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THE ROLE OF SPECIFIC IONS IN GLUTAMATE NEUROTOXICITY

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When the chick embryo retina is incubated in balanced salt solution containing glutamate (Glu) in 1 mM concentration, a neurodegenerative reaction occurs within 30 min. Here we report that the neurotoxic action of Glu on retinal neurons is dependent on the presence of Na^+ and Cl^-, but not Ca^{2+}, in the incubation medium. Also, we report that depolarizing concentrations of K^+ can induce a severe cytotoxic reaction in chick retina which, like the depolarization-linked neurotoxicity of Glu, is a Cl^--dependent phenomenon.

In recent decades, the common amino acid, glutamate (Glu), has become recognized as both a neurotoxin [13] and the leading neurotransmitter candidate at the majority of excitatory synapses in the mammalian central nervous system (CNS) [30]. Recent evidence [15, 17, 23, 27, 28], potentially implicating Glu neurotoxicity in several human neurodegenerative diseases, underscores the need for a more complete understanding of this type of neurotoxic process. Accumulating evidence [4, 12, 13, 15, 16, 23, 28, 30] supports the excitotoxic hypothesis [13, 16] that Glu neurotoxicity is mediated through Glu excitatory receptors and entails sustained depolarization of postsynaptic dendrosomal membranes, increased membrane permeability and impaired ion homeostasis. Although definitive evidence implicating specific ions has been lacking, several authors have proposed that excessive Ca^{2+} influx into the postsynaptic neuron may be responsible for this type of cell death [2, 4, 9]. Here we have used the in vitro chick embryo retina to study the role of various ions in Glu neurotoxicity.

We chose the chick embryo retina for these experiments because it is known to be sensitive to Glu neurotoxicity [22], and it can be studied in vitro as an intact sheet of organized tissue which is not disrupted or significantly altered from its in vivo state when transferred from the eye to the incubation chamber. However, before using this in vitro system to study mechanisms of Glu excitotoxicity (an in vivo phenomenon), we wanted to be sure that the same mechanisms are operative in the in vivo and in vitro situations. Toward this end, using a modified version of Reif-Lehrer et al.'s methods [22], we determined that incubation of 15-day-old chick embryo retina in balanced salt solution (BSS) containing an appropriate concentration of Glu (1 mM),

or its more potent excitotoxic analogues such as N-methylaspartate (NMA; 0.2 mM) or kainic acid (KA; 0.025 mM), results in a fully developed lesion within 30 min; that each compound is toxic in direct proportion to its known excitatory potency (e.g. KA > NMA > Glu); that agents known to antagonize excitatory amino acid (EAA) receptors [8, 19, 25, 30] effectively block the toxic reaction (e.g. α-aminophosphonopentanoate and Mg^{2+} block NMA; kynurenic acid blocks NMA, KA and Glu); and that the cytopathology induced in vitro is ultrastructurally identical to that described in vivo [12, 14, 16]. Thus, the essential features of Glu excitotoxicity, as defined by in vivo studies, are reproduced in the in vitro retina.

For ion substitution experiments, the eyes were removed from 15-day-old chick embryos and cut through the optical axis into quadrants after extrusion of the lens through a slit in the cornea. Retinal quadrants were gently separated from the pigment epithelium and incubated for 30 min at 37°C with Glu (1.0 mM) in standard BSS or in BSS variously modified to alter the ionic makeup (Table I). The medium was brought to pH 7.3 before incubation by a stream of 95% O_2–5% CO_2, and all incubations were terminated at 30 min. For histopathology evaluation, the retinal quadrants were initially fixed in a phosphate-buffered solution of 1.5% glutaraldehyde–1% paraformaldehyde, additionally fixed in OsO_4 and embedded in Araldite so

TABLE I

IONIC COMPOSTION OF INCUBATION MEDIA (mM)

	Standard	*Low Ca^{2+}*	*No Na^+*	*Low Cl^-*	*High K^+*	*High K^+, low Cl^-*
Ca^{2+}	0.5	–	0.5	0.5	0.5	0.5
K^+	5.0	5.0	5.0	5.0	90.0	90.0
Mg^{2+}	4.5	5.0	4.5	4. 5	4.5	4.5
Na^+	135.0	135.0	–	135.0	50.0	50.0
Benzoylcholine	–	–	135.0	–	–	–
Cl^-	150.0	150.0	150.0	1.0	150.0	10.0
SO_4^{2-}	–	–	–	7.0	–	70.0
Isethionate$^-$	–	–	–	135.0	–	–
Sucrose[a]	–	–	–	7.0	–	70.0
Glucose	5.6	5.6	5.6	5.6	5.6	5.6
Na/KPO_4^{3+}–HCO_3^- buffer	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0

[a]Added to maintain constant calculated osmolarity.

that sections could be cut for either light or electron microscopy as previously described [14].

Fig. 1a shows the normal appearance of the retina after a 30-min incubation in BSS containing no Glu. After a 30-min incubation with Glu (1 mM), striking acute edematous degeneration of neurons in the inner half of the retina was seen (Fig. 1b), and this reaction was totally prevented if the EAA antagonist, kynurenic acid, was included at 2 mM concentration.

When benzoylcholine, a cation to which the plasma membrane is impermeable, was substituted for Na^+ so that the incubation medium contained very little Na^+ (only < 1 mM contributed by the buffer), a 30-min incubation with Glu did not result in any toxic reaction (fig. 1d). Substituting the membrane-impermeable anion, isethionate, for Cl^- had the same effect, i.e. the toxic action of Glu was prevented (Fig. 1e). Eliminating Ca^{2+} from the medium or eliminating Ca^{2+} and adding ethyleneglycoltetraacetic acid (EGTA) (2 mM) to chelate residual Ca^{2+} failed to prevent or diminish the toxic action of Glu (Fig. 1f).

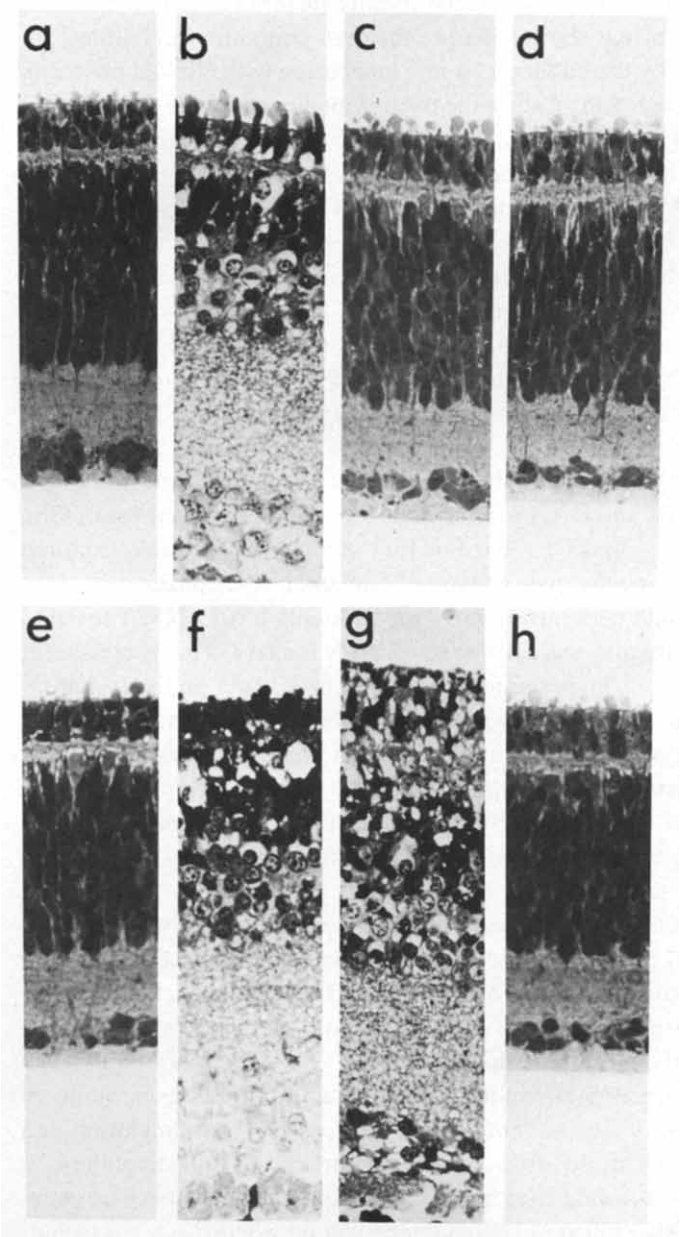
When a portion of the Na^+ was replaced by K^+, resulting in an abnormally high (90 mM) extracellular K^+ concentration (balanced by Cl^- as the major anion), and no Glu was added, the retina exhibited a dramatic cytotoxic response after 30 min incubation, resembling the edematous reaction to Glu (Fig. 1g). This toxic response to high K^+ was Cl^--dependent in that it was completely prevented when isethionate was substituted for Cl^- (Fig. 1h).

In other experiments [18], we have established that the retinotoxic activities of the potent Glu analogues, NMA and KA, have the same ionic properties as those of Glu, i.e. the toxic process is Na^+- and Cl^-- but not Ca^{2+}-dependent. We also explored the role of Mg^{2+} in modulating the toxic activity of Glu and its excitotoxic analogues and found that Mg^{2+}, in high concentration (20 mM), totally blocks NMA toxicity, partially blocks Glu toxicity and has little effect on KA toxicity. This is consistent with the findings of Evans et al. [6] pertaining to the effects of Mg^{2+} on the excitatory actions of these agents. The BSS used in these experiments was intentionally prepared with a high Mg^{2+} concentration in an effort to diminish synaptic release of endogenous excitotoxic transmitters (Glu and Asp), since such release might confound interpretation of our data. The 4.5–5.0 mM concentration of Mg^{2+} used, however, was not high enough to substantially interfere with the excitotoxic activity of exogenously applied Glu.

The hypothesis that acute Glu-induced neuronal necrosis is a Ca^{2+}-mediated process arises from several lines of evidence. The depolarizing action of some EAAs reportedly entails an inward conductance of Ca^{2+} [5], and exposure of rat cerebrocortical slices to excitotoxins in vitro results in a net intracellular uptake of Ca^{2+} [2]. Moreover, we [21] and others [9] have shown that Ca^{2+} selectively accumulates in neural elements undergoing excitotoxin-induced degeneration; however, none of these observations establishes a causal connection between Ca^{2+} accumulation and this type of cell death. A key study often cited in support of the Ca^{2+} hypothesis is that of Schaane et al. [26], showing that hepatocytes exposed to a variety of membrane toxins in vitro are either killed or spared depending on whether Ca^{2+} is includ-

ed in or excluded from the incubation medium. It should be noted, however, that 3 separate laboratories have recently reported failure to confirm Schaane et al. [1, 7, 29]. Here we have used an in vitro approach to directly test the role of Ca^{2+} in Glu neurotoxicity and have shown that this toxic process is dependent on Na^+ and Cl^- but not Ca^{2+}.

Our data agree with the recent observations of Rothman [24] that excitotoxin-induced necrosis of cultured dissociated hippocampal neurons is dependent on Na^+



and Cl^- but not Ca^{2+}. The similar results of the two studies, despite use of dissimilar in vitro preparations pertaining to neurons from different regions of the CNS, suggest that similar mechanisms may underlie excitotoxic phenomena in many if not all regions of the CNS. Our findings both complement and strengthen those of Rothman since there is less uncertainty in extrapolating to the in vivo CNS from our undisrupted retinal preparation than from cultured dissociated neurons. Also, we were able to use EGTA to remove residual Ca^{2+}, a manipulation Rothman was unable to perform due to the toxic action of chelating agents on cultured neurons. Together, the two sets of findings strongly support a hypothesis recently advanced by Miller and Slaughter [11] that passive Cl^- influx may play an important role in Glu neurotoxicity.

Very recently Choi reported [3] that exposure of cultured cerebrocortical neurons to Glu for only a few minutes, then removing Glu from the medium, results in delayed cell death in 12–24 h, provided that Ca^{2+} is present when the culture is initially exposed to Glu. Choi's findings do not contradict ours; rather they suggest that there may be two mechanisms by which excitotoxins can induce neuronal death, one being the traditionally recognized acute, fulminating, degenerative reaction studied by us and by Rothman, which is not Ca^{2+}-dependent, and the other being a slowly evolving neurotoxic process which may be Ca^{2+}-dependent. Recent evidence suggests that neuronal degeneration associated with common neurological disorders such as epilepsy [15] and anoxia (stroke) [23, 28], as well as less common conditions [17, 20, 27], may be mediated by excessive stimulation of Glu excitatory receptors. Future studies should be aimed at obtaining a detailed understanding of all possible mechanisms by which excitotoxins can destroy central neurons as this may facilitate the development of rational approaches for prevention or therapeutic management of Glu-linked neurodegenerative disorders.

The primary mechanism by which removal of Na^+ prevented toxicity in these experiments presumably relates to the role of Na^+ in the depolarization process, i.e.

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Fig. 1. The representative appearance of the 15-day-old chick embryo retina under various incubation conditions. All retinal quadrants were incubated for 30 min at pH 7.3, fixed in 1% paraformaldehyde–1.5% glutaraldehyde, postfixed in 1% OsO_4 and embedded in Araldite. The 1 μm sections illustrated here were cut with glass knives on a Porter Blum Mt-2 ultratome and were stained with 1:1 methylene blue and Azure II. In each experiment 3–4 retinal quadrants were incubated per experimental condition, and each condition was studied in at least 3 separate experiments. For a given condition the results did not vary, i.e. all sections either displayed a full lesion or all appeared normal. Incubation media were as follows: (a) standard BSS; (b) standard BSS + 1 mM Glu; (c) standard BSS + 1 mM Glu + 2 mM kynurenic acid; (d) low Na^+ medium + 1 mM Glu; (e) low Cl^- medium + 1 mM Glu; (f) no Ca^{2+} + 2 mM EGTA + 1 mM Glu; (g) high K^+ with Cl^-; (h) high K^+ without Cl^-. Note that the toxic action of Glu (b) affects many neurons and neuronal processes in the inner retina and causes the retina to appear massively swollen. A lesion having the same pattern and appearing at least as severe is seen in the retina incubated in Glu + EGTA and no Ca^{2+} (f) or Glu, no Ca^{2+} and no EGTA (not shown). The reaction to Glu does not occur if either Na^+ (d) or Cl^- (e) is removed from the medium. Incubating the retina in medium containing 90 mM K^+ with Cl^- results in an acute edematous reaction affecting neurons in nearly every retinal layer (g). Removing Cl^- from the medium prevents K^+ from inducing this reaction (h). a–h × 200.

an inward conductance of Na^+ is assumed to be the ionic event underlying Glu-induced membrane depolarization. Replacing Na^+ with an impermeant cation may have prevented Glu from inducing membrane depolarization. This interpretation can [10] and should be explored by electrophysiological methods. It is noteworthy that depolarizing concentrations of K^+ induced a cytotoxic reaction which, like Glu neurotoxicity, is a Cl^--dependent phenomenon. Thus, although cation-mediated membrane depolarization is the initiating event leading to either Glu-induced or K^+-induced neuronal degeneration, neither of these depolarizing mechanisms has toxic consequences unless an abundance of Cl^- is present in the extracellular compartment.

In summary, our findings indicate that acute, Glu-induced, neuronal necrosis results from a continuous, receptor-mediated, membrane depolarization which, in addition to initial Na^+ conductance changes, entails an unregulated passive influx of Cl^- balanced ionically by further Na^+ influx and osmotically by large increases in cell water. Ca^{2+} influx apparently does not play a critical role in this acute neurotoxic process, but the possible role of Ca^{2+} in a more delayed form of excitotoxin-induced neuronal death [3] warrants investigation.

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1 Acosta, D. and Sorensen, E.M.B., Role of calcium in cytotoxic injury of cultured hepatocytes, Ann. N.Y. Acad. Sci., 407 (1983) 78–92.
2 Berdichevsky, E., Riveros, S., Sanchez-Armass, S. and Orrego, F., Kainate, N-methylaspartate and other excitatory amino acids increase calcium influx into rat brain cortex cells in vitro, Neurosci. Lett., 36 (1983) 75–80.
3 Choi, D.W., Glutamate neurotoxicity in cortical cell culture is calcium-dependent, Neurosci. Lett., 58 (1985) 293–297.
4 Coyle, J.T., Neurotoxic action of kainic acid, J. Neurochem., 41 (1983) 1–11.
5 Dingledine, R., N-methylaspartate activates voltage-dependent calcium conductance in rat hippocampal pyramidal cell, J. Physiol. (London), 343 (1983) 385–405.
6 Evans, R.H., Francis, A.A. and Watkins, J.C., Selective antagonism by Mg^{2+} of amino acid-induced depolarization of spinal neurones, Experienta, 33 (1977) 489–491.
7 Fariss, M.W., Pascoe, G.A. and Reed, D.J., Vitamin E reversal of the effect of extracellular calcium on chemically induced toxicity in hepatocytes, Science, 227 (1985) 751–753.
8 Foster, A.C., Vezzani, A., French, E.D. and Schwarcz, R., Kynurenic acid blocks neurotoxicity and seizures induced in rats by the related brain metabolite quinolinic acid, Neurosci. Lett., 48 (1984) 273–278.
9 Jancsó, G., Karcsu, S., Kiraly, E., Szebeni, A., Toth, L., Bacsy, E., Joó, F.and Parducz, A., Neurotoxin induced nerve cell degeneration: possible involvement of calcium, Brain Res., 295 (1984) 211–216.
10 MacDonald, J.F., Substitution of extracellular sodium ions blocks the voltage-dependent decrease of input conductance evoked by L-aspartate, Can. J. Physiol. Pharmacol., 62 (1984) 109–115.
11 Miller, R.F. and Slaughter, M.M., Excitatory amino acid receptors in the vertebrate retina. In W.W. Morgan (Ed.), Retinal Transmitters and Modulators: Models for the Brain, Vol. II, CRC Press, 1985, pp. 123–160.
12 Olney, J.W., Glutamate-induced neuronal necrosis in the infant mouse hypothalamus. An electron microscopic study, J. Neuropathol. Exp. Neurol., 30 (1971) 75–90.
13 Olney, J.W., Neurotoxicity of excitatory amino acids. In E. McGeer, J. Olney and P. McGeer (Eds.), Kainic Acid as a Tool in Neurobiology, Raven Press, New York, 1978, pp. 95–122.

14 Olney, J.W., Glutamate-induced retinal degeneration in neonatal mice. Electron microscopy of the acutely evolving lesion, J. Neuropathol. Exp. Neurol., 28 (1969) 455–474.
15 Olney, J.W., Collins, R.C. and Sloviter, R.S., Excitotoxic mechanisms of epileptic brain damage. In A.V. Delgado-Escueta, A.A. Ward and D.M. Woodbury (Eds.), Basic Mechanisms of Epilepsy, Raven Press, New York, in press.
16 Olney, J.W., Ho, O.L. and Rhee, V., Cytotoxic effects of acidic and sulphur containing amino acids on the infant mouse central nervous system, Exp. Brain Res., 14 (1971) 61–76.
17 Olney, J.W., Misra, C.H. and de Gubareff, T., Cysteine-S-sulfate: brain damaging metabolite in sulfite oxidase deficiency, J. Neuropathol. Exp. Neurol., 34 (1975) 167–176.
18 Olney, J.W., Price, M.T., Samson, L. and Labruyere, J., The ionic basis of excitotoxin-induced neuronal necrosis, Soc. Neurosci. Abstr., 10 (1984) 24.
19 Perkins, M.N. and Stone, T.W., An iontophoretic investigation of the actions of convulsant kynurenines and their interaction with the endogenous excitant quinolinic acid, Brain Res., 247 (1982) 184–187.
20 Plaitakis, A., Berl, S. and Yahr, M., Abnormal glutamate metabolism in an adult-onset degenerative neurological disorder, Science, 216 (1982) 193–196.
21 Price, M.T., Olney, J.W., Samson, L. and Labruyere, J., Calcium influx accompanies but does not cause excitotoxin-induced neuronal necrosis in retina, Brain Res. Bull., (1985) 369–376.
22 Reif-Lehrer, L., Bergenthal, J. and Hanninen, L., Effects of monosodium glutamate on chick embryo retina in culture, Invest. Ophthalmol., 14 (1975) 114–124.
23 Rothman, S.M., Synaptic release of excitatory amino acid neurotransmitter mediates anoxic neuronal death, J. Neurosci., 4 (1984) 1884–1891.
24 Rothman, S.M., Excitatory amino acid neurotoxicity is produced by passive choride influx, J. Neurosci., 6 (1985) 1483–1489.
25 Samson, L., Olney., J.W., Price. M.T. and Labruyere, J., Kynurenate protects against excitotoxin-induced neuronal necrosis in chick retina, Soc. Neurosci. Abstr., 10 (1984) 24.
26 Schaane, F.A.X., Kane, A.B., Young, E.E. and Farber, J.L., Calcium dependence of toxic cell death: a final common pathway, Science, 206 (1979) 700–702.
27 Shoulson, I., Huntington's disease: anti-neurotoxic therapeutic strategies. In K. Fuxe, P. Roberts and R. Schwarcz (Eds.), Excitotoxins, Macmillan, London, 1983, pp. 343–353.
28 Simon, R.P., Swan, J.H., Griffiths, T. and Meldrum, B.S., Blockade of N-methyl-*D*-aspartate receptors may protect against ischemic damage in the brain, Science, 226 (1984) 850–852.
29 Smith, M.T., Thor, H. and Orrenius, S., Toxic injury to isolated hepatocytes is not dependent on extracellular calcium, Science, 213 (1981) 1257–1259.
30 Watkins, J.C., Excitatory amino acids. In E.G. McGeer, J. Olney and P. McGeer (Eds.), Kainic Acid as a Tool in Neurobiology, Raven Press, New York, 1978, pp. 37–69.