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Interactions of agonists and antagonists with a novel type of GABA receptor

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The projection from cortex to corpus striatum in rat is now widely considered to use glutamate (GLU) as a transmitter [1], and both receptor binding [2] and transmitter release studies [3] have demonstrated the presence of a γ-aminobutyric acid (GABA) receptor on cortico-striatal terminals. Activation of this presynaptic receptor produces a facilitation of stimulus-evoked release of [^{3}H]GLU recently accumulated in small prisms of rat striatum [3], and as such provides a convenient measurement of the functional response of the receptor. Although this receptor responds to a variety of GABA agonists, preliminary results [3] suggested their order of effectiveness to be unusual. Experiments were therefore carried out to define the functional responses of this receptor to a series of GABA agonists and antagonists.

Materials and methods

The release of [^{3}H]GLU was investigated using a continuous superfusion method described in detail previously [3–5]. 0.1 × 0.1 × 2.0 mm prisms of striatal tissue from male Wistar rats were rapidly prepared and suspended in buffered physiological medium. Suspensions were preincubated at 27° for 20 min then [^{3}H]GLU was added to a concentration of 0.3 μM and incubation continued for another 20 min to allow high affinity uptake. (The subphysiological temperature was used as it had been previously shown to permit greater reproducibility of the GABAergic responses [3].) Tissue (5 mg aliquots) was loaded onto filters in parallel glass chambers at controlled temperature, washed, and then continuously superfused with physiological medium at a rate of 0.5 ml/min. One minute fractions of perfusate were collected for scintillation counting of ^{3}H release and then, after some 12 min, the effects of submaximal depolarising stimuli (30 mM K^+) in combination with the GABAergic drugs were studied. The vast majority of ^{3}H released represented authentic [^{3}H]GLU and stimulus-evoked release was largely Ca^{2+}-dependent [4]. From each of the release curves obtained by plotting fractional release of ^{3}H against time, the peak K^+-evoked [^{3}H]GLU release in each case was calculated as percentage increase over basal efflux. These values in absence/presence of the drugs were then compared to reveal drug-induced modification of stimulus-evoked release [4].

Results and discussion

The responses of this GABA receptor to several agonists were examined over a broad concentration range (Fig. 1). 3-Amino propane sulphonic acid (APS), isoguvacine, GABA and muscimol all produced concentration-dependent facilitations of stimulus-induced GLU release with apparently parallel log concentration–response curves. The order of potency of these compounds was APS ≥ isoguvacine > GABA ≥ muscimol; thus clearly supporting the contention that this receptor shows novel structure–activity relationships, since at most other GABA receptors described, muscimol is by far the most potent of these agonists [6]. At very high concentrations of GABA agonists the responses became rather more variable (as reported in other systems, e.g. [7]), so no attempt was made to investigate near-maximal responses. The concentration of APS producing 50% of theoretical maximum response was calculated from a double reciprocal plot of the data to be 68.5 μM. Equieffective concentrations of the other agonists tested were estimated from Fig. 1 to be: isoguvacine, 100 μM; GABA, 180 μM; and muscimol, 225 μM.

An antagonsim of the responses of this receptor by bicuculline and picrotoxinin was indicated previously, but here experiments were carried out with ranges of agonist and antagonist concentrations in order to characterise the type of antagonism occurring in each case. An empirical indication of the type of antagonism can be given by the kind of alteration in agonist concentration–response curves (see inset of Fig. 2a). A competitive antagonist will cause a parallel rightwards shift whilst a true non-competitive antagonist will show a characteristic flattening of the curves. Theoretically, the effect of a competitive antagonist should be relatively reduced by increasing agonist concentrations whilst the effect of a true non-competitive antagonist should not be influenced. Therefore, a better diagnostic method for differentiating the type of antagonism should be a plot of fractional response remaining in the presence of a fixed antagonist concentration, against increasing concentrations of agonist (Fig. 2a). Bicuculline methiodide (at 10 μM) produced a parallel shift in the APS dose–response curve and an upward curving plot of fractional response against agonist concentration, consistent with competitive antagonism. Picrotoxinin (which is often considered to be a non-competitive antagonist of mammalian GABA receptor responses, e.g. [8]), also produced apparently parallel shifts in the APS dose–response curve and again upward curving plots in Fig. 2a, indicating that the antagonism could be reversed by increasing the agonist concentration. These effects were seen at picrotoxinin concentrations of both 4 μM (representing an APS dose ratio ≃ 2.2) and also 20 μM (APS dose ratio ≃ 5). Thus over at least a 5 fold

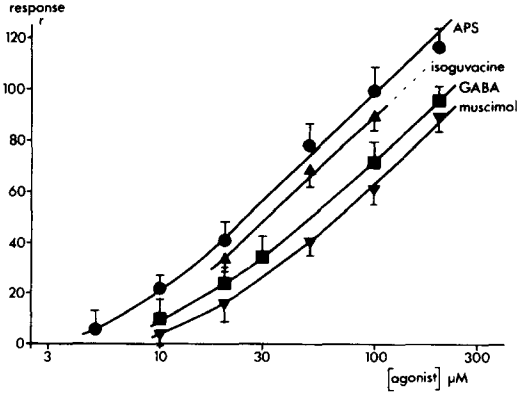

Fig. 1. Concentration dependence of the responses to a range of agonists at the GABA receptor on corticostriatal terminals. Response is measured as percentage facilitation of K^+-induced [^{3}H]GLU release and each point represents the mean ± S.E.M. of at least 3 separate experiments. (Control K^+-induced GLU release represented some 71.3 ± 4.1% increase over basal efflux, n = 26.) The sample of muscimol used in these experiments was confirmed to be an extremely potent agonist at another GABA receptor (the presynaptic autoreceptor in cerebral cortex, cf. [5], data not shown).

concentration range, picrotoxinin clearly did not display a true non-competitive type of antagonism of the APS response. Since both of the antagonists produced qualitatively similar shifts of the APS dose–response curve, further experiments were carried out to look for any possible differences in their modes of action by varying antagonist concentrations at a fixed agonist concentration. Fig. 2b shows concentration-dependent antagonsim of the response to 70 μM APS by bicuculline methiodide and picrotoxinin from which the antagonists IC_{50} values were estimated to be approximately 14 μM and 5.5 μM respectively. It was interesting that the inhibition curve for bicuculline methiodide appeared to be considerably steeper than that for picrotoxinin and so the results were analysed in the form of preliminary Hill plots from the smaller number of data points. Whilst picrotoxinin showed a Hill coefficient of approximately 0.9 (consistent with linear antagonism according to the law of mass action) the Hill coefficient for bicuculline methiodide was considerably greater than one (2.7) indicating the possible presence of cooperative effects. In an attempt to ascertain whether the two antagonists had independent sites of action, the effects of a combination of bicuculline methiodide and picrotoxinin were examined to look for additive responses. A combination of 5.5 μM picrotoxinin and 14 μM bicuculline methiodide (IC_{50} concentrations) elicited some 83.7 ± 6.9% inhibition, whilst a two fold increase in picrotoxinin concentration (which should in principle be indistinguishable from the combination if both compounds were acting at the same site) produced only 52.0 ± 3.1% inhibition ($P < 0.02$, calculated from raw data, $n = 3$). Therefore the combination of two antagonists produced a much greater inhibition than would be expected if they had an identical site of action.

The present experiments clearly confirm the preliminary report [3] of a novel class of GABA receptor with previously undescribed sequence of agonist potencies. APS and isoguvacine appear to be potent agonists at this site whilst the normally extremely potent GABA agonist muscimol is considerably less active. Such a structure–activity sequence has not yet been directly demonstrated in [^{3}H]GABA binding experiments.

This 'APS/isoguvacine receptor' is bicuculline-sensitive and does not show any marked response to baclofen and is therefore quite distinct from the baclofen-sensitive receptor recently described [9], (for which APS and isoguvacine also show very low affinity). A selective response to APS contradistinct from that to baclofen has been described on cerebellar NAergic terminals [10], but unfortunately other agonists were not investigated. The APS/isoguvacine receptor clearly must be designated as a sub-type of bicuculline-sensitive GABA receptors. As discussed previously, its agonist responses are not consistent with the high affinity GABA receptor revealed in Triton-treated synaptic membranes, and are quite distinct from those at the muscimol-activated GABA receptor mediating facilitation of [^{3}H]diazepam binding, where APS and isoguvacine are weak partial agonists. Since APS and isoguvacine show little affinity preference for either low or high affinity [^{3}H]GABA binding sites (in contrast to the marked preference of muscimol for the high affinity site) [11], it seems likely that these compounds would attach to both types of site. Interestingly, though, these compounds marked agonist activity at the present receptor contrasts notably with their partial agonist effects at the GABA receptor which modifies [^{3}H]diazepam binding [12, 13]. Whilst the latter experiments were performed on extensively washed membranes (a procedure reported to remove endogenous modulators of GABA binding and reveal high affinity binding sites [14]) the results reported here were carried out on whole fresh tissue and as such are likely to represent interactions with the low affinity GABA binding site which predominates in unwashed membranes [15]. A feasible explanation of all these data therefore is that whilst the high affinity GABA site recognises muscimol as a potent agonist, and APS/isoguvacine as just partial agonists; the low affinity site recognises APS/isoguvacine as more potent agonists than muscimol. It is suggested that the spectrum of bicuculline-sensitive GABAergic responses seen *in vivo*, in which muscimol and also APS and isoguvacine are potent agonists, may be explained in terms of responses from mixed populations of the two proposed receptor subtypes whose contributions might be controlled by endogenous modulators of these sites.

The APS/isoguvacine receptor is antagonised in a manner consistent with competitive inhibition by bicuculline methiodide and also picrotoxinin. Results from [^{3}H]GABA bind-

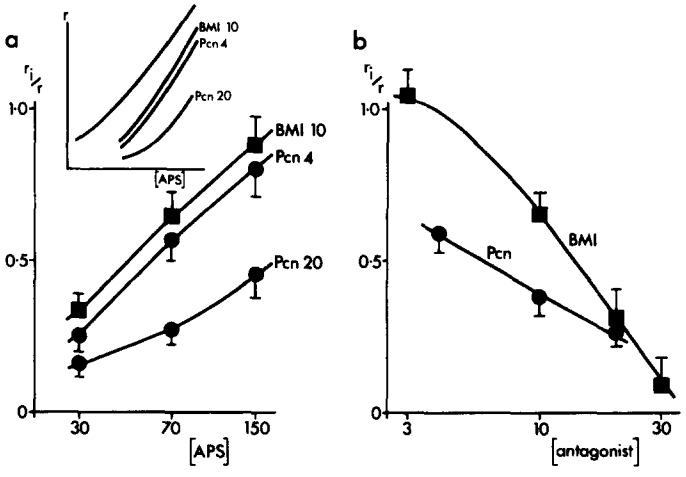

Fig. 2. Modifications by GABA antagonists, bicuculline methiodide (BMI) and picrotoxinin (Pcn), of the response to APS. Ordinate axes (r_i/r) represent response in presence of inhibitor as a fraction of control response at the same agonist concentration. All concentrations are μM. (a) Illustrates the effect of increasing agonist concentration on the inhibitory action of the antagonists (inset shows the resulting approximately parallel shifts in the APS dose–response curve). (b) Illustrates the effect of increasing antagonist concentrations on the response to a fixed concentration (70 μM) of APS. Each point represents the mean ± S.E.M. of 3 separate experiments.

ing experiments with mammalian CNS synaptic membranes are consistent with a close interaction of bicuculline and GABA recognition sites [16, 17]. Similar approaches however, have indicated that picrotoxinin recognition site may be relatively distinct from the GABA receptor, [14, 18]. Modifications of GABA receptor responses in the mammalian CNS showing parallel dose–response curve shifts with picrotoxinin have also been described previously, [7]. It is important to point out that although this is difficult to reconcile with a true non-competitive mode of action, these results could be explained in terms of a 'mixed' (or uncompetitive) type of antagonism. In the simplest model of such cases the antagonist combines with some species of the receptor which are themselves unable to combine with the ligand, producing kinetics with elements of both competitive and non-competitive antagonism. The present results provide some support for the concept of independent sites of action for picrotoxinin and bicuculline methiodide, consistent with the results of radioligand binding experiments. The antagonism by bicuculline methiodide showed a remarkably steep concentration dependence curve and high Hill coefficient, consistent with cooperative effects. Interestingly, radioligand binding studies have also provided data consistent with cooperative interactions between bicuculline and GABA [14, 17].

This investigation therefore provides evidence to propose a novel subtype of bicuculline-sensitive GABA receptor which is selectively activated by APS and isoguvacine, and which may represent the low affinity binding site for [^{3}H]GABA. Picrotoxinin and bicuculline methiodide inhibit this receptor's response, probably at separate sites, and in a fashion not at variance with their antagonism of other GABA receptor responses. It would therefore seem likely that the APS–isoguvacine receptor may utilise the same receptor–effector mechanism suggested for other bicuculline-sensitive GABA receptors.

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Pyrrolopyrimidine lethality in relation to ribonucleic acid synthesis in Sarcoma 180 cells *in vitro**

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Sangivamycin (SGM)† is the parent member of the pyrrolopyrimidine group of adenosine analogs [1]. This antitumor antibiotic has cytotoxic activity in a variety of experimental systems *in vitro* [2, 3] and *in vivo* [1, 4]. Following intracellular phosphorylation by adenosine kinase [5], SGM is incorporated into DNA [6, 7] and RNA [6, 7]. SGM has been shown to inhibit DNA [2] and RNA [2, 8] syntheses, *de novo* purine synthesis [9], and tRNA acylation [10]; however, it is not known whether these biochemical lesions are responsible for the lethal effects of the drug. SGM cytotoxicity is most pronounced following prolonged drug exposure [2]. Inhibition of nucleic acid synthesis by the drug occurs gradually, and RNA synthesis is inhibited to a lesser degree than DNA synthesis [2]. Recent studies have shown that SGM is preferentially incorporated into RNA, and that incorporation into poly(A)RNA correlates with cell killing by the drug [6].

Several pyrrolopyrimidine nucleoside analogs of SGM

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† Abbreviations: SGM, sangivamycin; TGM, thiosangivamycin; AXGM, sangivamycin-amidoxime; AMGM, sangivamycin-amidine; ADN, adenosine; and dCF, 2′-deoxycoformycin.