MOLECULAR PHARMACOLOGY, 19:27-30

Inhibition of γ-Aminobutyric Acid Release by γ-Aminobutyric Acid Agonist Drugs

Pharmacology of the γ-Aminobutyric Acid Autoreceptor

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Received June 17, 1980; Accepted July 28, 1980

SUMMARY

BRENNAN, M. J. W., R. C. CANTRILL, M. OLDFIELD, AND P. KROGSGAARD-LARSEN. Inhibition of γ-aminobutyric acid release by γ-aminobutyric acid agonist drugs: pharmacology of the γ-aminobutyric acid autoreceptor. *Mol. Pharmacol.* **19**:27-30 (1981).

The order of potency of several γ-aminobutyric acid (GABA) receptor agonists in inhibiting stimulated GABA release from rat cortical synaptosomes was the same as that for inhibiting specific [^{3}H]GABA binding in synaptic plasma membranes. This finding suggests that GABA autoreceptors are identical pharmacologically with sites labeled with [^{3}H]GABA in binding studies. There was a good correlation between receptor occupancy and inhibition of release, suggesting a direct coupling of the autoreceptors to the effector mechanism and the absence of "spare" autoreceptors.

INTRODUCTION

There is considerable evidence, in both the peripheral and central nervous systems, that presynaptic *alpha*-adrenergic receptors modulate norepinephrine release induced by both electrical and potassium ion stimulation; α-agonists inhibit and α-antagonists facilitate this release (1-4). There is also evidence that similar control mechanisms occur in dopaminergic (2, 5, 6) and cholinergic (7) neurons. Classic postsynaptic receptor agonists and antagonists have varying degrees of affinity for the corresponding autoreceptors; such pharmacological differences may open up the possibility of selective activation or blockade of one or other class of receptor by drugs.

The presence of autoreceptors capable of regulating the release of the important inhibitory transmitter GABA[3] from GABA-ergic neurons has recently been reported (8-11). These autoreceptors have been shown to be involved in the pathogenesis of the neural dysfunction characterizing acute attacks of the hereditary hepatic porphyrias (11, 12) and are therefore an important target in the development of new drugs. At present the selectivity of the GABA autoreceptors for agonists and antagonists is unknown. In the experiments reported herein we have examined the potency of a number of known GABA agonists in inhibiting K^+-stimulated release of [^{3}H]GABA from preloaded rat cortical synaptosomes and compared this with the potency of the drugs in inhibiting specific [^{3}H]GABA binding to cortical synaptic plasma membranes. These studies were aimed at the elucidation of any pharmacological differences between the autoreceptors and the sites labeled with [^{3}H]-GABA.

MATERIALS AND METHODS

Release of preloaded [^{3}H]GABA from rat cortical synaptosomes was monitored using a superfusion system described previously (13-15). Synaptosomes prepared from the cerebral cortices of adult male Wistar rats by a modification (16) of the method of Gray and Whittaker (17) were resuspended in 0.32 M glucose to yield a protein concentration (18) of approximately 5 mg/ml and diluted 1:10 with ice-cold incubation medium (final concentrations: 128 mM NaCl, 5 mM KCl, 2.7 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.1 mM amino-oxyacetic acid to prevent GABA catabolism, and 10 mM Tris-HCl buffer at pH 7.35). Aliquots (1 ml) of the suspension were preincubated at 37° for 15 min in a water bath in an air atmosphere. [2,3-^{3}H]GABA, specific activity 65 Ci/mmole (Radiochemical

These studies were supported in part by grants from the Danish and South African Medical Research Councils and the Atomic Energy Board of South Africa.

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[3] The abbreviations used are: GABA, γ-aminobutyric acid; THIP, 4,5,6,7-tetrahydroisoxazolo-(5,4-*c*)-pyridin-3-ol.

0026-895X/81/010027-04$02.00/0

Centre, Amersham), was diluted with unlabeled GABA to yield a specific activity of 10 Ci/mmole, and a small volume (1:100 of the final volume) was added to the incubation tubes to a final concentration of 0.5 μM. Incubation was continued for an additional 10 min, after which the synaptosome suspensions were layered on Millipore filters (0.45-μm pore) resting on filter supports constituting the bottoms of four parallel superfusion chambers (19) and thermostatically maintained at 37°. The chambers were connected to a multichannel peristaltic pump, the excess medium was drawn off at maximal flow rate, and the filters washed with 10 ml of control medium (concentrations as described above) containing 30 mM glucose. The flow rate was adjusted to 0.5 ml/min, and 1-min fractions were collected directly into scintillation counting vials. Superfusion was continued for 10 min with control medium to establish a baseline rate of GABA efflux; the control medium was then exchanged for the test medium containing the GABA agonists at various concentrations, and superfusion was continued for an additional 15 min. Aquagel I liquid scintillation fluid (Chemlab), 5 ml, was added to each vial and the radioactivity of the vials was counted in a Packard Tri-Carb 2660 spectrometer with automatic quench correction. Filters were solubilized in 10 ml of Aquagel I and counted in the same way. Stimulation of GABA release was calculated as the percentage increase in efflux over the baseline unstimulated level.

The sodium-independent binding of [^{3}H]GABA to rat cortical membranes was studied at 4° in Tris-citrate buffer by a modification (20) of the method of Enna and Snyder (21).

Adult Wistar rats were decapitated and the cortices were rapidly homogenized in 15 volumes of ice-cold 0.32 M sucrose. The homogenate was centrifuged at 1,000 × *g* for 10 min, the pellet was discarded, and the supernatant was centrifuged at 20,000 × *g* for 20 min. A suspension of the crude mitochondrial pellet in distilled water was dispersed with a Brinkmann Polytron PT-10 (setting 6) for 30 sec and centrifuged at 8,000 × *g* for 20 min. The supernatant fluid was collected, and the pellet, a bilayer with a soft, buffy coat, was rinsed carefully with the supernatant fluid to collect the upper layer. This suspension was centrifuged at 48,000 × *g* for 20 min. The final membrane pellets were submitted to two resuspensions in water and centrifugations at 48,000 × *g* for 20 min. The membranes were frozen rapidly at −70° and kept at −20° for at least 18 hr. Frozen pellets were resuspended in water, maintained at 25° for 20 min, and centrifuged at 48,000 × *g* for 10 min. This procedure was repeated once.

For the standard [^{3}H]GABA binding assay, aliquots of these membranes (0.8–1.2 mg of protein) were incubated in quadruplicate at 4° for 5 min in 2 ml of 0.05 M Tris-citrate buffer (pH 7.1) containing 5.8 nM [^{3}H]GABA alone or in the presence of 1 mM unlabeled GABA or other agonist drugs at various concentrations. After incubation, the tubes were centrifuged at 48,000 × *g* for 10 min, the supernatant fluid was decanted, and the pellets were rinsed superficially with two 5-ml aliquots of ice-cold distilled water. Bound radioactivity was extracted into 1 ml of water, 10 ml of scintillation fluid were added, and radioactivity was measured in a scintillation counter. Total specific [^{3}H]GABA binding was obtained by subtracting from the total bound radioactivity the amount not displaced by 1 mM unlabeled GABA. With this procedure of extensive washing and very rapid freezing of the synaptic membranes, approximately 80% of the total binding represented specifically bound GABA. Estimates of the concentrations of the agonists producing 50% inhibition of specific [^{3}H]GABA binding (IC_{50}) were made by examining the displacements produced by at least four different concentrations of the drugs and performing log-probit analyses of the results (22).

RESULTS

Muscimol, piperidine-4-sulfonic acid, isoguvacine, and THIP are potent GABA receptor agonists (20, 23). All agents reduced the K^+ (55 mM)-stimulated release of preloaded [^{3}H]GABA from rat cortical synaptosomes in a dose-dependent fashion (Fig. 1). In every case, at every drug concentration tested, the reduction in K^+-stimulated release was prevented by the GABA receptor antagonists (+)-bicuculline and picrotoxin at concentrations of 1 μM (Table 1). The muscarinic receptor antagonist atropine (1 μM) had no effect on the inhibition of GABA release caused by the agonist drugs. The demonstration that agonist drugs modify GABA release appro-

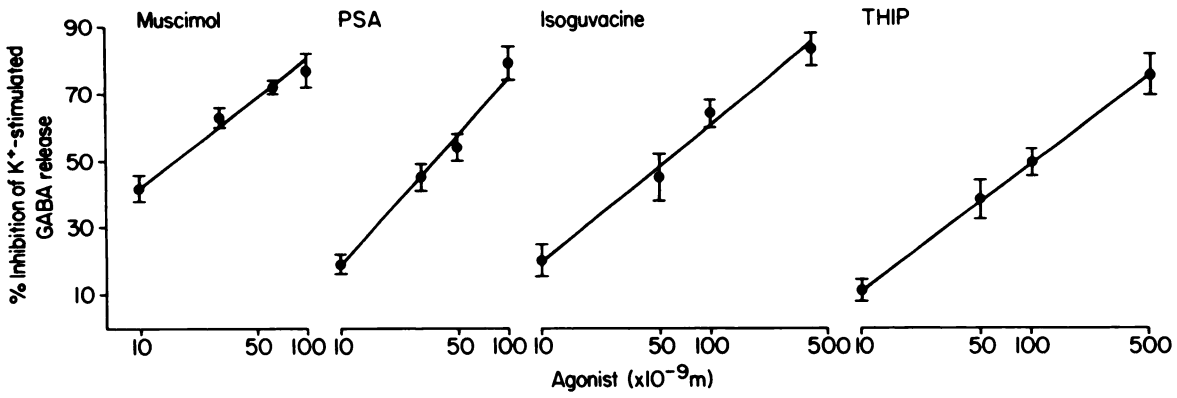

FIG. 1. *Dose-response curves for the inhibition of potassium-induced GABA release by GABA agonists*

The horizontal axes give concentrations of agonists (× 10^{-9} M) on a logarithmic scale. Represented on the vertical axis is the percentage inhibition of K^+ (55 mM)-stimulated GABA release. Each point represents the mean percentage inhibition ± standard error of the mean (n = 4). The curves are the best fits to the data by the method of least squares ($r^2 > 0.97$ for all curves). *PSA*, Piperidine-4-sulfonic acid.

TABLE 1

GABA agonist effects on the K^+-stimulated release of GABA from preloaded synaptosomes

Experiments were carried out as described in the text. The values in parentheses indicate the number of times the experimental conditions in the first column were repeated. Baseline efflux was calculated as percentage of total tissue stores released per fraction (0.57 ± 0.068%, n = 7). The second column refers to the percentage increase in efflux over this control baseline level; in each case the mean ± standard error of the mean is given. Statistical significance was calculated using the two-sided Student's t-test. The third column represents the average drug-induced change as compared with the release caused by 55 mM K^+ alone.

Drug present	Induced release (% increase over baseline control)	Mean drug effect on induced release
55 mM K^+ alone (6)	188 ± 26.9[a]	
10^{-7} M muscimol alone (4)	22 ± 4.9	
55 mM K^+ + 10^{-8} M muscimol (4)	107 ± 11.1[b]	−43%
55 mM K^+ + 10^{-8} M muscimol + 10^{-6} M bicuculline (2)	194 ± 13.7	+3%
55 mM K^+ + 5×10^{-8} M piperidine-4-sulfonic acid (4)	86 ± 6.1[b]	−54%
55 mM K^+ + 5×10^{-8} M piperidine-4-sulfonic acid + 10^{-6} M picrotoxin (2)	179 ± 12.1	−5%
55 mM K^+ + 10^{-7} M isoguvacine (4)	68 ± 7.8[b]	−64%
55 mM K^+ + 10^{-7} M isoguvacine + 10^{-6} M bicuculline (2)	184 ± 12.0	−2%
55 mM K^+ + 10^{-7} M THIP (4)	98 ± 5.0[b]	−48%
55 mM K^+ + 10^{-7} M THIP + 10^{-6} M picrotoxin (2)	177 ± 8.4	−6%

[a] Substitution of Mg^{2+} for Ca^{2+} in the medium did not significantly affect basal release of radioactivity, although 76 ± 2.2% (n = 4) of the K^+ (55 mM)-induced release was Ca^{2+}-dependent. The small Ca^{2+}-independent component of the K^+–stimulated release (24%) was not significantly affected by the agonist drugs (0.1 μM or 1 μM) or by bicuculline or picrotoxin (1 μM).

[b] $p < 0.001$, significant difference from 55 mM K^+ alone.

priately and that the effect is prevented by specific receptor antagonists is evidence for a receptor-mediated phenomenon. Piperidine-4-sulfonic acid, isoguvacine, and THIP at concentrations of up to 1 μM exhibited no effects on the basal release of GABA, although muscimol, at concentrations above 1 μM, stimulated GABA efflux in a dose-dependent fashion (Fig. 2). The IC_{50} value for inhibition of stimulated GABA release by muscimol (Table 2) might therefore be overestimated, since the combined effect of inhibition of stimulated release and stimulation of basal efflux should be taken into account. Neither bicuculline nor picrotoxin alone (1 μM or 10 μM) modified the basal release of radioactivity under the conditions used here. This was expected, as we consider that any GABA released from the tissue is rapidly recovered from the superfusion chamber by the flow of medium (19). The concentration of GABA in the medium therefore does not reach the levels necessary to stimulate presynaptic autoreceptors and hence the bicuculline or picrotoxin has no negative feedback effects to antagonize.

All of the GABA agonists used in this study inhibited specific [^{3}H]GABA binding to high-affinity receptor sites in synaptic membranes from rat cerebral cortex. The IC_{50}

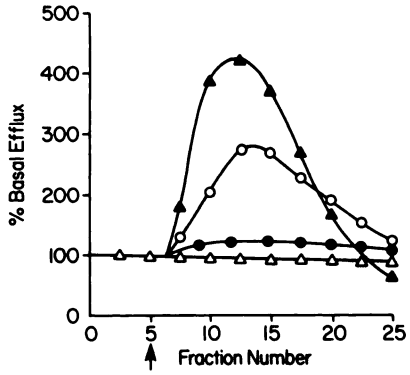

FIG. 2. *Stimulation of basal [^{3}H]GABA efflux from preloaded synaptosomes by muscimol*

The superfusion technique is described under Materials and Methods. Shown is the percentage change in efflux (baseline efflux = 100%) against fraction number. The *arrow* represents the changeover from control medium (△) to the test medium containing: ●, 0.1 μM muscimol; ○, 1 μM muscimol; ▲, 10 μM muscimol. The first few fractions were very variable in each case and are not shown. Each curve is an average of three superfusion experiments. Variation between individual curves was less than 10% of the mean.

values for the compounds are listed in Table 2. The order of potency of the compounds in inhibiting GABA receptor binding was the same as that for inhibiting K^+-stimulated GABA release. In addition, there was a good absolute correlation between receptor occupancy by agonist (IC_{50} for inhibiting binding) and inhibition of stimulated GABA release.

DISCUSSION

The data shown in Table 2 suggest that the GABA autoreceptors and the receptors labeled by [^{3}H]GABA in synaptic membranes are identical pharmacologically, at least insofar as the compounds tested here are concerned. Many binding studies using [^{3}H]GABA or [^{3}H]muscimol as ligands have been reported (9, 21, 24, 25). Our data

TABLE 2

Inhibition of K^+ (55 mM)-stimulated ^{3}H-GABA release from synaptosomes and specific [^{3}H]GABA binding in synaptic plasma membranes by GABA agonists

Release experiments were carried out as described in the text. IC_{50} values for the inhibition of K^+-stimulated GABA release were calculated from a best-fit line to the data by log-probit analysis. At every drug concentration tested, the inhibition of [^{3}H]GABA release was prevented by the GABA receptor antagonists bicuculline and picrotoxin (10^{-6} M). Sodium-independent binding of [^{3}H]GABA to membranes isolated from rat cerebral cortex was studied at 4° in Tris-citrate buffer using a centrifugation assay. The IC_{50} was estimated by varying the concentration over a range of values, using four data points for each of four inhibitor concentrations and calculating the line of best fit for a log-probit plot. Results are means ± standard error of the mean of four independent experiments.

	IC_{50}	
	GABA release	GABA binding
	nM	
Muscimol	16 ± 3	6 ± 0.2
Piperidine-4-sulfonic aicd	36 ± 4	34 ± 2
Isoguvacine	49 ± 5	37 ± 3
THIP	110 ± 8	131 ± 5

suggest that the interpretation of these studies must consider the labeling of both pre- and postsynaptic GABA receptor sites.

Although the release studies and the binding studies were carried out in media of different ionic compositions, there was a good absolute correlation between receptor occupancy by agonist and inhibition of stimulated GABA release. To date, strict quantitative comparison of the results of binding studies and neurophysiological data has proved difficult. This is due to the impossibility of precise regulation of drug concentrations in the extracellular fluid during microiontophoretic experiments. The results reported herein thus provide the first direct correlation between a GABA receptor-mediated effect and receptor occupancy. It appears from these data that the autoreceptor is coupled directly to the effector mechanism (presumably a chloride channel, since the inhibition of GABA release by agonist is picrotoxin-sensitive) and that there are no "spare" autoreceptors on the presynaptic terminals.

Recently the existence of bicuculline-insensitive presynaptic GABA receptors which regulate the release of norepinephrine, dopamine, and serotonin have been described in mammalian peripheral and central nervous systems (26–28). Our results suggest that the autoreceptors are distinct from this second class of presynaptic GABA receptors and more closely resemble the classic postsynaptic GABA binding site.

The GABA agonist THIP has recently been introduced for clinical trial in a number of neurological and psychiatric conditions. It is apparent from our data that such a drug will act on both pre- and postsynaptic receptors and that these two actions will tend to produce different effects. The development of agonists and antagonists selective for one class of GABA receptors is therefore of prime importance for the successful manipulation of brain GABA systems by drugs.

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