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THE EFFECT OF PHOSPHOLIPASES AND PROTEASES ON THE BINDING OF γ-AMINOBUTYRIC ACID TO JUNCTIONAL COMPLEXES OF RAT CEREBELLUM*

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SUMMARY

A preparation enriched in junctional complexes, as judged by marker enzymes and electron microscopy, was prepared from rat cerebellum. The junctional complexes were incubated with γ-amino[^{14}C]butyric acid at 25 °C for 10 min, using [^{3}H]sucrose as a marker for entrapped space. Total binding was determined in the absence of, and non-specific binding in the presence of, an excess of unlabelled γ-aminobutyric acid. The difference between the two binding values, i.e. the specific binding, was saturable and reversible, and showed positive cooperativity with a Hill number of about 2. The specific binding was inhibited by *N*-methylbicuculline, picrotoxinine and imidazole-4-acetic acid, but not by curare, strychnine or L-2,4-diaminobutyric acid. The above compounds had little effect on the non-specific binding, but addition of ethylenediaminetetraacetic acid decreased non-specific binding by 80 %. Trypsin, pronase, phospholipase A_2 (EC 3.1.1.4), lysolecithin and sodium dodecyl sulfate decreased binding. Phospholipase C (EC 3.1.4.3) increased the specific binding by 260 %. Phospholipids competed with γ-aminobutyric acid for binding, with phosphatidylethanolamine being more potent than phosphatidylcholine. These results lend support for Watkins' hypothesis that phosphatidylethanolamine competes with γ-aminobutyric acid for binding to the receptor protein.

INTRODUCTION

There is evidence supporting the role of γ-aminobutyric acid as an inhibitory transmitter in certain areas of the mammalian central nervous system, particularly in the cerebellum [1–4]. Selective uptake of γ-aminobutyric acid into stellate cells has been observed [5, 6]. When applied microiontophoretically, γ-aminobutyric acid also

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mimics the effects of stellate cell inhibitory action on frog cerebellar Purkinje cells, producing cessation of Purkinje cell discharge accompanied by hyperpolarization and decreased membrane resistance [7]. The inhibitory effect of γ-aminobutyric acid is antagonized by picrotoxin [8, 9], and bicuculline [10].

Besides its "functional" role as a neurotransmitter, γ-aminobutyric acid also participates metabolically in the "γ-aminobutyric acid" shunt of the tricarboxylic acid cycle [11]. In order to differentiate between the two pools, when studying the inhibitory function of γ-aminobutyric acid, it is essential to utilize a junctional complex preparation that is enriched in synapses and free of myelin, mitochondria and other cellular organelles. Here, we report the specific binding of γ-aminobutyric acid to the junctional complex isolated from the rat cerebellum.

Besides studying the binding of γ-aminobutyric acid to its postsynaptic receptor site, we were also interested in studying some of the biochemical interactions involved in the binding process. Watkins [12] noted the similarity in structure and charge distribution of acetylcholine, γ-aminobutyric acid and glutamic acid to the polar head groups of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine, respectively. He proposed that the membrane contains complexes between these lipids and proteins, and that the pharmacological actions of acetylcholine, γ-aminobutyric acid and glutamic acid result from dissociation of these complexes by these three substances and the permeability changes ensuing therefrom. In this paper, we attempted to test Watkins' hypothesis, using brain slices, synaptosomes and junctional complexes. While γ-aminobutyric acid, as a neurotransmitter, should interact at the postsynaptic site of these three tissue preparations, apparent γ-aminobutyric acid binding results using brain slices would also reflect transport and metabolic functions of γ-aminobutyric acid, while results with synaptosomes would be influenced by the active transport of γ-aminobutyric acid into the synaptosomes. In contrast, results using a junctional complex preparation would reflect mainly a postsynaptic receptor action of γ-aminobutyric acid. Our results on the effect of phospholipases and proteases on the binding of γ-aminobutyric acid to the junctional complexes are consistent with Watkins' hypothesis.

MATERIALS AND METHODS

Materials. Male, adult rats, 150–200 g, were used throughout this work. γ-amino[^{14}C]butyric acid, 21.5 Ci/M, was purchased from ICN Pharmaceuticals (Irvine, Calif.); sucrose ([1-^{3}H(N)]fructose, 4.79 Ci/mmol, in ethanol/water (7 : 3, v/v) from New England Nuclear (Boston, Mass.); phosphatidylserine from Nutritional Biochemicals Corp. (Cleveland, Ohio); phosphatidylethanolamine from Applied Science Laboratory (State College, Penn.); phosphatidylcholine (β,γ-dipalmitoyl) synthetic from California Corp. for Biochemical Research (Los Angeles, Calif.): picrotoxin from Fisher Scientific Co. (Fair Lawn, N.J.); trypsin from Mann Research Laboratories (New York, N. Y.).The tissue solubilizer and liquid scintillation counting fluids, Protosol and Aquasol, were obtained from New England Nuclear (Boston, Mass.). Curare and strychnine were purchased from K and K Laboratories (Plainview, N.Y.). Bicuculline and lysolecithin were obtained from Pierce Chemical Co. (Rockford, Ill.). The following compounds were purchased from Sigma Chemical Co. (St. Louis, Mo.): iodonitrotetrazolium violet (grade 1), phospholipase C (type 1,

from *Clostridium welchii*, 10 units/mg solid, 1 unit liberates 1 μmol of water-soluble organic phosphorus from egg yolk lecithin per min at pH 7.3 at 37 °C), and pronase (type V, from *Streptomyces griseus*, purified, 1.2 units/mg solid, 1 unit hydrolyzes casein to produce color equivalent to 1 μmol or 181 μg of tyrosine per min at pH 7.5 and 37 °C). Phospholipase A_2, purified from bee venom, was kindly provided by Mr. T. Lysz, and had an activity of 4.5 microequivalents of free fatty acid liberated from egg yolk phospholipids per 10 min per μg of solid.

Incubation of brain slices. Rat cerebral cortex slices (about 200 mg) were dissected free hand and incubated for 1 h with 5 ml of a 10 mM γ-aminobutyric acid solution in bicarbonate buffer, gassed with O_2/CO_2 (95 : 5, v/v), pH 7.4, at 37 °C. The buffer contained 122 mM NaCl, 3.1 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 25 mM $NaHCO_3$ and 10 mM glucose. The slices from the left hemisphere of the brain was used as control, while the right side was used for incubation with γ-aminobutyric acid. After incubation, the slices were homogenized in buffer. An aliquot was taken for protein analysis, the remainder was extracted with chloroform/methanol (2 : 1, v/v) and partitioned with 0.2 volume of water as described by Folch et al. [13]. Aliquots of the lipid extract were analyzed for: total lipid phosphorus [14], total lipid protein [15] and proteolipid protein [16]. The two measures of lipid protein differ in that total lipid protein is a direct measure of protein in a chloroform/methanol extract of the tissue while proteolipid protein is a measure of protein in a chloroform/methanol extract of the tissue, which has, however, been further purified by dialysis in order to remove loosely bound phospholipids.

Studies with synaptosomes. Rat cerebellum were homogenized in 0.32 M sucrose to give a 10 % homogenate, and centrifuged at $1000 \times g$ for 11 min. The supernatant was decanted, and the residue was washed with 0.32 M sucrose. The supernatant from the wash was pooled with the original supernatant, and centrifuged at $17\,000 \times g$ for 1 h. The residue was resuspended in 0.32 M sucrose and layered on a discontinuous sucrose gradient (5.5 ml of 1.2 M and 5.5 ml of 0.8 M sucrose). The layered gradients were centrifuged at $50\,000 \times g$ for 2 h. The fraction floating above 1.2 M sucrose, the synaptosomes, were collected for incubation with γ-aminobutyric acid.

1-ml aliquots of the synaptosomes (about 3 mg protein) were incubated with γ-amino[^{14}C]butyric acid (3, 16 and 1600 μM) at 25 °C for 10 min, in 14 ml of buffer (120 mM NaCl, 10 mM KCl, 2.5 mM $MgCl_2$ and 25 mM Tris, pH 7.4). After incubation, the samples were cooled and centrifuged at $31\,000 \times g$ for 30 min. The supernatant was decanted; the pellet rinsed lightly with 5 ml of cold buffer, and re-centrifuged. The rinsed pellet was solubilized with 1 ml of Protosol and counted with the addition of 10 ml of Aquasol in a Packard Tri-Carb liquid scintillation counter, using [^{14}C]toluene as internal standards. To test if any of the γ-aminobutyric acid taken up by the synaptosomes were bound to any of the membrane lipid components, the incubated synaptosomes were extracted with chloroform/methanol (2 : 1, v/v) and the lipid extract spotted on silica gel G thin-layer chromatography plates. The plates were developed with chloroform/methanol/water (120 : 70 : 17, v/v), twice in the same direction. Non-radioactive lipid extracts and unlabelled γ-aminobutyric acid were run along side of the radioactive lipid extract to locate the various lipid and γ-aminobutyric acid spots by spraying with ninhydrin or resorcinol or exposure to iodine. The radioactive strip was scraped off in 1 cm segments and each segment was

then counted with 10 ml of Aquasol in a Packard Tri-Carb liquid scintillation counter.

Preparation of the junctional complex. The methods of Cotman and Taylor [17] and Davis and Bloom [18] were followed. Whole rat cerebellum were homogenized in 10 volumes of 0.32 M sucrose (containing 50 μM $CaCl_2$ and 1 mM $MgCl_2$, pH 7) at 4 °C in a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle. The crude nuclei fraction was removed by centrifugation at $1000 \times g$ for 11 min, and washed once with 0.32 M sucrose. The combined supernatants were spun at $17\,600 \times g$ for 15 min to pellet the P_2 fraction which was then homogenized in 10 ml of 50 μM $CaCl_2$ per g of brain, and left at 4 °C for 15 min. The mixture was centrifuged at $23\,700 \times g$ for 30 min, to pellet the P_2 · water fraction which was then treated with 7–8 volumes of 0.08 M sodium succinate, 0.5 mg of iodonitrotetrazolium violet per ml, 50 μM $CaCl_2$ and 0.04 M sodium phosphate buffer, pH 7. After incubation at 30 °C for 20 min, the mixture was centrifuged at $23\,700 \times g$ for 20 min, and the pellet washed twice with 10 ml of 0.16 M sucrose containing 50 μM $CaCl_2$ and recentrifuged at $23\,700 \times g$ for 20 min. The pellet, P_2 · iodonitrotetrazolium violet, was layered onto a discontinuous sucrose gradient (3 ml of 0.8 M, 2.5 ml of 0.9 and 1.0 M and 3 ml of 1.2 M sucrose). After centrifuging at $50\,400 \times g$ for 2 h, the bands containing the synaptic plasma membranes were collected and diluted to 0.32 M with 50 μM $CaCl_2$ and pelleted at $23\,700 \times g$ for 45 min. This pellet was resuspended in a pH 7.5 solution containing 50 μM $CaCl_2$, 0.12 % Triton X-100 and 1 mM sodium ethylenediamine tetraacetic acid, such that the protein concentration was 1 mg/ml. After the Triton treatment (10 min at 4 °C), the solution was centrifuged at $50\,400 \times g$ for 30 min. The pellet was homogenized in 5 ml of 1 M sucrose and centrifuged at $159\,000 \times g$ for 35 min to float out the light broken membrane fragments. The pellet was rinsed with Ringers' solution once and then resuspended in Ringers' solution for incubation studies. The composition of the Ringers' solution is: 120 mM NaCl, 5 mM KCl, 1.24 mM $MgCl_2$, 2 mM $CaCl_2$ and 1 mM Tris, pH 7.4.

Enzyme assays. $(Na^+ + K^+)$-ATPase activity (in μmol of phosphorus/h per g) was measured by the method of Hosie [19]; acetylcholinesterase activity (in μmol of acetylcholine hydrolyzed/h per g) by the method of Ellman et al. [20]; fumarase activity (in units/g where 1 unit-change of absorbance of 0.01/min) by the method of Fonnum [21]; lactate dehydrogenase (in change of absorbance/min per g) by the method of Johnson [22]; 5′-nucleotidase activity (in μmol of phosphorus/h per g) by the method of Israel and Frachon-Mastour [23]. Protein was determined by the method of Lowry et al. [24].

Binding of γ-aminobutyric acid to the functional complex. To measure the total binding of γ-aminobutyric acid to the junctional complex, 0.5 ml of the junctional complex suspension (containing 0.1–0.2 mg of protein) was incubated with 0.5 ml of γ-amino[^{14}C]butyric acid (800 000 dpm), sufficient unlabelled γ-aminobutyric acid to reach the desired final concentration and 0.5 ml of [^{3}H]sucrose (3 000 000 dpm). The [^{3}H]sucrose, which when purchased was greater than 98 % pure, was further purified by paper chromatography prior to use, and was used as a marker of entrapped space in the tissue. After incubating at 25 °C for 10 min, the preparation was cooled and centrifuged at $176\,000 \times g$ for 20 min. The supernatant was removed and the pellet resuspended in three 0.2-ml aliquots of distilled water, solubilized with 1.4 ml of Protosol, 10 ml of Aquasol added and counted in a Packard Tri-Carb liquid scintillation counter. To measure non-specific binding, a 1000-fold excess of unlabelled

γ-aminobutyric acid was added, preincubated for 5 min, and then γ-amino[^{14}C]-butyric acid was added and incubated for 10 min. The difference between total and non-specific binding represents specific binding [25, 26]. All binding studies were carried out at least in duplicate, and the means are recorded in the tables. These duplicates varied less than 5 %.

To test the effect of antagonists, the junctional complexes were pretreated with the antagonists for 5 min, and then γ-amino[^{14}C]butyric acid was added and incubated for 10 min. The final concentrations of γ-aminobutyric acid and antagonists were 19 and 85 μM, respectively. *N*-Methylbicuculline was prepared from bicuculline by the method of Pong and Graham [27]. Picrotoxinine was separated from picrotin by the method of Jarboe and Porter [28]. To measure the effects of ions on the binding of γ-aminobutyric acid to the junctional complexes, Ringers' solution minus NaCl, or Ringers' solution minus $CaCl_2$ plus 10 mM EDTA were used for the incubation media.

Incubations with phospholipases and proteases. Aliquots of the junctional complex preparation (containing 0.1–0.2 mg of protein) were treated with the phospholipase solution at 25 °C for 20 min. Then γ-amino[^{14}C]butyric acid and [^{3}H]-sucrose were added, in the presence and absence of excess unlabelled γ-aminobutyric acid to measure non-specific and total binding as described above.

To measure the splitting of phospholipids by phospholipase A_2, incubated tissue pellets were extracted with butanol overnight [29], and then partitioned with *n*-butanol-saturated water. The butanol extract was evaporated to dryness, redissolved in chloroform/methanol (2 : 1, v/v) and spotted on 6×6 cm preactivated silica gel plates. The plates were developed with chloroform/methanol/water (13 : 5 : 0.8, v/v) in the first dimension, and with 3-heptanone/acetic acid/water (8 : 6 : 1, v/v) in the second dimension. The spots on the plates were detected with iodine vapor, scraped off and analyzed for phosphorus using Bartlett's method [14] adapted to a micro scale. To the silica gel in the test tube, 0.1 ml of concentrated H_2SO_4 was added, and then heated at 240 °C for 30 min. A few drops of 30 % H_2O_2 was added and heated again for 30 min to whiten the gel. The cooled hydrolyzate was then reacted with 0.1 ml of 1 % ammonium molybdate and 0.05 ml of Fiske-SubbaRow reagent, h ated at 100 °C for 10 min, cooled to room temperature, and absorbance read at 830 nm in a micro silica cuvette. The percent splitting of phospholipids by phospholipase A_2 was calculated as an increase in lysophosphatides over control, or as a decrease in the phospholipids over the control. In general, there was a good correlation between the two methods.

To measure the splitting of phospholipids by phospholipase C, the method of Rosenberg [30] was followed. Briefly, incubated tissue pellets were extracted with chloroform/methanol (2 : 1, v/v) overnight and then partitioned with 0.2 volume of water. The upper phase together with the interface rinses, and the lower phase were evaporated to dryness, combusted and then assayed for inorganic phosphorus. The tissue residue after the organic solvent extraction was extracted with acidified water. This aqueous extract was also combusted and assayed for phosphorus. The incubation medium was treated in the same manner as the tissue pellet described above. To calculate the percent splitting of phospholipids: total lipid phosphorus equals phosphorus of the lower phases from the tissue and incubation medium extracts; total aqueous phosphorus equals phosphorus of the upper phases of the tissue and incuba-

tion medium extracts plus the acidified water rinse; percent splitting equals increase in total aqueous phosphorus $\times 100$/total lipid phosphorus.

For incubation with pronase and trypsin, junctional complex aliquots (0.1–0.2 mg of protein) were pretreated with 0.9 mg of the enzyme per ml at 25 °C for 20 min and specific and non-specific binding of γ-aminobutyric acid determined. To measure the splitting of protein by the proteolytic enzymes, the incubated junctional complex was diluted with 8 ml of ice-cold Ringers' and centrifuged at 176 000 $\times g$ for 20 min. The pellet was resuspended in two 0.2-ml aliquots of distilled water and assayed for protein by the method of Lowry et al. [24].

Competition between γ-aminobutyric acid and phospholipids for binding to the junctional complex. Junctional complex aliquots (containing 0.1–0.2 mg protein) were incubated with 19 μM γ-amino[^{14}C]butyric acid in the presence and absence of sonicated phospholipids (71 μg of phosphatidylethanolamine and 57 μg of phosphatidylcholine and phosphatidylserine per ml) at 25 °C for 10 min. The specific and non-specific binding were determined as described above. In other experiments, solutions containing phosphatidylethanolamine only, and phosphatidylcholine only were used to assess the effect of each phospholipid on the binding.

RESULTS

The junctional complex preparation. In agreement with Davis and Bloom's finding [18], we found that iodonitrotetrazolium violet was very effective in eliminating mitochondrial contamination as judged by measurement of fumarase activity. Synaptic plasma membranes prepared with iodonitrotetrazolium violet contain 5 % of the total fumarase activity while synaptic plasma membranes prepared without iodonitrotetrazolium violet contain 29.6 % of the total activity. The junctional complex preparation contains no detectable fumarase activity.

Triton X-100 solubilizes about one-third of the synaptic plasma membrane protein, greater than 90 % of the ($Na^+ + K^+$)-ATPase activity and 62 % of the acetylcholinesterase activity, however, it solubilizes only about 8 % of the 5′-nucleotidase activity (Table I). When 1 M sucrose is used after treatment with Triton, to float out the lighter membrane fragments, the specific activity of 5′-nucleotidase is further increased to 10.65 μmol/h per mg protein (specific activity for synaptic plasma membranes is 3.92). The enzyme 5′-nucleotidase is used as a marker for our junctional complex preparation. The yield of the junctional complex fraction prepared with iodonitrotetrazolium violet, sucrose gradients, Triton X-100 and 1 M sucrose is 0.35 mg protein/g wet weight of cerebellum.

Electron microscopy of a Triton-treated synaptic plasma membrane fraction shows the junctional complex as dense barlike structures of pre- and postsynaptic membranes joined by a synaptic cleft.

Kinetics of γ-aminobutyric acid binding to the junctional complex. Our preliminary experiments showed that there is no difference in binding at 1 or 10 min of incubation with γ-amino[^{14}C]butyric at 25 °C. 10 min was chosen as the routine time for measurements because previous investigators have used this time period, and equilibrium has been reported to occur within 5 min [31]. It can be seen (Fig. 1) that the specific γ-aminobutyric acid binding appears saturable and sigmoidal in shape. This conclusion is, however, tentative since saturation was only observed at one

TABLE I

THE EFFECT OF TRITON X-100 ON VARIOUS ENZYME MARKERS

Synaptic plasma membranes were treated with Triton X-100 containing 3 mM $CaCl_2$, 3 mM Tris, pH 8, at a Triton/protein ratio of 1.2/1.0, at 4 °C for 10 min. Protein is expressed in mg/g brain; acetylcholinesterase in μmol of acetylcholine hydrolyzed/h per g; 5′-nucleotidase in μmol of phosphorus released/h per g; fumarase in units/g where 1 unit = change of absorbance of 0.01/min; lactate dehydrogenase in change of absorbance/min per g; ATPase in μmol of phosphorus/h per g. The relative specific activity is calculated as percent distribution of enzyme activity/percent distribution of protein after Triton treatment. When the synaptic plasma membrane fractions M(0.9), M(1.0), M(1.2) were pooled and then treated with Triton X-100, the fumarase activity was 3.249, indicating that 98.5 % of the original activity was solubilized by the detergent. n.d., not determined. M, original crude mitochondrial fraction; M(0.8) = myelin; M(0.9), M(1.0) and M(1.2) = synaptic plasma membrane; M(p) = mitochondria. The percent recoveries are: protein, 93; acetylcholinesterase, 43; 5′-nucleotidase, 90; ATPase, 96; fumarase, 102; lactate dehydrogenase, 96.

Measurement	Fraction					
	M	M(0.8)	M(0.9)	M(1.0)	M(1.2)	M(p)
Protein						
Before	30.691	8.341	4.271	3.238	4.481	6.984
After	n.d.	6.426	2.738	2.228	3.157	5.730
Solubilized (%)	n.d.	22.96	35.89	31.19	29.55	17.96
Acetylcholinesterase						
Before	36.70	6.16	4.33	2.01	2.19	1.03
After	n.d.	3.20	1.30	0.92	0.83	0.57
Solubilized (%)	n.d.	48.05	69.98	54.23	62.10	44.66
Relative specific activity	n.d.	1.01	1.76	1.08	0.85	0.26
5′-Nucleotidase						
Before	85.70	30.04	20.82	12.72	13.52	4.30
After	n.d.	26.99	19.57	11.78	11.98	3.80
Solubilized (%)	n.d.	10.15	5.98	7.40	11.4	11.63
Relative specific activity	n.d.	1.27	1.39	1.39	1.07	0.22
ATPase						
Before	623.40	37.40	158.5	211.8	187.0	3.77
After	n.d.	n.d.	6.03	6.77	17.6	n.d.
Solubilized (%)	n.d.	n.d.	96.2	96.8	90.6	n.d.
Fumarase						
Before	654.00	85.94	8.33	33.49	174.8	425.8
Lactate dehydrogenase						
Before	7.75	4.12	1.03	0.88	1.27	0.14

concentration, the highest used. The non-specific binding, on the other hand, is apparently not saturable. The maximal specific binding capacity is about 555 pmol/mg protein at 191 μM γ-aminobutyric acid concentration, K_m about 74 μM. Analysis of these equilibrium data by double-reciprocal plot (Fig. 2), and Hill plot (Fig. 3), suggest positive cooperativity; the Hill coefficient = 2.2.

While the data above show that the binding of γ-aminobutyric acid is specific in the sense that it is displaceable by excess unlabelled γ-aminobutyric acid, Table II shows that the binding is also physiologically specific, i.e. it binds to sites having affinity characteristics appropriate for a γ-aminobutyric acid receptor. The specific binding of γ-aminobutyric acid is antagonized by *N*-methylbicuculline, picrotoxinin and imidazoleacetic acid and unaffected by curare, strychnine and 2,4-diaminobutyric acid.

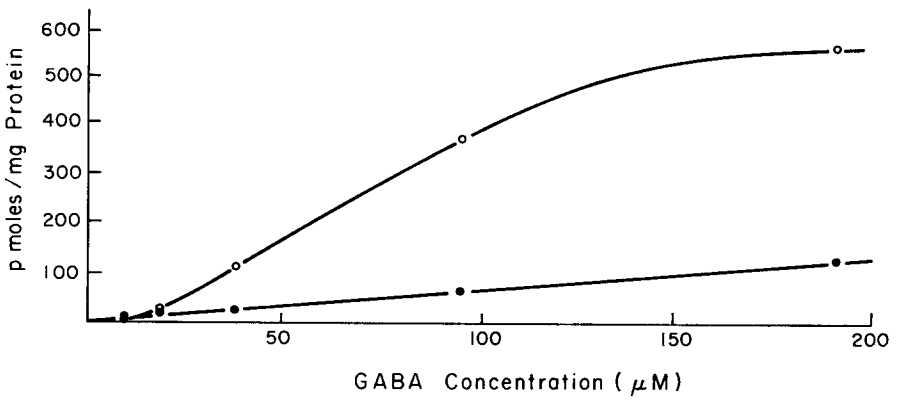

Fig. 1. Binding of γ-amino[^{14}C]butyric acid to the junctional complex. Junctional complex aliquots were incubated with γ-amino[^{14}C]butyric acid, unlabelled γ-aminobutyric acid and [^{3}H]-sucrose at 25 °C for 10 min as described in Materials and Methods. Non-specific binding (●–●) was measured in the presence of a 1000-fold excess unlabelled γ-aminobutyric acid. Specific binding (○–○) was obtained by subtracting the non-specific from the total binding. Binding is expressed as pmol/mg protein; concentration of γ-aminobutyric acid is in μmolar. Each point is the mean of duplicate determinations which varied less than 5 %.

The effect of ions on the binding of γ-aminobutyric acid to the junctional complex is shown in Table III. The omission of Na^+ or Ca^{2+} from the incubation medium has no effect on the specific binding, however, the omission of Ca^{2+} together with the addition of 10 mM EDTA decreased the non-specific binding by 80 %.

Effect of phospholipase on the binding of γ-aminobutyric acid to junctional complexes. The percent distribution of phospholipids in our preparation of junctional complexes (Table IV) is similar to that of the synaptic plasma membrane preparation [32]. The effect of phospholipase A_2 on phospholipids and on the binding of γ-aminobutyric acid to the junctional complex is shown in Fig. 4. There is a dose-related increase in the splitting of phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine with increasing phospholipase A_2 concentration, the splitting reaching a maximum at a phospholipase A_2 concentration of 250 μg/ml. The percent decrease in binding of γ-aminobutyric acid to the junctional complex preparation is also correlated with phospholipase A_2 concentration. The inhibition of binding reached 85 % at a phospholipase A_2 concentration of 250 μg/ml. Besides phospholipase A_2, detergents such as lysolecithin (50 μg/ml), and sodium dodecyl sulfate (100 μg/ml) also caused complete inhibition of binding, suggesting that the effects of phospholipase A_2 might be non-specific, and due to products of phospholipase A_2 treatment, i.e. lysophosphatides, which have detergent properties.

The effect of phospholipase C on phospholipids and on the binding of γ-aminobutyric acid to the junctional complex preparation is shown in Fig. 5. There is extensive splitting of phospholipids by phospholipase C. At the highest concentration of phospholipase C used (10 mg/ml), the following percentages of phospholipids are split: sphingomyelin, 74; phosphatidylcholine, 86; phosphatidylethanolamine, 63 and phosphatidylserine, 46. Concurrent with the splitting of phospholipids, the enzyme causes an increase in total and specific binding of γ-aminobutyric acid. Phospholipase

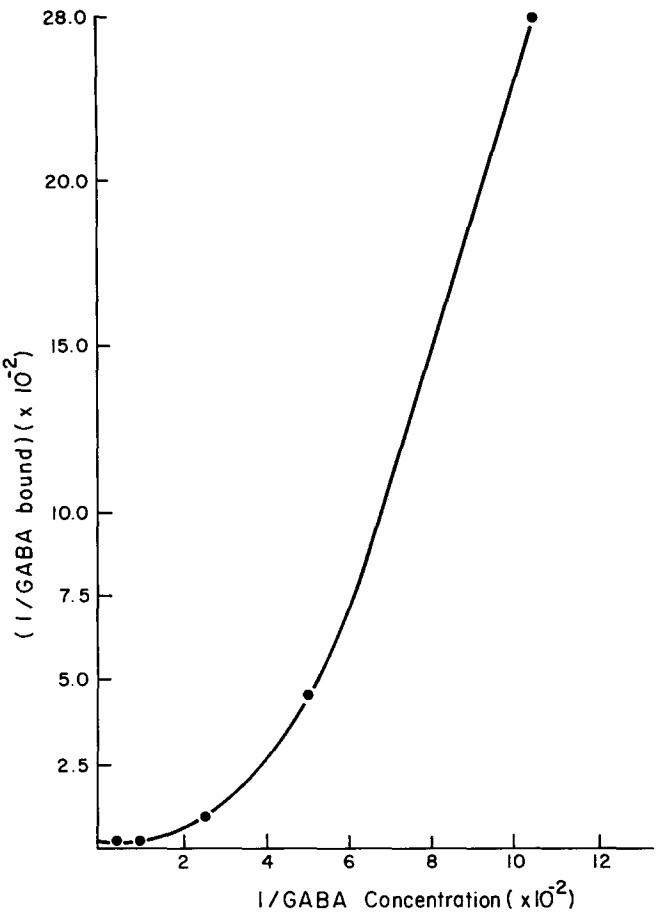

Fig. 2. Double-reciprocal plot of the specific binding of γ-aminobutyric acid to the junctional complex. Incubations were carried out at 25 °C for 10 min as described in the text. γ-Aminobutyric acid concentration is in μmolar; γ-aminobutyric acid bound is expressed as pmol/mg protein.

C (10 mg/ml) caused about a 70 % increase in total binding, and about a 260 % increase in specific binding, while the non-specific binding was abolished. Thus, the splitting of the polar head groups of the phospholipids seems to enhance the binding of γ-aminobutyric acid to its receptor.

Competition between phospholipids and γ-aminobutyric acid for binding to the junctional complexes. Phospholipids compete with γ-aminobutyric acid for binding to the receptor protein. In the presence of a mixture of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine (71, 57, 57 μg/ml, respectively), the specific binding decreased 65 % while the non-specific binding increased 88 %. The total binding was not significantly changed when individual phospholipids were used, phosphatidylethanolamine seemed to be more potent than phosphatidylcholine, decreasing specific binding 100 % while phosphatidylcholine decreased it by only 47 %.

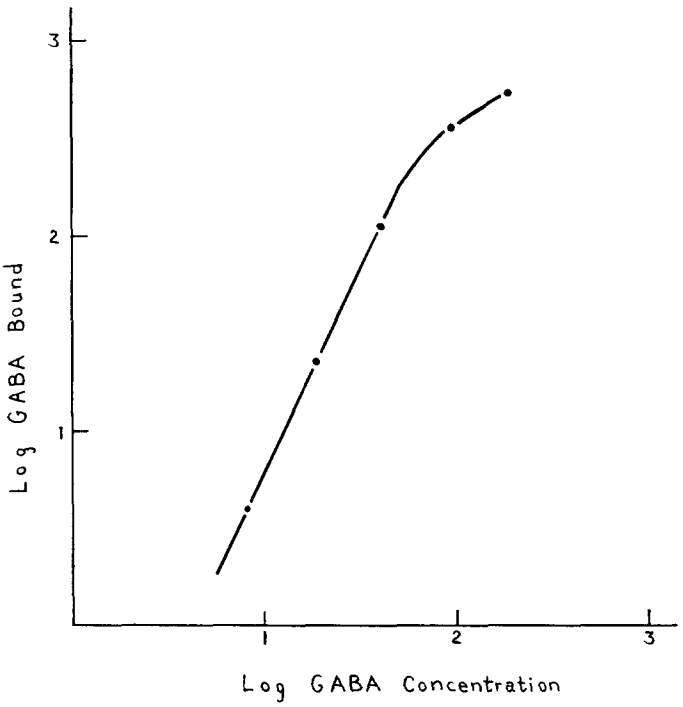

Fig. 3. Plot of the log of γ-aminobutyric acid concentration vs. the log of γ-aminobutyric bound (specific). The Hill coefficient, calculated from the linear portion of the graph, is 2.2. γ-Aminobutyric acid concentration is in μmolar; γ-aminobutyric acid bound is expressed as pmol/mg protein.

Effect of proteolytic enzymes on γ-aminobutyric acid binding to the junctional complexes. Pretreatment of the junctional complex preparation with trypsin and pronase (0.9 mg/ml) decreased the specific binding of γ-aminobutyric acid by 18 and 100 %, respectively. Trypsin also decreased non-specific binding by 52 %, while pronase had no effect. Trypsin split 30.5 % of the junctional complex protein, while pronase split 67.5 % of the junctional complex protein.

Studies with synaptosomes. We were interested to test if any γ-aminobutyric acid taken up by synaptosomes was associated with membrane lipids since according to Watkins [12], γ-aminobutyric acid would bind to the protein or to the phospholipid of a protein-phospholipid complex at the receptor site.

We found, in agreement with the literature [33, 34], that the uptake of γ-aminobutyric acid into cerebellar synaptosomes (25 °C, 10 min) is a saturable process. When incubated synaptosomes were extracted with chloroform/methanol (2 : 1, v/v) and spotted on thin-layer chromatographs, it was found that no radioactivity was associated with gangliosides or phospholipids. The percent distribution of radioactivity on the chromatograms of the lipid extract closely mimics that of free γ-aminobutyric acid containing approximately the same amount of radioactivity. This may be caused by a complete dissociation of the bound γ-aminobutyric acid to its free form during the process of organic solvent extraction. In any case, the uptake of γ-aminobutyric acid into synaptosomes is a different process from binding to receptor sites,

TABLE II

EFFECT OF DRUGS ON THE BINDING OF γ-AMINOBUTYRIC ACID

Junctional complexes (0.1–0.2 mg of protein) were incubated with 19 μM γ-aminobutyric acid and 86 μM drug at 25 °C for 10 min in the presence and absence of excess unlabelled γ-aminobutyric acid to determine non-specific and specific binding. All determinations were done in duplicate, which varied less than 6 %.

	Percent decrease in specific binding	Percent change in non-specific binding
Imidazoleacetic acid	95	+16
L-2,4-diaminobutyric acid	4	+15
N-Methylbicuculline	94	+ 1
Picrotoxinine	97	−11
Curare	5	− 4
Strychnine	0	− 2

TABLE III

EFFECT OF REMOVAL OF Na^+ AND Ca^{2+} FROM THE INCUBATION MEDIA ON THE BINDING OF γ-AMINOBUTYRIC ACID TO JUNCTIONAL COMPLEXES

Junctional complexes (containing 0.1–0.2 mg of protein) were incubated with 19 μM γ-amino[^{14}C]-butyric acid in the presence and absence of excess unlabelled γ-aminobutyric acid, at 25 °C for 10 min, in order to determine non-specific and specific bindings as described in the text. To test the effect of Na^+ on the binding, NaCl was omitted from the incubation medium; to test the effect of Ca^{2+}, Ca^{2+} was omitted from the incubation medium, and EDTA was added to a final concentration of 10 mM. All solutions were adjusted to pH 7.4.

	Total		Non-specific		Specific	Percent decrease in non-specific
Control	82.8,	76.1	36.6,	35.6	43.4	–
$-Ca^{2+}$, +EDTA	52.0,	51.0	9.1,	5.1	43.9	80
$-Na^+$	78.0,	77.8	32.6,	31.9	45.7	11

TABLE IV

PERCENT DISTRIBUTION OF PHOSPHOLIPIDS IN THE JUNCTIONAL COMPLEX

Phospholipids from junctional complexes were extracted with *n*-butanol as described in the text. The values are from two experiments. The junctional complex contained about 30 μg of lipid phosphorus per mg of protein.

Phospholipid	Distribution (%)	
Lysolecithin	3.10	4.51
Lysophosphatidylserine	1.17	1.86
Spingomyelin	9.89	7.94
Phosphatidylcholine	46.19	46.23
Phosphatidylserine	8.72	9.80
Phosphatidylethanolamine	30.93	29.66

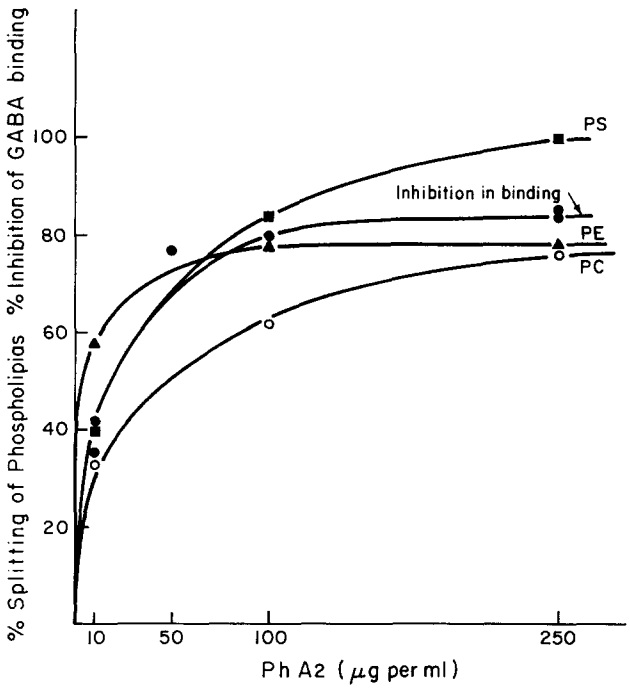

Fig. 4. Effect of phospholipase A_2 on junctional complexes. Aliquots of the junctional complex preparation were pretreated with phospholipase A_2 at 25 °C for 20 min. Then γ-amino[^{14}C]butyric acid was added, and the total binding was determined as described in the text. ●–●, the percentage inhibition in total binding. Also, incubated junctional complexes were extracted with butanol to determine the percent splitting of phospholipids: ■–■, phosphatidylserine; ▲–▲, phosphatidylethanolamine, and ○–○, phosphatidylcholine.

and whatever happens to γ-aminobutyric acid transported does not reflect on the events happening at the receptor site. Thus, while synaptosomes are the best preparation to study the transport phenomena, the junctional complex is more suitable for studying binding to receptor sites.

Studies with tissue slices. Cortical slices were incubated with γ-amino[^{14}C]-butyric acid and then extracted with chloroform/methanol to see if there is any change in total lipid phosphorus, total lipid protein or proteolipid protein. According to Watkins [12], γ-aminobutyric acid might bind to the protein of the protein-phospholipid complex, releasing the phospholipid; this dissociation of the protein-phospholipid complex should therefore result in a decrease in the level of extractable proteolipid. We found a significant decrease ($p < 0.05$), using a paired sample t test, in the level of proteolipid protein and total lipid protein, the mean percent decreases being 13.9±5.6 and 24.8±7.3 %, respectively, but no significant change in total lipid phosphorus. The control total lipid phosphorus, total lipid protein and proteolipid protein values (μg/mg total protein) were (mean±S. E.) 10.23±0.59, 16.25±1.36 and 6.54±0.90, respectively. Each of the above values and percent changes are the means of eight experiments.

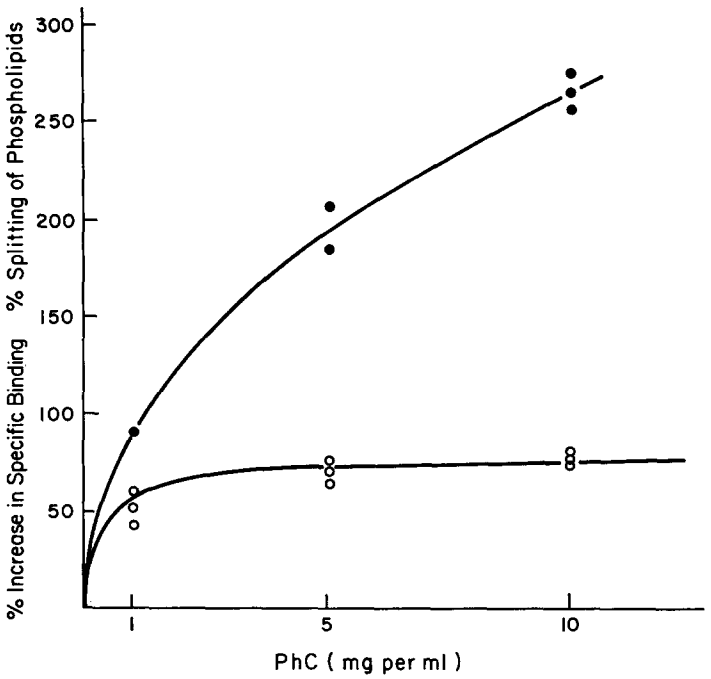

Fig. 5. Effect of phospholipase C on junctional complexes. Aliquots of junctional complex preparation (0.1–0.2 mg of protein) were pretreated with phospholipase C at 25 °C for 20 min, and then incubated with 19 μM γ-amino[^{14}C]butyric acid for 10 min, in the presence and absence of excess unlabelled γ-aminobutyric acid in order to measure non-specific and specific binding. The phospholipase C treated junctional complexes were also extracted with chloroform/methanol (2 : 1, v/v) to measure the splitting of phospholipids. ●–●, percent increase in specific binding: ○–○, percent splitting of total phospholipids.

DISCUSSION

Our goal in this study was to isolate a tissue fraction with high affinity for γ-aminobutyric acid and to study some possible biochemical interactions of the neurotransmitter and phospholipids with the receptor protein, as proposed by Watkins [12]. Incubation of cortical slices with 10 mM γ-aminobutyric acid for 1 h caused a significant (25 %) decrease in the level of proteolipid protein, which is in agreement with Watkins' hypothesis. However, the fact that the cortex is composed of various molecular layers with various cell populations that may have different affinities for γ-aminobutyric acid, and various amounts of proteolipids, makes it doubtful that the tissue slices are uniform enough for comparing changes in proteolipids after incubations with γ-aminobutyric acid. Therefore, we decided to use a more homogeneous system, the synaptosomes. When synaptosomes were incubated with μM concentrations of γ-aminobutyric acid, and then extracted with chloroform/methanol (2 : 1, v/v), it was found that γ-aminobutyric acid was present in the free, unbound form, none of it being bound to the membrane phospholipids, proteolipids or gangliosides. This may be due to the dissociation of binding induced by the extraction media; however even if truly reflecting the in vivo situation, this might only

represent γ-aminobutyric acid transported into the synaptosomes and not necessarily the interaction of γ-aminobutyric acid with postsynaptic sites, which might be a small fraction of the amount transported. The transported and postsynaptic actions of γ-aminobutyric acid have been shown to be two distinctly separate processes [34, 35].

The junctional complex is the best tissue preparation for studying receptors. It is most enriched in synaptic materials, and devoid of contaminations by mitochondria, axonal and membrane fragments. Electron micrographs showed that the preparation contained bars of presynaptic and postsynaptic membranes joined by the synaptic cleft. Enzymatic analysis indicated that it has no $(Na^+ + K^+)$-ATPase or fumarase activities. Since γ-aminobutyric acid also participates metabolically in the tricarboxylic acid cycle, the junctional complex preparation allows us to study the junctional receptor pool of γ-aminobutyric acid as distinct from its metabolic pool. Since there are no nerve ending vesicles present, it allows us also to measure receptor binding without interference from the transport process.

This junctional complex preparation binds γ-aminobutyric acid in a specific fashion. The binding is displaceable by excess unlabelled γ-aminobutyric acid, is readily reversible (almost all radioactivity was lost upon rinsing with Ringers' solution) and is rapid, whether at 4 or 37 °C (unpublished observations). The binding is also physiologically specific, being antagonized by its physiological antagonists, methylbicuculline, picrotoxinine and imidazoleacetic acid, and unaffected by curare (a cholinergic antagonist), strychnine (a glycine antagonist) and L-2,4-diaminobutyric acid. The effects of the γ-aminobutyric acid analogues (imidazoleacetic acid and L-2,4-diaminobutyric acid) on the specific binding also demonstrates that γ-aminobutyric acid is being bound to the postsynaptic receptor site rather than to the presynaptic uptake site. Imidazoleacetic acid, which acts postsynaptically to inhibit cortical neurons [36, 37] markedly inhibits the specific binding, while diaminobutyric acid, which inhibits uptake without any neurophysiological activity [38] has almost no effect on the specific binding. Note too that bicuculline, which has been shown to be a potent antagonist of γ-aminobutyric acid without any effect on the uptake [35] also blocks the specific binding.

By analyzing the kinetic data of binding, it was found that γ-aminobutyric acid exhibits positive cooperativity with a Hill coefficient of 2, suggesting that the γ-aminobutyric acid receptor is oligomeric, with multiple binding sites per molecule. Takeuchi and Takeuchi [8] proposed that the combination of two molecules of γ-aminobutyric acid is necessary to activate the γ-aminobutyric acid receptor in crayfish. Similar studies on locust muscle fibers suggest that three molecules may be required to activate the γ-aminobutyric acid receptor in this preparation [39]. Our data are in agreement with these physiological observations. From our kinetic data, we also calculated an approximate density of γ-aminobutyric acid binding sites of about 27 000 molecules/μm^2. For comparison, the density of α-toxin and α-bungarotoxin binding sites in electric tissue was estimated to be 35 000 and 10 000/μm^2, respectively [40, 41].

All the data above: the saturability and reversibility of binding, the pharmacological specificity and the resemblance to the physiological situation, support the conclusion that we are measuring the specific binding of γ-aminobutyric acid to the postsynaptic receptor site.

In studying the effect of ions on the binding of γ-aminobutyric acid to the junctional complex, we found that Na^+ or Ca^{2+} does not seem to be required for the

binding process. However, it is interesting to note that EDTA caused an 80 % decrease in non-specific binding. Lacombe and Hanoune [42] also observed an enhanced specificity of epinephrine binding to rat liver plasma membranes in the presence of EDTA.

In order to study the possible biochemical interactions of γ-aminobutyric acid and phospholipids with the receptor protein, phospholipases and proteases were used. Phospholipase A_2 inhibited the binding of γ-aminobutyric acid, an effect which is probably non-specific due to the disruption of the membrane structure and release of proteins by the detergent action of lysophosphatides, produced as a result of phospholipase A_2 action. Lysolecithin and sodium dodecyl sulfate also inhibited binding. This conclusion is similar to that of Rosenberg and coworkers [43–46] using the squid giant axon. In this regard, it is interesting to note that lysolecithin has, in fact, been utilized by Denburg [47] to solubilize the cholinergic binding macromolecule from the axon plasma membrane of the lobster walking leg.

The results with phospholipase C and proteases are in agreement with Watkins' hypothesis. Phospholipase C enhanced the specific binding by 260 % while pronase and trypsin abolished the specific binding. According to Watkins', γ-aminobutyric acid would be competing with the phospholipid polar head groups for binding to the receptor protein; a removal of the phospholipid polar head group should increase the number of γ-aminobutyric acid molecules able to bind to the protein receptor, and a destruction of the receptor protein should abolish binding. This is exactly what we have observed. We were also able to demonstrate a competition between γ-aminobutyric acid and phospholipid for binding to the receptor. In the presence of phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine, the specific binding decreased 65 %. Phosphatidylethanolamine is much more effective than phosphatidylcholine, which is also in accordance with Watkins' hypothesis. Note that only the specific binding is decreased by phosphatidylethanolamine, not the total binding, which provides evidence that the phospholipid is not simply interacting with the γ-aminobutyric acid in solution, decreasing the effective concentration of γ-aminobutyric acid available to the receptor. If this were the case, we would expect a decrease in total binding as well as the specific binding.

De Robertis and De Plazas [48] have measured the binding of γ-aminobutyric acid to hydrophobic proteins extracted from the shrimp muscle with chloroform/methanol (2 : 1, v/v). Though there are differences between their findings and ours (e.g. they found that picrotoxin has no effect on the binding and they did not observe any cooperativity in the binding), their conclusion that the γ-aminobutyric acid receptor is a hydrophobic protein is in agreement with our findings. Specifically, we have provided evidence in support of Watkins' hypothesis that γ-aminobutyric acid competes with phosphatidylethanolamine for binding to the receptor protein, thus dissociating the lipid-protein complex.

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