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FUNCTIONAL MEMBRANE VESICLES FROM THE NERVOUS SYSTEM OF INSECTS

I. SODIUM- AND CHLORIDE-DEPENDENT γ-AMINOBUTYRIC ACID TRANSPORT

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(1) A synaptosomal fraction obtained from locust nervous tissue has been shown to possess an active γ-aminobutyric acid transport mechanism. This activity is preserved and even enriched by the membrane vesicles derived from osmotically shocked synaptosomes. (2) Electron-microscopy examination indicates that the above membrane vesicles are derived predominantly from the neuronal plasma membrane and are devoid of any internal cellular organelles and components. Active transport of γ-aminobutyric acid into these vesicles has been demonstrated with artificially imposed ion gradients as the sole energy source. (3) γ-Aminobutyric acid transport can be driven by an Na^+ gradient (out>in) and/or by a gradient of Cl^- (out>in). This process is absolutely dependent on the simultaneous presence of both types of ion in the external medium. The stimulation of the process by valinomycin indicates that γ-aminobutyric acid transport is an electrogenic process which is stimulated by a membrane potential (interior negative).

Introduction

A certain structural and functional uniqueness of insect neuronal membranes was indicated by the detection of insect-selective toxins derived from scorpion venoms [1]. Those bind exclusively to insect neuronal membranes [2] and affect their sodium conductance [3,4]. Our knowledge of the biochemistry of insect neuronal membranes is limited when compared to those derived from mammalian sources. Any significant progress in insect neurochemistry demands a reliable functional preparation derived from the insect nervous system. In mammals the pinched-off nerve terminals (synaptosomes) [5,6] display the essential features of intact nerve endings, such as maintenance of transmembrane ion gradients and membrane potential [7,8], depolarization-induced neurotransmitter release [9,10] and its high affinity uptake [11], and action potential ion channels [10,12]. Recently synaptic plasma membrane vesicles have been obtained after osmotic shock of the mammalian synaptosomes. The vesicles are shown to be functional with regard to high affinity Na^+- and Cl^--dependent γ-aminobutyric acid transport [13,14].

Such functional preparations from insect neuronal tissues are not available. A preliminary but significant step in this direction was recently performed by Breer and Jeserich [15]. They used a flotation technique for the isolation of a synaptosomal fraction from the locust central nervous system. Its characterization was based on morphological (electron microscopy) and enzymatic criteria.

The aim of our study was to prepare pharmaco-

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logically functional insect neuronal membranes. Since γ-aminobutyric acid is a well-defined neurotransmitter in the insect central nervous system [16], we have used high-affinity active γ-aminobutyric acid transport as a criterion for the presence of closed functional vesicles. In order to clarify the dependence of this process on ion gradients and membrane potential we have also developed a preparation of membrane vesicles derived from the osmotically treated synaptosomes. Such preparation is of great advantage for further progress in insect neurochemistry and neuropharmacology.

Materials and Methods

Preparation of the synaptosomal fraction

The nervous system, comprising the dissected brains, subesophageal ganglia and the thoracic and abdominal regions of the ventral nerve cords, of adult *Locusta migratoria* locusts served as the source of neuronal tissue. The mean wet weight of one locust nervous system is 20.30 mg (S.D. ± 2.5(7)). All operations were performed in ice-cold mannitol buffer (0.25 M mannitol/1 mM EDTA, pH 7.3). The dissected tissues were manually homogenized in a volume of 25 ml per 100 nervous systems in a Wheaton Teflon glass homogenizer (10 up-and-down strokes). All centrifugations were performed with a Sorvall Superspeed RC2-B centrifuge, SS-34 rotor. The homogenate was centrifuged for 10 min at $1200 \times g_{av}$ (3250 rpm). The supernatant was collected and centrifuged for 15 min at $27000 \times g_{av}$ (15000 rpm). The pellet (P_2) was resuspended in a small volume (0.3–0.5 ml) of mannitol buffer and gently homogenized (three strokes). The synaptosomal fraction was further purified essentially according to the method of Breer and Jeserich [15]. To the resuspended P_2, 5 vol. Ficoll solution (12% w/v in mannitol buffer) were added and thoroughly mixed in a narrow centrifuge tube. The mixture was centrifuged for 75 min at $12100 \times g_{av}$ (10000 rpm). The floating pellicle was collected from the top of the Ficoll gradient using a Pasteur pipette. The pellicle was resuspended in mannitol buffer and centrifuged for 15 min at $27000 \times g_{av}$. The resulting pellet (P_2L), representing the enriched synaptosomal fraction, was finally resuspended in mannitol buffer. The pellet of the Ficoll gradient (P_2H), occasionally used, was washed and resuspended in mannitol buffer by the same procedure as described above.

Preparation of membrane vesicles

Membrane vesicles (mv) were prepared from the crude synaptosomal fraction (P_2) and from the enriched fraction (P_2L) by osmotic shock according to the method given by Kanner [13]. Each fraction was suspended in 5 mM Tris-HCl/1 mM EDTA (pH 7.4) (1:40,v/v) followed by slow stirring for 30 min at 4°C. The suspension was centrifuged for 15 min at $27000 \times g_{av}$. The resulting pellet was suspended in the loading solution (one locust nervous system equivalent per 200 μl) composed of 0.1 M potassium phosphate (pH 6.8)/1 mM $MgSO_4$ unless stated otherwise in the legends to the figures. After the loading step—incubation of 5 min at 37°C—the suspension was centrifuged for 15 min at $27000 \times g_{av}$. The resulting pellet was resuspended by gentle homogenization (three strokes) in a ratio of 10 μl loading solution per equivalent of nervous system.

It was noted that the membrane vesicles suspended in the loading solution (prior to the loading step) could be stored in liquid air without loss of transport activity for at least 6 weeks.

The indicated molarity of solutions always corresponds to the anion.

Transport assays

Transport assays were performed essentially as described for mammalian synaptosomes and membrane vesicles [11,13]. A sample of the resuspended P_2L synaptosomal fraction (10 μl) was added to 190 μl of a medium composed of 102.3 mM NaCl, 4.03 mM KCl, 1.05 mM $MgSO_4$, 20.16 mM Tris-HCl (pH 7.4), 8.87 mM glucose, 2.18 mM $CaCl_2$ and 1 μCi γ-amino[2,3-^{3}H]butyric acid (25 Ci/mmol) unless stated otherwise in the legends to the figures. In the case of membrane vesicles, 10 μl of the resuspended and loaded membrane vesicles (mvP_2 or mvP_2L) were added to 190 μl external solution consisting of 0.15 M NaCl plus 1 mM $MgSO_4$ and 1 μCi of γ-amino[2,3-^{3}H]butyric acid unless stated otherwise in the legends to the figures. Thus the buffer capacity of the external medium during transport is provided

by carry-over of the loading buffer (final concentration of 5 mM) upon dilution of the membrane vesicles. Ionophores, when present, were added to the external solution prior to the addition of the membranes. After incubation for various times at room temperature, reactions were terminated by the addition of 2 ml ice-cold 0.15 M NaCl and filtered through membrane filters (Schleicher and Schuell, 0.45 μm pore size). The filters were then washed with 2 ml ice-cold 0.15 M NaCl. Stopping the reaction, filtration and washing took about 15 s. The washed filters were dried and counted in a liquid scintillation counter. All experimental values were corrected for by subtracting zero-time values obtained by adding the membranes after the stop solution. Each experiment was performed at least three times. The results were reproducible, but the absolute values for transport varied from batch to batch. The radioactivity recovered on the filters was exclusively γ-aminobutyric acid. The radioactive substance was extracted from the filters with acid and subjected to thin-layer chromatography [11]. The R_F of this material was identical to that observed with authentic unlabelled or labelled γ-aminobutyric acid.

Protein determination

Protein was determined by a modified method of Lowry et al. [22].

Determination of total internal volume

This was performed according to Ref. 23. The external osmotic space and the total water were determined by [^{14}C]inulin and ^3H_2O, respectively. The total intravesicular space determined in this manner was found to be 5.6 ± 0.2 μl/mg protein.

Electron microscopy

Fractions P_2L, P_2H and mvP_2L were resuspended by gentle homogenization (three strokes) and fixed in ice-cold 2.5% gluteraldehyde in 0.1 M cacodylate/0.1 M sucrose buffer (pH 7.4) for 1 h at 4°C. The fixed material was sedimented by centrifugation for 15 min at $27000 \times g_{av}$. The pellets were subdivided into small fragments and postfixed in 1% osmium tetroxide in the above buffer for 1 h at 4°C; dehydrated in graded ethanols and stained by uranyl acetate at 70%

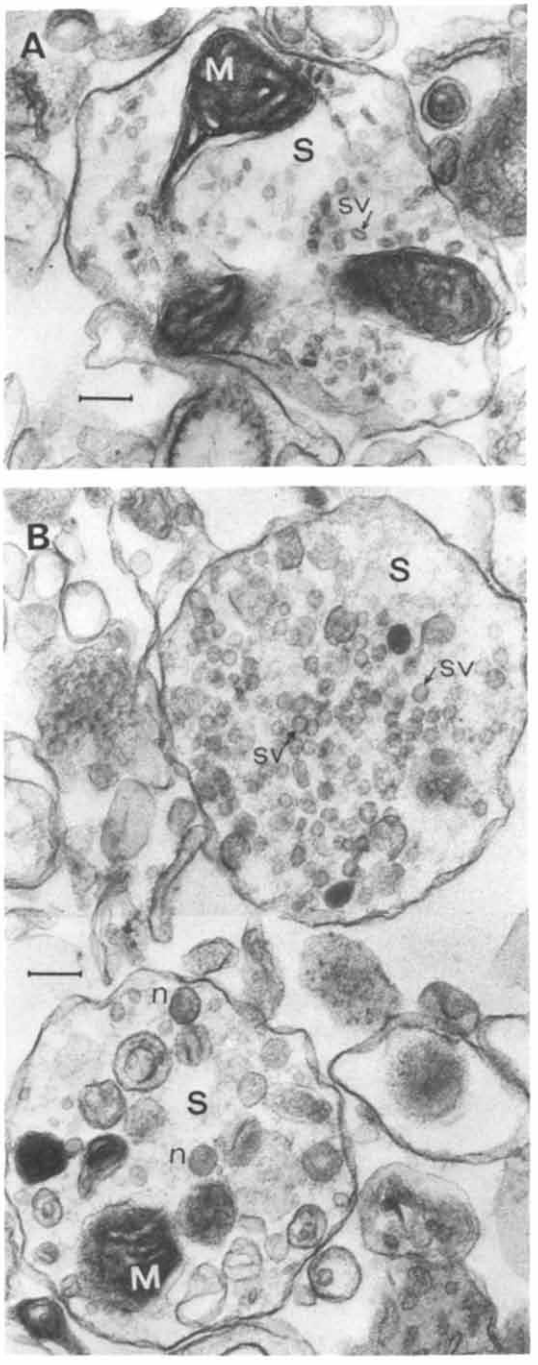

Fig. 1. Electron micrographs of typical synaptosomes obtained from the nervous system of *Locusta migratoria* by the flotation technique in the P_2L fraction. A. Note ellipsoidal synaptic vesicle profiles and mitochondria. B. Synaptosome with normal synaptic vesicles (top) and neurosecretory granules (bottom). M, mitochondria; n, neurosecretory granules; S, synaptosome; sv (arrows), synaptic vesicles. Bars correspond to 0.18 μm.

ethanol and embedded in Epon resin. Ultrathin sections (70–90 nm; LKB ultrotome) were stained with lead citrate prior to their examination in a JEOL JEM-100 electron microscope. The embedded fragments of pellets of fractions P_2L and mvP_2L which were cut in planes parallel and perpendicular to the centrifugal field have demonstrated identical results with regard to the form and distribution of the synaptosomes (Fig. 1) and membrane vesicles (Fig. 2A), respectively.

Materials

γ-Amino[2,3-^{3}H]butyric acid, ^3H_2O and [^{14}C]inulin were obtained from New England Nuclear. Valinomycin was purchased from Sigma Chemical Co. Nigericin was a generous gift from Dr. R. Hosley of Eli Lilly.

Results

Fractionation of the P_2 (crude synaptosomal) fraction of locust central nervous system by the flotation technique (see Materials and Methods) yielded a floating pellicle (P_2L) as well as material sedimenting through the gradient (P_2H). The floating pellicle is predominantly composed of closed

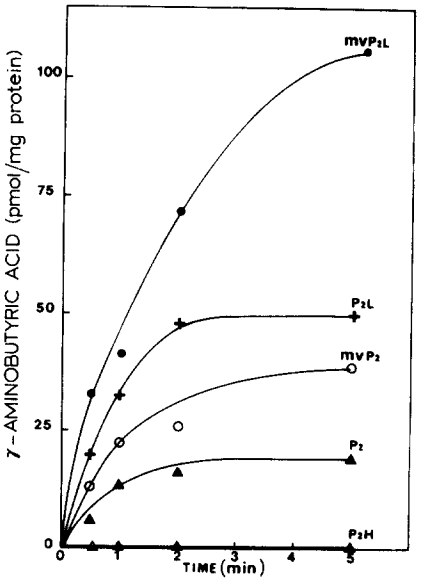

Fig. 3. Transport of γ-aminobutyric acid by the crude synaptosomal fraction (P_2, ▲——▲), a membrane vesicle derived from it (mvP_2, ○——○), the floating pellicle (P_2L, +——+), membrane vesicles derived from it (mvP_2L, ●——●) and the flotation pellet fraction (P_2H, △——△). Transport assays were performed as described in Materials and Methods. The following amounts (μg) of protein were used per time point: P_2, 120; mvP_2, 60; P_2L, 76; mvP_2L, 40 and P_2H, 80.

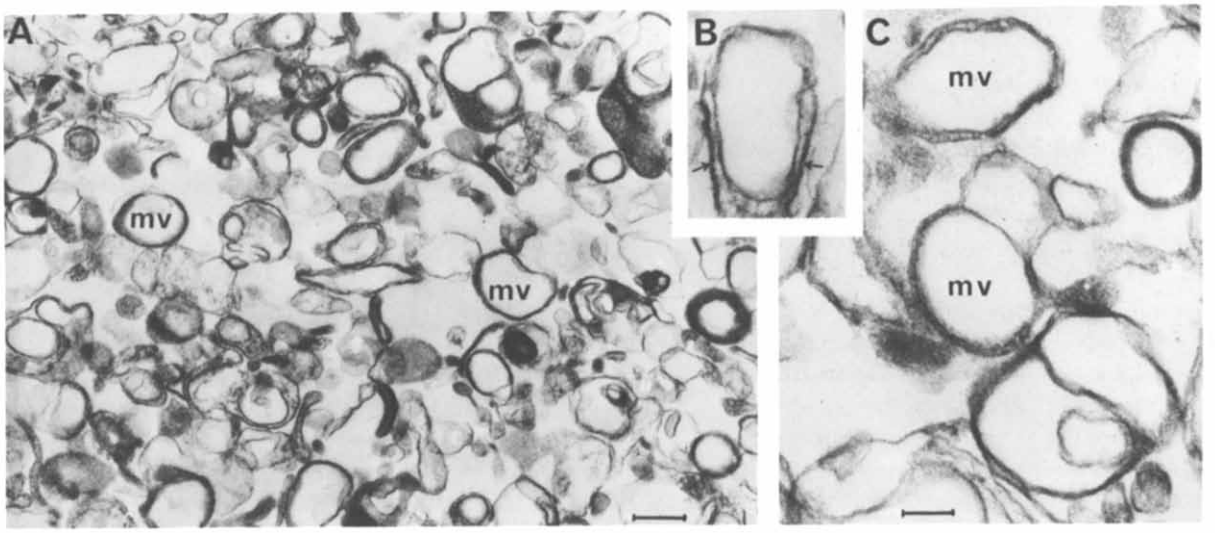

Fig. 2. Electron micrographs of membrane vesicles derived from the synaptosomal fraction P_2L. A. View in lower magnification of a collection of vesicular structures devoid of any cellular organelles. Bar = 0.3 μm. B. Vesicle derived from synaptosome, apparently with postsynaptic membranes still attached (arrows). Magnification as in C. C. View in high magnification of typical membrane vesicles. Bar = 0.12 μm. mv, membrane vesicle.

vesicular structures possessing the morphological characteristics of pinched-off nerve terminals (synaptosomes), illustrative examples of which are shown in Fig. 1. On the other hand the P_2H fraction was composed of mitochondria and other cellular fractions (data not shown). These results are in harmony with observations published by Breer and Jeserich [12]. Upon exposure of the synaptosomes to osmotic shock, closed membrane vesicles were obtained. These vesicles were devoid of any internal cellular organelles (Fig. 2). It is of interest to note that the diameter of these vesicles (0.2–0.5 μm) is significantly smaller than that of the intact synaptosomes (1–1.6 μm). This is probably due to a rearrangement of fragments of the synaptosomal plasma membranes.

The experiment depicted in Fig. 3 shows that the crude synaptosomal fraction (P_2) is able to catalyse γ-aminobutyric acid uptake. This uptake is apparently transport, since the accumulated radioactivity was found to be sensitive to the osmolarity of the medium (data not shown). Further fractionation yields a floating pellicle (P_2L) which is enriched in γ-aminobutyric acid transport activity, whereas the material sedimenting through the gradient (P_2H) is devoid of any such activity (Fig. 3). Furthermore, it can be seen (Fig. 3) that upon osmotic shock of both active fractions, a preparation, membrane vesicles, with an increased specific activity of γ-aminobutyric acid transport is obtained. It is of interest to note that although the diameter of the membrane vesicles is small in

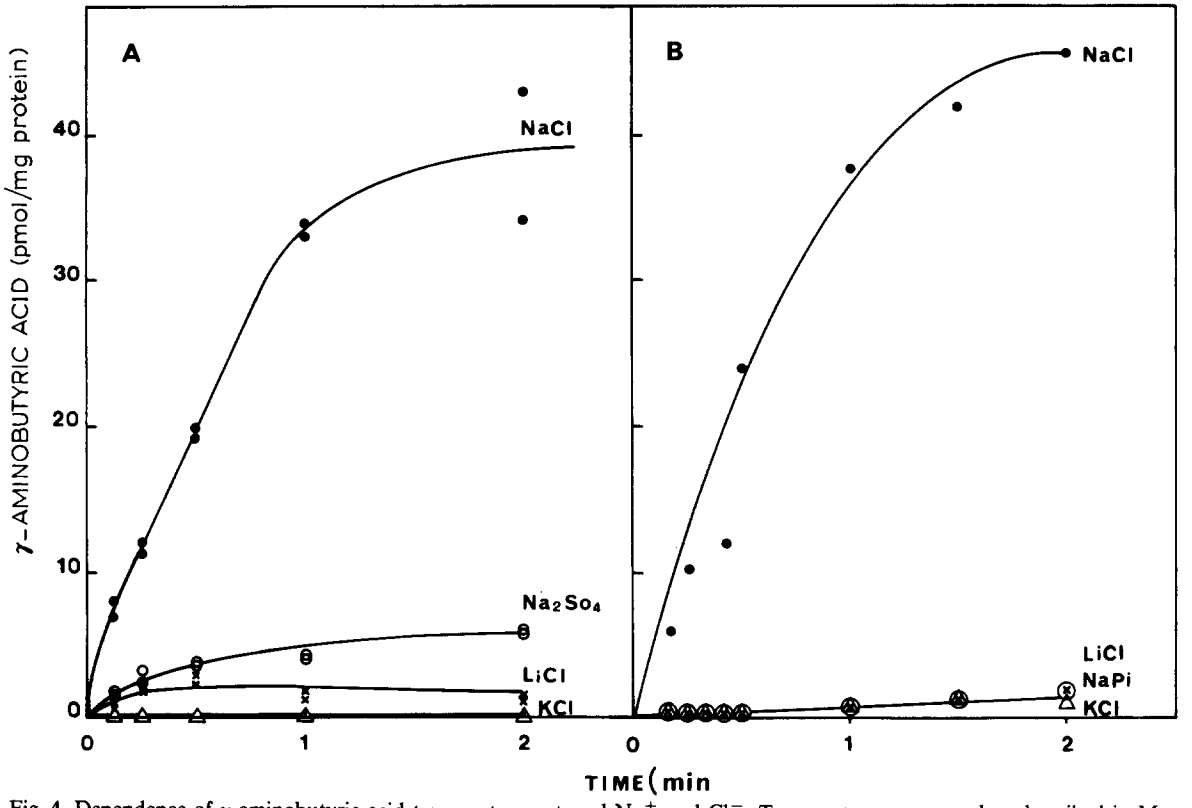

Fig. 4. Dependence of γ-aminobutyric acid transport on external Na^+ and Cl^-. Transport was measured as described in Materials and Methods. A. The flotation pellicle fraction (P_2L). The 102.3 mM NaCl (●———●) of the external medium was displaced by 51.15 mM Na_2SO_4 (○———○), 102.3 mM LiCl (×———×) or 102.3 mM KCl (△———△). Each reaction tube contained 96 μg protein. B. Membrane vesicles derived from the crude synaptosomal fraction (mvP_2). The loading medium was composed of 100 mM potassium phosphate (pH 6.8)/1 mM $MgSO_4$. The external media contained 1.25 μM valinomycin and were composed of: 150 mM NaCl/1 mM $MgSO_4$ (●———●); 150 mM KCl/1 mM $MgSO_4$ (△———△); 150 mM LiCl/1 mM $MgSO_4$ (×———×); 100 mM sodium phosphate/1 mM $MgSO_4$ (○———○). Each fraction tube contained 128 μg protein.

comparison to the pore size of the filters, all of the membrane protein was recovered on the filters (data not shown), probably due to electrostatic interactions.

The internal volume of the membrane vesicles (mvP_2L) was determined to be 5.6 μl per mg protein. In the experiment presented in Fig. 3 at least 100 pmol γ-aminobutyric acid per mg protein were accumulated. This represents an internal concentration of 17.5 μM. Since the external γ-aminobutyric acid concentration was 0.175 μM, at least 100-fold concentration of γ-aminobutyric acid against the concentration gradient was achieved. The transport of γ-aminobutyric acid displayed Michaelis-Menten kinetics. The Lineweaver-Burk plot yielded an apparent K_m of 3μM and a V_{max} of 3.2 nmol/min per mg protein.

As shown in Fig. 4 γ-aminobutyric acid transport is dependent on the simultaneous presence of external Na^+ and Cl^-. When Na^+ was omitted and replaced by other ions, no γ-aminobutyric acid transport was observed in the synaptosomal fraction (Fig. 4A) as well as in the membrane vesicles (Fig. 4B). When Cl^- was omitted in the same manner, γ-aminobutyric acid transport was completely prevented in the membrane vesicles (Fig. 4B) and greatly diminished in the synaptosomal fraction (Fig. 4A). The latter may be explained by the possible presence of endogenous Cl^- in the synaptosomes.

The ionophore nigericin is expected, under the conditions of the experiment, to exchange Na^+ for K^+ and thus to collapse the Na^+ gradient (out > in). In its presence a strong inhibition of γ-aminobutyric acid accumulation both in the synaptosomal fraction (Fig. 5A) and in the membrane vesicles (Fig. 5B) was observed. On the other hand, the ionophore valinomycin selectively enhances the membrane permeability to K^+. Under the conditions of the experiments (Fig. 5) with $[K^+]$ in $> [K^+]$ out, valinomycin is expected to enhance the magnitude of the membrane potential (interior negative) and this clearly stimulates γ-aminobutyric acid accumulation (Fig. 5A and B). Although significant stimulations of γ-aminobutyric acid uptake were observed with concentrations as low as 0.025 μM valinomycin (data not shown), the concentration yielding maximal stimulation (2.5 μM) was used (Fig. 5).

The importance of the Na^+ gradient is further

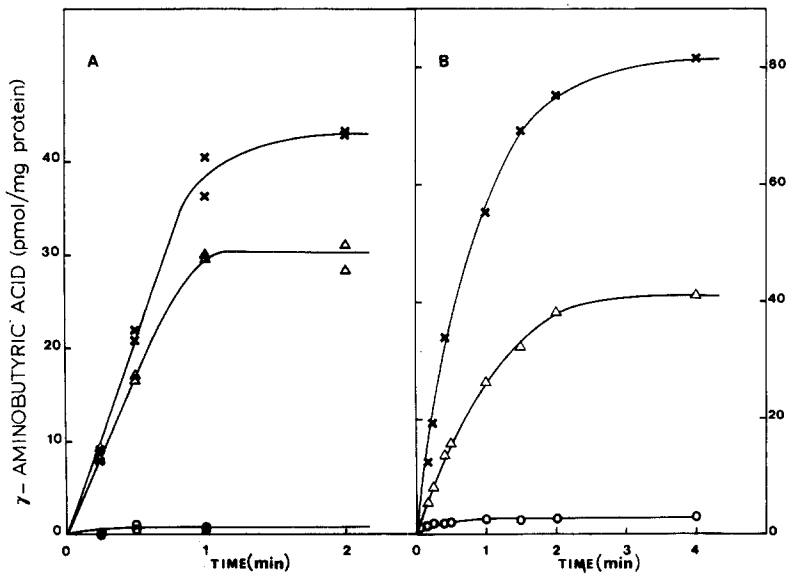

Fig. 5. The effect of ionophores on γ-aminobutyric acid transport. A. The flotation pellicle fraction (P_2L) and B. membrane vesicles derived from the crude synaptosomal fraction (mvP_2). Transport assays were performed as described in Materials and Methods. Ionophores: none (△——△); 2.5 μM valinomycin (×——×); 5 μM nigericin (○——○). 40 μg protein were used per assay in A, and 114 μg protein per assay in B.

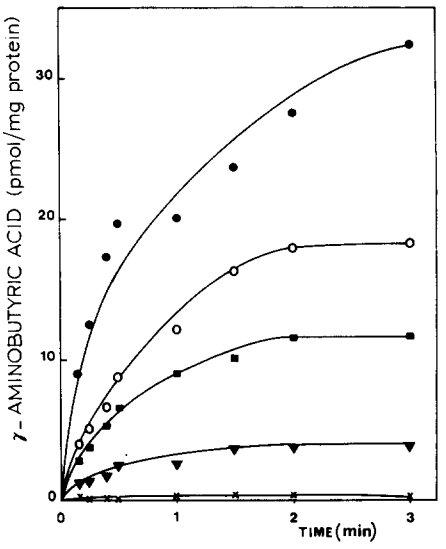

Fig. 6. Dependence of γ-aminobutyric acid transport on transmembranous Na^+ and Cl^- gradients as shown in the mvP_2 preparation. Transport was measured as described under Materials and Methods. The following compositions of the loading solutions were used: Na^+ and Cl^- gradients: 100 mM potassium phosphate (pH 6.8)/1 mM $MgSo_4$/45 mM sucrose (O——O, 98 μg protein per time point) or 100 mM Tris-phosphate (pH 6.8)/1 mM $MgSO_4$/45 mM sucrose (●——●, 106 μg protein). Cl^- gradient: 100 μM sodium phosphate (pH 6.8)/1 mM $MgSO_4$/45 mM sucrose (▼——▼, 102 μg protein). Na^+ gradient: 135 mM KCl/10 mM potassium phosphate (pH 6.8)/1 mM $MgSO_4$ (■——■, 88 μg protein). No gradient: 135 mM NaCl/10 mM sodium phosphate (pH 6.8)/1 mM $MgSO_4$ (×——×, 96 μg protein). The external medium was in all cases 0.15 M NaCl/1 mM $MgSO_4$.

illustrated by the experiment shown in Fig. 6. When the internal potassium phosphate is replaced by sodium phosphate (thus eliminating the Na^+ gradient) the γ-aminobutyric acid accumulation is greatly reduced (Fig. 6). The Cl^- gradient (out > in) represents an additional driving force; when the internal phosphate is replaced by Cl^-, a marked reduction in γ-aminobutyric acid transport is also observed. Furthermore when both Na^+ and Cl^- gradients are reduced (loading with NaCl) no accumulation of γ-aminobutyric acid is observed (Fig. 6). The presence of internal K^+ is not critical for γ-aminobutyric acid transport, since when it is replaced by Tris at least the same level of γ-aminobutyric acid accumulation is observed (Fig. 6).

Discussion

This study had demonstrated that the previously described [15] synaptosomal preparation obtained from locust nervous tissue by the flotation technique is functional, at least in one aspect of synaptic transmission, namely the reuptake mechanism. The high affinity of the system ($K_m = 3\,\mu M$) is very similar to that of its mammalian counterpart. This high affinity strongly suggests that the transport system under study is indeed involved in the reuptake process. The latter is thought to be involved in the termination of the process by removing the neurotransmitter from the synaptic cleft [24]. As shown, the synaptosomal fraction is very active in Na^+- and Cl^--dependent γ-aminobutyric acid uptake and this activity is preserved and even enriched by the membrane vesicles derived from the osmotically shocked synaptosomes. The synaptic plasma membrane vesicles, described here for the first time for insects, are a very useful tool in the study of transport phenomena in isolation from other interfering processes such as metabolism or sequestering into internal storage organelles (see Refs. 17–21 and Fig. 2).

When compared to the mammalian system, the results for the insect transport system are consistent with a mechanism in which γ-aminobutyric accumulation occurs via cotransport with Na^+ and Cl^-. The specific role of Cl^- was demonstrated when membrane vesicles loaded with potassium phosphate were diluted into sodium phosphate in the presence of valinomycin. Under these conditions it is expected that a membrane potential (interior negative) be generated. However, no transport at all was observed (Fig. 4B). It may thus be concluded that the function of Cl^- is not just limited to the generation of membrane potential, and that its specificity may be expressed, for instance, in a direct activation of γ-aminobutyric acid transporter molecule.

In order to explain the absolute dependency of γ-aminobutyric acid transport on both Na^+ and Cl^- and its stimulation by a membrane potential (interior negative) one possibility is to assume that the stoichiometry of the respective solutes by the transporter can be defined as n Na^+ : m Cl^- : γ-aminobutyric acid with $n > m$ (assuming that it is the zwitterionic form of γ-aminobutyric acid which

is transported). Alternative explanations are possible. For instance, the role of Cl^- may be dual; it is thought to be a rather permeant anion and therefore would be able to contribute to the membrane potential (interior negative). It might, also, bind just to the portion of the transporter facing the outside of the membrane, inducing a conformational change in the transporter, which now can bind Na^+ and γ-aminobutyric acid. Further study is required to distinguish between these possibilities.

At present it is not clear whether the membrane vesicles which transport γ-aminobutyric acid are derived from the synaptic plasma membrane or from glial membranes. Circumstantial evidence is consistent with the first possibility. When the crude synaptosomal fraction P_2 is fractionated by the flotation technique, a pellicle (P_2L) enriched in synaptosomes is isolated (Fig. 1). This fraction is also enriched in γ-aminobutyric acid uptake (Fig. 3). On the other hand, the material sedimenting through the gradient (P_2H) which is devoid of synaptosomal structures was also lacking γ-aminobutyric acid uptake activity.

To the best of our knowledge, this is the first description of a functional preparation derived from insect neuronal tissue. This preparation is a good starting point for study of unique features associated with the central nervous system of insects.

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