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Enhancement by benzodiazepines of the inhibitory effect of adenosine on skeletal neuromuscular transmission

L.C. Chiou, J.Y. Ling & [1]C.C. Chang

Department of Pharmacology, College of Medicine, National Taiwan University, Jen-Ai Rd., No. 1, Sec. 1, Taipei, Taiwan

1 Interactions of benzodiazepines with adenosine on the neuromuscular transmission were studied in mouse diaphragm preparations.

2 In tubocurarine (0.6–0.8 μM)-partially paralyzed preparations, diazepam (35 μM) and Ro 5-4864 (3~30 μM), a peripheral type benzodiazepine receptor agonist, potentiated the inhibitory effect of adenosine on indirect twitch responses.

3 The central type receptor agonist, clonazepam did not affect the inhibitory effect of adenosine.

4 The peripheral benzodiazepine receptor antagonist, PK11195 (1–10 μM) attenuated the adenosine inhibition and antagonized the potentiation by Ro 5-4864.

5 Ro 5-4864 failed to enhance further the inhibitory effect of adenosine in the presence of dipyridamole, an adenosine uptake inhibitor that also potentiated adenosine inhibition.

6 Neither Ro 5-4864 nor PK 11195 affected the inhibition produced by a stable adenosine analogue, 2-chloroadenosine, which is not a substrate for the adenosine uptake system.

7 Ro 5-4864 did not affect endplate potentials (e.p.ps) in the absence of adenosine, but reduced the amplitude of e.p.ps in the presence of adenosine without affecting miniature e.p.ps.

8 It is suggested that benzodiazepines potentiate the adenosine-effected presynaptic inhibition of neuromuscular transmission by an inhibition of adenosine uptake through activation of peripheral type benzodiazepine receptors.

Keywords: Benzodiazepines; adenosine; neuromuscular transmission; adenosine uptake; benzodiazepine receptors

Introduction

In addition to anxiolytic, anticonvulsant and sedative therapeutic effects, benzodiazepines are also used as muscle relaxants. Most of these effects are believed to be mediated by the central type benzodiazepine receptors that constitute the modulatory site of γ-aminobutyric acid $(GABA)_A$ receptor-chloride channel complex (Olsen, 1982; Haefely *et al.*, 1985). Nevertheless, several lines of evidence suggest that benzodiazepines exert some of their effects, if not all, through interaction with adenosine in the central nervous system (Dragunow *et al.*, 1985; Stone, 1986; Phillis & O'Regan, 1988a; Contreras & Germany, 1991; O'Connor *et al.*, 1991; Sierralta & Miranda, 1992). There are also reports (Roache & Griffiths, 1987; Roca *et al.*, 1988; Ruiz *et al.*, 1988) referring to interactions between benzodiazepines and methylxanthine adenosine receptor antagonists (Snyder *et al.*, 1981). Benzodiazepines have been shown to interfere with adenosine uptake by inhibiting the purine transporter system (Phillis *et al.*, 1980; Hammond *et al.*, 1983; Moritoki *et al.*, 1985; Bender & Hertz, 1986; Morgan & Stone, 1986) and hence to potentiate the pharmacological responses of adenosine in the central nervous system (Stone 1986; Phillis & O'Regan, 1988b) as well as in peripheral tissues, such as cardiac muscle (Clanachan & Marshall, 1980; Kenakin, 1982; Moritoki *et al.*, 1985), trachea (Devillier *et al.*, 1992), vas deferens (Clanachan & Marshall, 1980; Escubedo *et al.*, 1991), taenia coli (Moritoki *et al.*, 1985) and skeletal neuromuscular transmission (Mendonca & Ribeiro, 1989). In addition, benzodiazepines have also been reported to affect the binding of adenosine (Hawkins *et al.*, 1989; Kaplan *et al.*, 1992).

Two types of benzodiazepine receptor have been identified; the central type displaying nanomolar affinity for diazepam and the peripheral type receptors with affinity for diazepam in the 10 nanomolar range. The latter were first demonstrated in nonneuronal tissues (Braestrup & Squires, 1977) and later also in the central nervous system (Shoemaker *et al.*, 1981). Benzodiazepine binding sites appear to exist in the motor nerve-skeletal muscle system (Roeske & Yamamura, 1982; Wilkinson *et al.*, 1982) and diazepam interacted synergistically with adenosine-induced inhibition of neuromuscular transmission (Mendonca & Ribeiro, 1989). We have previously shown that peripheral type benzodiazepine receptor agonists but not central type agonists increased muscle contractility and antagonized the regenerative tonic endplate depolarization induced by train pulses in neostigmine-treated preparations (Chiou & Chang, 1993; 1994). In the present study, we further show that a peripheral benzodiazepine receptor agonist but not a central one potentiates adenosine-induced inhibition of mouse neuromuscular transmission. The mechanism of synergism was also examined.

Methods

Muscle contractions

Phrenic nerve-hemidiaphragm preparations isolated from ICR mice (20–25 g) of either sex were used. The preparation was incubated in an organ bath containing 15 ml Tyrode solution (composition in mM: NaCl 137, KCl 2.8, $CaCl_2$ 1.8, $MgCl_2$ 1.2, NaH_2PO_4 0.33, $NaHCO_3$ 11.9 and dextrose 11.2) kept at 36±0.2°C and oxygenated with 95% O_2 plus 5% CO_2. Indirect muscle contactions were elicited by stimulation of the phrenic nerve with supramaximal rectangular pulses of 0.05 ms width at 0.1 Hz and recorded isometrically with a transducer (BG25, Gould) coupled to a physiological recorder (Gould 3000). The safety factor for neuromuscular transmission in phrenic nerve-diaphragm preparations is high, being 3–5 (Paton & Waud, 1967; Chang *et al.*, 1975). In order to visualize the small magnitude of the inhibitory effect of drugs, the safety factor was deliberately decreased by adding 0.6–0.8 μM tubocurarine

[1] Author for correspondence.

that inhibited indirect twitch responses to 73–78% of control. This procedure reduced the safety margin close to one so that any inhibitory effect on neuromuscular transmission would result in a further decrease of the indirect contraction. Inhibitory effects were calculated in terms of percentage inhibition taking the response before treatment with adenosine as control. In the study of dose-inhibition relationships, adenosine or 2-chloroadenosine was applied cumulatively. The IC_{50} of adenosine or 2-chloroadenosine was determined by the interpolation from dose-response curves.

Electrophysiological studies

Transmembrane potentials and endplate potentials (e.p.ps) were measured by classical intracellular recording technique with 3 M KCl-filled microelectrodes (10–40 MΩ). Preparations were mounted horizontally in a perfusion chamber containing 4 ml oxygenated Tyrode solution and were perfused at a rate of 6–8 ml min^{-1} at 34±0.5°C. Signal inputs to the microelectrode were registered through a high impedance amplifier (Axoclamp-2A) and a computer-aided digitizer (D6100, Analogic). Endplate potentials were evoked by stimulation of the nerve at 1 Hz and monitored in cut muscle preparations (Barstad & Lilleheil, 1968) or in uncut muscle preparations treated with 3.2–3.6 μM tubocurarine. The amplitude of e.p.ps of cut muscle preparations was corrected for non-linear summation to −40 mV, assuming a reversal potential of 0 mV (Chang *et al.*, 1986).

Chemicals

Diazepam, clonazepam and flumazenil were generous gifts from Hoffmann-La Roche (Basle, Switzerland) and PK11195 (also coded as RP 52028) (1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline carboxamide) was from Rhone-Poulenc Rorer (Cedex, France). Adenosine, 2-chloroadenosine, dipyridamole and tubocurarine chloride were purchased from Sigma (U.S.A.) and Ro5-4864 (7-chloro-1,3-dihydro-1-methyl-5-(p-chlorophenyl)-2H-1,4-benzodiazepine-2-one) from Fluka (Switzerland). All benzodiazepines and PK11195 were dissolved in ethanol as stock solutions. Final vehicle concentrations added to the organ bath were less than 0.2% and had little effect on neuromuscular transmission. Dipyridamole was dissolved in a minimum volume of 1 N HCl and then diluted to 1 mM as a stock solution.

Statistics

All data are expessed as mean±s.e.mean. In electrophysiological experiments, data from 6–7 endplates for each preparation treated with tubocurarine were pooled for comparison. In cut muscle preparations, responses of the same endplate before and after drug treatment were compared. *n* refers to the number of preparations tested. Differences between means were analyzed by Student's paired *t* test. Contractile responses among different treatments were analyzed by non-paired *t* test.

Results

Effects on the inhibitory action of adenosine on twitch responses

In mouse diaphragm preparations partially paralyzed with 0.6–0.8 μM tubocurarine, adenosine inhibited the indirect twitch response within 10 min. The IC_{50} obtained from the cumulative concentration-inhibition curve was 74±7 μM ($n=22$). Diazepam, at 35 μM, increased the indirect twitches by 35±2% ($n=16$) when applied alone, but potentiated the inhibition caused by adenosine. The concentration-inhibition curve of adenosine was shifted to the left in the presence of 35 μM diazepam (Figure 1) and the IC_{50} for adenosine was decreased to 60±5 μM ($n=16$). Interestingly, diazepam at a lower concentration, 10 μM, shifted the inhibition curve rightward (Figure 1), indicating a biphasic nature of action. A higher concentration of diazepam (100 μM) was not used because axon conduction block occurred (Chiou & Chang, 1993).

Like 35 μM diazepam, Ro 5-4864, a selective peripheral benzodiazepine receptor agonist (Braestrup & Squires, 1977), enhanced the inhibitory effect of adenosine on neuromuscular transmission although it increased muscle contactility. At 3, 10 and 30 μM, Ro 5-4864 increased the twitch responses by 25±2, 29±2 and 51±3%, respectively. Unlike diazepam, Ro 5-4864 potentiated the adenosine-induced inhibition dose-dependently at all concentrations tested (3–30 μM) (Figure 2). The IC_{50} for adenosine in the presence of 3 and 30 μM Ro 5-4864 was decreased from 74±7 to 55±10 ($n=7$) and 38±3 μM ($n=10$), respectively. Ro 5-4864 also caused a conduction block at concentrations higher than 50 μM.

In contrast to diazepam and Ro 5-4864, clonazepam, a

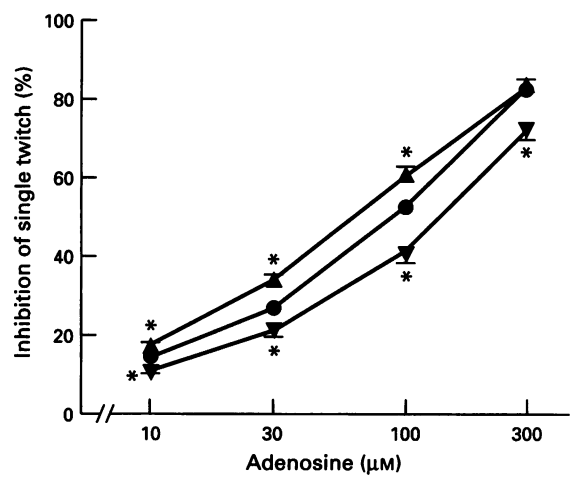

Figure 1 Effects of diazepam on adenosine-induced inhibition of indirect twitch responses. Indirect twitch responses were evoked at 0.1 Hz in mouse diaphragm preparations pretreated with tubocurarine (0.6–0.8 μM). Inhibitions (%) of twitch amplitude after cumulative addition of adenosine were obtained in the presence of vehicle (alcohol 0.1%, v/v) (●), 10 μM (▼) and 35 μM diazepam (▲). *$P<0.05$ vs. control. $n=12$–22.

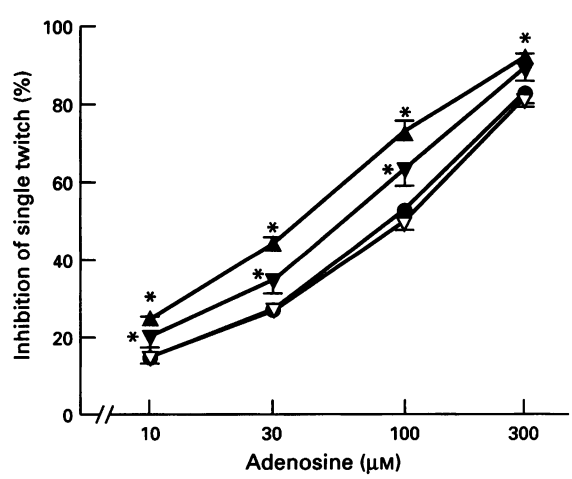

Figure 2 Effects of Ro 5-4864 and clonazepam on adenosine-induced inhibition of indirect twitch responses. Same experimental conditions as described in Figure 1 legend. Vehicle control (●); 3 (▼) or 30 (▲) μM Ro 5-4864; 10 μM clonazepam (▽). *$P<0.05$ vs. control. $n=7$–22.

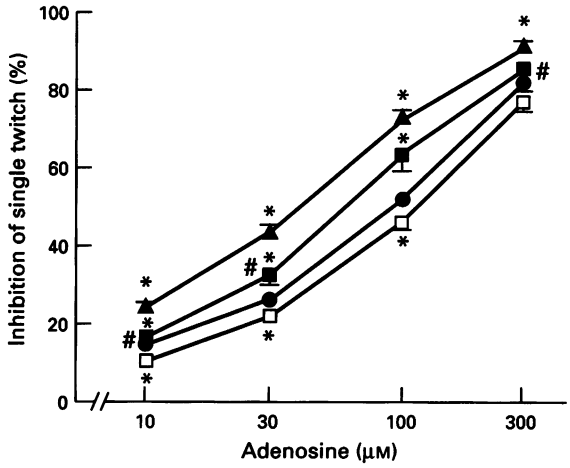

Figure 3 Effect of PK11195 on the adenosine-induced inhibition of indirect twitch responses in the presence or absence of Ro 5-4864. Same experimental conditions as described in Figure 1 legend. Vehicle control (●), 10 μM PK11195 (□), 30 μM Ro 5-4864 (▲); 10 μM PK11195 plus 30 μM Ro 5-4864 (■). $^*P<0.05$ vs. control. $^\#P<0.05$ vs. Ro 5-4864 alone. $n=10$–22.

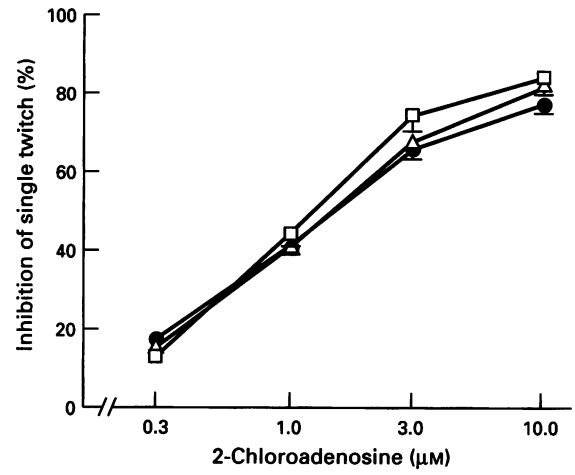

Figure 4 Effects of Ro 5-4864 and PK11195 on the inhibition by 2-chloroadenosine of indirect twitch responses. Similar experiments to those in Figure 3 using 2-chloroadenosine in place of adenosine. Control (●); 30 μM Ro 5-4864 (△); 10 μM PK11195 (□). $n=10$–22.

central benzodiazepine receptor agonist (Braestrup & Squires, 1977) affected neither the indirect twitch responses by itself, nor the inhibitory effect of adenosine (Figure 2).

To exclude the possibility that the interactions of benzodiazepines with adenosine described above were due to a nonspecific pharmacological action of these agents, unrelated to their binding to peripheral benzodiazepine receptors, we further studied the effect of PK11195, a peripheral receptor antagonist with a nonbenzodiazepine structure (Le Fur *et al.*, 1983a,b). PK11195 slightly attenuated the inhibition by adenosine and shifted the adenosine-inhibition curve to the right (Figure 3). PK11195, at 1 and 10 μM, caused the same magnitude of attenuation. The IC_{50} increased from 74 ± 7 ($n=22$) to 98 ± 9 ($n=12$) and to 95 ± 8 μM ($n=24$), respectively, after treatment with 1 and 10 μM PK11195. PK11195 also diminished the potentiation by Ro 5-4864 of adenosine inhibition (Figure 3). The IC_{50} for adenosine in the presence of 30 μM Ro 5-4864 was increased from 38 ± 3 ($n=10$) to 48 ± 3 ($n=10$) and 55 ± 8 μM ($n=11$), respectively, by 1 and 10 μM PK11195.

Effects on the inhibitory action of 2-chloroadenosine

2-Chloroadenosine, a nonhydrolyzable adenosine analogue which is not taken up by the nucleotide transporter system (Daly, 1983), inhibited indirect twitch responses dose-dependently, like adenosine, in preparations partially paralyzed with tubocurarine. The IC_{50} was 1.7 ± 0.2 μM, being about 40 fold more potent than adenosine. In contrast to adenosine-induced inhibition, Ro 5-4864 (30 μM) did not affect the inhibitory effect of 2-chloroadenosine on the indirect twitches (Figure 4). PK 11195, at 10 μM which attenuated the adenosine inhibition no matter whether Ro 5-4864 was present or not, had no significant effect on 2-chloroadenosine-induced inhibition (Figure 4).

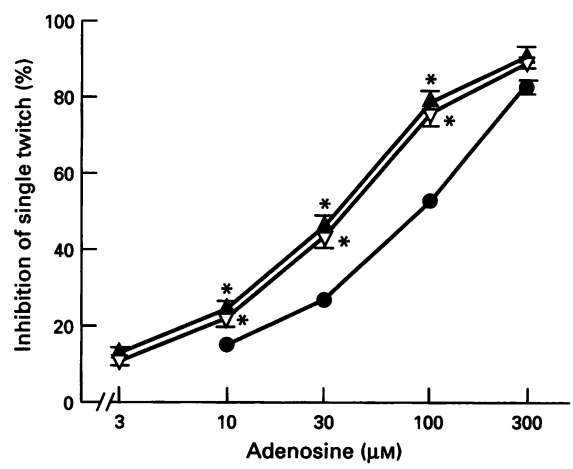

Figure 5 Effects of dipyridamole and Ro 5-4864 on adenosine-induced inhibition of indirect twitch responses. Same experimental conditions as described in Figure 1. Vehicle alone (●); 1 μM dipyridamole (△); 1 μM dipyridamole plus 30 μM Ro 5-4864 (▲). $^*P<0.05$ vs. control. $n=10$–22.

Effect on adenosine inhibition in the presence of dipyridamole

In the presence of 1 μM dipyridamole, an adenosine uptake inhibitor (Huang & Daly, 1974; Jarvis, 1986), the inhibitory effect of adenosine on the indirect twitches was markedly enhanced and the IC_{50} was reduced from 74 ± 7 to 36 ± 5 μM. In the presence of dipyridamole, Ro 5-4864 failed to enhance further the inhibitory effect of adenosine (Figure 5).

Table 1 Effects of adenosine on the endplate responses in the absence or presence of Ro 5-4864

	e.p.p. (mV)		*m.e.p.p.* (mV)
Treatment	*Cut muscle*	*Curare-treated*	*Intact muscle*
Control	15.4 ± 1.3	2.4 ± 0.2	1.4 ± 0.2
Adenosine, 30 μM	11.6 ± 1.0*	1.9 ± 0.2*	1.3 ± 0.2
Ro 5-4864, 30 μM	14.6 ± 2.7	–	1.5 ± 0.2
Ro 5-4864 + adenosine	9.5 ± 2.3*,**	1.5 ± 0.2*,**	1.5 ± 0.3

Endplate potentials (e.p.ps) were evoked at 1 Hz in 3.2–3.6 μM tubocurarine-treated preparations with membrane potentials within 75–80 mV, or in cut muscle preparations with membrane potentials at -39.1 ± 6.1 mV. Miniature e.p.ps (m.e.p.ps) were recorded in normal muscle preparations with membrane potential at -80 ± 0.8 mV.
$^*P<0.05$ vs. control; $^{**}P<0.05$ vs. adenosine alone. $n=7$–12.

Effect of Ro 5-4864 on the e.p.p. inhibition by adenosine

To elucidate the site of interaction between adenosine and benzodiazepines, their effects on endplate responses were examined. Both adenosine and Ro 5-4864, alone or in combination, had no effect on the amplitude of miniature e.p.ps (m.e.p.ps) in intact muscle endplates (Table 1). Adenosine, 30 μM, decreased the e.p.p. amplitude by 24±4% ($n=7$) in cut muscle preparations and by 16±6% ($n=12$) in preparations paralyzed with 3.2–3.6 μM tubocurarine. In parallel with its effect on indirect twitches, 30 μM Ro 5-4864 increased the depressant effects of adenosine on e.p.ps in the cut and tubocurarine-treated preparations from 16±6% to 37±4% and from 24±4% to 38±4%, respectively (Table 1). Ro 5-4864 did not affect the e.p.p. amplitude when adenosine was absent.

Discussion

We have found previously that benzodiazepine receptor ligands acting on the peripheral type receptor (such as diazepam, Ro 5-4864 and PK11195), but not those acting specifically on the central type receptor (such as clonazepam and flumazenil), inhibited the regenerative prolonged endplate depolarization caused by repetitive stimulation in the presence of anticholinesterase agents through a presynaptic mechanism (Chiou & Chang, 1993; 1994). In the present experiments we further showed that diazepam and Ro 5-4864, but not clonazepam, potentiated adenosine-induced inhibition of neuromuscular transmission. The potentiation by diazepam or Ro 5-4864 of adenosine-induced inhibition of indirect twitches was not due to an increase of muscle contractility (Chiou & Chang, 1994) since the latter should result in an attenuation rather than potentiation of adenosine inhibition.

Adenosine inhibition of neuromuscular transmission is produced through an inhibition of acetylcholine release from motor nerve terminals (Ginsborg & Hirst, 1972; Silinsky, 1984). Benzodiazepines at concentrations effective in potentiating adenosine inhibition have no direct effect on neuromuscular transmission at either pre- or postsynaptic sites (Chiou & Chang, 1993; 1994). In the presence of adenosine, the quantal content was further decreased by Ro 5-4864 since it further reduced the e.p.p. amplitude while not affecting the postsynaptic receptor sensitivity. The potentiation by benzodiazepines could reasonably be attributed to an enhancement of adenosine-induced inhibition of acetylcholine release, possibly through an action at the peripheral-type benzodiazepine receptors. Similar synergistic interactions between adenosine and peripheral benzodiazepine receptor ligands have been reported in other peripheral tissues (Davies & Huston, 1981; Escubedo *et al.*, 1991; Devillier *et al.*, 1992). It is interesting that PK11195, a peripheral benzodiazepine receptor antagonist (Le Fur *et al.*, 1983a, b), decreased the adenosine inhibition either in the absence or presence of Ro 5-4864, but not the 2-chloroadenosine-induced inhibition. Therefore, it seems unlikely that PK11195 directly affects adenosine receptors. Whether the attenuation by PK11195 of adenosine-induced inhibition in the absence of agonist is due to an antagonism against endogenous benzodiazepine (Verma & Snyder, 1989) remains to be elucidated. The reason why diazepam at 35μM potentiated the effect of adenosine while inhibiting it at lower concentrations is not clear.

The ineffectiveness of Ro 5-4864 in potentiating the inhibition induced by 2-chloroadenosine suggests that Ro 5-4864 potentiates adenosine inhibition by affecting the adenosine uptake system. Mendonca & Ribeiro (1989) proposed that diazepam potentiated adenosine-induced neuromuscular block in frog muscles through an inhibition of adenosine uptake. Diazepam and other benzodiazepines have been shown to inhibit adenosine uptake in neuronal and non-neuronal tissues (Phillis *et al.*, 1980; Hammond *et al.*, 1983; Moritoki *et al.*, 1985; Morgan & Stone, 1986). It has been suggested that peripheral type benzodiazepine receptors, present on human erythrocyte membranes, may be involved in the inhibition of nucleoside transport (Hammond *et al.*, 1983; Olson *et al.*, 1988). Our experiments with dipyridamole, which enhanced the inhibitory effect of adenosine on the neuromuscular transmission by itself (Chiou *et al.*, 1987; Ribeiro & Sebastiao, 1987), but negated the effect of Ro 5-4864, are in support of the above inference. It is inferred that peripheral type benzodiazepine receptors may modulate in some way the adenosine uptake mechanism at the neuromuscular junction.

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