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Commentary

Increase of the intracellular Ca^{2+} concentration mediated by transport of glutamate into rat hippocampal synaptosomes: characterization of the activated voltage sensitive Ca^{2+} channels

João O. Malva,[a,b]* António F. Ambrósio,[a] Arsélio P. Carvalho[a] and Caetana M. Carvalho[a]

[a]*Center for Neurosciences of Coimbra, Department of Zoology, University of Coimbra, 3000, Coimbra, Portugal*
[b]*Department of Biology, University of Minho, 4710, Braga, Portugal*

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Abstract

The changes in the intracellular free Ca^{2+} concentration, $[Ca^{2+}]_i$, mediated by glutamate and D-aspartate into rat hippocampal synaptosomes was studied. Glutamate increased the $[Ca^{2+}]_i$ in a dose-dependent manner with an EC_{50} of 1.87 μM and a maximal increase of 31.5 ± 0.9 nM. We also observed that stimulation of the synaptosomes with 100 μM α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), 100 μM kainate, or 100 μM D-aspartate increased the synaptosomal $[Ca^{2+}]_i$. The effect of either of these non-NMDA receptor agonists and of D-aspartate was additive, suggesting the activation of two different components (the ionotropic non-NMDA receptors or the glutamate transporters). Stimulation of synaptosomes with 100 μM glutamate increased the $[Ca^{2+}]_i$ and prevented the effect of either non-NMDA receptor agonists and the effect of D-aspartate. We also observed that incubation of the synaptosomes with D-aspartate induced the Ca^{2+}-independent release of glutamate, possibly through the reversal of the glutamate carrier. The aim of incubating the synaptosomes with D-aspartate was to avoid undesirable secondary activation of glutamate receptors. After incubating the synaptosomes with 100 μM D-aspartate (10 min at 37°C), the subsequent stimulation with D-aspartate increased the $[Ca^{2+}]_i$ due to glutamate transport. This increase in $[Ca^{2+}]_i$ induced by 100 μM D-aspartate was insensitive to 1 μM nitrendipine, but was inhibited by about 50% by the presence of both 500 nM ω-CgTx GVIA and 100 nM ω-Aga IVA or by 500 nM ω-CgTx MVIIC. We clearly identified two different processes by which glutamate increased the $[Ca^{2+}]_i$ in rat hippocampal synaptosomes: activation of non-NMDA receptors and activation of the glutamate transporters. We also characterized the voltage sensitive Ca^{2+} channels (VSCC) activated as a consequence of the glutamate transport, and determined that class B (N-type) and class A (P or Q-type) Ca^{2+} channels were responsible for about 50% of the signal. © 1998 Elsevier Science Ltd. All rights reserved

1. Introduction

Three different glutamate transporters from rat brain have been cloned. The glutamate transporter EAAC1 (Kanai and Hediger, 1992) is abundant in the rat hippocampus and appears to be exclusively located in neurons (Rothstein *et al.*, 1994; Swanson *et al.*, 1997). The other two types of glutamate transporters, GLT-1 (Pines *et al.*, 1992) and GLAST (Storck *et al.*, 1992) are expressed primarily in glial cells (Storck *et al.*, 1992; Chaudhry *et al.*, 1995; Lehre *et al.*, 1995; Schmitt *et al.*, 1996, 1997), and are of critical importance to prevent excitotoxicity caused by accumulation of glutamate in the extracellular space (Rothstein *et al.*, 1996).

The physiology of the glutamatergic neurotransmission is critically dependent on the efficient removal of glutamate from the synaptic cleft (Kanai *et al.*, 1993; Danbolt, 1994), in a process dependent on the existent Na^+ gradient through the plasma membrane

*Author to whom all correspondence should be addressed. Fax: 351 39 22776; E-mail: jomalva@cygnus.ci.uc.pt.

Abbreviations: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; VSCC, voltage sensitive Ca^{2+} channels; HEPES, N-2-hydroxyethyl piperazine-N''-2-ethane-sulfonic acid; BSA, bovine serum albumin; CNQX, 6-nitro-7-cyano-quinoxaline-2,3-dione; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10,imine maleate; t-PDC, L-trans-pyrrolidine-2,4-dicarboxylate.

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(Danbolt, 1994). The transport of glutamate and Na^+ may cause depolarization of the nerve terminals and activation of voltage-sensitive Ca^{2+} channels (VSCC), resulting in the increase of the $[Ca^{2+}]_i$ (McMahon *et al.*, 1989; Nicholls, 1989). The increase in the $[Ca^{2+}]_i$ may constitute a mechanism of short-term facilitation of further glutamate release (Nicholls, 1989). However, if the glutamate transport occurs into non-glutamatergic terminals through heterocarriers (Bonanno and Raiteri, 1994) the short-term effect may be either excitatory or inhibitory depending on the nature of the neurotransmitter released by the nerve terminal.

In some pathological conditions, resulting in ischaemia of the brain tissue, the concentration of glutamate in the extracellular space may increase, leading to the hyperactivation of glutamate receptors, causing neuronal toxicity (Danbolt, 1994; Schousboe and Frandsen, 1995). In some cases associated with the energetic depletion of the neurons, the major source of glutamate appears to be associated with non-exocytotic release of glutamate from nerve terminals, possibly involving the glutamate carriers working in the reverse manner (Kauppinen *et al.*, 1988; Sánchez-Prieto and González, 1988; Rubio *et al.*, 1991).

Although there is evidence for the involvement of VSCC in the increase of the $[Ca^{2+}]_i$ induced by the reuptake of glutamate in rat cortical synaptosomes, it is not clear which types of the VSCC mediate the influx of Ca^{2+} (McMahon *et al.*, 1989). In this work, we investigated the modulation of the $[Ca^{2+}]_i$ caused by stimulation of rat hippocampal synaptosomes with glutamate. Since glutamate is the natural substrate for glutamate receptors and also for glutamate transporters, one of our objectives was the identification of the two components responsible for the increase in the $[Ca^{2+}]_i$. The other major objective of the present work was the identification of the type(s) of VSCC responsible for the influx of Ca^{2+} associated with the reuptake of glutamate.

2. Experimental procedures

2.1. *Isolation of synaptosomes*

Male Wistar rats 45 days old were sacrificed by cervical dislocation and the hippocampi were used to isolate a crude synaptosomal fraction (P2).

A modification of the method previously described (McMahon *et al.*, 1992) was used. Six hippocampi were homogenized in 9 volumes of 0.32 M sucrose, 10 mM N-2-hydroxyethyl piperazine-N''-2-ethanesulfonic acid (HEPES), pH 7.4, by using a Thomas B homogenizer, and centrifuged at 3000***g*** for 2 min. The obtained pellet was washed by resuspension in the same sucrose medium and centrifuged as above. The combined supernatants were centrifuged at 14600***g***, for 12 min. From the obtained pellet (P2) the white and loosely packed layer was carefully removed with a spatula and resuspended in 0.32 M sucrose, 10 mM HEPES-Na, pH 7.4, at a protein concentration of about 10 mg ml^{-1}. The whole isolation procedure was performed with refrigerated solutions and materials (0–4°C).

Aliquots of 1 mg or 0.75 mg of protein were diluted in 1 ml of sucrose medium and pelleted in a refrigerated microfuge at 15800***g*** for 2 min. The drained pellets were stored on ice and used within 4 h. The protein concentration was determined by the biuret method (Layne, 1957).

2.2. *Measurements of the* $[Ca^{2+}]_i$

The determination of the $[Ca^{2+}]_i$ was performed by measuring the fluorescence of the Indo-1/Ca^{2+} complex essentially as previously described (Malva *et al.*, 1994). The loading of synaptosomes (0.75 mg of protein) was carried as previously described (Duarte *et al.*, 1991) with 3 μM Indo-1-acetoxymethyl ester (Indo-1/AM) in a medium containing 132 mM NaCl, 1 mM KCl, 1.4 mM $MgCl_2$, 100 μM $CaCl_2$, 1.2 mM H_3PO_4, 1 mg ml^{-1} fatty acid-free bovine serum albumin(BSA), 10 mM glucose and 10 mM HEPES, at pH 7.4. All drugs were added 5 min before the stimulation of the synaptosomes. In the case of ω-Aga IVA, ω-CgTx GVIA and ω-CgTx MVIIC the drugs were also present during the loading period of the synaptosomes. The measurement of the fluorescence of the loaded synaptosomes was carried using a Spex Fluoro Max spectrofluorimeter with excitation at 335 nm and two emission wavelengths of 410 nm and 485 nm, using 5 nm slits, in 2 ml of a medium containing 132 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES-Na, at pH 7.4. The calibration was performed as previously described (Malva *et al.*, 1994) and the conversion of fluorescence intensities into $[Ca^{2+}]_i$ values was performed using the calibration equation for double emission wavelength measurements (Grynkiewicz *et al.*, 1985).

2.3. *Endogenous glutamate release*

The release of endogenous glutamate was followed by using the continuous fluorimetric assay previously described (Nicholls and Sihra, 1986). Briefly, synaptosomes (1 mg of protein) were incubated during 20 min at 37°C in 0.75 ml of 132 mM NaCl, 1 mM KCl, 1.4 mM $MgCl_2$, 1.2 mM H_3PO_4, 100 μM $CaCl_2$, 10 mM glucose, 10 mM HEPES-Na, at pH 7.4, with 0.1% fatty acid free BSA. At the end of the incubation period the synaptosomes were centrifuged at 15800g, for 20 s, and resuspended in 1 ml of the same medium, without BSA and with 1 mM $CaCl_2$ or with 200 nM free Ca^{2+} (Vázquez *et al.*, 1994). The suspension was transferred to a stirred cuvette at 37°C followed by the addition of 1 mM NADP and 50 U of glutamate dehydrogenase. All drugs were

added 5 min before the stimulation of the synaptosomes. In the case of ω-Aga IVA, ω-CgTx GVIA and ω-CgTx MVIIC the drugs were also present during the 20 min incubation period. The fluorescence was measured using a Perkin Elmer model LS-5B luminescence spectrometer at 340 nm and 460 nm for the excitation and emission, respectively. The data were collected at 2 s intervals and at the end each experiment was calibrated by adding 5 nmol of L-glutamate.

2.4. *Materials*

Nitrendipine was obtained from Research Biochemical International (Natick, U.S.A.). ω-Conotoxin GVIA (ω-CgTx GVIA) was from Peninsula Laboratories Inc. (Belmont, CA, U.S.A.). ω-Conotoxin MVIIC (ω-CgTx MVIIC) was obtained from Peptide Institute Inc. (4-1-2 Ina, Minoh-shi, Osaka, 562 Japan). ω-Agatoxin IVA (ω-Aga IVA) was a kind gift from Dr Nicholas A. Saccomano, Central Research Division, Pfizer Inc., Eastern Point, Groton, CT 06340, U.S.A. 6-nitro-7-cyano-quinoxaline-2,3-dione (CNQX) was from NOVO, Nordisk, Denmark. The acetoxymethyl ester of Indo-1 (Indo-1/AM) was obtained from Molecular Probes Inc. Eugene, OR, USA. Ionomycin and fatty acid-free bovine serum albumin(BSA) were from Calbiochem-Boehringer Corp. (San Diego, U.S.A.). All other reagents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.) or from Merck-Schuchard (Germany). Stock solutions of Indo-1/AM, ionomycin, nitrendipine and CNQX were prepared in dimethyl sulfoxide.

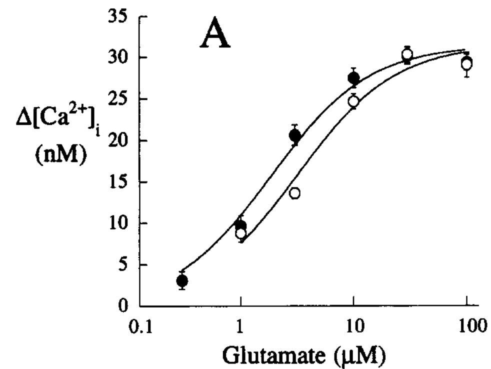
2.5. *Statistical analysis*

The results are presented as means ± standard error of the mean (s.e.m.) of the number of experiments indicated. Statistical significance was determined by ANOVA followed by Dunnet's post-test. Determination of kinetical parameters was performed by using GraphPad Prism (GraphPad, Intuitive Software for Science, San Diego, CA 92121, U.S.A.).

3. Results

3.1. *Modulation of the [Ca^{2+}]$_i$ caused by stimulation with glutamate*

Stimulation of rat hippocampal synaptosomes with glutamate increased the [Ca^{2+}]$_i$ in a dose-dependent manner [Fig. 1(A)]. The EC_{50} value calculated was 1.87 μM ($CI_{95\%}$ between 1.43 μM and 2.43 μM), and the maximum increase was 31.5 ± 0.9 nM. In the presence of 10 μM 6-nitro-7-cyano-quinoxaline-2,3-dione (CNQX) the EC_{50} value was 3.16 μM ($CI_{95\%}$ between 2.33 μM and 4.27 μM) and the maximum increase of the [Ca^{2+}]$_i$ was 31.6 ± 1.0 nM. The NMDA receptor antagonists (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10,imine maleate (MK-801) (1 μM) failed to significantly inhibit the increase in the [Ca^{2+}]$_i$ due to the stimulation with 10 μM glutamate, since the increase in the [Ca^{2+}]$_i$ was 27.7 ± 1.2 nM, or 29.0 ± 2.2 nM, in the absence or in the presence of 1 μM MK-801, respectively [Fig. 1(B)].



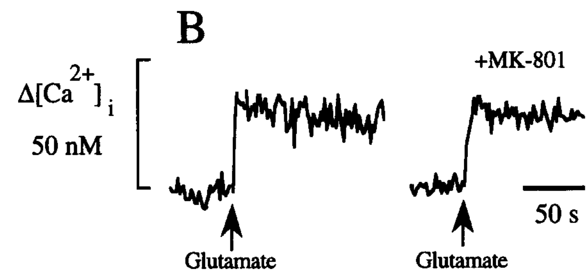


Fig. 1. Effect of glutamate in increasing the [Ca^{2+}]$_i$ in rat hippocampal synaptosomes. (A) Dose-response curve for the effect of glutamate in increasing the [Ca^{2+}]$_i$ in the absence (●) or in the presence of 10 μM CNQX (○). (B) Effect of 1 μM MK-801 on the increase in the [Ca^{2+}]$_i$ induced by 100 μM glutamate. The results presented in (A) are the mean ± s.e.m. of four independent experiments. The results in (B) are representative traces of four independent experiments.

The increase in the [Ca^{2+}]$_i$ due to glutamate was dependent on the presence of Na^+ in the external medium, since in *N*-methyl-D-glucamine (NMG^+) medium stimulation of the synaptosomes with 100 μM glutamate failed to induce a response [Fig. 2(A)]. The increase in the [Ca^{2+}]$_i$ induced by glutamate was also dependent on the extracellular Ca^{2+}, since in the absence of added Ca^{2+} to the assay medium, the increase in the [Ca^{2+}]$_i$ was not significant [Fig. 2(A)]. The addition of 20 μM L-trans-pyrrolidine-2,4-dicarboxylate (t-PDC), a competitive inhibitor of glutamate transport (Bridges *et al.*, 1991), prior to stimulation of the synaptosomes with glutamate increased the basal [Ca^{2+}]$_i$ and partially occluded the effect of 10 μM glutamate, which was only about 4 nM as compared to the control (28.0 ± 1.3 nM) [Fig. 2(B)]. Consistently, the addition of 20 μM t-PDC per se resulted

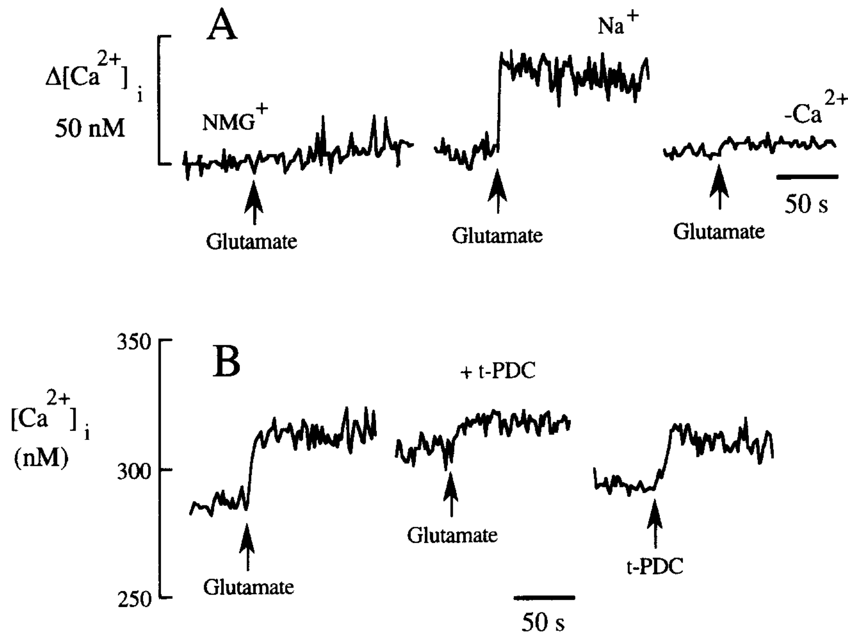


Fig. 2. Increase in the $[Ca^{2+}]_i$ induced by glutamate: dependence on extracellular Na^+ and Ca^{2+}, and inhibition by t-PDC. (A) In the absence of extracellular Na^+ (NMG^+ medium) and Ca^{2+}, stimulation with 100 μM glutamate failed to increase the $[Ca^{2+}]_i$. (B) The presence of 20 μM t-PDC prevents the increase in the $[Ca^{2+}]_i$ induced by 10 μM glutamate, but the compound is able, per se, to increase the $[Ca^{2+}]_i$. The results are representative of, at least, four independent experiments.

in a significant increase in the $[Ca^{2+}]_i$ of 25.4 ± 3.6 nM [Fig. 2(B)].

3.2. *Glutamate receptors and glutamate transporters mediated increases of the $[Ca^{2+}]_i$*

We previously identified a presynaptic kainate receptor which modulates the $[Ca^{2+}]_i$ in rat hippocampal synaptosomes (Malva *et al.*, 1995a). The presynaptic kainate receptor is activated by either kainate and AMPA, and activation by the latter agonist is more sensitive to the competitive inhibition by CNQX (Malva *et al.*, 1995a). The increase of the $[Ca^{2+}]_i$ induced by 100 μM glutamate occluded the responses to stimulation with non-NMDA receptor agonists (100 μM AMPA or 100 μM kainate), or with the glutamate transporter substrate D-aspartate (100 μM) (Fig. 3). However, when hippocampal synaptosomes were stimulated with non-NMDA receptor agonists (AMPA or kainate), or with D-aspartate, prior to glutamate addition, we observed two independent increases

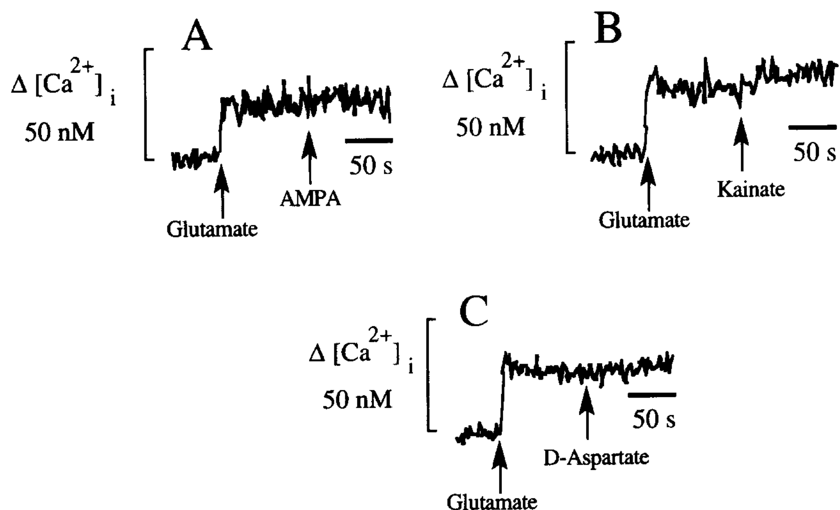


Fig. 3. Glutamate increases the $[Ca^{2+}]_i$ and prevents the response to either non-NMDA receptor agonists or to the glutamate transporter substrate D-aspartate. Response to the stimulation with 100 μM glutamate followed by the addition of 100 μM AMPA (A), by 100 μM kainate (B), or by 100 μM D-aspartate (C). The results are representative of at least four independent experiments.

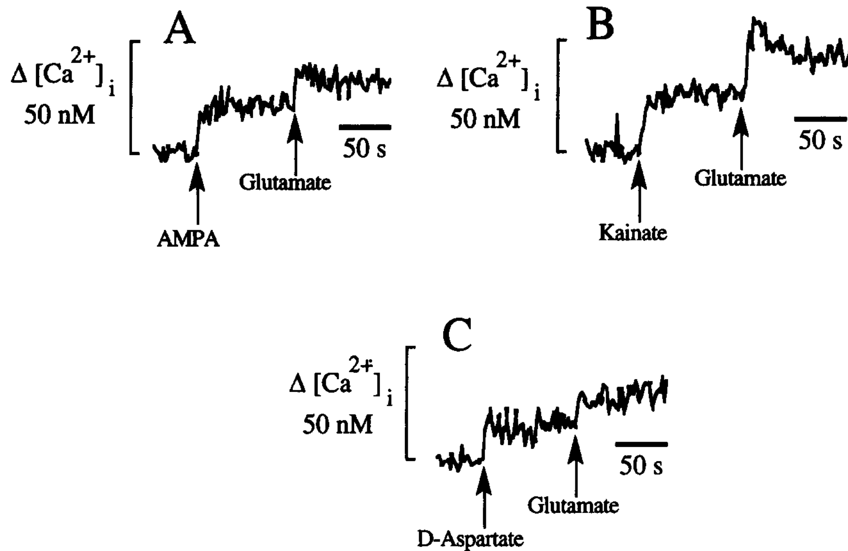


Fig. 4. Glutamate induces the increase in the $[Ca^{2+}]_i$ in addition to the effect of non-NMDA glutamate receptor agonists or in addition to the effect of the glutamate transporter substrate D-aspartate. A second stimulation was performed with 100 μM glutamate after the stimulation with (A) 100 μM AMPA, (B) 100 μM kainate, or (C) 100 μM D-aspartate. The results are representative of at least four independent experiments.

of the $[Ca^{2+}]_i$ (Fig. 4). The same was observed when the synaptosomes were stimulated with kainate prior to D-aspartate, or with D-aspartate prior to kainate, or with D-aspartate and then with AMPA (Fig. 5).

3.3. *The incubation with D-aspartate depleted the cytoplasmatic pool of glutamate*

After incubating the synaptosomes with 100 μM D-aspartate (10 min at 37°C), the glutamate released in response to the subsequent stimulation with 50 mM KCl, with 1 mM $CaCl_2$ in the assay medium, was reduced from the control value of 9.44 ± 0.40 nmol glutamate mg^{-1} protein $5 min^{-1}$ to 6.23 ± 0.90 nmol glutamate mg protein $5 min^{-1}$ (Fig. 6). In low Ca^{2+} medium (about 200 nM free Ca^{2+}), stimulation with 50 mM KCl induced the release of 3.58 ± 0.37 nmol glutamate mg^{-1} protein $5 min^{-1}$, and the incubation with D-aspartate reduced the release to 0.53 ± 0.21 nmol glutamate mg^{-1} protein $5 min^{-1}$ (Fig. 6). These values indicate that the incubation of the synaptosomes with D-aspartate reduced the Ca^{2+}-dependent release of glutamate by only 2.7% (5.86 nmol glutamate mg^{-1} protein $5 min^{-1}$ in control, and 5.7 nmol glutamate mg^{-1} protein $5 min^{-1}$ after incubation), but had a large effect (about 85% reduction) (3.58 nmol glutamate mg^{-1} protein $5 min^{-1}$ in control, and 0.53 nmol

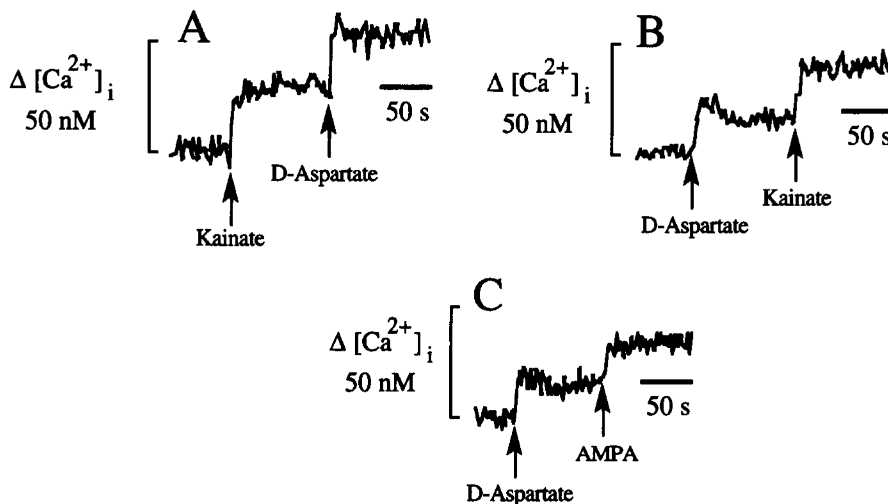


Fig. 5. The increase in the $[Ca^{2+}]_i$ induced by stimulation of rat hippocampal synaptosomes with non-NMDA glutamate receptor agonists and with the glutamate transporter substrate D-aspartate was additive. Effects of: (A) 100 μM kainate followed by 100 μM D-aspartate; (B) 100 μM D-aspartate followed by 100 μM kainate; (C) 100 μM D-aspartate followed by 100 μM AMPA. The results are representative of at least four independent experiments.

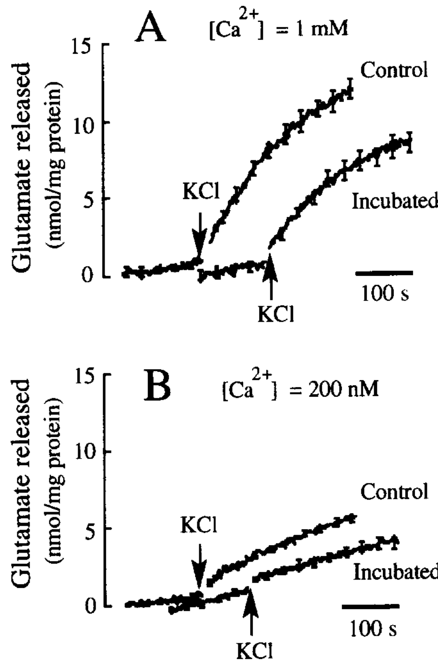


Fig. 6. Effect of incubation with D-aspartate on the release of endogenous glutamate induced by stimulation with 50 mM KCl. (A) Release of glutamate in the presence of 1 mM $CaCl_2$ in synaptosomes submitted or not to incubation with 100 μM D-aspartate (10 min at 37°C). (B) Release of glutamate in low Ca^{2+} medium (around 200 nM free Ca^{2+}; 50 μM EGTA and 38 μM $CaCl_2$, at pH 7.4) in synaptosomes submitted or not to incubation with D-aspartate. The results are the mean ± s.e.m. (every 30 s) of four independent experiments.

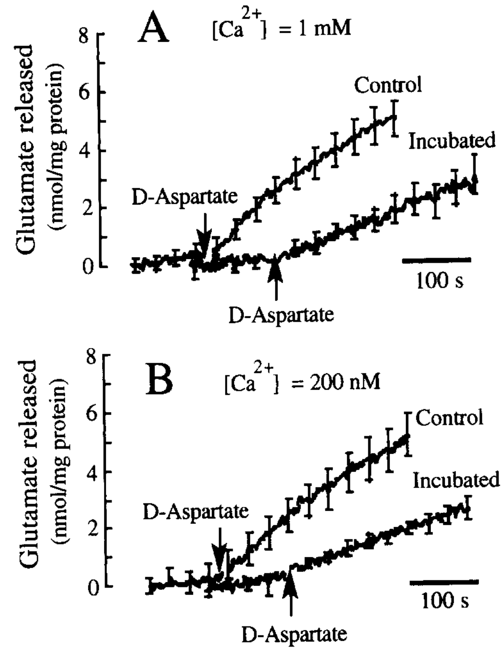


Fig. 7. Effect of incubation with D-aspartate on the release of endogenous glutamate induced by stimulation with 100 μM D-aspartate. (A) Release of glutamate in the presence of 1 mM $CaCl_2$ in synaptosomes submitted or not to incubation with 100 μM D-aspartate (10 min at 37°C). (B) Release of glutamate in low Ca^{2+} medium (around 200 nM free Ca^{2+}; 50 μM EGTA and 38 μM $CaCl_2$, at pH 7.4) in synaptosomes submitted or not to a incubation period with D-aspartate. The results are the mean ± s.e.m. (every 30 s) of four independent experiments.

glutamate mg^{-1} protein 5 min^{-1} after incubation) in the Ca^{2+}-independent release of glutamate. We also induced the release of glutamate by stimulating the synaptosomes with 100 μM D-aspartate (Fig. 7). In this case, the release of glutamate observed in medium containing 1 mM $CaCl_2$ was 4.49 ± 0.42 nmol glutamate mg^{-1} protein 5 min^{-1}, which was almost the amount of glutamate released in low Ca^{2+} medium (200 nM free Ca^{2+}) (4.41 ± 0.41 nmol glutamate mg^{-1} protein 5 min^{-1}), indicating that the stimulation with D-aspartate induced the release of glutamate essentially through a Ca^{2+}-independent process. The Ca^{2+}-independent release of glutamate was significantly reduced by the incubation with D-aspartate, since in these conditions the release observed was 2.15 ± 0.15 nmol glutamate mg^{-1} protein 5 min^{-1}, or 1.24 ± 0.21 nmol glutamate mg^{-1} protein 5 min^{-1} for medium containing 1 mM $CaCl_2$, or 200 nM free Ca^{2+}, respectively (Fig. 7). These observations indicate that the Ca^{2+}-independent release of glutamate, induced by 100 μM D-aspartate, was inhibited by about 72% after the incubation with D-aspartate.

After incubating the synaptosomes with D-aspartate, stimulation with 100 μM D-aspartate increased the $[Ca^{2+}]_i$ by 16.3 ± 1.9 nM, which was lower than the control (24.9 ± 1.5 nM) [Fig. 8(A)]. Similarly to what was observed for the stimulation with 100 μM glutamate, the increase in the $[Ca^{2+}]_i$ induced by 100 μM D-aspartate was dependent on the presence of Na^+ in the assay medium, since we did not observe any effect in NMG^+ medium [Fig. 8(A)]. The increase in the $[Ca^{2+}]_i$ induced by D-aspartate, in incubated synaptosomes, was dependent on the concentration of D-aspartate, with a maximal increase of 17.4 ± 0.8 nM and a EC_{50} value of 1.79 ($CI_{95\%}$ between 1.08 and 2.97 μM) [Fig. 8(B)].

3.4. *Involvement of voltage-sensitive Ca^{2+} channels on the increase of the $[Ca^{2+}]_i$ induced by stimulation with D-aspartate*

We used VSCC blockers to investigate the role of VSCC as mediators of the increase in the $[Ca^{2+}]_i$ induced by stimulation with D-aspartate. Nitrendipine (1 μM) failed to inhibit the signal due to stimulation with 100 μM D-aspartate (94.9 ± 4.2% as compared to the control). However, in the presence of 500 nM ω-CgTx GVIA the increase in the $[Ca^{2+}]_i$ was inhibited to 86.7 ± 4.9% of the control. Also, 100 nM ω-Aga IVA, or 500 nM ω-CgTx MVIIC, inhibited the increase of the $[Ca^{2+}]_i$ to 62.9 ± 2.1% or to 53.0 ± 3.9% of the control, respectively (Fig. 9). We also observed that the effects of 500 nM ω-CgTx GVIA and 100 nM ω-Aga IVA were additive, since in the presence of both toxins the signal was only 52.8 ± 5.2% of the control (Fig. 9). However, the effect

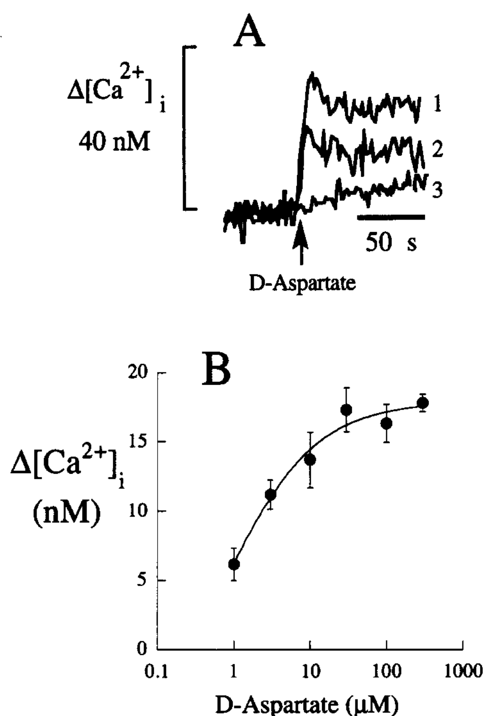


Fig. 8. Increase in the $[Ca^{2+}]_i$ induced by stimulation with D-aspartate in rat hippocampal synaptosomes incubated with D-aspartate. (A) Effect of 100 μM D-aspartate in non-incubated synaptosomes (1); or in incubated (100 μM D-aspartate for 10 min at 37°C) synaptosomes (2); or in incubated synaptosomes, followed by the monitoring of the $[Ca^{2+}]_i$ in NMG^+ medium (3). (B) Dose-response curve for the effect of D-aspartate in increasing the $[Ca^{2+}]_i$ in synaptosomes submitted to the incubation with D-aspartate. The results in (A) are representative of at least four independent experiments. The results in (B) are the mean ± s.e.m. of four independent experiments.

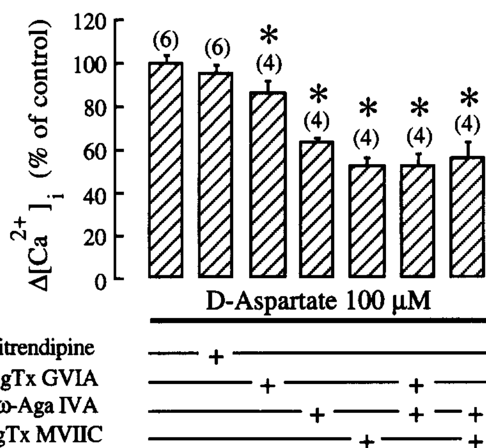


Fig. 9. Effect of VSCC toxins in inhibiting the increase in the $[Ca^{2+}]_i$ induced by 100 μM D-aspartate, in synaptosomes submitted to a incubation period of 10 min at 37°C in the presence of 100 μM D-aspartate. The toxins used were nitrendipine (1 μM), ω-CgTx GVIA (500 nM), ω-Aga IVA (100 nM) and ω-CgTx MVIIC (500 nM). All the toxins were present during the experiments. Also, ω-CgTx GVIA, ω-Aga IVA and ω-CgTx MVIIC were present during the loading period of the synaptosomes with the fluorescent probe. The results are the mean ± s.e.m. of the number of experiments indicated on the top of the bars. The results significantly different from the control are indicated (*).

of 100 nM ω-Aga IVA and 500 nM ω-CgTx MVIIC were not additive, since in the presence of both toxins the inhibition of the increase in the $[Ca^{2+}]_i$ was 56.9 ± 6.5% of the control, which was not different from the effect of 500 nM ω-CgTx MVIIC alone (Fig. 9).

4. Discussion

The results presented show that glutamate increased the $[Ca^{2+}]_i$ in rat hippocampal synaptosomes by two different processes: one involving the activation of non-NMDA ionotropic glutamate receptors, and the other involving the transport of glutamate. We also investigated the possible involvement of NMDA receptors in the $[Ca^{2+}]_i$ response to glutamate by using 1 μM MK-801, which is a non-competitive inhibitor of NMDA receptors (Huettner and Bean, 1988). The lack of inhibition by MK-801 [Fig. 1(B)] indicates that NMDA receptors are not significantly involved in the response to glutamate. Also, the effect of glutamate in increasing $[Ca^{2+}]_i$ was not mediated through metabotropic glutamate receptors, since in the absence of added Ca^{2+}, to the medium, glutamate failed to produce a visible response, indicating that intracellular Ca^{2+} reservoirs are not the source of the increase in $[Ca^{2+}]_i$. Although we were able to identify the inhibitory effect of 10 μM CNQX on the dose-response curve of glutamate in increasing the $[Ca^{2+}]_i$, the inhibition was smaller than expected for the exclusive involvement of non-NMDA receptors [Fig. 1(A)] (Honoré *et al.*, 1988; Egebjerg *et al.*, 1991), as we previously showed for the activation of kainate receptors with either kainate or AMPA (Malva *et al.*, 1995a).

We further observed that the competitive inhibitor of the glutamate transporters t-PDC (20 μM) (Bridges *et al.*, 1991) occluded the effect of 10 μM glutamate in increasing the $[Ca^{2+}]_i$ [Fig. 2(B)]. This result indicates that at least part of the effect of glutamate may be due to the activation of glutamate transporters. This hypothesis was further supported by the observation that in the presence of 20 μM t-PDC the basal $[Ca^{2+}]_i$ was consistently higher than in the absence of the compound, and also by the observation that t-PDC per se increased the $[Ca^{2+}]_i$ by mimicking the effect of glutamate. The increase in the $[Ca^{2+}]_i$ induced by 20 μM t-PDC is consistent with the idea that the compound is also a transportable substrate of the glutamate carriers, causing glutamate release through a carrier-mediated exchange process (Waldemeier *et al.*, 1993; Volterra *et al.*, 1996). The involvement of glutamate transporters in the glutamate-induced increase in the $[Ca^{2+}]_i$ is also consistent with the dependence on extracellular Na^+ [Fig. 2(A)], since the Na^+ gradient is the major driving force for the transport of glutamate (Danbolt, 1994).

We further characterized the dual effect of glutamate in increasing the $[Ca^{2+}]_i$ by studying the additivity of the

effects of substrates for both the glutamate transporters and non-NMDA ionotropic glutamate receptors. We observed that 100 μM glutamate prevents the effect of 100 μM AMPA, 100 μM kainate, or 100 μM D-aspartate, consistently with the capability of glutamate in activating non-NMDA receptors and the glutamate transporters (Fig. 3). However, when a specific substrate for non-NMDA receptors (AMPA or kainate), or for the glutamate transporters (D-aspartate) (Danbolt and Storm-Mathisen, 1986) was used, we could clearly identify a second process of increasing the $[Ca^{2+}]_i$, due to the activation of the glutamate transporters or to the activation of non-NMDA receptors, respectively (Figs 4 and 5).

The other major objective of the present work was the identification of the VSCC involved in the influx of Ca^{2+} caused by the activity of glutamate transporters. As discussed above, glutamate is not a good substance to investigate this mechanism, since it is also a substrate for glutamate receptors. Thus, we decided to use D-aspartate for this investigation, since D-aspartate is a non-metabolizable and is a specific substrate for the glutamate transporters (Danbolt and Storm-Mathisen, 1986). However, the addition of D-aspartate to synaptosomal preparations causes the release of cytoplasmatic glutamate through a carrier-mediated exchange process (Fig. 7) (Nicholls and Sihra, 1986). As a consequence of the carrier-mediated release of glutamate, we felt that the stimulation of the synaptosomes with D-aspartate may result in the indirect stimulation of glutamate receptors by the released glutamate. To reduce this possibility, we incubated the synaptosomes with 100 μM D-aspartate to maximally release glutamate from the cytoplasmatic pool, prior to the centrifugation of the synaptosomal suspension. Indeed, we observed that after incubating the synaptosomes with D-aspartate, the Ca^{2+}-independent release of glutamate was significantly reduced (Figs 6 and 7). This observation may indicate that the cytoplasmatic content of glutamate was reduced by the incubation with D-aspartate. Consistently, we observed that the incubation with D-aspartate caused a reduction of the increase in the $[Ca^{2+}]_i$ induced by the stimulation with 100 μM D-aspartate [Fig. 8(A)].

We then used specific toxins for the various VSCC to identify which type(s) of channels mediate the increase in the $[Ca^{2+}]_i$ due to the activation of the glutamate transporters. We used nitrendipine (1 μM) to inhibit L-type channels (Mori *et al.*, 1996), ω-CgTx GVIA (500 nM) to inhibit class B (N-type) Ca^{2+} channels (Olivera *et al.*, 1984), ω-Aga IVA (100 nM) or ω-CgTx MVIIC (500 nM) to inhibit class A (P or Q-type) Ca^{2+} channels (Mintz *et al.*, 1992; Sather *et al.*, 1993; Stea *et al.*, 1994; Malva *et al.*, 1995b). We failed to observe a nitrendipine-induced inhibition of the increase of the $[Ca^{2+}]_i$ stimulated by D-aspartate (Fig. 9). This result is consistent with other reports in which the authors did not observe significant involvement of L-type Ca^{2+} channels in the modulation of synaptosomal $[Ca^{2+}]_i$ (Tareilus *et al.*, 1993; Duarte *et al.*, 1996), or on the release of neurotransmitters (Carvalho *et al.*, 1995; Turner *et al.*, 1995). However, part of the increase in the $[Ca^{2+}]_i$ was inhibited by 500 nM ω-CgTx GVIA, 100 ω-Aga IVA, or 500 nM ω-CgTx MVIIC, indicating the involvement of class B (N-type) and class A (P or Q-type) Ca^{2+} channels. This is consistent with other reports showing the involvement of these channels in the modulation of the increase in synaptosomal $[Ca^{2+}]_i$ due to other stimulating agents (Malva *et al.*, 1995b; Duarte *et al.*, 1996), in the release of neurotransmitters (Malva *et al.*, 1994; Carvalho *et al.*, 1995; Turner *et al.*, 1995), or in the synaptic transmission in the hippocampus (Wheeler *et al.*, 1994; Wu and Saggau, 1994). The additivity of the effects of 500 nM ω-CgTx GVIA and 100 nM ω-Aga IVA (Fig. 9) indicates that the two toxins are indeed inhibiting different channels. However, that was not the case for 100 nM ω-Aga IVA and for 500 nM ω-CgTx MVIIC, since the inhibitory effects were not additive (Fig. 9). This is consistent with the inhibition of class A Ca^{2+} channels by the two toxins (Stea *et al.*, 1994). Our observation of the involvement of class B and class A Ca^{2+} channels on the increase in the $[Ca^{2+}]_i$ due to the activity of the glutamate transporter is in agreement with the presence of α_{1B} and α_{1A} subunits in nerve terminals (Westenbroek *et al.*, 1992; Westenbroek *et al.*, 1995). With toxins available it was not possible to inhibit about 50% of the increase in $[Ca^{2+}]_i$. One mechanism which may be involved in the Ca^{2+} influx resistant to the inhibition is the activation of the Na^+/Ca^{2+} exchanger working in reverse mode (Blaustein, 1988; Duarte *et al.*, 1991). Since the electrogenic uptake of glutamate is coupled to the influx of H^+ into the cytoplasm the resulting acidification may either affect the efficiency of the Na^+/Ca^{2+} antiporter, the activity of the plasma membrane Ca^{2+}-pump, or inducing the activity of the mitochondrial Ca^{2+}/H^+ exchanger which may, in this case, release Ca^{2+} from the mitochondria (DiPolo and Beaugé, 1990; Pietrobon *et al.*, 1990). However, the operation of the latter mechanism is not likely to occur in our preparation, since in the absence of Ca^{2+} in the assay medium glutamate did not induced detectable increase in $[Ca^{2+}]_i$, indicating that Ca^{2+} influx occurs through the plasma membrane.

It is well known that the type of synaptosomal preparation used in this work is able to accumulate exogenous glutamate, but the type of glutamate transporter(s) which are active remains to be clearly identified. Synaptosomes are structures of neuronal origin able to transport glutamate (Gundersen *et al.*, 1993; Kanai *et al.*, 1993), possibly through the neuronal EAAC1 type of glutamate transporters (Rothstein *et al.*, 1994). However, it is well known that P_2 fractions are also rich in non-neuronal membranes (Danbolt *et al.*, 1992), including membranes of glial origin rich in the glutamate transporter GLT-1 (Danbolt *et al.*, 1990; Pines *et al.*, 1992). Our results do

not allow us to definitively identify the nature of the glutamate transporter responsible for the increase in the $[Ca^{2+}]_i$, as well as the type of vesicles responsible for the accumulation of glutamate. However, part of the signal may be due the activity of synaptosomal glutamate transporters, since the incubation with D-aspartate reduced the Ca^{2+}-independent release of glutamate. The depletion of the cytoplasmic pool of glutamate probably was caused by the removal of the neurotransmitter from synaptosomes and not from vesicles of glial origin, since the concentration of glutamate in nerve terminals is much higher than that in glial cells, due to the intense glial metabolism of glutamate (Storm-Mathisen *et al.*, 1995).

In conclusion, we were able to identify two distinct processes by which glutamate may increase the $[Ca^{2+}]_i$ in rat hippocampal synaptosomes: one mechanism involving the activation of non-NMDA glutamate receptors, and the other mechanism involving the transport of glutamate. We also identified class B (N-type) and class A (P or Q-type) Ca^{2+} channels as the channels responsible for the increase in the $[Ca^{2+}]_i$ (about 50% of the signal) induced by stimulation with 100 μM D-aspartate. However, the channel(s) responsible for about 50% of the total increase in the $[Ca^{2+}]_i$ remain to be clearly identified, since we could not inhibit this fraction with the toxins available.

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