Presence of *N*-methyl-D-aspartate-like and kainate-like activities in bovine brain

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Searching for the natural ligands interacting with brain excitatory amino acid receptors, we have isolated from cow brain a low-M_r ampholyte fraction containing molecules with excitatory properties similar to those of *N*-methyl-D-aspartate and kainate, which cannot be accounted for by any of the known brain excitants. This finding supports the hypothesis of the existence of excitatory neurotransmitters other than L-glutamate and L-aspartate.

Excitatory amino acid *Receptor* *Brain endogenous ligand*
Putative excitatory neurotransmitter

1. INTRODUCTION

The identity of the neurotransmitter(s) responsible for the excitatory transactions of the mammalian central nervous system has been the topic of intensive research over the last 20 years. During this period, the attention has been focused mainly on two brain amino acids, L-Glutamate (L-Glu) and L-Aspartate (L-Asp) now considered by most investigators to be genuine neurotransmitters [1–3]. Recently, however, several observations have led to a reevaluation of the transmitter role of L-Glu and L-Asp, and to the question whether other endogenous excitatory substances might in fact be endowed with this function. Central to this new dilemma have been the demonstration of the heterogeneous nature of the receptors of excitatory amino acids [3–5] and the fact that only one receptor is activated preferentially by L-Glu and L-Asp (the L-Glu receptor) [3]. The natural ligands acting on the other receptors, namely the *N*-methyl-D-aspartate (NMDA) receptor, the kainate (KA) receptor and the Quisqualate (Quis) receptor remain so far unknown but their existence can be suspected. The case for the existence of a NMDA-like neurotransmitter is particularly strong since antagonists of NMDA, that are ineffective in blocking L-Glu or L-Asp-induced neuronal depolarizations, do block the postsynaptic excitations resulting from the stimulation of various presynaptic afferences [3,5,6].

To settle the question of the nature of the endogenous ligands of the various excitatory amino acid receptors, we have carried out a search for novel brain endogenous compounds capable of interacting with these receptors.

We here report the finding that extracts from cow brain contain molecules with excitatory properties similar to those of NMDA and KA (or Quis), which cannot be accounted for by any of the known brain excitants.

2. METHODS

2.1. *Preparation of the active extract*

Cow brain was obtained from a local slaughterhouse 20 min after the death of the animal. The brain was kept frozen at −80°C until use. For the preparation of the active extract, 600 g brain tissue were crushed into powder, homogenized in 2 l acidified acetone containing 0.2 ml of 10 N HCl, 2 ml pure 2-mercaptoethanol and trace amounts of $^{22}Na^+$ (1 μCi), L-[^{14}C]Glu (0.5 μCi), L-[^{14}C]Asp (0.5 μCi) and $^{36}Cl^-$ (0.5 μCi).

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The homogenate was stirred for 4 h at room temperature, filtered on Whatman 1 paper and the filtrate evaporated at 40°C under reduced pressure. The residue was dissolved in 100 ml deionized water, ultracentrifuged at 100000 × g for 1 h and filtered under suction. The clear yellow filtrate was then applied on a Dowex W50 X-8 H^+ form (100–200 mesh) column (30 × 5.5 cm) and eluted at a constant flow rate (12 ml/min), first with 3 l deionized water and then with 3 NH_4OH. The radioactivity in the eluent was monitored by the removal of 200-μl aliquots of each fraction (10 ml) and counting in a Packard autogamma scintillation using Triton-Toluene as a scintillation fluid. The fractions containing the ^{14}C-radioactivity as well as those eluted before and after the radioactive peak were pooled and evaporated. The non-radioactive residues were dissolved in 2 ml of 26 mM Tris–HCl (pH 7.3) and their osmolarity was measured in a Halbmikro osmometer (Knauer, Berlin). They were then divided into appropriate aliquots and kept frozen at −80°C until assayed for excitatory activity.

The residue from the pooled fractions containing ^{14}C-radioactivity was redissolved in 5 ml of 20 mM ammonium acetate (pH 6.8) applied on a 120 × 1.8 cm Biogel P_2 column equilibrated in the same solution and eluted at a flow rate of 0.6 ml/min. Aliquots of each fraction (8 ml) were analyzed for their absorbance at 230 nm and their ^{14}C-content. Fractions were pooled, lyophilized and prepared for the assay of excitatory activity as described above.

2.2. *Assay of excitatory activity*

A modification of the Na^+ flux assay developed in our laboratory [4] was used. The assay is based on the measure of the rate of release of $^{22}Na^+$ ions from $^{22}Na^+$-preloaded rat striatum slices. Excitatory amino acids increase this rate in a dose-dependent way and this effect is also expected from brain extracts containing excitatory ligands. One 300 μm thick slice from striata of a 40–60-day old SPD male rat is placed in a basket-shaped sieve kept in an oxigenated physiological medium [4] for 30–40 min at 37°C and, for the successive 45 min, at room temperature in 0.25 ml of the same medium containing 2 μCi $^{22}Na^+$. The sieve is then transferred every 2 min through a series of 10 tubes, each containing 2 ml non-radioactive physiological solution, including, from tube 5–9, 10^{-7} M tetrodotoxin, 0.02 mM Ca^{2+} and 10 mM Mg^{2+}. Tube 10 contains the brain extract to be tested in 300 μl of a medium identical to that in the preceding tubes except for the NaCl concentration, which is reduced by an amount corresponding, on an osmolarity basis, to that of the added brain extract. The NaCl is, however, never decreased below 60 mM, the concentration necessary for the proper functioning of the assay. In experiments of inhibition of the excitatory activity of brain extracts, the antagonist of excitatory amino acids was added in tube 9 and 10 and the brain extract in tube 10.

The amount of $^{22}Na^+$ in each wash out tube is measured, as well as that left in the slice at the end of the experiment and its rate of release is expressed in terms of a specific efflux rate $R_T = (C_{(t-\Delta)} - C_t)/(C_{(t-\Delta t)} \times \Delta t$ were C_t is the $^{22}Na^+$ content of the slices at time t and $C(t-\Delta t)$ at time $(t-\Delta t)$. The stimulatory effect of an effector is defined as Δ, a parameter equal to the difference between the R_T measured during the 2 min before and 2 min after the exposure to the effector. The amount of activity present in a given extract is expressed in terms of the amount of L-Glu which would produce the same Δ.

2.3. *Analytical procedures*

Amino acid analysis was performed by an automatic amino acid analyzer Dionex model D 550 (Palo Alto CA). For thin-layer chromatography (TLC), samples (5 μl) were applied on Kieselgel 60 F aluminium sheets (Merck) and chromatographed using either of the following solvents: ethanol–water (7:3, by vol.), butanol–acetic acid–water (8:2:2, by vol.) or chloroform–methanol–ammonia (2:2:1, by vol.).

3. RESULTS

3.1. *Extraction of neuroexcitatory activity*

After homogenization, 60% of the $^{22}Na^+$ and 80% of L-[^{14}C]Glu, and L-[^{14}C]Asp added as tracers, are recovered in the acidified acetone extract. Upon chromatography on a Dowex W50 X-8 resin in its H^+ form, the $^{36}Cl^-$ considered here as a label of the anions are eluted with water, whereas the ampholytes including L-[^{14}C]Glu and L-[^{14}C]Asp are eluted with 2 N ammonia. The $^{22}Na^+$

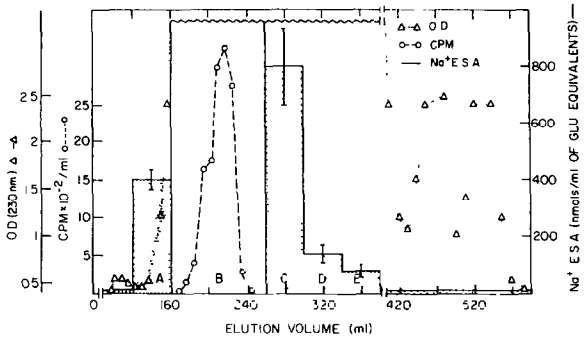

Fig. 1. Biogel P2 column chromatography of ampholyte fraction isolated from bovine brain on Dowex 50W column; 6 ml of extract containing trace amounts of L-[^{14}C]Glu and L-[^{14}C]Asp were chromatographed on a 1.8 × 120 cm column at a flow rate of 0.6 ml/min and eluted with 20 mM ammonium acetate pH 6.8; 8-ml fractions were collected and monitored for absorbance and radioactivity content. Groups of 5 successive fractions were pooled and assayed for Na^+ efflux stimulatory activity (Na^+ ESA).

ions are retained on the column under these conditions and therefore the ampholyte fraction is effectively desalted at this stage [7]. Since the fractions of the eluate that are devoid of [^{14}C] L-Glu and [^{14}C]L-Asp, do not display any excitatory activity, only the ampholyte fraction is processed further on a Biogel P2 column. Fig.1 shows its profile of elution. [^{14}C] L-Glu and [^{14}C] L-Asp emerge after 160 ml, together with the largest peak of absorbance at 230 nm. Optically dense material continues to appear well after the bed volume of the resin indicating the elution of molecules retarded by hydrophobic interactions with the Biogel P2 matrix.

A large amount of neuroexcitatory activity free from L-Glu and L-Asp (equivalent to that of 20 μmol of L-Glu) emerged from 260 ml to 300 ml (fraction C). Smaller amounts of excitatory activity are also present in fractions A, D and E. Accordingly, further analysis of the composition of fraction C was carried out. Amino acid analysis confirms the absence of L-Glu and L-Asp in fraction C and reveals the presence of urea NH_4OH and small amounts of tyrosine, phenylalanine and 4 unidentified molecules eluting between phenylalanine and ammonia. Neither the amino acids present nor urea possess any excitatory activity at the concentrations present in fraction C (~50 mM urea, 1.47 mM tyrosine, 3.6 mM phenylalanine and ~ NH_4OH). Analysis of fraction C by TLC reveals the presence of 5 ninhydrin-negative spots that are visualized with a sulfuric acid spray. Analysis by high pressure liquid chromatography (HPLC) using a C 18 column and a 1:1 mixture of acetonitrile and 10 mM triethylammonium sulfate at pH 3, as eluant, shows the presence of 3 major peaks absorbing at 250 nm.

3.2. *Reproducibility of the purification procedure*

In 5 different preparations, the activity found in fraction C varied from 32 to 7 μmol of L-Glu equivalents, with an average of 20. Such a variability could be accounted for by a chemical instability of the active molecules under the conditions used during the procedure. We have found that bubbling O_2 for 45 min in a solution containing fraction C, destroys 91% of the neuroexcitatory activity. This suggests that oxidation by atmospheric O_2 during the lengthy preparation could be responsible for the disappearance of part of the activity in some extracts.

Table 1

Antagonist	Fraction C	NMDA 30 μM	KA 100 μM	Quis 100 μM	Glu 0.5 μM
–	9.1 ± 0.5	13 ± 0.9	10 ± 1.3	8.2 ± 1	8.5 ± 0.6
2-APV 200 μM	3.4 ± 2[a]	1 ± 0.9[a]	9.7 ± 0.3	8.3 ± 0.5	7.9 ± 1
Secobarbital 1 mM	5.1 ± 1[a]	11 ± 1.5	2.5 ± 0.7[a]	2 ± 1.1[a]	8.2 ± 0.8

Inhibition by specific antagonists of the $^{22}Na^+$ efflux stimulation induced in brain slices by a purified brain extract and by selective agonists of the 4 classes of neuroexcitatory receptors. Each value (mean ± SD from at least 6 experiments) represents the difference between the basal specific efflux rate and the rate stimulated by an effector [4]

[a] $P < 0.01$ by Student's t-test

3.3. *Receptor specificity of fraction C*

Table 1 shows that the activity of fraction C is reduced by 60% in the presence of 2-APV, an NMDA antagonist and by 40% in the presence of Secobarbital, a KA and Quis inhibitor [8]. The extract thus displays a receptor specificity intermediate between those of NMDA and KA. Fraction C contains about 30 mM ammonia (present in the elution buffer of the Biogel column), which has been reported to affect the permeability to Na^+ of brain cells [9]. We have therefore tested its effect in the flux assay. At concentrations up to 60 mM, ammonia does not produce a stimulation of the specific Na^+ efflux rate higher than 10% of that caused by fraction C. This effect, in addition, could not be inhibited by 2-APV or by Secobarbital, which confirms that its contribution to the activity of fraction C can be considered as negligible.

4. DISCUSSION

Several observations mentioned in the introduction, have led to the suggestion that neuroexcitatory transmitters with pharmacological properties different from those of L-Glu and L-Asp might play an important role in the CNS [3]. We now report that compounds displaying neuroexcitatory activity (in the Na^+ flux assay) and different from all the known excitatory amino acids indeed exist in the mammalian brain.

Several problems were faced. The major one concerned the removal from brain of the disproportionately large amounts of Glu and Asp (13 and 3 μmol/g, respectively [1]). Another was the presentation of the chemical structure and properties of brain excitatory substances during all the isolation steps. Finally, it was essential to isolate the neuroactive substances in sufficient amounts to permit the assay of their pharmacological effects.

The total amount of activity found in the most active extract (fraction C), prepared from 600 g brain, corresponds to that of 37 μmol L-Glu, a value much lower than what is expected in the case of a neurotransmitter responsible for the bulk of brain excitatory transactions, whose levels should be in the order of magnitude of those of GABA (1.5 μmol/g), an inhibitory transmitter in the mammalian CNS [1]. This difference however can be explained by the low yield of the isolation procedure adopted so far, due possibly to the sensitivity of the excitatory molecules to oxidation, as well as to the extent of the overlap, in the elution from the Biogel P2 column, between the L-Glu and L-Asp peak and that of the material contained in fraction C.

The latter displays a receptor specificity which is intermediate between those of NMDA and kainate (or Quisqualate). These results are interpreted as being due to the presence of either two different compounds, a NMDA-like and a kainate-like material, or of a molecule with similar affinity for both receptors. The choice between the two possibilities has to await a chromatographic resolution of the components of fraction C.

Some information on the chemical properties of the active molecules can be inferred from their chromatographic behaviour on the Dowex and Biogel columns. The presence of a positively charged group is indicated by the absorption under acidic conditions on the Dowex cation exchange resin and the subsequent displacement in alkaline environment. The volume required the elute the active molecules from the Biogel column suggests that they are most probably retarded by hydrophobic interactions with the matrix. This phenomenon may be due to the presence of aromatic functions in the active molecules.

One ought to ask whether the neuroactive molecules present in fraction C interact directly with excitatory amino acid receptors or may increase the $^{22}Na^+$ efflux rate by other mechanisms. Indeed, the ouabain-like substance that is known to be present in brain [10] could possibly depolarize nerve terminals, as ouabain and K^+ ions do, and induce the synaptic release of endogenous excitatory neurotransmitters onto excitatory amino acid receptors [4] thus producing an increase in $^{22}Na^+$ efflux rate. Drastic ionic manipulations of the medium in which the brain slices are bathed in the $^{22}Na^+$ efflux assay, such as the reduction of Na^+ below 60 mM and the removal of Ca^{2+} (by possible Ca^{2+} chelators present in the brain extract) without a compensating increase in Mg^{2+} levels, could lead to the same result.

For the above reasons, we have routinely assayed the brain extracts under conditions known to prevent effectively the Ca^{2+}-dependent release of excitatory neurotransmitters, the activation of

the voltage-dependent Na^+ channel or damaging ionic imbalances [4]. It is therefore most likely that the $^{22}Na^+$ efflux produced by fraction C is indeed due to compounds acting directly on the excitatory amino acid receptors.

In conclusion, we have shown the presence in cow brain, of neuroexcitatory molecules with NMDA-like and kainate-like pharmacological properties. This finding supports, but does not prove conclusively, the hypothesis of the existence of so far unidentified neurotransmitters acting on the receptors of NMDA and kainate. It is now necessary to purify completely and identify chemically these neuroactive compounds, and subsequently establish whether they meet the criteria for their assignment as neurotransmitters.

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