NMDA RECEPTOR ACTIVITY AND ITS MODULATION BY SULFHYDRYL COMPOUNDS: CHEMILUMINESCENCE MEASUREMENTS IN RAT BRAIN CORTICAL SYNAPTOSOMES

(Received 4 February, 1999)

Belgin Küçükkaya, M.D. / Goncagül Haklar, M.D. / A. Süha Yalçın, Ph.D.

Department of Biochemistry, School of Medicine, Marmara University, Istanbul, Turkey.

ABSTRACT

Objective: We have used chemiluminescence (CL) measurements to investigate the effect of modulatory compounds on N-methyl-D-aspartate (NMDA) receptor activity in rat brain frontal cortex synaptosomes.

Methods: Freshly prepared synaptosomes (protein concentration: 5 mg/mL) were incubated with different modulatory molecules such as Zn^{++} (1 mM), Cd^{++} (1 mM), reduced glutathione, (GSH) (1 mM, 10 mM), oxidized glutathione (GSSG) (1 mM, 5 mM), dithiothreitol (DTT) (1 mM, 10 mM) and N-ethylmaleimide (NEM) (1 mM, 10 mM) for 5 minutes at room temperature. After preincubation, 0.1 mM NMDA was added either alone or in the presence of 65 mM KCl. CL measurements were taken with lucigenin as enhancer, at 10 second intervals for 1 minute.

Results: Synaptosomes showed a marked increase in CL upon addition of NMDA both in the presence and absence of KCl. When preincubated with the non-competitive NMDA receptor antagonist (Zn^{++}) or with the calcium channel blocker(Cd^{++}), CL formation was suppressed. DTT, a strong sulfhydryl group reducing agent, increased the activity of NMDA receptor, whereas NEM, a sulfhydryl group alkylating agent, decreased the NMDA receptor activity. Reduced and oxidized forms of glutathione decreased the receptor activity and were protective under excitotoxicity and depolarization conditions.

Conclusion: Our results demonstrated that endogenous and exogenous sulfhydryl compounds affect the redox modulatory site and play important roles in the generation of reactive oxygen species after activation of the NMDA receptor.

Key Words: Chemiluminescence; Cortical synaptosomes; Excitotoxicity; NMDA receptor; Sulfhydryl group modulators

INTRODUCTION

Glutamate is the major excitatory amino acid and plays a dominant role in fast information transfer, memory and learning through its membrane-associated receptors (1,2). Glutamate receptors can be grossly subdivided into ionotropic (N-methyl-D-aspartate (NMDA) and non-NMDA) receptors and metabotropic types (1). The predominant form of excitotoxic injury observed after a variety of neurologic insults is mediated by the activation of the NMDA receptor. NMDA related disorders include hypoxia/ischemia, trauma, epilepsy and possibly some neurodegenerative diseases such as HIV-associated dementia (3-5). A number of pharmacological sites modulate the activity of the NMDA receptor complex; these are the agonist-binding, the glycine-binding, the inhibitor cationic (zinc)-binding, the voltage-sensitive magnesium binding, the phencyclidine-binding and the polyamine-binding sites (1,6). One recently discovered site has been designated the redox modulatory site. This site is of particular interest since it acts as a control for current flux through NMDA receptor operated channels and can affect the degree of neurotoxicity produced by excessive NMDA receptor activation (7,8).

We have previously shown that chemiluminescence (CL) measurements may be used for investigating glutamate/NMDA excitotoxicity in rat brain homogenates (9). In this study, we have used rat brain frontal cortex synaptosomes and the CL assay to investigate the NMDA receptor activity upon addition of different modulators, especially those acting on the redox modulatory site.

MATERIALS AND METHODS

Male Sprague-Dawley rats (200-230 g) were used throughout the study. The animals were housed in a well-ventilated room at ambient temperature with a 12 hr light-dark cycle. Food and water were available ad

libitum. Prior to the experiments, rats were killed and their brains were immediately removed. Frontal cortices were freshy homogenized in 0.32 mM sucrose solution (10% w/v) using a motor driven Potter-Elvehjem glass homogenizer with a teflon pestle. Crude synaptosomal pellets (P2 fraction) were obtained by ultracentrifugation (10), suspended in Hank's buffer (130 mM NaCl, 5.2 mM KCl, 1.3 mM $CaCl_2$, 0.58 mM KH_2PO_4, 1.5 mM $NaHCO_3$, 10 mM glucose and 20 mM HEPES) and left at room temperature for 15 minutes to reach equilibrium. Synaptosomes were first preincubated with different modulatory molecules: zinc (1 mM), cadmium (1 mM), reduced glutathione (GSH, 1 mM and 10 mM), oxidized glutathione (GSSG, 1 mM and 5 mM), dithiothreitol (DTT, 1 mM and 10 mM) and N-ethyl maleimide (NEM, 1 mM and 10 mM) for 5 minutes at room temperature. Samples were then placed in glass scintillation vials at a final volume of 3 mL and a protein concentration of 5 mg/mL. CL measurements were taken with lucigenin as enhancer, at 10 second intervals for 1 minute, immediately after addition of 0.1 mM NMDA in the presence and the absence of 65 mM KCl (9). The area under curve (AUC) of each experiment was calculated (11).

Statistical analyses were performed with "Dunn's multiple comparison test". The results were considered significant when $p<0.05$.

RESULTS

The results of our study are summarized in Figure 1. We have observed significant increases in CL upon addition of 0.1 mM NMDA (excitotoxic concentration) to freshly prepared synaptosomal suspensions. CL counts were also increased with 65 mM KCl (chemical depolarization) and when NMDA and KCl were added together the increase was more pronounced. Preincubation with the NMDA receptor cationic inhibitor Zn^{++} (1 mM) and with the Ca^{++} - channel blocker Cd^{++} (1 mM), suppressed the formation of CL under excitotoxicity and depolarization conditions.

In the second part of our study, we have studied CL formation under excitotoxicity and depolarization conditions after preincubating synaptosomal suspensions with different sulfhydryl group modulators. DTT, a strong sulfhydryl reducing agent, increased CL measurements significantly at 1 mM and 10 mM concentrations. NEM, a sulfhydryl group alkylating agent, decreased the NMDA receptor activity at both concentrations. On the other hand, reduced and oxidized forms of lutathione decreased the receptor activity and were protective against CL formation under excitotoxicity and depolarization conditions.

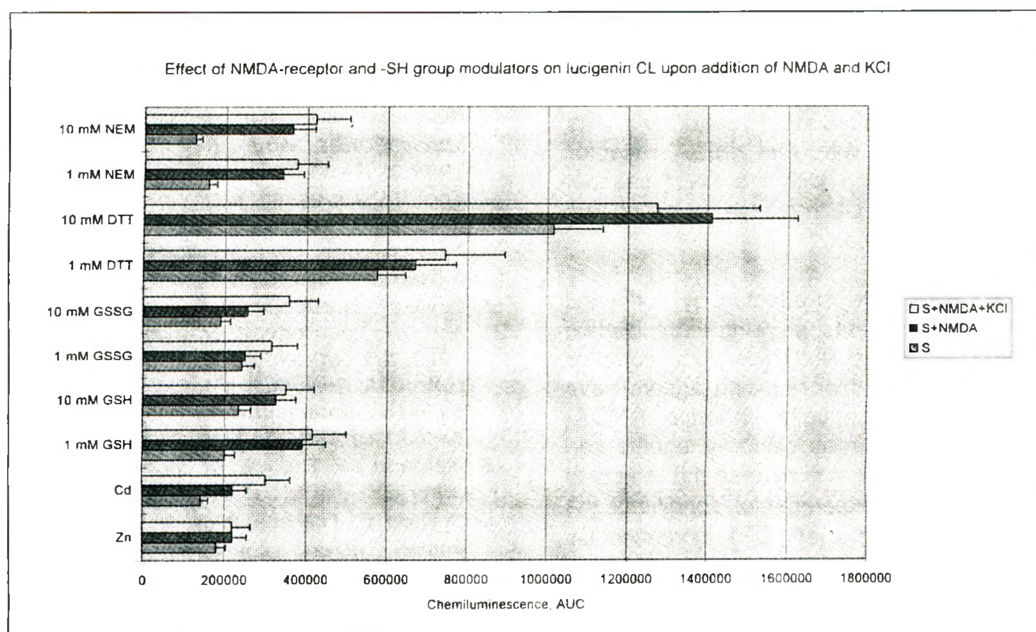

Fig.1.: NMDA and depolarization induced changes in NMDA-receptor activity upon addition of different modulator molecules.

Rat brain cortical synaptosomes (S) were first preincubated with different modulating molecules for 5 minutes at room temperature. CL measurements were taken after addition of 0.1 mM NMDA (excitotoxic concentration) in the absence and presence of 65 mM KCl (chemical depolarization). Results are expressed as the area under the curve (AUC) of CL. Further experimental details are described in materials and methods.

DISCUSSION

NMDA receptors have received the greatest attention with regard to excitotoxicity as they possess high permeabilty to Ca^{++} ions besides Na^{+} and K^{+} ions. It is known that Ca^{++} ions play important roles in the fast and slow intracellular signal transduction as well as cellular toxicity (2). We have previously shown that increasing concentrations of NMDA resulted in concomitant CL formation in rat brain homogenates. Moreover, upon addition of different NMDA receptor modulators, CL measurements were decreased or increased as expected (9). Lafon-Cazal et al. (13) have demonstrated by using "spin resonance trapping" that the activation of NMDA receptor occurred via superoxide radical formation. Patel et al. (14) have also shown the direct implication of superoxide generation in the pathogenesis of excitotoxic injury. This was confirmed by our CL measurements where lucigenin, the enhancer specific for superoxide radical, was more effective in following NMDA receptor activity (9).

The synaptosome is the simplest system which retains the full machinery for the uptake, storage, and release of neurotransmitters. Moreover, it also involves the presynaptic receptor-mediated signal transduction pathways which regulate release (12). In this report, with the CL assay described above, we aimed to study changes in the NMDA receptor activity by using the synaptosome model. We were particularly interested in the effects of different sulfhydryl group compounds on the redox modulatory site of the receptor. We have observed significant increases in CL under excitotoxicity and depolarization conditions. As expected, preincubation with the NMDA receptor cationic inhibitor Zn^{++} and with the Ca^{++}-channel blocker Cd^{++}, suppressed CL formation, revealing that CL follows receptor activation.

The redox modulatory site is affected by sulfhydryl reducing and oxidizing agents, showing the existence of disulfide bond(s) on the NMDA receptor complex (7,8,15,16). In particular, thiol reducing agents have been observed to dramatically enhance NMDA-mediated responses, whereas oxidizing agents can decrease native responses and reverse the effects of reductants (17). In our experimental conditions the strong sulfhydryl reducing agent DTT increased CL formation significantly, whereas the sulfhydryl blocking agent NEM was protective against excitotoxicity and depolarization induced CL formation. This implicates that, sulfhydryl group reducing agents act on the NMDA receptor-channel complex and enhance the activity of the receptor by breaking the extracellular disulfide bonds.

On the other hand, reduced and oxidized forms of glutathione had no effect on the receptor activity of synaptosomes before preincubation, but were protective against CL formation under excitotoxicity and depolarization conditions. Glutathione is the major endogenous non-protein sulfhydryl compound of the cell and occurs mainly in the reduced form. Following periods of oxidative stress or excitotoxicity, substantial GSH is converted to GSSG as the cell attempts to prevent further damage. GSSG is then actively transported out of the cell and accumulates in the extracellular space (16). Oxidizing agents like GSSG may also form disulfide bonds with vicinal free thiol groups and down regulate NMDA receptor. It was recently shown that both reduced and oxidized glutathione inhibit the binding of specific ligands to ionotropic glutamate receptors including the NMDA receptor complex (17). Thus, the effects are complex, involving both interaction with the agonist site and regulation of the receptor redox state.

In the present study, we have used a practical and sensitive method to investigate NMDA receptor activity and its modulation. Our results demonstrated that endogenous and exogenous sulfhydryl compounds affect the redox modulatory site and play important roles in the generation of reactive oxygen species after activation of the NMDA receptor. Similar studies may provide important clues regarding endogenous modulation and activation of the NMDA receptor under excitotoxicity and depolarization conditions.

REFERENCES

1. *Wroblewski JT, Danysz W. Modulation of glutamate receptors: molecular mechanisms and functional implications. Ann Rev Pharmacol Toxicol 1989;29:441-474.*
2. *Choi DW. Glutamate receptors and the induction of excitotoxic neuronal death. In: Bloom F, ed. Neuroscience: from the molecular to the cognitive. Amsterdam: Elsevier Science, 1994:47-51.*
3. *Coyle JT, Puttfarcken P. Oxidative stress, glutamate and the neurodegenerative disorders. Science 1993;262:689-695.*
4. *Lipton SA, Rosenberg PA. Excitatory amino acids as a final common pathway for neurologic disorders. N Engl J Med 1994;330:613-622.*
5. *Meldrum BS. The role of glutamate in epilepsy and other CNS disorders. Neurology 1994;44:514-523.*
6. *Scatton B. Pharmacology of the excitatory amino acids: novel therapeutic approaches in neurodegenerative disorders. In: Racagni G, Brunello A, Langer SZ, eds. Recent Advances in the Treatment of Neurodegenerative Disorders and Cognitive Dysfunction. Vol. 7. Basel:Karger, 1994:157-165.*
7. *Gozlan H, Ben-Ari Y. NMDA receptor redox sites: are they targets for neuronal protection. TIPS 1994;16:368-374.*
8. *Aizenman E, Reynolds IJ. Modulation of NMDA excitotoxicity by redox reagents. Ann NY Acad Sci 1992;648:125-131.*

9. Küçükkaya B, Haklar G, Yalçın AS. NMDA excitotoxicity and free radical generation in rat brain homogenates, application of a chemiluminescence assay. Neurochem Res 1996;21:1533-1536.
10. Dodd PR, Hardy JA, Oakley AE, Edwardson JA, Perry EK, Delaunoy JP. A rapid method for preparing synaptosomes: comparison with alternative procedures. Brain Res 1981;226:107-118.
11. Yalçın AS, Haklar G, Küçükkaya B, Yüksel M, Dalaman G. Chemiluminescence measurements for the detection of free radical species. In: Özben T, ed. Free Radicals, Oxidative Stress, and Antioxidants. New York: Plenum Press, 1998:189-193.
12. Nicholls DG. The glutamatergic nerve terminal. Eur J Biochem 1993;212:613-631.
13. Lafon-Cazal M, Pietri S, Culcasi M, Bockaert J. NMDA-dependent superoxide production and neurotoxicity. Nature 1993;364:535-537.
14. Patel M, Day BJ, Grapo JD, Fridovich I, Mc Namara JO. Requirement for superoxide in excitotoxic cell death. Neuron 1996;16:345-355.
15. Tang LH. Aizenman A. Long-lasting modification of N-methyl-D-aspartate receptor channel by a voltage dependent sulfhydryl redox process. Molec Pharmacol 1993;44:473-478.
16. Sucher NJ, Lipton SA. Redox modulatory site of the NMDA receptor channel complex: regulation by oxidized glutathione. J Neuroscience Res 1991;30:582-591.
17. Varga V, Jenei Zs, Janaky R, Saransaari P, Oja SS. Glutathione is an endogenous ligand of rat brain N-methyl-D-aspartate (NMDA) and 2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors. Neurochem Res 1997;22:1165-1171.