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Both protein kinase G dependent and independent mechanisms are involved in the modulation of glutamate release by nitric oxide in rat hippocampal nerve terminals

Sónia M. Sequeira, Arsélio P. Carvalho, Caetana M. Carvalho*

Center for Neuroscience of Coimbra, Department of Zoology and Faculty of Medicine, University of Coimbra, 3004-517 Coimbra, Portugal

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Abstract

We compared the effects of sodium nitroprusside (SNP), and of 8-bromo guanosine 3′,5′-cyclic monophosphate (8-BrcGMP), on the 4-aminopyridine (4-AP)-evoked Ca^{2+}-dependent release of glutamate from hippocampal nerve terminals and further investigated the role of protein kinase G (PKG) in this mechanism. SNP and 8-BrcGMP dose-dependently inhibited glutamate release, however SNP concentrations ([SNP]) $>$ 500 μM abolished the 4-AP evoked release, whereas 8-BrcGMP maximally inhibited the release by about 30%. The inhibition of glutamate release at low concentrations of SNP ($\leq$5 μM) was of about 20%, and was reversed by Rp-8(4-chlorophenylthio)guanosine-3′,5′-cyclic-monophosphorotioate) (RpCPTcGMP, 50 nM), but the inhibition at higher concentrations (5 $<$ SNP $\leq$ 50 μM) was insensitive to the PKG inhibitor, but sensitive to [1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one] (ODQ), which partially prevented the inhibition. [SNP] $>$ 50 μM strongly inhibited glutamate release, and this was not reversed by either inhibitor. Furthermore, [SNP] $\leq$ 50 μM enhanced cGMP formation, and the observed effects were not related to either decreased Ca^{2+} entry or ATP/ADP levels. Our results indicate that NO/PKG is the signaling pathway underlying the inhibition of glutamate release at low concentrations of NO, and imply that other NO-dependent, but PKG-independent, mechanisms are activated and have complementary roles at higher NO concentrations.

Keywords: Hippocampal synaptosomes; Glutamate release; NO donors; Nitric oxide; cGMP; PKG

Several studies argue in favor of nitric oxide (NO) acting as a retrograde messenger to regulate glutamate release in the brain [5,6,10–12,15]. However, because it potentially reacts with several intracellular systems, NO has multiple and often opposing effects at different concentrations of NO releasing agents. It is, therefore, essential to investigate the concentration-dependence of the effects of different NO donors on glutamate release as well as the signaling pathways involved. Nevertheless, a sensitive method of continuous measurement of NO concentration to clarify the physiological significance of the effects of NO is still lacking, whereas nitrate/nitrite formation has been generally used to determine NO production in higher yield, or longer time courses [9]. In this study, we used rat hippocampal synaptosomes, obtained as described elsewhere [4], in an attempt to elucidate the mechanisms that may be involved in the effects of NO on the 4-AP evoked Ca^{2+}-dependent release of glutamate, as affected by the NO donor SNP or by a cGMP analog, 8-BrcGMP.

The synaptosomal pellets (1 mg/ aliquot) were maintained on ice and used within 4 h. The glutamate release experiments were preceeded by an incubation period of 45 min, at 30ºC, in a medium containing (in mM): 132 NaCl, 1 KCl, 1 $MgCl_2$, 1.2 H_3PO_4, 0.1 $CaCl_2$, 10 glucose, 10 HEPES-Na^+, supplemented with 0.1 mg/ml BSA. During the incubation period, SNP, 8-BrcGMP, and/or the appropriate inhibitors were added at the 35th min, and were again included in the glutamate release experiments. The synaptosomes were collected by centrifugation (15 800 $\times$ *g* for 20 s), and resuspended in 1 ml fresh medium (in mM:

* Corresponding author. Tel.: +351-39-833369; fax: +351-39-822776; e-mail: cmcarvalho@gemini.ci.uc.pt

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132 NaCl, 1 KCl, 1 $MgCl_2$, 1.2 H_3PO_4, 1 $CaCl_2$, 10 glucose, 10 HEPES-Na). The release of glutamate was measured using a well established [8,11] continuous fluorimetric assay, based on the GDH-catalyzed reduction of $NADP^+$ in the presence of glutamate. We used a Perkin-Elmer Model LS-5B fluorimeter (λ_{exc} = 340 nm, λ_{em} = 460 nm) coupled to a thermostated (37ºC) chamber with continuous stirring, and collected data at 2-s intervals. In each experiment, the basal release of glutamate was followed for 5 min, after which 100 μM 4-AP was added to the suspension, and the 4-AP evoked release was further monitored for another 5 min, before 2.5 nmol of glutamate were added for calibration.

At this concentration, the 4-AP evoked glutamate release is mostly Ca^{2+}-dependent [11]. Fig. 1A shows that the NO donor SNP or the cGMP analog, 8-BrcGMP, both dose-dependently inhibited the Ca^{2+}-dependent release of glutamate, but to different extents; thus, SNP completely inhibited glutamate release at the higher concentrations tested (≥500 μM), but 8-BrcGMP maximally inhibited glutamate release by about 30%. In parallel experiments, SNP significantly increased the synaptosomal cGMP levels at low concentrations (SNP ≤ 50 μM), which rapidly decreased to basal levels at higher concentrations of SNP (Fig. 2). These results, obtained by radioimmunoassay, were performed on synaptosomes subjected to the same incubation conditions as in the glutamate release experiments, but always in the presence of 1 μM zaprinast, a specific cGMP-dependent phosphodiesterase inhibitor.

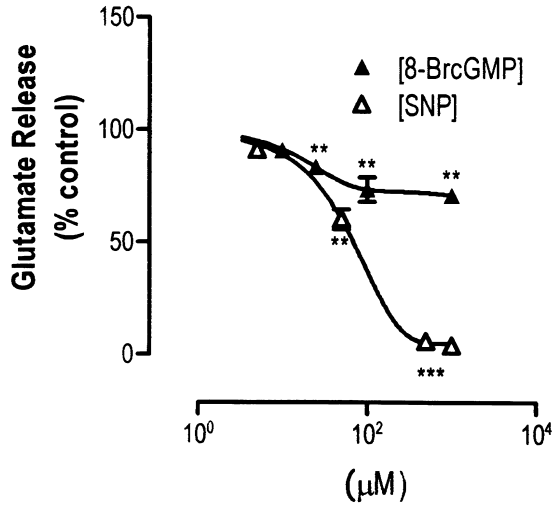

Fig. 1. Dose-dependent inhibition of the Ca^{2+}-dependent 4-AP evoked glutamate release by the NO donor, SNP, or the cGMP analog, 8-BrcGMP. In each situation, the synaptosomes were exposed to the drug for 10 min, during incubation, and another 5 min, during the assay, before addition of 4-AP, as detailed in the text. Data correspond to the % of glutamate release in the control (3.253 ± 0.08 nmol/5 min per mg protein), and each point is the mean ± SEM of 3–5 experiments performed in duplicate. Statistical significance was evaluated by ANOVA followed by Dunnet's post test: $^{**}P < 0.01$, $^{***}P < 0.001$.

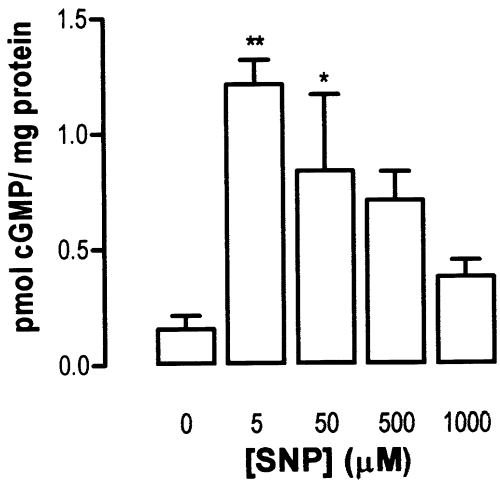

Fig. 2. Concentration-response curve for cGMP accumulation in SNP-treated synaptosomes. As shown, the lower concentrations of SNP (5 μM) induced the highest cGMP formation. Synaptosomes were subjected to the same conditions as in the glutamate release assay (Fig. 1) with the additional presence of 1 mM zaprinast in the assay medium. Each bar is the mean ± SEM of 3–5 experiments performed in duplicate. Statistical significance was evaluated by ANOVA followed by Dunnet's post test: $^{*}P < 0.05$, $^{**}P < 0.01$.

Hydroxylamine, another unrelated NO donor, had previously been shown by our laboratory to have a similar inhibitory effect on glutamate release and on cGMP levels in hippocampal synaptosomes [11]. In the previous study, we had also demonstrated the involvement of cGMP in the regulation of the 4-AP evoked glutamate release by the ability of ODQ, a specific guanylyl cyclase inhibitor, to reverse the inhibition of glutamate release by NO. In the present study, we show that the concentration of SNP which stimulated the formation of cGMP inhibited glutamate release by 20%. These observations strongly correlate with the inhibition induced by the cGMP analog, 8-BrcGMP (about 30% of the control) (Fig. 1), as well as by hydroxylamine [11], and imply a role for cGMP at low concentrations of NO. That protein kinase G (PKG) is a potential effector of cGMP-mediated effects in the brain is now widely accepted, and it has been demonstrated in a range of preparations [2,12,13,15]. In the present work, the specific PKG inhibitor, Rp-8(4-chlorophenylthio)guanosine-3′,5′-cyclic-monophosphorotioate) (RpCPTcGMP), at 50 nM, reversed the inhibition of glutamate release of SNP < 5 μM, but not that of higher concentrations, which do not involve significant cGMP formation (Fig. 3). Similar results were obtained with hydroxylamine, in which RpCPTcGMP reversed the inhibitory effect of 30 μM hydroxylamine on the 4-AP evoked release of glutamate by about 70% [1]. ODQ, however, completely reversed the effects of 5 μM SNP, and only partially reversed the inhibition by 50 μM SNP.

Higher concentrations of RpCPTcGMP did not further reverse the inhibitory effects of increasing concentrations of SNP, and, in fact, inhibited the 4-AP evoked release of

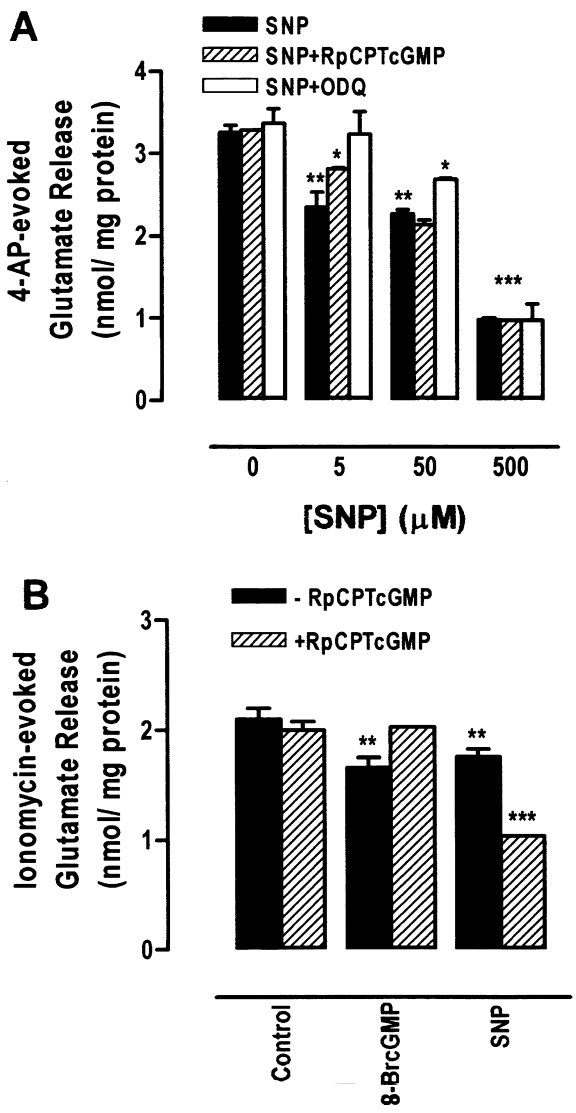

Fig. 3. Effect of RpCPTcGMP (50 nM) and/or ODQ (50 μM) on the release of glutamate evoked by (A) 100 μM 4-AP or (B) 5 μM ionomycin, in synaptosomes treated with either 8-BrcGMP or SNP. In (A), RpCPTcGMP, a specific PKG inhibitor, reversed the inhibition by 5 μM SNP, but not of higher concentrations. ODQ, a selective inhibitor of soluble GC, reversed the inhibition of release by [SNP] $\leq$ 50 μM. (B) Inhibition of glutamate release by 25 μM 8-BrcGMP and 50 μM SNP respectively, in conditions in which the activation of VDCC is bypassed by ionomycin, and reversal by RpCPTcGMP of the inhibition of release by 8-BrcGMP but not that of SNP. Each bar is the mean $\pm$ SEM of 3–5 experiments performed in duplicate. Statistical significance was evaluated by ANOVA followed by Dunnet's post test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

glutamate on it's own (data not shown), which may reflect the inhibition of cyclic nucleotide dependent channels (CNG) as previously suggested [13].

We interpret the results to indicate that cGMP may directly activate additional signaling effectors at high concentrations, which no longer effectively activate PKG, possibly due to autophosphorylation of PKG as a negative regulation of that pathway, as suggested in a recent study by El-Hussein et al. [3], which shows that in the thalamus, type II PKG is a substrate of NO/cGMP-dependent protein phosphorylation.

The Ca^{2+}-dependent release of glutamate occurs upon membrane depolarization and Ca^{2+} influx through voltage-dependent calcium channels (VDCC) [8] and therefore, one may postulate that NO inhibits glutamate release by directly, or indirectly through cGMP, blocking calcium entry. We had previously shown that the inhibition of glutamate release by hydroxylamine does not affect the $[Ca^{2+}]_i$ in response to 4-AP [11]. To further characterize the Ca^{2+} dependence of the inhibitory effects under study, we used ionomycin, a Ca^{2+} ionophore, to induce Ca^{2+} entry into synaptosomes surpassing the need for VDCC activation, and the effects of increasing concentrations of SNP or 8-BrcGMP were tested. As shown in figurehere>Fig. 3B, 50 μM SNP or 25 μM 8-BrcGMP inhibited the ionomycin-evoked glutamate release, in close resemblance with the results observed in depolarizing conditions (100 μM 4-AP) (figurehere>Fig. 3A). In these conditions, RpCPTcGMP reversed the inhibition of glutamate release by 8-BrcGMP but not that of SNP (50 μM) which in fact, slightly increased. These findings exclude the modulation of VDCC as a target for the cGMP-dependent inhibition of glutamate release. Similar results were obtained for higher concentrations of SNP (not shown), and, therefore, it is not likely altogether that VDCC are negatively modulated by NO in hippocampal synaptosomes. The question, however, remains as to which cGMP-independent mechanisms come into play at the higher concentrations of NO. Several studies have produced findings with respect to the direct involvement of NO as an effector molecule; S-nitrosylation has been proposed to affect the interactions among synaptic vesicle proteins and by bypassing Ca^{2+} entry, to increase exocytosis [6,7]. However, in the range of concentrations of SNP used in the present study, we did not observe enhanced glutamate release by NO, our data being in agreement with that of Sistiaga et al. [12], and thus refers to the inhibition of the Ca^{2+}-dependent release of glutamate, and not to the Ca^{2+}-independent component of release reported by others to be enhanced by SNP [7]. Yet another study shows that NO inhibits the glutamate vesicular proton pump by S-nitrosylation [14].

In conclusion, the present work shows evidence that in the hippocampus, PKG is involved in the inhibitory effect of low concentrations of NO on glutamate release, but does not account for the inhibition observed at higher concentrations of NO. However, the data suggest that the overall inhibition of release, and the signaling mechanisms activated, are dependent on NO concentration.

[1] Carvalho, C.M., Sequeira, S.M., Malva, J.O. and Carvalho, A.P., Involvement of the NO/cGMP signalling on the modulation by nitric oxide of glutamate release in hippocampal nerve terminals, FASEB J., 12 (1998) A460.

[2] Chen, C., Houchi, H., Ohnaka, M., Sakamoto, S., Niwa, Y. and Nakaya, Y., Nitric oxide activates Ca^{2+}-activated K^+ channels in

cultured bovine adrenal chromaffin cells, Neurosci. Lett., 248 (1998) 127–129.
[3] El-Husseini, A.El-D., Bladen, C., Williams, J.-A., Reiner, P.B. and Vincent, S.R., Nitric oxide regulates cyclicGMP-dependent protein kinase phosphorylation in rat brain, J. Neurochem., 71 (1998) 676–683.
[4] Malva, J.O., Carvalho, A.P. and Carvalho, C.M., Domoic acid induces the release of glutamate in the rat hippocampal CA3 subregion, NeuroReport, 7 (1997) 1330–1334.
[5] McNaught, K.S.P. and Brown, G., Nitric oxide causes glutamate release from brain synaptosomes, J. Neurochem., 70 (1998) 1541–1546.
[6] Meffert, M.K., Calakos, N.C., Scheller, R.H. and Schulman, H., Nitric oxide modulates synaptic vesicle docking/fusion reactions, Neuron, 16 (1996) 1229–1236.
[7] Meffert, M.K., Prenack, B.A. and Schulman, H., Nitric oxide stimulates Ca^{2+}-independent synaptic vesicle release, Neuron, 12 (1994) 1235–1244.
[8] Nicholls, D.G., Sihra, T.S. and Sánchez-Prieto, J., Calcium-dependent and -independent release of glutamate from synaptosomes monitored by continuous fluorometry, J. Neurochem., 49 (1987) 50–57.
[9] Schmidt, H.H.W. and Kelm, M., Determination of nitrite and nitrate by the Griess reaction. In M. Feelisch and J.S. Stamler, (Eds.), Methods in Nitric Oxide Research, Wiley, Chichester, 1996, pp. 491–497.
[10] Segieth, J., Getting, S.J., Biggs, C.S. and Whitton, P.S., Nitric oxide regulates excitatory amino acid release in a biphasic manner in freely moving rats, Neurosci. Lett., 200 (1995) 101–104.
[11] Sequeira, S.M., Ambrósio, A.F., Malva, J.O., Carvalho, A.P. and Carvalho, C.M., Modulation of glutamate release from hippocampal synaptosomes by nitric oxide, Nitric Oxide: Biol. Chem., 1 (1997) 315–329.
[12] Sistiaga, A., Miras-Portugal, M.T. and Sánchez-Prieto, J., Modulation of glutamate release by a nitric oxide/cGMP-dependent pathway, Eur. J. Pharmacol., 321 (1997) 247–257.
[13] Wei, J., Cohen, E.D., Genieser, H. and Barnstable, C.J., Substituted cGMP analogs can act as selective agonists of the rod photoreceptor cGMP-gated cation channel, J. Mol. Neurosci., 10 (1998) 53–64.
[14] Wolosker, H., Reis, M., Assreuy, J. and de Meis, L., Inhibition of glutamate uptake and proton pumping in synaptic vesicles by s-nitrosylation, J. Neurochem., 66 (1996) 1943–1948.
[15] Wu, J., Wang, Y., Rowan, M.J. and Anwyl, R., Evidence for involvement of the cGMP-protein kinase G signaling system in the induction of long-term depression, but not long-term potentiation, in the dentate gyrus in vitro, J. Neurosci., 18 (1998) 3589–3596