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Pharmacologically distinct $GABA_B$ receptors that mediate inhibition of GABA and glutamate release in human neocortex

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1 The release of endogenous γ-aminobutyric acid (GABA) and glutamic acid in the human brain has been investigated in synaptosomal preparations from fresh neocortical samples obtained from patients undergoing neurosurgery to reach deeply located tumours.

2 The basal outflows of GABA and glutamate from superfused synaptosomes were largely increased during depolarization with 15 mM KCl. The K^+-evoked overflows of both amino acids were almost totally dependent on the presence of Ca^{2+} in the superfusion medium.

3 The $GABA_B$ receptor agonist (−)-baclofen (1, 3 or 10 μM) inhibited the overflows of GABA and glutamate in a concentration-dependent manner. The inhibition caused by 10 μM of the agonist ranged from 45–50%.

4 The effect of three selective $GABA_B$ receptor antagonists on the inhibition of the K^+-evoked GABA and glutamate overflows elicited by 10 μM (−)-baclofen was investigated. Phaclofen antagonized (by about 50% at 100 μM; almost totally at 300 μM) the effect of (−)-baclofen on GABA overflow but did not modify the inhibition of glutamate release. The effect of (−)-baclofen on the K^+-evoked GABA overflow was unaffected by 3-amino-propyl (diethoxymethyl)phosphinic acid (CGP 35348; 10 or 100 μM); however, CGP 35348 (10 or 100 μM) antagonized (−)-baclofen (complete blockade at 100 μM) at the heteroreceptors on glutamatergic terminals. Finally, [3-[[(3,4-dichlorophenyl) methyl]amino]propyl] (diethoxymethyl) phosphinic aid (CGP 52432), 1 μM, blocked the $GABA_B$ autoreceptor, but was ineffective at the heteroreceptors. The selectivity of CGP 52432 was lost at 30 μM, as the compound, at this concentration, inhibited completely the (−)-baclofen effect both on GABA and glutamate release.

5 It is concluded that GABA and glutamate release evoked by depolarization of human neocortex nerve terminals can be affected differentially through pharmacologically distinct $GABA_B$ receptors.

Keywords: Human neocortex; $GABA_B$ receptor subtypes; GABA release; glutamate release; phaclofen; CGP 35348; CGP 52432

Introduction

Several studies have suggested that, in the rat brain, $GABA_B$ receptors exist as subtypes having distinct neuronal locations, functions and pharmacological properties (see, for reviews: Bonanno & Raiteri, 1993; Bowery, 1993; Mott & Lewis, 1994). $GABA_B$ receptors are often located presynaptically on axon terminals; accordingly, it is believed that one major function of $GABA_B$ receptors is to modulate, actually to inhibit, neurotransmitter release (Bowery *et al*., 1980; Bonanno & Raiteri, 1993; Bowery, 1993; Mott & Lewis, 1994). Due to these very properties, release from synaptosomes represents an appropriate model to study $GABA_B$ receptors. From previous data, comparing different selective antagonists at receptors of a given type that regulate release of different transmitters from superfused synaptosomes, it appears that this is a powerful strategy in the identification of native receptor subtypes (Raiteri *et al*., 1984; 1986; Gobbi *et al*., 1990; Bonanno & Raiteri, 1992; 1993; Clarke & Reuben, 1996; Schmid *et al*., 1996).

In rats, data from experiments with superfused cerebrocortical synaptosomes have suggested that the $GABA_B$ autoreceptors sited on γ-aminobutyric acid (GABA)-releasing terminals differ pharmacologically from the $GABA_B$ heteroreceptors sited on glutamate-releasing terminals (Bonanno & Raiteri, 1992). Such a difference was not, however, distinguishable in a cortical slice preparation where $GABA_B$ receptor antagonists affected similarly the release of [^{3}H]-GABA and of glutamate evoked by electrical stimulation (Waldmeier *et al*., 1994). On the other hand, Teoh *et al*. (1996) found that the $GABA_B$ receptor antagonist [3[[1-(**R**)-(3-carboxyphenyl)ethyl]amino] 2-(**S**)-hydroxy-propyl]-cyclohexylmethyl-phosphinic acid (CGP 56999A) antagonized the (−)-baclofen-induced inhibition of GABA release from rat spinal cord slices, whilst not affecting the release of glutamate. Furthermore, it was found that the effects of (−)-baclofen on the release of GABA and glutamic acid monitored during *in vivo* microdialysis of rat thalamic ventrobasal nucleus were differentially affected by $GABA_B$ receptor antagonists indicating $GABA_B$ receptor heterogeneity (Banerjee & Snead, 1995).

Although pharmacological differences between $GABA_B$ receptors regulating GABA and glutamate release seem therefore likely in the central nervous system (CNS) of the rat, no information exists in the literature about heterogeneity of $GABA_B$ receptors in the human brain. In fact, while GABA autoreceptors have been identified and characterized as the $GABA_B$ type in human neocortex (Bonanno *et al*., 1989; Fassio *et al*., 1994), very little is known about glutamate release in the human CNS (Smith *et al*., 1983) and the modulation of this release by GABA. Since there is increasing evidence for interspecies differences between receptors involved in the same function, we have compared the pharmacology of the $GABA_B$ receptors regulating, respectively, the release of GABA and glutamate in human brain by using synaptosomes from fresh specimens of cerebral cortex.

Methods

Human samples

Human cerebral cortex specimens were obtained from 8 female and 7 male patients (aged 45–65 years) undergoing neuro-

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surgery to reach deeply located tumours. The samples represented parts of frontal (5), temporal (4) and parietal (6) lobes. The tissues were obtained on different days and processed on the day of surgery. After premedication with atropine and meperidine, anaesthesia was induced with sodium pentobarbitone (Pentothal) and maintained with 70% nitrous oxide in 30% oxygen and 0.5–1% isoflurane. Pancuronium was employed to obtain muscular relaxation.

Synaptosome preparation

Immediately after removal, tissues were placed in a physiological salt solution (see below) kept at 0–4°C and synaptosomal preparations were obtained within 60–90 min. Tissue homogenization was conducted 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, 12 up-down strokes in about 1 min, 900 r.p.m.). The homogenate was first centrifuged at $1000 \times g$ for 5 min; synaptosomes were isolated from the supernatant by centrifugation at $12000 \times g$ for 20 min. All the preceding procedures were performed at 0–4°C. The synaptosomal pellet was then resuspended in a physiological medium having the following composition (in mM): NaCl 125, KCl 3, $MgSO_4$ 1.2, $CaCl_2$ 1.2, NaH_2PO_4 1.0, $NaHCO_3$ 22, glucose 10, aerated with 95% O_2 and 5% CO_2; pH 7.2–7.4. Protein was determined according to Bradford (1976).

Release experiments

Identical aliquots of the synaptosomal suspensions (0.4–0.6 mg of protein in the different experiments) were distributed on microporous filters placed at the bottom of 20 parallel superfusion chambers maintained at 37°C (Raiteri *et al.*, 1974). Superfusion was then started with standard medium at a rate of 0.5 ml min^{-1} and continued for a total of 48 min. After 36 min to equilibrate the system, fractions were collected according to the following scheme: two 3-min fractions (basal release) before and after one 6-min fraction (evoked release). A 90-s period of depolarization was applied after the first fraction had been collected. KCl (15 mM) was used to depolarize synaptosomes, NaCl substituting for an equimolar concentration of KCl. This time schedule derived from pilot experiments in which several 1-min fractions were collected and the time-course of amino acid release was monitored (see Figure 1). Synaptosomes were exposed to (−)-baclofen at the end of the first fraction collected. Phaclofen, 3-amino-propyl (diethoxymethyl)phosphinic acid (CGP 35348) or [3-[[(3,4-dichlorophenyl) methyl]amino]propyl] (diethoxymethyl) phosphinic acid (CGP 52432) was added to the superfusion medium 8 min before (−)-baclofen. The Ca^{2+}-free medium was introduced 18 min before K^+-depolarization. Samples collected were analysed for their endogenous transmitter content.

Amino acid determination

Endogenous glutamate or GABA was measured by high performance liquid chromatography (h.p.l.c.) analysis after pre-column derivatization with *o*-phthalaldehyde and fluorimetric detection. Amino acid resolution was obtained with a C_{18} reverse phase chromatography column (Chrompack, 10 cm × 4.6 mm, 3 μm) and a three-solvent discontinuous gradient, from 23% methanol in acetate buffer 0.1 M pH 6 to 46% methanol in acetate buffer 0.1 M pH 5.8 in 22 min at a flow rate of 0.9 ml min^{-1}.

Calculations

The amount of endogenous GABA or glutamic acid released in each fraction collected was expressed as pmol mg^{-1} synaptosomal protein. The depolarization-evoked overflow was estimated by subtracting the basal release, represented by the transmitter content in the two 3-min fraction collected, from the evoked release, represented by the 6-min fraction collected during and after the depolarization period. Drug effects were calculated as the ratio of the depolarization-evoked overflow calculated in the presence of the drugs versus that calculated under control conditions. Data presented in Table 1 and Figure 3 were compared by two tailed Student's *t* test. Statistical differences from control data in Figure 2 were tested by paired Student's *t* test. One-way ANOVA was used to analyse the concentration-response effects presented in Figure 2.

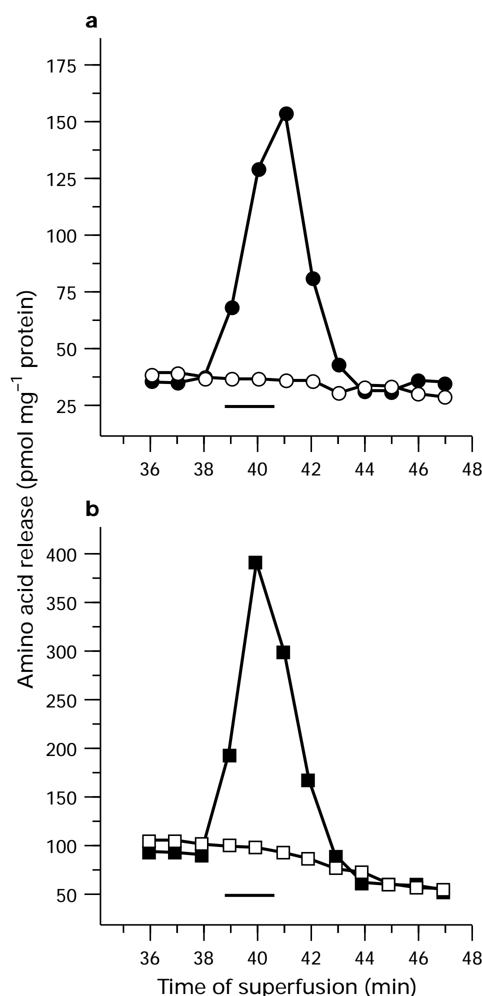

Figure 1 Time-course of the release of the endogenous (a) GABA or (b) glutamate from human brain cortex synaptosomes. One-min fractions were collected starting after 36 min of superfusion and a 90 s period of depolarization (15 mM K^+) was applied after 39 min (see horizontal bar). Points represent the mean of two experiments run in triplicate. Closed symbols: K^+-evoked release; open symbols: basal release (no depolarization applied).

Drugs

Phaclofen was obtained from Tocris Neuramin (Bristol, UK). (−)-Baclofen, 3-amino-propyl (diethoxymethyl)phosphinic acid (CGP 35348) and [3-[[(3,4-dichlorophenyl) methyl]amino]propyl] (diethoxymethyl) phosphinic acid (CGP 52432) were gifts from Ciba Geigy (Basel, Switzerland).

Results

Ca^{2+}-dependence of GABA and glutamate release

Human neocortical synaptosomes were depolarized by superfusion with 15 mM KCl; endogenous GABA and glutamate

Table 1 Calcium dependence of the release of endogenous GABA and glutamate from human brain cortex synaptosomes

Amino acid	*Basal release*[a] *1.2 mM Ca^{2+}*	*Ca^{2+}-free*	*K$^+$ (15 mM)-evoked release*[b] *1.2 mM Ca^{2+}*	*Ca^{2+}-free*
GABA	51±3.8 (3)	32±4.4* (3)	321±24 (3)	25±2.7** (3)
Glutamate	108±12 (3)	92±10 (3)	575±58 (3)	79±6.5** (3)

Values are means±s.e.mean of 3 experiments (3 different brain samples) in triplicate. [a]Measured as pmol mg^{-1} protein min^{-1}. [b]Measured as pmol mg^{-1} protein. *$P<0.05$; **$P<0.001$ when compared to the respective controls (two-tailed Student's t test).

were concomitantly measured in the superfusate samples by h.p.l.c. In the experiments presented throughout the work, the amino acid released in the 3-min fraction collected before the onset of K$^+$-stimulation (see Methods) amounted to 141±9.1, ranging from 60 to 202, (GABA) and to 273±43, ranging from 91 to 574, (glutamate) pmol mg^{-1} protein ($n=15$). The K$^+$ (15 mM)-evoked overflows were 378±24, ranging from 85 to 450, (GABA) and 540±48, ranging from 131 to 625, (glutamic acid) pmol mg^{-1} protein ($n=15$). In a set of experiments, the amino acid content of synaptosomes was measured at the end of superfusion: the synaptosomes contained 5.5±0.65 nmol mg^{-1} protein ($n=4$) of GABA and 21.0±2.3 nmol mg^{-1} protein ($n=4$) of glutamic acid.

The K$^+$-evoked overflow of both amino acids was strictly dependent on the presence of Ca^{2+} ions in the superfusion medium. The Ca^{2+}-dependency of the depolarization-evoked overflow of glutamic acid appeared slightly less pronounced than that of GABA. The basal outflow of GABA, but not that of glutamate, was significantly dependent on extracellular Ca^{2+} (Table 1).

Sensitivity to (−)-baclofen of the K$^+$-evoked GABA and glutamate overflows

Addition to the superfusion medium of (−)-baclofen (1, 3 or 10 μM), concomitantly with the high-K$^+$ pulse, significantly inhibited the depolarization-evoked overflow of both GABA and glutamate in a concentration-dependent manner (Figure 2). The patterns of inhibition for the two amino acids were similar to each other. Previous experiments with rat brain neocortex synaptosomes showed that the inhibition by (−)-baclofen of endogenous GABA and glutamate overflows was maximal when (−)-baclofen was added at 10 μM (Bonanno & Raiteri, 1992).

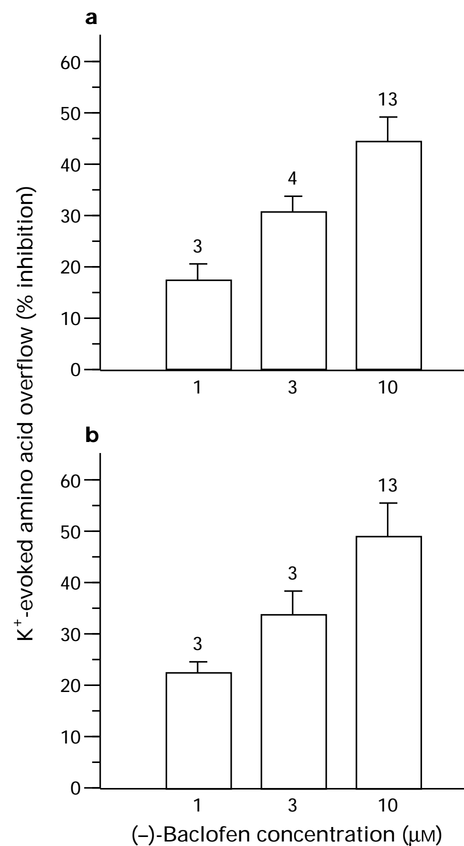

Figure 2 Inhibition by (−)-baclofen of the depolarization-evoked overflow of endogenous (a) GABA or (b) glutamate from human brain cortex synaptosomes. Data are expressed as % inhibition of the K$^+$ (15 mM)-evoked overflow. Synaptosomes were exposed to (−)-baclofen concomitantly with the depolarizing stimulus. Each column represents the mean±s.e.mean (vertical lines) of the number of experiments (performed in triplicate) indicated above each column. Statistical significance was tested by one-way ANOVA: $P<0.01$.

Differential antagonism of the effects of (−)-baclofen

Phaclofen antagonized the inhibition by 10 μM (−)-baclofen of the GABA overflow (Figure 3). The (−)-baclofen effect was halved by 100 μM phaclofen and almost abolished by 300 μM of this antagonist. In contrast, the effect of (−)-baclofen on the K$^+$-evoked overflow of glutamate could not be affected by phaclofen, up to 300 μM. The GABA$_B$ receptor antagonist CGP 35348 displayed the opposite behaviour. It was ineffective against (−)-baclofen at the GABA$_B$ autoreceptor regulating GABA release, but it reduced (at 10 μM) and abolished (at 100 μM) the effect of (−)-baclofen at the heteroreceptors on glutamatergic terminals. Figure 3 also shows that the recently introduced antagonist CGP 52432, added at 1 μM, blocked the effect of (−)-baclofen at the autoreceptors while leaving unaffected the inhibition of glutamate overflow. At 30 μM CGP 52432 appeared to lose its selectivity for the GABAergic system. All the antagonists, at the concentrations used, did not modify by themselves the depolarization-evoked amino acid overflow.

Discussion

As mentioned in the Introduction, GABA$_B$ autoreceptors in human brain had previously been investigated in this laboratory by monitoring release of radiolabelled GABA previously taken up into neocortex synaptosomes (Bonanno *et al.*, 1989; Fassio *et al.*, 1994). Since endogenously synthesized GABA can respond to releasing stimuli differently from GABA originating through reuptake (Szerb *et al.*, 1981; Szerb, 1984), endogenous GABA release has been measured in the present experiments. Moreover, the aim of this work was to compare some of the properties of GABA and glutamate release in the human brain; therefore, we thought that monitoring release of the two endogenous amino acids from the same synaptosomal samples would represent the most appropriate experimental approach.

When exposed to relatively mild depolarizing conditions (15 mM K$^+$), human neocortical synaptosomes released endogenous GABA and glutamate in a manner that was strictly dependent on the presence of Ca^{2+} ions in the superfusion medium, suggesting that the evoked release of GABA and glutamic acid is of neuronal origin.

The GABA$_B$ receptor agonist (−)-baclofen inhibited similarly the K$^+$-evoked overflows of GABA and glutamate. The

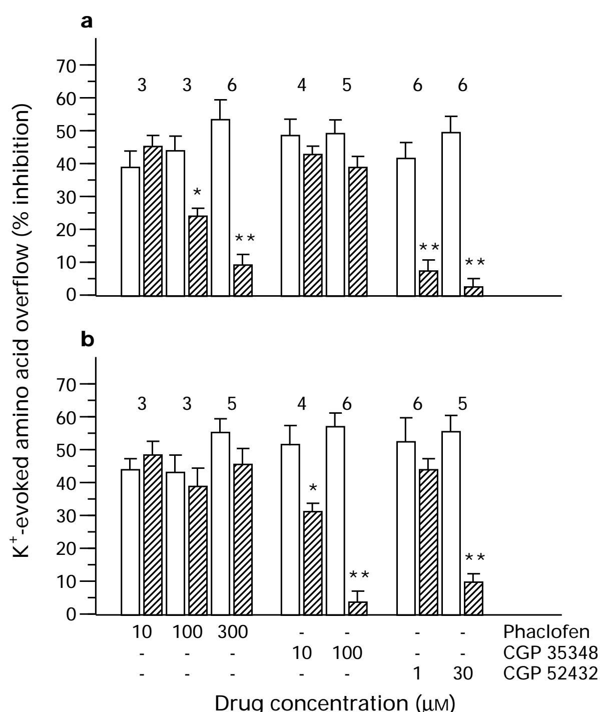

Figure 3 Antagonism by phaclofen, CGP 35348 or CGP 52432 of the (−)-baclofen (10 μM)-induced inhibition of the K^+ (15 mM)-evoked endogenous (a) GABA or (b) glutamate overflow. Data are expressed as % inhibition of the depolarization-evoked overflow. Synaptosomes were exposed to (−)-baclofen concomitantly with the depolarizing stimulus and to the antagonists 8 min before. Each column represents the mean ± s.e.mean (vertical lines) of the number of experiments (performed in triplicate) indicated above the column. Open columns: 10 μM (−)-baclofen; hatched columns: 10 μM (−)-baclofen + antagonist. *$P<0.05$ and **$P<0.001$ when compared to the effect of 10 μM (−)-baclofen alone (two tailed Student's *t* test).

technical characteristics of the superfusion system employed (a thin layer of crude synaptosomes up-down superfused under conditions in which released transmitters are immediately removed and indirect effects are therefore minimized) allow us to assume that (−)-baclofen acted directly on GABAergic and glutamatergic nerve terminals, respectively; the blockade of the (−)-baclofen effects by selective GABA$_B$ receptor antagonists, therefore, suggests that release-inhibiting GABA$_B$ receptors are located on both GABAergic and glutamatergic terminals of human neocortex.

The pharmacology of these two GABA$_B$ receptors was compared by an experimental paradigm often utilized in functional studies of receptor heterogeneity; the effects of a given concentration of agonist were challenged against varying concentrations of different antagonists. Needless to say, the concomitant measurement of endogenous GABA and glutamate released from the same superfusion chambers appears in this case particularly useful. The results obtained suggest that, in the human cerebrocortex, two distinct pharmacological subtypes of the GABA$_B$ receptor exist: (a) a phaclofen- and CGP 52432-sensitive, CGP 35348-insensitive autoreceptor, situated on the terminals of GABAergic neurones and mediating inhibition of GABA release; (b) a presynaptic heteroreceptor, located on the terminals of glutamatergic neurones and mediating inhibition of glutamic acid release; this receptor is insensitive to phaclofen and relatively insensitive to CGP 52432, but can be blocked by CGP 35348. In the absence of molecular cloning information, the two GABA$_B$ receptors characterized here appear to be very similar to those found in rats (Bonanno & Raiteri, 1992) which would, therefore, suggest that these models are useful for studying these GABA$_B$ receptors.

GABA and glutamate are major CNS transmitters and imbalances between the two systems are thought to play key roles in some pathological conditions including epilepsy and cognitive disorders. An involvement of GABA$_B$ receptors in absence seizures has been proposed (Bernasconi *et al*., 1992; Liu *et al*., 1992; Snead, 1992). Moreover, the *in vivo* release of GABA during microdialysis of the thalamus, an area which, together with the cortex, is thought to be intimately involved in generalized epilepsy (Gloor *et al*., 1990), was antagonized by phaclofen but not by CGP 35348, whereas the opposite was true for the release of glutamate, consistent with our *in vitro* results. Also consistent with the proposed existence of GABA$_B$ receptor subtypes is the finding that only a few out of several brain-permeable GABA$_B$ receptor antagonists could ameliorate cognitive functions in rats and monkeys (Fröstl *et al*., 1995). The present results showing that GABA$_B$ receptors with distinct pharmacological profiles also exist in the human brain make GABA$_B$ receptor antagonists a potential source of therapeutically useful drugs (Bittiger *et al*., 1996).

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