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Influence of adenosine receptor agonists on benzodiazepine withdrawal signs in mice

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Abstract

The involvement of adenosine receptor agonists in benzodiazepine withdrawal signs was evaluated as the seizure susceptibility of mice. The concomitant administration of subthreshold dose of pentetrazole (55.0 or 60.0 mg/kg, s.c.) with flumazenil (10.0 mg/kg, i.p.) in mice chronically treated with temazepam or diazepam induced the appearance of withdrawal signs: clonic seizures, tonic convulsions and death episodes. The administration of the selective A_1 (CPA-N^6-cyclopentyladenosine), A_{2A} (CGS 21680-2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine hydrochloride) and the non-selective A_1/A_{2A} (NECA-5′-*N*-ethylcarboxamidoadenosine) adenosine receptor agonists (i.p.) evoked the significant attenuation of benzodiazepine withdrawal signs, and these effects were more expressed in temazepam- than in diazepam-dependent mice. CPA has shown the most apparent and dose-dependent attenuating effect. The results confirm that adenosine A_1 and A_{2A} receptors are involved in benzodiazepine withdrawal signs, and adenosine A_1 receptor plays a predominant role in this phenomenon.

Keywords: Temazepam; Diazepam; Withdrawal sign; Adenosine agonist

1. Introduction

Benzodiazepines are the most frequently prescribed anxiolytic and sedative drugs and they have also anticonvulsant properties. γ-aminobutyric acid (GABA) and the GABA-ergic system is mainly involved in the effects of benzodiazepines. Flumazenil, a competitive antagonist of benzodiazepine sites, can block the benzodiazepine pharmacological effects. It is well known that the chronic benzodiazepine treatment may lead to the development of tolerance and dependence. In the experimental animals, benzodiazepine withdrawal signs include, among others, spontaneous seizures, an increase in muscle tone and a decrease in seizure threshold for convulsants (such as pentetrazole) (Woods et al., 1992; Mizoguchi et al., 1994; Tsuda et al., 1997a,b, 1998a,b).

The literature data have shown (for ref. see Allison and Pratt, 2003) that the development of benzodiazepine tolerance and dependence may result from the adaptive changes at the drug–receptor complex appearing during chronic treatment with benzodiazepines. The mentioned adaptive changes may be related to a decrease in reactivity of the $GABA_A$ receptor to the ligands or alterations in coupling between benzodiazepine and GABA sites on the $GABA_A$ receptor (Allison and Pratt, 2003). However, most studies have not found any changes in the number of benzodiazepine receptors after chronic treatment with benzodiazepines (Allison and Pratt, 2003). Additionally, Tietz et al. (1986) observed a decrease in benzodiazepine receptor density after chronic treatment with benzodiazepines, but they applied very large doses, which were unlikely to be related to the clinical situation.

Many neurotransmitters of the central nervous system (CNS) may be involved in the function of GABA-ergic complex and also in benzodiazepine dependence. For example, dopamine receptor agonists were able to potentiate

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the lorazepam withdrawal signs in rats (Nath et al., 2000), the stimulation of histamine receptors intensified the lorazepam withdrawal signs, and the blockade of these receptors induced the opposite effects (Nath and Gupta, 2001). Some NMDA receptor antagonists were able to attenuate benzodiazepine withdrawal signs in mice (Tsuda et al., 1997a), and the first group (1mGlu) of metabotropic glutamate receptor antagonist also induced the increase in seizure threshold after cessation of chronic treatment with diazepam (Suzuki et al., 1999).

Adenosine plays an important role in the regulation of neuronal activity acting via four adenosine receptor subtypes: A_1, A_{2A}, A_{2B}, A_3. The activation of adenosine A_1 and A_3 receptors induces the inhibition of adenylyl cyclase, while the stimulation of adenosine A_{2A} and A_{2B} receptors may lead to the activation of this enzyme (Fredholm et al., 2001). Adenosine modulates the activity of the other neurotransmitter systems. The important interactions of adenosine receptors with $GABA_A$, NMDA or dopamine receptors have been described (for ref. see Fredholm et al., 2001). The administration of adenosine and its analogs induces behavioral effects such as antinociception, sedation or anticonvulsive activity (Gupta et al., 2002; Ribeiro et al., 2003). Additionally, adenosine and its analogs are able to reduce morphine (Kaplan et al., 1994; Michalska and Malec, 1993) and ethanol (Kaplan et al., 1999; Malec et al., 1996) withdrawal signs in animals.

Several lines of evidence also suggest that the adenosinergic system may be involved in benzodiazepine activity, for example, it was demonstrated in vitro that benzodiazepines inhibited the adenosine uptake by rat brain cerebral cortical synaptosomes (Phillis et al., 1981). In vivo experiments have also shown that adenosine receptor antagonists, i.e., methylxanthines are able to antagonize the central actions of the benzodiazepines, both in rats (Phillis et al., 1980) and in humans (Arvidson et al., 1982). Hawkins et al. (1988) also demonstrated that chronic treatment with diazepam induced down-regulation of adenosine A_1 receptors in the rat hippocampus. All the published pharmacological experiments relating adenosine–benzodiazepine interactions were performed on rats, and there are no data concerning adenosine receptor ligands/benzodiazepine withdrawal signs interaction.

Thus, the clear anticonvulsant and neuromodulatory effects of adenosine and its analogs (described above) and the scarce data showing the pharmacological interactions between the adenosinergic system and benzodiazepines inspired us to evaluate the involvement of the adenosinergic system in benzodiazepine withdrawal syndrome in mice. Temazepam and diazepam were chosen as representatives of major clinically available short-acting ($T_{0,5}=11\pm6$ h) and long-acting ($T_{0,5}=43\pm13$ h) benzodiazepine, respectively (Charney et al., 2001). We first established an experimental model in which the seizure susceptibility developing after cessation of chronic treatment with temazepam or diazepam in mice can be clearly revealed. In the second step, we investigated the influence of adenosine receptor agonists on this withdrawal sign. The selective adenosine A_1 receptor agonist—CPA (N^6-cyclopentyladenosine), the selective adenosine A_{2A} receptor agonist—CGS 21680 (2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine hydrochloride), and the non-selective A_1/A_{2A} receptor agonist—NECA (5′-*N*-Ethylcarboxamidoadenosine) were used in our experiments.

2. Materials and methods

2.1. Animals

The experiments were carried out on male albino Swiss mice (20–30 g). The animals were kept 8–10 to a cage at room temperature of 22±1 °C, on natural day–night cycle (spring). Standard food (Murigran pellets, Bacutil, Motycz) and tap water were freely available. All the experiments were performed between 9 a.m. and 2 p.m.

The experiments were performed in accordance with the opinion of the Local Ethics Committee.

2.2. Drugs

The following drugs were used: temazepam (Signopam, Polfa-Poland), diazepam (Relanium, Polfa-Poland), flumazenil (Hoffman-La Roche, Swiss), pentetrazole (Cardiazol, Polfa-Poland), and adenosine receptor agonists: N^6-cyclopentyladenosine (CPA)—the selective A_1 receptor agonist; 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine hydrochloride (CGS 21680)—the selective A_{2A} receptor agonist; 5′-*N*-Ethylcarboxamidoadenosine (NECA)—A_1/A_2 receptor agonist, (all from Sigma-Aldrich, USA).

The temazepam and diazepam pellets were prepared according to the modified procedure described by Way et al. (1963) for morphine pellets.

Temazepam was suspended in 0.5% methylcellulose solution. Adenosine receptor ligands, diazepam and pentetrazole were dissolved in saline. Flumazenil was dissolved in the minimal volume of dimethylsulfoxide (DMSO), and diluted in saline. Adenosine analogs and flumazenil were administered intraperitoneally (i.p.), and other drugs were injected subcutaneously (s.c.).

All the drugs were administered in a volume of 10.0 ml/kg.

2.3. Procedure

The benzodiazepine dependence was produced by concomitant s.c. implantation of one pellet (containing

75.0 mg of temazepam or diazepam) per mouse, with s.c. injection of the drugs for 14 days. Temazepam (40.0 and 60.0 mg/kg) was injected twice daily at the doses of 40.0 mg/kg (on the 4th to 8th day of experiment) and 60.0 mg/kg (on 9th to 14th day of experiment). Diazepam was administered once daily at the doses of 25.0 and 50.0 mg/kg, similarly to temazepam injection procedure. Pellets were removed 26 h after the last injection.

All the experiments were conducted 26 h (temazepam) or 48 h (diazepam) after removing pellets. Then, the concomitant administration of subthreshold dose (55.0 or 60.0 mg/kg) of pentetrazole and flumazenil (10 mg/kg) induced the withdrawal signs (seizure incidents). Adenosine receptor agonists were injected 15 min before pentetrazole and flumazenil. The animals were observed after the administration of drugs for 1 h, and the number of mice developing clonic seizures, tonic convulsions and dead animals was recorded in that period.

The control animals were implanted with placebo pellets and received the same volume of the solvent at the respective time before the test.

2.4. Statistical analysis

The obtained data were analyzed statistically using χ^2-test with Yates correction. All the comparisons were performed on drug treated mice and appropriate control group. A probability (P) value of 0.05 or less was considered as statistically significant.

3. Results

Administration of pentetrazole (55.0 mg/kg) or pentetrazole with flumazenil (10.0 mg/kg) induced maximally 1 episode of clonic seizures in control (placebo pellets

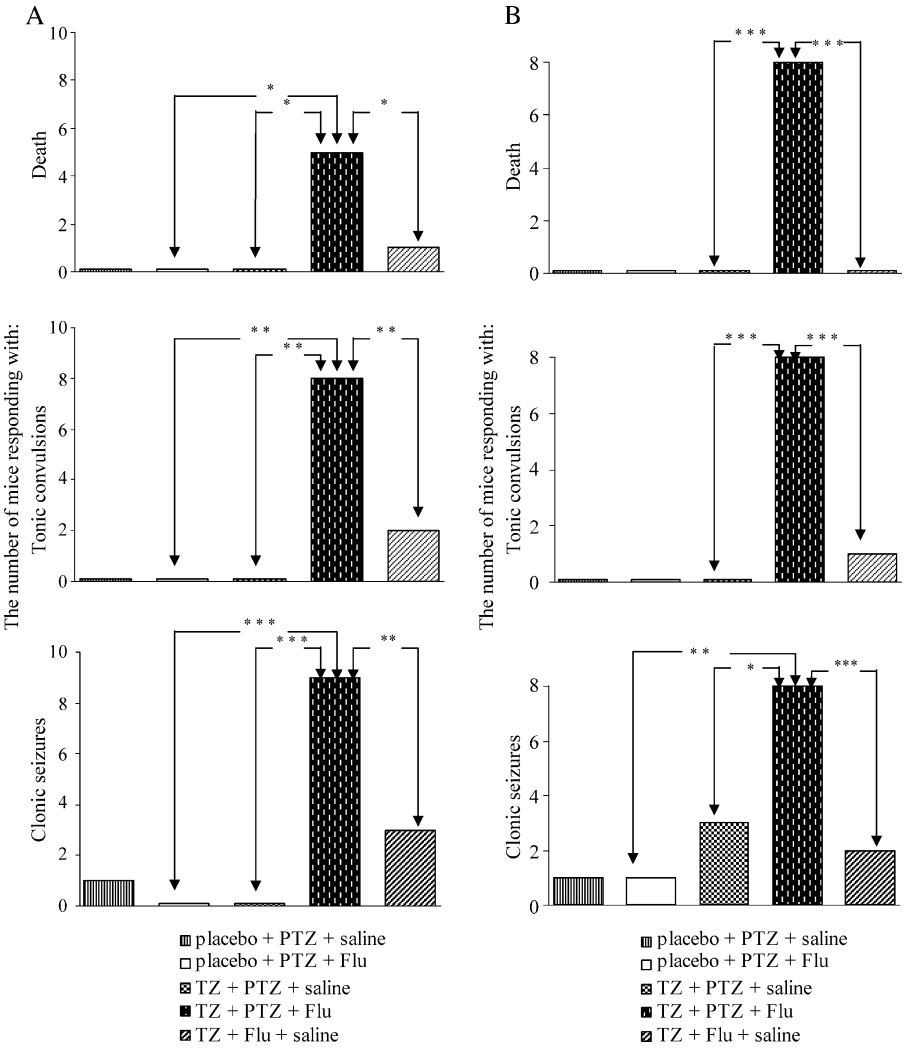

Fig. 1. (A) The effects of chronic treatment with temazepam (TZ) evaluated as the number of mice with seizures after administration of pentetrazole (PTZ)—55 mg/kg; or PTZ with flumazenil (Flu)—10 mg/kg. *P<0.05, **P<0.01, ***P<0.001 [χ^2 test with the Yates correction]. (B) The effects of chronic treatment with diazepam (DZ) evaluated as the number of mice with seizures after administration of pentetrazole (PTZ)—55 mg/kg; or PTZ with flumazenil (Flu)—10 mg/kg. *P<0.05, **P<0.01, ***P<0.001 [χ^2 test with the Yates correction].

implanted) mice. In mice chronically treated with temazepam or diazepam, simultaneous injection of pentetrazole and flumazenil evoked a significant increase in observed incidents (clonic seizures, tonic convulsions and death). Administration of flumazenil alone or pentetrazole alone evoked only slight seizure incidents in benzodiazepine mice (Fig. 1A and B).

3.1. The influence of CPA on temazepam or diazepam withdrawal signs

CPA (0.5 and 1.0 mg/kg) significantly and dose-dependently decreased the number of clonic seizures in mice chronically treated with temazepam ($P<0.01$ and $P<0.001$, respectively) or diazepam ($P<0.05$ and $P<0.001$, respectively). The episodes of tonic convulsions were significantly reduced ($P<0.05$) and incidents of mortality were completely prevented by the higher dose of CPA (1.0 mg/kg), (Fig. 2A and B).

3.2. The influence of CGS 21680 on temazepam or diazepam withdrawal signs

CGS 21680 significantly (2.0 mg/kg, $P<0.01$) and dose-dependently (1.0 and 2.0 mg/kg) reduced the number of clonic seizures in mice chronically treated with temazepam. The incidents of tonic convulsions and death were abolished by both doses of CGS 21680 (Fig. 3A).

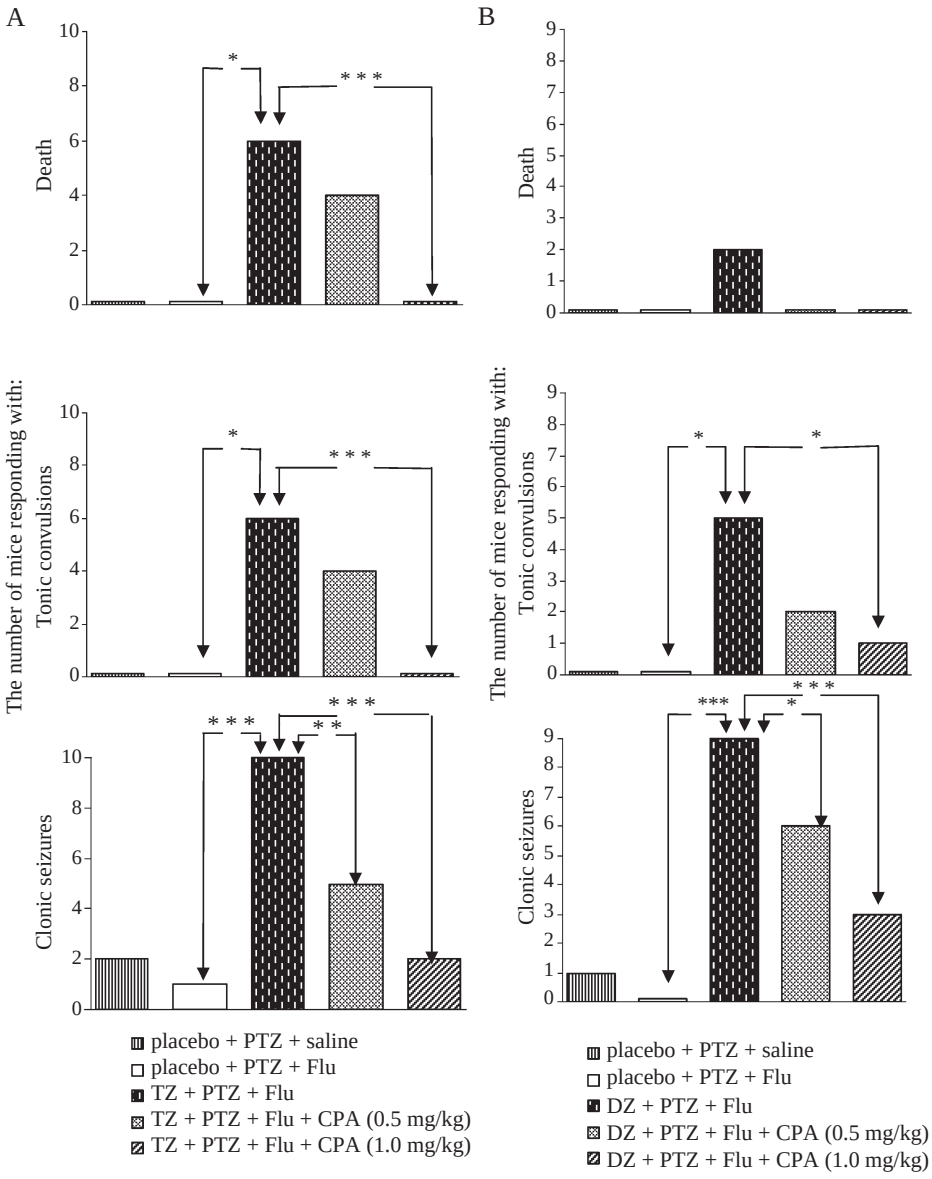

Fig. 2. (A) The influence of CPA on temazepam (TZ) withdrawal signs. PTZ—pentetrazole (60 mg/kg); Flu—flumazenil (10 mg/kg); *$P<0.05$, **$P<0.01$, ***$P<0.001$ [χ^2 test with the Yates correction]. (B) The influence of CPA on diazepam (DZ) withdrawal signs. PTZ—pentetrazole (60 mg/kg); Flu—flumazenil (10 mg/kg); *$P<0.05$, ***$P<0.001$ [χ^2 test with the Yates correction].

In chronically diazepam treated mice, CGS 21680 (2.0 and 4.0 mg/kg) significantly ($P<0.05$) reduced the number of clonic seizures and completely protected (2.0 mg/kg) against tonic convulsions and death (Fig. 3B).

3.3. The influence of NECA on temazepam or diazepam withdrawal signs

NECA (0.1 and 0.2 mg/kg) significantly and dose-dependently decreased the number of clonic seizures and abolished (at the higher dose) incidents of tonic convulsions and death in mice chronically treated with temazepam (Fig. 4A). In chronically diazepam treated mice, NECA (0.1 and 0.2 mg/kg) significantly ($P<0.05$) decreased the number of clonic seizures and, markedly but not significantly, reduced the number of tonic convulsions and death (Fig. 4B).

4. Discussion

The appearance of withdrawal signs confirms that the physical dependence has been developed, and the severity of this effect indicates the magnitude of this dependence (O'Brien, 2001). The present results confirm the literature data (Woods et al., 1992) showing that the half-life time of temazepam and diazepam did not play an important role in the severity of withdrawal signs, because we did not observe any significant differences in the severity of withdrawal signs between temazepam- (short-acting) or diazepam-(long-acting) dependent mice.

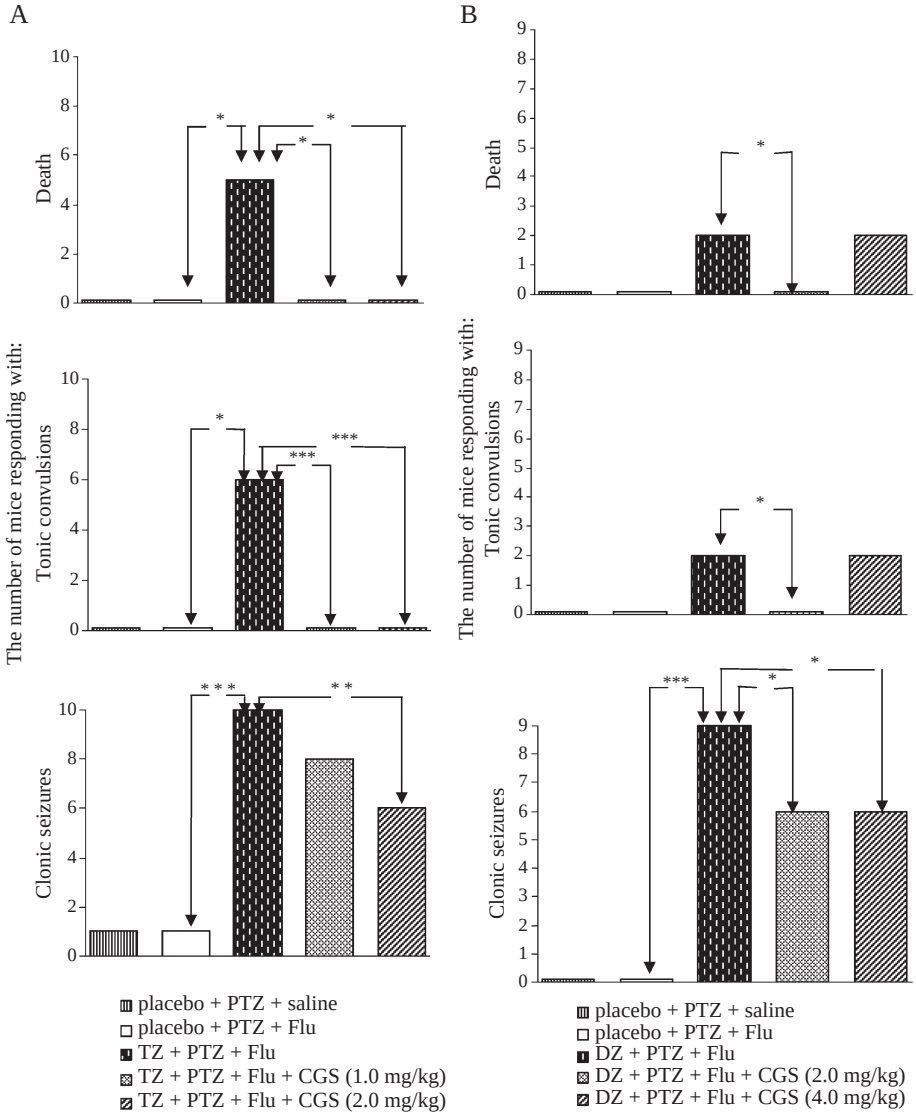

Fig. 3. (A) The influence of CGS 21680 (CGS) on temazepam (TZ) withdrawal signs. PTZ—pentetrazole (55 mg/kg); Flu—flumazenil (10 mg/kg); *$P<0.05$, **$P<0.01$, ***$P<0.001$ [χ^2 test with the Yates correction]. (B) The influence of CGS 21680 (CGS) on diazepam (DZ) withdrawal signs. PTZ—pentetrazole (55 mg/kg); Flu—flumazenil (10 mg/kg); *$P<0.05$, ***$P<0.001$ [χ^2 test with the Yates correction].

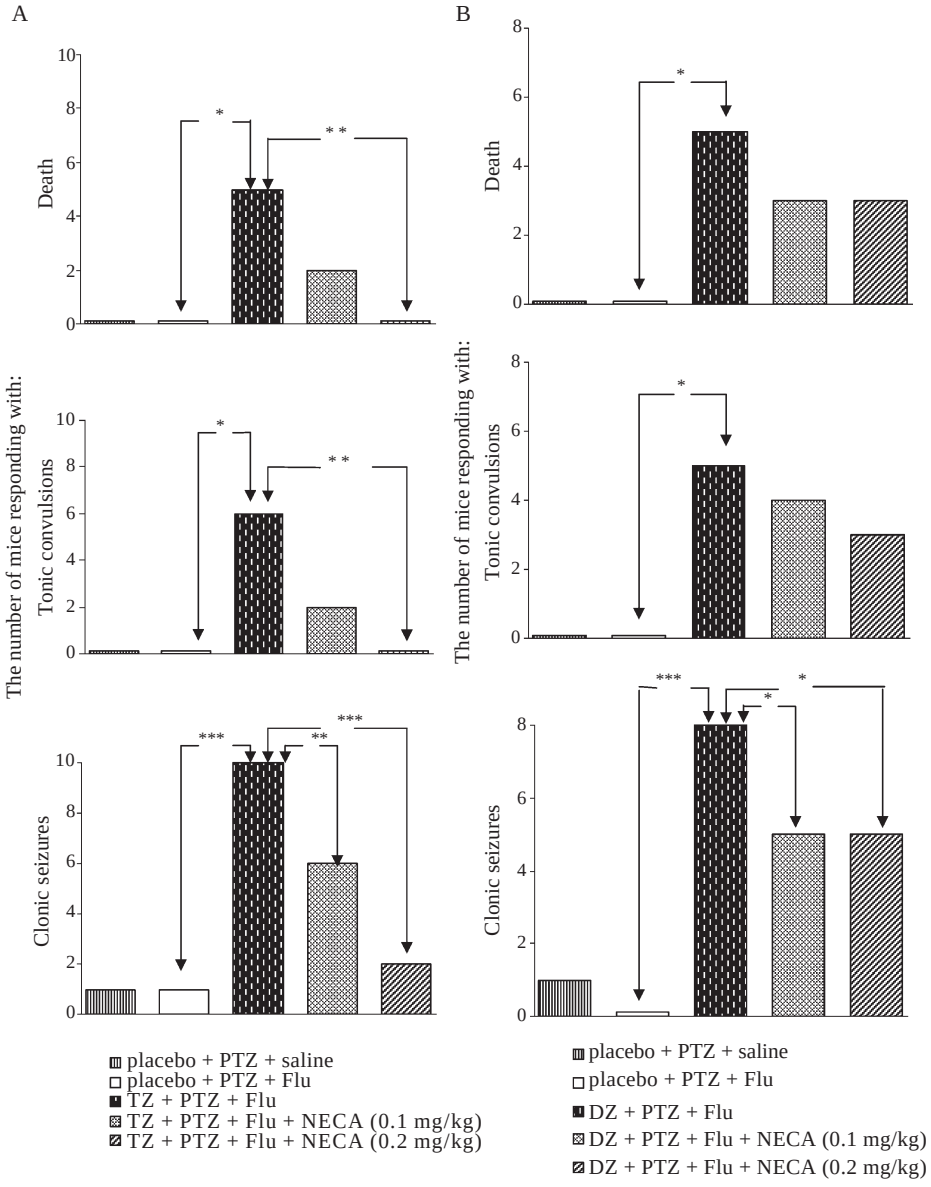

Fig. 4. (A) The influence of NECA on temazepam withdrawal signs. TZ—temazepam; PTZ—pentetrazole (55 mg/kg); Flu—flumazenil (10 mg/kg), $*P<0.05$, $**P<0.01$, $***P<0.001$ [χ^2 test with the Yates correction]. (B) The influence of NECA on diazepam (DZ) withdrawal signs. PTZ—pentetrazole (55 mg/kg); Flu—flumazenil (10 mg/kg); $*P<0.05$, $***P<0.001$ [χ^2 test with the Yates correction].

In our experiments, the concomitant administration of flumazenil (the benzodiazepine receptor antagonist) and pentetrazole (a chemical proconvulsive drug) allows us to obtain the intensive withdrawal signs immediately, both in temazepam- and diazepam-dependent mice: 100% of animals responded with clonic seizures, about 60% of mice with tonic convulsions and most of them reacted with death (Fig. 1A and B). The same effect was not obtained by the application of pentetrazole alone or flumazenil alone in benzodiazepine-dependent mice (Fig. 1A and B). Thus, all the observed effects demonstrated that simultaneous application of pentetrazole and flumazenil was necessary to perform the experiments concerning the influence of adenosine receptor agonists on benzodiazepine withdrawal signs.

Pentetrazole is a proconvulsant drug and its pharmacological effect is, at least partially, mediated by the interactions with the anion channel of $GABA_A$ receptor (Squires et al., 1984). Either single or repeated pentetrazole administration may modify $GABA_A$ receptor number and function, but the results of such studies are inconsistent. For example, Walsh et al. (1999) have shown that an acute injection of pentetrazole cause transient changes in $GABA_A$ receptor mRNA levels without altering receptor number. Ito et al. (1986) have found that an acute convulsive dose of pentetrazole, administered 30 min before assay, had little effect on the binding of several ligands to a various sites on the $GABA_A$ receptor in homogenates from seven regions of rat brain. However, Rocha et al. (1996) provided data in that

a single, subconvulsive injection of pentetrazole produced a decrease in [^{3}H] flunitrazepam binding throughout the rat brain. Psarropoulou et al. (1994) demonstrated in vitro that pentetrazole-induced seizures decrease GABA-mediated recurrent inhibition and enhance adenosine-mediated depression. It confirms that adenosine may have some protective effect in convulsive state.

The major finding of our experiments is that we observed, for the first time, a significant attenuation of all withdrawal signs (clonic seizures, tonic convulsions, death episodes) by the selective and non-selective adenosine receptor agonists in temazepam- and diazepam-dependent mice. The most apparent effects were obtained after application of CPA—selective adenosine A_1 receptor agonist. This drug evoked a significant and dose-dependent decrease in the number of all measured incidents. Furthermore, total protection against lethality was observed after administration of a higher dose of CPA (1.0 mg/kg) in both temazepam and diazepam-dependent mice. CGS 21680 (selective adenosine A_{2A} receptor agonist) and NECA–non-selective adenosine A_1/A_{2A} receptor agonist–also diminished the number of measured incidents: the completely protective effects against tonic episodes and mortality were observed after application of higher doses of these drugs in temazepam-dependent animals, while no significant changes were evoked by these drugs in diazepam-dependent mice. All these results also demonstrate that involvement of both adenosine receptors is more expressed in temazepam dependence than in diazepam dependence. The explanation of these differences between interaction of temazepam and diazepam with adenosine receptors is not clear and needs further experiments.

The obtained results demonstrate that adenosinergic system is involved in temazepam and diazepam withdrawal signs. CPA–the adenosine A_1 receptor agonist–has shown the most apparent and dose-dependent attenuating effect. It indicates that adenosine A_1 receptors may play the most important role in the expression of temazepam and diazepam withdrawal signs observed as the seizure susceptibility. These results confirm the literature data which have shown that adenosine A_1 receptors are mainly involved in anticonvulsant effects of adenosine (Murray et al., 1992; Zhang et al., 1994; Dunwiddie and Masino, 2001). However, the activation of adenosine A_{2A} receptors in some brain areas may induce similar effects, for example, audiogenic seizures in DBA/2 mice were inhibited by both A_1 and A_{2A} receptor agonists (de Sarro et al., 1999). In our experiments, the selective A_{2A} receptor agonist (CGS 21680) and the non-selective receptor agonist (NECA) reduced benzodiazepine withdrawal signs, although this action was less apparent than that induced by adenosine A_1 receptor agonist—CPA. It means that both adenosine A_1 and A_{2A} receptors are involved in the observed effects, although adenosine A_1 receptor plays the most important role in these effects. Thus, the attenuating effects of all adenosine receptor agonists on benzodiazepine withdrawal signs in mice are independent on their different biochemical effects on adenylyl cyclase (see Introduction).

The neurochemical mechanisms underlying the benzodiazepine withdrawal signs are not fully clarified. The numerous results of the studies demonstrated that adaptive changes of the GABA-ergic system are difficult to formulate and may be only one of the components of the mechanisms underlying the benzodiazepine withdrawal signs (for ref. see Allison and Pratt, 2003). For instance, the subsensitivity to GABA for several weeks after withdrawal is reported by one group of authors (Gonsalves and Gallager, 1987) while others report an increase in $GABA_A$ receptor function (Miller et al., 1988).

Some data suggest the involvement of the other, than GABA, neuronal systems in benzodiazepine dependence. The close interaction between GABA-A/NMDA neurotransmission in CNS has been documented (Chaudieu et al., 1994; Stelzer et al., 1987) and the neurophysiologic activity of the mammalian brain is maintained by the balance between inhibitory (such as GABA) and excitatory (such as glutamate) systems. After cessation of chronic treatment with benzodiazepines the excitatory mechanisms become more sensitive and this effect may be responsible for the expression of benzodiazepine withdrawal signs (Stephens, 1995). Then, the observed suppression of the benzodiazepine withdrawal syndrome by adenosine receptor agonists may be related (at least partially) to the antagonistic interaction between NMDA and adenosinergic systems, but this problem needs further studies.

Summing up, the results of the present study demonstrate that all adenosine receptor agonists are able to attenuate benzodiazepine withdrawal signs in mice, and the most apparent effects are observed after application of A_1 receptor agonist—CPA. It means that adenosine A_1 receptors play a predominant role in this behavior.

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