Nitric oxide mediates NMDA-evoked [^{3}H]GABA release from chick retina cells

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Abstract The stimulation of NMDA receptor increased [^{3}H]GABA release from preloaded cultured retina cells. This effect appears to be mediated by NO production, since addition of L-NA reduces NMDA-evoked [^{3}H]GABA release. Spermine/NO complex, an NO donor, mimics the effect produced by NMDA. The addition of zaprinast, a phosphodiesterase inhibitor, as well as 8-Br-cGMP enhances the NMDA-evoked [^{3}H]GABA release. These results agree with the existence in chick retina cells of NO/cGMP pathways and support a role for NO in NMDA-evoked events. The activation of this receptor complex through maturative stages of the retina together with the NO-mediated increase in GABA release may account for NMDA differentiative effect in culturing retina cells.

Key words: Nitric oxide; Chick embryo; Retina cell; NMDA; GABA release

1. Introduction

NO is a highly reactive radical species endowed with messenger functions in the central nervous system [1]. In the vertebrate brain the activation of NMDA subtype of glutamate receptor enhances neuronal Ca^{2+}-influx which in turn stimulates the neuronal type of NO synthase (nNOS) to generate NO and this is responsible for NMDA-mediated cGMP accumulation by stimulation of the soluble form of guanylate cyclase [2]. In addition, NO has been involved in other important NMDA receptor-mediated mechanisms including neurotransmitter release [3]. More recently, it has became apparent that a functional L-arginine-NO-cGMP pathway is present since the early stages of development in chick embryo retina [4,5]. For instance, we have previously shown that Ca^{2+}-dependent NOS activity is present in 9-day-old chick embryo retina and more importantly this can be stimulated through NMDA receptor activation [5,6]. Collectively these data led us to hypothesize that the occurrence of a functional NO/cGMP pathway, which can be modulated by NMDA receptor activation, may have a role in the maturation process of chick embryo retina. To test this hypothesis we have studied the effect of drugs which interfere with the NO/cGMP pathway on the NMDA-evoked [^{3}H]GABA release in chick embryo retina cells at different stages of culture differentiation. The results yielded by the present study support a role for NO/cGMP pathway in NMDA-evoked GABA release, and this may account for the differentiative effect of NMDA in cultured retina cells [7,8].

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Abbreviations: NO, nitric oxide; 8-Br-cGMP, 8-bromoguanosine 3′:5′-cyclic monophosphate; BH_4, tetra-hydrobiopterin; MK-801, (+)5-methyl-10,11-dihydro-5H-dibenzo-[a,d]cyclohepten-5,10-imine hydrogen maleate; LY 274614, ±3R*,4aS,8aR*-6-(phosphomethyl)-decahydro-isoquinoline-3-carboxylic; L-NA, N^{ω}-nitro-L-arginine; D-NA, N^{ω}-nitro-D-arginine; NMDA, *N*-methyl-D-aspartate; IBMX, isobutyl-methylxanthine; Hb, Haemoglobin

2. Materials and methods

L-NA, D-NA, MK-801, and spermine/NO complex were purchased from Research Biochemicals International (Natick, MA); L-[^{14}C]arginine (300 mCi/mmol) and [^{3}H]GABA (100 Ci/mmol) were from Amersham; BH_4 was from ICN Biomedicals (Aurora, OH). NMDA, 8-Br-cGMP, IBMX and other chemicals were from Sigma (St. Louis, MO). 2-O-propoxyphenyl-8-azapurine-6-one (Zaprinast), a phosphodiesterase inhibitor, was generously provided by Rhone-Poulenc (Dagenham, UK).

Primary cultures of cells were prepared using retinas from 8-day-old chick embryos, as reported by Nascimento and de Mello [9]. Trypsinized tissue was centrifuged at 500×*g* for 1 min and supernatant discarded. The pellet was resuspended in 5 ml of BME (Earle's salts) containing 5% fetal calf serum and mechanically dissociated. Then the cells were plated in 35 mm plastic dishes (30×10^6 cells/dish) and transferred to a 37°C incubator under humidified atmosphere of 95% air, 5% CO_2.

In order to evaluate NMDA response at different stages of culture differentiation we measured NO synthase activity after 3 (E8C3) and 6 days (E8C6) of cells in culture. Retina cells were collected and suspended in 20 mM HEPES salt buffer composed of 110 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 25 mM glucose and 58.4 mM sucrose (pH 7.4). Cells were distributed into assay tubes (1×10^6 cells) and incubated with L-[U-^{14}C]arginine (0.6 μCi, 80 nM), BH_4 (10 μM), and indicated agonist or drugs, for 45 min at 37°C. Reactions were stopped by addition of 750 μl of 20 mM HEPES salt buffer containing 4 mM EDTA. After centrifugation at 300×*g* for 1 min, cell pellets were lysed by addition of 1 ml of 0.3 M $HClO_4$. Appropriate aliquot of supernatant, obtained by centrifugation at 800×*g* for 5 min, was passed through Dowex AG50W-X8 (Na^- form), following by washing with 1 ml of water. [U-^{14}C]citrulline formation was measured using a liquid scintillation counter. Preliminary experiments showed that NMDA-stimulated NO synthesis was linear as a function of time (up to 45 min) as well as for protein (up to 300 μg).

[^{3}H]GABA release was assayed as described by Nascimento and de Mello [9]. The cultures at E8C3 and E8C6 stages were preincubated for 2 h in 1 ml of fresh BME containing [^{3}H]GABA (5×10^{-8} M), buffered at pH 7.35 with 20 mM HEPES. The medium was removed and the cultures washed 3 times with 2 ml Hanks' solution pH 7.4. Cells were then superfused with Hanks' solution at 37°C, in the presence of indicated drugs. Agonists or antagonists were used at the concentration able to give maximal effect as previously reported [6]. Experiments using NMDA were performed in Mg^{2+}-free Hanks' solution. The superfusion proceeded by performing successive changes of 0.5 ml of the bathing medium at intervals of 5 min. Samples of superfusates were collected and radioactivity was measured. The [^{3}H]GABA released at 5 min interval was plotted as the percentage of the total counts taken up by the cells. The total counts represent the radioactivity released during the superfusion plus the radioactivity remaining in the cells at the end of superfusion. cGMP contents were measured by radioimmunoassay (Amersham).

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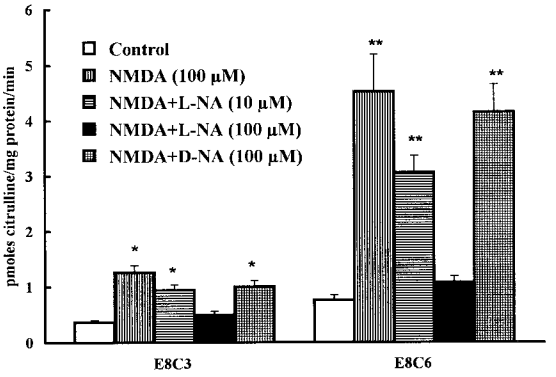

Fig. 1. Effect of NMDA on the NO synthesis from chick embryo retina cells at various stages of culturing. Cells from retinas of 8-day-old chick embryos were cultured for 3 (E8C3) or 6 days (E8C6). Intact cells were incubated with or without NMDA (100 μM) in the presence or absence of arginine analogues, such as L-NA (10–100 μM) and D-NA (100 μM). The NOS activity was measured by monitoring the conversion of L-arginine into citrulline. Data are means ± S.E.M. from 3–4 independent experiments performed in triplicate. $*P < 0.01$, $**P < 0.001$ in comparison to controls, unpaired two tailed Student's *t*-test.

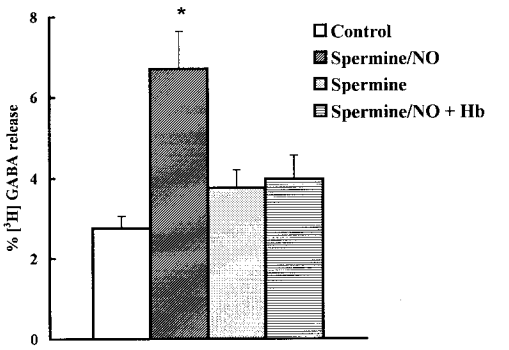

Fig. 2. NO-evoked release of [^{3}H]GABA from chick retina cell cultures. Cultures (E8C6) were loaded by incubation with [^{3}H]GABA for 2 h at 37°C. Then superfused with spermine/NO complex (100 μM), in the presence or absence of haemoglobin (Hb, 10 μM), or 100 μM spermine alone. For the evaluation of [^{3}H]GABA release see Section 2. Data are means ± S.E.M. from six experiments performed in triplicate. $*P < 0.01$ in comparison to controls, unpaired two tailed Student's *t*-test.

3. Results

In agreement with our previous observations made in intact chick embryo retina preparations, exposure of chick embryo retina cells (E8C6) to NMDA increases NOS activity by 0.5- and 6-fold according to the low (10 μM) and high (100 μM) dose used of the NMDA receptor agonist. In Fig. 1 we show that the stimulation of NOS activity seems to correlate with the age of cultures, since NMDA-induced increase of enzyme activity was greater in chick embryo retina cells cultured for 6 days than in those cultured for 3 days. The stimulated enzyme activity was inhibited in a stereoselective fashion by the NOS inhibitor, L-NA (10–100 μM).

As reported in Table 1, exposure of these cultures to NMDA (100 μM) also caused a 3-fold increase in [^{3}H]GABA release. This effect appears to be mediated through NMDA receptor-stimulated NO synthesis. In fact, both competitive (LY 274614; 100 μM) and non-competitive (MK-801; 10 μM) NMDA receptor antagonists and the NOS inhibitor L-NA (100 μM) significantly reduced NMDA-enhanced [^{3}H]GABA release.

To further involve NO in the mechanism underlying GABA release, the latter has been studied in chick embryo retina cell cultures exposed to spermine/NO (100 μM), a slow spontaneous releaser of this nitrogen radical, that appears to be more potent than sodium nitroprusside [10]. Under these conditions an approximately 3-fold increase in background [^{3}H]GABA release was observed (Fig. 2). The NO donor was added at the concentration able to produce the maximal effect on amino acid release from intact chick retina [6]. The effect of spermine/NO is due to NO released by the donor, because Hb (10 μM), an NO trapping agent, fully prevented the stimulated GABA release. This observation would exclude that under our present conditions a stimulatory effect of spermine acting on the polyamine modulatory site in the NMDA receptor complex [11] may take place. The latter deduction is strengthened by the evidence that in a concentration range as high as 0.5–1.5 mM, spermine weakly increased (22–37%) [^{3}H]GABA release in chick retina cell cultures. However, in Fig. 2 we show that no detectable increases were apparent when spermine was added at equimolar concentration of spermine/NO complex (100 μM). Interestingly, as reported in Fig. 3 application of NMDA (100 μM) or spermine/NO (100 μM) significantly increases cGMP levels, suggesting that the NMDA subtype of glutamate receptor modulates a fully functional NO/cGMP pathway in developing chick embryo retina cells. This hypothesis is further supported by the observed enhancement of NMDA-evoked [^{3}H]GABA release in E8C6 cultures exposed (10 min before and for duration of NMDA application) to inhibitors of phosphodiesterase, such as IBMX (100 μM) or zaprinast (10 μM). As reported in Table 2, phospho-

Table 1
[^{3}H]GABA release from chick embryo retina cells after NMDA activation

	% [^{3}H]GABA release	
	E8C3	E8C6
Control	1.92 ± 0.18	2.75 ± 0.41
NMDA (100 μM)	5.45 ± 0.61**	8.23 ± 0.78**
NMDA+MK-801 (10 μM)	2.45 ± 0.28	4.34 ± 0.51
NMDA+LY 274614 (100 μM)	2.73 ± 0.32	4.91 ± 0.47
NMDA+L-NA (100 μM)	2.98 ± 0.37	4.73 ± 0.63

Retina cells at E8C3 and E8C6 stages of culturing were incubated with [^{3}H]GABA for 2 h at 37°C. Then the non-incorporated radioactivity was washed out, and the cultures were superfused with Mg^{2+}-free Hanks' solution, or NMDA in Mg^{2+}-free Hanks' solution as described in Section 2. Antagonists of NMDA at indicated concentrations and L-NA were added to cultures 5 min before agonist addition. For the evaluation of [^{3}H]GABA release see Section 2. The values shown represent the mean ± S.E.M. from five experiments in triplicate.
$**P < 0.001$ in comparison to controls, unpaired two tailed Student's *t*-test.

diesterase inhibitors enhances NMDA-mediated release by approximately 22–55%, with no appreciable effect on basal [^{3}H]GABA release (data not shown). In similar experiments it has been found that zaprinast (10 μM) produces a 44% increase of spermine/NO-induced release. Not surprisingly, in unstimulated chick embryo retina cell cultures the exposure to 8-Br-cGMP (1 mM), the enzyme-resistant cGMP analogue, yielded a significant increase in [^{3}H]GABA release (see Table 2).

4. Discussion

The present experiments have shown that activation of the NMDA subtype of glutamate receptor stimulates the release of [^{3}H]GABA in chick embryo retina cell cultures. In fact, two selective antagonists (e.g. LY 274614 and MK-801) of this receptor subtype abolished NMDA-evoked release. A second important suggestion is that the mechanism through which NMDA receptor mediates this effect, involves stimulation of the NO/cGMP pathway because the selective NOS inhibitor L-NA, but not the less active isomer, D-NA prevented NMDA stimulation of [^{3}H]GABA release. In line with a role for NO, when retina cell cultures were exposed to the NO donor spermine/NO complex, an increase on [^{3}H]GABA release was observed, an effect, this latter, abolished by the NO trapping agent, Hb. Furthermore, the inhibitors of phosphodiesterase activity, such as zaprinast and IBMX, enhanced NMDA-evoked release likewise zaprinast did when co-applied with spermine/NO. Support for a role of NO/cGMP pathway in NMDA-evoked [^{3}H]GABA release also comes from the evidence that 8-Br-cGMP, an enzyme-resistant cGMP analogue, with low membrane permeability, increases the release of [^{3}H]GABA in unstimulated retina cell cultures. In addition to the findings on the modulation operated by NMDA receptor stimulation in a differentiative dependent fashion in chick embryo retina [12], our results show that NO/cGMP pathway may play a role in the control of [^{3}H]GABA release. This appears to be of great importance for our understanding of the mechanisms underlying retina cell differentiation and function. Several evidence implicate NO in fundamental processes concerned with nervous tissue cell development such as neurotransmitter release [3], synaptogenesis [13] etc. and the NO forming enzyme is known to undergo per se developmental changes in the retina and in several areas of the brain [5,14]. Furthermore, it is well established that differentiation of different nerve cells in culture operated by depolarizing conditions involves NMDA-mediated control of GABA release as well as the release of the neurotransmitters [8]. Therefore, it is conceivable that NO, a highly reactive and rapidly diffusible radical species, may represent the key messenger molecule through which maturative processes are controlled in chick embryo retina.

Table 2
Effect of 8-Br-cGMP and phosphodiesterase inhibitors on NO-evoked [^{3}H]GABA release from chick embryo retina cells

	% [^{3}H]GABA release
Basal	2.75 ± 0.40
8-Br-cGMP (0.5 mM)	3.98 ± 0.42
8-Br-cGMP (1.0 mM)	5.72 ± 0.65[a]
NMDA (100 μM)	8.23 ± 0.91
NMDA+Zaprinast (10 μM)	12.77 ± 1.35[b]
NMDA+IBMX (100 μM)	10.02 ± 1.25
Spermine/NO (100 μM)	6.76 ± 0.81
Spermine/NO+Zaprinast (10 μM)	9.73 ± 0.94[c]

Retina cells at E8C6 stages of culturing were incubated with [^{3}H]GABA (5×10^{-8} M) for 2 h at 37°C, and thereafter superfused in the presence of 8-Br-cGMP at indicated concentrations. Zaprinast and IBMX were added 10 min before the addition of NMDA or spermine/NO complex. For the evaluation of [^{3}H]GABA release see Section 2. Data are means ± S.E.M. from 5–6 independent experiments performed in triplicate.
[a] $P < 0.05$ in comparison to basal value.
[b,c] $P < 0.05$ in comparison to NMDA and NO donor alone, respectively. ANOVA with Newman-Keuls multiple comparison post-hoc test.

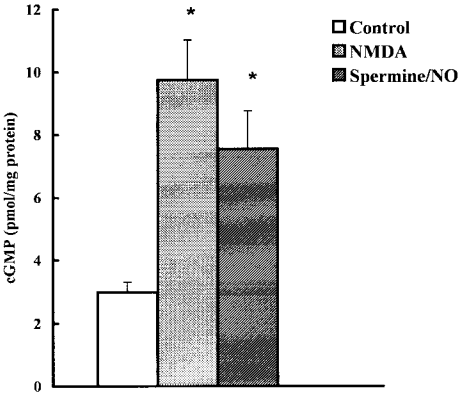

Fig. 3. Effects of NMDA (100 μM) and spermine/NO complex (100 μM) on cGMP levels in E8C6 chick embryo retina cells, during 20 min incubation. cGMP levels were measured by radioimmunoassay. Data are means ± S.E.M. from 3–4 independent experiments performed in triplicate. *$P < 0.01$ in comparison to controls, unpaired two tailed Student's t-test.

Previous experiments carried out in our laboratory using intact retina preparations failed to implicate cGMP in NMDA-mediated neurotransmitter release [6] seemingly because of technical difficulties generated by the presence of cytoarchitectural tissue organization in intact retina model. Conversely, in the present experiments, monolayer cell cultures from chick retina display characteristics of neuroblasts induced to differentiate showing at E8C6 a typical 'neuritic' pattern [15]. This observation together with our present results make chick embryo retina cell cultures more suitable for the characterization of neurochemical processes related to retina development and differentiation.

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