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Methylcobalamin attenuates the hypoxia/hypoglycemia- or glutamate-induced reduction in hippocampal fiber spikes in vitro

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Abstract

The effects of methylcobalamin, a vitamin B_{12} analogue, on the hypoxia/hypoglycemia- or glutamate-induced reduction in hippocampal CA1 presynaptic fiber spikes elicited by Schaffer collateral stimulation in rat brain slices were evaluated. Hippocampal slices were exposed to 15 min of hypoxia/hypoglycemia, and then these slices were returned to oxygenated and glucose-containing buffer for 3 h. Hypoxia/hypoglycemia reduced CA1 presynaptic potentials in vitro. Treatment with 10 μM methylcobalamin attenuated the impairment of CA1 presynaptic potentials induced by hypoxia/hypoglycemia or glutamate application (10 mM). Daily injection of methylcobalamin (0.5 mg/kg i.p./day) for 3 days in vivo also attenuated the hypoxia/hypoglycemia- or glutamate-induced reduction in presynaptic potentials in hippocampal slices. Pretreatment with cyanocobalamin at 10 μM failed to attenuate the impairment of CA1 presynaptic potentials. However, daily injection of cyanocobalamin (0.5 mg/kg i.p./day) for 3 days caused a protective action against the hypoxia/hypoglycemia- or glutamate-induced functional deficit. Furthermore, co-treatment of L-arginine (100 μM), a substrate for nitric oxide synthase, with methylcobalamin in vitro reversed the methylcobalamin-induced functional recovery. The present results demonstrate that methylcobalamin application in vivo or in vitro leads to functional recovery from hypoxia/hypoglycemia- or glutamate-induced impairment of CA1 presynaptic potentials. Neuroprotection was obtained by in vivo application of cyanocobalamin, but not by its in vitro application. It is reported that in vivo injected cyanocobalamin converted to methylcobalamin in the hepatic cells. Therefore, the results suggest that a transmethylation reaction in the hippocampal regions may be involved in the methylcobalamin-induced functional recovery from ischemic impairment.

Keywords: Vitamin B_{12}; Ischemia; Hippocampus; Neuroprotection; Glutamate; Hypoxia/hypoglycemia

1. Introduction

Vitamin B_{12} is an essential nutrient with important coenzyme functions in the metabolism of most tissues. The brain is relatively rich in vitamin B_{12} in humans (Inada et al., 1982), which indicates that vitamin B_{12} can be transported through the blood-brain barrier. Although the mechanisms responsible for the neurological lesions of vitamin B_{12} deficiencies are less well understood, vitamin B_{12} analogues, including methylcobalamin, have been widely used in the therapy of neurological diseases not only in the peripheral nervous system but also in the central nervous system (Chanarin et al., 1985).

The involvement of glumatate and NMDA receptors in ischemic neural damage was first demonstrated by Simon et al. (1984) reporting on the protective effect of an NMDA receptor antagonist. We (Shibata et al., 1992a,b, 1995a) have reported that the CA1 presynaptic fiber spikes of hippocampal slices exposed to oxygen/glucose-free medium are decreased, and this decrease is attenuated by co-treatment with Ca^{2+}-free medium, protein kinase C inhibitors and NMDA receptor antagonists. In addition, we recently demonstrated that a nitric oxide (NO) synthesis inhibitor attenuated this decrease (Shibata et al., 1995b). We have proposed that NO possibly synthesized in the postsynaptic neurons in response to NMDA receptor

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activation, diffuses back to the presynaptic terminal to inhibit spiking. Thus, the presynaptic fiber volley is suggested as a good marker to evaluate the mechanisms of presynaptic function (Dunwiddie and Miller, 1993). Recently Akaike et al. (1993) reported that incubation of methylcobalamin in the culture medium exerted a protective action in cortical neurons against NMDA receptor-coupled NO synthesis-mediated cytotoxicity. However, whether methylcobalamin possesses a neuroprotective action against the hypoxia/hypoglycemia-induced neurotoxicity is not known at present. Therefore, in this study we examined the effect of methylcobalamin on a hypoxia/hypoglycemia- or glutamate-induced decrease in CA1 presynaptic potential in hippocampal slices. In addition, we used cyanocobalamin as a positive control substance.

2. Materials and methods

2.1. Animals and slice preparations

Male Wistar rats weighing 300–400 g were used. The animals were decapitated and the brain was quickly removed. A tissue chopper was used to prepare parasagittal hippocampal slices (450 μm thickness) from the dorsal hippocampus of each animal. The composition of the control Krebs-Ringer solution which was equilibrated with 95% O_2-5% CO_2 gas mixture was (in mM) NaCl 129, $MgSO_4$ 1.3, $NaHCO_3$ 22.4, KH_2PO_4 1.2, KCl 4.2, glucose 10.0 and $CaCl_2$ 1.5. For hypoglycemia the glucose in the incubation medium was replaced by 10 mM sucrose. The hypoxia solution was equilibrated with a 95% N_2-5% CO_2 gas mixture for at least 1 h. The buffer had a pH of 7.3–7.4 and the temperature was kept at 37°C. Our chamber design, slice transfer methods and incubation procedures have been reported previously (Minamoto et al., 1994; Shibata et al., 1992a; Tanaka et al., 1994).

2.2. Presynaptic fiber spikes

Presynaptic potentials were recorded through a glass micropipette filled with normal physiological saline containing 5% fast green (DC resistance, 0.5–1 MΩ). To reduce the occurrence of fast excitatory postsynaptic potentials and/or population spikes during recording periods, the hippocampal CA1 presynaptic potential evoked by Schaffer collateral stimulation was recorded in a submerged chamber with perfusion of normal Krebs-Ringer solution (4 ml/min) containing 10 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX) (Dunwiddie and Miller, 1993) at 37°C. DNQX was not applied during hypoxic/hypoglycemic incubation to maintain the activation of glutamate receptor-dependent function (Shibata et al., 1995b). Stimulation pulses (0.2 Hz, 0.05 ms duration and 0.9 mA intensity) were reported to produce a supramaximal response in the normal non-hypoxia/hypoglycemia-treated group (Shibata et al., 1992a). A higher stimulus intensity was required to produce presynaptic fiber spikes than field EPSP. We obtained similar normal responses in this study using the same stimulus conditions.

Extracellular recordings of presynaptic fiber spikes in the stratum radiatum of the CA1 region were made and the latency of the negative portion of the presynaptic fiber spike amplitudes was quantitated as a peak-to-peak measurement between the negative peak of the fiber spike and the positivity which immediately preceded the negativity, or between the pre-stimulus baseline and the negative peak of the fiber spike.

2.3. Drugs

The drugs used in this study were methylcobalamin, cyanocobalamin and sodium nitroprusside. Methylcobalamin and cyanocobalamin were obtained from Eisai Corp. (Japan), and L-arginine and DNQX were from Sigma Chemical Co. (USA) and Funakoshi (Tokyo, Japan), respectively. All drugs were dissolved in distilled water.

2.4. Procedure

In in vitro experiments, the drug was added to the incubation medium 10 min prior to the incubation for hypoxia/hypoglycemia or with glutamate (10 mM) and during the 15-min hypoxia/hypoglycemia or glutamate (10 mM) after 30-min preincubation in the recirculation submersion chamber. L-Arginine (100 μM) was applied either alone or together with methylcobalamin for 25 min in total. In ex vivo experiments, rats were injected daily with methylcobalamin (0.5 mg/kg i.p.) or cyanocobalamin (0.5 mg/kg i.p.) for 3 days, then were decapitated 30 min after the last injection. Following 30-min preincubation, hippocampal slices were exposed to hypoxia/hypoglycemia or glutamate (10 mM) for 15 min. After a 3-h washout, the CA1 presynaptic potential was measured in both in vitro and ex vivo experiments. Normal presynaptic potentials were recorded after 3 h 55-min incubation (30-min preincubation, 25-min vehicle treatment and 3-h washout) in the control normal medium.

2.5. Statistical analysis

The data were expressed as means $\pm$ S.E.M. The significance of differences between groups was determined with the analysis of variance (ANOVA) followed by Dunnett's two-tailed test or Duncan's test for individual comparisons.

3. Results

The effects of methylcobalamin or cyanocobalamin on hypoxia/hypoglycemia-or glutamate-induced decreases in CA1 presynaptic potentials were examined. The CA1 presynaptic potentials of slices exposed to 15-min hypoxia/hypoglycemia were decreased by about 50–60% over a 3-h washout (Shibata et al., 1992a). Therefore, hippocampal slices were exposed to the drug-containing Krebs-Ringer for 10 min prior to the hypoxia/hypoglycemia and throughout the hypoxia/hypoglycemia (15 min). The slices were then returned to normal buffer for 3 h.

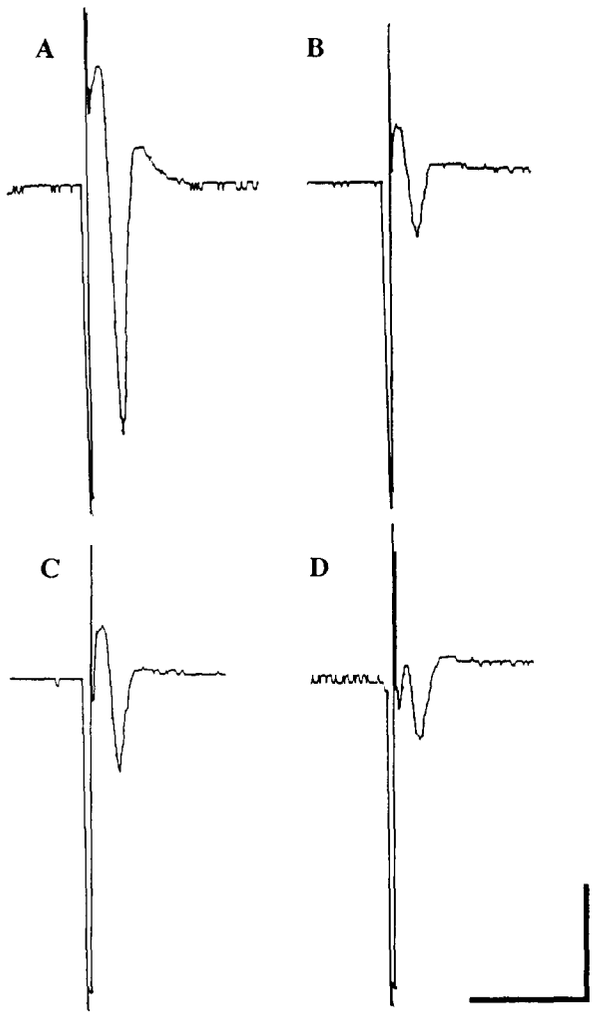

Fig. 1. Effect of methylcobalamin or cyanocobalamin on the hypoxic/hypoglycemic decrease of CA1 presynaptic potential in rat hippocampal slices. The drug effects were recorded 3 h after 15 min of combined hypoxia/hypoglycemia. A: CA1 presynaptic potential evoked by Schaffer collateral stimulation at 3 h without hypoxia/hypoglycemia application. B: Effect of vehicle on CA1 presynaptic potential evoked in hippocampal slices at 3 h after 15 min of hypoxia/hypoglycemia. C: Effect of methylcobalamin (10 μM) on hypoxia/hypoglycemia-induced decrease of CA1 presynaptic potential. D: Effect of cyanocobalamin (10 μM) on hypoxia/hypoglycemia-induced decrease of CA1 presynaptic potential. Hippocampal slices were exposed to Krebs-Ringer solution containing each of the drugs for 10 min before the induction of hypoxia/hypoglycemia and during the 15-min hypoxic/hypoglycemic period. Each trace is the average of 8 sweeps. Calibrations: 5 ms and 5 mV.

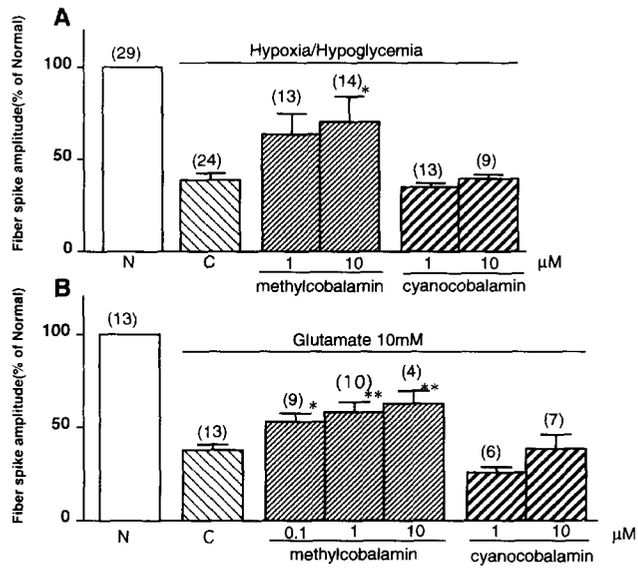

Fig. 2. Effect of methylcobalamin or cyanocobalamin on the decrease of the CA1 presynaptic potential induced by 15 min of hypoxia/hypoglycemia or glutamate (10 mM). The amplitude of the CA1 presynaptic potential in control slices subjected to normal conditions was regarded as 100%. The drug effects were recorded at 3 h after hypoxia/hypoglycemia. A: Effect of methylcobalamin or cyanocobalamin on hypoxia/hypoglycemia-induced decrease of CA1 presynaptic potential. N and C: Vehicle-treated normal and hypoxia/hypoglycemia slices, respectively. B: Effect of methylcobalamin or cyanocabalamin on glutamate-induced decrease of CA1 presynaptic potential. N and C: Vehicle-treated normal and glutamate (10 mM)-treated slices. The values are shown as the means ± S.E.M. Numbers in parentheses indicate the number of slices. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the hypoxic/hypoglycemic control group (C) (ANOVA followed by Dunnett's two-tailed test).

Representative examples of the CA1 presynaptic potentials elicited by stimulation of Schaffer collaterals are shown in Fig. 1. Trace B in Fig. 1 is an example of the response following the hypoxia/hypoglycemia treatment, while trace A is an example of a normal response. Trace C is an example of functional recovery with methylcobalamin, but trace D shows no recovery with cyanocobalamin.

Methylcobalamin significantly attenuated the hypoxia/hypoglycemia ($F(4,68) = 3.61$, $P < 0.01$)-or glutamate ($F(5,43) = 3.48$, $P < 0.01$)-induced decrease in amplitude of presynaptic fiber spikes (Fig. 2). However, cyanocobalamin failed to cause functional recovery from an impairment of the CA1 presynaptic potential induced by hypoxia/hypoglycemia or glutamate (Fig. 2). In contrast to the failure of cyanocobalamin to affect dysfunction in vitro, daily injection of cyanocobalamin (0.5 mg/kg i.p./day) for 3 days in vivo significantly attenuated the hypoxia/hypoglycemia

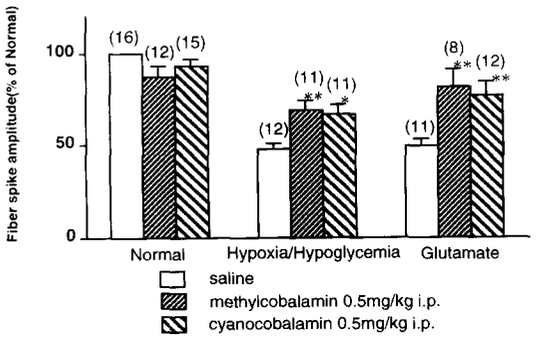

Fig. 3. Effect of daily injection of methylcobalamin or cyanocobalamin for 3 days in vivo on the decrease of the CA1 presynaptic potential induced in hippocampal slices by 15 min of hypoxia/hypoglycemia or glutamate (10 mM). The rats were injected daily with methylcobalamin (0.5 mg/kg i.p.) or cyanocobalamin (0.5 mg/kg i.p.) for 3 days, then were decapitated 30 min after the last injection. The amplitude of the CA1 presynaptic potential in vehicle-treated normal slices (N) was regarded as 100%. The drug effects were recorded at 3 h after hypoxia/hypoglycemia. The values are shown as means ± S.E.M. Numbers in parentheses indicate the number of slices. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. saline control group (ANOVA followed by Dunnett's two-tailed test).

($F(2,31) = 5.36$, $P < 0.01$)-or glutamate ($F(2,28) = 5.45$, $P < 0.01$)-induced decrease in amplitude in vitro (Fig. 3). Daily injection of methylcobalamin (0.5 mg/kg i.p./day) for 3 days also attenuated the hypoxia/hypoglycemia-or glutamate-induced dysfunction of CA1 presynaptic potentials (Fig. 3). However, repeated administration of methylcobalamin or cyanocobalamin did not affect the CA1 presynaptic potentials in normal Krebs-Ringer solution (Fig. 3).

L-Arginine at 100 μM, a substrate for NO synthase, did not affect the reduction in CA1 presynaptic potentials induced by hypoxia/hypoglycemia, if one compares column C and the thick-hatched column at the extreme right of Fig. 4. The recovery effect of methylcobalamin (1 μM or 10 μM) against the reduction in CA1 presynaptic potentials induced by hypoxia/hypoglycemia was significantly ($P < 0.01$, Duncan's test) reduced by co-treatment with L-arginine (Fig. 4).

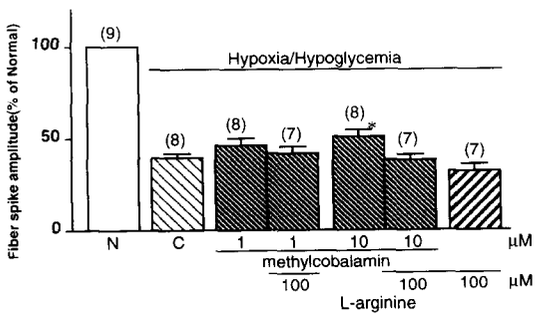

Fig. 4. Effects of L-arginine on methylcobalamin-induced protection against the decrease in CA1 presynaptic potential induced by 15 min of hypoxia/hypoglycemia. The amplitude of the CA1 presynaptic potential in non-hypoxic/hypoglycemic control slices was regarded as 100%. The drug effects were recorded at 3 h after hypoxia/hypoglycemia. N and C: Vehicle-treated normal and hypoxic/hypoglycemic slices, respectively. The values are shown as means ± S.E.M. Numbers in parentheses indicate the number of slices. Both agents were applied together from 10 min before the induction of hypoxia/hypoglycemia and during the 15-min hypoxic/hypoglycemic period. $^{*}P < 0.05$ vs. the hypoxic/hypoglycemic control group (C) (ANOVA followed by Duncan's test).

4. Discussion

The present results demonstrated that methylcobalamin when applied in vitro attenuated the reduction of CA1 presynaptic fiber spikes induced in hippocampal slices by hypoxia/hypoglycemia or glutamate, while this reduction was unaffected by exposure to cyanocobalamin. These results indicate that methylcobalamin induces functional recovery from the hypoxia/hypoglycemia- or glutamate-induced impairment of presynaptic function in hippocampal slices. The neuronal mechanisms of hypoxia/hypoglycemia- or glutamate-induced suppression of presynaptic activity are uncertain at present: a conduction block of Schaffer collaterals and/or a loss of CA3 neuronal excitability are possibilities. Our previous studies have demonstrated that these impairments are attenuated by Ca^{2+}-free medium, protein kinase C inhibitors, NO synthesis inhibitors, reducing agents such as glutathione and NMDA receptor antagonists (Shibata et al., 1992a,b, 1995a). At present, however we do not know the neuronal mechanism of the functional recovery induced by methylcobalamin.

Since cyanocobalamin did not attenuate the reduction of CA1 presynaptic fiber spikes induced in the hippocampal slices by hypoxia/hypoglycemia or glutamate (Fig. 2), it is possible that methylcobalamin caused functional recovery by acting as a methyl group donor. It has been found that cholinergic function plays an important role in learning and memory in animals (Levin and Bowman, 1986; Ridley et al., 1984; Spangler et al., 1986). Hall (1990) demonstrated that methylcobalamin acts as a methyl group donor for the conversion of homocysteine to methionine using cortical cultures. Methionine is transformed to *S*-adenosyl-L-methionine (SAM) in the methionine metabolism pathway. Methionine and its product, SAM, facilitate intracellular methylation reactions. SAM acts as a methyl donor in the reaction forming phosphatidylcholine in the phospholipid layer of the cell membrane (Bremer and Greenberg, 1961; Gibson et al., 1961). SAM has a beneficial effect on ischemia-induced neuronal degeneration (Matsui et al., 1987). This may be due to the action of SAM to prevent disturbances of phospholipids that would occur in the ischemic brain. The role that SAM has in accelerating synthesis of phospholipids may thus play a part in the amelioration of

neurotoxicity. Therefore, methylcobalamin may induce functional recovery from the hypoxia/hypoglycemia-induced deficit of CA1 presynaptic potential via the production of SAM.

Homocysteine has been shown to produce an increase in neuronal activity and convulsive seizures (Blennow et al., 1979; Dewhurst et al., 1983; Wuerthele et al., 1982). Thus homocysteine is thought to be a toxic agent in the central nervous system (Hall, 1990). Recently it has been reported that homocysteine levels are reduced by methylcobalamin and increased by methylcobalamin deficiency (Araki et al., 1993). Homocysteine is reported to decrease adenosine levels (Sciotti and Van Wylen, 1993). We recently demonstrated that adenosine receptor agonists cause neuroprotection (Tominaga et al., 1992) by inhibition of excitatory neurotransmitter release (Dunwiddie and Haas, 1985). Thus methylcobalamin may produce a neuroprotective action via reduction of the homocysteine levels and increasing of adenosine levels.

Recently, Dawson et al. (1991) have demonstrated, using cortical cultures, that nitric oxide (NO) mediates the NMDA receptor-induced neurotoxicity of glutamate. We have also demonstrated that the stimulation of NO production plays a detrimental role in the development of ischemic damage (Shibata et al., 1995b). Treatment with N^G-nitro-L-arginine methyl ester but not with N^G-nitro-D-arginine methyl ester produces attenuation of the hypoxia/hypoglycemia-induced decrease in CA1 presynaptic potential, and this neuroprotective effect was reduced by co-treatment with L-arginine. Since the protective effect of methylcobalamin against the reduced CA1 presynaptic potentials induced by hypoxia/hypoglycemia was reversed by L-arginine (Fig. 4), it is likely that prevention of the hypoxia/hypoglycemia-induced decrease in CA1 presynaptic potential by methylcobalamin may be mediated by the production of NO. The present results suggest that NO-related radicals such as hydroxy-and NO_2 free radical may play an important role in the ischemia-induced neurotoxicity, although the mechanism by which NO reduces hippocampal presynaptic fiber spikes is still unknown (Shibata et al., 1995b).

Methylcobalamin, when given to rats in vivo, by daily i.p. injections prior to the preparation of the brain slices (Fig. 3), was also found to attenuate the reduction of CA1 presynaptic potentials induced by hypoxia/hypoglycemia or glutamate. Although in vitro treatment with cyanocobalamin failed to protect from the hippocampal functional deficit induced by hypoxia/hypoglycemia or glutamate, in vivo exposure to cyanocobalamin produced an effect similar to that of methylcobalamin (Fig. 3). Under in vitro conditions, cyanocobalamin could not be converted to methylcobalamin. It is reported that, however, in vivo injected cyanocobalamin was easily converted to methylcobalamin in hepatic cells (Allen, 1976). Therefore the present results indicate that methylcobalamin causes neuroprotection through acting as a methyl group donor, although it was not elucidated how transmethylation is related to a protective effect in the central nervous system.

In conclusion, the present study demonstrated that methylcobalamin possesses neuroprotective effects against hypoxia/hypoglycemia-induced or a glutamate-induced functional deficit in hippocampal slices.

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