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Insulinostatic activity of cerebellin — Evidence from *in vivo* and *in vitro* studies in rats

Mathias Z. Strowski [a,1], Przemyslaw Kaczmarek [a,b,*,1], Stefan Mergler [c], Bertram Wiedenmann [a], Danuta Domin [b], Przemyslaw Szwajkowski [b], Tatiana Wojciechowicz [b], Marek Skrzypski [a,b], Dawid Szczepankiewicz [b], Tomasz Szkudelski [b], Marcin Rucinski [d], Ludwik K. Malendowicz [d], Krzysztof W. Nowak [b]

[a] *Medizinische Klinik mit Schwerpunkt Hepatologie, Gastroenterologie & Interdisziplinäres Stoffwechsel-Centrum: Endokrinologie, Diabetes und Stoffwechsel Charité - Universitätsmedizin Berlin, Campus Virchow-Klinikum, Augustenburger Platz 1, 13353 Berlin, Germany*

[b] *Department of Animal Physiology and Biochemistry, Poznan University of Life Sciences, 35 Wolynska St., 60-637 Poznan, Poland*

[c] *Augenklinik, Charité - Universitätsmedizin Berlin, Campus Virchow-Klinikum, 13353 Berlin, Germany*

[d] *Department of Histology and Embryology, K. Marcinkowski University of Medicine, 6 Swiecicki St., 60-781 Poznan, Poland*

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ABSTRACT

Cerebellin (CER) is a neuromodulatory hexadecapeptide that originates from the precursor protein precerebellin (Cbln1). Four highly homologous isoforms of Cbln are known (Cbln1–Cbln4), which are expressed in the central nervous system (CNS) and in peripheral tissues. CER modulates synaptic structure formation in the CNS, whereas in the peripheral tissues CER regulates catecholamine secretion. Cbln is also expressed in the pancreas; however, its function in the pancreas is unknown. Here, we demonstrate the role of CER in regulating insulin secretion *in vivo* and *in vitro*. We identified Cbln1 and Cbln3 transcripts in rat pancreatic islets and detected Cbln-immunoreactivity, predominantly located in the periphery of the rat endocrine pancreas. *In vivo*, CER reduced plasma insulin levels in rats after 1 and 2 h. CER decreased insulin secretion from isolated rat pancreatic islets at high (11 mM), but not at low (3.33 mM) glucose concentration. CER inhibited stimulated insulin secretion from clonal rat insulinoma (INS-1) cells, reduced forskolin-induced production of cAMP and intracellular calcium concentration. Our study demonstrates for the first time that Cbln1 and Cbln3 are expressed in the rat endocrine pancreas. Furthermore, we identify CER as an insulinostatic factor, which decreases intracellular cAMP production and calcium in INS-1 cells.

1. Introduction

The cerebellum-specific hexadecapeptide "cerebellin" (CER) was originally identified more than 20 years ago [1]. CER is derived from *precerebellin* (Cbln1), a member of the C1q/tumor necrosis factor superfamily, which also includes adiponectin, collagen X or the hibernation protein [2]. Four different isoforms of propeptides are known, which are termed Cbln1–Cbln4 [2–5]. Cbln1, 2 and 4 are secreted as glycoproteins, whereas Cbln3 retains in the endoplasmic reticulum [5]. Cbln can interact with each other by forming homo- or heterometric complexes, thereby changing their functional properties [6,7]. For example, a co-expression of Cbln1 together with Cbln3 results in a secretion of Cbln3, whereas a fraction of Cbln1 retains in the endoplasmic reticulum [8].

Functional studies of Cbln in the CNS provided evidence that these peptides act as signaling molecules and play a role in maintaining and developing of pre- and postsynaptic structures and in forming synaptic plasticity in various brain regions [2,9,10]. However, it is currently unknown which receptor interacts primarily with Cbln and what is the main intracellular signaling cascade. The glutamate receptor δ2 and the adiponectin receptor have been proposed to mediate the physiological effects of Cbln. However, convincing evidence in support of this hypothesis is still lacking.

Neither the expression pattern nor the function of Cbln in the peripheral tissues is well-characterized. The expression of Cbln on RNA and protein levels in the peripheral tissues has been shown in pituitary, heart, kidney, stomach, adrenal glands as well as in tumors such as phaeochromocytomas, cortisol-producing adrenocortical adenomas, ganglioneuroblastomas and neuroblastomas [11,12]. Burnet et al. reported a Cbln-like immunoreactivity in the rat pancreas [11], however the functional significance of Cbln expression in this tissue is unknown.

There are only a few studies available that evaluated the role of CER in regulating the function of peripheral tissues. Albertin et al. have demonstrated that CER can increase the secretion of norepinephrine, aldosterone and cortisol/corticosterone [13]. In addition, CER was able

* Corresponding author. Department of Animal Physiology and Biochemistry, Poznan University of Life Sciences, 35 Wolynska St., 60-637 Poznan, Poland. Tel.: +48 618487196; fax: +48 618487198.
E-mail address: przekacz@up.poznan.pl (P. Kaczmarek).

[1] Both authors contributed equally to the work.

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to facilitate the proliferation of adrenocortical cells [13–18]. These effects appeared to be mediated via the adenylate cyclase/protein kinase A-dependent pathway.

Aim of our current study was to characterize the effects of CER in regulating the endocrine pancreas function in rats. We tested primarily whether CER has an effect on insulin secretion by using *in vitro* and *in vivo* approaches.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich Biochemie GmbH (Hamburg, Germany). Cerebellin was purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland). Collagenase type 4 was from Worthington Biochemical Corporation (Lakewood, NJ, USA). Rat insulin RIA kit (RI-13K) and glucagon RIA kit (GL-32K) were purchased from Linco Res., Inc. (St. Charles, MO, USA). CAMP EIA (cAMP Biotrak Enzyme Immunoassay System) was from Amersham Biosciences (Piscataway, USA).

2.1.1. Animals

Studies were performed on adult female Wistar rats (180–190 g body weight, 8–12 weeks of age). Animals were maintained under standardized light cycle conditions (12 h light:12 h dark period) at a temperature of 22 ± 2 °C with free access to food (standard chow diet) and water. All animal studies and protocols were approved by the Local Ethical Commission.

2.1.2. Isolated rat pancreatic islets

Pancreata were cut into small pieces and islets were released by collagenase digestion as previously described [19]. The collagenase digestion was stopped by the addition of ice-cold Hank's balanced salt solution, pre-gassed with 95% O_2 and 5% CO_2. Islets were then separated from the remaining tissue by hand-picking under a stereomicroscope.

2.1.3. Cells

INS-1 cells were grown and maintained in DMEM containing 10% FCS and 100 U ml^{-1} penicillin and streptomycin sulfate in 10% CO_2 at 37 °C. Cell monolayers were grown in tissue culture 75-cm^2 flasks and passaged, when cells reached 75% confluency.

2.2. Methods

2.2.1. Expression analysis

2.2.1.1. Detection of Cbln expression in isolated pancreatic islets by RT-PCR. Total cellular RNA was extracted from freshly isolated rat pancreatic islets, whole brain and cerebellum using RNAwiz reagent (Ambion, Austin, TX, USA), according to the manufacturer's protocol. RT and PCR reaction were performed using the Access RT PCR System (Promega, Madison, WI, USA) and specific primers for rat Cbln1, Cbln2 and Cbln3 (primer sequences and expected size of products are presented in Table 1). After the RT-step (48 °C for 45 min), 38 PCR cycles for Cbln1 and Cbln2 or 34 PCR cycles for Cbln3 were performed. The conditions were as follows: 94 °C for 30 s, 58 °C for 1 min, and 68 °C for 2 min for Cbln1; 94 °C for 30 s, 56 °C for 1 min, and 68 °C for 2 min for Cbln2; and 94 °C for 30 s, 57 °C for 1 min, and 68 °C for 2 min for Cbln3. Control reactions were carried out with material prepared without the addition of reverse transcriptase. The PCR products were separated on a standard 1.5% ethidium bromide agarose gel, photographed and analyzed using Gel Logic 100 Imaging System and Kodak 1D Analysis Software (Eastman Kodak Company, New Haven, CT, USA).

Table 1
Nucleotide sequences of PCR primers.

Primer	Orientation	Sequence	PCR product size
Cbln1	Sense	5′ GAATGAGACAGAACCCATCGTC 3′	447 bp
	Antisense	5′ AGCTTGAGGTATGCTCGGTC 3′	
Cbln2	Sense	5′ GGTAAAGGCTCAGAACGACA 3′	270 bp
	Antisense	5′ GGTGCTACAAATATACTCGAGGC 3′	
Cbln3	Sense	5′ CAGTACCAGTGGTGCGATCT 3′	150 bp
	Antisense	5′ TGAACAGTCTGGCGGTTGT 3′	

2.2.1.2. Detection of Cbln expression in rat pancreas by immunohistochemistry. Samples of pancreas were fixed in Bouin's solution for 24 h, embedded in paraffin and sectioned with a microtome into 5–6 μm-thick specimens. The sections were deparaffinized, and incubated in 3% H_2O_2 for 30 min at room temperature to block endogenous peroxidase activity. After washing in PBS, sections were preincubated with normal goat serum (DAKO, Glostrup, Denmark) for 30 min and then incubated with a rabbit anti-cerebellin serum (rabbit anti-cerebellin polyclonal antibody, cat. no ZIB 1003, TCS Cellworks, Buckingham, U.K., rabbit anti-precerebellin (Cbln3) polyclonal antibody, Cat. No. ab36908, Abcam Inc., Cambridge, USA) or in theirs solvents as negative control, for 2–3 h. After washing in PBS, sections were incubated with secondary antibody and peroxidase (DAKO LSAB 2 System™ HRP, Code No. K0609). Reaction was amplified using Tyramide™ Signal Amplification System (TSA Biotin System, PerkinElmer, Waltham, MA, USA) — only cerebellin immunodetection. Peroxidase activity was detected using the 3, 3′-diaminobezidine technique (DAKO Liquid DAB Substrate-Chromogen System, Code No. K3466). Negative controls were carried out by similarly treating adjacent sections and omitting the primary antibody, as well as using primary antibody preabsorbed with antigen excess. Glucagon- and somatostatin-immunoreactivity was demonstrated with goat anti-glucagon and anti-somatostatin polyclonal antibody (Santa Cruz Biotechnology, Inc., California, USA).

2.2.2. In vivo experiments

Rats were injected daily with 0.2 ml of 0.9% NaCl (s.c.) for 14 days. On day 15, animals received s.c. injections of 0.2 ml of 0.9% NaCl (control groups), while experimental groups were treated with 0.5 or 1.5 nmol/100 g body weight CER. Rats were decapitated 60 or 120 min after injection. Blood was collected into ice-cold tubes containing EDTA (1 mg/ml) and analyzed for the concentration of glucose. Plasma was separated and stored at −80 °C for the determination of the insulin and glucagon concentration.

Insulin and glucagon concentrations in plasma and incubation media were measured by RIA, using specific rat insulin (RI-13K) and glucagon (GL-32K) kits. Intra- and interassay variations were 5% and 7%, and 6% and 8%, respectively. Blood glucose concentration was measured by the oxidase–peroxidase enzymatic method.

2.2.3. In vitro experiments

2.2.3.1. Secretion assays using isolated pancreatic islets. Groups of five islets matched by size were incubated for 90 min in 1 ml of Krebs–Ringer bicarbonate buffer (KRB, pH of 7.4) containing 0.5% BSA (fraction V, fatty acid-free) and 3.33 or 11.1 mmol/l glucose at 37 °C with or without the addition of CER (1, 10 or 100 nM). Incubations were performed in an atmosphere of 95% O_2 and 5% CO_2. At the end of incubations, aliquots of the medium were taken and stored at −20 °C for the subsequent determination of the insulin and glucagon concentrations. The incubations were performed in quintuplicates and the experiments were repeated twice.

2.2.3.2. Determinations of intracellular cAMP concentration in INS-1 cells. INS-1 cell monolayers were subcultured in 96-well plates and allowed to recover for 48 h before the start of the experiment. The

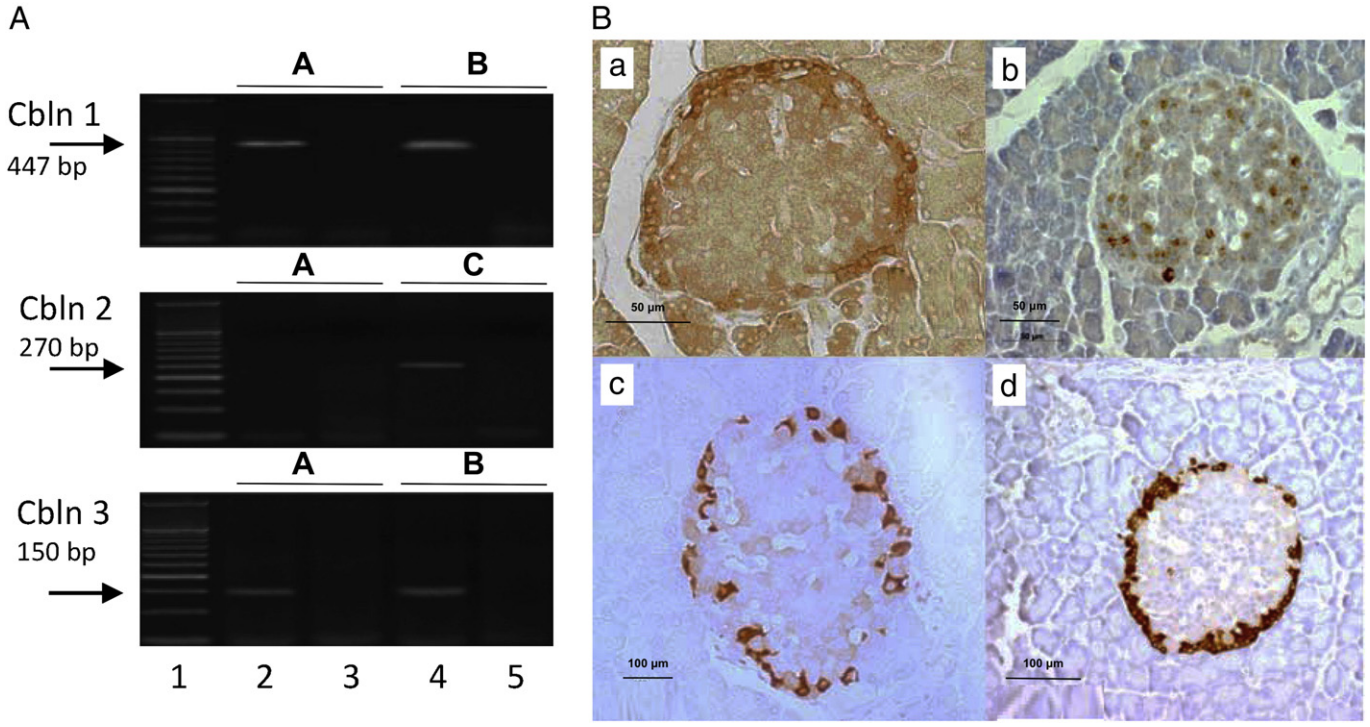

Fig. 1. A. Identification of *precerebellin 1*, *2* and *3* (Cbln1–3) in rat pancreatic islets by RT-PCR. A) Isolated rat pancreatic islets, B) rat cerebellum, and C) rat whole brain. RT-PCR products, generated by rat specific primes, were visualized by ethidium bromide-staining of 1.5% agarose gel. Molecular weights of the expected signals: 447 bp (Cbln1), 270 bp (Cbln2), and 150 bp (Cbln3). Lane 1 DNA ladder, lanes 3 and 5 negative controls (PCR without RT). B. Immunohistochemical localization of cerebellin (a), precerebellin — Cbln3, (b) glucagon (c) and somatostatin (d) in the endocrine rat pancreas. Note the Cbln-immunoreactivity predominantly localized at the A- and D-cell-rich periphery of pancreatic islets.

cells were incubated overnight in a serum-free environment at a cell monolayer confluency of 70%. Cells were washed with PBS and incubated in KRB containing 1% BSA and 0.5 mM isobutylmethyl-xanthine for 30 min at 37 °C. For forskolin-stimulated cAMP accumulation, the culture medium was removed and replaced with fresh KRB (1% BSA), with or without 10^{-6} M forskolin and test agents, the cells were then transferred to 37 °C for 5 min. After this incubation, KRB was replaced with 1 ml of 0.1 N HCl and the monolayers were frozen at −20 °C. The intracellular cAMP levels were measured by an EIA (cAMP Biotrak Enzyme Immunoassay System). Data obtained from the dose–response curves were analyzed by nonlinear regression analysis with GraphPad Prism® Software (San Diego, California, USA).

2.2.3.3. Measurements of intracellular calcium concentration in INS-1 cells. Changes of intracellular-free Ca^{2+} ($[Ca^{2+}]i$) were monitored by measuring fura 2 fluorescence. Cells were preincubated in a supplement-free culture medium (DMEM) containing 1 µM fura 2 AM for 30–45 min. Thereafter, cells were washed with the extracellular sodium- and potassium-free bath solution containing (mmol/l): N methyl D glucamine, 120; CsCl, 5.4; $MgCl_2$, 1.0; glucose, 10; and HEPES acid, 10 (pH adjusted to 7.3). Fluorescence measurements were performed at room temperature with a digital imaging system (T.I.L.L. Photonics, Munich, Germany). Fura 2 fluorescence was excited at 340 nm and 380 nm wavelengths and changes in intracellular-free Ca^{2+} levels were calculated based on the fluorescence ratio of the two excitation wavelengths according to Grynkiewicz et al. [20]. For displaying the quantitative changes of free $[Ca^{2+}]i$, fluorescence, levels were calculated in percent of control (basal line was set to 100%). When drugs were added from DMSO containing stock solutions, the solvent concentration did not exceed 0.1%. This low DMSO concentration did not change fura 2 fluorescence in control experiments (data not shown).

2.3. Statistical analysis

Results were averaged per experimental group and the standard error of the mean (SEM) was calculated. The statistical comparison of the data was done by ANOVA, followed by the Multiple Range Test of Duncan with GraphPad Prism® Software (San Diego, California, USA).

3. Results

3.1. Expression of Cbln in rat endocrine pancreas

Precerebellin expression in isolated rat pancreatic islets was studied by RT-PCR using specific primers for Cbln1, Cbln2, and Cbln3 (Table 1). We identified Cbln1 and Cbln3 in isolated rat pancreatic islets (Fig. 1A).

The amplified fragments corresponded to the expected sizes: 447 bp for Cbln1 and 150 bp for Cbln3. There was no evidence for Cbln2 expression in rat islets (Fig. 1A). Amplification of hypothalamic mRNA (positive control for Cbln2) by RT-PCR revealed a strong, clear signal of the expected size of 270 bp.

We used separate positive controls for Cbln1 and Cbln3 and for Cbln2, because the expression pattern of Cbln in various parts of the brain shows differences. For example, Cbln1 is expressed at high levels in the adult cerebellum, whereas Cbln2 is nearly undetectable [3]. In contrast, Cbln2 is expressed at relatively high levels in extracerebellar brain areas (e.g. hypothalamus) and in the fetal nervous system, whereas Cbln1 is either absent or only expressed at very low levels [2,3].

To confirm the expression of Cbln in the rat endocrine pancreas at protein level, immunohistochemical analyses of pancreatic sections were performed. Using a specific antibody, Cbln-like immunoreactive cells were identified predominantly at the periphery of islets (Fig. 1B), which is known to contain a high density of glucagon- and somatostatin-producing cells.

3.2. In vivo effects of CER on insulin, glucagon and glucose

To determine the *in vivo* effects of CER on the endocrine pancreas secretion, we administered a bolus of CER (0.5 and 1.5 nmol/100 g body weight, s.c.) into rats. We observed a significant decrease of plasma insulin concentration after 120 min at both doses of CER and at the 60 min time point at the low dose of CER ($n=6$ per time point) (Fig. 2). In addition, we also observed a moderate increase of plasma glucagon and a decrease of blood glucose concentration (Fig. 2). However, these effects were observed only after 60 min and at the highest tested dose of CER.

3.3. In vitro effects of CER on insulin secretion from isolated pancreatic islets

Incubation of isolated pancreatic islets with different concentrations of CER (1, 10 and 100 nM) at lower (3.33 mM) glucose in the incubation medium did not influence insulin secretion. Therefore, the effects of CER on insulin secretion, stimulated by higher glucose concentration, were characterized. At 11.1 mM glucose in the incubation medium, insulin secretion from isolated rat pancreatic islets increased by approximately 4-fold over the basal secretion (Fig. 3). CER at 10 nM and at 100 nM inhibited glucose 11.1 mM-stimulated insulin secretion.

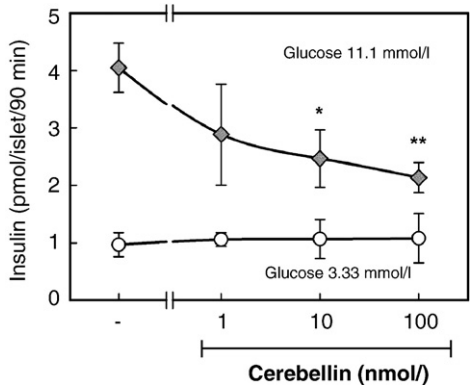

Fig. 3. Effect of cerebellin on insulin release from isolated rat pancreatic islets. Islets were incubated for 90 min with different concentrations of cerebellin either at 3.33 or 11.1 mmol/l glucose and the secreted insulin was assayed by RIA. Data points present means ± SEM, $n=10$. Statistically significant differences between cerebellin-treated groups and controls are marked * — $p<0.05$ and ** — $p<0.001$ vs. control.

3.4. Effects of CER on insulin secretion from INS-1 cells

To characterize the effects of CER at the cellular level, rat clonal INS-1 cells were exposed to CER and the amount of secreted insulin was determined. Since CER failed to affect insulin secretion from INS-1 cells at a low (3.3 mM) glucose concentration (data not shown), the effects of CER on stimulated insulin secretion were characterized. The secretion of insulin was induced by incubating the cells with a combination of 20 mM glucose, 10 nM exendin-4 (glucagon-like peptide-1 agonist). Exendin-4 is a known enhancer of insulin secretion at higher glucose concentrations. 20 mM glucose and exendin-4 effectively stimulated insulin secretion from INS-1 cells (Fig. 4). CER at 10^{-6} M and 10^{-7} M reduced glucose/exendin-4-stimulated insulin secretion from INS-1 cells (Fig. 4).

3.5. Effects of CER on cAMP and calcium levels in INS-1 cells

Intracellular calcium and cAMP-dependent pathways play a role in regulating the secretion processes. We therefore investigated the effects of CER on intracellular cAMP accumulation and intracellular calcium level in INS-1 cells. Since we failed to detect any effects of CER on basal cAMP, we used forskolin, a potent stimulus of adenylate cyclase activity. Forskolin stimulated cAMP production. CER (10^{-8} M–10^{-6} M) reduced forskolin-induced cAMP increase (Fig. 5A). In addition, the functional effect of CER was determined by measuring the changes of intracellular Ca^{2+} concentrations during exposure of INS-1 cells to this peptide. Fig. 5B shows that exposure of INS-1 cells to 10^{-7} M CER in the Ca^{2+}-containing solution reduced the f_{340}/f_{380} ratio

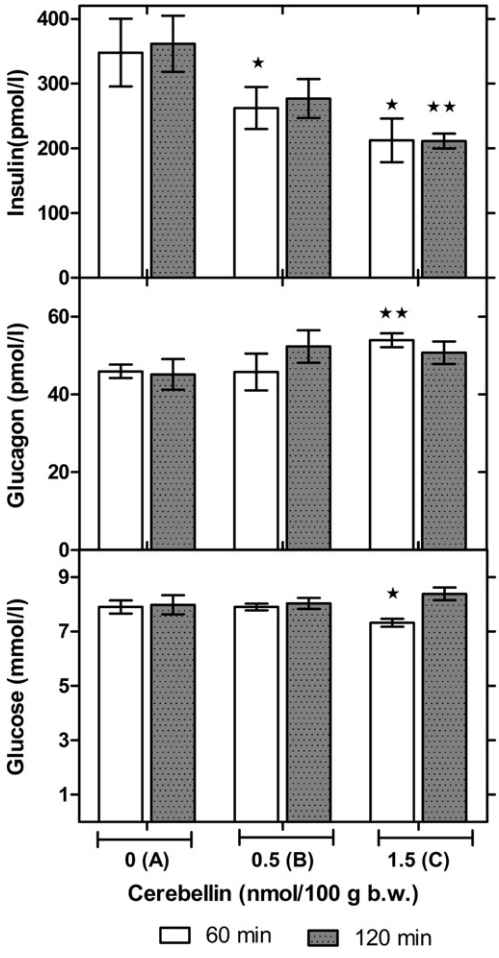

Fig. 2. Effects of bolus cerebellin (0.5 or 1.5 nmol/100 g body weight, s.c.) on blood glucose, plasma insulin and glucagon levels 60 or 120 min after administration. Bars present means ± SEM, $n=6$. The asterisks (* — $p<0.05$ and ** — $p<0.01$; Dunnett's Multiple Comparison Test) indicate a statistically significant difference between controls (without drug) and cerebellin bar chart.

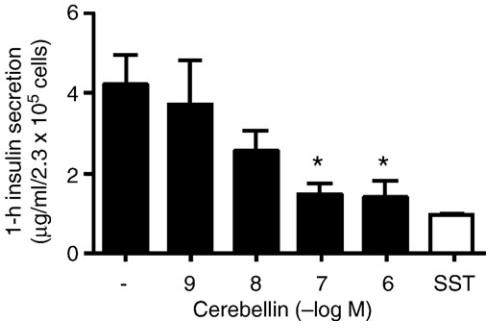

Fig. 4. Effect of cerebellin on 20 mM glucose/10 nM exendin-4-stimulated insulin secretion from INS-1 cells. Somatostatin-14 (SST), a well-known inhibitor of insulin secretion, was used as positive control. Data points present means ± SEM, $n=7$. Statistically significant differences between cerebellin-treated groups and controls are marked (* — $p<0.05$).

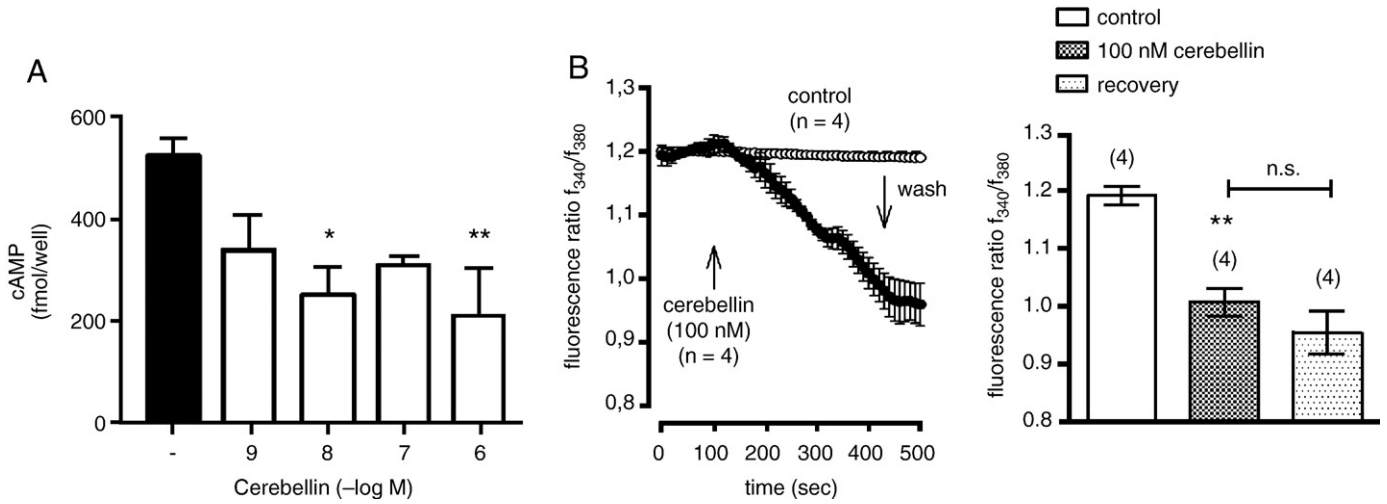

Fig. 5. Effects of cerebellin on cAMP production and intracellular calcium levels in INS-1 cells A: Inhibition of forskolin-induced increase of cAMP accumulation in INS-1 cells by cerebellin. Statistically significant differences between SST and cerebellin are marked ($n=4$; *$p<0.05$ and **$p<0.01$). B: Left panel: Irreversible reduction of intracellular calcium concentration by 100 nM cerebellin (filled circles, $n=4$). Control measurements performed with a vehicle (open circles, $n=4$). Changes in cytosolic free Ca^{2+} are depicted as the ratio of the fluorescence induced by the excitation wavelengths of 340 and 380 nm. Right panel. Summary of the experiments with cerebellin. The asterisks (**) indicate a statistically significant difference ($p<0.01$; paired *t*-test) between controls (Ca^{2+} base levels) and cerebellin.

from 1.194 ± 0.016 to 1.009 ± 0.024 ($n=4$; $p<0.01$). This effect was irreversible because, in the absence of CER (washout), calcium failed to recover ($f_{340}/f_{380} = 0.956 \pm 0.036$; $n=4$; $p=0.173$).

4. Discussion

Cbln fulfil important functions in the CNS as they contribute to the maintenance of synaptic structures and modulate their functions in the cerebellum. It is assumed that some of the effects of Cbln are mediated via the orphan glutamate receptor δ2 (Grid2) [9] but strong evidence has not so far been provided.

Little is known about the expression and function of Cbln in the peripheral tissues. A study of Burnet et al. [11] reported Cbln-like immunoreactivity in the rat pancreas and guinea pigs. However, in rats the intensity of pancreatic immunostaining was lower when compared to the staining in hypothalamic regions, while in guinea pigs the signal intensity in the pancreas resembled that of the hypothalamus [11].

In this study, we demonstrate for the first time the presence of Cbln1 and Cbln3 mRNA and Cbln-immunoreactivity in the endocrine pancreas of the rat. As detected by immunohistochemistry, Cbln-immunoreactive proteins are predominantly localized in the glucagon-producing A- and somatostatin-producing D-cell-rich periphery of the rat endocrine pancreas. However, Cbln and precerebellin immunoreactivity at lower signal intensity was also detected in the central part of islets. This region contains a high density of insulin-producing B-cells.

The expression pattern of Cbln subtypes in the rat islet is similar to that detected in mouse cerebellum and dorsal cochlear nucleus [5,11,21]. In all those structures, Cbln1 and Cbln3 are highly expressed while expression of Cbln2 is negligible. The expression pattern of Cbln1 and Cbln3 may be of functional relevance, as these isoforms have been reported to form heterodimers in the yeast two hybrid systems. As a consequence of this heterodimerization with Cbln1, a substantial fraction of Cbln3, which normally resides in the endoplasmic recticulum, is secreted, and escapes degradation [7]. However, a functional contribution of this interaction to mammalian physiology has not yet been demonstrated [8].

An additional novel finding of the present study is the demonstration of an insulinostatic activity of CER *in vivo* as well as *in vitro*. Furthermore, a moderate increase of glucagon and a decrease of glucose concentration have been observed. At the present stage, we do not know the reason for the lowering of the plasma glucose levels, despite a lowering of plasma insulin and an increase of plasma glucagon. Since CER is known to stimulate the secretion of catecholamines and corticosteroids, the mechanism leading to the fall of glucose must be caused by a different mechanism. It is possible that CER may directly enhance glucose utilization and/or decrease the rate of hepatic gluconeogenesis.

In agreement with the *in vivo* insulinostatic activity of CER, we detected a decrease of stimulated insulin secretion from isolated pancreatic islets, as well as from INS-1 cells. The expression of Cbln in the endocrine pancreas may indicate that Cbln may act via an autocrine and/or paracrine mechanism. However, the receptor for mediating the cellular effects of Cbln is not known and therefore we can only speculate by which mechanism(s) CER inhibits insulin secretion. Potentially, Cbln could interact with a membrane receptor and activate an intracellular signal transduction cascade in a manner analogous to TNF-alpha, as proposed before [22]. So far, however, no receptors for Cbln have been described in INS-1 cells. Therefore, the challenge remains to clone the Cbln receptor from insulinoma cells or the adrenal glands, where Cbln inhibits the catecholamine and corticosteroid secretion, as well.

Intracellular calcium and cAMP-dependent signal pathways play an important role in regulating insulin biosynthesis and secretion [23–25]. Additionally, previous studies have demonstrated that CER significantly increased cAMP release and stimulated norepinephrine output from adrenomedullary tissue via the adenylate cyclase/PKA-dependent signaling pathway [13,15]. Therefore, we focused on the investigation of this pathway using rat insulinoma cells. The present study shows that CER potently reduced forskolin-stimulated cAMP production and intracellular calcium concentration in INS-1 cells. These data suggest that CER could regulate insulin secretion through a modulation of cAMP- and calcium-dependent signal transduction. Both signaling pathways mediate the effects of other insulinostatic hormones such as somatostatin on insulin secretion [26,27].

In summary, we demonstrated the expression of Cbln1 and Cbln3 as well as Cbln-immunoreactivity in the endocrine rat pancreas for the first time. In addition, we provided evidence that CER exerts an insulinostatic activity *in vivo* and *in vitro*. Finally, we showed that cAMP and calcium-dependent pathways are negatively regulated by CER.

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