The Ca^{2+}/Cl^- dependent L-[^{3}H]glutamate binding: a new receptor or a particular transport process?

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Ca^{2+}/Cl^- increases the L-[^{3}H]glutamate binding to rat brain synaptic membranes. It was suggested that Ca^{2+}/Cl^- expresses a new class of glutamate receptors. We report several lines of evidence suggesting that Ca^{2+}/Cl^- in fact favours a glutamate transport into membrane vesicles. This finding may serve to reconcile most of the discrepancies found in the literature on the glutamate binding and its pharmacology.

Glutamate binding *Ca^{2+}/Cl^- effect on glutamate receptor/transport*

1. INTRODUCTION

L-Glutamate is thought to function as the major excitatory transmitter in the mammalian central nervous system [1,2]. Electrophysiological experiments have revealed that the actions of glutamate are mediated by multiple receptors, classically termed *N*-methyl-D-aspartate (NMDA), kainate (KA) and quisqualate (QA), according to their agonist specificities [3].

Recent biochemical studies have shown that Ca^{2+}/Cl^- increases the L-[^{3}H]glutamate ([^{3}H]GLU) binding by expressing a new class of receptors [4,5]. This Ca^{2+}/Cl^--sensitive population of GLU binding sites is potently inhibited by L-2-amino-4-phosphonobutyrate (L-APB) [4] and DL-[^{3}H]APB may label these sites [6]. Two hypotheses were proposed to explain this Ca^{2+}/Cl^- effect: (1) the linkage of some GLU sites to a membrane Cl^- ion channel (4,7) analogous to the GABA system [8], or (2) the existence of a desensitised form of receptor [5] with divalent cations promoting the transition from a low affinity to a high affinity state (possible analogy with the nicotinic receptor [9]).

We report here that Ca^{2+}/Cl^- enhanced [^{3}H]GLU binding seems to correspond to ligand accumulation into membrane vesicles and not to receptor occupancy. These data may also serve to reconcile the discrepancies between [^{3}H]GLU binding data performed under various experimental conditions. A preliminary account of this work has appeared as an abstract [10].

2. MATERIALS AND METHODS

Whole brains from male Wistar rats were homogenised in 20 vol. of ice-cold sucrose (0.32 M) and the homogenate centrifuged at $1000 \times g$ for 10 min at 4°C. The supernatant was recentrifuged ($17\,000 \times g$, 20 min) and the resultant pellet (P_2) lysed in 40 vol. of ice-cold distilled water (45 min). The membranes were centrifuged at $20\,000 \times g$ for 35 min, then washed three times by resuspension in 40 vol. of ice-cold water. The final pellet was resuspended in 50 mM Tris–citrate buffer, pH 7.4, and was diluted twice in the same buffer containing or not Ca^{2+}/Cl^- or another salt. Binding of [^{3}H]GLU was measured by incubating 100 μl aliquots of membrane suspension, 100 μl of [^{3}H]GLU (final concentration 100 nM) with 10 μl of buffer either free of added substances or containing unlabelled displacing substances at 1 mM. The final protein concentration ranged from 0.8 to 1.0 mg/ml. When Ca^{2+}/Cl^- or other salts were added, their final concentrations were 2.5 mM. Unless otherwise stated, the incubation was stopped after 10 or 50 min, for binding experiments performed

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at 37 or 0°C respectively, by adding 3.5 ml of 50 mM ice-cold Tris–citrate buffer pH 7.4 and filtration on Whatman glass filter (1 μM pore size). Filters were washed once with 3.5 ml of cold buffer and counted in a Kontron scintillation counter. Specific binding of [^{3}H]GLU was determined by subtracting the non specific component (binding in the presence of 1 mM unlabelled GLU) from the total binding.

Protein was assayed as described by Lowry et al. [11].

3. RESULTS AND DISCUSSION

The time course of Ca^{2+}/Cl^--dependent [^{3}H] GLU binding was biphasic at 37°C yet monophasic at 0°C (fig. 1). At 37°C, the equilibrium value was reached at 8 min and was maintained for a further 3–5 min, after which, the [^{3}H]GLU binding decreased exponentially. This pattern is quite similar to that reported for the time-course of glutamate, aspartate and GABA transport into membrane vesicles prepared from rat brain [12,13]. In fact, the decay of the amount of radioactive compound taken up presumably results from the dissipation of the artificially imposed ion gradient [14] by a background ion flux [12] and not from receptor degradation by proteases. Proteases inhibitors did not inhibit this decay (data not shown). At 0°C, the time-course of [^{3}H]GLU binding follows a classical Michaelis pattern (no decay after reaching the plateau).

The second point to be emphasised from these experiments was that Ca^{2+}/Cl^- increased the [^{3}H]GLU binding at 37°C, as previously reported [4], while Ca^{2+}/Cl^- slightly inhibited the [^{3}H]GLU binding at 0°C [15].

It is known that low temperatures (0–4°C) dramatically reduce or render transport processes across membranes undetectable, whereas low temperatures only modify the kinetic constant of the binding, but not the binding process itself. Therefore, our results suggest the presence of GLU transport into the membrane vesicles rather than ligand binding to membranes, as aspartate and glutamate can bind at 0°C as well as 37°C [14–17].

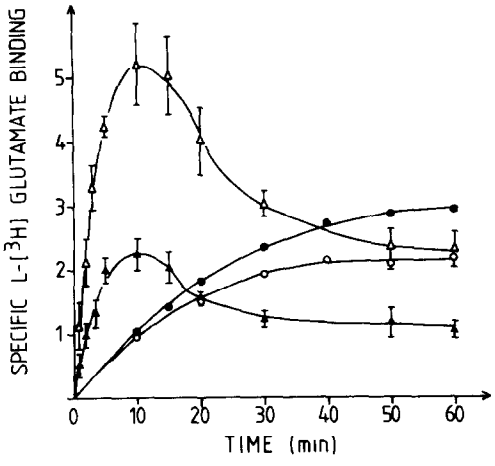

Fig. 1. Time course of [^{3}H]GLU binding to whole rat brain synaptic membranes at 0 or 37°C in absence or in presence of 2.5 mM Ca^{2+}/Cl^-. ●, 0°C, in absence of Ca^{2+}/Cl^-; ○, 0°C, 2.5 mM Ca^{2+}/Cl^-; ▲, 37°C, in absence of Ca^{2+}/Cl^-; △, 37°C, 2.5 mM Ca^{2+}/Cl^-. Membranes were prepared and specific binding of [^{3}H]GLU (100 nM) measured as described in Materials and Methods. Results are means ± SD from six separate experiments, each in duplicate and are expressed in pmol/mg protein. For clarity, the error bars have only been indicated in the figure for those experiments performed at 37°C. At 0°C the SEM of each point differed by less than 10%.

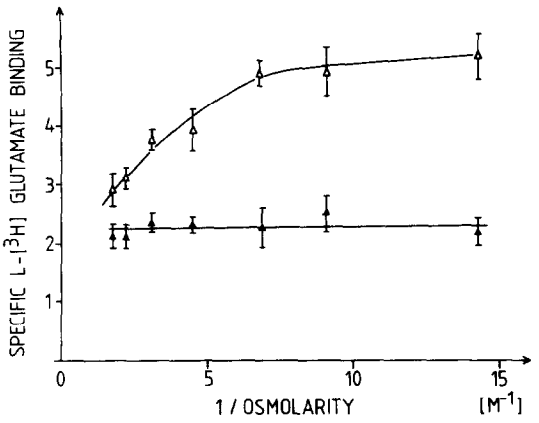

Fig. 2. Effect of external osmolarity on the [^{3}H]GLU binding to rat whole brain membranes. Binding experiments were performed at 37°C as indicated in Materials and Methods. The osmolarity of the incubation medium was increased by augmenting the sucrose concentration. Each point is a mean ± SD of three separate experiments, each in duplicate. ▲, [^{3}H]GLU binding (pmol/mg protein) at 37°C in absence of Ca^{2+}/Cl^-; △, [^{3}H]GLU binding (pmol/mg protein) at 37°C in presence of Ca^{2+}/Cl^-.

Furthermore, at 0°C, DL-[^{3}H]APB, a potent agonist at the Ca^{2+}/Cl^--sensitive GLU binding sites [6], does not bind to membranes even in the presence of Ca^{2+}/Cl^- [6].

In order to distinguish uptake from binding, the osmolarity of the incubation medium was augmented by the addition of increasing concentrations of sucrose. The increasing osmolarity reduced the intravesicular space of membrane vesicles. Thus, while uptake capacity decreases, ligand binding to receptor sites remains unaffected. At 37°C, the effect of Ca^{2+}/Cl^- on the [^{3}H]GLU binding depended on the osmolarity of the binding assay buffer (fig. 2), while the extent of binding in absence of Ca^{2+}/Cl^- was insensitive to the medium osmolarity (fig. 2). This provides additional evidence that the [^{3}H]GLU binding in the presence of Ca^{2+}/Cl^- does in fact correspond to transport of GLU into the membrane vesicles, as opposed to binding to the membrane.

To further support our hypothesis on the existence of a GLU transport mechanism, we examined the ion specificity (fig. 3). Fig. 3 shows that in addition to Ca^{2+}, several other divalent (Mg^{2+}, Mn^{2+}) and monovalent (K^+, Li^+) cations as their chloride salt and at a concentration of 2.5 mM, increase the [^{3}H]GLU binding when experiments are performed at 37°C in 50 mM Tris-citrate buffer, pH 7.4. These results suggest that there is no cation specificity. Moreover, this agrees with the fact that Mg^{2+}/Cl^- and Mn^{2+}/Cl^- were able to partially mimic the effects of Ca^{2+} on the DL-[^{3}H]APB binding as previously reported [6].

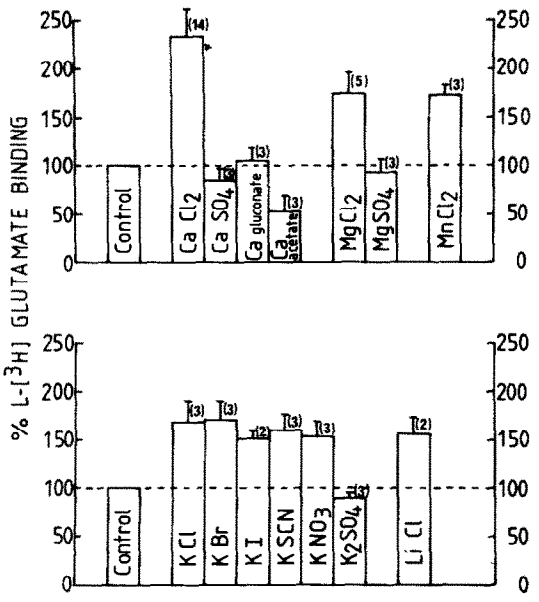

Fig. 3. Effects of various cations on [^{3}H]GLU binding to rat whole brain membranes. Binding in control samples was 2.22 ± 0.30 pmol/mg protein and taken as 100%. Binding experiments were performed at 37°C as described in Materials and Methods. Results are mean values ± SD of the percentage of binding as compared to control samples (100%). Number in parentheses refers to the number of separate experiments.

Several anions, such as bromide, iodide, nitrate and sulfocyanate promoted the enhancement of [^{3}H]GLU binding, as did chloride. They were all permeant through membrane vesicles, synaptosomes and cell membranes [14,19]. Impermeant anions, such as sulfate, gluconate and acetate were ineffective, whatever the cation considered. It is known that there is a high correlation between the degree of anion permeability, which creates a membrane potential (interior negative) and the enhancement of the initial rate of GLU transport into membrane vesicles. We observed that the anion order of potency which enhanced [^{3}H]GLU binding (fig. 3) is similar to that reported for increasing the glutamate/aspartate rate of uptake into membrane vesicles [20,21]. These results indicated an anion specificity permeant anions being effective in increasing [^{3}H]GLU binding, impermeant anions being inefficient. They revealed that Ca^{2+} is the most effective counter ion. This further strengthens the possibility of an electrogenic GLU transport into membrane vesicles. It may be suggested that the membrane potential (interior negative) is created by the differential permeability of the anion (e.g. Cl^-) with respect to the cation (e.g. Ca^{2+}) through the membranes of the vesicles. This implied that GLU, which presents a net charge of -1 at pH 7.4, is transported with a divalent cation or two monovalent cations in a complex possessing a net charge of $+1$.

The Ca^{2+}/Cl^- enhanced [^{3}H]GLU binding previously observed, was reported to be due to an increase in the number of binding sites, with no change in their affinity [4]. Incidentally, we found that the density of these sites was about 85 pmol/mg protein, which appears to closely correlate to the capacity of a transport mechanism, rather than to a maximal number of binding sites.

Electron micrograph (fig. 4) of the membrane preparation, used in our binding experiments, revealed the presence of several membrane vesicles, despite the multiple hypotonic shock (45 min in distilled ice-cold water followed by three main washings). Our membrane preparation was derived from that previously described [28] and commonly used for binding assays.

All our data suggested that the Ca^{2+}/Cl^- effect on the GLU binding was due to a GLU transport mechanism. However, L-GLU, L-aspartate and GABA are known to be sequestered in nerve terminal preparations (synaptosomes) [19,22,23], brain slices [24] and membrane vesicles [20]. A high affinity Na^+-dependent uptake system shared by acidic amino acid (GLU, aspartate) has been described [13,19,24]. The active transport of these compounds occurred with a net charge of +2 [13,26]. The problem of the transport mechanism specificity, revealed in the presence of Ca^{2+}/Cl^-, as compared to the classical high affinity GLU uptake, was raised. Thus, we examined the pharmacology of these phenomena. Although D-ASP, which is a poor displacing agent of the [^{3}H]GLU binding determined in the absence of Ca^{2+}/Cl^- and a relatively potent inhibitor of GLU uptake, displaced the Ca^{2+}/Cl^- dependent GLU binding effectively, D-GLU and quisqualate, both weak

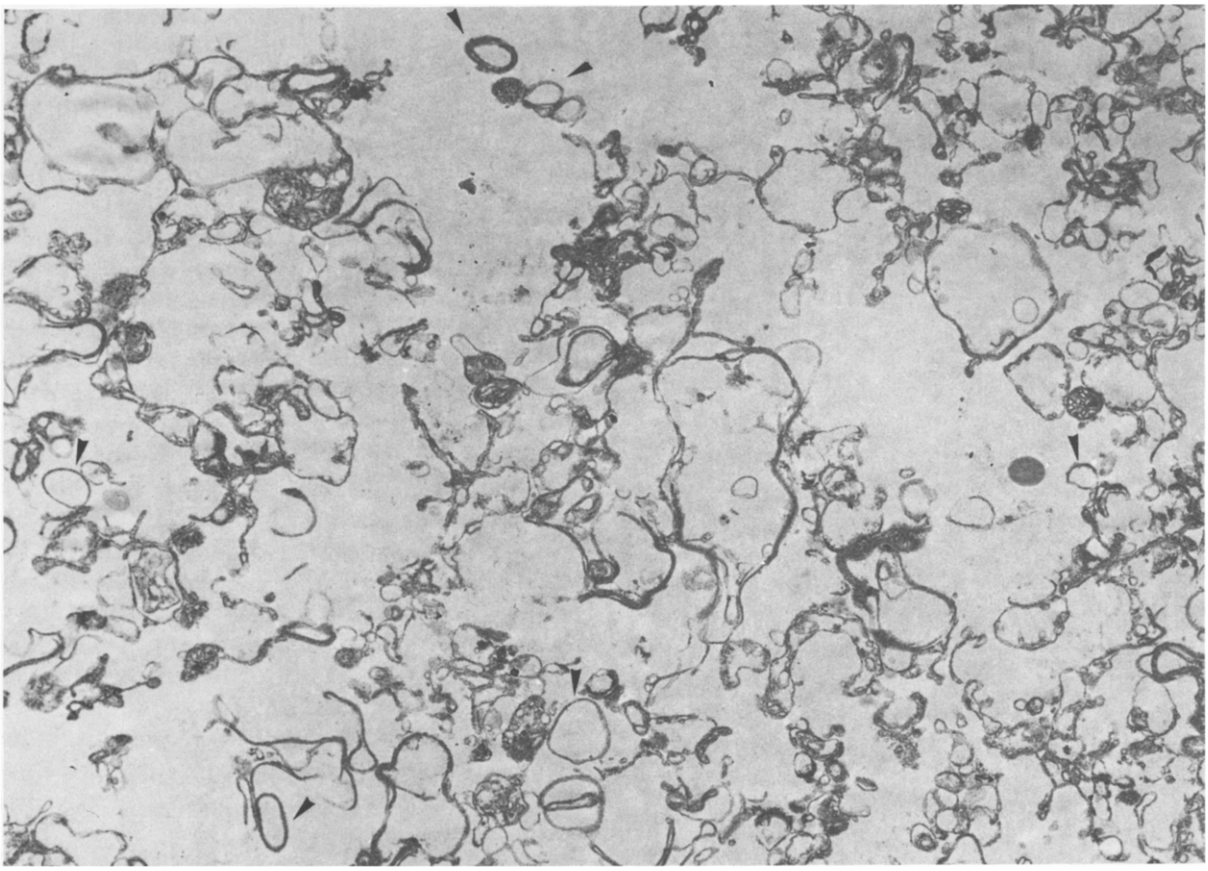

Fig. 4. Electron micrograph of the rat brain membrane at a magnification of 12 000 showing the presence of membrane vesicles in the preparation used for binding assays. The membrane preparation was performed as reported in Materials and Methods. After resuspension in 50 mM Tris-citrate buffer, pH 7.4, the membranes were centrifuged at 50 000 × g for 30 min. The resulting pellet was fixed with the Tris-citrate buffer containing 5% glutaraldehyde and post-fixed with 1% osmium tetroxyde in 0.2 M cacodylate buffer. After graduated dehydration with alcohol, the samples were embedded in spur resing. Ultrathin sections (70 nm) stained with uranyl acetate and lead citrate were examined with a 100 B Jeol electron microscope. Arrows indicate membrane vesicles.

GLU uptake inhibitors, displaced the Ca^{2+}/Cl^--dependent GLU binding with high affinity (data not shown, [4,5]). Moreover, DL-APB (1 mM) inhibited only 30% of the [^{3}H]GLU uptake into synaptosomes at appropriate protein concentrations (0.02–0.2 mg/ ml), for a 10 min incubation period (data not shown) in agreement with inhibition by APB of the GLU uptake in rat striatum [27]. We found that DL-APB displaced up to 80% of the Ca^{2+}/Cl^--dependent GLU binding, as previously reported [4,5]. It is clear, that the uptake mechanism, described in this paper, did not correspond to the classical high affinity uptake, in fact, it occurred in:

(1) a hypotonic medium (50 mM Tris–citrate) and not in a isoosmotic medium;

(2) the presence of only 2.5 mM Ca^{2+}/Cl^- and not in the presence of imposed $Na_o^+ > Na_i^+$ and $K_o^+ > K_o^+$ gradients like in membrane vesicles, or in presence of a gradient created by the ATPase like in synaptosomes;

(3) a membrane preparation (1 mg/ml protein) containing few vesicles, with a different membrane permeability due to the hypotonic medium and low temperature.

Finally, we suggest that Ca^{2+}/Cl^--enhanced [^{3}H]GLU binding does in fact correspond to transport of a complex GLU^-–Ca^{2+} into membrane vesicles in a hypotonic medium, the driving force being generated by the relative permeability of the anion (Cl^-) in relation to that of the cation (Ca^{2+}).

4. GENERAL DISCUSSION AND CONCLUSION

Our data strongly suggest that Ca^{2+}/Cl^--enhanced [^{3}H]GLU binding does in fact correspond to transport of GLU into membrane vesicles, as supported by the following points:

(1) the time course of Ca^{2+}/Cl^--dependent [^{3}H]GLU binding followed the pattern of uptake of excitatory amino acids into synaptic vesicles;

(2) low temperatures (0–4°C) completely abolish the Ca^{2+}/Cl^- effects;

(3) increasing the osmolarity of the incubation medium, decreases the Ca^{2+}/Cl^- enhancement of L-[^{3}H]GLU binding, without affecting the GLU binding itself;

(4) the cation and anion specificity coincide with the ability of these ions to create membrane potential, permeant anions being efficient (Cl^-, Br^-, SCN^-, NO_3), impermeant anions (SO_4^{2-}, gluconate, acetate) being ineffective.

(5) the density of 'binding sites' (80–100 pmol/ mg protein) fit better with an uptake capacity than with a number of receptor sites; the K_d is in the μmolar range;

(6) electron micrograph revealed the presence of membrane vesicles in extensively water-washed membranes.

These data may also serve to reconcile the discrepancies between GLU binding data [3,5,16, 17,28] at 0 and 37°C since the latter may contain a significant uptake component depending on the nature of the buffer used. They could explain several effects such as sonication, freezing, thawing, effects of triton on 'glutamate binding' performed in buffer containing Cl^- ions. It was reported that sonication increased glutamate binding up to 33 fold and it is known that sonication favours the vesiculation of membranes. Freezing and thawing, which breaks membrane vesicles, inhibited 'glutamate binding' by 90% [29–31]. When membranes are thawed rapidly at 38°C, (maintaining the integrity of membrane vesicles and the uptake capacity) only a slight decrease in glutamate binding was observed. It seems difficult to assess the physiological significance of the Ca^{2+}/Cl^--enhanced [^{3}H]GLU binding. APB, the most potent inhibitor of this binding, was reported to be synaptic depressant [32,33], although weak excitatory effects of APB have also been observed [34–36]. It has also been suggested that the distribution of these sites, determined by autoradiography in hippocampal slices, corresponds to the terminal fields of proposed glutamatergic pathways [37]. To explain these physiological effects of APB, it might be suggested that the transport phenomenon of the glutamate–calcium complex, different from the classical Na^+-dependent glutamate uptake, corresponds to one of the mechanisms which allow the Ca^{2+} entrance into the cells, leading, for example, to the well-known cell toxicity of glutamate, as recently reported [38]. This possibility is now being investigated.

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REFERENCES

[1] Johnson, J.L. (1972) Brain Res. 37, 1–19.
[2] Watkins, J.C. and Evans, R.H. (1981) Ann. Rev. Pharmacol. Toxicol. 21, 165–204.
[3] Watkins, J.C., Davies, J., Evans, R.M., Francis, A.A. and Jones, A.W. (1981) in: Advances in Biochemical Psychopharmacology (Di Chiara, G. and Gessa, G.L. eds,) vol. 27, pp. 263–273.
[4] Fagg, G.E., Foster, A.C., Mena, E.E. and Cotman, C.W. (1982) J. Neurosci. 2, 958–965.
[5] Fagg, G.E., Foster, A.C., Mena, E.E. and Cotman, C.W. (1983) Eur. J. Pharmacol. 88, 105–110.
[6] Butcher, S.P., Collins, J.F. and Roberts, P.J. (1983) Br. J. Pharmacol. 80, 355–364.
[7] Mena, E.E., Fagg, G.E. and Cotman, C.W. (1981) Brain Res. 243, 378–383.
[8] Hill, D.R. and Bowery, N.G. (1981) Nature (London) 280, 149–151.
[9] Lukas, R.J.H., Morimoto, H. and Bennett, E.L. (1979) Biochemistry 18, 2384–2395.
[10] Recasens, M., Pin, J-P. and Bockaert, J. (1984) in: IVth European Winter Conference on Brain Research, Courchevel, France.
[11] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randal, R.J. (1951) J. Biol. Chem. 193, 265–275.
[12] Roskoski, R., Rauch, N. and Roskoski, L.M. (1981) Arch. Biochem. Biophys. 207, 407–415.
[13] Recasens, M., Varga, V., Nanopoulos, D., Saadoun, F., Vincendon, G. and Benavides, J. (1982) Brain Res. 239, 153–173.
[14] Kanner, B.I. (1978) Biochemistry 17, 1207–1211.
[15] Recasens, M., Saadoun, F., Varga, V., De Feudis, F.V., Mandel, P., Lynch, G. and Vincendon, G. (1983) Neurochem. Int. 5, 89–94.
[16] Foster, A.C. and Roberts, P.J. (1978) J. Neurochem. 31, 1467–1477.
[17] Baudry, M. and Lynch, G. (1981) J. Neurochem. 36, 811–820.
[18] Werling, L.L. and Nadler, J.V. (1982) J. Neurochem. 38, 1050–1062.
[19] Roskoski, R. (1978) J. Neurochem. 31, 493–498.
[20] Kanner, B.I. and Sharon, I. (1978) Biochemistry 17, 3949–3953.
[21] Marvizon, J.G., Mayor, F., Aragon, M.C., Gimenez, C. and Valdivieso, F. (1981) J. Neurochem. 37, 1401–1406.
[22] Iversen, L.L. and Johnston, G.A.R. (1971) J. Neurochem. 18, 1939–1950.
[23] Levi, G. and Raiteri, M. (1974) Nature (London) 250, 735–737.
[24] Balcar, Y.J. and Johnston, G.A.R. (1972) J. Neurochem. 19, 2657–2666.
[25] Davies, L.P. and Johnston, G.A.R. (1976) J. Neurochem. 26, 1007–1014.
[26] Erecinska, M., Wantorsky, D. and Wilson, D.F. (1983) J. Biol. Chem. 258, 9069–9077.
[27] Vincent, S.R. and McGeer, E.G. (1980) Brain Res. 184, 99–108.
[28] Biziere, K., Thompson, H. and Coyle, J.T. (1980) Brain Res. 183, 421–433.
[29] Sharif, N.A. and Roberts, P.J. (1980) J. Neurochem. 34, 779–784.
[30] Recasens, M., Saadoun, F., Baudry, M., Maître, M. and Lynch, G. (1984) J. Neurosci. Res. 11, 157–169.
[31] Baudry, M., Smith, E. and Lynch, G. (1981) Mol. Pharmacol. 20, 280–286.
[32] Slaughter, M.M. and Miller, R.F. (1981) Science 211, 182–185.
[33] Koerner, J.F. and Cotman, C.W. (1982) Brain Res. 251, 105–115.
[34] Watkins, J.C., Curtis, D.R. and Brand, S.S. (1977) J. Pharm. Pharmacol. 29, 324.
[35] Evans, R.H., Francis, A.A., Hunt, K., Oakes, D.J. and Watkins, J.C. (1979) Br. J. Pharmacol. 67, 591–603.
[36] Davies, J. and Watkins, J.C. (1982) Brain Res. 235, 378–386.
[37] Monaghan, D.T., Holets, V.R., Toy, D.W. and Cotman, C.W. (1983) Nature (London) 306, 176–178.
[38] Retz, K.C. and Coyle, J.T. (1984) Neuropharmacology 23, 89–94.