Pergamon

0042-6989(95)00309-6

Vision Res., Vol. 36, No. 13, pp. 1867–1872, 1996

Printed in Great Britain
0042-6989/96 $15.00 + 0.00

On-line Detection of Glutamate Release from Cultured Chick Retinospheroids

CARLOS B. DUARTE,*‡ PAULO F. SANTOS,* J. SÁNCHEZ-PRIETO,† ARSÉLIO P. CARVALHO*

Received 26 May 1995; in revised form 3 October 1995; in final form 30 October 1995

A continuous fluorometric assay was adapted to measure the release of endogenous glutamate from cultured chick retinospheroids. The results obtained with this technique are compared with the release of [^{3}H]D-aspartate from monolayer cultures of chick retina cells. It is shown that although excitatory amino acids may be released in a Ca^{2+}-dependent manner, most of the neurotransmitter release from cultured retina cells occurs by reversal of the glutamate transporter. The presence of extracellular Ca^{2+} may actually inhibit glutamate release by the cells present in the retinospheroids, or the [^{3}H]D-aspartate release by cells in monolayers, when veratridine is the depolarizing agent.

Glutamate release [^{3}H]D-aspartate Retinospheroids Retina cells Intracellular Ca^{2+} Depolarization

INTRODUCTION

Glutamate is the major excitatory neurotransmitter in the retina. In the dark, the photoreceptors are depolarized and release glutamate from their terminals, at the outer plexiform layer (Massey, 1991; Barnstable, 1993). Depolarization of the bipolar cells, localized in the inner nuclear layer of the retina, also stimulates the release of glutamate, at the inner plexiform layer, and activates the glutamatergic ganglion cells (Massey, 1991; Barnstable, 1993).

The release of glutamate may occur by two different mechanisms: one mechanism requires Ca^{2+} entering during depolarization and probably occurs by exocytosis, while the other is Ca^{2+}-independent and requires the reversal of the glutamate transporter (Nicholls, 1989). Using an electrophysiological bioassay, it was shown that the release of excitatory neurotransmitters from the axon terminal of goldfish bipolar cells depends exclusively on Ca^{2+} influx through dihydropyridine-sensitive Ca^{2+} channels (Tachibana *et al.*, 1993). The entry of Ca^{2+} by voltage-sensitive Ca^{2+} channels and through a cGMP-gated channel was also shown to evoke exocytotic transmitter release by photoreceptors in the salamander retina (Rieke & Schwartz, 1994). However, in the toad retina, a sizeable component of synaptic transmission between photoreceptors and horizontal cells continues in the absence of Ca^{2+} influx through voltage-gated Ca^{2+} channels (Schwartz, 1986).

Very few direct attempts have been made to characterize the release of glutamate from retinal neurons. In the chick whole retina and in retinal synaptosomes the release of [^{14}C]glutamate evoked by K^+-depolarization or by kainate was shown to be strictly Ca^{2+}-independent (Tapia & Arias, 1982), suggesting that glutamate release probably originates from nonvesicular storage. We have modified a fluorometric method that has been utilized for detection of glutamate release from synaptosomes (Nicholls *et al.*, 1987), and have applied it to measure continuously the release of glutamate from retinospheroids. In this work, we investigated the Ca^{2+}-dependency of glutamate release from cultured chick retinospheroids, which contain areas homologous to all three retinal nuclear and both plexiform layers (Layer & Willbold, 1993). The results are compared with the release of [^{3}H]D-aspartate from monolayer cultures of chick retina cells, under the same experimental conditions.

METHODS

Materials

The acetoxymethylester of Indo-1 (Indo-1/AM) was obtained from Molecular Probes (Eugene, OR) and CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) from Tocris Cookson (Bristol, U.K.). Ionomycin was purchased from Calbiochem-Boehringer (San Diego, U.S.A.) and [^{3}H]D-aspartate from Amersham International (Buckinghamshire, U.K.). Fetal calf serum was from Irvine Scientific (Santa Ana, CA) and trypsin and gentamicin were from Gibco (Paisley, U.K.). All other reagents were from Sigma (St. Louis, MO).

*Center for Neuroscience of Coimbra, Department of Zoology, University of Coimbra, Coimbra, Portugal.
†Department of Biochemistry, Faculty of Veterinary, Complutense University, Madrid, Spain.
‡To whom all correspondence should be addressed [*Email* cbduarte @gemini.ci.uc.pt].

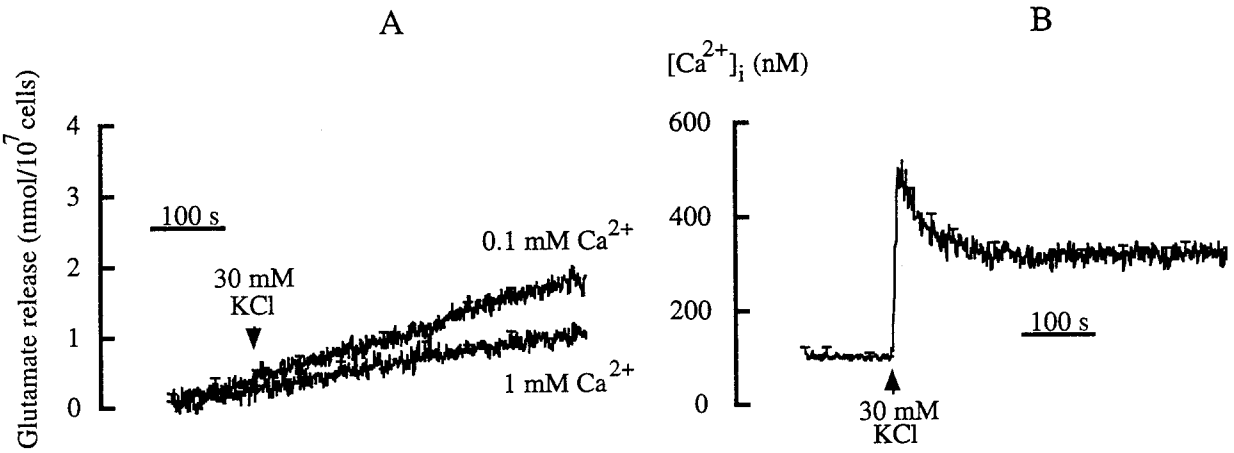

FIGURE 1. Glutamate release (A) and $[Ca^{2+}]_i$ response (B) evoked by KCl-depolarization of chick retinospheroids. In (A) the cells were resuspended in Na^+ medium with the indicated Ca^{2+} concentration, and transferred to fluorometric cuvettes containing $NADP^+$ and glutamate dehydrogenase. In (B) the Indo-1 loaded retinospheroids were resuspended in Na^+ medium containing 1 mM $CaCl_2$ and, where indicated, 30 mM KCl was added from a 3.5 M stock solution. The traces are means ± S.E.M. of four experiments, performed in independent preparations.

Preparation and culture of chick retina cells

Primary cultures of retinospheroids were prepared from 8-day-old chick (White Leghorn) embryos, according to Mello *et al.* (1991), with some modifications. Briefly, the retinas were incubated for 15 min at 37°C in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution, supplemented with 0.1% trypsin. The digested tissue was centrifuged at $140g_{av}$ for 1 min, and the pellet was resuspended in Basal Medium of Eagle (Earle's salts; BME), buffered with 25 mM $NaHCO_3$, and supplemented with 5% heat inactivated fetal calf serum and 15 μg/ml gentamicin. The tissue was dissociated mechanically by 10 aspirations with a large-bore 5 ml glass pipette. The cell suspension (1.4×10^6 cells/ml) was transferred to a rotary shaker operating at 50 r.p.m., under a humidified atmosphere of 95% air/5% CO_2, and cultured for 1 week.

Monolayer cultures of chick retina cells were prepared using cells dissociated as described above (Duarte *et al.*, 1992, 1993), and resuspended in BME buffered with 20 mM HEPES and 10 mM $NaHCO_3$, and supplemented with 5% heat inactivated fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were plated at a density of 0.4×10^6 cells/cm^2 in 35 mm plastic petri dishes, coated with poly-L-lysine (0.1 mg/ml), and cultured for 5 days.

Glutamate release

Retinospheroids (10.5×10^6 cells) were centrifuged at $140g_{av}$ for 5 sec, and resuspended in 0.75 ml of Na^+ medium, containing (in mM): 132 NaCl, 4 KCl, 1.4 $MgCl_2$, 1.2 H_3PO_4, 6 glucose, 10 HEPES-Na, pH 7.4, and 0.1 or 1 mM $CaCl_2$, as indicated. The cells were transferred to a thermostatted (37°C) stirred cuvette, and pre-incubated with 1 mM $NADP^+$ and 50 U of glutamate dehydrogenase for about 5 min. The fluorescence was measured using a computer-assisted Perkin–Elmer LS-5B Luminescence Spectrometer, with excitation at 340 nm and emission at 460 nm (Nicholls *et al.*, 1987). The traces were calibrated at the end of each experiment by adding 2.5 nmol of glutamate. The total intracellular glutamate, determined upon addition of 0.05% of Triton X-100 (w/vol), was 16.03 ± 0.47 nmol/10^7 cells ($n = 10$).

Indo-1 loading and $[Ca^{2+}]_i$ measurements

Retinospheroids (8.75×10^6 cells/ml) were incubated with 2 μM Indo-1/AM in BME buffered with 25 mM $NaHCO_3$ and 5 mM HEPES-Na, pH 7.4. Dye loading was performed for 45 min at 37°C, in a rotary shaker, and the cells were further incubated for 15 min in the same medium but without the indicator, in order to obtain a complete hydrolysis of the acetoxymethylester precursor of Indo-1. The cells were then centrifuged at $140g_{av}$ for 5 sec, and resuspended in Na^+ medium containing (in mM): 132 NaCl, 4 KCl, 1 $CaCl_2$, 1.4 $MgCl_2$, 6 glucose and 10 HEPES-Na, pH 7.4. The fluorescence was measured using a Perkin–Elmer LS-5B Luminescence Spectrometer, with excitation at 335 nm and emission at 410 nm, and using 5 nm slits. The $[Ca^{2+}]_i$ was calculated as described (Duarte *et al.*, 1992, 1993), except that 10 μM ionomycin was used to determine F_{max}.

[^{3}H]D-Aspartate uptake and release

Cultured chick retinal neurons were incubated in culture medium containing 40 nM [^{3}H]D-aspartate (1 μCi/ml), for 30 min at 37°C. The medium was then removed and the cells were washed six times with Na^+ medium containing (in mM): 132 NaCl, 4 KCl, 1 $CaCl_2$, 1.2 H_3PO_4, 1.4 $MgCl_2$, 6 glucose and 10 HEPES-Na, pH 7.4. The release of [^{3}H]D-aspartate was measured using a superfusion system, as previously described (Duarte *et al.*, 1992, 1993).

Statistical analysis

Results are presented as means ± S.E.M. of the indicated number of experiments. Statistical significance was determined using the two-tailed Student's *t*-test.

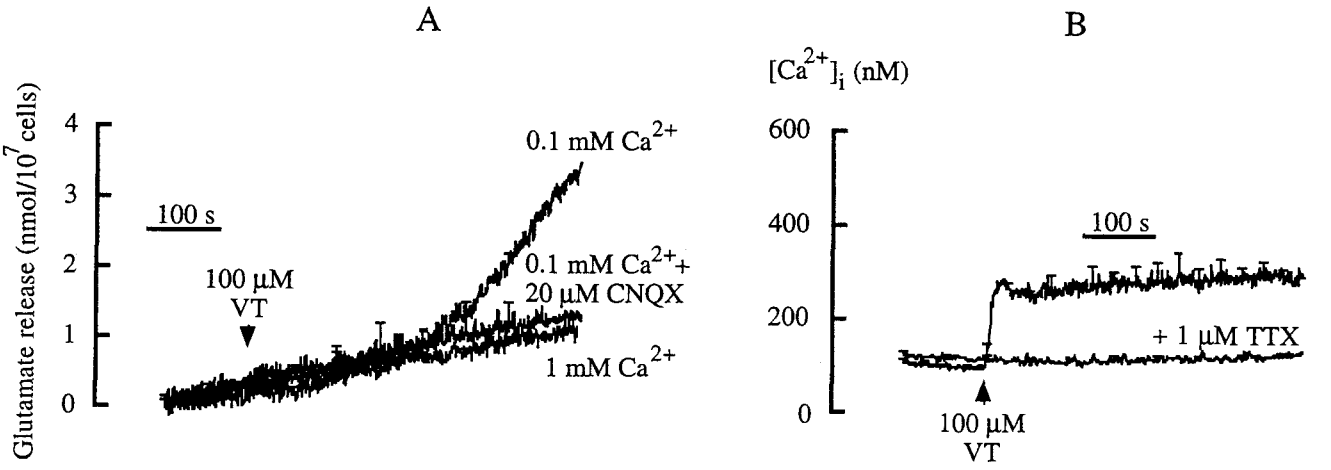

FIGURE 2. Release of endogenous glutamate (A) and $[Ca^{2+}]_i$ response (B) evoked by veratridine in cultured chick retinospheroids. The experiments were conducted similarly to those described in Fig. 1, and where indicated 100 μM veratridine was added. When the effect of tetrodotoxin (TTX) or CNQX (20 μM) were tested the cells were pre-incubated for 2 min with the drugs. The traces are means $\pm$ S.E.M. of four to six experiments, performed in independent preparations.

RESULTS

Effect of depolarizing agents on glutamate release from retinospheroids

Depolarization of cultured chick retinospheroids with 30 mM KCl, in a medium containing 0.1 mM $CaCl_2$, increased the release of endogenous glutamate to 1.65 ± 0.06 nmol/10^7 cells/7 min [10.3% of the total glutamate; Fig. 1(A)]. The basal release of glutamate under these experimental conditions, determined from the average trace depicted in Fig. 1(A), was 132.0 pmol/10^7 cells/min. Upon stimulation with 30 mM KCl, the rate of glutamate release increased to 199.7 pmol/10^7 cells/min. Similar results were obtained in media lacking added Ca^{2+} (not shown).

The presence of 1 mM $CaCl_2$ inhibited the release of glutamate induced by KCl depolarization to a value lower than that observed in low-Ca^{2+} medium [Fig. 1(A)]. The basal release of glutamate, calculated from the data of Fig. 1(A) was 66.3 pmol/10^7 cells/min, increasing to 111.4 pmol/10^7 cells/min upon K^+-depolarization.

Although the release of glutamate evoked by KCl-depolarization was inhibited by 1 mM extracellular Ca^{2+}, the stimulation of chick retinospheroids with 30 mM KCl increased the $[Ca^{2+}]_i$ by 382.5 ± 25.8 nm [Fig. 1(B)]. The initial $[Ca^{2+}]_i$ peak, which is probably due to the activation of glutamatergic and nonglutamatergic cells, was followed by a slow decrease of the $[Ca^{2+}]_i$ towards a plateau of 205.1 ± 20.8 nM ($n = 4$) above the resting concentration.

Stimulation of retinospheroids with 100 μM veratridine in low Ca^{2+} medium (0.1 mM $CaCl_2$) also increased significantly the release of endogenous glutamate, up to 2.86 ± 0.03 nmol/10^7 cells/7 min [17.8% of total glutamate; Fig. 2(A)]. The basal release of glutamate, calculated from the average trace shown in Fig. 2(A), was 82.3 pmol/10^7 cells/min. Upon stimulation with veratridine a lag-phase of about 4 min was observed, followed by the rapid release of glutamate, at a rate of 908.3 pmol/10^7 cells/min. Similar results were obtained when no Ca^{2+} was added to the extracellular medium (not shown). This effect was inhibited by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 20 μM), a nonselective blocker of non-NMDA glutamate receptors (Watkins *et al.*, 1990), suggesting that the glutamate released by veratridine stimulation activates glutamate receptors of glutamatergic cells, further enhancing the release of the neurotransmitter.

In the presence of 1 mM external Ca^{2+}, veratridine induced a rapid and sustained increase of the $[Ca^{2+}]_i$, by 171.2 ± 5.7 nm, in a tetrodotoxin-sensitive manner [Fig. 2(B)]. However, as observed for KCl-depolarized retinospheroids, the release of glutamate evoked by the alkaloid was significantly reduced when the extracellular Ca^{2+} concentration was increased from 0.1 to 1 mM [Fig. 2(A)].

Effect of KCl *and veratridine on [^{3}H]D-aspartate release from monolayer cultures of retina cells*

Taken together, the results suggest that the release of glutamate from retinospheroids is mainly Ca^{2+}-independent, probably mediated by reversal of the glutamate transporter (Nicholls, 1989). If there is any exocytotic release of the amino acid, it is probably masked by the effect of inhibitory factors released by neighbouring cells in a Ca^{2+}-dependent manner. In order to further investigate the Ca^{2+}-dependency of glutamate release from retina cells, in the absence of possible endogenous modulators released by other cells, we studied [^{3}H]D-aspartate release from perfused monolayer cultures of chick retina cells. In these studies, a low cell density was used to avoid strong interactions between the cells during depolarization with KCl (50 mM) or veratridine (50 μM). The superfusion of the cells would wash away any endogenous factor(s) released.

K^+-depolarization of monolayer cultures of chick retina cells, in a medium containing 50 mM KCl and 86 mM NaCl, released 3.46 ± 0.33% of the accumulated

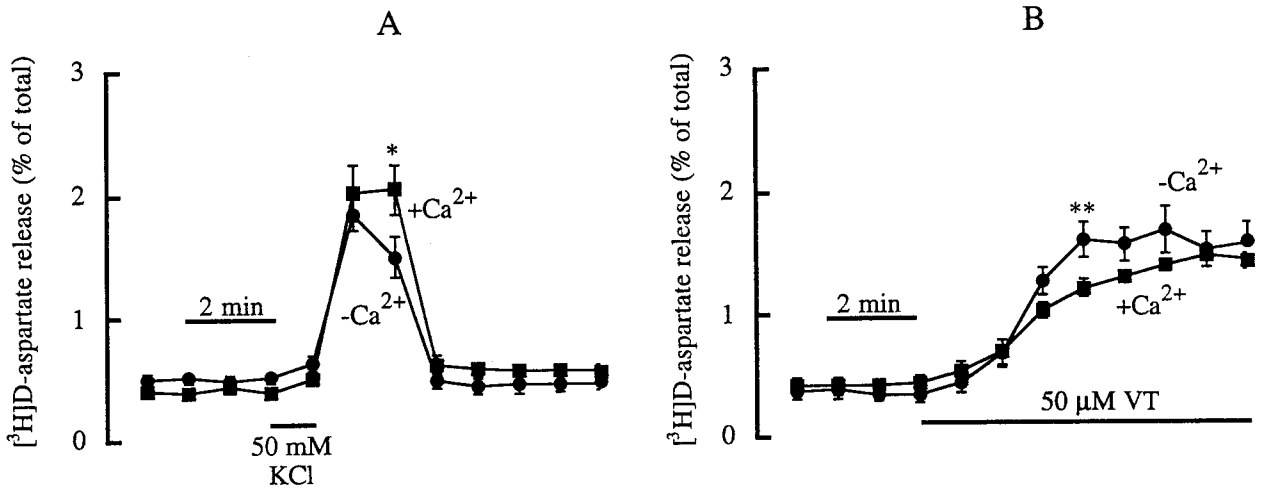

FIGURE 3. Ca^{2+}-dependence of the K^+- or veratridine-stimulated [^{3}H]D-aspartate release in cultured chick retinospheroids. The cells were pre-loaded with 40 nM [^{3}H]D-aspartate and perfused for 19 min with Ca^{2+}-containing (squares) or Ca^{2+}-free (circles) Na^+ medium. In (A), where indicated, the cells were challenged for 1 min with depolarizing media by replacing Na^+ isosmotically with 50 mM KCl, with (squares) or without (circles) 1 mM Ca^{2+}, and otherwise identical to the Na^+ medium. In (B), where indicated, the cells were stimulated with 50 μM veratridine, in the presence (squares) or in the absence (circles) of added Ca^{2+}. The Ca^{2+}-free media was prepared without adding Ca^{2+} or EGTA. Release of [^{3}H]D-aspartate is expressed as a percentage of the amount of radioactivity in the cells at the time when the release was measured. The results are means $\pm$ S.E.M. of five to nine experiments performed in independent preparations. $^*P = 0.06$; $^{**}P = 0.03$.

[^{3}H]D-aspartate, in the presence of 1 mM $CaCl_2$ [Fig. 3(A)]. In the absence of Ca^{2+}, 2.8 $\pm$ 0.42% of the total [^{3}H]D-aspartate was released [Fig. 3(A)], which suggests that, under these experimental conditions, a small fraction of the release may occur by exocytosis.

Stimulation of the perfused monolayer cultures with veratridine (50 μM), in the presence of 1 mM $CaCl_2$, evoked a sustained release of [^{3}H]D-aspartate of about 1.4% of the total intracellular radioactivity per minute [Fig. 3(B)]. When Ca^{2+} was absent from the perfusion medium, the alkaloid increased the release up to a higher value of 1.6% of the total intracellular [^{3}H]D-aspartate, and the difference was statistically significant for one data point [Fig. 3(B)].

DISCUSSION

Glutamate is the major excitatory neurotransmitter in the retina, and is released by photoreceptors, bipolar and ganglion cells (Ehinger, 1989; Massey, 1991). Surprisingly, very few direct attempts have been made to characterize the release of glutamate from retinal neurons.

In the intact retina, K^+-depolarization was found not to affect [^{14}C]glutamate release, whereas kainate slightly increased the amino acid release by a Ca^{2+}-independent mechanism (Tapia & Arias, 1982). However, the great disadvantage of using radioactive glutamate in neurotransmitter release studies is that the metabolism of labeled L-glutamate cannot be prevented. Therefore, it is essential, for long pre-incubations, that authentic glutamate is separated from its metabolites. In the present work, we have adapted a fluorometric assay (Nicholls *et al.*, 1987) to measure the release of endogenous glutamate from cultured chick retinospheroids. The advantages of this method, over HPLC techniques, is the rapid and continuous detection of glutamate release and the fact that there is no need to chromatograph multiple samples to obtain a single time-course (Nicholls, 1989).

The release of endogenous glutamate stimulated by depolarization of the retinospheroids with KCl or veratridine was significantly higher in low Ca^{2+} medium (0.1 mM) than in the presence of 1 mM $CaCl_2$. The Ca^{2+}-independent release of glutamate is thought to be due to the reversal of the electrogenic glutamate transporter, triggered by membrane depolarization (Nicholls, 1989). The magnitude of this component, especially upon stimulation with veratridine, may mask some exocytotic release of glutamate. Indeed, our observations contrast with the previously reported Ca^{2+}-dependent exocytotic release of excitatory amino acids from retina cells, determined using electrophysiological techniques (Tachibana *et al.*, 1993; Rieke & Schwartz, 1994; Gersdorff & Matthews, 1994). However, the lack of suitable noncompetitive blockers of the glutamate carrier makes difficult any pharmacological manipulations of this transport protein, in order to further dissect the exocytotic release of the transmitter under our experimental conditions.

The observation that extracellular Ca^{2+} inhibits the release of glutamate also contrasts with the $[Ca^{2+}]_i$ responses evoked by depolarization of the chick retinospheroids with KCl [Fig. 1(B)] or with veratridine [Fig. 2(B)]. Although smaller in magnitude, the $[Ca^{2+}]_i$ response to KCl-depolarization was similar to that reported for monolayer cultures of chick retina cells stimulated with 50 mM KCl (Duarte *et al.*, 1992). The transient $[Ca^{2+}]_i$ rise observed in the retinospheroids and in the monolayer cultures stimulated with KCl is in agreement with the reported inactivation of the $^{45}Ca^{2+}$

uptake after the initial peak increase (Wei *et al.*, 1989), probably due to the elevated $[Ca^{2+}]_i$ and/or plasma membrane depolarization (Chad, 1989; Duarte *et al.*, 1991; Gleason *et al.*, 1994).

The activation of ionotropic glutamate receptors is known to increase the $[Ca^{2+}]_i$ in cultured chick retina cells (Duarte *et al.*, 1993, in press). Therefore, since both KCl and veratridine stimulated the release of glutamate from the retinospheroids, it is possible that part of the $[Ca^{2+}]_i$ transients reported in Fig. 1(B) and Fig. 2(B) are due to the activation of glutamate receptors. This phenomenon may be particularly important upon stimulation with veratridine, since in this case we found that glutamate release was significantly inhibited by the non-NMDA receptor antagonist CNQX (Watkins *et al.*, 1990).

The unmetabolized analogue of L-glutamate, [^{3}H]D-aspartate, has been used as a radiochemical marker for glutamate release because it is thought to mix with the transmitter pool of glutamate and to mimic its behavior (Drejer *et al.*, 1983; Arqueros *et al.*, 1985; Benson *et al.*, 1991; Belhage *et al.*, 1992; Agostinho *et al.*, 1994). Depolarizing stimuli were shown to evoke a Ca^{2+}-dependent release of D-aspartate from synaptic terminals (Malthe-Sörenssen *et al.*, 1979; Potashner & Tran, 1984), and axotomy of glutamatergic neurons also diminishes the high-affinity uptake and depolarization-induced release of [^{3}H]D-aspartate (Potashner & Tran, 1984; Potashner *et al.*, 1988). Although D-aspartate may be a poor substrate for the vesicular glutamate transporter in the nerve terminals (Naito & Ueda, 1985; Maycox *et al.*, 1988), this has not so far been unequivocally documented (Fonnum, 1984; Nicholls & Attwell, 1990). Therefore, we further investigated the Ca^{2+}-dependency of glutamate release from the retina cells by measuring the release of [^{3}H]D-aspartate from monolayer cultures. In these studies, a low cell density (0.4×10^6 cells/cm^2) was used to avoid strong interactions between the cells, during depolarization with KCl or veratridine.

The small Ca^{2+}-dependent component of the [^{3}H]D-aspartate release, observed upon depolarization of the monolayer cultures with KCl, suggests that the excitatory amino acids may be also released from retinal neurons by exocytosis. However, most of the release appears to be due to the reversal of the excitatory amino acid transporter. In contrast, we observed that the release of [^{3}H]D-aspartate evoked by veratridine was inhibited in the presence of Ca^{2+}. These observations resemble the inhibitory effect of Ca^{2+} on the release of GABA by synaptosomes stimulated with veratridine (e.g. Santos *et al.*, 1992). This effect was attributed to an inhibition, by Ca^{2+}, of the alkaloid-mediated activation of the Na^+ channels (Levi *et al.*, 1980).

In conclusion, we have shown that in cultured chick retina cells glutamate is released mainly by a Ca^{2+}-independent mechanism, probably by reversal of the glutamate transporter (Nicholls, 1989). Similarly, the GABA carrier was found to play an important role on the neurotransmitter release in the retina (e.g. Schwartz, 1987; Duarte *et al.*, 1992, 1993), and a large component of the resting release of acetylcholine by the cholinergic amacrine cells is independent of extracellular Ca^{2+} and membrane potential (Masland & Tauchi, 1986). However, the physiological significance of these observations is not known.

The cultured chick retinospheroids contain areas homologous to all three retinal nuclear and both plexiform layers (Layer & Willbold, 1993). Since glutamatergic photoreceptors, bipolar and ganglion cells, in addition to glial Müller cells, are all present in these structures, it is not possible to determine which cell type(s), if not all, are responsible for the KCl- or the veratridine-induced release of glutamate reported here. Furthermore, since the glial cells are known to accumulate glutamate very quickly (Peng *et al.*, 1993), the neurotransmitter release detected by our assay is probably underestimated.

The presence of extracellular Ca^{2+} inhibited the release of endogenous glutamate by retinospheroids. Since these structures possess a heterogeneous neuron population, membrane depolarization may stimulate the release of modulatory substances by neighbouring neurons, which do not diffuse out easily and, therefore, may inhibit the release of glutamate from glutamatergic neurons. We are currently investigating the identity of this endogenous retinal inhibitory neurotransmitter/neuromodulator. Some of the possible candidates include GABA, the major inhibitory neurotransmitter in the retina (Barnstable, 1993), adenosine (Blazynski & Perez, 1991) and arachidonic acid, which can be released in a Ca^{2+}-dependent manner and is known to inhibit the glutamate transporter (Attwell *et al.*, 1993).

REFERENCES

Agostinho, P., Duarte, C. B., Carvalho, A. P. & Oliveira, C. R. (1994). Effect of oxidative stress on the release of [^{3}H]GABA in cultured chick retina cells. *Brain Research, 655*, 213–221.

Arqueros, L., Abarca, J. & Bustos, G. (1985). Release of [^{3}H]D-aspartic acid from the striatum. Effect of veratridine-evoked depolarization, frontoparietal cortex ablation, and striatal lesions with kainic acid. *Biochemical Pharmacology, 34*, 1217–1224.

Attwell, D., Miller, B. & Sarantis, M. (1993). Arachidonic acid as a messenger in the central nervous system. *Seminars Neuroscience, 5*, 159–169.

Barnstable, C. J. (1993). Glutamate and GABA in retinal circuitry. *Current Opinion in Neurobiology, 3*, 520–525.

Belhage, B., Rehder, V., Hansen, S. B., Kater, S. B. & Schousboe, A. (1992). ^{3}H-D-Aspartate release from cerebellar granule neurons is differentially regulated by glutamate- and K^+-stimulation. *Journal of Neuroscience Research, 33*, 436–444.

Benson, C. G., Chase, M. C. & Potashner, S. G. (1991). Decreased release of D-aspartate in the guinea pig spinal cord after lesions of the red nucleus. *Journal Neurochemistry, 56*, 1174–1183.

Blazynski, C. & Perez, M.-T. (1991). Adenosine in vertebrate retina: Localization, receptor characterization, and function. *Cellular Molecular Neurobiology, 11*, 463–484.

Chad, J. (1989). Inactivation of calcium channels. *Comparative Biochemistry & Physiology, 93A*, 95–105.

Drejer, J., Larsson, O. M. & Schousboe, A. (1983). Characterization of uptake and release processes for D- and L-aspartate in primary cultures of astrocytes and cerebellar granule cells. *Neurochemical Research, 8*, 231–243.

Duarte, C. B., Carvalho, C. M., Ferreira, I. L. & Carvalho, A. P. (1991).

Synaptosomal $[Ca^{2+}]_i$ as influenced by Na^+/Ca^{2+} exchange and KCl-depolarization. *Cell Calcium, 12*, 623–633.

Duarte, C. B., Ferreira, I. L., Santos, P. F., Oliveira, C. R. & Carvalho, A. P. (1992). Ca^{2+}-dependent release of [^{3}H]GABA in cultured chick retina cells. *Brain Research, 591*, 27–32.

Duarte, C. B., Ferreira, I. L., Santos, P. F., Oliveira, C. R. & Carvalho, A. P. (1993). Glutamate increases the $[Ca^{2+}]_i$ but stimulates Ca^{2+}-independent release of [^{3}H]GABA in cultured chick retina cells. *Brain Research, 611*, 130–138.

Duarte, C. B., Santos, P. F. & Carvalho, A. P. (1995). $[Ca^{2+}]_i$ regulation by glutamate receptor agonists in cultured chick retina cells. *Vision Research 36*, (in press).

Ehinger, B. (1989). Glutamate as a retinal neurotransmitter. In Weiler, R. & Osborne, N. (Eds) *Neurobiology of the inner retina*, (Vol. H21, pp. 1–14). Berlin: Springer.

Fonnum, F. (1984). Glutamate: A neurotransmitter in mammalian brain. *Journal of Neurochemistry, 42*, 1–11.

Gersdorff, H. & Matthews, G. (1994). Dynamics of synaptic vesicle fusion and membrane retrieval in synaptic membranes. *Nature, 367*, 735–739.

Gleason, E., Borges, S. & Wilson, M. (1994). Control of transmitter release from retinal amacrine cells by Ca^{2+} influx and efflux. *Neuron, 13*, 1109–1117.

Layer, P. G. & Willbold, E. (1993). Histogenesis of the avian retina in reaggregation culture: From dissociated cells to laminar neuronal networks. *International Review of Cytology, 146*, 1–47.

Levi, G., Gallo, V. & Raiteri, M. (1980). A re-evaluation of veratridine as a tool for studying the depolarization-induced release of neurotransmitters from nerve endings. *Neurochemical Research, 5*, 281–295.

Malthe-Sörenssen, D., Skrede, K. K. & Fonnum, F. (1979). Ca^{2+}-dependent release of [^{3}H]D-aspartate evoked by selective electrical stimulation of excitatory afferent fibers of hippocampal pyramidal cells *in vitro*. *Neuroscience, 4*, 1255–1263.

Masland, R. H. & Tauchi, M. (1986). The cholinergic amacrine cell. *Trends in Neuroscience, 9*, 218–223.

Massey, S. C. (1991). Cell types using glutamate as a neurotransmitter in the vertebrate retina. *Progress in Retinal Research, 9*, 399–425.

Maycox, P. R., Deckwerth, T., Hell, J. W. & Jahn, R. (1988). Glutamate uptake by brain synaptic vesicles. Energy dependence of transport and functional reconstitution in proteoliposomes. *Journal of Biological Chemistry, 263*, 15423–15428.

Mello, F. G., Hokoç, J. N., Ventura, A. L. & Gardino, P. F. (1991). Glutamic acid decarboxylase of embryonic avian retina cells in culture: Regulation by γ-aminobutyric acid (GABA). *Cellular Molecular Neurobiology, 11*, 485–496.

Naito, S. & Ueda, T. (1985). Characterization of glutamate uptake into synaptic vesicles. *Journal of Neurochemistry, 44*, 99–109.

Nicholls, D. G. (1989). Release of glutamate, aspartate, and γ-aminobutyric acid from isolated nerve terminals. *Journal of Neurochemistry, 52*, 331–341.

Nicholls, D. G. & Attwell, D. (1990). The release and uptake of excitatory amino acids. *Trends in Pharmacological Sciences, 11*, 462–468.

Nicholls, D. G., Sihra, T. S. & Sánchez-Prieto, J. (1987). Calcium-dependent and independent release of glutamate from synaptosomes monitored by continuous fluorimetry. *Journal of Neurochemistry, 47*, 50–57.

Peng, L., Hertz, L., Huang, R., Sonnewald, U., Petersen, S. B., Westergaard, N., Larsson, O. & Schousboe, A. (1993). Utilization of glutamine and of TCA cycle constituents as precursors for transmitter glutamate and GABA. *Developmental Neuroscience, 15*, 367–377.

Potashner, S. J. & Dymczyk, L. & Deangelis, M. M. (1988). D-aspartate uptake and release in the guinea pig spinal cord after partial ablation of the cerebral cortex. *Journal of Neurochemistry, 50*, 103–111.

Potashner, S. J. & Tran, P. L. (1984). Decreased uptake and release of D-aspartate in the guinea pig spinal cord after dorsal root section. *Journal of Neurochemistry, 42*, 1135–1144.

Rieke, F. & Schwartz, E. A. (1994). A cGMP-gated current can control exocytosis at cone synapses. *Neuron, 13*, 863–873.

Santos, M. S., Rodriguez, R. & Carvalho, A. P. (1992). Effect of depolarizing agents on the Ca^{2+}-independent and Ca^{2+}-dependent release of [^{3}H]GABA from sheep brain synaptosomes. *Biochemical Pharmacology, 44*, 301–308.

Schwartz, E. A. (1986). Synaptic transmission in amphibian retinae during conditions unfavorable for calcium entry into presynaptic terminals. *Journal of Physiology (London), 376*, 411–428.

Schwartz, E. A. (1987). Depolarization without calcium can release γ-aminobutyric acid from retinal neurons. *Science, 238*, 350–355.

Tachibana, M., Okada, T., Arimura, T., Kobayashi, K. & Piccolino, M. (1993). Dihydropyridine-sensitive calcium current mediates neurotransmitter release from bipolar cells of the goldfish retina. *Journal of Neuroscience, 13*, 2898–2909.

Tapia, R. & Arias, C. (1982). Selective stimulation of neurotransmitter release from chick retina by kainic and glutamic acids. *Journal of Neurochemistry, 39*, 1169–1178.

Watkins, J. C., Krogsgaard-Larsen, P. & Honoré, T. (1990). Structure–activity relationship in the development of excitatory agonists and competitive antagonists. *Trends in Neuroscience, 11*, 25–33.

Wei, X. Y., Rutledge, A., Zhong, Q., Ferreante, J. & Triggle, D. J. (1989). Ca^{2+} channels in chick neural retina cells characterized by 1,4-dihydropyridine antagonists and activators. *Canadian Journal of Physiology and Pharmacology, 69*, 506–514.

Acknowledgements—This work was supported by JNICT (Portugal) and by the Human Capital and Mobility Program (EU).