Sequential Involvement of Distinct Glutamate Receptors in Domoic Acid-Induced Neurotoxicity in Rat Mixed Cortical Cultures: Effect of Multiple Dose/Duration Paradigms, Chronological Age, and Repeated Exposure

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Received July 15, 2005; accepted September 28, 2005

The increasing occurrence of poisoning accidents in marine animals caused by the amnesic shellfish toxin, domoic acid (DOM), necessitates a better understanding of the factors contributing to DOM neurotoxicity. Here we evaluated the contribution and temporal involvement of NMDA, non-NMDA- and metabotropic-type glutamate receptors (GluRs) in DOM-induced neuronal death using rat primary mixed cortical cultures. Co-application of antagonists for AMPA/kainate- (NBQX) and NMDA-type GluRs (D-AP5) but not for metabotropic GluRs reduced DOM toxicity induced by either of three EC50 dose/duration exposure paradigms. Maximal protection offered by D-AP5 and NBQX either extended or not to the 30- to 60-min period after DOM exposure, respectively. Antagonists were ineffective if applied with a 2-h delay, indicating the presence of a critical time window for neuronal protection after DOM exposure. Early effects correlated with neuronal swelling was seen as early as 10 min post-DOM, which has been linked to non-NMDAR-mediated depolarization and release of endogenous glutamate. That DOM toxicity is dictated by iGluRs is supported by the finding that increased efficacy and potency of DOM with in vitro neuronal maturation are positively correlated with elevated protein levels of iGluR subunits, including NR1, GluR1, GluR2/3, GluR5, and GluR6/7. We determined the time course of DOM excitotoxicity. At >10 μM maximal neuronal death occurs within 2 h, while doses ≤10 μM continue to produce death during the subsequent 22-h washout period, indicating a quicker progression of the neuronal death cascade with high DOM concentrations. Accordingly, NBQX applied 30 min post-DOM afforded better protection against low dose/prolonged duration (3 μM/24 h) than against high dose/brief duration exposure (30 μM/10 min). Interestingly, prior exposure to subthreshold DOM dose-dependently aggravated toxicity produced by a subsequent exposure to DOM. These findings provide greater insight into the complex properties underlying DOM toxicity, including the sequential involvement of multiple GluRs, greater potency with increasing neuronal maturation and protein levels of iGluRs, varying efficacy depending on dose, duration, and prior history of DOM exposure.

Key Words: excitotoxicity; metabotropic glutamate receptors; AMPA receptors; NMDA receptors; development; amnesic shellfish poisoning.

Domoic acid (DOM) is a marine toxin produced by algal species that causes amnesic shellfish poisoning (ASP), a condition characterized by severe memory impairment and gastrointestinal and sensorimotor disturbances (Clayton et al., 1999; Perl et al., 1990; Wright et al., 1990). In severe cases of ASP, neurological symptoms develop within 48 h and may include seizures and even death. In experimental animals DOM has been shown to produce learning/memory deficits (Petrie et al., 1992). When transferred through the food chain, DOM causes accidental poisoning of humans (Perl et al., 1990; Teitelbaum et al., 1990) and marine animals (Gulland et al., 2002; Lefebvre et al., 1999; Scholin et al., 2000). DOM, a structural analogue of the excitatory neurotransmitter glutamate, activates glutamate receptors (Hampson et al., 1992; Hampson and Manalo, 1998; Verdoorn and Dimgledine, 1988) and produces excitatory responses throughout the mammalian central nervous system (Biscoe et al., 1976; Bureau et al., 1990; Stewart et al., 1990; Yool et al., 1992) as well as hippocampal neurodegeneration by binding to 2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)- type (AMPArs) and kainate (KA)-type GluRs (KARs) (Larm et al., 1997; Stewart et al., 1990).

DOM is highly potent at AMPARs and KARs (Doucette et al., 2003; Larm et al., 1997; Stewart et al., 1990), probably because of high-affinity binding (Hampson et al., 1992; Hampson and Manalo, 1998) and nondesensitizing responses at AMPARs (Carcache et al., 2003; Stewart et al., 1990) leading to persistent cellular ionic disturbances, swelling, and neurodegeneration (Attwell et al., 1993). In addition, DOM lacks a high-affinity uptake system (Suzuki and Hierlihy, 1993) and
can cause secondary release of nitric oxide (NO) and endogenous glutamate, further exacerbating neuronal death (Berman and Murray, 1997; Brown and Nijjar, 1995; Chandrasekaran et al., 2004; Dakshinamurti et al., 1993).

Calcium influx evoked by DOM, which is correlated with DOM-induced neuronal death, is sensitive to both NMDAR and non-NMDAR antagonists (Berman et al., 2002; Xi et al., 1997), suggesting involvement of NMDARs and Ca\(^{2+}\)-permeable AMPARs in DOM toxicity (Fischer et al., 2002; Hollmann et al., 1991; Jensen et al., 2001). Previous studies have shown that application of either AMPA/KAR or NMDAR antagonists confer significant protection against DOM (Berman and Murray, 1997; Jensen et al., 1998; Larm et al., 1997; Tasker and Strain, 1998), but it is not clear what the sequential involvement of each receptor subtype is, or whether mGluRs, which can either protect or exacerbate neuronal death (Conn and Pin, 1997; Koh et al., 1991; Strasser et al., 1998), contribute to DOM excitotoxicity. Moreover, it is unclear what the time course of DOM-induced neuronal death is and whether it is dependent on exposure duration and/or concentration.

Other factors such as advancing age have been correlated with increased severity of ASP (Auer, 1991; Chandrasekaran et al., 2004; Perl et al., 1990) and increased DOM-induced dysfunction in experimental animals (Dakshinamurti et al., 1993), while other studies indicate increased susceptibility of neonates to DOM toxicity (Xi et al., 1997). Because whole-animal studies are confounded by age-related changes in physiological parameters affecting DOM pharmacokinetics (Mayer, 2000; Xi et al., 1997), we used a barrierless preparation to compare DOM toxicity at different times in vitro and related differences to developmental changes in iGluR expression. We used cerebral cortical cultures, since they display excitotoxic responses earlier during neuronal maturation than CGCs (Frandsen and Schousboe, 1990). In this study different dose/duration paradigms of DOM treatment were used in order to provide insight into the complex mechanisms underlying DOM neurotoxicity. Our results show critical involvement of iGluRs, but not mGluRs, in DOM-induced neuronal death, especially during the first 2 h after DOM exposure, with AMPA/KARs playing a more prominent role earlier than NMDARs following DOM exposure and higher doses of DOM completing the neuronal death cascade more rapidly. We found a high correlation between DOM potency and subunit-specific iGluR expression during neuronal maturation in vitro. Finally, we explored the effect of prior DOM exposure on the effects of a subsequent exposure and found that toxicity was intensified. In combination, these data indicate a short critical window for neuroprotection by iGluR antagonists if applied following DOM exposure and implicate multiple factors influencing DOM toxicity, including dose, age, and prior history of DOM exposure. A preliminary subset of these data has been published previously (Quí and Curras-Collazo, 2002).

**MATERIALS AND METHODS**

**Mixed cortical cultures.** Animal care and use procedures were conducted in accordance with Guide and Care for the Use of Laboratory Animals (National Academy Press, Washington, DC, 1996) and were approved by the Institutional Animal Care and Use Committee of University of California, Riverside. We chose to study cortical cultures containing glia, since DOM has access to both neurons and glia in vivo, and astrocytes contain targets of DOM action such as glutamate transporters and AMPARs (Burnashev et al., 1992; Patneau et al., 1994) and may contribute to enhanced nitric oxide (NO) synthase activity after DOM exposure (Chandrasekaran et al., 2004). Moreover, astrocytes have been shown to both potentiate (Bal-Price and Brown, 2001; Dugan et al., 1995) and protect against excitotoxicity (Rothstein et al., 1996). Mixed cortical cultures were prepared similarly to that described previously (Curras et al., 1991) using embryonic pups obtained from pregnant Holtzman rats (Harlan, Chicago, IL) on embryonic day (ED) 16–18. Briefly, the fetal brains were dissected and collected in ice-cold Hank’s balanced salt solution (HBSS) under sterile conditions. Cerebral cortices including hippocampus were isolated and minced, and enzymatically digested in HBSS solution at 37°C for 30 min. Subsequently, the tissue was triturated in filter-sterilized Minimum Essential Medium (MEM) until completely dissociated into individual cells. The cells were then diluted with fortified MEM and plated on poly-D-lysine (0.1 mg/ml)-coated culture plates or 12-mm glass cover slips at a density of 4 × 10^5 cells/cm². Cortical cultures were grown in a humidified chamber maintained at 36.5°C containing air/CO\(_2\) (95/5%). At day in vitro (DIV) 5 and every 3 days thereafter, culture medium was replaced by 50% with serum-free MEM. Cytosine arabinoside (Ara-C, 10 μM) was added at DIV 5 to reduce glial proliferation.

**Cell culture medium and chemicals.** HBSS and MEM solutions were purchased from In Vitrogen. The MEM was fortified with 10% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals), 5% horse serum (GIBCO BRL), 1% (V/V) antibiotic and antimycotic mixture (GIBCO BRL), gentamycin sulfate (1 mg/ml), and sodium pyruvate (1 mg/ml), pH 7.22. Glutamate receptor ligands including domoic acid, D-2-amino-5-phosphonopentanoate (D-AP5), 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX), 1-aminooindan-1,5-dicarboxylic acid (AIDA), (S)-alpha-methyl-4-carboxyphenylglycine (MCPG), and (2S)-2-amino-2-(15,25)-2-carboxycycloprop-1-yl)-3-(xanth-9-yl) propanoic acid (LY341495) were purchased from Tocris Cookson. All other chemicals were obtained from Sigma.

**Treatment of cultures.** Unless stated otherwise, cultures at DIV 11 were used, and experiments were run at 37°C in a CO\(_2\) incubator. Prior to each experiment, the volume of culture medium in each well was calibrated and reset at original volume. Receptor ligands were applied using MEM as the diluent. After DOM or antagonist drug treatments, conditioned medium from untreated sister cultures was used to washout the drug-containing medium. In experiments examining the effects of multiple DOM exposure, cultures were pretreated with a subtoxic dose of DOM (1 μM DOM) on DIV 8 for 48 h and subsequently treated on DIV 11 with one of the above-mentioned DOM exposure paradigms, and neuronal survival was measured 24 h later. Neuronal survival in these treated cultures was compared with that in DIV 11 sister cultures not receiving 1 μM DOM pretreatment on DIV 8.

**Assessment of neuronal cell death.** Cultures at DIV 3–11 were treated with DOM (0.1–1000 μM) for varying durations (10 min–24 h) with or without antagonist, and cell survival was measured immediately after treatment or after washout. Cell viability was measured using an MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay, and absorbance values were normalized to values obtained from control sister cultures treated with medium replacement or antagonists alone in the absence of DOM. Neuronal death values
obtained using MTT assay were found to correspond proportionately with the number of dead or damaged neurons identified using the trypan blue dye exclusion test in response to DOM exposure. Neuronal counts and morphology were assessed, and digital pictures were taken using a Nikon Coolpix 990 camera under phase-contrast microscopy (Nikon TMS-F).

Cell survival was converted to percent of maximum neuronal death assessed 24 h after cultures were exposed to 10 mM NMDA for 2 h and subsequently washed with conditioned medium from unexposed sister cultures. NMDA applied for 24 h produces no injury in neocortical astrocyte cultures, since astrocytes do not contain functional NMDA receptors (David et al., 1996). Mixed cortical cultures show normal astrocyte morphology when grown on cover slips, treated in this manner with NMDA, and fixed at 24 h (data not shown). In some experiments, the effect of DOM on cell viability was measured at 2 h after treatment. In experiments comparing DOM efficacy across time in culture, percent neuronal cell death at each time point examined was calculated by normalizing death obtained at each DIV to maximum cell death (produced by 1 mM/24 h DOM at DIV 11). These values represented DOM efficacy and were compared to maximum cell death produced by NMDA (10 mM/2 h and measured 24 h later) at each DIV.

Maximal neuronal death and EC50 values from concentration-response curves for DOM and other GluR ligands were determined using nonlinear regression analysis of the dose-response relationship (Y = 100/(1 + 10((logEC50−X)/Hill Slope)^3), where Y and X represent the percentage of neuronal death and the logarithm of DOM concentration, respectively) using Prism software (GraphPad Software Inc., San Diego, CA). Based on initial concentration-response curves for 24-h, 2-h, and 10-min exposures (and cell survival measured at 24 h after the start of treatment), three equipotent EC50 paradigms were established in this study representing long, moderate, and brief exposure durations (3 μM for 24 h, 10 μM for 2 h, and 50 μM for 10 min; see the result sections).

Application of glutamate receptor antagonists. We tested an array of glutamate receptor antagonists, including those for the ionotropic receptor classes: NBQX (an AMPAR antagonist at 2 μM and a nonselective AMPAR/KAR antagonist at 10 μM), D-AP5 (an NMDA receptor antagonist; 10 and 100 μM), and a nonselective mGluR antagonist MCPG (500 μM; Bushell et al., 1996; Hayashi et al., 1994); AIDA (1 mM), a potent and selective antagonist for group I mGluRs (Strasser et al., 1998), and LY341495 (1 μM), a selective group II/III mGluR antagonist at this dose (Howson and Jane, 2003; Kingston et al., 1998). All drugs were dissolved in MEM and administered either 15 min before or at various times after the start of DOM application and in the continued presence of DOM.

Immunocytochemical analysis. Standard immunocytochemical and Western blot methods were used to examine glutamate receptor expression profiles at different times in vitro. For immunocytochemistry, mixed cultures grown on glass cover slips were fixed with 4% paraformaldehyde for 2 h. After washes with cold 0.01 M phosphate buffered saline (PBS), the cover slips were incubated in 0.05% H2O2 to block endogenous peroxidase activity. They were subsequently incubated with polyclonal antibodies (all purchased from Immobilon-P; Millipore). The membranes were then incubated overnight at 4°C in a blocking solution containing 5% nonfat dry milk and 1% bovine serum albumin. Primary and secondary antibodies were applied in a way similar to that described above for the immunocytochemistry procedure. The integrated optical density (IOD) of immunoreactive bands detected using enhanced chemiluminescence detection (Amersham) was quantified using ImagePro V4.5 software (Media Cybernetics) and was normalized to β-tubulin IOD values in the same sample to compensate for loading errors.

Statistical Analysis. Statistical differences were tested at p < 0.05 using Student’s t-test for two-group comparison, or one-way followed by Student-Neuman-Keuls or Tukey’s test for multiple-group comparisons. When two-way ANOVA analysis was conducted, a post hoc Bonferroni test was used for multiple comparisons. A Spearman bivariate linear model was used for testing correlation between GluR expression and excitotoxicity.

RESULTS

Characteristics of Mixed Cortical Cultures

Neurons gradually developed their mature morphology with increasing time in vitro, as manifested by the multipolar morphology and intricate neurite networks associated with phase-bright cell bodies. When results of immunocytochemical experiments were analyzed, the majority of neurons (>85%) at DIV 11 expressed NR1, GluR1, and GluR6/7, which are subunits for NMDA, AMPA, and KA receptors, respectively (Fig. 1). Immunoblotting data also indicated expression of GluR2/3 and GluR5 in our cultures (Fig. 8B). Using antibodies that are specific for either NeuN or GFAP proteins, and serve as specific markers for neurons or astrocytes, respectively. We found that cells constituted 13.3 ± 7.1% of total cell numbers in DIV 11 cultures (Fig. 1). This neuronal/glial ratio was kept consistent throughout the plates and throughout the cultures by treatment with Ara-C. Methodological controls receiving no primary antibody showed little or no immunoreactivity (data not shown).

Microscopic observation revealed virtually complete neuronal death in DIV 11 cultures exposed to 10 mM NMDA for 2 h (n = 5 experiments; data not shown). DOM (100 μM, 24 h)
produced maximal neuronal death (100.2 ± 5.9% of control, n = 6) similar to results shown by others using murine cortical cultures (Larm et al., 1997). Exposure of cultures to 50 μM/10 min DOM produced swelling in the majority of neurons examined. The swelling was prominent between 10 and 30 minutes after DOM washout but was reversed in the majority of neurons by 2 h after washout (Fig. 2, n = 4 experiments).

**Potency and Efficacy of DOM Relative to Other Excitatory Amino Acids (EAAs)**

Concentration-response curves were generated by measuring neuronal death responses to 24-h exposures of different DOM doses (0.1 μM to 1000 μM) and compared to those measured in DOM-naive cultures at DIV 11. Our results show that DOM induces neuronal death in a concentration-dependent manner, with an EC50 of 4.2 μM when applied for 24 h (Fig. 3A). This EC50 is similar to DOM affinity at [3H]-AMPA binding sites in rat brain membranes (IC50 of 2 μM; Hampson and Manalo, 1998). To compare the potency of DOM with that of other known prototypic EAAs, the dose-response profiles of NMDA, kainate, and glutamate were also examined. All agonists reduced neuronal survival dose dependently (Fig. 3A). EC50 values obtained for kainate, NMDA, and glutamate were 15.6 ± 6.2, 25.8 ± 7.1, and 36.4 ± 15.2 μM, respectively (n = 3–4), giving a rank order of potency of DOM > KA > NMDA ≈ glutamate (p < 0.05). Therefore, DOM was the most potent EAA measured using our cultured cortical neuron preparation, consistent with previous reports using in vivo and in vitro methods (Doucette et al., 2003; Koh and Choi, 1988; Larm et al., 1997). When exposed for 24 h, all EAAs examined produced maximum responses when compared to neuronal death produced by 10 mM/2 h NMDA. Neuronal death was 100.2 ± 5.9, 101.3 ± 5.2, 103.3 ± 4.5, and 99.4 ± 7.4% of control (n = 6).

**Effect of Concentration and Duration on DOM Toxicity**

We next tested the effects of different combinations of DOM concentration and exposure duration. Mixed cortical cultures were challenged with 3, 5, 10, or 50 μM DOM for a variety of exposure durations (10 min, 30 min, 1 h, or 2 h), after which DOM was washed out and the culture medium replaced with conditioned medium from unexposed sister cultures. In all cases neuronal death was measured at 24 h following the beginning of exposure. A two-way ANOVA analysis showed significant effects of both DOM exposure time (F3,126 = 66.2, p < 0.001) and DOM concentration (F3,126 = 102.3, p < 0.001), suggesting that DOM-induced neuronal death is determined by
both concentration and duration of DOM exposure. Figure 3B shows that, in general, neuronal survival decreased proportionately as a function of exposure time. However, DOM applied at the 50 \( \mu M \) concentration produced disproportionately greater neuronal death than the lower concentrations at every exposure time except that of 24 h. In the latter case, neuronal death was linearly related to DOM concentration. For example, after a 10-min exposure 50 \( \mu M \) DOM produced marked neuronal death of 47.4 \( \pm \) 5.1\% as compared to 2.8 \( \pm \) 2.2, 4.6 \( \pm \) 1.8, and 6.7 \( \pm \) 2.3\% triggered by slightly lower concentrations: 3, 5, and 10 \( \mu M \), respectively. By 1 h of treatment, 50 \( \mu M \) DOM produced near maximal neuronal death, and longer exposures did not increase neuronal death further. Post hoc Bonferroni analysis indicated no statistical difference in neuronal death produced by 50 \( \mu M \) DOM treatment for 60 min (81.6 \( \pm \) 6.8\%), 2 h (85.9 \( \pm \) 5.6\%), and 24 h (89.5 \( \pm \) 8.5\%; \( p > 0.05 \), \( n = 6 \)), suggesting that strong activation of iGluR receptors by high concentrations of DOM triggers near maximal neuronal death after shorter activation of iGluRs than that required for low doses. In contrast, 10 \( \mu M \) DOM required 24 h of exposure to produce greater than 50% neuronal death.

**Time Dependency of DOM-Induced Neuronal Death**

In order to examine the time course of neuronal death after different concentrations of DOM exposure, mixed cortical cultures were exposed to different doses of DOM for 2 h, and neuronal death was measured either immediately or 22 h post-DOM washout. A two-way ANOVA revealed time and concentration effects that were statistically significant (\( F = 13.6, \text{df} = 1, p < 0.001 \) for time effects; and \( F = 122.5, \text{df} = 5, p < 0.001 \) for concentration effects). The effect of exposure duration at different concentrations of DOM can be seen in Figure 3C. A significant outcome of our studies was the finding that low concentrations of DOM produced more neuronal death if this was measured 22 h after the washout of DOM than if measured immediately after DOM treatment (post hoc Bonferroni, \( p < 0.05 \)), while higher concentrations of DOM (20–100 \( \mu M \)) produced equivalent degrees of neuronal death when measured at these two time points (\( p > 0.05 \)). Accordingly, we observed that DOM potency is significantly greater in the 2 h DOM + 22 h wash (10.7 \( \pm \) 4.2 \( \mu M \)) than in the 2 h DOM group (19.6 \( \pm \) 5.9 \( \mu M \); \( n = 5 \), \( t = 2.75; p = 0.0251 \)).

In combination, these data suggest that low concentrations of DOM can reach their maximum efficacy even with weak activation of AMPAR/KARs if secondary events having delayed onset are allowed to occur. By producing stronger activation of AMPAR/KARs, the higher doses of DOM (50 and 100 \( \mu M \)) can result in maximal cell death by 2 h after exposure.

**Protective Effects of iGluR Antagonists against DOM-Induced Neuronal Death**

To evaluate the contribution of iGluRs targeted by different doses of DOM in cortical neurons we examined the effects of...
NBQX, an AMPA/KAR receptor antagonist (Sheardown et al., 1990), and D-AP5, an NMDA receptor antagonist (Davies and Watkins, 1982). The antagonists were applied prior to and concurrently with increasing concentrations of DOM applied for 24 h. Both antagonists acted in a dose-dependent manner to reduce DOM-induced neuronal death. Figure 4 shows that both NBQX and D-AP5 shifted the DOM dose-response curve to the right and decreased the maximum response to DOM as expected for competitive antagonism. At maximal concentrations, NBQX (10 μM) and D-AP5 (100 μM) each produced nearly complete suppression of neuronal death evoked by low to moderate concentrations (1−5 μM) of DOM. NBQX proved relatively more neuroprotective than D-AP5 with increasing DOM concentration. At high concentrations of DOM (50 and 100 μM), however, neither NBQX nor combined application of both antagonists could provide full protection, as expected for competitive antagonism.

Temporal Involvement of AMPA/KAR and NMDARs in DOM Toxicity

To address the temporal involvement of both AMPA/KA and NMDA receptors in DOM toxicity, we applied the above-tested iGluR antagonists with variable time delay and compared the effects on DOM-induced neuronal death. Each antagonist was applied alone or in combination with the other 15 min before or 30 min after the start of an EC$_{50}$ DOM exposure (10 μM/2 h, measured at 24 h). Our results, shown in Figure 5, revealed significant protection against DOM toxicity after preapplication of either 10 μM NBQX, 100 μM D-AP5, or both (#, Fig. 5), with preapplication of both antagonists affording the best protection against DOM exposure (102.6 ± 5.2% of total neuronal survival). In comparison, preapplication of D-AP5 (100 μM) provided significant less protection (77.4 ± 3.9%) when compared with combined effects of NBQX and D-AP5 ($n = 6$, $p < 0.001$). When given 30 min after DOM, but not if applied 2 h after DOM, but neuroprotection continued to be present during the following 22 h ($p < 0.05$ compared with DOM alone group). Bars represent means ± Sem of six experiments with six replicates per experiment.

![Figure 4](http://toxsci.oxfordjournals.org/)

**FIG. 5.** Critical time window for neuroprotection against DOM toxicity. Cultures at DIV 11 were treated with 10 μM DOM for 2 h, washed, and neuronal death measured 22 h later. This dose produced approximately 50% neuronal death (DOM alone). iGluR antagonists were either preapplied or applied 30 min, 60 min, or 2 h after the start of DOM treatment. Neuroprotection was greatest if 10 μM NBQX was preapplied (∼15 min; # $p < 0.05$ compared with DOM alone group) and exceeded (97.1 ± 4.1% neuronal survival) that provided by 100 μM D-AP5 (77.4 ± 3.9% neuronal survival). NBQX was increasingly less effective with increasing delay in application after DOM exposure; a significant decrease in protection was measured between groups receiving preapplication or 30-min delay in NBQX application ($**p < 0.01$). However, the effectiveness of D-AP5 was not significantly reduced when applied 30 min after DOM as compared to that produced by preapplication ($p > 0.05$). Combined application of both antagonists provided significant neuroprotection if applied 15 min before, or 30 or 60 min after, but not if applied 2 h after DOM, but neuroprotection continued to be present during the following 22 h ($p < 0.05$ compared with DOM alone group).
could be partially achieved even if concurrent antagonist treatment was delayed for 1 h after the start of DOM exposure. The additional protection by D-AP5 over that provided by NBQX (Figs. 4 and 5) points to a synergistic involvement of both NMDA receptors and AMPA/KAR 30 min after DOM exposure. Importantly, no protection was seen if either antagonist, alone or in combination, was applied 2 h after DOM application and continued to present during the following 22 h period.

Effect of Different DOM Dose/Exposure Paradigms

We next determined what dose-duration combinations yielded 50% DOM responses when measuring neuronal death 24 h after the start of exposure. We established three EC$_{50}$ exposure paradigms, which represent weak/prolonged exposure (3 μM/24 h), moderate concentration and duration exposure (10 μM/2 h), and strong/brief exposure (50 μM/10 min). Figure 6 shows that these DOM exposure conditions were equipotent and resulted in 58.2 ± 6.1, 54.5 ± 6.4, and 46.9 ± 4.6% neuronal death when measured at 24 h, respectively.

Next, we examined whether NMDARs and AMPA/KARs contributed to toxicity produced by these EC$_{50}$ paradigms. In each case D-AP5 (100 μM) or NBQX (10 μM) was applied 30 min after the start of DOM exposure, when both NBQX and D-AP5 offered significant protection against 10 μM/2h DOM (see Figs. 5 and 6). Figure 6 demonstrates that D-AP5 and NBQX each significantly rescued neurons from DOM-induced death. When using a brief, strong DOM challenge (50 μM/10 min), 100 μM D-AP5 (87.5 ± 5.2% of control) provided better protection than 10 μM NBQX (59.5 ± 4.4% of control, n = 5, p < 0.01). In comparison, NBQX rescued more neurons relative to D-AP5 during the 3 μM/24 h exposure conditions (97.4 ± 5.2 and 84.6 ± 5.8%, respectively, n = 5, p < 0.01). It is important to note, however, that the protection offered by D-AP5 against the three regimens was similar, suggesting a similar contribution and timing of NMDAR activity to DOM toxicity with low and high doses of DOM. The reduced AMPA/KAR involvement in response to high doses of DOM may represent a quicker transition to NMDAR activation or may be related instead to the absence of DOM at the binding sites, since the high dose was applied briefly and was washed out before the application of NBQX.

Lack of Contribution of Metabotropic Glutamate Receptors to DOM Toxicity

Since DOM has been shown to result in elevated levels of extracellular glutamate and aspartate in other culture preparations (Berman and Murray, 1997), and this may indirectly activate NMDA receptors in our cultures, we explored whether DOM recruits mGluR activity in producing excitotoxicity. Therefore, we examined the effects of several mGluR antagonists using the DOM exposure paradigms established above. The group I selective mGluR antagonist, aminoindan-1,5-dicarboxylic acid (AIDA) is neuroprotective against glutamate-induced excitotoxicity (Strasser et al., 1998). We tested the possible involvement of Group I, Group II, and Group III mGluRs using AIDA (1mM), MCPG (500 μM), and LY341495 (10 μM). None of these drugs significantly altered neuronal death induced by DOM, nor did they alter neuronal death by themselves (Fig. 7, p > 0.05 for each drug tested). Similar results were obtained with 10 μM LY341495, a non-selective mGluR antagonist at this dose. Therefore, it is unlikely that mGluRs, activated by DOM-induced secondary glutamate release, play an important role in DOM neurotoxicity under the in vitro conditions we tested.

Increased Neuronal Susceptibility to DOM during In Vitro Development: Correlation with iGluR Expression

Previous studies have shown that the susceptibility of central neurons to DOM neurotoxicity is age related in whole animals (Auer, 1991; Kerr et al., 2002; Mayer, 2000; Xi et al., 1997; Xi and Ramsdell, 1996), although the reasons for this are unclear. Using murine cortical cultures, Jensen et al. (1999) reported that 20 μM DOM (6 h exposure) produced ~10% more cell death at DIV 12 than at DIV 8. We have extended these findings by determining the developmental regulation of DOM potency and efficacy and correlating this to NMDAR, AMPAR, and KARs subunit expression. In Figure 8A neuronal survival is expressed as a percent of maximum cell death produced by 24-h exposure to 100 μM or 1 mM DOM at each DIV time point.
metabolotropic GluR antagonists do not protect against DOM-induced neurotoxicity. Cultures were treated with either 3 μM/24 h, 10 μM/2 h, or 50 μM/10 min DOM, washed at corresponding times, and neuronal death measured at 24 h post DOM treatment. mGluR ligands were applied 15 min before and continuously during DOM exposure and after washout until neuronal survival was measured at 24 h. mGluR antagonists AIDA (1 mM), MCPG (500 μM), and LY341495 (10 μM) produced no significant effects on neurotoxicity induced by various DOM exposure paradigms (p > 0.05, one-way ANOVA). Application of these mGluRs antagonists alone (control) for 24 h did not affect neuronal survival (p > 0.05, one-way ANOVA).

measured. DOM dose-response curves at DIV 3, DIV5, DIV8, and DIV 11 were constructed using a 24-h exposure paradigm (data not shown); calculated EC_{50} values are shown above bars in Figure 8A. Our results indicate that the potency of DOM increases gradually with successively increasing time in vitro until DIV 8 and then stabilizes; the calculated EC_{50} values were 40.8 ± 13.1, 33.2 ± 12.5, 4.93 ± 3.37, and 4.17 ± 2.11 μM for DIV 3, 5, 8, and 11, respectively (n = 3–5). In addition, DOM efficacy was assessed using three different dose/exposure duration paradigms and compared to that of a saturating NMDA exposure (10 μM/2 h). Using a 24-h exposure paradigm, DOM-induced cell death was maximal at all DIV time points examined, ranging from 96.6 ± 7.4% (at DIV 3) to 103.7 ± 9.9% (at DIV 11). NMDA acted similarly to DOM, in that it produced 100% neuronal death, except on DIV 3 when NMDA produced only 72% neuronal death.

A chronological increase in DOM efficacy was also observed for each of the three EC_{50} exposure paradigms (F_{3,97} = 34.5, p < 0.001 for time effects, two-way ANOVA). However, by DIV 8 mixed cortical cultures reach a maximum response to DOM regardless of the dosing paradigm used. For example, neuronal death achieved with 3 μM/24 h, 10 μM/2 h, and 50 μM/10 min DOM was 43.1 ± 4.6, 46.7 ± 4.1, and 43.1 ± 3.7% of maximum neuronal death observed at DIV 8 and 48.9 ± 3.8, 54.2 ± 3.2, and 51.6 ± 3.5% of maximum neuronal death observed at DIV 11, respectively. Therefore, no significant increase in DOM-induced maximum cell death and DOM potency could be detected between DIV 8 and 11 as could be detected between DIV 5 and 8 (p < 0.005).

Effects of Repeat Exposure to DOM

In order to examine the potential for increased risk to different DOM exposure paradigms following a previous prolonged, low-dose exposure, we exposed cultures to 1 μM DOM for 48 h (starting at DIV 8), washed them, subjected them to a second treatment of DOM, and measured neuronal death 24 h after the start of DOM application (at DIV 11). Figure 9 demonstrates that low-dose pretreatment did not produce significant neuronal death on its own when measured 24 h after washout (at DIV 11; 95.6 ± 5.3% of control neuronal survival). Preconditioned cortical cultures responded more vigorously to
a subsequent DOM test treatment. For example, neuronal survival values in cultures with or without DOM pretreatment were 31.3 ± 3.6 and 48.7 ± 4.2%, respectively, for a 3 μM/24 h exposure and 35.2 ± 4.7 and 51.7 ± 3.9%, respectively, for a 10 μM/2 h exposure. The effect of preconditioning was statistically significant for both exposure paradigms (**p < 0.01, Fig. 9). In contrast, preexposure to prolonged, low-dose DOM treatment did not augment cell death triggered by the 50 μM/10 min paradigm.

DISCUSSION

Temporal Involvement of iGluRs in DOM-Induced Excitotoxicity

Like glutamate neurotoxicity (Hartley et al., 1990; Prehn et al., 1995), DOM toxicity involves both NMDA and non-NMDA-type iGluRs as reported previously for cultured cerebellar granule cells (CGCs; Berman and Murray, 1997) and murine neocortical neurons (Jensen et al., 1999). NBQX completely rescued neurons if applied concurrently with DOM, but was 33% less effective if application was delayed by 30 min, pointing to an early phase mediated by AMPA/KARs after binding of DOM (Hampson and Manalo, 1998). Concurrently, DOM produced swelling that has been shown in CGCs to be dependent on AMPA/KAR activity (Berman and Murray, 1997). In contrast to NBQX, the NMDAR antagonist DAP5 was still neuroprotective at 1 h post-DOM.

NMDAR-mediated toxicity, therefore, occurs with greater delay (30–60 min later) and is observed even after removal of the toxin, suggesting that it occurs indirectly, presumably via elevations in extracellular glutamate subsequent to AMPA/KAR-mediated depolarization. In CGCs extracellular glutamate (and aspartate) is elevated within minutes after DOM exposure as a result of cell swelling and reversed glutamate uptake (Berman and Murray, 1997). The early phase may synergize with delayed NMDAR-mediated activity by providing depolarization-elicited removal of the Mg²⁺ block from
the receptor-channel (Mayer and Westbrook, 1987). Two hours after DOM exposure, iGluR antagonists do not afford significant neuronal protection, indicating a “critical window” for neuronal rescue after DOM. A summarizing figure concerning differential roles of GluRs and potential excitotoxicity mechanisms of DOM is presented in Fig. 10.

Effect of Different Exposure Paradigms

Each of the exposure paradigms, including 50 μM/10 min DOM, were equieffective, providing approximately 50% DOM-induced neuronal death. Results of experiments employing a 30-min delay in the application of iGluR antagonists indicated that low concentrations of DOM (3 μM/24 h and 10 μM/2 h) produced as much if not more toxicity mediated via AMPA/KARs activation as compared to 50 μM/10 μM DOM exposure during this early period (Fig. 6). The reduced protection provided by delayed NBQX treatment against high concentration of DOM (50 μM) may indicate a quicker progression from early to the late phase of DOM toxicity, evidenced by sensitivity to D-AP5 but not NBQX, since delayed NMDAR blockade protected equally well against DOM toxicity in each of the three exposure paradigms (82–88%). The similar efficacy of NMDAR blockade in all paradigms and the significant protection afforded by NMDA receptor blockade in vivo could be exploited in DOM poisoning accidents (Tasker and Strain, 1998).

In combination, these data indicate that early onset AMPAR/KAR activation occurs within the first 10 min after DOM application and is able in all three paradigms to trigger the secondary processes leading to NMDAR activation. NMDAR activation probably begins soon after DOM application, since glutamate and aspartate release reaches ~80% of maximum within the first 10 min in cultured CGCs (Berman and Murray, 1997), but may trigger maximum damage if continued for 30–60 min. At 2 h post-DOM, AMPAR and NMDAR activation no longer contribute to the ensuing DOM toxicity, which may result from NO-induced neurodegeneration (Chandrasekaran et al., 2004).

Timecourse of DOM Excitotoxicity

A novel finding is that the timecourse of DOM excitotoxicity is dependent on DOM concentration, with high concentrations producing complete neuronal death within just 2 h after exposure. At low doses the time course of cell death was more prolonged, and maximum cell death was not achieved until many hours later. Therefore, the potency of DOM increases with time of exposure (except at high doses), like that reported for NMDA and kainate (Koh and Choi, 1988). Rapid DOM toxicity that correlated with rapid swelling after high doses implicates acute processes related to iGluR activation, since iGluR antagonists rescue neurons only during this critical 2-h window post-DOM (Fig. 5). In support of this, glutamate induces neuronal swelling, intracellular Ca2+ deregulation, and neuronal death within 2 h (Tymianski et al., 1993). The slower effect of low DOM concentrations (3, 5, and 10 μM) may involve apoptosis, since excitatory amino acids can trigger either necrotic or apoptotic processes, depending on the intensity of the insult. The greater efficacy of DOM after brief application resembles more the effects of NMDA than kainate (Koh and Choi, 1988). In combination with the prominent NMDAR-mediated component of DOM (but not kainate toxicity; Jensen et al., 1999) and the similar requirement for NMDARs seen with low and high DOM concentrations (see Fig. 6), these data suggest that rapid DOM toxicity may involve expedited activation of downstream signaling pathways associated with NMDARs.

Lack of Significant Contribution of mGluRs to DOM Excitotoxicity

DOM does not bind directly to mGluRs (Hampson et al., 1992; Hampson and Manalo, 1998), but may activate mGluRs indirectly through release of endogenous EAAs. In our study, none of the mGluR antagonists changed DOM-induced neuronal death in any exposure paradigm. In contrast, similar concentrations of AIDA protect against NMDA receptor-mediated excitotoxicity in pure neuronal cortical cultures (Strasser et al., 1998).

Since Group I mGluRs produce presynaptic vesicular release of glutamate (Cartmell et al., 2000), and DOM-induced reversal of glutamate uptake (Berman and Murray, 1997) is likely to depend on glia, the inclusion of a moderate astrocytic component in our cortical cultures may mask a potential contribution...
by Group I mGluRs. Reduction of glutamate-induced EAA release by blocking Group I mGluRs, therefore, may not change effective levels of extracellular glutamate in the presence of DOM. Alternatively, receptors activated by DOM-triggered adenosine that inhibit exocytotic glutamate release (Berman and Murray, 1997) may compensate for release triggered by glutamate activation of Group I mGluRs. Presynaptic autoreceptors of inhibitory Group II and III mGluRs (Cartmell et al., 2000) do not appear to limit DOM toxic effects, and this cannot be attributed to lack of mGluR expression, since cortical cultures express Group I, II, and III mGluRs (Heck et al., 1997; Janssens and Lesage, 2001).

Developmental Susceptibility to DOM Excitotoxicity: Correlation with iGluR Expression

Saturating concentrations of DOM, if given for 24 h, induce maximal neuronal loss at all DIV examined. Using any of the three submaximal DOM treatment paradigms, we observed an increase in excitotoxicity with increasing time in vitro until DIV 8, as reported previously (Choi et al., 1987; Jensen et al., 1999). Regression analysis indicated that DOM potency is positively correlated with total iGluR expression (Figs. 8A and 8C). In particular, levels of the obligatory NMDAR subunit, NR1, are dramatically elevated between DIV 5 and 8, when DOM potency increases significantly. Previous studies have reported increased expression and function of NMDARs with time in vitro (Cheng et al., 1999; Hoffmann et al., 2000; Sinor et al., 1997), which could intensify NMDA receptor-mediated responses leading to augmented DOM potency.

Low to robust expression of some AMPAR/KAR subunit members (GluR1, GluR 2/3, and GluR 6/7) could be detected at DIV 3 when a 24-h exposure to DOM (100 μM–1 mM) produced maximal cell death (Fig. 8A). Large increases in expression were most notable for the AMPAR subunits GluR1 and GluR 2/3 as well as the KAR subunits GluR5 and GluR6/7, subunits shown to be predominantly expressed by cerebral cortex (Dai et al., 2002; Sommer et al., 1992). GluR2-containing AMPARs have been implicated in AMPAR-mediated excitotoxicity that increases during development (Janssens and Lesage, 2001) and may partially explain the developmental regulation of DOM potency. An alternative explanation is that DOM's enhanced effects during maturation may be due to activation of more Ca²⁺-permeable AMPARs or AMPAR isoforms generating non- or slowly desensitizing excitatory currents (Hampson and Manalo, 1998; Jensen et al., 1998; Patneau et al., 1994; Sommer et al., 1992; Stern-Bach et al., 1998). Increased functional expression of KARs, which has been implicated in DOM toxicity (Jensen et al., 1999), may also contribute. Moreover, increased glutamate
uptake activity at later times in vitro (Gaillet et al., 2001) may provide more capacity for “reversed glutamate uptake” (glutamate release) and increase the efficacy of DOM.

In line with our findings, whole-animal studies report that neonates are highly susceptible to DOM exposure (Xi et al., 1997) and that DOM potency is two-fold greater in the first week of postnatal development (Doucette et al., 2003). In contrast to whole-animal studies that are confounded by developmental changes in the blood–brain barrier and detoxifying mechanisms (Mayer, 2000; Xi et al., 1997), our results using a barrierless in vitro system indicate that intrinsic neuronal properties such as iGlur expression/function may be critical in determining the efficacy and potency of DOM.

**Increased Susceptibility to Subsequent DOM Exposure after Prior Preconditioning**

Another significant finding derived from this study is that a 48-h preconditioning to a sublethal dose of DOM increases the susceptibility of neurons to subsequent treatment with 3 μM/24 h or 10 μM/2 h DOM. DOM toxicity, therefore, is dependent on prior history of DOM exposure, a property that is not well appreciated today, and may constitute a significant risk factor for ASP poisoning. Potential mechanisms for increased susceptibility after preconditioning could be due to the fact that either cortical neurons are unable to mount a tolerance response, or cortical neurons in culture (as opposed to in vivo conditions) lack tolerance induction mechanisms. Our results differ from those of Kerr et al. (2002), who reported tolerance to the acute electrophysiological effects of DOM in hippocampus. However, the exposure times were much shorter, and neuronal death was not assessed in that study. A caveat, however, should be noted that the preconditioning stimuli used in this study differ from the stimuli used in other excitotoxicity studies, so that neurons may have been rendered fragile by the strength of the preconditioning stimulus. Our rationale in choosing a relatively longer DOM preconditioning duration was that longer exposure might reduce glutamate receptor expression and, therefore, lead to subsequent tolerance to DOM. Surprisingly, the latter proved not to be the case. Because the preconditioning concentration and duration might still produce sublethal effects on cultured cortical neurons, care should be taken in extrapolating these results to other excitotoxicity studies, especially when other confounding factors are present, such as neuronal developmental stages (this study) or animal age (Hesp et al., 2004). Interestingly, preconditioning did not alter the response to a subsequent high dose of DOM (50 μM/10 min). Increased DOM toxicity after repeat exposure is consistent with results from an in vivo study showing greater reductions in glial and neuronal enzyme activity associated with glutamate homeostasis or to increased Ca2+ influx in rats previously exposed in utero (Dakshinamurti et al., 1993; also see Clayton et al., 1999; Peng et al., 1997).

While these parameters, as well as increased extracellular glutamate levels, can change within 4 h after DOM treatment, a time frame comparable to our preconditioning period, our second treatment with DOM occurred after washout of preconditioned medium, ruling out increased glutamate background as a factor.

**ACKNOWLEDGMENTS**

We acknowledge funding from the University of California Toxic Substances Research and Teaching Program (UC TSR&TP) as a predoctoral fellowship to S.Q. and research grant to M.C.C.C. This study was also partly supported by the American Heart Association (M.C.C.C.).

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