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STUDIES ON BICUCULLINE BINDING SITES ON NEURONAL MEMBRANE USING FLUORESCENT ANTIBODY TECHNIQUE: COMPARATIVE BINDING OF GABA AND BICUCULLINE

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SUMMARY

Highly purified fluorescent labelled anti-bicuculline antibodies were used to mark bicuculline binding sites in cerebral cortex of monkey brain. Specific binding of bicuculline could be demonstrated in the synaptosomal fraction, when bicuculline was added both *in vitro* and *in vivo*. Addition of γ-aminobutyric acid (GABA) to the bicucullinised membrane led to a decrease in fluorescence indicating same receptor loci and establishing GABA-bicuculline antagonism at a molecular level.

INTRODUCTION

Accumulation of impressive evidence (1-3) indicates that γ-aminobutyric acid (GABA) acts as an inhibitory transmitter in central nervous system (CNS). A selective antagonism of GABA induced depression and of certain evoked inhibitions by bicuculline has been well documented (3,4). Bicuculline in all probability competes with GABA for a pharmacological receptor on the post-synaptic membrane, and the antagonistic effect in the vertebrate CNS has been attributed to a competitive antagonism (5,6), the nitrogen and three atom grouping of GABA carboxylate presumably competing for the same receptor site as the nitrogen and O=C-C grouping in bicuculline (7,8). Specific binding of [^{3}H] bicuculline with GABA receptor has been demonstrated (9,10). Hence

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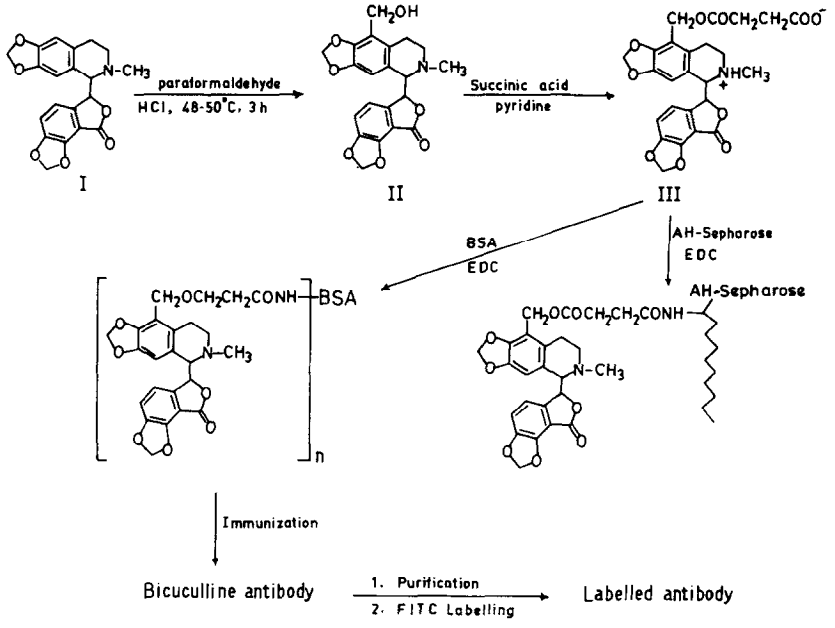
SCHEME-I



bicuculline may be used to mark the inhibitory post-synaptic GABA binding sites provided a sensitive method is available to detect the membrane-bound bicuculline. In the present study an immunofluorescent technique (11) has been developed to detect and study these bicuculline binding sites.

MATERIALS AND METHODS

Bicuculline (I) (250 mg) was functionalised to hydroxymethyl bicuculline (II) (yield 95%) with paraformaldehyde (300 mg) and conc. HCl (6 ml) (12), and II (100 mg) was converted to the hemisuccinate (III) (50 mg) with succinic acid (50 mg) in dry pyridine (10 ml) (13). The hemisuccinate (III) was conjugated to a carrier protein, bovine serum albumin (BSA) (14) for raising antibodies (Scheme I). The affinity column was prepared by coupling the hemisuccinate (III) (5 mM) with AH-Sepharose (sepharose coupled to 1,6-diamino hexane) (10 ml) (15). Amount of bicuculline bound to sepharose as measured by the method of Failla and Santi (16) was found to be 50μ g/ml sepharose. The γ-globulin fraction (10 mg), prepared from the antiserum after adsorption on BSA polymer (17), was loaded on the immunoadsorbant column (10 ml). The antibodies were eluted with 0.1 M acetic acid. The purified antibody (0.5 mg), after determining the protein components by gel electrophoresis (18), was conjugated with FITC (19) and again purified by

sephadex G25 chromatography (20). The amount of FITC was found to be 10 μg FITC/mg protein (20).

Bicuculline hydrochloride (0.6 mg) was administered to the cerebral cortex of anaesthetized monkey through a superoanterior opening into the skull, and after 10 min cortex was removed and subcellular fractions were obtained (21). Another monkey was similarly treated without administration of bicuculline to serve as control and for *in vitro* addition of bicuculline. The fractions were examined for their intactness by electron microscopy. The different fractions were incubated with fluorescent antibody and, after removing excess antibody by centrifugation, were examined by fluorescence spectrophotometry. Fractions from untreated monkey were incubated with bicuculline hydrochloride *in vitro* and similarly examined for fluorescence.

Synaptosomal fractions from bicucullinised cortex were treated with graded amounts of GABA, and after washing out the excess of bicuculline and GABA, treated with labelled antibody and after removing excess antibody, were examined for fluorescence to study the antagonism.

RESULTS AND DISCUSSION

The products II and III were identified by elemental (C,H,N) analysis and IR and NMR spectroscopy. The position of hydroxymethyl group in II was unambiguously ascribed from rigorous stereochemical considerations and NMR data (13). UV absorption at 296 nm showed the presence of bicuculline in the conjugate with BSA. Passive hemagglutination titres in two rabbits were 1:1280 and 1:640. Ouchterlony double diffusion (22) with the antiserum adsorbed thrice on BSA polymer showed no cross-reactivity with BSA and a high specificity for bicuculline. Immunoelectrophoresis also showed a clear single band.

It is interesting to note that gel electrophoresis of the purified antibody showed in addition to the main intense band, two other extremely weak bands. Since bicuculline can exist in three conformations (23), it is probable that bicuculline molecules landed up on the immunocompetent B cells in three distinct conformers to produce three different antibodies, one major conformer contributing the highest towards antibody formation.

Labelling of purified antibody with FITC was shown by shift of UV absorption maximum (20). Both the purified and labelled antibody were

Table-I

Emission intensities of subcellular fractions following treatment with fluorescent antibody

Test: Bicucullinised

Control: Without bicuculline

Fraction	Sample	Emission intensity	
		for *in vivo* administration of bicuculline	for *in vitro* addition of bicuculline
Synaptosomal	Test	74	77
Synaptosomal	Control	20	12
Mitochondrial	Test	0.5	1
Mitochondrial	Control	1	2
Microsomal	Test	10	3
Microsomal	Control	4	3
Nuclear	Test	10	5
Nuclear	Control	10	3

Microsomal, Mitochondrial and nuclear fractions from bicucullinised cortex were suspended uniformly in 1 ml of phosphate buffered saline, pH 7.4, and 0.2 ml aliquots were taken. The synaptosomal fractions (0.1 ml) were diluted with 0.1 ml PBS. A solution of fluorescent antibody (0.2 ml of 1 mg/ml solution in PBS, pH 7.4) was added to each tube and incubated (37°C) for 45 min. All samples were made upto 3 ml and centrifuged at 36,000 g for 10 min. The pellets were made upto 3.0 ml in PBS and fluorescence recorded. Controls were set up with fractions from normal cortex.

In the *in vitro* experiment suspensions from different fractions (0.2 ml) were treated with 10μg of bicuculline hydrochloride (0.1 ml of a 0.1 mg/ml solution), incubated for half an hour, and after removing excess of bicuculline by centrifugation, were treated with fluorescent antibody as above. Controls were set up without addition of bicuculline.

tested for specificity by Ouchterlony double diffusion and immunoelectrophoresis, giving a single band in both the cases.

Fluorescence spectrophotometric data for *in vitro* and *in vivo* addition of bicuculline are shown in Table I. The clear cut difference in the levels of fluorescence in the control and test synaptosomal fractions indicates a specific binding of the fluorescent antibody to the test sample and little binding in other fractions. These two

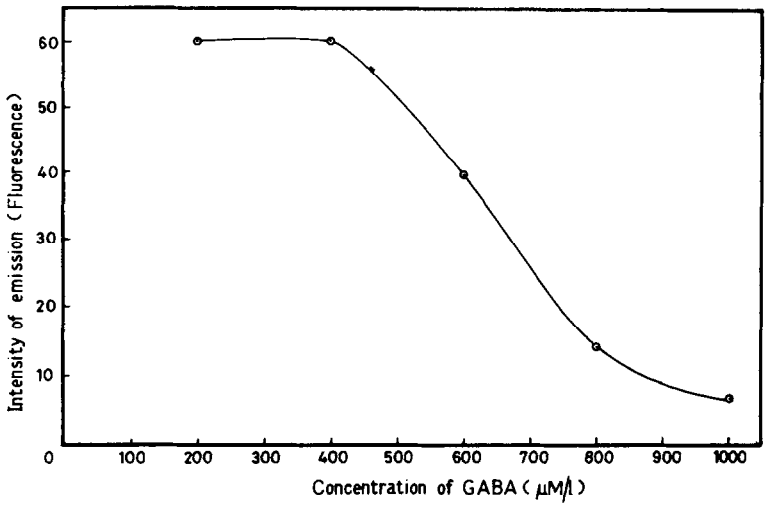


FIG. 1: Comparative Binding of Bicuculline and GABA

Suspensions of bicucullinised synaptosomal fraction (0.2 ml) were taken in different tubes and 0.04, 0.08, 0.12, 0.16 and 0.20 ml aliquots of a 0.015 M GABA solution were added in different tubes, incubated at room temperature for 30 min, made upto 3 ml with 0.1 M phosphate buffer, pH 7.4, and centrifuged at 36,000 g for 10 min. Fluorescent antibody solution (0.2 ml of a 1 mg/ml solution in PBS, pH 7.4) was added to the residue, incubated at 37° for 30 min. Mixtures were made up to 3.0 ml, centrifuged again and the residue made up to 3.0 ml with the buffer and fluorescence recorded.

experiments conclusively show that bicuculline does bind to specific synaptic membrane of cortical neurons of monkey brain.

The nature of the curve for comparative binding of bicuculline and GABA is shown in Fig.1. It is of utmost importance to add GABA before the addition of antibody, otherwise the antibody will act as a huge umbrella over bicuculline on the membrane hindering completely the approach of GABA. It is interesting to note that the displacement by GABA starts after a threshold concentration and that at higher concentration of GABA, bicuculline is completely displaceable. Thus bicuculline and GABA compete for the same receptor site.

The present study shows that it is possible to localise by fluorescent antibody technique antigens of molecular dimension of bicuculline, a very small molecule compared to viruses and bacteria.

The anatomical complexity of cerebral cortex, coupled with the difficulty of administration of bicuculline because of its low solubility, probably accounts for the differences of opinion as to the value of bicuculline in distinguishing GABA-mediated inhibition at cortical synapses (2,3). Our studies do reveal that bicuculline does bind to certain specific areas of cortical neurons and that, at least in vitro, it is replaceable by GABA. They also indicate the same site of action for both, and unambiguously establish the GABA-bicuculline antagonism in cortical neurons.

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