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Protection of cortical neurons against oxygen-glucose deprivation and *N*-methyl-D-aspartate by DIDS and SITS

Joseph S. Tauskela*, Geoff Mealing, Tanya Comas, Eric Brunette, Robert Monette, Daniel L. Small, Paul Morley

Institute for Biological Sciences, National Research Council of Canada, Montreal Road Campus, Building M-54, Ottawa, ON, Canada K1A 0R6

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Abstract

The Cl^- channel blockers, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) or 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) dose-dependently protected against oxygen-glucose deprivation in cultured rat cortical neurons. DIDS or SITS attenuated oxygen-glucose deprivation-induced increases in extracellular glutamate concentrations and intracellular Ca^{2+}. DIDS or SITS provided moderate protection against *N*-methyl-D-aspartate (NMDA) toxicity and decreased NMDA receptor-mediated increases in intracellular Ca^{2+}. Whole-cell NMDA receptor currents were attenuated 39 ± 2% and 21 ± 3% by 1 mM DIDS and SITS, respectively. Other Cl^- channel blockers as equipotent as DIDS and SITS did not decrease oxygen-glucose deprivation- or NMDA-mediated neuronal Ca^{2+} influx or toxicity. Neurotoxicity by exogenous glutamate was not prevented by SITS and was exacerbated by DIDS. Reductions in oxygen-glucose deprivation-induced increases in intracellular Ca^{2+} levels underlie neuroprotection by DIDS and SITS. This was a reflection of lower extracellular [glutamate], direct inhibition of Ca^{2+} influx through postsynaptic NMDA receptors, and possibly through other protective properties associated with DIDS and SITS.

Keywords: SITS; DIDS; Oxygen-glucose deprivation; NMDA (*N*-methyl-D-aspartate); Cortical neuron

1. Introduction

Disruption of ion homeostasis is an early hallmark of cerebral ischemia, which includes Na^+/Cl^-- and Ca^{2+}-dependent components (Choi, 1987). Ischemia/anoxia (Jiang et al., 1992; Inglefield and Schwartz-Bloom, 1998a) and NMDA receptor activation (Inglefield and Schwartz-Bloom, 1998b) may induce $[Cl^-]_i$ increases in neurons. The Ca^{2+}-dependent component of ischemic damage is generally attributed to excessive build-up of extracellular glutamate through processes which include exocytosis, reversal of glutamate transporters, astrocyte-swelling and cellular leakage.

Several features about 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) prompted us to investigate whether these compounds would be protective in a neuronal cell culture model of cerebral ischemia, oxygen-glucose deprivation. DIDS and SITS are inhibitors of Cl^- channels and Cl^-/HCO_3^- exchangers (Cabantchik and Greger, 1992), and are cerebral glutamate uptake inhibitors (Waniewski and Martin, 1983). DIDS and SITS are protective in cardiac models of ischemia/hypoxia (Tanaka et al., 1996; Heusch et al., 2000; Kawasaki et al., 2001; Ramasamy et al., 2001), which was attributed to Cl^--dependent and -independent properties. Cerebral ischemia may result in reversal of glutamate uptake transporters and cause glutamate efflux (Danbolt, 2001). Inhibition of transporters by dihydrokainate, combined with Cl^- channel blockade by 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS), reduces glutamate release more effectively than when either compound is administered alone (Seki et al., 1999). DIDS and SITS also protect against other neurodegenerative stimuli, possibly in a Cl^--dependent manner (Himi et al., 2002; O'Reilly et al., 2002; Small et al., 2002).

In the current study we document strong neuroprotection against oxygen-glucose deprivation by DIDS and SITS.

* Corresponding author. Tel.: +1-613-991-6752; fax: +1-613-941-4475.
E-mail address: joe.tauskela@nrc.ca (J.S. Tauskela).

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However, the basis for this protection differed from the initial rationale used to examine these compounds.

2. Methods

2.1. Materials

Tissue culture dishes and plates were purchased from either Du Pont-Life Technologies (Burlington, ON, Canada) or VWR Canlab (Mississauga, ON, Canada). Fetal bovine serum and minimal essential medium (MEM) were obtained from Wisent Canadian Laboratories (St-Bruno, QC, Canada). Horse serum was acquired from Hyclone Laboratories (Logan, UT, USA). NMDA, glutamate, (5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate (MK-801), tetrodotoxin, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and L-*trans*-pyrrolidine-2,4-dicarboxcylic acid (PDC) were purchased from Sigma (St. Louis, MO, USA). DIDS, SITS, DNDS, 4,4′-dibenzamidostilbene-2,2′-disulfonic acid (DBDS), $DiBAC_4(5)$, sytox-green, Fluo-4FF and 5-(and-6)-carboxynaphthofluorescein diacetate (CNF) were bought from Molecular Probes (Eugene, OR, USA). DL-*threo*-B-benzyloxyaspartic acid (DL-TBOA) was purchased from Tocris Cookson (Ellisville, MO, USA).

2.2. Preparation of rat cortical cultures

Cultures of E18 rat cortical neurons were prepared as described previously (Tauskela et al., 2001). Timed-pregnant Sprague–Dawley rats (Charles River Canada, St. Constant, QC, Canada) were anesthetized with halothane and killed by cervical dislocation. Following dissection of the cortical region of the fetal brain, cortical neurons were dispersed by trituration and centrifuged at $250 \times g$ for 5 min at 4 °C. Cells were plated at 1.0×10^6 (for electrophysiology experiments) or 1.7×10^6 cells/ml of medium consisting of MEM supplemented to 25 mM glucose, 10% fetal bovine serum, 10% horse serum and 2 mM glutamine on poly-L-lysine-coated 35-mm tissue culture dishes (for electrophysiology) or 12-well tissue culture plates. Cultures were treated with 15 μg/ml of 5-fluoro-2′-deoxyuridine and 35 μg/ml uridine after 4 days in vitro to minimize glial growth. At 7 days in vitro, one-half of the medium was replaced with medium consisting of MEM and 10% horse serum. Experiments were performed on cultures from 14 to 18 days in vitro.

2.3. Neurotoxic insults

2.3.1. Oxygen-glucose deprivation

Oxygen-glucose deprivation was performed as previously described (Tauskela et al., 2001) by placing cultures in a 37 °C incubator housed in an anaerobic glove box (Forma Scientific, Marjetta, OH, USA). Cultures were washed 2× in a glucose-free balanced salt solution (BSS) at 22 °C with the following composition (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 20 HEPES, 0.03 glycine, and maintained at pH 7.4. Cultures were then subjected to an anaerobic environment of 95% N_2/5% CO_2 for 75–95 min, producing an O_2 partial pressure equal to 10–15 Torr, as measured with an oxygen microelectrode (Microelectrodes, Londonberry, NH, USA). Oxygen-glucose deprivation was terminated by replacement of stored medium, and returning the cultures to a standard incubator maintained at 37 °C in 95% O_2/5% CO_2. Control cultures were exposed to BSS that contained 3 mM D-glucose and were maintained in the standard incubator. In experiments examining the effect of drugs, treated and untreated cultures were washed with BSS post-oxygen-glucose deprivation. For each 12-well plate subjected to oxygen-glucose deprivation, three to four wells were devoted to oxygen-glucose deprivation in the absence of drug, which eliminated concerns due to interplate variability in responses to oxygen-glucose deprivation.

2.3.2. Glutamate or NMDA exposures

Following washing in glucose-containing BSS, cultures were exposed to 40 μM NMDA or 80 μM glutamate for 15–20 min in this same buffer, washed, and culture medium replaced.

2.4. Assessment of neuronal injury

Neuronal injury was assessed 24 h following treatments by exposing cells to sytox-green, followed by measurement of fluorescence intensities with a fluorescence platereader. Specifically, media in 12-well plates were replaced with 0.5 ml/well of BSS containing glucose and 2.5 μM sytox-green, and incubated at 22 °C for 30 min. The fluorescence intensity (Ex = 480 ± 20 nm; Em = 530 ± 20 nm) from four locations within each well was then measured on a Cytofluor 2350 (Millipore, Bedford, MA, USA). The % sytox-green uptake was determined by subtracting the fluorescence measured in untreated sister cultures containing sytox-green, then normalizing values to the fluorescence representing 100% neuronal death, which was obtained by exposing sister cultures to 100 μM NMDA for 15 min. Thus, the % sytox-green uptake is equivalent to the percentage of dead cells above control levels (death in untreated control wells was < 5%).

2.5. Measurement of intracellular Ca^{2+}

Intracellular Ca^{2+} levels were determined using temperature-controlled platereader measurements of fluorescence intensity from 12-well plates containing cultures loaded with the Ca^{2+}-sensitive fluorescent dye Fluo-4FF (Ex = 485 ± 20 nm; Em = 530 ± 20 nm), and CNF (Ex = 590 ± 20 nm; Em = 645 ± 40 nm). Cultures were washed in glucose-containing BSS, then co-loaded with 4.5 μM Fluo-4FF and 15 μM CNF in BSS for 45 min at 37 °C,

washed 2×, and allowed to equilibrate for ≥60 min at 22 °C.

Prior to NMDA or glutamate treatment, cultures were washed in BSS. The fluorescence intensity from four locations within each well was measured in the platereader at 25 °C, and scanned following a 15-min exposure to NMDA or glutamate ± DIDS or SITS in BSS.

Prior to oxygen-glucose deprivation, cultures were washed 2× in glucose-free BSS, scanned in the platereader, and subjected to oxygen-glucose deprivation ± DIDS or oxygen-glucose deprivation ± SITS in glucose-free BSS. Immediately prior to termination of 75–90 min oxygen-glucose deprivation, each plate was tightly wrapped with parafilm while still in the anaerobic chamber to maintain a hypoxic atmosphere and scanned at 37 °C within 30 s of removal from the chamber.

Fluorescence intensities were background-corrected for the signals acquired from a cell-free plate containing dye-free buffer. The ratio of intensities of Fluo-4FF:CNF was calculated according to the formula

$$(\mathrm{FF4}_t - \mathrm{FF4}_{t=0})/\mathrm{CNF}_{t=0}$$

where $\mathrm{FF4}_{t=0}$ and $\mathrm{FF4}_t$ represent Fluo-4FF intensities for signals acquired pre- (time $t=0$) and post-insult (time t), respectively, and $\mathrm{CNF}_{t=0}$ is the CNF intensity obtained pre-insult. A CNF_t value was not included in this calculation since this dye did not remain homogenously distributed in the cytosol following NMDA receptor activation (R. Monette, unpublished observations). This microplatereader-based approach allowed objective high-volume/throughput analyses of relative changes in Ca^{2+} levels under conditions similar to those employed for neurotoxicity analyses. Fluo-4FF was chosen for several reasons. Neuronal $[Ca^{2+}]_i$ can be underestimated when using Ca^{2+}-sensitive dyes, such as with high affinity dyes (Hyrc et al., 1997), or with excessive dye loading, or photobleaching. Fluo-4FF is regarded as a low affinity dye ($K_d \sim 9.7$ μM). Fluo-4FF produces a stronger fluorescence signal than earlier generation dyes such as Fluo-3, minimizing dye loading. Ratioing the difference in Fluo-4FF signals to CNF compensated for factors such as variable neuronal density.

2.6. Whole-cell recording

The effects of Cl^- channel blockers on whole-cell NMDA-induced currents were measured as described previously (Mealing et al., 2001). Briefly, cortical neurons were perfused at 22 °C with bathing solution of the following composition (in mM): 140 NaCl, 5 KCl, 1 $CaCl_2$, 10 HEPES, 3 glucose, at pH 7.4. Also included in the bath were 1 μM tetrodotoxin to block Na^+ currents, and 10 μM glycine to saturate the glycine site on NMDA receptors. Patch pipettes (2–4 MΩ resistance) were constructed from 1.5 mm outer diameter/1.0 mm inner diameter Pyrex 7740 glass (Corning, Big Flats, MN, USA). The pipette solution contained (in mM): 140 CsCl, 1.1 EGTA, 10 HEPES, 2 Mg-ATP, at pH 7.2.

Whole-cell currents were acquired using an Axopatch 1-D amplifier equipped with a CV-4 headstage with a 1-GΩ feedback resistor (Axon Inst., Foster City, CA, USA). Voltage command and current acquisition were accomplished using a personal computer equipped with a Digidata 1200 interface and pClamp 7.0 software (Axon Inst.). A modified DAD-12 perfusion system (ALA Scientific Inst., Westbury, NY, USA) was used for rapid agonist or agonist–antagonist application.

2.7. Electrophysiological data analysis

The effect of Cl^- channel antagonists on NMDA-evoked currents was calculated according to the formula:

$$B = ((I - I_B)/I) \times 100$$

where I was determined by curve fitting the decay of the NMDA-evoked current during the NMDA application and extrapolating the fit to the end of the Cl^- channel antagonist co-application, and I_B was the current measured at the end of NMDA/Cl^- channel antagonist co-application. Current decays were fit to first-order exponential curves using a Chebyshev fit method and pClamp software.

2.8. Measurement of extracellular glutamate concentration

Immediately following exposure of cultures to oxygen-glucose deprivation, control wash or glutamate, buffer solutions were collected and stored at −80 °C. Glutamate concentrations were determined using a commercially available kit (Molecular Probes), in which glutamate is enzymatically converted to H_2O_2, which in turn reacts with Amplex Red reagent to produce the fluorescent product resorufin, and detected using a fluorescent platereader.

2.9. Statistical analysis

Three to six wells were investigated per condition from a minimum of three different platings. Data are presented as the mean ± S.E.M. Statistical comparisons were made by analysis of variance (ANOVA). When significant differences were observed, Scheffe's test was employed for multiple comparisons. Statistical significance was inferred at $P<0.05$.

3. Results

3.1. Effect of DIDS or SITS on oxygen-glucose deprivation-induced neurotoxicity

The effect of DIDS and SITS on neuronal toxicity by oxygen-glucose deprivation was determined on rat mixed

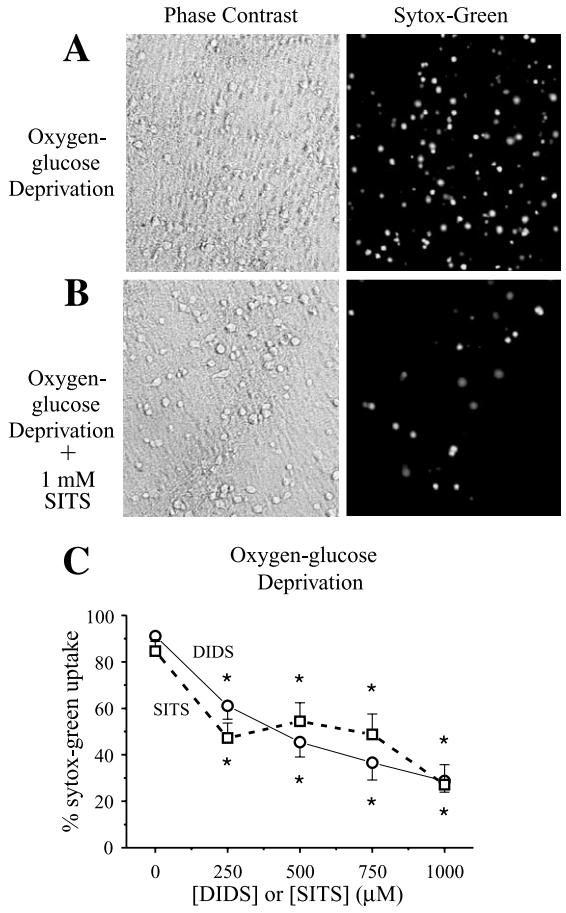

Fig. 1. (A, B) Representative microphotographs demonstrating protection against oxygen-glucose deprivation by co-incubation with 1 mM SITS. Cultures were subjected to 85 min oxygen-glucose deprivation ± SITS, followed by staining with sytox-green 24 h later. Phase contrast and fluorescence images of the same field, respectively, are shown for oxygen-glucose deprivation and oxygen-glucose deprivation + 1 mM SITS. (C) Dose-dependent protection against oxygen-glucose deprivation by 0.25–1.0 mM DIDS or SITS. DIDS or SITS were applied only during 75–90 min oxygen-glucose deprivation, and neurotoxicity was assessed 24 h later as the % sytox-green uptake. Data represent the means ± S.E.M. obtained from five different platings of neurons, each with four to six replicates. *Significant ($P<0.05$) difference in the % sytox-green uptake compared to oxygen-glucose deprivation performed in the absence of DIDS or SITS.

cortical neuronal–astrocyte cultures. Fig. 1A and B shows representative phase contrast and sytox-green images, respectively, for cultures exposed to 85 min oxygen-glucose deprivation ± 1 mM SITS. Subjecting cultures to oxygen-glucose deprivation for a duration of 75–90 min caused 91 ± 2% neuronal death, as measured by the % sytox-green uptake 24 h later. Inclusion of DIDS and SITS during oxygen-glucose deprivation dose-dependently protected cultures, with significant protection observed at 250 μM and a reduction in death by >50% at 1000 μM (Fig. 1C). A structurally similar Cl^- blocker, DNDS, applied at concentrations of 250–1000 μM did not significantly alter neurotoxicity caused by oxygen-glucose deprivation (data not shown). DIDS, SITS or DNDS (250–1000 μM) were not neurotoxic on their own (data not shown).

3.2. Effect of DIDS or SITS on NMDA neurotoxicity

NMDA receptor antagonists can protect against oxygen-glucose deprivation so we evaluated if DIDS or SITS protected against NMDA neurotoxicity alone. Application of 40 μM NMDA for 15–20 min caused 82 ± 4% sytox-green uptake. DIDS and SITS dose-dependently protected cultures, with significant protection observed at 250 μM and a reduction in toxicity by 21 ± 3% for SITS and 39 ± 2% for DIDS at 1 mM (Fig. 2A). DNDS and DBDS (Cabantchik and Greger, 1992), as well as a more potent Cl^-

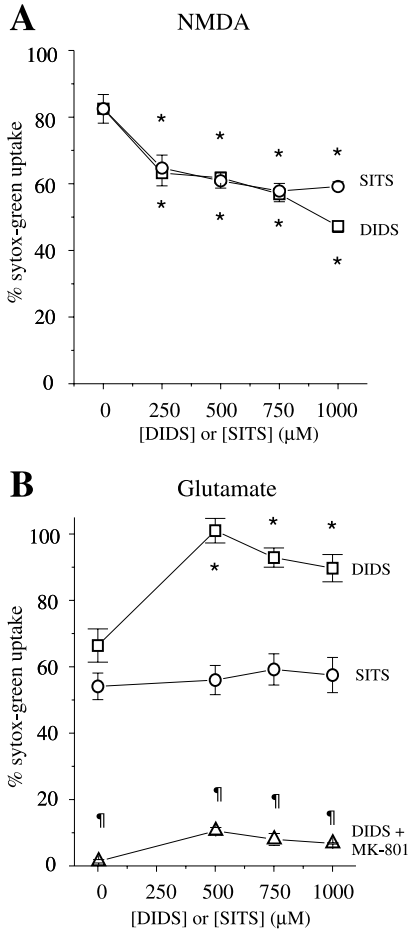

Fig. 2. (A) Dose-dependent protection against a 15–20 min application of 40 μM NMDA by 0.25–1.0 mM DIDS or SITS. (B) Lack of protection against a 15–20 min application of 80 μM glutamate by 0.5–1.0 mM DIDS or SITS. The effect of including the NMDA receptor antagonist, MK-801 (2.5 μM), with glutamate ± DIDS or SITS was also evaluated. In both (A) and (B), neuronal toxicity was evaluated as the % sytox-green uptake measured 24 h later. Data represent the means ± S.E.M. obtained from three to four different platings of neurons, each with four replicates. *Significant ($P<0.05$) difference in the % sytox-green uptake compared to NMDA or glutamate treatment in the absence of DIDS or SITS. ¶Significant ($P<0.05$) difference in the % sytox-green uptake compared to glutamate + DIDS.

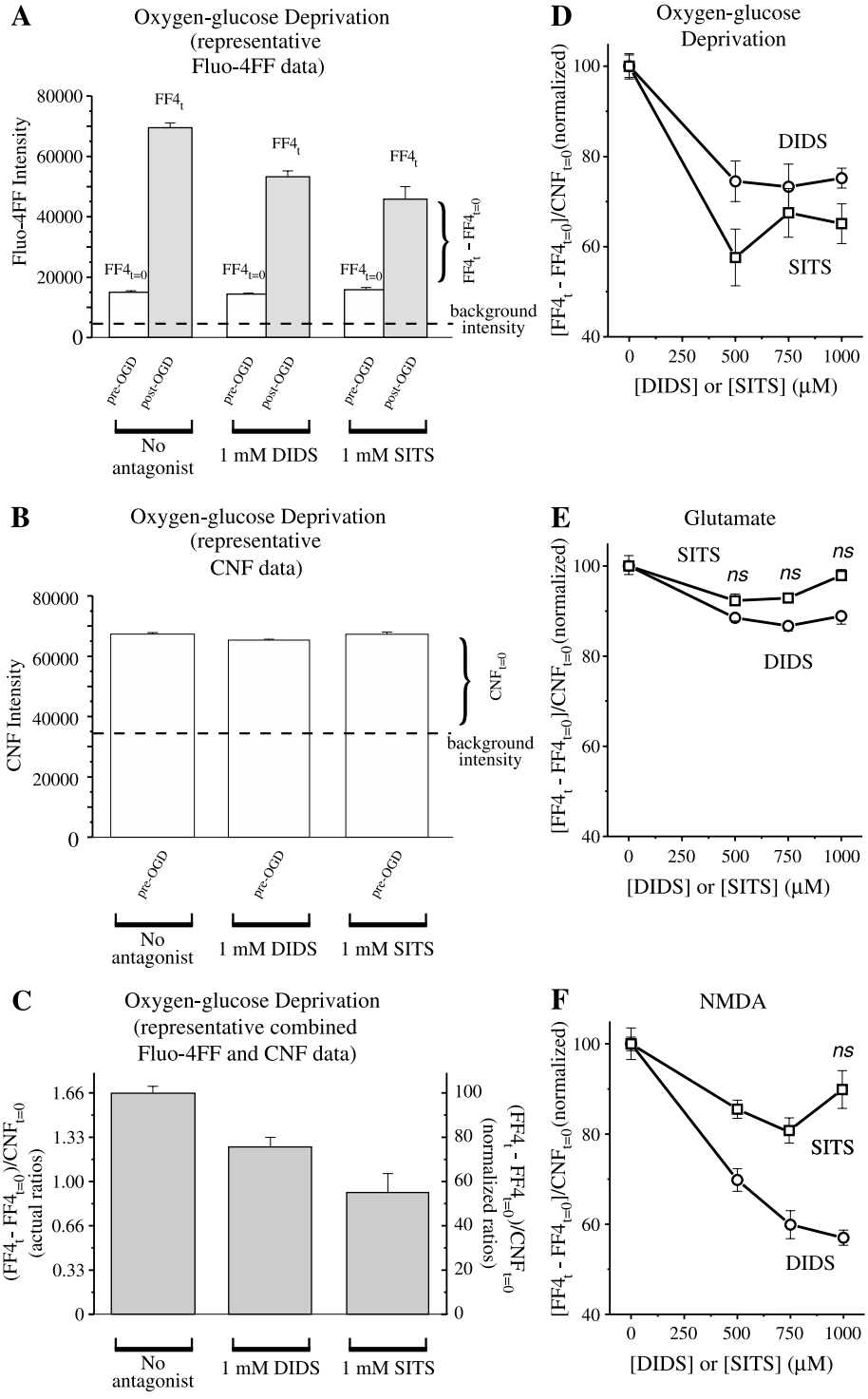

Fig. 3. Effect of DIDS or SITS on oxygen-glucose deprivation-, glutamate- or NMDA-induced increases in Ca^{2+} levels. Cultures in 12-well plates were co-loaded with 4.5 μM of a Ca^{2+} indicator, Fluo-4FF, and 15 μM 5-(and-6)-carboxynaphthofluorescein diacetate (CNF), and fluorescence intensities were measured using a fluorescence microplatereader. (A–C) A typical experiment examining the effect of 1 mM DIDS and 1 mM SITS on oxygen-glucose deprivation-induced increase in intracellular Ca^{2+}. (A) Representative Fluo-4FF fluorescence intensities were acquired pre-oxygen-glucose deprivation (designated as $FF4_{t=0}$), as well as at the end of 85 min oxygen-glucose deprivation ± 1 mM DIDS or 1 mM SITS ($FF4_t$). Dashed lines represent the background signal acquired from cultures containing buffer but no dye. Data represent the means ± S.E.M. obtained from four wells per condition within a single 12-well plate. (B) Representative CNF fluorescence intensities were acquired pre-oxygen-glucose deprivation (designated as $CNF_{t=0}$) from the same wells as in (A). (C) Representative actual and normalized ratios, calculated from the background-corrected intensities depicted in (A) and (B) above, according to the formula $(4FF_t - 4FF_{t=0})/CNF_{t=0}$. Summary of results for (D) $t = 75–90$ min oxygen-glucose deprivation ± 0.5–1.0 mM DIDS or SITS; (E) $t = 15$ min of 80 μM glutamate ± 0.5–1.0 mM DIDS or SITS; and (F) $t = 15$ min of 40 μM NMDA ± 0.5–1.0 mM DIDS or SITS. Data represent the means ± S.E.M. obtained from four different platings of neurons, each with three to four replicates. All values are significantly different ($P < 0.05$) from cultures treated in the absence of DIDS or SITS, unless indicated by *ns* (not significant).

channel blocker (Knauf et al., 1995), $DiBAC_4(5)$, did not significantly protect against NMDA (data not shown).

3.3. Effect of DIDS or SITS on glutamate neurotoxicity

DIDS and SITS have been characterized as glutamate uptake inhibitors (Waniewski and Martin, 1983) so we examined the effect of these drugs on neurotoxicity by exogenous glutamate. Application of 80 μM glutamate for 15–20 min resulted in 66 ± 5% sytox-green uptake, which was significantly augmented by 500–1000 μM DIDS (Fig. 2B). Blockade of the AMPA subtype of glutamate receptor did not prevent this enhancement, since 10 μM CNQX did not significantly alter these neurotoxicity profiles (data not shown). However, blockade of the NMDA receptor with 2.5 μM MK-801 completely prevented toxicity by glutamate ± DIDS (Fig. 2B).

In contrast, 500–1000 μM SITS did not significantly alter neurotoxicity of 15–20 min of 80 μM glutamate (Fig. 2B). CNQX did not alter this profile, while MK-801 was completely protective against glutamate ± SITS (data not shown).

A Cl^- blocker of similar potency, DNDS (Cabantchik and Greger, 1992), had no significant effect on glutamatergic neurotoxicity (data not shown).

3.4. Effect of DIDS or SITS on oxygen-glucose deprivation-, glutamate- or NMDA-induced Ca^{2+} increases

The effect of DIDS or SITS on oxygen-glucose deprivation-induced increases in neuronal Ca^{2+} levels was examined using cultures loaded with Fluo-4FF and CNF. In a typical experiment (Fig. 3A–C), pre-oxygen-glucose deprivation Fluo-4FF and CNF intensities acquired in the absence of drugs (designated $FF4_{t=0}$ and $CNF_{t=0}$, respectively) were ~3× and ~2× background signals acquired from dye- and cell-free buffer alone (Fig. 3A). In general, pre-oxygen-glucose deprivation CNF signals for each well were proportional to the Fluo-4FF signal, suggesting that CNF was a good marker of neuronal density. Relative to pre-oxygen-glucose deprivation Fluo-4FF intensities, oxygen-glucose deprivation (75–90 min) resulted in a ~7-fold increase in Fluo-4FF intensity, designated $FF4_t$, and this increase was attenuated 24% or 45%, respectively, by inclusion of 1 mM DIDS or SITS during oxygen-glucose deprivation (Fig. 3B). Data were processed as the ratio of the following fluorescence intensities, $(FF4_t - FF4_{t=0})/CNF_{t=0}$, and the ratio obtained in the absence of antagonist was normalized to 100 (Fig. 3C).

At concentrations of 500 μM, DIDS and SITS significantly attenuated the oxygen-glucose deprivation-induced increase in intracellular Ca^{2+} levels by 26% and 42%, respectively (Fig. 3D). DIDS and SITS did not alter the fluorescence signals during control wash (data not shown).

A rise in intracellular Ca^{2+} levels, measured following a 15-min exposure to 80 μM glutamate, was significantly attenuated 11–13% by DIDS (500–1000 μM) (Fig. 3E). By contrast, SITS did not significantly diminish the increase caused by glutamate (Fig. 3E).

The rise in neuronal Ca^{2+} levels measured following a 15-min application of 40 μM NMDA was reduced 30–43% by 500–1000 μM DIDS (Fig. 3F). SITS (500–750 μM) significantly attenuated the NMDA-induced increase in $[Ca^{2+}]_i$ by 15–20% (Fig. 3F).

3.5. Effect of DIDS or SITS on NMDA-induced currents

We evaluated if DIDS or SITS suppressed an oxygen-glucose deprivation- or NMDA-induced rise in Ca^{2+} influx by directly blocking the NMDA receptor. Whole-cell NMDA-induced currents were measured in cultured cortical neurons at a holding potential of −60 mV in response to a 2-s application of 10 μM NMDA immediately followed by a 3-s co-application of either DIDS, SITS or DNDS (Fig. 4). DIDS (500 μM) and SITS (500 μM) caused a 22 ± 2% and 16 ± 3% reduction in current amplitude, respectively. The block by DIDS and SITS was rapid and voltage-independent (data not shown). Increasing the concentration of DIDS or SITS to 1 mM further increased the antagonism to 39 ± 2% and 21 ± 3%. In contrast, ≤1 mM DNDS did not cause a significant reduction in current amplitude.

3.6. Effect of DIDS or SITS on extracellular glutamate levels during oxygen-glucose deprivation or exogenous glutamate application

We evaluated if DIDS and SITS resulted in altered extracellular glutamate levels during the neurotoxic maneuvers. Extracellular buffer was collected immediately following exposure of cultures to 0, 500, 750 or 1000 μM SITS or

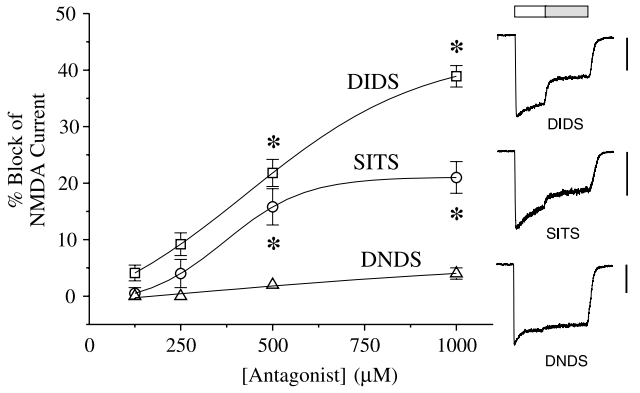

Fig. 4. The effect of three Cl^- blockers, DIDS, SITS and DNDS (0.25–1.0 mM) on whole-cell currents induced by 10 μM NMDA ($n = 5–8$ cells/compound). Significant ($P < 0.05$) blockade was observed at concentrations ≥500 μM for DIDS and SITS, but not for DNDS, at concentrations up to 1 mM. The inserts in the right panel show whole-cell currents in response to a 2-s application of 10 μM NMDA (open bar) immediately followed by a 3-s co-application (light grey bar) of either 1 mM DIDS, SITS or DNDS (top to bottom, respectively). Vertical scale bars indicate 200 pA.

DIDS added during 75–90 min of oxygen-glucose deprivation, 75–90 min normoxic control wash in glucose-free buffer or 15–20 min exposure to 80 μM glutamate. Oxygen-glucose deprivation resulted in an increase in extracellular glutamate concentration >10 μM, while the presence of SITS (500–1000 μM) or DIDS (500–750 μM) during oxygen-glucose deprivation significantly prevented these increases (Fig. 5A). In the controls, SITS and DIDS did not significantly increase extracellular [glutamate] following 75–90 min exposure of cultures to normoxic glucose-free buffer (Fig. 5B).

The concentration of glutamate in the 80 μM glutamate stock (not exposed to cells) was determined to be 78 ± 8 μM, confirming the accuracy of the glutamate assay. The glutamate concentration in buffer collected immediately following 15–20 min exposure of the cultures to 80 μM glutamate was significantly reduced to ≤ 40 μM (Fig. 5C). With the inclusion of 500–1000 SITS or DIDS during exposure of cultures to exogenous glutamate, the extracellular [glutamate] did not significantly vary from this value.

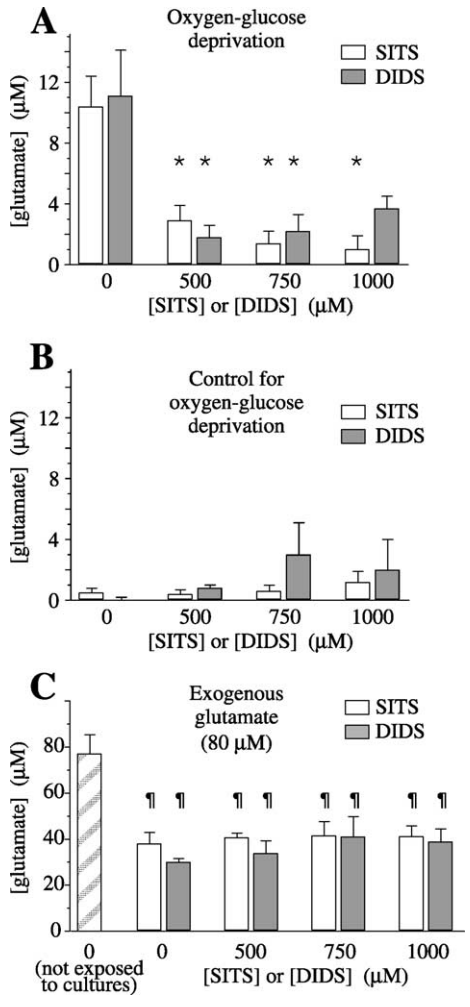

Fig. 5. Measurement of [glutamate] in buffer removed immediately following exposure of cultures to (A) 75–90 min oxygen-glucose deprivation ± 0.5–1.0 mM DIDS or SITS; (B) 75–90 min normoxic control wash in glucose-free buffer ± 0.5–1.0 mM DIDS or SITS or; (C) 15–20 min application of 80 μM glutamate ± 0.5–1.0 mM DIDS or SITS. Data represent the means ± S.E.M. obtained from three different platings of neurons, each with three replicates. *Significant ($P<0.05$) difference in [glutamate] compared to oxygen-glucose deprivation performed in the absence of DIDS or SITS. ¶Significant ($P<0.05$) difference in [glutamate] compared to an 80 μM glutamate stock which was not exposed to cultures.

4. Discussion

The rationale for examining whether DIDS or SITS would protect against oxygen-glucose deprivation was that these compounds may target several points along the neurotoxic cellular signaling cascade. We observed neuroprotection against oxygen-glucose deprivation, but in a manner that is likely independent of their ability to inhibit Cl^- channels or glutamate transporters. Instead, we observed that these compounds are weak NMDA receptor antagonists, which likely underlies a primarily glutamatergic-dependent mechanism of protection.

4.1. Protection against NMDA by DIDS and SITS by blocking the NMDA receptor

NMDA receptor antagonism by DIDS and SITS was verified by the electrophysiological observation of reductions in NMDA-induced currents (Fig. 4). Moreover, there was good agreement in the rank order of potency of DIDS and SITS in blocking NMDA-induced currents, increases in $[Ca^{2+}]_i$ (Fig. 3F), and toxicity (Fig. 2A). This indicates that DIDS and SITS protect against NMDA primarily by blocking the receptor. Reports of efficacy by DIDS or SITS in NMDA receptor-dependent models of toxicity may require re-interpretation (Zeevalk et al., 1989; Guarneri et al., 1998).

4.2. Lack of protection against glutamate by DIDS and SITS

The lack of protection against exogenous glutamate under normoxic conditions by SITS or DIDS (Fig. 2B) contrasted with the neuroprotection against oxygen-glucose deprivation (Fig. 1) and NMDA (Fig. 2A). Thus, a neurotoxic property of SITS, and especially DIDS, evidently overcame their ability to block the NMDA receptor when in the presence of added glutamate. Application of a more potent blocker of the NMDA receptor, MK-801, overcomes toxicity by glutamate + DIDS or SITS, indicating that the neurotoxic pathway ultimately proceeds via an NMDA receptor-linked mechanism.

SITS was not as effective in preventing an increase in intracellular Ca^{2+} by glutamate (Fig. 3E) compared to NMDA (Fig. 3F), which correlates with the protection against NMDA and not against glutamate. DIDS was also not as effective in preventing an increase in intracellular Ca^{2+} by glutamate (Fig. 3E) compared to NMDA (Fig. 3F), consistent with a Ca^{2+}-dependent neurotoxic component in

the presence of glutamate. However, unlike SITS, DIDS substantially exacerbated glutamate-induced toxicity (Fig. 2B), which was not accompanied by a corresponding increase in intracellular Ca^{2+} (Fig. 3E). This finding suggests that DIDS augments glutamate-induced injury by an additional undefined Ca^{2+}-independent mechanism. Glutamate also activates AMPA and metabotropic receptors. We have ruled out the possibility that DIDS or SITS augmented glutamatergic activation of AMPA receptors, since inclusion of CNQX did not prevent injury caused by glutamate ± DIDS or SITS (data not shown). Metabotropic glutamate receptors are structurally and functionally quite complex, with interactions with ionotropic receptors such as NMDA being described (Bruno et al., 2001). Although not investigated here, it is possible that neurotoxic interactions between certain metabotropic receptor subtypes with DIDS and SITS could be prevented by strong NMDA receptor block with MK-801.

In their capacity as inhibitors of glutamate uptake transporters (Waniewski and Martin, 1983), SITS and DIDS slightly augmented glutamate toxicity in cerebellar granule neurons by increasing the extracellular [glutamate] (Ohnishi et al., 1995). In the current study, mild inhibition of glutamate uptake may account for the slight Ca^{2+}-dependent neurotoxic component exerted by SITS and DIDS against glutamate. However, it is apparent that DIDS and SITS are less potent than more commonly employed inhibitors of glutamate uptake in cortical culture. A 1.5-h application of 1 mM DIDS or SITS was not harmful, while 0.5-h exposure to 100 μM DL-TBOA or to another glutamate uptake inhibitor, PDC, was lethal (data not shown). DIDS or SITS alone did not significantly increase extracellular [glutamate] (Fig. 5B,C).

4.3. Protection against oxygen-glucose deprivation by DIDS and SITS

Oxygen-glucose deprivation neurotoxicity in cortical culture results primarily from elevations in extracellular glutamate levels, with consequent Ca^{2+} entry through neuronal NMDA receptors initiating numerous deleterious signaling cascades. Thus, the reduced intracellular Ca^{2+} levels during oxygen-glucose deprivation (Fig. 3A–D) likely represents the primary mechanism of protection by DIDS and SITS. The seemingly paradoxical finding of oxygen-glucose deprivation neuroprotection by DIDS and SITS may be reconciled by the fact that the extracellular [glutamate] during oxygen-glucose deprivation is suppressed (Fig. 5A), thereby not allowing the neurotoxic action of DIDS and SITS to develop.

Reductions in an oxygen-glucose deprivation-induced increase in intracellular Ca^{2+} levels (Fig. 3A–D) by DIDS and SITS are a reflection of lower extracellular [glutamate], as well as direct inhibition of Ca^{2+} influx through postsynaptic NMDA receptors (Figs. 3F and 4). Other Cl^- channel blockers possess NMDA receptor antagonist activity (Lerma and Martin, 1992); however, DIDS and SITS may enjoy a decided advantage as potential therapeutics as a result of these and other neuroprotective properties.

DIDS or SITS may directly attenuate extracellular glutamate levels through several mechanisms. Postsynaptic NMDA receptor blockade enables maintenance of the resting membrane potential, thereby limiting depolarization/Ca^{2+}-dependent glutamate exocytosis. Cerebral ischemia may result in weakening of the electrochemical gradient, possibly culminating in reversal of glutamate transporters (reviewed by Danbolt, 2001). Reversal causes efflux of glutamate, and pharmacological inhibition of glutamate transporters can attenuate extracellular glutamate levels during ischemia in vivo (Seki et al., 1999; Phillis et al., 2000). However, in view of the weak action of DIDS and SITS on glutamate levels (Fig. 5B,C), blocking intracellular glutamate efflux through this pathway is not likely a dominant mechanism during oxygen-glucose deprivation. Glutamate release from astrocytes (Kimelberg et al., 1990a,b; Longuemare et al., 1999) or during cerebral ischemia in vivo (Phillis et al., 1997; Seki et al., 1999) can be inhibited by DIDS and SITS and other Cl^- channel blockers. In contrast, a Cl^--dependent mechanism of inhibiting glutamate release can likely be ruled out in the current study, since other Cl^- blockers did not protect against oxygen-glucose deprivation.

4.4. SITS and DIDS are pleiotropic protective agents

Other glutamate-independent properties may contribute to the neuroprotection against oxygen-glucose deprivation by DIDS or SITS. Anti-excitotoxic actions include inhibition of glycine release (Saransaari and Oja, 2001), augmentation of taurine release (Saransaari and Oja, 1998), or blockage of P2 receptors (Cavaliere et al., 2001). DIDS and SITS, but not DNDS, possess SCN groups which can react with lysine or cysteine residues, and differentially activate other receptors such as K_{ATP} channels (Furukawa et al., 1993), the activation of which can be neuroprotective. Finally, DIDS and SITS are protective in cardiac models of ischemia–reperfusion, attributed to inhibition of mitochondrial release of reactive oxygen species (Vanden Hoek et al., 1998), pH stabilization (Tanaka et al., 1996), and inhibition of fatty acid uptake (Ramasamy et al., 2001).

DIDS or SITS also protect against non-excitotoxic-based neurodegenerative stimuli. Particularly intriguing are the recent findings that SITS and DIDS protect against other neurotoxic stimuli such as the classical apoptotic agent, staurosporine, in cultured cortical (Small et al., 2002) and cerebellar granule neurons (Himi et al., 2002). Consequently, DIDS and SITS may target downstream deleterious cellular signaling that is common between oxygen-glucose deprivation and apoptotic neuronal signaling. In fact, we have identified no other Cl^- channel blockers besides SITS and DIDS that protect against apoptotic stimuli in neuroblastoma cells (O'Reilly et al.,

2002). As cerebral ischemia can culminate in a type of neuronal death which may be a hybrid of apoptosis and necrosis (MacManus and Buchan, 2000), the pleiotropic nature of the neuroprotection by DIDS or SITS provides additional rationale for investigating their neuroprotective properties against ischemia.

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