Quinolinate (QUIN), an endogenous dicarboxylic amino acid, structurally related to the putative retinal neurotransmitter aspartate, acts as a specific neurotoxin in the chick neural retina. Qualitative analysis of QUIN’s neurotoxic effects reveals that sensitivity to the amino acid is first detected in the 9-day-old embryonic chick retina. Nuclei and cytoplasm of some cells in the inner region of the inner nuclear layer and in the ganglion cell layer appear hypochromatic or electron lucent when examined by light or electron microscopy, respectively. Between day 10 and 12, the sensitivity of the embryonic retina to QUIN increases and remains around the day 12 level throughout the remaining embryonic and initial posthatching period. Cells in the inner half of the inner nuclear layer continue to be the most severely affected throughout retinal development, ganglion cells less so. Photoreceptor and most cells in the outer region of the inner nuclear layer remain undamaged. QUIN effects are partially reversible: retinas exposed to QUIN briefly in vitro and then transferred to fresh QUIN-free medium are not as severely affected as those allowed no recovery time. In day 1 posthatching chick retinas, similar patterns of QUIN-toxicity were observed in vitro (0.5–5 mM QUIN; 5–30 min) and in vivo (200–600 µg QUIN/eye; 0.5–24 hr following intravitreal injection). Invest Ophthalmol Vis Sci 26:50-57, 1985

Materials and Methods

In Vitro Culture Methods

White Leghorn chick (Gallus domesticus) eggs (Spafas Inc.; Norwich, CT) were kept in a humid, forced-air, temperature-controlled incubator (38°C). Eyes were removed from chicks on embryonic day 8–10, 12, 15, and 18 and posthatching day 1 and 2. After removal of the cornea and vitreous, a portion of the lower temporal quadrant between the periphery and the fundus was cut from the remaining tissue. The pigmented retinal epithelium was removed from the quadrant, leaving only isolated neural retina.
These quadrants were incubated for 1–30 min with various concentrations of QUIN (0.5–10 mM) (Sigma Chemical Co.; St. Louis, MO) in 15 ml of fresh Eagle's Basal Medium without glutamine (BME; Flow Labs Inc.; McLean, VA) containing 0.5% glucose and 5% commercially dialyzed fetal calf serum (GIBCO, Grand Island, NY; pH 7.6) in 25-cm Costar-tissue culture flasks (five to seven quadrants per flask) in a gyratory incubator-shaker (37°C; 50 rpm). Control retinal quadrants were incubated for 30 min in QUIN-free medium to which 5–10 mM NaCl had been added.

In “recovery” experiments, day 12 embryonic and day 1 posthatching retinas were exposed to QUIN for 1–15 min, washed 3 times with BME, and either fixed immediately or incubated in QUIN-free medium for another 30–60-min period.

Following incubation, all tissues were washed in fresh medium, fixed in glutaraldehyde (1.5% in 0.1 M cacodylate buffer; 37°C), postfixed in osmium tetroxide (1.0% in cacodylate buffer, room temperature), rinsed and stained en bloc with 2% uranyl acetate in 35% ethanol, and then dehydrated through a graded series of alcohols and two changes of propylene oxide. Samples were infiltrated with Embed 812 (Electron Microscopic Sciences; Ft. Washington, PA) and flat embedded. One-micron-thick transverse sections were cut from five to seven individual specimens in each treatment group using a Sorvall Ultramicrotome (MT5000), stained with toluidine blue, and viewed with a Zeiss Universal light microscope. Thin sections were cut with a diamond knife (Dupont), stained with uranyl acetate and lead citrate and viewed with a Philips 100 transmission electron microscope.

In Vivo Methods

Day 1–2 posthatching chicks were anesthetized with ether. Eyes were injected intravitreally through the center of the pupil with 200, 400, or 600 μg of QUIN dissolved in 10 μl of BME (pH 7.6). Controls were either not injected or sham injected with 10 μl BME. A 25-μl Hamilton syringe with a plastic stop secured 3 mm from the end of the needle tip assured that the needle was inserted to the same depth during each injection. Chicks were decapitated 0.5, 2, 6, or 24 hr after injection. The eyes were removed and retinal quadrants were dissected in BME and processed for microscopic analysis as described above for the in vitro experiments. The maintenance and use of animals for the experimental purposes described here was in compliance with the ARVO Resolution on the Use of Animals in Research.

The results for both in vitro and in vivo experiments are based on data gathered from five to seven retinal quadrants for each set of parameters tested. Key

Fig. 1. Top, Day 9 retina; 5 mM QUIN for 30 min. Arrows indicate affected cells in the INL and GCL. Note small vacuolations in IPL. Bar = 20 μm. All subsequent light micrographs are the same magnification. Fig. 2. Bottom, Day 10 retina exposed to 5 mM QUIN for 30 min. Hypochromatic nuclei are visible in the INL-1 and GCL (large arrows). Note vacuolations in the IPL and the normal appearance of the displaced amacrine cell (small arrow) in the IPL.
Fig. 3. Perinuclear region of an affected INL-I cell in a day-10 retina exposed to 5 mM QUIN for 30 min. The morphology of the mitochondria (M) is abnormal. Note the distended nuclear envelope (N) and numerous large membrane-limited cytoplasmic vesicles (V). An unaffected cell borders on the left (large arrow). Bar = 1 \mu m.

concentrations, experiment times and ages were reexamed in a second experiment with the same number of samples.

**Results**

**The Onset and Pattern of QUIN Effects In Vitro**

Untreated control retinas, for all ages used, showed no evidence of cellular changes.

The first evidence of QUIN sensitivity was seen in day 9 embryonic retinas exposed to 5 mM QUIN for 30 min: occasional hypochromatic nuclei appeared in the developing inner region of the inner nuclear layer (INL-I) and the ganglion cell layer (GCL); very small vacuolations appeared in the inner plexiform layer (IPL) (Fig. 1). As judged by the number of INL-I and GCL nuclei exhibiting hypochromicity, sensitivity to QUIN increased noticeably between days 9 and 10 (Fig. 2): more INL-I and GCL cells contained hypochromatic nuclei and vacuolated cytoplasm; larger vacuolations were present in the IPL. Displaced amacrine cells, as judged by their location in the day 10 IPL, were unaffected (Fig. 2, small arrow). Lower QUIN concentrations (1 mM) and shorter incubation times (5 min) elicited visible effects only in the INL-I and IPL (micrographs not shown). Ultrastructurally, affected nuclei contained very little stained nucleoplasm and their nuclear envelopes were distended. The cytoplasm in such cells was mostly electron lucent and contained a few abnormal, membrane-limited vesicles; mitochondria, when seen in affected cells, appeared abnormal (Fig. 3). In the INL-I and GCL, unaffected cells frequently bordered on affected cells on several sides. The IPL contained many large empty areas (probably the remains of neurites of degenerating cells) but retained some unaffected neurites.

QUIN effects were similar in day 12 retinas (cf, Figs. 2, 4). However, it appeared that more INL-I and GCL nuclei were hypochromatic, and the IPL was vacuolated more heavily. Day 12 was the youngest age at which any pyknotic nuclei were seen in the INL-I. At this time, very few cells in the outer region of the INL (INL-O), no cells in the outer nuclear layer (ONL) and no neurites in the forming outer plexiform layer (OPL) were affected. Even after the harshest exposure (30 min with 10 mM QUIN), no cellular or subcellular changes were visible in the ONL or OPL and only occasional affected nuclei were present in the INL-O.

The retina's rapid response to 5 mM QUIN was apparent in day 12 retinas: affected INL-I and GCL cell nuclei were hypochromatic, and the IPL was vacuolated after only a 5-min exposure to the compound (Fig. 5). Ultrastructurally, these nuclei were electron lucent except for clumped chromatin along the nuclear envelope (micrograph not shown).

Retinal sensitivity to 2.4-10.0 mM QUIN did not change from day 12 through hatching with respect to the type of cells affected. Following even the harshest treatment (30 min, 10 mM), only occasional hypochromatic cells with vacuolated cytoplasms were apparent in the GCL. There were no visible cellular or subcellular changes in the ONL, OPL, and except for a very few cells, in the INL-O (Fig. 6).

**Reversibility of QUIN Effects**

Both day 12 and posthatching retinas were able to recover partially from limited treatment with QUIN. Day-12 retinas, exposed to 5.0 mM QUIN for 1-5 min, washed and incubated in fresh medium for 29 days
or 25 min, respectively, showed no visible neurodegenerative effects whereas retinas so exposed and not permitted to recover contained hypochromatic nuclei.

Fig. 4. Top. Day 12 retina exposed to 5 mM QUIN for 30 min. A pyknotic nucleus (large arrow) is present in the INL-I. IPL vacuolations (small arrows) are more extensive than in the day 10 retina in Figure 2. Fig. 5. Bottom. Day 12 retina exposed to 5 mM QUIN for 5 min. Pyknotic nuclei are absent from the INL-I. Both INL-I and GCL affected nuclei are hypochromatic (arrows) and less extensive vacuolations are present in the IPL when compared with those in Figure 4.

Fig. 6. Day 1 posthatching retina exposed to 5 mM QUIN for 30 min. Some pyknotic nuclei (P) are present in the INL-I. The cytoplasm of some GCL cells contains vacuoles (arrow), which, when examined ultrastructurally, are large, electron lucent, membrane-limited vesicles.

in the INL-I and GCL, and small vacuolations in the IPL (cf, Figs. 5, 7). Effects on posthatching retinas so treated were only partially reversible: occasional pyknotic nuclei were present in the INL; however, the nuclei of cells in the GCL appeared normal, and their cytoplasm contained few vacuoles. (cf, Figs. 6, 8).

QUIN Effects In Vivo: Intravitreal Injection in Posthatching Chicks

Thirty minutes after intravitreal injection of 200–600 µg QUIN, retinas from posthatching chicks showed both hypochromatic as well as pyknotic nuclei in the INL-I, large vacuolations in the IPL and some hypochromicity in the GCL (Fig. 9). Other layers appeared similar to those in uninjected or sham injected controls. Qualitatively, these effects of QUIN paralleled those observed in posthatching retinas cultured in vitro (cf, Figs. 8, 9).

When the retinas from intravitreally injected eyes were examined at 2–24 hr postinjection, the hypochromatic nuclei seen 30 min after injection were no longer prevalent in the INL-I; the few affected INL-I
nuclei were pyknotic, and the IPL was vacuolated (cf. Figs. 9, 10). Hypochromatic GCL cells, while present in specimens examined at 0.5, 2, and 6 hr, could no longer be seen in retinas sampled 24 hr after injection. Ultrastructurally, electron lucent membrane-limited spaces, probably the remains of neurites of affected cells, were present in the IPL but not in the OPL (Fig. 11). Extracellular spaces were present in the IPL but not in the OPL of treated retinas; these spaces were not seen in the plexiform layers of control retinas (micrograph not shown).

Discussion

Our present qualitative data indicate that QUIN damage is largely limited to the INL-I, whereas GLU and KA damage is visible throughout the INL.11,12 All three compounds cause morphologic changes in cells of the INL-I (probably amacrine) and the GCL and in neurites in the IPL (possibly originating from amacrine and ganglion cells). However QUIN appears to be more restricted in its action than GLU or KA: when either the concentration of, or exposure to, GLU or KA is increased 10-fold beyond that required

Fig. 7. Top left. Day 12 retina exposed to 5 mM QUIN for 5 min, washed and further incubated in QUIN-free medium for 25 min. Top right, control retina incubated with 5 mM NaCl for 30 min. Staining patterns in the INL-I, GCL and IPL are similar for treated and control retinas. Fig. 8. Bottom. Day 1 posthatching retina exposed to 5 mM QUIN for 5 min, washed and further incubated in QUIN-free medium for 25 min. Some pyknotic nuclei (arrows) occur in the INL-I, and vacuolations remain in the IPL. The GCL appears normal.

Fig. 9. Day 1 posthatching retina 30 min after intravitreal injection with 600 μg QUIN in 10 μl BME; most INL-I cells are affected while the INL-O, the OPL and the ONL are not. Inset. GCL; note vacuoles in some GCL cells (arrows).
to elicit a definite response, cells of the middle and outer regions of the INL and neurites in the OPL are also damaged. Such a broadened response pattern could not be elicited with even the highest concentration of QUIN (10 mM) used in our experiments.

The observations reported here indicate other distinctions between the retinotoxic properties of QUIN, KA and GLU. Sensitivity to KA and GLU appears to precede sensitivity to QUIN developmentally by approximately 24 hr (unpublished observations). Retinal susceptibility to each compound, once established, increases substantially during the subsequent 24-hr period. However, unlike GLU and KA that affect additional retinal cell types during later embryonic development (unpublished observations), QUIN-induced damage, at the concentrations and exposure times used in this study, remains restricted to the INL-I, IPL, and GCL. In day 10 retinas, displaced amacrine cells, as judged by their location in the IPL, appear to be spared by QUIN, but damaged by GLU and KA.

As with KA, QUIN-toxicity was found to be reversible. When embryonic or posthatching retinas were exposed to QUIN for 5 min, followed by washing and 25-min recovery time in fresh medium, damage in all the retinal layers was less severe than in control retinas examined at the end of the 5-min treatment. This indicates that prolonged exposure to the toxin is necessary for irreversible, pathologic changes to occur.

The qualitative spectrum of retinal cells affected and the reversibility of QUIN effects studied in vitro also could be demonstrated in vivo. Thirty minutes following an intravitreal QUIN-injection, the INL-I, IPL, and GCL profiles were similar to those observed for the same exposure time in vitro. Ninety minutes later, ie, 2-hr postinjection, the pattern of retinal effects was similar to that seen in in vitro retinas exposed to QUIN and allowed to recover, thus suggesting that our in vitro observations of retinas reflect with reasonable accuracy the in vivo situation (cf, Figs. 6, 10).
Fig. 11. Day 2 posthatching retina: 24 hr after intravitreal injection with 600 μg of QUIN in 10 μl BME. Area of the IPL showing both affected (large arrows) and unaffected neurites (small arrows). The spaces (*) between cellular processes seen here are absent from the OPL and from the OPL and IPL of retinas from sham injected eyes. Bar = 1 μm.

Our data, which indicate that various amino acids affect the developing retina differently, support the current hypothesis that the toxic effects of KA and QUIN are mediated by different receptors. QUIN receptors may be confined to the IPL, whereas KA receptors seem likely to be present in both IPL and OPL.

Its selectivity in damaging retinal neurons renders QUIN a useful investigative tool. Our morphologic studies also may provide clues regarding the nature of putative retinal neurotransmitters. According to the excitotoxic hypothesis, there exists a mechanistic link between the neuroexcitatory and neurotoxic properties of certain acidic amino acids. Based on the selective toxic effects of QUIN on cells in the INL-I (probably amacrine) and GCL (probably ganglion cells), the excitotoxic hypothesis would predict that QUIN, or a QUIN-like compound present in the retina, could be contained in and released from cells whose axons impinge on both amacrine and ganglion cells. However, indirect mechanisms such as QUIN-evoked release of intraretinal GLU also may play important roles in the mediation of QUIN neurotoxicity in the retina.

Morphologic studies that examine the onset and pattern of sensitivity of the retina to various compounds can provide valuable insights into the physical distribution and function of receptors that are involved in retinal neurotransmission. While the neurotransmitter systems associated with a possible function of QUIN in the brain and retina are unknown at present, its CNS actions have been suggested to be mediated by a subclass of excitatory amino acid receptors, the N-methyl-D-aspartate-prefering receptor. Further histologic as well as pharmacologic studies will be necessary to evaluate QUIN as a selective retinal lesioning agent and as a useful tool to elucidate the complexities of excitatory amino acid neurotransmission in the retina.

Key words: Quinolinate, neurotoxin, embryonic retina, chicken, in vitro

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