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NMDA RECEPTOR-MEDIATED EXTRACELLULAR ADENOSINE ACCUMULATION IS BLOCKED BY PHOSPHATASE 1/2A INHIBITORS

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Abstract

We have previously demonstrated that NMDA receptor-mediated extracellular adenosine accumulation in neuronal cultures is receptor-mediated and requires calcium influx. Because protein kinase C (PKC) is a calcium-dependent enzyme, we hypothesized that activation of PKC might be involved in NMDA-mediated adenosine accumulation. PKC inhibitors however, did not block NMDA-evoked adenosine accumulation, but rather, stimulated basal adenosine accumulation. These data suggested the possibility that NMDA receptor mediated adenosine accumulation involves net dephosphorylation rather than phosphorylation of one or more substrates. Thus, inhibition of kinases would be expected to increase adenosine accumulation and inhibition of phosphatases would be expected to block adenosine accumulation. To test this hypothesis, we used the phosphatase 1/2A inhibitors calyculin A and okadaic acid. Both inhibitors significantly reduced NMDA-evoked adenosine accumulation. In contrast phosphatase 2B inhibitors did not block NMDA-evoked adenosine accumulation. These data suggest that NMDA-evoked adenosine accumulation is mediated by activation of phosphatase 1/2A. We have established previously that NMDA-mediated adenosine accumulation is associated with adenosine kinase inhibition. However, adenosine kinase is not a direct substrate for phosphatase 1/2A because inhibition of phosphatase 1/2A did not abolish NMDA-evoked adenosine kinase inhibition. Okadaic acid also had no effect on NO donor-evoked adenosine accumulation, which previously has been shown to be associated with adenosine kinase inhibition. Dephosphorylation of one or more proteins other than adenosine kinase as a consequence of NMDA receptor activation might play an important role in extracellular adenosine regulation, with important consequences for the regulation of excitatory synaptic transmission, plasticity, epileptogenesis, and excitotoxicity.

Keywords

protein kinase C; phosphatase 1/2A; adenosine; NMDA; neuroprotection; excitotoxicity; okadaic acid; epileptogenesis; plasticity

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1. Introduction

Adenosine is a ubiquitous neuromodulator in the central nervous system [18,23,42]. Its action is mediated by adenosine A₁, A_{2A}, A_{2B}, and A₃ receptors. Adenosine modulates neuronal excitability by decreasing neuronal firing [50] and inhibiting the release of neurotransmitters, such as glutamate [4,17], aspartate [6], acetylcholine [34], and γ -aminobutyric acid [3,7]. These effects are mediated by activation of presynaptic adenosine A₁ receptors.

Adenosine has been proposed as an endogenous neuroprotective agent in ischemia, stroke, epilepsy, Alzheimer's disease, and Parkinson's disease [13,32,53,61]. Adenosine receptor agonists have been reported to protect against ischemic cell death *in vivo* [2,11,14,66] and *in vitro* [22]. However, caffeine and adenosine A_{2A} receptor antagonists block β -amyloid-induced neurotoxicity in rat cultured cerebellar granule neurons [13]. Furthermore, in an animal model of Parkinson's disease, administration of A_{2A} receptor antagonists protected against the loss of nigral dopaminergic neuronal cells induced by 6-hydroxydopamine in rats and prevented the functional loss of dopaminergic nerve terminals in the striatum and the ensuing gliosis caused by MPTP in mice [32].

Protein phosphorylation is one the most important signaling mechanisms regulating cellular function. Phosphorylation is a dynamic process, involving shifting activities of kinases and phosphatases in the cell. Kinases play important, well documented, roles in the regulation of numerous cellular functions [10,43,67]. The physiological significance of serine/threonine protein phosphatases has also been appreciated, and these enzymes have been implicated in the regulation of ion channels, synaptic plasticity, exocytosis, and apoptosis [31,36,58,70].

Serine/threonine phosphatases can be divided into two major classes according to similarities in amino-acid sequence [51], the PPP and PPM classes. The PPP class shares a common phosphatase domain and includes PP1, PP2A, PP2B/calcineurin, PP4, PP5, PP6, and PP7. The PPM, or phosphatase 2C, class consists of several closely related isoforms that have very little sequence homology with the PPP family.

We have demonstrated previously that NMDA receptor activation evokes extracellular adenosine accumulation in cultured forebrain neurons, and that this effect is dependent upon calcium influx [38]. To further characterize the process or processes activated by elevation of intracellular calcium leading to extracellular adenosine accumulation, we tested the effect of inhibitors of PKC, a calcium-dependent kinase. Surprisingly, these agents had similar effects to adenosine as that of NMDA, suggesting that dephosphorylation rather than phosphorylation of one or more target proteins is associated with extracellular adenosine accumulation. Consistent with this view, we found that phosphatase 1/2A inhibitors blocked the effect of NMDA.

2. Results

Inhibition of Protein kinase C stimulated extracellular adenosine accumulation

To investigate the role of PKC in NMDA stimulated adenosine accumulation, we used several general and structurally unrelated PKC inhibitors, bisindolylmaleimide (BIS) [37,65] and calphostin C [30,37]. Neither of the PKC inhibitors blocked NMDA-evoked adenosine accumulation (Figure 1). On the contrary, both of the PKC inhibitors stimulated basal adenosine accumulation, with 101% (BIS, $p<0.01$) and 68% (calphostin C, $p<0.01$) increase in extracellular adenosine when compared to control. In four experiments of the type shown in Figure 1 that were performed, NMDA, BIS, and calphostin C stimulated a $95\pm14\%$ ($p<0.01$, $n=4$), $78\pm13\%$ ($p<0.01$, $n=4$), and $59\pm9\%$ ($p<0.01$, $n=4$) increase in adenosine

concentration, respectively. Furthermore, there was an additive effect on extracellular adenosine accumulation when both PKC inhibitors (BIS and calphostin C) and NMDA receptor agonist were present. Thus, in the presence of BIS and calphostin C, NMDA-evoked adenosine accumulation was further increased by 49% ($p<0.01$) and 24% ($p<0.05$), respectively, when compared to NMDA alone (Figure 1).

If PKC activation were involved in NMDA-evoked adenosine accumulation, inhibition of PKC would be expected to block the effect of NMDA. Since this did not occur, PKC does not seem to be involved in NMDA stimulated adenosine accumulation. A previous study showed that NMDA stimulation causes a decrease in adenosine kinase activity [38]. We found that PKC inhibition with BIS did not reverse the adenosine kinase inhibition mediated by NMDA receptor activation ($n = 2$). The fact that PKC inhibitors actually increased adenosine accumulation suggested that PKC may be involved in adenosine regulation. Phosphorylation of some target proteins of PKC might be associated with decreasing extracellular adenosine, and dephosphorylation of these target proteins might be associated with increasing adenosine accumulation.

Phosphatase 1/2A inhibitors, but not phosphatase 2B inhibitors, attenuated NMDA-evoked adenosine accumulation

It has been reported that phosphatases are involved in NMDA receptor signaling [8,46,59,69]. We observed that PKC inhibition caused an increase in adenosine accumulation; therefore, it is possible that modulation of phosphatase activity might affect adenosine production. To investigate the involvement of phosphatases in NMDA-evoked adenosine accumulation, we tested okadaic acid, a polyether compound with a C-38 structure, isolated from the black sponge *Halichondria okadai* [63], and calyculin A, an octamethylpolyhydroxylated C-28 fatty acid, isolated from the marine sponge *Disdermia calyx* [33]. We found that calyculin A (100 nM) and okadaic acid (100 nM) had no effect on basal adenosine, but blocked NMDA-evoked adenosine accumulation (Figure 2A and 2B). In all the experiments that were performed, calyculin A blocked $88 \pm 4.9\%$ ($n=6$) of NMDA-evoked adenosine accumulation, while okadaic acid blocked $61.3 \pm 7.2\%$ ($n=10$) ($p<0.01$) of NMDA-evoked adenosine accumulation. In addition, both PP1 and PP2A proteins were found to be present in these forebrain neuronal cultures, using Western blot analysis (Figure 2C). We tested the effect of lower doses of okadaic acid, and found minimal effects at 10 and 30 nM, suggesting that PP1 is the primary phosphatase mediating the effect of NMDA on NMDA receptor-mediated extracellular adenosine accumulation.

Calcineurin is a calcium activated phosphatase, and is involved in NMDA receptor activation [35,57]. To test the possible involvement of phosphatase 2B in NMDA-evoked adenosine accumulation, we used the phosphatase 2B inhibitor, bioallethrin [19]. As shown in Figure 3, bioallethrin (100 and 500 nM) did not block NMDA-evoked adenosine accumulation. At 500 nM, bioallethrin stimulated basal adenosine concentration significantly on its own ($p<0.001$). Similar results were observed in three experiments. Another phosphatase 2B inhibitor, cyclosporin A (25 μ M), did not block the effect of NMDA on adenosine accumulation ($n=2$) and did not increase basal adenosine concentration (data not shown). These data indicate that phosphatase 2B is not involved in NMDA-evoked adenosine accumulation.

Okadaic acid had no effect on NO donor stimulated extracellular adenosine accumulation

The evidence presented thus far suggested that NMDA-mediated adenosine accumulation is mediated by phosphatase 1/2A activation. Previously, we showed that NO stimulated extracellular adenosine accumulation and that this effect was mediated at least in part by adenosine kinase inhibition in neuronal cultures [54]. To test whether NO-induced

adenosine accumulation also requires phosphatase 1/2A activation, we tested the ability of okadaic acid to block the effect of NO. In the experiment shown in Figure 4, okadaic acid blocked 41% of NMDA-evoked adenosine accumulation ($p < 0.01$). However, okadaic acid had no effect on DEA/nonoate-evoked adenosine accumulation. Similar results were observed in four experiments that were performed. These data suggest that NMDA-evoked adenosine accumulation is mechanistically different from NO donor-evoked adenosine accumulation. Phosphatase 1/2A activation plays an important role in NMDA-mediated adenosine accumulation, but does not appear to be involved in NO donor-mediated adenosine accumulation.

Effect of okadaic acid on NMDA-induced adenosine kinase inhibition

We have demonstrated previously that NMDA-evoked extracellular adenosine accumulation is associated with inhibition of adenosine kinase activity [38]. This effect in itself could account for the stimulation of adenosine accumulation by NMDA, since an inhibitor of adenosine kinase (iodotubercidin) has been shown to be a sufficient stimulus for adenosine accumulation. To test the possibility that phosphatase 1/2A activation might be upstream of adenosine kinase inhibition, we investigated the effect of okadaic acid on NMDA-induced adenosine kinase inhibition. If phosphatase 1/2A activation were involved in adenosine kinase inhibition, then inhibition of phosphatase 1/2A activity should block adenosine kinase inhibition. However, as shown in Figure 5, okadaic acid had no effect on NMDA-induced adenosine kinase inhibition ($n = 8$). Similar negative results were also observed with calyculin A ($n = 2$). These data suggest that dephosphorylation of adenosine kinase by phosphatase 1/2A is not the cause of the adenosine accumulation that occurs.

3. Discussion

Regulation of extracellular adenosine by phosphatase 1/2A, but not phosphatase 2B

Data presented here demonstrate that NMDA receptor-mediated extracellular adenosine accumulation is blocked by protein phosphatase 1/2A inhibition. It has been reported that activation of NMDA receptors activates phosphatases, including calcineurin and phosphatases 1 and 2A [27,44,47,64]. OA and calyculin A, two structurally different yet potent phosphatase 1 and 2A inhibitors [29,33,62], significantly reduced NMDA-evoked adenosine accumulation (Figure 2A, 2B). Calyculin A blocked 86% of NMDA-evoked adenosine accumulation, while OA blocked 62% of the effect. Consistent with this observation, inhibition of protein kinase C promoted extracellular adenosine production (Figure 1). Although protein phosphatase inhibitors blocked the effect of NMDA on adenosine accumulation, this did not reverse the adenosine kinase inhibition produced by NMDA receptor activation (Figure 5).

Both PP1 and PP2A are present in our neuronal culture system. The concentration of OA and calyculin A that we used in most experiments, 100 nM, is not able to distinguish between PP1 and PP2A. The IC_{50} values for inhibition of PP1 and PP2A by OA are 60–500 nM and 0.5–1 nM respectively [33]. In some experiments, we tested okadaic acid at 10 and 30 nM, and found minimal inhibition of the effect of NMDA on adenosine accumulation at these concentrations. These data implicate PP1 and not PP2A in the regulation of extracellular adenosine concentrations by NMDA receptors. Calyculin A is equally potent against PP1 and PP2A, with IC_{50} values ranging from 0.5 to 2 nM [33].

PP2B/calcineurin is a dimeric phosphatase composed of a catalytic subunit (A) and a regulatory subunit (B) [25,55]. The catalytic subunit is activated by binding to the calcium/calmodulin complex. We have shown here that PP2B is unlikely to be involved in NMDA-evoked adenosine accumulation because two PP2B inhibitors did not block the NMDA effect on extracellular adenosine concentration.

Multiple pathways are likely involved in NMDA-mediated adenosine regulation

The data present herein together with our previous work [38] suggest that NMDA-mediated adenosine accumulation involves at least two pathways (Figure 6). As shown before [38], NMDA receptor activation causes inhibition of adenosine kinase, and adenosine kinase inhibition *per se* is associated with adenosine accumulation, as evidenced by the ability of iodotubericidin to promote adenosine accumulation. NMDA receptor activation is associated with depletion of ATP [38] (Figure 6; 1, 2, 4), and the accumulation of adenosine is likely to be due to a shift in the phosphorylation state of adenine nucleotides to dephosphorylated and monophosphorylated forms, the latter a substrate for cytoplasmic 5'-nucleotidase to produce adenosine. A rise in cytoplasmic adenosine concentration soon results in inhibition of adenosine kinase (Figure 6; 7), which leads to further accumulation of intracellular adenosine and ultimately extracellular adenosine through the operation of equilibrative transporters in the plasma membrane (Figure 6; 8). Nitric oxide has been found to produce adenosine accumulation in neuronal cultures by a similar pathway, that is, dependent upon ATP depletion and adenosine kinase inhibition [54]. In the present work we found that phosphatase 1/2A inhibitors had no effect on NMDA-induced adenosine kinase inhibition, yet significantly reduced NMDA receptor-mediated adenosine accumulation. Furthermore, as shown previously [38] (Figure 5G), iodotubericidin at a concentration that would be expected to completely block adenosine kinase activity does not occlude NMDA receptor induced adenosine accumulation. Therefore, it seems likely that although a portion of the effect of NMDA to induce adenosine accumulation is due to ATP depletion and adenosine kinase inhibition, the bulk of the effect is independent of inhibition of adenosine kinase. These data suggest the existence of a second pathway, dependent on dephosphorylation of one or more substrates (Figure 6; 3, 5, 6). Nitric oxide stimulated adenosine accumulation is entirely independent of this second pathway, because phosphatase 1/2A inhibitors have no effect on nitric oxide induced adenosine accumulation. These considerations leave open the question of what are the phosphorylated proteins that are dephosphorylated as a consequence of NMD receptor activation and are involved in adenosine accumulation. PP1/2A could down-regulate adenosine deaminase activity, inhibiting adenosine degradation and leading to adenosine accumulation. PP1/2A could also up-regulate cellular 5'-nucleotidase [1,5,9,16,21,24,26,28,35,39,41,45,48,49,52,56,60,71], which would stimulate adenosine production by hydrolyzing AMP to adenosine. It is also possible that PP1/2A regulate extracellular adenosine production by affecting the phosphorylation state of the NMDA receptor. It has been shown that treatment of rat nucleus accumbens slices with calyculin A caused a several fold increase in NR1 phosphorylation [59], and the phosphorylation state of the NMDA receptor may be linked to extracellular adenosine accumulation.

Adenosine in LTP and LTD

Serine/threonine protein phosphatases have been implicated in the regulation of synaptic plasticity [70]. A possible role for adenosine in synaptic plasticity is not adequately appreciated given that regulation of extracellular adenosine levels may have important effects in paradigms used to demonstrate plasticity. For example, it has been suggested that β -adrenergic receptor activation enables the induction of LTP during the low frequency stimulation in the hippocampal CA1 region by inhibition of phosphatase 1 and 2A [64]. Adenosine is known to suppress LTP in the CA1 neurons of guinea pig hippocampal slices [20]. We have shown here that NMDA stimulated extracellular adenosine accumulation is significantly reduced by phosphatase 1 and 2A inhibitors. Therefore in the slice preparation, it is possible that β -adrenergic receptor-mediated inhibition of phosphatase 1 and 2A may enable LTP production by inhibiting extracellular adenosine accumulation. This hypothesis could be easily tested by the use of appropriate adenosine receptor antagonists in the

paradigm in which β -adrenergic receptor mediated enabling of LTP is observed. Adenosine is also able to attenuate LTD in the hippocampus [12,15].

In summary, we have shown here that NMDA-evoked extracellular adenosine accumulation is mediated by activation of phosphatase 1/2A. Adenosine kinase is not likely to be the downstream target of phosphatase 1/2A, because inhibition of phosphatase 1/2A does not block the decrease in adenosine kinase activity produced by NMDA receptor activation. Our prior experiments showed that maximal inhibition of adenosine kinase with iodotubericidin did not occlude the effects of NMDA on extracellular adenosine [38], consistent with the present results that suggest a pathway regulating extracellular adenosine that is independent of adenosine kinase. This pathway appears to be subject to dephosphorylation stimulated by NMDA receptor activation, and this dephosphorylation is causally linked to the accumulation of adenosine. The target or targets for the dephosphorylation activity stimulated by NMDA receptor activation and that promote extracellular adenosine accumulation remain to be determined.

4. Experimental Procedures

Neuronal Cultures

Forebrain neuronal cultures were prepared from 16-day Sprague-Dawley rat embryos (Charles River laboratories, Boston, MA) as previously described [68]. Cultures were initially plated on poly-L-lysine coated 24 well plastic plates using an 80/10/10 (v/v) mixture of Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY), Ham's F-12 (Sigma, St. Louis, MO), heat-inactivated iron supplemented calf serum (Hyclone, Logan, UT), containing 2 mM glutamine, 25 mM HEPES, 24 U/ml penicillin and 24 μ g/ml streptomycin, incubated in a 5% CO₂/95% air at 36°C. Cell proliferation was inhibited by 5 μ M cytosine arabinoside exposed for 72 hours after the initial 24 hours incubation. On the fourth day of culture, the medium was removed and replaced with 90% modified Eagle's medium (Gibco), 10% NuSerum IV (Collaborative Research, Bedford, MA), containing 2 mM glutamine, 5 mM HEPES, 10 μ g/ml superoxide dismutase (Boehringer-Mannheim, Indianapolis, IN), 1 μ g/ml catalase (Sigma), 11 mM glucose, 9.3 mM sodium bicarbonate, and 2% B27 supplement (Gibco). Medium was not subsequently changed. Using this technique, the cultures contained less than 1% astrocytes [68], typically about 0.2%. Neuronal cultures were used at 2–3 weeks after plating. Cells were washed three times with Hank's Balanced Salt Solution (HBSS; Gibco) containing 0.1% bovine serum albumin and replaced with 0.5 ml of Earle's Balanced Salt Solution (EBSS; Gibco). The cultures were pre-incubated in EBSS for 2 hours, then chemicals were added to the medium at various concentrations and incubated for selected times as indicated in the figure legends. In all cases, control wells received the same vehicles as the test wells, in the same volume. For NMDA, solvent was water, and 100X stocks were used. For all other drugs, 1000X solutions were made in DMSO, and final concentration of DMSO was 0.1–0.2%.

Determination of extracellular adenosine

An aliquot (450 μ l) of medium was taken to determine the extracellular adenosine concentration by HPLC at the end of the experiment exactly as described previously [38].

Determination of adenosine kinase activity

For measurement of adenosine kinase activity, a previously published procedure [40] was used and replicated in detail. This procedure monitors *in vivo* phosphorylation of [U-¹⁴C] adenosine (489 mCi/mmol, Amersham, Arlington Heights, IL, USA), to AMP by adenosine kinase [38,54]. Cultures were exposed to NMDA for 5 minutes, and then radioactivity was added (0.04 μ M adenosine, final concentration 5 μ Ci/10⁻⁶ μ mol). At selected intervals,

medium was removed, and 200 μ l of ice-cold lysis buffer (20 mM sodium acetate pH 4.0, 2 mM EDTA) was added to each well. The cells were immediately frozen using ethanol/dry ice and subsequently thawed, and the cell lysate (150 μ l) was spotted onto ion exchange discs (Whatman DE-81; Fisher Scientific, Pittsburgh, PA, USA). The discs were then washed with 2 mM ammonium formate, rinsed successively with distilled water, methanol, and acetone, dried in room air, exposed to a solution of 0.1 M HCl/0.4 M KCl, and bound radioactivity was measured by liquid scintillation. The results of this assay were similar to those obtained by measuring adenosine kinase activity directly in cell lysates [38].

Western Blot analysis of PP1 and PP2A in neuronal cultures

Neuronal cultures were washed twice with 20 mM Tris, pH 7.4. Cells were lysed using lysis buffer from Qiagen (Cat. No. 37101; Qiagen Inc., Valencia, CA91355, USA) in the presence of protease inhibitors. Specifically, cells were incubated on ice for 30 minutes with vortexing briefly every ten minutes. Cell lysate was collected, spun at 10,000g at 4°C for 30 minutes. The supernatant was collected for protein determination and for Western blot analysis. Equal amount of proteins were separated on 12% SDS-PAGE, transferred to PVDF membrane. Membranes were blocked with TBST plus 5% non-fat-dry-milk for 1hr at room temperature with gentle shaking, washed three times (20 minutes each) with TBST, and probed with anti-PP1 (rabbit, polyclonal antibody, 2.5 μ g/mL, Upstate, Waltham, MA 02451, USA) and anti-PP2A (mouse, monoclonal antibody, 1 μ g/mL, Upstate) antibodies in TBST plus 5% non-fat-dry-milk overnight at 4°C with gentle rocking. Membranes were then washed three times (20 minute each) with TBST, exposed to secondary antibodies (1:5,000 dilution, donkey anti-rabbit for PP1, sheep anti-mouse for PP2A, Amersham Bioscience Corp., Piscataway, NJ 08855, USA) in TBST plus 5% non-fat-dry-milk for 1 hr at room temperature with gentle shaking. Membranes were visualized with ECL plus (PerkinElmer, Boston, MA USA 02118, Cat. Number NEL 103) following manufacture's instructions.

Statistics

Statistical comparisons were performed by analysis of variance with the *post hoc* Tukey-Kramer Multiple Comparisons test using the Instat2 program from GraphPad Inc. (San Diego, CA, USA) and by two-tailed Student's t-test (unpaired). In figures, the following symbols are used for indicating statistical significance of test result compared with basal levels: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Test results are compared with basal levels unless indicated otherwise by a line above bars. In this case, the bars at the ends of the line are being compared. Experiments were performed with 3–4 replicate samples in each experiment. For each particular experiment, the number of replicate samples is shown in the figure legend. Each experiment was performed a minimum of 3 times, unless as indicated. Data for individual experiments are presented as mean $\pm$ standard deviation (S.D.), and for pooled experiments, as mean $\pm$ standard error of the mean (S.E.M.). In general, one representative experiment is presented in figures, unless indicated otherwise. Pooled results, representing dispersion of data across all experiments, are presented in the text.

Material

2'-Deoxyadenosine 3'5'-cyclic monophosphate and NMDA were purchased from Research Biochemicals International (Natick, MA). Okadaic acid and cyclosporin A were obtained from Sigma Chemical Co. (St. Louis, MO). Calphostin C, bisindolylmaleimide (BIS), and calyculin A were purchased from Calbiochem (La Jolla, CA). Bioallethrin was purchased from Alexis Corp. (San Diego, CA). Diethylamine NONOate was purchased from Cayman Chemical (Ann Arbor, MI).

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Figure 1. Effect of PKC inhibitors on NMDA-evoked extracellular adenosine accumulation BIS and calphostin C not only stimulated basal adenosine level ($p < 0.01$), but had an additive effect on NMDA-evoked extracellular adenosine accumulation ($p < 0.01$). Cultures were pre-incubated with PKC inhibitors (BIS, 80 nM; calphostin C, “Cal”, 800 nM) for 30 minutes, exposed to NMDA (10 μ M) for 30 minutes, and extracellular adenosine determined by HPLC. The experiment is representative of four similar ones that were performed.

Figure 2. Phosphatase 1/2A inhibitors blocked NMDA-evoked extracellular adenosine accumulation

A&B. Neuronal cell cultures were pre-incubated with okadaic acid (A, 100 nM) or calyculin A (B, 100 nM) for 30 minutes, then stimulated with NMDA (10 μ M) for 30 minutes. Extracellular adenosine was determined by HPLC. The experiment is representative of six and ten for calyculin A and okadaic acid, respectively.

C. Phosphatase 1 and 2A were detected in neuronal cultures. Cell lysates from neuronal cultures were assayed for the presence of phosphatase 1 and phosphatase 2A by immunoblot analysis. The experiment shown is representative of two that were performed.

Figure 3. A phosphatase 2B inhibitor had no effect on NMDA-evoked extracellular adenosine accumulation

Neuronal cultures were pre-incubated with bioallethrin (A, 100 and 500 nM) for 30 minutes, then stimulated with NMDA (10 μ M) for another 30 minutes. Extracellular adenosine was measured by HPLC. The experiment shown here is representative of three that were performed.

Figure 4. Okadaic acid had no effect on NO donor stimulated extracellular adenosine accumulation

Neuronal cultures were pre-incubated with okadaic acid (OA) for 30 minutes, then stimulated with NMDA (10 μ M) and DEANONOate (100 μ M) for an additional 30 minutes. Extracellular adenosine was determined by HPLC. The experiment shown here is representative of four that were performed.

Figure 5. Okadaic acid had no effect on NMDA-evoked adenosine kinase inhibition

Cultures were *pre*-incubated with okadaic acid (100 nM) for 30 minutes, exposed to NMDA (10 μ M) for 5 minutes, and then 14 C-adenosine for 25 minutes. Cellular adenosine kinase activity was determined. The experiment is representative of the eight that were performed.

Figure 6. Two pathways produce extracellular adenosine accumulation in response to NMDA receptor activation

NMDA receptor activation (1) causes calcium influx and elevation of intracellular calcium (2). This increase in calcium activates two pathways. One is dependent upon activation of protein phosphatase 1/2A activity (3) and one is not, but is associated with depletion of intracellular ATP probably mediated by uptake of calcium by mitochondria (4) and production of 5'-AMP that is dephosphorylated to adenosine by cytoplasmic 5' nucleotidase.

Dephosphorylation (5) by phosphatase 1/2A of one or more unknown substrates (6), upon which adenosine accumulation depends, can be blocked by okadaic acid and other phosphatase 1/2A inhibitors. Adenosine accumulation itself is inhibitory to adenosine kinase (7), promoting the further accumulation of adenosine. Iodotubercidin, a pharmacological inhibitor of adenosine kinase, causes adenosine accumulation. Equilibrative transporters in the plasma membrane facilitate the diffusion of adenosine down its concentration gradient and the accumulation of adenosine outside the cell (8). Nitric oxide, which readily crosses cell membranes (9), also stimulates adenosine accumulation, by acting as a metabolic inhibitor and depleting intracellular ATP (10). Extracellular adenosine acts in a negative feedback loop to inhibit transmitter release from presynaptic terminals (11).