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Calcium ions induce glutamate transport into rat brain membrane vesicles in the absence of sodium and chloride

Evidence for a novel uptake site?

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Evidence is presented that glutamate binding to rat brain synaptic plasma membranes measured in Cl^-- and Na^+-free but Ca^{2+}-supplemented buffers is partly due to uptake of glutamate into resealed membrane vesicles. An intravesicular volume of 7.9 μl/mg protein was measured. Ca^{2+}-induced glutamate binding to synaptic plasma membranes was found to be sensitive to low temperatures as well as to an increase in osmolarity and was abolished by short pulses of ultrasonication. None of several glutamate receptor agonists tested discriminated between basal and Ca^{2+}-induced binding, but 4 out of 5 glutamate uptake inhibitors did. The Ca^{2+}-induced increase in glutamate binding was the same irrespective of whether calcium acetate, calcium sulfate or calcium gluconate in either Tris-acetate of Tris-citrate buffer was used.

Ca^{2+}; Glutamate uptake; Glutamate receptor binding; Cl^--independent transport

1. INTRODUCTION

Triggered by the pioneering work of Pin et al. [1] in 1984, several recent publications questioned the long-held opinion that Cl^--dependent glutamate binding to brain synaptic plasma membranes (SPM) really reflects postsynaptic receptor binding [2–5]. Instead, these authors accumulated a convincing body of evidence suggesting that this 'receptor binding' actually represents uptake into resealed membrane vesicles. This new form of glutamate transport was found to differ in many features from the long-established Na^+-dependent uptake system [1,5].

For our work on postsynaptic glutamate receptors these reports were the signal to change our standard buffer system from Tris-chloride to Tris-acetate in order to avoid uptake artifacts. Additionally, we set out to check whether we could detect any indications for glutamate uptake when employing the chloride-free Tris-acetate buffer system. To our surprise, we found a Ca^{2+}-induced 'binding' stimulation, usually of around 140%, which exhibited all properties of a transport phenomenon but was absolutely independent of either Cl^- or Na^+ added exogenously. The purpose of this paper is to describe some properties of this novel uptake mechanism. Furthermore, we wish to point out the necessity to reconsider carefully receptor binding data measured in the presence of calcium.

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2. MATERIALS AND METHODS

2.1. *Membrane preparations*

Crude cortex SPM from 3-month-old male Wistar rats were obtained according to Enna and Snyder [6] while purified SPM were prepared by the procedure of Jones and Matus [7]. All final membranes were washed 3× with 50 mM Tris-acetate, pH 7.1 at 37°C (Tris-Ac), and were stored frozen in Tris-Ac at −80°C for up to 2 months without significant loss of any binding properties.

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2.2. *Glutamate-binding assay*

L-[^{3}H]Glutamate binding to SPM was measured by a filtration assay. Briefly, 100 μl SPM (= 70–100 μg protein) were incubated in Tris-Ac at 37°C for 60 min in a total volume of 300 μl with L-[^{3}H]glutamate (Amersham, 53 Ci/mmol) added to give a final concentration of 50 nM. According to the specific experimental requirements, salts and/or glutamate-binding inhibitors had been added from 10-fold concentrated stock solutions prior to the membranes. Addition of ice-cold Tris-Ac and rapid filtration onto nitrocellulose filters (Schleicher & Schuell BA85) terminated the incubation period. Filters were washed once with ice-cold Tris-Ac, solubilized in scintillator (ACS, Amersham) and counted. Specific binding was calculated as the difference between total binding and unspecific binding which was measured in the presence of a 2000-fold excess (100 μM) of unlabeled L-glutamate. All determinations were made in triplicate. Standard errors were less than 10%. All inhibitors used were from Sigma (St. Louis) except DL-*threo*-hydroxyaspartate, which was from Calbiochem (San Diego).

2.3. *Estimation of intravesicular space*

Intravesicular volumes of resealed vesicles in SPM preparations were measured utilizing the differential distribution of a membrane-permeable (^3H_2O) and a membrane-impermeable ([^{14}C]inulin) tracer according to Rudnick [8]. The enrichment of ^3H_2O over [^{14}C]inulin in the membrane pellet relative to the control value of the membrane supernatant provided a measure of the intravesicular space.

Protein was estimated according to Peterson [9].

3. RESULTS

3.1. *Characterization of basal and Ca^{2+}-induced binding*

Basal [^{3}H]Glu binding in Tris-Ac buffer at 37°C saturated within 45 min, was linearly dependent on the protein concentration and exhibited biphasic kinetics (see fig.1), with a high-affinity site (K_d = 838 ± 99 nM, b_{max} = 4.5 ± 0.5 pmol/mg protein) and a low-affinity site (K_d = 1954 ± 245 nM, b_{max} = 8.8 ± 1.3 pmol/mg protein). Upon addition of 5 mM calcium acetate, binding was increased by an average of 138%, from 229 ± 6 to 544 ± 8 fmol/mg protein (mean of 32 determinations ± SE using the standard binding assay). This increase in binding was clearly due to an increase in the number of low-affinity binding sites, leaving their affinity unchanged (K_d = 1850 ± 87, b_{max} = 23.0 ± 1.1, see fig.1). High-affinity sites were not detectable in the presence of calcium. Increased binding was elicited by calcium concentrations ranging from 0.5 to 10 mM (not shown). Ca^{2+}-induced binding was temperature sensitive – no significant increase could be produced at 0°C

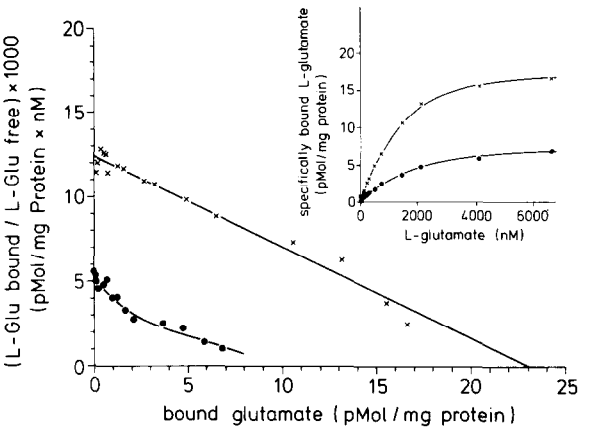

Fig.1. Saturation curves (inset) and Scatchard plots of L-glutamate binding to crude rat cortex SPM in the absence (●) or presence (×) of 5 mM calcium acetate. Binding was assayed at 37°C for 60 min (see section 2) with glutamate concentrations ranging from 5 to 6640 nM. Data are means of 3 determinations, all done in triplicate.

(140 ± 14 and 164 ± 14 fmol/mg protein with and without Ca^{2+}, respectively; mean of 10 determinations ± SE). Preincubation of SPM at 37°C followed by extensive washings did not reduce Ca^{2+}-induced binding stimulation, rendering unlikely the possibility that contaminating Cl^- interacting with Ca^{2+} could be responsible for the increase in binding.

These data led us to the speculation that the Ca^{2+}-induced elevation of binding might represent glutamate uptake into resealed membrane vesicles rather than receptor binding.

3.2. *Determination of intravesicular space in SPM preparations*

Membrane vesicles, a prerequisite for any form of uptake, have been reported to exist in SPM preparations [1,3,7]. In fact, procedures used to prepare SPM are very similar to those designed to produce vesicles for uptake experiments [8,10–12]. We therefore measured the intravesicular space in our SPM preparations. We found 7.9 ± 0.3 μl/mg protein (mean of 12 determinations ± SE), which is almost identical with the volume reported for a vesicle preparation from rat brain membranes [10]. Incubation of the SPM with 5 mM Ca^{2+} did not increase the intravesicular space (6.5 ± 0.2 μl/mg). Thus, uptake cannot be due to an increase in vesicle size, as one might speculate in view of the

known property of Ca^{2+} to enhance fusion of proteoliposomes [13]. Incubation with 0.3 M sucrose or 5 s of ultrasonication with a tip sonicator (Branson, microtip, setting 3) reduced the intravesicular volume by about 50%, while incubation of SPM with 0.5% Triton X-100 abolished it completely.

3.3. *Influence of osmolarity and ultrasonication on Ca^{2+}-induced binding*

To test our uptake hypothesis, we assayed both basal as well as Ca^{2+}-induced binding, in the presence of increasing amounts of sucrose, in order to reduce the intravesicular space by osmotic compression of vesicles [1,9,10]. As can be seen in fig.2, basal binding is hardly affected by this treatment whereas Ca^{2+}-induced binding is abolished completely. Preincubation of SPM with 0.9 M sucrose followed by 3 × washing with Tric-Ac did not return Ca^{2+}-induced binding to sucrose-free control levels, indicating that it was not an inhibitory influence of the sucrose itself that abolished the Ca^{2+}-induced binding.

Ultrasonication of SPM for 5 s, either prior to the [^{3}H]glutamate incubation period or immediately afterwards, led to a substantial suppression of Ca^{2+}-induced binding, whereas basal binding was not significantly affected under these conditions.

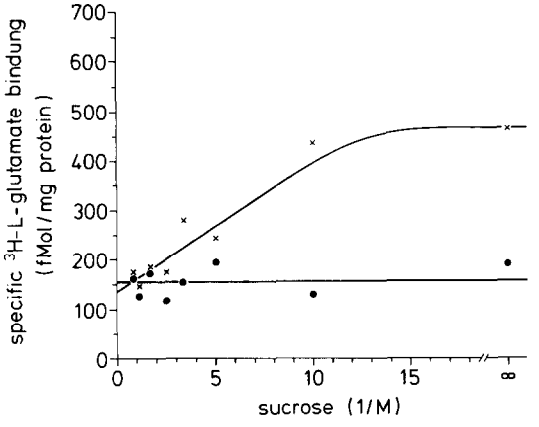

Fig.2. Influence of increasing concentrations of sucrose on L-[^{3}H]glutamate binding to crude rat cortex SPM, measured at 37°C in the absence (●) or presence (×) of 5 mM calcium acetate. SPM were preincubated for 25 min with the indicated sucrose concentrations, then L-[^{3}H]glutamate was added and incubation continued for another 60 min. Data are means of 3 determinations, done in triplicate. SE of all data was less than 15%.

Without sonication we found a basal binding of 243 ± 26 fmol/mg, which was increased in the presence of 5 mM calcium to 603 ± 46 fmol/mg, whereas after sonication we measured 187 ± 18 and 295 ± 31 fmol/mg, respectively (means of 2 determinations ± SE). This finding is in agreement with the marked reduction by ultrasonication of intravesicular space, as reported above, providing additional evidence for an uptake process being responsible for the Ca^{2+}-induced elevation of binding.

3.4. *Pharmacology and influence of anions on Ca^{2+}-induced binding*

In order to characterize and compare the pharmacology of basal and Ca^{2+}-induced binding sites we tested the inhibitory action of several glutamate receptor agonists and some known glutamate uptake inhibitors (see table 1). Out of all compounds

Table 1

Comparison of inhibition of basal and Ca^{2+}-induced glutamate binding by receptor agonists and uptake inhibitors

	Inhibition of basal binding	Inhibition of Ca^{2+}-induced binding
L-Glutamate	100	100
L-Aspartate	93 ± 12	74 ± 9
D-Aspartate	25 ± 13	9 ± 9
L-Cysteinesulfinate	60 ± 8	63 ± 11
L-Homocysteinate	72 ± 19	107 ± 11
Ibotenate	108 ± 19	99 ± 10
Quisqualate	110 ± 8	84 ± 8
N-Methyl-D-aspartate	8 ± 10	11 ± 8
Kainate	−16 ± 10	4 ± 13
2-Amino-4-phosphono-butyrate	24 ± 15	67 ± 10
DL-*threo*-Hydroxy-aspartate	6 ± 8	40 ± 8
DL-Aspartate-β-hydroxamate	4 ± 19	50 ± 10
SITS (4-acetamido-4'-isothiocyanato-stilbene-2,2'-disulfonate)	42 ± 11	79 ± 8

Potency of inhibitors (all tested at 100 μM) was assayed in the absence or presence of 1 mM calcium acetate as described in section 2, using crude rat cortex SPM. Inhibitor-displaceable glutamate binding in the absence of calcium (= basal binding) was subtracted from inhibitor-displaceable binding in the presence of calcium to obtain Ca^{2+}-induced binding. All data are given in percent, inhibition by L-glutamate being set to 100%. Data are means ± SE of 2 determinations done in triplicate

tested, only 2-amino-4-phosphonobutyric acid, DL-*threo*-hydroxyaspartate, DL-aspartate-β-hydroxamate and SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid) discriminated between basal and Ca^{2+}-induced binding, in effectively inhibiting the latter while leaving basal binding unchanged or much less affected. This finding is interesting, since the latter 4 compounds have been reported to be inhibitors of glutamate uptake [2,14–16]. However, another inhibitor of Na^+- as well as Cl^--dependent glutamate uptake, D-aspartate [1,16], did not inhibit Ca^{2+}-induced binding.

We observed the Ca^{2+}-induced increase in glutamate binding not only in crude SPM [6] but also in sucrose gradient-purified SPM [7], and in Tris-Ac buffer as well as Tris-citrate. Furthermore, binding stimulation was the same irrespective of whether we used calcium acetate, calcium gluconate or calcium sulfate. Calcium salts with membrane-permeable counter anions (Cl^-, Br^-, NO_3^-) gave an additional, sucrose-sensitive 10-fold binding stimulation, indicative of activation of the recently described [1–5] Cl^--dependent/Na^+-independent uptake system (not shown).

4. DISCUSSION

From the data presented above we conclude that Ca^{2+}-induced stimulation of glutamate binding to SPM in the absence of Cl^- and Na^+ represents uptake into resealed membrane vesicles. This view is supported by the following observations:

(i) No Ca^{2+} induction of binding is found at 0°C;

(ii) Ca^{2+}-induced binding, as opposed to basal binding, is sensitive to an increase in osmolarity;

(iii) Ca^{2+}-induced binding, as opposed to basal binding, is abolished by short ultrasonication;

(iv) The intravesicular volume of the SPM preparations used is not negligible but represents a volume comparable to that found in vesicle preparations;

(v) Several known glutamate uptake inhibitors abolish Ca^{2+}-induced binding, as opposed to basal binding;

(vi) Dissociation of Ca^{2+}-induced binding in the presence of a 20000-fold excess of unlabeled glutamate proceeds with a half-life of about 30 min (not shown), which is at least 30-times slower than reported for postsynaptic receptor binding [16].

Several glutamate transport mechanisms in brain tissue have been described. Among these, Na^+-dependent uptake is the most prominent. However, participation of this type of uptake can be excluded because it is absolutely dependent on Na^+ and has a different pharmacology. Notably, it is not inhibited by quisqualate [17], whereas Ca^{2+}-induced uptake is almost completely abolished by quisqualate.

The glutamate uptake process found in synaptic vesicles [18] differs from Ca^{2+}-induced uptake in being Ca^{2+}-independent, not inhibitable by quisqualate and in having a much lower affinity (K_d = 1.6 mM).

Mitochondrial glutamate uptake [19] is also unrelated to the Ca^{2+}-induced uptake, having a 1000-fold lower affinity (K_d = 4 mM) concomitant with a much higher binding site density (b_{max} = 23 nmol/min per mg, measured at 25°C).

The recently described [1–5] Cl^--dependent/Na^+-independent uptake system usually is addressed as 'Cl^-/Ca^{2+}-dependent binding' [1,2], reflecting the observation that Ca^{2+} enhances Cl^--dependent uptake. However, it has been completely overlooked that calcium itself induces considerable uptake even in the absence of Cl^-. Whether the type of uptake described here represents a novel mechanism of glutamate transport or is just a special case of the Cl^--dependent process is not clear at present. Published affinity data for the Cl^--dependent uptake process vary broadly, ranging from K_d = 0.2 μM to 5 μM [2,4]. Thus, comparison with the Ca^{2+}-induced uptake is difficult. The pharmacology shows some similarities, although the lack of inhibition by D-aspartate in the case of Ca^{2+}-induced uptake is a notable difference. To clarify this question, work along these lines is in progress.

The results discussed above strongly suggest that glutamate receptor binding data obtained in Cl^-/Na^+-free buffers in the presence of Ca^{2+} at assay temperatures above 0°C should be urgently reevaluated.

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