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The β isoenzyme of Ca^{2+}/calmodulin-dependent kinase type II as possible mediator of somatostatin functions in pituitary tumour cells

Davide Cervia

Department for Innovation in Biological, Agro-food and Forest systems, University of Tuscia, Viterbo, Italy

Abstract. Somatostatin or somatostatin release inhibiting factor (SRIF) analogues are indicated for the treatment of somatotropinomas that hypersecrete growth hormone (GH). Indeed, SRIF inhibits intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), thus allowing the inhibition of GH secretion. In the present study, our hypothesis was that Ca^{2+}/calmodulin-dependent kinase type II (CaMKII), a multifunctional serine/threonine protein kinase, is part of those signalling mechanisms mediating SRIF functions.

All four CaMKII isoenzymes (termed α, β, γ and δ) are expressed in rat somatotroph GC cells, although only CaMKIIβ is inhibited by SRIF at both mRNA and protein levels. Similarly to SRIF, the specific knockdown of CaMKIIβ by RNA interference induces a decrease of $[Ca^{2+}]_i$. The effects of SRIF and those of CaMKIIβ knockdown are non-additive. These results are confirmed by the pharmacological blockade of CAMKII. We also observed that, similarly to SRIF, the specific knockdown of CaMKIIβ induces a decrease of both GH content/secretion.

These results raise the hypothesis that CaMKIIβ may mediate, at least in part, the SRIF-induced control of $[Ca^{2+}]_i$. In addition, CaMKIIβ seems to play a positive role in maintaining the exocytosis of GH. Our data provide a framework for better elucidating the pathophysiological role of SRIF transduction network in somatotropinomas.

Key words: Somatotropinomas — Somatostatin — Ca^{2+} — Growth hormone — siRNA

Abbreviations: CaMKII, Ca^{2+}/calmodulin-dependent kinase type II; DMEM-F12, dulbecco's MEM nutrient mix F-12; D-PBS, dulbecco's phosphate buffered saline; EIA, enzymoimmunoassay; FBS, foetal bovine serum; GH, growth hormone; HBSS, hank´s buffered salt solution; HRP, horseradish peroxidase; OD, optical density; siRNA, small interfering RNA; SDS, sodium dodecyl sulfate; SRIF, somatostatin or somatotropin release inhibiting factor; $sst_{1\text{-}5}$, SRIF receptor subtype 1-5; TBST, tris-buffered saline-0.1% (v/v) tween.

Introduction

The cyclic peptide somatostatin or somatotropin release inhibiting factor (SRIF) has been discovered as a hypothalamic factor which inhibits the secretion of growth hormone (GH) from the pituitary (Brazeau et al. 1973). The inhibition of a large number of secretory processes (releasing of GH, thyrotropin, corticotrophin releasing hormone, adrenocorticotrophic hormone, insulin, glucagon, secretin and vasoactive intestinal peptide hormone) (Olias et al. 2004) is a key property of SRIF, thus representing the rationale for the therapeutic use of SRIF analogues in different pathologies (Melmed 2006; Cervia and Bagnoli 2007; Grozinsky-Glasberg et al. 2008). Among them, acromegaly is one of the most important indication for SRIF analogues, being an insidious neuroendocrine disease that, in most cases, is a result of a pituitary adenoma that hypersecretes GH. The goals of the therapy are to control excessive GH secretion and limit, if not reverse, the long-term medical consequences and risk of premature mortality associated with acromegaly (Katznelson 2006).

Upon activation by ligand binding, SRIF receptors (named sst_1 to sst_5) couple to G-proteins which relay signals to multiple downstream signalling pathways (Cervia and Bagnoli 2007). However, our current understanding of SRIF receptor signal-

Correspondence to: Davide Cervia, Department for Innovation in Biological, Agro-food and Forest systems, University of Tuscia, Largo dell'Università snc, blocco D, 01100 Viterbo, Italy
E-mail: d.cervia@unitus.it

ling reflects a rather complicated picture and further studies are needed to clarify the pathophysiological role of this transduction network. For instance, results obtained in human and rat somatotropinomas, indicate that SRIF induces the inhibition of GH secretion through the activation of K^+ current, the reduction of Ca^{2+} influx from voltage-gated Ca^{2+} channels and the subsequent decrease of intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ (Cervia and Bagnoli 2007; Yang et al. 2007). Secretion of GH from pituitary cells is critically dependent on the level of $[Ca^{2+}]_i$, which is controlled mainly by Ca^{2+} influx through voltage-gated Ca^{2+} channels and release from intracellular Ca^{2+} storage sites. In somatotropinomas, sst_2 is the main receptor inhibiting voltage-gated Ca^{2+} current (Yang et al. 2007) and it has also been suggested to decrease GH release through the inhibition of the voltage-dependent Ca^{2+} channels (Florio et al. 2003). These evidences are confirmed by previous studies performed in rat anterior pituitary GC cells, an adenoma cell line which exhibits pacemaker activity and concomitant spontaneous oscillations of $[Ca^{2+}]_i$ and GH secretion (Cervia et al. 2002b). In GC cells, sst_2 activation is responsible for the $[Ca^{2+}]_i$ decrease and the block of Ca^{2+} transients which, in turn, results in the sst_2-mediated decrease of GH secretion (Petrucci et al. 2000; Cervia et al. 2002a,b, 2003).

Ca^{2+}/calmodulin-dependent kinase type II (CaMKII), a multifunctional serine/threonine protein kinase, is highly expressed in neuronal tissues (Takeuchi et al. 2000; Griffith 2004; Schulman 2004; Ishida et al. 2005; Yamauchi 2005; Kamata et al. 2006; Wayman et al. 2008). Four isoenzymes, termed α, β, γ and δ, are encoded by distinct genes in eukaryotes (Hudmon and Schulman 2002). The unique ability of CaMKII to modulate and to respond to both the amplitude and the frequency of Ca^{2+} fluctuations (Molkentin 2006; Grueter et al. 2007) makes this enzyme an important transducer of Ca^{2+} signals. In the present study, our hypothesis was that CaMKII is part of those signalling mechanisms mediating SRIF functions in somatotropinomas. In particular, the possibility that CaMKII is coupled to the control of Ca^{2+} and mediates the SRIF-induced decrease of $[Ca^{2+}]_i$ was investigated in GC cells. Whether CaMKII modulation can influence the production of GH was also studied. At present, no evidence is available regarding SRIF coupling to CaMKII although, functionally, CaMKII has been found to be involved in a variety of Ca^{2+}-mediated cellular processes including biosynthesis and release of neurotransmitters and hormones (Easom 1999; Takeuchi et al. 2000; Osterhoff et al. 2003; Wang et al. 2005; Yamauchi 2005; Kolarow et al. 2007; Liu et al. 2007).

Materials and Methods

Cell culture

GC rat anterior pituitary tumour cells were cultured in a complete medium composed of DMEM-F12 (dulbecco's MEM nutrient mix F-12) supplemented with 10% (v/v) horse serum, 5% (v/v) FBS (foetal bovine serum), 2 mM glutamine, 100 UI/ml penicillin and 100 μg/ml streptomycin at 37°C, 5% CO_2 in an humidified atmosphere. The cells were detached from the culture flask by washing with D-PBS (dulbecco's phosphate buffered saline) and brief incubation with trypsin/EDTA (ethylene diamine tetraacetic acid). For the experiments, the cells were cultured in 100-mm Petri dishes.

RT-PCR experiments

GC cells were detached from 100-mm Petri dishes. When indicated, GC cells were treated in the absence or in the presence of 100 nM SRIF (30 min) before the assay. Using published protocols (Cervia et al. 2007, 2008), total RNA was extracted with the RNeasy Mini Kit with DNAse digestion (Qiagen, Hilden, Germany), according to the manufacturer's recommended procedure. After solubilization in RNase-free water, total RNA was quantified by Bio-Rad SmartSpec 3000 spectrophotometer (Hercules, CA, USA). First-strand cDNA was generated from 3 μg of total RNA using iScript cDNA Synthesis Kit (Bio-Rad). As show in Table 1, a set of primer pairs amplifying at

Table 1. Primer pairs designed for PCR analysis

Gene	Primer sequence	Amplicon length (bp)	Gene accession N°	Source
CaMKIIα	F: 5'-gctctcagccagagatcacc-3' R: 5'-ctggcatcagcctcactgta-3'	195	NM_012920	Primer3 software*
CaMKIIβ	F: 5'-gaccagcacaagctgtacca-3' R: 5'-agcttcctccttgcattgaa-3'	248	NM_001042354	Primer3 software*
CaMKIIγ	F: 5'-gccaagagacagtggagtgcttacgca-3' R: 5'-cagcggtgcagcaggggctcctgagcagtgata-3'	728	NM_133605	Balla et al. 2002
CaMKIIδ	F: 5'-ctggcacacctgggtatctt-3' R: 5'-cactgtgtcccattctggtg-3'	200	NM_012519	Primer3 software*
cyclophilin A	F: 5'-tcgagctgtttgcagacaaagt-3' R: 5'-caatgctcatgccttctttcac-3'	351	NM_017101	Primer3 software*

* Rozen and Skaletsky 2000

195–728 bp fragments were designed to hybridise to unique regions of the gene sequence of either CAMKII isoenzymes or cyclophilin A. The reverse transcription polymerase chain reactions (RT-PCR) were carried out using 1 μl of cDNA in a 15 μl total volume of PCR buffer (Invitrogen, Milano, Italy), containing 3 mM $MgCl_2$, 300 μM dNTPs, 300 nM of appropriate primers and Taq polymerase (0.35 U). The amplification reactions were carried out in a thermal gradient cycler (Bio-Rad) and the relationship between the intensity of the signal and the PCR cycle number was analyzed to determine the linear range for the PCR product formation. The intensities of the signals within the linear range of amplification were used for data analysis. For each amplification, two types of controls were performed: i) RT-PCR mixture with no reverse transcriptase to control for genomic DNA contamination, and ii) PCR mixture with no cDNA template, to check for possible external contamination. CaMKII isoenzyme mRNAs were coamplified with cyclophilin A mRNA (house-keeping gene). A 5 μl sample of the PCR reaction was electrophoresed on an ethidium bromide-containing 2% (w/v) agarose gel by the use of the Bio-Rad Sub-cell GT system. After migration, bands corresponding to the amplified products were visualized with Gel Doc 2000 System.

Western blotting

GC cells were detached from 100-mm Petri dishes, washed twice with D-PBS and suspended in homogenisation buffer (10 mM Tris-HCl (pH 7.5), 3 mM EGTA, 5 mM EDTA, 250 mM sucrose) supplemented with protease inhibitors (Protease Inhibitor Cocktail Complete) and phosphatase inhibitors (PhosStop). When indicated, GC cells were treated in the absence or in the presence of 100 nM SRIF (30 min) before the assay. After sonication, the supernatant (cytosolic extracts) was obtained by centrifugation at 20 000 × *g* (30 min, 4°C). Protein concentration was determined using the Bradford method. Using published protocols (Cervia et al. 2006, 2007), sodium dodecyl sulfate (SDS) and β-mercaptoethanol was added to samples before boiling, and equal amount of proteins (40 μg/lane) was separated by 10% (w/v) SDS-PAGE with the Bio-Rad Mini-PROTEAN 3 system. After transfer of proteins onto polyvinylidene difluoride membranes (Amersham Biosciencies, Milano, Italy) blots were blocked for 1 h with TBST (tris-buffered saline-0.1% (v/v) tween) containing 5% (w/v) skimmed-milk powder. The incubation with a rabbit polyclonal, anti-CaMKIIβ primary antibody (1 : 1000) was performed overnight at 4°C with TBST-5% (w/v) skimmed-milk powder. After reaction in TBST-5% (w/v) skimmed-milk powder for 3 h with an anti-rabbit IgG, horseradish peroxidase (HRP)-conjugated secondary antibody (1 : 1000), bands were visualized using Millipore Immobilon Western Chemiluminescent HRP Substrate (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions and exposure to autoradiography Cl-Xposure films (Thermo Fisher Scientific). To monitor potential artefacts in loading and transfer among samples in different lanes, the blots were routinely treated with Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) and reprobed with a mouse monoclonal, anti-β-actin primary antibody (1 : 20 000) and an anti-mouse IgG, HRP-conjugated secondary antibody (1 : 20 000). By means of Quantity One software (Bio-Rad), the semi-quantitative analysis of blots was performed by measuring the optical density (OD) of the CaMKIIβ band with respect to the OD of the band corresponding to the respective β-actin. After statistics (raw data), data resulting from different experiments were represented and averaged in the same graph.

siRNA transfection

Chemically synthesized small interfering RNA (siRNA) for rat CaMKIIβ (gene accession N°: NM_001042354) have been designed by Qiagen (HP GenomeWide siRNA) using the HiPerformance Design Algorithm (Table 2). Briefly, GC cells (at a density of 10^6) were plated in 100-mm dishes and transiently transfected for 24, 48, 72 or 96 h with siRNA duplexes at 20 nM, the most effective concentration to knockdown CaMKIIβ (as assessed in initial transfection experiments). HiperFect Transfection reagents (Qiagen) have been used, according to the manufacturer's instructions. A negative control siRNA (a nonsilencing siRNA

Table 2. siRNA duplexes for CaMKIIβ

Name	Sequence	Target sequence	Cat. N°*
CaMKIIβ site-1 siRNA	F: 5'-GGUCCAUGUGAGGGUUUGAdTdT-3' R: 5'-UCAAACCCUCACAUGGACCdTdG-3'	CAGGTCCATGTGAGGGTTTGA	SI01494871
CaMKIIβ site-2 siRNA	F: 5'-GAGCCUUGUAGGUGUACAAdTdT-3' R: 5'-UUGUACACCUACAAGGCUCdGdG-3'	CCGAGCCTTGTAGGTGTACAA	SI01494878
CaMKIIβ site-3 siRNA	F: 5'-CACUUUAAAUUCACGUCAUdTdT-3' R: 5'-AUGACGUGAAUUUAAAGUGdGdG-3'	CCCACTTTAAATTCACGTCAT	SI01494885

*HP GenomeWide siRNA from Qiagen (Hilden, Germany)

from Qiagen with no homology to any known mammalian gene) was performed in every experiment.

$[Ca^{2+}]_i$ measurements

Using published protocols (Petrucci et al. 2000; Cervia et al. 2002a, 2003, 2005, 2006), GC cells (transfected or untransfected) were detached from 100-mm Petri dishes, suspended in incubation medium HBSS (hank´s buffered salt solution) containing 137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 4.1 mM $MgSO_4$, 4.4 mM KH_2PO_4, 4.9 mM $MgCl_2$, 300 mM Na_2HPO_4, 55 mM D-glucose and supplemented with 20 mM HEPES, pH 7.4. Cells were then washed in HBSS/HEPES supplemented with 0.2% (w/v) bovine serum albumin and loaded for 30 min at 37°C, 5% CO_2 with the permeant fluorescent $[Ca^{2+}]_i$ indicator fura-2/AM (2 μM). For fluorescence measurements, cells were resuspended in HBSS/HEPES supplemented with 250 μM sulfinpyrazone, to prevent dye leakage, and 1% (v/v) FBS, transferred to a quartz thermostated cuvette (37°C) and maintained under continuous stirring. Each cuvette contained about 1.5×10^6 cells, as determined with a standard hemocytometer chamber and trypan blue exclusion. During cell treatment (i.e., 100 nM SRIF, 10/100 μM KN-62), the fluorescence intensity of fura-2/AM was quantified with a LS-50B Luminescence Spectrometer (Perkin-Elmer, Norwalk, CT, USA) with a double excitation wavelength set at 340 nm and 380 nm, respectively, and an emission wavelength monitored at 510 nm. The photomultiplier was coupled to a personal computer equipped with the Perkin-Elmer FL WinLab software for data acquisition. Calibration of fura-2/AM data in terms of $[Ca^{2+}]_i$ was performed as reported by Traina et al. (1996). After statistics (raw data), data resulting from different experiments were represented and averaged in the same graph.

GH secretion enzymoimmunoassay (EIA)

Using published protocols (Cervia et al. 2002b, 2003), GH was detected and quantified by means of a commercially available EIA kit (SPI Bio, Montigny Le Bretonneux, France) utilizing the double-antibody sandwich technique, according to the manufacturer's recommended procedure. Briefly, GC cells (transfected or untransfected) were detached from 100-mm dishes, and seeded in new dishes at a concentration of 10^7 cells. After plate adesion, cells were treated in the absence or in the presence of 100 nM SRIF for 24 h. EIA was then carried-out both on supernatant (GH release) and in the collected cells (GH content). In particular, GH content was measured in the lysed cell extracts obtained as indicated above. Similar approaches to measure intracellular GH were reported in previous papers (Luna et al. 2005; Petkovic et al. 2007). The color intensity of the reaction product (proportional to the GH concentration) was quantified spectrophotometrically by the Microplate reader 680 XR (Bio-Rad) at λ = 405 nm. Data were expressed as ng GH *per* 1 ml of medium. After statistics (raw data), data resulting from different experiments were represented and averaged in the same graph. The number of viable cells cultured in the 100-mm Petri dishes before EIA was about 10^7/well, as determined with a standard hemocytometer chamber and trypan blue exclusion. Thus, we calculated that the GH output by 10^7 cells after 24 h was 2.5–3.5 mg/ml (basal GH release).

Statistics

Upon verification of normal distribution, statistical significance of raw data between the groups in each experiment was evaluated using paired Student's *t*-test or ANOVA followed by Newman-Keuls Multiple Comparison post-test. The GraphPad Prism software package (GraphPad Software, San Diego, CA, USA) was used. The results were expressed as means ± SD or SEM of the indicated *n* values.

Chemicals

DMEM-F12 and glutamine were purchased from Euroclone (Pavia, Italy). Horse serum, FBS, penicillin-streptomycin and D-PBS were obtained from Lonza (Basel, Switzerland). Fura-2/AM and skimmed-milk powder were obtained from Merck-Calbiochem (Darmstadt, Germany). Primer pairs for CaMKIIα, CaMKIIβ, CaMKIIγ, CaMKIIδ, and cyclophilin A were purchased from MWG Biotech (Ebersberg, Germany). Agarose was purchased from Eurobio (Les Ulis, France). Protease Inhibitor Cocktail Complete and PhosStop were obtained from Roche Applied Science (Penzberg, Germany). The rabbit polyclonal, anti-CaMKIIβ primary antibody was purchased from Abcam (Cambridge, UK). The anti-rabbit IgG, HRP-conjugated secondary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). SRIF (SRIF-14) was obtained from Bachem (Bubendorf, Switzerland). If not specified, chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Results

Expression of CaMKII isoenzymes

To identify the isoenzymes of CaMKII expressed in GC cells, RT-PCR experiments were carried out. As shown in Fig. 1A, we detected the amplified product corresponding to CaMKIIα, CaMKIIβ, CaMKIIγ and CaMKIIδ mRNAs. In another set of experiments, we found that 30 min SRIF application at 100 nM, a concentration giving maximal receptor occupancy in our system (Petrucci et al. 2000; Cervia et al.

2003), decreased the expression of the band corresponding to CaMKIIβ mRNA while the bands corresponding to CaMKIIα, CaMKIIγ and CaMKIIδ mRNAs were not affected (Fig. 1B,C). As shown in the Western blot experiments of Fig 1D, the levels of CaMKIIβ protein decreased after 30 min treatment with 100 nM SRIF. In particular, the OD of CaMKIIβ protein bands in the presence of SRIF was 19.6 ± 1.2% lower than in control conditions.

Knockdown of CaMKIIβ

Our findings suggest the possibility that selective inhibition of CaMKIIβ can mimick SRIF functional effects in somatotropinomas. To validate this hypothesis, we used double-stranded siRNA technology in order to drastically decrease CaMKIIβ expression in GC cells, without affecting the other related isoenzymes. After 48 h of transfection, among three

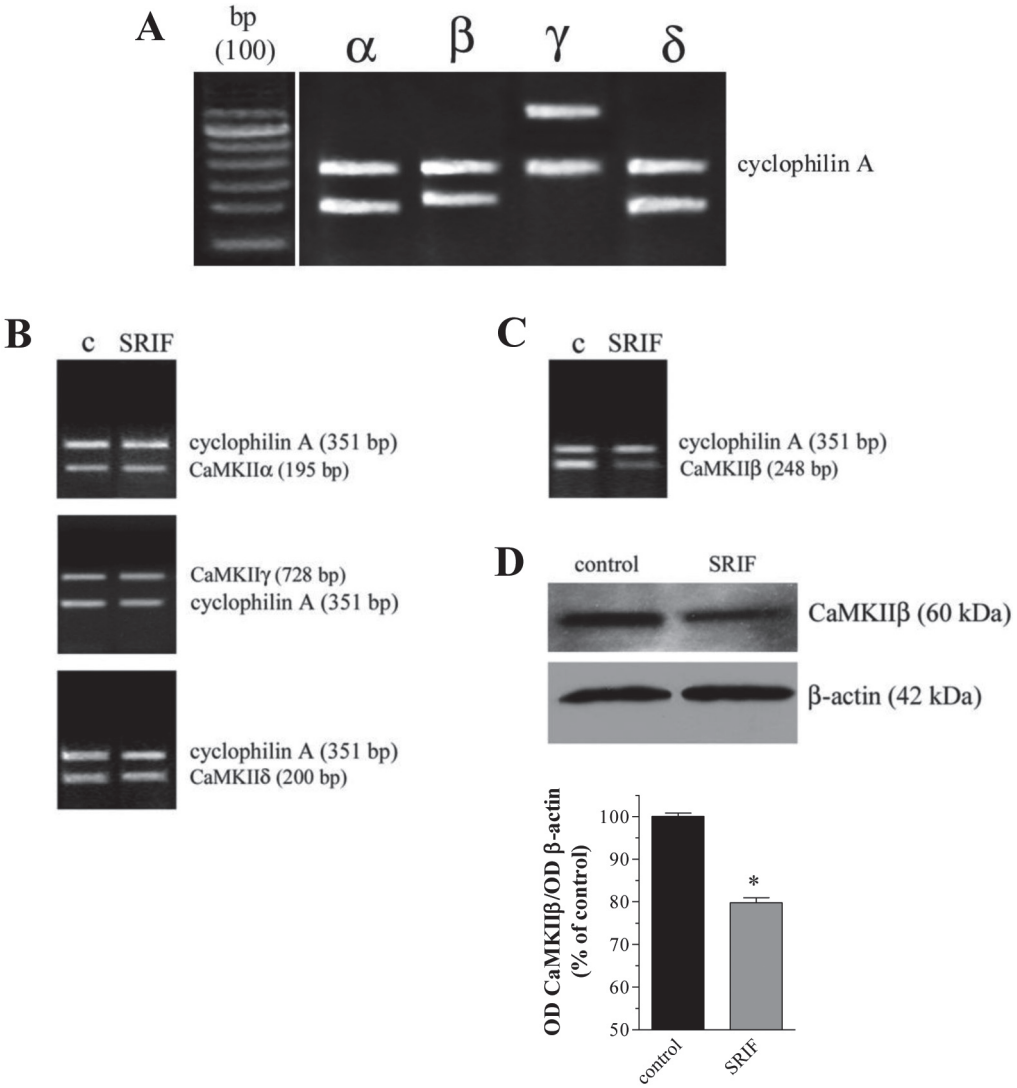

Figure 1. A. RT-PCR products of distinct CaMKII mRNAs (different isoenzymes) co-amplified with cyclophilin A mRNA in GC cells (n = 4). RT-PCR products of the mRNA for CaMKIIα, γ, δ (**B**) and β (**C**) (co-amplified with cyclophilin A mRNA) in GC cells treated in the absence (control: "c") or in the presence of 100 nM SRIF (30 min) (n = 4). **D.** GC cells were treated in the absence (control) or in the presence of 100 nM SRIF for 30 min before Western blot experiments for CaMKIIβ protein. β-actin was used as internal standard. The upper panel is representative of 4 independent experiments. The histograms in the lower panel represent the analysis of Western blot products performed by measuring the OD of the bands corresponding to CaMKIIβ with respect to the OD of the band corresponding to β-actin. Each histogram represents the mean ± SD of data from 4 independent experiments. * $p < 0.001$ *vs.* control (paired Student's t-test on raw data). Data are expressed by setting the OD in control conditions as 100%.

available pairs of siRNAs the most effective knockdown of CaMKIIβ mRNA was obtained with 20 nM CaMKIIβ site-1 siRNA (Fig. 2A). Similar values of CaMKIIβ mRNA were found in control cells (untransfected or transfected with negative control siRNA, data not shown). As shown in Fig. 2B, CaMKIIβ mRNA was inhibited by 20 nM CaMKIIβ site-1 siRNA in a time-dependent manner with the most effective knockdown obtained after 96 h of transfection when the levels of CaMKIIβ protein decreased by 75.0 ± 2.0% as compared with controls (Fig. 2C). In order to verify the selective knockdown of CaMKIIβ, cells were transfected with 20 nM CaMKIIβ site-1 siRNA for 96 h, before the measurement of

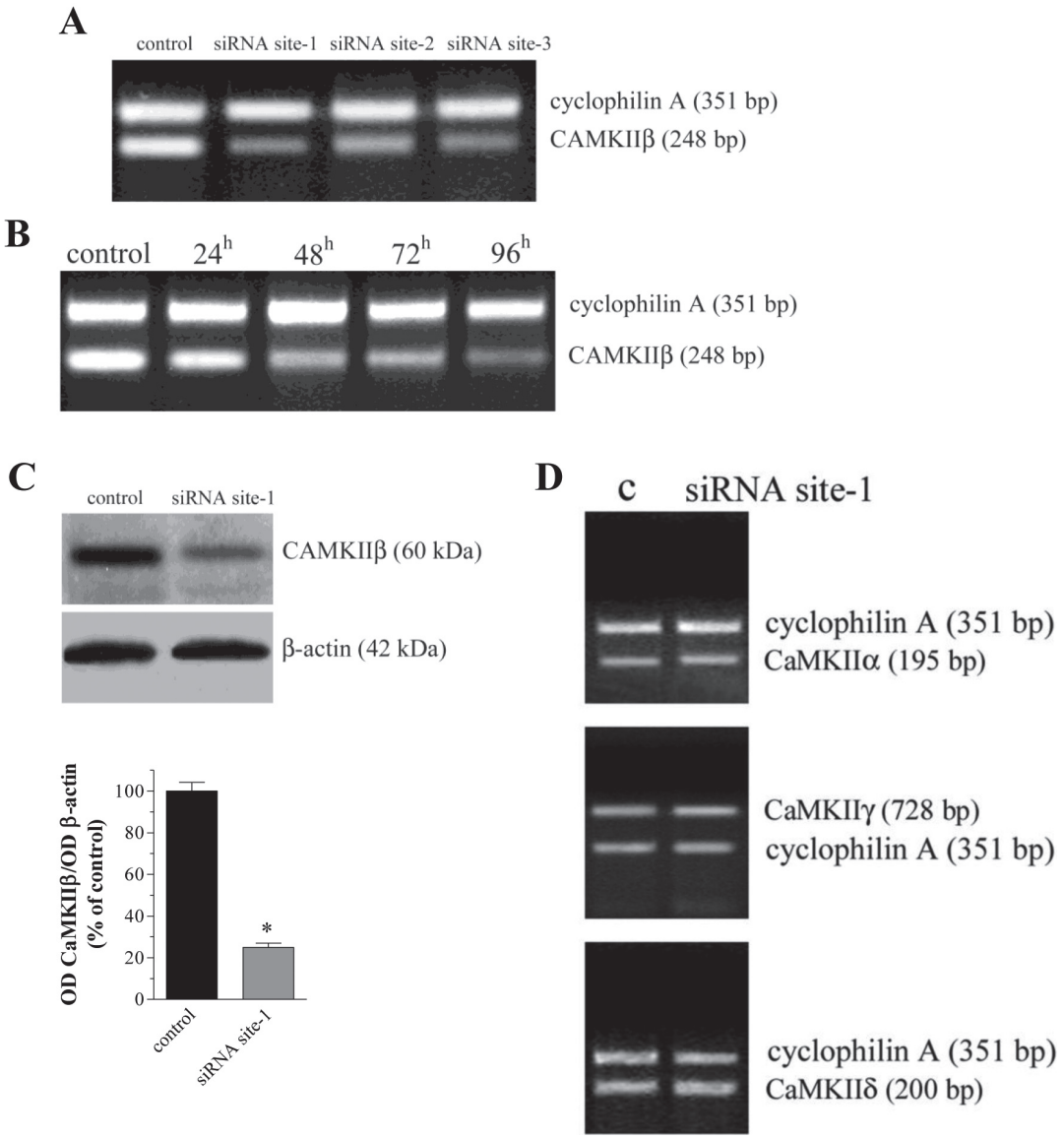

Figure 2. A. RT-PCR products of CaMKIIβ mRNA (co-amplified with cyclophilin A mRNA) in GC cells transfected for 48 h with 3 different siRNAs for CaMKIIβ at 20 nM ($n = 3$) **B.** RT-PCR products of CaMKIIβ mRNA (co-amplified with cyclophilin A mRNA) in GC cells transfected for increasing times with 20 nM CaMKIIβ site-1 siRNA ($n = 3$) **C.** GC cells were transfected with CaMKIIβ site-1 siRNA (20 nM, 96 h) before Western blot experiments for CaMKIIβ protein. β-actin was used as internal standard. The upper panel is representative of 3 independent experiments. The histograms in the lower panel represent the analysis of Western blot products performed by measuring the OD of the bands corresponding to CaMKIIβ with respect to the OD of the band corresponding to β-actin. Each histogram represents the mean ± SD ($n = 3$), * $p < 0.001$ *vs.* control (paired Student's *t*-test on raw data). Data are expressed by setting the OD in control conditions as 100%. **D.** RT-PCR products of the mRNA for CaMKIIα, γ, and δ (co-amplified with cyclophilin A mRNA) in GC cells transfected with CaMKIIβ site-1 siRNA (20 nM, 96 h) ($n = 3$).

other CaMKII isoenzymes. As shown in Fig. 2D, the expression levels of CaMKIIα, γ, or δ mRNAs were not affected by cell transfection.

$[Ca^{2+}]_i$ levels

Levels of $[Ca^{2+}]_i$ were measured by spectrofluorometry in fura-2/AM loaded GC cells. Similar values of basal $[Ca^{2+}]_i$ were found in control cells (untransfected or transfected with negative control siRNA, data not shown). In agreement with previous studies (Petrucci et al. 2000; Cervia et al. 2002a), 5-s SRIF application at 100 nM resulted in a decrease of basal $[Ca^{2+}]_i$ by 20.2 ± 1.9% (Fig. 3A,C). In order to establish whether CaMKIIβ is involved in the control of Ca^{2+} homeostasis, $[Ca^{2+}]_i$ levels were investigated in cells transfected with CaMKIIβ site-1 siRNA (20 nM, 96 h). As shown in Fig. 3B and C, the knockdown of CaMKIIβ by siRNA site-1 resulted in a 10.0 ± 2.7% decrease of $[Ca^{2+}]_i$ when compared with controls. It is important to note that this modest inhibition of $[Ca^{2+}]_i$ was clearly detectable and that similar $[Ca^{2+}]_i$ changes have been previously shown in GC and other pituitary cells (Cervia et al. 2003, 2005). In

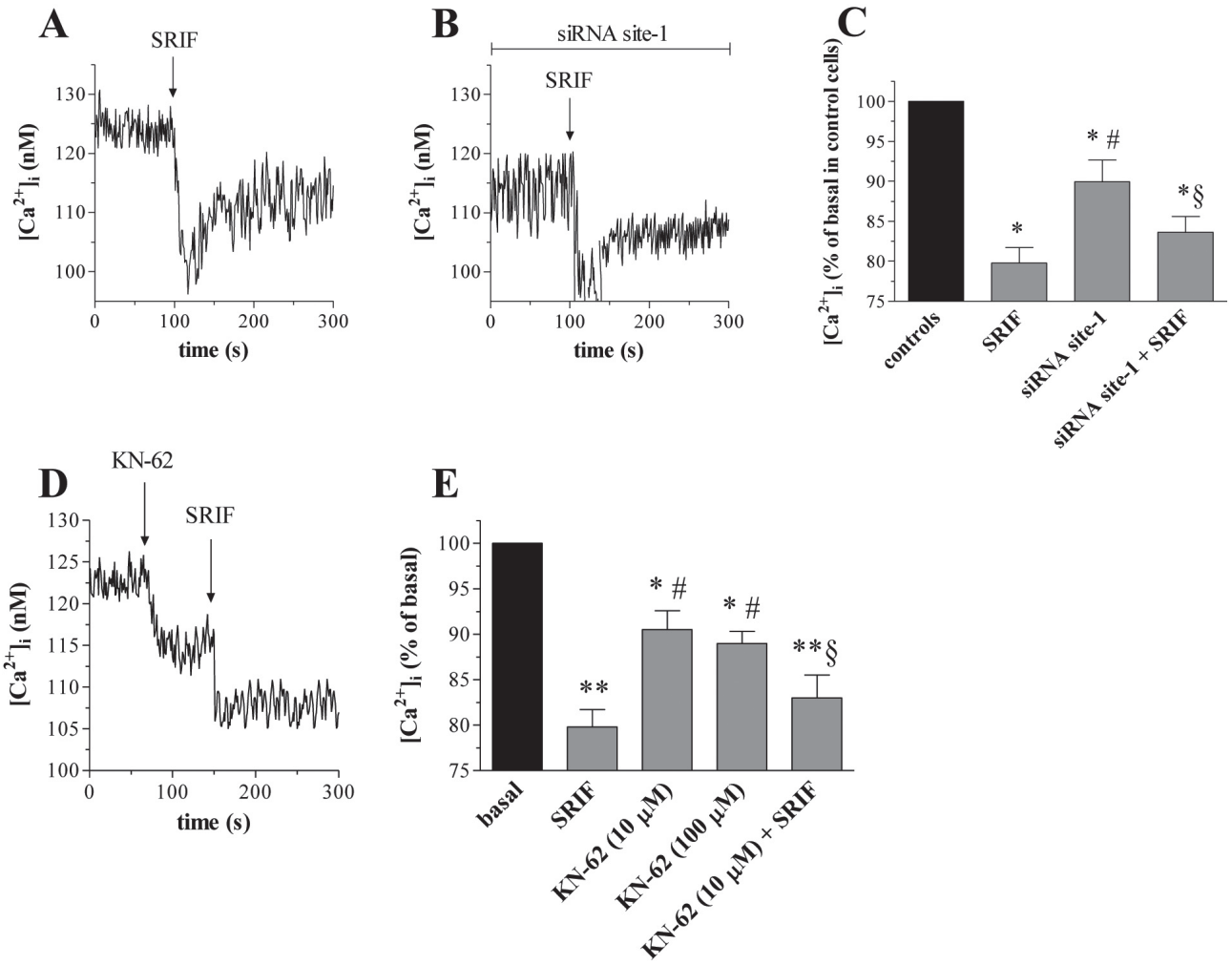

Figure 3. $[Ca^{2+}]_i$ measurements in GC cells treated with 100 nM SRIF in control conditions (**A**) or transfected with 20 nM CaMKIIβ site-1 siRNA for 96 h (**B**). Each trace shown is typical of the recording made in 7–8 independent experiments. Data collected were calculated as a percentage of basal $[Ca^{2+}]_i$ in control cells and then reported in the histograms showed in (**C**). Each histogram represents the mean ± SEM, $n = 7$. * $p < 0.001$ *vs.* control values; # $p < 0.01$ *vs.* SRIF values; § $p < 0.05$ *vs.* CaMKIIβ site-1 siRNA values (ANOVA followed by Newman-Keuls post test on raw data). $[Ca^{2+}]_i$ measurements in GC cells treated with 100 nM SRIF in the presence of KN-62 (10 μM). **D.** The trace shown is typical of the recording ($n = 6$). Data collected were calculated as a percentage of basal $[Ca^{2+}]_i$ and then reported in the histograms showed in (**E**). Each histogram represents the mean ± SEM, $n = 6$. * $p < 0.01$ and ** $p < 0.001$ *vs.* basal values; # $p < 0.05$ *vs.* SRIF values; § $p < 0.05$ *vs.* KN-62 values (ANOVA followed by Newman-Keuls post test on raw data).

addition, the subsequent application of 100 nM SRIF in cells transfected with CaMKIIβ site-1 siRNA induced a $[Ca^{2+}]_i$ decrease comparable to that obtained with SRIF alone in control cells. Similar results were obtained with the use of the isoquinoline sulfonamide blocker of CaMKII, KN-62. This compound competitively inhibits the binding of calmodulin to CaMKII by interacting with the calmodulin binding site and thus inactivates the enzyme (Hidaka and Yokokura 1996). KN-62 was applied at 10 μM, a concentration widely used in different systems, including rat anterior pituitary cells, to inhibit specifically CAMKII activity (Cui et al. 1996; Roberson et al. 2005; Murakami et al. 2007). As shown in Fig. 3D and E, KN-62 decreased basal $[Ca^{2+}]_i$ by 9.5 ± 2.1%. This was the maximum effect, as similar results were obtained when KN-62 was applied at 100 μM. In addition, the subsequent application of SRIF in the presence of 10 μM KN-62 induced a $[Ca^{2+}]_i$ decrease comparable to that obtained after SRIF alone.

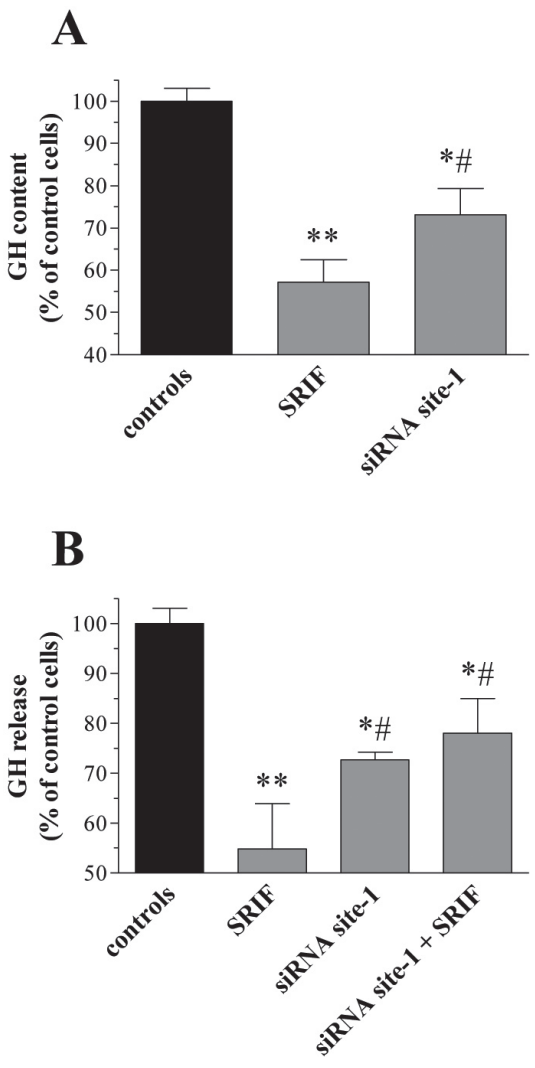

Figure 4. EIA evaluation of GH content (**A**) and release (**B**) in GC cells cultured for 24 h in the presence of 100 nM SRIF or cultured 24 h after the transfection with CaMKIIβ site-1 siRNA (20 nM, 96 h). Each histogram represents the mean ± SEM, $n = 3$. * $p < 0.05$ and ** $p < 0.01$ *vs.* control values; # $p < 0.05$ *vs.* SRIF values (ANOVA followed by Newman-Keuls post test on raw data). Data are expressed by setting GH in control conditions as 100%.

GH production

In another set of experiments we established whether CaMKIIβ levels are related to the expression of GH (content and release). Thus, GC cells were transfected with CaMKIIβ site-1 siRNA (20 nM, 96 h). Then, the medium was changed and after 24 h of cell culture EIA was carried-out. Comparable values of GH were found in control cells (untreated or transfected with negative control siRNA, data not shown). SRIF application (24 h) at 100 nM reduced both content and release of GH by 42.9 ± 5.4% and 45.2 ± 9.2%, respectively (Fig. 4A, B). The knockdown of CaMKIIβ by siRNA site-1 resulted in a reduction of GH content and secretion by 26.9 ± 6.3% and 27.3 ± 1.5%, respectively, when compared with controls. In addition, SRIF treatment did not significantly decrease GH release in cells transfected with CAMKIIβ site-1 siRNA.

Discussion

Among the CaMKII isoenzymes, the γ and δ isoenzymes have a widespread distribution in the body whereas the α and β isoenzymes are the predominant forms in the brain (Hudmon and Schulman 2002; Schulman 2004; Yamauchi 2005). In the present study, we have demonstrated that all four CaMKII isoenzymes are expressed in GC cells at the mRNA level. However, we found for the first time that only the CaMKIIβ isoenzyme is inhibited by SRIF both at mRNA and protein levels. The extremely fast downregulation of CaMKIIβ exerted by SRIF in our system may account for the rapid mRNA and protein turnover which often characterize cancer/*in vitro* cells. Accordingly, in tumoral cell models, SRIF compounds have been recently reported to rapidly modulate the expression (both at mRNA and protein level) of different genes (Horiguchi et al. 2009; Xing et al. 2010).

In agreement with previous results, we found that SRIF inhibits basal $[Ca^{2+}]_i$ (Petrucci et al. 2000) in GC cells. This effect is mimicked by the specific knockdown of CaMKIIβ and the CAMKII pharmacological blocking with KN-62, indicating that CaMKIIβ takes a part in the control of $[Ca^{2+}]_i$. There are recent results demonstrating suppression of endogenous CaMKIIβ by RNA interference in rat hippocampal neurons (Okamoto et al. 2007) although, to the best of our knowledge, no data have been reported on CaMKII knockdown in pituitary. On the other hand, experimental approaches based on

RNA interference have been used in tumour pituitary cells to examine signal molecules/receptors involved in SRIF functions (Theodoropoulou et al. 2006; Ben-Shlomo et al. 2007). In different experimental models (Nie et al. 2007; Hao et al. 2008; Wheeler et al. 2008), including neuroendocrine cells (Basavappa et al. 1999), CaMKII plays an important role in maintaining basal activity of Ca^{2+} channels and in regulating their activation, thereby increasing Ca^{2+} currents and $[Ca^{2+}]_i$. In GC cells, the effects of SRIF on $[Ca^{2+}]_i$ and those of CaMKIIβ knockdown or KN-62 are non-additive. These results raise the hypothesis that CaMKIIβ may mediate, at least in part, the SRIF-induced $[Ca^{2+}]_i$ modulation. Indeed, in GC cells, SRIF induces a decrease in $[Ca^{2+}]_i$ through a down-regulation of Ca^{2+} channels and extracellular Ca^{2+} influx (Petrucci et al. 2000). However, we have clearly demonstrated that SRIF influences CaMKIIβ levels at the level of regulation of transcription although the fast kinetic of SRIF effects on $[Ca^{2+}]_i$ suggest also a direct interaction between SRIF and CaMKIIβ. Further data are needed to exploit this possibility.

GC cells display cycles of regularly spaced spontaneous action potentials with concomitant intracellular Ca^{2+} transients which have been demonstrated as an effective manner to maintain GH exocytosis (Cervia et al. 2002b; Dominguez et al. 2007). In neurons, exocytosis seems to depend on multiple factors, including the activation of CaMKII signalling (which causes an increase of $[Ca^{2+}]_i$) and proceeds *via* delayed fusion pore opening (Kolarow et al. 2007). In line with these results, we have demonstrated here that the isoenzyme β of CaMKII plays a positive role in maintaining the exocytosis of stored GH in somatotropinomas, possibly through its effect on $[Ca^{2+}]_i$. Indeed, in GC cells, GH production (both content and release) is decreased after CaMKIIβ specific knockdown. CaMKII activation and GH secretion are reported to be correlated also in rat GH_3 tumor pituitary cells (Kanasaki et al. 2002). Homologous normal and tumoral pituitary cells, including GC cells, display the same type of secretory mechanisms *in vitro* (Cervia et al. 2002b; Stojilkovic et al. 2005). Thus, CAMKIIβ activity may be required for GH production also in normal somatotropes of mammals, as demonstrated in fishes (Wong et al. 2005). Some evidence collected in different experimental models (normal and tumoral pituitary cells) suggest that the pathways responsible for the regulation of GH production in mammals involve signals other than Ca^{2+}, as for instance K^+/Na^+ currents, nitric oxide, inositol phosphate/protein kinase C, arachidonic acid or adenylyl cyclase (Voss et al. 2001; Baratta et al. 2002; Gracia-Navarro et al. 2002; Xu et al. 2002; Anderson et al. 2004; Cervia and Bagnoli 2007; Dominguez et al. 2007; Kineman and Luque 2007; Yang et al. 2008). CAMKIIβ may add further complexity to this pathway system, although its role remains to be established. For instance, through its unique regulatory properties, CaMKII can be predicted to serve in specialized aspects of the secretory process. In particular, the ability of CaMKII to remain active may represent a mechanism by which releasable pools of vesicles are replenished between secretory stimuli (Easom 1999).

In the present study we have confirmed that SRIF reduces GH production (both content and release) in GC cells, in agreement with previous results (Cervia et al. 2002b, 2003). Neuropeptides may affect GH system through different mechanisms. For instance, orexin-A has been suggested to play an important role in increasing GH secretion in somatotropes through the enhancement of the Ca^{2+} current (Xu et al. 2002). Generally, the control of $[Ca^{2+}]_i$ accounts for the SRIF-induced inhibition of hormone secretion in different neuroendocrine cell lines (Yang et al. 2007; Smith 2008). In particular, Ca^{2+} (and adenylyl cyclase/cAMP/protein kinase A pathway) is involved in the SRIF-mediated inhibition of GH in both normal and tumoral pituitary cells (Florio et al. 2003; Cervia and Bagnoli 2007). SRIF coupling to CaMKII has been hypothesized in several studies in the hippocampus and in the pancreatic acinar AR42J cells of rats (Liu and Wong 2005; Pittaluga et al. 2005). In GC cells, the fact that CaMKIIβ knockdown mimicks SRIF effects on GH production and that CaMKIIβ levels are rapidly decreased after SRIF application suggests that CaMKIIβ inhibition would possibly mediate antisecretory effects of SRIF. Consistently, SRIF did not decrease GH release when CAMKIIβ is knocked-down, further suggesting the crucial role played by this enzyme in SRIF mechanisms. In line with these findings, insulin secretion, which is inhibited by SRIF, is under the control of CaMKIIβ and Ca^{2+} in neuroendocrine β-cells of pancreas (Easom 1999; Wang et al. 2005; Smith 2008). However, additional studies are required to elucidate fully the functional implications of this complex mechanism in GC cells.

Conclusion

This study provides a framework for better elucidating the signalling of the neuropeptide SRIF and its functional correlates in a *in vitro* model of somatotropinoma. Our present results, in combination with those of previous papers (Petrucci et al. 2000; Cervia et al. 2002a,b, 2003), suggest that SRIF is coupled to the inhibition of adenylyl cyclase pathway, $[Ca^{2+}]_i$, and CAMKIIβ. These mediators seem to be coupled to SRIF-induced control of GH production. Acromegaly is the most important indication for SRIF analogues being a result of a somatotropinoma that hypersecretes GH. The therapy of acromegalic patients is well established with regard to current generation of SRIF analogues which may be useful in approximately 60–70% of the patient population (Hofland et al. 2005; Bronstein 2006; Katznelson 2006; Melmed 2006; Tolis et al. 2006; van der Hoek et al. 2007). On the other hand, there is certainly a need for novel SRIF analogues for

the treatment of neuroendocrine tumours, which possibly target specific signalling regulators. Thus, the elucidation of the complexity of SRIF signalling coupled to GH control may offer a new therapeutic perspective with the development of specific pharmacological signalling modulators that could be incorporated into the therapeutic arsenal.

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