Cell Tissue Res (2001) 304:43–49
DOI 10.1007/s004410100364

REGULAR ARTICLE

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Differential localization of prohormone convertases PC1 and PC2 in two distinct types of secretory granules in rat pituitary gonadotrophs

Received: 16 October 2000 / Accepted: 24 January 2001 / Published online: 7 March 2001

Abstract Prohormone convertases PC1 and PC2 are endoproteases involved in prohormone cleavage at pairs of basic amino acids. There is a report that prohormone convertase exists in the rat anterior pituitary gonadotrophs, where it had previously been considered that proprotein processing does not take place. In addition to luteinizing hormone and follicle-stimulating hormone, rat pituitary gonadotrophs contain chromogranin A (CgA) and secretogranin II (SgII), two members of the family of granin proteins, which have proteolytic sites in their molecules. In the present study we examined whether there is a close correlation between subcellular localization of prohormone convertases and granin proteins. Ultrathin sections of rat anterior pituitary were immunolabeled with anti-PC1 or -PC2 antisera and then stained with immunogold. Immunogold particles for PC1 were exclusively found in large, lucent secretory granules, whereas those for PC2 were seen in both large, lucent and small, dense granules. The double-immunolabeling also demonstrated colocalization of PC2 and SgII in small, dense granules and of PC1, PC2, and CgA in large, lucent granules. These immunocytochemical results suggest that PC2 may be involved in the proteolytic processing of SgII and that both PC1 and PC2 may be necessary to process CgA.

Keywords PC1 · PC2 · Granin · Chromogranin A · Secretogranin II · Pituitary gland · Immunocytochemistry · Rat (Wistar)

This work was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture of Japan to S.T.

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Introduction

Recently, two mammalian prohormone convertases, PC1 (also called PC3) and PC2, were identified by cDNA cloning (Seidah et al. 1990, 1991; Smeekens and Steiner 1990; Hakes et al. 1991; Nakayama et al. 1991; Smeekens et al. 1991) and shown to be involved in the cleavage of many protein precursors at paired basic sites in neuroendocrine cells and neurons (Benjannet et al. 1991; Korner et al. 1991; Nakayama et al. 1991; Thomas et al. 1991). In the anterior pituitary gland, these enzymes are restricted to the corticotrophs, because these cells are the only type of anterior pituitary cell expressing a prohormone, i.e., pro-opiomelanocortin (POMC), which is endoproteolytically processed into mature peptides (Day et al. 1992). However, Marcinkiewicz et al. (1993) have demonstrated immunohistochemically the expression of PC1 in gonadotrophs; and more recently Muller et al. (1998) have reported from an immunoelectron-microscopic study that mammotrophs express both PC1 and PC2. Since proprotein processing does not take place in these cells, the presence of these enzymes raises an important question as to what kinds of substrates are cleaved by them.

It is well known that neuroendocrine secretory granules contain a group of secretory proteins, the "granin" (chromogranin/secretogranin) family, which include chromogranin A (CgA) and secretogranin II (SgII). These proteins undergo proteolytic processing before secretion, although their exact function has not yet been elucidated (Huttner et al. 1991; Winkler and Fischer-Colbrie 1992). Several studies using gene transfer techniques have suggested that PC1 and PC2 may be involved in the processing of granin proteins (Arden et al. 1994; Dittie and Tooze 1995; Hoflehner et al. 1995; Eskeland et al. 1996; Laslop et al. 1998). Immunocytochemical studies show that rat gonadotrophs contain two distinct secretory granules containing different granin proteins (Watanabe et al. 1991). However, these granules might not contain PC1 and/or PC2 in situ. As immunoelectron-microscopic examination might provide signifi-

cant information on the possible roles of PC1 and PC2 in gonadotrophs, we investigated the subcellular distribution of prohormone convertases and CgA or SgII in rat gonadotrophs and observed their colocalization in the intracellular granules.

Materials and methods

Animals

Normal adult male rats of the Wistar strain, weighing 150–200 g, were housed in a temperature-controlled room (22±2°C), with automatically controlled lights (lights on, 0600–1800 hours daily), and were fed food and water ad libitum for at least 2 weeks before use. Newborn male rats (1 week old) were also used. All animal experiments were in compliance with the NIH *Guide for the Care and Use of Laboratory Animals*.

Antibodies

The antibodies used in this study were prepared in rabbits and characterized as described previously: anti-PC1 serum (ST-28) and anti-PC2 serum (ST-29) against synthetic peptides covering amino acids 442–459 and 613–629 of the respective mouse proteins (Tanaka et al. 1996), the sequences of which are identical to those of the rat (Hakes et al. 1991); anti-rat CgA serum (ST-83), which was raised against the peptide corresponding to amino acid residues 126–141 of the rat protein (produced by S. Tanaka); and anti-SgII serum (a gift from Dr. T. Watanabe).

Immunofluorescence

The rats were decapitated under anesthesia, and their pituitary glands were quickly removed, fixed for 2 days in Bouin-Hollande fixative, dehydrated, and embedded in paraffin. Frontal sections (4 μm) were cut and mounted on gelatin-coated slides. The deparaffinized sections were rinsed with distilled water and phosphate-buffered saline (PBS). For single-labeling of PC1 and PC2, immunofluorescence staining was performed essentially as described previously (Tanaka et al. 1997). For amplification of the signals for PC1 or PC2, the sections were then treated with Gomori's oxidation mixture according to the procedure of Kurabuchi and Tanaka (1997). The sections were sequentially incubated with 1% bovine serum albumin (BSA)-PBS, rabbit anti-PC1 or PC2 serum (1:500), and lissamine rhodamine sulfonyl chloride (LRSC)-labeled affinity-purified donkey anti-rabbit IgG (Jackson Immunoresearch, West Glove, Pa., USA). For double-labeling of prohormone convertase and luteinizing hormone-β (LHβ), the sections were incubated sequentially with 1% BSA-PBS for 1 h and then reacted overnight with a mixture of rabbit anti-PC1 or PC2 and mouse monoclonal anti-ovine LHβ, both diluted to 1:500. After being rinsed with PBS, the sections were incubated with a mixture of LRSC-labeled affinity-purified donkey anti-rabbit IgG (Jackson Immunoresearch, West Glove, Pa., USA) and 5-([4,6-dichlorotriazin-2-yl]amino)-fluorescein (DTAF)-labeled affinity-purified donkey anti-mouse IgG (Jackson Immunoresearch, West Glove, Pa., USA) for 1.5 h. The sections were washed with PBS, mounted in Permafluor (Immunon, Pittsburgh, Pa., USA), and examined with an Olympus BX50 microscope equipped with a BX-epifluorescence attachment (Olympus Optical, Tokyo, Japan).

Immunogold electron microscopy

Pituitary glands were fixed with a mixture of 0.2% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, for 2 h at 4°C. They were dehydrated in ethanol, embedded in LR White (London Resin, Basingstoke, UK), and then sectioned on a Reichert Ultracut-E (Reichert-Jung, Vienna, Austria). For immunolabeling of PC1 or PC2, ultrathin sections were subjected to Gomori's oxidation as described. Double-immunogold labeling of the sections with different antibodies was performed as described previously (Tanaka et al. 1996). Briefly, the two faces of the grids were incubated with different antibodies (anti-Cg A, 1:8000; anti-SgII, 1:8000; anti-PC1, 1:300; anti-PC2, 1:500) and then with anti-rabbit IgG conjugated with different sizes of gold particles (10 nm and 5 nm; Biocell Research Laboratory, Cardiff, UK). The specificity of anti-PC1 and PC2 sera was assessed in preabsorption tests at the immunoelectron-microscopic level. For these tests diluted antisera were preabsorbed with their antigen peptides at a final concentration of 0.01 mg/ml, for 16 h at 4°C, prior to immunolabeling. The immunolabeled sections were fixed with 1% osmium tetroxide, and then stained with a mixture of uranyl acetate and methyl cellulose according to a published protocol (Roth et al. 1990). They were then examined with a Hitachi 7500 electron microscope at 80 kV.

To examine the degree of colocalization of the prohormone convertases with granin proteins in the secretory granules, we performed semiquantitative analysis on the double labeling data. Granules were also classified into two types, i.e., CgA-positive and SgII-positive. Secretory granules containing four or more gold particles were considered to be positively labeled and were counted in photomicrographs (final magnification ×30,000). The number of PC1- or PC2-positive granules was scored for each granule type, and the ratio of the number of PC1 or PC2-positive granules per total number of CgA- or SgII-positive granules in each gonadotroph was calculated. The results were expressed as the means ± standard error of 15 gonadotrophs.

Results

Immunofluorescence detection of PC1 and PC2 in anterior pituitary glands

When the histological sections of rat pituitary glands were immunostained with anti-PC1 or -PC2 serum, PC1 immunoreactivity was observed in the pars distalis, pars intermedia, and pars nervosa; whereas PC2 was preferentially found in the pars intermedia and pars nervosa (Fig. 1a, b). These findings are consistent with a previous report on the mouse pituitary gland (Marcinkiewicz et al. 1993). To examine whether PC1 exists in gonadotrophs, we carried out a double-immunofluorescence staining of sections of the anterior pituitary for PC1 and LHβ. Most of the immunoreactive LHβ cells corresponded to PC1-positive cells (Fig. 1c, d). No signal for PC2 was detectable in the pars distalis with anti-PC2 serum; however, signals were found in the pars intermedia and pars nervosa (Fig. 1b).

Subcellular localization of PC1 and PC2 in gonadotrophs

By conventional electron-microscopic investigation, typical gonadotrophs in adult male rats can be characterized by two types of secretory granules, i.e., large granules with a low electron density and small ones with a high electron density (Inoue and Kurosumi 1984; Childs 1986; Tougard and Tixier-Vidal 1988). We performed electron-microscopic immunocytochemistry using immunogold to examine the subcellular distribution of PC1

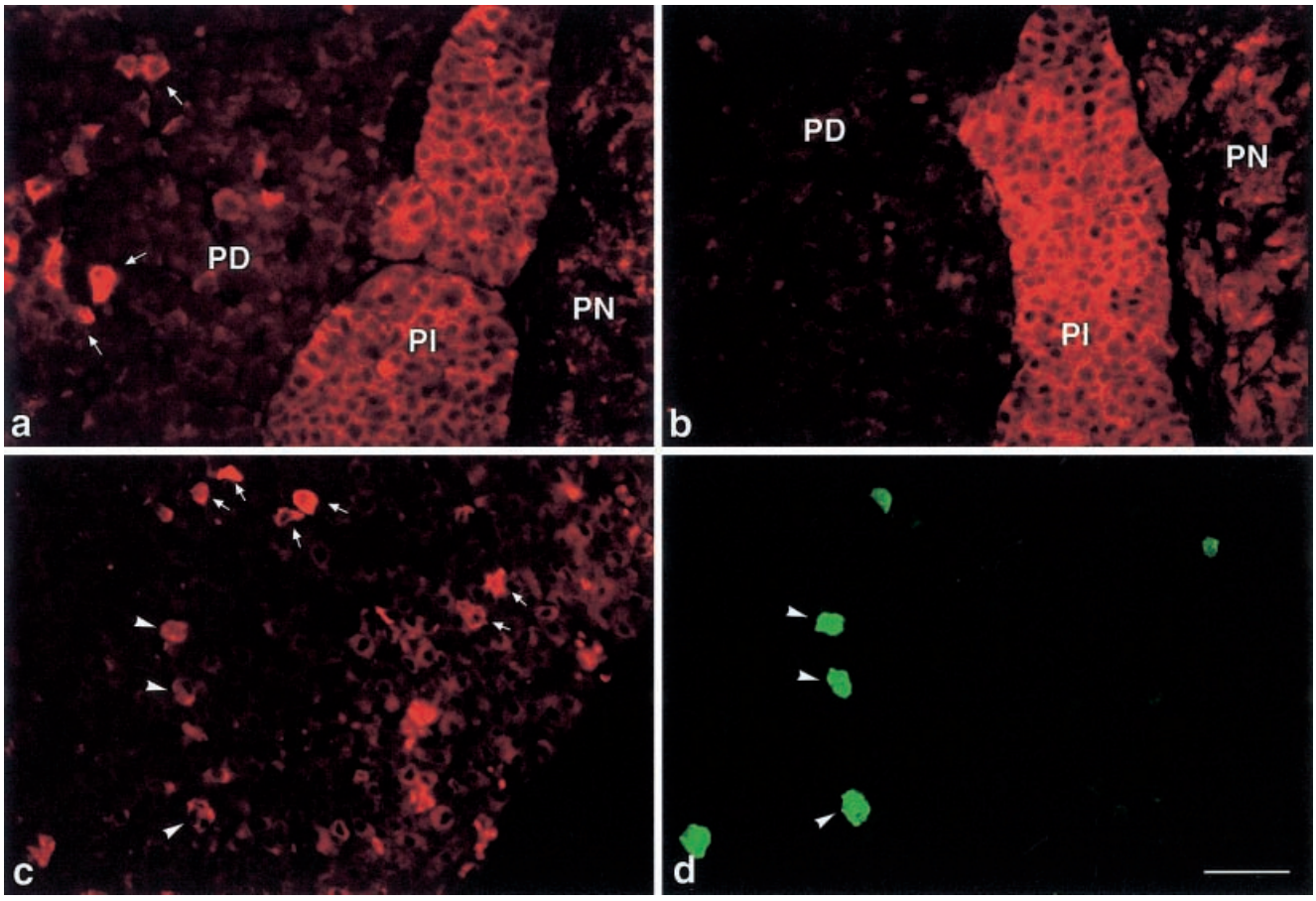

Fig. 1a–d Immunofluorescence localization of prohormone convertases PC1 and PC2, and luteinizing hormone-β (LHβ) in the male rat pituitary gland. **a** PC1 is seen in some cells of the pars distalis (*PD*; *arrows*), most cells of the pars intermedia (*PI*), and in nerve fibers and terminals of the pars nervosa (*PN*). **b** PC2 is observed in the pars intermedia and the pars nervosa, but not in the pars distalis. **c** Double-immunostaining for PC1 and **d** LHβ indicates that most of LHβ-positive cells also contain PC1 (*arrowheads*). *Arrows* indicate PC1-positive cells devoid of label for LHβ. ×230. *Bar* 50 μm

and PC2 in gonadotrophs. The immunogold particles indicating the presence of PC1 were preferentially localized in the large granules in the gonadotrophs having large, lucent and small, dense granules, whereas PC2 was distributed in both large and small granules in the cells (Fig. 2). In the absorption test, positive immunolabeling obtained with each antiserum was completely eliminated, when 0.01 mg/ml of their antigen peptides were used as an adsorbent (data not shown).

To investigate the possible colocalization of prohormone convertases and CgA or SgII, we used double-immunolabeling. When ultrathin sections were double-immunolabeled for PC1 and CgA or SgII, immunogold particles for PC1 were exclusively observed in CgA-positive, large granules, as only a low background level of immunolabeling for PC1 was detectable in the SgII-positive, small granules (Fig. 3a, b). On the other hand, when ultrathin sections were double-immunolabeled with anti-PC2 and anti-CgA or anti-SgII, PC2 was located in both CgA-positive, large granules and SgII-positive, small granules devoid of immunolabels for CgA (Fig. 3c, d).

To confirm this relationship between prohormone convertases and granin proteins, we performed semi-quantitative analysis of the data from the double labeling. The ratio of the number of PC1- and PC2-positive granules per total number of CgA-positive granules was 69.8±3.2% and 32.8±3.4%, respectively. Similarly, the ratio of PC2-positive granules per SgII-positive granules was 80.6±2.5%, and the colocalization of PC1 and SgII was negligible.

In addition, we frequently observed secretory granules with a dense core surrounded by a clear halo in the gonadotrophs of newborn rats. Immunogold particles for PC2 were preferentially localized in the dense core region, where SgII was concomitantly detected (Fig. 4).

Discussion

The exact function of the granin proteins is not yet definitely established, but these proteins are considered to be the peptide precursors for various biologically active peptides, e.g., parastatin, pancreastatin, chromostatin, and vasostatin I and II from CgA, and secretoneurin from SgII (reviewed by Huttner et al. 1991; Winkler and Fischer-Colbrie 1992; Natori and Huttner 1994). The pri-

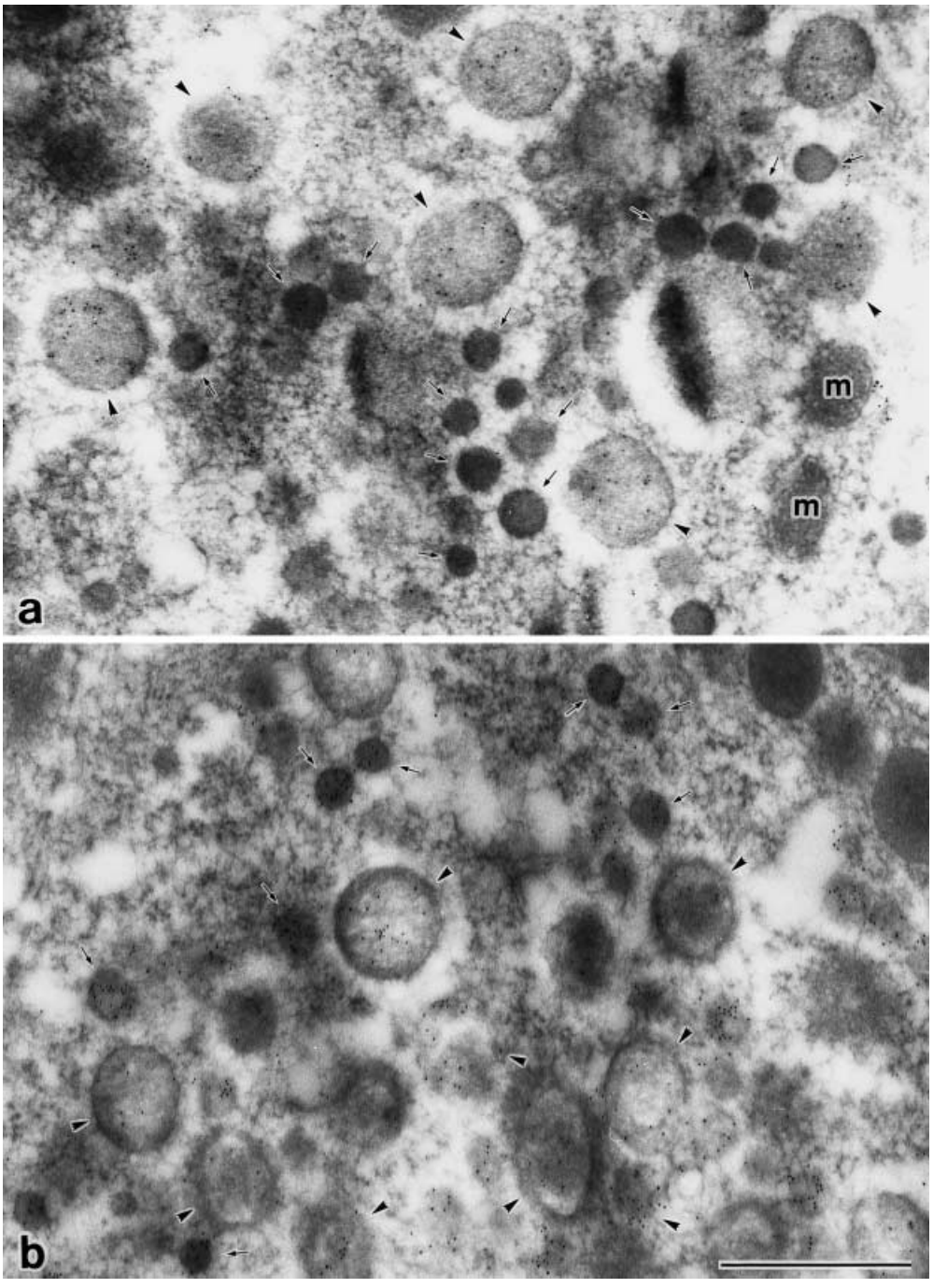

Fig. 2a, b Electron micrographs of typical adult male gonadotroph immunolabeled for PC1 (**a**) and PC2 (**b**). Immunogold particles indicating the presence of PC1 are seen in large granules (*arrowheads*), but not in small granules (*arrows*). Immunogold particles for PC2 are observed in small (*arrows*) and large granules (*arrowheads*) (*m* mitochondria). ×5,000. *Bar* 500 nm

mary structure of CgA and SgII, as well as that of other members of the granin family, contains multiple pairs of basic amino acids, which are potential sites for cleavage by endoproteases PC1 and PC2. To better understand the involvement of PC1 and PC2 in the processing of the granin proteins, it is important to define their exact subcellular distribution. It is well known that typical gonadotrophs in adult male rats exhibit two distinct subsets of secretory granules, i.e., small, dense granules rich in LH and large, lucent granules rich in follicle-stimulating hormone (FSH; Inoue and Kurosumi 1984; Childs 1986; Tougard and Tixier-Vidal 1988). Recently, Watanabe et al. (1991, 1993, 1998) have indicated that large FSH-rich granules contain CgA and small LH-rich granules, SgII, in the male rat gonadotrophs, and suggested that these granins are involved in the formation of the two types of granule. A report showing the existence of prohormone convertases in gonadotrophs (Marcinkiewicz et al. 1993) has prompted us to formulate the hypothesis that not only granin protein but also its derived peptides, which are processed by PC1 and/or PC2, may participate in the formation of secretory granules. In the present immunocytochemical study, we demonstrated that both PC1 and PC2 immunoreactivities were localized in the large,

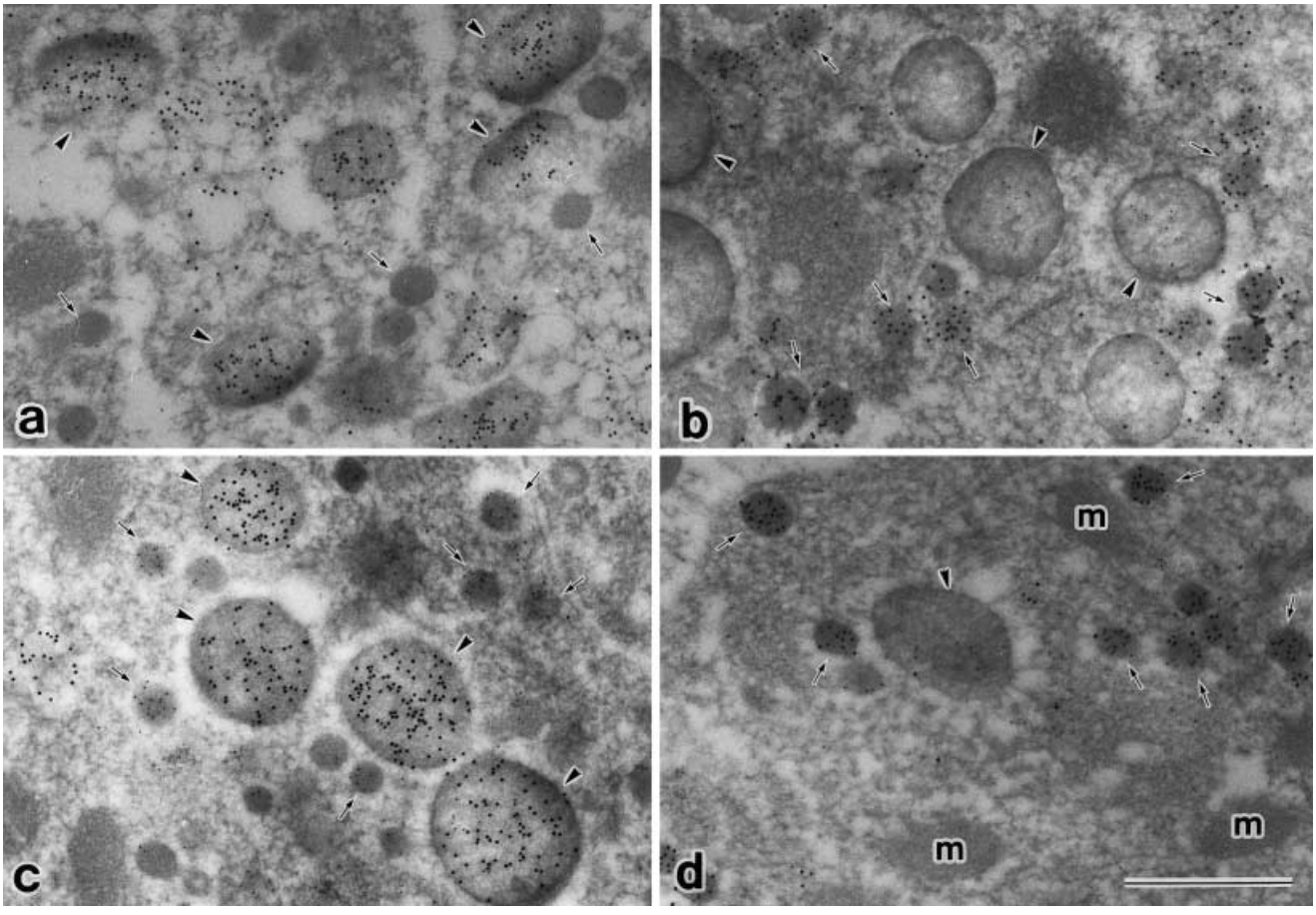

Fig. 3a–d Electron micrographs of typical adult male gonadotroph doubly immunolabeled for PC1 and chromogranin A (CgA in **a**) or secretogranin II (SgII in **b**), or for PC2 and CgA (**c**) or SgII (**d**). *Small gold particles* indicating the presence of PC1 and PC2 are localized in CgA-positive (*large gold particles*), large granules (*arrowheads*), whereas PC2 (*small gold particles*) is seen in SgII-positive (*large gold particles*), small granules (*arrows*) (*m* mitochondria). ×4,500. *Bar* 500 nm

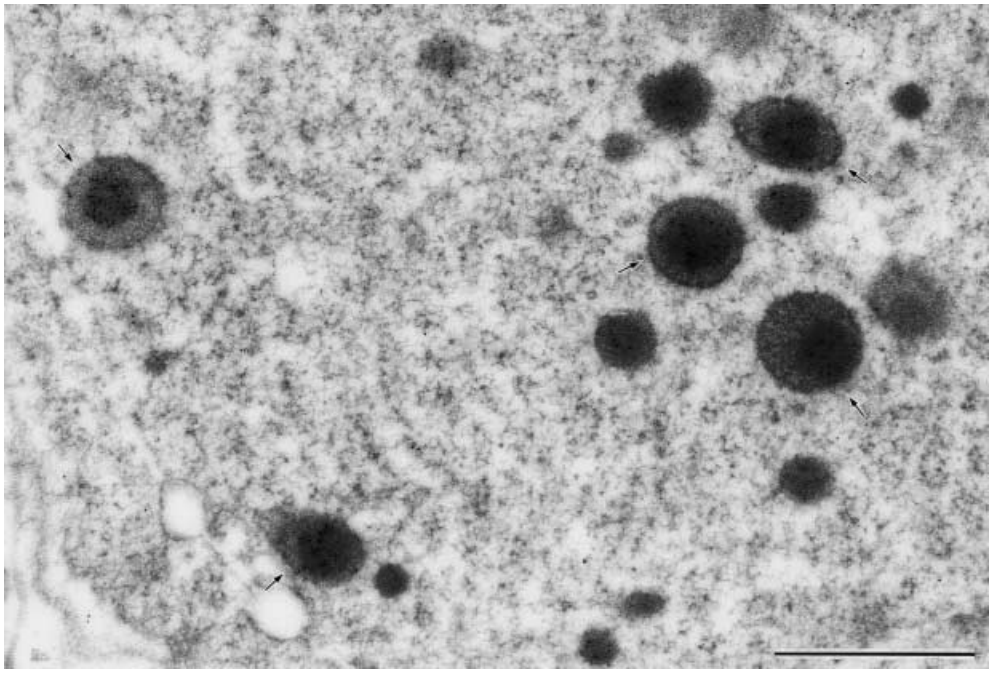

Fig. 4 Electron micrograph of a gonadotroph from a newborn male rat immunolabeled for PC2. PC2 is localized in the dense core of the secretory granules (*arrows*). ×5,000. *Bar* 500 nm

CgA-containing granules, but that only PC2 was detectable in the small, SgII-containing granules. This finding was also confirmed by the present semiquantitative analysis, though the labeling ratio of PC2 in CgA-positive granules was less than that of PC1.

There are several studies on the specificity of PC1 and PC2 for proteolytic cleavage of CgA and SgII, but the results remain controversial. By expressing antisense nucleotides against PC1 mRNA in AtT-20 cells, which contain a high amount of endogenous PC1, Eskeland et al. (1996) have concluded that PC1 could proteolytically cleave CgA. On the other hand, a CgA-converting activity has been shown in the ion-exchange fractions of PC2, which are collected from insulin granules, but not in those of PC1 (Arden et al. 1994). Our immunocytochemical observation indicates the colocalization of PC1, PC2, and CgA in large granules and thus suggests that both convertases might process CgA in situ.

The present immunocytochemical study also revealed that small SgII-containing granules also contained PC2 but not PC1. Because a stably transfected PC12 cell line expressing PC2 processed SgII (Dittie and Tooze 1995), PC2 may be an endoprotease responsible for the cleavages of SgII. To support this possibility, we examined atypical secretory granules with an electron-dense core surrounded by a clear halo in newborn rat gonadotrophs. Since the dense-core materials mainly contain SgII (Watanabe et al. 1993), we expected to define more precisely the relationship between PC2 and SgII. The immunoelectron-microscopic study showed that the localization of PC2 was restricted to the SgII-positive, dense-core region of secretory granules with a halo. Therefore, this finding strongly suggests that there is a close correlation between subcellular localization of PC2 and SgII. Hoflehner et al. (1995) report that PC1 overexpressed in pituitary prolactinoma GH4C1 cells by vaccinia virus cleaves SgII. In addition, Egger et al. (1993) have shown that large, dense-cored vesicles from adrenal medulla and sympathetic axons have PC1 and PC2 activities capable of cleaving SgII. However, these data obtained by biochemical studies with gene transfection are somewhat unreliable, because such systems may not always reflect physiological events (Bloomquist et al. 1991).

In the present study, we cannot demonstrate immunoreactivity for PC2 in the pars distalis using immunofluorescence. A similar result was also obtained when the peroxidase-antiperoxidase method was used (unpublished data). An in situ hybridization study indicates that a substantial amount of PC2 transcript is present in the anterior pituitary gland (Day et al. 1992); and an immunohistochemical study has shown that PC2 is localized in the mouse gonadotrophs, although there are no data on colocalization (Marcinkiewicz et al. 1993). Moreover, Muller et al. (1998) have shown, by immunoelectron microscopy, that PC2 is present in rat mammotrophs. Therefore, these discrepancies might be due to a difference in resolving power between light and electron microscopy. On the other hand, we obtained intense immunoreactivity in the pars intermedia, the pars nervosa, and pancreatic endocrine cells of rats, and in anterior pituitary cells of amphibians when we used the same antibody (Tanaka et al. 1996; Kurabuchi and Tanaka 1997). Therefore, the epitope of the PC2 molecule in the rat gonadotrophs may possibly be hidded by surrounding molecules, and the LR White-embedding procedure might give favorable conditions for exposing the epitope. In the present immunoabsorption test, indeed, the specificity of immunolabeling for PC1 and PC2 in the rat gonadotrophs was confirmed at the electron-microscopic level. However, exactly why light-microscopic immunocytochemistry does not detect PC2 immunoreactivity in any cells of rat anterior pituitary glands remains unknown. The present findings provide further argument for substrate specificity of these prohormone convertases in the processing of granin proteins.

Acknowledgement We thank Dr. T. Watanabe for supplying the rabbit anti-rat secretogranin II serum.

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