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PC2 and 7B2 Null Mice Demonstrate That PC2 Is Essential for Normal Pro-CCK Processing

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Analysis of CCK content in extracts of whole forebrain from PC2 and 7B2 null mouse brain showed a significant decrease relative to wild-type brains. More detailed analysis revealed that CCK 8 amide levels in cerebral cortex and forebrain regions were more decreased than in hypothalamus. CCK 8 content in PC2 null mouse intestines was identical to control. Null mutant brains contained less CCK 8 than wild type and no other forms were seen when analyzed by gel filtration chromatography. No brain area examined was completely devoid of CCK, suggesting that other enzymes can partially compensate for the loss of PC2. This is the first demonstration that any endoprotease is important for CCK processing but also suggest the presence of a redundant system to ensure production of active CCK in the brain. © 2000 Academic Press

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Cholecystokinin (CCK) is produced by neurons in the brain and by neurons and endocrine cells in the gut. A large body of evidence has shown that CCK may act as a neurotransmitter and play a crucial role in anxiety, memory and satiety (1). CCK is one of the peptides with the most dramatic tissue-specific pattern of processing. The major difference is between how pro CCK is processed in the brain and the gut. The brain makes mainly CCK 8 amide, while the gut makes larger forms like CCK 12, 22, 33, 39, 58, 83 amide. Like many hormones and neuropeptides, CCK is produced initially as a large pre-prohormone which passes through the regulated secretory pathway, undergoes a number of post-translational modifications including tyrosine sulfation, endoproteolytic cleavages, trimming by carboxypeptidase, and C-terminal amidation (2).

The endoproteolytic enzymes which are responsible for the processing of pro-CCK have not been definitively identified, but recent studies have shown that the subtilisin-like proconvertases, PC1, PC2, and PC5 may be involved in processing of pro-CCK. PC1, PC2, and PC5 are widely distributed in neural and endocrine tissues and have been shown to cleave a number of pro-peptides including pro CCK (3), POMC (4, 5), proinsulin (6), proenkephalin (7), proglucagon (8), and prosomatostatin (9). Although detailed studies of the colocalization of CCK with these enzymes have not been performed, PC1, PC2, and PC5 are found in CCK-ergic cell types and neuronal projections in both in the brain and intestine. PC2 is more abundant than PC1 in most tissues. PC1, PC2, and PC5 are colocalized with oxytocin in the supraoptic and paraventricular nuclei of the hypothalmus. The oxytocin cells are known to also contain CCK (10) and thus these enzymes have a distribution which is consistent with a role in CCK processing (11, 12).

Earlier studies using antisense methodology supported a role of PC1 and PC2 in pro CCK processing in endocrine tumor cells in culture. Inhibition of PC1 protein expression by antisense PC1 mRNA in mouse intestinal (STC-1) and rat pancreatic (RIN 5F) cells caused a selective depletion of CCK 8 while sparing CCK 22 (13). Inhibition of PC2 protein expression by similar strategy depleted CCK 22 sparing CCK 8 (14). These results support a role for PC1 in production of CCK 8 and PC2 for production of CCK 22 in these cell lines and suggested that PC2 activity may be responsible for the production of larger forms of amidated CCK. However, in a separate study recombinant PC2 was found to efficiently cleave synthetic CCK 33 (15), a form found in the intestine, producing CCK 8.

The physiological relevance of these enzymes in pro CCK processing was difficult to examine until the production of knockout mice in which the specific enzymes were deleted. PC2 knockout mice are viable and fertile and have defective processing of insulin, somatostatin, glucagon (16) and enkephalin (17). In this study, we have examined the possible role of PC2 in CCK pro-

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TABLE 1

CCK Concentrations (pg/μg Protein) in PC2 and 7B2 Null and Control Mouse Forebrain and Cerebellum, Expressed as Mean ± SEM

Strain	CCK ng/mg protein
PC2 control mice	(♀) 5.7; (♀) 5.1
PC2 null mice	(♀) 5.2; (♀) 3.1
7B2 control mice	(♀) 5.2; (♂) 5.8; (♂) 8.2
7B2 null mice	(♀) 1.5; (♂) 4.1; (♂) 3.7
Data pooled from PC2 and 7B2 controls	6 ± 0.6
Data pooled from PC2 and 7B2 null mice	3.5 ± 0.6*

Note. Controls, $N = 5$; null, $N = 5$.

cessing in PC2 knockout mice. Production of active PC2 is dependent on an additional protein called 7B2, their complex association was reviewed recently (18). 7B2 null mice are known to have no demonstrable PC2 activity, are deficient in processing of islet hormones, and display hypoglycemia, hyperinsulinemia and hypoglucagonemia (19). Unlike PC2 null mice, 7B2 null mice develop and die of Cushing's disease, with multiple sequelae of hypercorticosteronism. In this report, we have also examined the levels of CCK 8 amide in 7B2 null mice to understand its role in the processing of pro CCK.

MATERIALS AND METHODS

Animals. PC2 knockout mice were purchased from Jackson Labs, homozygous alleles were confirmed by PCR of genomic DNA samples from tail. Mice were eutharized with CO_2 vapor and rapidly dissected with tissues quickly frozen on dry ice. Brain tissues were sonicated in 0.1M HCl, intestinal tissues were homogenized in Tekmar Ultra-Turrax. Aliquots were removed for total protein estimation, protein concentrations were assayed by the Lowry assay (20). Samples were clarified by centrifugation prior to RIA. Whole brain including cerebellum, pons, medulla from 7B2 null mice were obtained from the laboratory of Dr. Iris Lindberg along with some additional PC2 and control mouse brains.

Chromatography. Brain extracts were separated by gel filtration chromatography on a 50 × 2.5-cm column of Spherilose GCL-90 (Isco) in a run at 4°C in 50 mM Tris, 200 mM NaCl, pH 7.8, containing 0.1% BSA and 0.02% sodium azide. Fractions of 1.0 mL were collected and aliquots removed for the radioimmunoassay (RIA). The column was calibrated with synthetic peptide standards, their elution was determined by RIA.

RIA. The CCK 8 RIA was performed as previously described (21), using the rabbit polyclonal CCK 8 antibody (R5) that is specific for amidated forms of CCK. The RIA used [^{125}I]gastrin-17 as tracer, produced by iodination with chloramine-T (22).

RESULTS AND DISCUSSION

CCK amide levels were lower in whole brain from PC2 null mice and 7B2 null mice than controls, but this difference was not statistically significant because of the small number of animals tested (Table 1). Because the CCK levels of whole brain of the two wild type strains did not differ between each other, when these values were pooled and compared against data pooled from PC2 null and 7B2 nulls, the nulls animals had less CCK than the controls (Table 1). The single female 7B2 null tested appeared to have much lower levels of CCK than the male 7B2 nulls tested, suggesting a possible sex difference in the severity of the phenotype. Data from more animals would be required to draw this conclusion.

To further examine the possibility that the lack of PC2 causes a bigger decrease in levels of CCK amide in some brain and gut regions than others, we performed a regional dissection of the brains and intestines of some additional PC2 null mice and controls (Table 2). In cerebral cortex and forebrain remaining (minus hypothalamus and cerebral cortex) of null mice, CCK levels were decreased relative to controls (40% and 73% respectively). Levels of CCK in PC2 null mice hypothalamus, duodenum and ileum also showed a tendency to be lower, but this was not significant.

Low pressure gel filtration chromatography and CCK RIA was used to examine the form of CCK found in these mice brain extracts. As previously demonstrated (23), control mouse brain contained mainly CCK 8. This was also true for PC2 null mice and 7B2 null mice (Fig. 1). In both cases, in agreement with tissue levels, less CCK 8 was seen in null mice brains and but no additional forms of amidated CCK were detected.

These results demonstrate that PC2 (acting directly or indirectly) is important for the production of normal levels of active CCK in most regions of the brain, particularly cerebral cortex and in the forebrain minus cerebral cortex and hypothalamus. PC2 appears to be less important in the intestine and hypothalamus. Despite the large decrease in CCK content in some tissues, in no tissue examined was CCK completely absent, suggesting that

TABLE 2

CCK Concentrations (pg/μg Protein) in PC2 Null and Control Mouse Brain and Gut Regions; Expressed as Mean ± SEM

Tissue	PC2 null mice	Control mice
Hypothalamus	1.8 (±0.3)	2.7 (±0.4)
Cerebral cortex	11.0 (±1.7)*	18.4 (±1.4)
Forebrain†	1.6 (±0.7)*	5.9 (±1.2)
Duodenum	1.05 (±0.2)	2.0 (±0.4)
Jejunum	0.9 (±0.3)	1.2 (±0.4)
Ileum	1.6 (±0.9)	1.2 (±0.4)

Note. Controls, $N = 6$; null, $N = 4$.
* $P < 0.05$.
† Forebrain without hypothalamus and cerebral cortex.

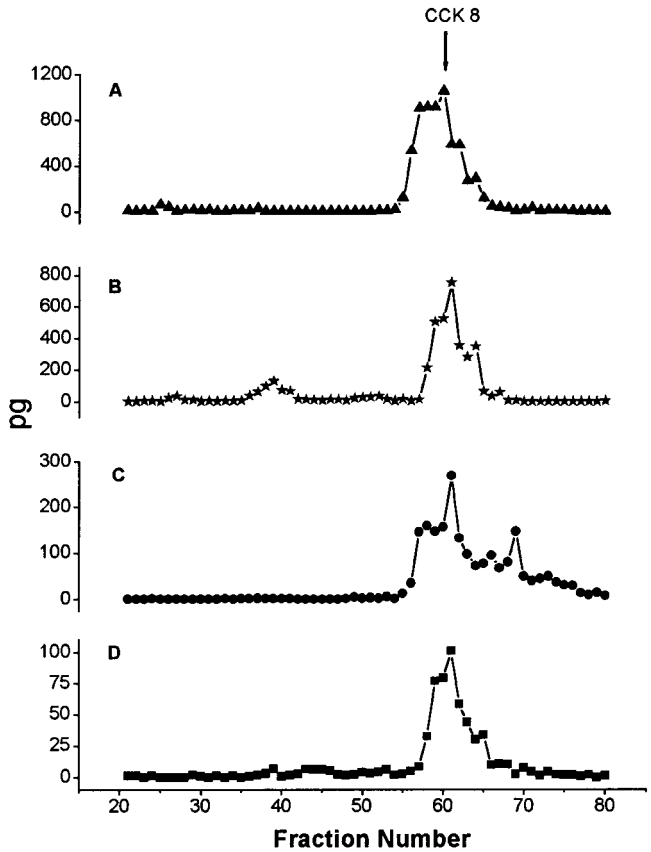

FIG. 1. GCL-90 chromatography of (A) PC2 control, (B) PC2 null, (C) 7B2 control, and (D) 7B2 null brain extracts detected with CCK RIA. Elution of CCK 8 standard is indicated.

some other enzyme or enzymes possibly PC1 and/or PC5 were able to partially compensate for the loss of PC2. Studies in progress on the co-localization of CCK with PC1, PC2 and PC5 may shed light on this hypothesis. 7B2 null mice appear to have a similar reduction in CCK levels as PC2 mice, although a more detailed examination may reveal differences. It is likely that this decrease in CCK levels in 7B2 null mice is due to the absence of active PC2 in these mice.

PC2 is abundant and widely expressed in the rodent brain, so it is not surprising that its loss does affect production of active CCK. PC2 is particularly abundant in the cerebral cortex, hippocampus and thalamus and is less abundant in the hypothalamus (12). PC2 is much less abundant in the intestine where PC5 is most abundant (24). It may be that PC5 may more effectively replaces PC2 in the intestine than it does in the brain. This is the first demonstration that the loss of a specific endo proteolytic enzyme alters the processing of pro CCK. That the loss of PC2 does not completely eliminate CCK processing suggests that a partially effective redundant system exists, which may involve PC1 or PC5 to ensure production of active CCK.

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