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Oxidative stress induced by glutamate receptor agonists

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The effect of various selective glutamate agonists upon the rate of generation of reactive oxygen species (ROS), was examined in an isolated synaptoneurosomal (microsac) fraction derived from rat cerebral cortex. The rates of ROS generation were determined by a fluorescent probe. Agonists specific for each of the three major glutamate inotropic receptor sites (NMDA, kainic acid, α-amino-3-hydroxy-5-methyl-4-isoxalolpropionic acid, AMPA), were able to enhance rates of ROS generation. The metabotropic glutamate agonist *trans*-1-aminocyclopentane-1,3-dicarboxylic acid, (ACPD), was inactive in this regard. Stimulation of ROS was most pronounced in the case of kainate. Such results could not be replicated by use of ion-channel active agents, veratridine and A23817. Pretreatment with the kainate antagonist, 6-cyano-7-quinoxaline-2,3-dione (CNQX), was not able to block the kainate-induced elvation of ROS. Domoic acid, a kainate agonist, also enhanced microsac ROS generation. Neurological damage may result from generation of excess free radicals, and this may be effected by glutamate agonists acting by means independent of their ionotropic properties.

INTRODUCTION

Excess neuronal activity is known to have the potential to effect neuronal death, especially in the hippocampus. In addition to the frank neuronal hyperactivity apparent in epilepsy, several other neurological disorders are associated with hyperexcitatory events within the brain.

Various mechanisms have been proposed as forming the basis of such induced damage. Excessive utilization of metabolic processes without timely replenishment of cellular energy reserves, can lead to depletion of ATP and disruption of anabolic processes. Such a deficit can result in partial collapse of ion gradients. The consequent increase in cytosolic levels of calcium and depletion of potassium can exacerbate initiating events, by elevation of rates of neuronal firing. In addition, lowered levels of substrates may retard mitochondrial oxidative phosphorylation and also impair the efficiency of electron transfer. Intracellular calcium overload can set off a cascade of other events such as phospholipase activation, potentially leading to elevation of free radical production[20,34]. Phospholipase A_2-generated oxidative activity has been shown to inhibit functioning of the γ-aminobutyrate receptor gated channel, the major inhibitory receptor complex of the cell[38].

Recently, evidence suggesting a role for free radicals in excitotoxic events has been accumulating. Free radicals may play a role in seizure-related brain damage[22,31], and superoxide anion is generated within the brain during seizure activity[3]. Attenuation of convulsive activity by phenytoin or corticosteroids reduces cerebral levels of lipid peroxidation[46]. There is also evidence of a more causal relation between the generation of ROS and excitotoxic damage. Free radical generation during cerebral ischemia may underlie delayed neuronal death[25,42]. Ischemic injury and edema within the CNS have frequently been found to involve excessive oxidative activity as judged by lipid peroxidation, induction of superoxide dismutase, and the protection afforded by antioxidant chemicals, such as a-tocopherol, iron chelators, or 21-aminosteroids[12,19,23,29,33,44,45,47].

The purpose of this study was to both to examine the generality of the relation between neuronal erxcitation and free radical generation, and to gain information on the mechanisms underlying this relation. This report describes the elevation of rates of generation of reactive oxygen species (ROS) within an isolated sub-

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cellular cerebral fraction (microsacs), in response to several agonists specific for various subclasses of the glutamate receptor.

MATERIALS AND METHODS

Tissue preparation

Adult male CR 1 CD rats (250–300 g) were obtained from Charles River Breeding Laboratories (Wilmington, MA), and were maintained in the animal facility in clear polypropylene cages with water and food provided ad libitum.

Rats were decapitated, the brains rapidly excised and placed on ice, and the whole brain minus the cerebellum and pons-medulla was dissected out. Microsacs, which are morphological elements, composed of presynaptic nerve endings in conjunction with attached postsynaptic cytosolic inclusions, were prepared using the method described by Allan and Harris[2]. Fresh cerebral tissue was homogenized in 20 volumes HEPES buffer, and centrifuged at 900 g for 15 min. The resulting pellet was resuspended in the same volume of buffer and recentrifuged using the same conditions. The final precipitate was then suspended in sufficient buffer to a final protein concentration of 1.7 mg/ml.

Assay for oxygen reactive species formation

Reactive oxygen species were assayed using 2′,7′-dichlorofluorescin diacetate (DCFH-DA), which is de-esterified within cells to the ionized free acid, DCFH. This is trapped within cells and thus accumulated[4]. DCFH is capable of being oxidized to the fluorescrent 2′,7′-dichlorofluorescein by reactive oxygen. The utility of this probe in isolated subcellular cerebral systems has been documented[5]. Microsacs (0.5 ml) were diluted in 9 volumes of HEPES buffer. The diluted fractions (5.0 ml) were then incubated with 5 μM DCFH-DA (added from a stock solution of 1.25 mM in ethanol) at 37°C for 15 min. After this loading with DCFH-DA, the fractions were incubated for a further 60 min together with various glutamate receptor agonists or antagonists. At the beginning and at the end of incubation, fluorescence was monitored on a Farrand Spectrofluorometer, with excitation wavelength at 488 nm (bandwidth 5 nm), and emission wavelength 525 nm (bandwidth 20 nm). The rate of generation was found to be linear over the incubation period[26].

Autofluorescence of fractions was corrected for by the inclusion in each experiment of parallel blanks with no DCFH-DA. The correction for autofluorescence was always less than 11% of the total. Oxygen reactive species formation was quantitated from a DCF standard curve (0.05–1.0 μM) and results were expressed as nmol DCF formed/hour/mgprot.

Materials

2′,7′-Dichlorofluorescin diacetate was purchased from Molecular Probes, Inc. (Eugene, OR), while 2′,7′-dichlorofluorescein (DCF) required for calibration was obtained from Polysciences, Inc. (Warrington, PA). The glutamate-related pharmacological agents were obtained from Tocris Neuramin, Bristol, England. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Statistical analyses

Differences between groups were assessed by one-way Analysis of Variance followed by Fisher's Least Significant Difference Test. The acceptance level of significance was $P < 0.05$ using a two-tailed distribution.

RESULTS

The intensity of ROS generation within cerebrocortical microsacs was greatly increased in the presence of the glutamate agonist, kainic acid in a dose-dependent manner while the effect of NMDA was much smaller (Fig. 1). In order to test the effect of stimulation of the quisqualate site, the more selective agonist α-amino-3-hydroxy-5-methyl-4-isoxalolpropionic acid (AMPA), was utilized. This agent also had a marked capacity to stimulate the generation of ROS by microsacs. Thus agonists for the three major inotropic glutamate binding sites all enhanced rates of ROS generation within microsacs. The shellfish neurotoxin, domoic acid, which predominantly acts as a glutamate agonist at the kainate site[43], was also able to enhance synaptosomal ROS (Fig. 2). Both d- and l-glutamate stimulated ROS, the effect of the d-isomer being the most pronounced (Fig. 2). The metabotropic agonist, *trans*-1-aminocyclopentane-1,3-dicarboxylic acid, *trans*-ACPD[27], however, did not enhance ROS at a concentration of 1 mM (Fig. 2).

Since kainate was a relatively powerful stimulant of ROS, further experiments were focussed on this well-characterized receptor site. A relatively selective antagonist of non-NMDA glutamergic sites, 6-cyano-7-quinoxaline-2,3-dione (CNQX)[21], failed to inhibit the effect of kainate in stimulating ROS production (Fig.

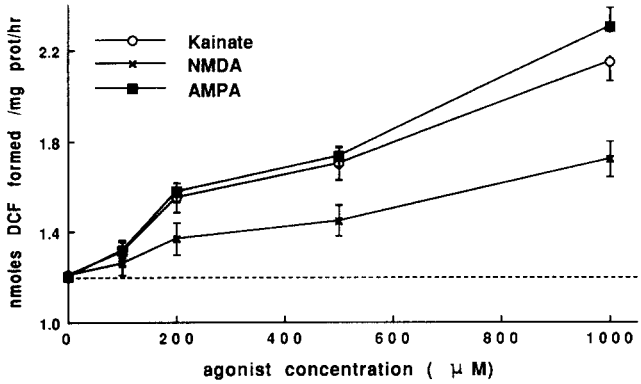

Fig. 1. Stimulation of basal rate of generation of reactive oxygen species in cerebral microsacs, by several glutamate receptor agonists. Each value represents the mean of 3 individual determinations ± S.E.M.

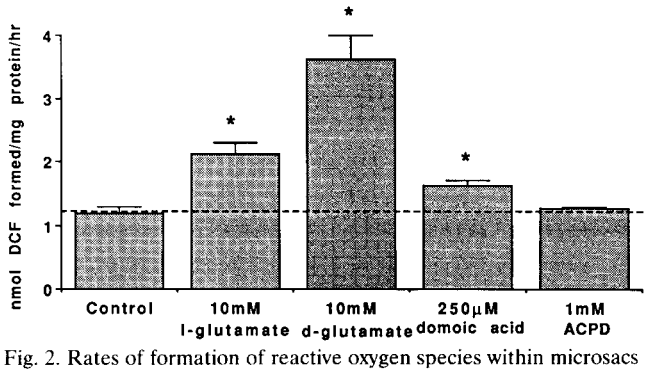

Fig. 2. Rates of formation of reactive oxygen species within microsacs treated with glutamate isomers, domoic acid, or with a metabotropic glutamate receptor agonist (*trans*-ACPD). Each value represents mean of 5–8 experiments derived from separate animals ± S.E.M.
* Differs significantly from corresponding control value ($P < 0.05$).

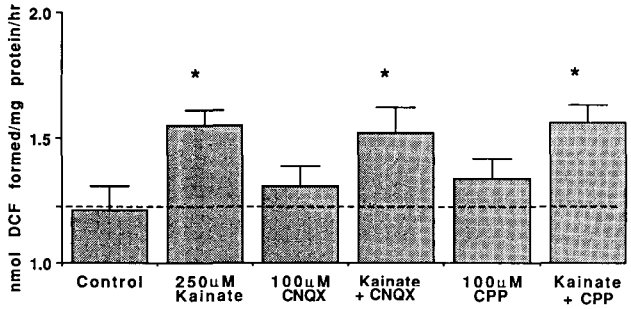

Fig. 3. Kainate-stimulated ROS generation in microsacs: effect of selective glutamate antagonists. Each value represents mean of 5–6 determinations ± S.E.M. * Differs significantly from corresponding control value ($P < 0.05$).

3). The selective antagonist of the NMDA binding site 3-(*RS*-carboxypiperazine-4-yl)-propyl-1-phosphonic acid (CPP)[13], also had no inhibitory effect on kainate-stimulated ROS.

The glutamate-activated site predominantly stimulated by kainate, largely involves facilitation of sodium influx into the cell. In view of this, the effect of a selective agonist of sodium gating, veratridine, was also examined. In this case, no enhancement of ROS was apparent at either of the concentrations of veratridine used (Fig. 4). When calcium entry into the cytosol was enhanced by the presence of the calcium ionophore A23187, no change in the rate of ROS formation was apparent (Fig. 4). The ROS-enhancing potency of kainate was not diminished when a sodium-free incubation buffer was employed, substituting choline chloride for NaCl (Fig. 4).

There is evidence suggesting that nitric oxide mediates the neurotoxicity of glutamate, Kainate-induced oxidative activity was assayed in the presence of 1 mM nitroarginine, an inhibitor of NO synthetase. No inhibition of ROS was observed (data not shown), suggesting that NO does not mediate the kainate elicited enhancement of ROS reported here.

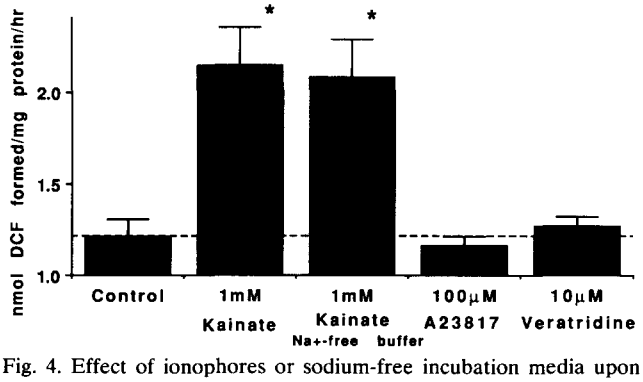

Fig. 4. Effect of ionophores or sodium-free incubation media upon ROS generation in microsacs. Each value represents mean of 6 determinations ± S.E.M. * Differs significantly from corresponding control value ($P < 0.05$).

DISCUSSION

Although this crude synaptoneurosome fraction has many presynaptic elements, cytosolic postsynaptic inclusions are also present, and these may constitute the major sites of glutamate receptors. However, there also is evidence that kainate receptors can have a significant presynaptic location[16].

All ionotropic glutamate agonists studied were able to elevate rates of synaptosomal ROS generation. Two agonists of the kainate site, kainic acid and domoic acid, and an agonist of the quisqualate site (AMPA) were more active in this regard than was NMDA. When administered to mice, domoic acid has been found to elevate cerebral levels of superoxide dismutase[6], which also suggests an ability to promote oxidative stress. The ability of domoic acid to mimic the ROS-inducing effects of kainate, suggests that the stimulation of ROS caused by kainate and domoate, is by way of their action at the kainate subclass of the glutamate receptor, rather than at some other undefined site. However, the failure of CNQX to block the stimulation of ROS caused by kainate, implies that some additional unknown kainate-responsive mechanism may exist.

Several reports indicate that there may be a direct relation between activation of various classes of glutamate receptor and free radical formation. Glutamate toxicity in a neuronal cell line has been attributed to inhibition of cystine transport and consequent oxidative stress[30]. Activation of the N-methyl-d-aspartate (NMDA) receptor site has been implicated in the postischemic elevation of lipid peroxidation in the hippocampus[18]. There is a report of exacerbation of NMDA toxicity in glutathione-deficient cortical cultures[8]. Calcium stimulation of phospholipase A_2 and thence the arachidonic acid cascade, followed by the consequent activation of ROS by lipoxygenases, may be a means by which excitatory events promote excess generation of ROS[36]. NMDA agonists are especially potent in the stimulation of nitric oxide synthetase[24], and nitric oxide can interact with superoxide to form the intensely oxidant nitroperoxyl radical. Transgenic mice overexpressing human copper-zinc superoxide dismutase have been of great value in demonstrating a relation between glutamate toxicity and reactive oxygen species. Glutamate-induced swelling and death are significantly reduced in cultures of primary cortical neurons of these mice, relative to normal non-transgenic littermates[11]. A recent report has found kainic acid administered in vivo, to elevate rates of lipid peroxidation in both cerebellum and hippocampus[41].

While there is a correlation between excitotoxicity

and free radical generation, an unequivocal causal relation remains to be uncovered. Transient ischemia elevates cerebral levels of both excitatory amino acids and rates of hydroxyl radical formation[15]. Whether one of these events gives rise to the second is not well established. Excitatory events may stimulate ROS, but there is also evidence that ROS can lead to release of excitatory amino acids[35]. Glutamine synthetase appears to be especially sensitive to ROS-induced damage, and this may increase glutamate concentrations[39]. The ensuing combination of oxidative and excitatory stress has been proposed to underlie some of the behavioral deficits associated with aging[10]. A bidirectional cooperation between excess neuronal activity and free radicals may therefore be relatively common[32], but the functional value of such a potentially synergistic, deleterious association is unclear.

The kainate-activated glutamate receptor is predominantly involved in gating of sodium channels, rather than the calcium channel regulatory role played by the NMDA binding site. Thus the mechanism by which ROS production is elevated by kainate is unlikely to be solely by way of elevation of cytosolic calcium although calcium has frequently been invoked as an inducer of ROS. For example, elevated levels of calcium can enhance free radical-induced damage to cerebral morphological fractions[7], and antioxidants can protect against cytotoxicity induced by high intracellular levels of calcium in the CNS[1]. Conversely, elevation of lipid peroxidation asscociated with ischemia can be prevented by administration of calcium antagonists such as verapamil[44]. Reduction of cytosolic levels of ionic calcium has been reported to reduce damage to DNA effected by hydrogen peroxide[9].

However, the relation between calcium and free radicals seems complex in that extracellular calcium may be an important defence against lipid peroxidation[17,44].

There is evidence implicating excessive levels of intracellular sodium as effectors of prooxidant status[37,40]. However, since application of veratridine did not elevate ROS generation in the microsac fraction, influx of sodium ions through the channel gated by the inotropic kainate receptor probably cannot by itself account for the kainate-induced increase in ROS reported here.

The failure of activation of sodium or calcium channels by selective pharmacological agents acting separately or in combination, to act in a manner resembling glutamate agonists suggests that the effects of kainate may not be solely explicable in terms of facilitation of ion fluxes. There may be a general property of the so-called inotropic glutamatergic agents to act by additional means than modulation of ion-specific cation channels at the cell surface. For example, the possibility exists that kainate enters the cell and exerts effects directly on cytosolic loci, or that "inotropic" agonists may activate metabotropic receptors. Such additional properties would account for the finding that NMDA toxicity cannot be prevented by maintenance of low concentrations of cytosolic calcium[28].

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