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Ca^{2+} influx through glutamate receptor-associated channels in retina cells correlates with neuronal cell death

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Abstract

We studied the effect of glutamate, *N*-methyl-D-aspartate (NMDA), kainate or K^+ depolarization, on neurotoxicity in cultured chick retinal cells, under conditions in which we could discriminate between Ca^{2+} entering through ionotropic glutamate receptors and voltage-sensitive Ca^{2+} channels (VSCCs). When neurons were challenged with NMDA, kainate or glutamate, in Na^+-containing medium, a decrease in cell survival was observed, whereas K^+ depolarization did not affect the viability of the cells. The Mg^{2+} ion completely prevented the toxic effect mediated by the NMDA receptor, and had a small but significant protective effect at the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate (AMPA/kainate) receptor-induced cell death. We observed that, in a Na^+-free *N*-methyl-D-glucamine (NMG) medium, to avoid the activation of VSCCs indirectly by the glutamate receptor agonists, stimulation of the glutamate receptors causes Ca^{2+} influx only through NMDA and AMPA/kainate receptor-associated channels, and that Ca^{2+} entry correlates well with subsequent cell death. These results show that the activation of NMDA or AMPA/kainate receptors can cause excitotoxicity in retinal neurons by mechanisms not involving Na^+ influx, but rather depending on the permeation of Ca^{2+} through glutamate receptor-associated channels. For small Ca^{2+} loads the entry of Ca^{2+} through the NMDA receptor-associated channel was more efficient in triggering cell death than the influx of Ca^{2+} through the AMPA/kainate receptor.

Keywords: Retinal cell; Glutamate receptor; Neurotoxicity; Ca^{2+}; MK-801; CNQX (6-cyano-7-nitroquinoxaline-2,3-dione)

1. Introduction

Since the pioneer observation of Lucas and Newhouse (1957) of retinotoxic properties of glutamate, accumulating evidence indicates that the increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in the neuronal cells leads to cell death (Choi and Rothman, 1990; Meldrum and Garthwaite, 1990; Choi and Hartley, 1993). Although most excitatory synaptic transmission in the nervous system is probably mediated by glutamate, substantial evidence now suggests that glutamate neurotoxicity contributes to the pathogenesis of central neuronal cells induced by acute insults. In fact, conditions that lead to endogenous glutamate release increase the $[Ca^{2+}]_i$, which activates a large number of Ca^{2+}-dependent processes and causes neuronal damage, both of which promote further increase in glutamate release (Choi, 1988; Louzada-Junior et al., 1992; Osborne and Herrera, 1994) and a decreased glutamate uptake (Yu et al., 1987). Thus, the excitotoxic insult is amplified by the continued stimulation of glutamate receptors which promotes the spread of excitotoxicity to other neurons. The mechanisms underlying glutamate excitotoxicity are not fully understood, but the loss of Ca^{2+} homeostasis due to the activation of glutamate receptors probably plays an important role (Choi and Hartley, 1993).

The organization of the retina is well-understood, consisting of essentially five types of neurons and one type of glial cells, and glutamate is a retinal neurotransmitter used by photoreceptors, bipolar, and ganglion cells (Barnstable, 1993). In the inner plexiform layer of the retina, the bipolar cells, which are glutamatergic, provide the excitatory input to the amacrine and ganglion cells (Barnstable, 1993), and amacrine cells have been shown to respond to both *N*-methyl-D-aspartate (NMDA) and non-NMDA receptor agonists (Hofmann and Möckel, 1991; Ferreira et al., 1994). NMDA receptors form ion channels highly permeable to Ca^{2+}, whereas non-NMDA ionotropic recep-

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tors, activated by kainate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), have traditionally been thought to be Ca^{2+}-impermeable (Mayer and Westbrook, 1987; Asher and Nowak, 1988). However, it is now clear that several types of AMPA/kainate receptors may also be Ca^{2+}-permeable, these receptors can be important sources of Ca^{2+} influx in some types of neurons and glia (Ogura et al., 1990; Iino et al., 1990; Glaum et al., 1990; Gilbertson et al., 1991; Pruss et al., 1991; Burnashev et al., 1992), and may also be important mediators of excitotoxicity (Sheardown et al., 1990; Brorson et al., 1994). The NMDA receptor-associated Ca^{2+} channels have been regarded as the predominant route of retinal glutamate neurotoxicity (Facci et al., 1990; Louzada-Junior et al., 1992; Zeevalk and Nicklas, 1992a; Abu El-Asrar et al., 1992; Kashii et al., 1994), and may be the primary route of Ca^{2+} entry during acute cerebral ischaemia (Choi, 1988) and retinal metabolic stress (Zeevalk and Nicklas, 1991, 1992b). However, the non-NMDA receptors also probably play an important role in Ca^{2+}-mediated death of chick retinal cells, since Ca^{2+}-permeable AMPA/kainate receptors exist in the retina (Gilbertson et al., 1991; Rörig and Grantyn, 1993; Carvalho et al., 1995; Duarte et al., 1996).

Although the Ca^{2+} overload may contribute to a series of membrane, cytoplasmic, and nuclear events that result in neurotoxicity, Ca^{2+} alone may not be sufficient to trigger this series of events. Elevation of the $[Ca^{2+}]_i$, to the same level as that attained by glutamate, but with cyanide or by K^+ depolarization to allow Ca^{2+} influx through voltage-gated calcium channels, causes less permanent neuronal damage than does glutamate itself (Michaels and Rothman, 1990; Dubinsky and Rothman, 1991), suggesting that the location or the mode of Ca^{2+} entry may be critical (Tymianski et al., 1993). Thus, it seems that excitotoxicity mediated by pathways coupled to receptor-associated Ca^{2+} channels might be particularly important to the vulnerability of retinal cells. In chick retinal cells, the agonists of the ionotropic glutamate receptors, NMDA, kainate and AMPA increase the $[Ca^{2+}]_i$ through a composite effect, comprising Ca^{2+} permeating the receptor-associated channels and the Ca^{2+} entering through the voltage-sensitive calcium channels (VSCCs) (Duarte et al., 1996). Since the Ca^{2+} signals provided by activation of the glutamate receptors are diverse, they are likely to have distinct effects on neurotoxicity in these cells.

In the present work, we determined the $^{45}Ca^{2+}$ influx through the glutamate receptor-associated channels in the absence of Ca^{2+} entry through the VSCCs, and cell death was then measured in identical conditions of exposure to each agonist. We found that glutamate receptor activation causes excitotoxicity, and that the Ca^{2+} influx through the receptor-associated channels seems to be the most important route of Ca^{2+}-induced cell death in chick retinal cells in culture, when compared with the influx of Ca^{2+} via the VSCCs opened by K^+ depolarization.

2. Materials and methods

2.1. Cell culture

Primary cultures of chick retinal neurons were prepared from 8-day-old chick (White Leghorn) embryos, as described previously (Duarte et al., 1992). Retinas were dissected free from other ocular tissues, and the cells were incubated for 15 min at 37°C in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (CMF), supplemented with 0.1% trypsin. The digested tissue was centrifuged at $140 \times g_{av}$, for 1 min, and the pellet was resuspended in basal medium of Eagle (Earle's salts; 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). After mechanical dissociation, cells were seeded at 0.45×10^6 cells/cm^2 in 12-well plates for $^{45}Ca^{2+}$ uptake and neuronal injury studies, or in 35-mm plastic Petri dishes for [^{3}H]D-aspartate release measurements. Cells were cultured for 5 days in BME at 37°C, in a humidified atmosphere of 95% air/5% CO_2. A preparation similar to the one used in this work was shown to be highly enriched in amacrine-like neurons (Huba and Hofmann, 1990). However, all the preparations contained a significant number of neurons resembling bipolar cells.

2.2. Exposure to excitatory amino acids

After a washing step in Na^+ or *N*-methyl-D-glucamine (NMG) buffers, as described in figure captions, cell cultures were exposed at 37°C to NMDA, kainate or glutamate, with or without (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate (MK-801), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) or glycine, in Na^+ or NMG buffers with the following composition (in mM): 132 NaCl or NMG, 4 KCl, 6 glucose, 10 HEPES-Tris pH 7.4, in the presence or in the absence of 1.4 mM $MgCl_2$ or 1 mM $CaCl_2$. Where indicated in figure captions, $MgCl_2$ was omitted from the media during the exposure to the agonists in order to potentiate the contribution of NMDA receptors (Nowak et al., 1984) to the observed neurotoxicity. When MK-801, CNQX or glycine were tested, a pre-incubation of 1 min was performed prior to stimulation with the agonists.

2.3. $^{45}Ca^{2+}$ uptake studies

The cells were washed with Na^+ or NMG medium, and then incubated with $^{45}CaCl_2$ (0.7 μCi/μmol) in the presence of glutamate, NMDA, kainate or KCl (50 mM), and with or without MK-801, CNQX or glycine. After the desired time, as indicated in the figure captions, the drug containing solutions were quickly washed out, and the Ca^{2+}-uptake was stopped by adding to the cells an ice-cold Ca^{2+}-free Na^+ or NMG medium with 1 mM $LaCl_3$. The

cells were further washed with a Ca^{2+}-free Na^+ or NMG medium supplemented with 1 mM EGTA (Duarte et al., 1993a). Cells were lysed by addition of 1 ml of H_2O, and the radioactivity was measured using Universol scintillation cocktail (ICN) and a Packard 2000 spectrometer provided with dpm correction. All $^{45}Ca^{2+}$ uptake studies were made in duplicates.

2.4. Assessment of neuronal injury

Cultures were washed and then incubated with the agonists in the presence or in the absence of the antagonists of the glutamate receptors, as indicated. After the desired time, as indicated in the figure captions, the stimulating media were removed and the cells washed in the basal medium in order to remove the stimulating agent. Cultures were immediately assessed for cell survival or alternatively the cells returned to the incubation chamber in culture medium lacking serum for 18–20 h.

Assessment of neuronal injury was made by a colorimetric assay for cell survival using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl (MTT) and a modification of the method of Mosmann (1983). MTT (0.5 mg/ml) in BME medium without serum was added to the cultures and incubated for 3 h at 37°C in the incubation chamber. MTT, when taken up by live cells with active mitochondria, is converted from yellow to a water-insoluble blue-coloured product. The precipitated dye was dissolved in 1.5 ml 0.04 M HCl in isopropanol and colorimetrically (absorbance at 570 nm) quantitated. All experiments were made in triplicates.

2.5. [^{3}H]D-Aspartate release

Cells were loaded with 40 nM [^{3}H]D-aspartate (1 μCi/ml) for 30 min at 37°C in Ca^{2+}-containing Mg^{2+}-free Na^+ medium. Cells were then washed in order to remove the radioactive medium, and then were perfused during 15 min in Na^+ medium using the superfusion method previously described (Drejer et al., 1987), modified by Duarte et al. (1992). The release of [^{3}H]D-aspartate was determined in Na^+ medium for 4 min, and thereafter the cells were perfused with NMG medium for 20 min. The samples were collected every minute, and at the end of the experiment, the cells were lysed with 0.2 M HCl in order to determine the [^{3}H]D-aspartate that remained inside the cells. The radioactivity was measured using Universol scintillation cocktail (ICN) and a Packard 2000 spectrometer provided with dpm correction.

2.6. Other methods

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.

3. Results

3.1. Delayed neuronal death induced by glutamate receptor activation

Chick retinal cells in culture exposed for 2 h to 100 μM NMDA, kainate or glutamate in Ca^{2+}-containing Mg^{2+}-free Na^+ medium showed an increased death with time, after the initial exposure period (Table 1). After 15 min of exposure to the agonists, neuronal swelling, the appearance of dark granules in the somata, and dendrite blebbs were observed by phase contrast microscopy (not shown), but even after 2 h exposure only a slight decrease in cell survival, as measured by the MTT assay, occurred (NMDA 93.3 ± 4.0%; kainate 79.5 ± 4.5%; glutamate 85.4 ± 5.2%). However, when the toxic effects were measured 18–20 h later, after the 2 h of exposure, a significant decrease in cell survival was observed (Table 1). The results show that, in a Na^+-containing medium, kainate (51.7 ± 3.1%) was more potent than NMDA (72.6 ± 2.1%), and glutamate (62.2 ± 3.4%) had an intermediate efficacy in inducing cell death. Based on this observation, we subsequently determined the effect of glutamate receptor agonists on cell viability 18–20 h after the 2 h exposure to the agonists.

3.2. Glutamate receptor agonists-induced cell death: effect of glutamate receptor antagonists and extracellular Mg^{2+}

Glutamate increased cell death, either in the presence (74.0 ± 4.8%) or in the absence (62.2 ± 3.4%) of Mg^{2+}. In both conditions, the cell death was almost completely prevented by the NMDA receptor antagonist MK-801 (10 μM) (Wong et al., 1986) as shown in Fig. 1A, suggesting that glutamate-induced cell death was mostly mediated through the NMDA receptor. In the presence of MK-801 plus the AMPA/kainate receptor antagonist CNQX (20 μM) (Watkins et al., 1990), cell death was completely

Table 1
Effect of time interval between the end of exposure to agonists and the measurement of cell damage

	2 h exposure to agonists	2 h exposure to agonists + 18–20 h in culture medium
Control	100.0 ± 0.03% (5)	100.8 ± 0.7% (11)
NMDA (100 μM)	93.3 ± 4.0% (4)	72.6 ± 2.1% (8) [a]
Kainate (100 μM)	79.5 ± 4.5% (5)	51.7 ± 3.1% (11) [a]
Glutamate (100 μM)	85.4 ± 5.2% (3)	62.2 ± 3.4% (8) [a]

Retinal cells were treated for 2 h with NMDA, kainate or glutamate in Ca^{2+}-containing Mg^{2+}-free Na^+ medium. Cultures were immediately assessed for cell survival using the MTT method, as described in section 2, or, alternatively, they were returned to the incubator in culture medium lacking serum. In this case, cell survival was assessed 18–20 h after the stimulation with the agonists. The values in parentheses indicate the number of experiments made in triplicates in different cultures. Data are presented as mean ± S.E.M. values. [a] Significantly lower ($P < 0.05$) when compared with the results of the first column.

prevented, suggesting a small but significant involvement of the non-NMDA receptors in the glutamate response.

In order to further characterize the glutamate receptors involved in chick retinal cell death, we studied the response to NMDA and kainate, and the protective effect of glutamate receptor antagonists. We did not observe any reduction in cell survival when the cells were exposed to NMDA in the presence of physiological concentrations of Mg^{2+} (Nowak et al., 1984) (Fig. 1B), but omission of Mg^{2+} during the stimulation of the NMDA receptor decreased the cell survival to 72.6 ± 2.1%. As expected, MK-801 completely prevented cell death induced by NMDA in the absence of Mg^{2+}.

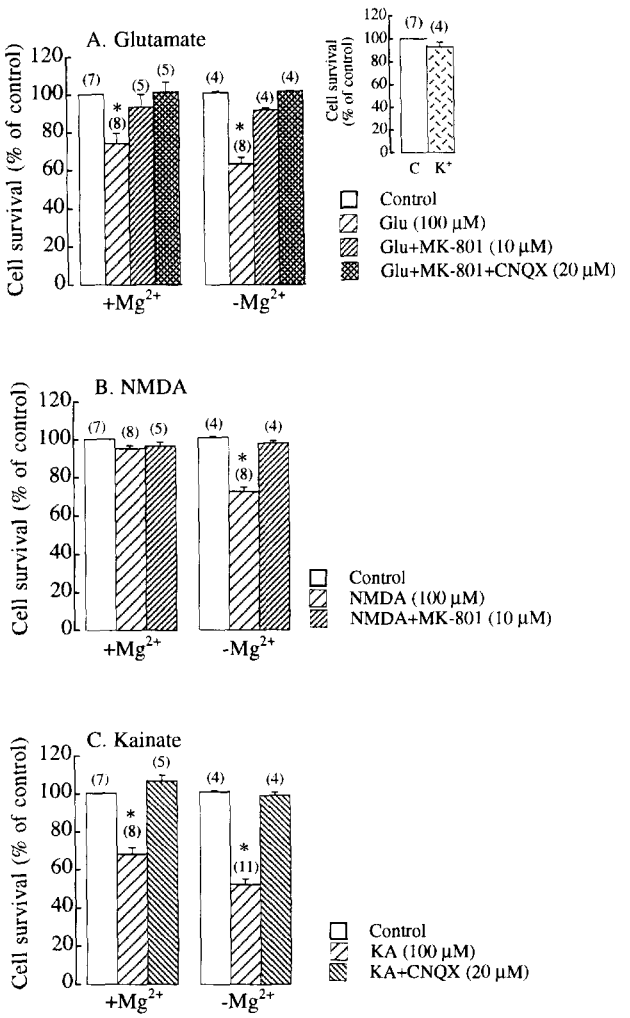

Fig. 1. Survival of retinal cells stimulated with glutamate, NMDA or kainate in Ca^{2+}-containing Na^+ medium, in the presence or in the absence of Mg^{2+}. Cells were exposed to glutamate (A), NMDA (B) or kainate (C), with or without the glutamate receptor antagonists MK-801 (10 μM) and CNQX (20 μM), or K^+ (50 mM) (inset of A), during 2 h. After a wash step in Na^+ medium without drugs, the cells returned to incubator chamber for 18–20 h, in serum-free culture medium. Cell survival was assessed as described in the methods and bars represent mean ± S.E.M. values of the indicated number of experiments, performed in triplicates in independent cultures. * Significantly different from each control value ($P < 0.05$).

Table 2
$^{45}Ca^{2+}$ influx induced by glutamate or K^+ depolarization, with or without the glutamate receptor antagonists MK-801 and CNQX

	dpm/2 × 10^6 cells
Control	715.0 ± 87.0 (3)
Glutamate	1534.3 ± 184.8 (3) [a]
Glutamate + MK-801 + CNQX	717.0 ± 108.1 (3)
50 mM KCl + MK-801 + CNQX	2675.0 ± 559.5 (3) [a]

Retinal cells were treated for 2 h with 100 μM glutamate or 50 mM KCl in the presence or in the absence of MK-801 (10 μM) and CNQX (20 μM) in Ca^{2+}, Mg^{2+} containing Na^+ medium, in the presence of $^{45}Ca^{2+}$ (0.7 μCi/μmol). The values in parentheses indicate the number of experiments made in triplicates in different cultures. Data are presented as mean ± S.E.M values expressed in dpm. [a] Significantly higher ($P < 0.05$) when compared with control.

The agonist of the non-NMDA glutamate receptors, kainate, also reduced cell viability, in the presence or in the absence of Mg^{2+}, and this effect was antagonized by CNQX (Fig. 1C). The Mg^{2+} ion seemed to have a small but significant ($P < 0.05$) protective effect on the response to kainate exposure (Fig. 1C). In fact, in the presence of Mg^{2+} the neurotoxicity induced by kainate was reduced by ~ 14.1%, as compared with that determined when the cation was not present in the medium. This observation suggests that Mg^{2+} interacts with non-NMDA receptors, as reported previously (Gilbertson et al., 1991). The protective effect of Mg^{2+} on the response to kainate is not due to modulation of the NMDA receptor-mediated toxicity, because either in the presence or in the absence of Mg^{2+}, MK-801 did not affect the kainate response in Na^+ medium (not shown).

Incubation of the cells with high K^+ (50 mM) in the presence of MK-801 plus CNQX, to avoid toxicity phenomena due to the activation of glutamate receptors in response to endogenous glutamate release, did not affect significantly the cell survival, despite the observation that a significant increase of $^{45}Ca^{2+}$ occurred in identical experimental conditions (Table 2). Indeed, only 7.3% of neuronal death was observed ($P > 0.05$) when the cells were depolarized by high K^+ (Fig. 1A, inset). Although glutamate reduced significantly the cell survival under the same experimental conditions, it was less efficient than KCl depolarization in stimulating the accumulation of $^{45}Ca^{2+}$ (Fig. 1, Table 2).

3.3. Role of Ca^{2+} entry through NMDA or AMPA / kainate receptor-associated channels on cell death

The functional consequences of the Ca^{2+} entry through the glutamate receptor-associated channels can be investigated by stimulating the cells in Na^+-free NMG medium, which prevents membrane depolarization and the activation of the VSCCs (Iino et al., 1990; Segal and Manor, 1992; Carvalho et al., 1995; Duarte et al., 1996). However, a 2-h exposure of retina cells to NMG medium, reduced

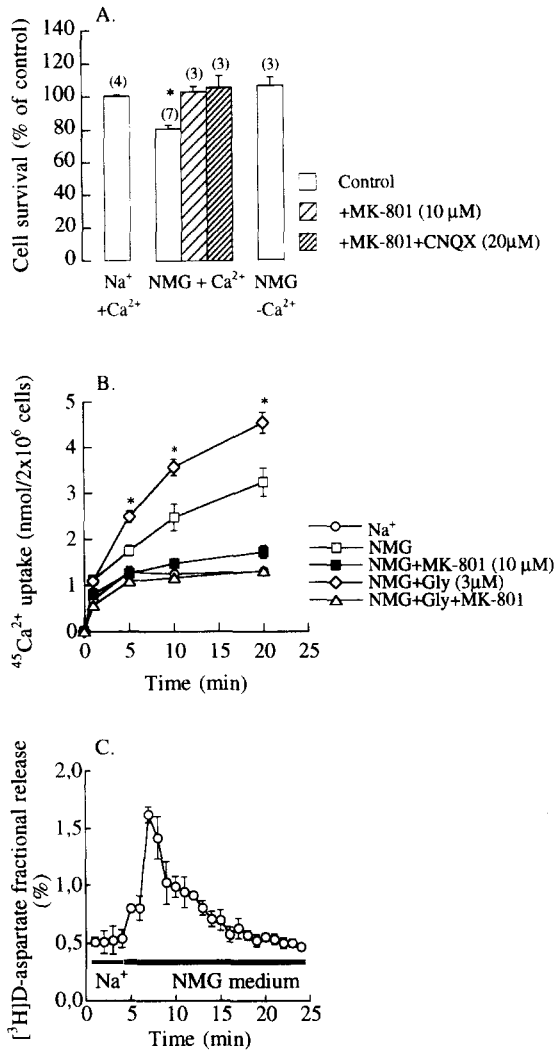

Fig. 2. Correlation between $^{45}Ca^{2+}$ influx, neuronal survival and [^{3}H]D-aspartate release in cultured retina cells exposed to NMG medium. (A) Neuronal survival in cells exposed to Na^+ (control) or to NMG medium, with or without 1 mM Ca^{2+}. The experiments were performed during 20 min, in the absence of Mg^{2+}, with or without MK-801 (10 μM) and CNQX (20 μM). * Significantly different from cell survival in Na^+ medium ($P < 0.05$). (B) Time course for $^{45}Ca^{2+}$ uptake by cells exposed to Na^+ or NMG medium with Ca^{2+} in the absence of Mg^{2+}, with or without added glycine (3 μM) and MK-801 (10 μM). * Significantly higher than the control in the absence of glycine ($P < 0.05$). (C) [^{3}H]D-Aspartate release in cells exposed to NMG medium during 20 min. All results were presented as mean ± S.E.M. values ($n = 3$–8) performed in duplicates for $^{45}Ca^{2+}$ influx, triplicates for cell survival and $n = 3$ in independent cultures for [^{3}H]D-aspartate release.

the cell survival by ~ 50%, as determined 18–20 h later. Moreover, the cell viability was not further reduced by incubating the cells with glutamate or glutamate receptor agonists, NMDA or kainate, in NMG medium (not shown).

The results depicted in Fig. 2A show that when retina cells were exposed to NMG medium during 20 min, in the presence of external Ca^{2+}, a decrease of 20% on cell survival was observed, as compared with control conditions (Na^+ medium). The neuronal damage observed in these conditions was completely abolished by MK-801, and CNQX had no additional protective effect (Fig. 2A). However, when Ca^{2+} was absent from the extracellular medium, we did not observe any toxic effect in Na^+-free medium (Fig. 2A).

The reduction of cell survival observed in NMG medium correlates with an increased accumulation of $^{45}Ca^{2+}$ under these experimental conditions, as compared with that determined in Na^+ medium. Thus, after 20 min in NMG medium, the retina cells accumulated up to ~ 4.5 nmol/2 × 10^6 cells, whereas only ~ 1.3 nmol/2 × 10^6 cells were accumulated in Na^+ medium. The increased $^{45}Ca^{2+}$ uptake observed in NMG medium was potentiated by glycine (Johnson and Asher, 1987), and completely inhibited by MK-801 (Fig. 2B), suggesting that it is mediated by the activation of the NMDA receptors. The accumulation of Ca^{2+} inside the cells that occur in NMG medium is probably responsible for the decrease in cell survival, because we never observed any effect of NMG medium exposure on cell survival, in the absence of external Ca^{2+} (Fig. 2A). The results presented in Fig. 2C show that the accumulated [^{3}H]D-aspartate was in fact released when the cells were transferred from a Na^+ to NMG medium in the

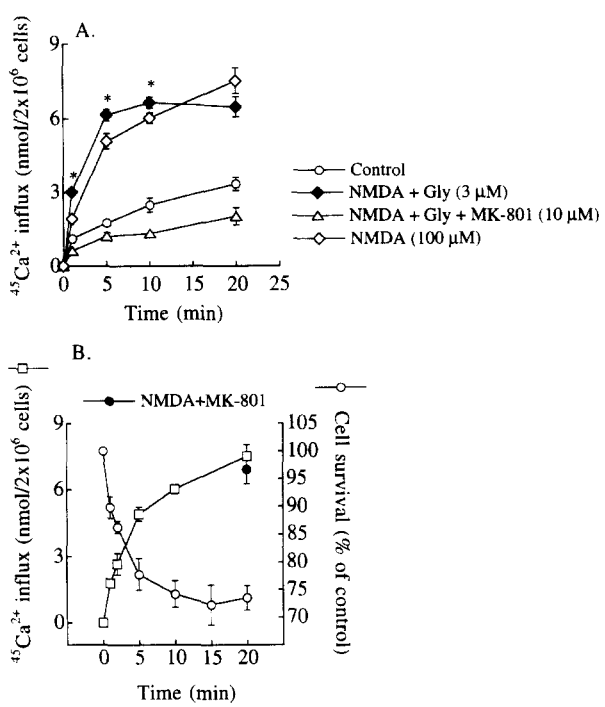

Fig. 3. Time course for $^{45}Ca^{2+}$ influx and cell survival due to Ca^{2+} entry through NMDA receptor-associated channel in chick retinal cells. (A) $^{45}Ca^{2+}$ influx induced by NMDA (100 μM) in Ca^{2+}-containing Mg^{2+}-free NMG medium, with or without glycine (3 μM) and MK-801 (10 μM). * Significantly higher than the control in the absence of glycine ($P < 0.05$). (B) Correlation between cell survival and $^{45}Ca^{2+}$ influx in retinal cells due to NMDA stimulation in NMG medium, without glycine. Filled circle represent the effect of MK-801 on cell survival induced by NMDA. Results represent the mean ± S.E.M. values ($n = 3$–6) performed in duplicates for $^{45}Ca^{2+}$ influx, and triplicates for cell survival measurements.

presence of extracellular Ca^{2+}. [^{3}H]D-Aspartate is believed to label the transmitter glutamate pool and, therefore, has been extensively used as a glutamate release marker (e.g., Belhage et al., 1992).

In order to further evaluate the role of Ca^{2+} entry through the NMDA receptor-associated channel to cell death, we stimulated the cells with NMDA in NMG medium (Fig. 3). In these conditions, NMDA evoked an increase in $^{45}Ca^{2+}$ uptake with time, up to 7 nmol/2 × 10^6 cells after 20 min, and this effect was again potentiated by glycine and was completely blocked by MK-801 (Fig. 3A). Fig. 3B correlates the NMDA stimulated $^{45}Ca^{2+}$ influx with the percentage of cell survival, as function of the incubation time. Our results show that when the cells are stimulated with NMDA, the increase in $^{45}Ca^{2+}$ influx correlates with the decrease in cell survival in a time-dependent manner.

The effect of Ca^{2+} entry through the AMPA/kainate receptor-associated channels on cell death was determined in the presence of MK-801 in order to eliminate any effect mediated by the NMDA receptors activated by endogenous glutamate released in NMG medium. Fig. 4 shows that

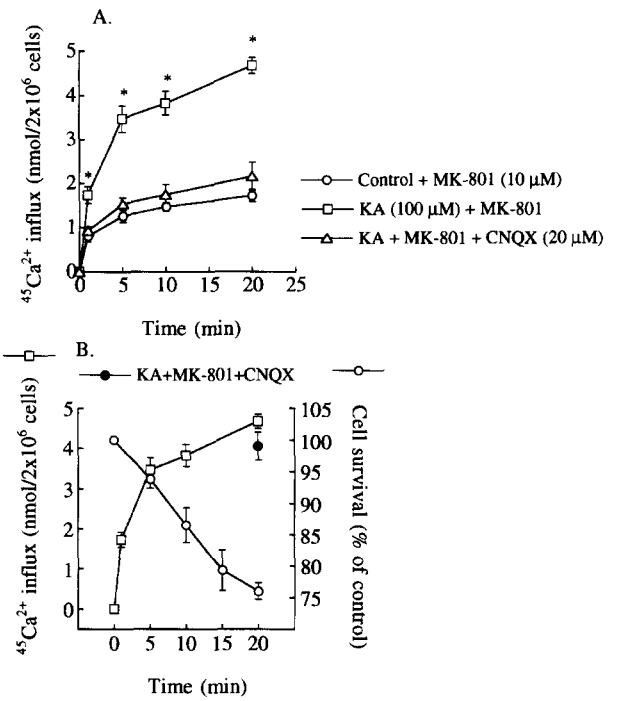

Fig. 4. Time course for $^{45}Ca^{2+}$ influx and cell survival due to Ca^{2+} entry through kainate receptor-associated channel in chick retinal cells. (A) $^{45}Ca^{2+}$ influx induced by kainate (100 μM) in Ca^{2+}-containing Mg^{2+}-free NMG medium, in the presence of MK-801 (10 μM) with or without CNQX (20 μM). * Significantly higher than the control ($P < 0.05$). (B) Correlation between cell survival and $^{45}Ca^{2+}$ influx in retinal cells due to kainate stimulation in the presence of MK-801. Filled circle represents the effect of CNQX in protecting from cell death induced by kainate. Results represent the mean ± S.E.M. values ($n = 3$–6) performed in duplicates for $^{45}Ca^{2+}$ influx, and triplicates for cell survival measurements.

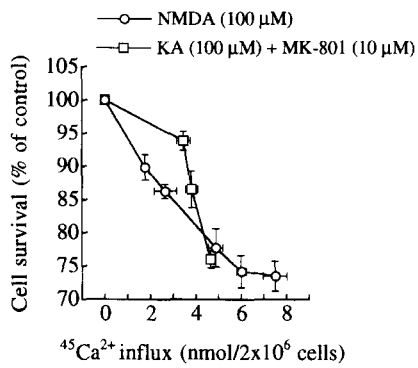

Fig. 5. Correlation between $^{45}Ca^{2+}$ influx and survival of retinal cells induced by NMDA or kainate. The graph is a replot of data from Fig. 3B and Fig. 4B.

stimulation of chick retinal cells with kainate, in the presence of MK-801, leads to an influx of $^{45}Ca^{2+}$ up to 4.5 nmol/2 × 10^6 cells after 20 min and this influx was completely blocked by CNQX (Fig. 4A). In this case, we also observed a time-dependent decrease in cell survival (Fig. 4B). However, for short incubation periods (up to 5 min), the large influx of Ca^{2+} produces a small effect on cell survival (Fig. 4B).

In order to determine how the influx of $^{45}Ca^{2+}$ through the NMDA and non-NMDA receptor-associated channels is correlated with the reduction of cell survival, the data from Fig. 3B and Fig. 4B were reploted in Fig. 5. For small Ca^{2+} loads, the influx of Ca^{2+} through the NMDA receptor-associated channel appears to be more effective in reducing cell survival than Ca^{2+} entry through the AMPA/kainate receptor-associated channel (Fig. 5). However, for long incubation periods with the agonists, which cause an extensive accumulation of Ca^{2+}, the uptake of $^{45}Ca^{2+}$ stimulated by the two agonists was equally related to cell death (Fig. 5).

4. Discussion

The excessive entry of Ca^{2+} is thought to be the major cause of glutamate toxicity in nerve cells, but Ca^{2+} may enter through either the VSCCs or through the glutamate receptor-associated channels. In the present study, we correlated the effects of glutamate receptor agonists on the influx of $^{45}Ca^{2+}$ and on neurotoxicity of cultured chick retina cells. Furthermore, we compared the efficiency of Ca^{2+} entering through VSCCs and through the glutamate receptor-associated channels in stimulating neurotoxicity.

The levels of average somatic $[Ca^{2+}]_i$ may only loosely reflect the amounts of Ca^{2+} influx, since Ca^{2+} entering a neuron may become bound and not contribute to $[Ca^{2+}]_i$. Conversely, only small quantities of Ca^{2+} influx may be required to elevate $[Ca^{2+}]_i$ if sequestration into compartments is limited. Furthermore, release from intracellular

stores can elevate $[Ca^{2+}]_i$ independent of Ca^{2+} influx, and may play an important role in the glutamate-induced neuronal degeneration (Frandsen and Schousboe, 1991). Several studies of acute hypoxia (Goldberg et al., 1989) and glutamate toxicity (Kurth et al., 1989; Eimerl and Schramm, 1992, 1994; Hartley et al., 1993) showed a close correlation between $^{45}Ca^{2+}$ influx and toxicity. For these reasons, measurement of total Ca^{2+} influx might provide a better predictor of subsequent neuronal death related to Ca^{2+} entry than is provided by measurements of somatic $[Ca^{2+}]_i$.

We have previously shown that glutamate, NMDA and kainate increase the $[Ca^{2+}]_i$ in cultured retina cells, through a composite effect, comprising Ca^{2+} permeating the receptor-associated channels and Ca^{2+} entering through VSCCs (Carvalho et al., 1995; Duarte et al., 1996). Our results show that, in Na^+-containing medium glutamate, NMDA and kainate decreased the cell survival, and that glutamate-induced cell death was mostly mediated through the activation of the NMDA receptor. The Mg^{2+} ion protected against the toxicity resulting from the activation of the NMDA receptor, as expected from the well-known Mg^{2+} blockade of the NMDA receptor that occurs in a voltage-dependent manner (Nowak et al., 1984). The reduction of cell survival induced by glutamate or kainate was also partially prevented by Mg^{2+}, suggesting that the cation promote desensitization of the non-NMDA receptors in a voltage-independent way. A similar effect of Mg^{2+} was reported in retinal bipolar cells (Gilbertson et al., 1991), but is at variance with the previously reported lack of effect of Mg^{2+} on the kainate-induced toxicity in the embryonic day 13 chick retina (Zeevalk and Nicklas, 1992b). However, preliminary results showed that the influx of $^{45}Ca^{2+}$ through the AMPA/kainate receptor-associated channels can be diminished by physiological concentrations of Mg^{2+} (not shown).

Exposure of chick retinal neurons to the K^+ (50 mM) depolarization evokes large $[Ca^{2+}]_i$ transients due to Ca^{2+} entry through nitrendipine-sensitive and dihydropiridine-insensitive Ca^{2+} channels (Duarte et al., 1992). However, we show that the depolarization of chick retina cells with 50 mM KCl did not affect their viability, despite the increase of $^{45}Ca^{2+}$ accumulation. Pharmacological evidence indicates that depolarizing concentrations of K^+ can promote the survival of cilliary ganglion neurones by opening dihydropiridine-sensitive Ca^{2+} channels (Collins et al., 1991). The relevant parameter suppressing programmed cell death in sympathetic neurons depolarized with high potassium appeared to be Ca^{2+} influx rather than depolarization (Franklin et al., 1995) and, probably, the influx of Ca^{2+} induced by chronic depolarization can substitute for trophic factors in promoting survival (Franklin et al., 1995). Therefore, it is possible that the transient Ca^{2+} influx observed in depolarized retina cells would trigger subsequent events leading to increased neuronal survival. The failure of depolarizing concentrations of K^+ to kill retinal neurons in our experiments, again point out the small role of Ca^{2+} entry through VSCCs in this type of neurotoxicity.

Despite the observation that K^+ depolarization is more efficient in elevating the intracellular Ca^{2+} (Duarte et al., 1992; Carvalho et al., 1995) than glutamate, NMDA or kainate (Duarte et al., 1993b; Ferreira et al., 1994) more neurons died upon activation of glutamate receptors. These results reflect clearly that although Ca^{2+} triggers neurotoxicity, not all sustained elevations in $[Ca^{2+}]_i$ are equally neurotoxic, and that toxicity clearly depends on the source of Ca^{2+} influx. Our results are in agreement with those previously reported in spinal neurons (Tymianski et al., 1993), and show that neurotoxicity of glutamate is triggered primarily by Ca^{2+} entry through the NMDA receptor-gated channels. Therefore, it is concluded that the NMDA receptor play an important role in the glutamate-mediated neurotoxicity in chick retinal cells.

In order to confirm that Ca^{2+} permeation through the NMDA and AMPA/kainate receptor-associated channels is directly related to the neurodegeneration of retinal cells, we stimulated the cells with NMDA and kainate in the absence of extacellular Na^+ (NMG medium), in order to prevent any effect of depolarization and activation of voltage-gated Ca^{2+} channels. The effects of NMDA were potentiated by glycine and were completely inhibited by MK-801, and the accumulation of $^{45}Ca^{2+}$ induced by the agonist was correlated with a decrease of cell survival. We also found that the Ca^{2+} permeability of the AMPA/kainate receptor is lower than that of the NMDA receptor-associated channel. This observation correlates with the fact that the activation of non-NMDA receptors in NMG medium for short periods of time was not as toxic as the activation of the NMDA receptors under the same conditions. However, these results are at odds with previous reports, where we have evaluated the relative Ca^{2+} permeability of the NMDA and AMPA/kainate receptor channels by measuring the $[Ca^{2+}]_i$ responses in NMG medium (Carvalho et al., 1995; Duarte et al., 1996). The higher Ca^{2+} permeability of the non-NMDA receptors observed in these studies may be due to the fact that the actual level of $[Ca^{2+}]_i$ measured may not accurately reflect the total amount of Ca^{2+} entering the cell. Moreover, this apparent discrepancy between $[Ca^{2+}]_i$ measurements and the results of $^{45}Ca^{2+}$ uptake may be due to different buffering systems being triggered depending on the pathway by which Ca^{2+} enters the cell.

As shown in Fig. 5, for short incubation periods, corresponding to smaller Ca^{2+} loads, the entry of Ca^{2+} through the NMDA receptor-associated channel was more efficient in triggering cell death than the influx of Ca^{2+} through the AMPA/kainate receptor. These results suggest that the NMDA and AMPA/kainate receptors may have a distinct distribution in the cell, being the NMDA receptors closer to the regions where the cytotoxic events are triggered. Other possible explanations for the observed differences in the profile of the agonists, despite similar Ca^{2+} loads,

should be considered: (1) KCl-induced $^{45}Ca^{2+}$ influx may not have occurred in the 'critical compartment'; (2) different agonists could produce comparable Ca^{2+} loads by utilizing different Ca^{2+} sources and/or pathways. For example, glutamate but not KCl have also been shown to release Ca^{2+} from dantrolene-sensitive intracellular compartments in cerebral cortical neurones (Frandsen and Schousboe, 1991). The total Ca^{2+} load, source and site of $[Ca^{2+}]_i$ increase, the ability of a cell to buffer the $[Ca^{2+}]_i$ load and the other second messenger pathways activated by an agonist may, therefore, be more important determinants of neurotoxicity of an agonist than the peak of the $[Ca^{2+}]_i$ change. Indeed, depending on the route of Ca^{2+} entry into a neuron, distinct signal transduction pathways can be activated, leading to different patterns of gene expression and different physiological responses (Gallin and Greenberg, 1995).

Exposure of the cells to NMG medium stimulated the accumulation of $^{45}Ca^{2+}$ from the extracellular medium, and this accumulation was potentiated by glycine and was completely inhibited by MK-801, suggesting that the observed Ca^{2+} influx was mediated by the NMDA receptor channel. The incubation of retinal neurons in NMG medium for 20 min reduced cell survival by 20%, and this effect was completely antagonized by MK-801. These results, and the observation that exposure to NMG medium evoked the release of [^{3}H]D-aspartate, lead us to conclude that endogenous excitatory amino acids (possibly glutamate) are released when retinal cells are exposed to NMG medium. Under these conditions the release of [^{3}H]D-aspartate is probably due to the reversal of the plasma membrane transporter, due to changes on the Na^+ electrochemical gradient (Nicholls and Attwell, 1990). The glutamate released in NMG medium appears to stimulate preferentially the NMDA receptors, probably due to the higher affinity of the NMDA receptor to glutamate (Olverman et al., 1984).

It is important to note that the neurotoxicity observed in NMG medium was not dependent on extracellular Na^+, but rather was dependent on extracellular Ca^{2+}. The demonstration of a Ca^{2+} dependency for the glutamate-stimulated neurotoxicity has generally been interpreted as a requirement for Ca^{2+} influx (Tymianski et al., 1993). However, it should be noted that if Ca^{2+} was required for the binding of glutamate to its receptors we should also not observe neurotoxicity in Ca^{2+}-free media. This is not probably the case, because the binding of L-glutamate to kainate receptors is actually facilitated by removing extracellular Ca^{2+}, whereas the binding of agonists to other glutamate receptors is not greatly affected by Ca^{2+} (Dingledine et al., 1988). Therefore, the role of Ca^{2+} in neurotoxicity cannot be explained in terms of the Ca^{2+} effect on the glutamate binding to its receptors.

We have shown in this report that, in cultured chick retina cells, activation of the NMDA or non-NMDA receptors leads to an excitotoxic insult. The uptake of $^{45}Ca^{2+}$ and the decrease in cell survival was maximal when the excitotoxic insult was performed by NMDA, suggesting that these receptors are more permeable to Ca^{2+} than are the non-NMDA receptor. The neurodegeneration in neocortical cell cultures induced by brief activation of the NMDA receptors is also Ca^{2+}-dependent (Hartley et al., 1993; Weiss et al., 1994), and probably is triggered by Ca^{2+} influx through the Ca^{2+}-permeable NMDA receptor-gated channel. In contrast, much more prolonged activation of the Ca^{2+}-permeable AMPA/kainate receptor-gated channels is required to induce comparable cortical degeneration (Koh et al., 1990; Weiss et al., 1994). The fact that cultured chick retinal cells released preloaded γ-amino [^{3}H]butyric acid ([^{3}H]GABA) upon stimulation with glutamate (Ferreira et al., 1994) and that GABA seems to be the most important endogenous amino acid released in response to excitatory amino acids receptor activation (unpublished data), suggests that the retinal GABAergic neurons are sensitive to excitotoxic damage. Accordingly, these cells were found to be affected by in vivo and in vitro ischemic insults to the rabbit retina (Osborne and Herrera, 1994).

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