Pharmacological relevance of peripheral type benzodiazepine receptors on motor nerve and skeletal muscle

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- 1 Effects of agonists and antagonists of peripheral and central benzodiazepine receptors (pBZR and cBZR) on neuromuscular transmission were studied in mouse isolated phrenic nerve-diaphragm preparations.
- 2 Ro5-4864, a pBZR agonist, had no effect on the neuromuscular transmission but increased muscle contractility and antagonized the tetanic fade induced by neostigmine.
- 3 Ro5-4864 inhibited the regenerative tonic endplate depolarization caused by repetitive stimulation in the presence of neostigmine without affecting the amplitude and decay time of miniature and evoked single endplate potentials.
- 4 All the effects of Ro5-4864 were shared by PK11195, a pBZR antagonist, but not by clonazepam and flumazenil, a cBZR agonist and antagonist, respectively.
- 5 It is suggested that peripheral type benzodiazepine receptors modulate presynaptic function and muscle contraction.

Keywords: Peripheral benzodiazepine receptors; benzodiazepines; Ro5-4864; PK11195; neuromuscular transmission

Introduction

Benzodiazepine receptors (BZR) have been classified into central and peripheral types according to their relative affinities for specific ligands (Braestrup & Squires, 1977; Rampe & Triggle, 1986). The central type (cBZR) displays high affinity for diazepam (nanomolar range) and is involved in the anxiolytic, muscle relaxing, sedative and hypnotic actions of benzodiazepines (Haefely *et al.*, 1979). Peripheral BZR (pBZR), having tens of nanomolar affinity for diazepam (Braestrup & Squires, 1977), were originally demonstrated in various peripheral nonneuronal tissues by a selective binding ligand Ro5-4864 (7-chloro-1, 3-dihydro-1-methyl-5-(p-chlorophenyl)-2H-1,4-benzodiazepine-2-one) and later also found in the central nervous system (Schoemaker *et al.*, 1981). The functional roles of pBZR in the peripheral nervous system are far from clear, although involvement in steroidogenesis, cell proliferation and immunoreactivity of non-neuronal tissues have been proposed (Ruff *et al.*, 1985; Anholt, 1986; Mukhin *et al.*, 1989; Papadopoulos *et al.*, 1990; Bruce *et al.*, 1991). In addition, a third type nonselective binding site for benzodiazepines with micromolar affinity has been demonstrated in the brain (Bowling & DeLorenzo, 1982) and leech neurones (Johansen *et al.*, 1985). This type of receptor providing no selectivity for either pBZR or cBZR ligands was suggested to be related to the inhibition of Ca²⁺ channel in synaptosomes (Taft & DeLorenzo, 1984) and inhibition of electric shock-induced seizure by benzodiazepines (Bowling & DeLorenzo, 1982).

Since diazepam improves the peripheral neuromuscular transmission impaired by anticholinesterase agents and enhances the muscle contractility (Chiou & Chang, 1993), it seems possible that benzodiazepine receptors exist in the peripheral motor nerve and skeletal muscle and have modulatory function on synaptic transmission. Specific binding sites for [³H]-flunitrazepam (Wilkinson *et al.*, 1982) and [³H]-Ro5-4864 (Roeske & Yamamura, 1982) have been demonstrated in rat diaphragm and it has been proposed that they are the peripheral type. The present study was undertaken to compare the functional activities of both cBZR and pBZR agonists and antagonists and to elucidate the possible functional role(s) of pBZR in neuromuscular junction.

Methods

Muscle contractions

Muscle contractions and electrophysiological experiments were performed in phrenic nerve-hemidiaphragm preparations isolated from ICR mice (20–25 g) of either sex. The organ bath contained 15 ml Tyrode solution (composition in mM: NaCl 137, KCl 2.8, CaCl₂ 1.8, MgCl₂ 1.2, NaH₂PO₄ 0.33, NaHCO₃ 11.9 and dextrose 11.2) oxygenated with 95% O₂ plus 5% CO₂ and kept at 36 ± 0.2°C. Tetanic contraction and single twitches were evoked at 50–100 Hz for 3 s and 0.1 Hz, respectively. Indirect muscle contractions were elicited by stimulation of the phrenic nerve with supramaximal rectangular pulses of 0.05 ms width. Direct muscle contractions were elicited by field stimulation of the diaphragm with 0.5 ms bipolar pulses in the presence of 4 µM tubocurarine to exclude any possible indirect component. Contractions were recorded isometrically via a transducer (BG25, Gould) coupled to a physiological recorder (Gould 3000). The tetanic performance was evaluated by measuring the area under the contractile curves (AUC).

Electrophysiological studies

Electrophysiological experiments were performed 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. Transmembrane potentials were recorded through a high impedance amplifier (Axoclamp-2A) and registered with a computer-aided digitizer of 100 kHz bandwidth (D6100, Analogic). Endplate potentials (e.p.ps) were evoked by nerve stimulation at 0.1 Hz or 75 Hz and recorded at endplate area of cut muscle preparations (Barstad & Lilleheil, 1968). The amplitude of e.p.p. was corrected for non-linear summation to –40 mV, assuming a reversal potential of 0 mV (Chang *et al.*, 1986).

A regenerative tonic depolarization was provoked by stimulation with repetitive pulses at 75 Hz. This was determined by the upstroke depolarization and the associated shut-off of phasic e.p.ps (Chang & Hong, 1986). The duration of regenerative depolarization was measured from the upstroke to the resumption of phasic e.p.ps. The extent of depolarization was

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determined by the maximal changes of the endplate membrane potential.

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). Ro5-4864 was purchased from Fluka (Switzerland) and neostigmine bromide, tubocurarine chloride and γ -aminobutyric acid (GABA) from Sigma (USA). 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 the neuromuscular transmission.

Statistics

All data are expressed as mean $\pm$ s.e. Differences between means were analyzed by Student's *t* test.

Results

Effect on muscle contractions in the absence of neostigmine

Like diazepam, Ro5-4864, a pBZR agonist, dose-dependently increased the direct single twitch response at 5–50 μ M in the presence of 4 μ M tubocurarine (Figure 1). The indirect single twitch response was increased to the same extent by Ro5-4864 at 5–30 μ M. At 50 μ M, a stepwise decrease of the indirect twitch tension, implicating nerve conduction block, was observed in some preparations after the initial potentiation (Figure 2). The tetanic contractions evoked directly or indirectly at 50–100 Hz were also enhanced by Ro5-4864 in the same concentration-range. However, the higher the stimulation rate, the less was the enhancement. A slight tonic contracture was observed when Ro5-4864 was at concentrations higher than 30 μ M.

The effects of PK11195, a pBZR ligand nominally regarded as an antagonist (Le Fur *et al.*, 1983a,b; Benavides *et al.*, 1984; Mizoule *et al.*, 1984; Ruff *et al.*, 1985; Slobodyansky *et al.*, 1989; Bruce *et al.*, 1991), were compared. Interestingly, this compound also increased the muscle contractility evoked directly or indirectly by either single or repetitive pulses, at the same concentration-range. Muscle contracture and axonal conduction block were also observed at 100 μ M. Ro5-4864 and PK11195, though classified as agonist and antagonist, respectively, appeared to act additively with no sign of antagonism. Their effects were analogous to diazepam (Chiou & Chang, 1993) but were more potent.

In contrast, clonazepam and flumazenil, the cBZR agonist and antagonist (Braestrup & Squires, 1977; Laurent *et al.*, 1981), respectively, did not affect the direct or indirect twitch responses at concentrations up to 50 μ M (Figure 1). Moreover, the effects of Ro5-4864, PK11195 or diazepam were not antagonized after pretreatment with flumazenil.

Effects on muscle contractions in the presence of neostigmine

Three characteristic actions of anticholinesterase agents on isolated nerve-skeletal muscle preparations, i.e., spontaneous muscle fasciculation, twitch potentiation and tetanic fade, an inability to maintain a tetanus during high frequency stimulation of motor nerve (Hobbiger, 1976), were reproduced by pretreating the diaphragm with 0.3 μ M neostigmine. Ro5-4864, PK11195 and diazepam at 1–20 μ M dose-dependently inhibited the twitch potentiation (Figure 3). All three ligands appeared equipotent. Clonazepam was effective only at con-

centrations higher than 20 μ M while flumazenil was ineffective at concentrations up to 50 μ M.

The tetanic fade was antagonized by Ro5-4864 and PK11195 (Figure 4) in a manner analogous to diazepam (Chiou & Chang, 1993) with a bell-shape dose-response relation (Figure 5). The potencies were in the order of PK11195 $\geq$ Ro5-4864 > diazepam. By contrast, both cBZR ligands were ineffective in restoring tetanic fade and did not affect the restoration by Ro5-4864, PK11195 and diazepam.

Similar to the effects on twitch potentiation and tetanic fade, the spontaneous muscle fasciculation was abolished by Ro5-4864 and PK11195 at 10 μ M and by clonazepam at 20 μ M but not by flumazenil at concentrations up to 50 μ M.

Figure 1 Dose-response curves of benzodiazepine receptor ligands on the direct twitch responses. Twitch responses were evoked at 0.1 Hz directly in the presence of 4 μ M tubocurarine; diazepam (○); Ro5-4864 (□); PK11195 (▽); clonazepam (◆); flumazenil (△). *n* = 3–6.

Figure 2 Effect of Ro5-4864 on the indirect twitch response of mouse isolated phrenic nerve-diaphragm preparation. The indirect twitch response was evoked at 0.1 Hz. Ro5-4864, 50 μ M was added at ▲. Calibration: 5 min, 1 g.

Figure 3 Effect of benzodiazepine receptor ligands on the indirect twitch response in the presence of neostigmine. Indirect twitch response evoked at 0.1 Hz in the presence of 0.3 μ M neostigmine is expressed as % of control in the absence of drugs. Concentration zero represents the average responses produced by neostigmine alone; diazepam (●); Ro5-4864 (□); PK11195 (▽); clonazepam (◇); flumazenil (△). *n* = 3–8.

Effect on the endplate responses

Ro5-4864, at concentrations up to 30 μM , did not affect the amplitude and decay time of e.p.p. or m.e.p.p. In the presence of neostigmine, single e.p.ps as well as m.e.p.ps that were enlarged and prolonged were also unaffected by Ro5-4864 (Table 1). The regenerative depolarization, a unique phenomenon provoked by high frequency stimulation in anticholinesterase-treated endplates (Chang & Hong, 1986; Hong & Chang, 1989) was inhibited by Ro5-4864 and PK 11195. The incidence of tonic depolarization, as characterized by the upstroke depolarization followed by complete shut-off of phasic e.p.ps was decreased from about 95% to two-thirds of junctions after treatment with 10 μM Ro5-4864, and the duration, if it occurred, was shortened (Table 1). The extent of depolarization was decreased resulting in a restoration of train e.p.ps (Figure 6). PK11195, at 5 μM , produced the same degree of inhibition as 10 μM Ro5-4864. The effects of both

Figure 4 Effect of Ro5-4864, PK11195 and flumazenil on the tetanic fade in the presence of neostigmine. Phrenic nerve was stimulated at 75 Hz for 3 s in preparations before (left column) and after treatment with 0.3 μM neostigmine (middle column) and after further treatment (right column) with 10 μM Ro5-4864 (a), 5 μM PK 11195 (b), or 10 μM flumazenil (c). Calibration: 3 s, 5 g.

Ro5-4864 and PK11195 were again similar to that of diazepam (Chiou & Chang, 1993) but the potency of diazepam was lower. Again, in contrast to pBZR ligands, both clonazepam and flumazenil did not affect the neostigmine-induced regenerative depolarization.

Figure 5 Effect of benzodiazepine receptor ligands on tetanic contraction in the presence of neostigmine. Tetanic contractions induced by 75 Hz nerve stimulation for 3 s in the presence of 0.3 μM neostigmine (Neo) were measured from the area under the curve (AUC) of contraction and expressed as % of control before drug treatment. Figures below each column are the concentrations of BZR ligands in μM . $n = 4-7$. * $P < 0.05$ vs. the next smaller concentration in paired t test.

Figure 6 Effect of Ro5-4864 on the endplate responses during repetitive stimulation in the presence of neostigmine. The preparation was treated with 0.3 μM neostigmine and stimulated at 75 Hz. The responses at the endplate were recorded from the same junction before (a) and after (b) treatment with 10 μM Ro5-4864. Resting membrane potential denoted by the broken lines was about -46 mV. Calibration: 200 ms, 10 mV.

Table 1 Effect of Ro5-4864 on the endplate responses in the presence of neostigmine

Treatment	m.e.p.p. ^a		e.p.p. ^b		Regenerative depolarization ^c		
	Amplitude (mV)	t_1 ^d (ms)	Amplitude (mV)	t_1 (ms)	Incidence	Duration (ms)	Extent (mV)
Control	2.9 $\pm$ 0.2	3.1 $\pm$ 0.1	16.0 $\pm$ 0.2	6.1 $\pm$ 0.1	21/22	887 $\pm$ 134	19.6 $\pm$ 1.3
Ro5-4864 (10 μM)	3.0 $\pm$ 0.2	3.0 $\pm$ 0.2	15.8 $\pm$ 0.2	6.4 $\pm$ 0.1	12/18*	451 $\pm$ 73*	14.0 $\pm$ 1.4*

Neostigmine (0.3 μM) was present throughout.
^aRecorded in uncut muscle preparations (resting membrane potentials: -82 ± 2 mV) in the presence of 1 μM tetrodotoxin.
^bEvoked at 0.6 Hz and recorded in cut muscle preparations (resting membrane potentials: -41 ± 1 mV).
^cProvoked by stimulating nerve at 75 Hz for 2.5 s and calculated as described in Methods.
^dHalf decay time.
* $P < 0.05$.

Effect of GABA on the actions of pBZR ligands

GABA, at 100 μM , did not affect the neuromuscular transmission in the presence or absence of neostigmine nor did it affect the enhancement of muscle contraction and the restoration of neostigmine-impaired tetanic contraction caused by diazepam, Ro5-4864 or PK11195.

Discussion

BZR of the peripheral type have been demonstrated in skeletal muscle by binding studies (Roeske & Yamamura, 1982; Wilkinson *et al.*, 1982). Clonazepam, a cBZR ligand, was 3~4 order less potent than Ro5-4864 in displacing the specific binding of benzodiazepine ligands. In this study, we found that there was a clear-cut distinction between the pBZR and cBZR ligands in their effects on neuromuscular transmission. The pBZR ligands Ro5-4864 and PK11195, irrespective of agonist or antagonist activity, shared with diazepam the same effects on mouse phrenic nerve-diaphragm (Chiou & Chang, 1993), i.e., an enhancement of muscle contractility and a restoration from anticholinesterase-induced neuromuscular transmission disorders. The cBZR ligands, clonazepam and flumazenil, were much less effective or ineffective. This pattern of ligands selectively suggested that the receptor involved was pBZR but not cBZR or the third type. The effects of the pBZR ligands were unlikely to be due to a nonspecific membrane action of benzodiazepine derivatives since clonazepam and flumazenil are congeners of benzodiazepines but were ineffective while PK11195, a non benzodiazepine, was effective. It is noteworthy that these pBZR ligands have no appreciable effect on the process of neuromuscular transmission in the absence of neostigmine, as judged from the unchanged single or train e.p.s. Accordingly, it may be inferred that pBZR exist in motor nerve-skeletal muscle system and have functional relevance at least in opposing pathophysiological or pharmacological distresses. These effects of pBZR ligands seem unrelated to GABA receptors since GABA showed neither action of its own nor interaction with pBZR ligands.

PK11195, though classified as a pBZR antagonist in cardiac and other tissues (Le Fur *et al.*, 1983a,b; Benavides *et al.*, 1984; Mizoule *et al.*, 1985; Ruff *et al.*, 1985; Slobodyansky *et al.*, 1989; Bruce *et al.*, 1991), probably acts as an agonist in motor nerve and skeletal muscle as in PC12 cells (Ohara-Imazumi *et al.*, 1991), coronary blood vessel (Grupp *et al.*, 1987), bronchiole (Marao *et al.*, 1990), duodenum (Escubedo *et al.*, 1992) and steroidogenic cells (Mukhin *et al.*, 1989; Papadopoulos *et al.*, 1990).

The effective concentrations of diazepam and Ro5-4864 for neuromuscular actions were two orders of magnitude higher than their K_D value. In general, however, the effective concentrations for pharmacological action on intact organ preparations were much higher than their binding affinity constant measured in homogenized membranes (cf. Hullihan *et al.*, 1983). The difference may be due to penetration barrier and/or to higher receptor occupancy required to generate the

functional responses. Compared with brain or other peripheral tissues such as liver, kidney or heart, the specific binding sites for [^3H]-diazepam or [^3H]-Ro5-4864 in skeletal muscle are rather sparse (Braestrup & Squires, 1977; Roeske & Yamamura, 1982; Anholt, 1986). Therefore, higher concentrations may be required to produce pharmacological effects. Another reason may be the decreased binding of diazepam or Ro5-4864 when the temperature was elevated from 0~4°C to 37°C (Braestrup & Squires, 1977; Le Fur *et al.*, 1984b; Raeburn *et al.*, 1988; Mihara & Fujimoto, 1989).

The restoration of neostigmine-induced tetanic fade by Ro5-4864 and PK11195 can be explained by their inhibition of the train pulse-induced tonic depolarization. This depolarization, a novel phenomenon produced by anticholinesterase agents and resulting in tetanic fade, is attributed to a regenerative ACh release triggered by the accumulated ACh through activation of presynaptic nicotinic ACh receptor and Ca^{2+} channels (Chang & Hong, 1986; Hong & Chang, 1989). How the pBZR ligands inhibit this regenerative depolarization is not known. Since the ligands did not affect the postsynaptic expression of e.p.s., a presynaptic interaction seems more likely. Whether a Ca^{2+} channel inhibition is involved remains to be elucidated. Indeed, various pharmacological effects of benzodiazepines have been related to an inhibition of Ca^{2+} channels, e.g. inhibition of dopamine release in pheochromocytoma cells (Nakazawa *et al.*, 1991; Ohara-Imazumi *et al.*, 1991), ileal longitudinal muscle contraction (Hullihan *et al.*, 1983; Rampe & Triggle, 1987), vas deferens constriction (Escubedo *et al.*, 1992), action potential duration and contractility of cardiac muscle (Mestre *et al.*, 1984; Holck & Osterrieder, 1985) and β -endorphin release from At T20 cells (Bisserbe *et al.*, 1986). In addition, Ro5-4864 competitively inhibited the specific binding of dihydropyridine Ca^{2+} channel blockers on Ca^{2+} channels (Bender & Hertz, 1985; Holck & Osterrieder, 1985; Rampe & Triggle, 1987) and *vice versa* (Cantor *et al.*, 1984). However, opposite results were also reported (cf. Rampe & Triggle, 1986; Raeburn *et al.*, 1988).

The enhancement of muscle contractility by pBZR ligands could be attributed to an effect on dihydropyridine receptors located on the T-tubular membrane of skeletal muscle, which probably function mostly as a voltage sensor in the process of excitation-contraction coupling (Rios & Pizarro, 1991). Several L-type Ca^{2+} channel antagonists have been shown to enhance the contractility of skeletal muscle (Chang *et al.*, 1988; Dulhunty & Gage, 1988; Neuhaus *et al.*, 1990) and shift the activation curve of excitation-contraction coupling (Dulhunty & Gage, 1988; Neuhaus *et al.*, 1990). The possibility remains that the pBZR ligands modulate the excitation-contraction coupling and enhance muscle contractility by acting on this voltage sensor.

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