New isoforms of Ca^{2+}/calmodulin-dependent protein kinase II in smooth muscle

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Four novel isoforms of Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) were found in rat aorta smooth muscle. Two of them were related to γ-isoform of brain CaM kinase II (γ-a). Differences in the primary structure of these isoforms were located in the variable region. One of them (γ-b) contained 23 unique amino acid residues, whereas the other (γ-c) did not contain this sequence. Both isoforms lacked the two segments (Val-316 to Gln-337 and Lys-353 to Leu-362) present in γ-a. The DNA sequence of these γ-isoforms except the variable region was exactly the same, suggesting that they are produced by alternative splicing. Another two isoforms were related to the δ-isoform of brain CaM kinase II (δ-a). δ-b contained a unique 11-residue sequence in the variable region whereas δ-c did not. As found for γ-isoforms, the sequence analysis suggested that the three δ-isoforms are also produced by alternative splicing. Analysis of RNA by reverse transcription PCR confirmed the existence of specific messages for γ-b, δ-a and δ-b. The variety of isoforms of CaM kinase II suggest that each isoform may play a specialized role in cell regulation.

INTRODUCTION

Increases in the cytosolic Ca^{2+} concentration mediate the various transmembrane signal-transduction responses. One of the most important intracellular acceptors of the Ca^{2+} signal is calmodulin (CaM), which exerts a modulating influence on the function of many intracellular proteins (Cohen and Klee, 1988). Ca^{2+}/CaM-dependent protein kinases play an important role in regulating various aspects of Ca^{2+}/CaM-dependent processes. Among them, Ca^{2+}/CaM-dependent protein kinase II (CaM kinase II) shows a broad substrate specificity and has been thought to be a multifunctional protein kinase (Colbran et al., 1989; Hanson and Schulman, 1992). It was originally isolated from brain tissues (Fukunaga et al., 1982; Bennett et al., 1983; Goldenring et al., 1983; Kuret and Schulman, 1984) and has been extensively studied using brain enzyme. Five isoforms from brain have been identified termed α, β, β′, γ and δ. The amino acid sequences of these isoforms have been deduced from their cDNA structures (Lin et al., 1987; Hanley et al., 1987; Bennett and Kennedy, 1987; Bulleit et al., 1988; Tobimatsu et al., 1988; Tobimatsu and Fujisawa, 1989). Although these isoforms are encoded in four independent genes, the amino acid sequence is highly conserved. The α- and β-isoforms are primarily expressed in brain (Lin et al., 1987; Bulleit et al., 1988) as the major components of brain CaM kinase II, whereas the γ- and δ-isoforms are expressed in various tissues (Tobimatsu and Fugisawa, 1989).

Biochemical characterization has also been well studied for brain CaM kinase II. It has been demonstrated to have a molecular mass of approx. 550 kDa and is composed of two distinct subunits with molecular masses of 54 kDa (α-subunit) and 60 kDa (β-subunit) (Goldenring et al., 1983; Bennett et al., 1983; Kuret and Schulman, 1984). Recently, it was revealed by electron microscopy that the holoenzymes of brain CaM kinase II are octamers and decamers in which the catalytic part of the enzyme is tethered together by a central association domain (Kanaseki et al., 1991).

A role for CaM kinase II in the regulation of smooth-muscle function at several steps of signal transduction has been suggested, i.e. modulation of the voltage-dependent Ca^{2+} current (McCarron et al., 1992), possible modulation of ryanodine receptor channel (Witcher et al., 1991; Furutsuka et al., 1992; Wang and Best, 1992), modulation of myosin light-chain kinase activity (Ikebe and Reardon, 1990a; Hashimoto and Soderling, 1990; Stull et al., 1990) and modulation of the function of the thin-filament-binding proteins, caldesmon (Ikebe and Reardon, 1990b) and calponin (Winder and Walsh, 1990). In smooth muscle, CaM kinase II was first isolated as caldesmon kinase (Ikebe et al., 1990) with a molecular mass of the major subunit of 56 kDa. Isolated smooth-muscle CaM kinase II has enzymological properties similar to that of brain (Ikebe et al., 1990); however, smooth-muscle CaM kinase II is a tetramer according to its native molecular mass rather than a decamer or octamer as are the brain enzymes (Zhou and Ikebe, 1992). Furthermore, the major autophosphorylation site responsible for the activation of the enzyme has been identified as a serine six amino acid residues upstream of Thr-286 (Zhou and Ikebe, 1992). The differences in the properties of smooth-muscle kinase from those of the brain enzyme are likely to be due to the difference in the primary structure.

In this study, we cloned smooth-muscle CaM kinase II isoforms, and found four new CaM kinase II isoforms expressed in rat aorta smooth muscle. A preliminary version of this work has appeared in an abstract form (Zhou and Ikebe, 1993).

EXPERIMENTAL

Materials

Sequenase 2.0 was obtained from United States Biochemical Corp. [α-[^{35}S]]dATP (specific radioactivity > 1000 Ci/mmol), [α-^{32}P]dCTP (specific radioactivity, 3000 Ci/mmol) and enhanced chemiluminescence (ECL) Western-blot detection reagents were purchased from Amersham. The enzymes and PCR kit were obtained as follows: restriction endonucleases and DNA polymerase I Klenow fragment, from New England

Abbreviations used: LB medium, Luria–Bertani medium; CaM, calmodulin; CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; 1 × SSC, 0.15 M NaCl + 0.015 M sodium citrate; ECL, enhanced chemiluminescence.

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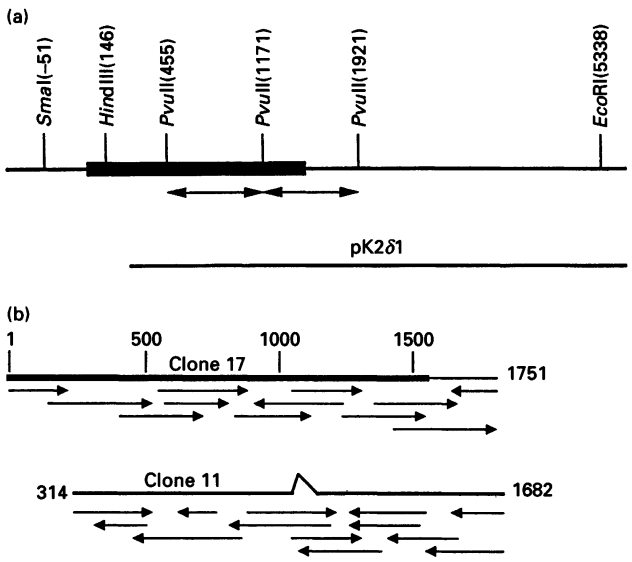

Figure 1 Cloning of rat aorta smooth-muscle CaM kinase II

(**a**) cDNA probe used for cDNA library screening. cDNA of rat brain CaM kinase II δ-isoform (pK2δ1) was digested with *Pvu*II. This produced two fragments, 0.7 kb and 0.75 kb, which were gel-purified, random-priming labelled, and used as probes for library screening. The protein-coding region of cDNA is indicated by a bold line. The region of the cDNA probe is indicated by the horizontal arrows. The relative positions of the restriction sites are also shown. (**b**) Sequencing strategy of aorta γ-CaM kinase II clones. The bold line indicates the coding region of aorta γ-isoforms. The deletion of 69 bp in clone 11 (γ-c) which is present in clone 17 (γ-b) is shown. Arrows indicate the direction and extent of sequence determination.

Biolabs; reverse transcriptase-AMV (from avian myeloblastosis virus), exonuclease III and Mung bean nuclease, from Boehringer-Mannheim Corp.; PCR kit, from Perkin–Elmer. Oligo(dT)-primed rat aorta cDNA library, constructed in the λZapII vector, was a gift from Dr. Arnold Schwartz, University of Cincinnati. Two rat brain CaM kinase II cDNA clones, pK2γ1 of brain γ-isoform (Tobimatsu et al., 1988) and pK2δ1 of brain δ-isoform (Tobimatsu and Fujisawa, 1989), were kindly given by Dr. Hitoshi Fujisawa, Asahikawa Medical College. Two anti-(rat brain CaM kinase) monoclonal antibodies, CBα-2 (specific to α-subunit) and CBβ-1 (specific to β-subunit), were kind gifts from Dr. Howard Schulman, Stanford University.

Isolation of full-length rat aorta CaM kinase II cDNA

Rat aorta λZapII cDNA library was screened using two *Pvu*II fragments of the cDNA of brain δ-isoform as probes, corresponding to nucleotides 455–1171 and 1171–1921 of CaM kinase II δ-subunit (Figure 1a). The DNA fragment was labelled by the random-priming procedure with a specific radioactivity of 1×10^9 c.p.m./μg of DNA. DNA–DNA hybridization for cDNA library screening was carried out at 42 °C for 20–24 h in Church's buffer (Church and Gilbert, 1984) and was followed by a final wash of 0.2–0.5 × SSC and 0.1 % SDS at 42 °C.

The 5′ region of clone 17 (γ-b) was obtained by reverse transcription PCR: a primer pair was selected so that 600 bp of the N-terminal region of the coding region would be amplified. It consisted of nucleotides 24–59 of brain γ-isoform (5′-CCGCCCGCCCATATGGCCACCACCGCCACCTGCACC-3′) and complement of nucleotides 615–638 (5′-CCCGCA GGCCCAGATATCCACAGG-3′). Aorta total RNA (5 μg) was reverse-transcribed with 20 pmol of complementary primer and 25 units of reverse transcriptase at 42 °C for 60 min. The product was then PCR-amplified for 30 cycles (60 s at 94 °C, 60 s at 55 °C, and 90 s at 72 °C).

DNA sequencing

pBluescript SKII(−) containing positive inserts was excised automatically from λ phage on M13 helper phage infection (pBluescript Exo/Mung DNA sequencing system; Stratagene). For each CaM kinase II clone, deletion mutants were produced by exonuclease III and Mung bean nuclease digestion (Henikoff, 1984; Barnes, 1987). DNA sequencing was performed using the dideoxynucleotide chain-termination method (Sanger et al., 1977) with Sequenase 2.0.

Immunoblot analysis

Rat brain and aorta were homogenized in 5 vol. of 2 % SDS and boiled for 5 min. The homogenates (2 μg of protein/lane) were subjected to SDS/PAGE and proteins were electrotransferred to Immobilon membrane (Millipore) at 35 V for 2 h and visualized by the ECL detection method (Amersham).

PCR analysis of CaM kinase II isoforms in rat aorta cDNA library

Total DNA from the rat aorta cDNA library was as follows: 10–20 μl of phage solution (1×10^5 plaque-forming units/μl) of the λZapII cDNA library was added to 50 μl of overnight XL1-Blue culture pellet resuspended in 2.5 ml of 10 mM $MgCl_2$. After incubation at room temperature for 10 min, 2 ml of Luria–Bertani (LB) medium containing 10 mM $MgCl_2$ was added to the phage/bacteria mixture which was left to shake vigorously for 5 h at 37 °C. The lysate was then mixed with an equal volume of LB medium-equilibrated DEAE-Sephacel to remove genomic DNA, and 100 μl of 5 M NaCl and 540 μl of propan-2-ol were added to the supernatant to precipitate phage DNA. Phenol/chloroform was used to finally extract the library cDNA. Some 0.1 μg of the extracted rat aorta total cDNA was used as template for 30 cycles of the amplification process using PCR (30 s at 94 °C, 30 s at 55 °C, 60 s at 72 °C) with the following primers (degenerate positions indicated by parentheses): (1) V$\delta\gamma$5′-5′-CGGAATTC(*Eco*RI site)ACCCATGGG(A)TCTG-TCAACGG(T)TC-3′; (2) V$\delta\gamma$3′-5′-CGGGATCC(*Bam*HI site)TCAAAGGA(C)AGTGAGGCCTGGG(A)TCAC-3′. The sequence of these primers corresponds to the following regions of the rat brain CaM kinase II: nucleotides 804–827 of both γ- and δ-subunits(V$\delta\gamma$5′); nucleotides 1264–1287 of γ-subunit/ nucleotides 1219–1244 of δ-subunit(V$\delta\gamma$3′). The PCR products were subcloned into pBluscript II SK(+) via *Eco*RI and *Bam*HI sites on the primers and sequenced.

Northern-blot analysis

Total RNA (15 μg) from various rat tissues was size-fractionated by agarose-gel electrophoresis and subsequently blotted on to a hybridization transfer membrane (GeneScreen). RNA was hybridized with γ-c cDNA (nucleotides 841–1325 of γ-a, with the two deletions shown in Figure 4) (Tobimatsu et al., 1988) or δ-a cDNA (nucleotides 1028–1466) (Tobimatsu and Fujisawa, 1989) probe at 60 °C, overnight in Church's buffer, and washed with 0.2 × SSC at 60 °C for 1 h. Autoradiography of the blot was carried out at −80 °C for 2 weeks.

Southern-blot analysis

Two primer pairs were selected so that each (γ or δ) variable domain would be amplified. Primer pair γ consisted of

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ATG GAG ACC ACC GCC ACC TGC ACC CGC TTC ACC GAC GAC TAC CAG CTT TTC GAG GAG CTC GGC AAG GGT GCC TTC  75
Met Glu Thr Thr Ala Thr Cys Thr Arg Phe Thr Asp Asp Tyr Gln Leu Phe Glu Glu Leu Gly Lys Gly Ala Phe  25

TCG GTG GTC CGG AGG TGC GTG AAG AAA ACG TCG ACG CAG GAG TAT GCA GCA AAA ATC ATC AAT ACC AAG AAG TTG 150
Ser Val Val Arg Arg Cys Val Lys Lys Thr Ser Thr Gln Glu Tyr Ala Ala Lys Ile Ile Asn Thr Lys Lys Leu  50

TCT GCC CGA GAT CAT CAG AAA CTA GAA CGA GAG GCC CGG ATA TGC CGA CTT CTG AAA CAT CCA AAC ATC GTG CGT 225
Ser Ala Arg Asp His Gln Lys Leu Glu Arg Glu Ala Arg Ile Cys Arg Leu Leu Lys His Pro Asn Ile Val Arg  75

CTC CAT GAC AGT ATT TCT GAA GAA GGG TTT CAC TAC CTC GTG TTT GAC CTT GTT ACG GGA GGG GAG TTG TTT GAA 300
Leu His Asp Ser Ile Ser Glu Glu Gly Phe His Tyr Leu Val Phe Asp Leu Val Thr Gly Gly Glu Leu Phe Glu 100

GAC ATC GTG GCC AGA GAA TAC TAC AGC GAA GCT GAT GCC AGC CAC TGT ATA CAT CAG ATC CTG GAG AGC GTC AAC 375
Asp Ile Val Ala Arg Glu Tyr Tyr Ser Glu Ala Asp Ala Ser His Cys Ile His Gln Ile Leu Glu Ser Val Asn 125

CAC ATC CAC CAG CAT GAC ATC GTC CAC CGG GAC CTG AAG CCC GAG AAC TTG CTG CTG GCG AGT AAA TGC AAG GGT 450
His Ile His Gln His Asp Ile Val His Arg Asp Leu Lys Pro Glu Asn Leu Leu Leu Ala Ser Lys Cys Lys Gly 150

GCT GCG GTC AAG CTA GCT GAT TTT GGC CTG GCC ATC GAA GTG CAG GGA GAG CAG CAG GCT TGG TTT GGT TTT GCT 525
Ala Ala Val Lys Leu Ala Asp Phe Gly Leu Ala Ile Glu Val Gln Gly Glu Gln Gln Ala Trp Phe Gly Phe Ala 175

GGC ACC CCA GGT TAC TTG TCC CCT GAG GTC TTG AGG AAA GAT CCC TAT GGA AAA CCT GTG GAT ATC TGG GCC TGC 600
Gly Thr Pro Gly Tyr Leu Ser Pro Glu Val Leu Arg Lys Asp Pro Tyr Gly Lys Pro Val Asp Ile Trp Ala Cys 200

GGG GTC ATC CTG TAT ATC CTC CTG GTG GGC TAC CCT CCC TTC TGG GAT GAG GAT CAG CAC AAG CTC TAT CAG CAG 675
Gly Val Ile Leu Tyr Ile Leu Leu Val Gly Tyr Pro Pro Phe Trp Asp Glu Asp Gln His Lys Leu Tyr Gln Gln 225

ATC AAA GCT GGA GCC TAC GAT TTC CCA TCA CCA GAA TGG GAC ACA GTC ACT CCT GAA GCT AAG AAC TTG ATC AAC 750
Ile Lys Ala Gly Ala Tyr Asp Phe Pro Ser Pro Glu Trp Asp Thr Val Thr Pro Glu Ala Lys Asn Leu Ile Asn 250

CAG ATG TTG ACC ATA AAC CCT GCA AAG CGT ATC ACA GCT GAC CAG GCT CTC AAG CAC CCA TGG GTC TGT CAA CGG 825
Gln Met Leu Thr Ile Asn Pro Ala Lys Arg Ile Thr Ala Asp Gln Ala Leu Lys His Pro Trp Val Cys Gln Arg 275

TCA ACA GTG GCA TCC ATG ATG CAT CGC CAA GAG ACA GTG GAG TGC TTA CGC AAG TTC AAC GCC CGG AGA AAA CTG 900
Ser Thr Val Ala Ser Met Met His Arg Gln Glu Thr Val Glu Cys Leu Arg Lys Phe Asn Ala Arg Arg Lys Leu 300

AAG GGT GCC ATC CTC ACA ACC ATG CTT GTC TCC AGG AAC TTT TCA GCT GCC AAG AGC CTA TTG AAC AAG AAG TCA 975
Lys Gly Ala Ile Leu Thr Thr Met Leu Val Ser Arg Asn Phe Ser Ala Ala Lys Ser Leu Leu Asn Lys Lys Ser 325

GAT GGC GGC GTC AAG CCA GAG CAA CAA CAA AAA CAG TCT CGT AAG CCC AGC CCA AGA GCC CGC GAT CCC TTG CAG 1050
Asp Gly Gly Val Lys Pro Glu Gln Gln Gln Lys Gln Ser Arg Lys Pro Ser Pro Arg Ala Arg Asp Pro Leu Gln 350

ACG GCC ATG GAG CCA CAA ACC ACC GTG GTA CAT AAT GCT ACA GAT GGG ATC AAG GGC TCC ACA GAG AGC TGC AAC 1125
Thr Ala Met Glu Pro Gln Thr Thr Val Val His Asn Ala Thr Asp Gly Ile Lys Gly Ser Thr Glu Ser Cys Asn 375

ACC ACC ACA GAA GAC GAA GAT CTC AAA GTG CGA AAA CAG GAG ATC ATT AAG ATC ACA GAA CAG CTG ATA GAA GCC 1200
Thr Thr Thr Glu Asp Glu Asp Leu Lys Val Arg Lys Gln Glu Ile Ile Lys Ile Thr Glu Gln Leu Ile Glu Ala 400

ATC AAC AAT GGG GAC TTT GAG GCC TAC ACG AAG ATT TGT GAC CCA GGC CTC ACT TCC TTT GAG CCA GAA GCC CTT 1275
Ile Asn Asn Gly Asp Phe Glu Ala Tyr Thr Lys Ile Cys Asp Pro Gly Leu Thr Ser Phe Glu Pro Glu Ala Leu 425

GGT AAC CTC GTG GAG GGG ATG GAT TTC CAT AAG TTT TAC TTT GAG AAT CTC CTG TCC AAG AAC AGC AAG CCT ATC 1350
Gly Asn Leu Val Glu Gly Met Asp Phe His Lys Phe Tyr Phe Glu Asn Leu Leu Ser Lys Asn Ser Lys Pro Ile 450

CAC ACC ACC ATC CTA AAC CCA CAT GTC CAT GTC ATC GGC GAG GAC GCA GCT TGC ATC GCC TAC ATC CGG CTC ACC 1425
His Thr Thr Ile Leu Asn Pro His Val His Val Ile Gly Glu Asp Ala Ala Cys Ile Ala Tyr Ile Arg Leu Thr 475

CAG TAC ATC GAT GGG CAG GGT CGG CCT CGC ACC AGT CAG TCA GAG GAG ACC CGG GTG TGG CAC CGC CGG GAT GGC 1500
Gln Tyr Ile Asp Gly Gln Gly Arg Pro Arg Thr Ser Gln Ser Glu Glu Thr Arg Val Trp His Arg Arg Asp Gly 500

AAG TGG CTC AAT GTC CAC TAT CAC TGC TCA GGA GCC CCT GCT GCA CCG CTG CAG TGA GCT CAG GCA CAG GGG CAT 1575
Lys Trp Leu Asn Val His Tyr His Cys Ser Gly Ala Pro Ala Ala Pro Leu Gln end                         517

TAG GAG ATT CCA GCT GGA GGC TGA ACC TTC GAA GTC AGT GAC TCT GGA GGG CCT GAG TGA CAG CCG CGG TCC TGT 1650
CCA TTT GAG GTT TAA ACA ATT ACA TTA CAA AGC GCA GCA GCC AAT GAC GCC CCT GCA TGC AGC CCT CCC GCC CGC 1725
CCT TCG TGT CTG TCT CTG CTG TAC TGA GGT GTT TTT TAC                                                 1751
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Figure 2 Nucleotide and deducad amino acid sequence of aorta smooth-muscle clone γ-b

The boxed segment is present in clone 17 (γ-b) but not in clone 11 (γ-c). The CaM-binding site is indicated by a dashed underline. The autophosphorylation residues responsible for production of CaM-independent activity in brain CaM kinase II (Thr) and in the smooth-muscle isoform (Ser) are indicated by solid underlines.

nucleotides 895–914 of brain γ-isoform (5′-CAGTGGAGTG CTTACGCAAG-3′) and complement of nucleotides 1197–1217 (5′-CACTTTATCTTCGTCTTC-3′). Primer pair δ consisted of nucleotides 1082–1101 of brain δ-isoform (5′-CTGTAGACTGCTTGAAGAAA-3′) and complement of nucleotides 1339–1359 (5′-TGCTTTCACGTCTTCATCCTC-3′). Aorta total RNA (5 μg) was reverse-transcribed with 20 pmol of complementary primers, and the products were then PCR-amplified as described above. Standard procedures were used to transfer the PCR fragments from agarose gels to GeneScreen hybridization transfer membrane. The fragments amplified with primer pair γ were probed with two γ subtype-specific oligonucleotide probes, corresponding to the unique insertions of the variable region in γ-isoforms (5′-CCAGAGCAACAACAAAAACAGTCTCGTAAG-3′ of γ-b and 5′-GTTGGCAGGCAGAGCTCCGC-3′ of γ-a). Two δ subtype-specific oligonucleotide probes, corresponding to the unique insertions of the variable region in δ-isoforms, were also used as probes for primer-pair-δ-amplified PCR products. Radiolabelling of the primers was accomplished using [γ-^{32}P]ATP and T_4 polynucleotide kinase (New England Biolabs). Hybridization was carried out at 45 °C overnight in Church's buffer and washed in 0.5 × SSC at 45 °C for 30 min.

RESULTS

The rat aorta cDNA library was screened using *Pvu*II fragments of the cDNA of rat brain CaM kinase II δ-isoform as probes. Of 18 positive clones obtained, nine showed high sequence identity to known rat brain CaM kinase II, as judged from partial sequencing analysis. It was found that five of them shared sequence identity with γ-isoform (clones 3, 5, 11, 16, 17) and four shared sequence identity with δ-isoform (clones 1, 6, 7, 10). Clones 1, 6 and 10, and clones 3 and 16, were identical. The nucleotide sequence of clone 17 is shown in Figure 2. The 5′ region of the sequence was obtained by reverse transcription PCR as described in the Experimental section. Although the majority of the sequence of clone 17 was the same as brain γ

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     His Pro Trp Ile Cys Gln Arg Ser Thr Val Ala Ser Met Met His Arg Gln Glu Thr Val Asp Cys Leu Lys Lys  25
δ-a  CAC CCA TGG ATC TGT CAA CGT TCT ACT GTT GCC TCC ATG ATG CAC AGG CAG GAG ACT GTA GAC TGC TTG AAG AAA  75
δ-b  CAC CCA TGG ATC TGT CAA CGT TCT ACT GTT GCC TCC ATG ATG CAC AGG CAG GAG ACT GTA GAC TGC TTG AAG AAA
δ-c  CAC CCA TGG ATC TGT CAA CGT TCT ACT GTT GCC TCC ATG ATG CAC AGG CAG GAG ACT GTA GAC TGC TTG AAG AAA
                                          *                                   *

     Phe Asn Ala Arg Arg Lys Leu Lys Gly Ala Ile Leu Thr Thr Met Leu Ala Thr Arg Asn Phe Ser Ala Ala Lys  50
δ-a  TTT AAT GCT CGA CGG AAA TTG AAG GGT GCC ATC TTG ACA ACT ATG CTG GCT ACG AGA AAT TTT TCA GCA GCC AAG 150
δ-b  TTT AAT GCT CGA CGG AAA TTG AAG GGT GCC ATC TTG ACA ACT ATG CTG GCT ACG AGA AAT TTT TCA GCA GCC AAG
δ-c  TTT AAT GCT CGA CGG AAA TTG AAG GGT GCC ATC TTG ACA ACT ATG CTG GCT ACG AGA AAT TTT TCA GCA GCC AAG

     Ser Leu Leu Lys Lys Pro Asp Gly Val Lys Lys Arg Lys Ser Ser Ser Ser Val Gln Met Met Ile Asn Asn Lys  75
δ-a  AGT TTG TTG AAG AAA CCG GAT GGG GTA AAG --- --- --- --- --- --- --- --- --- --- ATA AAC AAC AAA 225
δ-b  AGT TTG TTG AAG AAA CCG GAT GGG GTA AAG AAA AGG AAG TCC AGT TCG AGT GTT CAG ATG ATG --- --- --- ---
δ-c  AGT TTG TTG AAG AAA CCG GAT GGG GTA AAG --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

     Ala Asn Val Val Thr Ser Pro Lys Glu Asn Ile Pro Thr Pro Ala Leu Glu Pro Gln Thr Thr Val Ile His Asn 100
δ-a  GCC AAC GTG GTA ACC AGC CCC AAA GAA AAT ATT CCT ACC CCG GCG CTG GAG CCC CAA ACT ACT GTA ATC CAC AAC 300
δ-b  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
δ-c  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

     Pro Asp Gly Asn Lys Glu Ser Thr Glu Ser Ser Asn Thr Thr Ile Glu Asp Glu Asp Val Lys Ala Arg Lys Gln 125
δ-a  CCT GAT GGA AAC AAG GAG TCA ACT GAG AGC TCA AAT ACC ACC ATT GAG GAT GAA GAC GTG AAA GCA CGA AAG CAA 375
δ-b  --- --- --- --- --- GAG TCA ACT GAG AGC TCA AAT ACC ACC ATT GAG GAT GAA GAC GTG AAA GCA CGA AAG CAA
δ-c  --- --- --- --- --- GAG TCA ACT GAG AGC TCA AAT ACC ACC ATT GAG GAT GAA GAC GTG AAA GCA CGA AAG CAA

     Glu Ile Ile Lys Val Thr Glu Gln Leu Ile Glu Ala Ile Asn Asn Gly Asp Phe Glu Ala Tyr Thr Lys Ile Cys 150
δ-a  GAG ATC ATC AAA GTC ACT GAG CAG CTG ATT GAA GCT ATC AAC AAT GGG GAC TTC GAG GCT TAC ACG AAA ATC TGT 450
δ-b  GAG ATC ATC AAA GTC ACT GAG CAG CTG ATT GAA GCT ATC AAC AAT GGG GAC TTC GAG GCT TAC ACG AAA ATC TGT
δ-c  GAG ATC ATC AAA GTC ACT GAG CAG CTG ATT GAA GCT ATC AAC AAT GGG GAC TTC GAG GCT TAC ACG AAA ATC TGT

     Asp Pro Gly Leu Thr Ala Phe Glu 158
δ-a  GAT CCA GGC CTC ACT GCC TTT GAA 474
δ-b  GAT CCA GGC CTC ACT GCC TTT GAA
δ-c  GAT CCA GGC CTC ACT GCC TTT GAA
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Figure 3 Partial nucleotide and deduced amino acid sequence of the variable domain of aorta smooth-muscle δ-isoforms

Sequences are aligned for maximum identity and gaps are indicated by dashed lines. The CaM-binding region is indicated by a solid underline. The autophosphorylation sites responsible for the activation of the kinase are shown by asterisks (Thr for brain enzyme and Ser for smooth-muscle kinase).

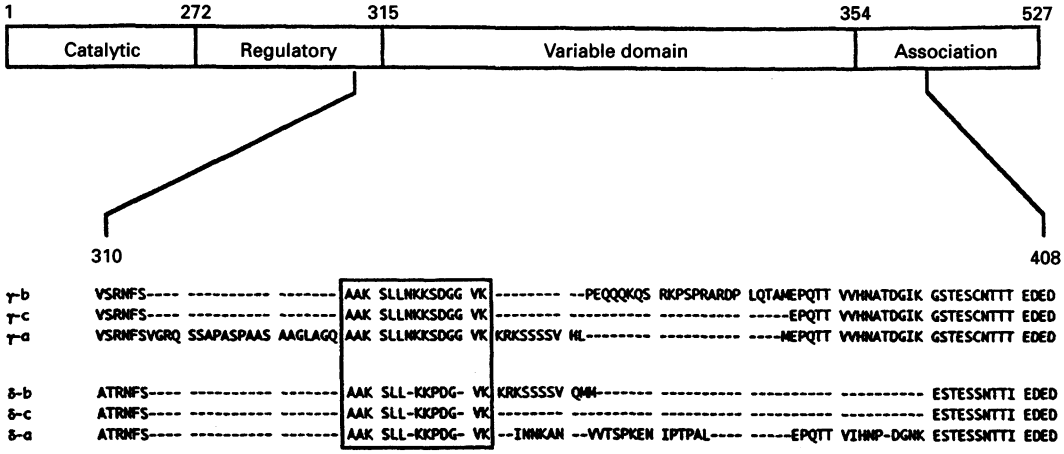

Figure 4 Alignment of the deduced amino acid sequence of various smooth-muscle γ- and δ-isoforms of CaM kinase II

Amino acids are respresented by the single-letter code. The sequences are aligned for maximum identity, and gaps are indicated by dashed lines. Identical residues among the isoforms are boxed.

(γ-a), clone 17 (γ-b) was distinct from the brain γ-isoform in two respects. First, clone 17 contained a unique 69 nucleotides (23 amino acids; boxed sequence in Figure 2) downstream of the regulatory region. Second, the two segments present in the brain γ-isoform at the variable region were deleted in clone 17 (Figure 4). The sequence of clone 3 was shorter and completely included in clone 17. Clone 11 (γ-c) did not contain the sequence from position 988 to 1056 (the boxed segment in Figure 2). On the other hand, two clones that shared sequence identity with the brain δ-isoform (δ-a) contained a cDNA insert that did not cover the variable region. Therefore, to examine whether or not smooth muscle expresses novel variants of δ-isoform and other γ-isoforms, we performed PCR using the total cDNA of the rat aorta library as template. The primers were designed so as to sandwich the variable region of both γ- and δ-isoform (see the Experimental section).

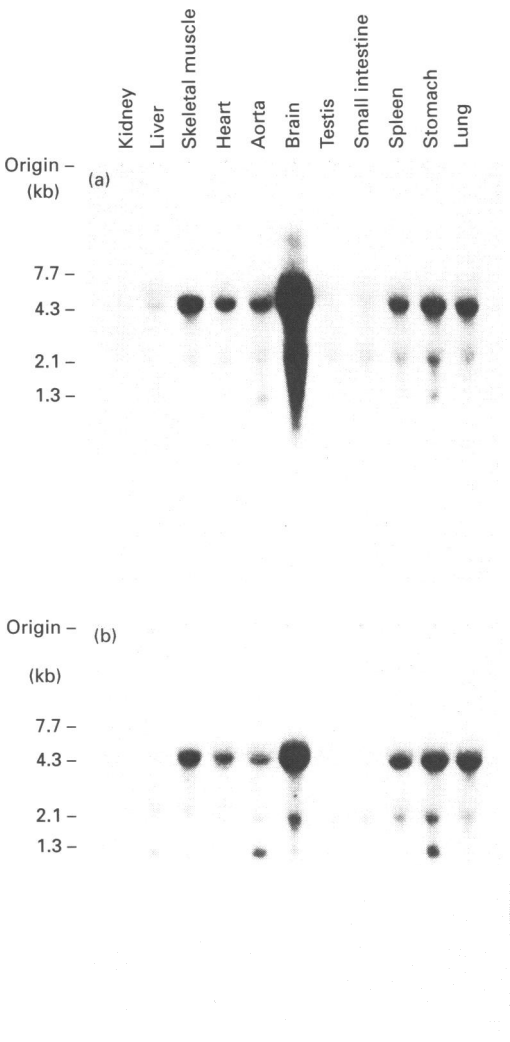

Figure 5 Northern-blot analysis of mRNA level of γ- and δ-isoforms in various tissues

Total RNA (15 μg) from various rat tissues was hybridized with labelled (**a**) γ-c cDNA (nucleotides 841–1325 of γ-a, with two deletions shown in Figure 4), and (**b**) δ-a cDNA (nucleotides 1028–1466) at 60 °C in Church's buffer as described in the Experimental section. Autoradiography was carried out at −80 °C for 2 weeks.

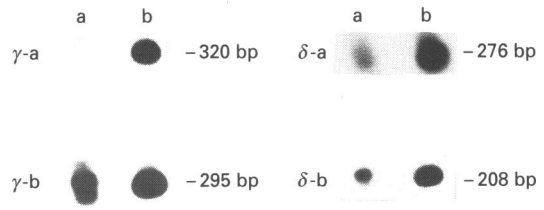

Figure 6 Southern-blot analysis of mRNA expression level of specific aorta CaM kinase II subtypes

Reverse transcription-PCR was performed using primer pairs to amplify the variable region in either the γ- or δ-isoform as described in the Experimental section. The fragments amplified with primer pair γ were probed respectively with two γ-subtype-specific oligonucleotide probes, corresponding to the unique insertions of the variable region in γ-isoforms. The cDNAs amplified with primer pair δ were probed with the corresponding δ subtype-specific oligonucleotides at 45 °C in Church's buffer and washed with 0.5 × SSC. Autoradiography was carried out at −80 °C for 2–20 h. Lane a, reverse transcription PCR products; lane b, PCR-amplified fragments using corresponding cloned CaM kinase II cDNAs as templates (positive control).

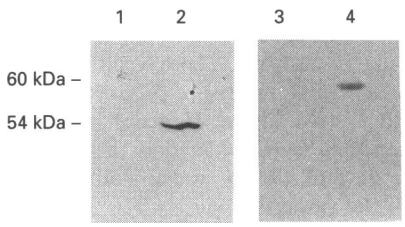

Figure 7 Western-blot analysis with α- and β-isoform-specific monoclonal antibodies

Total tissue homogenates of rat brain and aorta (2 μg) were applied. α- and β-CaM kinase II were detected as described in the Experimental section. Lanes 1 and 2, incubated with anti-CaM kinase II α monoclonal antibody (CBα-2); lanes 3 and 4, incubated with anti-CaM kinase II β monoclonal antibody (CBβ-1). Lanes 1 and 3, rat aorta homogenate; lanes 2 and 4, rat brain homogenate.

PCR products obtained were subcloned into pBluescript II SK(+) vector. Plasmid DNAs were prepared from 51 individual clones. Of these, 27 were δ-isoform variants (12 were δ-a, 11 were δ-b and four were δ-c) and 24 were γ-isoform variants (one was γ-b and 23 were γ-c). These clones were sequenced, and three δ-isoform variants and two γ-isoform variants were found. Of the three δ-isoforms, one (δ-a) had a sequence identical with that of the brain δ isoform (Figure 3). However, the other two clones were found to be novel δ-isoforms. One (δ-b) lacked 102 nucleotides present in δ-a but contained 33 novel nucleotides in the same region (Figure 3). The other clone (δ-c) also lacked 102 nucleotides at the same position but no insertion was found (Figure 3). The sequences of the two γ-isoform variants were the same as the sequence of clones 17 (γ-b) and 11 (γ-c) obtained from library screening. However, we did not detect the γ-isoform found in brain (γ-a). The amino acid sequence alignment of the three γ-isoform variants and three δ-isoform variants at the variable region deduced from the nucleotide sequence is shown in Figure 4. γ-a contained two unique segments, 21 and 11 amino acid residues, which were deleted in both γ-b and γ-c. In γ-b, however, 23 unique residues were inserted instead. δ-a contained a unique 34-amino-acid sequence which was not present in either δ-b or δ-c. δ-b contained 11 unique amino acid residues instead of the deleted segment. It is interesting that the inserted unique segment in δ-b shared significant sequence identity with the second unique segment of γ-a (Figure 4). These results clearly show that mRNAs encoding the five CaM kinase II isoforms (γ-b, γ-c, δ-a, δ-b and δ-c) are present in rat aorta smooth muscle. The structural properties of the variable region of these isoforms were analysed. Hydropathy analysis revealed that the γ-b unique sequence was quite hydrophilic and had a high surface probability whereas the γ-a unique sequence was rather hydrophobic with a low surface probability. On the other hand, the unique sequence of δ-a and δ-b did not show any particular hydrophobicity or hydrophilicity properties (results not shown).

To establish whether γ- and δ-isoforms were coded for by differently sized mRNAs, and to study the tissue distribution of the two isoforms, a Northern-blot analysis of total RNA from various rat tissues was carried out (Figure 5). Using the PCR product of either γ-c (nucleotides 841–1325 of γ-a, with two deletions) or δ-a (nucleotides 1028–1466) as a probe for RNA blotting, a 4.3 kb message was detected in rat skeletal muscle, heart, aorta, brain, spleen, stomach and lung, but not much was detected in kidney, liver, testis and small intestine. As isoform-

specific insertions were present in γ-a, γ-b, δ-a and δ-b, and the level of expression for both γ- and δ-isoforms was relatively low (2-week exposure of Northern-blot membrane), the aorta RNA was amplified by reverse transcription PCR, and Southern-blot analysis was performed using specific oligonucleotide probes. The results showed the existence of specific messages for γ-b, δ-a and δ-b in rat aorta smooth muscle (Figure 6). No γ-a message was detected, which was consistent with the finding that, of 51 individual clones, no γ-a clone was found.

It is known that the major CaM kinase II isoforms expressed in brain are α and β. To see if these isoforms are also expressed in rat aorta, the expression of α- and β-isoforms was examined by Western-blot analysis using isoform-specific monoclonal antibodies as probes. Neither α- nor β-isoform was detected in rat aorta (Figure 7).

DISCUSSION

We have demonstrated four novel CaM kinase II isoforms in rat aorta smooth muscle. Two were γ-isoform variants and two were δ-isoform variants. The difference between the nucleotide sequences of these variants was centred at the variable domain (Figure 4); the rest of the sequence was exactly the same as the γ- or δ-isoform found in rat brain. These results strongly suggest that the γ- and δ-isoform variants are produced by alternative splicing of γ- and δ-CaM kinase II genes respectively. This notion is also supported by the fact that the positions of the deletion and insertion of the γ- and δ-isoform variants are common for all of the variants found.

It has been previously shown (Zhou and Ikebe, 1992) that smooth-muscle CaM kinase II has a tetrameric holoenzyme structure in contrast with the brain CaM kinase II isoforms, the holoenzyme structure of which is a decamer or octamer (Kaneseki et al., 1991). It was also found (Zhou and Ikebe, 1992) that the major autophosphorylation site of smooth-muscle CaM kinase II is a serine residue seven residues upstream of the major autophosphorylation site of the brain enzyme, Thr-286. The present results imply that the difference in the biochemical properties of the smooth-muscle CaM kinase II from those of brain enzymes previously reported is likely to be due to the differences in the structure of the variable region of the kinase. For example, the inserted sequence of γ-b was hydrophilic and predicted to have a high surface probability whereas the γ-a specific sequence was rather hydrophobic with low surface probability. These differences may cause the change in the holoenzyme structure which may be important to the function of each CaM kinase II isoform.

The present results suggest that the majority of CaM kinase II isoforms in smooth-muscle cells are novel CaM kinase II isoforms because: (1) α- and β-isoform-specific monoclonal antibodies (prepared against rat brain enzymes) failed to detect the expression of α- and β-isoforms in rat aorta (Figure 7); (2) Northern-blot analysis revealed that mRNA corresponding to γ- and δ-isoforms was present in aorta (Figure 5); (3) reverse transcription PCR revealed that γ-b and δ-b were present in aorta (Figure 6); (4) mRNA corresponding to the isoforms γ-b, γ-c, δ-a, δ-b and δ-c must be present, as the cDNA library contained these clones (Figures 2 and 3).

While this manuscript was in preparation, Nghiem et al. (1993) reported two novel γ-isoform variants from human T-lymphocytes. One of them is the same as the γ-c isoform found in rat aorta smooth muscle and the other has a 23-amino-acid-residue insert at the same position as found in the γ-b isoform described in this study. However, the inserted sequence in human T-lymphocyte CaM kinase II, i.e. Pro-Gln-Ser-Asn-Asn-Lys-Asn-Ser-Leu-Val-Ser-Pro-Ala-Gln-Glu-Pro-Ala-Pro-Leu-Gln-Thr-Ala-Met, is distinct from the inserted sequence found in the present study, i.e. Pro*-Glu-Gln-Gln-Lys*-Gln-Ser*-Arg-Lys-Pro-Ser-Pro-Arg-Ala-Arg-Asp-Pro*-Leu*-Gln*-Thr*-Ala*-Met*, where the common residues are shown with asterisks and the unique residues are underlined.

The physiological function of each isoform of CaM kinase II is obscure, but, it is likely that the various isoforms have specific roles for modulation of cell processes. This might be characterized by possible differences in substrate specificity and subcellular localization. It is known that CaM kinase II exists in both a soluble form in the cytosol and a membrane-bound form in the cytoskeletal compartment (Kennedy et al., 1983; Goldenring et al., 1983; Kelly et al., 1984; Miller and Kennedy, 1985). The membrane-associated domain of the cytoskeletal enzyme must reside in the C-terminal domain (including variable region), as a 30 kDa constitutively active fragment produced from the membrane-associated enzyme containing an N-terminal catalytic domain but not a CaM-binding site is soluble and does not bind to the membrane (Levine and Sahyoun, 1987). The association sites of the CaM kinase II with the membrane are obscure. However, because the primary structure (except the variable region) is highly conserved among various isoforms including α and β (approx 85% of residues are identical among the isoforms), it is reasonable to assume that the variable region may be responsible for the membrane association of certain isoforms. Taking into account the fact that the variation of the structure of CaM kinase II isoforms is centred in the variable domain, this region may modulate the differential subcellular localization of each isoform of CaM kinase II, although this requires further investigation.

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