Solubilization and purification of a putative quisqualate-sensitive glutamate receptor from crustacean muscle

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Two high-affinity glutamate-binding proteins have been solubilized and purified from crustacean muscle membranes. L-[³H]Glutamate binding to intact membranes gave IC₅₀ values (concn. giving 50 % inhibition) of 0.35 μM for glutamate, 9.0 μM for quisqualate and 36.2 μM for ibotenic acid. Kainate, domoate, N-methyl-D-aspartate, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate, D-glutamate and L-aspartate were poor inhibitors of glutamate binding. The two proteins were purified approx. 600-fold from muscle membranes by use of concanavalin A and gel-filtration column chromatography. One protein showed an apparent Stokes radius of 5.4 nm, as determined by gel filtration, with a subunit of M_r 74000 as shown by SDS/polyacrylamide-gel electrophoresis. Analysis of glutamate binding to this protein revealed a K_D of 2.4 μM and a B_{max} of 15.5 nmol/mg, and a K_i for quisqualate inhibition of glutamate binding of 1.0 μM. The second protein was eluted anomalously from the gel-filtration column and showed a subunit of M_r 65000 on SDS/polyacrylamide gels. Glutamate binding to this protein showed a K_D of 1.3 μM and a B_{max} of 14.5 nmol/mg, and was only slightly sensitive to the presence of quisqualate (K_i = 0.8 mM). Glutamate binding to both proteins was insensitive to kainate. It is concluded that the M_r-74000 protein is a prime candidate for the quisqualate-sensitive receptor of the crustacean neuromuscular junction.

INTRODUCTION

Glutamate is the major excitatory transmitter at both the crustacean and insect neuromuscular junctions (Takeuchi, 1987; Usherwood, 1978). In the vertebrate central nervous system three different types of glutamate receptor coupled to cation channels have been identified, on the basis of their relative affinities for three specific agonists (Watkins & Evans, 1981). The three agonists now used to define these receptors are N-methyl-D-aspartate, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate. In addition, a G-protein-linked receptor, named the metabotropic receptor, has recently been described (Sugiyama *et al.*, 1989). The pharmacology of the crustacean neuromuscular-junction glutamate receptor is similar to that of the vertebrate AMPA receptor, since quisqualate is a potent depolarizing agent in crustacean muscle (Shinozaki & Shibuya, 1974), as well as being active at the vertebrate AMPA receptor. By contrast, N-methyl-D-aspartate has no effect (McBain & Wheal, 1984), and kainate acts at an extra junctional receptor which is present on the muscle plasma membrane (Shinozaki, 1980). Despite extensive studies on the pharmacology and electrophysiology of the glutamate receptors in both vertebrates and invertebrates (Fonnum, 1984; Takeuchi, 1987), relatively little biochemical or structural information was known until recently. The solubilization and isolation of the kainate receptor from chicken brain (Gregor *et al.*, 1988) and frog brain (Hampson & Wenthold, 1988) and a glutamate-binding protein from rat brain (Kuonen & Roberts, 1987; Chen *et al.*, 1988) have all now been reported. In addition the DNAs encoding two kainate-binding proteins (of approx. M_r 52000) have been cloned and sequenced (Wada *et al.*, 1989; Gregor *et al.*, 1989), and a rat kainate-sensitive-receptor DNA that specifies a protein of M_r 99800 has been functionally expressed (Hollman *et al.*, 1989). This in turn has led to the isolation of three more glutamate

receptor DNAs, which code for AMPA-sensitive receptors in rat brain (Keinanen *et al.*, 1990).

We considered it likely that crustacean muscle would be a rich source of the quisqualate-sensitive receptor, which could be isolated in the absence of the other excitatory amino acid receptors. We report here the characterization, solubilization and purification of a quisqualate-sensitive glutamate-binding protein from crustacean muscle.

EXPERIMENTAL

Materials

All standard laboratory chemicals were obtained from either Sigma or BDH (both of Poole, Dorset, U.K.). [³H]Glutamate (46 Ci/mmol) was from Amersham International, and *Nephrops norvegicus* (langoustines) were obtained from the University Marine Station, Millport, Cumbrae, Scotland, and kept in aerated artificial seawater at 12 °C. Ibotenic acid was kindly given by Dr. Ian Harrow, Pfizer Central Research, Sandwich, Kent, U.K. Domoic acid was from Tocris Neuramin, Buckhurst Hill, Essex, U.K.

Preparation of crude muscle membranes

Approx. 50 g of *Nephrops* abdominal muscle was finely chopped and homogenized in 0.4 M-sucrose/0.4 M-glycine, pH 7.4, containing 1 mM-EDTA, 1 mM-phenylmethanesulphonyl fluoride (PMSF) and 1 mM-benzamidinium in a rotating (640 rev./min) glass/Teflon homogenizer at 4 °C. After centrifugation at 1000 g for 10 min to remove cell debris, the supernatant was re-centrifuged at 15000 g for 45 min. The resultant pellet was then hypo-osmotically lysed with 240 ml of 1 mM-EDTA/1 mM-PMSF/1 mM-benzamidinium and re-homogenized. After re-centrifugation at 48000 g for 10 min, the membrane

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; IC₅₀, concn. giving 50 % inhibition.

pellet was washed four times by resuspension with 50 mM-Tris acetate buffer, pH 7.4, containing 1 mM-EDTA, 1 mM-PMSF and 1 mM-benzamidine, with centrifuging between washes. The final membrane pellet (P2) was resuspended in 20 ml of 50 mM-Tris acetate (protein concn. approx. 0.6 mg/ml) and used in the membrane-binding assays.

Solubilization and purification of glutamate-binding proteins

Solubilization conditions were optimized by addition of various detergents to the muscle membranes and stirring slowly for 1 h at 4 °C. Routinely this involved the addition of (final concns.) sodium cholate (0.25 %, w/v), NaCl (1.5 M), PMSF (1 mM), EDTA (1 mM), benzamidine (1 mM), pepstatin A (10 mM), E-64 (20 mM), leupeptin (20 mM), *N*-tosyl-L-phenylalanylchloromethane (100 mM) and *N*-*p*-tosyl-L-lysylchloromethane (10 mM). The solubilized membranes were subsequently centrifuged at 100 000 *g* for 1 h, and the supernatant was then recirculated for 16 h at 4 °C through a 4 ml column of concanavalin A-Sepharose. Samples of the solubilized membranes were kept for assay and dialysed for 16 h against 2 litres of buffer A (10 mM-Tris adjusted to pH 7.4 with acetic acid, 0.5 M-NaCl, 1 mM-CaCl₂, 1 mM-MgCl₂, 0.05 % cholate, 1 mM-EDTA, 1 mM-PMSF, 1 mM-benzamidine).

The concanavalin A-Sepharose column was drained, washed with 200 ml of Buffer A, and the bound proteins were eluted with 5 × 4 ml of Buffer A containing 0.5 M- α -methyl mannoside. The first three eluates were pooled and dialysed for 3 h against 3 litres of 50 mM-NaCl/10 mM-Tris acetate (pH 7.4)/1 mM-CaCl₂/1 mM-MgCl₂/0.05 % cholate/1 mM-EDTA/1 mM-PMSF/1 mM-benzamidine. The dialysed sample was concentrated to 0.8 ml under vacuum (without freezing) and loaded on a 50 cm × 0.9 cm AcA34 column equilibrated with Buffer A. The column was run at 3.0 ml/h at 4 °C, and 1 ml fractions were collected. The column was calibrated with the following marker proteins: urease, alcohol dehydrogenase, bovine serum albumin and carbonic anhydrase. Blue Dextran was used to measure the void volume and *N*-acetyltyrosine the total column volume.

Glutamate-binding assays

Glutamate binding to muscle membranes was based on the method of Sharif & Roberts (1984). Samples (0.225 ml) of membranes were incubated with 0.025 ml of competing ligand or 50 mM-Tris acetate, pH 7.4, for 30 min at 20 °C. Then 0.025 ml of [³H]glutamate (25 nM final concn.) was added and the incubation continued for a further 30 min. The reaction was terminated by centrifugation for 5 min in an MSE Microcentaur (8000 rev./min) and the supernatant removed by aspiration. Pellets were superficially washed twice with ice cold Tris acetate buffer and then counted for radioactivity by liquid-scintillation counting. Displacement curves for various ligands were carried out by preincubating the membranes with a range of ligand concentrations (1 mM–1 nM) before addition of the labelled glutamate.

Glutamate binding to solubilized proteins was measured by preincubation for 30 min at 20 °C of 0.1 ml or 0.2 ml samples, with or without 0.025 ml of competing ligand, in a total volume of 0.25 ml, in 50 mM-Tris acetate, pH 7.4. After addition of 0.025 ml of [³H]glutamate (25 nM final concn.) and a further incubation for 30 min at 20 °C, the soluble proteins were precipitated by addition of 0.5 ml of bovine γ -globulins (1.5 mg/ml) in 0.1 M-phosphate buffer (pH 7.4) followed by 0.5 ml of ice-cold poly(ethylene glycol) (25 %, w/v), and centrifuged at 2000 *g* for 10 min. After a rinse with 0.5 ml of 9 % poly(ethylene glycol) the pellets were re-centrifuged, and subjected to liquid-scintillation counting. Non-specific [³H]glutamate binding, defined as that occurring in the presence of 1 mM-

quisqualate or -glutamate, amounted to approx. 50 % of the total binding. Most of this was due to entrapment in the poly(ethylene glycol) pellet.

The binding observed to intact and solubilized membranes with various concentrations of glutamate fitted a two-site model. The B_{max} and K_d values were derived by using a non-linear least-squares method to give the best fit.

Polyacrylamide-gel electrophoresis

Protein samples were precipitated in 10 % (v/v) trichloroacetic acid, reduced with 6.5 mM-dithiothreitol, heated at 100 °C for 5 min and electrophoresed as described by Laemmli (1970). The gels were subsequently silver-stained by the method of Ansorge (1982). Protein was assayed by the method of Bradford (1976).

RESULTS

Glutamate-binding sites in crustacean muscle membranes

Crude membranes were prepared from *Nephrops* muscle as described above, and their ability to bind [³H]glutamate was determined. The ability of glutamate and five of its analogues to inhibit glutamate binding is shown in Fig. 1. Glutamate was the

Fig. 1. Displacement of [³H]glutamate binding to solubilized (a) and intact (b) *Nephrops* muscle membranes

Results are means $\pm$ range (domoate and ibotenate, $n = 2$) or means $\pm$ S.E.M. of between 4 and 12 experiments in (a) or between 3 and 17 experiments in (b). They are expressed as percentages of the binding seen with 25 nM-[³H]glutamate, after subtraction of the non-specific binding seen in the presence of 1 mM-glutamate. This non-specific component amounted to between 40 and 50 % of the total binding. Key: ●, glutamate; ■, quisqualate; □, kainate; ▲, AMPA; △, ibotenate; ○, domoate. Total specific binding in (a) was approx. 4000 d.p.m., and that in (b) 6000 d.p.m.

Fig. 2. Glutamate binding to intact (a) and solubilized (b) *Nephrops* muscle membranes

[³H]Glutamate binding was measured over a range of glutamate concentrations, and the results are expressed as means $\pm$ S.E.M. of five experiments. Scatchard analysis of the binding data is shown in the insets, K_d and B_{max} values being obtained by a non-linear least-squares method: B, bound (pmol/mg); F, free (μ M).

most potent inhibitor, with an IC_{50} (concn. giving 50% inhibition) of 0.3μ M, whereas quisqualate and ibotenate had IC_{50} values of 9μ M and 36μ M respectively. Interestingly, AMPA, which is thought to bind specifically to quisqualate-sensitive

receptors in the vertebrate central nervous system (Monaghan *et al.*, 1984), was a poor ligand in this system. It showed an IC_{50} of 0.76 mM and, like domoate and kainate, was only able to inhibit glutamate binding to a sub-population of sites. No inhibition of binding was seen with *N*-methyl-D-aspartate, D-glutamate or L-aspartate at concentrations up to 1 mM . Fig. 2 shows a Scatchard analysis of the glutamate-binding sites, which suggests the presence of two major ligand-binding sites, with K_d values of $3.7 \pm 1.0 \mu$ M and $33.7 \pm 3.3 \mu$ M and B_{max} values of 26.50 ± 2.4 and $74.1 \pm 7.2 \text{ pmol/mg}$ of protein respectively.

Solubilization of the quisqualate-sensitive binding sites

A number of detergents were tested for their ability to solubilize the quisqualate-sensitive glutamate-binding sites. The best conditions found involved the use of 0.25% cholate in the presence of 1.5 M-NaCl , which resulted in the solubilization of approx. 50% of the binding sites (Table 1). Attempts to detect [³H]glutamate-binding sites in the unsolubilized material were unsuccessful. In order to check that the quisqualate-sensitive sites had indeed been solubilized, the ability of glutamate, quisqualate, ibotenate, domoate, kainate and AMPA to inhibit [³H]glutamate binding was ascertained. No significant difference was observed between the IC_{50} values determined for the solubilized and membrane-bound binding sites (Fig. 1, Table 2). Similarly, Scatchard analysis (Fig. 2) revealed the presence of two binding sites (K_d $4.4 \pm 2 \mu$ M and $35.0 \pm 3.8 \mu$ M). This analysis suggested that the solubilized binding sites displayed the same pharmacological properties as the membrane-bound sites.

Purification of the quisqualate-sensitive binding sites

The solubilized binding sites were subsequently purified on a concanavalin A-Sepharose column, which resulted in a 178-fold purification with a yield of 57% (Table 3). The glutamate-binding sites that were not retained by this lectin column amounted to $14.3 \pm 4.0 \text{ pmol/50 g}$ of tissue. The specific activity of this fraction was 1.4 pmol of [³H]glutamate bound/mg of protein. The glutamate-binding activity which did not bind to the concanavalin A column was screened for its sensitivity to quisqualate and kainate. No such sensitivity was observed (results not shown). The polypeptide composition of the α -methyl mannoside eluate of the concanavalin A column is shown in Fig. 4, where it is apparent that few of the major solubilized proteins were specifically eluted from this column by α -methyl mannoside, except for a polypeptide of M_r 74000. The first 12 ml of α -methyl mannoside eluate containing the glutamate-binding activity was then subjected to gel filtration. Three peaks of glutamate-binding activity were observed (Fig. 3). The first, which was eluted with an apparent Stokes radius of 5.4 nm , was detected in every

Table 1. Detergent solubilization of crustacean muscle membranes

Membranes were isolated from *Nephrops* abdominal muscle as described in the Experimental section. The membranes were solubilized by gentle stirring for 1 h at 4°C , in the above detergents, centrifuged at 100000 g for 1 h, and the solubilized supernatant was dialysed overnight against $5 \times 1 \text{ l}$ batches of Buffer A. The dialysed supernatant was assayed as described in the Experimental section. Results are expressed as percentages of the total activity initially present in P2 (mean $\pm$ range) ($n = 2$).

Condition	Solubilization of [³ H]glutamate-binding activity (%)	Total protein solubilized (%)
0.1% sodium deoxycholate	18.6 ± 1.2	30.0 ± 3.5
0.1% sodium deoxycholate + 1.5 M-NaCl	30.2 ± 4.2	42.2 ± 2.1
0.25% sodium cholate	27.8 ± 3.2	45.0 ± 3.8
0.25% sodium cholate + 1.5 M-NaCl	51.4 ± 4.2	85.0 ± 4.1
0.3% CHAPS	20.0 ± 2.5	32.0 ± 2.6
0.1% Triton X-100	22.0 ± 1.5	34.0 ± 3.5

Table 2. Inhibition constants for L-[³H]glutamate binding to membranes, solubilized membranes and purified proteins

The values given are means $\pm$ range of two determinations or means $\pm$ S.E.M. for the numbers of determinations shown in parentheses: NI, inhibition of binding of less than 50% at 1 mM (kainate) or 10 μ M (domoate).

Ligand	K_i (μ M)			
	Membranes	Solubilized membranes	M_r -74000	M_r -65000
L-Glutamate	0.35 $\pm$ 0.15 (10)	0.25 $\pm$ 0.2 (4)	2.0 $\pm$ 0.9 (3)	1.6 $\pm$ 0.5 (3)
Quisqualate	25.4 $\pm$ 11.3 (17)	15.3 $\pm$ 4.1 (12)	1.0 $\pm$ 0.2 (4)	8000 $\pm$ 4200 (4)
Ibotenate	36.2 $\pm$ 14.3 (6)	45.0 $\pm$ 22.5 (2)	—	—
AMPA	760 $\pm$ 90 (8)	760 $\pm$ 390 (4)	—	—
Kainate	250 $\pm$ 90 (8)	244 $\pm$ 122 (4)	NI (3)	NI (3)
Domoate	NI (3)	NI (3)	—	—

Table 3. Purification of glutamate-binding proteins from crustacean muscle

Glutamate binding to the fractions was measured by using 25 nM-[³H]glutamate as described in the Experimental section. The values are means $\pm$ S.E.M. of 11 experiments.

	Protein (mg)	Binding (pmol)	Specific activity (pmol/mg)	Purification (fold)	Yield (%)
Solubilized membranes	12.9 $\pm$ 2.1	23.7 $\pm$ 5.1	2	1	100
Concanavalin A eluate	0.042 $\pm$ 0.1014	13.4 $\pm$ 3.4	320	178	57
Gel-filtration eluates					
M_r 74000	0.003 $\pm$ 0.001	3.3 $\pm$ 1.0	1100	611	14
M_r 65000	0.0025 $\pm$ 0.001	3.3 $\pm$ 1.0	1320	733	14

Fig. 3. Gel-filtration chromatography

The concanavalin A-Sepharose column eluate was submitted to gel filtration on an Aca34 column as described in the Experimental section: 1 ml fractions were collected and assayed for glutamate-binding activity. The column was calibrated with the following proteins: Blue Dextran (BD), void volume; urease trimer (U), M_r 272000, Stokes radius 5.0 nm; alcohol dehydrogenase (AD), M_r 150000, Stokes radius 4.6 nm; serum albumin (SA), M_r 66000, Stokes radius 3.5 nm; carbonic anhydrase (CA), M_r 29000, Stokes radius 2.0 nm; *N*-acetyltyrosine (AT), total column volume.

Fig. 4. SDS/polyacrylamide-gel electrophoresis of glutamate-binding protein during purification

A, Solubilized membranes; B, concanavalin A-Sepharose eluate. The numbered tracks, which correspond to fractions collected from the gel-filtration column, contain the three peaks of glutamate-binding activity shown in Fig. 3. Electrophoresis was carried out under reducing conditions as described in the Experimental section.

experiment and consisted of one major polypeptide of M_r 74000 as observed by SDS/polyacrylamide-gel electrophoresis (Fig. 4). A variable amount of glutamate-binding activity was eluted near the middle of the column run and consisted of two major polypeptides (Fig. 4). The size of this peak was greatly enhanced, at the expense of the first peak, if the protease inhibitors were omitted from the solubilization buffer (results not shown). A third peak of glutamate-binding protein was eluted with *N*-acetyltyrosine at the end of the column run. SDS/polyacrylamide-gel electrophoresis revealed a polypeptide of

Fig. 5. Glutamate binding to purified proteins of M_r 74000 (a) and 65000 (b).

[^3H]Glutamate binding was measured over a range of glutamate concentrations, and the results are means $\pm$ S.E.M. of three experiments. Where no error bars are indicated, the S.E.M. values are smaller than the symbols. Scatchard analysis of the binding data is shown in the insets: B, bound (nmol/mg); F, free (μM).

M_r 65000 (Fig. 4). This polypeptide had presumably bound loosely to the column matrix and so was eluted anomalously. Table 3 illustrates the final yield (14%) and purification of the M_r -74000 and the M_r -65000 glutamate-binding proteins.

Characterization of the purified glutamate-binding proteins

In order to determine if either of these proteins were part of the quisqualate-sensitive glutamate receptor, their binding abilities were further characterized. Scatchard analysis of glutamate binding to these proteins revealed a single binding site for each (Fig. 5). The protein of M_r 74000 had a K_d of $2.0 \pm 0.6 \mu\text{M}$ and a B_{max} of $15.5 \pm 0.2 \text{ nmol/mg}$, whereas that of M_r 65000 had a K_d

Fig. 6. Displacement of [^3H]glutamate binding to purified proteins of M_r 74000 (a) and 65000 (b).

Results are expressed as percentages of the specific binding seen with 25 nM [^3H]glutamate and are means $\pm$ S.E.M. of four separate experiments for quisqualate (■) or three for glutamate (●) and kainate (▲). The non-specific binding component, i.e. that in the presence of 1 mM-glutamate, amounted to 50% of the total binding.

of $1.3 \pm 0.1 \mu\text{M}$ and a B_{max} of $14.5 \pm 3.1 \text{ nmol/mg}$. If it is assumed that the molecular mass of the M_r -74000 protein in non-denaturing conditions is approx. 300 kDa [i.e. between that of urease and the exclusion limit (350 kDa) of the gel-filtration matrix], then the B_{max} of this protein is consistent with 4.6 mol of glutamate binding/mol of protein, whereas the B_{max} of the M_r -65000 polypeptide implies the binding of 0.9 mol of glutamate/mol of protein. The sensitivity of these glutamate-binding proteins to quisqualate and kainate was also examined. As shown in Fig. 6, the protein of M_r 74000 was sensitive to quisqualate (IC_{50} for glutamate binding was $1 \mu\text{M}$) but not to kainate, whereas the M_r -65000 was sensitive to neither analogue.

DISCUSSION

Central-nervous-system glutamate receptors are currently the focus of considerable attention, since they have been implicated in processes such as long-term potentiation (Collingridge *et al.*, 1983) and ischaemic damage (Onodera *et al.*, 1986), and in invertebrates glutamate receptors may be potential sites for pesticide action.

Previous attempts to isolate excitatory amino acid receptors

have met with mixed fortunes. Hampson & Wenthold (1988) used a domoic acid affinity column to isolate a 48 kDa kainate receptor from frog brain. The low $B_{\max}$ values (1.7 and 4.4 nmol/mg of protein) suggested that the preparation may not have been homogeneous. Gregor *et al.* (1988) also reported the purification of an M_r -49000 putative kainate receptor from chick brain. The authors suggested that the low degree of purification (41-fold) may have been due to the co-purification of denatured protein. Chen *et al.* (1988) purified two proteins (M_r 71000 and 63000) from rat brain whose glutamate-binding characteristics suggested that the larger was part of the glutamate receptor, although both ibotenate and quisqualate were relatively poor displacers of bound glutamate (IC_{50} 10–100 μ M). Chen *et al.* (1988) were unable to assign an approximate molecular size to the intact receptor, owing to the interaction of the gel-filtration matrices with these polypeptides.

We chose to attempt the purification of the crustacean receptor for a number of reasons: (1) the dense innervation (Florey & Cahill, 1982) suggested the presence of large numbers of receptor molecules; (2) the receptor is predominantly of one type; (3) tissue is available in relatively large quantities; and (4) it was known that concanavalin A interacted with the receptor (Mathers & Usherwood, 1976, 1978; Shinozaki & Ishida, 1979).

The preliminary characterization of the glutamate receptor in the muscle membrane as reported here is consistent with the presence of a quisqualate-sensitive receptor. The rank order of potency observed in this preparation, i.e. glutamate > quisqualate > ibotenate > kainate, is similar to that observed when the post-synaptic action of these agonists is measured electrophysiologically in crustacean muscle. In such analyses quisqualate is more potent than glutamate, perhaps because it is not metabolized by the tissue, whereas ibotenate and kainate have relatively little effect (Shinozaki, 1980). Similarly, the ability of domoate to modulate depolarization of crayfish muscle is much greater than that of kainate (Shinozaki, 1988). This is reflected in the greater ability of domoate to displace glutamate in this preparation, i.e. the IC_{20} (concn. giving 20% inhibition) for domoate was 0.1 μ M and the IC_{20} for kainate was 1 μ M. Interestingly, the ibotenate-induced hyperpolarization of crayfish muscle has a half-maximal effect at 0.2 mM (Shinozaki & Ishida, 1980), whereas its IC_{50} for glutamate displacement is 0.09 mM (Fig. 1). The relative order of potencies shown in Fig. 1 differs considerably from that observed by Syvertsen & Fonnum (1989). This could be due to the freezing of membranes before assay by these workers, a process reported to decrease glutamate binding to membranes (Sharif & Roberts, 1984; Filbin *et al.*, 1985). Alternatively, performing the binding assay at 4 °C and in the presence of Cl^- may also contribute to the observed differences. In our hands, freezing the *Nephrops* muscle membranes and subsequently assaying at 4 °C in Tris/HCl, as described by Syvertsen & Fonnum (1989), abolished quisqualate-sensitivity. However, we were unable to detect an increase in sensitivity to ibotenate (results not shown). We did, however, observe considerable variation in the quisqualate-sensitivity of glutamate binding under our assay conditions (the IC_{50} values ranged between 1 and 100 μ M). We have been unable to determine the reason for the variation, although the complex displacement curves shown in Fig. 1 imply that variable yields of the different quisqualate-sensitive glutamate-binding sites may have been responsible. Much less variation in quisqualate-sensitivity was observed after solubilization (IC_{50} values ranged between 1 and 20 μ M). The weak inhibition of glutamate binding to *Nephrops* muscle membrane by AMPA, *N*-methyl-D-aspartate and L-aspartate is consistent with previous results in the crayfish (Shinozaki, 1988; Syvertsen & Fonnum, 1989) and locust (Usherwood & Machili, 1968; Shinozaki, 1988) muscles.

Purification of the solubilized glutamate-binding sites achieved with concanavalin A was due to the small number of membrane proteins retained by, and specifically eluted from, the column (Fig. 4). A similarly small number of concanavalin A-positive proteins were found by Breer & Knipper (1985) in insect synaptic membranes. A substantial proportion (approx. 60%) of the glutamate-binding sites were not retained by the concanavalin column. These quisqualate- and kainate-insensitive sites may correspond to the lower-affinity sites detected in the membranes.

Subsequent gel filtration resulted in the identification of three sizes of glutamate-binding site. The middle peak appeared to be the product(s) of proteolysis, since it increased in the absence of the wide range of protease inhibitors used in this preparation. The polypeptide composition (Fig. 4) would be consistent with such a proposition. The high- M_r peak has a Stokes radius of 5.4 nm and an approximate M_r of 300000, with a subunit M_r of 74000. The subunit M_r is similar to the radiation-inactivation measurement of the glutamate-binding site in cockroach and mammalian synaptic membranes (Bardsley & Roberts, 1985; Sepulveda & Sattelle, 1989), but not with the AMPA-binding site (M_r 43200; Honore & Nielsen, 1985). The third peak, containing a polypeptide of M_r 65000, ran anomalously on the gel-filtration column, in a manner similar to that described for two polypeptides (M_r 71000 and 63000) which are thought to be part of a rat glutamate receptor (Chen *et al.*, 1988). The presumptive interaction with the AcA34 matrix was fortuitous in that it has permitted the separation of this protein, which showed no quisqualate-sensitivity, from the M_r -74000 protein, which was quisqualate-sensitive.

The result of the Scatchard analysis of glutamate binding to the M_r -74000 protein (i.e. 4.6 mol of glutamate to 1 mol of protein) suggested that the intact protein may consist of four similar subunits of M_r 74000, each of which binds to glutamate. Such a homotetrameric structure for a neurotransmitter receptor has been suggested for the locust acetylcholine receptor (Breer *et al.*, 1985). However, further analysis will be required to prove such a structure, since some aggregation may have occurred in the low concentrations of detergent used, the estimate of the M_r is inaccurate, and the ratio obtained (4.6:1) is not very convincing. We were unable to detect any co-operativity in the glutamate binding to either protein, even though biophysical experiments have suggested that there is co-operativity in channel gating of the excitatory receptor in both the locust muscle (Kerry *et al.*, 1988) and at the crayfish neuromuscular junction (Dudel, 1977). It is therefore conceivable that this protein is not involved in excitatory responses to glutamate, but rather in inhibitory responses, since chloride- and quisqualate-sensitive binding proteins have recently been described (Brose *et al.*, 1989).

In summary, we have isolated a protein with a subunit M_r of 74000, which may consist of four polypeptides each having a quisqualate-sensitive glutamate-binding site. The data suggest that this protein is a crustacean muscle glutamate receptor. The relationship, if any, between this protein and the M_r -65000 protein is unknown. It is conceivable that the latter is a breakdown product which has lost its quisqualate-sensitivity as a result of proteolysis.

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REFERENCES

- Ansorge, W. (1982) in *Electrophoresis* (Stathakos, D., ed.), pp. 235–242. de Gruyter, Berlin.
- Bardsley, M. E. & Roberts, P. J. (1985) *Biochem. Biophys. Res. Commun.* **126**, 227–232.

- Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254
- Breer, H. & Knipper, M. (1985) in *Neurochemical Techniques in Insect Research* (Breer, H. & Miller, T. A., eds.), pp. 125–155, Springer-Verlag, Berlin
- Breer, H., Kleene, R. & Hinz, G. (1985) *J. Neurosci.* **5**, 3386–3392
- Brose, N., Halpain, S., Suchanek, C. & Jahn, R. (1989) *J. Biol. Chem.* **264**, 9619–9625
- Chen, J.-W., Cunningham, M. D., Galton, N. & Michaelis, E. K. (1988) *J. Biol. Chem.* **263**, 417–426
- Collingridge, G. L., Kohl, S. J. & McLennan, H. (1983) *J. Physiol. (London)* **334**, 33–46
- Dudel, J. (1977) *Pflügers Arch.* **368**, 49–54
- Filbin, M. T., Eldefrawi, M. E. & Eldefrawi, A. T. (1985) *Life Sci.* **36**, 1531–1539
- Florey, E. & Cahill, M. (1982) *Cell Tissue Res.* **224**, 527–541
- Fonnum, F. (1984) *J. Neurochem.* **42**, 1–11
- Gregor, P., Eshhar, N., Ortega, A. & Teichberg, V. I. (1988) *EMBO J.* **7**, 2673–2679
- Gregor, P., Mano, I., Maoz, I., McKeown, M. & Teichberg, V. I. (1989) *Nature (London)* **342**, 689–692
- Hampson, D. R. & Wenthold, R. J. (1988) *J. Biol. Chem.* **263**, 2500–2505
- Hollman, M., O'Shea-Greenfield, A., Rogers, S. W. & Heinemann, S. (1989) *Nature (London)* **342**, 643–648
- Honore, T. & Nielsen, M. (1985) *Neurosci. Lett.* **54**, 27–32
- Keinanen, K., Wisden, W., Sommer, B., Werner, P., Herb, A., Verdoorn, T. A., Sakmann, B. & Seeburg, P. H. (1990) *Science* **249**, 556–560
- Kerry, C. J., Ramsey, R. L., Sansom, M. S. P. & Usherwood, P. N. R. (1988) *Biophys. J.* **53**, 39–52
- Kuonen, D. R. & Roberts, P. J. (1987) *J. Neurochem.* **49**, 272–281
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Mathers, D. A. & Usherwood, P. N. R. (1976) *Nature (London)* **259**, 409–411
- Mathers, D. A. & Usherwood, P. N. R. (1978) *Comp. Biochem. Physiol.* **59C**, 151–155
- McBain, A. E. & Wheal, H. V. (1984) *Comp. Biochem. Physiol.* **77C**, 357–362
- Monaghan, D. T., Yao, D. & Cotman, C. W. (1984) *Brain Res.* **324**, 160–164
- Onodera, H., Sata, G. & Kogure, K. (1986) *Neurosci. Lett.* **68**, 169–174
- Sepulveda, M.-I. & Sattelle, D. B. (1989) *Neurosci. Lett.* **100**, 210–214
- Sharif, N. A. & Roberts, P. J. (1984) *Neurochem. Res.* **9**, 81–101
- Shinozaki, H. (1980) *Progr. Neurobiol.* **14**, 121–155
- Shinozaki, H. (1988) *Progr. Neurobiol.* **30**, 399–435
- Shinozaki, H. & Ishida, M. (1979) *Brain Res.* **161**, 493–501
- Shinozaki, H. & Ishida, M. (1980) *Brain Res.* **198**, 157–165
- Shinozaki, H. & Shibuya, I. (1974) *Neuropharmacology* **13**, 663–672
- Sugiyama, H., Ito, I. & Watanake, M. (1989) *Neuron* **3**, 129–132
- Syvetsen, C. & Fonnum, F. (1989) *J. Neurochem.* **52**, 755–760
- Takeuchi, A. (1987) *Jpn. J. Physiol.* **37**, 559–572
- Usherwood, P. N. R. (1978) *Adv. Comp. Biochem. Physiol.* **7**, 227–309
- Usherwood, P. N. R. & Machili, P. (1968) *J. Exp. Biol.* **49**, 341–361
- Wada, K., Dechesne, C. J., Shimasaki, S., King, R. G., Kusano, K., Buonanno, A., Hampson, D. R., Banner, C., Wenthold, R. J. & Nakatani, Y. (1989) *Nature (London)* **342**, 684–689
- Watkins, J. C. & Evans, R. H. A. (1981) *Rev. Pharmacol. Toxicol.* **21**, 165–204

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