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Co-administration of adenosine kinase and deaminase inhibitors produces supra-additive potentiation of *N*-methyl-D-aspartate-evoked adenosine formation in cortex

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Abstract

Activation of glutamate receptors triggers the release of adenosine, which exerts important inhibitory actions in the brain. Evoked adenosine release is potentiated when either adenosine kinase or adenosine deaminase are inhibited. We studied the effects of concurrent inhibition of adenosine kinase and adenosine deaminase on *N*-methyl-D-aspartate (NMDA)-evoked formation of extracellular adenosine in slices of rat parietal cortex, to determine if combinations of inhibitors of adenosine kinase and adenosine deaminase can produce supra-additive potentiation of this adenosine formation. Combinations of low concentrations of the adenosine kinase inhibitors 5′-amino-5′-deoxyadenosine (0.2 μM) or 5′-iodotubercidin (0.01 μM) with a low concentration of the adenosine deaminase inhibitor 2′-deoxycoformycin (0.2 μM) produced additive potentiations of NMDA-evoked adenosine release from slices of rat parietal cortex. However, combinations of low concentrations of 5′-amino-5′-deoxyadenosine (0.2 μM) or 5′-iodotubercidin (0.01 μM) with a maximal concentration of 2′-deoxycoformycin (200 μM) produced supra-additive potentiation of NMDA-evoked adenosine release. These findings suggest that such combinations of adenosine kinase inhibitors with adenosine deaminase inhibitors may provide useful strategies for developing therapies to treat disorders associated with excessive NMDA receptor activation, such as seizures, ischemic damage and neurodegenerative diseases. © 1998 Elsevier Science B.V.

Keywords: 2′-Deoxycoformycin; 5′-Amino-5′-deoxyadenosine; 5′-Iodotubercidin; Adenosine; NMDA (*N*-methyl-D-aspartate)

1. Introduction

Excessive activation of excitatory amino acid receptors has been implicated in numerous neurological disorders, including those involving seizures, excitotoxicity and neurodegeneration (Lipton and Rosenberg, 1994). Activation of excitatory amino acid receptors in the central nervous system promotes the formation of extracellular adenosine, which in turn acts at adenosine A_1 receptors to provide an inhibitory threshold for excitatory amino acid-evoked transmission (Craig and White, 1992; White et al., 1993; Manzoni et al., 1994). This depression of excitation may be due to both pre- and post-synaptic actions at adenosine A_1 receptors (Okada and Ozawa, 1980; Corradetti et al., 1984; Garaschuk et al., 1992). The adenosine formed when *N*-methyl-D-aspartate (NMDA) receptors are activated appears to modulate normal excitatory amino acid transmission (Craig and White, 1992; Mitchell et al., 1993; Manzoni et al., 1994) and provides some protection from excessive stimulation, seizures and excitotoxicity (Von Lubitz et al., 1993). However, this protection afforded by endogenous adenosine appears to be incomplete in so far as the acute administration of adenosine and various adenosine agonists provides additional protection (Von Lubitz et al., 1993). Unfortunately, adenosine agonists produce numerous unacceptable side-effects which preclude their chronic use therapeutically for the treatment of seizure disorders or neurodegeneration. On the other hand, there may be value in elevating endogenous extracellular adenosine levels in an event-specific manner, where the basal levels of extracellular adenosine remain largely unaltered but the formation of extracellular adenosine evoked by excessive glutamate receptor activation or conditions of metabolic stress are potentiated substantially. Under these conditions, extracellular adenosine levels would not be increased chronically but only intermittently during periods of excessive activity or metabolic stress. The increased

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extracellular adenosine might then provide protection against seizures and the development of excitotoxicity.

There are three primary ways to potentiate the formation of extracellular adenosine in the central nervous system: first, by inhibiting the conversion of adenosine to inosine via adenosine deaminase, secondly by inhibiting the conversion of adenosine to 5′AMP by adenosine kinase and finally by blocking the uptake of extracellular adenosine. Previously, we studied the effects of inhibitors of adenosine deaminase and adenosine kinase on the formation of extracellular adenosine evoked by NMDA, kainate and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) in rat cortical slices (White, 1996). Inhibition of adenosine kinase with either 5′-amino-5′-deoxyadenosine or 5′-iodotubercidin had a much greater potentiating effect on evoked adenosine release than on basal release, indicating that this potentiation is event-specific. Moreover, adenosine kinase inhibitors appear to be much more effective and selective than adenosine deaminase inhibitors, such as 2′-deoxycoformycin and erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), at potentiating NMDA-evoked adenosine formation. Significantly, Zhang et al. (1993) have also shown that adenosine kinase inhibitors are much more effective than adenosine deaminase inhibitors as anticonvulsants in a bicucculline model of epilepsy. However, when they combined an adenosine kinase inhibitor (5′-amino-5′-deoxyadenosine) at a dose that had negligible anticonvulsant activity with a high dose of an adenosine deaminase inhibitor (2′-deoxycoformycin), full anticonvulsant efficacy was obtained. This suggests that combinations of adenosine deaminase inhibitors with adenosine kinase inhibitors might produce supra-additive anticonvulsant effects as a consequence of supra-additive potentiations of extracellular adenosine formation. In the present study, we examined the effects of combinations of adenosine deaminase and adenosine kinase inhibitors on the formation of extracellular adenosine produced when NMDA receptors were activated in slices of rat parietal cortex.

2. Materials and methods

2.1. Preparation of cortical slices

All procedures were approved by the Dalhousie University Animal Care Committee. Male Sprague–Dawley rats (250–350 g, Charles River Canada) were killed by decapitation and their brains rapidly excised into ice-cold Krebs–Henseleit bicarbonate medium containing 111 mM NaCl, 26.2 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$ and 11 mM glucose gassed with 95% O_2–5% CO_2 to maintain a pH of 7.4. A lateral 1–1.5 mm portion of parietal cortex was removed from both hemispheres with a recessed tissue slicer and 0.4 mm coronal slices were prepared with a McIlwain tissue chopper. Adjacent slices were placed into two tissue baths so that each bath contained 6 slices (3 each from the left and right hemispheres).

2.2. Superfusion of slices

Cortical slices rested on nylon mesh platforms in two tissue baths adjusted to internal volumes of 0.5 ml. The two tissue baths were run in parallel and assigned in alternate experiments as either ‘test’ or ‘control’ treatments. The slices were perfused from top to bottom with oxygenated Krebs–Henseleit medium at 36°C at a flow rate of 0.75 ml/min. Control baths were equilibrated with Krebs–Henseleit medium for 60 min, whereas treated baths were equilibrated for 50 min with Krebs–Henseleit medium, followed by an additional 10 min of preincubation with Krebs–Henseleit medium and one or both of the inhibitor drugs. After equilibration/preincubation for 60 min, 3 serial 2.5 min fractions were collected from both baths and processed for analysis of adenosine content. After the third fraction was collected, the superfusing medium in the control bath was switched from Krebs–Henseleit to Krebs–Henseleit + NMDA (100 mM) and that in the treated bath was switched from Krebs–Henseleit + drug to Krebs–Henseleit + drug + NMDA (100 μM). Four serial 2.5 min fractions were then collected from each bath and processed for analysis of adenosine content. After collecting the fourth fraction, the superfusing media were switched back to those before the NMDA stimulus (to Krebs–Henseleit for control and Krebs–Henseleit + drug for treated). Three final serial 2.5 min fractions were then collected and processed for analysis of adenosine content. Following collection of all fractions, the slices were removed from the tissue baths for determination of wet tissue weights.

2.3. Determination of adenosine release

Samples of superfusate were deproteinated with $Ba(OH)_2$ and $ZnSO_4$ and then reacted with chloroacetylaldehyde to form 1-*N*6-ethenoadenosine, which was assayed by high performance liquid chromatography (HPLC) with fluorescence detection as described previously (Hoehn and White, 1990; Hoehn et al., 1990). Adenosine standards in Krebs–Henseleit medium were assayed identically. Adenosine standards in Krebs–Henseleit plus drug were also assayed. The amount of adenosine in the samples was quantitated by comparison of peak heights with the standards and the rate of adenosine release calculated and expressed as pmol/g cortex per min. The basal rate of adenosine release was taken as the rate for the sample immediately preceding NMDA exposure (time 0). Evoked

adenosine release was obtained by subtracting the basal rate of release from all other samples. The total evoked adenosine (nmol/g cortex) was the total amount of adenosine released during the entire 17.5 min period following exposure to NMDA. Because there was substantial variability in the values obtained between separate series of experiments, values from individual treatments were normalized to percents of NMDA-evoked adenosine release alone (control). The mean percents of control for a particular series of experiments were calculated to determine the overall effect of the various drug treatments on extracellular adenosine formation. This permitted the comparison of results from separate series of experiments and construction of concentration–response curves for adenosine kinase and deaminase inhibitors on NMDA-evoked formation of extracellular adenosine. Values are expressed as mean % of control ± S.E.M.

2.4. Drugs and chemicals

Adenosine, *N*-methyl-D-aspartate (NMDA) and 5′-amino-5′-deoxyadenosine were purchased from Sigma Chemical Co., Saint Louis, MO. 5′-Iodotubercidin was purchased from Research Biochemicals International, Natick, MA. Chloracetylaldehyde was purchased from Aldrich Chemical Co., Milwaukee, WI. 2′-Deoxycoformycin was a gift from Parke-Davis Pharmaceutical Research Division, Ann Arbor, MI. 5′-Iodotubercidin was dissolved in dimethyl sulfoxide and diluted 500-fold in Krebs–Henseleit medium. All other drugs dissolved freely in Krebs–Henseleit medium. Controls were superfused identically with the corresponding solvent-containing media.

2.5. Statistical analysis

Total evoked adenosine was initially calculated in pmol/g tissue/min. In order to eliminate inter-experimental variation in adenosine release, raw values of total-evoked adenosine release obtained from the treated baths were normalized to percents of the control baths. Total evoked adenosine release was compared between groups using a 1-way analysis of variance (ANOVA), followed by Newman–Keuls post-hoc analysis. Significance was assumed when $P < 0.05$.

3. Results

3.1. Concentration–response relationships for adenosine kinase and deaminase inhibitors to potentiate NMDA-evoked formation of extracellular adenosine

5′-Iodotubercidin was 10-fold more potent than 5′-amino-5′-deoxyadenosine at potentiating the formation of extracellular adenosine evoked by NMDA (Fig. 1). The adenosine deaminase inhibitor 2′-deoxycoformycin appeared to be much less effective than the adenosine kinase inhibitors in potentiating NMDA-evoked adenosine formation, confirming that inhibition of adenosine kinase results in substantially greater potentiation of evoked adenosine formation than does inhibition of adenosine deaminase (White, 1996).

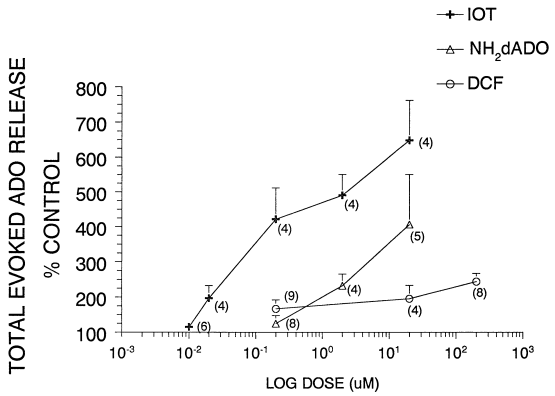

Fig. 1. Concentration–response relations for the potentiation of NMDA-evoked adenosine release by adenosine deaminase inhibitors and adenosine kinase inhibitors. Abbreviations: IOT, 5′-iodotubercidin; NH_2dADO, 5′-amino-5′-deoxyadenosine; DCF, 2′-deoxycoformycin. The adenosine kinase inhibitors 5′-iodotubercidin and 5′-amino-5′-deoxyadenosine appeared to be more effective than the adenosine deaminase inhibitor 2′-deoxycoformycin in potentiating NMDA-evoked formation of extracellular adenosine. 5′-Iodotubercidin was more potent than 5′-amino-5′-deoxyadenosine in potentiating adenosine formation. Numbers in parentheses indicate the number of experiments.

3.2. Effects of combinations of adenosine kinase inhibitors and adenosine deaminase inhibitors on NMDA-evoked adenosine release

Low concentrations of 5′-amino-5′-deoxyadenosine (0.2 μM) or 2′-deoxycoformycin (0.2 μM) potentiated adenosine release to 132 ± 12.7 and 177 ± 19.8% of control values, respectively (Fig. 2). The combination of a low concentration of 5′-amino-5′-deoxyadenosine (0.2 μM) with a low concentration of 2′-deoxycoformycin (0.2 μM) appeared to produce additive effects, potentiating NMDA-evoked adenosine formation to 204 ± 19.5% of control (Fig. 2). There was no significant difference between the values obtained for the low concentration of 2′-deoxycoformycin and the combination of 5′-amino-5′-deoxyadenosine and 2′-deoxycoformycin ($P > 0.05$). A high (maximal) concentration of 2′-deoxycoformycin (200 μM) potentiated adenosine release to 236 ± 21.1% of control (Fig. 2). When a low concentration of 5′-amino-5′-deoxyadenosine (0.2 μM) was combined with this maximal concentration of 2′-deoxycoformycin (200 μM), the resulting potentiation of NMDA-evoked adenosine release was greater than the sum of the effects of the individual drugs, indicating supra-additive potentiation of NMDA-evoked adenosine release (465 ± 110% of control, $P < 0.05$; Fig. 2).

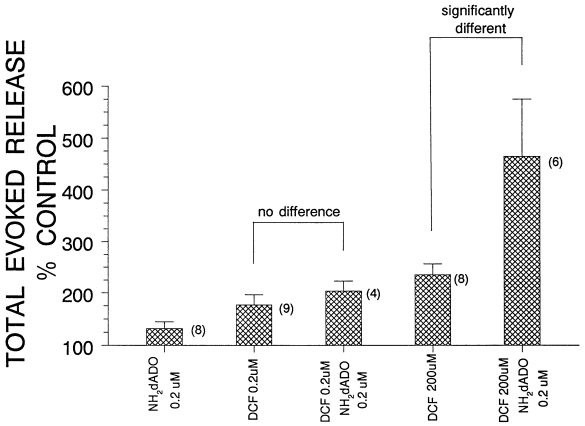

Fig. 2. Effects of combinations of 5′-amino-5′-deoxyadenosine with 2′-deoxycoformycin on NMDA-evoked formation of extracellular adenosine. Abbreviations: NH_2dADO, 5′-amino-5′-deoxyadenosine; DCF, 2′-deoxycoformycin. The effect of a combination of 0.2 μM 5′-amino-5′-deoxyadenosine with 0.2 μM 2′-deoxycoformycin on NMDA-evoked formation of extracellular adenosine was not statistically different than the effect of 0.2 μM 2′-deoxycoformycin alone ($P > 0.05$). However, the effect of the combination of 0.2 μM 5′-amino-5′-deoxyadenosine with 200 μM 2′-deoxycoformycin on NMDA-evoked adenosine formation was significantly greater than the effect of 200 μM 2′-deoxycoformycin alone ($P < 0.05$). Numbers in parentheses indicate the number of experiments.

When a low concentration of the adenosine kinase inhibitor, 5′-iodotubercidin (0.01 μM), which on its own potentiated adenosine release to $114 \pm 14.9\%$ of control, was combined with a low concentration of 2′-deoxycoformycin (0.2 μM), potentiation of NMDA-evoked adenosine release was additive ($253 \pm 63.9\%$ of control; Fig. 3). However, when the same low concentration of 5′-iodotubercidin was combined with a maximal concentration of 2′-deoxycoformycin (200 μM), supra-additive potentiation of NMDA-evoked adenosine release was observed ($368 \pm 50.9\%$ of control, $P < 0.05$; Fig. 3).

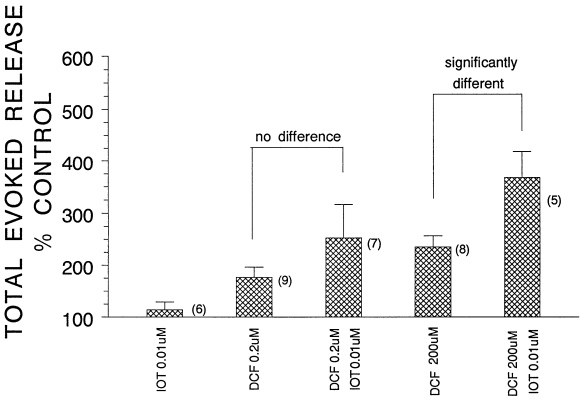

Fig. 3. Effects of combinations of 5′-iodotubercidin with 2′-deoxycoformycin on NMDA-evoked formation of extracellular adenosine. Abbreviations: IOT, 5′-iodotubercidin; DCF, 2′-deoxycoformycin. The effect of a combination of 0.01 μM 5′-iodotubercidin with 0.2 μM 2′-deoxycoformycin on NMDA-evoked formation of extracellular adenosine was not statistically different than the effect of 0.2 μM 2′-deoxycoformycin alone ($P > 0.05$). However, the effect of a combination of 0.01 μM 5′-iodotubercidin with 200 μM 2′-deoxycoformycin was significantly greater than 200 μM 2′-deoxycoformycin alone ($P < 0.05$). Numbers in parentheses indicate the number of experiments.

4. Discussion

Adenosine is released from post-synaptic neurons in response to glutamate receptor activation and is thought to provide inhibitory neuromodulation via activation of pre- and post-synaptic adenosine A_1 receptors (Okada and Ozawa, 1980; Corradetti et al., 1984; Garaschuk et al., 1992). Potentiation of extracellular adenosine formation in the central nervous system (CNS) may offer therapeutic benefit by suppressing excitotoxicity due to ischemic/hypoxic or hypoglycemic conditions (Choi, 1988), as well as diminishing epileptogenic (DiFiglia, 1990) and neurodegenerative (Choi, 1988; DiFiglia, 1990; Beal et al., 1991) disorders. We have shown previously that adenosine kinase inhibitors produce substantial potentiation of NMDA-evoked formation of extracellular adenosine with much less effect on basal adenosine release, suggesting that their effects are event-specific (White, 1996). In the present study, the adenosine kinase inhibitors 5′-iodotubercidin and 5′-amino-5′-deoxyadenosine were more potent and effective than the adenosine deaminase inhibitor 2′-deoxycoformycin at potentiating NMDA-evoked formation of extracellular adenosine in rat cortical slices. This confirms previous studies that indicated that adenosine kinase plays a greater role than adenosine deaminase in regulating extracellular adenosine levels in the CNS (Lloyd and Fredholm, 1995; White, 1996). Moreover, Zhang et al. (1993) showed that inhibition of adenosine kinase is much more effective than inhibition of adenosine deaminase in preventing bicuculline-evoked seizures in rats. The present results support this observation.

Parkinson and Geiger (1996) have recently shown that, besides inhibiting adenosine kinase, 5′-iodotubercidin also inhibits adenosine transport and the binding of nitrobenzylthioinosine to nucleoside transporters in cultured smooth muscle cells. This raises the possibility that part of the potentiating effect of 5′-iodotubercidin on NMDA-evoked formation of extracellular adenosine might be due to effects on adenosine transport rather than effects on adenosine kinase. However, the K_i values for 5′-iodotubercidin to inhibit adenosine transport are in the micromolar range (Parkinson and Geiger, 1996), much greater than the nanomolar concentrations which potentiated NMDA-evoked adenosine release in the present study. Thus, inhibition of nucleoside transport likely contributed little to 5′-iodotubercidin's capacity to potentiate NMDA-evoked formation of extracellular adenosine observed in the present study.

Zhang et al. (1993) showed that the combination of a low dose of an adenosine kinase inhibitor with a maximal

dose of an adenosine deaminase inhibitor produced supra-additive suppression of bicuculline-induced seizures in the rat. We found that such combinations caused supra-additive potentiation of NMDA-evoked adenosine formation, thus supporting the proposition that the anticonvulsant actions observed by Zhang et al. (1993) may be mediated by the formation of extracellular adenosine, possibly as a consequence of NMDA receptor activation. It should be noted that the combination of a low dose of an adenosine kinase inhibitor with a low dose of a deaminase inhibitor does not produce supra-additive potentiation of NMDA-evoked adenosine formation. When the adenosine deaminase and kinase pathways are both only partially inhibited, adenosine deaminase may play a greater role in adenosine metabolism. However, when the adenosine deaminase pathway is completely blocked, inhibition of adenosine kinase can then promote supra-additive potentiation of NMDA-evoked adenosine formation.

In conclusion, the combination of a low concentration of adenosine kinase inhibitor with a maximal concentration of an adenosine deaminase inhibitor produces supra-additive potentiation of NMDA-evoked formation of extracellular adenosine in the cortex. These findings support the notion that the combination of adenosine kinase inhibitors with adenosine deaminase inhibitors may provide a useful approach in the development of new therapeutic strategies for treating disorders associated with excessive NMDA receptor activation in the brain, such as seizures, excitotoxicity and various neurodegenerative disorders.

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