Volume 238, number 1, 142–146 FEB 06069 September 1988

The effect of pepstatin A, an inhibitor of the pro-opiomelanocortin (POMC)-converting enzyme, on POMC processing in mouse intermediate pituitary

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Received 24 May 1988

In our previous studies, we have purified a unique, paired basic residue-specific, prohormone-converting enzyme from pituitary intermediate lobe secretory vesicles. This enzyme, an aspartyl protease, was shown to cleave the intermediate lobe prohormone, pro-opiomelanocortin (POMC), to adrenocorticotropin, β-endorphin and a 16 kDa NH_2-terminal glycopeptide, in vitro [(1985) J. Biol. Chem. 260, 7194–7205]. To provide some evidence that this enzyme plays a role in prohormone conversion in the intact cell, the ability of pepstatin A, an aspartyl protease inhibitor, to block POMC processing in the mouse intermediate pituitary was investigated. By the use of a radioactive pulse-chase paradigm, [^{3}H]POMC processing was found to be inhibited by 36.4% in pepstatin A-treated intermediate lobes. This result is consistent with the inactivation of pro-opiomelanocortin-converting enzyme by pepstatin A in the intact pituitary and further supports a role of this enzyme in POMC processing in vivo.

Prohormone processing enzyme; Intermediate lobe; ACTH/endorphin prohormone; Aspartyl protease; Protease inhibitor

1. INTRODUCTION

The conversion of pro-opiomelanocortin (POMC, ACTH/endorphin prohormone) in the intermediate pituitary to yield biologically active hormones requires a sequence of enzymatic post-translational processing steps which occur within secretory vesicles [1–3]. The first cleavages take place at pairs of basic amino acid residues which flank the hormones to be cleaved. In a previous study, we reported the purification from bovine intermediate pituitary secretory vesicles, a PCE that is specific for paired basic residues of POMC [4]. The enzyme processed POMC to yield ACTH, β-endorphin and a 16 kDa NH_2-terminal glycopeptide [4]. Recently PCE was also shown to cleave NH_2-terminal POMC 1–76 at the Arg-Lys pair to yield products, identified by retention times on high-performance liquid chromatography and immunoreactivity, as Lys-γ_3-MSH and NH_2-terminal POMC 1–49 (Estivariz, F., Birch, N.P. and Loh, Y.P., in preparation). PCE was characterized as a 70 kDa glycoprotein with a pH optimum of 4.0–5.0 [4]. It was inhibited by pepstatin A and diazoacetyl-norleucine methyl ester, both aspartyl protease inhibitors, but not by thiol or serine protease inhibitors [4,5]. An enzyme activity with similar specificity and characteristics has also been found in rat intermediate and anterior pituitary secretory vesicles [6,7].

The involvement of PCE in POMC processing in vivo is supported by (i) its localization in secretory vesicles, the intracellular site of processing [3]; (ii) its specificity for paired basic residues of POMC; (iii) its acidic pH optimum, consistent with the

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Abbreviations: POMC, pro-opiomelanocortin; ACTH, adrenocorticotropin; α-MSH, α-melanotropin or N^{α}-acetyl-$ACTH_{1-13}$ NH_2; PCE, pro-opiomelanocortin-converting enzyme; DMSO, dimethyl sulfoxide

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

acidic internal environment of secretory vesicles [4,8] and (iv) its co-secretion with α-MSH (a POMC-derived hormone), in a co-ordinately regulated manner [9]. To provide some evidence for a physiological role of PCE in prohormone conversion in the intact cell, the ability of pepstatin A, an inhibitor of this enzyme, to interfere with POMC processing in pituitary cells was examined. In this study we show that POMC processing was inhibited in pepstatin A-treated mouse pituitary intermediate lobe cells.

2. MATERIALS AND METHODS

2.1. *Animals*

White, male mice (NIH strain) weighing 25–30 g were obtained from the National Institutes of Health animals facilities (Bethesda, MD).

2.2. *Analysis of neurointermediate lobes treated with pepstatin A and pulse-chase incubated in [^{3}H]arginine*

Pituitary neurointermediate lobes were dissected from mice. Since only the intermediate lobe synthesizes pro-opiomelanocortin, and the neural lobe does not, the two lobes were not separated for these biosynthesis studies. Neurointermediate lobes were pre-incubated at 37°C for 45 min in mammalian Ringer's solution [3] in the presence or absence of pepstatin A (10^{-4} M). Lobes were then pulse incubated for 10 min in Ringer's solution containing 18 μM [^{3}H]arginine (spec. act. 18 mCi/mmol, purchased from Dupont-New England Nuclear Corp., Boston, MA) with or without pepstatin A, followed by a chase incubation for 2 h in Ringer's solution containing 1 mM unlabelled arginine ± pepstatin A. The lobes were then homogenized in 0.1 N HCl. Homogenates were either immunoprecipitated with antiserum specific for the mid-portion of ACTH (DP4), or analyzed directly on 11% polyacrylamide acid-urea gels [10]. The medium from the chase incubation was also collected, acidified to a final concentration of 0.1 N HCl and analyzed by acid-urea gels. In some experiments, lobes were inhibited from secretion by dopamine (5×10^{-5} M), included in the chase-incubation medium [11]. Dopamine does not affect the processing of POMC [11]. Pulse-chase incubation and immunoprecipitation procedures have been described in detail elsewhere [3]. Since pepstatin A was solubilized in DMSO, the incubation medium contained a final concentration of 0.1% DMSO. Control experiments indicate that 0.1% DMSO did not affect the lobes' ability to incorporate [^{3}H]arginine, the morphology of the cells as evidenced by electron microscopy and the ability of the cells to show regulated secretion.

2.3. *Preparation and characterization of subcellular fractions*

Twenty neurointermediate lobes were pooled after a 45 min preincubation, 10 min pulse in [^{3}H]arginine and a 2 h chase incubation in medium containing pepstatin A, as described above. Dopamine (5×10^{-5} M) was also included in the chase incubation to block secretion. The lobes were homogenized in 0.25 M sucrose/10 mM Tris-Cl, pH 7.4 at 0°C and the

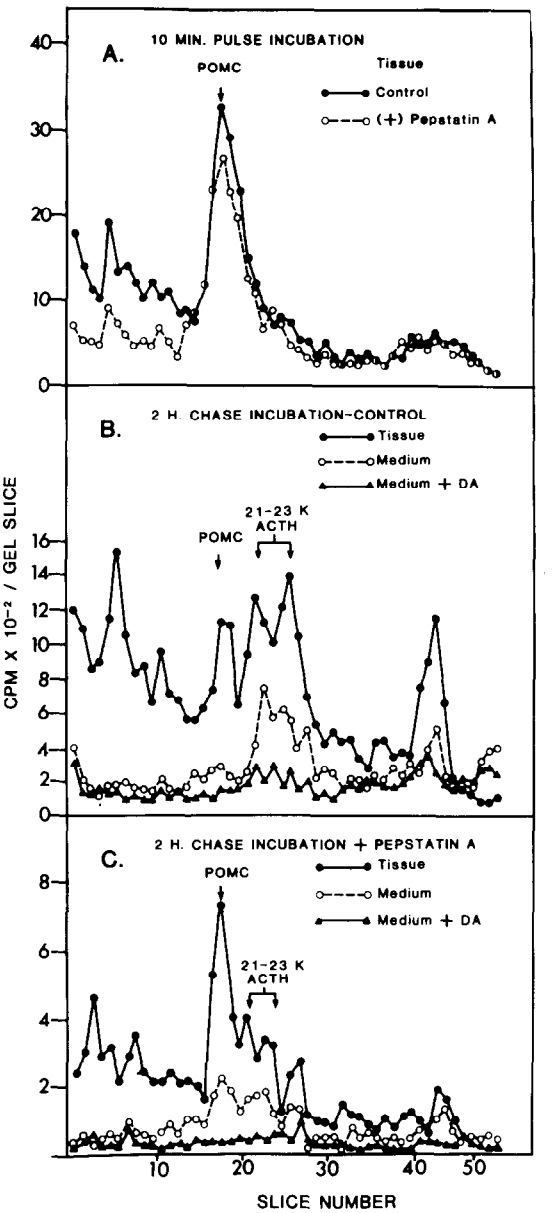

Fig.1. Acid-urea gel profiles of [^{3}H]arginine-labelled proteins from control and pepstatin A-treated neurointermediate lobes and secretion medium. (A) Acid-urea gel profiles of lobes pulse labelled in [^{3}H]arginine for 10 min with (○---○) and without pepstatin A (●——●). (B) Acid-urea gel profiles of a lobe pulse labelled in [^{3}H]arginine for 10 min and chase incubated for 2 h (●——●), chase medium (○---○) and chase medium from a lobe pulse labelled for 10 min and chase incubated for 2 h in the presence of dopamine (▲——▲). (C) Acid-urea gel profiles of a lobe pulse labelled in [^{3}H]arginine for 10 min and chase incubated for 2 h in the presence of pepstatin A (●——●), chase medium from the pepstatin A-treated lobe (○---○) and chase medium from a lobe pulse labelled for 10 min and chase incubated for 2 h in the presence of pepstatin A and dopamine (▲——▲).

homogenate was then subjected to differential centrifugation at 4°C (described in detail elsewhere) [3]. The fractions generated were: P I (cell debris and nuclei), P II (mitochondria/secretory vesicle fraction), P III (secretory vesicle fraction), P IV (microsomal fraction) and the supernatant. The fractions were characterized by the distribution of monamine oxidase (mitochondria marker) and α-MSH (secretory vesicle marker). Each fraction was suspended in 0.1 N HCl, immunoprecipitated with ACTH antiserum and analyzed for POMC by acid-urea gel electrophoresis as described previously [4].

3. RESULTS

Neurointermediate lobes were pulse labelled for 10 min and homogenized, or chase incubated for 2 h. Fig.1A shows that with a pulse incubation alone, POMC, previously identified by its electrophoretic and immunological properties [3], was the major peak seen in the acid-urea gel profiles, in both control and pepstatin A-treated lobes. Incorporation of [^{3}H]arginine into POMC in pepstatin A-treated lobes was quantitatively within the same range as control lobes (not shown), indicating that pepstatin A did not affect POMC synthesis. In control lobes that were pulse labelled and chase incubated for 2 h (fig.1B), POMC was significantly processed (table 1) to various products including 21–23 kDa ACTH intermediates, which had previously been identified by immunoprecipitation [3]. These products were secreted into the medium and the secretion was inhibited by dopamine. In contrast, pepstatin A-treated lobes, after a 2 h chase incubation, showed a major POMC peak and significantly less processed products (fig.1C). The small amount of processed products formed were similar to those found in control lobes (fig.1B and C) and these products were secreted into the medium, in a dopamine inhibitable manner. However, most of the unprocessed POMC was not secreted (fig.1C). Quantitative analysis (table 1) revealed a 36.4% inhibition of POMC processing with pepstatin A treatment, relative to control. A similar level of inhibition of POMC processing by pepstatin A was observed in lobes treated in the same manner, except that dopamine was added to inhibit secretion (not shown).

The lack of secretion of the unprocessed POMC prompted the investigation into whether pepstatin A treatment interfered with the packaging of the prohormone into secretory vesicles for processing, which normally occurs in untreated lobes [3]. Pepstatin A-treated lobes that were pulse labelled and chase incubated in dopamine for 2 h, were subcellularly fractionated to determine the distribution of [^{3}H]POMC within the cell. Fig.2 shows the gel profile of ^{3}H-labelled, anti-ACTH immunoprecipitated, POMC and various

Table 1

Analysis of inhibition of POMC processing by pepstatin A in mouse neurointermediate lobes

Treatment	% $\left(\frac{\text{POMC cpm}}{\text{POMC cpm + products cpm}}\right)^a$	% POMC conversion	% inhibition
Control lobes (10 min pulse)	71.5 ± 5.6 (2)		
Control lobes + medium (10 min pulse-2 h chase)	15.9 ± 2.0 (4)	77.8	
Pepstatin A-treated lobes (10 min pulse)	67.9 ± 4.8 (2)		
Pepstatin A-treated lobes + medium (10 min pulse-2 h chase)	34.3 ± 1.4 (4)	49.5	36.4

[a] Values shown are the mean ± SD. The number of lobes analyzed is shown in parentheses. cpm in POMC peak (gel slices 16–20) and product peaks (gel slices 21–50) were taken from acid-urea gel profiles as shown in fig.1

$$\%\ \text{POMC conversion} = 100\% - \left[\frac{\%\ (\text{POMC/POMC + products})t_{\text{chase}} = 2\ \text{h}}{\%\ (\text{POMC/POMC + products})t_{\text{chase}} = 0\ \text{h}} \times 100\right]$$

$$\%\ \text{inhibition} = 100\% - \left[\frac{\%\ \text{conversion in pepstatin A-treated lobes}}{\%\ \text{conversion in control lobes}} \times 100\right]$$

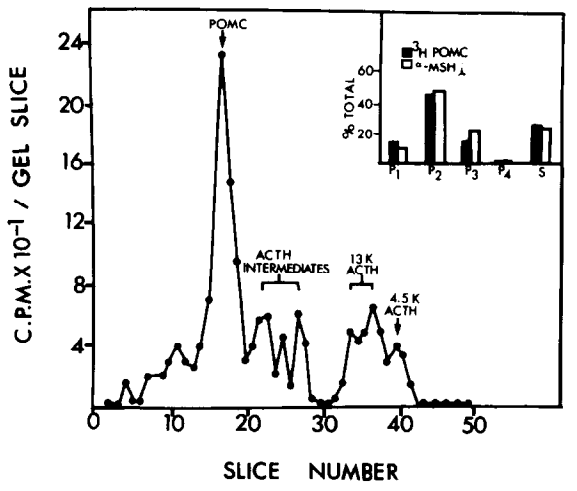

Fig.2. Acid-urea gel profile of anti-ACTH immunoprecipitated proteins from pepstatin A-treated neurointermediate lobes. Lobes were pulse labelled in [^{3}H]arginine for 10 min and chase incubated for 2 h in the presence of dopamine and pepstatin A. (Inset) Subcellular distribution of anti-ACTH immunoprecipitated [^{3}H]POMC and immunoreactive α-MSH (see section 2).

21–23 kDa ACTH intermediates and ACTH products (13 and 4.5 kDa) from pepstatin A-treated lobes. Note that POMC is the predominant peak, indicating inhibition of processing in these lobes. Fig.2 (inset) shows that anti-ACTH immunoprecipitated POMC was primarily present in fractions (P II and P III) which contain α-MSH, the marker for secretory vesicles. Essentially no POMC was found in the microsomal fraction (P IV, which contains Golgi and rough endoplasmic reticulum). These results suggest that POMC in pepstatin A-treated lobes was intracellularly transported into secretory vesicles and not retained in the Golgi apparatus or rough endoplasmic reticulum.

4. DISCUSSION

Many mammalian prohormone processing enzymes that cleave at paired basic residues have been reported [4,12–18]. While evidence such as the specificity, localization in secretory vesicles and the acidic pH optimum have suggested that some of these enzymes may play a physiological role in prohormone processing, none have been shown directly to function in the intact cell. Evidence for direct participation of an enzyme in prohormone processing in the intact cell may be obtained by several ways: by showing (i) that a mutant that fails to process the prohormone lacks the enzyme; (ii) that an anti-sense mRNA to the enzyme introduced into the cell blocks the expression of the enzyme and hence processing of the prohormone, or (iii) that treatment of intact cells with an inhibitor of the enzyme prevents prohormone processing. The lack of appropriate mammalian mutants and the experimental difficulties inherent in these approaches have rendered the demonstration of the involvement of a putative processing enzyme in prohormone conversion in the intact tissue, a formidable task. In this study, we have attempted to show that the secretory vesicle, paired basic residue-specific, PCE [4] participates in prohormone processing in the intact pituitary using the third approach. Pepstatin A is a potent inhibitor of PCE [4] and it is a hydrophobic peptide which is capable of entering cells and accumulating in organelles [19]. We showed that treatment of mouse neurointermediate lobes with pepstatin A resulted in a 36.4% inhibition of POMC processing as compared to untreated lobes (fig.1, table 1). Subcellular fractionation studies indicate that POMC was transported and packaged into secretory vesicles. Hence, the inhibition of POMC processing in pepstatin A-treated lobes was not due to an effect on intracellular routing of POMC into secretory vesicles, but rather an inhibitory effect of this agent on an aspartyl protease, presumably the PCE activity [4] in secretory vesicles. The level of inhibition of POMC processing by pepstatin A is dependent on the efficiency of entry of the inhibitor into the cells and accumulation in sufficient concentrations in the secretory vesicles. Complete inhibition of POMC processing would occur only when there is total inactivation of all PCE molecules within the cells. Such conditions are probably not experimentally achievable, especially since the intact intermediate lobe has only a finite time of viability in vitro and therefore, the treatment time cannot be more extensive. In view of these experimental constraints, the incomplete inhibition of POMC processing by pepstatin A observed in intact mouse intermediate lobes was perhaps not surprising. Nevertheless, the inhibitory effect of pepstatin A was significant and highly reproducible.

The release studies showed that the unprocessed POMC in the pepstatin A-treated lobes was not secreted. Preliminary autoradiographic studies at the electron microscopic level on pepstatin A-treated mouse intermediate lobes that were pulsed in [^{3}H]arginine for 10 min and chase incubated for 2 h, revealed silver grains associated with secretory vesicles that were primarily localized in the proximity of the Golgi apparatus. In correlation with the subcellular fractionation data, these vesicles probably contain unprocessed POMC, raising the hypothesis that the prohormone processing event within the secretory vesicle may be a prerequisite for its mobilization towards the plasmalemma for secretion.

In summary, this study has demonstrated that an inhibitor of PCE can block processing of POMC in intact intermediate pituitary cells. These data add to the existing evidence (described in section 1) in providing strong support for the participation of PCE in POMC processing in intermediate pituitary cells in vivo.

Acknowledgements: I thank Drs Nigel Birch, Mark Whitnall and Elaine Neale for helpful discussions and Ms Maxine Schaefer for typing the manuscript. The electron microscopic autoradiography was performed by Dr Urmi Patel-Vaidya, Ultratone Inc., Silver Spring, MD.

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