BIOPHYSICS AND BIOCHEMISTRY

Activation of Nitric Oxide Pathway Mediated by Metabotropic Glutamate Receptors in Primary Cultures of Cerebellar Granule Cells

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Stimulation of L-glutamate receptors (GluRs) is thought to produce neuron death through the elevation of free intracellular Ca^{2+} levels, leading to activation of Ca^{2+}/calmodulin-dependent synthesis of cytotoxic amounts of NO·. In the present study, NO synthase activation mediated by mGluR stimulation is investigated in primary cultures of granule cells (CGrC). It is found that a selective agonist of mGluRs, DL-1-aminocyclopentane-trans-1,3-dicarboxylic acid (ACPD), raises both the cGMP and nitrite (NO_2^-) levels, which are used as a biochemical index to study the enzymatic NO· release from L-arginine. This effect is abolished by applying both Nω-nitro-L-arginine methyl ester (NAME) and DL-2-amino-4-phosphonobutyric acid (AP4), and is independent of Ca^{2+}. In contrast, the α-amino-3-hydroxy-5-methylisoxarole-4-propionnate (AMPA)-induced increase in cGMP content is eliminated by the preincubation of CGrC with 4 mM EGTA-chelated Ca^{2+}.

Key Words: *nitric oxide; L-glutamate receptor; granule cells; cerebellum*

Activation of L-glutamate receptors (GluRs) triggers various transmembrane biochemical signals, which include an increase in both calcium ion (Ca^{2+}) flux and turnover of inositol phospholipids and a rise of the levels of cyclic nucleotides [11,14,16]. A number of observations have shown that the postsynaptic activation of constitutive calcium/calmodulin (Ca^{2+}/CM)-dependent nitric oxide synthase (NOS) and nitric oxide (NO·) release in the CNS are also coupled with the glutamatergic system [2,6]. The link between the activation of GluRs and the stimulation of NOS activity is thought to be a rise in the intracellular level of Ca^{2+} [2,6]. It has been shown that NOS is present primarily in discrete neuronal populations, with the highest densities occurring in the cerebellum [2,6,17]. In turn, L-glutamate (Glu) is believed to function as a neurotransmitter for ascending and parallel fibers in the cerebellum [17]. *In vivo* stimulation of these fibers elicits a prompt and massive increase in the level of NO·, which activates the soluble form of guanylate cyclase (sGC) to produce cGMP [6]. In neurons, the glutamate stimulus is transmitted both by metabotropic receptors (mGluRs), which belong to the G protein-associated receptor family, and by ionotropic receptors (iGluRs), which represent a particular class of ligand-gated

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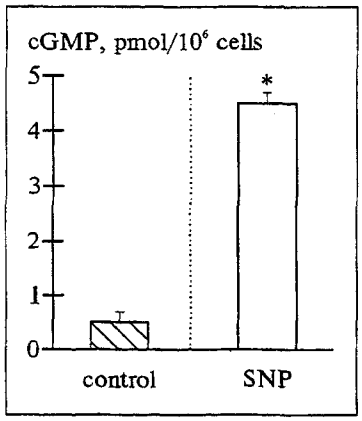

Fig. 1. Effect of SNP (100 μM) on cGMP production in cultures of granule cells. The data represent as $M \pm m$ of 6 observations. *$p<0.01$ *vs.* the control by Tukey's test.

ion channels and are subdivided into receptors for N-methyl-D-aspartate (NMDA), and non-NMDA receptors for α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) and kainate (KA) [3,10]. Agonists at iGluRs activate NOS, promoting an influx of Ca^{2+} from the extracellular space, whereas agents stimulating the mGluRs activate the enzyme by mobilizing Ca^{2+} from intracellular sources [12, 15]. Since the pattern of expression of GluRs varies during differentiation, maturation, and aging of neurons [9], regulation of cellular activity by Glu may be the result of the coordinated interplay of the various GluRs operating through NO· generation. Granule cells in culture have been shown to express specific recognition sites for excitatory amino acids, just as they do in the intact cerebellum [8]. Activation of these sites by Glu triggers Ca^{2+} uptake and stimulation of sGC [8].

In the present study, NOS activation mediated by mGluRs stimulation was investigated in primary cultures of granule cells (CGrC). It was found that a selective agonist of mGluRs, DL-1-aminocyclo-

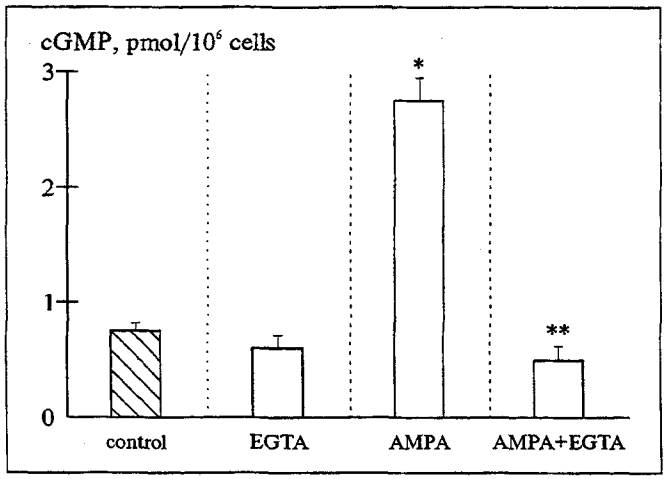

Fig. 2. Effect of EGTA (4 mM) on AMPA–dependent (0.1 mM) cGMP production in cultures of granule cells. *$p<0.01$ *vs.* the control by Tukey's test. **$p<0.05$ ($F_{int}=3.07$) by two–way ANOVA.

pentane-trans-1,3-dicarboxylic acid (ACPD) [7], raises both the cGMP and nitrite (NO_2^-) levels, which are biochemical indexes of the enzymatic release of NO· from L-arginine.

MATERIALS AND METHODS

Cell cultures. Cerebellar granule cell cultures, obtained as previously described [5], were generously provided by Dr. A. Riccio (Catholic University, Rome). Cells were plated (2×10^6 cells per 35-mm dish) on dishes previously coated with poly-L-lysine (10 μg/ml). The culture dishes were incubated with basal Eagle's medium (BME) (Flow) at 37°C in a B 6000 microbiological incubator (Heraeus). Humidified 95% air and 5% CO_2 0 was supplied automatically. Experiments were performed on CGrC at 8 days *in vitro*.

CGrC were preincubated with or without 4 mM EGTA (Sigma) as described in [7], 0.1 mM Nω-nitro-L-arginine (NAME) (Sigma), 0.1 mM isobutylmethylxanthine (IBMX), and 0.1 mM guanine for 10 min, after which L-arginine (0.1 mM) (Sigma) and NADPH (0.1 mM) (Merck) were added. After preincubation for another 15 min, 200 μM ACPD, 50 μM DL-2-amino-4-phosphonobutyric acid (AP4), and 100 μM AMPA were applied for 15 min to study the coupling of mGluRs with the NO· pathway. Sodium nitroprusside (SNP) (100 μM) was used for 15 min after preincubation of CGrC with IBMX and guanine, as described above.

Measurement of cGMP content. After the drug application, the incubation medium was removed, CGrC were washed with 0.2 M phosphate buffer solution (PBS) (pH 7.4), and 0.5 ml of 0.4 N $HClO_4$ was added [11]. Then the cells were harvested and the suspension was centrifuged at 10,000 g for 10 min. The supernatants, after $HClO_4$ precipitation by K_2CO_3, were processed for cGMP assay using the Cyclic GMP [^{3}H] Assay System (Amersham).

Measurement of NO_2^- level. After the drug application, the incubation medium was removed, CGrC were washed with 0.2 M PBS (pH 7.4), and 0.5 ml of 0.05 N NaOH was added. Then the cells were harvested and the suspension was centrifuged at 10,000 g for 10 min. The proteins were pelleted by centrifugation. For assay of NO_2^- by the Griss reaction, 400 μl of supernatant were incubated with 400 ml of 1% sulfanylamide (Sigma) and 400 μl of 1% N-(1-naphthyl)ethylenediaminedihydrochloride (Sigma) in 2.5% H_3PO_4 0 at room temperature for 5 min. Optical density at 543 nm was measured. The results were expressed as $M \pm m$. of 5-6 observations. Statistical analysis was performed by two-way analysis of variance

(ANOVA). The F-test for significant interaction (F_{int}) was followed by Tukey's test to compare the experimental group with controls. In some experiments Dunnett's test was used as well. A p value of <0.05 was considered significant. AP4, AMPA, and ACPD were from Research Biochemicals International; the other reagents were chemically pure.

RESULTS

In the preliminary set of experiments the $NO^{\cdot}$-dependent activity of sGC in CGrC incubated with Ca^{2+}-depleted BME was tested. With this objective 100 μM SNP, a donor of $NO^{\cdot}$, were applied in the presence of 4 mM EGTA. The stimulation of guanylate cyclase under these conditions raised the cGMP level in CGrC from 0.56±0.17 pmol/10^6 cells to 4.41±0.13 pmol/10^6 cells (n=6) (Fig. 1), which assumes a sGC response also to the endogenously produced nitric oxide. There are two pathways of Ca^{2+}/CM-dependent production of nitric oxide via GluRs: 1) influx of extracellular Ca^{2+} induced by stimulation of both NMDA and AMPA/KA types of iGluRs; 2) Ca^{2+} release from the intracellular stores (calcitosomes) induced by stimulation of mGluRs. Incubation of CGrC with 100 μM AMPA raises the cGMP level, which drops in the presence of 4 mM EGTA (Fig. 2) (F_{int}=3.07, p<0.01; n=6). The effect of AMPA on NOS activity may be realized via two mechanisms triggered by membrane depolarization as a result of an effect on the AMPA/KA receptors. The first of these is the Ca^{2+} flux via the potential-dependent calcium channels [1], while the second is associated with the release of endogenous Glu [4]. Removal of extracellular Ca^{2+} by applying EGTA prevented the involvement of iGluRs in NOS activation (Fig. 2) [7].

The addition of 200 μM ACPD to CGrC induced a pronounced increase in cGMP formation that was completely reversed by the selective NOS inhibitor NAME (100 μM) (F_{int}=60.91, p<0.01; n=6) (Fig. 2). The effect of ACPD was abolished by AP4 (50 μM; p<0.01; n=6; Fig. 3), thus indicating that mGluR were involved in the sGC stimulation via the $NO^{\cdot}$ pathway. Indeed, incubation of CGrC with 200 μM ACPD produced an increase in the NO_2^- content from 0.75±0.17 nmol/10^6 cells to 1.81±0.21 nmol/10^6 cells (n=5), which was blocked (0.88±0.19 nmol/10^6 cells; F_{int}=9.16; p<0.05; n=5) by applying 200 mM AP4. The mGluR antagonist AP4 was found to inhibit the increase of phosphoinositol turnover induced by excitatory amino acids. In the present study, AP4 blocked the ACPD-induced increase in both NO_2^- and cGMP formation, suggesting a role for intracellular Ca^{2+} in the control of $NO^{\cdot}$ synthesis in CGrC.

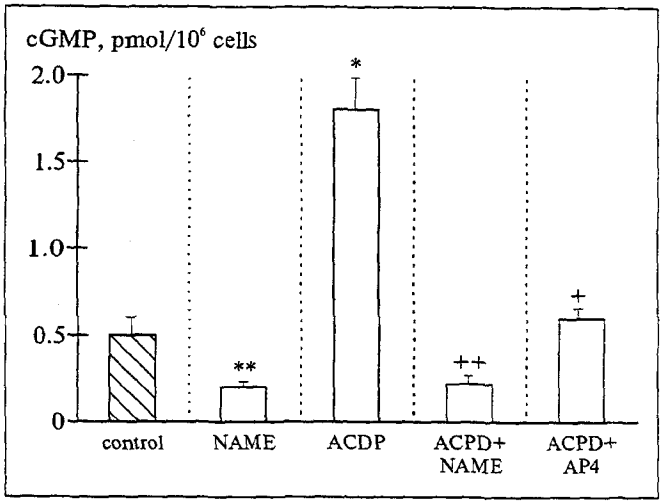

Fig. 3. Effect of NAME (0.1 mM) and AP4 (0.05 mM) on ACPD-dependent cGMP in cultures of granule cells. $^{*}p$<0.01; $^{**}p$<0.05 *vs.* the control, ^{+}p<0.01 *vs.* ACPD by Tukey's test, ^{++}p<0.01 (F_{int}=60.91) by two-way ANOVA.

In conclusion, glutamate is known to be implicated in the pathogenesis of the "excitotoxic" neuronal damage associated with ischemia, hypoglycemia, and hypoxia. Stimulation of GluRs is thought to produce neuron death through the elevation of free intracellular Ca^{2+} levels, leading to activation of Ca^{2+}/CM-dependent synthesis of cytotoxic amounts of $NO^{\cdot}$. Recent experiments have demonstrated that the mGluR-coupled $NO^{\cdot}$ pathway could be a suitable target for novel pharmacological strategies aimed at the treatment of neurodegenerative processes in the cerebellum.

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Aldosterone Binding with Corticosteroid Brain Receptors in Rats: Effect of Behavioral Typology and Stress

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Differences in ^{3}H-aldosterone binding with hippocampus cytosol receptors were found to be dependent on the behavioral type of male Wistar rats in the "emotional resonance" test. These differences were not observed in the cytosol analysis of the remaining part of the brain. Control rats and rats subjected to short-term stress by painful electrical stimulation showed a long-term drop of ^{3}H-aldosterone binding with hippocampus cytosol in active as compared to passive animals preferring a closed space.

Key Words: *corticosteroid receptors; aldosterone; brain; hippocampus; stress; individual behavior*

In recent years a great deal of attention has been paid to the role of brain corticosteroids (including aldosterone, AS) and corticosteroid receptors (CR) in various functions of the central nervous system. Steroids are able to directly interact with the neuron membrane, but most of the effects of glucocorticoids and mineralocorticoids are determined by their binding with intracellular receptors and subsequent alterations in the expression of certain genes [7]. The highest CR density in the brain is specific for the limbic system and above all the hippocampus. Mineralocorticoid receptors respond to the same signal as glucocorticoid receptors (corticosterone or cortisol). Mineralocorticoid receptors of the limbic system are believed to interact predominantly with corticosterone even if the blood level of the latter is very low [7]. CR of the limbic system and other brain areas maintain the ionic balance, reactions to neurotransmitters, and neuron excitability, as well as participate in the stress response, information processing, emotional reactions, and the execution of behavioral strategies [7,9].

These findings indicated that the brain CR probably play a role in individual behavior and prompted an investigation of AS-CR binding in the brain of rats with different characteristics of behavior. The "emotional resonance" phenomenon (ER) is a useful tool for behavioral typology in rats [3]. Since it was shown earlier that neurochemical differences among animals divided in the ER test were more pronounced after short-term stress [2], it seemed advisable to study both the basal and post-stress characteristics of the brain CR.

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