Cloning and quantitative determination of the human Ca^{2+} /calmodulin-dependent protein kinase II (*CaMK II*) isoforms in human beta cells

H. Rochlitz¹, A. Voigt¹, B. Lankat-Buttgereit², B. Göke², H. Heimberg³, M. A. Nauck⁴, U. Schiemann⁵, H. Schatz¹, A. F. H. Pfeiffer¹

¹ Department of Internal Medicine, University Hospital Bergmannsheil, University of Bochum, Bochum, Germany

² Clinical Research Unit for Gastrointestinal Endocrinology, Department of Internal Medicine, Philipps-University of Marburg, Marburg, Germany

³ Diabetes Research Centre, Vrije Universiteit Brussels, Brussels, Belgium

⁴ Department of Internal Medicine, Knappschafts Hospital, University of Bochum, Bochum, Germany

⁵ Department of Internal Medicine B, University Hospital of Münster, Münster, Germany

Abstract

Aims/hypothesis. The Ca^{2+} /calmodulin-dependent protein kinase II (CaMK II) is highly expressed in pancreatic islets and associated with insulin secretion vesicles. The suppression of CaMK II disturbs insulin secretion and insulin gene expression. There are four isoforms of CaMK II, α to δ , that are expressed from different genes in mammals. Our aim was to identify the isoforms of CaMK II expressed in human beta cells by molecular cloning from a human insulinoma cDNA library and to assess its distribution in humans.

Methods. The previously unknown complete coding sequences of human *CaMK II β* and the kinase domain of *CaMK II δ* were cloned from a human insulinoma cDNA library. Quantitative determination of CaMK II isoform mRNA was carried out in several tissues and beta cells purified by fluorescence activated cell sorting and compared to the housekeeping enzyme pyruvate dehydrogenase.

Results. We found *CaMK II β* occurred in three splice variants and was highly expressed in endocrine tis-

sues such as adrenals, pituitary and beta cells. Liver showed moderate expression but adipose tissue or lymphocytes had very low levels of *CaMK II β* -mRNA. In human beta cells *CaMK II β* and δ were expressed equally with pyruvate dehydrogenase whereas tenfold lower expression of *CaMK II γ* and no expression of *CaMK II α* were found.

Conclusion/interpretation. Although *CaMK II δ* is ubiquitously expressed, *CaMK II β* shows preferential expression in neuroendocrine tissues. In comparison with the expression of a key regulatory enzyme in glucose oxidation, pyruvate dehydrogenase, two of the four CaM kinases investigated are expressed at equally high levels, which supports an important role in beta-cell physiology. These results provide the basis for exploring the pathophysiological relevance of *CaMK II β* in human diabetes. [Diabetologia (2000) 43: 465–473]

Keywords Insulin secretion, protein kinase, insulin secreting cells, human CaMK II, cloning of new subtypes.

The Ca^{2+} /calmodulin-dependent protein kinase II (CaMK II) is a multifunctional enzyme which is widely expressed from yeast to mammals and occurs in four different isoforms termed α , β , γ and δ

which are derived from different genes [1, 2]. The highest concentrations are in the CNS where the α -isoform and β -isoform are involved in neurotransmitter release [1, 2] whereas the γ -isoform and δ -isoform occur in most peripheral cells [3, 4] and participate in control of cell cycle, metabolism, gene expression and membrane excitability. Insulin secretion in response to glucose and other nutrient stimuli is dependent on Ca^{2+} and CaMK II seems to play an important part in this process [5–7]. Impairment of insulin secretion in response to glucose is an early event in the development of adult Type II (non-insulin-dependent) diabetes mellitus making it

Received: 20 August 1999 and in revised form: 11 January 2000

Corresponding author: Dr. A. Pfeiffer, Medizinische Klinik und Poliklinik, Berufsgenossenschaftliche Kliniken Bergmannsheil, Universitätsklinik, Bürkle-de-la-Camp-Platz 1, D-44789 Bochum, Germany

Abbreviations: CaMK II, Ca^{2+} /calmodulin-dependent protein kinase II; PDH, pyruvate dehydrogenase.

possible that this kinase contributes to the disease [8, 9].

Isoforms of CaMK II are encoded as single polypeptides each containing a catalytic, a regulatory, a variable and an association domain with molecular masses between 50 000 and 60 000 M_r. The catalytic domain has features similar to most serine threonine protein kinases. The kinase domain is located in the N-terminal half of the molecule followed by a regulatory domain which is the site of calmodulin binding and autophosphorylation, through which CaMK II can sustain its own activity after a Ca²⁺ surge [10–14]. The regulatory domain is followed by a region of high variability where most differences between the 4 CaMK II genes are found. In this region extensive splicing occurs generating between 2 and 12 subtypes of each isoform [15–18]. The precise role of this region is not known but it is thought it enables the protein to associate with subcellular organelles and cytoskeletal structures [19]. The C-terminal part of the enzyme is responsible for oligomerisation of CaMK II allowing for the association of 6 to 12 subunits [1, 20]. Cells can contain more than a single isoform of the kinase and both, homomultimers and heteromultimers, of the kinase can exist.

It has been proposed that CaMK II participates in the regulation of insulin secretion from beta cells [5, 6, 21, 22]. Glucose was shown to activate CaMK II in beta cells and to cause its autophosphorylation [23]. An antagonist of CaMK II, KN-62, was shown to inhibit insulin secretion [24, 25] but also to inhibit voltage-regulated Ca²⁺ channels which can, by itself, prevent insulin secretion [26]. Studies at the single cell level have shown the inhibition of insulin exocytosis in response to glucose on treatment of beta cells with a peptide inhibitory to CaMK II [27, 28]. Indeed, CaMK II has been localised on insulin granules both in fish [21] and rat islets and in INS-1 cells [29]. Further studies have confirmed an association with insulin granules and have proposed synapsin 1 and microtubule-associated protein (MAP)-2 as important substrates [30–32]. In INS-1 cells suppression of *CaMK II* caused a decreased mRNA expression of insulin, glucokinase and GLUT-2 suggesting that CaMK II not only has a role in exocytosis but also in the regulation of gene expression of molecules which are involved in glucose-stimulated insulin secretion. This was confirmed by western blot analysis and colorimetric measurement of glucose metabolism [33, 34].

Partial human sequences of *CaMK II* α , β and δ have been identified as sequence tagged sites in a shotgun approach of random cDNA sequencing [35]. The human CaMK II γ and several splice variants thereof were previously cloned from a lymphocyte cDNA library [15].

Very recently while this work was in progress another study [36] used a PCR approach to amplify the variable region of all subtypes of *CaMK II* from sev-

eral human tumour cell types and identified two splice variants of *CaMK II* β , four of *CaMK II* γ and two of *CaMK II* δ .

The aim of this study was (1) to identify the isoforms of *CaMK II* present in human beta cells, (2) to obtain their complete cDNA sequences, (3) to determine quantitatively their contribution to *CaMK II* types in beta cells and (4) to obtain an idea of their expression in other tissues to define them as either largely beta-cell specific or widely expressed molecules. The latter could be of relevance when searching for associations between single nucleotide polymorphisms of *CaMK II* isoforms and human diseases such as Type II diabetes.

Methods

Materials. We obtained *Taq* DNA polymerase and primers for PCR from Gibco, Gaithersburg, Md., USA, or MWG Biotech, Munich, Germany, reverse transcriptase M-MuLV-RT and random primers pd(N)₆ from Boehringer, Mannheim, Germany, RNasin and dNTPs from Promega, Madison, Wis., USA, the ABI-PRISM Dye Terminator cycle sequencing kit from Applied Biosystems, Foster City, Calif., USA and Polymorphprep from Nycomed, Oslo, Norway. Restriction endonucleases and T4 ligase were from Boehringer, Mannheim. Chemicals were purchased from Merck, Darmstadt, Germany, except where indicated otherwise.

Molecular cloning of human *CaMK II* cDNA. The human insulinoma cDNA library in a λ -gt11 vector was prepared as described previously [37]. Inserts of plaque-purified clones were released by *Eco*RI-digest and subcloned into pBluescript KS with or without phagemid (Stratagene, La Jolla, Calif., USA) using T4 ligase. Sequencing was done on a Perkin Elmer sequencer (Foster City, Calif., USA) using the ABI-PRISM Dye Terminator cycle sequencing mix.

Tissue and cell preparation. Human adipose tissue and muscle were obtained from patients undergoing hip surgery. Pituitary tissue was from hypophysectomy of non-functioning pituitary adenomas. Patients gave their informed consent and an ethical committee approved the study. Bone cells were cultured from primary bone explants as described [38] and consisted of passage 2 osteoblasts. Bone cells were grown in Hams F12K medium (Seromed, Berlin, Germany) containing 10% fetal calf serum (Gibco). Fibroblasts were cultured from non-transformed tissue explants obtained from patients undergoing surgery for colon carcinomas using DMEM medium (Gibco) supplemented with 10% fetal calf serum. Normal adrenal cortex tissue was obtained during bilateral adrenalectomy from patients operated because of ectopic ACTH syndrome. A second sample was obtained from normal adrenal cortex during adrenalectomy because of a pheochromocytoma. Liver tissue was obtained from a control biopsy during cholecystectomy which showed histologically normal liver tissue. Human beta cells were isolated from three patients by fluorescence-activated cell sorting as described previously [39].

Mononuclear leucocytes were obtained by density gradient centrifugation of whole blood using Polymorphprep as described in the user manual. Thrombocytes were obtained by centrifugation of EDTA blood for 10 min at 350 $\times$ g followed by a second centrifugation of the supernatant for 10 min at

Table 1. List of primers which were used to amplify human *CaMK II* isoforms or PDH and for construction of internal DNA standards

No.	Name	Starting point within the CDS	Annealing temp. (°C)	Sequence	Length of amplicates (bp)
1.	hCamK β 1	4	58	CGGGATCCCG-GCCACCACGGTGACCTGCAC ①	282
2.	hCamK β 2	285 c	58	GGAATTC-ACCAGTGACCAGATCGAAGACCA ②	
3.	hCamK β 3	260	56	ACCTGGTCTTCGATCTGGTC	406
4.	hCamK β 4	665 c	56	AGCTTGTGCTGGTCTCGTC	
5.	hCamK β 5	606	55	GATCCTGTACATCCTGCTCG	322
6.	hCamK β 6	927 c	55	CAGCATGGTGGTGAGGATGG	
7.	hCamK β 7	908	55	CCATCCTCACCACCATGCTG	170–287 ④
8.	hCamK β 8	1149 c	55	GATGAGCTGCTCCGTGGTCT	
9.	hCamK β 9	1130	58	AGACCACGGAGCAGCTCATC	384
10.	hCamK β 12	1513 c	58	TTCATGTCAGCGGGGCCACA	
11.	hCamK β 3ex	342	56	ACCTGGTCTTCGATCTGGTC-ACTGTATCCAGCAGATCC ③	341
12.	hCamK γ 1	104	53	TCCGCAGGTGTGTGAAGAAA	316
13.	hCamK γ 2	419 c	53	ACGATGTCATGCTGGTGGAT	
14.	hCamK γ 1ex	193	53	TCCGCAGGTGTGTGAAGAAA-CTAGAACGTGAGGCTCGGA ③	248
15.	hCamK δ 1	6	68	TTCGACCACAACCTGCACCA	193
16.	hCamK δ 2	198 c	68	ACGGCAGATTCTAGCTTCTC	
17.	hCamK δ 1ex	107	68	TCGACCACAACCTGCACCA-CTGGACAAGAATATGCTGCC ③	111
18.	hCamK α 1		58	GGTCACTGGTGGGGAATGC	311
19.	hCamK α 2		58	CCACAGGCTTCCCGTACGGG	
20.	PDH 1	4294	60	GGTATGGATGAGGAGCTGGA	102
21.	PDH 2	4478 c	60	CTTCCACAGCCCTCGACTAA	
22.	PDH 5	5351	60	TCTCCATGCAAGCCATTGAC	265
23.	PDH 6	6035 c	60	TCATTCTCTAGCACCACCAC	
24.	PDH 5ex	5351	60	TCTCCATGCAAGCCATTGAC-GCCTGTGCCTATAGTCTTCA ③	215

① primer contains a BamH I-recognition site as 5' extension
 ② primer contains an EcoR I-recognition site as 5' extension
 ③ primer for generation of shortened internal DNA standards for the competitive PCR

④ length of the amplicates differ in different splice variants of *CaMK II β*
 c = start nucleotide on the complementary strand

800 $\times$ g. Thrombocytes, mononuclear leucocytes, osteoblasts and pituitary adenomas were homogenised by ultrasound, other tissues by an Ultra Turrax homogeniser (Janke and Kunkel, Staufen, Germany).

RNA isolation and reverse transcription. Total RNA from tissues was isolated by the acid guanidinium thiocyanate method [40]. Total RNA was quantified by determination of the O.D. at 260 and 280 nm. Reverse transcription (RT) was done by using 0.5–1 μ g of total RNA and the following additions (final concentration): 20 U RNasin, 40 pmol (0.5 μ g) random primer pd(N)₆, 20 pmol dNTP, reaction buffer as supplied, ultrapure H₂O to a total volume of 20 μ l. The reaction mix was heated to 65 °C for 5 min and cooled to 25 °C for 10 min to allow random primers to anneal. We added 20 units of M-MuLV-RT for 60 min at 37 °C. The reaction was stopped by denaturing at 95 °C for 5 min.

Polymerase chain reaction. All primers were created with Primer Designer 2.0 (Scientific & Educational Software 1991, Durham, N. C., USA). We mixed 10 to 200 ng of reverse transcribed total RNA with the following additions to a final volume of 100 μ l (final concentrations): 1.5–3 mmol/l MgCl₂, 20 pmol dNTP, 30 pmol of upstream and downstream primer and 2 U of *Taq*-DNA-polymerase, reaction buffer as supplied and ultrapure water. The PCR was started by denaturing for 2 min at 95 °C followed by up to 45 cycles of 40 s at 95 °C, 30 s at the specific annealing temperature, 1 min at 72 °C. Final elongation was done for 10 min at 72 °C. We then analysed 8 to 16 μ l

of the PCR reaction on a 2% agarose gel by submarine electrophoresis at 5 V/cm. Detection was done with ethidium bromide staining.

Sequencing of PCR products. Polymerase chain reactions, which showed a single band were sequenced after purification by the High Pure PCR Product Purification Kit from Boehringer. If there were two or more bands in a PCR reaction, the single bands were excised from the agarose gel and prepared by the High Pure PCR Product Purification Kit. The purified amplicates were sequenced twice with the ABI-PRISM Dye Terminator cycle sequencing mix and either the 5'-primer or 3'-primer and then analysed on a Perkin Elmer sequencer. Expected bands were sequenced at least once for confirmation, new bands were sequenced three times.

Semiquantitative comparative PCR with internal cDNA standards. For use as internal standards, primers were designed for pyruvate dehydrogenase (PDH) (E.C.1.2.4.1) β -subunit sequence (NID g219983/GenBank) (Table 1). These primers were used for amplification together with the target *CaMK II* primer. At the end of cycle number 21, 24, 27, 30, 33, 36, 39, 42 and 45 the entire reaction was cooled on ice for 1 min and 8 μ l were removed for analysis by submarine gel electrophoresis. The linearity of the amplifications was controlled by densitometric scanning of the gels and plotting of the density against the cycle number. The amplifications were analysed in the linear range as described in detail previously [41].

A

Homology View: 30 bases/block.

B

*rCaMK II*β (1) MATTVTCTRFTDEYQLYEDIGKGAFSVVRRCVKLCTGHEYAAKIINTKKLSARDHQKLEREARICRLKHSNIVRLHDSISEEGFHYLVFDLVTGGELFE

*hCaMK II*β1 (1) MATTVTCTRFTDEYQLYEDIGKGAFSVVRRCVKLCTGHEYAAKIINTKKLSARDHQKLEREARICRLKHSNIVRLHDSISEEGFHYLVFDLVTGGELFE

*hCaMK II*β2 (1) MATTVTCTRFTDEYQLYEDIGKGAFSVVRRCVKLCTGHEYAAKIINTKKLSARDHQKLEREARICRLKHSNIVRLHDSISEEGFHYLVFDLVTGGELFE

*hCaMK II*β4 (1) MATTVTCTRFTDEYQLYEDIGKGAFSVVRRCVKLCTGHEYAAKIINTKKLSARDHQKLEREARICRLKHSNIVRLHDSISEEGFHYLVFDLVTGGELFE

(101) DIVAREYYSEADASHCIQQILEAVLHCHQMGGVVRDLKPENLLASCKGAAVKLADFGLAIEVQGDQQAWFAGTGPYLSPEVLRKEAYGKPVDIWAC

(101) DIVAREYYSEADASHCIQQILEAVLHCHQMGGVVRDLKPENLLASCKGAAVKLADFGLAIEVQGDQQAWFAGTGPYLSPEVLRKEAYGKPVDIWAC

(101) DIVAREYYSEADASHCIQQILEAVLHCHQMGGVVRDLKPENLLASCKGAAVKLADFGLAIEVQGDQQAWFAGTGPYLSPEVLRKEAYGKPVDIWAC

(101) DIVAREYYSEADASHCIQQILEAVLHCHQMGGVVRDLKPENLLASCKGAAVKLADFGLAIEVQGDQQAWFAGTGPYLSPEVLRKEAYGKPVDIWAC

(201) GVILYILLVGYPFWEDEDQHKLYQKIKAGAYDFPSPPEWDTVTPEAKNLINQMLTINPAKRITAHEALKHPWVCQRSTVASMMHRQETVECLKKFNARRKL

(201) GVILYILLVGYPFWEDEDQHKLYQKIKAGAYDFPSPPEWDTVTPEAKNLINQMLTINPAKRITAHEALKHPWVCQRSTVASMMHRQETVECLKKFNARRKL

(201) GVILYILLVGYPFWEDEDQHKLYQKIKAGAYDFPSPPEWDTVTPEAKNLINQMLTINPAKRITAHEALKHPWVCQRSTVASMMHRQETVECLKKFNARRKL

(201) GVILYILLVGYPFWEDEDQHKLYQKIKAGAYDFPSPPEWDTVTPEAKNLINQMLTINPAKRITAHEALKHPWVCQRSTVASMMHRQETVECLKKFNARRKL

(301) KGAILTTMLATRNFSVGRQTAPATMSTAASGTTMGLVEQAKSLNKKADGVKPTNSTKNSAATSPKGLPPLAALPQTIVIHNPVDGIKESSDSANT

(301) KGAILTTMLATRNFSV-----AKSLNKKADGVKPTNSTKNSAATSPKGLPPLAALPQTIVIHNPVDGIKESSDSANT

(301) KGAILTTMLATRNFSV-----AKSLNKKADGVKPTNSTKNSAATSPKGLPPLAALPQTIVIHNPVDGIKESSDSANT

(301) KGAILTTMLATRNFSV-----AKSLNKKADGVKPTNSTKNSAATSPKGLPPLAALPQTIVIHNPVDGIKESSDSANT

(401) TIEDEDAKARKQEI IKTTTEQLIEAVNNGDFEAYAKICDPGLTSFEPEALGNLVEGMDFHRFYFENLLAKNSKPIHTTILNPHVHVIGEDAACIAYIRLTQ

(362) TIEDEDAKARKQEI IKTTTEQLIEAVNNGDFEAYAKICDPGLTSFEPEALGNLVEGMDFHRFYFENLLAKNSKPIHTTILNPHVHVIGEDAACIAYIRLTQ

(338) TIEDEDAKARKQEI IKTTTEQLIEAVNNGDFEAYAKICDPGLTSFEPEALGNLVEGMDFHRFYFENLLAKNSKPIHTTILNPHVHVIGEDAACIAYIRLTQ

(377) TIEDEDAKARKQEI IKTTTEQLIEAVNNGDFEAYAKICDPGLTSFEPEALGNLVEGMDFHRFYFENLLAKNSKPIHTTILNPHVHVIGEDAACIAYIRLTQ

(501) YIDGQGRPRTSQSEETRVWHRHEDGKWQNVHFHCSGAPVAPLQ

(462) YIDGQGRPRTSQSEETRVWHRHEDGKWQNVHFHCSGAPVAPLQ

(438) YIDGQGRPRTSQSEETRVWHRHEDGKWQNVHFHCSGAPVAPLQ

(477) YIDGQGRPRTSQSEETRVWHRHEDGKWQNVHFHCSGAPVAPLQ

C

D

Fig. 1A–D. *CaMK II β* . **A** Graphical alignment of *CaMK II β* cDNA sequence with homologous clones from a human insulinoma cDNA library. Clones No. 2 and 3 have a 72 bp deletion at position 988 compared with clone No. 1 and 4. **B** Schematic homology map of the aligned cDNA sequences of the complete rat and human *CaMK II β* coding sequences. A high degree (92% overall) of homology of the nucleotide sequences is shown. ■ > 90%; ▒ > 80%, □ > 70%. **C** Alignment of the deduced amino acid sequences of rat *CaMK II β* and the human *CaMK II β* -subtypes β_1 , β_2 and β_4 . Mismatching amino acids are highlighted and were located within the variable domain with one exception. Arrows indicate the variable regions. **D** RT-PCR of *CaMK II β* and pyruvate dehydrogenase (PDH) in human beta cells. Amplified DNA fragments which were obtained by using overlapping primer pairs for the complete coding sequence of the *CaMK II β* -isoforms (lane 1–5) are shown. Lane 4 shows three different splice variants of the *CaMK II β* -subtypes which were detected and sequenced in this study. PDH: amplification of PDH used to show mRNA/cDNA integrity

Competitive PCR. For competitive PCR [42] internal DNA standards were generated for *CaMK II β* , γ , δ and for PDH. Shortened standard fragments of these sequences were generated by deleting 50 to 80 bp of internal sequence. The standards contained the same sequences within the primer binding region and the remaining internal cDNA sequence. These standards were added at different concentrations to the PCR mix and comparative PCR was done at each concentration. The concentration at which the shortened standard was amplified similarly to the target sequence was taken as indicating similar concentrations of standard and target cDNA. The use of comparative PCR at each quantity of competitive standard assured that similar amplification was obtained over a wide range of cycles thus avoiding a potential source of error in competitive PCR.

Results

To identify subtypes of *CaMK II* expressed in insulin-producing cells a human insulinoma λ -gt11 cDNA library was screened. The complete coding sequence of rat *CaMK II δ_2* was used as a probe because this type of *CaMK II* was the major isoform in rat islets and in INS-1 and RINm5F rat insulinoma cells [29]. A screen at intermediate stringency (42°C hybridisation temperature) allowed the identification of ten positive clones which were subcloned and sequenced. Of the ten, seven clones were 92% homologous to the rat *CaMK II β* cDNA sequence (Fig. 1A), two were 93% homologous to rat *CaMK II δ* cDNA (Fig. 2) and one corresponded to the known sequence of human *CaMK II γ* [43].

The clones representing *CaMK II β* contained a complete coding sequence of 1509 bp encoding for 503 amino acids (EMBL Accession No. AJ252236). This molecule, termed human *CaMK II β_1* differed from the rat subtype β by the absence of the sequence in the first and the third variable domain termed V1 and V3 (Fig. 1B,C) [44]. Furthermore we found a sec-

ond coding sequence of 1437 bp, which had the same nucleotide sequence as the subtype β_1 but had a 72 bp deletion at position 988 (EMBL AJ252237). This subtype, termed β_2 , had deletions of all three variable domains V1 to V3 (Fig. 1B,C).

An alignment of the amino acid sequences of the rat and human *CaMK II β* that were deduced showed an extreme degree of sequence conservation. There was only one amino acid difference outside the variable domains which was C-terminal in the association domain. There was no difference in amino acid sequence of the complete catalytic and regulatory domains from amino acids 1–315. The six other variations were within the variable domains and four of those within the differently spliced fragments (Fig. 1C).

The clones representing *CaMK II δ* contained the previously unknown 727 bp of the 5'-region including the translational start codon and the kinase domain of the enzyme (EMBL AJ252239). The human nucleotide sequence was 93% homologous (Fig. 2) and the amino acid sequence deduced was 100% identical to that of the rat. Additionally 427 bp of the 5' untranslated sequence were cloned (Fig. 2).

To obtain an idea whether human *CaMK II β* is preferentially expressed in the central nervous system as shown in rats, we searched for *CaMK II β* subtypes which might occur in peripheral tissues. To allow scanning of the entire coding sequence five pairs of primers were designed for amplification of overlapping fragments. Screening of different human tissues showed an additional subtype of *CaMK II β* which had an insertion of 45 bp, which corresponds to the V₃ region, immediately following the V₂ region which differed between subtype β_1 and β_2 . This subtype termed *CaMK II β_4* had 1552 bp and was coding for 518 amino acids (EMBL AJ252238) (Fig. 1B,D). All human tissues and cell lines we investigated contained the subtypes β_1 and β_2 . We also detected *CaMK II β_4* in all tissues except for a pheochromocytoma. We were not able to identify a human *CaMK II β_3* in any of the primary human tissues investigated [45].

The *CaMK II β* isoform was extremely difficult to amplify from leucocytes due to very low expressions of the mRNA. A correct amplification of the complete coding sequence was only obtained in 10 out of 30 cDNA samples of human mononuclear cells investigated. Low expression was found in fat and muscle tissue, primary fibroblasts and in primary osteoblast cultures [38]. We additionally tested endocrine tissues and found a strong expression of *CaMK II β* in adrenal cortex, pheochromocytomas and pituitary. To obtain a measure of *CaMK II β* gene expression the number of cycles of PCR needed to obtain a detectable positive signal relative to the signal of the house-keeping enzyme pyruvate dehydrogenase (comparative PCR) was determined. In beta cells, pituitary and a pheochromocytoma a positive signal was ob-

B

We report the complete human cDNA sequence of *CaMK II β* . Remarkably the human *CaMK II* se-

Fig. 3. Semiquantitative and comparative PCR: the coamplification of *CaMK IIβ* and PDH in endocrine and non-endocrine tissues by using the housekeeping enzyme PDH as reference gene (internal standard) is shown. The expression of *CaMK IIβ* was similar to that of PDH in pituitary, pheochromocytoma and beta cells whereas lower levels of *CaMK IIβ* than of PDH were observed in adrenal cortex, liver and leucocytes as indicated by the much earlier appearance of PDH amplification

Fig. 4. Competitive quantitative PCR of *CaMK IIβ*, γ , δ and PDH in human beta cells. Similar amounts of cDNA were amplified in the presence of the indicated amounts of internal standards using specific primers for each *CaMK II* isoform and for PDH. The concentrations of standards shown were adapted for effective competition. The gels are representative for three to seven experiments of each *CaMK II* isoform and PDH

sequence is extremely conserved compared with that of the rat: 99.4% homology at the amino acid level suggesting a strong selection pressure of the protein. There was no difference within the first 316 amino acids comprising the entire kinase and regulatory domains. The differences between rat and human concerned the presence or absence of the variable domains. The V_1 -region [43, 44] was absent in all three human β -subtypes identified. The variations between

the 3 β -subtypes described here all occurred in the V_2 -region and V_3 -region. The β_1 -subtype has deleted V_1 -domains and V_3 -domains. The β_2 -subtype, which has not previously been detected in other species, lacked all variable regions (V_1 – V_3). This is rather unusual for most *CaMK II* subtypes and was previously observed only in the δ_2 subtype.

A third subtype was detected by RT-PCR which only lacked the V_1 -region compared with the rat brain subtypes and was termed β_4 to avoid confusion with the β_3 -subtype described before [17, 45].

We were unable to find the β_3 isoform in human tissue. Another study described a complete nucleotide sequence identity of the rat and human β_3 insertion [45]. This is unusual as we and others [15] observed about 8% and higher variations between rats and humans at the nucleotide level, which was evenly distributed over the entire sequence. The β_3 isoform also was not identified by others [36] who analysed the human *CaMK II* variable region by PCR. Because the β_3 -isoform contains a relatively long insert it could be difficult, however, to amplify by PCR, particularly in the presence of larger quantities of *CaMK IIβ₁* due to competition.

The clone representing the human sequence of *CaMK IIδ* comprised the kinase domain and showed 93% identity at the nucleotide and 100% at the amino acid levels between the rat and the human sequence again showing an extreme degree of sequence conservation. A partial sequence of *CaMK IIδ* was previously reported to be widely expressed in human tissues [36] as shown by PCR analysis of its regulatory domain and has been found in all cell types examined until now.

The distribution of *CaMK IIβ* differed quite considerably between humans and rats where the enzyme is largely confined to the central nervous system and is not expressed in the periphery [3]. In humans the *CaMK IIβ* sequence was detectable by PCR in numerous cell types but the expression differed widely.

The determination of *CaMK II* expression by PCR only does not permit an estimation of relative and absolute expression. For this reason two different methods of quantitative PCR [42] were used, because the semiquantitative (comparative) PCR offers the ability to determine the expression of CaM kinases by comparison with the expression of a reference gene whereas absolute concentrations of amplified cDNA can be quantified by competitive PCR. In this study the pyruvate dehydrogenase was chosen as a reference gene because it is present in most tissues and thought to have a key regulatory role in glucose metabolism and aerobic energy production (housekeeping enzyme). The state of activation of PDH was shown to be increased by high glucose concentrations in beta cells and vice versa [46] and in adipose tissues a correlation of PDH activity and expression was ob-

served [47], indicating a direct influence of carbohydrates on the protein content of PDH.

When compared with PDH very low expression of *CaMK II β* was found in leucocytes where the mRNA was only marginally detectable. Fibroblasts, osteoblasts and tissues such as striated muscle and fat had low expression. In contrast, high expression of mRNA was found in two types of tissue: endocrine cells such as pituitary, adrenal glands and beta cells and, although to a lesser extent, in liver tissue.

In this context it is notable that some genes share high expression in beta cells and the liver and are involved in metabolic regulation such as GLUT1/2 and glucokinase [48]. The high abundance of CaM kinase II β and δ mRNA could lead to the conclusion that these isoforms are important molecules in beta-cell physiology, compared with CaM kinase II γ , but this has to be proven.

At this time the exact role of CaMK II in glucose-stimulated insulin release is not clear. In mammalian brain, *CaMK II β* is highly expressed in synapses where it accounts for about 2 % of the cellular protein [49]. A function in neurotransmitter exocytosis seems probable but its precise function is not known. It seems CaMK II β forms heterooligomers with CaMK II α in the brain and could account for the binding of the complex to cytoskeletal and synaptic structures. A similar function can be presumed for CaMK II β/δ in beta cells. In the insulinoma cell lines INS-1 and RINm5F, CaMK II δ is predominantly associated with insulin granules and attached cytoskeletal structures [29]. In clonal beta cells, synapsin I and microtubule-associated protein (MAP)-2 are substrates which are phosphorylated by CaMK II [31] and which could be related to its effects on insulin secretion [50].

Another possible role of CaMK II is the regulation of gene expression in beta cells.

Antisense suppression of *CaMK II δ* gene expression in INS-1 cells resulted in the loss of glucokinase, GLUT-2 and insulin gene expression, indicating that CaMK II participates in the regulation of these genes [33].

In humans, the mRNAs of the *CaMK II β* and δ -subtypes are highly expressed in beta cells and represent the major isoforms.

Acknowledgements. This study was supported by the German Research Foundation (DFG) (Pf 164/13-2), the German Diabetes Association and by "FoRUM" of the Ruhr-University Bochum.

The Central Unit of Beta-Cell Transplant receives financial support from a shared-cost action of the Medical and Health Research of the European Community (BMH-CT95-1561) and from the Juvenile Diabetes Foundation International (JDF-DIRP 95-97).

H. Heimberg is the recipient of a post-doctoral research fellowship from the Fund for Scientific Research, Flanders (Belgium).

We thank Professor Pipeleers for general support and discussion of this study.

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