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

ARTICLE *in* DIABETOLOGIA · MAY 2000

Impact Factor: 6.67 · DOI: 10.1007/s001250051330 · Source: PubMed

9 AUTHORS, INCLUDING:

Brigitte Lankat-Buttgereit
Philipps University of Marburg
54 PUBLICATIONS **1,595** CITATIONS
SEE PROFILE

Burkhard Göke
University Medical Center Hamburg - Eppe…
762 PUBLICATIONS **17,157** CITATIONS
SEE PROFILE

Michael A Nauck
Diabeteszentrum Bad Lauterberg
348 PUBLICATIONS **20,426** CITATIONS
SEE PROFILE

Andreas Pfeiffer
Charité Universitätsmedizin Berlin
313 PUBLICATIONS **5,282** CITATIONS
SEE PROFILE

Diabetologia (2000) 43: 465–473

Diabetologia

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

H. Rochlitz[1], A. Voigt[1], B. Lankat-Buttgereit[2], B. Göke[2], H. Heimberg[3], M. A. Nauck[4], U. Schiemann[5], H. Schatz[1], A. F. H. Pfeiffer[1]

[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 IIβ* and the kinase domain of *CaMK IIδ* 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 IIβ occurred in three splice variants and was highly expressed in endocrine tissues such as adrenals, pituitary and beta cells. Liver showed moderate expression but adipose tissue or lymphocytes had very low levels of *CaMK IIβ*-mRNA. In human beta cells *CaMK IIβ* and δ were expressed equally with pyruvate dehydrogenase whereas tenfold lower expression of *CaMK IIγ* and no expression of *CaMK II*α were found.
Conclusion/interpretation. Although *CaMK IIδ* is ubiquitously expressed, *CaMK IIβ* 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 IIβ 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 Ca^{2+} 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 Ca^{2+} 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 IIα*, *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 several human tumour cell types and identified two splice variants of *CaMK IIβ*, four of *CaMK IIγ* and two of *CaMK IIδ*.

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 amplificates (bp)
1.	hCamK β1	4	58	CGGGATCCCG-GCCACCACGGTGACCTGCAC ①	282
2.	hCamK β2	285 c	58	GGAATTC-ACCAGTGACCAGATCGAAGACCA ②	
3.	hCamK β3	260	56	ACCTGGTCTTCGATCTGGTC	406
4.	hCamK β4	665 c	56	AGCTTGTGCTGGTCCTCGTC	
5.	hCamK β5	606	55	GATCCTGTACATCCTGCTCG	322
6.	hCamK β6	927 c	55	CAGCATGGTGGTGAGGATGG	
7.	hCamK β7	908	55	CCATCCTCACCACCATGCTG	170–287 ④
8.	hCamK β8	1149 c	55	GATGAGCTGCTCCGTGGTCT	
9.	hCamK β9	1130	58	AGACCACGGAGCAGCTCATC	384
10.	hCamK β12	1513 c	58	TTCACTGCAGCGGGGCCACA	
11.	hCamK β3ex	342	56	ACCTGGTCTTCGATCTGGTC-ACTGTATCCAGCAGATCC ③	341
12.	hCamK γ1	104	53	TCCGCAGGTGTGTGAAGAAA	316
13.	hCamK γ2	419 c	53	ACGATGTCATGCTGGTGGAT	
14.	hCamK γ1ex	193	53	TCCGCAGGTGTGTGAAGAAA-CTAGAACGTGAGGCTCGGA ③	248
15.	hCamK δ1	6	68	TTCGACCACAACCTGCACCA	193
16.	hCamK δ2	198 c	68	ACGGCAGATTCTAGCTTCTC	
17.	hCamK δ1ex	107	68	TCGACCACAACCTGCACCA-CTGGACAAGAATATGCTGCC ③	111
18.	hCamK α1		58	GGTCACTGGTGGGGAACTGC	311
19.	hCamK α2		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 amplificates differ in different splice variants of *CaMK IIβ*
c = start nucleotide on the complementary strand

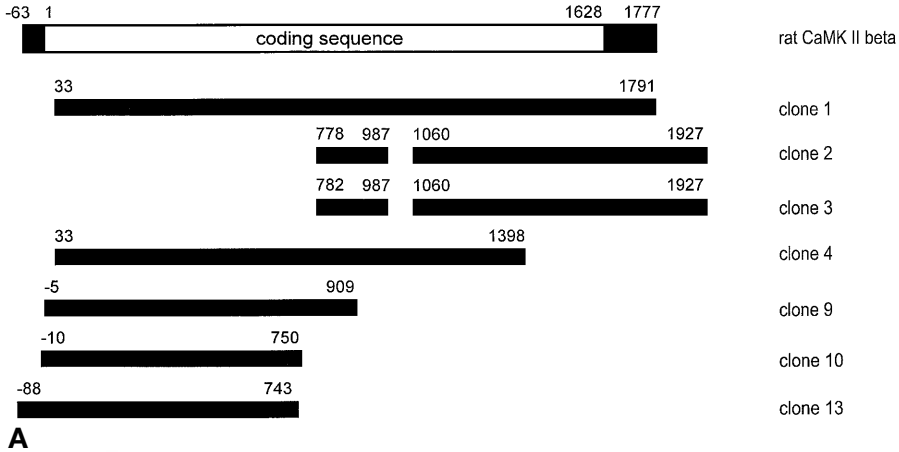
$800 \times g$. Thrombocytes, mononuclear leucocytes, osteoblasts and pituitary adenomas were homogenised by ultrasound, other tissues by an Ultra Turrax homogeniser (Janke and Kunkel, Staufen, Germany).

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 μg of total RNA and the following additions (final concentration): 20 U RNasin, 40 pmol (0.5 μg) random primer $pd(N)_6$, 20 pmol dNTP, reaction buffer as supplied, ultrapure H_2O to a total volume of 20 μl. 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.

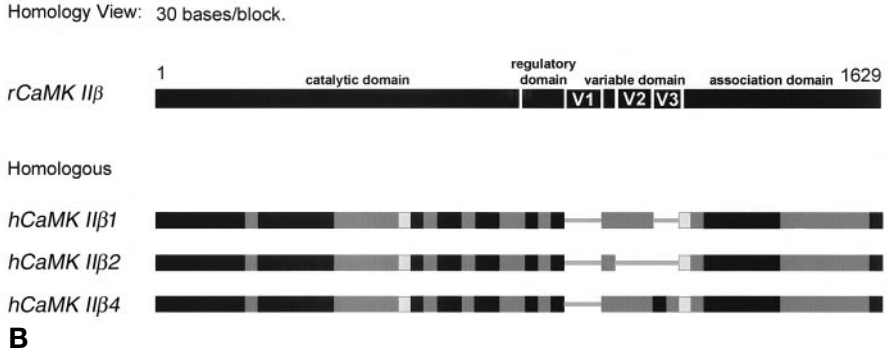
Polymerase chain reaction. All primers were created with Primer Designer 2.0 (Scientific & Educational Software 1991, Durham, N.C., USA). We mixed 10 to 200 ng of reverse transcribed total RNA with the following additions to a final volume of 100 μl (final concentrations): 1.5–3 mmol/l $MgCl_2$, 20 pmol 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 μl 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 amplificates 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 μl 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].



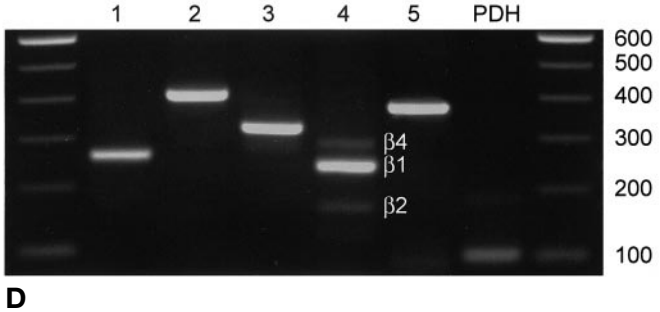
A



B

```
rCaMK β (   1) MATTVTCTRFTDEYQLYEDIGKGAFSVVRRCVKLCTGHEYAAKIINTKKLSARDHQKLEREARICRLLKHSNIVRLHDSISEEGFHYLVFDLVTGGELFE
hCaMK β1(   1) MATTVTCTRFTDEYQLYEDIGKGAFSVVRRCVKLCTGHEYAAKIINTKKLSARDHQKLEREARICRLLKHSNIVRLHDSISEEGFHYLVFDLVTGGELFE
hCaMK β2(   1) MATTVTCTRFTDEYQLYEDIGKGAFSVVRRCVKLCTGHEYAAKIINTKKLSARDHQKLEREARICRLLKHSNIVRLHDSISEEGFHYLVFDLVTGGELFE
hCaMK β4(   1) MATTVTCTRFTDEYQLYEDIGKGAFSVVRRCVKLCTGHEYAAKIINTKKLSARDHQKLEREARICRLLKHSNIVRLHDSISEEGFHYLVFDLVTGGELFE

        (101) DIVAREYYSEADASHCIQQILEAVLHCHQMGVVHRDLKPENLLLASKCKGAAVKLADFGLAIEVQGDQQAWFGFAGTPGYLSPEVLRKEAYGKPVDIWAC
        (101) DIVAREYYSEADASHCIQQILEAVLHCHQMGVVHRDLKPENLLLASKCKGAAVKLADFGLAIEVQGDQQAWFGFAGTPGYLSPEVLRKEAYGKPVDIWAC
        (101) DIVAREYYSEADASHCIQQILEAVLHCHQMGVVHRDLKPENLLLASKCKGAAVKLADFGLAIEVQGDQQAWFGFAGTPGYLSPEVLRKEAYGKPVDIWAC
        (101) DIVAREYYSEADASHCIQQILEAVLHCHQMGVVHRDLKPENLLLASKCKGAAVKLADFGLAIEVQGDQQAWFGFAGTPGYLSPEVLRKEAYGKPVDIWAC

        (201) GVILYILLVGYPPFWDEDQHKLYQQIKAGAYDFPSPEWDTVTPEAKNLINQMLTINPAKRITAHEALKHPWVCQRSTVASMMHRQETVECLKKFNARRKL
        (201) GVILYILLVGYPPFWDEDQHKLYQQIKAGAYDFPSPEWDTVTPEAKNLINQMLTINPAKRITAHEALKHPWVCQRSTVASMMHRQETVECLKKFNARRKL
        (201) GVILYILLVGYPPFWDEDQHKLYQQIKAGAYDFPSPEWDTVTPEAKNLINQMLTINPAKRITAHEALKHPWVCQRSTVASMMHRQETVECLKKFNARRKL
        (201) GVILYILLVGYPPFWDEDQHKLYQQIKAGAYDFPSPEWDTVTPEAKNLINQMLTINPAKRITAHEALKHPWVCQRSTVASMMHRQETVECLKKFNARRKL

        (301) KGAILTTMLATRNFSVGRQTTAPATMSTAASGTTMGLVEQAKSLLNKKADGVKPQTNSTKNSSATTSPKGSLPPAALEPQTTVIHNPVDGIKESSDSTNT
        (301) KGAILTTMLATRNFSA------------------------AKSLLNKKADGVKPQTNSTKNSSATTSPKGTLPPAAL---------------ESSDSTNT
        (301) KGAILTTMLATRNFSA------------------------AKSLLNKKADGVK-----------------------------------------ESSDSTNT
        (301) KGAILTTMLATRNFSA------------------------AKSLLNKKADGVKPQTNSTKNSSATTSPKGTLPPAALEPQTTVIHNPVDGIKESSDSTNT

        (401) TIEDEDAKARKQEIIKTTEQLIEAVNNGDFEAYAKICDPGLTSFEPEALGNLVEGMDFHRFYFENLLAKNSKPIHTTILNPHVHVIGEDAACIAYIRLTQ
        (362) TIEDEDAKARKQEIIKTTEQLIEAVNNGDFEAYAKICDPGLTSFEPEALGNLVEGMDFHRFYFENLLAKNSKPIHTTILNPHVHVIGEDAACIAYIRLTQ
        (338) TIEDEDAKARKQEIIKTTEQLIEAVNNGDFEAYAKICDPGLTSFEPEALGNLVEGMDFHRFYFENLLAKNSKPIHTTILNPHVHVIGEDAACIAYIRLTQ
        (377) TIEDEDAKARKQEIIKTTEQLIEAVNNGDFEAYAKICDPGLTSFEPEALGNLVEGMDFHRFYFENLLAKNSKPIHTTILNPHVHVIGEDAACIAYIRLTQ

        (501) YIDGQGRPRTSQSEETRVWHRRDGKWQNVHFHCSGAPVAPLQ
        (462) YIDGQGRPRTSQSEETRVWHRRDGKWQNVHFHCSGAPVAPLQ
        (438) YIDGQGRPRTSQSEETRVWHRRDGKWQNVHFHCSGAPVAPLQ
        (477) YIDGQGRPRTSQSEETRVWHRRDGKWQNVHFHCSGAPVAPLQ
```

C



D

Fig. 1 A–D. CaMK IIβ. **A** Graphical alignment of *CaMK IIβ* 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 IIβ* 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 IIβ* and the human *CaMK IIβ*-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 IIβ* 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 IIβ*-isoforms (lane 1–5) are shown. Lane 4 shows three different splice variants of the *CaMK IIβ*-subtypes which were detected and sequenced in this study. PDH: amplication of PDH used to show mRNA/cDNA integrity

Competitive PCR. For competitive PCR [42] internal DNA standards were generated for *CaMK IIβ*, 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 IIδ_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 IIβ* cDNA sequence (Fig. 1A), two were 93% homologous to rat *CaMK IIδ* cDNA (Fig. 2) and one corresponded to the known sequence of human *CaMK IIγ* [43].

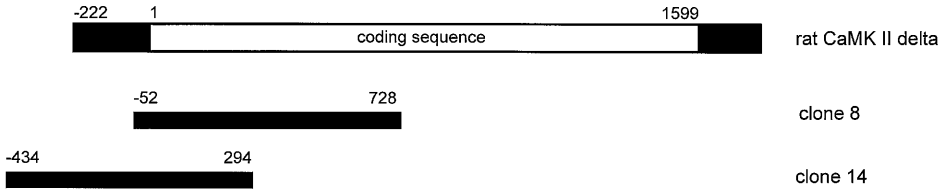
The clones representing *CaMK IIβ* contained a complete coding sequence of 1509 bp encoding for 503 amino acids (EMBL Accession No. AJ252236). This molecule, termed human *CaMK IIβ_1* differed from the rat subtype β by the absence of the sequence in the first and the third variable domain termed V1 and V3 (Fig. 1B,C) [44]. Furthermore we found a second 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 IIβ* 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 IIδ* 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 IIβ* is preferentially expressed in the central nervous system as shown in rats, we searched for *CaMK IIβ* 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 IIβ* which had an insertion of 45 bp, which corresponds to the V_3 region, immediately following the V_2 region which differed between subtype β_1 and β_2. This subtype termed *CaMK IIβ_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 IIβ_4* in all tissues except for a pheochromocytoma. We were not able to identify a human *CaMK IIβ_3* in any of the primary human tissues investigated [45].

The *CaMK IIβ* 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 IIβ* in adrenal cortex, pheochromocytomas and pituitary. To obtain a measure of *CaMK IIβ* gene expression the number of cycles of PCR needed to obtain a detectable positive signal relative to the signal of the housekeeping enzyme pyruvate dehydrogenase (comparative PCR) was determined. In beta cells, pituitary and a pheochromocytoma a positive signal was ob-



A

```
hCaMK IIδ     1 ATGGCTTCGACCACAACCTGCACCAGGTTCACGGACGAGTATCAGCTTTTCGAGGAGCTTGGAAAGGGGGCATTCTCAGTGGTGAGAAGATGTATGAAAA
rCaMK IIδ     1 .............C.........C.......C.............C...........C................................C.......

              101 TTCCTACTGGACAAGAATATGCTGCCAAAATTATCAACACCAAAAAGCTTTCTGCTAGGGATCATCAGAAACTAGAAAGAGAAGCTAGAATCTGCCGTCT
              101 .C..............G.....................................................G.....G...................

              201 TTTGAAGCACCCTAATATTGTGCGACTTCATGATAGCATATCAGAAGAGGGCTTTCACTACTTGGTGTTTGATTTAGTTACTGGAGGTGAACTGTTTGAA
              201 C..........C.........A..........C........C...........C..T.............C...........T..C.....C......

              301 GACATAGTGGCAAGAGAATACTACAGTGAAGCTGATGCCAGTCATTGTATACAGCAGATTCTAGAAAGTGTTAATCATTGTCACCTAAATGGCATAGTTC
              301 ...................T........G.....................A...........G.....A.........................

              401 ACAGGGACCTGAAGCCTGAGAATTTGCTTTTAGCTAGCAAATCCAAGGGAGCAGCTGTGAAATTGGCAGACTTTGGCTTAGCCATAGAAGTTCAAGGGGA
              401 ..............................................A..............C..........C......................C..

              501 CCAGCAGGCGTGGTTTGGTTTTGCTGGCACACCTGGATATCTTTCTCCAGAAGTTTTACGTAAAGATCCTTATGGAAAGCCAGTGGATATGTGGGCATGT
              501 ....................................G.................CC.....................A........C............

              601 GGTGTCATTCTCTATATTCTACTTGTGGGGTATCCACCCTTCTGGGATGAAGACCAACACAGACTCTATCAGCAGATCAAGGCTGGAGCTTATGATTTTC
              601 ..C.....C.....C..CT.G..G.....A..C...................T..G..T.....G.....................G..C.......

              701 CATCACCAGAATGGGACACGGTGACTCC
              701 ..................A.....A..
```

B

Fig. 2. A Graphical alignment of rat *CaMK IIδ* cDNA sequence with homologous clones from a human insulinoma cDNA library. **B** Alignment of the rat and human *CaMK IIδ* nucleotide sequence (homology 93%). Differing bases are shown for the rat *CaMK II* subtype

tained at the same cycle number as PDH whereas in leucocytes at least 10 additional cycles were needed to detect a *CaMK II β*-cDNA signal (Fig. 3). This indicates an about 2^{10}-fold (= 1024) difference in gene expression between these tissues. The highest expression relative to PDH of *CaMK IIβ* was seen in cDNA obtained from purified human beta cells (Fig. 3). Moderate expressions were observed in liver (Fig. 3).

We were particularly interested in investigating the isoforms of *CaMK II* present in human beta cells where *CaMK II* is a possible candidate gene for Type II diabetes. The isoforms present in beta cells were determined because a precise knowledge of the isoforms and relative quantities of the *CaMK II* subtypes should be helpful in deciding which isoform of this enzyme should be considered as a candidate gene for causing Type II diabetes. Primers for detection of human *CaMK IIα*, *β*, *γ* and *δ* were designed and used in RT-PCR of the human beta-cell RNA. Human beta cells expressed *CaMK IIγ*-mRNA and *δ*-mRNA in addition to *CaMK IIβ*. We did not, on the contrary, find *CaMK IIα*, but a positive control for the successful amplification of human *CaMK IIα* was obtained in pheochromocytoma cDNA (data not shown). We identified the subtypes β_1, β_2 and β_4 of *CaMK IIβ* but could not find a PCR product corresponding to the subtype β_3 [45]. The β_1 signal greatly exceeded, however, that of the other isoforms.

In view of the importance of CaMK II in human beta cells it was of interest to obtain information on the quantitative expression of the isoforms. Internal standards were designed for *CaMK IIβ*, *γ* and *δ* and additionally for PDH. Competitive quantitative PCR was carried out using the same amount of beta-cell cDNA in the presence of different internal standard concentrations. On the resulting gels the intensity of the *CaMK II* amplificates (upper bands) and the standard amplificates (lower bands) were compared. For CaM kinase II *β* the competition phenomenon was detected between 10 and 50 fg of internal standard DNA, for CaM kinase II *γ* at 1 fg, for CaM kinase II *δ* and PDH between 50 and 100 fg (Fig. 4). Altogether nine experiments of competitive quantitative PCR with RNA from three subjects were done. In these experiments the average amount of mRNA in human beta cells (means ± SEM) equalled 47.9 fg + 7.86 SEM of *CaMK IIβ*, 41.3 fg + 1.33 SEM of *CaMK IIδ*, 2.4 fg + 0.56 SEM of *CaMK IIγ* and 138.9 fg + 30.9 SEM of the PDH standard DNA. This agrees very well with the results obtained in semiquantitative comparative PCR for *CaMK IIβ* as described above (Fig. 3) and shows that the mRNAs coding for *CaMK IIβ* and *δ* have a high abundance equalling the mRNA of PDH in beta cells.

Discussion

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

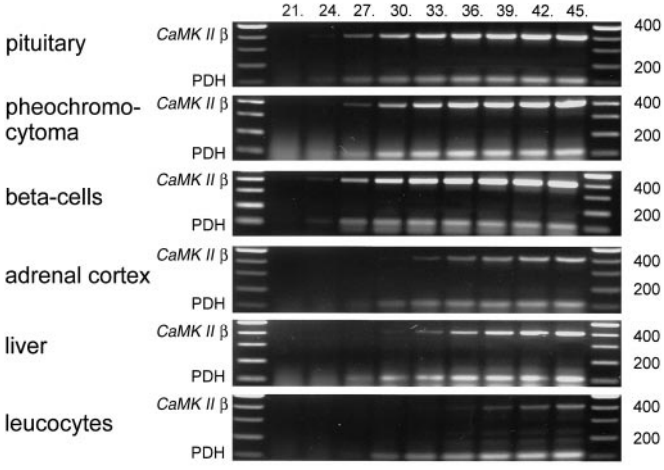


Fig. 3. Semiquantitative and comparative PCR: the coamplification of *CaMK IIβ* and PDH in endocrine and non-endocrine tissues by using the housekeeping enzyme PDH as reference gene (internal standard) is shown. The expression of *CaMK IIβ* was similar to that of PDH in pituitary, pheochromocytoma and 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

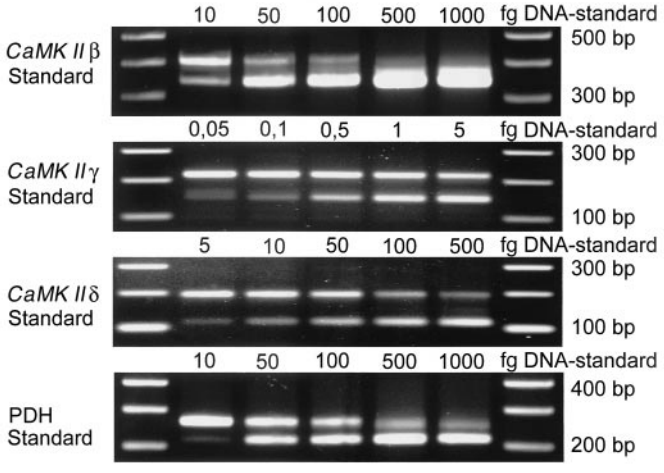


Fig. 4. Competitive quantitative PCR of *CaMK IIβ*, *γ*, *δ* and PDH in human beta cells. Similar amounts of cDNA were amplified in the presence of the indicated amounts of internal standards using specific primers for each *CaMK II* isoform and for PDH. The concentrations of standards shown were adapted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

quence 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 IIβ*$_1$ 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 IIβ* 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 extend, 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 IIβ and δ mRNA could lead to the conclusion that these isoforms are important molecules in beta-cell physiology, compared with CaM kinase IIγ, 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 IIβ* 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 IIβ forms heterooligomers with CaMK IIα 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 IIβ/δ in beta cells. In the insulinoma cell lines INS-1 and RINm5F, CaMK IIδ 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 IIδ* 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 IIβ* 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.

References

1. Schulman H, Hanson PI (1993) Multifunctional Ca2 + / Calmodulin-dependent protein kinase. Neurochem Res 18: 65–77
2. Braun AP, Schulman H (1995) The multifunctional calcium/calmodulin-dependent protein kinase: from form to function. Annu Rev Physiol 57: 417–445
3. Tobimatsu T, Fujisawa H (1989) Tissue specific expression of four types of rat calmodulin dependent protein kinase II mRNAs. J Biol Chem 264: 17907–17912
4. Tobimatsu T, Kameshita J, Fujisawa H (1988) Molecular cloning of the cDNA encoding the third polypeptide (gamma) of brain calmodulin-dependent protein kinase II. J Biol Chem 263: 16082–16086
5. Ashcroft SJH (1994) Protein phosphorylation and β-cell function. Diabetologia 37[Suppl 2]:S21–S29
6. Howell SL, Jones PM, Persaud SJ (1994) Regulation of insulin secretion: the role of second messengers. Diabetologia 37[Suppl 2]:30–35
7. Easom RA (1999) CaM kinase II: a protein kinase with extraordinary talents germane to insulin exocytosis. Diabetes 48: 675–684
8. Rasmussen H, Zawalich KC, Ganesan S, Calle R, Zawalich WS (1990) Physiology and pathophysiology of insulin secretion. Diabetes Care 13: 655–666
9. Levy J, Gavin JR, Sowers JR (1994) Diabetes mellitus: a disease of abnormal cellular calcium metabolism. Am J Med 96: 260–273
10. Schulman H, Lou L (1989) Multifunctional calcium/calmodulin dependent protein kinase: domain structure and regulation. Trends Neurosci 14: 62–66
11. Colbran RJ, Schworer CM, Hashimoto Y et al. (1989) Calcium/calmodulin dependent protein kinase II. Biochem J 258: 313–325
12. Hanson PI, Kapiloff MS, Lou LL, Rosenfeld MG, Schulman H (1989) Expression of a multifunctional calcium/calmodulin-dependent protein kinase and mutational analysis of its autoregulation. Neuron 3: 59–70
13. Colbran RJ, Fong YL, Schworer CM, Soderling TR (1988) Regulatory interactions of the calmodulin-binding, Inhibitory, and autophosphorylation domains of Ca2 + /calmodulin-dependent protein kinase II. J Biol Chem 263: 18145–18151
14. Lickteig R, Shenolikars S, Denner L, Kelly PT (1988) Regulation of Ca2 + /calmodulin-dependent protein kinase II by Ca2 + /calmodulin-independent autophosphorylation. J Biol Chem 263: 19232–19239
15. Nghiem P, Saati SM, Marten SCL, Gardner P, Schulman H (1993) Cloning and analysis of two new isoforms of multifunctional Ca2 + /Calmodulin-dependent protein kinase C. J Biol Chem 268: 5471–5479
16. Mayer P, Möhlig M, Schatz H, Pfeiffer A (1993) New isoforms of multifunctional calcium/calmodulin kinase II. FEBS Lett 333: 315–318
17. Urquidi V, Ashcroft SJ (1995) A novel pancreatic beta cell isoform of calcium/calmodulin-dependent protein kinase II (beta 3 isoform) contains a proline-rich tandem repeat in the association domain. FEBS Lett 358: 23–26
18. Mayer P, Mohlig M, Schatz H, Pfeiffer A (1994) Additional isoforms of multifunctional calcium/calmodulin kinase II in rat heart. Biochem J 298: 757–758
19. McNeill RB, Colbran RJ (1995) Interaction of autophosphorylated Ca2 + /calmodulin-dependent protein kinase II with neuronal cytoskeletal proteins. Characterization of binding to a 190-kDa postsynaptic density protein. J Biol Chem 270: 10043–10049

20. Fährmann M, Möhlig M, Schatz H, Pfeiffer A (1998) Purification and characterization of a Ca2 + /calmodulin-dependent protein kinase II from hog gastric mucosa using a protein-protein affinity chromatographic technique. Eur J Biochem 255: 516–525
21. Watkins DT (1991) Ca2 + /calmodulin-dependent phosphorylation of islet secretory granule proteins. Diabetes 40: 1063–1068
22. Ämmälä C, Eliasson L, Bokvist K et al. (1994) Activation of protein kinases and inhibition of protein phospatases play a central role in the regulation of exocytosis in mouse pancreatic β-cells. Proc Natl Acad Sci USA 91: 4343–4347
23. Wenham RM, Landt M, Easom RA (1994) Glucose activates the multifunctional Ca2 + /calmodulin-dependent protein kinase II in isolated rat pancreatic islets. J Biol Chem 269: 4947–4952
24. Niki I, Okazaki K, Saitoh M et al. (1993) Presence and possible involvement of Ca/Calmodulin-dependent protein kinases in insulin release from the rat pancreatic β cell. Biochem Biophys Res Commun 191: 255–261
25. Wenham RM, Landt M, Walters SM, Hidaka H, Easom RA (1992) Inhibition of insulin secretion by KN-62, a specific inhibitor of the multifunctional Ca2 + /Calmodulin-dependent protein kinase II. Biochem Biophys Res Commun 189: 128–133
26. Li G, Hidaka H, Wollheim CB (1992) Inhibition of voltage-gated Ca2 + channels in insulin secretion in HIT cells by the Ca2 + /Calmodulin-dependent protein kinase II inhibitor KN-62: comparison with antagonists of calmodulin and L-type Ca2 + channels. Mol Pharmacol 42: 489–498
27. Smith MK, Colbran RJ, Brickey DA, Soderling TR (1992) Functional determinants in the autoinhibitory domain of calcium/calmodulin-dependent protein kinase II. J Biol Chem 267: 1761–1768
28. Ämmälä C, Eliasson L, Bokvist K, Larsson O, Ashcroft FM, Rorsman P (1993) Exocytosis elicited by action potentials and voltage clamp calcium currents in individual mouse pancreatic B-cells. J Physiol (Lond) 472: 665–688
29. Möhlig M, Wolter S, Mayer P et al. (1997) Insulinoma cells contain an isoform of Ca2 + /calmodulin dependent protein kinase II delta associated with insulin secretion vesicles. Endocrinology 138: 2577–2584
30. Matsumoto K, Fukunaga K, Miyazaki J-I, Shichiri M, Miyamoto E (1995) Ca2 + /calmodulin-dependent protein kinase II and synapsin I-like protein in mouse insulinoma MIN6 cells. Endocrinol 136: 3784–3793
31. Matsumoto K, Ebihara K, Yamamoto H et al. (1999) Cloning from insulinoma cells of synapsin I associated with insulin secretory granules. J Biol Chem 274: 2053–2059
32. Krueger KA, Bhatt H, Landt M, Easom RA (1997) Calcium-stimulated phosphorylation of MAP-2 in pancreatic betaTC3-cells is mediated by Ca2 + /calmodulin-dependent kinase II. J Biol Chem 272: 27464–27469
33. Osterhoff M, Möhlig M, Ortmann J, Bikashagi F, Schatz H, Pfeiffer A (1999) Antisense-RNA suppression of Ca + + / calmodulin dependent protein kinase IId2 (CaMK II) in INS-1 cells causes a loss of insulin, GLUT-2, glukokinase, and IAPP gene expression. Diabetologia 42[Suppl 1]:A25 (Abstract)
34. Leibiger IB, Leibiger B, Moede T, Berggren P-O (1998) Exocytosis of insulin promotes insulin gene transcription via the insulin receptor/PI-3 kinase/p70 s6 kinase and CaM kinase pathways. Mol Cell 1: 933–938
35. Adams MD, Kerlavage AR, Fleischmann RD et al. (1995) Initial assessment of human gene diversity and expression patterns based upon 83 million nucleotides of cDNA sequence. Nature 377[6547 Suppl]:3–174
36. Tombes RM, Krystal GW (1997) Identification of novel human tumor cell-specific CaMK-II variants. Biochim Biophys Acta 1355: 281–292
37. van-Eyll B, Lankat-Buttgereit B, Bode HP, Goke R, Goke B (1994) Signal transduction of the GLP-1-receptor cloned from a human insulinoma. FEBS Lett 348: 7–13
38. Battmann A, Battmann A, Jundt G, Schulz A (1997) Endosteal human bone cells (EBC) show age-related activity in vitro. Exp Clin Endocrinol Diabetes 105: 98–102
39. Delaney CA, Pavlovic D, Hoorens A, Pipeleers DG, Eizirik DL (1997) Cytokines induce deoxyribonucleic acid strand breaks and apoptosis in human pancreatic islet cells. Endocrinology 138: 2610–2614
40. Chomczynski P, Sacchi N (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159
41. Pfeiffer D, Spranger J, Al-Deiri M et al. (1997) mRNA expression of ligands of the epidermal-growth-factor-receptor in the uterus. Int J Cancer 72: 581–586
42. Köhler T, Laßner D, Rost A-K, Thamm B, Pustowoit B, Remke H (1995) Quantitation of mRNA by polymerase chain reaction – nonradioactive PCR methods. Springer, Berlin Heidelberg New York, p: 143 ff
43. Bennet MK, Kennedy MB (1987) Deduced primary structure of the β-subunit of brain type II calcium/calmodulin-dependent protein kinase determined by molecular cloning. Proc Natl Acad Sci USA 84: 1794–1798
44. Bulleit RF, Bennett MK, Molloy SS, Hurley JB, Kennedy MB (1988) Conserved and variable regions in the subunits of brain type II Ca2 + /calmodulin-dependent protein kinase. Neuron 1: 63–72
45. Breen MA, Ashcroft SJ (1997) Human islets of Langerhans express multiple isoforms of calcium/calmodulin-dependent protein kinase II. Biochem Biophys Res Commun 236: 473–478
46. McCormack JG, Longo EA, Corkey BE (1990) Glucose-induced activation of pyruvate dehydrogenase in isolated rat pancreatic islets. Biochem J 267: 527–530
47. Maury J, Kerbey AL, Priestman DA, Patel MS, Girard J, Ferre P (1995) Pretranslational regulation of pyruvate dehydrogenase complex subunits in white adipose tissue during the suckling-weaning transition in the rat. Biochem J 311: 531–535
48. De-Vos A, Heimberg H, Quartier E et al. (1995) Human and rat beta cells differ in glucose transporter but not in glucokinase gene expression. J Clin Invest 96: 2489–2495
49. Strack S, Choi S, Lovinger DM, Colbran RJ (1997) Translocation of autophosphorylated calcium/calmodulin-dependent protein kinase II to the postsynaptic density. J Biol Chem 272: 13467–13470
50. Krueger KA, Ings EI, Brun A-M, Landt M, Easom RA (1999) Site-specific phosphorylation of synapsin I by Ca2 + /calmodulin-dependent protein kinase II in pancreatic betaTC3 cells. Diabetes 48: 499–506