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Enhanced neuronal damage by co-administration of quinolinic acid and free radicals, and protection by adenosine A_{2A} receptor antagonists

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1 Quinolinic acid may be an important endogenous excitotoxin, but its concentrations in brain are low. We have therefore attempted to determine whether its neurotoxicity can be increased by the simultaneous presence of free radicals.

2 Quinolinic acid was injected into the hippocampus of anaesthetized rats at doses of 40 and 80 nmols which produced little neuronal loss, and 120 nmols which produced over 90% neuronal loss.

3 A mixture of xanthine and xanthine oxidase, a known source of free radical reactive oxygen species, also generated little damage alone, but killed over 80% of CA1 neurons when combined with 80 nmols of quinolinic acid. Similarly, the nitric oxide donor S-nitroso-N-acetylpenicillamine (SNAP) potentiated the damage produced by quinolinic acid.

4 The glutamate antagonist 5,7-dichlorokynurenic acid prevented the damage produced by 120 nmols of quinolinic acid, but not that produced by quinolinic acid plus xanthine/xanthine oxidase, indicating that damage was not simply the result of free radical enhancement of NMDA receptor activation.

5 Three chemically dissimilar antagonists at adenosine A_{2A} receptors prevented the damage caused by quinolinic acid and xanthine/xanthine oxidase or by quinolinic acid plus SNAP.

6 It is concluded that reactive oxygen species can potentiate the neurotoxicity of quinolinic acid. The site of interaction is probably distal to the NMDA receptor. Blockade of adenosine A_{2A} receptors can protect against this combined damage, suggesting potential value in the prevention of brain damage.

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Abbreviations: AIDS, acquired immunodeficiency syndrome; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CGS 15943, 5-amino-9-chloro-2-(2-furyl)-1,2,4-triazolo[1,5-c]quinazoline; CGS 21680, 2-[4-(2-carboxyethyl)-phenylethylamino]-5′N-ethyl-carboxamido-adenosine; CNS, central nervous system; CSC, 8-(3-chlorostyryl)caffeine; DMPA, *N6*-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)-ethyl]-adenosine; HIV, human immunodeficiency virus; NMDA, N-methyl-D-aspartate; NOS, nitric oxide synthase; SCH58261, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3e]-1,2,4-triazolo[1,5c]pyrimidine; SNAP, S-nitroso-N-acetylpenicillamine; ZM241385, 4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}(1,3,5}triazin-5-yl-amino]ethyl)phenol

Introduction

The activation of several subtypes of glutamate receptor can produce damage to neurones in the central nervous system (CNS). As a result, it has been suggested that a rise in the level of an endogenous excitotoxin could contribute to the initiation or development of the neuronal damage which occurs in degenerative disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease or AIDS-dementia (Foster & Schwarcz, 1989; Kerr *et al*., 1998; Stone, 2001). One of the best studied compounds which could act as an endogenous excitotoxin at the N-methyl-D-aspartate (NMDA) receptors is quinolinic acid (Stone & Perkins, 1981; Schwarcz *et al*., 1983; 1984; Stone, 1993, 2001), a tryptophan metabolite produced by metabolism along the kynurenine pathway from tryptophan to nicotinic acid. Quinolinic acid has a potency comparable with that of NMDA in producing neurotoxicity but is relatively weak when producing neuronal excitation or displacement of glutamate receptor ligands in binding studies (Schwarcz *et al*., 1983; Perkins & Stone, 1983; Foster & Schwarcz, 1989; Stone, 1993). Furthermore, the concentrations of quinolinic acid in the CNS are normally substantially lower than those which can directly cause excitotoxicity (Moroni *et al*., 1984; Heyes *et al*., 1989). For these reasons, doubt has been expressed as to whether quinolinic acid could indeed contribute to any recognized CNS neuropathology.

There are, however, several reasons to believe that it can. Firstly, the concentration of quinolinic acid in the brain can increase several hundred-fold in response to pathological conditions and infection by agents such as HIV-1 (Heyes *et*

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al., 1989, 1991). Secondly, it has been demonstrated that even low micromolar concentrations of quinolinic acid can produce damage in cell cultures or *in vivo*, especially if those levels are maintained for several weeks (Khaspekov *et al*., 1989; Whetsell & Schwarcz, 1989; Galarraga *et al*., 1990; Kerr *et al*., 1998). Thirdly, neurones in some parts of the CNS are known to be exquisitely sensitive to injury by quinolinic acid, with damage produced at concentrations as low as 100 nM (Giulian *et al*., 1990, 1993).

In addition, it is possible that the excitotoxic damage produced by quinolinic acid, even at low concentrations, could be potentiated by other endogenous and injurious molecules. Quinolinic acid in the brain is produced mainly by the increased kynurenine pathway activity which is induced in microglia and invading macrophages by immune activation (Heyes *et al*., 1992a, 1996; Espey *et al*., 1997). These cells, when activated, also generate increased quantities of free radicals such as reactive oxygen species and we have therefore considered the possibility that free radicals generated by activated immune cells could interact with quinolinic acid to produce enhanced damage.

Methods

Intrahippocampal injections

Male Wistar rats weighing between 200 and 250 g were used. All animals were housed singly and provided with free access to food and water. Animals were anaesthetised with chloral hydrate (400 mg kg^{-1}, intraperitoneally) and placed in a stereotaxic frame. The scalp was incised and a burr hole made through the skull to permit access of the injection needle into the hippocampus at the desired co-ordinates (anteroposterior: 3.0 mm behind the bregma suture, dorsoventral: 2.8 mm below the cortical surface and lateral: 3.0 mm from the midline suture; Paxinos & Watson, 1986). The needle was then lowered to these co-ordinates and left in place for 2 min before the injection of test agents. The compounds used in this study were all introduced through a 29-gauge needle, injections being made in a volume of between 1 and 2 μl at a constant rate of 0.3 ml min^{-1} using a Sage infusion pump (Jones *et al*., 1998a, b). The injection needle was allowed to remain in place for 2 min after ending the injection so as to prevent leakage of drug along the needle track. The scalp was then sutured and the animals left to recover for 7 days.

Quinolinic acid was injected at doses of 40, 80 and 120 nmols. A mixture of xanthine (100 μM) and xanthine oxidase (0.1 U ml^{-1}) was co-administered by mixing solutions of the individual agents with the stock quinolinic acid solution in the appropriate proportions. The dose of xanthine and xanthine oxidase was limited by the solubility of the compounds, so that higher dosage was not practicable. Quinolinic acid was dissolved in 1 N NaOH and then diluted with 165 mM NaCl solution. The pH of the solution was then adjusted using 1 N HCl to between 7 and 7.6 before making up to volume by the addition of further saline. S-nitroso-N-acetylpenicillamine (SNAP) was injected at doses of 100 and 500 nmols.

Tissue fixing and slicing

Seven days after recovery from the intrahippocampal injections, rats were killed by an overdose of sodium pentobarbitone. The chest was opened to expose the heart and 10 ml of 0.9% sodium chloride solution was injected *via* a 26-gauge needle inserted into the left cardiac ventricle to wash blood from the cerebral vessels. This was immediately followed by 4% formaldehyde in phosphate buffered saline. The brain was then removed and stored in fixative for up to 1 week. A coronal slice of brain, approximately 3 mm thick, was prepared to include the location of the injection track, which was normally apparent from the residual dimpling of the cortical surface produced by the needle penetration. The block of brain was dehydrated and impregnated with paraffin wax throughout before embedding in wax. Sections were cut 6 μm thick, mounted on slides and stained with cresyl fast violet.

Sections were subsequently examined under a light microscope and areas CA1 and CA3 examined for damage. The damage was quantified in the CA1 region by selecting three sections approximately 2000–2500 μm from the site of the needle track and taking the average number of intact, surviving neurones at a magnification of $100\times$. A comparable count was made of neurons in the contralateral, unaffected side of the hippocampus, and the number of intact cells on the damaged side (a mean of the three sections counted) was then expressed as a percentage of the control side. As an indication of the number of cells per field counted for analysis, the number counted in a series of 10 control brains was 282×14. In all cases, the damaged and control sides were examined in the same coronal sections. Four animals were used for each data point, except for the preliminary data with quinolinic acid, where $n=3$. In a separate preliminary series of five brains, it was confirmed that there was no significant difference between the number of neurons in a CA1 field of view from an animal which had received no treatment or surgery, and a field from the contralateral side of a test animal.

Data analysis

The results are presented as mean$\pm$s.e.mean. Analysis of variance (ANOVA) followed by the Bonferroni post-test for multiple comparisons was used to determine any statistical significance. Significance refers to results where $P<0.05$ was obtained.

Drugs

All compounds were obtained from Sigma Chemicals, with the exception of 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3e]-1,2,4-triazolo[1,5c]pyrimidine (SCH58261) and 4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}(1,3,5}triazin-5-yl-amino]ethyl)phenol (ZM241385), which were partly obtained as gifts from Dr E. Ongini, Schering-Plough Research Institute, and Dr S. Poucher, Zeneca Pharmaceuticals respectively. The latter compound was also purchased from Tocris Chemicals. 8-(3-chlorostyryl)caffeine (CSC) was obtained from Research Biochemicals, Inc. All compounds were dissolved initially in dimethyl-sulphoxide and subsequently diluted to the required concentration for injection. The final concentration of dimethyl-sulphoxide was never more than 0.1%, which in earlier pilot experiments did not influence neuronal viability.

Results

Effects of quinolinic acid

Normal intact pyramidal neurones had a clearly rounded appearance with a clear nucleus and nucleolus (Figure 1). Quinolinic acid produced a dose-dependent effect in the CA1 and CA3 regions of the hippocampus with no obvious or significant damage being seen at the lower doses of 40 nmols (4.3% cell loss $\pm$2.4, $n=3$) and 80 nmols (14.1% loss $\pm$3.3, $n=3$) but a substantial and usually almost total destruction at the highest dose (91.1% loss $\pm$6.5, $n=3$). This relationship was consistent with our previous studies (Behan *et al*., 1999; Behan & Stone, 2000), in which a dose of 120 nmols was found to produce a substantial degree of damage against which neuroprotective agents could be assessed. The border between damaged areas of CA1 and adjacent undamaged areas was sharply defined, as noted by Foster & Schwarcz (1989). There was also an infiltration of the pyramidal cell layers and surrounding tissue by microglial cells after the higher dose. Of the damaged areas, the CA1 area was selected for the quantification of the damage and protection as described in Methods, and the intermediate 80 nmols dose of quinolinic acid was selected for some of the later combination studies with potentially neuroprotective agents.

Effects of free radical generators

At the dose employed in this study, which was largely determined by the limited solubility of the compounds used, the mixture of xanthine and xanthine oxidase produced no obvious neuronal damage when injected alone (Figure 2). Similarly, the nitric oxide donor S-nitroso-N-acetylpenicillamine (SNAP) produced no significant neuronal loss when administered alone into the hippocampus at either of the doses tested (100 and 500 nmols). The higher dose was therefore selected for further experiments (Figure 3).

Combined administration

The two lower doses of quinolinate were tested in combination with the xanthine/xanthine oxidase mixture. With the lowest dose damage was apparent as a loss of healthy cells in the CA1 area when the two agents were administered simultaneously, with a proportion of cells showing general shrinkage, with enlarged and disrupted but darkly-staining nuclei or nuclear remnants (Figure 1). The damage induced by the combinations of xanthine/xanthine oxidase with either dose of quinolinic acid was very significantly greater than that produced by either agent alone (Figure 2), the combination based on quinolinic acid at 40 nmols producing a combined loss of 35% of neurons, and the combination involving 80 nmols of quinolinic acid yielding a loss of 81% of CA1 neurones.

The combined administration of quinolinate and SNAP (500 nmols) also produced significant damage with either the

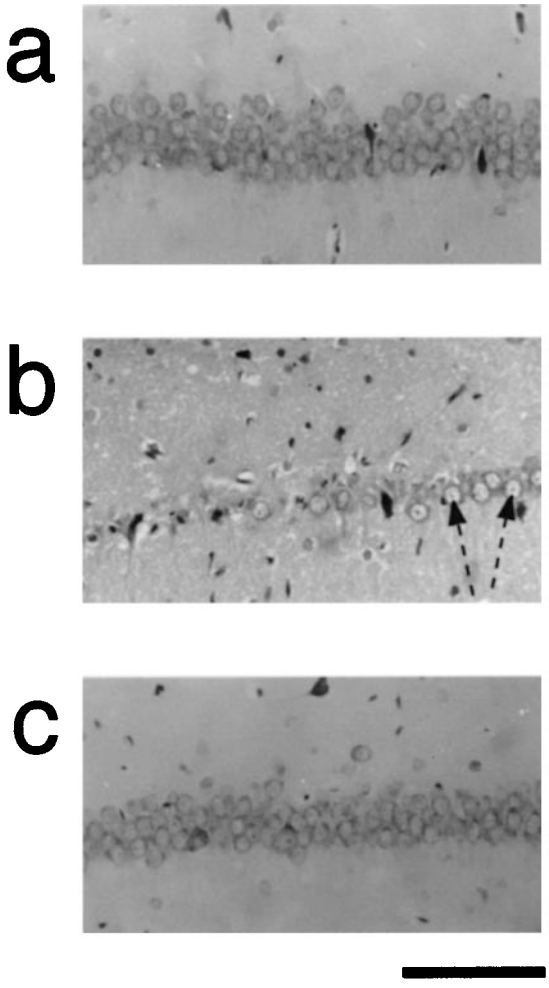

Figure 1 Photomicrographs of the CA1 area of the rat hippocampus. (A) Control section, showing the CA1 neurons as clear, rounded cells in a well-organized layer. (B) Following the administration of a combination of quinolinic acid 80 nmols with xanthine/xanthine oxidase (100 μM with 0.1 U ml^{-1}), the CA1 layer is largely devoid of neurons, leaving a disorganized layer with a few neurons remaining intact (arrows). (C) The co-administration of ZM241385 together with quinolinic acid and the xanthine/xanthine oxidase mixture protects against the damage, leaving a largely intact cell layer in this example. Scale bar:-100 μM.

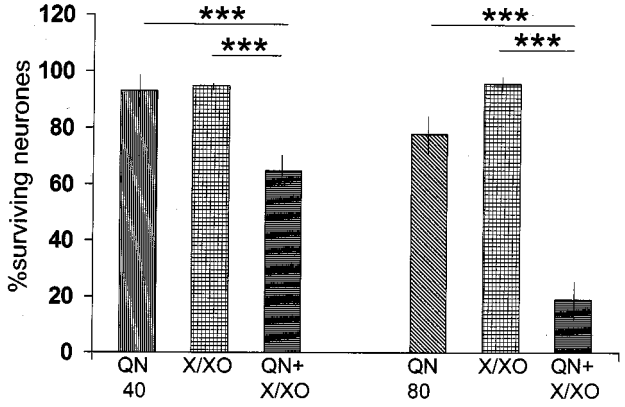

Figure 2 Histogram illustrating the extent of neuronal damage induced by intrahippocampal injections of quinolinic acid at doses of 40 or 80 nmols (QN40, QN80) or a mixture of xanthine 100 μM with xanthine oxidase (0.1 U ml^{-1}) (X/XO). When administered alone, neither injection induced a significant degree of cell loss, but when administered simultaneously, a highly significant loss of neurons was observed (QN + X/XO). The columns show the mean $\pm$ s.e.mean ($n=4$) of the percentage of neurons surviving in the CA1 region of the counted sections. ***$P<0.001$.

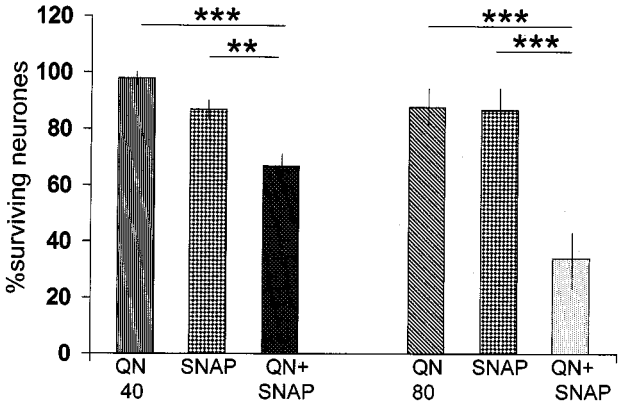

Figure 3 Histogram illustrating the extent of neuronal damage induced by intrahippocampal injections of quinolinic acid at doses of 40 or 80 nmols (QN40, QN80) or SNAP 500 nmols. When administered alone, neither injection induced a significant degree of cell loss, but when administered simultaneously, a highly significant loss of neurons was observed. The columns show the mean ± s.e.mean ($n=4$) of the percentage of neurons surviving in the CA1 region of the counted sections. $^{**}P<0.01$; $^{***}P<0.001$.

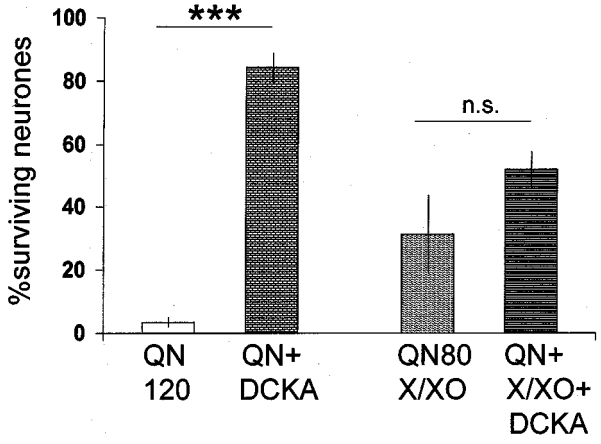

Figure 4 Histogram illustrating the extent of neuronal damage induced by intrahippocampal injections of quinolinic acid at a dose of 120 nmols (QN120) or a mixture of quinolinic acid 80 nmols with xanthine 100 μM and xanthine oxidase (0.1 U ml^{-1}). Both these injections induced a highly significant degree of cell loss. When administered simultaneously, 5,7-dichlorokynurenic acid, 1 nmol (DCKA) was able to block completely the damage produced by quinolinic acid (QN + DCKA) but not that produced by the combination of quinolinic acid and the free radical-generating mixture (QN + X/XO + DCKA). The columns show the mean ± s.e.mean ($n=4$) of the percentage of neurons surviving in the CA1 region of the counted sections. $^{***}P<0.001$.

40 or 80 nmol doses of the excitotoxin (Figure 3), the combination with the higher dose producing a loss of 66% of neurons in the CA1 region.

Effect of 5,7-dichlorokynurenic acid

The substantial degree of neuronal loss resulting from the administration of quinolinic acid at the high dose of 120 nmols is indicated in Figure 4. In this series of animals, only 3.5% of neurons remained intact after 7 days. 5,7-dichlorokynurenic acid was administered at a dose of 1 nmol, which had been found in preliminary experiments to block the damage produced by 120 nmols of quinolinic acid. This activity was confirmed in the present series, in which 5,7-dichlorokynurenate produced a highly significant protection when combined with 120 nmols of quinolinic acid compared with the administration of quinolinic acid alone (Figure 4).

A combination of 80 nmols of quinolinic acid with the xanthine/xanthine oxidase free-radical generating mixture generated a loss of 69% of the CA1 neurons, a value not significantly different from that seen in the experimental group illustrated in Figure 2. Against this combination, however, 5,7-dichlorokynurenate was not able to afford a significant degree of protection, yielding only a tendency towards protection and a neurone count of 52% of that in control sections (Figure 4).

Protection by purines

Using the combination of 80 nmols quinolinic acid with xanthine/xanthine oxidase, the potentially protective effects of antagonists at adenosine receptors were also examined. Administration of the A_{2A} receptor antagonist ZM241385 at a dose of 2.5 pmols, which we have previously reported to protect against damage induced by kainic acid (Jones *et al.*, 1998a, b) produced a small change in the proportion of surviving neurons from 27% ± 5.4 to 40% ± 7.7 ($n=3$) which was not statistically significant. At a higher dose of 25 pmols, significant protection was seen compared with the control quinolinic acid and xanthine/xanthine oxidase mixture (Figure 5). Similarly, another non-xanthine compound SCH58261 had little effect at a dose of 5 pmols, increasing the proportion of surviving neurons from 22% ± 8.1 to 38% ± 6.4, whereas a higher dose of 50 pmols did produce a significant protection as illustrated in Figure 5. The xanthine-derived A_{2A} receptor antagonist chlorostyrylcaffeine (CSC) also protected against the damage produced by the toxic combination, at a dose of 100 pmols (Figure 5).

Exactly comparable results were obtained when the adenosine receptor ligands were examined against the combination of quinolinic acid and SNAP (Figure 6). Chlorostyrylcaffeine and the higher doses of ZM241385 and SCH58261 were able to protect against cell loss in the CA1 region induced by the administration of the 500 nmols dose of SNAP (Figure 6).

Discussion

Injury to the brain results in glial cell proliferation and infiltration both by activated glial cells, and by activated peripheral macrophages which can cross the blood–brain barrier. These activated cells exhibit a greatly enhanced secretion of quinolinic acid synthesized from tryptophan (Heyes *et al.*, 1992a, 1996; Espey *et al.*, 1997; Pemberton *et al.*, 1997; see Stone, 1993, 2001). Since quinolinic acid is a selective agonist at NMDA-sensitive glutamate receptors (Stone & Perkins, 1981; Stone & Burton, 1988), one consequence of its activation of NMDA receptors is excitotoxicity (Schwarcz *et al.*, 1983; 1984), and the massive elevation of its concentration within the brain in neuro-inflammatory states or in AIDS dementia (Heyes *et al.*, 1989, 1991, 1992b), has raised the possibility

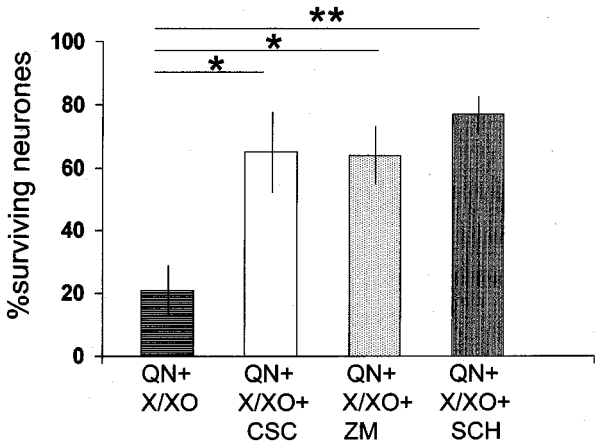

Figure 5 Histogram illustrating the extent of neuronal damage induced by intrahippocampal injections of quinolinic acid 80 nmols in combination with a mixture of xanthine 100 μM with xanthine oxidase (0.1 U ml^{-1}). A highly significant degree of neuronal loss is produced by this combination (QN+X/XO), which is prevented by the simultaneous administration of chlorostyrylcaffeine, 100 pmols (CSC), ZM241385, 25 pmols (ZM) or SCH58261, 50 pmols (SCH). The columns show the mean$\pm$s.e.mean ($n=4$) of the percentage of neurons surviving in the CA1 region of the counted sections. *$P<0.05$; **$P<0.01$.

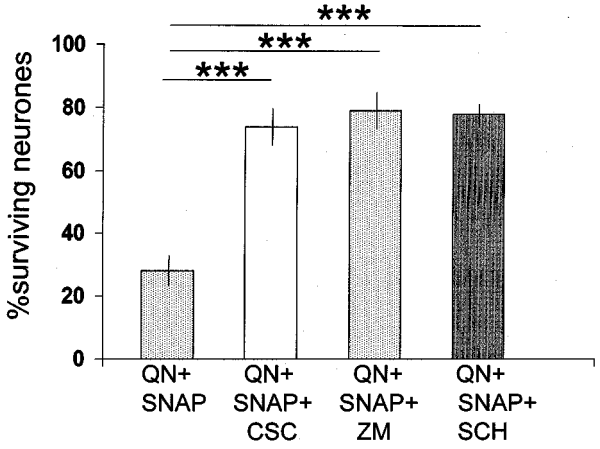

Figure 6 Histogram illustrating the extent of neuronal damage induced by intrahippocampal injections of quinolinic acid 80 nmols in combination with SNAP 500 nmols. A highly significant degree of neuronal loss is produced by this combination, which is prevented by the simultaneous administration of chlorostyrylcaffeine (CSC), ZM241385 (ZM) or SCH58261 (SCH). The columns show the mean$\pm$s.e.mean ($n=4$) of the percentage of neurons surviving in the CA1 region of the counted sections. ***$P<0.001$.

that it may contribute to the brain damage and cognitive dysfunction that can occur in these and neurodegenerative conditions.

In general, however, the amount of quinolinate in brain is below the level which is sufficient to produce damage, and the question arises of whether quinolinate-induced damage may be potentiated by other compounds secreted during the neuro-inflammatory reaction. In this study, we have demonstrated that doses of quinolinate which alone produced no signs of overt cell damage, will destroy a large proportion of hippocampal neurones in the additional presence of xanthine and xanthine oxidase, a well-recognized generator of reactive oxygen species such as superoxide and hydroxyl radicals.

It has been suggested that the free radicals generated by the xanthine/xanthine oxidase combination can promote the release of excitatory amino acids in hippocampal slices (Pellegrini-Giampietro *et al*., 1988), so that the combination tested here could still be acting *via* glutamate receptors, partly by the direct action of quinolinic acid, and partly by the indirect action of free radicals releasing glutamate. However, the glutamate antagonist 5,7-dichlorokynurenate did not reduce significantly the mean level of neuronal damage, despite the fact that it could substantially reduce the damage produced by a higher dose of quinolinic acid alone. This suggests that the site of potentiation between quinolinic acid and free radical-induced damage is distal to activation of the NMDA receptor, and is not simply the result of free radical-enhanced glutamate release or a free radical-mediated enhancement of NMDA receptor toxicity. Alternatively, damage could result from a completely different mechanism of one or both of the agents. It is unlikely that non-NMDA receptors are involved, since there is no evidence for an action of quinolinic acid at such sites, and 5,7-dichlorokynurenic acid has high selectivity for the strychnine-resistant glycine site of the NMDA receptor (IC_{50} 200 nM) compared with kainate ($IC_{50}>300$ μM), quisqualate ($IC_{50}>30$ μM) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (IC_{50} 75 μM) (Leeson *et al*., 1991). Since the activation of NMDA receptors by NMDA itself or by quinolinic acid can increase the generation of free radicals (Hammer *et al*., 1993; Lafon-Cazal *et al*., 1993; Rios & Santamaria, 1991; Behan *et al*., 1999) the combination of agents used here may induce mutually potentiated form of oxidative stress.

The involvement of free radicals is supported by the combinations of nitric oxide and quinolinic acid, since nitric oxide and related species such as peroxynitrite are potent free radicals able to produce lipid peroxidation. Several studies have shown that nitric oxide donors can produce neuronal damage *in vivo* (Loiacono & Beart, 1992; Gross *et al*., 1994) and *in vitro* (Dawson *et al*., 1994b; Palluy & Rigaud, 1996). Similarly, despite some reports to the contrary, a majority of studies indicate that NOS inhibitors reduce ischaemic damage (Dawson *et al*., 1994a; Panahian *et al*., 1996; Margaill *et al*., 1997). These views are consistent with the present finding of increased damage in the presence of quinolinic acid and a nitric oxide donor.

The present data are also consistent with the report by Guidetti & Schwarcz (1999) showing potentiation of striatal damage by a combination of quinolinic acid and 3-hydroxykynurenine. The latter is another member of the kynurenine pathway which produces neuronal damage *via* the generation of free radicals (Eastman & Guilarte, 1990; Okuda *et al*., 1996). Taken together, all these data suggest that the neurotoxic properties of even low concentrations of quinolinic acid can be enhanced substantially by the simultaneous presence of reactive oxygen species. Since the production of both is raised in microglia and CNS macrophages following damage, inflammation or immune activation, this combination could account for a significant proportion of the delayed neurodegeneration which occurs after excitotoxic or ischaemic insults.

Neuroprotection by adenosine antagonists

Adenosine or its analogues can reduce excitotoxicity and ischaemic damage *via* A_1 receptors (von Lubitz *et al*., 1988; Macgregor & Stone, 1993; Heron *et al*., 1994) or A_{2A} receptors (Sheardown & Knutsen, 1996; Jones *et al*., 1998a, b). Evidence for neuroprotection by an A_{2A} receptor antagonist was first suggested by Gao & Phillis (1994). This was later confirmed and extended to other ischaemic models and excitotoxic damage, using a range of A_{2A} receptor antagonist ligands (Phillis, 1995; von Lubitz *et al*., 1995; Ongini *et al*., 1997; Monopoli *et al*., 1998; Jones *et al*., 1998a, b). The selective adenosine antagonist ZM241385, which is 80-fold selective for A_{2A} *versus* A_{2B} receptors, and 500–1000-fold selective for A_{2A} *versus* A_1 receptors (Palmer *et al*., 1995), was reported to protect the hippocampus against kainic acid to a similar degree as the agonist CGS21680 (Jones *et al*., 1998a, b). In support, work using A_{2A} receptor knockout mice has revealed that ischaemic brain injury is decreased compared with normal mice (Bona *et al*., 1997; Chen *et al*., 1999).

In the present study three chemically distinct antagonists at A_{2A} receptors, including the xanthine CSC and non-xanthines ZM241385 and SCH58261, protected against quinolinic acid. The doses used were chosen to target A_{2A} receptors with some selectivity at the sections used for cell counting. The dilution factor of an agent injected in a volume of 1 μl but subsequently diluting into a sphere of radius 2500 μm (the distance selected for assessment, see Methods) is approximately 30. This would yield a concentration of around 4 μM for CSC, the K_i for which at A_{2A} receptors is 54 nM, while the K_i at rat A1 receptors is 28 μM (Jarvis & Williams, 1989; Jacobson *et al*., 1993). Similarly, ZM241385 is likely to reach a concentration of about 1 μM, compared with its K_i at A_{2A} receptors of approximately 1 nM, a K_i of 3 μM at A1 receptors and 100 μM at A3 receptors (Poucher *et al*., 1995). Both these compounds should, therefore, completely saturate A_{2A} receptors, while leaving A1 receptors relatively unaffected. SCH58261 was used at a relatively high concentration which is expected to reach around 2 μM at the site or analysis, compared with a K_i of 1 nM at striatal A_{2A} receptors and over 100 nM at A1 receptors (Cunha *et al*., 1996; Ongini *et al*., 1997). The high dose was chosen because of evidence that the hippocampal A_{2A} receptor is less sensitive to SCH58261 than the striatal receptors (Johansson *et al*., 1993; Johansson & Fredholm, 1995; Lindstrom *et al*., 1996). In all cases, however, blockade of A1 receptors should increase cell damage, rather than protect, and would tend to oppose, rather than explain, the present data.

The most likely mechanism by which blockade of A_{2A} receptors can produce protection is the suppression of glutamate release. Activation of A_{2A} receptors has an excitatory action on neurones, partly *via* an increased release of glutamate (Simpson *et al*., 1992; Sebastião & Ribeiro, 1992; Popoli *et al*., 1995). The blockade of A_{2A} receptors, therefore, may reduce the extracellular concentrations of glutamate below a threshold necessary for cell damage. In addition, since A_{2A} receptors suppress responses mediated by A_1 sites (Lopes *et al*., 1999; O'Kane & Stone, 1998), A_{2A} receptor antagonists will 'unmask' the A_1 receptors and thus permit a degree of protection by A_1 receptors.

There may also be effects of A_{2A} receptor antagonists on glial cell function. Activation of A_{2A} receptors suppresses phagocytic activity (Zalavary *et al*., 1994); antagonists should therefore facilitate the removal of dying cells and toxic materials from a region of injury. In addition, although A_{2A} receptors inhibit the production of several pro-inflammatory cytokines from cells (Dianzani *et al*., 1994), they can also potentiate the pro-inflammatory effects of those compounds (Scholz-Pedretti *et al*., 2001). The protective effect of A_{2A} receptor antagonists may therefore reflect a net reduction of pro-inflammatory activity in the damaged region. Activation of A_{2A} receptors can promote glial proliferation after brain injury (Rathbone *et al*., 1999; Hindley *et al*., 1994). Since these cells produce anti-inflammatory cytokines as well as pro-inflammatory compounds and kynurenines, the net balance of these may influence neuronal survival.

Whatever the mechanism, the ability of A_{2A} receptor antagonists to protect not only aginst excitotoxins, but also against combined excitotoxic/free radical injury suggests that this class of compounds could be of value in preventing neuronal damage resulting from a variety of insults.

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