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Searching for Mechanisms of *N*-Methyl-D-Aspartate-Induced Glutathione Efflux in Organotypic Hippocampal Cultures*

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Abstract

N-Methyl-D-aspartate (NMDA)-receptor stimulation evoked a selective and partly delayed elevated efflux of glutathione, phosphoethanolamine, and taurine from organotypic rat hippocampus slice cultures. The protein kinase inhibitors H9 and staurosporine had no effect on the efflux. The phospholipase A₂ inhibitors quinacrine and 4-bromophenacyl bromide, as well as arachidonic acid, a product of phospholipase A₂ activity, did not affect the stimulated efflux. Polymyxin B, an antimicrobial agent that inhibits protein kinase C, and quinacrine in high concentration (500 μM), blocked efflux completely. The stimulated efflux after but not during NMDA incubation was attenuated by a calmodulin antagonist (W7) and an anion transport inhibitor (DNDS). Omission of calcium increased the spontaneous efflux with no or small additional effects by NMDA. In conclusion, NMDA receptor stimulation cause an increased selective efflux of glutathione, phosphoethanolamine and taurine in organotypic cultures of rat hippocampus. The efflux may partly be regulated by calmodulin and DNDS sensitive channels.

Keywords

NMDA; glutathione; phosphoethanolamine; taurine; efflux

INTRODUCTION

Acute cerebral insults such as ischemia and brain trauma are associated with excessive release and extracellular accumulation of the neurotransmitter glutamate (1), leading to persistent activation of glutamate receptors and neurodegeneration (2–5). The glutamate receptor mediated increase of reactive oxygen species (ROS) (6–8) may be a triggering event in the excitotoxic cell death (9–12). The reduced form of glutathione (GSH) is a major component in cellular protection against ROS (for reviews see (13–17)). Glutathione is also essential for maintenance of the thiols of proteins and of other antioxidants, for example, ascorbate and α-tocopherol (18,19). A decreased glutathione level, through inhibition of its synthesis, is accompanied by increased excitotoxic response to NMDA, degeneration of mitochondria, and larger infarct areas in stroke models (20–22). Extracellularly, glutathione has been suggested to have multifaceted electrophysiological effects by binding to its own receptors and by modulating glutamatergic excitatory neurotransmission by displacing glutamate from its ionotropic receptors (23–25). Glutathione may also increase NMDA receptor responses by interacting with its redox sites (26–28). Furthermore, because the breakdown products of

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glutathione include cysteine, glycine, and cysteinylglycine, extracellular breakdown may supply surrounding cells with these glutathione precursors (29–31). Despite the key function of glutathione in intracellular redox regulation and the putative extracellular effects in brain, the mechanisms and factors determining the efflux remain unknown.

Glutathione efflux from brain cells is increased by potassium depolarization, iron (Fe^{2+}), inhibitors of mitochondrial respiration (malonate, 1-methyl-4-phenylpyridinium (MPP^+), 3-nitropropionic acid and cyanide), and after ischemia (32–38). In a previous study, we showed that the NMDA-mediated glutathione efflux was dependent on extracellular calcium but unrelated to dantrolene sensitive intracellular calcium release, osmotic change and glutathione-, or nitric oxide-synthesis (39). In this study our main goals were (i) to evaluate if organotypic cultures of rat hippocampus may be used as a model system in characterization of NMDA-mediated efflux of glutathione and (ii) to initiate mechanistic studies using pharmacological tools for manipulation of activities and functions of protein kinases, phospholipase A_2 , calmodulin, anion channels, glutathione synthesis, and intracellular calcium. One of the potentials using cultured slices compared to acutely isolated slices (which we will make use of in coming studies) is the possibility to correlate release of glutathione and similar compounds after an acute insult to delayed excitotoxicity one or more days after the acute insult.

In parallel to glutathione we analysed the efflux of the neurotransmitter glutamate, phosphoethanolamine (PEA, also named phosphorylethanolamine), the polar soluble component of phosphatidylethanolamine, and taurine. The latter two were included because previous studies by others and us have shown that these amines are released after NMDA-receptor stimulation and other insults (4,39–43).

EXPERIMENTAL PROCEDURE

Drugs and Solutions

HEPES-buffered salt solution (HBSS) contained (in mM): 143.4 NaCl, 5 HEPES, 5.4 KCl, 1.2 MgSO_4 , 1.2 NaH_2PO_4 , 2.0 CaCl_2 , and 10 glucose. Culture medium consisted of 50% Eagle's Basal Medium (BME), 23% horse serum, 25% Earl's balanced salt solution, 7.5 mg/ml D-glucose, 25 U/ml Pest, and 1 mM glutamine. Acivicin, arachidonic acid (AA), 4-bromophenacyl bromide (BPB), DL-buthionine-[S,R]-sulfoximine (BSO), dantrolene, phorbol 12-myristate 13-acetate (PMA), polymyxin B-sulfate (PMXB), quinacrine, and staurosporine were obtained from Sigma (St. Louis, MO, USA). 4,4'-dinitrostilbene-2,2'-disulfonic acid disodium salt (DNDS), *N*-(2-aminoethyl)-5-isoquinoline-sulfoamide dihydrochloride (H9) and *N*-(6-aminoethyl)-5-chloro-1-naphthalene sulfonamide hydrochloride (W7) were from Molecular Probes (Eugene, OR, USA). Arachidonic acid, 4-bromophenacyl bromide, dantrolene, PMA and staurosporine were prepared as stock solutions in dimethyl sulfoxide (DMSO) and stored at -20°C .

Preparation of Organotypic Cultures

All animal procedures were approved by the local ethics committee in Göteborg, Sweden. Organotypic hippocampal slice cultures were prepared according to the method of Stoppini et al. (44) with minor modifications. Briefly, the hippocampi of 7–9-day-old Sprague-Dawley rat pups were dissected and sectioned transversely at 350–400 μm . Four slices were transferred to 30 mm diameter porous membrane inserts (Millicell-CM 0.4 μm). These were placed in 6-well tissue culture plates containing 1.2 ml of culture medium. The slices were maintained in a humidified 95% air/5% CO_2 atmosphere at 36°C . Cultures were allowed to develop for 2 weeks before use, with media changed every 3–4 days.

Efflux of Glutathione and Amino Acids

After removal of the culture medium the cultures were washed twice with prewarmed HBSS (~36°C), 1 ml was placed above and 1 ml below the membrane. The slices were thereafter preincubated for 60 min and washed again in HBSS. For preincubation and for incubation during the efflux studies 1 ml HBSS was placed below and 0.6 ml above the membrane. The cultures were incubated in 95% air/5% CO₂ atmosphere at 36°C. The incubation procedure was similar to that reported earlier (45). In these studies, no toxicity due to the incubation was observed 24 h after the incubation. During the experiment HBSS was renewed every 15 min, 300 µl of the medium above the membrane was collected for analysis. β-Mercaptoethanol (β-ME), Na₂EDTA, and NaN₃ were added to the samples to final concentrations of 20, 1, and 5 mM, respectively. Stimulation was performed with NMDA (10 µM)/glycine (10 µM) for 15 min. When renewing the medium after NMDA exposure the cultures were washed with 1 ml HBSS above the membrane and 1 ml below. When included, acivicin (0.2 or 2.0 mM) and BSO (5 mM) were present during the preincubation and throughout the experiment. Arachidonic acid (30 µM), BPB (100 µM), dantrolene (40 µM), PMA (1 µM), PMXB (10 µM), quinacrine (50 and 500 µM), staurosporine (1 µM), DNDS (1mM), H9 (100 µM), W7 (100 µM), or calcium-free medium were introduced 30 min before the introduction of NMDA. Experiments were also performed in which 500 µM quinacrine was introduced simultaneously with NMDA/glycine. When the drugs prepared in DMSO were used, the same concentrations of DMSO (0.05 or 0.1%) were present in the control experiments. In calcium-free medium NaCl substituted CaCl₂.

Glutathione, Amino Acid and Protein Measurements

Glutathione and amino acids were determined using reversed phase HPLC employing automated precolumn fluorogenic labeling with OPA/β-mercaptoethanol as described earlier (42). The column (300 × 4.6 mm) was packed with Nucleosil 100-5 C₁₈ (Macherey-Nagel, Germany). The derivatives were eluted with a gradient from 0 to 90% methanol (containing 1.25% tetrahydrofuran [v/v]) in a Na-phosphate buffer (50 mM set at pH 5.40 with 1M NaOH and containing 2.5% tetrahydrofuran [v/v]). The autoinjector (Waters Wisp 717 plus) was programmed to add 50 µl OPA/β-ME to 100 µl sample. The methodology does not discriminate between thiols and disulfides; the term glutathione therefore refers to the total concentration of reduced glutathione (GSH), glutathione dimers (GSSG), and mixed glutathione disulfides (GSX).

The hippocampal slices were dissolved in 1 M NaOH, and the protein content was determined using BSA protein assay reagent (Pierce, Rockford, IL, USA) with bovine serum albumin as standard. Absorbance was measured at 570 nm in a microplate reader (Emax, Molecular devices, Sunnyvale, CA, USA).

Statistical Analysis

Data are expressed as mean ± SEM and were analyzed for significance using the Wilcoxon matched pairs rank sum test or the Mann-Whitney U-test for unpaired sample groups. Data presented on efflux rates are from n wells. A *P* value of <.05 was considered statistically significant.

RESULTS

The efflux rates (see experimental procedures for details) of glutathione, taurine, PEA, and glutamate from organotypic hippocampal slice cultures were analysed before (0–60 min), during (61–75 min) and after (76–120 min) incubation with 10 µM NMDA/10 µM glycine (Fig. 1). Maximal efflux rates of glutathione and PEA were observed after removal of NMDA (75–90 min, 390.4 ± 48.9% and 809.6 ± 107.4%, respectively), whereas the maximal efflux

rate of taurine was observed during the 15 min incubation period with NMDA/ glycine (60–75 min, $905.9 \pm 102.4\%$). Aspartate, as-paragine, glutamate, glutamine, ethanolamine, valine, or isoleucine efflux rates were not affected by NMDA incubation (data shown for glutamate, Fig. 1).

The efflux rate of glutathione increased during the incubation period with HBSS prior to NMDA/glycine introduction (Fig. 2). This effect was blocked by BSO, the glutathione synthesis inhibitor (Fig. 2). The stimulus evoked efflux rate of glutathione in cultures incubated in BSO increased by 41.5 ± 3.4 pmol/min/mg protein, which was significantly lower than in control experiments (77.5 ± 11.3 pmol/min/mg, Fig. 2). The corresponding percentage increase (compared to basal efflux) with and without BSO were during NMDA/glycine incubation $604.7 \pm 70.1\%$ and $356.8 \pm 40.1\%$, respectively, and after removal of NMDA $217.0 \pm 19.6\%$ and $390.4 \pm 48.9\%$, respectively. Efflux rates of amino acids were not affected by BSO incubation (data not shown).

The protein kinase C inhibitor PMXB (10 μ M) and the phospholipase A₂ inhibitor quinacrine (500 μ M) completely abolished the NMDA-mediated increases in glutathione, PEA, and taurine efflux rates (Fig. 3). No effect was observed when 50 μ M quinacrine was used (Fig. 3). Introducing 500 μ M quinacrine simultaneously with NMDA/glycine did not alter the efflux rates during NMDA/glycine incubation, but decreased the efflux rates of glutathione and PEA after NMDA incubation (Fig. 3). Basal glutathione, PEA, and taurine efflux rates were not altered by 10 μ M PMXB or by 50 or 500 μ M quinacrine (data not shown). Incubation of the cultures with the phospholipase A₂ inhibitor BPB did not alter the efflux rates of PEA or taurine ($n = 4$, data not shown). It was not possible to evaluate the effect of BPB on glutathione efflux rate as BPB interfered with the analysis of glutathione.

The calmodulin antagonist W7 and the anion transport inhibitor DNDS had no effect on the basal or stimulated efflux rates of glutathione, PEA, and taurine *during* the incubation with NMDA, but attenuated the efflux rates *after* NMDA removal (Fig. 3).

Arachidonic acid, dantrolene, PMA, staurosporine, or H9 produced no effects on basal (data not shown) or NMDA-mediated efflux rates of glutathione, PEA, or taurine (Fig. 3).

Omitting calcium from the medium increased the efflux rates of glutathione, PEA, and taurine (Fig. 4). The effect was most prominent for glutathione with an increase similar to that induced by NMDA/glycine in calcium-containing medium. The efflux rate of glutathione was not further increased when NMDA was included in calcium-free medium. Calcium-free medium abolished the NMDA-mediated increased efflux rates of PEA and taurine.

Preincubation of the organotypic hippocampal slice cultures with 0.2 or 2.0 mM acivicin, an inhibitor of γ -glutamyl transpeptidase, did not significantly alter the glutathione efflux rate (data not shown).

DISCUSSION

The basal efflux rates and the quantitative as well as the qualitative effects of NMDA on efflux of glutathione and amino acids from organotypic hippocampal slices were similar to those reported earlier for efflux from acutely isolated hippocampus slices (39). In accordance with the previous results (39), maximal glutathione and PEA efflux rates were reached after NMDA withdrawal, whereas maximal taurine efflux rate was observed during NMDA incubation. This implies that organotypic cultures is a relevant model to search for the mechanisms of NMDA-mediated efflux. One advantage with the organotypic culture is that long-term toxicity studies can be performed. If the mechanisms of acute NMDA-stimulated glutathione efflux are elucidated, blockage of such release will be employed to evaluate its influence on the delayed

toxicity following NMDA receptor overactivation. The present results indicate that calmodulin and DNDS-sensitive channels, but not PKC or PLA₂ activities, may partly be involved in the regulation of stimulated glutathione, taurine, and PEA efflux after but not during NMDA receptor stimulation.

Some differences in the efflux comparing the two models are notable. The basal efflux and the NMDA-stimulated efflux of glutathione were affected by the glutathione synthesis blocker BSO in organotypic slices but not in acute slices (39). We have no obvious explanation for this discrepancy, but it is clear that with the present protocol a greater proportion of the efflux from the organotypic cultures appears to be derived from a glutathione pool that turns over very rapidly (46). This may, hypothetically, be due to differences in maturity of the organotypic cultures compared to the acutely isolated slices. This is not surprising as several aspects of NMDA-mediated efflux of taurine from brain slices has been shown to be dependent on the age of the animal (43). In contrast to our previous results (39), omission of Ca²⁺ from the medium massively increased the basal efflux rates of glutathione. Smaller but significant increases were observed for PEA and taurine. The discrepancy between the two models is probably due to different sensitivity to calcium-free medium in the acute hippocampal slice preparation and cultured hippocampal slices. Incubation in calcium-free medium can cause depolarization and spontaneous bursts of population spikes in hippocampal CA1 neurons (47,48). The increased glutathione efflux rate in the present experiments could thus be due to depolarization, which can increase glutathione efflux (36). However, taurine and PEA efflux increased to a much lower extent than glutathione, indicating that another factor, at present unknown, is involved in mediating glutathione efflux during calcium-free conditions. The effect of calcium omission reported here is not an isolated finding as incubation of hepatocytes and tumor cells in calcium-free medium also induces increased efflux of reduced glutathione (49,50). Similar to our previous experiments no additional efflux rate of glutathione, PEA or taurine was detected when NMDA was included in Ca²⁺-free medium showing that the NMDA stimulation efflux also in organotypic cultures is calcium-dependent.

Another major difference between the models is that in our previous work, using acute hippocampal slice preparations, incubation with the γ -glutamyl transpeptidase inhibitor acivicin increased basal and stimulation-induced glutathione efflux (39,51,52). This effect is most likely due to blockage of extracellular breakdown of glutathione. Unexpectedly, incubation of organotypic hippocampal cultures with acivicin was not accompanied by increased glutathione efflux. The activity of this enzyme in neuronal/glial cell membranes starts to appear at postnatal day 7 (53). We can thus expect very low levels of γ -glutamyl transpeptidase in the slices at the start of the culturing period. The development of the activity of γ -glutamyl transpeptidase in microvessels of brain is regulated by the status of thyroid hormones (53). As far as we know the gene regulation of the enzyme in neuronal/glial membranes is unknown. Although the cultures show a well-developed maturity of several aspects of brain function (54), the expression of some genes, such as that encoding γ -glutamyl transpeptidase, is likely to be dependent on factors others than those present in the adult horse serum that is used as additive to the culture medium.

The mechanisms involved in glutathione efflux in the brain are unknown. Efflux of glutathione in liver cells is altered by PKC activity. Activation of PKC increase, whereas inhibition of PKC decrease glutathione efflux rate from liver cells in vivo and in vitro (55,56). In our experiments, the PKC inhibitor PMXB completely abolished the increased NMDA-mediated efflux rates of glutathione, PEA, and taurine. However, incubation with the protein kinase inhibitors staurosporine or H9 had no effect on the NMDA-mediated efflux rates, nor did incubation with the PKC activating phorbol ester PMA, implying that the effect of PMXB is not achieved through inhibition of PKC. Although PMXB is often used as a PKC inhibitor it is not selective; it also inhibits Ca²⁺-activated K⁺-channels (57,58) and calmodulin (59), and thereby enzymes

such as calcium/calmodulin-dependent kinase II and calcineurin. We cannot exclude that PMBX is acting of some isoform of PKC that is not activated by PMA and not inhibited by H9 or staurosporin. However the most plausible explanation is that PMBX exerts its effect by some other mechanism than blockage of PKC, possibly by reducing calcium influx after NMDA receptor activation.

Glutamate receptor activation increases PLA₂ activity, resulting in increased levels of ROS and in arachidonic acid release. The PLA₂ inhibitor quinacrine attenuates the generation of ROS, arachidonic acid release, glutamate cytotoxicity, and stroke injury (6,60–62). In our experiments, quinacrine (500 μM) completely abolished the increased NMDA-mediated efflux rates of glutathione, PEA, and taurine. BPB, another PLA₂ inhibitor, had no effect on PEA or taurine efflux rates (glutathione was not determined due to interference of BPB with the analysis), nor did direct addition of the PLA₂ product arachidonic acid alter glutathione, PEA, or taurine efflux rates. These results, together with the lack of effect of 50 μM quinacrine, a concentration generally used to inhibit PLA₂ activity, indicates an effect of quinacrine in high concentrations unrelated to PLA₂ inhibition. One conceivable mechanism is by inhibition of Ca²⁺-influx and/or Ca²⁺-release from intracellular stores. Quinacrine decrease Ca influx in synaptosomes (63) and blocks Ca²⁺-induced Ca²⁺-release in the sarcoplasmic reticulum (64). When quinacrine (500 μM) was introduced simultaneously with NMDA, there was no effect on glutathione, PEA, or taurine efflux rates, indicating that quinacrine per se did not inhibit NMDA-receptor activation. During the incubation period after NMDA removal, glutathione and PEA efflux rate were reduced compared to control experiments, possibly because quinacrine must reach a certain intracellular concentration to exert its action.

Part of the cytosolic Ca²⁺-increase after NMDA receptor activation results from intracellular stores (65). Dantrolene, a blocker of ryanodine receptor-dependent Ca²⁺-release, attenuates the NMDA-mediated rise in intracellular Ca²⁺ (66). As in our previous experiments with acute hippocampal slices, dantrolene did not change glutathione, PEA, or taurine efflux rates, suggesting that the efflux rate is not regulated by ryanodine receptor-dependent Ca²⁺-release. This is at variance with studies of the CA1 area of the rat hippocampus (67). In this report, NMDA-mediated taurine efflux was partly, but not completely, reduced by dantrolene. The discrepancy may be related to the differences in preparations, sampling area, and collection technique.

The calmodulin inhibitor W7 attenuated the efflux rate of glutathione, PEA, and taurine *after* NMDA removal. Increased intracellular calcium concentrations leads to activation of calmodulin, which in turn stimulates several enzymes such as Ca²⁺/calmodulin-dependent kinase II (CaMKII), neuronal NO synthetase, adenylate cyclase, and calcineurin (32,68–70). Several responses to glutamate-receptor activation are altered by the calmodulin inhibitor W7. Glutamate-mediated elevations of cAMP and Ca²⁺ are attenuated by W7, presumably via inhibition of calmodulin-dependent activation of adenylate cyclase and CaMKII, respectively (71). Increased Na⁺,K⁺-ATPase after glutamate application to cultured cerebellar neurones was reduced by W7 and by the calcineurin inhibitor cyclosporin, suggesting an involvement of calmodulin-calcineurin-dependent dephosphorylation (72). Interestingly, W7 prevents glutamate-induced depletion of ATP and neuronal death in cultured cerebellar neurones (73). Also the anion transport inhibitor DNDS attenuated the efflux rate of glutathione, PEA, and taurine *after* NMDA removal. DNDS decrease the permeability of glutathione through the cystic fibrosis transmembrane conductance regulator (CFTR) (74,75), a phosphorylation and ATP-dependent membrane transport protein, permeable both to chloride and larger organic anions (74,75). Interestingly, the CFTR mRNA and protein is expressed in the hippocampus (76). Another disulphonic stilbene derivative, DIDS, inhibits glutathione transport in yeast by Ycf1p (77), the yeast orthologue of the multidrug resistance associated proteins (MRP). DIDS also inhibits glutathione transport in retinal Müller cells (78). The similarity in effect of DNDS

and W7 on glutathione, PEA, and taurine efflux rates in our study may indicate a relationship between calmodulin and an anion transport system. CFTR as well as other anion channels have phosphorylation sites that can alter their activities (79–81). A speculative working hypothesis is thus that a least two different processes are involved in efflux of glutathione, PEA, and taurine efflux after NMDA-stimulation. One such process appears to occur via a DNDS-sensitive channel that is regulated by a Ca^{2+} -calmodulin dependent activity. Further experiments using specific inhibitors against calcineurin, CaMKII, NO synthase, and adenylate cyclase as well as other chloride-channel blockers (but see below) may help to clarify the mechanisms in NMDA-stimulated efflux of glutathione, PEA, and taurine.

The basal efflux rates of glutathione, PEA, and taurine were not affected by PMXB, quinacrine, W7, or DNDS, and the efflux during NMDA-stimulation was unchanged by W7 and DNDS, suggesting differently regulated effluxes of glutathione, PEA, and taurine *before*, *during*, and *after* NMDA receptor stimulation. Alternatively, the efflux have different cellular origin. Glutathione is present in both neurons and glia (29,30,82–86), but as no functional NMDA receptors appear to be located on hippocampal glia cells (87), the most straightforward explanation for the increase in glutathione efflux is neuronal release. However, it cannot be excluded that NMDA-receptor stimulation induces a release of a substance from neurons that evokes glutathione release from the glial cells. One such substance may be K^+ , which is released following NMDA receptor overactivation (88). Because high K^+ -evoked efflux of taurine from glial cells appear to be dependent on calmodulin (89,90), we can hypothesize the efflux *after* NMDA may, at least partly, be due to glial uptake of K^+ , which leads to passive osmotic uptake of water and opening of DNDS-sensitive volume activated anion channels via calmodulin (89,90).

The likely unselective effects of the drugs quinacrine and PMXB on NMDA-mediated calcium influx is not unique. Several of the drugs used in studying the mechanisms of organic anion transport, such as furosemide, piretamide, and bumetanide, as well as niflumic and flufenamic acids, appear to block NMDA-induced currents by binding to the NMDA-receptor (91), a finding that often is neglected. Similarly, it was recently reported that combinations of antioxidants such as TEMPO, catalase, trolox, and ascorbate had profound blocking effects on NMDA currents (92). In our experiments, we have used concentrations of drugs that have been shown effective against their supposed single targets in other studies. However, we realize that findings of inhibited efflux by any drug needs additional information to rule out unselective effects on NMDA receptors and/or calcium influx. One way to at least partly get around this problem is to use different types of drugs, which we have done in the case of protein kinase C and phospholipase A_2 . In the case of DNDS and W7, we do not suspect unselective effects because the efflux during NMDA application was unchanged.

In conclusion, NMDA-receptor stimulation cause an increased efflux of glutathione, PEA, and taurine in organotypic cultures of rat hippocampus. The present experiments indicate that efflux *during* and *after* NMDA incubation may be regulated by different mechanisms. The organotypic cultures appear to be a suitable model for further studies on the mechanisms of NMDA-stimulated efflux, particularly if coupled to parallel measurements of toxicity.

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Fig. 1. Effect of 15-min incubation of organotypic hippocampal slice cultures with 10 μ M NMDA/10 μ M glycine on the efflux rates of glutathione, PEA, taurine, and glutamate ($n = 19$). $*P < .05$ comparing NMDA-mediated efflux rates with the efflux rates before NMDA introduction (45 – 60 min).

Fig. 2.

Glutathione efflux rate from organotypic hippocampal slice cultures in the presence (○) or absence (▲) of 5 mM BSO ($n = 5$). BSO was present during preincubation and throughout the experiment. The black bar indicates the incubation period with NMDA/glycine. $*P < .05$ comparing efflux rate with and without BSO. $\#P < .05$ comparing efflux rate with that during the first incubation period (0–15 min).

Fig. 3.

Effect of indicated drugs on NMDA-mediated efflux rates of glutathione, PEA, taurine, and glutamate. Values are expressed as percent of basal efflux rate, comparing the efflux rates before introduction of NMDA (45–60 min) with the efflux rates during NMDA/glycine incubation (gray bars, 60–75 min), and with the efflux rates after NMDA incubation (black bars, 75–90 min). All drugs were introduced 30 min before NMDA introduction and were present throughout the experiment except in experiments denoted 500 Quin*, in which quinacrine was introduced simultaneously with NMDA. Used concentrations: PMXB, 10 μ M; 500 Quin, 500 μ ; staurosporine, 1 μ M; W7, 100 μ M; H9, 100 μ M; DNDS, 1 mM; AA, 30

μM; 50 Quin, 50 μM; dantrolene, 40 μM; PMA, 1 μM. * $P < .05$ comparing efflux rates with and without the indicated treatments ($n \geq 4$).

Fig. 4. Glutathione, PEA, taurine, and glutamate efflux rates in the presence (▲) or absence (○) of calcium in the medium (n = 5). Calcium-free medium was introduced 30 min before NMDA/glycine introduction. The black bar indicates the incubation period with NMDA/glycine. * $P < .05$ comparing efflux rates with and without calcium.