L-Arginine Uptake and Release by Cultured Avian Retinal Cells: Differential Cellular Localization in Relation to Nitric Oxide Synthase

*†Marcelo Cossenza and *Roberto Paes de Carvalho

*Department of Neurobiology and Program of Neuroimmunology, Federal Fluminense University, Niterói; and †Institute of Biophysics Carlos Chagas Filho, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

Abstract: The availability of L-arginine is of pivotal importance for the synthesis of nitric oxide, a signaling molecule in the CNS. Here we show the presence of a high-affinity L-arginine uptake system (K_m of 4.4 ± 0.5 μM and a V_max of 26.0 ± 0.9 fmol/well/min) in cultured chick retinal cells. Different compounds, such as G-4-monomethyl-L-arginine and L-lysine, were able to inhibit the uptake that was also inhibited 60–70% in the absence of sodium and/or calcium ions. No trans stimulation was observed when cells were preloaded with L-lysine. The data indicate that the L-arginine uptake in cultured retinal cells is partially mediated by the y^+ system, but has a great contribution of the B^0,+ system. Autoradiographic studies revealed that the uptake is predominant in glial cells and can also be detected in neurons, whereas immunocytochemistry of nitric oxide synthase and L-citrulline showed that the enzyme is present in neurons and photoreceptors, but not in glial cells. L-[3H]Arginine is released from purified glial cultures incubated with high concentrations of potassium in the extracellular medium. Moreover, the amino acid released from preloaded glial cells was taken up by purified neuronal cultures. These results indicate that L-arginine released from glial cells is taken up by neurons and used as substrate for the synthesis of nitric oxide. Key Words: Chick—Transport—Citrulline—Glial cells—Release.


The synthesis of nitric oxide (NO) is catalyzed by the enzyme NO synthase (NOS), which promotes the transformation of L-arginine to L-citrulline and NO. NOS is present in several cell types, especially in endothelial cells, macrophages, and neurons (Mitchell et al., 1992; Busconi and Michel, 1993; Egberongbe et al., 1994; Nussler et al., 1994). In the CNS, NO plays important roles in the regulation of neurotransmitter release (for review, see Zhang and Snyder, 1995) and of developmental events such as neuronal proliferation and death (Peunova and Enikolopov, 1995). The production of NO is importantly regulated by the influx of calcium ions through NMDA receptor channels stimulated by the neurotransmitter glutamate (Garthwaite et al., 1988; Southam and Garthwaite, 1991).

One of the limiting points in the regulation of NO synthesis is the availability of the precursor L-arginine, which depends on the presence of a specific uptake system for this amino acid. The transport of cationic amino acids across cell membranes is mediated by a group of well described transport systems (y^+, y^+_L, b^0,+ and B^0,+) in mammals, the transport is mediated predominantly by the y^+ system, which facilitates sodium-independent cationic amino acid transport. Two genes, mCAT-1 and mCAT-2, encode multiple membrane-spanning related proteins that, when expressed in Xenopus oocytes, are functionally indistinguishable from the y^+ system (MacLeod et al., 1994). The systems y^+_L and b^0,+ also mediate the sodium-independent transport of cationic amino acids, and two genes were identified that encode the shared glycoproteins rBAT and 4F2hc (MacLeod et al., 1994). The B^0, + system mediates a sodium-dependent transport, but no putative protein has yet been described for this system (Kakuda and MacLeod, 1994; MacLeod et al., 1994; MacLeod and Kakuda, 1996). Several L-arginine analogues that block NOS are also able to block the uptake of this amino acid (Westergaard et al., 1993; Schmidlin and Wiesinger, 1994).

Immunocytochemical evidence indicates that L-arginine is localized predominantly in glial cells (Aoki et al., 1991; Pow, 1994; Pow and Crook, 1997), and the properties of L-arginine transport in these cells suggest the participation of the y^+ system (Schmidlin and Wiesinger, 1994). Recent work has also shown that L-arginine is released from glial cells stimulated by α-amino-3-hydroxy-5-methylisoxazole-
4-propionate (AMPA) glutamate receptor agonists (Grima et al., 1997, 1998).

Several neurotransmitter systems have been characterized in the chick retina and their regulation studied during development (Paes de Carvalho and de Mello, 1982, 1985; do Nascimento and de Mello, 1985; Hokç et al., 1990; Paes de Carvalho et al., 1990, 1992; Ventura and de Mello, 1990; do Nascimento et al., 1992; Gardino et al., 1993; Calvet and Ventura, 1995). Previous studies showed a calcium-dependent NOS activity in the early stages of chick retina development that decreased at subsequent ages until hatching (de Faria et al., 1995; Paes de Carvalho and Mattos, 1996; Paes de Carvalho et al., 1996; Ientile et al., 1999).

In the present work, we demonstrate the presence of a high-affinity sodium- and calcium-dependent L-arginine uptake system in cultured retinal cells. Autoradiographic experiments show L-[3H]arginine uptake in glial cells as well as in neurons. The glial cells incubated with high concentrations of potassium are able to release the amino acid that can be taken up by the neurons. Together with immunocytochemical evidence for the presence of NOS and L-citrulline in cultured neurons, but not in glial cells, our results indicate the extracellular recycling of L-arginine between glial cells and neurons.

**MATERIALS AND METHODS**

**Materials**

L-[3H]Arginine (53 Ci/mmol) was purchased from Amer sham. Basal medium Eagle and medium 199 were from GIBCO. HEPES, L-arginine, N6-nitro-L-arginine (L-NA), and L-lysine were from Sigma. Nω-Monomethyl-L-arginine (L-NMMA), N-iminoethyl-L-ornithine (L-NIO), and Nω-nitro-L-arginine methyl ester (L-NAME) were from Research Biochemicals Inc. All other reagents used were of analytical grade. Fertilized White Leghorn chick eggs were obtained from a local hatchery and incubated at 37°C in a humidified atmosphere up to the appropriate age. The antibodies against L-citrulline were kindly supplied to us by Dr. S. Vincent (Department of Psychiatry, University of British Columbia, Vancouver, British Columbia, Canada). This antibody was tested extensively, and its colocalization with NOS was determined in several structures of the CNS (Vincent and Kimura, 1992). The antibodies against neuronal NOS were kindly supplied to us by Dr. V. Riveros-Moreno (Wellcome Research Laboratories, Beckenham, Kent, U.K.). This antibody was raised against the sequence 519–540 (LPLLLQANGNDPELFQIPPELC) of the neuronal rat enzyme.

**Preparation of high-density mixed primary cultures of retina cells**

Monolayer cultures of chick retina cells were prepared as previously described (de Mello, 1978). In brief, retinas from 8-day-old chick embryos were dissected from other ocular tissues, including the pigment epithelium, and digested with 0.1% trypsin, in calcium- and magnesium-free Hank’s balanced salt solution for 20 min at 37°C. The cells were suspended in basal medium Eagle supplemented with 5% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml), and seeded in 35-mm tissue culture plastic dishes or 16-mm wells at a density of 2 × 10⁴ cells/mm². The cells were maintained at 37°C in a humidified incubator with 95% air and 5% CO₂ and were used after 6 days in culture. The medium was changed every other day.

**Preparation of glial cell cultures**

Retinas from 11-day-old embryos were dissected and the cells dissociated as described for the mixed cultures. The cells were seeded in 16-mm multwell plates at a density of 2 × 10³ cells/mm² and cultured for 21 days. After this time, the neurons die and the glial cells turn confluent. The medium was changed every 3 days.

**Preparation of purified cultures of neurons and photoreceptors**

Glia-free cultures of retina neurons and photoreceptors from 8-day-old chick embryos were prepared as previously described (Adler et al., 1984). In brief, the retinas were dissected, and the cells were dissociated after mild trypsinization, suspended in medium 199, and seeded in 35-mm tissue culture plastic dishes or over glass coverslips (484 mm²) precoated with poly-L-ornithine. The cell density was 8.3 × 10⁵ cells/mm². The culture medium used was medium 199 supplemented with 1% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). The cultures were incubated for 3–6 days at 37°C in a humidified atmosphere of 5% CO₂/95% air, with no change of medium.

**1-[3H]Arginine uptake**

For the uptake studies, cells were incubated in Hanks’ solution (140 mM NaCl, 5 mM KCl, 20 mM HEPES, 4 mM glucose, 1 mM MgCl₂, 2 mM CaCl₂) containing L-[3H]arginine at 37°C. After the incubation time, the cells were washed and lysed with water and the intracellular radioactivity determined by liquid scintillation. To test the effect of different substances on the uptake, the cultures were preincubated for 15 min in the absence or presence of these substances, before the addition of 1-[3H]arginine, and maintained during the uptake period. The effect of the absence of sodium was tested by the isosmotic substitution of sodium chloride by lithium chloride. The effect of the absence of calcium ions was studied in Hanks’ solution without calcium and with 2 mM EGTA.

**1-[3H]Arginine release from glial cultures**

For the release experiments, glial cultures were loaded with 1-[3H]arginine (2.65 µCi/ml) for 60 min. After this time, the cultures were washed and incubated in Hanks’ solution for four successive periods of 3 min and one period of 10 min (basal release). The cultures were then incubated for another period of 10 min in Hanks’ solution containing 50 mM KCl (stimulated release) and lysed with water for determination of the remaining intracellular radioactivity. The radioactivity of all samples was determined by liquid scintillation spectroscopy, and the results were expressed as a percentage of the radioactivity present inside the cells at each period (% fractional release).

**1-[3H]Arginine release in cocultures of purified glial and neuronal cells**

In this case, glial cultures were first loaded with 1-[3H]arginine (2.65 µCi/ml) for 60 min. After washing and incubation of these cultures in Hanks’ solution for four successive periods of 3 min, coverslips containing purified neuronal cultures were transferred to dishes containing the glial cells and accommodated over a plastic apparatus, avoiding the direct contact between the two cultures but immersed in the same medium. The Hanks’ solution was then added, and the cocultures were incubated for a period of 10 min (basal release). After this time,
the coverslips with the neurons were transferred to different dishes and lysed with water for determination of intracellular radioactivity. A second set of coverslips were then transferred to the same dishes with the glial cells, and a subsequent period of 10 min of release was performed with Hanks’ solution containing 50 mM KCl. After this time, the coverslips were transferred and lysed as above, and the intracellular radioactivity remaining inside the glial cells was also determined for the calculation of the % fractional release.

Analysis of released radioactivity

The basal and potassium-stimulated released radioactivity was analyzed using TLC plates as described by Olken and Marletta (1993) using the solvent mixture chloroform/methanol/ammonium hydroxide (2:2:1). Standard solutions of l-arginine, l-ornithine, and l-citrulline were run in parallel as internal standards. The $R_f$ values for l-arginine, l-ornithine, and l-citrulline were 0.19, 0.28, and 0.57, respectively. After staining with ninhydrin, silica gel was scraped off the plates from the corresponding stains and the radioactivity determined by scintillation counting.

Immunocytochemistry

Chick retina cells, cultured on glass coverslips at a density of $2 \times 10^4$ cells/mm$^2$ for 6 days, were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 1 h. After several washes in PB, the cells were treated sequentially with PB containing 3% hydrogen peroxide and 0.1% Triton X-100 for the inactivation of endogenous peroxidases and cellular permeabilization. After blocking the nonspecific sites with normal goat serum (1:100 solution in PB containing 2.5 mg/ml bovine serum albumin), the cultures were washed and incubated for 48 h with primary antibody (anti-NOS, 1:10,000, or anti-citrulline, 1:20,000). The cultures were then washed and incubated with secondary antibody (1:400 solution of biotinylated anti-rabbit antibody in PB containing bovine serum albumin and normal goat serum, 1:200) for 90 min. The cultures were incubated with avidin–biotin–peroxidase complex (ABC Vector Elite kit) for 90 min and the staining developed with diaminobenzidine.

Autoradiography

Cultures were incubated for 30 min with l-[3H]arginine (2.65 $\mu$Ci/ml), washed, and fixed for 1 h with a solution of 2.5% glutaraldehyde in 0.2 M PB. After this time, the cultures were covered with autoradiographic emulsion (Amersham, Hypercoat EM I) and maintained in the dark for 40–50 days at 4°C. After development, the cultures were coverslipped and analyzed in a Zeiss Axioscope microscope.

RESULTS

Autoradiographic localization of l-[3H]arginine uptake in cultured retinal cells

When a high number of chick embryo retina cells were dissociated and cultured for 6 days, the glial cells proliferated and formed a sheet of flat cells over which the neurons aggregated and extended neurites (Fig. 1A). When these cultures were incubated with l-[3H]arginine for 30 min and processed for autoradiography, most of the grains were localized over the glial cells (Fig. 1B). The labeling was weak over the neurons, especially in cells close to or over the glial cells (Fig. 1C). To demonstrate the existence of a transporter system for l-arginine in neurons, we used the purified cultures in which only neurons differentiated, including photoreceptors (Fig. 2A). When these cultures were incubated with l-[3H]arginine as above and processed for autoradiography, an extensive labeling over the neurons was observed, although occasionally neurons without labeling

FIG. 1. Autoradiography of l-[3H]arginine uptake by mixed cultures of chick retina cells. A: Phase-contrast micrograph of a culture incubated for 6 days (C6) showing the presence of neurons (long arrows) and glial cells (short arrow). B and C: Photomicrographs of C6 cultures incubated for 30 min with l-[3H]arginine and processed for autoradiography. Notice the extensive labeling of glial cells (short arrows), whereas the neurons (long arrows) are much less labeled especially when in close contact with glial cells (white long arrows). Scale bars = 30 $\mu$m.
could also be observed (Fig. 2B). The autoradiographic grains could be observed over the soma and neurites of both neurons and photoreceptors (Fig. 2B and C).

**Kinetic analysis of \( \text{L-}[^3\text{H}]\text{arginine} \) uptake**

The uptake of \( \text{L-}[^3\text{H}]\text{arginine} \) by retinal cells in mixed high-density cultures was measured and found to attain equilibrium after 20 min of incubation at 37°C (Fig. 3).

The uptake was of high affinity, and saturation was observed at concentrations of \( >30 \mu\text{M} \) (Fig. 4A). The Lineweaver–Burk plot of these data was linear up to the concentrations studied, showing a \( K_m \) of \( 4.4 \pm 0.5 \mu\text{M} \) and \( V_{\text{max}} \) of \( 26.0 \pm 0.9 \text{ fmol/well/min} \) (Fig. 4B).

Several arginine analogues and amino acids are able to inhibit the uptake of \( \text{L-arginine} \) in other systems. To characterize the pharmacological properties of the retinal uptake of \( \text{L-arginine} \), we investigated the effects of the...
amino acid L-lysine and some arginine analogues on the uptake of L-[3H]arginine by cultured cells. As illustrated in Fig. 5, L-NMMA was the most potent analogue tested after L-arginine itself, followed by L-lysine, L-NA, L-NIO, and L-NAME.

In several cellular systems, the uptake of L-arginine is mediated by the y\textsuperscript{+} transport system (White, 1985; Westergaard et al., 1993; Schmidlin and Wiesinger, 1994; Schmidt et al., 1995). One of the features of this system is the phenomenon of trans stimulation (White, 1985), which is characterized by a higher rate of L-arginine transport after intracellular accumulation of substrates such as L-lysine or L-arginine. As our experiments were all performed by first preincubating the cells with the inhibitor, it was possible that the inhibitory effect of some of those analogues was masked due to trans stimulation of the cells. To test this possibility, we preloaded the cultures with 10 mM L-lysine for 30 min, washed the cultures to remove the remaining extracellular L-lysine, and then incubated the cells for 30 min with L-[3H]arginine. No increase in the uptake of L-[3H]arginine was observed in the preloaded cultures, suggesting that no trans stimulation occurs in these cultures (Fig. 6A). In fact, we observed a decrease in the uptake rate of L-[3H]arginine under these conditions that could be due to an inhibitory effect of L-lysine if this amino acid diffused out of the cells through its concentration gradient (Fig. 6A). To test if the preincubation protocol was inducing an inhibition of L-[3H]arginine uptake, we further incubated the cultures with two concentrations of L-lysine (10 and 500 µM) added 15 min before or together with L-[3H]arginine. No decrease in the uptake of L-[3H]arginine into cells was observed with 10 µM L-lysine in both incubation protocols, as compared with control. A decrease of ~50% in the uptake was observed with 500 µM L-lysine, but no difference was observed between the two incubation protocols (Fig. 6B).

**Ionic dependence of the uptake**

The uptake of L-arginine by cultured retinal cells was found to be partially dependent on the presence of calcium and sodium ions in the extracellular medium. As shown in Fig. 7, the uptake was inhibited by 63.6 ± 4.5% and 59.6 ± 3.5% in the absence of sodium and calcium, respectively. In parallel experiments, the uptake of GABA was shown to be blocked completely in the absence of sodium ions (data not shown). The removal of both ions promoted an inhibition of 72.8 ± 2.2% of L-arginine uptake, indicating the existence of an uptake component that is not dependent on the presence of both ions. The uptake was also inhibited by 53.1 ± 1.4% in the presence of high concentrations of potassium chloride (50 mM).

**Release of L-[3H]arginine**

Purified cultures of glial cells were prepared to test if these cells were able to release L-[3H]arginine in response to depolarization induced by high potassium concentrations. Figure 8A shows the aspect of these cultures when the glial cells were grown in the absence of neurons. The glial cultures showed a threefold increase of...
released radioactivity in the presence of 50 mM KCl (Fig. 8B). The basal and stimulated released radioactivity was also analyzed by TLC. Most of the radioactivity was found as L-arginine with smaller amounts of L-ornithine and L-citrulline (Table 1).

Uptake by neurons of radioactivity released by glial cells

To test if the radioactivity released by glial cells could be taken up by neurons, we performed a series of experiments using cocultures of purified glial and neuronal cells. In this case, the glial cultures were first exposed for 1 h to L-[3H]arginine to allow these cells to take up the amino acid. Thereafter, the release experiment was performed in a same way as previously described, but in the presence of coverslips containing purified neuronal cultures (see Materials and Methods). After the experiment, the released radioactivity, as well as the intracellular radioactivity of the glial and neuronal cultures, was measured. As observed in Fig. 8C, under these conditions the release was very similar to that shown by purified glial cultures in the absence of neuronal cultures. Furthermore, relatively high amounts of radioactivity could be found inside the neurons (Fig. 8C) after the periods of basal and stimulated release from glial cells. The uptake by these cells was lower in the presence of high concentrations of extracellular potassium in a similar manner as observed with mixed cultures (Fig. 7).

Identification of NO-producing cells in the cultures

To study the cellular localization of the NO system in retinal cultures, we performed immunocytochemical studies using antibodies against NOS and L-citrulline. Both NOS (Fig. 9A) and L-citrulline (Fig. 9B and D) immunoreactivities were present in a large proportion of neurons, including photoreceptors, with no staining observed in glial cells. The staining for L-citrulline was greatly reduced when the cultures were preincubated with 500 μM L-NA 1 h before fixation (Fig. 9C). These results suggest that, in the present conditions, the synthesis of NO occurs in neurons, but not in glial cells. Together with the autoradiographic results of L-[3H]arginine uptake shown in Fig. 1, these experiments reveal that the synthesis of NO is performed in neurons and not in glial cells, but the uptake of L-arginine is more evident in glial cells when both cell types coexist in the cultures.

DISCUSSION

In the present work, we demonstrated the presence of an uptake system for L-arginine in cultured chick retina cells. The uptake is present in both glial and neuronal cells, but seems to be predominant in glial cells when both types of cells coexist in culture. Moreover, we showed the release of L-[3H]arginine from glial cells after depolarization with high potassium concentrations. The released amino acid could then be taken up by the neurons and used in the synthesis of NO.

![Fig. 7](image-url) Ionic dependence of L-[3H]arginine uptake by cultured retinal cells. The cultures were incubated in the presence or absence of sodium (substituted by lithium) and/or calcium and with normal or high concentrations of potassium (50 mM). The cultures incubated in the absence of calcium also contained 1 mM EGTA. The results are expressed as the means ± SEM of three separate experiments. *p < 0.001.

![Fig. 8](image-url) Release of L-[3H]arginine in mixed cultures of chick retinal cells and in purified cultures of glial retinal cells. A: Phase-contrast photomicrograph of a purified culture of glial cells showing the almost complete absence of neurons. B: Purified glial cultures were incubated for 30 min with L-[3H]arginine and processed for release studies in the absence (Basal) or presence of 50 mM KCl (K+). C: Purified glial cultures were incubated with L-[3H]arginine and processed for release studies as in B except for the presence of coverslips containing purified neuronal cultures during the periods of basal (B) and stimulated (K+) release. After each period, the coverslips were transferred to different dishes and the intracellular radioactivity determined. The results are expressed as the means ± SEM of three separate experiments. *p < 0.001 when compared with basal levels.
Characterization of L-arginine uptake by retinal cells

The kinetic analysis of the uptake revealed several differences between the retinal and the y⁺ system described in other cell types: (a) The first important difference is the observation that L-lysine and L-arginine have distinct potencies to inhibit L-[³H]arginine uptake into retinal cells, whereas the y⁺ system is described as a cationic amino acid transport system that does not distinguish between L-arginine and L-lysine (White, 1985). (b) Another difference is that the calculated Km in our cultures was 4.4 ± 0.5 μM, a value that is far lower than that described for the y⁺ system in which the calculated Km ranges between 30 and 100 μM (Westergaard et al., 1993). (c) In retinal cells, the uptake is partially dependent on the presence of sodium ions in the extracellular medium, whereas the y⁺ system is strictly sodium-independent (White, 1985; Kakuda and MacLeod, 1994). (d) Another important difference between the uptake of L-arginine by retinal cells and the y⁺ system is that no trans stimulation was observed in our experiments. (e) The analogues L-NA, L-NNAME, and L-NIO were able to inhibit ~50% of L-arginine uptake in the retinal cultures, although with a low affinity, whereas these compounds are not able to compete for the y⁺ system. In conclusion, our data strongly suggest that the uptake of L-arginine by chick retinal cells is partially mediated by a system different from the y⁺ system and fits better with the B⁰⁺ system found in some cells (Hosoya et al., 1997).

Kinetics of inhibition of L-arginine uptake by different compounds

The analysis of the inhibition curves shown in Fig. 5 indicates that unlabeled L-arginine potently inhibits the uptake of L-[³H]arginine by retinal cells. In contrast, the affinity of other tested substances, such as L-NA and L-NNAME, is much lower, showing IC₅₀ values in concentrations higher than 500 μM. This observation is in good agreement with the finding that these substances have a low affinity for the y⁺ system due to the presence of an electron-withdrawing nitro group (Westergaard et al., 1993; Schmidlin and Wiesinger, 1994). However, these compounds are also able to compete for the B⁰⁺ system (Kakuda and MacLeod, 1994). L-Lysine and L-NIO also have a low affinity for the uptake, showing IC₅₀ values of ~500 μM. In contrast, the arginine analogue L-NMMA presents an interesting inhibition pattern, showing a much higher potency for the inhibition of L-arginine uptake with an IC₅₀ of ~10 μM. Additionally, the inhibition curve for L-NMMA appears to show two components, one with an affinity similar to that of L-arginine itself and another with an affinity similar to those of the other analogues and L-lysine.

Ionic dependence of the uptake

Although the Lineweaver–Burk plot of the concentration curve of L-arginine uptake has indicated the presence of only one class of transporters, our results obtained in the absence of sodium and/or calcium indicate the existence of a component that does not depend on the presence of these ions. Therefore, it is possible that more than one uptake system exists in the cultures with different degrees of dependence on the presence of sodium and calcium ions.

The uptake of L-arginine into retinal cells was found to have a very significant calcium dependence. Calcium-dependent L-arginine uptake was reported previously in fetal hypothalamic cultures (Wayte et al., 1996). We do not know at present the significance of this finding, but it will have interesting physiological consequences if both neuronal NOS and the uptake of the precursor L-arginine are regulated by the influx of calcium ions after stimulation of NMDA receptors. The possibility that the transporter protein is regulated by calmodulin-dependent kinases deserves further research. One alternative explanation for the effects of calcium removal would be the cell depolarization observed in this condition and the consequent inhibition of L-arginine transport (Kavanaugh, 1993). The depolarization of lung epithelial cells during hypoxia was shown to correlate with the inhibition of L-arginine transport by the y⁺ system (Zharikov et al., 1997).

Comparison of L-arginine uptake autoradiography with immunocytochemistry for NOS and L-citrulline

We have shown in the present work that the uptake of L-arginine is predominant in glial cells in the retinal cultures. This finding is in accordance with previous studies showing the presence of L-arginine in glial cells (Schmidlin and Wiesinger, 1994) and its release by activation of non-NMDA glutamate receptors (Grima et al., 1997, 1998). In our mixed cultures, the presence of grains over neurons could be visualized, but in a much smaller amount than over glial cells, especially in the vicinity of these cells. We suggest that these data can be explained if the uptake of L-arginine is performed by both glial cells and neurons, but with a lower affinity and/or capacity in the latter cells. However, detailed
kinetic experiments are necessary to clarify this point. Moreover, our results with purified neuronal cultures (Fig. 2) showed the presence of the uptake system in a large proportion of neurons in culture, including photoreceptors.

Although a high level of uptake could be demonstrated in glial cells, we found that neither NOS nor L-citrulline could be detected by immunocytochemistry in these cells, indicating the absence of inducible NOS activity in glial cells under the present conditions. In contrast, a large population of neurons, including photoreceptors, were found to contain both NOS and the product L-citrulline. One possible explanation for these results is that the L-arginine that is taken up by glial cells is released and taken up by the neurons, which could use it as a precursor for the synthesis of NO. Alternatively, L-arginine taken up by glial cells could be used in other metabolic pathways, such as protein synthesis.

Analysis of L-arginine release from retinal cells in culture

Our results showed the release of radioactivity from cultures of glial retinal cells previously loaded with L-[3H]arginine. The chromatographic analysis of the released radioactivity showed that ~80% was due to L-arginine with lower levels of L-ornithine and L-citrulline. The presence of L-citrulline is due probably to the presence of a small population of neurons contaminating the glial cultures or, alternatively, the production of small amounts of this amino acid in glial cells that could not be detected by the immunocytochemical methods used in this study. The release was also observed in mixed cultures (data not shown), and in both cases the incubation with a high potassium concentration induced a threefold increase in the release as compared with the basal levels. These data indicate that glial cells are able to release L-arginine in response to depolarization. The release mechanism is presently unknown, but it is possible that it is mediated by the reversal of the cationic amino acid transporter present in these cells. A similar mechanism of release was suggested for the neurotransmitter GABA in retinal neurons (Yazulla and Kleinschmidt, 1983). In experiments using cocultures, we have demonstrated that the radioactivity released from glial cells can be taken up readily by neurons. The uptake by the neurons in this
case was higher than when L-[3H]arginine was given directly to the neurons in the absence of glial cells. The explanation for this result is presently unknown, but one possibility that deserves further research is the stimulation of neuronal uptake of L-arginine by factors secreted by glial cells.

The stimulation of L-arginine release from cortical glial cells in culture was observed after activation of non-NMDA ionotropic glutamate receptors (Grima et al., 1997, 1998). Our recent unpublished results show that, in glial cultures and also in mixed cultures, the release of L-arginine is stimulated by glutamate (data not shown), suggesting that the activation of receptors for this neurotransmitter can trigger mechanisms involved in the release process in glial cells.

In conclusion, our results have demonstrated the presence of a high-affinity calcium- and sodium-dependent L-arginine uptake system in cultured retinal cells. This system is strongly detected in glial cells that lack NOS activity but can also release the amino acid, indicating the existence of an extracellular recycling of L-arginine between retinal neurons and glial cells in culture.

Acknowledgment: We thank Dr. Ana Lúcia M. Ventura for critical review of the manuscript and Luzeli Ribeiro de Assis for technical assistance. We also thank Drs. V. Riveros-Moreno and S. Vincent for providing us with the antibodies against neuronal NOS and L-citrulline. This work was supported by grants from CNpq, FINEP, FAPERJ, and MCT/PRONEX. M.C. was the recipient of a fellowship from CAPES.

REFERENCES


Ventura A. L. M. and de Mello F. G. (1990) D1 dopamine receptors in neurite regions of embryonic and differentiated retina are highly coupled to adenylate cyclase in embryonic but not in the mature tissue. Brain Res. 530, 301–308.


