The Effects of Glutamate and Kainate on Cell Proliferation in Retinal Cultures

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Two different monolayer culture preparations were used to investigate the effects of glutamate and kainate on retinal cell proliferation. Glial monolayer cultures were produced from 8-day-old chick embryo neural retina. Glial monolayers were exposed to glutamate (5 mM) or kainate (0.4 mM) at either 3 or 5 days in vitro. It was found that exposure to glutamate at 3 days in vitro significantly reduced the number of glia present at 6 days, but a day 5 exposure or exposure to kainate did not significantly affect the number of glia. This glutamate-induced decrease in cell number was seen even though thymidine kinase (EC 2.7.75) and thymidylate synthetase (EC 2.1.1-) activities were higher in treated cultures. Neuroblast monolayer cultures were produced from 6-day-old chick embryo neural retina. These cultures also were exposed to glutamate (5 mM) or kainate (0.4 mM). Glutamate, but not kainate treated cultures, showed a decrease in neuroblast survival and proliferation compared with controls. Thus, at the concentrations tested, glutamate appears to be a general retinal toxin, while the survival of immature neurons or glia is not affected by kainate. Invest Ophthalmol Vis Sci 25:558-563, 1984

Monosodium glutamate is known to have retinotoxic effects, most noticeably neuronal degeneration, through a mechanism not yet understood. Recent studies have shown that glutamate also has toxic effects on Müller cells and that neuron–glia interactions may be important in the expression of glutamate toxicity. Although the effects of glutamate and related analogs on the developing retina have been well-documented, less attention has been paid to the effects of glutamate on the proliferation of glioblasts or neuroblasts. A recent study by this investigator demonstrated that glutamate injected into chick embryos subsequently will decrease the ability of glial cells, of retinal origin, to proliferate in vitro. Furthermore, in vitro exposure to glutamate (at 1 and 3 days in vitro) will also inhibit glial proliferation in these cultures.

The purpose of this study is to further examine the effects of glutamate on glial proliferation in neural retinal cultures and to extend the study to kainate, a rigid glutamate analog. In addition, a modification of our basic monolayer tissue culture system is described, which produces a monolayer of neuroblast from the chick embryo neural retina. These neuroblast cultures are used in this study to determine whether glutamate or kainate influence neuroblast proliferation in vitro.

Materials and Methods

Monolayer Cultures

The preparation of glia-containing monolayer cultures from chick embryo neural retina has been described. Briefly, neural retinas from 8-day, White Leghorn, chick embryos were dissected free of other ocular tissue and mechanically dissociated after a 10 min incubation of Ca²⁺ — Mg²⁺ free Hank’s balanced salt solution (CMF) and a second 20-min incubation with 0.25% trypsin (1:250, Nutritional Biochemical; Cleveland, OH) in CMF. Cells (5 × 10⁵) were suspended in 2 ml of culture medium consisting of modified Eagles Basal Medium supplemented with 10% fetal bovine serum. Cells were seeded on 35 mm tissue culture plastic dishes (Nunc, Denmark). Cultures were incubated at 37.5°C in a humidified atmosphere of 5% CO₂ in air. Culture medium was replaced at 3 and 5 days in vitro.

Neuroblast cultures were prepared from 6-day chick embryo neural retina. This tissue was dissected and dissociated as described above. Cells (4 × 10⁵) were suspended in 2 ml of modified Eagles Basal Medium supplemented with serum-free N1 (consisting of insulin, selenium, progesterone, transferrin and putrescine) plus 5% heart extract prepared from 14-day-old chick embryos. Cells were seeded on 35-mm tissue culture dishes previously coated with a highly adhesive collagen.
Treatment and Analysis of Cultures

At the times described in the Results section, cultures received aliquots from stock solutions of NaCl, glutamate or kainate necessary to achieve final concentrations of 5 mM NaCl or glutamate or of 0.4 mM kainate. At the times indicated, cultures were fixed by adding 1.5 ml of 2% glutaraldehyde to the culture medium for 15 min. Cultures were examined using phase microscopy. Techniques used for the quantitation of cell number have been described. This quantitation involved counting the number of cells per strip in at least three diametral strips 2.0 X 0.1 mm. This value was then converted to total number of cells per dish.

Some neuroblast cultures were exposed to 1 μCi/ml [3H]-thymidine (6.7 Ci/mmol, New England Nuclear; Boston, MA) by adding it directly to the culture medium at 2 days in vitro. At 3 or 6 days, these cultures were fixed and processed for autoradiography as previously described.

Thymidine kinase and thymidylic synthetase activities were assayed as previously described. Cells were removed from the dish by a 20-min incubation in CMF and then manually scraped from the dish. The total number of cells per sample was determined by counting an aliquot from the cell suspension in a hemocytometer. Cells were then removed from suspension by 5-min centrifugation at 4,000 g. Cells were disrupted by a cell disruptor (Heat Systems Ultrasonics; Plainview, New York) for 3 sec. Enzyme activity was expressed as femtomoles of thymidine monophosphate formed per minute per cell.

Results

The Effect of Glutamate and Kainate on Glial Proliferation

When neural retinal cells are seeded at a high density, clumps of cells form on the culture dish. After about 3 days in vitro, glial cells begin to migrate from these clumps and proliferate until a confluent layer of cells is formed across the surface of the dish. In this study, these cultures were treated with either NaCl, glutamate, or kainate. The results of these treatments on glial proliferation are described in Table 1. The number of glial cells that migrate from the clumps and proliferate in vitro is reduced greatly when cultures are treated with glutamate at 3 days in vitro. Treatment at 5 days or treatment with kainate failed to significantly affect glial proliferation. In contrast, kainate treatment at 3 days caused a consistent, but nonsignificant increase in glial number.

Figure 1 is a time lapse sequence of representative fields from two, 5-day, glial cultures treated at 3 days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time of treatment</th>
<th>Gliarial number ( \times 10^{3} )</th>
</tr>
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<tbody>
<tr>
<td>NaCl</td>
<td>3 days in vitro</td>
<td>106.4 ± 15.4</td>
</tr>
<tr>
<td>Glutamate</td>
<td>10.4 ± 4.6</td>
<td></td>
</tr>
<tr>
<td>Kainate</td>
<td>3 days in vitro</td>
<td>132.0 ± 15.6</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 days in vitro</td>
<td>107.6 ± 21.4</td>
</tr>
<tr>
<td>Glutamate</td>
<td>92.0 ± 22.8</td>
<td></td>
</tr>
<tr>
<td>Kainate</td>
<td>111.2 ± 14.2</td>
<td></td>
</tr>
</tbody>
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Table 1. Effects of glutamate and kainate on glial number at 6 days in vitro

With NaCl (A, B, C) or glutamate (1, 2, 3), respectively. This sequence of photographs clearly illustrates two effects of glutamate exposure on glial cells. First, there are fewer cells in the glutamate treated cultures at 5 days. Secondly, glial cells in glutamate treated cultures tend to have an elongated shape. These two responses to glutamate result in fewer areas of cell contact in glutamate treated cultures compared with controls. These changes remain at 6 days in vitro even with the removal of glutamate containing medium at 5 days.

Thymidine Kinase and Thymidylic Synthetase Activity

Thymidine kinase (EC 2.7.75) and thymidylic synthetase (EC 2.1.1.--) activities (Table 2) were determined in glial cultures treated at 3 days in vitro as described in Materials and Methods. Thymidine kinase activity was higher in both glutamate- and kainate-treated cultures compared with controls. Thymidylic synthetase activity was higher only in the glutamate-treated cultures.

Neuroblast Proliferation

By seeding neural retina from 6-day-old embryos at a relatively low density (500 cells/mm²) on a highly adhesive collagen, a purified neuroblast culture results. Figure 2 shows the in vitro development of a neuroblast culture. Figure 2A is a neuroblast culture at 1 day in vitro; notice the single dispersed cells. Figure 2B is a culture at 3 days; in addition to individual neurons, clusters of cells have formed. By 6 days in vitro (Fig. 2C), there are many clusters of cells, as well as individual neurons present. It is noteworthy that these cultures lack glial cells as seen in glia-containing cultures from 8-day chick embryos (Fig. 1).

[3H]-thymidine was added to neuroblast cultures at 2 days in vitro and they were processed autoradio-
Fig. 1. Time lapse photographs of 5-day glial cultures. A, B, C show a single field of a 5-day, in vitro, glial culture treated with 5 mM NaCl at 3 days in vitro (A = 0 hr; B = 2 hr; C = 7 hr). Photographs 1, 2, 3 show a single field of a 5-day glial culture treated with 5 mM glutamate at 3 days in vitro (1 = 0 hr; 2 = 2 hr; 3 = 7 hr). Magnification bar = 50 μm.

Graphically at 3 or 6 days in vitro. When processed at 3 days, it was observed that the clusters of cells were dividing cells, based on the incorporation of thymidine.

Table 2. The effects of glutamate and kainate on thymidine kinase and thymidylate synthetase activity in glial cultures at 6 days in vitro

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Thymidine kinase (fmol/min/cell)</th>
<th>Thymidylate synthetase (fmol/min/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>101 ± 20</td>
<td>259 ± 164</td>
</tr>
<tr>
<td>Glutamate</td>
<td>135 ± 17*</td>
<td>772 ± 164*</td>
</tr>
<tr>
<td>Kainate</td>
<td>154 ± 16*</td>
<td>260 ± 147</td>
</tr>
</tbody>
</table>

Thymidylate synthetase and thymidine kinase activity in glial cultures. At 3 days in vitro, glial cultures were treated as described in Table 1. At 6 days in vitro, glial cultures were assayed for enzyme activities in triplicate dishes from two different experiments (see text for details). Enzyme activity is expressed as femtomoles of thymidine monophosphate formed per minute per cell under the conditions of the test. Values are means ± SD.

* P < 0.05 by Student's t-test as compared with control values.

(Fig. 3A[1], 3A[2]). These clusters of cells, therefore, referred to as proliferation clusters. Within proliferation clusters both cells with and without neurites can be seen (Figs. 2B, 3A[1]). By 6 days in vitro, many thymidine labeled neurons can be seen, an example of which is shown in Figure 3B (1, 2).

The number of proliferation clusters was determined at 3 days in vitro in neuroblast cultures treated 24 hr earlier with either NaCl, glutamate, or kainate. Exposure to glutamate significantly reduced the number of proliferation clusters compared with controls; whereas, there was not a significant difference between controls and kainate treated cultures (Fig. 4).

Discussion

This study demonstrates that glutamate, but not its analog kainate, can inhibit proliferation of both glioblasts and neuroblasts in chick embryonic neural retina.
cultures. Previous work demonstrated that when glial cultures were exposed to glutamate at 1 and 3 days in vitro, the number of glia cells present at 6 days in vitro was reduced. In this study it was shown that glutamate exposure only at 3 days in vitro will produce this effect, while later exposure at 5 days failed to significantly reduce the number of glia present (Table 1). Three days in vitro is a significant stage in the development of glia in this system. At this time, glia that were slowly dividing in clumps of round cells begin to migrate out of these clumps and form a rapidly dividing layer of flat shaped cells. Glutamate has its effect during this period of morphologic change and onset of rapid growth.

Glutamate exposure at 3 days in vitro affects both the rate of proliferation and results in glia with a slightly elongated shape (Fig. 1). These responses remain 72 hr after glutamate exposure, even with the culture medium being changed to medium without glutamate, as it was done routinely at 5 days in vitro. This observation, along with previous results, demonstrates that these effects are long lasting. It is of interest to note that glutamate-treated cultures divide slower than controls, even though thymidine kinase and thymidylate synthetase activities are above normal levels (Tables 1, 2). Furthermore, glutamate is known to decrease thymidine phosphorylase (EC 2.4.2.4) activity, which is responsible for the degradation of thymidine to thymine. Therefore, thymidine, a key precursor in DNA synthesis, should be readily available to these cells. Thus, it appears that glutamate does not inhibit glia proliferation by decreasing DNA synthetic enzyme activity, but by some other mechanism. It is possible that the elongation of glial cells in vitro results in a physical reorganization less suitable for cell division.

The identity of glia and neurons in monolayer culture from the retina has been established. Neuroblast cultures from 6 day chick embryo neural retina have been designated as such because their morphology is similar to that of cultured neurons, their morphology and behavior is similar to other CNS neuroblast cultures, and they are tetanus toxin positive (unpublished observations).

Glutamate is also toxic to neuroblast cultures (Fig. 4). A 24-hr exposure to glutamate decreases the number of proliferation clusters by 50%. It is of interest to note that kainate does not decrease the number of proliferation clusters in neuroblast cultures. Kainate has been suggested to be a specific neurotoxin and does have a powerful toxic action on postmitotic neurons originating from retinal and other CNS tissue. However, neuroblasts do not appear to be affected by kainate in this manner. The neurotoxic action of kainate is complex and may require interaction with glutamate receptors or neurotransmitter systems. The concentration of glutamate used in this study (5 mM) was chosen because it was shown previously to kill 50% of the neurons grown in purified cultures, while the kain-
Fig. 3. [3H]-thymidine incorporation detected autoradiographically in neuroblast cultures. At 2 days in vitro, neuroblast cultures received [3H]-thymidine (1 μCi/ml) and were processed autoradiographically after 3 days (A) or 6 days (B) in vitro. A1, 2 or B1, 2 are darkfield and brightfield photographs of the same field. Open arrows indicate unlabeled neurons; closed arrows indicate labeled neurons.

Fig. 4. Effects of glutamate and kainate on neuroblast cultures. After 2 days in vitro, neuroblast cultures were treated with either NaCl (5 mM), glutamate (5 mM), or kainate (0.4 mM). At 3 days in vitro, the number of proliferation clusters per culture was determined. Values are means with standard deviations from duplicate dishes from three different experiments (see text for details). * P < 0.05 by Student’s t-test. P is a comparison between glutamate and control values.

Glutamate concentration that used (0.4 mM) was twice the amount required to kill such neurons. Although kainate can be 100 times more potent than glutamate as a neurotoxin in retina and other CNS tissue, it had no such effect on neuroblast or glioblast at the concentration tested in this study.

This study indicates that the cellular requirements for kainate toxicity may not exist in the retinal neuroblast. However, these requirements are present soon after these cells become postmitotic. Furthermore, kainate does not affect glia proliferation or morphology as does glutamate.

Glia sensitivity to glutamate occurs during a limited period of in vitro development. This study suggests that glutamate and kainate sensitivity change during development: glia losing sensitivity and neurons gaining sensitivity. This may reflect changes in receptors, membrane properties, or cell metabolism as these cells develop. It requires further study to determine the
mechanisms responsible for the observations seen in this and related studies.\textsuperscript{5,14} It appears that glutamate is a neuronal-glial toxin, while kainate only affects mature neurons (Fig. 4).\textsuperscript{14}

Understanding the complexity of glutamate toxicity is important because it has several characteristics in common with various CNS degenerative diseases,\textsuperscript{21,22} and is a widely used food additive.\textsuperscript{23} Because recent studies suggest glutamate sensitivity may change with maturation or particular cellular or biochemical interactions, our present knowledge makes it difficult to assess health risks or to make many of the necessary links between glutamate toxicity and various neurodegenerative diseases without further study.

**Key words:** glutamate, kainate, retina, cell proliferation, cultures

**References**

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