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Processing of secretogranin II by prohormone convertases: importance of PC1 in generation of secretoneurin

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Abstract Secretoneurin is a recently characterized neuropeptide present in the primary amino acid sequence of secretogranin II. We investigated the proteolytic processing of secretogranin II by prohormone convertases in vivo in a cellular system using the vaccinia virus system. Both PC1 and PC2 can cleave the secretogranin II precursor at sites of pairs of basic amino acids to yield intermediate-sized fragments. Other convertases like PACE4, PC5 and furin were not active. For the formation of the free neuropeptide secretoneurin a different pattern was found. Only PC1 but none of the other convertases tested including PC2 were capable of generating secretoneurin. Our results demonstrate that the prohormone convertases PC1 and PC2 are involved in proteolytic processing of secretogranin II. The neuropeptide secretoneurin can only be generated by PC1 suggesting tissue-specific processing of secretogranin II in neurons expressing different subsets of the prohormone convertases.

Key words: Chromogranin; Neuroendocrine; Proteolytic processing

1. Introduction

Secretoneurin is a novel neuropeptide which is generated in vivo by proteolytic processing from its precursor secretogranin II [1]. Secretogranin II was initially recognized in pituitary slices as a protein strongly labeled by sulfate [2] and later shown to occur in many endocrine and nervous tissues [3,4]. Together with the chromogranins A and B, secretogranin II belongs to the group of acidic proteins generally called chromogranins [5].

In bovine adrenal chromaffin granules secretogranin II is proteolytically processed to smaller peptides [3,4,6] including secretoneurin [7]. In the central and peripheral nervous system secretoneurin is found in high concentrations in large dense core vesicles [7–10]. As first biological effects a dose-dependent release of dopamine from rat striatal slices [11] and a potent chemotactic effect towards monocytes [12] have been described recently.

The enzymes involved in the processing of secretogranin II have not yet been established. Recently a class of subtilisin-like endopeptidases responsible for prohormone processing was identified by its relationship to the kexin protease from yeast. This class of proteases, which were named prohormone convertases (PC), comprises several members i.e. PC1/PC3, PC2, PACE4, PC4, PC5/PC6 and furin (for reviews see [13–15]). We and others recently demonstrated the presence of PC1 and PC2 in chromaffin granules of the bovine adrenal medulla [16–18]. Since members of the PC family are found in chromaffin granules together with secretogranin II we tried to answer in the present study the following questions. (i) Are members of the PC family involved in endoproteolytic processing of secretogranin II? (ii) Which are the main cleavage sites in the secretogranin II precursor? (iii) Is formation of secretoneurin from secretogranin II the result of sequential cleavage by multiple endopeptidases or is one convertase alone sufficient to generate this neuropeptide?

2. Materials and methods

2.1. Infection of cells and analysis of extracts

A fragment (bases 79–2373) of the bovine secretogranin II (SgII) cDNA clone [19] containing the entire coding region was subcloned into the vaccinia virus (VV) PMJ601 vector into the 5′ *Sma*I/*Bam*HI 3′ site. For mouse PC1 and mouse PC2, a full-length transcript was subcloned into the vaccinia virus vector pVV3 [20]. The recombinant VV:hfurin, VV:hPACE4 and VV:mPC5 were prepared from the PMJ 601 vector in a similar fashion to PC1 and PC2 [21,22].

Pituitary prolactinoma GH_4C_1 cells were infected with either VV:SgII alone or VV:SgII plus the respective prohormone convertases PC1, PC2, PACE4, PC5 or furin for 2 h. After infection, the medium was removed from cells by aspiration. Then, 500 μl of distilled water was added to cells followed by a brief sonication step. The cell extracts were transferred to Eppendorf vials and boiled immediately for 10 min. Boiled samples (cell extracts and media) were centrifuged at 14,000 × *g* for 20 min. The supernatants were subjected to one- and two-dimensional immunoblotting as described in detail [23]. Briefly, cell extracts (60 μg) and media (130 μg protein determined according to [24]) from infected cells were separated on a 10–17% polyacrylamide gradient gel, electrotransferred to nitrocellulose sheets (Schleicher and Schüll, Germany, BA83, 0.2 μm) and incubated with antibody and radioiodinated Protein A (NEN, USA). The secreted proteins in the medium are likely to represent more directly the peptides in the secretory granules, in total cell extracts some peptides retained in the Golgi compartment are also included. However, we also analyzed cell extracts and the results matched those obtained with media extracts. Antisera (AR-16, LF-19, secretoneurin, EL-17 see [7,12] and anti PC1 [22]) were used at a dilution of 1:200. Immunoreactive proteins were visualized by autoradiography and quantified using a Fuji BAS 1000 phosphoimager system.

2.2. Gel-filtration HPLC

Extracts from infected cells and media were applied to a Superose 12 HR 10/30 gel-filtration column (Pharmacia LKB, Sweden) at a flow rate of 0.4 ml/min. As column buffer 37.5 mM sodium phosphate, pH 7.4 containing 37.5 mM NaCl was used. One minute fractions were collected and analyzed by a radioimmunoassay (RIA) as described previously in detail [7]. The specific [7] secretoneurin RIA recognized the free peptide as well as its precursor secretogranin II equally well.

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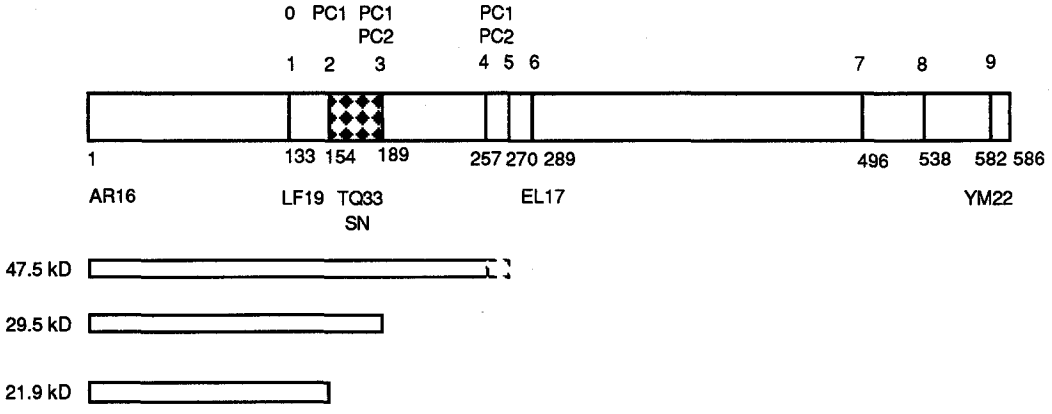

Fig. 1. Schematic drawing of the secretogranin II precursor. The sequence of bovine secretogranin II [19] is presented. The nine proteolytic cleavage sites (pairs of basic amino acids) are indicated by numbers on the top. The putative cleavage at these sites by PC1 or PC2 is indicated. Site 1 (indicated by 0) is not used for proteolytic cleavage in vivo [7]. The numbers on the bottom represent the positions of peptides flanked by pairs of basic amino acids in the secretogranin II precursor. The abbreviations below, i.e. AR16, LF19, TQ33 (= SN; secretoneurin), EL17 and YM22 indicate the first and last amino acid of the peptide given in the single letter code plus the number of total amino acids present which were used for generation of peptide antisera. The bars below represent identified intermediate-sized cleavage products of secretogranin II and their apparent molecular weight as obtained from SDS gels (see Figs. 2 and 3).

3. Results and discussion

3.1. Proteolytic processing of secretogranin II by the precursor convertases (PC's)

The processing of secretogranin II by the various prohormone convertases in vivo was investigated by co-expression of these components in GH_4C_1 cells using the vaccinia virus expression system. The pituitary prolactinoma cell-line GH_4C_1 contains very little endogenous secretoneurin-IR as determined by RIA. After infection with a recombinant vaccinia virus expressing bovine secretogranin II (VV:SgII) a 100-fold increase in immunoreactivity was found (not shown). By immunoblotting the molecular form of secretogranin II obtained after virus infection was further characterized. An antibody directed against a peptide (LF19) representing amino acids 133–151 of bovine secretogranin II (see Fig. 1) revealed the presence of intact secretogranin II (apparent M_r on SDS gels: 87,000) and a minor band of M_r 75,000 (see Fig. 2). No further processing products resulting from a putative cleavage by proteases endogenous to GH_4C_1 cells was evident. Co-infection of these cells with VV:SgII plus VV:PC1 resulted in a significant proteolysis of secretogranin II. Three additional bands were gener-

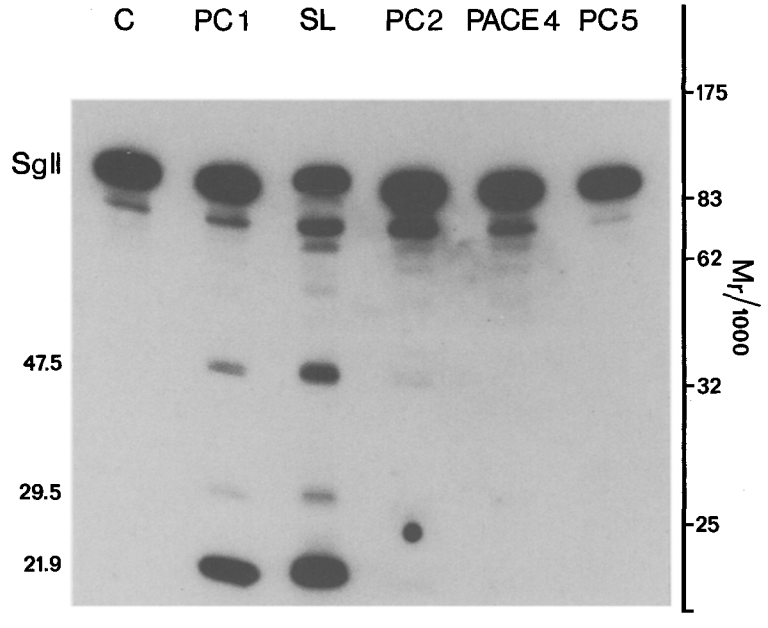

Fig. 2. Immunoblots with LF19 antiserum. Media secreted from GH_4C_1 co-infected with recombinant vaccinia virus vectors to express secretogranin II and prohormone convertases were separated in one-dimensional SDS polyacrylamide gels and subjected to immunoblotting with an antiserum against the LF19 peptide (see Fig. 1). C, control cells expressing secretogranin II; PC1, PC2 PACE4, PC5, cells expressing the indicated prohormone convertases plus secretogranin II; SL, soluble content of bovine chromaffin granules (50 μg). The position of secretogranin II (SgII) and intermediate sized-forms plus their respective apparent molecular weight are indicated on the left. The experiments were performed in triplicate, one representative sample is given here.

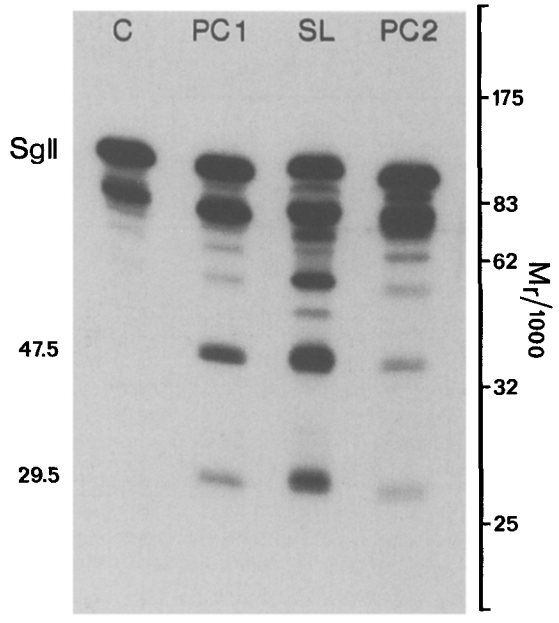

Fig. 3. Immunoblot with secretoneurin antiserum. Media secreted from GH_4C_1 co-infected with recombinant vaccinia virus vectors to express secretogranin II and prohormone convertases were separated in one-dimensional SDS polyacrylamide gels and subjected to immunoblotting with an antiserum against secretoneurin (see Fig. 1). C, control cells expressing secretogranin II; PC1, PC2 cells expressing the indicated prohormone convertases plus secretogranin II; SL, soluble content of bovine chromaffin granules (50 μg). The position of secretogranin II (SgII) and intermediate sized-formes plus their respective apparent molecular weight are indicated on the left.

ated, two bands with apparent M_r of 47,500 and 29,500 and one major band of M_r 21,800 (see Fig. 2). All three bands generated by PC1 are endogenously found in the soluble content of chromaffin granules (see Fig. 2) indicating that PC1-induced cleavage of secretogranin II closely resembles that occurring in vivo in the adrenal medulla. Co-infection of VV:SgII and VV:PC2 yielded the 47,500 and 29,500 intermediate-sized bands (Fig. 2). The prominent 21,900 band generated by PC1 was not formed by PC2. Co-infection with PACE4, PC5 (Fig. 2) or furin (not shown) demonstrated that these proteases were not capable of cleaving secretogranin II to any significant degree. Using an antiserum against secretoneurin a comparable result, i.e. formation of the 47,500 and 29,500 bands by PC1 and PC2 was obtained (see Fig. 3). The prominent band of 21,900 formed by PC1 cannot be detected with the secretoneurin antiserum apparently due to a lack of this peptide in its sequence (see below and Fig. 1). A co-expression of PC1 plus PC2 together with SgII did not alter the pattern of cleavage products in comparison to PC1 infection alone (not shown).

By immunoblotting with antisera directed against an N-terminal peptide (AR16, see Fig. 1) and a peptide located in the middle of secretogranin II (EL17) the relative position of the intermediate-sized cleavage products was established. The 47,500 band was labeled with the AR16, LF19, SN but not with the EL17 antiserum and is therefore generated by cleavage at sites 4 or 5 (for nomenclature see Fig. 1). Due to a lack of further specific antisera the exact cleavage position (either site 4 or 5) could not be further ascertained. The 29,500 band was labeled by the AR16, LF19 and SN antiserum and is formed by proteolysis at position 3. The 21,900 band cross- reacts with the AR16 and LF19 antiserum and therefore results from cleavage at position 2. Taken together, these results indicate that

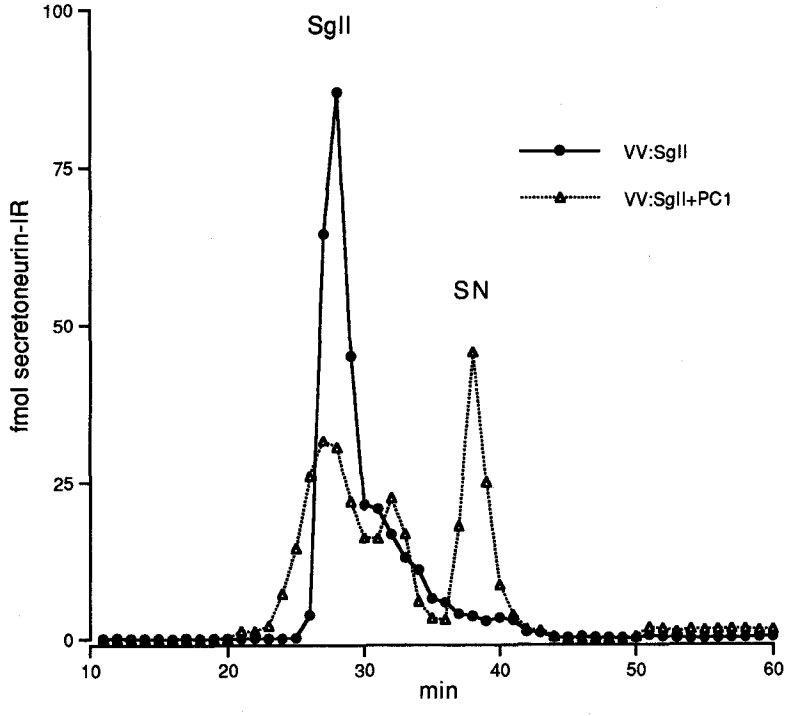

Fig. 4. Gel-filtration HPLC analysis of GH_4C_1 cells. GH_4C_1 cells were infected with VV:SgII (secretogranin II cDNA subcloned into vaccinia virus) or VV:SgII plus VV:PC1. The individual fractions were analysed for secretoneurin-IR with a specific RIA. The positions of secretogranin II (SgII) and secretoneurin (SN) in the eluate are indicated. The experiments were performed in triplicate, one representative sample is given here.

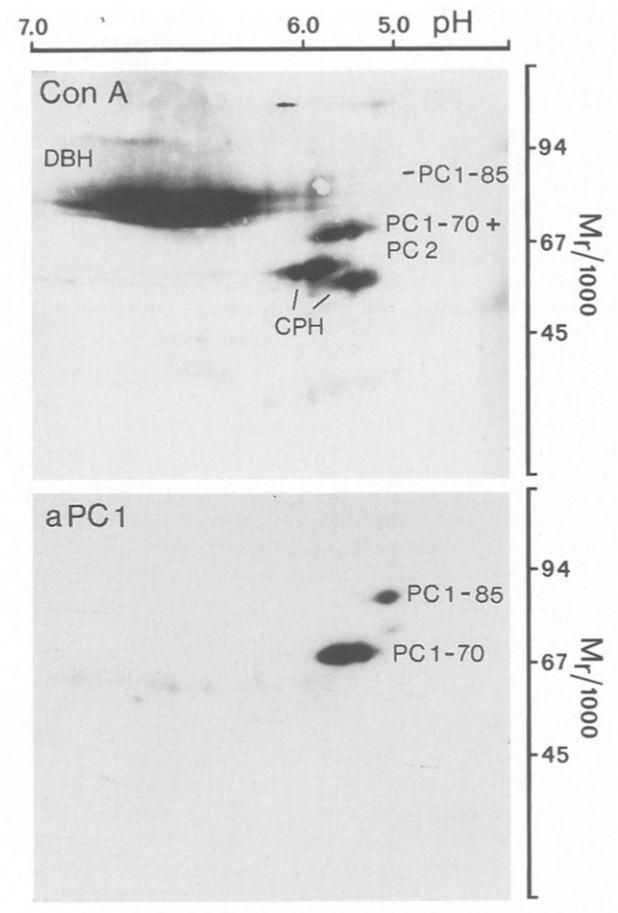

Fig. 5. Two-dimensional immunoblot of bovine chromaffin granule membranes. Membrane proteins were separated by two-dimensional electrophoresis. After transfer to nitrocellulose proteins were first immunolabeled with an antiserum directed against the N-terminal region of PC1 (aPC1, lower panel) followed by labeling for glycoproteins with concanvalin A (Con A, upper panel). The antiserum recognizes two molecular forms of PC1 labeled PC1–85 and PC1–70. The spot corresponding to PC1–85 is barely visible in the lectin blot. DBH, dopamine β-hydroxylase; CPH, carboxypeptidase H; PC2, prohormone convertase 2 (compare two-dimensional blots in [18] for identification).

both PC1 and PC2 can cleave the secretogranin II precursor protein at sites 3 and 4/5. Site 2 on the other hand is used only by PC1 but not by PC2. This is consistent with a recent finding that a proline residue at P4 favors the cleavage by PC1 of human prorenin at the ProMetLysArg site↓ [25] a sequence similar to that of site 2 ProPheLysArg↓. In the present experiments a putative cleavage of the secretogranin II precursor at sites 7 to 9 was not investigated. In two recent studies, however, peptide fragments resulting from cleavage at sites 8 and 9 were isolated [26,27]. Thus, one can assume that these sites might also be used in vivo by prohormone convertases.

3.2. *Formation of secretoneurin by endopeptidases*

Secretoneurin is a 33 amino acid peptide present in the primary amino acid sequence of secretogranin II [7]. The putative generation of secretoneurin by prohormone convertases was investigated by gel-filtration HPLC followed by RIA. Media from cells infected with VV:SgII contained one peak of secretoneurin-IR corresponding to secretogranin II (Fig. 4). Very little, if any immunoreactivity was found in the fractions corresponding to intermediate-sized proteins and secretoneurin. In contrast, in cells co-infected with VV:SgII plus VV:PC1 significant amounts of secretoneurin (roughly 50%) were detected indicating formation of secretoneurin from its precursor secretogranin II by PC1. In cells co-infected with VV:PC2, VV:PACE4, VV:PC5 or VV:furin secretoneurin was not generated (not shown). These results indicate that PC1 but none of the other convertases tested, i.e. PC2, PACE4, PC5 and furin, is capable of generating the neuropeptide secretoneurin from its precursor secretogranin II to any significant degree.

One prerequisite for a generation of secretoneurin by PC1 in vivo is the co-localization of both secretogranin II plus PC1 in large dense secretory vesicles. For chromaffin granules the presence of PC1 as detected by immunoblotting is given in Fig. 5. Two main bands of M_r 85,000 and 70,000 are labeled with an antiserum directed against the N-terminal region of PC1. Previously, using an antiserum directed against the C-terminus only the 85 kDa form was detected [18]. Apparently a significant amount of PC1 in chromaffin granules is already processed by cleavage of the C-terminal end as established for ATt-20 and GH_3 cells [21,25,28,29]. Thus, the results presented here, i.e. the formation of secretoneurin induced by overexpression of secretogranin II plus PC1 with vaccinia virus in a tumor cell-line containing large dense secretory vesicles plus the established co-localization of PC1 with secretogranin II in secretory vesicles of adrenal medulla, sympathetic splenic nerve and the pituitary [17,30,31] suggests that PC1 is responsible for generation of secretoneurin in vivo. The rather unexpected strict dependence of the secretoneurin formation on PC1 deserves some further comments. In the central nervous system PC1 and PC2 are co-localized in various areas but also distinctly expressed in others [32]. Thus, in these neurons a differential processing of the secretogranin II precursor to small peptides seems possible. Neurons expressing PC1 can generate secretoneurin from secretogranin II. In neurons expressing PC2 but no PC1 secretogranin II is also processed to smaller fragments but apparently not to secretoneurin. It is at present not established whether any of these other smaller fragments posses a biological activity as it has been established for secretoneurin.

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