European Journal of Neuroscience, Vol. 19, pp. 2669–2681, 2004

Elevation of intracellular cAMP evokes activity-dependent release of adenosine in cultured rat forebrain neurons

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Keywords: cGMP, homeostatic feedback, NMDA, phosphodiesterase, plasticity, zaprinast

Abstract

Adenosine is an important regulator of neuronal excitability. Zaprinast is a cyclic nucleotide phosphodiesterase inhibitor, and has been shown in the hippocampal slice to suppress excitation. This action can be blocked by an adenosine receptor antagonist, and therefore is presumably due to adenosine release stimulated by exposure to zaprinast. To explore the mechanism of this phenomenon further, we examined the effect of zaprinast on adenosine release itself in cultured rat forebrain neurons. Zaprinast significantly stimulated extracellular adenosine accumulation. The effect of zaprinast on adenosine appeared to be mediated by increasing intracellular cyclic adenosine monophosphate (cAMP) and activation of protein kinase A (PKA): (i) zaprinast stimulated intracellular cAMP accumulation; (ii) a cAMP antagonist (Rp-8-Br-cAMP) significantly reduced the zaprinast effect on adenosine; (iii) an inhibitor of phosphodiesterase (PDE)1 (vinpocetine) and an activator of adenylate cyclase (forskolin) mimicked the effect of zaprinast on adenosine. We also found that zaprinast had no effect on adenosine in astrocyte cultures, and tetrodotoxin completely blocked zaprinast-evoked adenosine accumulation in neuronal cultures, suggesting that neuronal activity was likely to be involved. Consistent with a dependence on neuronal activity, NMDA receptor antagonists (MK-801 and D-APV) and removal of extracellular glutamate by glutamate–pyruvate transaminase blocked the effect of zaprinast. In addition, zaprinast was shown to stimulate glutamate release. Thus, our data suggest that zaprinast-evoked adenosine accumulation is likely to be mediated by stimulation of glutamate release by a cAMP- and PKA-dependent mechanism, most likely by inhibition of PDE1 in neurons. Furthermore, regulation of cAMP, either by inhibiting cAMP–PDE activity or by stimulating adenylate cyclase activity, may play an important role in modulating neuronal excitability. These data suggest the existence of a homeostatic negative feedback loop in which increases in neuronal activity are damped by release of adenosine following activation of glutamate receptors.

Introduction

Adenosine is an inhibitory neuromodulator in the CNS (Greene & Haas, 1991). Presynaptically, it inhibits the release of a number of neurotransmitters, including glutamate (Dolphin & Archer, 1983), aspartate (Jackisch *et al*., 1984), acetylcholine (Burke & Nadler, 1988) and γ-aminobutyric acid (GABA; Bonci & Williams, 1996) by activating the A1 receptor. Postsynaptically, it causes hyperpolarization of the membrane potential by activating a barium-sensitive potassium channel (Trussel & Jackson, 1987). It increases the amplitude of the action potential afterhyperpolarization (I_{AHP}), by activating the calcium-dependent potassium current that underlies the long-lasting afterhyperpolarization and the accommodation of firing seen in many cortical neurons (Haas & Greene, 1984). Furthermore, adenosine inhibits the hyperpolarization-activated cation current (I_h) (Pape, 1992; Rainnie *et al*., 1994). The consequence of these effects is a decrease in neuronal firing. Thus, adenosine is an important regulator of excitability, and understanding how extracellular adenosine concentrations are controlled is important in order to understand the modulation of excitability of neurons in the CNS.

Cyclic nucleotide phosphodiesterases (PDEs) are key enzymes regulating intracellular cyclic nucleotide metabolism and thus play an important role in regulating various cellular functions (Beavo, 1995; Dousa, 1999). Cyclic adenosine monophosphate (cAMP) and cGMP are inactivated by PDEs via hydrolytic cleavage of the 3′-ribose-phosphate bond. PDEs are a large superfamily of enzymes with at least 11 different subfamilies (Beavo, 1995; Dousa, 1999; Fawcett *et al*., 2000). The subfamilies differ in their affinities for cAMP or cGMP, their expression patterns and their responses to cellular factors (Beavo, 1995; Dousa, 1999).

A number of selective PDE inhibitors have been described, and are valuable tools for the *in vitro* and *in vivo* study of the function of PDEs as well as for therapeutic purposes (Beavo, 1995; Dousa, 1999). Zaprinast is a PDE inhibitor; it inhibits PDE1 ($K_i = 10\,\mu M$; Ahn *et al*., 1992), PDE5 ($K_i = 0.14\,\mu M$; Gillespie & Beavo, 1989), PDE9 ($IC_{50} = 35\,\mu M$; Fisher *et al*., 1998) and PDE11 ($IC_{50} = 12\,\mu M$; Fawcett *et al*., 2000). The presence of PDE1 (Sharma *et al*., 1984), PDE5 (Kotera *et al*., 1997) and PDE9 (Fisher *et al*., 1998; Andreeva *et al*., 2001) in the CNS has been reported. In the hippocampus, zaprinast was shown to induce depression of field excitatory potentials (Boulton *et al*., 1994), and this effect was blocked by an adenosine A1

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Received 15 September 2003, revised 5 March 2004, accepted 19 March 2004

doi:10.1111/j.1460-9568.2004.03396.x

receptor antagonist (Broome *et al*., 1994). This work suggested that zaprinast might stimulate the accumulation of extracellular adenosine, although direct evidence for this was lacking. The present study was undertaken to characterize the effect of zaprinast on extracellular adenosine in the simplified system of neuronal cultures derived from embryonic rat forebrain in order to better understand the mechanisms underlying the physiological regulation of extracellular adenosine levels. We found that zaprinast evoked extracellular adenosine accumulation in neuronal cultures. This effect was mediated by stimulation of glutamate release and activation of *N*-methyl-D-aspartate (NMDA) receptors, shown previously to evoke extracellular adenosine accumulation (Lu *et al*., 2003). This effect of zaprinast appears to occur by a cGMP-independent and cAMP-dependent mechanism. Thus, there appears to be a mechanism in neurons by which increased excitability is damped by the release of adenosine secondary to the activation of glutamate receptors. Part of this work was published previously in the form of an abstract (Lu *et al*., 1999).

Materials and methods

Neuronal cultures

Neuronal cultures were prepared from 16-day-old Sprague–Dawley rat embryos (Charles River, Boston, MA, USA), as previously described (Wang *et al*., 1998). Pregnant rats were anaesthetized with CO_2 narcosis and then killed by cervical dislocation, animal care was in accordance with the Institutional Animal Care and Use Committee at Children's Hospital. Cultures were initially plated on poly-L-lysine-coated 24-well plastic plates using an 80/10/10 (v/v/v) mixture of Dulbecco's modified Eagle's medium (Gibco, 11960-010, Grand Island, NY, USA), Ham's F-12 (Sigma, St Louis, MO, USA; N-4888), heat-inactivated iron-supplemented calf serum (Hyclone A2151, Logan, UT, USA), containing 2 mM glutamine, 25 mM Hepes, 24 U/mL penicillin and 24 μg/mL streptomycin, incubated in a 5% CO_2/95% air at 36 °C. Cell proliferation was inhibited by 5 μM cytosine arabinoside for 72 h after the initial 24-h incubation. On the fourth day of culture, the medium was removed and replaced with a maintenance medium consisting of 90% modified Eagle's medium (Sigma M0275), 10% NuSerum IV (Collaborative Research, 55004, Bedford, MA, USA), 2 mM glutamine, 5 mM Hepes, 50 unit/mL superoxide dismutase (Boehringer-Mannheim, 837–113, Indianapolis, IN, USA), 20 unit/mL catalase (Sigma C-40), 11 mM glucose, 9.3 mM sodium bicarbonate and 2% B27 supplement (Gibco 17504-044). Medium was not subsequently changed. Using this technique, the cultures contained less than 1% astrocytes (Wang *et al*., 1998), typically about 0.2%. Neuronal cultures were used at 2–3 weeks after plating. For astrocyte-rich (AR) cultures [mixed cultures of astrocytes and neurons with 90–95% astrocytes (Rosenberg, 1991)], cytosine arabinoside was added at day 15 after plating, medium was changed on a Monday, Wednesday and Friday, and cultures were used from 24 to 56 days after plating. Cells were washed three times with Hank's balanced salt solution (Gibco 14025-092) containing 0.1% bovine serum albumin (Boehringer Mannheim, 100-069) and replaced with 0.5 mL Earle's balanced salt solution (Sigma E3024). The cultures were preincubated in Earle's balanced salt solution for 2 h, then chemicals were added to the medium at various concentrations and incubated for selected times, as indicated in the figure legends.

Astrocyte cultures were prepared using cortex derived from P4 animals that were minced, trypsinized and plated on collagen/poly-L-lysine-coated plates (Rosenberg & Dichter, 1989). Oligodendroglia were removed by shaking, and the remaining cells were trypsinized and replated. This procedure yielded cultures that contained no neurons.

Determination of extracellular adenosine

An aliquot (450 μL) of medium was taken to determine the extracellular adenosine by high-performance liquid chromatography (HPLC) at the end of the experiment, as described previously (Rosenberg *et al*., 1994). Briefly, each sample was collected and mixed with 4.5 μL of 1 M Na_2EDTA to yield a final concentration of 10 mM. For derivatization, 22.5 μL of 10% chloroacetaldehyde and 22.5 μL of 1 N HCl, 45 μL of 4.25 μM 2′-deoxyadenosine 3′5′-cyclic monophosphate (as an internal standard) in 2 M potassium phosphate (pH 5.6) were added, and samples were incubated at 37 °C for 24 h. The etheno derivatives were then measured by reverse-phase HPLC with a fluorescence detector. Samples were run on a C18 column (ODS Hypersil, 3 μm, 100 × 4.6 mm, 103-33, Keystone Scientific, Bellefonte, PA, USA) using a mobile phase containing 50 mM ammonium acetate buffer (pH 5.5), 0.2 mM tetrabutylammonium hydrogen sulphate and 1 mM Na_2EDTA/methanol (9/1 v/v). Adenosine was identified by its retention time relative to an internal standard, and the amount in the samples was quantified using Beckman System Gold software by determining the ratio of the peak height of adenosine and other adenine nucleotides with the peak height of the internal standard in each sample, and comparing the values obtained with standard curves constructed of ratios of external standards with the internal standard. Extracellular adenosine concentration is expressed in figures as pmol/100 μL as 100 μL was the size of the aliquot actually assayed using a 100-μL loop.

Measurement of extracellular glutamate

Extracellular glutamate concentration was determined by an enzymatic fluorometric assay adapted from Nicholls *et al*. (1987). Five-hundred microlitres of sample medium was mixed with 500 μL fluorometric reagent containing 33 mM trizma base, 17 mM trizma acetate, 100 μM adenosine 5′-diphosphate and 400 μM β-nicotinamide adenine dinucleotide. A 15-μL aliquot of glutamate dehydrogenase (1.5 unit/μL, Boehringer Mannheim, 127-710) was added to the mixture and incubated for 20 min at room temperature. At the end of incubation, fluorescence intensity was determined by a fluorometer with excitation at 335 nm and emission at 430 nm. Glutamate standards were included in the assay, and extracellular glutamate concentration was determined by interpolation.

Measurement of cellular cGMP and cAMP

Cellular cGMP and cAMP were determined by enzyme-linked immunoassay using kits from Assay Designs (Catalogue nos 900-066 and 900-014; Ann Arbor, MI 48108, USA).

cAMP turnover was measured by labelling the cellular ATP pool with radioactive adenine and by measuring radioactivity associated with cAMP (Salomon, 1979; Johnson *et al*., 1994). Cells were incubated with 2,8-^{3}H-adenine (25 Ci/mmol, 0.5 μCi/well) for 2 h to label the ATP pool. Cells were then exposed to isobutylmethylxanthine (IBMX) and zaprinast for 30 min. Medium was then removed and cellular cAMP was extracted with 500 μL of ice-cold 0.3 M $HClO_4$ for 30 min. Acid samples were neutralized with 1/10 v/v of 3 M $KHCO_3$, incubated for 15 min on ice, and then centrifuged at 2500 ***g*** for 5 min. cAMP contained in these samples was assayed using SpinZyme Acidic Alumina devices from Pierce (catalogue # 29541; Rockford, IL 61105, USA) following the manufacturer's procedure.

Electrophysiology

Electrophysiological recordings were performed at room temperature (25 °C) using the whole-cell configuration of the patch-clamp technique, utilizing both voltage-clamp and current-clamp settings. The membrane potential was clamped to −60 mV. In current-clamp mode,

the starting membrane potential was not recorded. Current was passed through the electrode to maintain the membrane potential near −60 mV. Recording was performed on cortical neurons in AR cultures. Cells were bathed in external solution containing (in mM): NaCl, 150; $CaCl_2$, 1.0; KCl, 2.8; Hepes, 10. In addition, the solution contained: 10 μM glycine, 250 nM tetrodotoxin (TTX; Calbiochem, La Jolla, CA, USA), and pH was adjusted to 7.2 with NaOH. Electrodes were pulled on a Sutter P-87 electrode puller (Sutter Instruments, Novato, CA, USA) to a resistance of 1.5–3 MΩ when filled with internal solution containing (in mM): CsCl, 140; EGTA/CsOH, 10; $CaCl_2$, 1; Hepes, 10 (pH adjusted to 7.2 with CsOH). Signals were amplified using an Axopatch 200B integrating patch-clamp amplifier (Axon Instruments, Foster City, CA, USA), filtered using an 80 dB/decade filter at 1 kHz, and digitized at 2 kHz with a DigiData 1200b (Axon Instruments) computer interface. The reference electrode was an Ag/AgCl wire bridged with 2 M KCl/1% agarose in PE-90 tubing. Drugs were applied via a perfusion system with a stepper motor for fast solution changes (Warner Instruments, Hamden, CT, USA). NMDA and zaprinast were dissolved in external solution for recording. Data were collected and analysed using commercially available software (pCLAMP, Axon Instruments).

Materials

Zaprinast, IBMX, dipyridamole, vinpocetine and My5445 were purchased from Biomol (Plymouth Meeting, PA, USA). 8-Bromoguanosine-3′,5′-cyclicmonophosphate (8-Br-cGMP), 8-(4-chlorophenylthio) guanosine-3′,5′-cyclic monophosphorothioate, Sp-isomer (Sp-8-pCPT-cGMPS), β-phenyl-1-N2-etheno-8-bromoguanosine-3′,5′-cyclic monophosphate (8-Br-PET-cGMP), β-phenyl-6-N2-etheno-8-bromoguanosine-3′,5′-cyclic monophosphorothioate, Sp-isomer (Sp-8-Br-PET-cGMPS), 8-(4-chlorophenylthio) guanosine-3′,5′-cyclic monophosphate (8-pCPT-cGMP) and 8-bromoadenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer (Rp-8-Br-cAMPS) were purchased from Biolog (La Jolla, CA, USA). Forskolin, GPT, pyruvate, trizma base, trizma acetate, adenosine 5′-diphosphate and β-nicotinamide adenine dinucleotide were purchased from Sigma. (+)-5-Methyl-10,11-dihydro-5H-dibenzo [a, d] cyclohepten-5,10-imine hydrogen maleate (MK-801), D-2-amino-5-phosphonovaleric acid (D-APV), TTX, bicuculline and 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulphonamide (NBQX) were purchased from Research Biochemicals International (Natick, MA, USA). Glutamate dehydrogenase was purchased from Boehringer Mannheim. UK114,542 was a gift from Pfizer.

Statistics

Statistical comparisons were performed by analysis of variance with the post-hoc Tukey–Kramer Multiple Comparisons Test using the Instat2 program from GraphPad (San Diego, CA, USA) and by two-tailed Student's *t*-test (unpaired). All experiments were repeated a minimum of three times unless indicated otherwise. In general, experiments were performed with three–four replicate samples. Error bars represent intra-assay variation expressed as the SD. In data pooled from several experiments, SEM is used. The following symbols are used for indicating statistical significance in figures: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Results

Zaprinast stimulated extracellular adenosine accumulation in rat forebrain neuronal cultures

In initial experiments, we found that zaprinast evoked a concentration-dependent increase in extracellular adenosine (Fig. 1A), with an EC_{50} value of 5.6 ± 0.4 μM ($n = 3$). In all subsequent experiments, 10 μM zaprinast was used. IBMX is a non-specific PDE inhibitor that is an effective antagonist of PDE1, 2, 3, 4, 5, 6, 10 and 11 (Beavo *et al*., 1970; Wells *et al*., 1975; Manganiello *et al*., 1995; Dousa, 1999; Fawcett *et al*., 2000). Previously, we described that the effect of isoproterenol-stimulated extracellular adenosine accumulation in cortical cultures was potentiated by IBMX, presumably by blocking intracellular PDE activity and raising intracellular cyclic nucleotide levels (Rosenberg *et al*., 1994). Therefore, we tested the effect of IBMX on zaprinast-evoked adenosine accumulation (Fig. 1B). In this series of experiments, zaprinast increased adenosine by 35 ± 5% ($P < 0.05$, $n = 3$), IBMX increased adenosine by 25 ± 6% ($P < 0.05$, $n = 3$), and zaprinast plus IBMX increased adenosine by 138 ± 13% ($P < 0.01$, $n = 3$). The effect of zaprinast was maximal at 150 min in the presence of IBMX (Fig. 1C). Because 30 min exposure to 10 μM zaprinast plus 100 μM IBMX consistently evoked significant extracellular adenosine accumulation, most of the experiments carried out subsequently used these parameters to evoke adenosine accumulation.

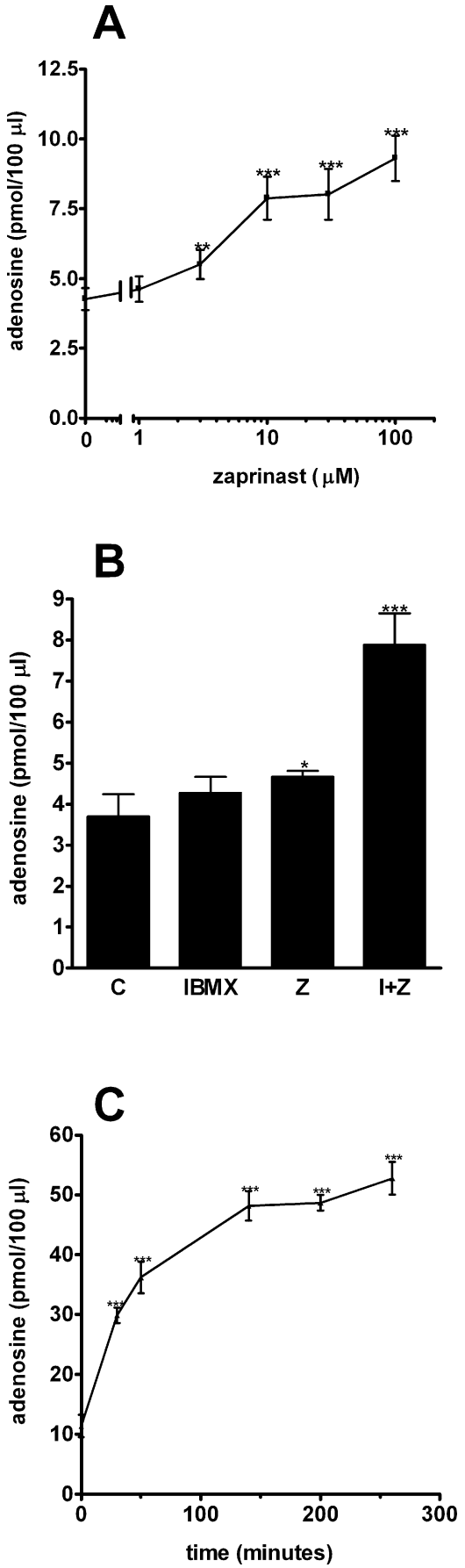
Role of cGMP in zaprinast-evoked adenosine accumulation

Zaprinast is an inhibitor of cGMP-specific PDEs (Gillespie & Beavo, 1989). To investigate the role of cGMP in zaprinast-evoked adenosine accumulation, we first measured intracellular cGMP after exposure to zaprinast. In five experiments that were performed, there was no consistent increase in cGMP content after zaprinast treatment (Fig. 2A). IBMX itself did cause a significant increase of intracellular cGMP content (Fig. 2A), and in the presence of IBMX, zaprinast did not produce a further increase. Next, we used 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a soluble guanylate cyclase inhibitor (Garthwaite *et al*., 1995), to reduce the endogenous level of cGMP. In the presence of ODQ, the intracellular cGMP level was significantly reduced (73–83% decrease, Fig. 2B). However, ODQ had no effect on zaprinast-evoked extracellular adenosine accumulation (Fig. 2C, $n = 4$). Finally, we used two cell-permeable and PDE-resistant cGMP analogues, CPT-cGMP and 8-Br-cGMP, to test their effect on extracellular adenosine concentration (Fig. 2D). Neither 8-Br-cGMP (1 mM) nor CPT-cGMP (1 mM) stimulated adenosine accumulation in rat forebrain cultures in the presence (data not shown) or absence of IBMX (Fig. 2D). Addition of other cell-permeable and PDE-resistant cGMP analogues (Sp-8-pCPT-cGMPS, 8-Br-PET-cGMP and Sp-8-Br-PET-cGMPS), all at 100 μM, also did not elicit any statistically significant increase in extracellular adenosine (data not shown, $n = 2$ each).

Zaprinast was originally characterized as an inhibitor of PDE5, a cGMP-specific PDE (Gillespie & Beavo, 1989). We investigated whether the effect of zaprinast on adenosine accumulation was mediated by PDE5 inhibition by testing the effect of two other PDE5 inhibitors, dipyridamole (25 μM) and My5445 (25 μM). Neither of these PDE5 inhibitors had any effect on extracellular adenosine (data not shown, $n = 2$ each). In addition, UK114,542, another PDE5 inhibitor, at concentrations from 6 to 200 μM, had no effect on extracellular adenosine (data not shown, $n = 4$). These data suggest that the effect of zaprinast on adenosine accumulation is unlikely to be mediated by inhibition of cGMP-specific PDEs.

Role of cAMP in zaprinast-evoked adenosine accumulation

Zaprinast also inhibits PDEs (PDE1 and PDE 11) that hydrolyse cAMP (Ahn *et al*., 1992; Fawcett *et al*., 2000). To investigate a possible role of cAMP in zaprinast-evoked adenosine accumulation, we measured the intracellular cAMP concentration after zaprinast stimulation in the presence of IBMX (100 μM). In 11 experiments that were performed, an average increase of 62 ± 14% was observed ($P < 0.001$, Fig. 3A). Similar results were obtained by monitoring cAMP turnover, in which



an average increase of $42 \pm 11\%$ was detected when compared with control (data not shown, $n = 6$, $P < 0.01$).

Because zaprinast increased intracellular cAMP levels, it seemed possible that zaprinast might stimulate adenosine accumulation by activation of protein kinase A (PKA). If the increase of cAMP was responsible for the extracellular adenosine accumulation by this pathway, we would expect that a PKA antagonist would block zaprinast-stimulated extracellular adenosine accumulation. This was indeed the case. The PKA antagonist, Rp-8-Br-cAMPS (500 µM, Gjertsen *et al.*, 1995), had no effect on basal adenosine concentration (data not shown, $n = 3$), but significantly reduced zaprinast-evoked adenosine accumulation (Fig. 3B) ($53 \pm 13\%$ decrease when compared with zaprinast alone, $n = 8$, $P < 0.05$). Taken together, these data suggest that zaprinast-evoked extracellular adenosine accumulation is likely mediated by inhibition of cAMP-specific PDE activity, accumulation of cellular cAMP and activation of PKA.

Is zaprinast acting as a PDE inhibitor?

The previous results suggested that the effect of zaprinast in neuronal cultures was mediated by an increase in intracellular cAMP but not cGMP. We next asked the question whether this effect of zaprinast was due to PDE inhibition. If this was the case, then other PDE inhibitors should have similar effects. To investigate whether the effect of zaprinast was mediated by inhibiting cAMP-specific PDEs, we tested the effect of inhibitors of PDE2 [erythro-9-(2 hydroxy-3-nonyl)adenine.HC1. (EHNA), 25 µM, $n = 4$], PDE3 (milrinone, 20 µM, $n = 3$; trequinisin, 1 µM, $n = 3$; and quazinone, 20 µM, $n = 3$) and PDE4 (RO20-1724, 20–200 µM, $n = 4$) on extracellular adenosine concentration. We found that these PDE inhibitors had no effect on extracellular adenosine level. Next, we investigated the effect of a PDE1 inhibitor, vinpocetine ($K_i = 10$ µM, Ahn *et al.*, 1992), on extracellular adenosine concentration. Vinpocetine (100 µM) produced a $94 \pm 16\%$ increase of extracellular adenosine (Fig. 3C, $n = 4$, $P < 0.001$), very close to the effect of zaprinast plus IBMX on extracellular adenosine (an increase of $112 \pm 9\%$ above control). Another PDE1 inhibitor, 8-methoxymethyl-IBMX (100 µM, Ahn *et al.*, 1989), also stimulated adenosine accumulation ($66 \pm 6\%$ increase, data not shown, $n = 4$). In addition, there was no additive effect when zaprinast and vinpocetine were added together in the presence of IBMX (Fig. 3C), indicating that zaprinast and vinpocetine were likely acting on the same target, possibly PDE1.

FIG. 1. Zaprinast-stimulated extracellular adenosine accumulation in rat forebrain neuronal cultures. (A) Concentration dependence of zaprinast stimulation of extracellular adenosine accumulation. Neuronal cultures were exposed to selected concentrations of zaprinast (1, 3, 10, 30 and 100 µM) in the absence of IBMX for 30 min. Extracellular adenosine in the medium was determined by HPLC. Values are the mean ± SD from quadruplicate samples. Zaprinast evoked a concentration-dependent increase in extracellular adenosine accumulation, with an EC_{50} value of 6 µM. The experiment is representative of three that were performed. (B) IBMX potentiated zaprinast-evoked extracellular adenosine accumulation. Neuronal cultures were exposed to IBMX (I, 100 µM), zaprinast (Z, 10 µM) and zaprinast plus IBMX for 30 min; extracellular adenosine was determined by HPLC. Values are the mean ± SD from quadruplicate samples. Zaprinast itself increased adenosine by 26% ($P < 0.05$) when compared with control. IBMX itself slightly stimulated basal extracellular adenosine concentration (15% increase over control, $P > 0.05$). Zaprinast plus IBMX increased adenosine by 112% ($P < 0.001$) when compared with control. The experiment is representative of three that were performed. (C) Time dependence of zaprinast-stimulated extracellular adenosine accumulation. Neuronal cultures were exposed to zaprinast (10 µM) in the presence of IBMX for 0, 30, 60, 130, 200 and 260 min; extracellular adenosine in the medium was determined by HPLC. Values are the mean ± SD from quadruplicate samples. The effect of zaprinast was maximal at 150 min. The experiment is representative of three that were performed.

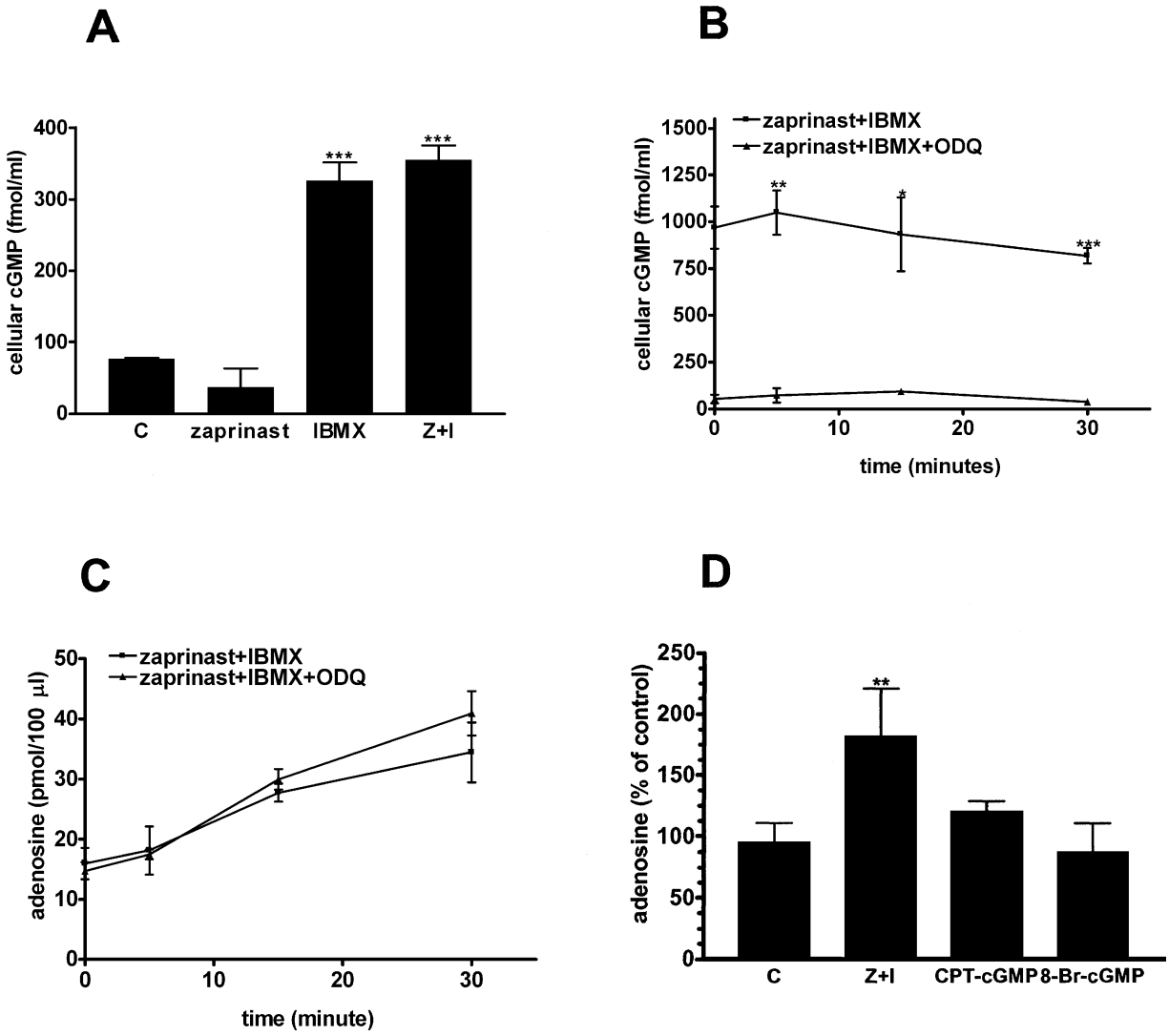


FIG. 2. Zaprinast-stimulated extracellular adenosine accumulation is not mediated via a cGMP-dependent pathway. (A) Zaprinast did not induce intracellular cGMP accumulation. Neuronal cultures were incubated with zaprinast (Z, 10 μM) in the presence or absence of IBMX (I, 100 μM) for 30 min, culture medium was removed, and cellular cGMP content was measured by a direct cGMP enzyme immunoassay. Values are the mean ± SD from triplicate samples. Zaprinast had no effect on cGMP, while IBMX caused significant cGMP accumulation ($P < 0.001$). This experiment is representative of five that were performed. (B) and (C) Reduction of intracellular cGMP concentration had no effect on zaprinast-evoked adenosine accumulation. Neuronal cultures were preincubated with ODQ (10 μM) and IBMX (100 μM) for 2 h, and then were exposed to zaprinast (10 μM) for 0, 5, 15 and 30 min. Cellular cGMP content was determined by a direct cGMP enzyme immunoassay (B), and culture medium was collected for measurement of adenosine by HPLC (C). Values are the mean ± SD from triplicate samples. ODQ significantly reduced cellular cGMP content, but had no effect on extracellular adenosine level. These experiments are representative of two (B) and four (C) that were performed. (D) Cyclic GMP analogues did not stimulate extracellular adenosine accumulation. Neuronal cultures were incubated with zaprinast (10 μM) plus IBMX (100 μM), CPT-cGMP (1 mM) or 8-Br-cGMP (1 mM) for 30 min. Supernatant was collected for measuring adenosine by HPLC. Values are the mean ± SD from quadruplicate samples. CPT-cGMP and 8-Br-cGMP had no effect on extracellular adenosine levels. The control value of the extracellular adenosine was 5.9 ± 1.9 pmol/100 μL. This experiment is representative of four that were performed.

We have shown previously that intracellular cAMP is a significant source for extracellular adenosine accumulation (Rosenberg & Dichter, 1989; Rosenberg *et al*., 1994; Rosenberg & Li, 1995a, b, 1996), in that intracellular cAMP is transported outside of cells and degraded to adenosine by cAMP-specific PDE activity and 5′-nucleotidase. This pathway has been shown to be operative in several slice models (Gereau & Conn, 1994; Bonci & Williams, 1996). To investigate whether cAMP was the source for extracellular adenosine in the present experiments, we used RO20-1724, a cAMP-specific PDE inhibitor that has been shown previously to block cAMP-dependent adenosine accumulation in cortical cultures and in other systems as well (Gereau & Conn, 1994; Rosenberg *et al*., 1994; Bonci & Williams, 1996). In the presence of low concentrations of RO20-1724 (6, 20 μM), zaprinast-evoked cellular cAMP accumulation was further increased (data not shown, $n = 2$), indicating that at these concentrations, RO20-1724 inhibited cAMP degradation in these cultures. At higher concentrations (60, 200 μM), RO20-1724 failed to cause further increase in cellular cAMP (data not shown, $n = 2$). On the contrary, it caused a decrease in cAMP. If cAMP was the source for the extracellular adenosine that was observed, then 20 μM RO20-1724 would be expected to diminish zaprinast-evoked adenosine accumulation. However, we found that 20 μM RO20-1724 had no effect on zaprinast-evoked extracellular adenosine accumulation (data not shown, $n = 3$). Furthermore, we tested the effect of probenecid (1 mM), a cAMP transport inhibitor, on zaprinast-evoked adenosine accumulation. In four experiments that were performed, zaprinast plus

IBMX stimulated a $188 \pm 18\%$ ($P < 0.01$, $n = 4$, data not shown) increase in extracellular adenosine when compared with control. In the presence of probenecid, zaprinast plus IBMX stimulated a $170 \pm 15\%$ ($P < 0.05$, $n = 4$, data not shown) increase in extracellular adenosine. These data suggested that the increase of intracellular cAMP produced by exposure to zaprinast was not likely itself to be the source for extracellular adenosine.

The role of NMDA and non-NMDA receptors in zaprinast-evoked extracellular adenosine accumulation

To investigate whether the effect of zaprinast was specific to neurons, we tested the effect of zaprinast on astrocyte and AR cultures. We found that zaprinast did not stimulate extracellular adenosine accumulation in astrocyte or AR cultures in the presence or absence of IBMX (data not shown, $n = 2$ each).

Because the effect of zaprinast on extracellular adenosine was only observed in neuronal cultures, the relationship between neuronal activity and zaprinast-evoked adenosine accumulation was investigated. TTX, a blocker of voltage-dependent sodium channels, was used to investigate the role of neuronal activity on zaprinast-stimulated adenosine accumulation. TTX (10 μM) completely blocked zaprinast-induced extracellular adenosine accumulation ($P < 0.001$, Fig. 4A, $n = 4$). TTX also reduced basal adenosine concentration (a 62% decrease compared with control, $P < 0.05$, Fig. 4A).

We found previously that TTX completely inhibited NMDA-evoked adenosine accumulation (Lu *et al.*, 2003). Therefore, it seemed possible that zaprinast might evoke adenosine accumulation by stimulating neuronal activity, causing glutamate release and NMDA receptor activation. If the response to zaprinast was secondary to such an effect, TTX should block it. To investigate the role of NMDA receptor activation in zaprinast-evoked adenosine accumulation, MK-801 (10 μM), a non-competitive NMDA receptor antagonist, and D-APV (100 μM), a competitive NMDA receptor antagonist, were used. We found that MK-801 completely blocked zaprinast plus IBMX-induced extracellular adenosine accumulation (Fig. 4B, $n = 3$). MK-801 also caused a significant decrease in basal adenosine concentration (42% decrease, $P < 0.05$, Fig. 4B). D-APV (100 μM) blocked 84% of the zaprinast plus IBMX-evoked adenosine accumulation (Fig. 4C, $P < 0.01$, $n = 2$). These data support the hypothesis that zaprinast-stimulated extracellular adenosine accumulation requires NMDA receptor activation.

Interestingly, NBQX (100 μM), a non-NMDA glutamate receptor antagonist, which has no effect on basal adenosine concentration (Fig. 4B), reduced the effect of zaprinast on extracellular adenosine accumulation, a $31 \pm 2\%$ decrease when compared with zaprinast alone ($P < 0.001$, $n = 5$, Fig. 4B). To eliminate the possibility that the effect of NBQX was mediated by acting upon NMDA receptors [by blocking the glycine co-agonist site (Yu & Miller, 1994)], we tested the effect of NBQX on zaprinast-evoked adenosine accumulation in the presence of glycine (1 mM, Fig. 4D). Glycine had no effect on basal adenosine concentration, did not potentiate zaprinast-stimulated extracellular adenosine accumulation and did not eliminate the effect of NBQX on zaprinast-evoked adenosine accumulation ($n = 3$). These

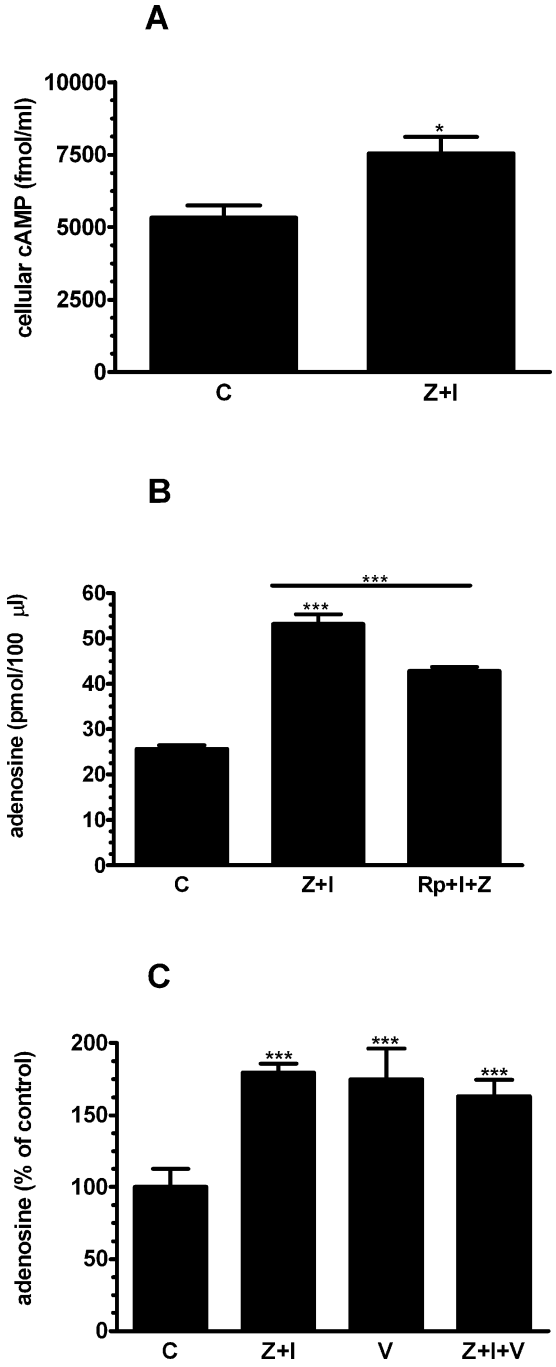


FIG. 3. Zaprinast-evoked extracellular adenosine accumulation is mediated via a cAMP-dependent pathway. (A) Zaprinast-stimulated cellular cAMP accumulation. Neuronal cultures were incubated with zaprinast (Z, 10 μM) in the presence of IBMX (I, 100 μM) or with IBMX alone (C) for 30 min. Culture medium was removed, and cellular cAMP content was measured by a direct cAMP enzyme immunoassay. Values are the mean ± SD from triplicate samples. Zaprinast (10 μM) significantly stimulated intracellular cAMP accumulation (42% increase) in the presence of 100 μM IBMX. This experiment is representative of 11 that were performed. (B) cAMP antagonist, Rp-8-Br-cAMP, significantly reduced zaprinast-evoked extracellular adenosine accumulation. Cultures were preincubated with Rp-8-Br-cAMP (Rp, 500 μM) for 30 min; then exposed to zaprinast (Z, 10 μM) for 30 min. IBMX (I, 100 μM) was present in all groups for the same length of time as that of zaprinast. Extracellular adenosine was determined by HPLC. Values are the mean ± SD from quadruplicate samples. Rp-8-Br-cAMP caused a 40% decrease in extracellular adenosine when compared with zaprinast alone. This experiment is representative of eight that were performed. (C) Vinpocetine mimicked zaprinast-evoked extracellular adenosine accumulation and occluded the effect of zaprinast. Cultures were incubated with IBMX (I, 100 μM) plus zaprinast (Z, 10 μM), vinpocetine (V, 100 μM), or IBMX plus zaprinast plus vinpocetine for 30 min. Extracellular adenosine was determined by HPLC. Values are the mean ± SD from quadruplicate samples. Vinpocetine stimulated adenosine accumulation to the same extent as that of zaprinast plus IBMX. No additive effect between vinpocetine and zaprinast plus IBMX was observed. This experiment is representative of four that were performed.

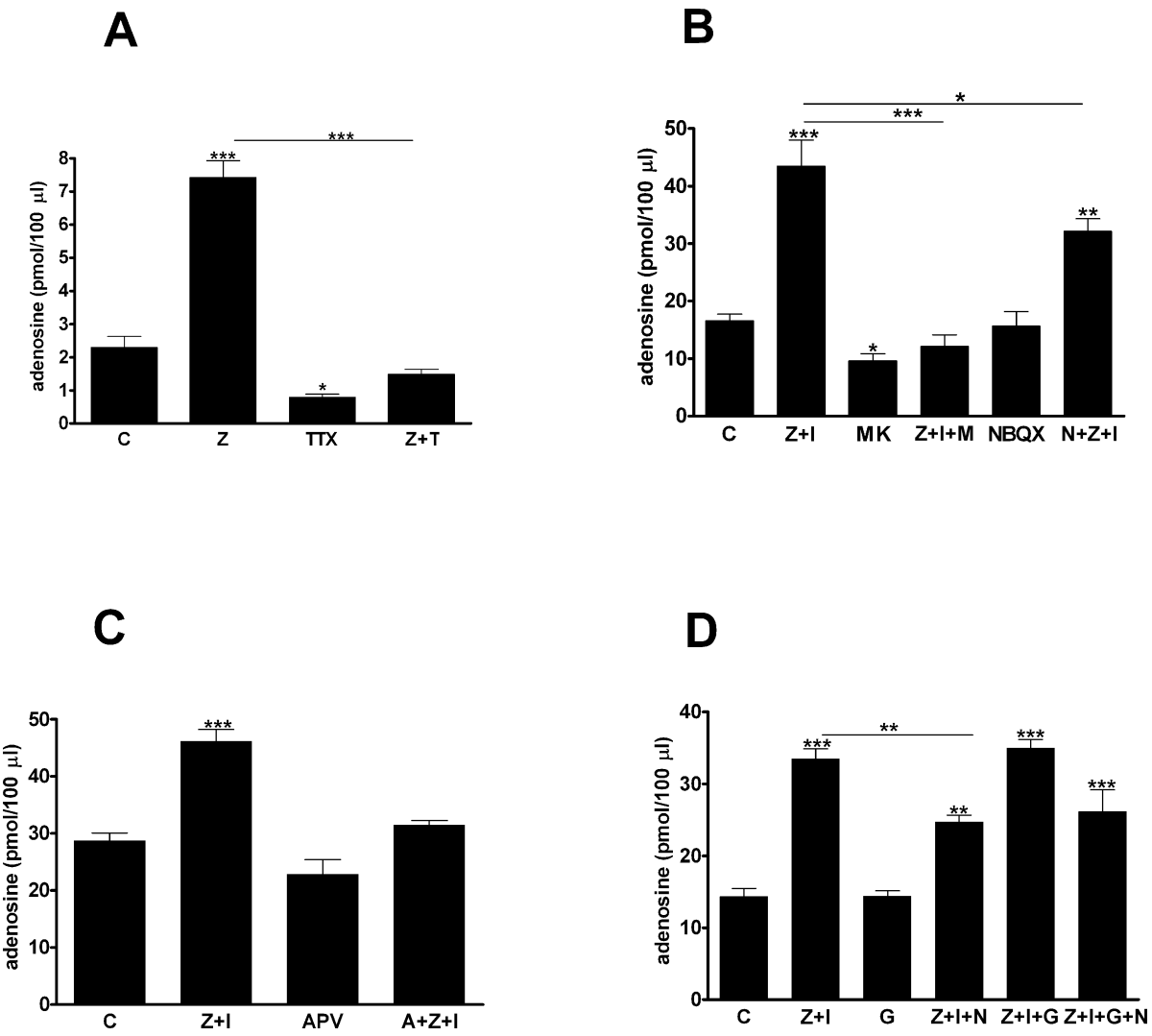
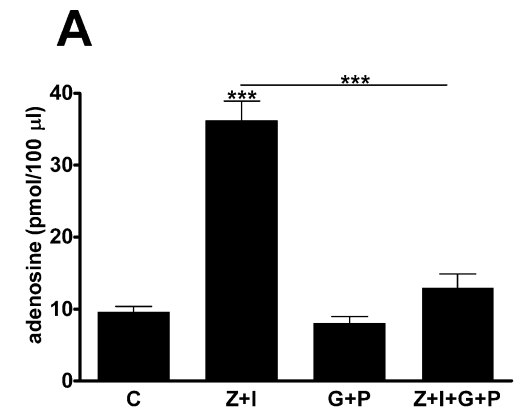


FIG. 4. Zaprinast-evoked extracellular adenosine accumulation was mediated by stimulating glutamate release. (A) TTX blocked zaprinast-evoked extracellular adenosine accumulation. Cultures were preincubated with TTX (10 µM) for 15 min, exposed to zaprinast (Z, 10 µM) for 30 min, and then extracellular adenosine was determined by HPLC. IBMX was not present in this experiment. Values are the mean ± SD from quadruplicate samples. TTX reduced basal adenosine level and completely blocked zaprinast-evoked adenosine accumulation. This experiment is representative of four that were performed. (B) MK-801 blocked while NBQX reduced zaprinast-evoked extracellular adenosine accumulation. Cultures were preincubated with MK-801 (M, 10 µM) or NBQX (N, 100 µM) for 15 min, and then exposed to zaprinast (Z, 10 µM) plus IBMX (100 µM) for 30 min. Supernatant was collected for measuring extracellular adenosine by HPLC. Values are the mean ± SD from quadruplicate samples. NBQX reduced the effect of zaprinast on extracellular adenosine accumulation, a 26% decrease when compared with zaprinast alone ($P < 0.05$). This experiment is representative of five that were performed. (C) APV blocked zaprinast-evoked extracellular adenosine accumulation. Cultures were preincubated with APV (A, 100 µM) for 15 min, exposed to zaprinast (Z, 10 µM) plus IBMX (100 µM) for 30 min, and extracellular adenosine was determined by HPLC. Values are the mean ± SD from quadruplicate samples. APV blocked 84% of zaprinast-evoked adenosine accumulation. This experiment is representative of two that were performed. (D) NBQX significantly reduced zaprinast-induced extracellular adenosine accumulation in the presence of glycine. Cultures were preincubated with NBQX (N, 100 µM) and/or glycine (G, 1 mM) for 15 min, and then exposed to zaprinast (10 µM) plus IBMX (100 µM) for 30 min. Supernatant was collected for measuring extracellular adenosine by HPLC. Values are the mean ± SD from quadruplicate samples. NBQX blocked 26% of zaprinast-evoked adenosine accumulation. Glycine had no significant effect on the ability of NBQX to reduce zaprinast-evoked adenosine accumulation. This experiment is representative of three that were performed.

results are consistent with the hypothesis that zaprinast-stimulated extracellular adenosine accumulation involves NMDA and non-NMDA receptor activation. Taken together, these data suggest that zaprinast-stimulated extracellular adenosine accumulation might be the result of an effect of zaprinast on glutamate release.

To investigate whether zaprinast stimulates glutamate release, we tested the effect of enzymatic removal of glutamate (O'Brien & Fischbach, 1986; Blitzblau *et al*., 1996; Wang *et al*., 1998) on zaprinast-evoked adenosine accumulation (Fig. 5A). Glutamate is converted to α-ketoglutarate and alanine by glutamate-pyruvate transaminase (GPT) in the presence of pyruvate (O'Brien & Fischbach, 1986). If glutamate release was a necessary intermediate step in zaprinast-evoked adenosine accumulation, we would expect that GPT would block the effect of zaprinast on extracellular adenosine. In the presence of pyruvate (2 mM) and GPT (10 U/mL), the basal adenosine level was decreased, a 34 ± 11% decrease compared with control ($n = 4$, $P < 0.05$, Fig. 5A). As expected, zaprinast-evoked extracellular adenosine accumulation was almost completely blocked (95 ± 7%, $n = 4$, $P < 0.01$) in the presence of pyruvate and GPT (Fig. 5A). Next we investigated whether an increase in extracellular glutamate stimulated by zaprinast could be demonstrated. Using a fluorometic assay to measure extracellular glutamate concentration



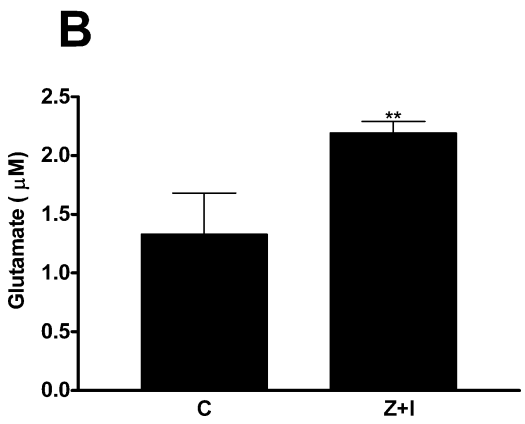
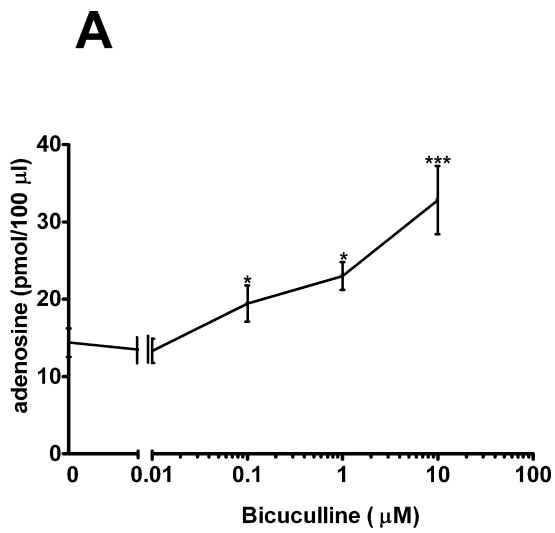


FIG. 5. Glutamate release is the intermediate step in zaprinast-evoked extracellular adenosine accumulation. (A) GPT blocked zaprinast-evoked extracellular adenosine accumulation. Cultures were preincubated with GPT/pyruvate (G, 10 U/mL; P, 2 mM) for 30 min, exposed to zaprinast (Z, 10 µM) and IBMX (I, 100 µM) for 30 min, and extracellular adenosine was determined by HPLC. Values are the mean ± SD from quadruplicate samples. In the presence of pyruvate and GPT, zaprinast-evoked extracellular adenosine accumulation was almost completely blocked (88%, $P < 0.001$). Pyruvate plus GPT caused a 16% decrease in basal adenosine ($P > 0.05$). This experiment is representative of four that were performed. (B) Zaprinast-stimulated glutamate release. Cultures were incubated with IBMX (I, 100 µM) and zaprinast (Z, 10 µM) for 30 min, and extracellular glutamate was determined by a fluorometric assay. Values are the mean ± SD from quadruplicate samples. Zaprinast stimulated extracellular glutamate accumulation (65% increase, $P < 0.01$). This experiment is representative of five that were performed.



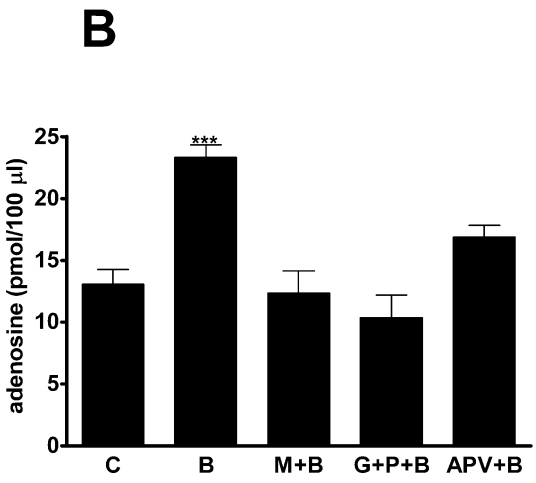


FIG. 6. Bicuculline-stimulated extracellular adenosine accumulation by causing the release of glutamate. (A) Bicuculline-stimulated extracellular adenosine accumulation. Cultures were exposed to 0, 0.01, 0.1, 1 and 10 µM bicuculline for 30 min, and extracellular adenosine was determined by HPLC. Values are the mean ± SD from quadruplicate samples. Bicuculline stimulated extracellular adenosine accumulation, with an EC_{50} value of 0.76 µM. This experiment is representative of three that were performed. (B) Bicuculline-stimulated adenosine accumulation was blocked by MK-801, APV and pyruvate plus GPT. Cultures were preincubated with GPT/pyruvate (G, 10 U/mL; P, 2 mM) for 30 min, MK-801 (M, 10 µM) and APV (100 µM) for 15 min, then exposed to bicuculline (B, 10 µM) for 30 min. Extracellular adenosine was determined by HPLC. Values are the mean ± SD from quadruplicate samples. MK-801 and pyruvate plus GPT blocked bicuculline-evoked adenosine accumulation. APV significantly reduced bicuculline-evoked adenosine accumulation, a 63% decrease when compared with bicuculline alone ($P < 0.01$). This experiment is representative of four that were performed.

(Nicholls *et al.*, 1987), we found that zaprinast stimulated glutamate release with an increase of 61 ± 12% in the extracellular medium ($n = 5$, $P < 0.001$, Fig. 5B). Taken together, these results suggest that zaprinast-evoked adenosine accumulation is mediated through increased glutamate release and subsequent activation of glutamate receptors.

Potential role of inhibitory neurotransmission in zaprinast-evoked extracellular adenosine accumulation

If the effect of zaprinast was due to stimulation of glutamate release, this might be due to a direct effect on glutamate release, possibly at excitatory synapses, or to a decrease in inhibition in the neuronal network comprising the neuronal culture, leading indirectly to an increase in synaptic glutamate release. If the latter hypothesis was correct, one would expect that blocking inhibitory neurotransmission might have the same consequences as exposure to zaprinast. To test this hypothesis, we used bicuculline, a specific $GABA_A$ receptor antagonist (Curtis *et al.*, 1970). Bicuculline stimulated extracellular adenosine accumulation, with a mean EC_{50} value of 3.3 ± 2.3 µM ($n = 3$, Fig. 6A). To further test whether the bicuculline effect was

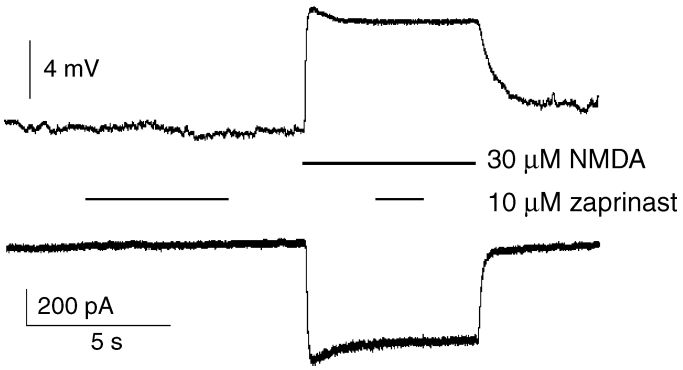


FIG. 7. Effects of direct application of zaprinast on neurons during physiological recordings. Zaprinast (10 μM) induced no changes in membrane potential nor did it antagonize 30 μM NMDA-induced depolarizations during current-clamp recordings (top trace). Similarly, zaprinast did not induce a current of its own, nor did it alter NMDA-induced currents in voltage-clamped recordings (bottom trace). Top and bottom traces correspond to different cells. Virtually identical results were obtained from a total of three cells under current-clamp and four cells under voltage-clamp.

the consequence of increased glutamate release, NMDA receptor antagonists (MK-801 and D-APV) and GPT were used to attempt to block the effect (Fig. 6B). In fact, both GPT and MK-801 completely blocked bicuculline-evoked adenosine accumulation (Fig. 6B, $n = 4$). D-APV also significantly reduced bicuculline-evoked adenosine accumulation (a $64 \pm 17\%$ decrease compared with bicuculline alone, $P < 0.01$, $n = 4$, Fig. 4B).

Because zaprinast-evoked adenosine release is ultimately mediated by activation of NMDA receptors, one possibility is that zaprinast interacts directly with NMDA receptors, resulting in their activation. To test this, we analysed the effect of zaprinast on neurons in culture under voltage- as well as current-clamp conditions (Fig. 7). We found that zaprinast had no effect on the membrane potential ($n = 3$ cells), and had no effect on NMDA-evoked currents ($n = 4$ cells). These data demonstrate that zaprinast is unlikely to act by a direct action on NMDA receptors.

Given that vinpocetine mimicked the effect of zaprinast plus IBMX (Fig. 3), and that the effect of zaprinast was blocked by MK-801, we would expect that MK-801 should block vinpocetine-evoked adenosine accumulation if the vinpocetine effect was mediated by the same mechanism as that of zaprinast. In fact, MK-801 reduced $70 \pm 17\%$ of vinpocetine-evoked adenosine accumulation ($n = 4$, Fig. 8A). Taken together, these data suggest that zaprinast-evoked extracellular adenosine accumulation is likely to be mediated by inhibition of PDE1, accumulation of cAMP, activation of PKA and stimulation of glutamate release.

To test the critical role of cAMP in zaprinast-evoked adenosine accumulation, we used a different approach to increase intracellular cAMP concentration. Specifically, we tested the effect of forskolin (Seamon *et al*., 1981), a known adenylate cyclase activator, on extracellular adenosine (Fig. 8B). Forskolin (100 μM) caused a significant increase in extracellular adenosine ($P < 0.01$, $n = 3$). The effect of forskolin on adenosine was similar to that of zaprinast plus IBMX. These data suggest that increase of cAMP, whether it is mediated by cAMP-specific PDE inhibition or by activation of adenylate cyclase, is responsible for the accumulation of adenosine. In addition, to verify that the forskolin-evoked adenosine accumulation is mechanistically similar to the zaprinast/IBMX-evoked increase in adenosine, we tested the effect of MK-801 (Fig. 8B; $n = 3$). Indeed, MK-801 blocked the forskolin-induced increase in extracellular adenosine.

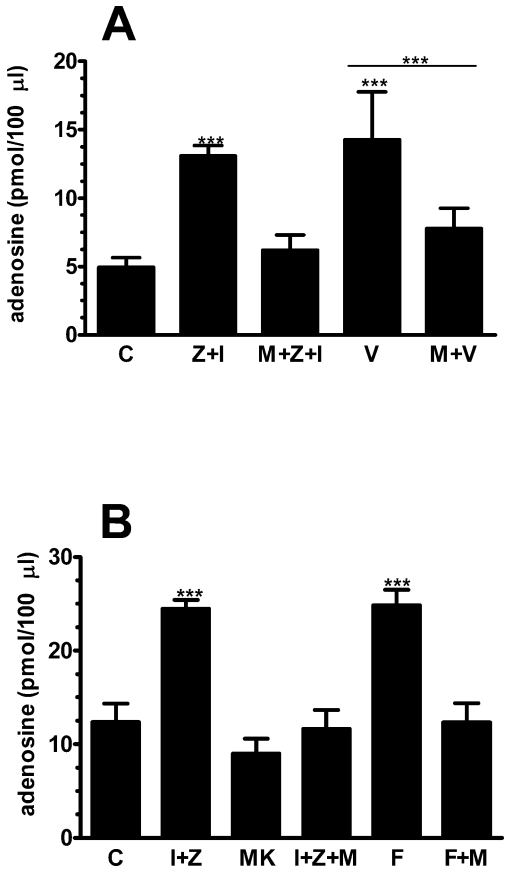


FIG. 8. Zaprinast-evoked extracellular adenosine accumulation was mimicked by vinpocetine and forskolin, and blocked by MK-801. (A) Vinpocetine-evoked extracellular adenosine accumulation was blocked by MK-801. Cultures were preincubated with MK-801 (M, 10 μM) for 15 min, then IBMX (I, 100 μM) and zaprinast (Z, 10 μM), or vinpocetine (V, 100 μM) for 30 min. Extracellular adenosine was determined by HPLC. Values are the mean ± SD from quadruplicate samples. MK-801 blocked 70% ($P < 0.001$) of the extracellular adenosine accumulation evoked by vinpocetine. This experiment is representative of four that were performed. (B) Forskolin-evoked extracellular adenosine accumulation was blocked by MK-801. Cultures were preincubated with MK-801 (M, 10 μM) for 15 min, exposed to zaprinast (Z, 10 μM) plus IBMX (I, 100 μM), forskolin (F, 100 μM) for 30 min, and then extracellular adenosine was determined by HPLC. Values are the mean ± SD from quadruplicate samples. This experiment is representative of four that were performed.

Discussion

Non-involvement of cGMP in zaprinast-evoked adenosine accumulation

It has been reported that zaprinast induced depression of excitatory synaptic transmission in the Schaffer collateral-commissural pathway in rat hippocampal slices (Boulton *et al*., 1994). This effect appeared to be mediated by adenosine receptor activation as it was blocked by an adenosine A1 antagonist (Broome *et al*., 1994). Our results directly demonstrate that zaprinast stimulates extracellular adenosine accumulation (Fig. 1), although not necessarily by the same mechanism in neuronal cultures as in hippocampal slices. Boulton *et al*. (1994) reported that the activation of the nitric oxide-cGMP signalling pathway by SNAP also evoked depression of excitatory transmission, and that 8-Br-cGMP mimicked this effect implying, but not proving, that the effect of zaprinast was mediated by an increase in intracellular cGMP. In neuronal cultures, zaprinast-evoked extracellular adenosine accumulation was not mimicked by cGMP analogues (8-Br-cGMP,

CPT-cGMP, Sp-8-pCPT-cGMP, 8-Br-PET-cGMP and Sp-8-Br-PET-cGMPS) (Fig. 2D and data not shown), and zaprinast did not stimulate intracellular cGMP accumulation. Furthermore, reducing intracellular cGMP level by ODQ had no effect on zaprinast-evoked extracellular adenosine accumulation (Fig. 2B and C). Finally, in the same system, nitric oxide stimulated adenosine release, but by a cGMP-independent mechanism (Rosenberg *et al*., 2000). Therefore, in neuronal cultures it is unlikely that a cGMP-dependent pathway mediates zaprinast-evoked extracellular adenosine accumulation. For this reason, cGMP-mediated mechanisms affecting excitatory (Sistiaga *et al*., 1997; Wei *et al*., 2002) or inhibitory (Wei *et al*., 1999) synaptic transmission are unlikely to be involved in zaprinast-evoked extracellular adenosine accumulation in neuronal cultures. Consistent with our observations, Staveren *et al*. (2001) showed that zaprinast had no effect on cGMP levels in rat hippocampal slices. In addition, Kruuse *et al*. (2001) found that 30 μM zaprinast increased cAMP levels in cerebral arteries, and that zaprinast dilated arteries even in the presence of ODQ, which greatly suppressed cGMP levels.

Role of cAMP in zaprinast-stimulated adenosine release

Zaprinast-evoked adenosine release in neuronal culture appears, instead, to be mediated by increase of intracellular cAMP and activation of PKA. Zaprinast stimulated intracellular cAMP accumulation, and a PKA antagonist, Rp-8-Br-cAMP, significantly reduced zaprinast-evoked adenosine accumulation. Furthermore, activation of adenylate cyclase by forskolin also stimulated adenosine accumulation. These data suggest that a PKA-dependent pathway is likely to be involved. The effect of zaprinast to increase cAMP is most likely due to inhibition of cAMP PDE activity. Because the effect of zaprinast is mimicked and occluded by vinpocetine, it appears likely that the target for zaprinast is PDE1.

PDE1 is a calcium/calmodulin-dependent enzyme that plays a key role in the complex interactions between the cyclic nucleotide and calcium second messenger systems. Three genes encoding PDE1 have been identified: PDE1A, PDE1B and PDE1C. Multiple alternative splice variants of PDE1A (Sonnenburg *et al*., 1993, 1995) and PDE1C (Yan *et al*., 1995, 1996) have been identified with different enzyme kinetics, tissue and cellular localization. Different substrate affinities for cAMP and cGMP of the multiple PDE1 isozymes have also been reported (Yan *et al*., 1995, 1996). For example, bovine PDE1A2 has a Km of 112.7 μM for cAMP and 5.1 μM for cGMP, bovine PDE1B1 has a Km of 24.3 μM for cAMP and 2.7 μM for cGMP, and rat PDE1C2 has a Km of 1.2 μM for cAMP and 1.1 μM for cGMP. PDE1 mRNA and protein have been found in various brain regions (Bentley *et al*., 1992; Sonnenburg *et al*., 1995; Yan *et al*., 1996), suggesting an important role in neuronal function. It has been shown that PDE1C splice variants with a high affinity for both cAMP and cGMP were expressed in cerebellar granule cells in culture (Yan *et al*., 1996). The present data implicating PDE1 in zaprinast-evoked adenosine accumulation are compatible with previous observations that PDE1 is present in cortex (Bentley *et al*., 1992; Sonnenburg *et al*., 1993). However, previous studies have failed to demonstrate a role for PDE1 in cyclic nucleotide metabolism in cortical neuronal cultures (Yamashita *et al*., 1997; Suvarna & O'Donnell, 2002).

We have shown that zaprinast-evoked adenosine accumulation is significantly potentiated by IBMX (Fig. 1B). IBMX has dual activity, acting as an antagonist of adenosine receptors as well as inhibiting cAMP PDE (Choi *et al*., 1988). IBMX potentiation of the effect of zaprinast might be due to: (i) blocking adenosine receptor activation, therefore decreasing inhibitory tone in the neuronal network; (ii) binding to the same PDE enzyme as zaprinast, but at a different site, potentiating the inhibitory effect of zaprinast on PDE activity; (iii) inhibiting PDE enzymes that are not inhibited by zaprinast, thus affecting overall PDE activity.

Zaprinast-evoked extracellular adenosine accumulation is mediated by glutamate release

We have previously shown that NMDA receptor activation evoked adenosine release in neuronal cultures (Lu *et al*., 2003). Extracellular glutamate accumulation and glutamate receptor activation in neurons are likely to be responsible for the accumulation of extracellular adenosine by zaprinast observed in the present study for the following reasons: (i) MK-801, a non-competitive NMDA receptor antagonist, and D-APV, a competitive NMDA receptor antagonist, blocked zaprinast-evoked adenosine accumulation (Fig. 4A and B), indicating that NMDA receptor activation is required for zaprinast-evoked adenosine accumulation; (ii) zaprinast-evoked extracellular adenosine accumulation was blocked by an enzyme system degrading extracellular glutamate (Fig. 5A), demonstrating that glutamate accumulation in the extracellular medium is required for zaprinast-evoked adenosine accumulation; (iii) zaprinast-stimulated extracellular glutamate accumulation was confirmed by measuring extracellular glutamate (Fig. 5B); (iv) blocking inhibitory neurotransmission with bicuculline, which indirectly stimulates glutamate release, also mimicked the effect of zaprinast (Fig. 6); (v) zaprinast-evoked extracellular adenosine accumulation occurs only in neuronal rat forebrain cultures, which contain less than 1% astrocytes, and not in astrocyte cultures, suggesting that zaprinast-stimulated extracellular adenosine accumulation is a neuron-specific phenomenon. Interestingly, no effect of zaprinast in AR cultures was observed. In AR cultures, the effect of zaprinast stimulation of glutamate release from a small number of neurons (10% of total cells) may be abrogated by the presence of astrocytes, which are plentiful, surround excitatory synapses in these cultures (Harris & Rosenberg, 1993) and rapidly take up released glutamate (Rosenberg, 1991). These data indicate that the effect of zaprinast on adenosine accumulation in neuronal cultures is due to glutamate release and NMDA receptor activation. The electrophysiological experiments performed herein exclude a direct activation of NMDA receptors by zaprinast. Other effects of zaprinast on the membrane and synaptic properties of neurons are possible and, in fact, likely as an explanation for this compound's effect on glutamate release. However, a detailed investigation of this issue is beyond the scope of the present report.

NMDA-stimulated extracellular adenosine accumulation has been reported *in vivo* in rat striatum (Melani *et al*., 1999) and hippocampus (Chen *et al*., 1992), as well as *in vitro* in guinea pig hippocampal (Manzoni *et al*., 1994) and rat cortical slices (Craig & White, 1993). Extracellular adenosine accumulation has also been shown to be stimulated by non-NMDA receptor activation *in vivo* in rat hippocampus (Carswell *et al*., 1997), as well as *in vitro* in rat cortical slices (Craig & White, 1993). In our studies, it appears that most of the zaprinast effect was mediated by NMDA receptor activation. The contribution of non-NMDA receptor activation may be to depolarize the membrane and potentiate NMDA receptor activation by releasing the voltage-dependent magnesium block.

If glutamate release and NMDA receptor activation account for the effect of zaprinast in adenosine accumulation, it remains to be determined how zaprinast causes glutamate release. We hypothesize that zaprinast-stimulated glutamate release is contingent upon the increase in intracellular cAMP and activation of PKA. We have demonstrated that increase of cellular cAMP, whether it is mediated by zaprinast plus IBMX, by vinpocetine, or by forskolin, causes accumulation of adenosine. In all three cases, NMDA receptor antagonists blocked the adenosine effect, suggesting that glutamate receptor activation is involved. Consistent with this hypothesis, it has been

reported that dopamine D1 receptor activation stimulates the release of glutamate in the hippocampus via increasing cAMP levels (Bouron & Reuter, 1999), and cAMP analogues have been shown to enhance the release of glutamate from slices of rat dentate gyrus (Dolphin & Archer, 1983), in cerebrocortical nerve terminals (Herrero & Sanchez-Prieto, 1996) and in the entorhinal cortex (Evans *et al*., 2001). In addition, presynaptic group III mGluRs facilitate release of glutamate via activation of adenylate cyclase and subsequent PKA activation (Evans *et al*., 2001). Although the molecular mechanisms that regulate exocytosis by PKA are not well characterized, it has been shown that PKA phosphorylates synaptic vesicle proteins (cysteine string protein and snapin), and thus modulates the vesicle priming (snapin) and vesicle fusion (cysteine string protein) stages of exocytosis (Evans & Morgan, 2003).

Zaprinast-evoked adenosine accumulation might also be mediated by inhibition of inhibitory neurotransmission as bicuculline mimics the effect of zaprinast. The role of cAMP in regulating inhibitory neurotransmission is not clear. It has been reported that cAMP analogues both stimulate (Wang *et al*., 1997; Cunha & Ribeiro, 2000) and inhibit (Weiss, 1988) GABA release. Increase in intracellular cAMP has been reported to enhance or inhibit the $GABA_A$ receptor response (McDonald *et al*., 1998; Brunig *et al*., 1999).

Role of adenosine in long-term depression and long-term potentiation

In the hippocampus, zaprinast-induced depression of field excitatory potentials, elicited by stimulation of the Schaffer collateral-commissural pathway, is mediated by adenosine A1 receptors (Boulton *et al*., 1994; Broome *et al*., 1994), and we show here that zaprinast stimulates adenosine release in rat forebrain neuronal cultures. It has been reported that zaprinast induces long-term depression in the corticostriatal (Calabresi *et al*., 1999) and cerebellar brain slice preparation (Hartell, 1996). However, the role of adenosine in the generation of long-term depression by zaprinast has not been explored. In contrast, adenosine is known to suppress long-term potentiation of the CA1 neurons in hippocampal slices (Arai *et al*., 1990; De Mendonca & Ribeiro, 1990; Fujii *et al*., 2000).

In summary, we demonstrate here that zaprinast stimulates extracellular adenosine accumulation in rat forebrain neuronal cultures by causing the release of glutamate via a cGMP-independent, cAMP-dependent process. Inhibition of inhibitory neurotransmission also caused extracellular adenosine accumulation, suggesting that the effect of zaprinast on extracellular adenosine accumulation may be either to directly stimulate glutamate release or to indirectly stimulate glutamate release by suppressing inhibitory synaptic transmission. These results link intracellular cAMP levels with the regulation of network activity, as illustrated in Fig. 9. Inhibition of PDE1 either by zaprinast/IBMX or by vinpocetine and activation of adenylate cyclase by forskolin causes intracellular cAMP accumulation (A). cAMP may increase neuronal activity either directly (B^1) or indirectly by decreasing inhibitory neurotransmission (B^2). Increased neuronal activity leads to glutamate receptor activation (C), which evokes adenosine release (D). Adenosine exerts a negative feedback effect on neuronal activity (E). Activation of glutamate receptors not only regulates extracellular adenosine levels, as shown here, but also selectively activates transcription factors (West *et al*., 2002), which in turn might be expected to have a profound effect on network activity over a longer time scale. The present results suggest the existence of multiple mechanisms to control the excitability of ensembles of neurons, reminiscent of the direct (mediated by activation of adenylyl cyclase) and indirect (mediated by the production of adenosine) effects of dopamine in the midbrain slice preparation (Bonci & Williams, 1996). Characterizing these mechanisms further may be important in understanding synaptic plasticity, behavioural state regulation, excitotoxicity and epileptogenesis.

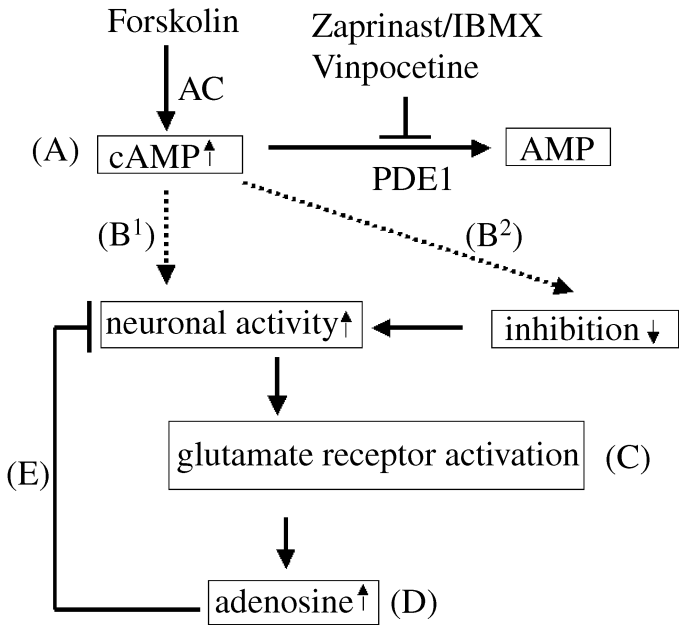


FIG. 9. Augmentation of cAMP leads to increased neuronal activity and homeostatic release of adenosine. Inhibition of PDE1 by zaprinast/IBMX or vinpocetine, and activation of adenylate cyclase by forskolin, cause the accumulation of intracellular cAMP. cAMP may increase neuronal activity either directly or indirectly by decreasing inhibitory neurotransmission. Increased neuronal activity causes glutamate receptor activation, which evokes adenosine release. Adenosine exerts a negative feedback effect on neuronal activity.

Acknowledgements

This work was supported by grants from the Medical Research Council of Canada/Muscular Dystrophy Association of Canada (Y.L.), the National Heart, Lung, and Blood Institute (HL59595 and HL60292; P. A. R.), and the National Institute of Child Health and Human Development (HD 18655) to Children's Hospital.

Abbreviations

AR, astrocyte-rich; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; GABA, γ-aminobutyric acid; GPT, glutamate-pyruvate transaminase; HPLC, high-performance liquid chromatography; IBMX, isobutylmethylxanthine; NMDA, *N*-methyl-D-aspartate; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PDE, phosphodiesterase; PKA, protein kinase A; TTX, tetrodotoxin.

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