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REVIEW ARTICLE

The Tissue-Specific Processing of Pro-Opiomelanocortin

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It is just over 30 years since the definitive identification of the adrenocorticotrophin (ACTH) precursor, pro-opiomelanocotin (POMC). Although first characterised in the anterior and intermediate lobes of the pituitary, POMC is also expressed in a number of both central and peripheral tissues including the skin, central nervous tissue and placenta. Following synthesis, POMC undergoes extensive post-translational processing producing not only ACTH, but also a number of other biologically active peptides. The extent and pattern of this processing is tissue-specific, the end result being the tissue dependent production of different combinations of peptides from the same precursor. These peptides have a diverse range of biological roles ranging from pigmentation to adrenal function to the regulation of feeding. This level of complexity has resulted in POMC becoming the archetypal model for prohormone processing, illustrating how a single protein combined with post-translational modification can have a diverse number of roles.

Key words: pro-opiomelanocortin, ACTH, N-POMC, prohormone convertase.

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In 1967, a new era in endocrinology was born. A set of pulse chase experiments performed by Steiner and Oyer (1) elegantly showed, for the first time, that insulin was derived from a larger precursor molecule, proinsulin. The radical revelation, that bioactive peptides are derived from larger precursors, forever changed endocrinology and, from then on, the concept of hormone precursors (or prohormones) has become a central dogma. Subsequently, the use of both traditional protein sequence studies and recombinant DNA technology have provided definitive structures for the precursors of all the known bioactive peptides as well as identifying several previously unknown precursors, the function of which are still not fully understood. In general, all these molecules contain within their sequence one or more copies of the active peptide while some contain several different bioactive peptides. In addition, they undergo a series of highly regulated post-translational events that includes specific proteolytic cleavage and modifications such as amidation, acetylation and phosphorylation. These post-translational processing events are especially interesting because there are a number of examples where the extent of processing can yield very different bioactive peptides from the same precursor. Probably the best example of this phenomenon, and arguably the most studied prohormone, is the ACTH precursor pro-opiomelanocortin (POMC). This short review aims to highlight why it has been of such interest to (neuro)endocrinologists and suggests that it still has a few more secrets to yield.

Discovery of POMC

The discovery of POMC (Fig. 1) was preceded by a number of what, at the time, appeared to be unrelated observations. Both ACTH and α-melanocyte-stimulating hormone (α-MSH) had been purified and sequenced from the pituitaries of various species. However, the fact that α-MSH has the same amino acid sequence as the first 13 residues of ACTH was not seen to be of much significance until the isolation of corticotrophin-like intermediate peptide (CLIP) (2), the peptide that comprises the C-terminal of ACTH. This, together with the identification of larger molecular weight forms of immunoreactive ACTH (3, 4), began to suggest that ACTH and α-MSH were indeed derived from the same molecule.

A further observation suggesting the existence of a common precursor to a number of pituitary hormones was that the sequence of the newly-discovered pituitary peptide, β-endorphin (5, 6), which had strong opiate-like activity, shared the same sequence as the C-terminal of β-lipotrophin (β-LPH), another pituitary hormone released under the same conditions as ACTH. It was subsequently shown that β-LPH was expressed in the same pituitary cells as α-MSH and CLIP (7).

In 1976, the first published report came that suggested ACTH and β-LPH were derived from the same molecule (8) and 1 year later, in two separate studies, Mains *et al.* (9) and Roberts and Herbert (10) both demonstrated that ACTH and β-LPH were present in a single molecule. Mains *et al.* (9) used a pulse chase approach

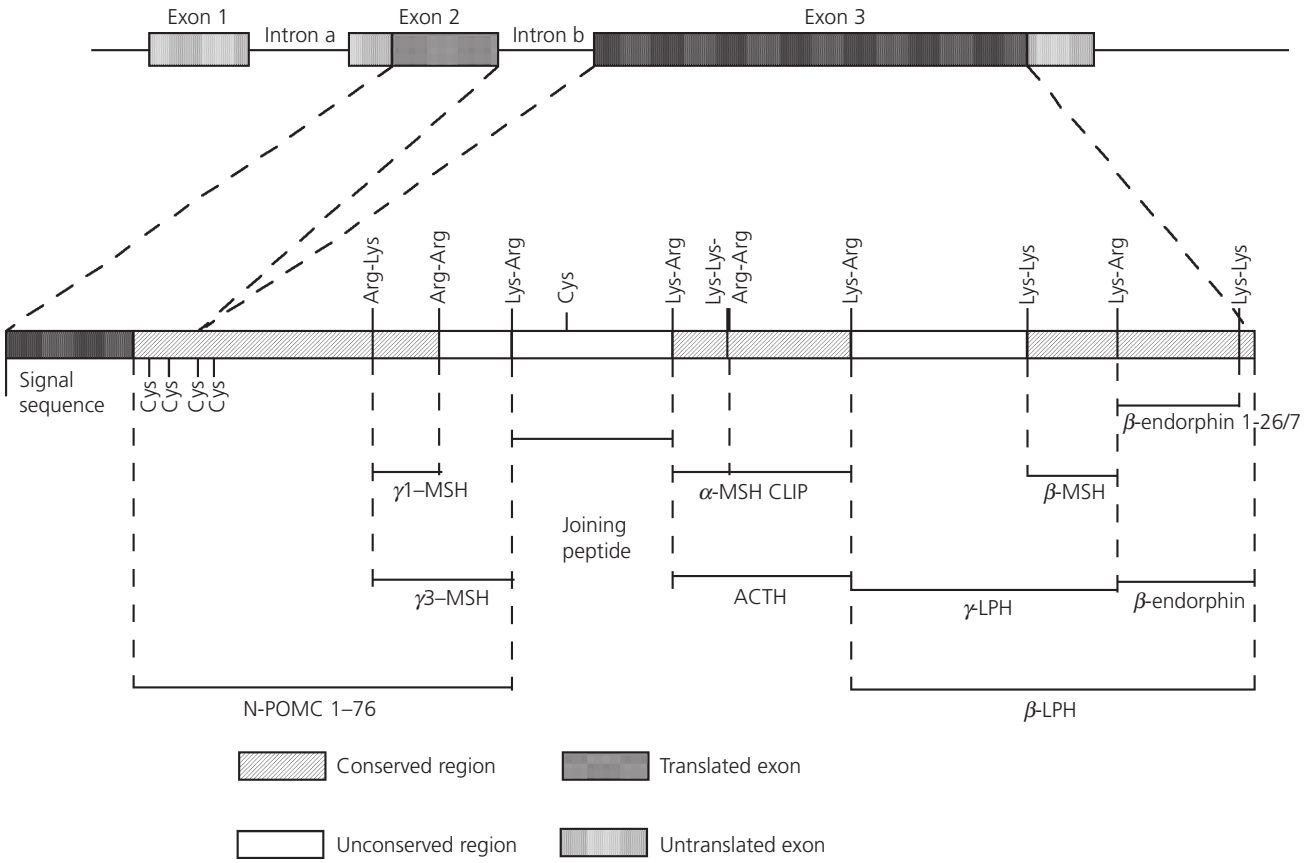

Fig. 1. Structure of the pro-opiomelanocotin gene and protein. The location of the various peptides together with the dibasic amino acid residue cleavage sites and the areas conserved between species are shown. ACTH, adrenocorticotrophin; CLIP, corticotrophin-like intermediate peptide; LPH, lipotrophin; MSH, melanocyte-stimulating hormone; POMC, pro-opiomelanocotin.

with the mouse pituitary tumour cell line AtT-20 that secretes large amounts of ACTH. They incubated the cells with radiolabelled amino acids (the pulse) and then washed the radioactivity away after a short period (the chase), thus labelling all proteins synthesised during the period of the pulse. By taking the cells at different times after the chase and immuno-precipitating the cellular protein using either an ACTH or β-endorphin antiserum followed by electrophoretic analysis using SDS-PAGE, they identified a common 31-kDa protein that became progressively smaller with time after the pulse until it became fully processed.

Roberts and Herbert (10) also used the AtT-20 cell line together with a cell free translation system. Messenger RNA was purified from the cells and expressed in the cell free system. In this way, a 28-kDa protein was identified that was recognised by both an ACTH and β-endorphin antiserum.

Although these experiments provide conclusive evidence that indeed ACTH and β-LPH were derived from the same protein, it was not until 1979 when the cDNA encoding POMC was cloned, from both the bovine intermediate lobe (11) and from the mouse pituitary cell line AtT-20 (12), that the true structural relationship between ACTH and β-LPH was revealed. Since then, the POMC gene has been cloned and sequenced from a variety of organisms. In every species examined, it has been found that there is a single functional copy of the gene and the overall gene structure, with the exception of the loss of γ-MSH in the salmon (13), has remained remarkably conserved throughout evolution. The precursor molecule (pre-pro-opiomelanocortin) has a 26 amino acid signal sequence followed by an N-terminal peptide (16K fragment or pro-γ-MSH) containing the first (γ) MSH sequence (a peptide that had previously not been identified). In the centre of the molecule is ACTH, the sequence of which contains the second MSH sequence (α-MSH). Finally, at the C-terminus, is β-LPH, the sequence of which includes that for β-endorphin and the third (β) MSH sequence. All the MSH peptides contain a common core sequence of amino acids, MEHFRW (although, in γ-MSH, the glutamic residue is substituted by a glycine) and are collectively known as the melanocortins. Dibasic amino acid residues (e.g. Arg-Lys, Arg-Arg, Lys-Arg or Lys-Lys) flank all the bioactive peptides within POMC and are cleaved by specific proteases to release the active peptides.

The melanocortin receptors

The known roles of ACTH in steroid production from the adrenal and α- and β-MSH in skin pigmentation suggested the existence of at least two receptors specific for the melanocortin peptides. Using a cloning strategy based on the sequence of known G-protein

coupled receptors, the first receptor specific for α-MSH was cloned in 1992 (14, 15). In the following 2 years, a further four closely-related receptors were identified and were subsequently named melanocortin receptors 1–5 (MC1-5-R). All members of the family are typical rhodopsin-like seven transmembrane spanning receptors that couple through cAMP, although it has been demonstrated that some members can use other signalling pathways (16, 17). The MC1-R is expressed predominantly in the skin where it mediates the effects of α-MSH on pigmentation. It is also expressed in cells involved with the inflammatory response where it plays a role in mediating the anti-inflammatory effects of α-MSH (14, 15). The MC2-R is expressed in the adrenal cortex where it stimulates steroidogenesis and is expressed also in the adipocytes of rodents where it stimulates liploysis. It is unique amongst the family for being highly specific for ACTH (15, 18). The MC3-R is expressed mainly in the hypothalamus and also in gut, kidney and placenta. This receptor is the only MCR with significant affinity for γ-MSH, but can also bind α-MSH and ACTH with approximately equal affinity (19). This receptor plays a role in feeding behaviour by influencing fat mass; its role in the periphery is still not clear. The MC4-R is expressed mainly in the brain and spinal cord. Its principle ligand is α-MSH (20) and it plays a key role in the regulation of appetite. The MC5-R is expressed at low levels in a wide variety of tissues with α-MSH showing the greatest binding affinity (21). Its function remains obscure, although studies in the MC5-R null mouse have implicated a role in exocrine gland function (22). There is currently significant interest in the MC-Rs as drug targets for obesity, resulting in the development of a number of selective agonist and antagonists. A detailed review of these, together with the pharmacology of the MC-Rs, can be found in MacNeil *et al.* (23).

Sites of expression of POMC and the concept of tissue specific processing

The POMC gene is predominantly expressed in the anterior and intermediate lobes of the pituitary and its mRNA has been detected in several other tissues, including the brain, lymphocytes, skin, testis, thyroid, placenta, pancreas, gut, kidney adrenal and liver (24). It is generally accepted that the vast majority of POMC peptides found in the circulation are derived from the pituitary whereas, by contrast, peptides produced in extra-pituitary tissues act in an autocrine or paracrine fashion.

One consequence of producing peptides from a larger precursor is that the precursor can be cleaved in a tissue specific manner to give a different set of products depending in which tissue the precursor is synthesised. POMC is the classical example of this so-called tissue specific processing and has been studied extensively.

Processing of POMC in the pituitary

POMC is expressed in both the anterior and intermediate lobes of the pituitary. In the anterior lobe, it is produced in a sub-population of cells called corticotrophs representing approximately 1% of the total protein content whereas, in the intermediate lobe, it is produced in cells called melanotrophs (Fig. 2). A clearly defined

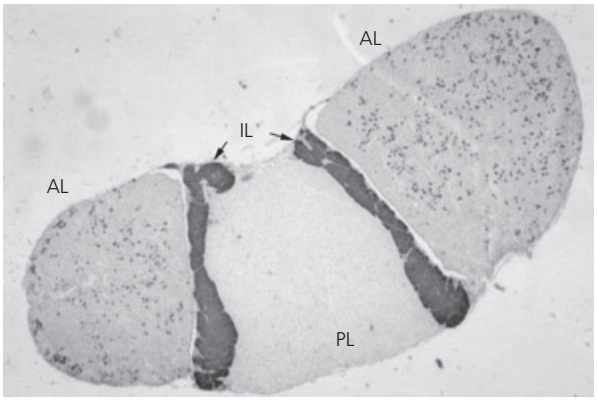

Fig. 2. Section of a rat pituitary stained with an antibody to γ-melanocyte-stimulating hormone. Expression of pro-opiomelanocotin (POMC) can be seen in numerous corticotroph cells scattered throughout the anterior lobe (AL) whereas, in comparison, expression is more extensive in the intermediate lobe (IL), which is almost completely comprised of melanotroph cells. No cells expressing POMC can be observed in the posterior lobe (PL).

intermediate lobe is not present in humans, where it regresses soon after birth.

The processing in each of the lobes is quite different and has led to the use of the term 'tissue specific processing' that describes the concept of different secreted products being generated from the same precursor. The processing of POMC in each of these tissues is shown in Fig. 3 along with the melanocortin receptor subtypes (MCX-R where *X* is a number from 1–5) responsible for the effects of the processed peptides. An interesting observation is that the processing of POMC in the pituitary is not 100% efficient, resulting in significant amounts of intact precursor being released into the circulation (25).

Anterior lobe

The AtT-20 cell line has been used extensively to characterise the processing pattern in the anterior pituitary and has been a good model, reflecting accurately the processing events that occur in anterior pituitary corticotrophs (26). In these cells, processing of POMC results in the generation of pro-γ-MSH, ACTH and β-LPH, with little processing to the smaller peptides. This pattern of processing also occurs in the anterior pituitary (27–29), although a significant proportion of the β-LPH is further cleaved to β-endorphin (30, 31). There is some evidence to suggest that, in the rat and the sheep, a proportion of the ACTH is cleaved to α-MSH and CLIP.

Intermediate lobe

Processing of POMC in the intermediate lobe is more extensive with further cleavage of the peptides produced in the anterior lobe. ACTH is cleaved to α-MSH and CLIP whereas β-LPH is virtually completely processed to β-endorphin and γ-LPH (28, 32). β-endorphin$_{1\text{-}31}$ is cleaved at the C-terminus to give β-endorphin$_{1\text{-}27}$, β-endorphin$_{1\text{-}26}$

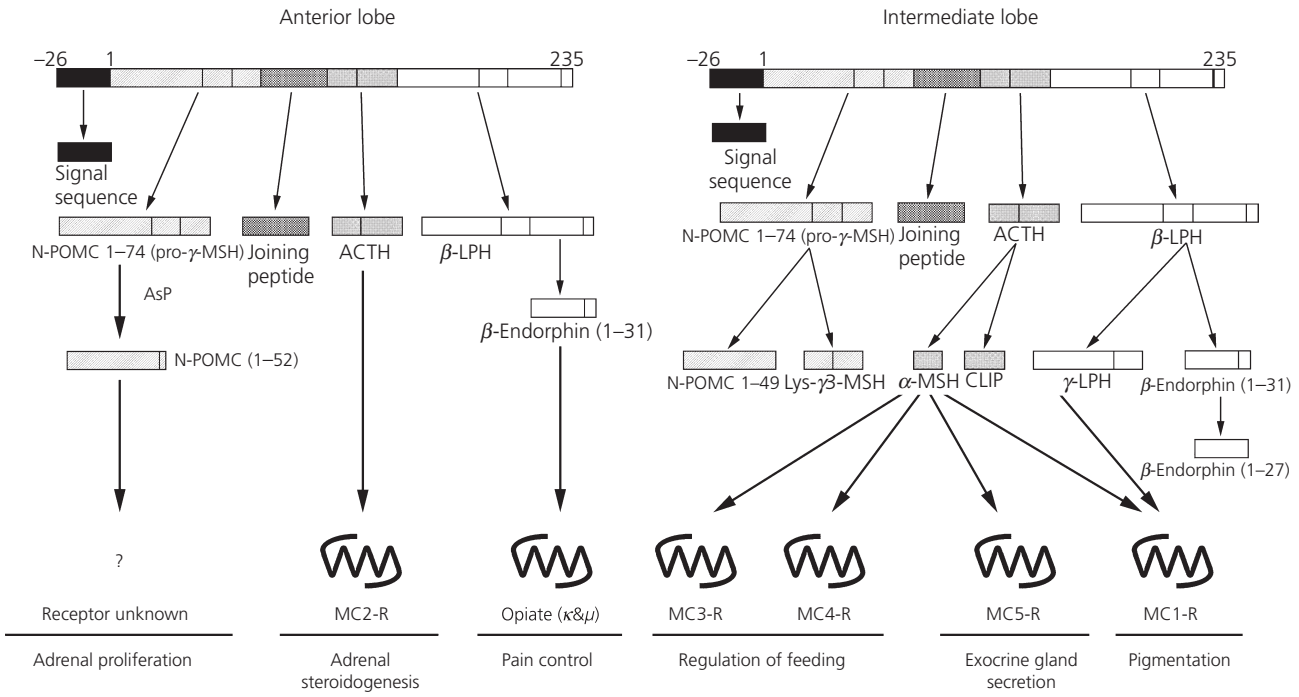

Fig. 3. Processing of pro-opiomelanocotin (POMC) in the anterior and intermediate lobes of the pituitary and the physiological roles of the resulting peptides. POMC peptides act mainly through a family of five G-protein coupled receptors known as the melanocortin receptors (MCX-R where *X* is a number from 1–5). β-endorphin acts via the κ and μ opiate receptors and the receptor through which the N-terminal peptides stimulate adrenal mitogenesis remains to be identified. ACTH, adrenocorticotrophin; CLIP, corticotrophin-like intermediate peptide; LPH, lipotrophin; MSH, melanocyte-stimulating hormone.

and a dipeptide glycylglutamine (31). The majority of the β-endorphin is *N*-acetylated (27, 33), which destroys its opiate activity (34). The N-terminal fragment pro-γ-MSH is also partially cleaved at an Arg-Lys site to give N-POMC 1–49 and Lys-γ_3-MSH (35). This latter peptide contains an additional dibasic site (although not in all species) but does not appear to be cleaved to any significant extent.

Processing of POMC in other tissues

As previously stated, the mRNA of POMC can be detected in a number of non-pituitary tissues, although, in many of these, the transcripts are not full length and cannot be efficiently translated, leading to extremely low levels of protein (36). However, there are a number of exceptions to this where significant amounts of POMC are produced. In general, processing in these tissues is similar to that observed in the intermediate lobe of the pituitary, although there are subtle differences between tissues.

Brain

It has been known for many years that the brain produced POMC-derived peptides (37), although it was initially assumed these were of pituitary origin. It is now known that this is not the case and that POMC is expressed mainly in the hypothalamus, specifically the arcurate nucleus, although it is expressed also at lower levels in both the hippocampus and cortex (24, 38).

Processing in the brain is generally accepted to follow a pattern similar to the intermediate lobe, although the majority of α-MSH expressed in the hypothalamus is not acetylated (39). Interestingly, in a more recent report, it has been suggested that there are significant amounts of the larger anterior lobe peptides (40). The physiological role of POMC peptides in the brain is currently an area of active research as it has been shown that α-MSH is central to the regulation of appetite, being part of an endocrine feedback loop with leptin (a hormone produced by adipocytes) together with the MC3 and MC4 receptors (41). The complete architecture of this system is still far from clear, but has clear potential as a therapeutic target for the treatment of obesity.

Skin

The role of α-MSH and β-MSH in lower vertebrates, such as frogs, has been known for many years where it causes darkening of the skin to match the environmental background. Interestingly, frog skin was used for many years as a sensitive bioassay for α-MSH (42, 43). The role of pituitary-derived α-MSH in the pigmentation of humans is not clear because, without an intermediate lobe, this peptide cannot be found in the circulation, although, in conditions resulting in overproduction of POMC such as Cushing's syndrome, Nelson's syndrome or Addison's disease, there is a distinct darkening of the skin. There is local production of POMC and its peptides in melanocytes, keratinocytes (44, 45) and dermal microvascular

endothelial cells (46) with processing in an intermediate lobe pattern. The synthesis and release of these peptides is regulated by both cytokines and ultraviolet radiation, suggesting the existence of an autocrine/paracrine role for POMC peptides in skin pigmentation acting via the MC1 receptor (45, 46).

Lymphocytes

Lymphocytes and macrophages express a number of melanocortin receptors (38, 47), as well as certain classes of opiate receptors, and it has been known for a long time that POMC peptides can influence immune function. The expression of POMC in lymphocytes was discovered in the late 1970s due to ACTH-like bioactivity being detected in lymphocyte-derived preparations of interferon-α (48), although, due to the low expression levels, it took some considerable time to definitively show expression of POMC at both the mRNA and protein level. Interestingly, processing of POMC in lymphocytes appears to be more like that observed in the anterior pituitary with the generation of ACTH (49). The functional significance of POMC expression in lymphocytes is still not entirely clear, and it is generally asssumed that it forms part of a biochemical loop linking the immune, nervous and endocrine systems (38, 48).

Placenta

In comparison to the pituitary, the placenta expresses the POMC gene at a relatively low level, although the large mass of the placenta means that it can secrete significant amounts of POMC-derived peptides into the circulation. Processing of POMC in the placenta is unique, and leads to the release of significant amounts of both unprocessed and partially processed precursor, as well as ACTH, β-LPH, α-MSH and β-endorphin (50, 51). Secretion levels are relatively low throughout pregnancy and tend to rise towards term, which leads to increased levels of ACTH, although these usually remain within the normal range (52). The physiological role of POMC derived from the placenta remains unknown, although it was suggested recently that many placental peptides (including POMC) are modified with phosphocholine, a molecule implicated in immunomodulation (53).

Molecular basis of POMC tissue specific processing

As shown in Fig. 1, POMC is processed at pairs of basic amino acids. Within the sequence of POMC, there are eight pairs and one quadruplet. In the anterior pituitary, four Lys-Arg pairs are cleaved whereas, in the intermediate lobe (and most of the other tissues), all nine sites are cleaved. Despite the knowledge of the necessity of dibasic residues at the cleavage sites, it took until the 1990s to finally identify the enzymes responsible. The first breakthrough came with the cloning of a yeast gene called KEX2 (54, 55). The product of the KEX2 gene, known as kexin is a protease that cleaves the yeast α factor mating pheromone. Cellular expression of the gene demonstrated that the enzyme belongs to the subtilisin family of Ca^{2+} dependent serine proteinases. KEX2 has the ability to selectively cleave a number of mammalian prohormones and this led to the hypothesis that similar mammalian counterparts existed.

The first mammalian gene to be identified as a potential processing enzyme came as a consequence of computer alignment of the amino acid sequences surrounding the serine and catalytically important asparagine of kexin against known protein sequences. By this method, a human gene of unknown function was identified, named *fur*, that lies upstream from the tyrosine kinase *fps/*fes proto-oncogene. The product of this gene, known as furin, was to become the first mammalian processing enzyme identified (56).

Using the sequence of the two eukaryotic proteases and several other subtilisin sequences, Smeekens and Steiner (57) designed degenerate oligonucleotides corresponding to the conserved active site. By utilising these in the polymerase chain reaction (PCR) with cDNA isolated from a human insulinoma library as template, a fragment was generated that was then used to probe that library. A full-length cDNA was identified that encoded a protease that became known as PC2. Concurrently Seidah *et al.* (58, 59) used a similar procedure with the sequence of furin and a mouse pituitary library identified two cDNA clones that were to become known as PC2 and PC1.

Elegant studies performed *in vitro* using cell based assays and recombinant enzymes (60–64), together with studies looking at PC1 (65) and PC2 (66) knockout animals, have revealed the molecular

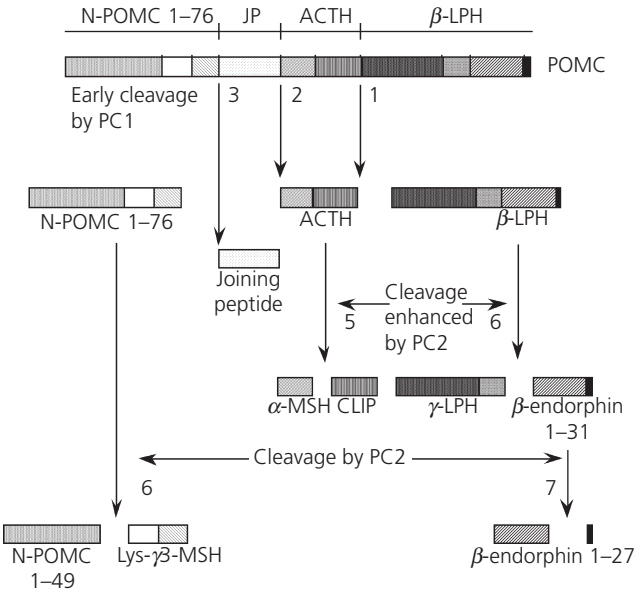

Fig. 4. Processing of pro-opiomelanocotin (POMC) by prohormone convertases 1 and 2. The dibasic sites are cleaved in the specific order illustrated by the numbers, showing that PC2 acts on the products of PC1 cleavage. ACTH, adrenocorticotrophin; CLIP, corticotrophin-like intermediate peptide; LPH, lipotrophin; MSH, melanocyte-stimulating hormone. Adapted from Zhou A *et al.* (64), Journal of Biological Chemistry. Copyright 1993 by American Society for Biochemistry & Molecular Biology. Reproduced with permission of American Society for Biochemistry & Molecular Biology in the format Journal via Copyright Clearance Center.

basis of the tissue specific processing observed in the anterior and intermediate lobes. In the anterior lobe, PC1 is the predominant enzyme present, which, due to its more limited proteolytic activity, results in the generation of ACTH. However, in the intermediate lobe, both PC1 and PC2 are present and their co-ordinate actions result in the generation of the smaller POMC peptides (50, 51). Cleavage occurs in a specific order (Fig. 4) with some of the dibasic sites being more labile than others (54). Accordingly, the age of a specific secretory granule has an influence on the proportion of the various peptides, with older granules containing more extensively processed peptides than younger granules.

Post-secretional processing of POMC peptides

A number of elegant studies undertaken in the early 1980s demonstrated that peptides derived from the N-terminal of pro-γ-MSH, lacking the γ-MSH sequence are involved with the maintenance of adrenal cortex size and have the ability to promote adrenal mitogenesis (67–69). Since these peptides were not products of anterior pituitary processing, it was proposed that circulating pro-γ-MSH was cleaved, most likely at the adrenal itself. More recently, a putative serine protease has been cloned from the adrenal that is up-regulated during adrenal growth. Initial studies using synthetic substrates suggest that it could potentially cleave pro-γ-MSH to generate a 52 residue N-terminal fragment, which could then potentially stimulate adrenal mitogenesis (70). The generation of this fragment, independent of the pituitary, represents another level of regulation of POMC peptide function giving adrenal size independence from the demands of steroidogenesis.

Summary

Most peptide hormones are produced as inactive precursor molecules that are subsequently cleaved at dibasic residues to release the active peptides. As the first multi-hormone to be discovered over 30 years ago, POMC has been extensively studied and characterised. Expressed mainly in the anterior and intermediate lobes of the pituitary, it is also expressed and processed in a number of central and peripheral tissues. POMC is the precursor to a number of bioactive peptide hormones, the exact combination produced being dependent on the tissue in which it is expressed. This 'tissue specific' processing results in a huge diversity of biological actions from a single molecule that has been of keen interest to endocrinologists since POMC was discovered. However, we are still some way off fully understanding the biology of POMC and its peptides and, as a consequence, there is still plenty to keep endocrinologists occupied for several more years to come.

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