Molecular cloning and expression of the calmodulin gene from guinea pig hearts

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Abstract. The aim of the present study was to isolate and characterize a complementary DNA (cDNA) clone encoding the calmodulin (CaM; GenBank accession no. FJ012165) gene from guinea pig hearts. The CaM gene was amplified from cDNA collected from guinea pig hearts and inserted into a pGEM®-T Easy vector. Subsequently, CaM nucleotide and protein sequence similarity analysis was conducted between guinea pigs and other species. In addition, reverse transcription-polymerase chain reaction (RT-PCR) was performed to investigate the CaM 3 expression patterns in different guinea pig tissues. Sequence analysis revealed that the CaM gene isolated from the guinea pig heart had ~90% sequence identity with the CaM 3 genes in humans, mice and rats. Furthermore, the deduced peptide sequences of CaM 3 in the guinea pig showed 100% homology to the CaM proteins from other species. In addition, the RT-PCR results indicated that CaM 3 was widely and differentially expressed in guinea pigs. In conclusion, the current study provided valuable information with regard to the cloning and expression of CaM 3 in guinea pig hearts. These findings may be helpful for understanding the function of CaM3 and the possible role of CaM3 in cardiovascular diseases.

Introduction

Calmodulin (CaM) is a ubiquitous, multifunctional Ca^{2+}-binding protein that is involved in the regulation of numerous important physiological functions, including neural activity, gene expression, enzyme regulation and muscle contraction (1). A previous study demonstrated that the elevation of CaM levels in transformed cells directly affected the rate of cell proliferation (2). Numerous CaM-binding proteins have been identified, a number of which have been shown to be critical for the regulation of cell functions (3-5). CaM regulates the activity of the majority of its binding partners in a Ca^{2+}-dependent manner (6). For example, CaM binds to L-type Ca^{2+} channels, resulting in CaM-channel complexes that are essential for Ca^{2+}-dependent facilitation and inactivation (7). Furthermore, CaM plays a vital role in numerous diseases by participating in signaling pathways that regulate multiple crucial physiological processes. It has previously been reported that CaM may be an important mediator for Ca^{2+} homeostasis in Alzheimer's disease (8). In addition, defects in CaM functions disrupt important calcium signaling events in the heart, affecting membrane ion channels and inducing arrhythmias (9).

CaM is a relatively small protein with only 148 amino acids. The protein is highly conserved across different species, and comprises four EF hands that form two structurally similar domains connected by a flexible central linker (10). A previous study demonstrated that CaM is encoded by multiple genes in vertebrates and invertebrates, as was first reported in chickens (11). Subsequently, CaM 1, 2 and 3 have been cloned, sequenced and characterized in rats (12-14) and humans (15-17). Furthermore, single-copy genes of CaM have been identified and cloned in *Dictyostelium discoideum* (18), *Chlamydomonas* (19) and yeasts (20,21). However, to the best of our knowledge, the genetic information of CaM in guinea pigs has never been established.

Guinea pigs are one of the most widely used models for various diseases, including pulmonary, gastrointestinal and other life-threatening infections (22-24). The electrophysiological features of cardiac Ca^{2+} channels have been extensively studied in guinea pig cardiomyocytes (25,26). Moreover, numerous findings have highlighted the importance of CaM in the regulation of cardiac Ca^{2+} channel-based activities (25,26). Therefore, it is necessary to identify the molecular fundamentals of CaM in guinea pig hearts.

In order to ascertain the CaM gene information in the guinea pig genome, CaM genes were isolated from guinea pig hearts and characterized. Therefore, the expression pattern of CaM 3 in guinea pigs was investigated with the aim to improve the understanding of CaM 3 functions.

Materials and methods

Bacterial strains, vectors and media. In order to clone the CaM gene from guinea pig hearts, *E. coli* JM109 (Takara Bio Inc., Otsu,

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Key words: calmodulin, cloning, guinea pig, gene expression

Table I. Nucleotide sequences of the primers used in polymerase chain reaction amplification.

Primers	Primer sequences
CaM 3 coding region	Forward: 5'-ATGGCTGACCAGCTGAC-3' Reverse: 5'-CTTTGCAGTCATCATC-3'
CaM 3 3'-UTR	Forward: 5'-ATGGCTGACCAACTGACTGAAGAG-3' Reverse: 5'-TACCGTCGTTCCACTAGTGATTT-3'
CaM 3 5'-UTR	Forward: 5'-GCCGGAGGAACCTTG-3' Reverse: 5'-GCTCTGCTTCAGTGGG-3'
β-actin	Forward: 5'-CCAACTGGGACGACATGGAG-3' Reverse: 5'-CGTAGCCCTCGTAGATGGGC-3'

CaM, calmodulin; UTR, untranslated region.

Japan) was employed as the host cell, with the pGEM®-T Easy TA cloning vector (Promega Corporation, Madison, WI, USA) used as the host-vector system. The *E. coli* cells were grown at 37˚C in lysogeny broth agar plates containing ampicillin, with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for the selection of positive clones. The plasmid mini kit and gel extraction kit were purchased from Axygen (Union City, CA, USA).

Molecular cloning of CaM cDNA from guinea pig hearts. Experiments were carried out following approval from the Committee of Animal Experimentation at China Medical University (Shenyang, China). Six guinea pigs (either gender) were used in this study. They were purchased from the Department of Laboratory Animal, China Medical University (Shenyang, China). Following anesthetization by ether (Tiangen Biotech Co., Ltd., Beijing, China), adult guinea pigs (weight, 250-300 g) were sacrificed by decapitation, and the left ventricular myocardium was quickly removed, frozen in liquid nitrogen and stored at -80˚C. Total RNA from the tissue was isolated using TRIzol® reagent (Invitrogen Life Technologies, Grand Island, NY, USA), and the RNA obtained was reverse transcribed to cDNA using avian myeloblastosis virus reverse transcriptase with an RNA polymerase chain reaction (PCR) kit (version 3.0; Takara), oligo-(dT) and random primers, according to the manufacturer's instructions. The cDNA was then subjected to normal PCR amplification with Taq DNA polymerase (Takara), or rapid amplification of the cDNA end (3'-RACE) with a 3' full RACE kit (Takara. Since the nucleotide sequences of CaM, including the untranslated regions (UTRs), were known to be highly conserved among mammals, nucleotide oligomers based on multiple alignments of the highly conserved areas from humans and rats were employed as primers for the PCR to amplify the coding region and the 5'-UTR. With regard to the cloning of the 3'-UTR, 3'-RACE was carried out with the gene-specific forward primer corresponding to the N-terminal structure of the coding region, while GeneRacer Oligo dT (Takara) was used as the reverse primer. The primers used are shown in Table I. The amplification conditions included an initial denaturation for 3 min at 94˚C, followed by 30 cycles of denaturation for 1 min at 94˚C, annealing for 1 min according to the melting temperature of the primers, extension for 1 min at 72˚C, and a final extension for 10 min at 72˚C. PCR products of the expected size were purified from the agarose gel using a gel extraction kit. The cDNA fragments obtained were subcloned into the pGEM®-T Easy vector, and sequenced by Sangon Biotech Co., Ltd. (Shanghai, China). The sequence of each cDNA was determined from more than three independent clones, which was subsequently used to deduce the full length cDNA sequence.

Bioinformatics analysis. Analyses for nucleotide and protein sequence similarities were conducted with the BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). Multiple comparisons were conducted using DNASTAR software (DNASTAR, Madison, WI, USA).

Reverse transcription PCR (RT-PCR). The mRNA expression levels of CaM 3 were analyzed semi-quantitatively using RT-PCR. Total RNA was isolated from 50-100-mg tissue samples collected from the left ventricle, cerebral cortex, cerebellum, small intestine, aorta, kidney, lung, liver, skeletal muscle and spleen of the guinea pigs, as described previously. Aliquots of the RNA solutions were added to the RT mixture prepared from the RNA PCR kit, and following the RT reaction, PCR was conducted for 30 cycles. The primer pairs were specific for CaM 3, and the sequences were as follows: Forward, 5'-AAGGATGGAGATGGCACTATTACCA-3'; and reverse, 5'-AGGGGAGTGAAGGAGAGAAAAGAGC-3'; the gene product was 461 bp. GAPDH, a constitutively expressed gene, was used as an internal standard to verify the RT-PCR assay. The sequences of the GAPDH primers were as follows: Forward, 5'-TTCCAGTATGATTCTACCCACG-3'; and reverse, 5'-CCCTCCACAATGCCGAAG-3'. These primers were used to amplify a 400-bp fragment of the guinea pig GAPDH cDNA. Diethylpyrocarbonate-water for the replacement of the cDNA template was used as a negative control. PCR products were analyzed on a 1.2% agarose gel.

Results

Cloning of the CaM 3 gene in guinea pigs. In order to clone the CaM gene from guinea pigs, the primers were designed based on the regions of the highest reported homologous nucleotide sequences of CaM from humans, mice and rats, as shown in Table I. The encoding region of CaM cDNA from the guinea pig hearts was subsequently isolated and amplified

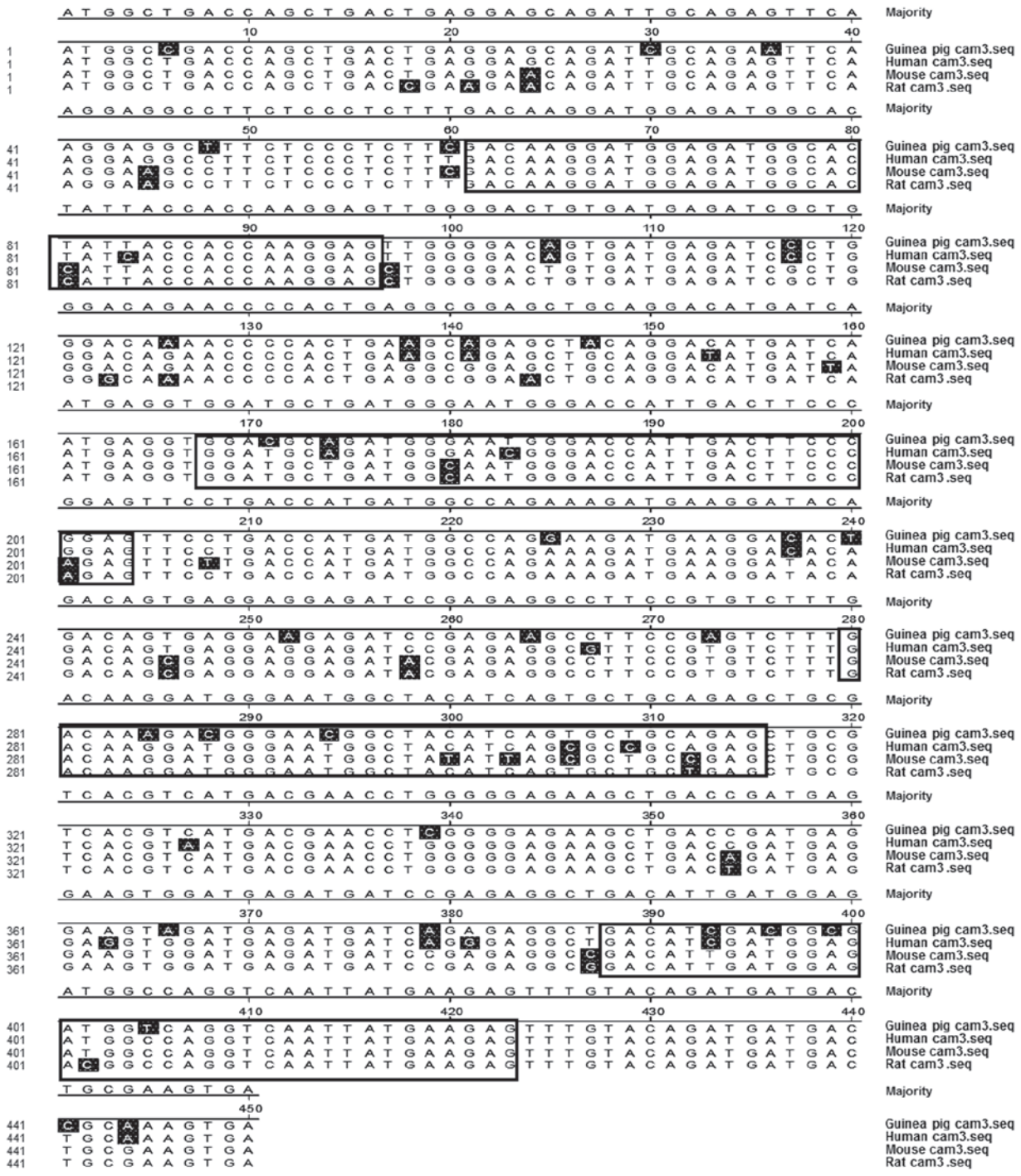

Figure 1. Sequence alignment of the CaM coding regions from different species. The CaM 3 cDNA sequences from guinea pigs, humans, mice and rats were aligned to show the high degree of homology. Shaded residues were different from the consensus. The black frames indicate the coding regions for the Ca^{2+}-binding sites. CaM, calmodulin.

using RT-PCR, after which the genetic information was inserted into the vectors. Nucleotide sequencing of CaM was determined in three independent clones, which revealed identical sequences (Fig. 1).

The comparison of CaM 3 sequences between different species indicated that the coding regions of CaM were similar to those from humans, mice and rats (Fig. 1). Bioinformatics analysis further indicated that the CaM gene in guinea pigs shared high sequence homology with humans (93.1% similarity), mice (89.8%) and rats (89.8%) (Fig. 2A). In addition, the phylogenetic tree revealed close evolutionary associations between these groups of CaM 3 genes (Fig. 2B). These results indicated that the CaM gene isolated from the guinea pigs was likely to be CaM 3.

The deduced amino acid sequences of the CaM 3 coding nucleotide sequences isolated from the guinea pigs revealed 100% similarity to those products of the CaM 3 genes from humans, mice and rats (Fig. 3). The sequences contained four highly conserved Ca^{2+}-binding domains that were characteristic of CaM (Fig. 3). Based on these results, even though

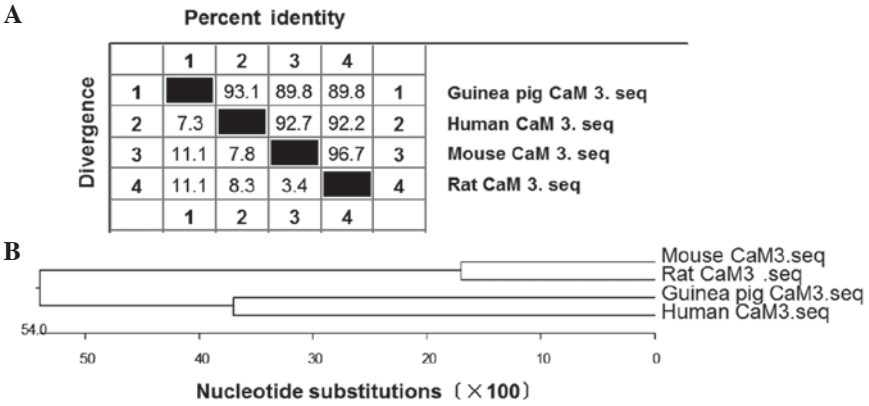

Figure 2. Phylogenetic analysis of CaM 3 genes from different species. (A) Percentage identity of alignment showing the homology of the CaM 3 sequences from guinea pigs compared with those from humans, mice and rats. (B) Phylogenetic tree of the CaM 3 cDNA coding regions from the alignment. CaM, calmodulin.

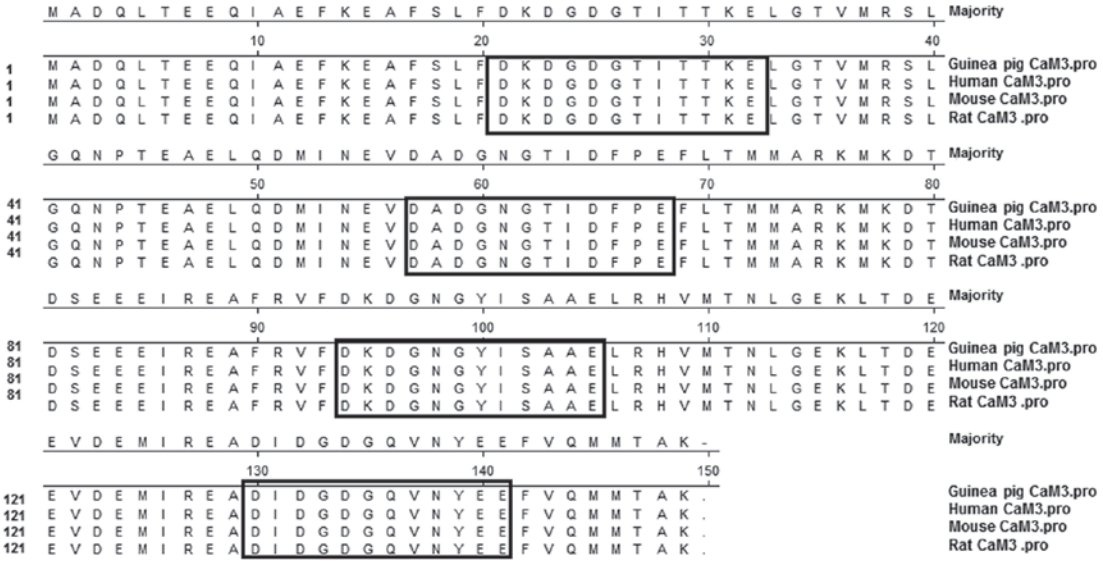

Figure 3. Peptide alignment of CaM 3 from different species. The amino acid sequences of CaM 3 from guinea pigs, humans, mice and rats are consistent. Amino acid sequences for the Ca^{2+}-binding sites are marked with black frames. CaM, calmodulin.

the base sequences were not exactly the same, the predicted amino acid sequences of the guinea pig CaM 3 showed 100% homology to the CaM proteins from other species.

Cloning of the 5'- and 3'-UTR sequences of CaM 3 in guinea pigs. To obtain more information on the CaM gene obtained from guinea pig hearts, the 5'- and 3'-UTR sequences were determined through the methods of RT-PCR and 3'-RACE PCR, respectively. The cDNA fragments obtained were subsequently inserted into vectors. The 5'-UTR of the cloned CaM cDNA was relatively short (32 bp); however, the sequence showed high homology with the CaM 3 genes in humans, mice and rats (data not shown). In addition, the 3'-UTR sequence of the guinea pig CaM was compared with those from the CaM 3 genes in humans, mice and rats, and the nucleotide sequence homology similarities were determined as 95.3, 93.2 and 94.7%, respectively (Fig. 4). These results demonstrated that the guinea pig CaM cDNA clones (32, 450 and 154 bp for the 5'-UTR, coding region and 3'-UTR, respectively) exhibited high homology with the previously reported cDNA sequences of CaM 3 genes in humans, mice and rats. Based on these results, sequence data of the guinea pig CaM 3 gene, isolated in the current study, have been registered in GenBank (accession no. FJ012165; 636 bp), and show high homology with the counterparts from other animals.

Homology analysis of the guinea pig CaM 3 gene with CaM 1 and 2 genes in other animals. Considering that there are three known CaM genes in a number of species, the CaM 3 sequences obtained in the guinea pig hearts were compared with the CaM 1 and 2 genes from humans, mice and rats. As shown in Fig. 5, the results demonstrated that the coding sequence similarities between the guinea pig CaM 3 gene and the CaM 1 gene in humans, mice and rats were 80.0, 82.9 and 82.5%, respectively. When compared with the CaM 2 gene in humans, mice and rats, the nucleotide sequence homologies were determined to be 81.4, 82.3 and 82.7%, respectively. Therefore, the guinea pig CaM 3 gene was found to share extensive homologies with the CaM 1 and 2 genes from other animals, although the degree of

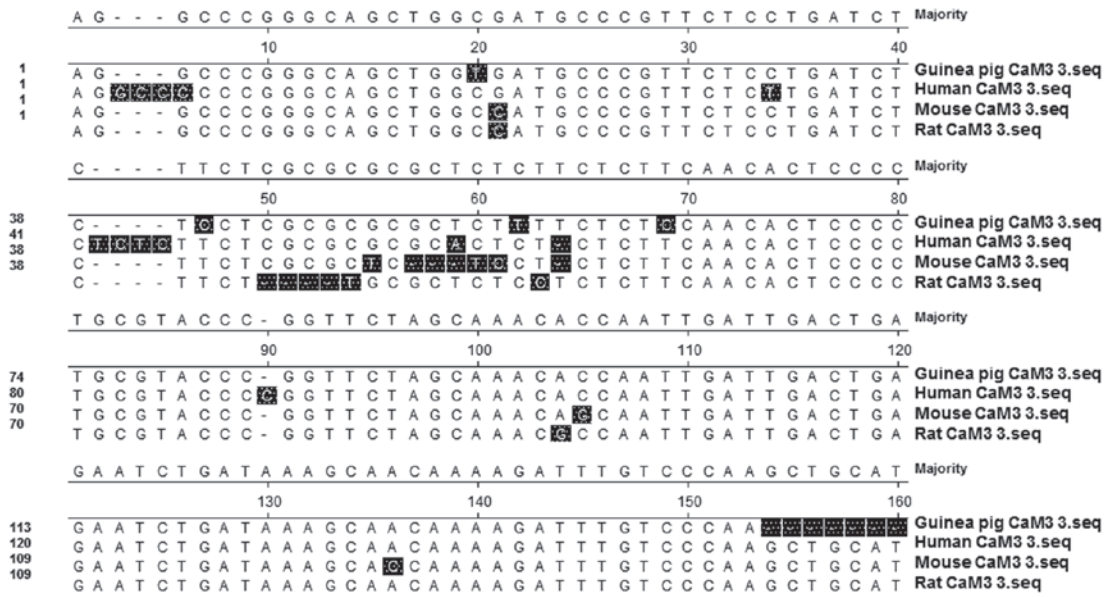

Figure 4. Alignment of the 3'-UTR of CaM 3 from different species. Nucleotide sequences of the 3'-UTR of the CaM 3 gene from guinea pigs, humans, mice and rats are aligned to show the similarities. Residues different from the consensus are indicated in the shaded boxes. CaM, calmodulin; UTR, untranslated region.

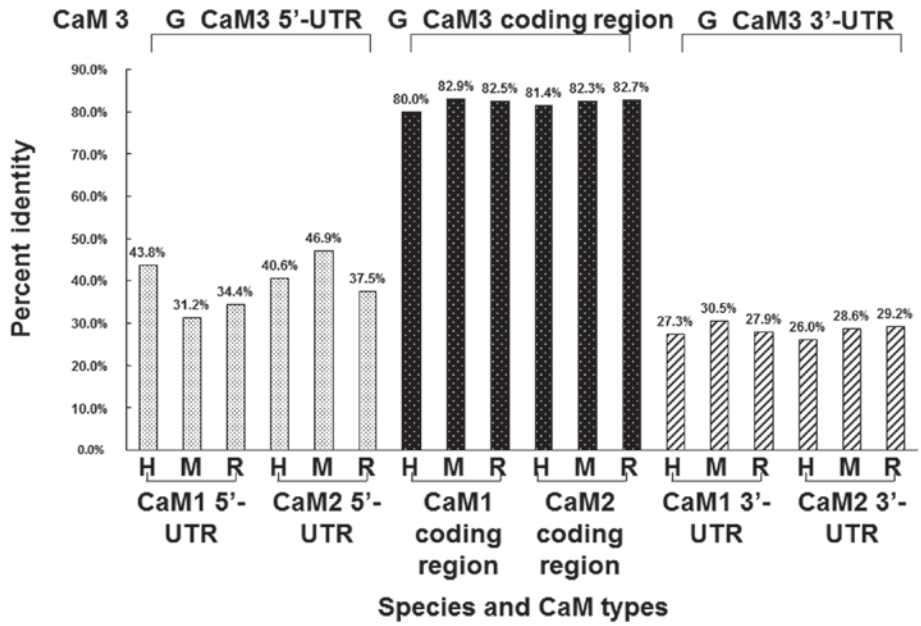

Figure 5. Comparison of the CaM 3 sequence from guinea pigs with the CaM 1 and CaM 2 sequences from humans, mice and rats. The coding regions of CaM 1, CaM 2 and CaM 3 show high homology, while the 5'- and 3'-UTRs of the CaM 3 gene from guinea pigs show a low degree of homology with the CaM 1 and CaM 2 genes from humans, mice and rats. H, humans; M, mice; R, rats; G, guinea pigs; CaM, calmodulin; UTR, untranslated region.

homology was not as high as that for the CaM 3 genes. However, the 5'- and 3'-UTRs of the CaM 3 mRNA were highly diverged when compared with the respective CaM 1 and 2 sequences from other animals; the nucleotide sequence homologies varied between 26 and 47%. These results indicated that the coding regions of the guinea pig CaM 3 gene were highly conserved when compared with the CaM 1, 2 and 3 genes in other animals; however, the sequences of the UTRs were diverged among the CaM 1, 2, 3 genes. By contrast, the homologies of the CaM protein sequences between CaMs 1, 2 and 3 were 100% (data not shown).

CaM 3 expression in different guinea pig tissues. To investigate the expression pattern of CaM 3 in guinea pig tissues, the mRNA expression levels of CaM 3 in various tissues were detected using a RT-PCR method. As shown in Fig. 6, CaM 3 was widely distributed in the guinea pig tissues, with expression at different levels. The expression of CaM 3 was relatively abundant in the tissues of the cerebral cortex, aorta and lung, while moderate levels of expression were observed in the left ventricle, small intestine and kidney. In addition, low mRNA expression levels of CaM 3 were detected in the skeletal muscle, cerebellum and spleen. These results demonstrated

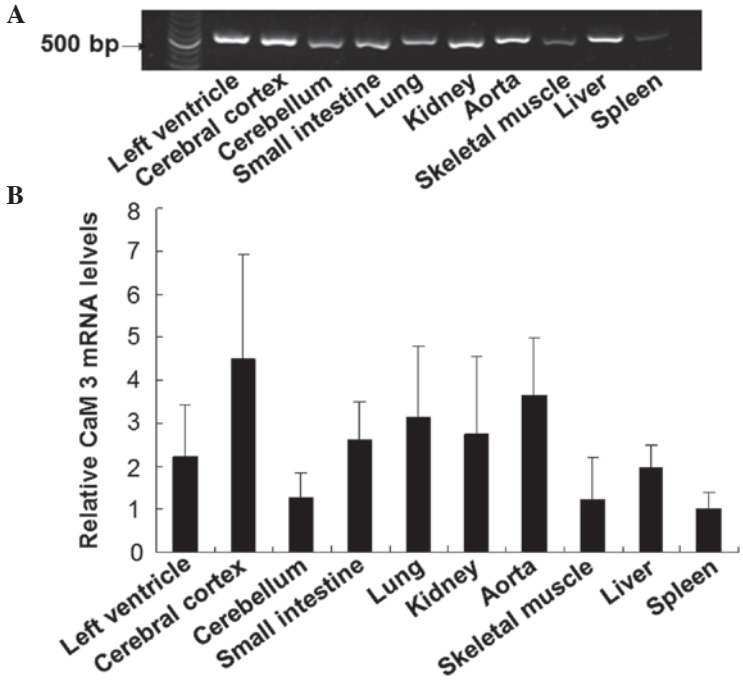

Figure 6. Expression levels of CaM 3 mRNA in various tissues from guinea pigs. (A) Detection of the mRNA expression levels of CaM 3 in various tissues collected from guinea pigs was conducted using reverse transcription-polymerase chain reaction. (B) Statistical analysis of the relative mRNA expression levels of CaM 3. Data are presented as the mean ± standard deviation (n=4-5). CaM, calmodulin.

the wide, but differential distribution of CaM 3 in guinea pig tissues.

Discussion

CaM, a ubiquitous Ca^{2+}-binding protein, has a highly conserved amino acid sequence across a number of species, indicating the pivotal role of the protein in the regulation of basic cellular functions. In vertebrates and invertebrates, CaM is always encoded by a multigene family, exhibiting complex regulation. The same also holds true for plants (27,28). One of the exceptions is *Scoparia dulcis*, in which CaM protein is encoded by only one specific gene (27). In mammals, CaM is generally encoded by three different genes (29-31). These CaM genes share a high degree of conservation with each other, within a species, as well as across species. In the present study, a CaM cDNA clone from guinea pig hearts was obtained and characterized. The results demonstrated that the CaM cDNA clone exhibited the highest degree of homology with the previously reported cDNA of CaM 3 genes, indicating that the isolated gene was CaM 3. Notably, the amino acid sequence of the CaM 3 cDNA clone was identical to the previously reported sequences of the CaM 1, 2 and 3 proteins from other mammals.

It is well known that CaM functions as a key element in the signaling mechanisms underlying the regulation of numerous Ca^{2+}-mediated cellular functions (32). The guinea pig is a widely used model for diseases; however, little information is available with regard to the genetic information of CaM in guinea pigs. To the best of our knowledge, the present study was the first to clone the guinea pig CaM 3 gene. When comparing the coding region of the CaM 3 gene in guinea pigs with that from other animals (humans, mice and rats), the homologies varied between 89 and 93%. In addition, the sequences of the 5'- and 3'-UTRs of CaM 3 exhibited high homologies across these species. These results indicated a high similarity in CaM 3 genes among different species. Furthermore, the protein product of the CaM 3 gene in guinea pigs was the same as that in humans, mice and rats. In addition, the sequence of the CaM 3 gene was compared with those of the CaM 1 and 2 genes. In the coding regions, the nucleotide sequence homologies varied between 81 and 83%; however, the UTRs exhibited a lower degree of homology (26-46.9%). Thus, the data indicated that the distinct types of CaM were generally different from each other in the UTRs. The CaM gene family has previously been reported to be comprised of three non-allelic members in mammals, including humans and rats. By contrast, in the non-coding regions, there were no marked sequence similarities among these three CaM genes (33,34). Therefore, the results of the present study were consistent with the aforementioned studies. It can be hypothesized that there are three CaM genes in guinea pig hearts, and the gene that was isolated and characterized was the specific CaM 3 gene. Thus, further studies are required to identify the genes corresponding to CaM 1 and 2 in guinea pigs. In addition, the amino acid sequence of CaM 3 in the guinea pigs was shown to be identical to those of the CaM 1, 2 and 3 proteins in other mammals (humans, mice and rats). Therefore, further investigation into whether multigene families for the same CaM protein in guinea pigs, in the way that they do for CaM in humans and rats, is required.

It is unknown whether the CaM 3 gene is differentially expressed in various tissues of guinea pigs. In the present study, the mRNA expression levels of CaM 3 in different tissues from guinea pigs were detected by RT-PCR. Gene expression occurred predominantly in the cerebral cortex, aorta and lung,

whilst lower expression was observed in the skeletal muscle, cerebellum and spleen. In addition, CaM 3 was expressed at a moderate level in the left ventricle, small intestine and kidney. A previous study reported that distinct CaM genes are widely expressed throughout the mid-brain stem region (35). Furthermore, Zhou *et al* (36) studied the regional distribution of CaM activity in the rat brain, while Solà *et al* (37) investigated the distribution pattern of CaM 1, 2 and 3 genes in the mouse brain. The two studies found that CaM activity did not necessarily correlate with the amount of CaM present. In the present study, the results from the RT-PCR detection revealed the differences in the relative abundance of CaM 3 mRNA expression levels among the various tissues, which indicated that the CaM 3 gene may be differentially regulated in these tissues. Therefore, CaM 3 may have distinct functions according to the different tissues/regions in guinea pigs.

In conclusion, the present study identified and characterized a CaM 3 cDNA clone obtained from guinea pig hearts. CaM is involved in the activation of CaM-dependent protein kinase II, which is associated with the pathogenesis of ischemia-reperfusion injury (12). Future research should investigate the role of CaM 3 in cardiovascular diseases. Therefore, the present study has provided valuable information with regard to the cloning and expression of CaM 3 in guinea pigs. CaM 3 was demonstrated to be expressed in various tissues, indicating the extensive effects of the protein in corresponding regions. The present results may help to improve the understanding of CaM 3 function and the possible role of CaM 3 in cardiovascular diseases.

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