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Effects of pyrroloquinoline quinone on glutamate-induced production of reactive oxygen species in neurons

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Abstract

Pyrroloquinoline quinone may act as a free radical scavenger and also as a modulator of the NMDA receptor associated redox modulatory site. Using the oxidation sensitive dye dihydroethidium, we examined the effects of pyrroloquinoline quinone on free radical production in cultured forebrain neurons following glutamate receptor activation. Both glutamate (100 μM) and hydrogen peroxide (30 mM) produced a rapid increase in dihydroethidium fluorescence indicating dye oxidation. Pyrroloquinoline quinone (5–200 μM) effectively inhibited dihydroethidium fluorescence induced by glutamate but not by hydrogen peroxide. Glutamate-induced dihydroethidium fluorescence was inhibited by the thiol oxidant 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB). Pyrroloquinoline quinone (50 μM) inhibited glutamate responses in control and in dithiothreitol treated neurons. However, pyrroloquinoline quinone did not further decrease the response to glutamate in DTNB treated neurons. These results suggest that pyrroloquinoline quinone inhibits the free radical-generating response to glutamate by oxidizing the NMDA receptor redox site and not by scavenging reactive oxygen species.

Keywords: Free radical; Glutamate; Pyrroloquinoline quinone; NMDA receptor; Redox modulatory site; Dihydroethidium

1. Introduction

Trauma and stroke, as well as other neurodegenerative diseases, are associated with a massive release of glutamate into the extracellular medium (Benveniste et al., 1984; Liu et al., 1991). Glutamate has been demonstrated to be neurotoxic both in vivo and in vitro and this appears to be dependent on ionotropic glutamate receptor activation (Rothman and Olney, 1987; Choi et al., 1988). Elevated levels of reactive oxygen species in the brain have also been associated with neuronal damage following trauma, stroke, ischemic/reperfusion injury and other neurodegenerative diseases (Choi, 1990; Halliwell, 1992; Hall and Braughler, 1989; Braughler and Hall, 1989). Following NMDA receptor activation in vitro, an increase in reactive oxygen species has been observed (Bindokas et al., 1996; Dugan et al., 1995; Reynolds and Hastings, 1995; Lafon-Cazal et al., 1993). In addition, free radicals can enhance glutamate release (Pellegrini-Gampietro et al., 1990), perhaps by inhibiting glutamate uptake (Volterra et al., 1994), and antioxidants can partially protect against glutamate injury (Chan et al., 1990; Monyer et al., 1990). Thus, one of the key events in glutamate neurotoxicity may be the production of reactive oxygen species. However, it is still uncertain how or if glutamate and free radicals interact to produce neuronal damage.

The methods presently available to study this complex interaction between glutamate excitotoxicity and free radical production are limited. Oxidation sensitive dyes can detect, with the use of confocal microscopy, the production of reactive oxygen species at an intracellular level. Among these oxidative sensitive dyes, 2′,7′-dichlorofluorescin (Reynolds and Hastings, 1995; LeBel et al., 1992), dihydrorhodamine (Dugan et al., 1995) and dihydroethidium (Bindokas et al., 1996) are currently used to evaluate oxidant stress in cells. Dichlorofluorescin is believed to be selectively oxidized by hydroperoxides, but may also include lipid peroxides and hydroxyl radicals (Cathcart et al., 1983; Bass et al., 1983). Dihydrorhodamine has been reported to be a selective indicator for peroxynitrite (Kooy et al., 1994) but also appears to be sensitive to hydrogen peroxide (Dugan et al., 1995). This study employed the use of dihydroethidium to measure free radical production in

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cultured forebrain neurons. Dihydroethidium is a freely permeable, blue fluorescent dye that when oxidized forms ethidium which intercalates with DNA and becomes trapped inside the cell (Bucana et al., 1986). Dihydroethidium is selectively oxidized by superoxide (Rothe and Valet, 1990; Bucana et al., 1986) and thus would detect free radical production at a step prior to that detectable by the other known oxidation sensitive dyes. This is an important advantage for using dihydroethidium in studying the sequence of events surrounding neuronal death since it is not clearly understood whether the production of reactive oxygen species contributes to the early or late stages in this process.

Pyrroloquinoline quinone is a water soluble redox cofactor capable of efficient and reversible transfer of electrons between reductants and oxidants (Gallop et al., 1989). Pyrroloquinoline quinone is believed to be an essential nutrient since studies have shown that animals given pyrroloquinoline quinone deficient diets display a variety of illnesses (Killgore et al., 1989). In addition, this compound has been found in a variety of animal cells and cell products such as milk, serum and synovial fluid (Gallop et al., 1993). Studies, both in vivo and in vitro, have shown that pyrroloquinoline quinone can protect against several types of oxidative damage and toxic injury (Bishop et al., 1994; Smidt et al., 1991) including excitotoxicity and stroke damage (Jensen et al., 1994; Aizenman et al., 1992), as well as inhibit free radical production (Hamagishi et al., 1990).

The main purpose of the present study was to test the hypothesis that pyrroloquinoline quinone can act as a free radical scavenger. As pyrroloquinoline quinone can oxidize the redox modulatory site of the NMDA receptor (Aizenman et al., 1994), it is possible that the neuroprotective effects produced by pyrroloquinoline quinone could arise from either free radical scavenging or redox modulation, or both. We examined the effects of pyrroloquinoline quinone on the production of reactive oxygen species following an excitotoxic glutamate stimulus as well as following the addition of an exogenous free radical, hydrogen peroxide.

2. Materials and methods

2.1. Cell culture

Primary cultures of forebrain neurons from embryonic rats were used throughout this study. Forebrains were obtained from embryonic day 17 Sprague-Dawley rats and dissociated according to White and Reynolds (1995). Briefly, tissue was incubated in 0.005–0.01% trypsin in Ca^{2+}-free, Mg^{2+}-free media (in mM: 116 NaCl, 5.4 KCl, 26.2 $NaHCO_3$, 11.7 NaH_2PO_4, 5 glucose, 0.001% Phenol Red and minimum essential medium amino acids; pH adjusted to 7.4 with NaOH) for 30 min at 37°C. Viability determinations were made with the trypan blue (0.08%) exclusion method. The plating suspension was diluted to 300 000 cell/ml using plating medium (v/v solution of 90% Dulbecco's modified Eagle's medium, 10% heat-inactivated fetal bovine serum, 24 U/ml penicillin, 24 μg/ml streptomycin; final glutamine concentration 3.1 mM). Cells were plated onto poly-L-lysine-coated (40 μg/ml) 35 mm glass coverslips which were then inverted 1 day later in a maintenance medium (horse serum substituted for fetal calf serum, all other constituents identical). Inversion of the coverslips prevents glial proliferation. Cells were maintained under 95% air/5% CO_2 until utilization 14 or 21 days later. Only those coverslips containing healthy neurons (rounded-oval, smooth and bright cell bodies under phase contrast microscopy) were used.

2.2. Fluorescence microscopy

Production of reactive oxygen species was measured in forebrain neurons using the oxidation sensitive dye, dihydroethidium (Bindokas et al., 1996). Dihydroethidium fluorescence was recorded using a Meridian ACAS 570c laser scanning confocal imaging system (Meridian Instruments, Okemos, MI, USA). Cells were illuminated with the 488 nm line of an argon laser producing 200 mW of light that was attenuated by passage through a 7% neutral density filter and to a further 8% by the acousto-optical modulator to yield a total attenuation of 0.56% of the starting intensity. Emitted light was collected by a 40× phase contrast objective and an 80 μm pinhole resulting in an optical slice of about 2.5 μm through the middle of the neurons, which average around 20 μm in diameter.

On the day of experimentation, culture medium was removed and replaced with HEPES-buffered salt solution (HBSS) of the following composition (mM): 137 NaCl, 5 KCl, 0.9 $MgSO_4$, 1.4 $CaCl_2$, 3 $NaHCO_3$, 0.6 Na_2HPO_4, 0.4 KH_2PO_4, 5.6 glucose and 20 HEPES; adjusted to pH 7.4 with NaOH. Coverslips were then inverted (cell layer facing upwards) and incubated for 15 min at 37°C with dihydroethidium (10 μM) diluted in HBSS supplemented with 5 mg/ml bovine serum albumin. A stock solution of dihydroethidium (4 mM) in anhydrous dimethyl sulfoxide (DMSO) was prepared fresh daily and stored under nitrogen. The coverslips were mounted on the stage of an ACAS 570c imaging system and fluorescence was recorded at room temperature from a single field of cells (180 × 180 μm) per coverslip typically containing 5–15 neurons. Dihydroethidium (10 μM) was included in HBSS throughout the experiment. After acquiring two images (one scan per minute) of baseline fluorescence, dihydroethidium fluorescence is recorded over an additional 10 images following the addition of 100 μM glutamate plus 1 μM glycine (using 21-day-old cells) or 30 mM hydrogen peroxide (using 14-day-old cells). Pyrroloquinoline quinone (5–200 μM), dithiothreitol (5 mM) and 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB, 100 μM) were examined for their

effects on free radical production. Dithiothreitol and DTNB were dissolved in HBSS. Pyrroloquinoline quinone was initially dissolved in DMSO and then diluted with water to generate a stock concentration of 5 mM. The DMSO concentration in this stock was 15.5% (v/v), and the highest concentration of DMSO applied to neurons was 0.6% (with 200 μM pyrroloquinoline quinone). Dithiothreitol and DTNB were preincubated with the cells for approximately 10 min prior to but not during the addition of glutamate. For all experiments using pyrroloquinoline quinone, cells were exposed to pyrroloquinoline quinone for approximately 10 min prior to and during addition of glutamate or hydrogen peroxide. All agents were added by exchanging the chamber solution.

2.2.1. Data analysis and statistics

Fluorescence was normalized to the intensity measured in the first scan. Data were normalized to account for problems in equal dye loading. Data were presented for each test condition as the change in normalized fluorescence (mean ± S.E.M.) over time (minutes). All experiments were performed on at least three coverslip from no less than two different culture dates. Statistical analysis was performed using analysis of variance followed by a Bonferroni post-hoc analysis.

3. Results

3.1. Glutamate- or hydrogen-peroxide-induced dye oxidation

Glutamate (100 μM) and hydrogen peroxide (30 mM) increased the fluorescence of dihydroethidium indicating dye oxidation (Fig. 1). Glutamate produced a more robust response (typically a 5 fold increase from baseline) than hydrogen peroxide (3 fold increase from baseline). Dihydroethidium is reported to be more sensitive to superoxide than to other reactive oxygen species. However, our results indicate that dihydroethidium may also be sensitive to

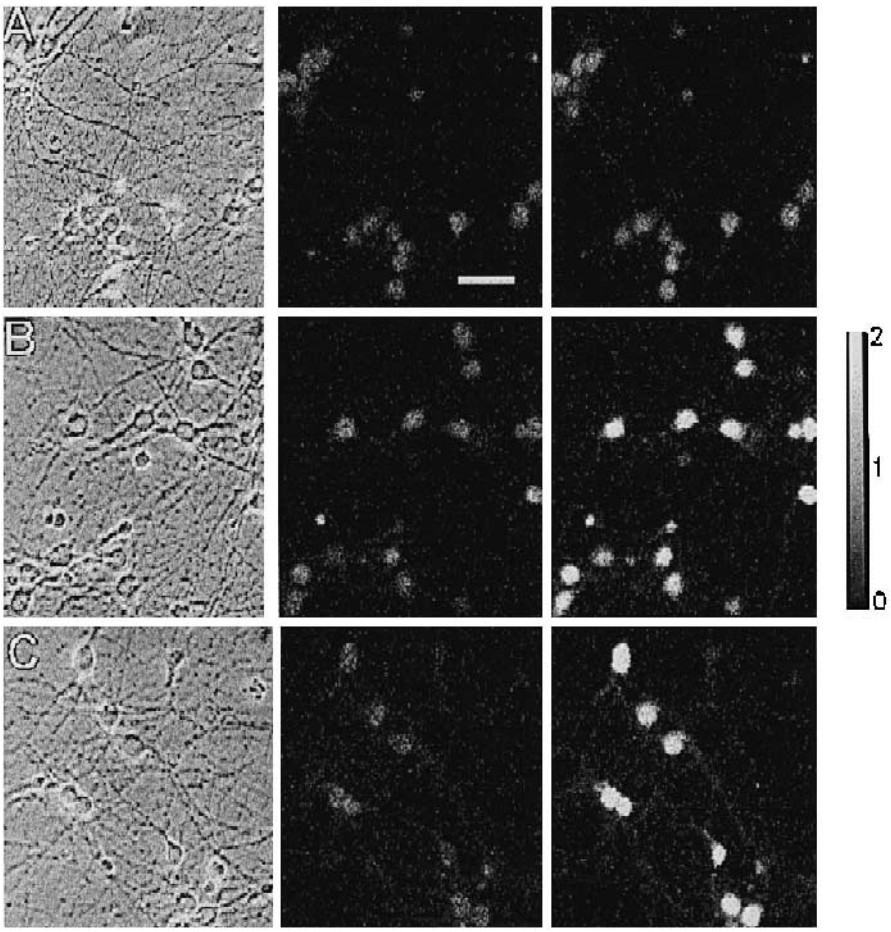

Fig. 1. Oxidant-induced increase of dihydroethidium fluorescence. The images represent fields of neurons treated with HBSS (A), 30 mM hydrogen peroxide (B), or 100 μM glutamate with 1 μM glycine (C). Two images of baseline fluorescence were taken prior to the addition of HBSS, hydrogen peroxide, or glutamate and measurements were taken for an additional 10 min at one scan every minute. The left panel shows a phase contrast image of the cortical neuronal field, the middle panel shows fluorescence at time zero and the right panel shows fluorescence following a 10-min exposure to HBSS, hydrogen peroxide, or glutamate. The grey scale represents arbitrary fluorescence units. The scale bar represents 40 μm.

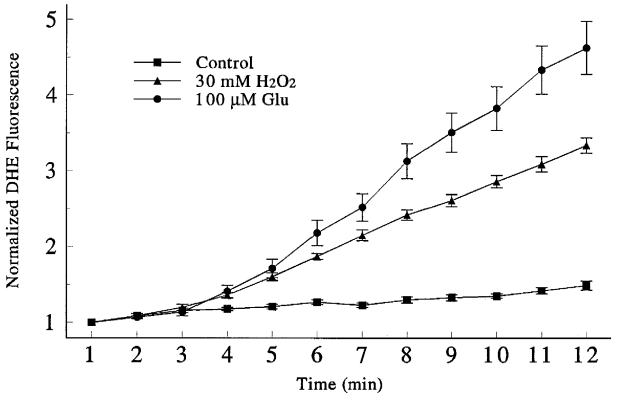

Fig. 2. Effect of oxidants on dihydroethidium fluorescence. Data of the type shown in Fig. 1 were quantitated and normalized to fold change from the fluorescence intensity at the first scan. The curves show the time course of the fluorescence increase during a typical experiment. Each line represents the mean ± S.E.M. from a field of 10–15 neurons from a single coverslip.

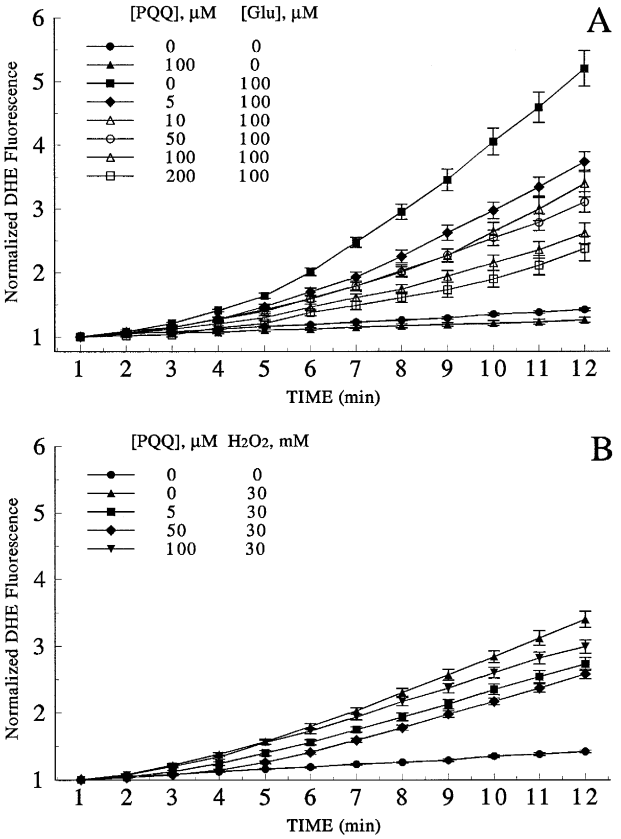

Fig. 3. Effects of pyrroloquinoline quinone on glutamate- or hydrogen-peroxide-induced dihydroethidium fluorescence. Primary cultures of rat forebrain neurons were utilized at day 21 or day 14 in vitro for glutamate (A) or hydrogen peroxide (B) exposure, respectively. Dihydroethidium was present in the buffer for the duration of the experiment. Pyrroloquinoline quinone (5–200 μM) was added approximately 5 min prior to and during glutamate or hydrogen peroxide exposure. (A) Glutamate (100 μM with 1 μM glycine) or (B) hydrogen peroxide (30 mM) was added after obtaining 2 min of basal fluorescence. Dihydroethidium fluorescence was normalized to fluorescence intensity at the start of the experiment. Points represent the mean ± S.E.M. from 20–150 neurons obtained from 2–14 coverslips from no less than two culture dates.

oxidation by exogenously applied hydrogen peroxide. Both glutamate and hydrogen peroxide produced dye fluorescence that was evenly distributed throughout the cell body (Fig. 1). Dihydroethidium did not oxidize over the course of the experiment in the absence of added oxidizing stimuli (Fig. 2).

3.2. Effects of pyrroloquinoline quinone on free radical scavenging

Pyrroloquinoline quinone (5–200 μM) inhibited the glutamate-induced dihydroethidium fluorescence in a concentration-dependent manner (Fig. 3A). However, the effects of pyrroloquinoline quinone on hydrogen-peroxide-induced dihydroethidium fluorescence was minimal and was not concentration-dependent (Fig. 3B). Incubation of the neurons with pyrroloquinoline quinone (50–100 μM) alone did not produce dye oxidation when compared to controls (Fig. 3). These results would therefore suggest that pyrroloquinoline quinone alone does not lead to the production of superoxide in our model system.

3.3. Modulation of glutamate responses by dithiothreitol or DTNB

Various thiol reductants and oxidants are known to affect glutamate responses by altering the redox state of NMDA receptors. Two redox agents (dithiothreitol and DTNB) were examined for their effect on glutamate-induced free radical production. Either dithiothreitol or DTNB was incubated for approximately 10 min prior to

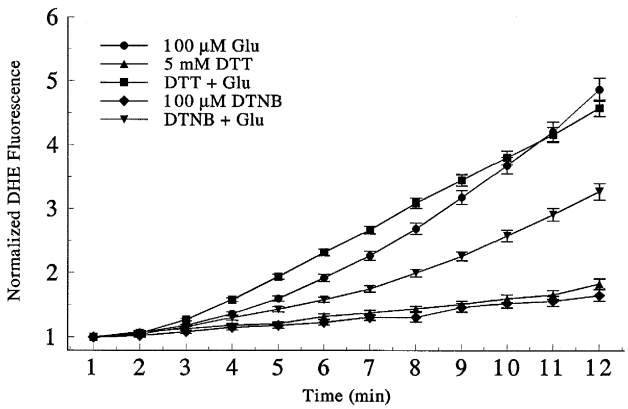

Fig. 4. Effects of redox agents on glutamate-induced dihydroethidium fluorescence. Primary cultures of rat forebrain neurons were utilized at day 21 in vitro. Dihydroethidium was present in the buffer for the duration of the experiment. Immediately following loading with dihydroethidium cells were exposed to either 5 mM dithiothreitol or 100 μM DTNB for approximately 10 min prior to the addition of 100 μM glutamate/1 μM glycine or HBSS as a control. Dithiothreitol and DTNB were both removed from the extracellular medium during glutamate exposure. Dihydroethidium fluorescence was normalized to fluorescence intensity at the start of the experiment. Points represent the mean ± S.E.M. from 20–150 neurons obtained from 2–14 coverslips from no less than two culture dates.

but not during glutamate exposure. The thiol reductant dithiothreitol (5 mM) produced a very modest enhancement of glutamate responses while the thiol oxidant DTNB (100 μM) partially inhibited glutamate responses (Fig. 4). However, the response produced by glutamate in dithiothreitol-pretreated neurons was not significantly different from control responses with glutamate ($P > 0.05$, analysis of variance). Previous studies have shown that after potentiating an NMDA-evoked response with dithiothreitol, the NMDA receptor was noted to undergo spontaneous oxidation (Sinor et al., 1997; Tang and Aizenman, 1993), and presumably sufficient time had elapsed for spontaneous oxidation to occur in our treatment paradigm. The slight increase in fluorescence produced by the addition of dithiothreitol alone may be due to an enhancement of receptor activation by endogenous glutamate.

3.4. Modulation of the NMDA redox site by pyrroloquinoline quinone

The effects of pyrroloquinoline quinone on glutamate evoked responses following exposure to dithiothreitol and DTNB were also examined. Neurons were exposed to pyrroloquinoline quinone and either dithiothreitol or DTNB for approximately 10 min prior to the addition of glutamate. During glutamate exposure either redox agent was removed from the cells while pyrroloquinoline quinone was still present. Pyrroloquinoline quinone (50 μM) significantly ($P < 0.001$) decreased the glutamate-induced dihydroethidium oxidation in dithiothreitol-treated cells (Fig. 5). One would expect that if DTNB and pyrroloquinoline quinone were inhibiting glutamate-induced production of reactive oxygen species by different mechanisms, then the addition of pyrroloquinoline quinone and DTNB together would have an additive effect. However, pyrroloquinoline quinone (50 μM), DTNB (100 μM) and pyrroloquinoline quinone plus DTNB were all equally efficient in inhibiting glutamate-induced free radical production (Fig. 6).

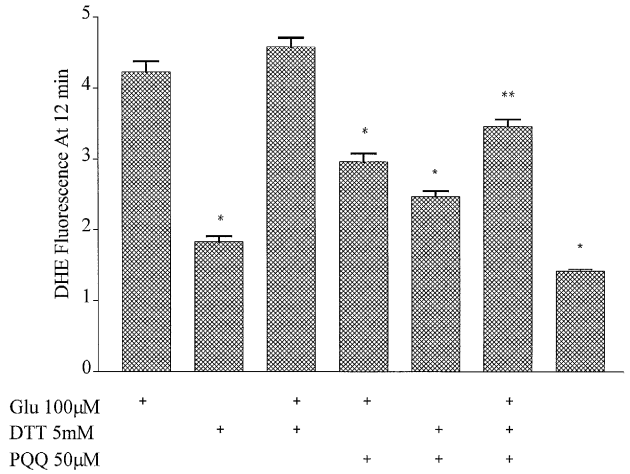

Fig. 5. Effects of pyrroloquinoline quinone on the potentiation of glutamate responses by dithiothreitol. Each bar represents the mean ± S.E.M. of dihydroethidium fluorescence at 12 min normalized to the intensity of the first scan. Neurons were exposed to 5 mM dithiothreitol or dithiothreitol plus 50 μM pyrroloquinoline quinone for approximately 10 min prior to the addition of 100 μM glutamate/1 μM glycine. Pyrroloquinoline quinone but not dithiothreitol remained in solution during glutamate exposure. Two images of baseline fluorescence were taken prior to the addition of HBSS (control) or glutamate and measurements were continued an additional 10 min at one scan per minute. Dihydroethidium fluorescence was normalized to baseline fluorescence. * Significantly different from the response to glutamate alone, * * significantly different from glutamate with dithiothreitol, $P < 0.001$, one-way analysis of variance with Bonferroni correction for multiple comparisons.

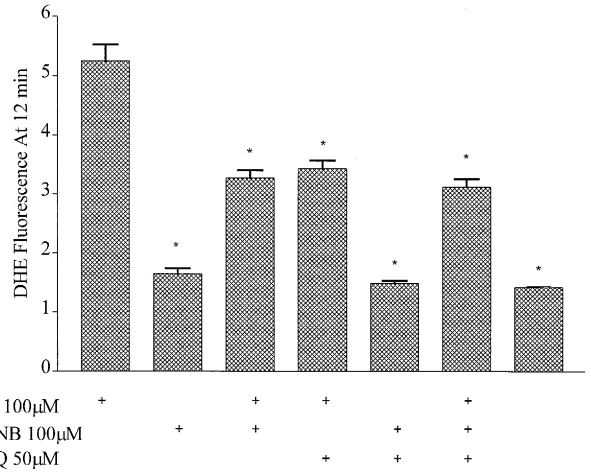

Fig. 6. Effects of pyrroloquinoline quinone on the inhibition of glutamate responses by DTNB. Each bar represents the mean ± S.E.M. of dihydroethidium fluorescence at 12 min normalized to the intensity of the first scan. Neurons were exposed to 100 μM DTNB or DTNB plus 50 μM pyrroloquinoline quinone for approximately 10 min prior to the addition of 100 μM glutamate/1 μM glycine. Pyrroloquinoline quinone but not DTNB remained in solution during glutamate exposure. Two images of baseline fluorescence were taken prior to the addition of HBSS (control) or glutamate and measurements were continued an additional 10 min at one scan per minute. Dihydroethidium fluorescence was normalized to baseline fluorescence. By the end of the 12-min scanning period DTNB, pyrroloquinoline quinone, or DTNB + pyrroloquinoline quinone each significantly ($P < 0.001$) inhibited glutamate-evoked responses. However, these effects were not significantly different from each other. * Significantly different from the response to glutamate alone, $P < 0.001$, one-way analysis of variance with Bonferroni correction for multiple comparisons.

4. Discussion

The NMDA redox modulatory site is sensitive to sulfhydryl redox agents. Disulfide reducing agents will potentiate an NMDA response whereas sulfhydryl oxidizing agents have the opposite effect (Aizenman et al., 1989). These agents can also influence several NMDA receptor mediated events including delayed cell death (Levy et al., 1990; Aizenman et al., 1990), epileptiform activity in hippocampal slices (Tolliver and Pellmar, 1987) and long-term potentiation (Tauck and Ashbeck, 1990). It has also been shown in vivo that tissue redox systems are

reduced during ischemia (Auer and Siesjö, 1988). Our results show that while dithiothreitol, a disulfide reducing agent, did not significantly enhance glutamate-induced dihydroethidium fluorescence, DTNB, a sulfhydryl oxidizing agent, inhibited glutamate responses (Fig. 4). These results suggest that the glutamate receptors found in our neuronal cell cultures are in a heterogeneous redox state and are not entirely oxidized nor reduced. This supports previous results that the redox state of native receptors varies among neurons (Tang and Aizenman, 1993; Aizenman et al., 1989; Sinor et al., 1997). The failure of dithiothreitol to produce a significant increase in the glutamate response is most likely due to the pretreatment paradigm employed in these studies, which allows ample opportunity for spontaneous re-oxidation to occur.

Since we found that pyrroloquinoline quinone could partially inhibit glutamate responses in untreated and dithiothreitol treated neurons, our data would support previous observations that pyrroloquinoline quinone can regulate NMDA receptor activity by directly interacting with the redox site of the receptor (Aizenman et al., 1992, 1994). Pyrroloquinoline quinone in the presence of a reducing agent may lead to the spontaneous production of superoxide (Aizenman et al., 1992), and this may account for the slightly larger dihydroethidium response in the combined presence of dithiothreitol and pyrroloquinoline quinone compared to dithiothreitol or pyrroloquinoline quinone alone (Figs. 3 and 5). However, pyrroloquinoline quinone significantly prevented the effects of dithiothreitol on the dihydroethidium response to glutamate even in the presence of this glutamate-independent signal (Fig. 5). Pyrroloquinoline quinone can oxidize sulfhydryl groups in several proteins (Park and Churchich, 1992) including the NMDA redox modulatory site in rats (Jensen et al., 1994; Aizenman et al., 1992). Our results strongly suggest that pyrroloquinoline quinone was not acting as a free radical scavenger in the present paradigm which is contradictory to previous reports that pyrroloquinoline quinone can inhibit free radical production both in vivo and in vitro (Hamagishi et al., 1990). Thus, the oxidation of the NMDA redox modulatory site via pyrroloquinoline quinone may be responsible for the neuroprotective effects of pyrroloquinoline quinone seen both in vitro and in vivo (Jensen et al., 1994; Aizenman et al., 1992). These results give additional strength to the idea that pyrroloquinoline quinone and other quinone-containing proteins can offer therapeutic protection against NMDA receptor-mediated neurotoxicity, by a unique mechanism, namely a direct interaction with the receptor.

One of the potential biochemical roles of pyrroloquinoline quinone is to nonenzymatically oxidize superoxide and reduce NAD(P) and by doing so, in pharmacologic doses, protect against reduced glutathione depletion (Fluckiger, 1993). For example, pyrroloquinoline quinone has been shown to protect against oxidative damage induced by hydrocortisone, a compound which decreases the levels of reduced glutathione (Nishigori et al., 1989). Since reduced glutathione is needed for the removal of hydrogen peroxide, pyrroloquinoline quinone could protect against glutamate-induced neuronal injury by inhibiting the depletion of reduced glutathione and thus indirectly act as an antioxidant. However, our results would imply that pyrroloquinoline quinone is not acting as a free radical scavenger indirectly by increasing reduced glutathione levels because if this were true then one would expect pyrroloquinoline quinone to have a greater effect on inhibiting production of reactive oxygen species by hydrogen peroxide (Fig. 3). The reduced and semiquinone forms of free pyrroloquinoline quinone are known to react with oxygen to form superoxide (Fluckiger, 1993; Davidson et al., 1992; Duine et al., 1987). For this reason there has been concern that in vivo this process must be under careful regulation and may be another possible target for drugs designed to reduce excessive superoxide production (Gallop et al., 1989). However, pyrroloquinoline quinone by itself did not oxidize dihydroethidium (Fig. 3), suggesting that in this model system pyrroloquinoline quinone alone does not produce superoxide nor is it capable of oxidizing dihydroethidium directly. It is possible that in vivo the redox milieu is different from the artificial conditions extant in our cell culture model, and that the in vivo state would result in a different predominant effect of pyrroloquinoline quinone. For example, if additional reductants were present it is possible that pyrroloquinoline quinone might more effectively generate superoxide (although note that superoxide does not appear to be the critical oxidant of the NMDA receptor; Aizenman, 1995). However, as we are currently unable to perform such assays in vivo we cannot perform a direct comparison to address this point.

Frequently, colorimetric and luminescent assays are used to detect cellular production of reactive oxygen species. However, these methods are limited due to the requirement that the reactive oxygen species either exit cells or are produced extracellularly for detection. This would particularly limit the detection of superoxide which passes poorly across cellular membranes. The advantage of using oxidative sensitive dyes is that one can detect, with the use of confocal microscopy, the production of reactive oxygen species at an intracellular level. In this study we have demonstrated that the production of reactive oxygen species in cortical neurons following glutamate receptor activation can be decreased by the endogenous oxidant cofactor pyrroloquinoline quinone. Dihydroethidium has been reported to be a selective indicator of superoxide. Superoxide can then be converted to hydrogen peroxide spontaneously or enzymatically with superoxide dismutase. Since the production of superoxide occurs prior to that of other reactive oxygen species and since dihydroethidium is believed to be specific to oxidation by superoxide, it is perhaps unexpected that dihydroethidium would be sensitive to oxidation by hydrogen peroxide. However, our

results demonstrate that both glutamate and hydrogen peroxide were capable of increasing dihydroethidium fluorescence (Fig. 1). In addition to the possibility that dihydroethidium may also be sensitive to oxidation by exogenously applied hydrogen peroxide, prior studies have suggested that hydrogen peroxide can secondarily generate superoxide (Carter et al., 1994) and thus can indirectly increase dihydroethidium fluorescence possibly through the inhibition of Cu/Zn superoxide dismutase (Bindokas et al., 1996). Dye oxidation was more robust following glutamate receptor activation than from an exogenous free radical source, hydrogen peroxide (Fig. 2), suggesting that dihydroethidium more effectively detects the reactive oxygen species produced by glutamate than peroxide.

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