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**Involvement of the cAMP-dependent pathway in the reduction of epileptiform
bursting caused by somatostatin in the mouse hippocampus**

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Abstract

The cAMP pathway is major signal transduction system involved in hippocampal neurotransmission. Recently, the peptide somatostatin-14 (SRIF) has emerged as a key signal that, by activating its receptors, inhibits epileptiform bursting in the mouse hippocampus. Little is known on transduction mechanisms which may mediate SRIF function in native cell/tissues. Using a well established model of epileptiform activity induced by Mg^{2+} -free medium with 4-aminopyridine (0 Mg^{2+} /4-AP) in mouse hippocampal slices, we demonstrated that PKA-related signaling is upregulated by hippocampal bursting and that treatment with SRIF normalizes this upregulation. We also demonstrated that the SRIF-induced inhibition of PKA impairs phosphorylation of the NMDA receptor subunit NR1. Extracellular recordings of the 0 Mg^{2+} /4-AP-induced hippocampal discharge from the CA3 region demonstrated that treating slices with compounds which interfere with PKA activity prevent SRIF inhibition of epileptiform bursting. Our results suggest that SRIF modulation of hippocampal activity may involve PKA-related signaling.

Key words: transduction pathway, epilepsy, hippocampal slices, PKA modulation, electrophysiological recordings

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Introduction

A major signal transduction system which is related at various levels with hippocampal neurotransmission is the adenylyl cyclase (AC)/cyclic AMP (cAMP)/ cAMP-dependent protein kinase A (PKA) pathway (see for review Nguyen and Woo 2003). The cAMP signaling system is regulated by hippocampal seizures (Higashima et al. 2002; Iwasa et al. 2000; Jang et al. 2001), and changes in PKA phosphorylation of glutamate receptors have been found to be related to epileptic seizures in the rat hippocampus (Arias et al. 2002; Chang et al. 2005; Huo et al. 2006; Moussa et al. 2001; Niimura et al. 2005). On the other hand, there is evidence that the PKA pathway plays a role in the control of glutamatergic and GABAergic transmission in the rat hippocampus (Negrete-Diaz et al. 2006; Pita-Almenar et al. 2006; Poisbeau et al. 1999), while cAMP has been shown to mediate epileptiform generation in rat hippocampal slices (Higashima et al. 2002). The possibility that the PKA pathway may affect the epileptic state is further strengthened by the finding that inhibition of the PKA activity may display an antiepileptic effect (Vazquez-Lopez et al. 2005).

The peptide somatostatin-14 or SRIF (somatotropin release inhibiting factor) is widely distributed in the mammalian brain where it mediates numerous functions through binding to its receptors (sst₁-sst₅), all belonging to the G protein-coupled receptor family (see for review Hannon et al. 2002; Olias et al. 2004). Beside their initially described neuroendocrine role, SRIF systems have been shown to subserve neuromodulatory roles in the brain, influencing motor activity, sleep, sensory processes and cognitive functions, and are altered in brain diseases like affective disorders, Alzheimer's disease and epilepsy (see for review Cervia and Bagnoli 2007; Viollet et al. 2008). In this respect, numerous evidence suggests that SRIF is a potent endogenous anticonvulsant (see for review Tallent and Qiu 2008). In particular, functional data on the hippocampal SRIF system of rodents have provided compelling evidence that SRIF compounds play an important role in inhibiting excitatory transmission, including hyperexcitability underlying seizures (Binaschi et al. 2003; Fu and van den Pol 2007; Tallent and Siggins 1999; Vezzani and Hoyer 1999). In this

respect, SRIF inhibitory effects on hippocampal bursting has been recently demonstrated in mouse hippocampal slices in a well-established model of interictal-like activity in mouse hippocampal slices (Cammalleri et al. 2004, 2006; Cervia et al. 2005; Ristori et al. 2008a).

Many transduction mechanisms have been described to mediate SRIF function in transfected systems (see for review Lahlou et al. 2004; Olias et al. 2004), however most physiological actions of the native SRIF receptors *in situ* remain to be established. There is recent evidence that the cyclooxygenase-2 (COX-2)/prostaglandin E₂ (PGE₂) pathway is involved in the SRIF-induced decrease of epileptiform bursting in the mouse hippocampus (Ristori et al. 2008a). An important component in SRIF transduction mechanisms in recombinant systems as well as in native cells/tissues is the modulation of the PKA pathway (see for ref. Cervia and Bagnoli 2007; Cervia et al. 2005). In particular, inhibition of AC by SRIF has been demonstrated in ovine and mouse retinas, in the rat frontoparietal cortex and in the rat hippocampus (Colas et al. 1992; Izquierdo-Claros et al. 2002, 2004; Ristori et al. 2008b). Recently, SRIF-mediated inhibition of AC activity has been shown to parallel SRIF-induced reduction of epileptiform bursting in the mouse hippocampus (Cammalleri et al. 2006). Whether SRIF inhibition of hippocampal bursting may involve the cAMP-PKA signaling cascade remains to be established.

In the present study, we have used a well-established model of epileptiform bursting in rat and mouse hippocampal slices (Cammalleri et al. 2004, 2006; Kilb et al. 2007; Ristori et al. 2008a; Sanna et al. 2000; Siniscalchi et al. 1997) in order to investigate whether SRIF inhibition of hippocampal discharge involves modulation of the PKA pathway.

Methods

Animals

Experiments were performed on 84 mice (C57BL/6 strain) of both sexes at 6-8 weeks after birth (18-25 g body weight). Animals were kept in a regulated environment ($23\pm 1^{\circ}\text{C}$, $50\pm 5\%$ humidity) with a 12 h light/dark cycle (lights on at 8 a.m.) with food and water ad lib. In all experiments, mice were anesthetized with halothane (4%). Experiments were performed in compliance with the Italian law on animal care N° 116/1992 and in accordance with the European Community Council Directive (EEC/609/86). All efforts were made to reduce both animal suffering and the number of animals used.

Preparation of hippocampal slices

Slices of dorsal hippocampus were prepared as previously described (Cammalleri et al. 2004, 2006; Cervia et al. 2005; Ristori et al. 2008a). Mice were anaesthetized and decapitated. Their brains were rapidly removed, placed in ice-cold artificial cerebrospinal fluid (aCSF; 130 mM NaCl, 3.5 mM KCl, 1.25 mM NaH_2PO_4 , 1.5 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 24 mM NaHCO_3 , 10 mM glucose, pH 7.4) and gassed with 95% O_2 and 5% CO_2 . Transverse hippocampal slices (400 μm in thickness, 6 slices from each mouse) were prepared with the use of a vibratome (Campden Instruments, Loughborough, UK) and kept individually. They were incubated for 1 h at room temperature in aCSF. Epileptiform activity was obtained using the potassium channel blocker 4-aminopyridine (4-AP) in the absence of external Mg^{2+} (0 Mg^{2+}) (Cammalleri et al. 2004, 2006; Kilb et al. 2007; Ristori et al. 2008a; Sanna et al. 2000, Siniscalchi et al. 1997). In this model, epileptiform activity consisted of ictal discharges which disappeared within 2 h after 0 Mg^{2+} /4-AP and were replaced by continuous interictal events, which remained synchronous in the hippocampal regions, as previously described (Barbarosie and Avoli, 1997). This treatment has been described to overcome apparent discrepancies resulting from studies using either the 0 Mg^{2+} model or the 4-AP model alone (Siniscalchi et al. 1997). Slices were incubated for 3 h in either aCSF or 0 Mg^{2+} /4-AP. Then, the medium was replaced with fresh aCSF, 0 Mg^{2+} /4-AP or 0 Mg^{2+} /4-AP + SRIF and the

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3 slices incubated for additional 5, 10, 15 or 20 min. SRIF was used at 1 μ M, a concentration giving
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5 maximal receptor occupancy in our system (Cammalleri et al. 2004, 2006; Cervia et al. 2005), that
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7 was also used in previous studies on the effects of SRIF compounds in the rodent hippocampus
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9 (Boehm and Betz 1997; Cammalleri et al. 2004, 2006; Ristori et al. 2008a; Schweitzer et al. 1998;
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11 Tallent and Siggins 1997, 1999). For measurements of AC and PKA activities, messengers and
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13 proteins, 3-6 samples were used for each experimental condition. Each sample consisted of 3 slices
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15 collected at the same anterior-posterior location from the hippocampal formation of different
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17 animals and pooled together. All measurements were conducted in duplicate for each sample. In
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19 immunohistochemical studies, 6 slices kept individually from different animals were used for each
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21 experimental condition. Before collection, slices were randomly evaluated electrophysiologically to
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23 ascertain SRIF inhibition of epileptiform activity. SRIF inhibitory effects on hippocampal bursting
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25 were found to be independent from slice location. In functional studies, for each experimental
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27 condition we used 6 slices kept individually from different animals to remove potential animal
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29 specific responses.
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Measurements of adenylyl cyclase (AC) activity

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43 AC activity was measured in agreement with Cammalleri et al. (2006). Briefly, slices were
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45 homogenized in ice-cold buffer (10 mM HEPES/Tris, 10% sucrose, pH 7.4). The homogenate was
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47 centrifuged at 1,000xg for 10 min, the supernatant was collected and stored. The pellet was re-
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49 centrifuged at 1,000xg for 10 min, the supernatant was collected and stored. The pellet was re-
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51 suspended, re-homogenized and centrifuged as above. The two supernatants were combined and
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53 centrifuged at 11,000xg for 20 min. The pellet was re-suspended in 30 mL of ice-cold buffer
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55 without sucrose and centrifuged at 27,000xg for 10 min. Finally, the pellet was re-suspended in the
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57 same buffer supplemented with 20 μ g/mL aprotinin and 20 μ g/mL leupeptin and used immediately.
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59 All steps of the above procedure were performed at 4°C. Protein concentration was determined by
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the method of Bradford (1976). The standard mixture for AC assay (0.4 mL/tube: 75 mM

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HEPES/Tris, 1 mM EGTA, 1 mM MgSO₄, 10 μM 3-isobutyl-1-methylxanthine, 500 μM GTP, pH 7.4) was supplemented with 20 μg of membrane protein/tube. Stimulation of AC activity was produced by addition of increasing concentrations of Ca²⁺ in combination with 2 μM calmodulin. Membranes were preincubated for 20 min at 0°C, the reaction was then initiated by the addition of 500 μM ATP, carried out for 10 min at 30°C and stopped by boiling. After centrifugation of samples, cAMP was measured by a protein-binding method on the clear supernatant, using [³H]cAMP, according to Brown et al. (1972). The data are expressed as cAMP pmoles/min/mg of protein.

Measurements of mRNAs with semiquantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA extraction was performed in Trizol reagent (Invitrogen, Carlsbad, CA, USA). After solubilization in diethyl pyrocarbonate-treated water, total RNA was quantified by SmartSpec 3000 spectrophotometer (Bio-Rad, Hercules, CA, USA). First-strand cDNA was generated from 2 μg of total RNA using random primers and the reverse transcriptase of Moloney murine leukemia virus (Invitrogen). Forward and reverse primers were chosen from mouse sequences to hybridize to unique regions of the respective gene sequence and are listed in Table 1. The housekeeping gene cyclophilin B was used as an internal standard in agreement with previous studies in the mouse hippocampus (Cammalleri et al. 2004, 2006; Ristori et al. 2008a). Indeed, cyclophilin B mRNA is presumably stable and its hippocampal level does not appear to be influenced by epileptiform treatments (Cammalleri et al. 2004, 2006). The PCR reactions were carried out using 1 μl of cDNA in a 25 μl mixture containing 1.5 mM MgCl₂, 200 μM dNTPs, and 400 nM of appropriate primers. Taq polymerase (0.5 U, Invitrogen) was also added. The amplification reactions were carried out in a thermal gradient cycler (Bio-Rad) for 25 (AC1 and cyclophilin B), 29 (AC8) or 31 (AC3 and Cα

subunit of PKA) cycles. Each cycle consisted of denaturation for 60 sec at 94°C, annealing for 60 sec and an extension for 60 sec at 72°C. The temperature of annealing was 60°C for AC1, AC8 and cyclophilin B, 58°C for AC3 and 55°C for C α subunit. A final extension step at 72°C for 10 min terminated the amplification. For each amplification, two types of controls were performed: (i) RT-PCR mixture with no reverse transcriptase to control genomic DNA contamination; and (ii) PCR mixture with no cDNA template added to check for possible external contamination. A 5 μ l sample of the PCR reaction was electrophoresed on an ethidium bromide-containing 2% agarose gel. After migration, bands corresponding to the amplified products were analysed with Gel Doc 2000 System equipped with Quantity One software (Bio-Rad). Semiquantitative analysis of PCR products was performed by measuring the optical density (OD) of the bands corresponding to AC1, AC3 and AC8 isoforms or to the C α subunit of PKA with respect to the OD of the band corresponding to the respective cyclophilin B.

Measurements of cAMP-dependent protein kinase A (PKA) activity

Slices were homogenized in ice-cold buffer (25 mM Tris-HCl, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulphonyl fluoride, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, pH 7.4). Hippocampal cytosol fraction was isolated by centrifugation at 14,000xg for 10 min at 4°C. Protein concentration was determined according to Bradford (1976). PKA activity was measured with the PepTag nonradioactive PKA assay kit (Promega, Madison, MI, USA), according to the manufacturer's instructions. Briefly, PKA activity in the hippocampal cytosol fractions (10 μ g protein/sample) was measured at 30°C for 30 min, in a mixture containing 2 μ g of the PKA substrate kemptide and 1 μ M cAMP. The reaction was stopped by placing the samples on a 95°C heating block for 10 min. Agarose gel electrophoresis (0.8%) was used to separate the phosphorylated (net charge -1) from non-phosphorylated (net charge +1) kemptide. The phosphorylated kemptide was excised from the gel and PKA activity was quantified

spectrophotometrically using a SmartSpec 3000 spectrophotometer (Bio-Rad). Data are expressed as incorporated picomoles of phosphate/min/mg of protein, and values represent the mean of duplicate determination.

Measurements of protein levels with Western blot

To measure the protein levels of the C α subunit of PKA, hippocampal extracts were prepared as previously described (Cammalleri et al. 2006, 2004). Briefly, the hippocampus was homogenized in 10 mM Tris-HCl, pH 7.6 containing 5 mM EDTA, 3 mM EGTA, 250 mM sucrose, 1 mM phenylmethylsulphonyl fluoride, 1 μ M peptistatin, 10 μ g/mL leupeptin, 2 μ g/mL aprotinin and centrifuged at 22,000xg for 30 min at 4°C. The supernatant was used for Western blot analysis. On the same samples, the expression of α -tubulin was evaluated as an internal standard.

To measure the protein levels of the NMDA receptor (NMDAR) subunit NR1 and of its phosphorylated form, hippocampal extracts were prepared according to Niimura et al. (2005). Briefly, the hippocampus was homogenized in 0.32 M sucrose containing leupeptin, aprotinin, and antipain (5 μ g/mL each), 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium orthovanadate, 20 mM each of β -glycerophosphate and p-nitrophenylphosphate. The homogenate was centrifuged at 22,000xg for 30 min at 4°C and the supernatant was used for Western blot analysis. Protein concentration in each sample was determined according to Bradford (1976).

Aliquots of each sample containing equal amounts of protein (40 μ g) were subjected to 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis, according to Laemmli (1970), and transblotted onto polyvinylidene difluoride membrane. Membranes were blocked 1 h in 3% non-fat milk and incubated with primary antibodies. To evaluate levels of the C α subunit of PKA, membranes were incubated overnight at 4°C with a rabbit polyclonal antibody directed against the C-terminus (1:200 dilution) or a goat polyclonal antibody directed against the N-terminus (1:200 dilution) of the C α subunit. To evaluate phosphorylation of NR1 subunit of NMDA receptors by

PKA, we examined serine phosphorylation on the PKA phosphorylation site Ser897 (pS897 NR1). Membranes were incubated overnight at 4°C with a rabbit polyclonal antibody directed against pS897 NR1 (1:500 dilution). The membranes were then incubated for 2 hours at 4°C with horseradish peroxidase-linked secondary antibodies directed against rabbit IgG (1:2,500 dilution) or goat IgG (1:2,500 dilution), as appropriate, and developed by the enhanced chemiluminescence detection method (Supersignal West Pico, Pierce, Rockford, IL, USA). Subsequently, membranes were stripped and re-probed with a rabbit polyclonal antibody directed against α -tubulin (1:200 dilution) or against NR1 subunit (1 μ g/ml dilution). OD of the bands was evaluated by using Quantity One software (Bio-Rad). OD of C α subunit was normalized to the level of α -tubulin whereas OD of pS897 NR1 was normalized to the level of NR1.

Immunohistochemistry

Slices were fixed for 1 h in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at 4°C. They were then rinsed in 0.1 M PB and incubated for 48 h in 1:200 of a rabbit polyclonal antibody directed against the C-terminus of the C α subunit of PKA diluted in 0.1 M PB containing 0.3% Triton X-100 at 4°C. The slices were then rinsed in 0.1 M PB and incubated overnight in secondary antibody conjugated with Alexa Fluor 488 at a dilution of 1:200 in 0.1 M PB containing 0.3% Triton X-100. Finally, the slices were rinsed in 0.1 M PB, mounted on gelatin-coated glass slides and coverslipped in a 0.1 M PB-glycerine mixture. The specificity of C α -immunoreactivity (-ir) was evaluated by omitting the primary antibody. The slices were examined with a confocal laser scanning microscope (Leica Microsystems Heidelberg, Mannheim, Germany) using 10x or 40x objective lens. Optical sections were scanned through the thickness of each slice, and single 1 μ m thick optical sections at the same predetermined z-axis were collected for the analysis of C α -ir intensity. Methods for measuring immunofluorescence intensity from blindcoded confocal images were adapted from Gazzaley et al. (1996a, b; 1997) and are detailed in previous works (Cammalleri

et al. 2006; Ristori et al. 2008a). The software package KS-300 (Carl Zeiss Vision, München-Hallbergmoos, Germany) was used to determine the average pixel intensity (grey scale) of single fields in the CA1, CA3 and in the dentate gyrus. Within each field, the contribution of unlabelled portions was removed, normalizing the data to the background fluorescence. Mean intensity values for each field were expressed as a percentage of the average fluorescence intensity of the corresponding field of hippocampal slices in basal conditions (aCSF). The data represent the mean intensity values from 6 slices for each experimental condition.

Electrophysiology

Using published protocols (Cammalleri et al. 2004, 2006; Cervia et al. 2005; Ristori et al. 2008a), at the end of the incubation in 0 Mg^{2+} /4-AP hippocampal slices were transferred to an interface recording chamber containing fresh 0 Mg^{2+} /4-AP. They were continuously superfused at 3 ml/min with gassed bathing solution at 32°C; the drugs were added to this superfusion solution.

Extracellular recordings of hippocampal discharge were performed from the stratum pyramidale of the CA3 region by using 2-5 $M\Omega$ microelectrodes filled with aCSF. Epileptiform bursts induced by 0 Mg^{2+} /4-AP were amplified with an Axoclamp-2B amplifier (Axon Instruments, Foster City, CA, USA), filtered (DC 3-10 kHz) and displayed on an oscilloscope. Signals were recorded for 20 min. The interval between SRIF application and the recording of an observable effect was 6-8 min. All traces were analysed using the LabView software package (National Instruments, Austin, TX, USA). The data represent the mean discharge frequency (burst frequency/min) over the registration period from 6 slices for each experimental condition. Inhibition of burst frequency was calculated as a percentage of the burst frequency in the presence of 0 Mg^{2+} /4-AP.

Analysis of data

Upon verification of normal distribution, statistical significance of data was evaluated using ANOVA followed by multiple comparison Bonferroni's post-test. GraphPad Prism software package (Graph Software, San Diego, CA, USA) was used. Differences with $P < 0.05$ were considered significant. EC_{50} and IC_{50} were determined by non-linear regression curve analysis of the concentration-effect responses using GraphPad Prism. Results are given as means $\pm$ SEM.

Chemicals

SRIF was purchased from Bachem (Bubendorf, Switzerland). Primer pairs for AC1, AC3, AC8, $C\alpha$ and cyclophilin B were purchased from Eurobio (Les Ulis, France). [3H]cAMP (20 Ci/mmol) was purchased from Perkin-Elmer Life Sciences (Waltham, MA, USA). Antibodies directed against the C-terminus or the N-terminus of $C\alpha$ subunit or against α -tubulin as well as the horseradish peroxidase-linked secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies conjugated with Alexa Fluor 488 were purchased from Molecular Probes (Eugene, OR, USA). Antibodies directed against NR1 or pS897 NR1 were from Upstate Biotechnology (Lake Placid, NY, USA). Where not specified, chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Results

SRIF effects on both AC activity and expression of Ca^{2+} -stimulated AC isoforms

In the present study, we evaluated whether SRIF inhibitory action on epileptiform bursting involves modulation of AC activity. AC activity in hippocampal slices was significantly higher after 0 Mg^{2+} /4-AP ($136.2 \pm 15.4\%$, $P < 0.001$) and this increase was diminished by SRIF, which recovered AC activity levels measured in aCSF conditions (Fig. 1a). The increase in intracellular Ca^{2+}

concentration plays a critical role in seizure etiology (DeLorenzo et al. 2006). Thus, we evaluated whether SRIF inhibitory action on epileptiform bursting involves modulation of Ca^{2+} -stimulated AC activity. As shown in figure 1b, the effects of Ca^{2+} on the AC activity in hippocampal membranes were bidirectional, a typical trend reflecting the main involvement of Ca^{2+} -stimulated AC isoforms (namely AC1, AC3 and AC8) in the hippocampal AC activity (Guillou et al. 1999). Maximal stimulation occurred at micromolar concentrations of Ca^{2+} . Curve-fitting analyses revealed that Ca^{2+} produced a concentration-dependent stimulation of AC activity with an EC_{50} which was significantly lower in 0 Mg^{2+} /4-AP than in 0 Mg^{2+} /4-AP + SRIF and in aCSF (0.018 μM in 0 Mg^{2+} /4-AP vs 0.052 μM in 0 Mg^{2+} /4-AP + SRIF and 0.039 μM in aCSF, $P < 0.001$ and $P < 0.05$, respectively). No statistical differences were observed between the EC_{50} values of aCSF-treated slices and 0 Mg^{2+} /4-AP + SRIF-treated slices. Changes in the catalytic properties of Ca^{2+} -stimulated AC isoforms could result from alterations in the subtype of AC being expressed. We therefore examined whether treatment with 0 Mg^{2+} /4-AP could enhance the expression of the mRNAs for the Ca^{2+} -stimulated AC isoforms, and whether SRIF application could counteract this effect. As shown in figure 2, we found that treatment with 0 Mg^{2+} /4-AP significantly increased the mRNA levels of AC1, AC3 and AC8 as compared to mRNA levels measured in aCSF conditions (40.4 $\pm$ 5.5%, $P < 0.01$; 49.1 $\pm$ 6.7%, $P < 0.05$; 45.4 $\pm$ 3.5%, $P < 0.01$, respectively). These increases were not influenced by 5, 10 or 15 min treatment with SRIF. In contrast, 20 min treatment with SRIF abolished the 0 Mg^{2+} /4-AP-induced increase in mRNAs of the three isoforms.

SRIF effects on both PKA activity and expression of the C α subunit of PKA

PKA is the main effector of cAMP regulation, thus we may expect that variations in both AC activity and expression of distinct AC isoforms result in concomitant changes of PKA activity. We examined whether SRIF-induced decrease of both AC activity and AC isoform expression in hippocampal slices treated with 0 Mg^{2+} /4-AP was accompanied by a down-stream decrease in PKA

activity. As shown in figure 3, after 0 Mg^{2+} /4-AP, PKA activity was significantly increased ($45.8 \pm 10.6\%$; $P < 0.001$). In 0 Mg^{2+} /4-AP-treated slices, SRIF administration restored values of PKA activity similar to those observed in aCSF.

PKA is a tetrameric enzyme consisting of two regulatory and two catalytic subunits. cAMP promotes PKA activity by binding to regulatory subunits, thereby releasing active catalytic subunits including $C\alpha$ and $C\beta$ both of which are expressed in the rodent hippocampus (Shobe 2002). In order to determine whether SRIF inhibition of epileptiform bursting influences $C\alpha$ expression in the mouse hippocampus, we evaluated the effects of 0 Mg^{2+} /4-AP either alone or in combination with SRIF on both messenger and protein of the $C\alpha$ subunit in hippocampal slices. We found that 0 Mg^{2+} /4-AP treatment increased $C\alpha$ mRNA levels by $35.7 \pm 5.2\%$ ($P < 0.01$) with respect to aCSF (Fig. 4a). The 0 Mg^{2+} /4-AP-induced increase in the mRNA level of the $C\alpha$ subunit was not influenced by 5 or 10 min treatment with SRIF. In contrast, 15 or 20 min treatment with SRIF abolished the 0 Mg^{2+} /4-AP-induced increase in $C\alpha$ subunit mRNA level. Semiquantitative Western blot analysis of hippocampal lysates using antibodies directed against either the C-terminus (Fig. 4b) or the N-terminus (Fig. 4c) of the $C\alpha$ subunit revealed a substantial increase in levels of the $C\alpha$ protein after 0 Mg^{2+} /4-AP treatment ($60.7 \pm 9.0\%$, $P < 0.01$ and $74.6 \pm 8.9\%$, $P < 0.01$, respectively). This 0 Mg^{2+} /4-AP-induced increase in the $C\alpha$ protein was abolished by SRIF when applied for 20 min but not for 5, 10 or 15 min (Fig. 4b, c). Our immunohistochemical observations confirmed these data. In addition, they provided novel insights on $C\alpha$ subunit localization in the mouse hippocampus. As shown by the confocal image of figure 5a, constitutive $C\alpha$ protein expression, as detected by the use of an antibody directed against the C terminus of the $C\alpha$ subunit, could be observed in many areas of the hippocampus. In particular, prominent staining was in the CA3 and in the dentate gyrus. As shown by higher magnifications (insets of Fig. 5a), in CA3 immunolabeling was in pyramidal cell somata and in their dendritic fields of the stratum lucidum where the mossy fiber pathway terminates; in the dentate gyrus immunolabeling was in granular cell somata and in the stratum moleculare. Less staining was observed in CA1, where it was localized in the pyramidal cell somata

and their dendritic field of the stratum radiatum. After 0 Mg^{2+} /4-AP treatment, immunolabeling intensity was observed to increase in both CA3 and the dentate gyrus when compared with the basal condition, and this increase was abolished by SRIF (Fig. 5b, c). This was confirmed by the quantitative analysis showing that no effects of 0 Mg^{2+} /4-AP on $C\alpha$ -ir were found in the pyramidal layer of CA1 (Fig. 5d), whereas $C\alpha$ -ir significantly increased in the pyramidal layer of CA3 ($93.1 \pm 12.6\%$, $P < 0.01$, Fig. 5e) and in the molecular layer of the dentate gyrus ($86.2 \pm 25.2\%$, $P < 0.05$, Fig. 5f). SRIF completely restored $C\alpha$ -ir in both CA3 and dentate gyrus.

SRIF effects on phosphorylation of NMDA receptors

In the hippocampus, NMDA receptors (NMDARs) are tetrameric complexes consisting of NR1, NR2A and/or NR2B subunits (Hawkins et al. 1999). Seizure activity is associated with enhanced phosphorylation of hippocampal NMDAR subunits (Arias et al. 2002; Huo et al. 2006; Moussa et al. 2004; Niimura et al. 2005). In particular, PKA-induced phosphorylation of NR1 has been demonstrated in rat hippocampal models of status epilepticus (Niimura et al. 2005). In order to determine whether specific NR1 phosphorylation by PKA is inhibited by SRIF in the mouse hippocampus, a semiquantitative Western blot analysis was performed. As shown in figure 6, after 0 Mg^{2+} /4-AP, phosphorylation of NR1 at the PKA-dependent site serine 897 was significantly increased ($84.4 \pm 4.2\%$; $P < 0.001$). SRIF administration did not alter total protein levels of NR1 subunit of NMDAR. However, it did markedly reduce the level of NR1 phosphorylation ($33.3 \pm 2.4\%$; $P < 0.001$).

PKA involvement in the SRIF-mediated inhibition of hippocampal bursting

The cAMP cascade is one of the major second messenger systems regulating hippocampal activity (Arnsten et al. 2005; Nguyen and Woo 2003). Also, our observations that SRIF inhibits the

cAMP/PKA transducing system activated by epileptiform treatment (see above), led us to test whether this system was involved in the inhibition of epileptiform bursting induced by SRIF. If a decrease of cAMP levels is necessary for the SRIF-mediated inhibition of epileptiform bursting, preventing this decrease should block this effect. Therefore, in these experiments, we treated slices with the membrane-permeable cAMP analog dibutyryl cAMP (dbcAMP; Henion et al. 1967) that should “clamp” PKA activity at a high constant level. dbcAMP was used at 0.5 mM, according to previous works (Diogenes et al. 2004; Slack and Walsh, 1995). As shown in figure 7, interictal spontaneous discharges developed within 40–60 min in the CA3 region of hippocampal slices treated with 0 Mg^{2+} /4-AP. The frequency ($29.7 \pm 0.2/\text{min}$) and duration (150–200 msec) of the discharge became stable after 2 h and did not change up to 5 h (data not shown). In agreement with previous results (Cammalleri et al. 2004, 2006; Cervia et al. 2005; Ristori et al. 2008a), SRIF (20 min treatment) decreased significantly the bursting frequency by $35.6 \pm 2.1\%$ ($P < 0.001$). By itself, dbcAMP (2 h pretreatment and continuously perfused in the recording bath), did not affect the 0 Mg^{2+} /4-AP-induced bursting frequency, but in combination with SRIF (20 min treatment) it completely abolished the inhibitory effect of SRIF on bursting discharge. The fact that dbcAMP itself did not influence hippocampal activity is in agreement with previous results demonstrating that excitatory synaptic transmission is not influenced by dbcAMP in the rat hippocampus (Diogenes et al. 2004). To further test the hypothesis that the PKA pathway may mediate the action of SRIF on epileptiform bursting, we increased PKA activity by enhancing cAMP production through AC activation using forskolin. Slice incubation with forskolin (30 μM) for 1 h caused a $58.2 \pm 2.6\%$ ($P < 0.001$) increase of 0 Mg^{2+} /4-AP-induced hippocampal bursting. This observation is in agreement with previous results demonstrating that forskolin enhances afterdischarge generation in rat hippocampal slices (Higashima et al. 2002) and increases interictal bursting in hippocampal slices from neonatal mice (Agmon and Wells 2003). In forskolin-stimulated slices, the inhibitory action of SRIF was abolished (Fig. 7).

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In another set of experiments, we tested the effects of SRIF on bursting discharge in the presence of H-89, which directly inhibits the catalytic subunit of PKA. Given the large variability of H-89 concentrations reported to inhibit PKA activity (Cunha-Reis et al. 2005; Li et al. 2007; Munno et al. 2003; Welsby et al. 2007; Young and Yang 2004), we assessed H-89 efficacy to inhibit PKA in our experimental model by measuring PKA activity in 0 Mg²⁺/4-AP-treated hippocampal slices after their incubation in increasing concentrations of H-89. We found that increasing concentrations of H-89 produced a dose-dependent inhibition of 0 Mg²⁺/4-AP-stimulated PKA activity with an IC₅₀ of 0.5 µM and an E_{max} of 90.6% (Fig. 8a). As shown in figure 8b, by itself, 3 µM H-89 (2 h pretreatment and continuously perfused in the recording bath), did not affect the 0 Mg²⁺/4-AP-induced bursting frequency in agreement with the observation that H-89 does not influence ictal activity in rat hippocampal slices (Higashima et al. 2002). When SRIF was applied in the presence of H-89, bursting discharge was not significantly different from that recorded after the application of 0 Mg²⁺/4-AP alone, indicating that H-89 abolished the inhibitory effect of SRIF on epileptiform bursting.

Discussion

There is general agreement that SRIF exerts important inhibitory actions in the hippocampus and acts as an endogenous antiepileptic. However, the molecular events linking SRIF receptors to their inhibitory effects on hippocampal bursting are poorly understood. The results of the present study suggest that the PKA pathway may be involved in the reduction of epileptiform bursting caused by SRIF in the mouse hippocampus.

As shown by our results, SRIF counteracts the increase of AC activity and expression associated with epileptiform bursting. Our findings that both AC activity and the mRNA expression of distinct AC isoforms are increased by epileptiform treatment is in line with previous observations showing increased cAMP levels in seizures (see for ref. Higashima et al. 2002), increased levels of AC

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3 isoform mRNAs in the hippocampus following kindling or chronic electroconvulsive shock (Iwasa
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5 et al. 2000; Jang et al. 2001) and involvement of increased AC expression in the onset of glutamate-
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7 induced epilepsy in the rat hippocampus (Wang et al. 2004). Our data demonstrate that these
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9 epileptiform bursting-related increases of AC activity and expression are inhibited by SRIF,
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11 suggesting an involvement of the AC transduction pathway in mediating the SRIF anti-epileptic
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13 effect. This SRIF inhibition of the 0 Mg^{2+} /4-AP -induced increase in AC activity is in line with
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15 previous studies demonstrating that anticonvulsant action of peptides as, for instance, galanin, is
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17 generally associated with inhibition of AC activity (Saar et al. 2002). In addition, anticonvulsant
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19 pharmacological treatments, including carbamazepine, have been demonstrated to inhibit AC
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21 activity as well as the downstream effects of AC activation (Chen et al. 1996).
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27 It is difficult to determine whether the decrease of AC isoform expression following SRIF
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29 administration is a consequence of a direct SRIF effect at the transcriptional level or a consequence
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31 of the SRIF-induced reduction in bursting activity. The possibility exists that different AC isoforms
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33 are differently regulated by SRIF and/or bursting activity. For instance, AC3 is not directly
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35 regulated by Gi/Go-coupled receptors; therefore an activity-dependent regulation may be present in
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37 this case. On the other hand, for AC1 and AC8 regulation both SRIF- and bursting activity-based
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39 mechanisms may be of importance.
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44 In the AC cascade, PKA is a major modulator of synaptic transmission likely to be involved in
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46 molecular and cellular events leading to epileptogenesis (see for ref. Vazquez-Lopez et al. 2005).
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49 Although conflicting results have been reported in different models of hippocampal seizures
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51 (Vazquez-Lopez et al. 2005; Yechikhov et al. 2001), our data demonstrate an increased PKA
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53 activity in 0 Mg^{2+} /4-AP-treated hippocampal slices. Most importantly, we have shown that SRIF
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55 treatment inhibits this increase, suggesting that PKA pathway may be involved in the transduction
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57 of the SRIF effects on epileptiform bursting. This is in agreement with evidence reporting that
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59 modulation of hippocampal activity by neuroactive substances may involve AC/cAMP/PKA
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pathway. For instance, positive coupling to PKA mediates the effects of vasointestinal peptide and

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3 brain derived neurotrophic factor on synaptic transmission in the rat hippocampus (Cunha-Reis et al.
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5 2005; Diogenes et al. 2004). In addition, elevation of PKA activity in mice underlies effects of
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8 corticotropin-releasing factor on hippocampal neuronal excitability (Blank et al. 2003). On the other
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11 hand, negative coupling to AC/cAMP/PKA pathway appears to be required for induction of long
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13 term depression induced by the activation of glutamate and adenosine receptors or the application of
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15 endocannabinoids in the rodent hippocampus (Chevalleyre et al. 2007; Santschi et al. 2006).
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17 We have shown that both the 0 Mg^{2+} /4-AP-induced increase and the SRIF-related decrease of PKA
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19 activity are paralleled by consensual changes in mRNA and protein levels of the $\text{C}\alpha$ subunit of
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21 PKA. The possibility exists that post-translational changes following SRIF application may change
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23 epitope availability, and therefore prevent the recognition of the appropriate protein levels in
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25 Western blotting. This does not seem to be the case, since the same amount of $\text{C}\alpha$ subunit was
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27 detected with the use of two different antibodies directed against either the C-terminus or the N-
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29 terminus of the protein. The data of protein expression obtained with Western blot are confirmed by
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31 immunohistochemistry. These observations also contribute important information on $\text{C}\alpha$ subunit
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33 localization in the mouse hippocampus. Indeed, the only available information is in a previous
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35 paper reporting $\text{C}\alpha$ subunit expression in both the dentate gyrus and pyramidal cell layers, and $\text{C}\beta$
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37 subunit localization to the dentate gyrus (Cadd and McKnight 1989). Our findings show that 0
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39 Mg^{2+} /4-AP treatment increases $\text{C}\alpha$ -ir in both the CA3 and the dentate gyrus, but not in CA1,
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41 suggesting a regional specificity of the PKA signaling in the hippocampus, as also indicated, for
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43 instance, by the observation that long term potentiation is regulated by PKA in CA1, but not in
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45 CA2–3 of the mouse hippocampus (Nguyen and Woo 2003).
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48 One way by which SRIF-induced inhibition of PKA activity may result in inhibition of epileptiform
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50 bursting is through reduced PKA phosphorylation of glutamatergic receptors, causing a decrease of
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52 postsynaptic excitatory currents with a consequent hyperpolarization of pyramidal neurons, and/or
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54 presynaptic modulation of glutamate release by pyramidal cells. Indeed, it has been reported that
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56 SRIF reduces NMDA receptor-mediated excitatory postsynaptic currents in the mouse hippocampus
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(Tallent and Siggins 1997), whereas presynaptic SRIF receptors have been shown to mediate excitatory transmission in the rodent hippocampus (Boehm and Betz 1997; Cammalleri et al. 2006; Tallent and Siggins 1997). Consistent with this hypothesis, following 0 Mg^{2+} /4-AP treatment, we have observed phosphorylation of a PKA-dependent site on NMDA receptor subunit NR1, in line with previous data (Niimura et al. 2005), and phosphorylation decrease in the presence of SRIF. Our data suggest that SRIF administration to 0 Mg^{2+} /4-AP treated hippocampal slices may reduce AC isoform and/or Ca subunit expression acting at transcriptional level. The changes, both in mRNA expression and in protein levels, are detected within a 20 min time span, which is compatible with reported short-term regulation of PKA at both messenger and protein levels (Lee et al. 2008; Liu et al. 2008). Our time course experiments show that AC isoforms and Ca subunit mRNA levels are decreased by SRIF application within 10-15 minutes and that the decrease of Ca subunit protein levels follows within the next 5 minutes, consistent with an effect at transcriptional level.

Data from our functional studies investigating SRIF effects on epileptiform bursting after manipulation of PKA activity can be interpreted as evidence in favor of SRIF-induced effects on hippocampal bursting being mediated by the PKA signal transduction pathway. However, the possibility exists that other, alternative mechanisms are involved. For instance, there are numerous indications that SRIF may reduce pyramidal cell excitability through synergistic pre- and post-synaptic actions and, in particular, through augmentation of the voltage-sensitive M-current (Qiu et al. 2008). In our electrophysiological studies, we interfered with PKA activity using dbcAMP, forskolin or H-89. dbcAMP was used to “clamp” PKA activity at constant levels, forskolin to induce a continuous increase of AC activity (resulting in increased PKA activation), H-89 to inhibit PKA activity. In all cases, the application of the compound before SRIF administration completely abolished SRIF effects on epileptiform bursting. Since each of the three compounds prevents, although in different ways, possible modulation of PKA activity, it seems that SRIF can exert its effects on epileptiform bursting only in the presence of a PKA activity that can be modulated. Our

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3 interpretation of these results is that SRIF functional effects on epileptic bursting are likely to be
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5 mediated by the PKA pathway rather than by other SRIF-related effectors (for instance, ion
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7 channels).

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10 In a previous study, we demonstrated that application of exogenous PGE₂ blocks SRIF inhibition of
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12 epileptiform bursting, indicating that the COX-2/PGE₂ pathway is involved in the SRIF-induced
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14 decrease of epileptiform bursting in the mouse hippocampus (Ristori et al. 2008a). The present
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16 results provide evidence that SRIF inhibition of hippocampal bursting may also require PKA
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18 signaling in the mouse hippocampus, suggesting that multiple signaling actions may subserve SRIF
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20 inhibition of hippocampal bursting. In particular, PGE₂ acts through G protein-coupled receptors
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22 which are linked to the AC-cAMP-PKA signaling system (Rocca 2006). For instance, the PKA
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24 pathway has been shown to regulate PGE₂-induced modulation of hippocampal excitatory
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26 transmission in rat CA1 pyramidal neurons (Chen and Bazan 2005). Overall, these observations
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28 suggest that a cross-talk between the two signaling mechanisms activated by SRIF may exist at
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30 some level in the PKA pathway.

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33 The present results imply the coupling of SRIF receptors to a second messenger cascade involving
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35 regulation of PKA, although SRIF receptors mediating antiepileptic action of SRIF in the 0 Mg²⁺/4-
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37 AP hippocampal model still remain to be elucidated. Indeed, scarce and controversial data have
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39 been produced on SRIF receptor localization in the rodent hippocampus (see for ref Viollet et al.
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41 2008), however there is indication that in rodents, sst₂ and sst₄ mediate the majority of SRIF
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43 antiepileptic actions, with a main role of sst₂ in rat and of sst₄ in mouse (see for ref Tallent and Qiu,
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45 2008, Qiu et al. 2008). On the other hand, a role of sst₁ in mediating anticonvulsant effects of SRIF
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47 has been also demonstrated in the 0 Mg²⁺/4-AP hippocampal model by Cammalleri et al. (2004)
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49 although this has not been confirmed by Qiu et al. (2008).

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51 In conclusion, we demonstrated that PKA-related signaling is upregulated in a functional model of
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53 hippocampal bursting and that SRIF inhibition of bursting discharge is associated to
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55 downregulation of the same pathway. In addition, our functional studies strongly suggest that SRIF
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effects on epileptiform bursting require modulation of PKA activity. Together, these findings concur to demonstrate that SRIF coupling to cAMP signaling may mediate SRIF inhibitory effects on epileptiform discharge.

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Table 1 Primer pairs used for RT-PCR analysis

Gene	Primer sequence	Product length (bp)	Gene Bank Accession No.	Ref.
Cyclophilin B	F: 5'-CCATCGTGTCAATCAAGGACTTCAT-3' R: 5'-TTGCCATCCAGCCAGGAGGTCT-3'	216	M60456	Cammalleri et al. 2004, 2006
AC1	F: 5'-AGCTATGAGCCAATCATGGC-3' R: 5'-GACATGAGGAAGTGCTGTGC-3'	206	AF053980	Al-Hakim et al. 2004
AC3	F: 5'-CCTGTGTGCTATCATCGTGG-3' R: 5'-TCATCTAGGTAGTCGCAGCG-3'	753	NM 138305	Al-Hakim et al. 2004
AC8	F: 5'-TCAGTGGTGGCAGGTGTAAT-3' R: 5'-TTTGCCTGTTGAGAGACTGG-3'	337	NM 009623	Designed by Primer3 software ^a
PKA Cα	F: 5'-AGCCATACCCAATCTTGCTG -3' R: 5'-AAGGGAAGGTTGGCGTACT -3'	126	NM 008854	Designed by Primer3 software ^a

^a, Rozen and Skaletsky (2000)

Figure captions

Fig. 1 a Measurements of AC activity in hippocampal slices treated with aCSF, 0 Mg^{2+} /4-AP and 0 Mg^{2+} /4-AP + 1 μM SRIF. Each histogram represents the mean $\pm$ SEM (bars). * $P < 0.001$ vs aCSF (ANOVA followed by Bonferroni's post-test). Six samples were used for each experimental condition. For each sample, measurements were conducted in duplicate. **b** Effects of increasing Ca^{2+} concentrations on AC activity of membranes from hippocampal slices treated with aCSF (filled dots), 0 Mg^{2+} /4-AP (filled triangles) and 0 Mg^{2+} /4-AP + 1 μM SRIF (filled squares). The data points represent the mean $\pm$ SEM (bars) of 6 samples measured in duplicate. Nine mice were used

Fig. 2 Measurements of AC1 (a), AC3 (b) and AC8 (c) mRNAs in hippocampal slices treated with aCSF, 0 Mg^{2+} /4-AP and 0 Mg^{2+} /4-AP + 1 μM SRIF. Representative RT-PCR products corresponding to AC isoforms or cyclophilin B (internal standard) are shown in the left panels, whereas the semiquantitative RT-PCR analysis is in the right panels. Each histogram represents the mean $\pm$ SEM (bars). Four samples were used for each experimental condition. For each sample, measurements were conducted in duplicate. Twelve mice were used. * $P < 0.05$ and ** $P < 0.01$ vs aCSF (ANOVA followed by Bonferroni's post-test)

Fig. 3 Measurements of PKA activity in cytosol fraction from hippocampal slices treated with aCSF, 0 Mg^{2+} /4-AP and 0 Mg^{2+} /4-AP + 1 μM SRIF. PKA activity was measured using the fluorescent-tagged kemptide as a phosphorylation substrate. **a** Representative agarose gel containing phosphorylated and non-phosphorylated kemptide bands. In the negative control, PKA activity was measured in the absence of the hippocampal cytosol fraction. **b** PKA activity as quantified spectrophotometrically from excised bands. Each histogram represents the mean $\pm$ SEM (bars). Three samples were used for each experimental condition. For each sample, measurements

were conducted in duplicate. Five mice were used. *P<0.001 vs aCSF (ANOVA followed by Bonferroni's post-test)

Fig. 4 Measurements of messenger and proteins of the PKA subunit C α in hippocampal slices treated with aCSF, 0 Mg²⁺/4-AP and 0 Mg²⁺/4-AP + 1 μ M SRIF. **a** Representative RT-PCR products corresponding to the C α subunit or cyclophilin B (internal standard) and the semiquantitative RT-PCR analysis. Each histogram represents the mean $\pm$ SEM (bars). Samples used in this study were the same as in Fig. 2. For each sample, measurements were conducted in duplicate. **b, c** Western blot showing immunoreactivities for the C α subunit, as detected with antibodies directed against either the C-terminus (in **b**) or the N-terminus (in **c**) and analysis of Western blot products performed by measuring the optical density (OD) of the bands corresponding to the C α protein with respect to the OD of the band corresponding to α -tubulin. The antibodies used in these studies labeled proteic bands at the expected molecular weights. Each histogram represents the mean $\pm$ SEM (bars). Three samples were used for each experimental condition. For each sample, measurements were conducted in duplicate. Nine mice were used. *P<0.05 and **P<0.01 vs aCSF (ANOVA followed by Bonferroni's post-test)

Fig. 5 a Regional distribution of C α -ir in a hippocampal slice treated with aCSF. This low magnification image is a reconstruction of 7 confocal optical sections acquired with a 10x objective. Scale bars: 300 μ m for the low magnification image, 40 μ m for the images in the insets. DG: dentate gyrus; Gr: granular layer; Mol: stratum moleculare; Mos: mossy fiber pathway in the stratum lucidum; Or: stratum oriens; Pyr: pyramidal layer. **b, c** Representative images of the regional distribution of C α -ir in hippocampal slices treated with 0 Mg²⁺/4-AP and 0 Mg²⁺/4-AP + 1 μ M SRIF. Similar to the image in the upper panel, these images are reconstructions from multiple confocal optical sections. Scale bar: 300 μ m. **d-f** Relative fluorescence intensity of C α -ir in distinct regions of hippocampal slices under the different experimental conditions. Each histogram

represents the mean $\pm$ SEM (bars) of data from 6 measurements in different fields of 6 different slices for each experimental condition. Six mice were used. * $P < 0.05$ and ** $P < 0.01$ vs aCSF (ANOVA followed by Bonferroni's post-test)

Fig. 6 Measurements of NR1 phosphorylation in hippocampal slices treated with aCSF, 0 $\text{Mg}^{2+}/4\text{-AP}$ and 0 $\text{Mg}^{2+}/4\text{-AP}$ + 1 μM SRIF. Western blot showing immunoreactivities, as detected with antibodies to either pS897 NR1 or NR1 and analysis of Western blot products performed by measuring the optical density (OD) of the bands corresponding to pS897 NR1 with respect to the OD of the bands corresponding to NR1. The antibodies used in this study labeled proteic bands at the expected molecular weights. Each histogram represents the mean $\pm$ SEM (bars) expressed as a percentage of aCSF values. Three samples were used for each experimental condition. For each sample, measurements were conducted in duplicate. Five mice were used. * $p < 0.001$ vs aCSF (ANOVA followed by Bonferroni's post-test)

Fig. 7 Measurements of epileptiform bursting in hippocampal slices treated with 0 $\text{Mg}^{2+}/4\text{-AP}$ in the absence or in the presence of 1 μM SRIF either alone or in combination with 0.5 mM dbcAMP or 30 μM forskolin. Each histogram represents the mean $\pm$ SEM (bars) of data from 6 different slices for each experimental condition. * $P < 0.001$ vs 0 $\text{Mg}^{2+}/4\text{-AP}$ (ANOVA followed by Bonferroni's post-test). Eighteen mice were used. Representative traces depicting electrophysiological recordings of epileptiform bursting are shown in the right panel

Fig. 8 a Measurements of PKA activity in cytosol fraction from hippocampal slices treated with 0 $\text{Mg}^{2+}/4\text{-AP}$ in the presence of increasing concentrations of H-89. PKA activity was measured using the fluorescent-tagged kemptide as a phosphorylation substrate. The data points represent the mean $\pm$ SEM (bars) of three samples. For each sample, measurements were conducted in duplicate. Eight mice were used. **b** Measurements of epileptiform bursting in hippocampal slices treated with 0

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Mg²⁺/4-AP in the absence or in the presence of 1 μM SRIF either alone or in combination with 3 μM H-89. Each histogram represents the mean ± SEM (bars) of data from 6 different slices for each experimental condition. *P<0.001 vs 0 Mg²⁺/4-AP (ANOVA followed by Bonferroni's post-test). Twelve mice were used. Representatives traces depicting electrophysiological recordings of epileptiform bursting are shown in the right panel

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