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FLUORESCENT LABELLING OF STRYCHNINE : A NOVEL APPROACH FOR RECOGNITION OF STRYCHNINE BINDING SITES ON NEURONAL MEMBRANE

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SUMMARY

Strychnine was coupled to fluorescein isothiocyanate to mark strychnine binding sites in spinal cord of rat. Specific binding of strychnine could be demonstrated in synaptosomal fraction. Addition of glycine to the strychninised membrane led to a decrease in fluorescence indicating same receptor loci.

INTRODUCTION

Glycine has been well established as an inhibitory transmitter in central nervous system (1-3). Strychnine specifically antagonises the action of glycine by interacting with membrane sites at or near glycine receptors (1-3). Specific binding of [^{3}H] strychnine to synaptosomal preparations has been demonstrated (4,5). Hence strychnine may be used to mark the inhibitory post-synaptic glycine binding sites provided a sensitive method is available to detect membrane bound strychnine. Earlier work from this laboratory (12) involving structure-specific anti-strychnine fluorescent antibodies located the strychnine binding sites on spinal motoneurons. In this work strychnine has been directly attached to a fluorescent probe to detect and study these binding sites.

MATERIALS AND METHODS

Strychnine (I) was converted to 3-nitro strychnine (II) and was reduced to 3-amino strychnine (III) (Scheme I) (6) which was purified by TLC using $CHCl_3$:CH_3OH = 4:1 on silica gel. The

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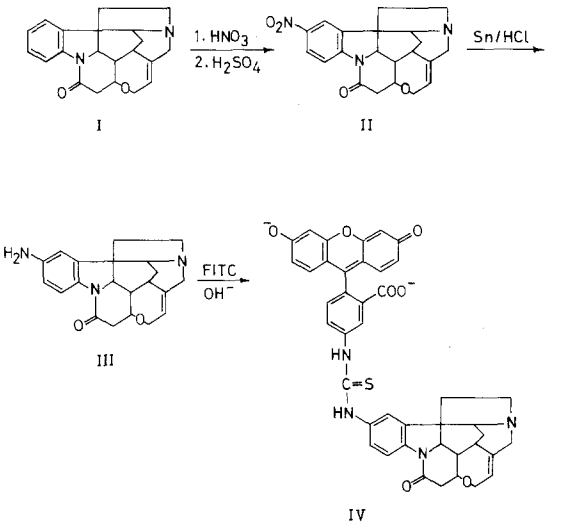

Scheme 1.

purified compound (III) (25 mg) in 5 ml of 0.1 M phosphate buffer (PB), pH 5.7, was slowly added at 7-9°C to a solution of fluorescein isothiocyanate (FITC) (25 mg) in 5 ml of 0.5 M carbonate bicarbonate buffer, pH 9.5 and the mixture stirred for 4h at 7-9°C. After leaving overnight at 0°C the mixture was passed through sephadex G 10 or G 15 which was eluted with 0.005 M PB, pH 8.0. The fractions containing the first coloured band were pooled and lyophilized to get the FITC labelled strychnine (IV) (40 mg) along with some phosphates.

Adult rats (12) were decapitated and spinal cord and brain stem rapidly removed and combined together and subcellular fractions were obtained (7). Different fractions were treated with labelled strychnine (test), fluorescein (control), strychnine (control) and after washing thoroughly, were examined by fluorescence spectrophotometry.

Synaptosomal fractions were treated with the labelled strychnine and graded amounts of glycine, and after a thorough wash, were examined for fluorescence to study the antagonism.

RESULTS AND DISCUSSION

The products II and III were identified by IR and NMR spectroscopy. UV absorption of IV showed absorption due to both strychnine ($\lambda_{max} \approx 285$ nm) and FITC ($\lambda_{max} \approx 490$ nm). IR absorption of FITC at ≈ 2050 cm^{-1} (8) due to the isothiocyanate group (9) was completely absent in IV (Fig.1) showing the completion of the reaction.

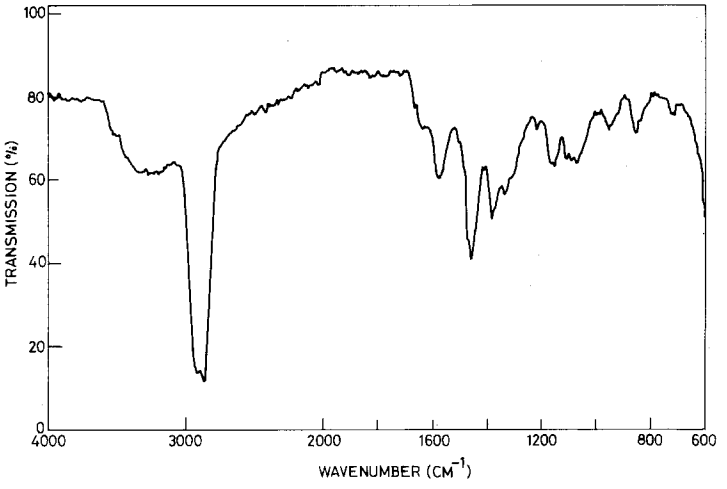

Fig.1. Infrared Spectrum of Labelled Strychnine (IV) (Nujol).

Table I

Emission Intensities of Subcellular Fractions

Test : Labelled Strychnine

Control : Fluorescein (Strychnine)

Fraction	Emission Intensity	
	Test	Control
Synaptosomal	70.5	7.5 (4.5)*
Myelin	16.5	3 (2)
Mitochondrial	21.5	3.5 (2)
Nuclear	13.5	4.5 (2)

*Controls in brackets indicate fluorescence levels with strychnine.

Different fractions were suspended uniformly in 10 ml of 0.1 M phosphate buffered saline, pH 7.4, and 0.2 ml aliquots were taken. The fractions were incubated with 10 μg of labelled strychnine (100 μg/ml solution in 0.005 M phosphate buffer, pH 8.0) (test) or equimolecular amount of fluorescein (100 μg/ml solution in 0.005 M PB, pH 8.0) (control) or strychnine (100 μg/ml solution in 0.1 M PB, pH 5.7) (control) for 45 min at 37°C. All samples were made upto 3.0 ml with the PBS, washed thoroughly and centrifuged at 36,000 g for 10 min. The washing was repeated and the final pellet was made upto 3.0 ml in PBS and fluorescence recorded.

Fluorescence spectrophotometric data (Table I) shows a clear cut difference in the levels of fluorescence in the control and test synaptosomal fractions indicating specific binding of fluorescent strychnine to the synaptosomal, and little binding to other fractions. Thus strychnine does bind to specific synaptic membrane of spinal cord of rat.

Slight increase of fluorescence in the test samples of other fractions (Table I) are probably due to nonspecific binding of labelled strychnine (having hydrophobic strychnine and hydrophobic and ionic fluorescein moieties) with membrane proteins. It may be pertinent to mention here that FITC cannot be used in the control as it would combine covalently with the membrane proteins (8) as shown in Table II. However, the excitation and emission wavelengths of fluorescein (Fig.2) are almost in the same region as those of FITC (8) allowing its use as control.

Table II

Binding of FITC with Membrane Proteins

Sample	Emission Intensity
Synaptosome + Labelled Strychnine	80.5
Synaptosome + FITC	50.5*
Synaptosome + Fluorescein	7.5

*variable to higher values

Aliquots of synaptosomal fraction (0.2 ml) were incubated with labelled strychnine (10 μg) or equimolecular amount of FITC or fluorescein, and after washing fluorescence was recorded as before. The fraction treated with FITC shows high level of fluorescence due to covalent attachment of FITC with membrane. The same phenomenon is observed with myelin, mitochondrial and nuclear fractions (not shown in the table).

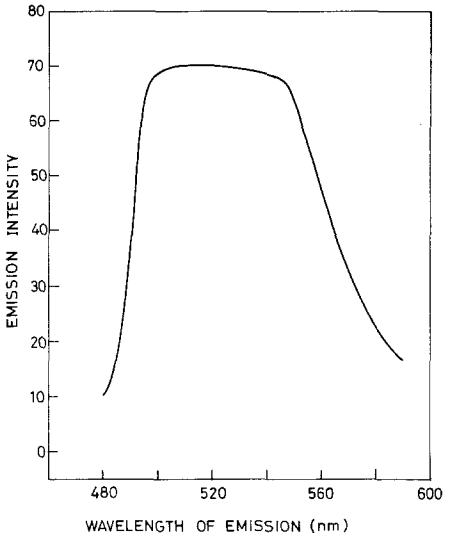

Fig.2. Fluorescence Spectrum of Fluorescein in 0.005 M phosphate buffer, pH 8.0, showing broad emission around 520 nm (excitation wavelength = 493.5 nm). The corresponding values for FITC are 520 nm (emission) and 490 nm (excitation).

Addition of glycine to the strychninised synaptosome leads to a decrease in fluorescence (Fig.3) indicating displacement of labelled strychnine by glycine. Thus glycine and strychnine compete for the same receptor site. However the amount of glycine required for the displacement is rather high probably

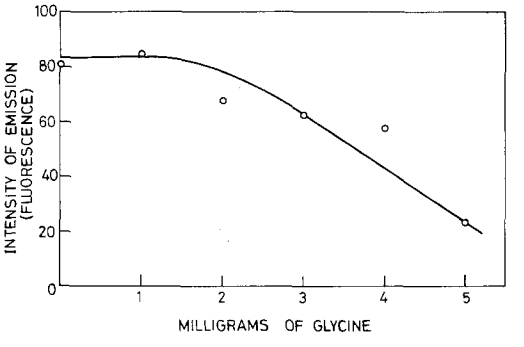

Fig.3. Comparative Binding of Strychnine and Glycine. Suspensions of synaptosomal fraction (0.2 ml) containing 10 μg of labelled strychnine were taken in different tubes and 0.1, 0.2, 0.3, 0.4, 0.5 ml aliquots of a 10 mg/ml glycine solution in water were added in different tubes, incubated for 45 min (37°C), washed as before and fluorescence recorded.

because : (i)approach of glycine is sterically hindered by the FITC molecule shielding the membrane bound strychnine (ii)FITC molecule attached to strychnine also strongly interacts with membrane via charged groups thus apparently increasing the binding affinity of labelled strychnine. It is important that addition of γ-amino butyric acid to the test synaptosomal fraction did not have any effect on the level of fluorescence, further confirming the specific binding of labelled strychnine.

Addition of strychnine or 3-amino strychnine to the test synaptosomal fraction leads to an unusual enhancement of fluorescence (Table III) for reasons not very clear. The fluorescence again decreases by addition of excess of glycine (Table III). Addition of strychnine to labelled strychnine in aqueous solution, however, did not affect the fluorescence level. Probably,

Table III

Effect of Addition of Strychnine

Synaptosome (Test) : Synaptosome (0.2 ml) + Labelled Strychnine (10 μg)

Sample	Emission Intensity
Synaptosome (Test)	28
Synaptosome (Test) + Strychnine (+Glycine)	90 (55.5)*
Synaptosome (Test) + 3-Amino Strychnine (+Glycine)	85 (50)

*values in brackets indicate the fluorescence after addition of glycine.

Aliquots of synaptosome (test) were treated with 1.6 μg of strychnine or 1.6 μg of 3-amino strychnine (10 μg/ml solution in 0.1 M phosphate buffer, pH 5.7) and incubated for 45 min at 37°C. Another lot was incubated with addition of glycine (0.4 ml of a 10 mg/ml solution in water). Fluorescence was recorded after washing as before.

the additional strychnine forms a complex with bound labelled strychnine on the membrane enhancing the fluorescence level.

Although fluorescent antibody technique (8) has been used for localisation of small molecules like bicuculline (10,11) and strychnine (12), the use of direct fluorescent labelled molecules as probes is rather few (13-16). The present study affords a simple technique to localise a molecule of small dimension selectively functionalised at a position remote from the probable recognition site so as not to interfere with the receptor interaction.

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