Journal of Cellular Biochemistry 68:259–268 (1998)

Differentiation-Dependent Expression of Cardiac δ-CaMKII Isoforms

Brigitte Hoch, Hannelore Haase, Wolfgang Schulze, Dirk Hagemann, Ingo Morano, Ernst-Georg Krause, and Peter Karczewski*

Max Delbrück Centre for Molecular Medicine, Robert-Rössle-Strasse, Berlin-Buch, Germany

Abstract Despite their important role in controlling the cardiac Ca^{2+} homeostasis, presence and functions of individual isoforms of the multifunctional Ca^{2+}/calmodulin-dependent protein kinase in the heart are not well studied. Here we report on expression of isoforms of the δ class in two differentiation states of the embryonic rat heart-derived cell line H9c2 compared to adult rat heart. Reverse transcription coupled polymerase chain reaction analysis revealed specific expression patterns of four variants of the δ class (δ_B, δ_C, δ_4, δ_9) in adult rat heart, H9c2 myoblasts, and skeletal muscle-like H9c2 myotubes. δ_C was identified as a common isoform with higher amounts in H9c2 cells and the prominent one in myoblasts. In contrast, expression of δ_9 accompanied cardiac as well as skeletal muscle differentiation. Expression of δ_B, however, was representative for differentiated cardiac muscle, whereas δ_4 expression coincided with differentiation into the skeletal muscle-like state. Our results demonstrate differentiation-dependent isoform expression of the δ class of the multifunctional Ca^{2+}/calmodulin-dependent protein kinase of muscle. The identification of cardiac target proteins for this kinase, e.g. the α_1-subunit of the L-type Ca^{2+} channel, the sarcoplasmic reticulum Ca^{2+}-ATPase, phospholamban and the ryanodine receptor define H9c2 myoblasts as a suitable model system for further functional characterization of the identified cardiac δ isoforms. J. Cell. Biochem. 68:259–268, 1998.

Key words: multifunctional Ca^{2+}/calmodulin-dependent protein kinase; cardiac isoforms; muscle differentiation; cell line Hgc2; adult rat heart

The multifunctional Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) is a complex regulated mediator of the Ca^{2+}-signal [Braun and Schulman, 1995] and takes part in the regulation of gene expression and cell cycle control [Nghiem et al., 1994; Planas-Silva and Means, 1992]. Specificity of the transposition of the Ca^{2+} signal is guaranteed not only by complex regulation of CaMKII activity but by localization of the holoenzyme in different compartments of the cell as well. Localization is determined through holoenzyme composition of 8–12 subunits of identical or various CaMKII isoforms [Kabasaki et al., 1991]. A majority of subunits of the δ_B-CaMKII isoform containing a nuclear localization signal therefore directs the holoenzyme to the nucleus [Srinivasan et al., 1994]. Diversity of CaMKII isoforms is believed to originate from alternative splicing of precursor RNAs encoded by four genes (α, β, γ, δ) [Tobimatsu and Fujisawa, 1989; Brocke et al., 1995]. Members of the α- and β-CaMKII classes are expressed only in neuronal tissues. In addition to their neuronal presence, γ- and δ-CaMKII isoforms are expressed in non-neuronal tissues [Tobimatsu and Fujisawa, 1989; Schworer et al., 1993; Tombes and Krystal, 1997; Singer et al., 1997].

CaMKII was shown to influence differentiation processes in various non-muscle cell types [Wang and Simonson, 1996; Masse and Kelly, 1997]. However, there are no studies elucidating the influence of individual CaMKII isoforms on muscle differentiation, including cardiac muscle differentiation. In cardiac tissue, transcript and protein expression data revealed the presence of δ_B and δ_C isoforms [Edman and Schulman, 1994; Baltas et al., 1995]. Other cardiac δ-CaMKII isoforms were described as partial cDNA sequences [Mayer et al., 1995]. Immunoprecipitation studies with

Contract grant sponsor: Deutsche Forschungsgemeinschaft; Contract grant number: Ka 939/5-1; Contract grant number: Ha 1779/3-1; Contract grant sponsor: Sonnenfeld-Stiftung.

*Correspondence to: Dr. Peter Karczewski, Max Delbrück Centre for Molecular Medicine, Robert-Rössle-Str. 10, 13122 Berlin-Buch, Germany. E-mail: pkarcze@mdc-berlin.de

Received 21 July 1997; Accepted 22 September 1997

γ-CaMKII subunit-specific antibodies gained evidence for the formation of heteromultimer holoenzymes in the heart, composed of abundantly expressed γ-CaMKII isoforms and δ-CaMKII subunits, expressed to a much lower amount [Singer et al., 1997]. Until now, none of the non-neuronal CaMKII isoforms could be linked to a cardiac-specific function and their expression during heart development was not investigated. As a regulator of Ca^{2+} homeostasis CaMKII is of essential importance for normal heart function. Established or putative cardiac CaMKII substrates are the voltage-dependent L-type Ca^{2+} channel [Anderson et al., 1994], the ryanodine receptor (Ca^{2+} release channel, CRC) [Hohenegger and Suko, 1993], the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) [Hawkins et al., 1994], its regulator protein phospholamban [Simmerman et al., 1986], adenylyl cyclase [Wei et al., 1996] and the cardiac muscle C-protein [Hartzell and Glass, 1984]. The contribution of different isoforms of cardiac CaMKII to the regulation of these proteins is unknown.

The clonal cell line H9c2 was derived from embryonic rat heart ventricle [Kimes and Brandt, 1976] and retains elements and properties of signaling pathways of adult cardiomyocytes [Hescheler et al., 1991; Mejia-Alvarez et al., 1994; Dangel et al., 1996]. H9c2 cells preserve these traits characterized as the myoblast state over long periods of cultivation [Hescheler et al., 1991]. Defined changes of culture conditions initiate cell differentiation resulting in cell fusion and generation of morphologically skeletal muscle-like myotubes [Hescheler et al., 1991; Mejia-Alvarez et al., 1994]. Here we report on (1) the expression of δ-CaMKII isoforms in adult rat heart compared to distinct differentiation states of H9c2 cells, and (2) the expression of identified or putative cardiac CaMKII substrates in H9c2 cells revealing the suitability of this cell line to study molecular aspects of cardiac CaMKII isoforms.

METHODS

Cell Culture

The cell line H9c2 (2–1) [Kimes and Brandt, 1976] was obtained from the European Collection of Animal Cell Cultures and was cultured in the myoblast state in Dulbecco's modified Eagle's medium (Gibco BRL, Eggenstein, Germany) supplied with 10% (v/v) fetal calf serum (FCS) (Biochrom, Berlin, Germany), penicilline (100 IU/ml) and streptomycine (100 µg/ml) (Gibco BRL) under an atmosphere of 6.4% CO_2 at 37°C. Myoblasts were passaged and harvested before reaching the confluent state. For differentiation to myotubes, cells were grown as above until reaching confluence, then serum content was reduced to 1% and cells were grown additionally for 14 days.

Extraction of RNA

Total RNA from H9c2 cells was isolated with the RNAeasy system (Qiagen, Hieden, Germany) and from adult rat heart according to Kingston et al. [1994]. After isolation, remaining DNA contaminations were digested in 40 mM Tris/HCl, pH 7.5, 6 mM $MgCl_2$ and 10 µl RNase-free DNaseI (Pharmacia, Freiburg, Germany) for 10 min at 37°C in a total volume of 100 µl. RNA was re-extracted with the RNA clean up procedure of the RNeasy system. Total digestion of genomic DNA was assessed by polymerase chain reaction (PCR) without the reverse transcription (RT) step (data not shown).

Reverse Transcription-Coupled Polymerase Chain Reaction

For reverse transcription, RNA solution was heated and immediately chilled on ice. Random primed cDNA synthesis for PCR was carried out in a total volume of 60 µl containing up to 6 µg of total RNA in the presence of 6 µl 10× PCR buffer (Eurogentec, Seraing, Belgium), 4.8 µl 25 mM $MgCl_2$, 16 µl dNTP (2.5 mM each nucleotide; United States Biochemical, Cleveland, U.S.A.), 1.5 µl RNase inhibitor (RNAguard, Pharmacia), 1.5 µl 10× hexanucleotide mix (Boehringer-Mannheim, Mannheim, Germany) and 3 µl Superscript II $RNaseH^-$ Reverse transcriptase (Gibco BRL). After a 10 min incubation at room temperature reverse transcription was performed for 1 h at 42°C. For each PCR, an aliquot of the reverse transcription reaction equivalent to that containing originally 500 ng total RNA was used. The volume of this aliquot was increased to 100 µl and to a final amount of 10 µl 10× PCR buffer, 8 µl 25 mM $MgCl_2$, 4 µl dNTP, 50 pmol of each specific primer (Table I), and 0.2 µl thermostable DNA polymerase (GoldStar red DNA polymerase, Eurogentec). Standard PCR was performed in a TRIO thermocycler (Biometra, GöHingen, Germany) as follows: 3 min 94°C denaturation step, 35 cycles (30 sec 94°C, 1 min 55°C, 1 min 72°C) of amplification and a final 7 min elongation step at 72°C. After

TABLE I. Primers Used for PCR*

Primer specific for	Direction	Sequence	Product length (bp)	Accession number/Ref.
Common 3′ CaMKII	3′	TCAGATGTTTTGCCACAAAGAGGTGCCTCCT		J05072
δ_B-CaMKII	5′	AAAAGGAAGTCCAGTTCGAGTGTTCAGATGAT	549	L13407
δ_C-CaMKII	5′	CCGGATGGGGTAAAGGAGTCAACTGAGAGCT	531	L13406
δ_9-CaMKII	5′	GTAAAGGAGCCCCAAACTACTGTAA	564	Mayer et al. [1995]
δ_4-CaMKII	5′	CTACCCCGGCGCTGGAGTCAAC	530	Schworer et al. [1993]
GAPDH	5′	CAGTCCATGCCATCACTGCC	609	M17701
	3′	GGGTCTGGGATGGAATTGTG		M17701
α_{1C} subunit Ca^{2+} channel	5′	GCAGAAGACATCGACCCTGAG	264	S74397
	3′	CATCACCAGCCAGTAGAAGAC		S74397
α_{1S} subunit Ca^{2+} channel	5′	CGCGAGGTCATGGACGTGGAG	201	Mejia-Alvarez et al. [1994]
	3′	GATGACCAGCCAGTAGAAGAC		
Ryanodine receptor	5′	CACAATGGCAAACAGCT	389	Michaels et al. [1996]
	3′	CACATGAAGCATTTGGTCTCCAT		
Phospholamban	5′	GCGGAATTCATGGAAAAAGTCCAATACCTTACTCGCT	399	L03382
	3′	GCGGAATTCGAGCCACACTGAGGAAACGGGCA		L03382

*All primers were HPLC-purified and purchased from BioTez (Berlin, Germany). Primers for phospholamban contain restriction sites at the ends in addition to the gene-specific sequences. CaMKII isoforms δ_B and δ_C [Edman and Schulman, 1994] were also referred to as δ_3 and δ_2 [Schworer et al., 1993; Mayer et al., 1995].
HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction.

PCR, 8 µl of the 100 µl reaction mixtures were loaded onto 2% agarose gels containing ethidiumbromide. As a size marker for gel electrophoresis, the ready-load 100 bp DNA standard (Gibco BRL) was used. After agarose gel electrophoresis, stained DNA bands were visualized under ultraviolet (UV) light. Background-substracted optical densities from peak areas were obtained with the PCBas system (raytest Isotopenmeßgeräte GmbH, Straubenhardt, Germany). For comparison of different reactions, glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific PCRs were carried out in parallel.

Preparation of Total Homogenates and Subcellular Fractions

Total protein of cells harvested in phosphate-buffered saline (PBS) and sedimented by centrifugation or heart tissue of neonatal and adult rats were isolated by homogenization in the presence of 50 mM Tris/HCl pH 6.8, 10% glycerol and 6% sodium dodecyl sulfate (SDS). Cells were homogenized by sonication for 5 min at maximal power and tissue samples were homogenized in a glass/glass potter. After centrifugation (5 min, 14,000*g*) protein content of the supernatant was determined with the DC Assay kit (BioRad, Mŭnchen, Germany). For preparation of subcellular fractions, cell pellets of 10–15 million cells were homogenized in 0.75 ml homogenization buffer (HB; 10 mM Hepes pH 7.5, 0.2 mM phenylmethylsulfonylfluoride (PMSF), 0.1 mM dithiothreitol (DTT)) with an Ultra Turrax at 30,000 rpm for 3 × 5 s and centrifuged at 600*g* for 10 min. The resulting crude nuclear pellet was suspended in buffer B1 (0.75 ml of 10 mM Hepes pH 7.5, 250 mM sucrose, 0.2 mM PMSF and 0.1 mM DTT) and quickly frozen. The supernatant was centrifuged at 10,000*g* for 10 min. The resulting supernatant was put on ice, the pellet rehomogenized in 0.75 ml HB and sedimented by centrifugation as above. The supernatants of both spins were combined and centrifuged at 150,000*g* for 20 min. The final microsomal pellet was dissolved in 0.3 ml B1. The supernatant of the last spin is referred to as cytosolic fraction. All subcellular fractions were aliquoted and stored at -80°C.

Generation of Antibodies Specific for δ-CaMKII and for the α_{1C}-Subunit of the L-Type Ca^{2+} Channel

For antibody production in rabbits, peptides were commercially synthesized and purified (BioTeZ, Berlin, Germany). Antigenic epitopes comprised the following amino acid sequences: KENFSGGTSLWQNI corresponding to the C-terminus unique for a subclass of isoforms of δ-CaMKII from rat [Mayer et al., 1995] and

EEEEKERKKLARTASPEKK of the cytoplasmic linker between repeat II and III of the α_{1C}-subunit of the L-type Ca^{2+} channel [Mikami et al., 1989]. Peptide conjugation and immunization protocol was according to Haase et al. [1993]. The resulting polyclonal antibodies were affinity purified on peptide affinity columns [Calovini et al., 1995].

Immunoblotting

Protein samples solubilized in SDS sample buffer were electrophoresed through SDS polyacrylamide gels [Laemmli, 1970] or SDS urea gels [Swank and Munkres, 1971]. Separated proteins were electrotransferred onto polyvinylidenedifluoride membranes. Processing for immunoblotting was performed as described in Towbin et al. [1979]. Antibodies were diluted as follows: 1 µg/ml for anti-δ-CaMKII, 0.5 µg/ml for anticardiac-α_1 Ca^{2+} channel, 1/1,000 for anti-SERCA2a (BioMol, Hamburg, Germany) and 0.5 µg/ml for anti-phospholamban antibodies (BioMol). For detection the second antibody was anti-rabbit IgG or anti-mouse IgG (both obtained from Sigma, Deisenhofen, Germany) conjugated with peroxydase. The immunoreaction was visualized using the enhanced chemoluminescence kit (Amersham, Braunschweig, Germany) and autoradiography on X-ray films. Densitometric analysis of autoradiograms was performed with the PDI imaging system (PDI, New York, New York).

Immunocytochemistry of δ-CaMKII

H9c2 myoblasts grown on glass chamber slides were fixed according to standard procedures. As primary antibody the anti-δ-CaMKII antibody described above was used in a 5 µg/ml dilution. Primary antibodies were visualized with a dichlotriazinyl aminofluorescein-(DTAF)-conjugated species-specific secondary antibody (Dianova, Hamburg, Germany). Evaluation of immunofluorescence labeling was done with an Axioskop microscope equipped with optics and appropriate filter systems (Zeiss, Oberkochen, Germany). Specificity of the signal was checked by preincubation of the primary antibody solution with the peptide used for generation of the antibody in a 300-fold molar excess of the peptide, resulting in suppression of the signal (data not shown).

Statistical Evaluation

Results are given as means ± standard errors (±SEM). Mean values were compared using the unpaired t-test assuming $p < 0.05$ as statistically significant.

RESULTS

Differentiation-Dependent Expression of δ-CaMKII Isoforms

The presence of δ-CaMKII isoforms was investigated in adult rat heart, H9c2 myoblasts and myotubes. A representative result of RT-PCR with primer pairs specific for the δ-CaMKII isoforms δ_B, δ_C, δ_4, δ_9 and the housekeeping gene GAPDH is shown in Figure 1a. In adult rat heart, δ_B- and δ_9-CaMKII are the predominantly expressed isoforms. δ_C-CaMKII is expressed to a lower extent and amplification of δ_4-CaMKII is near the detection limit. In H9c2 myoblasts, δ_C is the dominant CaMKII isoform. Isoforms δ_9 and δ_4 are expressed to a lower extent. For δ_B-CaMKII transcripts only a very weak signal is detectable. Differentiation of myoblasts to myotubes is paralleled by an increase of expression of isoforms δ_9- and δ_4, whereas δ_C-CaMKII expression remained unchanged and the δ_B-CaMKII signal is no longer visible. Figure 1b and 1c illustrate the differentiation-dependent expression of δ-CaMKII transcripts. Expression of δ-isoforms in terminally differentiated rat heart tissue and in skeletal muscle-like myotubes is compared to their expression in myoblasts. In rat heart tissue, isoforms δ_B and δ_9 are significantly elevated, whereas isoforms δ_C and δ_4 are significantly reduced compared to their expression in H9c2 myoblasts (Fig. 1b). Differentiation from myoblasts to myotubes does not change δ_C-CaMKII expression. Isoforms δ_4- and δ_9, however, were significantly higher expressed than in myoblasts. Expression of δ_B-CaMKII is reduced near the detection limit (Fig. 1c).

In order to analyze δ-CaMKII protein expression, an anti-peptide antibody was generated, directed against a partial amino acid sequence of the second variable domain of δ-CaMKII. The antigenic determinant used is specific for the neuronal δ-CaMKII isoform δ_1 and the non-neuronal isoforms δ_B, δ_C, δ_4, and δ_9 [Mayer et al., 1995]. This antibody recognized different δ-CaMKII isoforms in preparations from H9c2 cells and sarcoplasmic reticulum vesicle-fractions of adult heart (Fig. 2a, left). In H9c2 cells, the size of the main immunostained protein was in the range as expected for δ_C-CaMKII (56.4 kDa) [Edman and Schulman, 1994]. Myotubes showed additionally a faint signal above

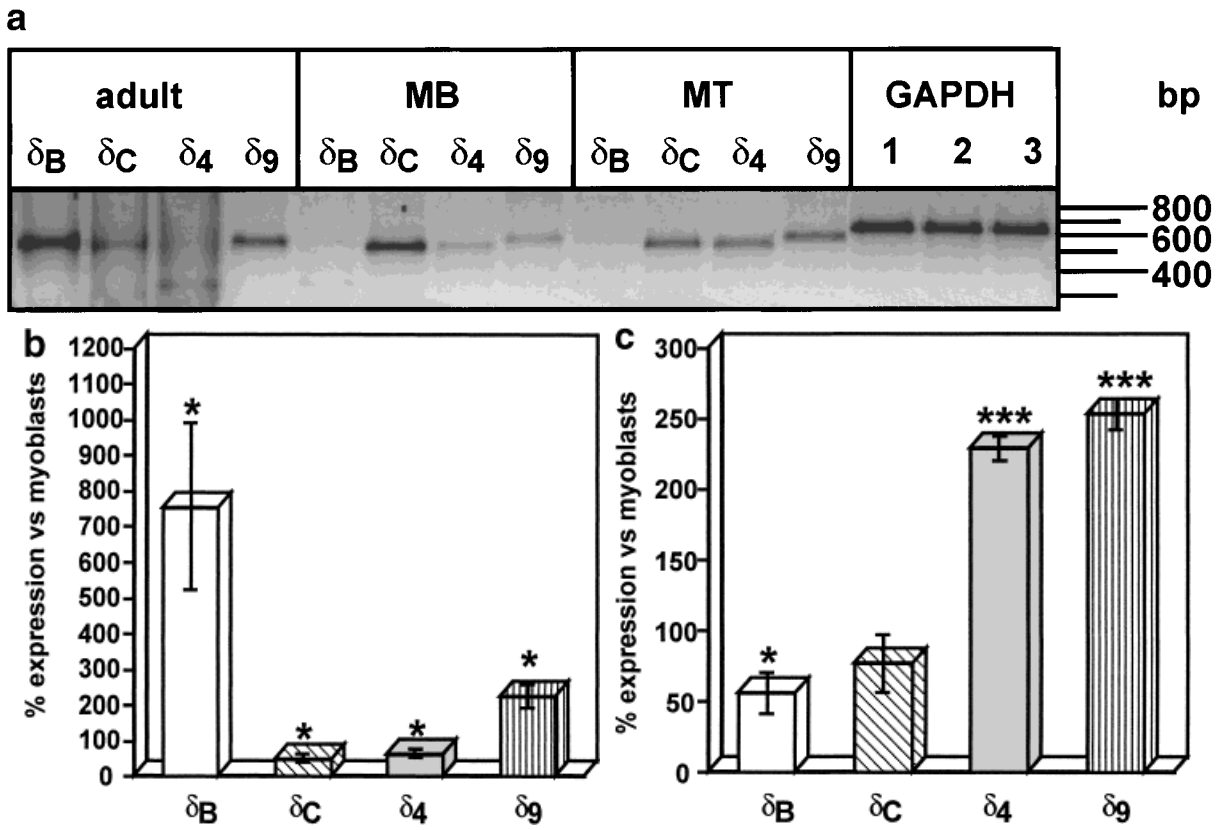

Fig. 1. Differentiation-dependent expression of δ-CaMKII isoforms. **a:** δ-CaMKII isoform specific transcripts in adult rat heart (adult), myoblasts (MB), and myotubes (MT). Representative RT-PCR analysis with primer combinations specific for δ_B-, δ_C-, δ_4-, and δ_9-CaMKII and GAPDH. GAPDH-specific amplification products were obtained from adult rat heart (1), H9c2 myoblasts (2), and myotubes (3). Specificity of the amplification products for δ_B-, δ_C-, δ_4- and δ_9-CaMKII isoforms was confirmed by sequencing (data not shown). **b:** Data from three independent RT-PCR analyses comparing amplification of adult rat heart and H9c2 myoblast-derived cDNAs. Expression levels were normalized to GAPDH expression in myoblasts. Expression of the single CaMKII isoforms in myoblasts were set 100%. Asterisks indicate *p*-values ($*p < 0.05$) versus expression in myoblasts. **c:** Data from 3–4 independent RT-PCR analyses comparing amplification of H9c2 myoblast and myotube-derived cDNAs. Calculation was done as in b. Asterisks indicate *p*-values ($*p < 0.05$; $***p < 0.0001$) versus expression in myoblasts.

which may result from δ_4- or δ_9-CaMKII, isoforms being 20 and 14 amino acids longer than δ_C-CaMKII. This result confirmed our data on the transcriptional level (Fig. 1a). In the fraction from adult rat heart, a strong signal was observed corresponding to the molecular mass expected for δ_B-CaMKII (57.7 kDa) [Edman and Schulman, 1994] and for δ_9-CaMKII, which differs from δ_B only by three amino acids. A second smaller band was detected with the expected size for δ_C-CaMKII. This is in line with our data on the mRNA level in adult rat heart (Fig. 1a). The specificity of the immunodetection was shown by preincubation of the antibody with the peptide used for immunization (Fig. 2a, right). Location of δ-CaMKII in H9c2 myoblasts by immunofluorescence (Fig. 2b) revealed its presence near the plasma membrane, in smooth endoplasmic reticulum and the perinuclear region.

Expression of Putative Cardiac CaMKII Substrates in H9c2 Myoblasts and Myotubes

Expression of the cardiac L-type Ca^{2+} channel in H9c2 cells on protein level is shown by immunoblotting of total protein extracts from H9c2 cells, neonatal and adult rat heart tissue (Fig. 3a). The immunodetection gives signals at 250 kDa in all three systems and at 220 kDa in H9c2 cells and neonatal heart, depicting similarities between H9c2 cells derived from embryonic heart and neonatal heart. SERCA2a and phospholamban were detected in subcellular fractions of H9c2 myoblasts and were compared with the sarcoplasmic reticulum fraction of adult rat heart (Fig. 3b). As expected for these

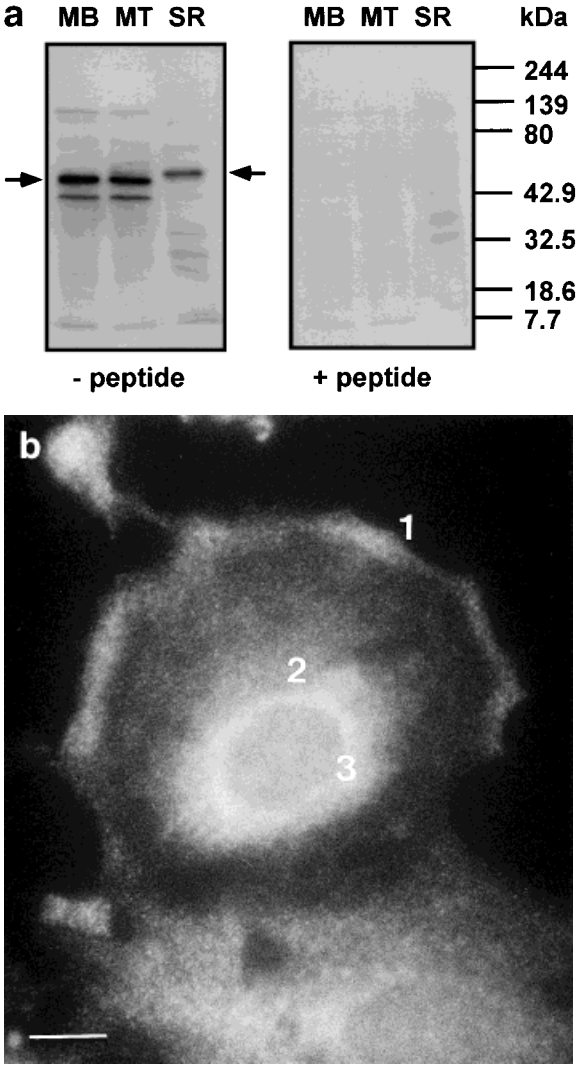

Fig. 2. Expression of δ-CaMKII isoforms on the protein level. **a:** Presence of δ-CaMKII isoforms in protein extracts from H9c2 cells and adult rat heart and specificity of the used anti-peptide antibody; 15 µg of total protein from H9c2 myoblasts (MB) or myotubes (MT) and 12.5 µg of a sarcoplasmic fraction (SR) from adult rat heart were separated on a 10% SDS-Laemmli gel. After blotting, immunodetection was carried out with a δ-CaMKII-specific anti-peptide antibody. Left, - peptide. Arrows, dominant δ-CaMKII specific signals. Right, + peptide shows the immunoreaction where the primary antibody was preincubated overnight at 4°C in the presence of a 150-fold molar excess of the peptide used for generation of the antibody. **b:** Immunofluorescence detection of δ-CaMKII in H9c2 myoblasts. The immunoreaction of the primary anti-δ-CaMKII-peptide antibody was visualized with a secondary species-specific DTAF-labeled antibody (1, plasma membrane; 2, smooth endoplasmic reticulum; 3, perinuclear space). Scale bar = 10 µm.

membrane-associated proteins, there were no signals in the cytosolic fraction of myoblasts. Both proteins were found in the microsomal fraction. SERCA2a was also detected in the crude nuclear pellet. Compared to adult heart, SERCA2a and phospholamban proteins are expressed at very low levels in H9c2 cells. RT-PCR studies with total RNA from adult rat heart and H9c2 cells in the presence of a primer pair specific for the cardiac ryanodine receptor revealed expression of its transcripts in H9c2 cells, but with reduced amounts compared to adult rat heart (Fig. 3c).

Differentiation of H9c2 myoblasts to myotubes is demonstrated by the induction of the skeletal isoform (α_{1S}) of the α_1-subunit of the L-type Ca^{2+} channel compared to H9c2 myoblasts (394.5% ± 16.5% in four independent experiments) (Fig. 4). Besides the expected amplification products of 264 bp for the cardiac isoform (α_{1C}) and 201 bp for α_{1S}, longer fragments were detected but were not regarded when quantifying the amounts of α_{1C} and α_{1S}-specific products. Amplification of at least one unspecific product using the α_{1C}-specific primer pair was also described by Mejia-Alvarez et al. [1994]. There are no differences in the amount of the α_{1C}-specific transcript between H9c2 myoblasts and myotubes. On the protein level, however, differentiation is accompanied by a reduction of α_{1C}-subunit on the protein level (Fig. 3a). Corresponding RT-PCR analysis in the presence of a primer pair specific for phospholamban demonstrated reduced transcription levels after differentiation to myotubes compared to myoblasts (68% ± 7%; n = 3) (data not shown). RT-PCR studies with total RNA from H9c2 myoblasts and myotubes in the presence of a primer pair specific for the cardiac ryanodine receptor revealed reduced expression of its transcripts in myotubes (Fig. 3c).

DISCUSSION

In order to establish a cellular model delivering defined differentiation states of high reproducibility and that is suitable for the study of molecular aspects of cardiac CaMKII isoforms, we investigated the clonal embryonic rat heart-derived cell line H9c2 [Kimes and Brandt, 1976]. The use of a clonal cell line also encompassed disadvantages of primary cultures of cardiomyocytes as cultures of neonatal cardiomyocytes are always composed of different cell types [Henning et al., 1996], and adult cardiomyocytes dedifferentiate and change their phenotype during cultivation [Volz et al., 1991].

The cell line was characterized in regard to cardiac Ca^{2+} cycling systems which are known as or supposed to be controlled by CaMKII. As shown here, H9c2 cells express the cardiac

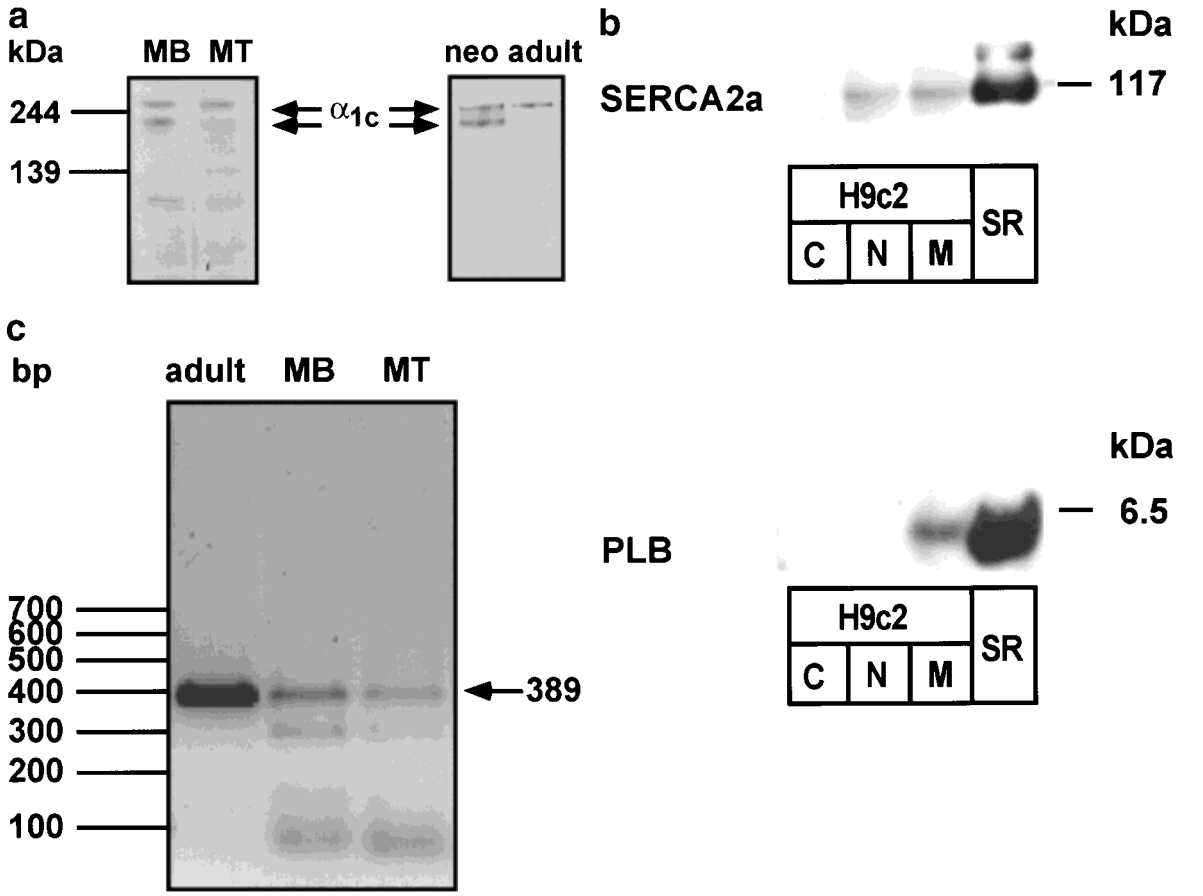

Fig. 3. Expression of cardiac Ca^{2+} cycling systems in H9c2 cells. **a:** Immunodetection of the cardiac δ_1-subunit of the L-type Ca^{2+} channel (δ_{1C}). 25 µg total protein from myoblasts (MB), myotubes (MT) and 30 µg total protein from neonatal (neo) and adult rat heart (adult) were separated on 6.5% SDS-Laemmli gels. After blotting, detection was carried out with an antibody specific for α_{1C}. **b:** Immunodetection of SERCA2a and phospholamban (PLB) in subcellular fractions of myoblasts (H9c2) and the sarcoplasmic reticulum (SR) from adult rat heart; 20 µg of total protein of subcellular fractions (C = cytosolic fraction; N = nuclear pellet; M = microsomal fraction) from myoblasts and 5 µg SR-fraction were separated on SDS gels. After blotting, detection was performed with commercially available specific antibodies. **c:** Presence of the cardiac isoform of the ryanodine receptor in adult rat heart (adult), myoblasts (MB), and myotubes (MT). RT-PCR analysis with a primer pair specific for the cardiac ryanodine receptor results in the amplification of a 389-bp product as described for the ryanodine receptor in rat heart.

L-type Ca^{2+} channel on the protein level confirming results on its electrophysiology and transcription in H9c2 cells [Hescheler et al., 1991; Mejia-Alvarez et al., 1994]. Expression of the cardiac isoform of the ryanodine receptor, SERCA2a and phospholamban was also demonstrated in H9c2 cells. The cells express these cardiac genes to lower extent than observed in adult heart. H9c2 cells do not show a cardiomyocyte morphology [Kimes and Brandt, 1976]. However, the demonstrated expression of cardiac Ca^{2+} cycling systems in H9c2 cells strongly qualifies them as a useful model to study a specific spectrum of cardiomyocyte functions, e.g. the CaMKII controlled part of the cardiac Ca^{2+} homeostasis.

Myoblasts represent dividing mononucleated precursors of the muscle lineage. Their differentiation to polynucleated myotubes results, among others, in changes in the expression of some of the investigated Ca^{2+} cycling proteins. These changes reflect complex regulatory mechanisms on transcriptional and post-transcriptional level accompanying the morphological changes. Clear separation of the two differentiation states is defined by the strong induction by about 400% of the skeletal subunit of the L-type Ca^{2+} channel in myotubes compared to myoblasts. Only a very low accumulation of transcripts for this isoform could be detected in myoblasts. Mejia-Alvarez et al. [1994] reported on the simultaneous expression

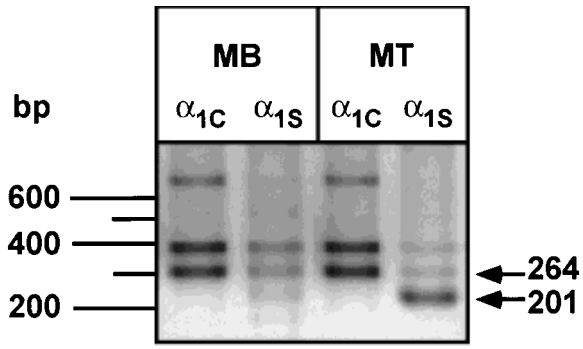

Fig. 4. Expression of transcripts for the cardiac α_{1C}) and skeletal (α_{1S}) α_1-subunit of the L-type Ca^{2+} channel. RT-PCR analysis of total RNA from H9c2 myoblasts (MB) and myotubes (MT) with primer combinations specific for the cardiac α_{1C} and skeletal α_{1S} subunits of the L-type Ca^{2+} channel. Lengths of the expected specific products are 264 bp for the α_{1C}-derived amplification products and 201 bp for the α_{1S}-derived amplification products.

of the cardiac and the skeletal subunit in a less defined differentiation state. In contrast to this study, we demonstrated that differentiation to the myotube state is accompanied by a strong accumulation of transcripts for the skeletal subunit. Differentiation of H9c2 cells to myotubes resulted in the expression of both subunits on the transcriptional level. This is in line with a study on subunit expression in primary skeletal muscle cell cultures from hindlimbs of neonatal rats [Bulteau et al., 1997] demonstrating co-expression of both subunits in earlier states of myotube formation during in vitro myogenesis. Reduced transcript levels for phospholamban and the cardiac ryanodine receptor and diminished protein expression of the cardiac subunit of the L-type Ca^{2+} channel furthermore characterize the skeletal muscle-like phenotype of H9c2 myotubes.

Recently we have demonstrated that CaMKII in the sarcoplasmic reticulum of porcine heart is composed of distinct δ-subunits [Baltas et al., 1995]. We therefore compared in adult rat heart and H9c2 cells δ-CaMKII isoforms, which were already described at the transcript level to be present in the myocardium [Mayer et al., 1995]. The rational of this attempt was to compare δ-CaMKII isoform pattern of the terminally differentiated heart with two defined states of H9c2 cells representing qualitatively different stages of muscle differentiation in order to (1) identify the dominant cardiac isoform, and (2) gain insights into the position of distinct isoforms in muscle differentiation.

Our data show a differentiation-specific isoform pattern of the δ-CaMKII isoforms δ_B, δ_C, δ_4, and δ_9. On the transcriptional level we used PCR conditions that fulfill several in part counteracting demands in order to obtain a semiquantitative result concerning the expression of these isoforms. The various isoforms show strongly different levels in the investigated differentiation states. Consequently, it was necessary to extend the number of PCR cycles to 35, which enabled us to amplify products for all isoforms simultaneously. A potential disadvantage of this method concerns strongly expressed isoforms that could be amplified out of the linear range of the reaction. This would lead to a possible underestimation of the real differences. However, this underestimation should change the given relative differences in expression levels (Fig. 1b,c) to even higher significance. Despite this limitation of the method significant differences were detected although the expression values may differ much more. The δ_C-subunit predominantly expressed in H9c2 myoblasts remained at high level in myotubes and, with a significant decrease, in adult heart. Amount of transcripts for δ_9-CaMKII was increased relative to myoblasts in both myotubes and terminally differentiated adult rat heart. Thus, expression of δ_9 apparently accompanies cell differentiation in general and does not distinguish between the cardiac and skeletal muscle lineage. δ_4-CaMKII was identified in skeletal muscle [Schworer et al., 1993]. We observed a significant increase in the amount of δ_4 transcripts in myotubes that parallels the expression of the skeletal Ca^{2+} channel α_1 subunit. Thus, our data support the notion that δ_4 is the skeletal muscle isoform of δ-CaMKII. The δ_B-subunit transcript was one of the dominantly expressed isoforms in the heart, which is in agreement with our protein data. Therefore, we propose δ_B-CaMKII to be the characteristic form of the terminally differentiated cardiomyocyte.

Intracellular localization of δ-CaMKII determined by immunofluorescence demonstrated its preferential location close to membrane structures of H9c2 myoblasts. Some of these structures (here identified as smooth endoplasmic reticulum and perinuclear region) are accounted by others as sarcoplasmic reticulum-like [McCartney et al., 1995]. Thus, the isoform δ_C, the prominent CaMKII subunit in myoblasts, apparently determined the location of the enzyme

to these structures. From our immunofluorescence data it is obvious that the cell nucleus of H9c2 myoblasts is void of δ-CaMKII. This is in line with the very low expression level for the nuclear localization signal-containing isoform δ_B [Srinivasan et al., 1994]. In adult heart, CaMKII is mainly located in the sarcoplasmic reticulum [Baltas et al., 1995] and the cytosolic compartment [Gupta and Kranias, 1989] although, as shown here, the isoform δ_B is one of the dominantly expressed cardiac CaMKII isoforms. We therefore propose that the ratio of the two identified dominant cardiac δ-CaMKII pools, δ_B and δ_9 and additionally the minor expressed isoform δ_C accounts for localization of the enzyme to different cellular compartments. Elucidation of changes in holoenzyme composition of CaMKII with respect to cell growth and differentiation as well as alterations in Ca^{2+} cycling proteins will be a subject of our further investigations.

ACKNOWLEDGMENTS

We thank Mrs. I. Ameln and Mrs. D. Riege for expert technical assistance. This work was supported by grants Ka 939/5-1 (to P.K.) and Ha 1779/3-1 (to H.H.) from the Deutsche Forschungsgemeinschaft and by a grant from the Sonnenfeld-Stiftung to D.H.

REFERENCES

Anderson ME, Braun AP, Schulman H, Premack BA (1994): Multifunctional Ca^{2+}/calmodulin-dependent protein kinase mediates Ca^{2+}-induced enhancement of the L-type Ca^{2+} current in rabbit ventricular myocytes. Circ Res 75:854–861.

Baltas L, Karczewski P, Krause EG (1995): The cardiac sarcoplasmic reticulum phospholamban kinase is a distinct δ-CaM kinase isozyme. FEBS Lett 373:71–75.

Braun AP, Schulman H (1995): The multifunctional calcium/calmodulin-dependent protein kinase: From form to function. Annu Rev Physiol 57:417–445.

Brocke L, Srinivasan M, Schulman H (1995): Developmental and regional expression of multifunctional Ca^{2+}/calmodulin-dependent protein kinase isoforms in rat brain. J Neurosci 15:6797–6808.

Bulteau L, Cogné M, Cognard C, Raymond G (1997): Reversal of the relative expression of cardiac and skeletal alpha1 subunit isoforms of L-type calcium channel during in vitro myogenesis. Pflügers Arch-Eur J Physiol 433:376–378.

Calovini T, Haase H, Morano I (1995): Steroid hormone regulation of myosin subunit expression in smooth and cardiac muscle. J Cell Biochem 59:69–78.

Dangel V, Giray J, Ratge D, Wisser H (1996): Regulation of β-adrenoceptor density and mRNA levels in the rat heart cell-line H9c2. Biochem J 317:925–931.

Edman CF, Schulman H (1994): Identification and characterization of δ_B-CaM kinase and δ_C-CaM kinase from rat heart, two new multifunctional Ca^{2+}/calmodulin-dependent protein kinase isoforms. Biochim Biophys Acta 1221: 89–101.

Gupta RC, Kranias EG (1989): Purification and characterization of a calcium-calmodulin-dependent phospholamban kinase from canine myocardium. Biochemistry 28: 5909–5916.

Haase H, Karczewski P, Beckert R, Krause EG (1993): Phosphorylation of the L-type calcium channel β-subunit is involved in β-adrenergic signal transduction in canine myocardium. FEBS Lett 335:217–222.

Hartzell HC, Glass DB (1984): Phosphorylation of purified cardiac muscle C-protein by purified cAMP-dependent and endogenous Ca^{2+}/calmodulin-dependent protein kinases. J Biol Chem 259:15587–15596.

Hawkins C, Xu A, Narayanan N (1994): Sarcoplasmic reticulum calcium pump in cardiac and slow twitch skeletal muscle but not fast twitch skeletal muscle undergoes phosphorylation by endogenous and exogenous Ca^{2+}/calmodulin-dependent protein kinase. J Biol Chem 269: 31198–31206.

Henning U, Wolf WP, Holtzhauer M (1996): Primary cultures of cardiac muscle cells as models for investigation of protein glycosylation. Mol Cell Biochem 160/161:41–46.

Hescheler J, Meyer R, Plant S, Krautwurst D, Rosenthal W, Schultz G (1991): Morphological, biochemical and electrophysiological characterization of a clonal cell (H9c2) line from rat heart. Circ Res 69:1476–1486.

Hohenegger M, Suko J (1993): Phosphorylation of the purified cardiac ryanodine receptor by exogenous and endogenous protein kinases. Biochem J 296:303–308.

Kabasaki T, Ikeuchi Y, Sugiura H, Yamauchi T (1991): Structural features of Ca^{2+}/calmodulin-dependent protein kinase II revealed by electron microscopy. J Cell Biol 115:1049–1060.

Kimes BW, Brandt BL (1976): Properties of a clonal muscle cell line from rat heart. Exp Cell Res 98:367–381.

Kingston RE, Chomczynski P, Sacchi N (1994): Guanidinium methods for total RNA preparation. In Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Struhl K (eds): "Current Protocols in Molecular Biology." Suppl 23. New York: John Wiley & Sons, pp 4.2.1–4.2.8.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Masse T, Kelly PT (1997): Overexpression of Ca^{2+}/calmodulin dependent protein kinase II in PC12 cells alters cell growth, morphology, and nerve growth factor induced differentiation. J Neurosci 17:924–931.

Mayer P, Möhlig M, Idlibe D, Pfeiffer A (1995): Novel and uncommon isoforms of the calcium sensing enzyme calcium/calmodulin dependent protein kinase II in heart tissue. Basic Res Cardiol 90:372–379.

McCartney S, Little BM, Langeberg LK, Scott JD (1995): Cloning and characterization of A-kinase anchor protein 100 (AKAP100). J Biol Chem 270:9327–9333.

Mejia-Alvarez R, Tomaselli GF, Marban E (1994): Simultaneous expression of cardiac and skeletal muscle isoforms of the L-type Ca^{2+} channel in a rat heart muscle cell line. J Physiol 478.2:315–329.

Michaels K, Cho CH, Moraru I, Spoerel N, Kim DH (1996): Quantification of Ca^{2+} release channel mRNA in pressure-

overload induced hypertrophic rat heart by competitive reverse transcription and competitive polymerase chain reaction. Mol Cells 6:528–533.

Mikami A, Imoto K, Tanabe T, Niidome T, Mori Y, Takeshima H, Narumiya S, Numa S (1989): Primary structure and functional expression of the cardiac dihydropyridine-sensitive calcium channel. Nature 340:230–233.

Nghiem P, Ollick T, Gardner P, Schulman H (1994): Interleukin-2 transcriptional block by multifunctional Ca^{2+}/calmodulin kinase. Nature 371:347–350.

Planas-Silva MD, Means AR (1992): Expression of a constitutive form of calcium/calmodulin dependent protein kinase II leads to arrest of the cell cycle in G_2. EMBO J 11:507–517.

Schworer CM, Rothblum LI, Thekkumkara TJ, Singer HA (1993): Identification of novel isoforms of the δ-subunit of Ca^{2+}/calmodulin-dependent protein kinase II. J Biol Chem 268:14443–14449.

Simmerman HKB, Collins JH, Theibert JL, Wegener AD, Jones LR (1986): Sequence analysis of phospholamban: Identification of phosphorylation sites and two major structural domains. J Biol Chem 261:13333–13341.

Singer HA, Benscoter HA, Schworer CM (1997): Novel Ca^{2+}/calmodulin-dependent protein kinase II γ-subunit variants expressed in vascular smooth muscle, brain, and cardiomyocytes. J Biol Chem 272:9393–9400.

Srinivasan M, Edman CF, Schulman, H (1994): Alternative splicing introduces a nuclear localization signal that targets multifunctional CaM kinase to the nucleus. J Cell Biol 126:839–852.

Swank RT, Munkres KD (1971): Molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide gel with sodium dodecyl sulphate. Anal Biochem 39:462–477.

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.

Tombes RM, Krystal GW (1997): Identification of novel human tumor cell-specific CaMK-II variants. Biochim Biophys Acta 1355:281–292.

Towbin H, Staehelin T, Gordon J (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and applications. Proc Natl Acad Sci USA 76:4350–4354.

Volz A, Piper HM, Siegmund B, Schwartz P (1991): Longevity of adult ventricular rat heart muscle cells in serum-free primary culture. J Mol Cell Cardiol 23:161–173.

Wang Y, Simonson MS (1996): Voltage-insensitive Ca^{2+} channels and Ca^{2+}/calmodulin-dependent protein kinases propagate signals from endothelin-1 receptors to the c-fos promoter. Mol Cell Biol 16:5915–5923.

Wei J, Wayman G, Storm DR (1996): Phosphorylation and inhibition of type III adenylyl cyclase by calmodulin-dependent protein kinase II in vivo. J Biol Chem 271: 24231–24235.