Expression of precerebellins in cultured rat calvaria osteoblast-like cells

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Abstract. Cerebellin (CER), originally isolated from rat cerebellum, is a hexadecapeptide derived from the larger precursor called precerebellin 1 (Cbln1). At present 4 propeptides designated as Cbln1, Cbln2, Cbln3 and Cbln4 are recognized. They belong to precerebellin subfamily of the C_1q family proteins. Precerebellins act as transneuronal regulators of synapse development and synaptic plasticity in various brain regions. Initially CER was thought to be a cerebellum specific peptide, however subsequent studies revealed its presence in other brain regions as well as in extraneuronal tissues. We investigated whether precerebellins are expressed and involved in regulation of cultured rat calvarial osteoblast-like (ROB) cells. Classic RT-PCR revealed the presence of Cbln1 and Cbln3 mRNA in fragments of rat calvaria, in freshly isolated ROB cells and in ROB cells cultured for 7, 14 and 21 days. Cbln2 and Cbln4 mRNA, on the other hand, could not be demonstrated in ROB cells but was found to be present in the brain. In freshly isolated ROB cells expression of Cbln1 gene was very low and gradually increased in relation to the duration of culture. Expression of Cbln3, on the other hand, was very low in fragments of rat calvaria, and increased notably after digestion with collagenase-I. The highest expression of this precerebellin was observed at day 14 of culture while at days 7 and 21 levels of expressions were notably lower. Neither Cbln2 nor Cbln4 was found to be expressed in the ROB cells. Neither CER nor des-Ser[1]-CER (10^{-10}-10^{-6}M) affect osteocalcin production and proliferation rate of studied cells. The above findings suggest that CER, which theoretically would be derived from Cbln1, modulate neither differentiated (osteocalcin secretion) nor basic (proliferation) functions of cultured rat osteoblast-like cells. The obtained data raise an intriguing hypothesis that precerebellins may be involved in regulating of spatial organization of osteoblastic niches in the bone.

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Introduction

Cerebellin (CER), originally isolated from rat cerebellum, is a hexadecapeptide derived from the larger precursor called precerebellin 1 (Cbln1) (1,2). Initially CER was thought to be a cerebellum specific peptide, however, subsequent studies revealed its presence in other brain regions as well as in extraneuronal tissues. At present 4 propeptides designated as Cbln1, Cbln2, Cbln3 and Cbln4 are recognized (3-6). Their nucleotide sequences are highly homologous, moreover entire family of precerebellins demonstrates high homology to the family of atypical solluble collagens (types VII and X) (7,8) as well as to A, B and C subunits of the component complex C_1q (9-11). Cbln1-Cbln4 are highly expressed in the brain and recent data suggest that the family of cbln proteins acts as transneuronal regulators (transneuronal) of synapse development and synaptic plasticity in various brain regions (6,12).

Apart from the central nervous system, precerebellins are also expressed at periphery. Immunoreactive CER was reported to be present in the alimentary tract, heart and kidney of the rat and guinea pig and in normal and pathological human adrenals (4,13,14). Those earlier observations were confirmed and extended by means of molecular biology techniques (6). At the mRNA and/or protein level expression of Cbln1 was found in testes, ovaries, adrenals and pancreatic islets, Cbln2 in testes and Cbln3 in testes, ovaries and adrenals (5,6,15).

It has been reported that numerous neuropeptides acting via endocrine, paracrine or autocrine routes and through the nervous system regulate biological activity of the major bone cell types. To this class of biologically active peptides among others belong arginine-vasopressin, substance P, VIP, PACAP, GIP, leptin, ghrelin and neuro-medin U (16-29). The present study aimed to investigate whether CER may be included into the class of bone regulating peptides. To address this issue, on cultured rat calvaria osteoblast-like (ROB) cells we studied expression of precerebellins and we

Table I. QPCR analysis of precerebellins (Cbln1-4) and HPRT (hypoxanthine-guanine phosphoribosyl transferase) mRNAs in cultured rat calvarial osteoblast-like (ROB) cells.[a]

cDNA	Genbank accession no.	Primers	Primer sequence (5'-3')	Position	PCR product size (bp)
Cbln1	XM 001067551	S	CCCTACGGGCACTGCTCTG	144-162	171
		A	CTGCGTTCTGAGTCAAAGTTG	294-314	
		probe	CTGCCATC	203-210	
Cbln2	NM 001012740	S	TGGCCTTCTCCGCTACTC	457-474	119
		A	CGAGGCAAGGTCAAAGTGGT	556-575	
		probe	TGAGCAAC	502-509	
Cbln3	XM 001056593	S	ACCATGAACCAGCAGGAGAA	483-502	86
		A	AAAGCCACCGCCCTCATT	551-568	
		probe	TGCGATCT	523-531	
Cbln4	XM 001053640	S	GAGCAACAAGACTCGCATCAT	1859-1879	80
		A	CAAAGACGGATTCCAGTGTG	1919-1938	
		probe	CTGGTTAA	1896-1903	
HPRT	NM 012583	S	CAGTCAACGGGGGACATAAAAG	391-412	146
		A	ATTTTGGGGCTGTACTGCTTGA	515-536	
		probe	CCTCATG	451-457	

[a]Oligonucleotide sequences for sense (S) and antisense (A) primers, and for probes are shown. Also the sequence of the probes is shown.

tested effects of CER and des-Ser[1]-CER (desCER) on proliferative activity and osteocalcin secretion by these cells.

Materials and methods

Animals and reagents. Two day-old Wistar rats, born in our animal facilities, were used. The local Ethics Committee for Animal Studies approved the study protocol. Cerebellin (CER) and desCER were purchased from Bachem AG, Bubendorf, Switzerland. If not otherwise stated, the reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) or from POCh (Gliwice, Poland).

Rat calvaria osteoblast-like (ROB) cell culture. The technique was that described earlier (30-34) with few modifications. Briefly, calvarias of eight 2-day-old rats were placed in DMEM (Gibco, UK), and the connective tissue was removed. Calvarias were then cut into small fragments, which were digested with 0.1% collagenase-I (Sigma-Aldrich) for 30 min at 37˚C. ROB cells were harvested by centrifugation and resuspended in DMEM supplemented with $NaHCO_3$, 6% FCS (fetal calf serum) and antibiotic-antimycotic solution. Cells were then plated in culture dishes (Nalge Nunc International, Denmark, $1x10^4$ cells/dish), and cultured for 21 days at 37˚C in an humidified atmosphere of 95% air-5% CO_2, medium being changed every other day (34). The experiments were performed on freshly isolated cells before and after digestion (RT-PCR) and cells harvested at days 7, 14 and 21 of culture. Forty-eight hours before sampling cells were maintained in medium without FCS. In such medium osteoblasts were exposed for 48 h to various concentrations of CER and desCER.

Osteocalcin determination. Osteocalcin concentrations in the culture medium were estimated using a rat osteocalcin EIA kit (Biomedical Technologies Inc., Stoughton, MA, USA). The sensitivity of the assay was 0.5 ng/ml, and inter- and intra-assay CVs: 7 and 4%, respectively.

Cell proliferation. Proliferation rate of ROB cells was measured by the EZ4U Non-radioactive Cell Proliferation and Cytotoxic Assay kit (Biomedica, Vienna, Austria) (35). Cultured cells were incubated for 90 min with EZ4U, and the formazan derivative production, which is linearly related to the cell number, was measured at 490 nm wavelength in a microplate autoreader EL-13 (Bio-Tek Instruments, Winooski, VT, USA).

Conventional RT-PCR and qPCR. Methods applied were described earlier (15,36-40). Total RNA from harvested cells and fragments of calvaria was extracted, by a method using TRI Reagent (Sigma) and purified on columns (Rneasy Mini Kit, Qiagen) (15,41,42). Contaminating DNA was eliminated by DNase-I treatment (Rnase-Free DNase Set; Promega, Madison, WI), and the amount of total RNA was determined by measuring optical density at 260 nm and purity was estimated by 260/280 nm absorption ratio, which was consistently >1.8. RT was performed using Verte M-MLV reverse transcriptase

(Novazym) with Oligo-dT (PE Biosystems, Warrington, UK) as primers. Reaction was performed at 42˚C for 60 min. Quality of obtained product was evaluated by electrophoresis in 1% agarose gel with etidium bromide. Applied primers for studies of precerebellin gene expression (Table I) were purchased from the Laboratory of DNA Sequencing and Oligonucleotide Synthesis, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw. HPRT (hypoxanthine-guanine phosphoribosyl transferase) gene was used as reference to normalize data.

To determine efficiency of target and reference qPCR reactions, for all studied genes and HPRT - [reference gene - hypoxanthine guanine phosphoribosyltransferase (EC 2.4.2.8)] gene standard curves were prepared with the products of traditional RT-PCR reaction. These products were subsequently separated on 2% agarose gel. Specific bands were extracted by DNA gel extraction kit (Millipore). Amount of extracted DNA was spectrophotometrically quantified. Standard curves were generated by dilution of isolated cDNA in 1:10 dilution steps.

QPCR was performed on Lightcycler 2.0 instrument (Roche) with software version 4.0. PCR product was detected by LNA probe hydrolysis from Universal Probe Library of Roche. They are labeled at the 5'-end with fluorescein (FAM) and at the 3'-end with a dark quencher dye. Primers and probes were designed using ProbeFinder Version 2.33 of Roche. Each 20 μl of reaction mixture contained: pair of specific primers at concentration of 0.5 μM, 4 μl of LightCycler TaqMan Master Mix, 0.5 μM of gene specific probe and 4 μl of cDNA sample (or standards and control reactions). Each sample was assayed in triplicate. The qPCR program included 10-min denaturation step to activate the Taq DNA polymerase followed by a three-step amplification program: denaturation step at 95˚C for 10 sec, an annealing step at 56˚C for 5 sec, and extension step at 72˚C for 2 sec. Detection of the PCR amplicons was performed by size fractionation on 2% agarose gel electrophoresis.

Statistical analyses. Data are expressed as the means ± SEM. Two-sample statistical comparisons were performed by the unpaired Student's t-test. When more than two groups were to be compared, one-way analysis of variance (ANOVA) was used, followed by the Tukey test for multiple comparisons. The analysis was performed by using the statistical package GraphPad Prism 3.02 (GraphPad Software Inc., San Diego, CA).

Results

Classic RT-PCR revealed the presence of Cbln1 and Cbln3 mRNA in fragments of rat calvaria, in freshly isolated ROB cells and in ROB cells cultured for 7, 14 and 21 days (Fig. 1). Cbln2 and Cbln4 mRNA, on the other hand, could not be demonstrated in ROB cells but was found to be present in the brain. With specific primers RNA amplification by means of RT-PCR revealed the presence of reaction products with expected size: Cbln1, 171 bp; Cbln2, 119 bp; Cbln3, 86 bp; Cbln4, 80 bp.

As demonstrated in Fig. 2, in freshly isolated ROB cells expression of Cbln1 gene was very low but significantly

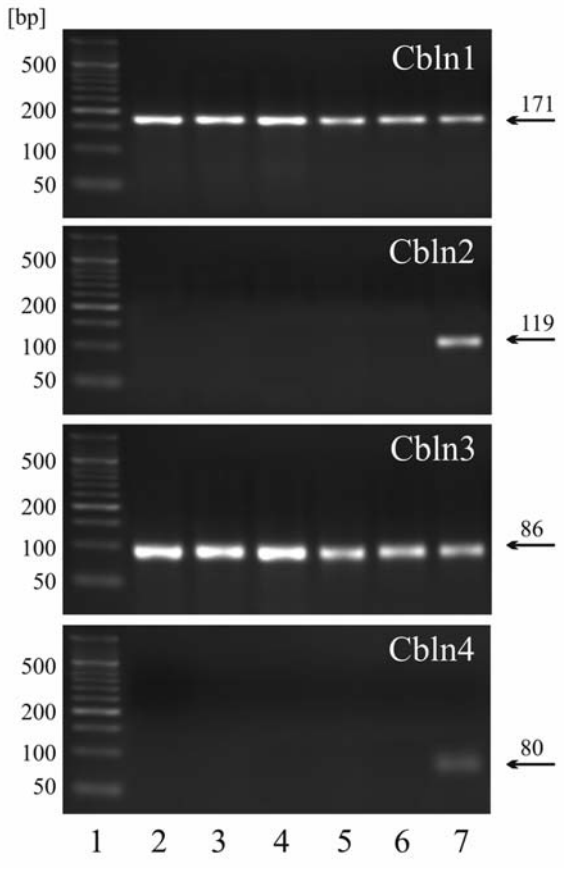

Figure 1. Ethidium bromide-stained 2% agarose gel showing cDNA amplified with rat specific primer from RNA of ROB cells cultured for 7, 14 and 21 days. Lane 1, size marker (O'Range Ruler 50-bp DNA Ladder; MBI Fermentas, Lithuania); lane 2, fragments of rat calvaria; lane 3, freshly isolated ROB cells; lane 4, day 7 of culture; lane 5, day 14 of culture; lane 6, day 21 of culture; lane 7, positive controls (cerebellum for Cbln1 and Cbln3; brain for Cbln2 and Cbln4). Reaction products with expected size are seen: Cbln1, 171 bp; Cbln2, 119 bp; Cbln3, 86 bp; Cbln4, 80 bp.

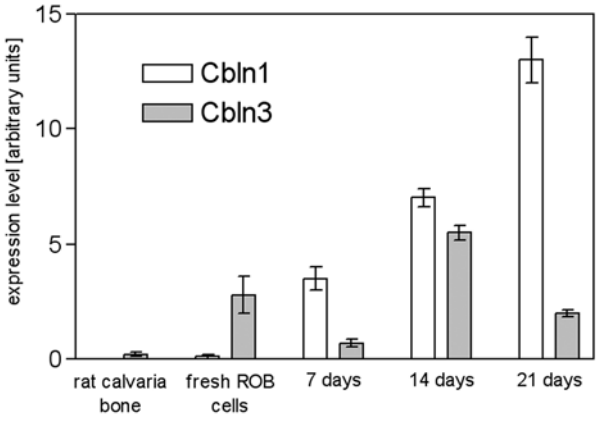

Figure 2. QPCR analyses of Cbln1 and Cbln3 gene expression in fragments of rat calvaria bone, in freshly isolated cells and in the ROB cells at day 7, 14 and 21 of culture, respectively. All samples were amplified in triplicate, and HPRT gene expression was used as reference to normalize data. Each group n=3.

increased (One-way ANOVA: $F_{3,8}$=85.46, p<0.01) in relation to the duration of culture (Fig. 2). Expression of Cbln3, on the other hand, was very low in fragments of rat calvaria, and

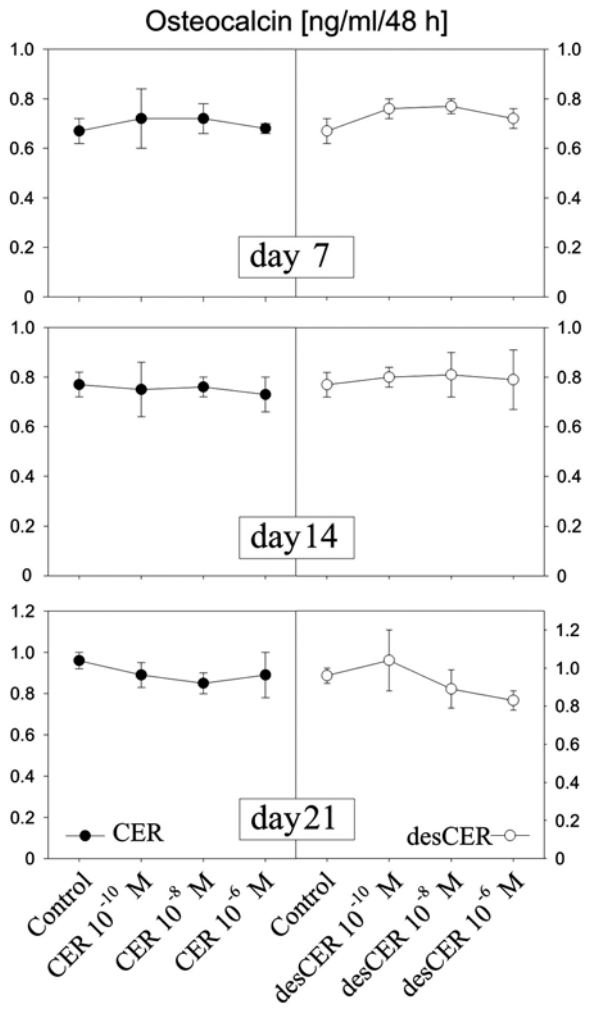

Figure 3. Effects of CER and desCER on osteocalcin secretion (ng/ml/48 h/ $1x10^5$ cells) by cultured ROB cells. Cells were exposed to tested compounds for 48 h in FCS stripped of medium. Neuropeptides were added to culture 48 h before medium collection at days 7, 14 and 21. Data are expressed as means ± SEM, n=6. Statistical comparisons (in relation to control) were done by the unpaired Student's t-test - all differences statistically non-significant.

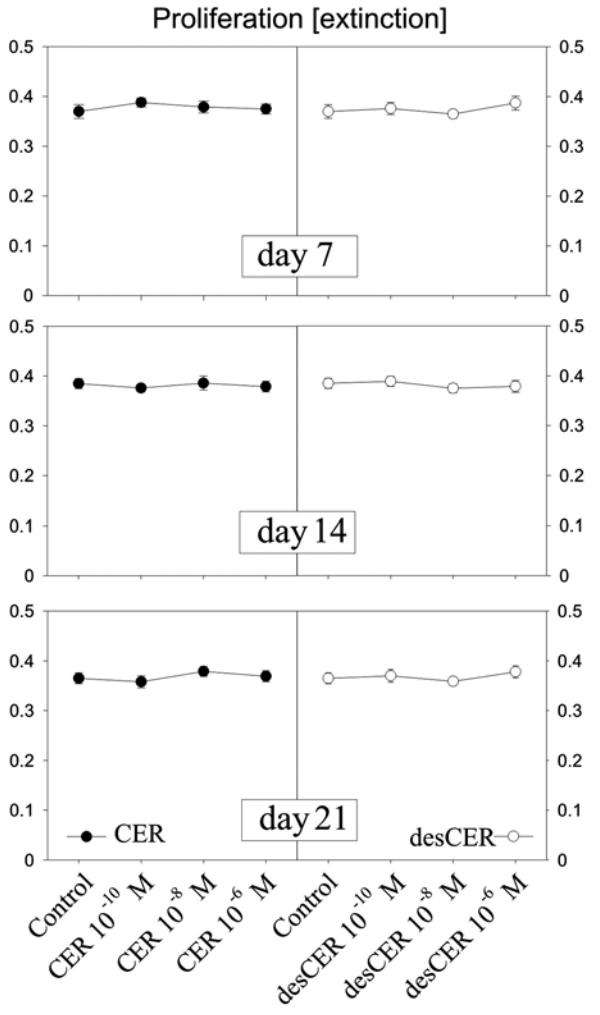

Figure 4. Effects of CER and desCER on proliferative activity (absorbance) of cultured ROB cells. Cells were exposed to tested compounds for 48 h in FCS stripped of medium. Neuropeptides were added to culture 48 h before medium collection at days 7, 14 and 21. Data are expressed as means ± SEM, n=6. Statistical comparisons (in relation to control) were done by the unpaired Student's t-test - all differences statistically non-significant.

increased notably after digestion with collagenase-I. The highest expression of that precerebellin was observed at day 14 of culture while at days 7 and 21 levels of expression were significantly lower ($p<0.05$, Student's t-test). Neither Cbln2 nor Cbln4 were found to be expressed in the ROB cells.

To investigate whether CER or desCER affect osteocalcin production, cultured ROB cells were exposed for 48 h to various concentrations of neuropeptides (10^{-10}-10^{-6} M). Neither CER nor desCER changed osteocalcin concentrations in media analyzed at days 7, 14 and 21 of culture (Fig. 3). Likewise, both cerebellins did not change the proliferative activity of studied cells (Fig. 4).

Discussion

Data on expression of CER and CER mRNA outside the central nervous system are limited and controversial. In this study for the first time we demonstrated expression of Cbln1 and Cbln3 at the mRNA level in freshly isolated and cultured ROB cells, while expression of the remaining precerebellins (Cbln2 and Cbln4) could not be demostrated.

As presently known, entire family of precerebellins is comprised of Cbln1, Cbln2, Cbln3 and Cbln4. These propeptides are expressed in the brain, predominantly in cerebellum, and belong to the C_1q and tumour necrosis factor superfamily of signalling molecules (4,43). Cbln1-derived peptide, CER and its truncated form des-Ser^1-CER (desCER) were originally isolated from rat cerebellum (1,2). It has been suggested that desCER is an apparent metabolite of CER, formation of which is probably catalysed by the serin aminopeptidase (1,13). Recent data, however, demonstrate that precerebellins may be released from heterologous cells and may form heteromeric complexes with each other. These findings suggest that precerebellins are not only precursor proteins but may function also as signalling molecules. In this regard studies on $cbln1^{-/-}$ mice suggest that in cerebellum Cbln1 may function as an adhesive molecule regulating synaptic structures between parallel fibres and Purkinje cells (11,12,44,45). Apart from a possible physiological role of

precerebellins, it is expected that Cbln1-derived peptides would also be involved in regulating biological activity of various cell types.

Both CER and desCER are present at high concentrations in cerebellum and other brain regions. Moreover, these peptides were identified in such organs as alimentary tract, heart, kidney and endocrine glands (4,13,14). However, no CER receptor was identified, although both CER and desCER exert a secretagogue action on catecholamine and aldosterone and cortisol/corticosterone secretion as well as on proliferative activity of adrenocortical cells (15,46-50). Moreover, exogenous CER exerts inhibitory effects on thyroid function in the rat (51). In this regard, Takachashi *et al* (52) recently reviewed possible CER involvement in the regulation of neuroendocrine axis function. Furthermore, it has been found that CER increased cAMP release by human and rat adrenal slices and these effects were annulled by the protein kinase A inhibitor but not by phospholipase C and protein kinase C inhibitors (46,47). Thus, the above presented evidence suggests the possibility of direct, protein kinase A mediated CER action on secretory activity of adrenocortical cells.

Despite a distinct expression of Cbln1 and Cbln3 mRNA in rat osteoblast-like cells, we failed to demonstrate CER and desCER (concentrations 10^{-10}-10^{-6} M) effects on osteocalcin secretion and proliferative activity of these cells. The above findings suggest that CER, which theoretically would be derived from Cbln1, modulate neither differentiated (osteocalcin secretion) nor basic (proliferation) functions of cultured rat osteoblast-like cells. However, we have to consider also the other possible role of precerebellins in these cells. High expression of Cbln1 and Cbln3 in cultured ROB cells suggest that the cells may release precerebellins which are known to form heteromeric signalling complexes. In cerebellum, Cbln1 may function as an adhesive molecule regulating synaptic structures between parallel fibres and Purkinje cells (11,12,44). Likewise, in the bone precerebellins could be responsible for spatial organization of the osteoblastic niches forming a specific bone microenvironment (53-55).

Our findings raise an intriguing hypothesis that precerebellins may be involved in regulating of spatial organization of osteoblastic niches in the bone. This assumption however requires further investigation.

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