ELSEVIER

European Journal of Pharmacology 362 (1998) 143–148

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$GABA_B$ receptors as potential targets for drugs able to prevent excessive excitatory amino acid transmission in the spinal cord

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Received 18 June 1998; revised 13 October 1998; accepted 16 October 1998

Abstract

The effects of $GABA_B$ receptor activation on the Ca^{2+}-dependent depolarization-induced overflow of endogenous glutamic acid and γ-aminobutyric acid (GABA) was studied in rat spinal cord nerve terminals exposed in superfusion to 15 mM KCl. The $GABA_B$ receptor agonist (−)-baclofen inhibited the K^+-evoked overflow of glutamate (EC_{50} = 0.098 μM) but was almost inactive against that of GABA. The overflow of both transmitters could be quite similarly inhibited by two other $GABA_B$ receptor agonists, 3-APPA (3-aminopropylphosphonous acid; EC_{50} = 0.087 and 0.050 μM in the case of GABA and glutamate, respectively) and CGP 44532 (3-amino-2(*S*)-hydroxypropyl)methylphosphinic acid; EC_{50} = 0.81 and 0.50 μM). The $GABA_B$ receptor antagonist CGP 35348 [3-amino-propyl(diethoxymethyl)phosphinic acid] blocked the effect of 3-APPA (1 μM) at the autoreceptors ($IC_{50} \simeq 1$ μM), but not at the heteroreceptors. In contrast, the effects of 3-APPA at both autoreceptors and heteroreceptors could be similarly prevented by another $GABA_B$ receptor antagonist, CGP 52432 [3-[[(3,4-dichlorophenyl)methyl]amino]propyl](diethoxymethyl) phosphinic acid ($IC_{50} \simeq 10$ μM). The data suggest that, in the spinal cord, $GABA_B$ autoreceptors on GABA-releasing terminals differ pharmacologically from $GABA_B$ heteroreceptors on glutamatergic terminals. Selective $GABA_B$ receptor ligands may be helpful for conditions characterized by excessive glutamatergic transmission in the spinal cord.

Keywords: $GABA_B$ receptor subtype; Spinal cord; GABA (γ-aminobutyric acid) release; Glutamic acid release; Excitotoxicity

1. Introduction

It is generally believed that one major function of $GABA_B$ receptors is to modulate, actually to inhibit, neurotransmitter release. Accordingly, $GABA_B$ receptors are often located presynaptically on axon terminals (Bonanno and Raiteri, 1993b; Bowery, 1993; Mott and Lewis, 1994). Several studies have suggested that, in rat as well as in human brain, $GABA_B$ receptors exist as subtypes having distinct neuronal locations, functions and pharmacological properties (Dutar and Nicoll, 1988; Calabresi et al., 1991; Davies et al., 1991; Banerjee and Snead, 1995; see, for reviews, Bonanno and Raiteri, 1993b; Bowery, 1993; Mott and Lewis, 1994).

It was previously reported that the selective $GABA_B$ receptor agonist (−)-baclofen potently inhibited the depolarization-evoked release of [^{3}H]γ-aminobutyric acid ([^{3}H]GABA) from cerebral cortex synaptosomes, but it was almost ineffective against the [^{3}H]GABA release elicited by depolarization of spinal cord synaptosomes, suggesting that cortical and spinal cord $GABA_B$ autoreceptors might represent different receptor subtypes (Raiteri et al., 1989). This idea was strengthened by the subsequent finding that spinal cord and cortical $GABA_B$ autoreceptors could be differentially blocked by selective $GABA_B$ receptor antagonists (Bonanno and Raiteri, 1993a).

Excessive activation of glutamate transmission is known to play an important role in neurodegenerative diseases. In the spinal cord of patients with amyotrophic lateral sclerosis a selective decrease of the glial glutamate transporter GLT-1 is thought to lead to an increase in extracellular glutamate concentration with consequent excitotoxic degeneration of motor neurons (Rothstein et al., 1990, 1992; Krieger et al., 1996; Rothstein, 1996). Enhancement of GABA release and/or reduction of glutamate release would probably limit the effects of excessive glutamatergic excitation.

If pharmacologically distinct $GABA_B$ autoreceptors exist on cortical and spinal cord GABAergic axon terminals,

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0014-2999/98/$ - see front matter
PII: S0014-2999(98)00759-6

antagonists at the spinal autoreceptor could selectively enhance the release of GABA in the spinal cord. Furthermore, as release-inhibiting $GABA_B$ heteroreceptors are present on glutamatergic nerve endings of the spinal cord (see, for references, Malcangio and Bowery, 1996), this antagonist-induced GABA release might in turn inhibit the release of glutamate, provided that the antagonist does not concomitantly block $GABA_B$ heteroreceptors on glutamatergic terminals. Interestingly, previous results obtained with several selective $GABA_B$ receptor antagonists indicate that, in the cerebral cortex, $GABA_B$ autoreceptors differ pharmacologically from the $GABA_B$ heteroreceptors regulating glutamate release, both in rats (Bonanno and Raiteri, 1992, 1993b) and humans (Bonanno et al., 1997).

The aim of the present work was to establish whether, in the spinal cord, $GABA_B$ autoreceptors and $GABA_B$ heteroreceptors regulating GABA and glutamate release, respectively, also exhibit pharmacological diversity.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (200–250 g) were used. Animals were housed at constant temperature (22 ± 1°C) and relative humidity (50%) under a regular light–dark schedule (light: 7 a.m.–7 p.m.). Food and water were freely available.

2.2. Preparation of synaptosomes

Rats were killed by decapitation and the spinal cord was rapidly dissected. Synaptosomes were prepared as described previously (Raiteri et al., 1984). Briefly, the whole spinal cord was homogenized in 40 volumes of 0.32 M sucrose, buffered at pH 7.4 with phosphate, using a glass-teflon tissue grinder (clearance 0.25 mm). The homogenate was centrifuged (5 min, $1000 \times g$ at 0–4°C) to remove nuclei and debris and synaptosomes were isolated from the supernatant by centrifugation at $12{,}000 \times g$ for 20 min. The synaptosomal pellet was then resuspended in a physiological medium of the following composition (mM): NaCl, 125; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1; $NaHCO_3$, 22; glucose, 10 (aerated with 95% O_2 and 5% CO_2); pH 7.2–7.4. Protein was measured according to Bradford (1976), using bovine serum albumin as a standard.

2.3. Release experiments

Identical aliquots of the synaptosomal suspensions (about 0.5 mg protein) were distributed onto 0.65-μm Millipore filters placed at the bottom of a set of parallel superfusion chambers maintained at 37°C (Raiteri et al., 1974) and layered under moderate vacuum filtration. Superfusion was then started (flow rate 0.5 ml/min) with standard medium aerated with 95% O_2 and 5% CO_2. After 36 min to equilibrate the system, fractions were collected according to the following scheme: two 3-min samples (basal release), one before and one after a 6-min sample representing the evoked release. A 90-s period of depolarization with 15 mM KCl was applied after collection of the first 3-min basal sample; KCl replaced an equimolar concentration of NaCl. (−)-Baclofen, 3-aminopropylphosphonous acid (3-APPA) or (3-amino-2(*S*)-hydroxypropyl) methylphosphinic acid (CGP 44532) was added to the superfusion medium concomitantly with the depolarizing stimulus. The $GABA_B$ receptor antagonists, 3-aminopropyl(diethoxymethyl)phosphinic acid (CGP 35348) and [3-[[(3,4-dichlorophenyl)methyl]amino] propyl](diethoxymethyl)phosphinic acid (CGP 52432), were added 8 min before 3-APPA. *N*-[4,4-diphenyl-3-butenyl]-guvacine (SK&F 100330A), a selective GABA uptake inhibitor (Yunger et al., 1984), was present in the superfusion medium at a final concentration of 30 μM to minimize carrier-mediated exchange of endogenous GABA with 3-APPA. When appropriate, calcium was omitted 18 min before the depolarizing stimulus. Fractions collected were analyzed for their endogenous amino acid content.

2.4. Glutamate and GABA determination

Endogenous GABA and glutamate were determined by high performance liquid chromatography (HPLC) analysis following automatic derivatization (Waters 715 Ultrawisp; Milford, MA, USA) with *o*-phthalaldehyde and separation on a C_{18} reverse-phase chromatographic column (Chrompack, Middleburg, The Netherlands; 10×4.6 mm, 3 μm; 30°C) coupled with fluorimetric detection (Shimadzu, Tokyo, Japan; RF551, excitation wavelength 350 nm; emission wavelength 450 nm). Buffers and the gradient program were as follows: solvent A, 0.1 M sodium acetate (pH 5.8)/methanol, 80: 20; solvent B, 0.1 M sodium acetate (pH 5.8)/methanol, 20: 80; solvent C, 0.1 M sodium acetate (pH 6.0)/methanol 80: 20; gradient program, 100% C for 4 min from the initiation of the program; 90% A and 10% B in 1 min; isocratic step for 2 min, 78% A and 22% B in 2 min; isocratic step for 6.50 min; 66% A and 34% B in 1.10 min; isocratic step for 1.50 min; 42% A and 58% B in 1.10 min; isocratic step for 3.5 min; flow rate 0.9 ml/min (Waters 600 MS gradient system). Homoserine was used as internal standard.

2.5. Calculations

The amounts of endogenous GABA or glutamate measured in the superfusate fractions are expressed as pmol/mg protein/6 min. The KCl-evoked overflow was

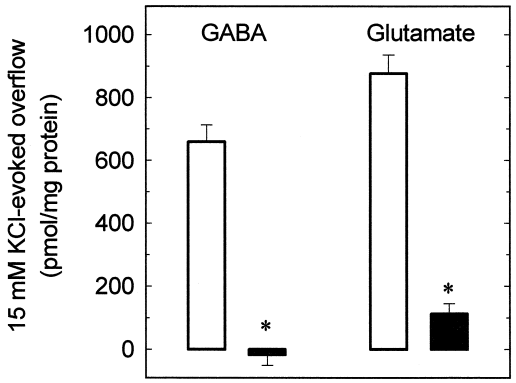

Fig. 1. Overflow of endogenous GABA and glutamate evoked by depolarization with 15 mM KCl from rat spinal cord synaptosomes in standard (open bars) or Ca^{2+}-free (solid bars) medium. The depolarization pulse (90 s) was applied after collection of the first fraction. The Ca^{2+}-free medium was introduced 18 min earlier. See Section 2 for other technical details. The basal release in the two 3-min fractions collected before and after the evoked release amounted to 321 ± 27.3 pmol/mg protein/6 min ($n = 3$) and 605 ± 43.1 pmol/mg protein/6 min ($n = 3$) for GABA and glutamate, respectively. Means ± S.E.M. of three separate experiments in triplicate are shown. $* P < 0.001$ when compared to the respective overflow in standard medium.

estimated by subtracting the amount of neurotransmitter in the 6-min samples representing the basal release from the neurotransmitter content in the 6-min fraction collected during and after the depolarization pulse. The effects of drugs were evaluated as the ratio of the depolarization-evoked overflow calculated in the presence of drugs vs. that obtained under control conditions and expressed as percent inhibition of neurotransmitter overflow. Appropriate controls with antagonists alone were always run in parallel.

EC_{50} values were determined by analyzing the experimental data with the following four parametric logistic equation, using a function-fitting routine provided by the software Sigma Plot version 3.0:

$$y = a + \left((b - a) / \left(1 + \left(10^{c} / 10^{x} \right)^{d} \right) \right)$$

where a was the minimum and b the maximum value of the data; c the EC_{50}; d the slope of the curve.

The two-tailed Student's t-test was used for statistical comparison of the data.

2.6. Drugs

3-APPA was purchased from Tocris Cookson (Bristol, UK). The following drugs were gifts from the Companies indicated: SK&F 100330A (Smith Kline and French, Welwyn, UK); (−)-baclofen, 3-APPA, CGP 44532, CGP 52432 and CGP 35348 (Novartis, Basel, Switzerland). Stock solutions (10 mM) were prepared in water except in the case of SK&F 100330A, which was first dissolved in methanol before it was diluted (the concentration of methanol in the 10 mM stock solution was 0.5%).

3. Results

Depolarization of isolated spinal cord nerve terminals with 15 mM KCl caused the overflow of endogenous GABA and glutamic acid. The overflow of both amino acids was abolished when Ca^{2+} ions were omitted from the superfusion medium (Fig. 1).

When the selective $GABA_B$ receptor agonist (−)-baclofen was added in various concentrations (0.003–30 μM), the K^+-evoked overflow of glutamate was inhibited in a concentration-dependent manner ($EC_{50} = 0.098$ μM; maximal effect ≃ 50%; Fig. 2). In contrast, the K^+-evoked overflow of endogenous GABA was poorly sensitive to (−)-baclofen, which caused a not significant 10% inhibition when added to the superfusion medium at 30 μM (Fig. 2).

The $GABA_B$ receptor agonist 3-APPA, added at 0.003–3 μM, concentration dependently inhibited both the K^+-evoked overflow of glutamate and that of GABA (Fig. 3). The efficacy and apparent affinity of 3-APPA were similar in the two release systems ($EC_{50} = 0.087$ μM for GABA and 0.050 μM for glutamate overflow; maximal inhibition ≃ 50%).

The K^+-evoked overflow of glutamate and GABA was also inhibited by the $GABA_B$ receptor agonist CGP 44532 (Froestl et al., 1995). As illustrated in Fig. 4, the concentration-inhibition curves for glutamate and GABA release

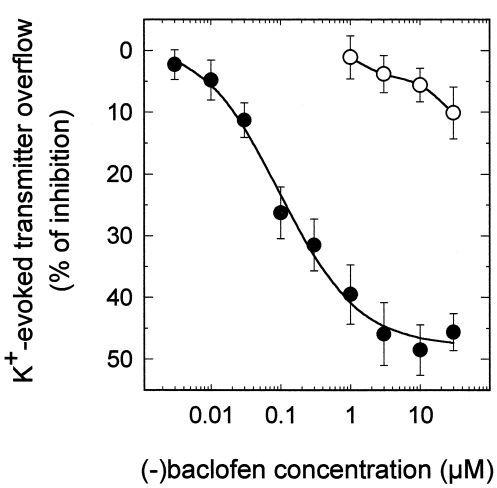

Fig. 2. Effects of (−)-baclofen on the K^+ (15 mM)-evoked release of endogenous GABA or glutamate from rat spinal cord synaptosomes. Synaptosomes were exposed to (−)-baclofen concomitantly with the depolarizing stimulus. See Section 2 for other technical details. The data are means ± S.E.M. of 4–10 separate experiments in triplicate. Open circles: effects of (−)-baclofen on GABA overflow; solid circles: effects of (−)-baclofen on glutamate overflow.

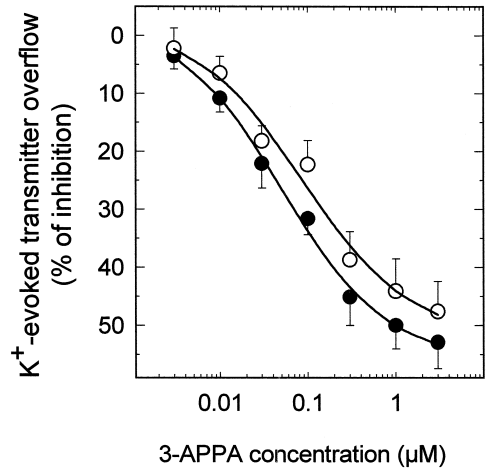

Fig. 3. Effects of 3-APPA on the K^+ (15 mM)-evoked release of endogenous GABA or glutamate from rat spinal cord synaptosomes. Synaptosomes were exposed to 3-APPA concomitantly with the depolarizing stimulus. See Section 2 for other technical details. The data are means ± S.E.M. of 3–20 separate experiments in triplicate. Open circles: effects of 3-APPA on GABA overflow; solid circles: effects of 3-APPA on glutamate overflow.

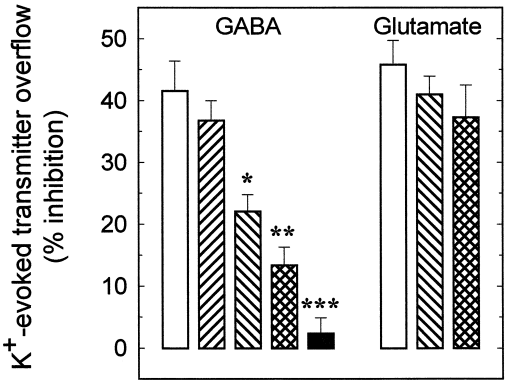

Fig. 5. Effect of CGP 35348 on the 3-APPA (1 μM)-induced inhibition of endogenous GABA or glutamate overflow from rat spinal cord synaptosomes depolarized in superfusion by a 90-s pulse of 15 mM KCl. Synaptosomes were exposed to 3-APPA concomitantly with the depolarizing pulse and to CGP 35348 8 min before the pulse of 15 mM KCl. See Section 2 for other technical details. The data are means ± S.E.M. of 3–9 experiments in triplicate. Open bars: 1 μM 3-APPA; right-hatched bars: 1 μM 3-APPA + 0.1 μM CGP 35348; left-hatched bars: 1 μM 3-APPA + 1 μM CGP 35348; cross-hatched bars: 1 μM 3-APPA + 10 μM CGP 35348; solid bars: 1 μM 3-APPA + 100 μM CGP 35348. $* P < 0.05$; $** P < 0.005$ and $*** P < 0.001$ when compared to the effect of 1 μM 3-APPA alone on GABA overflow.

were superimposable. CGP 44532 appeared to act as a full agonist, like 3-APPA and (−)-baclofen (on glutamate release); it was however about 10-fold less potent than 3-APPA as inhibitor of GABA and glutamate release (EC_{50} = 0.81 and 0.50, respectively).

The effect of 1 μM 3-APPA on the release of GABA was concentration dependently blocked by CGP 35348, a selective $GABA_B$ receptor antagonist (Fig. 5). Added at 1 μM, CGP 35348 halved the effect of 1 μM 3-APPA on the release of GABA. In contrast, CGP 35348, up to 10 μM, was unable to prevent the action of 3-APPA at the $GABA_B$ heteroreceptor regulating the release of glutamate from spinal cord axon terminals. When added at 100 μM, CGP 35348 inhibited, on its own, the K^+-evoked overflow of glutamate (not shown).

Fig. 6 shows that the selective $GABA_B$ receptor antagonist CGP 52432 prevented in a concentration-dependent

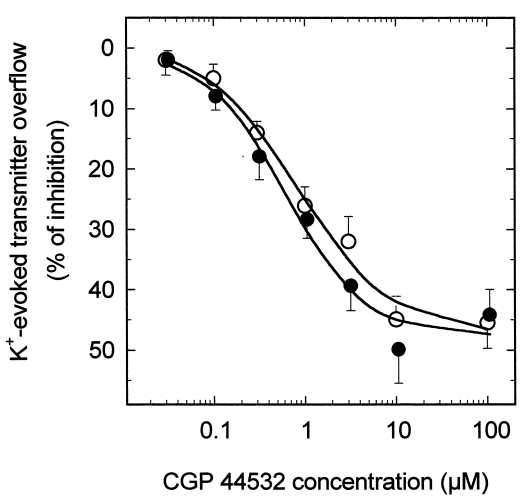

Fig. 4. Effects of CGP 44532 on the K^+ (15 mM)-evoked release of endogenous GABA or glutamate from rat spinal cord synaptosomes. Synaptosomes were exposed to CGP 44532 concomitantly with the depolarizing stimulus. See Section 2 for other technical details. The data are means ± S.E.M. of 4–8 separate experiments in triplicate. Open circles: effects of CGP 44532 on GABA overflow; solid circles: effects of CGP 44532 on glutamate overflow.

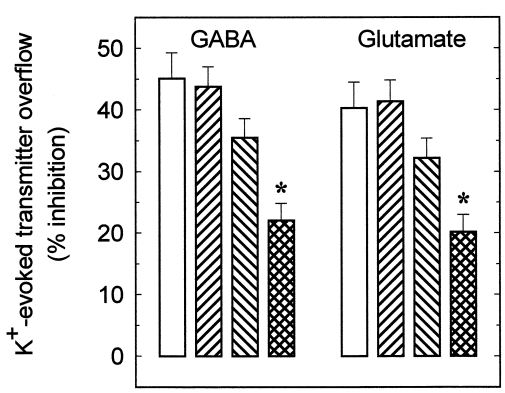

Fig. 6. Effect of CGP 52432 on the 3-APPA (1 μM)-induced inhibition of endogenous GABA or glutamate overflow from rat spinal cord synaptosomes depolarized in superfusion with a 90-s pulse of 15 mM KCl. Synaptosomes were exposed to 3-APPA concomitantly with the depolarizing pulse and to CGP 52432 8 min before the pulse of 15 mM KCl. See Section 2 for other technical details. The data are means ± S.E.M. of 4–8 experiments in triplicate. Open bars: 1 μM 3-APPA; right-hatched bars: 1 μM 3-APPA + 0.1 μM CGP 52432; left-hatched bars: 1 μM 3-APPA + 1 μM CGP 52432; cross-hatched bars: 1 μM 3-APPA + 10 μM CGP 52432. $* P < 0.01$ when compared to the respective control overflow.

manner and to the same extent the inhibition of the evoked overflow of GABA and glutamate caused by 1 μM 3-APPA. The effect of 3-APPA on the release of both amino acids was almost halved by 10 μM CGP 52432.

4. Discussion

The release of endogenous GABA and glutamate from spinal cord synaptosomes evoked by a relatively mild K^+ depolarization (15 mM) was totally dependent on the presence of external Ca^{2+} and is therefore compatible with exocytotic release from nerve terminals.

Monitoring neurotransmitter release by a technique in which thin layers of synaptosomes, immobilized on microporous filters, are up–down superfused under conditions which minimize indirect effects makes it likely that the K^+-evoked overflow of GABA and glutamate originates directly from the corresponding axon terminals. By the same token, the overflow modulation produced by the $GABA_B$ receptor agonists added to the superfusion medium occurred through the activation of $GABA_B$ receptors that are probably localized on GABAergic (autoreceptors) and glutamatergic (heteroreceptors) nerve endings, respectively.

In previous studies GABA autoreceptors in rat spinal cord were studied by monitoring the synaptosomal release of preaccumulated [^{3}H]GABA in the presence of 50 μM of the GABA transaminase inhibitor aminooxyacetic acid (Raiteri et al., 1989; Bonanno and Raiteri, 1993a). These studies yielded a very surprising result: although the K^+-evoked release of [^{3}H]GABA in the spinal cord was potently inhibited by GABA ($EC_{50} = 1.01$ μM) and by the $GABA_B$ receptor agonist 3-APPA ($EC_{50} = 0.078$ μM), as was the release in the cerebral cortex, the release of [^{3}H]GABA in the spinal cord was very poorly sensitive to (−)-baclofen ($EC_{50} = 425$ μM) which, on the other hand, inhibited potently ($EC_{50} = 1.37$ μM) the release in the neocortex. The idea that the 'baclofen-insensitive' autoreceptor in the spinal cord could represent a $GABA_B$ receptor subtype different from the cortical autoreceptor was subsequently strengthened by results obtained with the $GABA_B$ receptor antagonists phaclofen and CGP 35348: phaclofen blocked the cortical but not the spinal autoreceptor, whereas the opposite was true for CGP 35348 (Bonanno and Raiteri, 1993a).

The above data for 3-APPA and the $GABA_B$ receptor antagonist CGP 35348 confirmed that GABAergic nerve terminals in the spinal cord possess $GABA_B$ autoreceptors; however the inability of (−)-baclofen, the prototype $GABA_B$ receptor agonist, to activate these receptors remained quite puzzling. Considering that preaccumulated [^{3}H]GABA and endogenous GABA may respond differently to releasing stimuli (see Szerb, 1983) and to release-modulating agents (Pittaluga and Raiteri, 1987), it was important to ascertain if, in the spinal cord, the release of endogenous GABA also was insensitive to (−)-baclofen. The results shown in Fig. 2 confirm that GABA-releasing axon terminals in the spinal cord possess $GABA_B$ autoreceptors that are (−)-baclofen-insensitive. It should be noted that, in the same synaptosomal preparation, the release of glutamate was strongly inhibited by (−)-baclofen.

These data for (−)-baclofen raised the possibility that, in the spinal cord, $GABA_B$ autoreceptors might differ pharmacologically from the $GABA_B$ heteroreceptors regulating glutamate release. To strengthen this view, other $GABA_B$ receptor ligands, receptor antagonists in particular, had to be tested.

The potent $GABA_B$ receptor agonist 3-APPA did not reveal differences between auto- and heteroreceptors because it inhibited the K^+-evoked release of endogenous GABA and glutamate from the same preparations of spinal cord synaptosomes with concentration-response curves that were almost superimposable. Another selective agonist at $GABA_B$ receptors, CGP 44532, behaved as a full agonist and exhibited the same potency at autoreceptors and heteroreceptors ($EC_{50} = 0.81$ and 0.50 μM, respectively). This compound has been found to inhibit the electrically induced release of [^{3}H]GABA from rat neocortex slices where it was about equipotent to (−)-baclofen (EC_{50} = 0.40 vs. 0.33 μM; Froestl et al., 1995). In a comparative study with baclofen, CGP 44532 produced greater muscle relaxation than baclofen, without the sedation, reduced vigilance or gastrointestinal effects sometimes observed with baclofen (Froestl et al., 1995).

The $GABA_B$ receptor antagonist CGP 35348 was clearly able to discriminate between $GABA_B$ autoreceptors and heteroreceptors in the spinal cord. The compound could prevent almost completely the effect of 3-APPA on the K^+-evoked release of endogenous GABA at a concentration (10 μM) that was ineffective on the release of glutamate. In a study with rat dorsal horn slices, Teoh et al. (1996) observed that the $GABA_B$ receptor antagonist CGP 56999A, though not other $GABA_B$ receptor antagonists, affected only GABA release from dorsal horn interneurons, but not glutamate released from primary sensory afferent terminals. The finding of these authors that (−)-baclofen could inhibit both GABA and glutamate release is in apparent contrast with the present data and can be explained by the different experimental conditions used (dorsal horn slices vs. spinal cord synaptosomes). According to Teoh et al. (1996), electrical stimulation of dorsal roots elicits the direct release of glutamate from primary afferents onto GABA interneurons which, in turn, release GABA. Thus inhibition by (−)-baclofen of GABA release in dorsal horn slices may simply reflect inhibition of glutamate release. Accordingly, antagonists selective for $GABA_B$ autoreceptors (like CGP 56999A) would disinhibit the stimulated release of GABA while increasing the inhibition of stimulated glutamate release through GABAergic recurrents, whereas antagonists selective for

$GABA_B$ heteroreceptors on glutamate terminals would augment the stimulated release of both amino acids. In superfused synaptosomes, where GABA and glutamate should not interfere with each other's release (see above), the different sensitivity to (−)-baclofen of autoreceptors vs. heteroreceptors could be clearly detected.

The data obtained with CGP 52432 deserve some comment. This $GABA_B$ receptor antagonist is reported to be a potent and selective $GABA_B$ autoreceptor antagonist in rat cerebral cortex (Lanza et al., 1993). Indeed, CGP 52432 blocked the (−)-baclofen inhibition of GABA release from cerebrocortex synaptosomes with an $IC_{50} = 0.085$ μM, whereas it was 100-fold less potent at cortical $GABA_B$ heteroreceptors regulating glutamate release ($IC_{50} = 9.26$ μM). As shown in Fig. 6, CGP 52432 was much weaker at the spinal ($IC_{50} \simeq 10$ μM) than at the cortical autoreceptors, while its potency at the heteroreceptors regulating glutamate release was identical in cortex and spinal cord.

To conclude, spinal cord nerve terminals that release GABA and glutamate during depolarization in the presence of Ca^{2+} ions possess release-inhibiting $GABA_B$ autoreceptors and heteroreceptors that exhibit a different pharmacology. The autoreceptors are baclofen-insensitive $GABA_B$ receptors that can be blocked by CGP 35348; the heteroreceptors are potently activated by (−)-baclofen but appear to be insensitive to CGP 35348. The results of the present study suggest that the pharmacological differences existing between $GABA_B$ autoreceptors and heteroreceptors in the spinal cord, together with those between telencephalic and spinal cord receptors (see Section 1), could allow for the development of drugs potentially useful in neurodegenerative diseases, like amyotrophic lateral sclerosis, which are characterized by excessive glutamatergic excitatory transmission.

Acknowledgements

The financial support of Telethon-Italy (Grant Number 869) is gratefully acknowledged. The authors wish to thank Mrs. Maura Agate for editorial assistance.

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