HYDROPHOBIC PROTEINS ISOLATED FROM CRUSTACEAN MUSCLE HAVING GLUTAMATE AND γ-AMINOBUTYRATE RECEPTOR PROPERTIES

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1. Introduction

Muscle tissue of crustacea has a double innervation with excitatory and inhibitory nerve fibres. Several evidences have suggested that the excitatory transmitter is glutamic acid. They refer to the stimulation of contraction by the applied drug [1, 2] experiments of stimulus release [3] and effects of glutamate on membrane potentials [4]. By radioautography the uptake of [^{3}H]glutamate was found to be greater at the neuromuscular junction and to be enhanced by electrical stimulation [5]. A considerable literature supports the view that γ-aminobutyric acid (GABA) is the inhibitory transmitter (see [6, 7]). The effect of GABA may be blocked by picrotoxin [8, 9] and bicuculline [10]. The inhibitory nerves of crustacea contain 100-fold more GABA than those of the excitatory [11] and the synthesizing enzyme is only present in the former [12]. Furthermore GABA appears in the superfusate after stimulation of the inhibitory nerves [13].

The separation of glutamate receptors from insect muscle has been carried out by Lunt [14], using both the methods of solubilization by detergents [15] and the extraction with organic solvents [16].

In the present work we will describe the separation of two hydrophobic protein fractions (i.e. proteolipids) one binding glutamate, the other γ-aminobutyrate.

2. Materials and methods

Muscles of shrimps, *Artemisia longinaris* from the family *Penalidae*, were homogenized in 0.05 M Tris–Cl buffer pH 7.5; then centrifuged at 100 000 *g* for 30 min and the sediment was lyophilised. The sediment contained 25.6 mg total protein per g tissue [17]. After extraction in chloroform–methanol (2:1) 154.5 μg of hydrophobic protein per g tissue was separated. The best results were obtained after submitting the extract to precipitation with 4 vol of diethyl-ether at −20°C. The sediment had 50% of the protein and only 5% of the lipid phosphorus. Both the sediment and the supernatant were used for binding experiments and for the isolation of receptor proteins. The radioactive drugs used were: [U-^{14}C]L-glutamic acid, 270 mCi/mmole; [1-^{14}C]γ-aminobutyric acid, 3.14 mCi/mmole; [U-^{14}C]L-glutamine, 45 mCi/mmole and [U-^{14}C]L-aspartic acid, 231 mCi/mmole (all from Amersham).

3. Results

3.1. *Binding of [^{14}C]L-glutamate and column chromatography*

Preliminary binding studies, using a centrifugation–washing method [18], were carried out on homogenized fresh tissue incubated with [^{14}C]L-glutamate, at a final concn. of 9×10^{-8} M. The pellets were lyophilise extracted with chloroform–methanol [16] and chromatographed in a Sephadex LH-20 column. The results obtained were identical to those shown in fig. 1, in which the binding was carried out directly on the lipid extract at a concn. of 3.6×10^{-7} M [^{14}C]L-glutamate.

The hydrophobic proteins emerged in three peaks in the chloroform and two smaller ones in the chloroform–methanol (4:1) (fig. 1). The radioactivity appeared in a sharp peak between 16–26 ml of chloro-

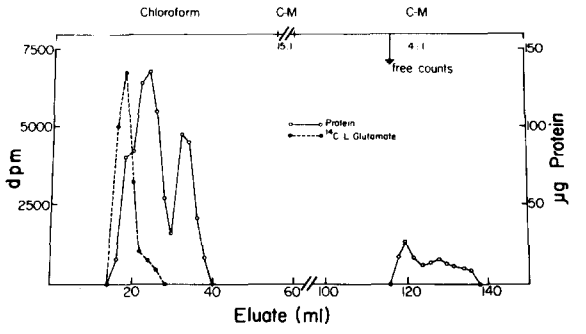

Fig. 1. Chromatography on Sephadex LH-20 of the total hydrophobic proteins from shrimp muscle and binding of [^{14}C]L-glutamate. 500 mg of lyophilised sediment, corresponding to 3.3 g fresh tissue, were extracted with chloroform–methanol as in [16]. The extract was treated with activated charcoal to eliminate the red pigment. It was then concentrated to 3 ml and submitted to binding with 3.6×10^{-7} M [^{14}C]L-glutamate. Observe that the radioactivity is coincident with the beginning of the protein elution. Each tube contains 2 ml of eluate. (See further details in the text).

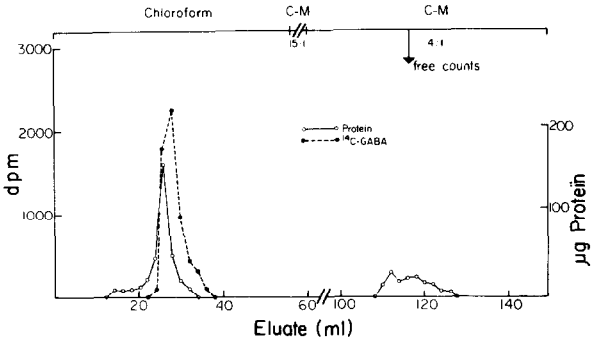

Fig. 2. Chromatograaohy on Sephadex LH-20 of the hydrophobic proteins contained in the diethyl-ether precipitate of the shrimp muscle and binding of [^{14}C]GABA. 1 g of lyophilized sediment was extracted as in fig. 1. The concentrated extract was precipitated 1:4 with diethylether at −20°C. The precipitate, redissolved in 3 ml of chloroform–methanol (2:1), was submitted to binding with 2×10^{-6} M [^{14}C]GABA. Observe that the radioactivity appears in coincidence with a sharp peak of protein which does not contain lipid phosphorus. Each tube contains 2 ml of elutae. (See further details in the text.)

form in coincidence with the first protein fraction that is eluted. The amount of protein in this fraction is about 27 µg/g. The lipid phosphorus was eluted in a large peak between 16–34 ml of chloroform and a small peak in chloroform–methanol (4:1). Experiments of binding carried out with the supernatant of the ether precipitate gave similar results. The radioactivity now appeared with a better defined peak of protein eluting between 12–20 ml of chloroform.

Control experiments done with [^{14}C]L-glutamate processed in a similar way, but in the absence of extract, showed that the free counts appeared in the chloroform–methanol (4:1) (fig. 1).

3.2. *Binding of [^{14}C]L-glutamate by partition*

The technique described by Weber et al. [19] for a fluorescent cholinergic probe was adapted to study the binding of glutamate. In the organic phase there were 40 µg of the protein fraction, known to bind this ligand, dissolved in 2 ml of 0.05 M Tris–Cl buffer pH 7.5 and 0.2 M NaCl. Control experiments showed that the partition of glutamate was 100% favorable to water. The binding experiments were done at concentrations of free glutamate between 1.8×10^{-6} and 1.0×10^{-4} M. Saturation was reached between 6.5×10^{-5} and 1×10^{-4} M and the binding was of 3.07 pmoles/µg protein. This corresponds to the binding of one mole of glutamate per 320 000 g of protein.

The specificity of this binding was investigated carrying similar experiments with 2.5×10^{-4} M [^{14}C]L-aspartate and [^{14}C]L-glutamine. In both cases there was no binding to the protein in the lower phase.

3.3. *Binding of [^{14}C]GABA and column chromatography*

Preliminary experiments suggested that [^{14}C]GABA was related to a hydrophobic protein fraction eluted at higher effluent volumes than glutamate. However, the most clear cut results were obtained using the ether precipitate. This was redissolved in 3 ml of chloroform–methanol (2:1) and [^{14}C]GABA was added to a final concn. of 2×10^{-6} M. After 30 min the extract was chromatographed in a Sephadex LH-20 column. As shown in fig. 2 the radioactivity appeared in coincidence with a peak of protein between 24–32 ml of chloroform. This peak is devoid of lipid phosphorus. In control experiments the free [^{14}C]GABA was eluted at the end of the chromatogram. The ether precipitate was apparently devoid of receptor protein for glutamate since it showed no binding for this ligand.

The amount of protein binding GABA corresponds to 21.5 μg/g fresh tissue.

3.4. *Saturation of the binding of [^{14}C]GABA*

The protein fraction eluted between 24–32 ml was separated and submitted to binding with [^{14}C]GABA in concentrations between 2.1×10^{-6} and 2.4×10^{-5} M. Then the extracts were rechromatographed using small columns of Sephadex LH-20 (10 × 0.8 cm) which were eluted with chloroform. The saturation of the binding was reached at a concentration in between 1.5×10^{-5} and 2.4×10^{-5} M with 12.8 pmoles/μg protein. At this level 1 mole of GABA binds to approx. 80 000 g of protein.

3.5. *Inhibition by bicuculline of GABA binding*

Experiments done as in sect. 3.4 using a fixed concentration of [^{14}C]GABA (i.e. 8×10^{-6} M) in the presence of increasing concentrations of bicuculline (i.e. $1 \times 10^{-5} - 5 \times 10^{-4}$ M) produced an inhibition of the binding of [^{14}C]GABA of the order of 50–80.5%.

4. Discussion

Since, in 1967, De Robertis et al. [16] reported the isolation of a hydrophobic protein fraction (i.e. proteolipid) from cerebral cortex binding cholinergic ligands considerable progress has been made in the isolation and characterization of central and peripheral receptors (see [20]). Most work has been carried out on the electric tissue of the *Electrophorus* and *Torpedo* using either organic solvents [21] or detergents for solubilization of receptor proteins [15, 22]. With organic solvents a cholinergic receptor fraction was isolated from skeletal muscle [23] and adrenergic binding proteins were separated from spleen [24] and heart [25]. More recently a muscarinic binding protein was isolated from the circular layer of the intestine [26].

Regarding amino acid binding proteins, to our knowledge, the only work is that of Lunt [14] who used muscles of the locust (*Shistocerca gregaria*). He extracted a crude detergent solubilized protein that by equilibrium dialysis showed high affinity for glutamate and practically no binding for glutamine or aspartate. Using organic solvent extraction [16], followed by Sephadex LH-20 chromatography, he found two protein fractions showing specific binding for glutamate. He concluded that these hydrophobic proteins could constitute part of the glutamate receptor at the insect neuromuscular junction.

The results here described suggest that in shrimp muscle both glutamate and GABA bind to two distinct hydrophobic protein fractions which can be separated by their chromatographic characteristics and also by their different properties regarding the diethyl-ether precipitation. The maximal amount of protein binding glutamate corresponds to 27 μg/g, a figure that is considerably lower than that given by Lunt [14] for the locust. The degree of purification achieved in our case is higher than 900-fold, since in the total sediment used for the extraction we had 25.6 mg total protein. Our glutamate-binding protein saturates at 1 mole per 320 000 g and does not bind GABA, aspartate or glutamine. The GABA receptor protein appeared in a better defined peak, without lipid phosphorus, and at a concentration of 21.5 μg/g which corresponds to a purification of about 1200 times. Here the saturation is achieved at 1 mole per 80 000 g of protein. The specificity of this protein fraction was demonstrated by the lack of binding of glutamate, glutamine, aspartate and by the competition with the specific inhibitor bicuculline. Further work is being conducted to better ascertain the specificity of the binding of glutamate and GABA with the use of other competitors, to separate receptor proteins from other species and to obtain information about the size and nature of the macromolecules involved in the specific binding of these amino acids.

The work done so far permits us to conclude that the two hydrophobic proteins isolated from shrimp muscle have some of the properties that are characteristic of receptors for glutamate and GABA.

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References

[1] J. Robbins, J. Physiol. 148 (1959) 39.
[2] A. Van Harrevel and M. Mendelson, J. Cell Comp. Physiol. 54 (1959) 85.
[3] G.A. Kerkut, L.D. Leake, A. Shapira, S. Cowan and R.J. Walker, Comp. Biochem. Physiol. 15 (1965) 485.
[4] R. Beránek and P.L. Miller, J. Exptl. Biol. 49 (1968) 83.
[5] J.R. Faeder and M.M. Salpeter, J. Cell Biol. 46 (1970) 300.
[6] E. Florey, Federation Proc. 26 (1967) 1164.
[7] F.E. Bloom, Neur. Res. Progr. Bull. 10 (1972) 127.
[8] J. Robbins and W.G. Van der Kloot, J. Physiol. 148 (1958) 39.
[9] H. Grundfest, J.P. Reuben and W.H. Rickles, Jr., J. Gen. Physiol. 42 (1959) 130.
[10] D.R. Curtis, A.W. Duggan, D. Felix and G.A.R. Johnston, Nature 228 (1970) 676.
[11] E.A. Kravitz and D.D. Potter, J. Neurochem. 12 (1965) 323.
[12] Z.W. Hall, M.D. Bownds and E.A. Kravits, J. Cell Biol. 46 (1970) 290.
[13] M. Otsuka, L.L. Iversen, Z.W. Hall and E.A. Kravitz, Proc. Natl. Acad. Sci. U.S. 56 (1966) 1110.
[14] G.G. Lunt, Compt. Gen. Pharmacol. (1973) in press.
[15] J.P. Changeux, M. Kasai, M. Huchet and J.C. Meunier, Compt. Rend. 270 (1970) 2864.
[16] E. De Robertis, S. Fiszer and E.F. Soto, Science 158 (1967) 928.
[17] O.H. Lowry, N.J. Rozebrough, A.L. Farr and P.J. Randall, J. Biol. Chem. 193 (1951) 265.
[18] J.M. Azcurra and E. De Robertis, Int. J. Neuropharmacol. 6 (1967) 15.
[19] G. Weber, D.P. Borris, E. De Robertis, F.J. Barrantes, J.L. La Torre and M.C. Llorente de Carlin, Molec. Pharmacol. 7 (1971) 530.
[20] E. De Robertis, Science 171 (1971) 963.
[21] J.L. La Torre, G.S. Lunt and E. De Robertis, Proc. Natl. Acad. Sci. U.S. 65 (1970) 716.
[22] R. Miledi, P. Molinoff and L.T. Potter, Nature 229 (1971) 554.
[23] G.G. Lunt, E. Stefani and E. De Robertis, J. Neurochem. 18 (1971) 1545.
[24] S. Fiszer de Plazas and E. De Robertis, Biochim. Biophys. Acta 266 (1972) 246.
[25] E. Ochoa, M.C. Llorente de Carlin and E. De Robertis, Europ. J. Pharmacol. 18 (1972) 367.
[26] E. Ochoa and E. De Robertis, Biochim. Biophys. Acta 295 (1973) 528.