct the trafficking of the heterologous DsRed protein to secretory vesicles. Furthermore, neuroendocrine types of cells, represented by PC12 cells, apparently possess mechanisms to route cathepsin L to secretory vesicles. Thus, expression of cathepsin L in PC12 cells that contain the regulated secretory pathway, results in trafficking of cathepsin L to secretory vesicles for the production, storage, and secretion of the (Met)enkephalin peptide neurotransmitter.

**DISCUSSION**

Expression of cathepsin L with proenkephalin (PE) in neuroendocrine PC12 cells resulted in the conversion of PE to enkephalin-containing intermediates to mature (Met)enkephalin (fig. 8) in the regulated secretory pathway. Cathepsin L generated high molecular weight PE-derived intermediates of 26 kDa to ~4.5 kDa which were similar to those present in vivo in enkephalin-containing secretory vesicles of adrenal medullary chromaffin cells (11, 31). Cathepsin L processing of PE resulted in elevated levels of cellular (Met)enkephalin, with increased amounts of nicotine-induced secretion of (Met)enkephalin via the regulated secretory pathway. Furthermore, cathepsin L was colocalized to enkephalin-containing secretory vesicles. Expression of cathepsin L-DsRed resulted in its trafficking to secretory vesicles that contain the chromogranin A (a marker for secretory vesicles), whereas DsRed expression alone (not fused to cathepsin L) resulted in the localization of DsRed throughout the cell in nuclei and cytoplasm. Clearly, cathepsin L expression in neuroendocrine PC12 cells allows trafficking of cathepsin L to secretory vesicles. In addition, in vivo colocalization of cathepsin L with enkephalin in adrenal medulla was demonstrated. These findings indicate that cellular cathepsin L participates in the processing...
of proenkephalin to produce the active (Met)enkephalin peptide neurotransmitter in the regulated secretory pathway. These results demonstrate that cathepsin L functions as a novel processing enzyme that converts a neuropeptide precursor into (Met)enkephalin that functions as a peptide neurotransmitter. These findings provide significant evidence for cathepsin L as a cysteine protease pathway for the biosynthesis of enkephalins and possibly other neuropeptides including the POMC-derived peptide hormones (unpublished observations).

Importantly, cellular cathepsin L participates in the proteolytic processing of PE, as illustrated by expression of cathepsin L with PE in neuroendocrine PC12 cells (derived from rat adrenal medullary pheochromocytoma). Immunoblot analyses with anti-(Met)enkephalin, and anti-synenkephalin that recognizes the NH$_2$-terminal domain of PE (bovine), showed that cathepsin L generated NH$_2$-terminal domain-containing intermediates of 26, 22-23, 18-19, and 8-9 kDa (fig. 8). Moreover, these PE intermediates are present in vivo in chromaffin secretory vesicles (11, 31). Clearly, cathepsin L converts PE to intermediates and peptide products in neuroendocrine PC12 cells that resemble those in vivo.

Secretory vesicle cathepsin L has been shown to cleave proenkephalin and enkephalin-containing intermediates at the NH$_2$-terminal side of paired basic residues, and occasionally between the dibasic residues (32-35). Such cleavage specificity would result in the production of enkephalin peptide intermediates with basic residue extensions at NH$_2$- and COOH-termini. These basic residues at NH$_2$- and COOH-termini can be removed by the exopeptidases aminopeptidase B (AP-B) and carboxypeptidase E/H that are present in PC12 cells (28, 36). AP-B removes NH$_2$-terminal basic residues from neuropeptides in secretory vesicles (37-40), and has been demonstrated to be colocalized by electron microscopy with enkephalin in regulated secretory vesicles of adrenal medullary chromaffin cells (37). Furthermore, removal of COOH-terminal basic residues of neuropeptides is accomplished by carboxypeptidase E/H (41, 42) that is also present in secretory vesicles with enkephalin and neuropeptides. Thus, expression of proenkephalin with cathepsin L in PC12 cells, combined with endogenous AP-B and CPE/H, yields mature, processed (Met)enkephalin measured radioimmunoassay in this study.

Significantly, expression of cathepsin L promotes the production and secretion of (Met)enkephalin in the regulated secretory pathway, demonstrated by increased nicotine-stimulated secretion of (Met)enkephalin. Colocalization of cellular cathepsin L to enkephalin-containing secretory vesicles in transfected PC12 cells, as well as in adrenal medullary chromaffin cells in vivo, supports the hypothesis that cathepsin L functions within secretory vesicles for production of (Met)enkephalin. These results demonstrate a biological role of cathepsin L for production of an active peptide neurotransmitter.

The secretory vesicle function of cathepsin L for production of an active neuropeptide is distinct from its well known lysosomal function for protein degradation. It is notable that nearly all of the expressed cathepsin L in neuroendocrine PC12 cells was localized to neuropeptide-containing secretory vesicles. It was of interest that the cathepsin L/DsRed fusion protein was routed to secretory vesicles, but DsRed alone (without signal peptide) was present throughout the cell in nuclei and cytoplasm. Thus, cathepsin L directs the trafficking of the heterologous DsRed protein to secretory vesicles. It is notable that cathepsin L in PC12 cells is routed almost entirely to secretory vesicles, based on its colocalization with the secretory vesicle markers enkephalin and chromogranin A. These results suggest that PC12 cells route the majority of cathepsin L to secretory vesicles, rather than to lysosomes. It is apparent that in neuroendocrine PC12 cells, cathepsin L is routed to secretory vesicles that produce, store, and secrete neuropeptides.

These studies of cathepsin L expression in PC12 cells for (Met)enkephalin production complement cathepsin L gene knockout studies that demonstrate reduction of (Met)enkephalin in brain by approximately 50% (14). The direct expression experiments of this study demonstrate active participation of cathepsin L in the proteolytic processing of PE that is required for the biosynthesis of (Met)enkephalin. Thus, the cellular expression studies of cathepsin L, combined with the cathepsin L gene knockout
data, together provide support for a role of cathepsin L in producing (Met)enkephalin.

The role of cathepsin proteases in secretory vesicles has been extended in several studies, demonstrating biological roles for cathepsins in these vesicles. For example, cathepsin B has been found to be present in granules of rat islets of Langerhans where proinsulin is converted to insulin (43,44). In the pancreatic secretory pathway, cathepsin B is involved in trypsinogen activation during hereditary pancreatitis (45). In juxtaglomerular secretory granules, cathepsin B participates in processin prorenin (46,47). With respect to neuronal functions, cathepsin B has been identified in neurosecretory vesicles of adrenal medullary chromaffin cells as a candidate β-secretase for converting APP (amyloid precursor protein) into β-amyloid peptides that are secreted (48); extracellular Aβ peptides accumulate in aged brains as notable amyloid deposits in Alzheimer’s disease (49-51). Moreover, the cysteine cathepsins B, L, and H are localized in Golgi and regulated secretory pathways of several neuroendocrine tissues (52-54). These secretory vesicle functions of cysteine cathepsins contrast with the well known functions of cathepsin proteases in lysosomes for protein degradation. Clearly, cathepsins participate in biological functions of neuroendocrine systems.

Overall, this study demonstrates that cathepsin L functions as a proneuropeptide processing enzyme for the conversion of proenkephalin to mature (Met)enkephalin, an opioid peptide neurotransmitter and hormone. The cathepsin L processing pathway represents an alternative route for the biosynthesis of active neuropeptides, in addition to the well known proprotein convertase family of processing proteases. Among the neuroendocrine members of the proprotein convertase family, PC2 has been shown to be involved in proneuropeptide processing for the production of (Met)enkephalin (55,56) and other peptide neurotransmitters and hormones. Findings from this study lead to the hypothesis for distinct protease pathways for processing proneuropeptide and related prohormones—the cysteine protease cathepsin L, and the subtilisin-like proprotein convertases. It will be of interest in future studies to assess the coordinate regulation of these protease pathways for the biosynthesis of active peptide neurotransmitters.
FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Elevated (Met)enkephalin production during cathepsin L expression. Elevation of cellular content of (Met)enkephalin in PC12 cells was observed after coexpression of cathepsin L (CL) and proenkephalin (PE). The radioimmunoassay (RIA) for (Met)enkephalin measures processed (Met)enkephalin since the RIA does not crossreact with PE (14). Controls included cells transfected with vector alone (no insert, control), PE alone, and cathepsin L (CL) alone. Experiments were conducted by transfection of triplicate wells of cells for each group, with RIA assay of (Met)enkephalin conducted in duplicate assays performed twice. Results are expressed as x ± s.e.m. (mean ± standard error of the mean).

Fig. 2. Elevation of nicotine-stimulated secretion of (Met)enkephalin by cathepsin L in PC12 cells. Nicotine-stimulated secretion of (Met)enkephalin was assessed in non-transfected control cells and in cells cotransfected with proenkephalin (PE) and cathepsin L (CL). Cells expressing PE and CL showed elevated levels of nicotine-stimulated (Met)enkephalin secretion, indicating that cathepsin L increased the production of (Met)enkephalin in the regulated secretory pathway. Experiments were conducted by transfection of triplicate wells of cells for each group, with RIA assay of (Met)enkephalin conducted in duplicate assays performed twice. Results are expressed as x ± s.e.m. (mean ± standard error of the mean).

Fig. 3. Cathepsin L processing of cellular proenkephalin (PE) in PC12 cells. (a) Anti-enkephalin immunoblot detection of PE-derived products. PE-expressing PC12 cells (lane 2) were transfected with cathepsin L (lane 3) as described in the methods. Control cells transfected with vector alone (lane 1) was included. PE-derived products were analyzed in PC12 cell homogenates with anti-(Met)enkephalin immunoblots. Arrows indicate the PE-derived products of 23 kDa and 18/19 kDa present in cathepsin L transfected cells (lane 3). Arrows also indicate expression of preproenkephalin (PPE) and proenkephalin (PE). (b) Anti-synenkephalin immunoblot detection of PE-derived products. PC12 cells expressing PE with or without cathepsin L (lanes 2 and 1, respectively) were analyzed by anti-synenkephalin immunoblots for PE-derived intermediate products. Anti-synenkephalin recognizes the NH2-terminal domain of bovine PE that lacks enkephalin. Expression of cathepsin L resulted in the conversion of PE to intermediates of 26, 23, 18/19, and 8/9 kDa (shown by arrows for bands whose intensities were increased in cells expressing cathepsin L). (c) Cathepsin L expression demonstrated by anti-cathepsin L immunoblot. Immunoblotting with anti-cathepsin L confirmed expression of cathepsin L in transfected PC12 cells (lane 2), but not in cells transfected with vector alone (lane 1).

Fig. 4. Similar proenkephalin-derived intermediates in chromaffin granules in vivo and in cathepsin L transfected PC12 cells in vitro. Proenkephalin (PE)-derived intermediates in purified chromaffin granules detected by anti-(Met)enkephalin immunoblots (lane a) were similar to those observed in PC12 cells cotransfected with cathepsin L (CL) and PE (lane b). Chromaffin granules were isolated from bovine adrenal medullary chromaffin cells that contain enkephalin peptides. Chromaffin granules (CG, lane a) contain PE-derived intermediates of 23, 18/19, 8/9, and ~4.5 kDa (shown by arrows). These enkephalin-containing intermediates were also present in transfected PC12 cells (lane b, see arrows).

Fig. 5. Colocalization of cathepsin L with (Met)enkephalin in secretory vesicles of PC12 cells. (a) Cathepsin L/DsRed expression in PC12 cells. A cathepsin L/DsRed fusion construct was expressed in PC12 cells. Cathepsin L/DsRed (CL/DsRed) (red immunofluorescence) illustrates expression of cathepsin L, demonstrated by confocal microscopy. (b) Enkephalin in PC12 transfected with cathepsin L/DsRed and proenkephalin. Enkephalin was detected in PC12 cells transfected with proenkephalin by anti-enkephalin (mouse), detected with anti-mouse IgG Alexa Fluor 488 by confocal immunofluorescence microscopy (green immunofluorescence).
(c) Colocalization of cathepsin L/DsRed and enkephalin in PC12 cells. Colocalization of cathepsin L/DsRed and enkephalin was illustrated by confocal immunofluorescence microscopy (yellow immunofluorescence).

**Fig. 6.** In vivo cathepsin L colocalization with enkephalin in adrenal medullary chromaffin cells. In vivo colocalization of cathepsin L with enkephalin in adrenal medullary chromaffin cells (bovine) was examined by immunohistochemistry of tissue sections with rabbit anti-cathepsin L and mouse anti-(Met)enkephalin. Cathepsin L was detected with anti-rabbit IgG conjugated to Alexa Fluor 488 (green fluorescence), and (Met)enkephalin immunofluorescence was detected with anti-mouse IgG conjugated to Alexa Fluor 594 (red fluorescence). Merging of images demonstrated colocalization of cathepsin L and (Met)enkephalin, illustrated by yellow fluorescence. Nuclei were stained with DAPI blue.

**Fig. 7.** Trafficking of cathepsin L/DsRed to chromogranin A-containing secretory vesicles in PC12 cells. (a) Colocalization of cathepsin L/DsRed with chromogranin A. PC12 cells transfected with the cathepsin L/DsRed construct (CL/DsRed) were stained with anti-chromogranin A, a marker for secretory vesicles. CL/DsRed was visualized as DsRed immunofluorescence (panel i), and chromogranin A (CgA) was visualized with anti-rabbit IgG Alexa Fluor 488 (green immunofluorescence (panel ii). Colocalization of CL/DsRed and CgA was indicated by the merged images showing yellow immunofluorescence (panel iii). (b) Expression of DsRed alone. DsRed was expressed in PC12 cells, and its cellular distribution was observed by DsRed immunofluorescence.

**Fig. 8.** Proenkephalin-derived products generated by cathepsin L. Proenkephalin is schematically illustrated at the top of this figure, containing active enkephalin peptides consisting of (Met)enkephalin (ME) and (Leu)enkephalin (LE), as well as the octapeptide (ME-Arg-Gly-Leu) and heptapeptide (H, ME-Arg-Phe) forms of enkephalin. Cathepsin L conversion of proenkephalin to enkephalin-containing intermediates products is illustrated, based on the detection of intermediates with anti-(Met)enkephalin or anti-synenkephalin in figures 1 and 2. The observed molecular weight on SDS-PAGE is illustrated for each predicted PE-derived intermediate that was generated by cathepsin L. These cathepsin L-generated PE-derived intermediates are similar to those found in vivo in adrenal medulla.
(Met)enkephalin Secreted
pg/µl media

Control

PE/CL

- nicotine
+ nicotine
Figure 3

(a) Enkephalin immunoblot

(b) Synenkephalin immunoblot

(c) Cathepsin L immunoblot
Figure 4

CG

PC12: PE-CL
Figure 5

(a) CL/DsRed

(b) enkephalin

(c) CL/DsRed-enkephalin
Figure 6
Figure 7

(a) CL/DsRed    CgA                          CL/DsRed - CgA

(b) DsRed

10 µm
Figure 8

- 26 kDa
- 22/23 kDa
- 18/19 kDa
- 8/9 kDa
- ~4.5 kDa, Peptide F
- (Met)enkephalin
Cathepsin L expression is directed to secretory vesicles for enkephalin neuropeptide biosynthesis and secretion
Shin-Rong Hwang, Christina Garza, Charles Mosier, Thomas Toneff, Eric Wunderlich, Paul Goldsmith and Vivian Hook

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