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Research report

Creatine increases hippocampal Na^+,K^+-ATPase activity via NMDA–calcineurin pathway

Leonardo Magno Rambo[a,b], Leandro Rodrigo Ribeiro[b], Vanessa Grigoletto Schramm[a], Andriely Moreira Berch[a], Daniel Neis Stamm[d], Iuri Domingues Della-Pace[d], Luiz Fernando Almeida Silva[b], Ana Flávia Furian[c], Mauro Schneider Oliveira[c], Michele Rechia Fighera[b], Luiz Fernando Freire Royes[a,b,d,*]

[a] Departamento de Fisiologia e Farmacologia, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[b] Programa de Pós-graduação em Ciências Biológicas, Bioquímica Toxicológica, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[c] Universidade Federal do Pampa, Itaqui, RS, Brazil
[d] Departamento de Métodos e Técnicas Desportivas, Centro de Educação Física e Desportos, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil

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ABSTRACT

Achievements made over the past few years have demonstrated the important role of the creatine and phosphocreatine system in the buffering and transport of high-energy phosphates into the brain; however, the non-energetic processes elicited by this guanidine compound in the hippocampus are still poorly understood. In the present study we disclosed that the incubation of rat hippocampal slices with creatine (10 mM) for 30 min increased Na^+,K^+-ATPase activity. In addition, intrahippocampal injection of creatine (5 nmol/site) also increased the above-mentioned activity. The incubation of hippocampal slices with *N*-methyl-D-aspartate (NMDA; MK-801, 10 μM) and NMDA Receptor 2B (NR2B; ifenprodil, 3 μM) antagonists but not with the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA)/kainate antagonist (DNQX, 10 μM) and nitric oxide synthase inhibitor (NOS; L-NAME, 100 μM), blunted the effect of creatine on Na^+,K^+-ATPase activity. Furthermore, the calcineurin inhibitor (cyclosporine A, 200 nM) as well as the Protein Kinase C (PMA, 100 nM) and Protein Kinase A (8-Br-cAMP, 30 μM) activators attenuated the creatine-induced increase of Na^+,K^+-ATPase activity. In addition, the incubation of hippocampal slices with creatine (10 mM) for 30 min increased calcineurin activity. The results presented here suggest that creatine increases Na^+,K^+-ATPase activity via NMDA–calcineurin pathway, proposing an putative underlying non-energetic role of this guanidine compound. However, more studies are needed to assess the contribution of this putative alternative role in neurological diseases that present decreased Na^+,K^+-ATPase activity.

1. Introduction

Creatine (*N*-[aminoiminomethyl]-*N*-methyl glycine) is a guanidine compound synthesized in the kidneys, liver, pancreas, and brain or obtained from alimentary sources like meat and fresh fish (Wyss and Kaddurah-Daouk, 2000). Experimental and clinical findings indicate that creatine-induced high-energy phosphate maintenance protects against ATP depletion in a number of pathological conditions including Alzheimer's (Burklen et al., 2006), Parkinson's (Bender et al., 2006, 2008) and Huntington's diseases (Ferrante et al., 2000; Hersch et al., 2006; Ryu et al., 2005); amyotrophic lateral sclerosis (Ellis and Rosenfeld, 2004; Rosenfeld et al., 2008; Shefner et al., 2004), and traumatic brain injury (Sullivan et al., 2000; Sakellaris et al., 2006; Scheff and Dhillon, 2004). Although there is the notion that the mechanisms of neuronal function improvement and neuroprotection exerted by creatine include enhanced energy buffering, a non-energetic neuromodulatory role for creatine has also been proposed (Persky and Brazeau, 2001). Creatine is not only synthesized and taken up by neurons, but that it is also released in an action-potential-dependent manner (Almeida et al., 2006). Furthermore, the bath application of creatine increases both amplitude and number of population spike in the stratum radiatum of the hippocampal CA1 subfield, an effect reverted by the selective NMDA receptor antagonist 2-amino-5-phosphonopentanoic acid (AP5) (Royes et al., 2008). In line with this view, creatine increases [^{3}H]MK-801 binding to hippocampal membranes by 55% (Royes et al., 2008) and leads to spatial learning improvement, possibly by modulating

* Corresponding author at: Departamento de Métodos e Técnicas Desportivas, Centro de Educação Física e Desportos, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil. Tel.: +55 55 3220 9378; fax: +55 55 3220 8031.
E-mail address: nandoroyes@yahoo.com.br (L.F.F. Royes).

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polyamine binding site at the NMDA receptor (Oliveira et al., 2008). However, the downstream effectors of the creatine-induced modulation of the NMDA receptor are still unknown.

NMDA receptors stimulation leads to the activation of Na^+,K^+-ATPase (Marcaida et al., 1996; Munhoz et al., 2005; Bersier et al., 2008), a key enzyme involved in the transmembranal transport of sodium and potassium which plays a pivotal role in the cellular ionic gradient maintenance (Skou and Esmann, 1992) and may be modulated by a complex and not completely understood phosphorylation cascade of regulatory proteins. PKA activators, such as forskolin and Sp-5,6-DCI-cBIMPS - as well as the PKC activator phorbol 12,13-dibutyrate – significantly reduce Na^+,K^+-ATPase activity in neurons or COS cells (Cheng et al., 1999, 1997a,b; Nishi et al., 1999a). On the other hand, α-adrenergic as well as NMDA receptor activation increases Na^+,K^+-ATPase activity through the activation of the calcium-dependent protein phosphatase 2B, calcineurin (Aperia et al., 1992; Marcaida et al., 1996). The glutamate-induced calcineurin activation counteracts the PKC-mediated phosphorylation of Na^+,K^+-ATPase leading to an increase of pump activity (Marcaida et al., 1996).

In this research we take into account that creatine may play a putative role as a neuromodulator in the brain (Almeida et al., 2006). In this sense, previous works from our group suggest that creatine may modulate brain NMDA receptors (Royes et al., 2008; Oliveira et al., 2008). Furthermore, in the same way, there are data in literature showing that glutamatergic agonists increase Na^+,K^+-ATPase activity in cultured neurons (Inoue et al., 1999). Therefore, we decided to investigate whether creatine alters Na^+,K^+-ATPase activity in rat hippocampal slices. Moreover, given that PKA, PKC and calcineurin are major downstream kinases/phosphatases involved in the Na^+,K^+-ATPase activity regulation (Cheng et al., 1999; Nishi et al., 1999a,b), we also investigated the effect of creatine on calcineurin activity and whether those pathways are involved in the effect exerted by creatine on Na^+,K^+-ATPase activity.

2. Materials and methods

2.1. Animals and reagents

Adult male Wistar rats (250–300 g) maintained under controlled light and environment (12 h light/dark cycle, 24 ± 1 °C, 55% relative humidity) with free access to food and water were used. Animal utilization reported in this study was conducted in accordance with the policies of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised in 1996) and with the Institutional and National regulations for animal research. All efforts were made to reduce the number of animals used, as well as to minimize their suffering.

Phorbol 12-myristate 13-acetate (PMA) was dissolved in 100% ethanol and then diluted with aCSF (artificial cerebrospinal fluid) in such a way that ethanol concentration did not exceed 0.006%. 6,7-Dinitroquinoxaline-2,3(1H,4H)-dione (DNQX) was dissolved in 100% dimethyl sulfoxide (DMSO) and then diluted with aCSF so that DMSO concentration would not exceed 0.003%. Creatine, creatinine, 3-guanidinopropionic acid (3-GPA), tetrodotoxin (TTX), *N*-nitro-L-arginine methyl ester (L-NAME), (5R,10S)-(+)5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801), ifenprodil, PMA, 8-bromoadenosine-3′,5′-cyclic monophosphate (8-Br-cAMP), DNQX, cyclosporin A and all other reagents were purchased from Sigma (St. Louis, MO, USA) and solutions were prepared in aCSF. Calcineurin assay kit was purchased from Enzo Life Sciences (Farmingdale, NY, USA). To avoid unspecific actions induced by contaminants, creatine identity and purity was checked by nuclear resonance methods and found to be greater than 99%.

2.2. In situ experiments

Animals were sacrificed by decapitation and their hippocampus was immediately dissected and used for the preparation of slices (400 μm thick) with a McIlwain tissue chopper. Slices were suspended in an aCSF containing (in mM): 1.25 NaH_2PO_4; 22 NaH_2CO_3; 1.8 $MgSO_4$; 129.0 NaCl; 1.8 $CaCl_2$; 3.5 KCl; 10 D-glucose and 10 Trizma base. Afterwards, their contents were aerated with carbogen [95% O_2, 5% CO_2]; pH was adjusted with carbogen and HCl to 7.4. Osmolarity ranged from 290 to 310 mOsm was checked with an osmometer (ADI instruments, model 3320). The viability of hippocampal slices was assessed at 0, 30, 60 and 90 min after preparation by measuring lactate dehydrogenase (LDH) activity with a standard commercial kit (Labtest, Porto Alegre, RS, Brazil). Considering that the hippocampal slices were viable for more than 60 min after preparation, all experiments were performed within this time window (data not shown).

The effect of creatine on hippocampal Na^+,K^+-ATPase activity was investigated by incubating 10–12 slices for 30 min at 37 °C with increasing concentrations of creatine (0, 0.1, 1 or 10 mM). The concentrations of creatine used in the current study were chosen according to previous biochemical and electrophysiological studies (Royes et al., 2008; Balestrino et al., 1999; Kass and Lipton, 1986) as well as because they are comparable to creatine doses used in previous *in vivo* studies (Oliveira et al., 2008). The effect of creatine on hippocampal calcineurin activity was investigated by incubating 10–12 slices for 30 min at 37 °C with creatine (10 mM).

To test the effect of creatine metabolite, creatinine, on Na^+,K^+-ATPase activity, hippocampal slices were incubated with increasing concentrations (0, 0.1, 1 or 10 mM). The role of NMDA and AMPA/kainate receptors in the Na^+,K^+-ATPase activity alteration elicited by creatine, hippocampal slices were co-incubated with MK-801 (10 μM) or ifenprodil (3 μM) or DNQX (10 μM) plus creatine (10 mM). In order to test the participation of the creatine transporter and sodium channel in the effect exerted by this guanidine compound on Na^+,K^+-ATPase activity, hippocampal slices were bathed with 3-GPA (10 mM), a creatine uptake inhibitor, or TTX (0.1 μM), a voltage-gated sodium channel blocker, plus creatine (10 mM) (Lunardi et al., 2006). In addition, the involvement of nitric oxide synthase (NOS), PKA/PKC and calcineurin pathways in the effect exerted by creatine on Na^+,K^+-ATPase was tested by incubating hippocampal slices with L-NAME (100 μM) or PMA (100 nM) or 8-Br-cAMP (30 μM) or cyclosporin A (200 nM) plus creatine (10 mM).

After the incubation period, the medium was discarded and slices were gently homogenized (7–10 strokes) in ice-cold 10 mM Tris–HCl buffer, pH 7.4, for determination of Na^+,K^+-ATPase activity. In a separate set of experiments, designed to determine whether creatine increased Na^+,K^+-ATPase activity by directly interacting with the enzyme, creatine (10 mM) was added directly to the reaction medium containing hippocampal homogenates.

2.3. Preparation of plasma membrane from hippocampal slices

After the incubation period with creatine (10 mM) or aCSF, the medium was discarded and the slices were used to prepare plasma membranes according to Mayrand et al. (1982), with some modifications. The hippocampal slices were homogenized in a sucrose solution containing (in mM) 250 Sucrose, 10 Tris and 1 EDTA (pH 7.7). The homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C to remove excess debris. The supernatant was removed and centrifuged at 7700 × *g* for 20 min at 4 °C, followed by centrifugation of the supernatant at 7700 × *g* for 5 min at 4 °C. The supernatant obtained was centrifuged at 55,000 × *g* for 30 min at 4 °C. The white membrane portion of the pellet was retained, discarding the brown pellet. The white membrane pellet was homogenized in the sucrose solution and then frozen at −20 °C. Specific Na^+,K^+-ATPase activity was measured in this samples of purified membranes according to the follow method.

2.4. Na^+,K^+-ATPase activity measurements

Na^+,K^+-ATPase activity was measured according to Wyse et al. (2000). Briefly, the assay medium consisting of 30 mM Tris–HCl buffer, pH 7.4; 0.1 mM EDTA; 50 mM NaCl; 5 mM KCl; 6 mM $MgCl_2$ and 50 μg of protein in the presence or absence of ouabain (1 mM); summing up to a final volume of 250 μL. The reaction was started by the addition of adenosine triphosphate to a final concentration of 5 mM. After 30 min at 37 °C, the reaction was stopped by the addition of 50 μL of 60% (w/v) trichloroacetic acid. Saturated substrate concentrations were used and the reaction was linear with protein (1 mg/mL) and time (30 min). Appropriate controls were included in the assays for non-enzymatic hydrolysis of ATP. The amount of inorganic phosphate (Pi) released was quantified colorimetrically, as described by Fiske and Subbarow (1925), using KH_2PO_4 as a reference standard. Specific Na^+,K^+-ATPase activity was calculated by subtracting the ouabain-insensitive activity from the overall activity (in the absence of ouabain) and expressed in nmol Pi/mg protein/min.

In a separate set of experiments, we investigated whether some Na^+,K^+-ATPase α isoforms are selectively modulated by creatine. For this purpose, we used a classical pharmacological approach based on the isoform-specific sensitivity to ouabain (Nishi et al., 1999a). We determined whether creatine increased ouabain-sensitive ATPase activity using 3 μM or 4 mM ouabain (so as to inhibit only the Na^+,K^+-ATPase isoforms containing α_2 and α_3 subunits, or to inhibit every isoform, respectively). Furthermore, Mg^{2+}-ATPase activity was measured in the presence of 3 mM ouabain, according to Munhoz et al. (2005).

2.5. Calcineurin enzymatic activity assay

After the incubation period, the medium was discarded and slices were gently homogenized (7–10 strokes) in calcineurin assay kit buffer. Briefly, calcineurin activity assays were performed on hippocampal slices homogenates (6 mg/mL) using a colorimetric calcineurin assay kit according to the manufacturer's protocol (Enzo Life Sciences, kit no. BML-AK804). Reactions were stopped after 30 min, and absorption was read spectrophotometrically at a wavelength of 620 nm. Calcineurin activity was expressed in nmol Pi/mg protein/min.

2.6. In vivo experiments

To determine whether the creatine-induced increase on Na^+,K^+-ATPase activity also occurred *in vivo*, animals were anesthetized with Equithesin (1% phenobarbital, 2% magnesium sulfate, 4% chloral hydrate, 42% propylene glycol, and 11% ethanol, 3 mL/kg, i.p.) and placed in a rodent stereotaxic apparatus. Two cannula were inserted 1 mm above the CA1 region of the dorsal hippocampus, bilaterally (coordinates relative to bregma: AP 4 mm, ML 3 mm, V 2 mm from the dura) under stereotaxic guidance (Paxinos and Watson, 1986). Chloramphenicol (200 mg/kg, i.p.) was administered immediately before the surgical procedure. Three days after the surgical procedure, animals were injected with creatine (5 nmol/0.5 μL) and 30 min thereafter animals were sacrificed. The hippocampi were rapidly removed and gently homogenized (7–10 strokes) in ice-cold 10 mM Tris–HCl buffer, pH 7.4 and Na^+,K^+-ATPase activity measured as described above. The dose of creatine used in this set of experiments was chosen based on previous studies (Oliveira et al., 2008) and is comparable to the concentrations of creatine used in the in situ experiments.

2.7. Protein determination

The protein content was colorimetrically determined by the method of Bradford (1976) using bovine serum albumin (1 mg/mL) as a standard.

2.8. Statistical analyses

Data were analyzed by a *t* test, one- or two-way ANOVA, and post hoc analyses were carried out by the Student-Newman-Keuls test, when appropriate. Correlation analysis was carried out using the Pearson's correlation coefficient. A probability of $P < 0.05$ was considered significant. All data are expressed as mean + SEM.

3. Results

Fig. 1A shows the effect of creatine (0, 0.1, 1 or 10 mM) on Na^+,K^+-ATPase activity in rat hippocampal slices. Statistical analysis disclosed that creatine (10 mM) increases Na^+,K^+-ATPase activity in rat hippocampal slices [$F(3, 32) = 2.99$; $P < 0.05$, Fig. 1A]. Fig. 1B shows the effect of creatine (10 mM) on Na^+,K^+-ATPase activity of purified plasma membranes from hippocampal slices. In purified plasma membranes from hippocampal slices creatine also increase Na^+,K^+-ATPase activity [$t(8) = 4.055$; $P < 0.05$, Fig. 1B] in spite of the higher specific activity. To determine the effect of creatine in a cell free system, we added creatine directly to hippocampal homogenates. In this experimental condition, creatine (10 mM) did not alter the Na^+,K^+-ATPase enzyme activity [$F(1, 8) = 0.47$; $P > 0.05$, Fig. 1C]. Using a classical pharmacological approach based on the isoform-specific sensitivity to ouabain concentration (Nishi et al., 1999a), we investigated whether some α isoforms of Na^+,K^+-ATPase are selectively activated by creatine. The results presented in this report suggest that the stimulatory effect of creatine on Na^+,K^+-ATPase activity is specific for $\alpha_{2/3}$ isoform, since the effect of creatine on the enzyme activity was not observed in the presence of 3 μM ouabain (a concentration that inhibits $\alpha_{2/3}$, but not α_1 isoforms). Furthermore, such creatine effect was extended to Mg^{2+}-ATPase [$F(7, 40) = 231.2$; $P < 0.05$, Fig. 1D]. Statistical analysis also disclosed that the intra-hippocampal injection of creatine (5 nmol/site) increased Na^+,K^+-ATPase activity by 21% in the hippocampus [$F(1, 18) = 7.03$; $P < 0.05$, Fig. 1F], confirming the results obtained in situ.To test whether the creatine-induced increase in Na^+,K^+-ATPase activity may be mediated by trans-cellular creatine transport and for its metabolite (creatinine), hippocampal slices were incubated with increasing concentrations of creatinine (0, 0.1, 1 or 10 mM) and 3-GPA (10 mM). In our experimental conditions, creatinine [$F(3, 20) = 0.75$; $P > 0.05$] (Table 1) had no effect on Na^+,K^+-ATPase activity. Accordingly, statistical analysis disclosed that the creatine-induced Na^+,K^+-ATPase activity increase was not attenuated by 3-GPA [$F(3, 36) = 2.21$; $P < 0.05$, Fig. 2A].

In the present study, we showed that such increase was attenuated by a voltage-gated sodium channel blocker (TTX, 0.1 μM) [$F(3, 24) = 6.56$; $P < 0.05$, Fig. 2B], NMDA antagonist (MK-801, 10 μM) [$F(3, 20) = 5.75$; $P < 0.05$, Fig. 2C] and the selective antagonist for NR2B subunit (ifenprodil, 3 μM) [$F(3, 28) = 6.42$; $P < 0.05$, Fig. 2D].

Table 1
Effect of creatinine (0, 0.1, 1 or 10 mM) on Na^+,K^+-ATPase activity of rat hippocampal slices.

Treatment (mM)	Na^+,K^+-ATPase activity (nmol Pi/mg protein/min) Creatinine
aCSF	72.38 ± 4.81
0.1	79.32 ± 4.74
1	78.14 ± 4.79
10	80.88 ± 2.17

Data are mean ± SEM for $n = 6$ in each group.

However, statistical analysis disclosed that the stimulatory effect exerted by creatine on Na^+,K^+-ATPase activity was not reverted by co-incubation with AMPA/kainate antagonist (DNQX, 10 μM) [$F(3, 16) = 0.12$; $P > 0.05$, Fig. 2E]. In addition, although the Na^+,K^+-ATPase activity is regulated by NMDA–NOS pathway (Munhoz et al., 2005), the results presented in this report showed that a non-specific isoform NOS inhibitor (L-NAME, 100 μM) did not alter the studied enzyme activity [$F(3, 24) = 0.82$; $P > 0.05$, Fig. 2F].

The results presented in this report showed that slices prefunded with activator of PKC (PMA; 100 nM) and PKA (8-Br-cAMP; 30 μM) attenuated the creatine-induced increase in Na^+,K^+-ATPase activity [$F(3, 32) = 4.79$; $P < 0.05$ and $F(3, 20) = 4.47$; $P < 0.05$, Fig. 2G and H, both respectively]. In addition, statistical analysis disclosed that the incubation with cyclosporin A (200 nM) attenuated the stimulatory effect of creatine on Na^+,K^+-ATPase activity [$F(3, 20) = 7.08$; $P < 0.05$, Fig. 2I]. In line of this view, we demonstrated that hippocampal slices incubated with creatine had higher both Na^+,K^+-ATPase [$F(1, 8) = 7.86$; $P < 0.05$, Fig. 3A] and calcineurin [$F(1, 8) = 24.16$; $P < 0.05$, Fig. 3B] activities. Moreover, correlation analysis (Pearson's) disclosed that Na^+,K^+-ATPase activity positively correlates with calcineurin activity ($r = 0.874$; $P < 0.05$, Fig. 3C).

4. Discussion

In the current study we showed that the incubation with creatine (10 mM) increases Na^+,K^+-ATPase activity in rat hippocampal slices homogenates and plasma membranes and that this effect seems to be $\alpha_{2/3}$ isoform-specific, taking into account the isoform-specific sensitivity to ouabain, according to our pharmacological approach. Furthermore, the stimulatory effect of this guanidine compound on Na^+,K^+-ATPase activity was evidenced *in vivo*, after intrahippocampal injection, but not in hippocampal homogenates of naive animals incubated directly with creatine, indicating that it requires an intact cellular system. Regarding this point, it is also possible that tissue homogenizing disrupts critical anchoring protein linkages between NMDA receptors, kinases, and Na^+,K^+-ATPase, resulting in the uncoupling of enzyme modulatory components. The results presented in this report disclosed that NR2B-containing NMDA receptors, voltage-gated Na^+ channels and subsequent calcineurin pathway activation are involved in the stimulatory effect exerted by this guanidine compound. Our results also evidenced pharmacologically that the increase of Na^+,K^+-ATPase activity elicited by creatine is independent of its uptake. Besides, such effect possibly involves a mechanism independent of changes in the bioenergetic status of the hippocampus, since creatine needs to be transported into the cell to be phosphorylated by creatine kinase and thus to exert its role as an energy buffer.

The creatine concentrations chosen for concentration/response curve were based on previous data. It was reported that exogenous creatine at high concentration (25 mM) protects in vitro brain slices from hypoxic damage (Kass and Lipton, 1986). Incubation of hippocampal slices with different concentrations of creatine (0.5, 1, 10, 25 mM) results in a dose-dependent increase in intracellular

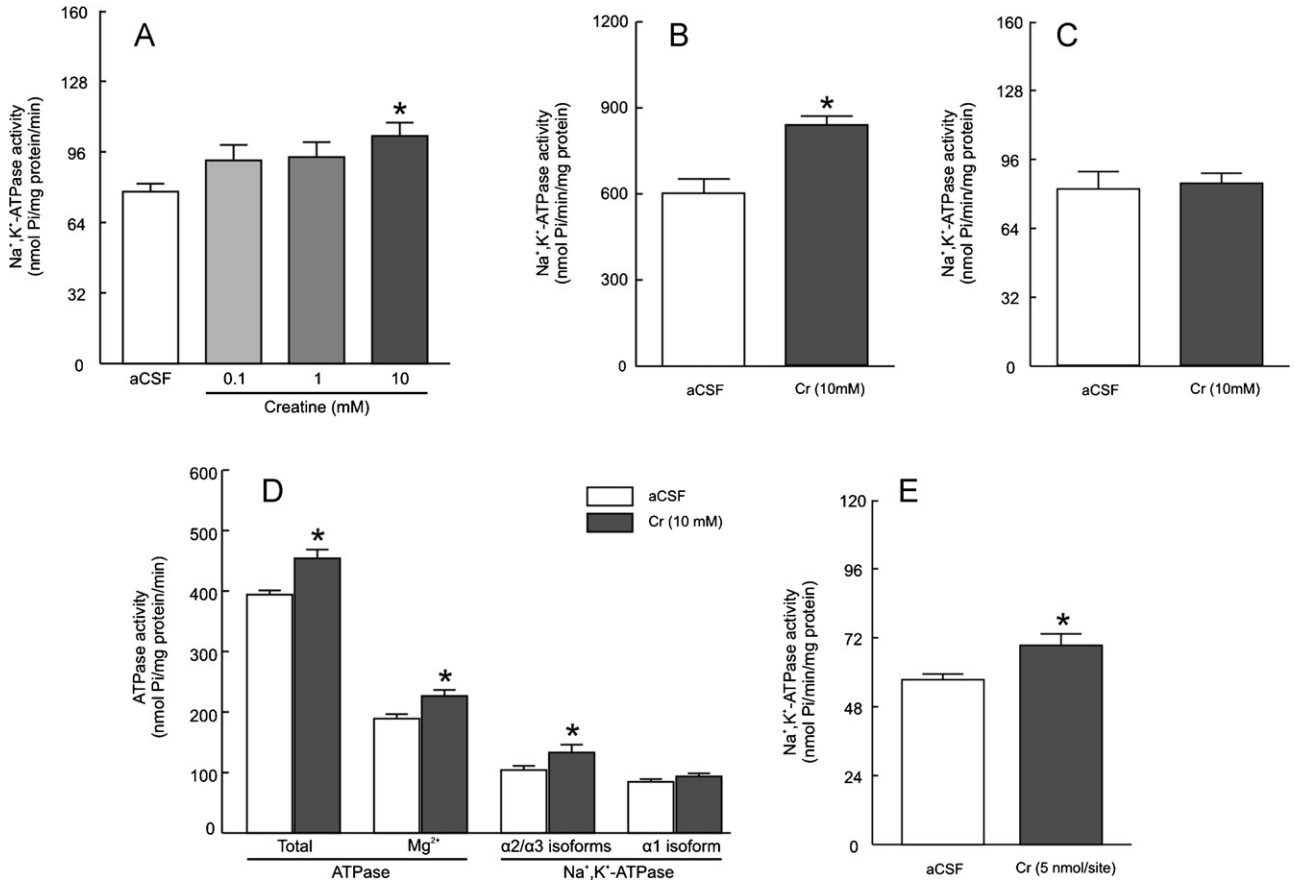

Fig. 1. Effect of creatine (0, 0.1, 1 or 10 mM) on Na^+,K^+-ATPase activity of rat hippocampal slices homogenate (A), purifyed plasma membrane from rat hippocampal slices (B) and homogenates from total rat hippocampus tissue (C). It is also shown the effect of creatine (10 mM) on the activity of different Na^+,K^+-ATPase isoforms (according to isoform-specific sensitivity to ouabain) and Mg^{2+}-ATPase (D) and the effect of intracerebroventricular injection of creatine (5 nmol/site) on Na^+,K^+-ATPase activity in rat hippocampus *in vivo* (E). Data are mean + SEM for $n = 5–10$ in each group. *A significant difference compared with aCSF group.

phosphocreatine, reaching a plateau with roughly 10 mM creatine, a condition associated with delayed anoxic depolarization (Balestrino et al., 1999). Such creatine concentration range exerts excitatory action on hippocampal slices (Royes et al., 2008).

We showed here that creatine (10 mM) induced an increase on $\alpha_{2/3}$ Na^+,K^+-ATPase activity, by a classical pharmacological approach based on the isoform-specific sensitivity to ouabain, without to employ specific antibodies for enzyme isoforms. Therefore, our discussion is based only in our pharmacological data. In the brain, α_2 and α_3 isoforms are found in glial and neuronal cells (in same order), contributing to membrane potential generation control, K^+ re-uptake after depolarization (Lecuona et al., 1996; Mobasheri et al., 2000; Peng et al., 1997). In this sense, Na^+,K^+-ATPase is a crucial enzyme involved in the control of neuronal excitability and its inhibition may be associated with several neurological disorders. In line of this view, it has been demonstrated experimentally that the Na^+,K^+-ATPase inhibitor ouabain increases Ca^{2+} entry into brain slices (Fujisawa et al., 1965), and causes electrographically recorded seizures in mice (Jamme et al., 1995), glutamate release by reversal of the Na^+-dependent transporter (Li and Stys, 2001), and cell death in rat hippocampi (Lees et al., 1990). Furthermore, a mutation in the α_3 isoform – that reduces Na^+,K^+-ATPase activity about 42% in mice brain – was associated with increased hyperexcitability in the central nervous system, suggesting that Na^+,K^+-ATPase may be involved in control of epileptogenic activity (Clapcote et al., 2009). In this sense, a mutation in the α_2 isoform has been related with sporadic hemiplegic migraine and epileptic seizures in humans (Gallanti et al., 2008). Considering such fact, it is plausible to propose that the stimulation of $\alpha_{2/3}$ Na^+,K^+-ATPase elicited by creatine may be a parallel mechanism involved in the neuroprotective effects of this compound in neurological diseases.

Although it is believed that the mechanism underlying creatine-induced neuronal function improvement and neuroprotection involves enhanced energy storage in a variety of experimental models of neurological disease (Klein and Ferrante, 2007; Magni et al., 2007; Royes et al., 2003, 2006), a direct neuromodulatory role for creatine has also been proposed (Persky and Brazeau, 2001). In this context, it has been shown that creatine is not only synthesized and taken up by neurons, but also released in such a manner that it depends on the action potential of the brain cells (Almeida et al., 2006). This guanidino compound also augments cerebral blood flow after stroke (Prass et al., 2007) and reduces inhibitory GABA and glycine responses in mice neurons in cell culture (De Deyn and Macdonald, 1990). Recently, experimental findings from our group suggest that creatine not only seems to be involved in energy metabolism but may also play an important role in the early consolidation of spatial learning in hippocampus with participation of polyamines binding site at the NMDA receptor (Oliveira et al., 2008). Accordingly, the incubation of hippocampal slices with MK-801 and ifenprodil, respectively selective antagonists for NMDA and NMDA/NR2B subunit receptors attenuated the creatine-induced enzyme activity increase, suggesting that this effect is dependent of NMDA/NR2B activation. Furthermore, although the glutamate–NMDA–NOS–cGMP–PKG pathway is known to stimulate $\alpha_{2/3}$ Na^+,K^+-ATPase activity (Munhoz et al., 2005), in our experimental conditions we did not find this pathway involved in the stimulatory effect of creatine on Na^+,K^+-ATPase.

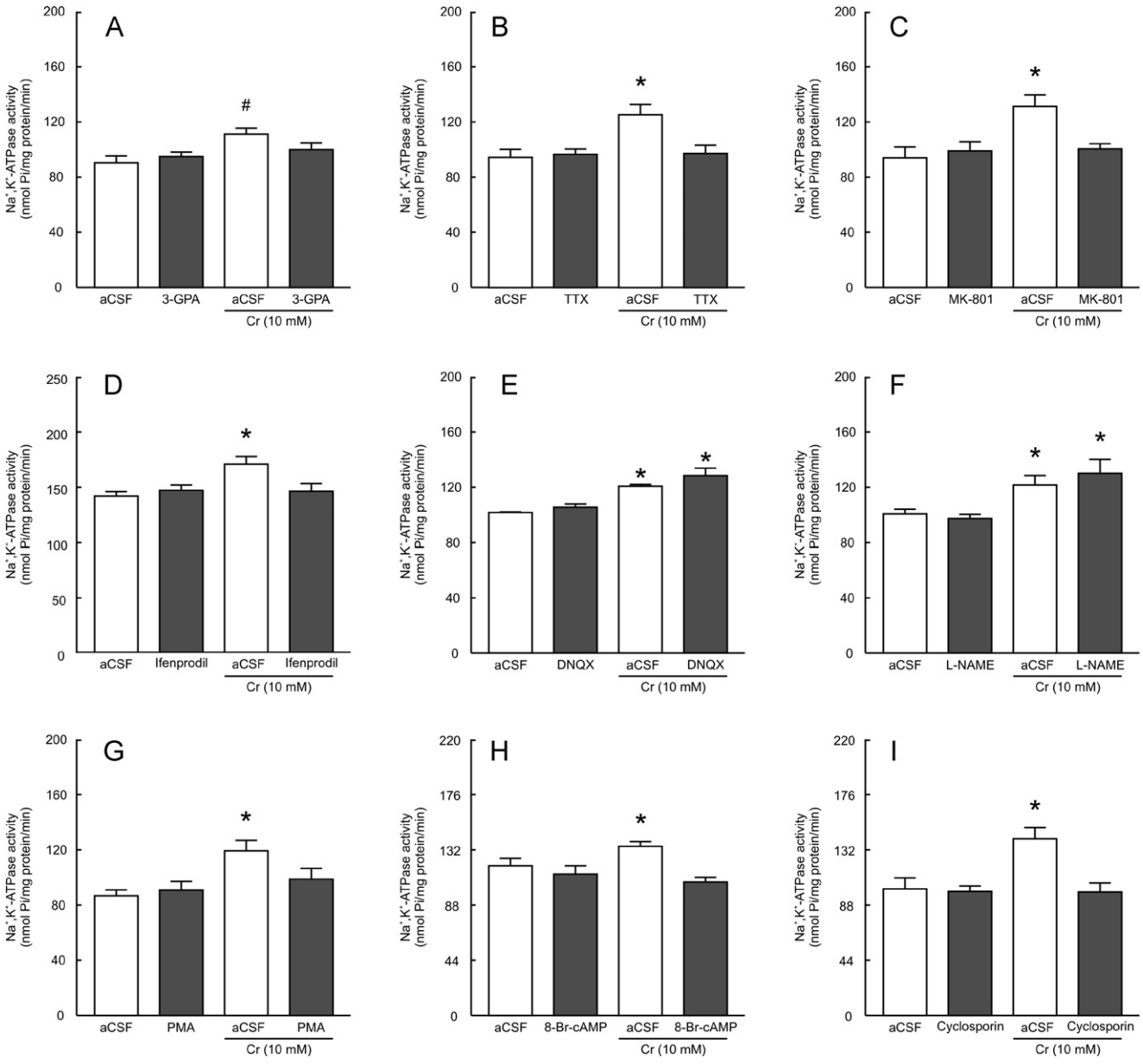

Fig. 2. Effect of 3-GPA (10 mM, A), TTX (0.1 μM, B), MK-801 (10 μM, C), ifenprodil (3 μM, D), DNQX (10 μM, E), L-NAME (100 μM, F), PMA (100 nM, G), 8-Br-cAMP (30 μM, H) and cyclosporin A (200 nM, I) on the creatine-induced (10 mM) increase in Na^+,K^+-ATPase activity of rat hippocampal slices. Data are mean + SEM for $n = 5–9$ in each group. *A significant difference compared with all groups.

In this context, considering that another intracellular pathway could be involved in the present stimulatory effect of creatine, we decided to investigate the involvement of calcineurin and PKA/PKC pathways. This particular experiment data showed that the stimulatory effect exerted by creatine was attenuated by incubation with cyclosporin A (a calcineurin inhibitor). Furthermore, the increase of calcineurin activity as well as the positive correlation between Na^+,K^+-ATPase and calcineurin activity in hippocampal slices incubated with creatine reinforces the assumption that creatine stimulates Na^+,K^+-ATPase by increasing calcineurin activity. These findings agree with previous observations showing that the glutamate activation of NMDA receptors followed by calcineurin leads to stimulation of Na^+,K^+-ATPase in cerebellar neurons in culture (Marcaida et al., 1996). Furthermore, it has been proposed that the stimulation of the α-adrenergic receptor activates the phosphatase calcineurin, leading to an increase of Na^+,K^+-ATPase activity in renal tubular cells (Aperia et al., 1992). In the same way, calcineurin-induced Na^+,K^+-ATPase dephosphorylation is the mechanism proposed by which norepinephrine induces Na^+,K^+-ATPase increase in rat brain (Mallick et al., 2000). In the present study we disclosed that the incubation with the PKC activator (PMA) or the PKA activator (8-Br-cAMP) attenuated the stimulatory effect exerted by creatine on Na^+,K^+-ATPase. These data presented here leads us to speculate that the increase in Na^+,K^+-ATPase activity can occur in two ways: directly, by the activation of calcineurin and the counteraction of the PKC-mediated phosphorylation of the Ser-23 in the α subunit (Bertuccio et al., 2003, 2007); or indirectly, by dephosphorylation of the phosphorylation site for PKA (Thr-34) in the dopamine and cyclic AMP-regulated phosphoprotein (Nishi et al., 1999b). However, it is merely an speculation and further studies are needed to clarify this point.

Summing up, we provide, for the first time, pharmacological evidences of a putative non-energetic pathway by which creatine stimulates Na^+,K^+-ATPase activity in rat hippocampal slices.

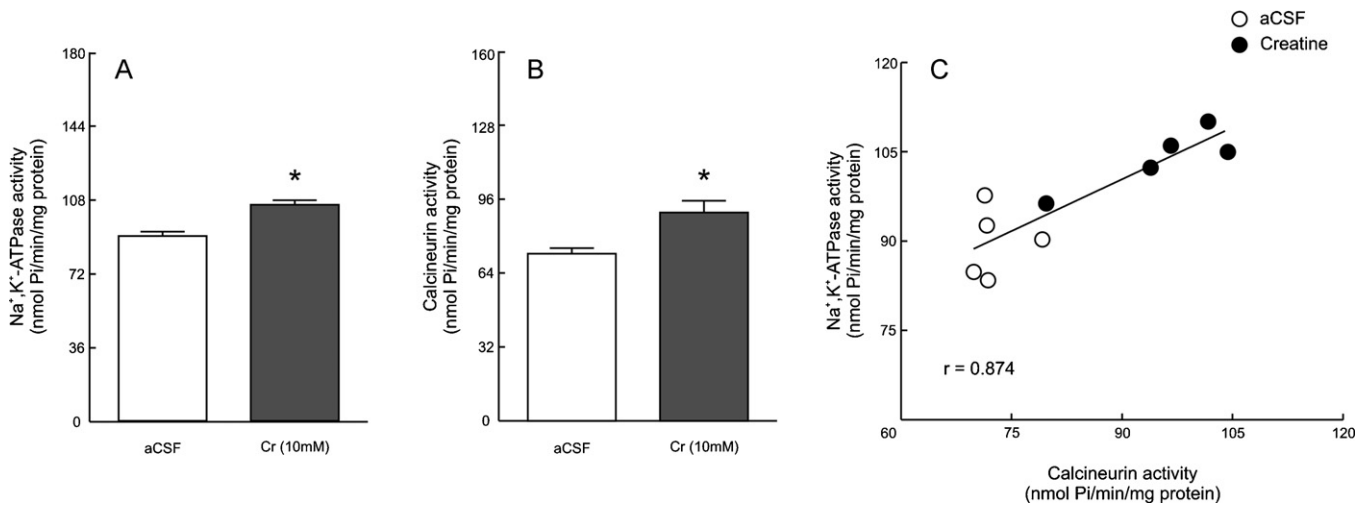

Fig. 3. Effect of creatine (10 mM) on Na^+,K^+-ATPase activity (A), calcineurin activity (B) and the correlation analysis between Na^+,K^+-ATPase and calcineurin activity (C). Data are mean + SEM for $n = 5$ in each group. *A significant difference compared with aCSF group.

Furthermore, the results presented in this report suggest that at least some of these effects are mediated by NR2B-containing NMDA receptors, voltage-gated Na^+ channels and subsequent calcineurin pathway activation. The activation of this phosphatase, in turn, counteracts the PKC- and PKA-mediated Na^+,K^+-ATPase phosphorylation, leading to the stimulation of $\alpha_{2/3}$ Na^+,K^+-ATPase activity. Therefore, in addition to its role as a precursor of energy-rich compounds that maintains nearly all cellular function and induces neuronal function improvement, the Na^+,K^+-ATPase activity increase induced by creatine suggests an underlying non-energetic role of this guanidine compound in neuronal plasticity and possibly in several neurological diseases. However, more studies are required to assess the contribution of this alternative role in protection exerted by creatine in neurological diseases that present decreased Na^+,K^+-ATPase activity.

Conflict of interest

The authors declare that they have no conflicts of interest.

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