

Research report

Picolinic acid protects against quinolinic acid-induced depletion of NADPH diaphorase containing neurons in the rat striatum

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Abstract

Previous studies in our laboratory have demonstrated that focal injections of picolinic acid (PIC) protect the cholinergic neurons of the nucleus basalis magnocellularis (nbm) against quinolinic acid (QUIN)-induced neurotoxicity. The present study was designed to examine the effects of chronic infusions of QUIN and PIC on nicotinamide adenine dinucleotide (NADPH) diaphorase containing neurons of the rat striatum. Using osmotic minipumps, QUIN (6 nmol/h) and PIC (18 nmol/h) were infused alone or in combination to examine the neurotoxic effects of QUIN and the potential anti-neurotoxic action of PIC. Exposure to QUIN for 7 days severely depleted NADPH diaphorase-positive neurons. When co-infused with this neurotoxic dose of QUIN, PIC attenuated the depletion of NADPH diaphorase neurons induced by QUIN. The infusion of PIC alone did not affect the number of these neurons. These results indicate that PIC itself is not neurotoxic and effectively prevents chronic QUIN-induced neurotoxicity in the rat striatum. Since PIC and QUIN are derived from the same metabolic pathway, a balance between endogenous compounds that produce neurotoxicity and those antagonizing these effects may be important in normal neuronal function.

Keywords: Quinolinic acid; Picolinic acid; NADPH diaphorase; Striatum; Osmotic minipump

1. Introduction

Focal injections of excitotoxins into the central nervous system (CNS) cause profound activation of excitatory amino acid (EAA) receptors [11,39]. Overstimulation of these receptors can produce axon sparing lesions [24,35,37] which mimic the biochemical features of various neurodegenerative disorders [4,28]. A sub-type of EAA receptors, the *N*-methyl-D-aspartate (NMDA) receptor, has been implicated in ischemic [4,25,26] and hypoglycaemic [27,47] brain damage, neurodegenerative disease [26,48], epilepsy [6,7] and the neurological manifestations of acquired immune deficiency syndrome [16].

In the CNS, the kynurenine pathway of tryptophan metabolism produces both excitotoxic and neuropro-

TECTIVE agents. Quinolinic acid (QUIN), a pyridine dicarboxylic acid derived from this pathway [40], produces neurotoxicity by overstimulation of the NMDA receptor [31,37]. Microinjections of QUIN into several regions of the CNS produce lesions which are attenuated by a number of NMDA receptor antagonists [11–13,37]. Interestingly, specific neurons in various regions of the CNS exhibit differential sensitivity to QUIN and NMDA [9,31,36] and it has therefore been suggested that QUIN may be acting on a specific sub-type of NMDA receptor [31]. As well, QUIN, unlike NMDA [22], requires intact presynaptic glutamatergic input for expression of its neurotoxic effects [13,14,35,40].

In contrast to QUIN, kynurenic acid, another metabolite yielded by the kynurenine pathway, is an allosteric modulator of the NMDA receptor at the glycine site [20,46]. Kynurenic acid blocks the neurotoxic effects of both QUIN and NMDA, however, it is much more effective against the neurotoxic actions of QUIN [13]. Despite its neuroprotective properties,

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kynurenic acid administration can produce behavioural abnormalities [43] and has been reported to cause the depletion of γ -aminobutyric acid containing neurons in the rat striatum [32].

Recent investigations with other tryptophan metabolites have demonstrated that picolinic acid (PIC), a pyridine monocarboxylic acid, protects cholinergic neurons of the nucleus basalis magnocellularis (nbm) against QUIN-induced neurotoxicity [5,18]. As well, PIC has been found to attenuate striatal dopaminergic neurotoxicity produced by nigral injections of QUIN [3]. Unlike kynurenic acid and other NMDA antagonists, PIC appears to effectively block neurotoxicity without apparently affecting normal excitation [3,34]. Also, PIC, unlike some other pyridine carboxylic acids, was found to be non-toxic when injected alone into the nbm [5,18].

Previous studies involving QUIN neurotoxicity and the protective effects of antagonists, including PIC, have generally relied on acute high dose microinjections of these compounds into specific brain regions. However, chronic exposure to low doses of QUIN may be of more pharmacological relevance. Recent studies have demonstrated that long term exposure of the rat striatum to QUIN, delivered via osmotic minipumps, results in depletion of histological and biochemical neuronal markers, including nicotinamide adenine dinucleotide (NADPH) diaphorase, glutamic acid decarboxylase, somatostatin and cholineacetyl transferase [10,41]. The NADPH diaphorase containing neurons in the rat striatum appear to be very sensitive to the effects of long term exposure to QUIN [10,41]. A single co-injection of PIC with QUIN protects basal forebrain cholinergic neurons [5,18] and dopaminergic nigrostriatal neurons [3] against QUIN-mediated neurotoxicity. However, the ability of PIC to protect striatal NADPH diaphorase neurons against QUIN-mediated neurotoxicity produced by chronic infusion has not been investigated.

The goal of this study was to investigate the effects of chronic infusions of lower doses of QUIN on striatal NADPH diaphorase containing neurons and to determine if PIC can influence QUIN-induced neurotoxicity. An associated goal was to determine if PIC has the potential to exert a neurotoxic action on these striatal neurons. Although the relative vulnerability of NADPH diaphorase positive neurons to QUIN has been a subject of controversy, a recent study has demonstrated that NADPH diaphorase neurons are depleted by a single injection of QUIN [33]. Additionally, continuous intrastriatal infusions of QUIN have been found to produce NADPH diaphorase neuron toxicity [10,41]. Thus, a continuous infusion model was considered appropriate to study QUIN-PIC interactions on NADPH diaphorase neurons. A preliminary report of this work has appeared in abstract form [19].

2. Materials and methods

2.1. Surgical procedure

Male Sprague–Dawley rats (Charles River, St. Constant, Que.) weighing 250–275 g were used in all experiments. Rats were housed in wire cages and maintained on a 12 h light/dark cycle at 21°C. Food (Purina Rodent Chow 5001; Ralston Purina, Toronto, Ont.) and water were provided ad libitum. Prior to surgery, stainless steel cannulas (28 gauge) were connected to an osmotic minipump (Alzet Model 2002; ALZA Corp., Palo Alto, CA) with polyethylene tubing. The minipumps were filled with QUIN and/or PIC or saline and stabilized in 0.9% saline at room temperature for 24 h before implantation. Animals were anaesthetized with 4% halothane and anaesthesia was maintained by the delivery of 2–2.5% halothane. Rats were placed in a stereotaxic frame with the incisor bar set at –3.3 mm. The cannula was implanted into the striatum according to coordinates from Paxinos and Watson [30] (1 mm anterior, 2.5 mm lateral, 5 mm ventral from bregma) and secured using 2 small stainless steel screws and acrylic dental cement. The minipump was inserted subcutaneously at the back of the animal's neck. Continuous infusions were carried out at a constant rate of 0.5 μ l/h. Control animals received an infusion of 0.9% saline for 7 days. QUIN was delivered at a rate of 6 nmol/h for 1–7 days to examine the time-course of its effect on NADPH diaphorase neurons. PIC was infused at 18 nmol/h alone or in combination with 6 nmol/h QUIN for a period of 7 days.

2.2. NADPH diaphorase histochemistry

At the end of the infusion period, animals were anaesthetized with pentobarbital (50 mg/kg) and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.4). Brains were removed and immersed in 30% sucrose in PBS overnight. Coronal sections of rat striatum were cut in a cryostat set at 40 μ m. Every third section in slices containing the cannula tract and every section anterior and posterior to the cannula tract were stained for NADPH diaphorase using a modified procedure of Sims et al. [38]. Free floating sections were incubated in PBS containing 2.4 mM NADPH, 1.1 mM nitro blue tetrazolium and 12% dimethyl sulfoxide for 15–30 min and subsequently rinsed with saline. Sections were mounted on glass slides, coverslipped and viewed under a light microscope.

Cells that stained positive for NADPH diaphorase were counted in the plane of the cannula over a circular area totalling 4.9 mm²/section. For each animal, 5 sections of those containing the cannula tract were counted and the sum of the cell counts for the cannulated side of each animal were expressed as a percentage of the sum of counts of similar sites on the contralateral sides of each section. The size of the lesion was measured in both anterior and posterior directions for a distance extending 1 mm from the cannula tract. Cell counts for each section were expressed as a percentage of counts on the contralateral side and were taken at 40 μ m intervals. For cell counts, all histological slides were coded such that the investigator was unaware of the treatment condition or the position of the section relative to the cannula tract.

2.3. Drugs and chemicals

Halothane was obtained from Benson Medical Industries Inc. (Markham, Ont.), QUIN, PIC, NADPH, Nitro blue tetrazolium and paraformaldehyde were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were at least reagent grade quality and supplied by either Fisher Scientific (Ottawa, Ont.) or Canlab (Pointe Claire, Que.).

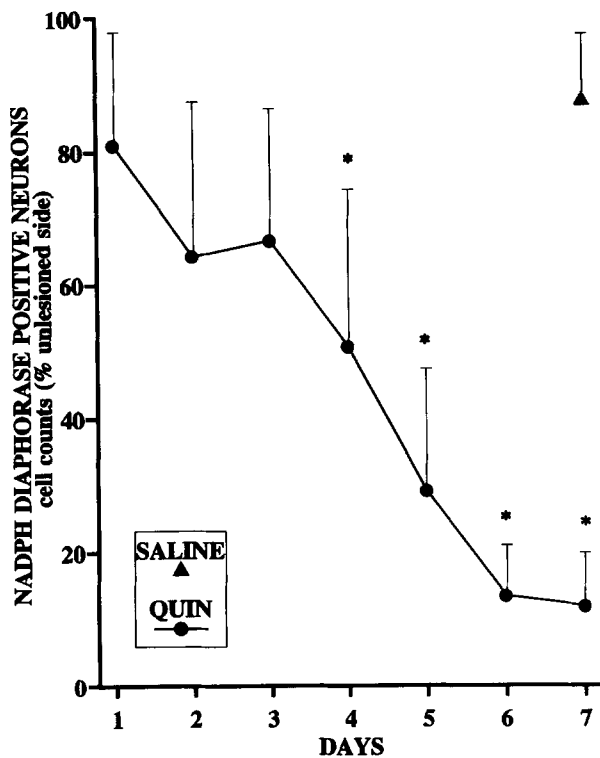


Fig. 1. Cell counts of NADPH diaphorase positive staining cells of rat striatum following infusions of QUIN (6 nmol/h) for 1–7 days and saline (0.9%) for 7 days. Cell counts are expressed as a percentage of the contralateral side. Each point represents the mean \pm S.D. of 4–6 experiments. A significant difference from saline infusion is represented by * $P < 0.05$.

QUIN was dissolved initially in 1 N NaOH, the pH was adjusted to 7.4 with HCl and the desired concentration was then achieved by the addition of 0.9% saline. PIC was dissolved in 0.9% saline and the pH adjusted to 7.4 with NaOH. All solutions were passed through a

Millex-GS 0.22 μ m syringe tip filter (Millipore, Bedford, MA) prior to filling the minipumps.

2.4. Statistical analysis

Results are expressed as the mean \pm standard deviation (S.D.). All data were assessed for homogeneity of variance using Cochran's test and subsequent parametric statistical analysis was performed using a randomized one-way ANOVA followed by Dunnett's test to determine which groups were significantly different from control. Differences in mean values were considered significant if $P < 0.05$.

3. Results

3.1. Effects of QUIN on NADPH diaphorase-positive neurons

Fig. 1 depicts the effects of 7 days of intrastriatal saline infusions and the effects of QUIN (6 nmol/h) infused for specific time periods (1–7 days) on the number of rat striatal NADPH diaphorase positive neurons. The total number of cell counts on the contralateral sides of each treatment group ranged from 460.4 ± 83.5 to 546.8 ± 70.1 . Saline infusion resulted in $87.3 \pm 9.7\%$ ($n = 5$) of cells remaining when compared to the contralateral side. Following 4 days of exposure to QUIN (6 nmol/h), the number of NADPH diaphorase neurons was significantly reduced ($50.6 \pm 25.0\%$ of the contralateral side; $n = 4$) when compared to the saline infused animals. After 5 and 6 days of QUIN infusion cell counts were further reduced to $29.2 \pm 17.8\%$ and $13.4 \pm 7.9\%$ of the contralateral side, respectively ($n = 4$). Seven days following the start of QUIN infusion, the number of NADPH diaphorase

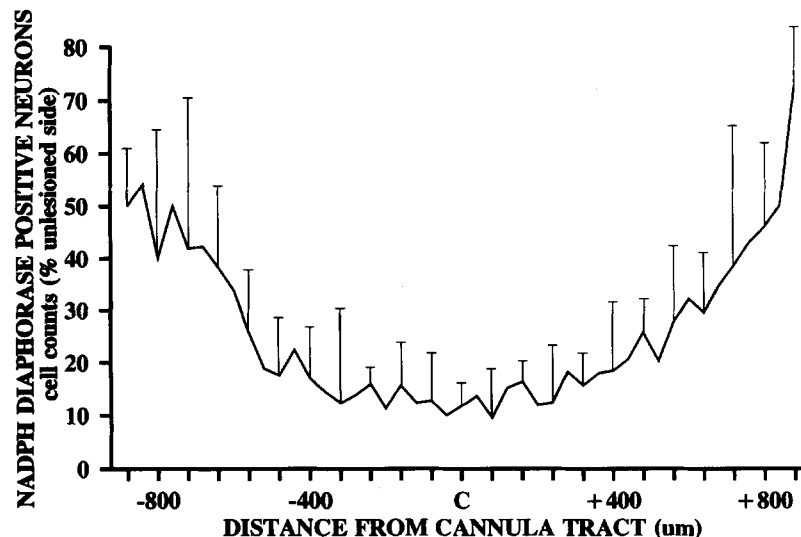
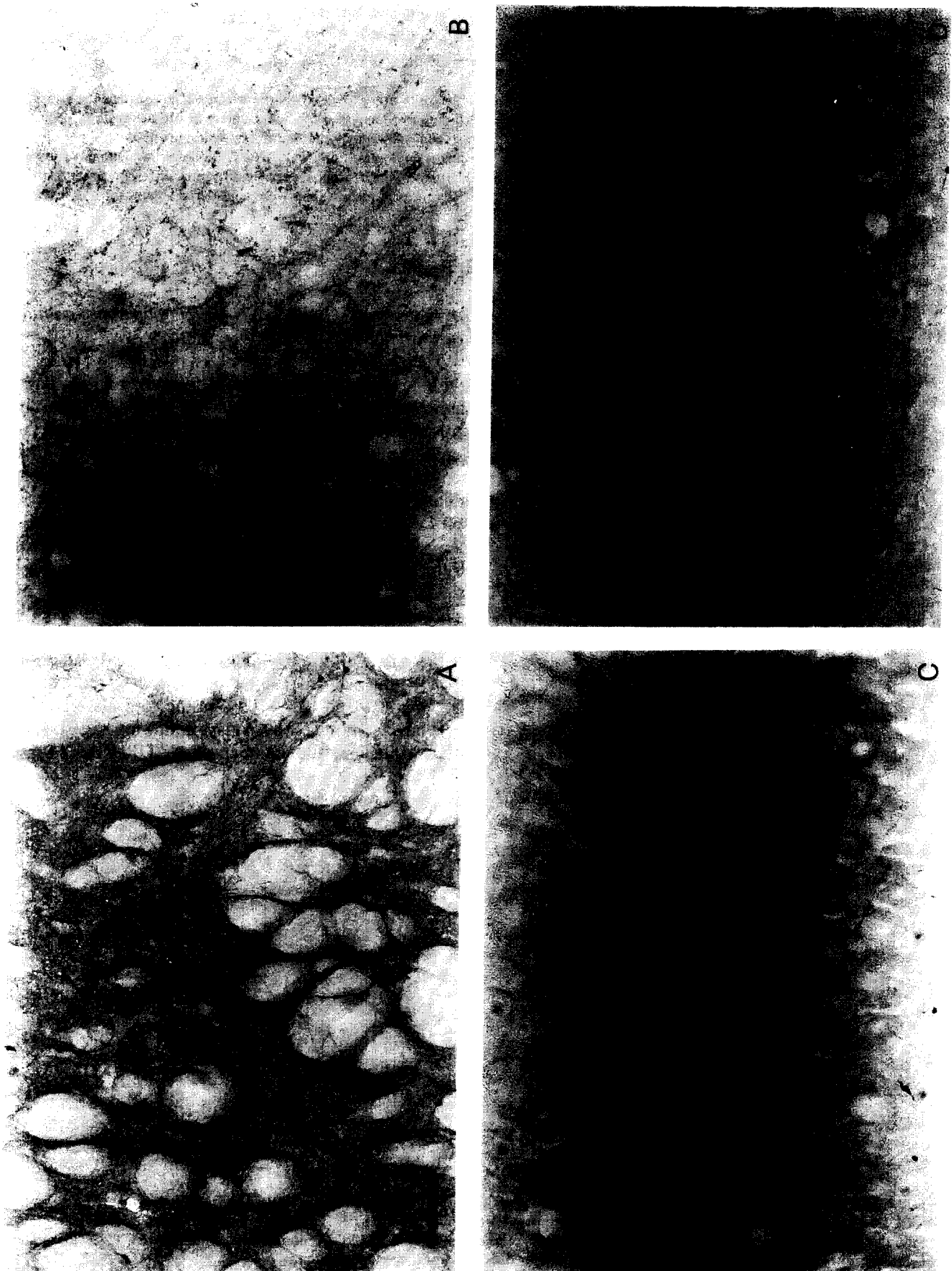


Fig. 2. Cell counts for NADPH diaphorase positive staining cells taken at 40 μ m intervals throughout the rat striatum following the infusion of 6 nmol/h QUIN for 7 days. Cell counts are expressed as a percentage of the contralateral side. Each point represents the mean of 4–6 sections. For clarity, the S.D. was shown for points at 80 μ m intervals. C, cell counts taken in sections containing the cannula tract. Positive numbers indicate anterior direction and negative numbers indicate posterior direction from the cannula tract.



neurons remaining was only $10.7 \pm 6.9\%$ ($n = 6$) of the unlesioned side.

As the loss of cells was extensive following 7 days of QUIN infusion, in subsequent work, this infusion rate and time period was chosen for experiments testing the action of PIC on QUIN-mediated NADPH diaphorase cell depletion. The extent of neuronal damage following 7 days of QUIN infusion into the rat striatum was calculated by counting NADPH diaphorase positive staining cells every 40 μm , from 1 mm anterior to and 1 mm posterior to the plane of the cannula tract. As seen in Fig. 2, the loss of NADPH diaphorase positive staining cells was greatest at the site of injection. The number of positive staining cells (% of contralateral side) within the 1.25 mm radius examined, increased with increasing distance from the plane of the cannula. The number of cells was approximately 50% in the anterior and posterior direction of the striatum at a distance of approximately 0.8 mm from the cannula tract. The diameter of the cannula tract itself was approximately 0.8 mm.

3.2. Effects of PIC on QUIN-induced depletion of NADPH diaphorase-positive neurons

Since previous experiments in the rat nbm determined that acute cholinergic neurotoxicity produced by QUIN is attenuated by PIC at a molar ratio of 3:1 (PIC to QUIN) [5], the present study used this ratio in infusion experiments. Fig. 3 depicts representative photomicrographs of the rat striatum stained for NADPH diaphorase containing neurons following a 7 day infusion of saline, QUIN (6 nmol/h), PIC (18 nmol/h), and the combination of QUIN with PIC. Tissue from saline treated animals exhibited cell staining throughout the striatum, including the area of the cannula tract (Fig. 3A). In contrast, the infusion of QUIN resulted in the presence of cell staining only along the outer margins of the striatum (Fig. 3B). Continuous infusion of PIC alone (Fig. 3C) or in combination with QUIN (Fig. 3D) yielded a staining pattern which resembled that seen in the saline controls, in which positive staining cells were seen throughout the striatum as well as along the cannula tract. The cumulative results of cell survival in the infusion experiments are depicted graphically in Fig. 4. The infusion of saline for 7 days decreased NADPH diaphorase cell counts by 14.5% ($n = 5$) of the contralateral side in the plane of the cannula within a 1.25 mm radius of the infusion site. QUIN, produced an 89.3% ($n = 6$) depletion of striatal NADPH diaphorase neurons relative to the unlesioned side, in the vicinity of the infusion. The

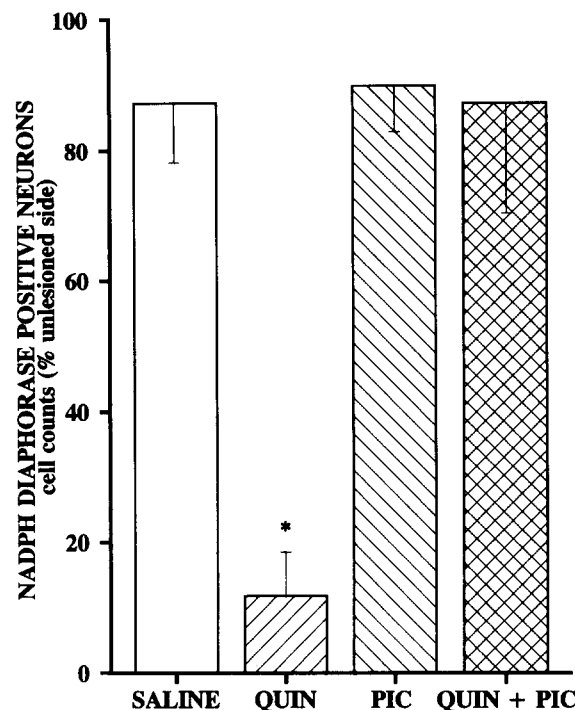


Fig. 4. Cell counts of NADPH diaphorase-positive neurons of the rat striatum following continuous infusions of saline (0.9%), QUIN (6 nmol/h) and PIC (18 nmol/h) for 7 days. Cell counts are expressed as percentage of the contralateral side. Each bar represents the mean \pm S.D. of 5–7 experiments. A significant difference from saline infusion is represented by * $P < 0.05$.

infusion of PIC alone did not significantly affect the number of NADPH diaphorase neurons (12.0% depletion; $n = 5$), while the infusion of PIC with QUIN prevented the decrease in these neurons induced by exposure to QUIN (16.5% depletion; $n = 5$). The marginal decrease in cell counts seen when PIC was infused alone or in combination with QUIN was not significantly different from cell counts resulting from saline infusion. Thus, in these infusion experiments, PIC itself was not toxic and when co-infused with QUIN, prevented the QUIN-induced depletion of NADPH diaphorase staining neurons in the rat striatum.

4. Discussion

The objective of this study was to investigate the effects of long-term low dose infusions of QUIN and PIC, alone or in combination, on NADPH diaphorase containing neurons in the rat striatum. The infusion dose of QUIN was fixed at 6 nmol/h as this dose was

Fig. 3. Photomicrograph of 40 μm coronal sections of rat striatum stained for NADPH diaphorase following infusions of (A) saline (0.9%), (B) QUIN (6 nmol/h), (C) PIC (18 nmol/h) and (D) QUIN with PIC. Bar = 100 μm .

previously reported to produce profound neuronal degeneration in Nissl stained sections following 7 days of exposure [44]. A previous study has demonstrated that PIC protects against acute QUIN-induced neurotoxicity at a molar ratio of 3:1 [5], therefore, this ratio was used in the present study. The results of the present study demonstrate that low dose QUIN infusion is toxic to NADPH diaphorase positive neurons and that PIC, while exerting no toxic action, effectively protects these neurons against the neurotoxic action of QUIN.

The effect of infusions of QUIN on striatal NADPH diaphorase neurons in our study is in agreement with previous reports on continuous intrastratial infusions [10,41], however, much higher doses of QUIN were infused into the striatum over a longer time period. A low dose of QUIN (7.5 nmol/h), administered for 21 days via chronic intrastratial microdialysis, was also reported to decrease the number of NADPH diaphorase staining cells [1]. However, this population of cells was relatively spared when compared with the depletion of Nissl stained cells. The lesion core (including the microdialysis tract) demonstrated a complete loss of cells, while the transition zone (previously described by Beal et al., [2]) demonstrated extensive loss of Nissl staining cells with a relatively small loss of NADPH diaphorase positive neurons. In the present study we focused on the NADPH diaphorase containing neurons and determined that these neurons are indeed severely depleted by a 7 day exposure to 6 nmol/h QUIN. In the present report, the lesion core and transition zone were included in total cell counts. Cells were present in both the lesion core and transition zone following chronic saline infusion and were present only in the outer margins of the transition zone following QUIN infusion. In addition, the area of damage described by Bazzett et al. [1], assessed by Nissl staining, did not extend beyond 400 μ m from the lesion core. The area of damage, as seen in Fig. 2 and qualitatively assessed in Nissl stained sections (data not shown), in the present study is much larger, where extensive depletion of NADPH diaphorase positive neurons occurs up to 800 μ m anterior to and posterior to the plane of the cannula.

In the microdialysis study carried out by Bazzett and co-workers [1], chronic implantation of the microdialysis probe produced significant non-specific neuronal damage, as infusion of vehicle alone significantly reduced Nissl staining. This observation corresponds with the findings of Georgieva et al. [15], who reported that chronic *in vivo* microdialysis produces marked tissue changes in the rat striatum. In contrast, our results demonstrate that saline infusion via osmotic minipumps attached to cannulas produces little non-specific damage as assessed qualitatively by examining Nissl stained sections (data not shown) and quantitatively by counting NADPH diaphorase neurons (see Fig. 3A). The

non-specific damage resulting from chronic microdialysis would explain the lack of staining observed in the lesion core and could bias the assessment of QUIN neurotoxicity reported by Bazzett and co-workers [1].

Previously, in the nbm cholinergic model of QUIN toxicity, exposure to focal injections of QUIN severely depleted cortical choline acetyltransferase activity and PIC antagonized this neurotoxic action of QUIN when co-injected at a dose 3-fold higher than QUIN [5]. However, PIC at this molar ratio only partially restored choline acetyltransferase activity. In the present study, this molar ratio of PIC co-infused with QUIN restored NADPH diaphorase cell counts to control levels. This protection was also observed qualitatively in Nissl stained sections. While cell survival of NADPH diaphorase neurons was comparable to saline, not all neurons protected by PIC exhibited features comparable to saline controls (Fig. 3). However, PIC clearly prevented the cell death observed following a 7 day infusion of QUIN.

We have examined the effects of QUIN on a biochemical marker for NADPH diaphorase neurons. It has been demonstrated that neuronal NADPH diaphorase in the rat is the same enzyme as nitric oxide synthase (NOS) [8,17]. However, in order to detect a significant level of NOS in striatal homogenates, a much larger area of the striatum than that used in morphological assessments was required. Slices taken for biochemical measurements were 3 mm thick and as seen in Fig. 2, would contain tissue that was not exposed to neurotoxic concentrations of QUIN. With minipump infusions the flow rate is fixed and the only way to produce a greater area of damage extending from the injection site is to increase the concentration of the neurotoxin being infused [44]. In this study, higher doses of QUIN (24 nmol/h) were necessary in order to consistently reveal NOS depletion in the rat striatum (unpublished observations) and these higher doses of QUIN and the larger tissue area required for biochemical assessment would not accurately reflect what is seen histologically in the plane of the cannula tract. As well, a much higher dose of PIC would then be required to attempt to reduce the effect of QUIN.

The mechanism(s) by which PIC attenuates QUIN excitotoxicity is not known. It may be a receptor-mediated event, as PIC can displace [3 H]glutamate binding [21]. However, PIC action does not appear to involve competition with QUIN at its binding site, as the competitive NMDA antagonist [3 H]2-amino-7-phosphonoheptanoic acid was displaced by QUIN but not by PIC [21]. PIC has been shown to act as a weak partial agonist at the strychnine-sensitive glycine receptor site [42] but not at the strychnine-insensitive site of the NMDA receptor complex [20]. Therefore, it is not clear if this is the basis of its protective action or if PIC can protect by interfering with other some other modu-

latory sites (e.g. zinc or polyamine site) on the QUIN-sensitive NMDA receptor.

An interesting feature of PIC action is that it selectively protects against QUIN and kainic acid-induced neurotoxicity but not ibotenate or quisqualate induced neurotoxicity [5]. Since, the neurotoxicity produced by QUIN and kainic acid is dependent on the presence of an intact glutamatergic input to target neurons [13,14,23,29,35], PIC may be interfering with presynaptic glutamate input to influence this neurotoxicity. Kainic acid-induced glutamate release is blocked by PIC, however, PIC alone can enhance glutamate release from rat striatal slices [45]. This PIC-induced increase in glutamate release does not appear to be consistent with its neuroprotective action. Thus, the neuroprotective mechanism(s) of PIC remains to be determined.

Previous studies have demonstrated that a single injection of PIC can attenuate the excitotoxic action of QUIN [3,5] and the present study has demonstrated that continuous infusion of PIC protects against the neurotoxic effects of continuous infusion of QUIN. While the action of PIC on other types of striatal cells, such as enkephalinergic neurons, remains to be elucidated, the fact that PIC protects NADPH diaphorase, cholinergic [5] and dopaminergic [3] neurons from QUIN neurotoxicity, indicates that this endogenous agent has a protective action on several neuron populations. At present, there are no reports of PIC producing neurotoxicity and only one study has demonstrated that continuous infusion of kynurenic acid, another neuroprotective kynurenine metabolite, is toxic to striatal neurons [32]. At this point, it is not clear if higher doses of PIC infusion alone can produce neurotoxicity. Thus, the action of high doses of PIC infusion on striatal neuron populations needs to be investigated. The present findings also suggest the need for determining the sites or mechanisms of action of PIC. Since QUIN and PIC originate from the same precursor in the kynurenine metabolic pathway [28] and QUIN has been detected in the CNS, it is conceivable that PIC could act as an endogenous neuroprotective agent in the brain. Therefore, it would be of considerable interest to determine if endogenous PIC exists in the brain and to devise ways of raising its levels in the CNS to counteract the neurodegenerative effects associated with elevated levels of QUIN.

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