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Stimulation of *N*-Methyl-D-aspartate Receptor-Mediated Calcium Entry into Dissociated Neurons by Reduced and Oxidized Glutathione

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SUMMARY

The effects of GSH (γ-glutamylcysteinylglycine) and GSSG on intracellular calcium levels ($[Ca^{2+}]_i$) were investigated using fura-2-loaded dissociated brain cells from newborn rat pups. Both produced concentration-dependent increases in $[Ca^{2+}]_i$ (EC_{50} values of 914.3 ± 190.5 and 583.0 ± 97.2 μM for GSH and GSSG, respectively), similar to that observed with *N*-methyl-D-aspartate (NMDA) and other agonists at the NMDA receptor. Maximum response (expressed as percentage change in $[Ca^{2+}]_i$ relative to basal) was significantly greater for GSSG (37.5 ± 1.6%) than for GSH (25.3 ± 1.6%). The response to both agents was prevented or reversed by competitive (100 μM) (−)-2-amino-5-phosphonovalerate and noncompetitive (400 nM) MK-801 or 1.0 mM Mg^{2+} antagonists of NMDA receptor-mediated calcium entry, even at concentrations of GSH and GSSG normally producing maximal response. The idea that these effects are mediated, at least in part, by interaction with the NMDA receptor was supported by the effects of GSH and GSSG on the binding of the NMDA receptor ligand [^{3}H]CGP-39653 to membranes isolated from hippocampal and cortical homogenates. Both GSH and GSSG displaced bound [^{3}H]CGP-39653, with IC_{50} values of 0.93 ± 0.18 and 11.02 ± 1.22 μM, respectively, and produced an increase in the apparent K_d of binding (control, 8.92 ± 0.83 nM, and GSH, 13.31 ± 1.19 nM; control, 11.59 ± 0.35 nM, and GSSG, 18.73 ± 0.66 nM). However, both also produced modest reductions in B_{max} (control, 1265 ± 69 fmol/mg of protein, and GSH, 901 ± 73 fmol/mg of protein; control, 1068 ± 30 fmol/mg of protein, and GSSG, 730 ± 18 fmol/mg of protein) and Hill slopes (GSH, 0.66 ± 0.02; GSSG, 0.62 ± 0.04). This suggests complex kinetics for the interaction of GSH and GSSG with the NMDA receptor. Taken together, the results suggest the potential for modulation of the NMDA receptor complex by GSH and GSSG.

Glutamate is a major excitatory neurotransmitter in the central nervous system (1) that is known to stimulate NMDA receptors as well as other glutamate receptor subtypes. NMDA receptors are thought to be involved in important physiological processes such as neuronal development, learning and memory, and excitotoxicity. Although glutamate itself is thought to be the physiological neurotransmitter for these receptors, specific interactions with γ-glutamyl peptides, several of which are known to exist in the brain (2), have also been reported (3, 4). The endogenous peptide GSH (γ-glutamylcysteinylglycine) is particularly interesting with respect to NMDA receptors, because its constituent amino acids bear a close relationship to those known to participate in NMDA receptor activation. Glycine, for example, is known to be a coagonist for the activation of NMDA receptors by glutamate (5). Moreover, sulfhydryl-reducing agents, such as dithiothreitol, are known to enhance NMDA receptor activation (6). Thus, the sulfhydryl-containing amino acid cysteine within glutathione could conceivably contribute to such an action at the NMDA receptor complex. Furthermore, glutathione is found in concentrations of approximately 1–2 mM in whole brain (7) and is localized predominantly in glial cells (8). Recent evidence suggests that cultured glial cells may release glutathione into the surrounding medium (9).

In this study, we have investigated the effects of GSH and GSSG on NMDA receptor-mediated calcium entry into dissociated neurons. The results presented here suggest that glutathione may have a physiological role in the activation of NMDA receptors in the brain.

Materials and Methods

Isolation of dissociated brain cells and fura-2/acetoxymethyl ester loading. Pregnant Sprague-Dawley female rats were provided

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSS, balanced salt solution; HBH, HEPES-buffered Hanks'; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; APV, (−)-2-amino-5-phosphonovalerate.

through a permanent breeding colony at the Animal Resources Center at The University of Texas at Austin. All experiments were conducted on 1-day-old neonatal rat pups obtained from this breeding colony. Brain cells were isolated from whole brains of rat pups by the method of Raizada *et al.* (10), with some modifications (11). Six pups were used for each experiment. After decapitation, whole brains were removed and placed in a 35-mm culture dish on ice. Each brain was rinsed with ice-cold BSS, pH 7.4, containing (in mM) NaCl, 137; KCl, 5.4; Na_2HPO_4, 0.17; KH_2PO_4, 0.22; glucose, 5.5; and sucrose, 59. Meninges and blood vessels were meticulously removed, and each brain was placed in cold BSS until all dissections were completed.

After a wash step with BSS, each brain was transferred to a glass Petri dish and minced. The minced tissue was placed in a trypsinizing flask containing 8 ml of 0.05% trypsin, 0.5 mM EDTA, in BSS. The brain tissue was stirred gently for 10 min at 37°, with careful monitoring of pH at 7.4. After 10 min of trypsinization, the undissociated tissue was allowed to settle, and the dissociated cells were collected and transferred to a second trypsinization flask containing 10 ml of warm Dulbecco's modified Eagle's medium (high glucose, 4.5 g/liter) plus 10% plasma-derived horse serum and 3.3 μg/ml DNase I. After 5 min of gentle stirring at 37°, the contents of this flask were divided equally into two 10-ml centrifuge tubes, taking care to exclude any small tissue fragments. The cells were centrifuged at 300 × *g* for 5 min. Supernatants were decanted, and the cells were resuspended in a total of 4 ml of warm (37°) HBH, pH 7.4, containing (in mM) HEPES, 20; NaCl, 137; $CaCl_2$, 1.3; $MgSO_4$, 0.4; $MgCl_2$, 0.5; KCl, 5.0; KH_2PO_4, 0.4; Na_2HPO_4, 0.6; $NaHCO_3$, 3.0; and glucose, 5.6. Cell suspensions taken for counting and trypan blue staining consistently showed 90–95% cellular viability (11).

The cell suspensions were further diluted to a total of 8 ml with 37° HBH, divided into two 4-ml aliquots, and placed in a Dubnoff shaking water bath for 5 min at 37°. A final concentration of 5 μM fura-2/acetoxymethyl ester in dimethyl sulfoxide was added to one aliquot and the same volume of dimethyl sulfoxide was added to the other aliquot of cells, which served as a control. The cell suspensions were returned to the shaking water bath for 45 min. The fura-2-loaded cells and the control cells were each transferred to centrifuge tubes and diluted with 4 ml each of 37° Mg^{2+}-free HBH. After a 5-min centrifugation at 300 × *g*, the supernatant was decanted and the pellet was resuspended in a volume of 37° Mg^{2+}-free HBH, which resulted in approximately 3.5 × 10^6 cells/2 ml. Cells were used for approximately 60 min after fura-2 loading.

A SPEX Fluorolog spectrophotofluorometer (model CM2 cation measurement system), with a water-jacketed cuvette compartment maintained at room temperature, was used for fluorescence determinations. Suspensions of dissociated cells were kept at room temperature. For each sample, a 2-ml aliquot was placed in polystyrene cuvettes and incubated for 2 min at 37° before fluorescence measurements. Cells were then placed in the cell compartment, and fluorescence intensity was scanned for 3 min. Basal resting levels were established after 50 sec, at which time GSH/GSSG addition was made. A magnetic stir bar was placed in each cuvette, to keep cells from settling and to provide an adequate mixing of drugs.

GSH/GSSG concentration-response and antagonist studies. Concentration-effect experiments on dissociated cells were conducted with GSH or GSSG (0.125–8.0 mM). Each 2-ml sample containing approximately 3.5 × 10^6 cells was warmed for 2 min in a 37° water bath and subsequently monitored with dual monochromators (excitation at 340 nm and 380 nm, emission at 505 nm) for 50 sec, to determine resting $[Ca^{2+}]_i$. Resting levels recorded were the average $[Ca^{2+}]_i$ over this 50-sec period. GSH or GSSG was then added directly into the cuvette, in a volume of 20 μl. After 50 sec had passed, allowing for a plateau in the response, another 50-sec average was taken and recorded as the stimulated value. $\Delta[Ca^{2+}]_i$ was then determined by subtracting the resting value from the stimulated value. Percentage of $\Delta[Ca^{2+}]_i$ was calculated by dividing each $\Delta[Ca^{2+}]_i$ by its respective resting value and multiplying by 100. The percentage $\Delta[Ca^{2+}]_i$ values were analyzed using the computer program ALLFIT (12), to generate the best fitting logistic concentration-response curves and to make statistical comparisons between the GSSG curve and the GSH curve. Additional studies were conducted with the NMDA antagonists Mg^{2+} (1 mM), D-APV (100 μM), and MK-801 (400 nM). The purpose of these studies was to test for the abilities of the compounds to prevent and reverse GSH- and GSSG-induced increases in $[Ca^{2+}]_i$. Each 2-ml sample containing approximately 3.5 × 10^6 cells was monitored, as described above, for 50 sec, to determine resting $[Ca^{2+}]_i$. Antagonists were then added 50 sec before or after the addition of 2 mM GSH or GSSG. Drug injection volumes of 20 μl each were added directly into the cuvette while monitoring continued. Cytosolic Ca^{2+} values were measured every second and averages were recorded every 3 sec. $\Delta[Ca^{2+}]_i$ and percentage increases in $[Ca^{2+}]_i$ were determined as described above. Statistical analyses were determined by analysis of variance with repeated measures, followed by a Newman-Keuls *post hoc* analysis.

Calcium concentration-response studies were also conducted in which 2 mM GSH was tested against 0–1600 μM calcium. The purpose of these studies was to determine the extent to which increased $[Ca^{2+}]_i$ produced by GSH was dependent on extracellular calcium. For these studies, dissociated cells were isolated in calcium-free medium and then incubated with 0, 50, 100, 200, 400, 800, or 1600 μM calcium for 2 min. GSH (2 mM) was then added, and the change in $[Ca^{2+}]_i$ was then measured.

Fluorescence parameter measurements and experimental calculations. $[Ca^{2+}]_i$ was determined according to the method of Grynkiewicz *et al.* (13), using a K_d of 224 nM: $[Ca^{2+}]_i = K_d \times S_{f/2}/S_{b/2}\,(R - R_{min}/R_{max} - R)$. Parameter determinations and calculations were as follows. Maximum fluorescence (R_{max}) was determined by exposing fura-2-loaded and non-fura-2-loaded cells to 0.1% sodium dodecyl sulfate and 4 mM $CaCl_2$. Fluorescence intensity at 340 and 380 nm in non-fura-2-loaded cells was subtracted from that in fura-2-loaded cells. The resulting net ratio of 340/380-nm fluorescence was used for R_{max}.

Minimum fluorescence (R_{min}) was determined by exposing fura-2-loaded and non-fura-2-loaded cells to minimum fluorescence conditions of 0.1% sodium dodecyl sulfate, zero calcium, and 5 mM EGTA. Fluorescence intensity at 340 and 380 nm in non-fura-2-loaded cells was subtracted from that in fura-2-loaded cells. The resulting net ratio of 340/380-nm fluorescence was used for R_{min}.

The R value was determined by quenching the extracellular fura-2-Ca^{2+} signal in each experimental sample with 40 μM Mn^{2+} at the end of each scan. The net decrease in fluorescence intensity at 340 nm was taken as the Mn^{2+} correction. The Mn^{2+} correction was added to the 340-nm fluorescence intensity of non-fura-2-loaded cells, and the sum was used as a 340-nm correction factor. The 340-nm correction factor was subtracted from the 340-nm fluorescence intensity of all experimental samples. The 380-nm fluorescence intensity of non-fura-2-loaded cells was used as a 380-nm correction factor for the 380-nm fluorescence of all experimental samples. The ratio of 340/380-nm fluorescence intensity after correction factor subtraction was used as the R value. The final 380-nm R_{min} value was used as $S_{f/2}$ and the final 380-nm R_{max} value was used as $S_{b/2}$.

[^{3}H]CGP-39653 binding assay. The hippocampus and cortex were isolated from male Sprague-Dawley rats (250–300 g) after decapitation. The tissue was prepared for [^{3}H]CGP-39653 (specific activity, 37.2–51.3 Ci/mmol) binding assays according to the method of Sills *et al.* (14). Briefly, the tissue was homogenized in 50 volumes of 5 mM Tris·HCl, pH 8, at 4° with a Polytron (Tekmar Tissuemizer rheostat setting 35; 20 sec). The membranes were then centrifuged at 48,000 × *g* for 10 min. The pellets were resuspended in 50 volumes of cold Tris·HCl containing 10 mM EDTA. The resuspension was incubated at 37° for 10 min and then centrifuged at 48,000 × *g* for 10 min. The pellet was resuspended in 50 volumes of Tris·HCl and centrifuged. The final pellet was resuspended in 50 volumes of Tris·HCl and stored in the freezer (−20°) for 4–10 days.

On the day of assay, the tissue was thawed and then centrifuged for 10 min at 48,000 × *g*. The pellet was resuspended in Tris·HCl and

centrifuged for 10 min at 48,000 × g two additional times. The final pellet was resuspended in 12.5 volumes of Tris·HCl, pH 8, at 4°.

Saturation assays were performed using concentrations of [^{3}H]CGP-39653 from 0.47 to 60 nM. In the control experiments, total binding tubes contained 100 μl of [^{3}H]CGP-39653 and 800 μl of buffer. Nonspecific binding tubes contained 100 μl of [^{3}H]CGP-39653, 700 μl of buffer, and 100 μl of glutamate (100 μM final concentration). Experimental samples containing GSH (1 μM) or GSSG (10 μM) (final concentration) were added in 100-μl aliquots to both total and nonspecific binding tubes, with an appropriate reduction in buffer to yield a final volume of 1 ml. The binding reaction was initiated with the addition of 100 μl of tissue to each tube.

In the competition experiments, 100-μl aliquots of varying concentrations of GSH (0.1 nM to 3.0 mM) or GSSG (0.01 nM to 10 mM) were added to tubes containing 700 μl of buffer and 100 μl of [^{3}H]CGP-39653 (30 nM). The reaction was initiated by the addition of 100 μl of tissue to each tube, to yield a final volume of 1 ml.

After the addition of the tissue, the binding reaction was continued for 1–2 hr at 0°. Bound radioactivity was obtained after a vacuum filtration of the contents of each sample on a Schleicher and Schuell no. 32 glass fiber filter, using a Brandel cell harvester. Three 5-ml washes with cold Tris·HCl were used. Filters were placed in scintillation vials, and 5 ml of Ready Value were added. The radioactivity was determined using liquid scintillation counting, after the filters had equilibrated with the cocktail overnight. Protein values were determined using the Lowry protein assay (15), with bovine serum albumin as the standard.

Analysis of glutathione purity. The purity of GSH and GSSG (Sigma Chemical Co.) used in this study was tested at the Protein Sequencing Center at the University of Texas. The Applied Biosystems derivatizer-analyzer system at the Protein Sequencing Center was used to form phenylthiocarbamyl-amino acid derivatives of GSH and any other amino acid that might contaminate the commercial GSH preparation. Derivatized amino acids were separated by high performance liquid chromatography, using a reverse phase C18 bonded-phase silica column, and detection of amino acids was accomplished with a flow cell from the column to a fluorescence detector.

Measurement of γ-glutamyl transpeptidase activity. A 20-μl aliquot containing 5 × 10^7 dissociated cells was diluted to a total incubation volume of 0.5 ml, and γ-glutamyl transpeptidase activity was measured as described in Sigma Technical Bulletin 545.

Results

Both GSSG and GSH produced a concentration-dependent increase in $[Ca^{2+}]_i$. The least-squares best fitting curves (12) are shown in Fig. 1. GSSG produced a greater stimulation of $[Ca^{2+}]_i$ than did GSH. ALLFIT analysis (Table 1) showed that the maximum response produced by GSSG was significantly greater than that produced by GSH. EC_{50} values for the GSSG and GSH curves were not different, nor were the slopes or the minimum responses produced by GSSG and GSH. Fig. 2 shows the results of a typical experiment in which the change in $[Ca^{2+}]_i$ over time was monitored after the addition of 2 mM GSH. The rate of calcium entry after the addition of 2 mM GSH was similar to that previously observed in dissociated neurons after the addition of 50 μM NMDA (16). The magnitude of GSH and GSSG enhancement of $[Ca^{2+}]_i$ was also similar to maximum responses produced by NMDA in the dissociated neuron preparation. As shown in Table 2, 2 mM GSSG and GSH produced a 113.5 ± 10.2 nM and 55.3 ± 9.6 nM increase in $[Ca^{2+}]_i$, respectively. Maximum responses to NMDA in this preparation resulted in an approximately 120 nM increase in $[Ca^{2+}]_i$ (11).

Fig. 3 shows the results of calcium concentration-response studies with 2 mM GSH. The purpose of these studies was to

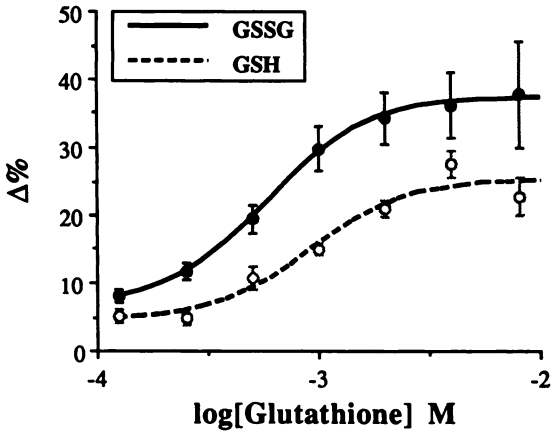

Fig. 1. Concentration-effect curves for GSSG- and GSH-mediated increases in $[Ca^{2+}]_i$. [GSSG] and [GSH] = 125 μM to 8 mM. Percentage change in $[Ca^{2+}]_i$ was calculated as described in Materials and Methods. *Curves* shown were generated by the computer program ALLFIT. Each data point for the GSSG curve represents the mean ± standard error of 11–23 experiments, whereas those of the GSH curve are the mean ± standard error of seven to 20 experiments.

TABLE 1

Concentration-effect curve parameters for GSSG- and GSH-mediated increases in cytosolic Ca^{2+}

Parameters were estimated by fitting the concentration-effect curve data plotted in Fig. 1 to a logistic equation, using the unweighted fitting routines in the computer program ALLFIT. Differences between the individual parameters of the GSH and GSSG curves were tested by comparing the residual of fits sharing the parameters across the two curves with the residual of the completely unconstrained fit. Values shown are means ± standard errors determined by ALLFIT.

Parameter	GSSG	GSH
EC_{50} (μM)	583.0 ± 97.2	914.3 ± 190.5
Maximum (% change)[a]	37.5 ± 1.6	25.3 ± 1.9[b]
Slope	1.9 ± 0.6	2.0 ± 0.8
Minimum (% change)[a]	6.5 ± 3.1	4.4 ± 2.1

[a] Percentage change in internal calcium ion concentration, compared with resting levels.

[b] Significantly different from the maximum response for GSSG ($p < 0.05$).

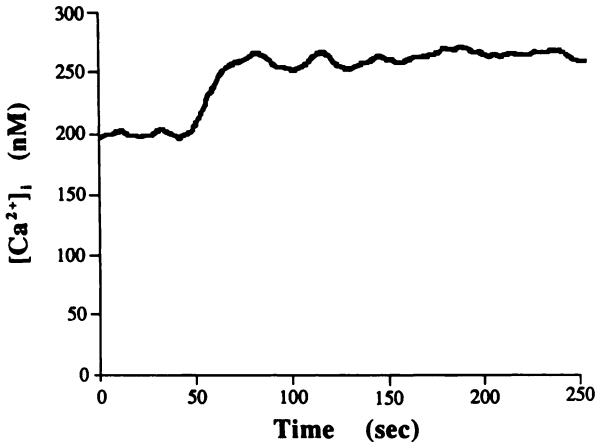

Fig. 2. Effects of GSH on a sample of dissociated brain cells. Data represent changes in $[Ca^{2+}]_i$ in response to 2 mM GSH in a sample of dissociated cells (3.5 × 10^6 cells/2 ml). The time course of the GSH-stimulated $[Ca^{2+}]_i$ increase was followed at 340- and 380-nm excitation and 505-nm emission, and nanomolar $[Ca^{2+}]_i$ was calculated as described in Materials and Methods. GSH was added directly to the sample cuvette at the 50-sec time point and after a stable resting $[Ca^{2+}]_i$ base line was attained.

TABLE 2

Effect of Mg^{2+}, APV, and MK-801 on glutathione-mediated increases in $[Ca^{2+}]_i$

Two sets of experiments were performed, one involving GSSG and the other GSH. Each 2-ml sample, containing approximately 3.5×10^6 cells, was initially monitored for 50 sec, to determine $[Ca^{2+}]_i$ at rest. An NMDA antagonist, either Mg^{2+} (1.0 mM), APV (100 μM), or MK-801 (400 nM), was then added either 50 sec before or 50 sec after the addition of 2 mM GSSG or GSH. Final $[Ca^{2+}]_i$ was determined approximately 50 sec after the final addition. $\Delta[Ca^{2+}]_i$ represents the net change in $[Ca^{2+}]_i$ after subtracting resting levels; values represent mean ± standard error increases in $[Ca^{2+}]_i$ of three experiments. Percentage of change values were calculated by dividing each $\Delta[Ca^{2+}]_i$ by its respective resting value and then multiplying by 100. Analysis of variance with repeated measures and Newman-Keuls *post hoc* analysis were run on actual $[Ca^{2+}]_i$ (nM) values.

Addition at 50 sec	Addition at 100 sec	$\Delta[Ca^{2+}]_i$			
		GSSG (2 mM)		GSH (2 mM)	
		nM	%	nM	%
Glutathione		113.5 ± 10.2	48.7 ± 2.0	55.3 ± 9.6	29.8 ± 5.1
Mg^{2+}	Glutathione	4.0 ± 4.5*	2.1 ± 2.4*	5.0 ± 3.0*	2.8 ± 1.4*
Glutathione	Mg^{2+}	20.0 ± 11.4*	12.3 ± 7.0*	11.7 ± 2.9*	6.0 ± 1.5*
APV	Glutathione	−0.3 ± 4.7*	0.3 ± 2.8*	4.7 ± 1.5*	3.0 ± 1.0*
Glutathione	APV	31.3 ± 2.0*	16.0 ± 1.8*	16.5 ± 0.5	9.8 ± 0.6
MK-801	Glutathione	−1.0 ± 7.0*	−0.2 ± 3.7*	7.7 ± 2.4*	3.9 ± 0.9*
Glutathione	MK-801	57.7 ± 3.3*	32.1 ± 4.6*	29.0 ± 6.2	15.3 ± 1.8

* Significance at the $p < 0.05$ level, compared with glutathione addition alone.

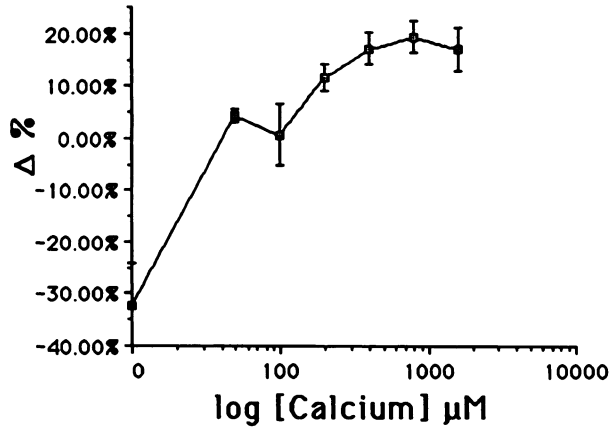

Fig. 3. Concentration-effect curve for the calcium dependence of GSH-mediated increases in $[Ca^{2+}]_i$. Dissociated cells were preincubated for 2 min with 0, 50, 100, 200, 400, 800, or 1600 μM calcium. GSH (2 mM) was then added, and the peak effect on $[Ca^{2+}]_i$ was determined. Percentage change in $[Ca^{2+}]_i$ was calculated as described in Materials and Methods. The results represent the mean ± standard error of three experiments.

determine the extent to which changes in $[Ca^{2+}]_i$ in response to GSH are dependent on extracellular calcium. The results show that GSH did not increase $[Ca^{2+}]_i$ in the absence of extracellular calcium. On the contrary, $[Ca^{2+}]_i$ was decreased when 2 mM GSH was added to cells preincubated in the absence of extracellular calcium. The decreased $[Ca^{2+}]_i$ was completely reversed upon the addition of 50 μM calcium before the addition of GSH. The significance of the apparent decrease in $[Ca^{2+}]_i$ caused by GSH in the absence of extracellular calcium is not clear.

GSH (γ-glutamylcysteinylglycine) contains three amino acids, which have been demonstrated to interact individually with glutamate receptors of the NMDA type. GSSG is composed of two molecules of glutathione connected by a disulfide bond. Thus, studies were conducted to test the possibility that GSH and/or GSSG may increase $[Ca^{2+}]_i$ by interacting with NMDA receptors. Table 2 shows the results of experiments that examined the effects of known antagonists of NMDA receptor function on GSH- and GSSG-stimulated $[Ca^{2+}]_i$. In agreement with the results shown in Fig. 1, Table 2 shows that GSSG produced a greater stimulation of $[Ca^{2+}]_i$ than did GSH. Pretreatment of dissociated neurons with the noncompetitive NMDA antagonist Mg^{2+} (1 mM) for 50 sec before the addition of glutathione resulted in a complete inhibition of GSSG- and GSH-stimulated $[Ca^{2+}]_i$. To study further the effects of Mg^{2+}, glutathione was added first, followed by Mg^{2+} after 50 sec, when the peak GSSG or GSH response had occurred. This resulted in the reversal of both GSSG- and GSH-stimulated $[Ca^{2+}]_i$. D-APV is a potent and selective competitive antagonist of the NMDA receptor (17). Table 2 shows that exposure of dissociated neurons to APV (100 μM) prevented GSSG- and GSH-stimulated $[Ca^{2+}]_i$. Furthermore, addition of APV after GSSG and GSH had produced their maximal increase in $[Ca^{2+}]_i$ resulted in a reversal of the glutathione response. Table 2 also shows the results of similar experiments with MK-801, a noncompetitive antagonist that binds to the phencyclidine site in the ion channel of the NMDA receptor (18). Exposure of dissociated neurons to MK-801 (400 nM) prevented GSSG or GSH stimulation of $[Ca^{2+}]_i$. As with the other antagonists, MK-801 also reversed the elevation of $[Ca^{2+}]_i$ produced by GSSG or GSH, although the reversal of the $[Ca^{2+}]_i$ increase appeared to be less complete than that observed with Mg^{2+} or APV. D-APV, Mg^{2+} and MK-801 had no effect on resting $[Ca^{2+}]_i$ before glutathione addition.

Because the individual amino acid components of GSH and GSSG are known to be agonists or coagonists at the NMDA receptor, it is possible that presence of amino acid impurities in the purchased material or enzymatic production of free glutamate may have been responsible for stimulation of $[Ca^{2+}]_i$. GSH (Sigma Chemical Co.) eluted with a retention time of 11.65 min in a single peak. A small peak observed at 6.75 min was found to represent approximately 8% GSSG. Stock preparations of glutathione (prepared as 2 mM solutions) were assayed for possible glutamate contamination. Ten microliters of a 2 mM glutathione solution were assayed with a detection sensitivity for glutamate and aspartate of 15 pmol. Thus, the assay would have detected, at the lower limits of sensitivity, 0.075% glutamate or aspartate contamination. No glutamate or aspartate was detected. In addition, the 2-ml cell suspension samples were assayed for glutamate and aspartate before and after 100 sec of exposure to glutathione. The cells were filtered, and the media were assayed for glutamate and aspartate. Again, no glutamate was detected. The GSSG (Sigma Chemical Co.) used in this study was also tested for purity and was found to contain approximately 5% GSH. The dissociated cell prepara-

tion was also analyzed for γ-glutamyl transpeptidase activity. γ-Glutamyl transpeptidase is known to metabolize glutathione by cleaving glutamate from the tripeptide. There was no detectable γ-glutamyl transpeptidase activity in the dissociated cell preparation. Thus, it is highly unlikely that the enhancement of $[Ca^{2+}]_i$ occurred as a result of a contaminant or metabolite of glutathione.

Receptor binding studies were consistent with an action of glutathione at the NMDA site. First, both GSH and GSSG displaced [^{3}H]CGP-39653 (30 nM) binding, with IC_{50} values of 0.98 ± 0.18 and 11.26 ± 1.37, respectively (Fig. 4). Second, 1 μM GSH and 10 μM GSSG significantly increased the apparent K_d for binding, consistent with a competitive interaction. However, both GSH and GSSG also produced a modest decrease in the B_{max} for [^{3}H]CGP-39653 binding, of approximately 30% (Table 3). In addition, Hill slopes were significantly less than 1 for displacement of [^{3}H]CGP-39653 binding by both GSH and GSSG (0.66 ± 0.02 and 0.62 ± 0.04, respectively). This suggests that more complex phenomena may contribute to the pattern of binding than can be explained by a purely competitive type of interaction.

Finally, saturation curves describing [^{3}H]CGP-39653 binding in the present study were consistent with the initial characterization of this ligand by Sills *et al.* (14). In the present study, Scatchard plots were linear ($r^2 = 0.96$). K_d values of 6.2 ± 0.6 nM were reported by Sills *et al.* (14), compared with 8.92 ± 0.83 and 11.59 ± 0.35 nM in our experiments (Table 3). B_{max} values reported by Sills *et al.* (14) were 970 ± 220 fmol/mg of protein, compared with B_{max} values in the present report of 1265 ± 69 and 1068 ± 30 fmol/mg of protein (Table 3).

Discussion

Glutathione is known primarily for its role as the most abundant antioxidant/free radical scavenger in the body. It is found in high concentrations, approximately 2 mM, in the brain (7). Furthermore, glutathione is localized almost exclusively in glial cells (8, 19), although it may also exist in nerve terminals and some neurons (7, 19). Concentrations ranging from 8 to 20 mM have been reported in cultured astrocytes (9). Oxidized glutathione levels were found to be 1.2% or less of the total glutathione (7). Thus, glutathione exists almost exclusively in the reduced form in brain. The physiological implications of these observation are that neurons have a relatively low antioxidant defense mechanism, which may account for their fragility, and that glial cells may serve to protect neurons through the release of glutathione (8, 9).

The results of the present investigation, as well as previous reports by others (3, 4), suggest that glutathione may also be involved functionally in the activation of glutamate receptors in the brain. Our experiments focused specifically on the ability of GSH and GSSG to interact with the glutamate binding site of the NMDA receptor. Both GSH and GSSG stimulated calcium influx, in a concentration-dependent manner, across a physiological range of 125 μM to 8 mM. The competitive NMDA antagonist APV blocked and reversed GSH- and GSSG-stimulated calcium entry into dissociated neurons. The noncompetitive antagonists Mg^{2+} and MK-801 blocked the response in a predicted manner. Moreover, GSH and GSSG displaced binding of the specific NMDA receptor ligand [^{3}H]CGP-39653, in a competitive manner, at concentrations considerably less (IC_{50} values of 1 and 10 μM, respectively) than those required to stimulate calcium influx. However, the B_{max} values for [^{3}H]CGP-39653 binding were reduced by both GSH and GSSG in equilibrium binding studies. Furthermore, Hill slopes were significantly less than 1 for displacement of [^{3}H]CGP-39653 binding by both GSH and GSSG. These observations suggest that the interaction of the peptide with the NMDA receptor may occur in a more complex fashion than can be described by simple competition with the NMDA site. One explanation for the apparent decrease in B_{max} for [^{3}H]CGP-39653 binding may be that binding of glycine and cysteine within the glutathione molecule to sites (e.g., glycine binding site and a sulfhydryl binding site for cysteine) on the NMDA receptor complex may produce an allosteric alteration of the competitive binding site of the receptor complex, thus reducing maximal binding.

GSSG was slightly more potent than GSH in stimulating calcium entry. Furthermore, the elevation of $[Ca^{2+}]_i$ by GSH and GSSG was equally sensitive to inhibition by Mg^{2+}, APV, and MK-801. Thus, in contrast to recent evidence that sulfhydryl-reducing agents, such as dithiothreitol, enhance NMDA receptor activation (6), the results of the present study suggest that the cysteinyl SH groups of glutathione may not contribute significantly to its actions on the NMDA receptor.

These findings, along with those of Yudkoff *et al.* (9) demonstrating release of glutathione from astrocytes, suggest that glutathione may have a physiological role in activation or modulation of NMDA receptors. Competitive binding studies described in the present report show that GSH and GSSG displace the binding of 30 nM [^{3}H]CGP-39653 with IC_{50} values

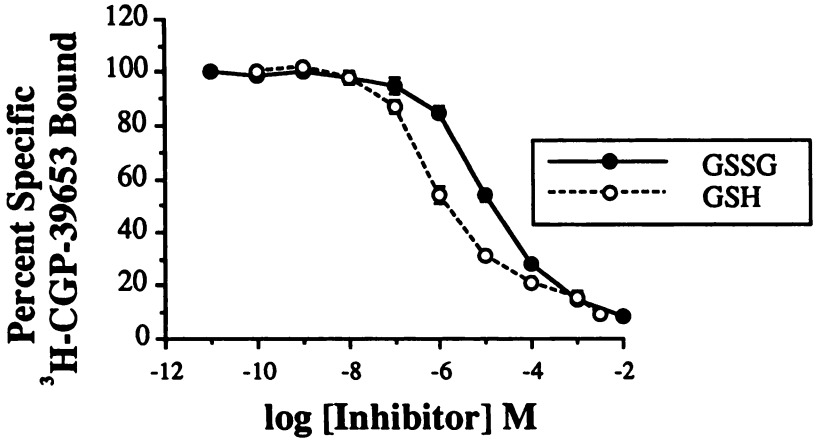

Fig. 4. GSH and GSSG displacement of [^{3}H]CGP-39653 binding. Competition curves are for 30 nM [^{3}H]CGP-39653 in adult rat combined cortical and hippocampal brain regions. These data represent four experiments (mean ± standard error) performed in duplicate. The IC_{50} for GSH was 0.93 ± 0.18 μM, and the IC_{50} for GSSG was 11.02 ± 1.22 μM. The IC_{50} values for GSH and GSSG were significantly different ($p < 0.05$), as measured by the paired *t* test.

TABLE 3

Alteration of the binding parameters for [^{3}H]CGP-39653 by glutathione

Binding parameters were generated from adult rat brain cortical and hippocampal tissues. Saturation experiments were performed using [^{3}H]CGP-39653 in a concentration range of 0.47–60 nM. r^2 values were >0.90. Data represent results of six experiments (mean ± standard error) performed in duplicate.

Condition	K_d	B_{max}
	nM	fmol/mg
Control	8.92 ± 0.83	1265 ± 69
GSH (1 μM)	13.32 ± 1.19[a]	901 ± 73[a]
Control	11.59 ± 0.35	1068 ± 30
GSSG (10 μM)	18.73 ± 0.66[a]	730 ± 18[a]

[a] Significant ($p < 0.05$) change versus respective control, using analysis of variance with a split plot design, followed by simple effect tests. The control values for the GSH and GSSG experiments were not significantly different.

of approximately 1 μM and 10 μM, respectively, suggesting apparent K_d values for the two forms of the peptide of 0.23 and 2.29 μM (20). EC_{50} concentrations of GSH and GSSG for increasing $[Ca^{2+}]_i$ in dissociated neurons were approximately 900 μM for GSH and 600 μM for GSSG. Thus, a difference of approximately 4000-fold for GSH and 300-fold for GSSG exists in their ability to displace [^{3}H]CGP-39653 binding, compared with stimulation of calcium influx. This difference appears to be considerably less (approximately 2–4-fold) for NMDA, which displaces [^{3}H]CGP-39653 binding with a K_i of 3.66 μM (14) and stimulates calcium entry into dissociated neurons with an EC_{50} of approximately 7–16 μM (11, 16). Thus, physiologically, low micromolar concentrations of glutathione released from glial cells may serve to modulate glutamate binding to NMDA receptors in such a way as to regulate, modify, or prevent neuronal glutamate responses. High micromolar concentrations, on the other hand, may activate NMDA receptors directly to increase intracellular calcium. Glutathione (4) and other γ-glutamyl peptides found in the brain (3) have been reported to alter [^{3}H]glutamate binding in the mammalian nervous system. Furthermore, recent work by Levy *et al.* (21) demonstrates that glutathione prevents NMDA receptor-mediated toxicity in rat retinal ganglion cells. Taken together, these findings suggest that glutathione, as well as other glutamate-containing peptides, may regulate and/or activate other glutamate receptors in addition to NMDA receptors.

Because glutathione is localized predominantly in glial cells, the finding that glutathione may regulate or activate NMDA receptors raises the question of the physiological role that glial cells may play in the regulation of functions thought to be linked with NMDA receptor activation. NMDA receptor activation appears to play a role in neuronal development (22, 23). Recent evidence has shown that, during neuronal development, central nervous system neurons migrate on astroglial fibers (24). The migration of neurons occurs on highly elongated astroglial processes and is characterized by the movement of the neuronal cell body along the glial projections, rather than neurite outgrowth (24). Silver *et al.* (25) reported that axonal guidance during development of the great cerebral commissure is directed by a glial "sling," which forms a bridge-like structure along which neurons migrate toward the contralateral side of the brain. The cellular mechanism(s) involved in astroglial control of neuronal migration are not known. However, it is tempting to speculate, in light of the findings of the present investigation, that glutamate-containing peptides elaborated from glial cells, such as glutathione, may play some role in these processes through an interaction with neuronal NMDA receptors. Consistent with the suggestion that glutathione may play some role in neuronal development are the findings that inborn errors of glutathione metabolism and glutathione synthetase deficiency are accompanied by mental retardation and severe brain damage (26, 27) (see also Ref. 8). Irregularities associated with NMDA receptor activation could contribute to these developmental abnormalities. In addition, such developmental abnormalities could also be due to the diminished contribution of glutathione to the maintenance of redox homeostasis.

NMDA receptor activation may also be linked with long term potentiation, a process very likely involved in learning and memory (28–30) and excitotoxicity (31), which may mediate such processes as neuronal injury in hypoxia-ischemia (32–34) and sustained seizures (35). Regulation and/or activation of NMDA receptors by glutathione and other γ-glutamyl peptides in the brain may also play a role in these processes.

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