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Polyhydroxylated C_{60}, Fullerenol, a Novel Free-radical Trapper, Prevented Hydrogen Peroxide- and Cumene Hydroperoxide-elicited Changes in Rat Hippocampus In-vitro

M-C. TSAI, Y. H. CHEN* AND L. Y. CHIANG†

*Department of Pharmacology, *College of Medicine and †Centre for Condensed Matter Sciences, National Taiwan University, Taipei, Taiwan*

Abstract

The role of polyhydroxylated C_{60} (fullerenol), a novel free-radical trapper, in prevention of hydrogen peroxide- and cumene hydroperoxide-elicited damage was studied in hippocampal slices from the rat in-vitro. The interactions of polyhydroxylated C_{60}, adenosine and 6,7-dinitroquinoxaline-2,3-dione (DNQX) were also compared.

Hydrogen peroxide (0·006–0·02%) and cumene hydroperoxide (0·5–1·0 mM) both reversibly reduced the amplitudes of CA1-evoked population spikes in the hippocampal slices. Deferoxamine (1 mM) had little effect on the population spikes. Deferoxamine (1 mM) significantly prevented the hydrogen peroxide (0·006%)-elicited inhibition of the population spikes. Polyhydroxylated C_{60} (0·1 mM) significantly prevented hydrogen peroxide- or cumene hydroperoxide-elicited reduction of the population spikes and also prevented the effects of hydrogen peroxide and cumene hydroperoxide on paired-pulse facilitation in the hippocampal slice. Adenosine reduced the amplitude of population spikes and promoted paired-pulse facilitation in the CA1 region of the hippocampus. Polyhydroxylated C_{60} did not alter either of the effects of adenosine on the population spikes. DNQX reduced the amplitude of the population spikes in the CA1 region but did not affect the ratio of paired-pulse facilitation. Fullerenol did not alter either effect of DNQX on the population spikes.

These results suggested that polyhydroxylated C_{60} prevented hydrogen peroxide- and cumene hydroperoxide-elicited damage in the hippocampus slices. These effects might be associated with the free-radical scavenging activity of polyhydroxylated C_{60}.

Evidence is growing that free radicals are involved in the pathophysiology of ischaemia/reperfusion injury (Cooper et al 1980; Rehncrona et al 1980) and in other neurological conditions including epilepsy (Abbott et al 1990) and Parkinson's disease (Adams & Odunze 1991). The hippocampus has been used as a model of local connectivity patterns (Bernard & Wheal 1994). The electrophysiological consequences of free-radical exposure have been extensively examined in hippocampal slice preparations by use of a variety of generating systems (Pellmar 1986, 1987; Colton et al 1989; Pellmar et al 1992). Exposure of hippocampal tissue to hydrogen peroxide resulted in the production of free radicals through the Fenton reaction (Pellmar et al 1989). It is assumed that the hydroxyl radical, and not peroxide, causes the reduction in synaptic potentials and the ability of synaptic potentials to generate action potentials because dimethylsulphoxide (DMSO, Trolox-C; a free-radical scavenger) and deferoxamine (an iron chelator) prevented most of the peroxide damage (Pellmar et al 1989). These electrophysiological deficits were reversible if the damage was not too severe. Cumene hydroperoxide is also widely used as a free radical generator because it has been reported to cause lipid peroxidation by a free-radical mechanism and electrophysiological alterations in isolated cardiac tissue (Nakaya et al 1987; Gill et al 1995).

Correspondence: M-C. Tsai, Department of Pharmacology, College of Medicine, National Taiwan University, No.1., Sec.1., Jen-Ai Road, Taipei, Taiwan. E-Mail: t9403001@ccms.ntu.edu.tw

Cage molecules, e.g. (60) fullerene (C_{60}) and analogues show high reactivity toward organic radical addition (Krusic et al 1991; Taylor & Walton 1993). This reactivity was, presumably, correlated with the intrinsically large electronegativity of C_{60}. We recently synthesized the water-soluble polyhydroxylated C_{60} by a sequence of reactions involving, as the key step, the electrophilic attack of nitronium tetrafluoroborate on fullerenes in the presence of carboxylic acid (Chiang et al 1992b). By chemical derivatization to fullerene oxide, C_{60} (Fig. 1) was structurally characterized as containing 18–20 hydroxyl groups on average (Chiang et al 1992a, 1993). The reduced chemical reactivity of the remaining, conjugated double bonds in polyhydroxylated C_{60} certainly reduced their inherent biological toxicity to less than that of the parent C_{60}. Combination of the moderate electron affinity and the allylic hydroxyl functional groups of C_{60} made it an appropriate candidate for applications such as a free-radical remover or a water-soluble antioxidant in biological systems. Although we demonstrated that polyhydroxylated C_{60} showed extremely efficient superoxide-radical scavenging activity (Chiang et al 1995), it is unclear whether polyhydroxylated C_{60} can provide protection against free-radical-induced damage in biological systems.

In this study hydrogen peroxide and cumene hydroperoxide were used as free-radical-generating systems to evaluate the preventive effect of polyhydroxylated C_{60} on free-radical-induced damage in the hippocampus in-vitro.

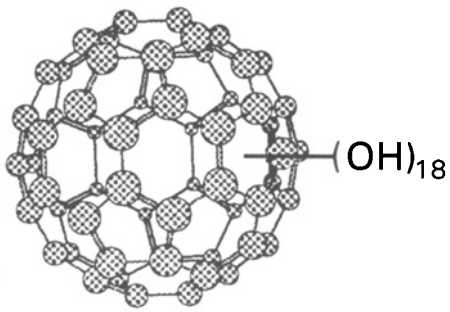

FIG. 1. The chemical structure of polyhydroxylated C_{60} (fullerenol).

Materials and Methods

Preparation and maintenance of slices

Male Wistar rats, 100–140 g, were anaesthetized with ether and decapitated. The hippocampi were rapidly removed and placed in ice-cold, oxygenated (95% O_2 and 5% CO_2) artificial cerebrospinal fluid of composition (mM): NaCl 124, KCl 4, KH_2PO_4 1·0, $MgCl_2 \cdot 6H_2O$ 1·0, $CaCl_2$ 2·5, $NaHCO_3$ 26, glucose 10 (Smith et al 1993). Slices 400 μm thick were cut from the mid-septo-temporal regions of both hippocampi, perpendicularly to the long axis of this structure, with a Sorval tissue chopper, then placed in a recording chamber which was kept at 33–35°C. Ninety-five % O_2 and 5% CO_2 was bubbled through the water bath surrounding the chamber, and the fluid level was maintained above the upper surface of the slice. The perfusion rate was 6 mL min^{-1}. The slices were incubated for 1 h before use (Dunwiddie & Lynch 1978). Drugs were administered through a three-way tap and complete exchange of the chamber volume occurred in 3 min.

Electrophysiology

Stimulation and recording paradigms in the hippocampal slice were followed. Bipolar stimulating electrodes consisted of 300 μm-diameter Teflon-insulated stainless-steel wire, with the return path provided by an Ag-AgCl wire in the bath. Stimulation electrodes were positioned, with visual guidance by use of a dissecting microscope, in the stratum radiatum for orthodromic stimulation of the Schaffer collateral pathway to the CA1 pyramidal cells (Pellmar 1986; Bernard & Wheal 1994). Stimulation conditions were 0·1 ms rectangular pulses of 5–15 V. Low-frequency stimulation was elicited at a frequency of 0·2 Hz. Extracellular recording electrodes with a tip resistance of 1–5 MΩ were filled with 2 mM NaCl and placed in the cell body layer of CA1 to record the somatic response. The amplitude of the population spikes was a function of stimulus intensity. Increasing the intensity of the stimulation also increased the amplitudes of the population spikes. In these experiments the stimulation intensity which elicited half-maximum amplitudes was used. The intensity of the stimulation was maintained constant throughout the experiment. For the paired-pulse stimulation an interval of 50 ms was used. The value for facilitation (paired-pulsed facilitation) was obtained by finding the difference between the amplitudes of the second and the first population spikes; it was recorded as the percentage increase (Nathan et al 1990).

Recorded signals were amplified via a Grass HIP5 probe and Grass P5 AC preamplifier (Model P511) and monitored by means of a Hameg, HM-205-3 oscilloscope. The wave forms were then printed by use of a Hameg HD 148 graphic printer or measured with a Data 6500 (sampling frequency 3–5 kHz). The criterion for accepting a slice and starting a recording session was that maximum stimulation evoked a single population spike with an amplitude at least 5 mV. Stimulation voltages adjusted to elicit baseline population spike amplitudes of half-maximum were approximately 3 to 5 mV (Dunwiddie & Hoffer 1980). The amplitude of the population spike was measured as the distance from the negative peak to the following positive peak (Matsuoka et al 1993).

The mean amplitudes of the population spikes after various treatments were compared by means of Student's two-tailed *t*-test. Differences were considered significant at $P < 0.05$.

All applications of drugs for electrophysiological studies were made through the medium perfused. Except for DNQX and cumene hydroperoxide, all drugs were water-soluble. Stock solutions of drugs were often prepared at high concentrations in distilled water (10 mM) and frozen until use. DNQX and cumene hydroperoxide were dissolved in DMSO and ethanol, respectively, and diluted to the concentration used. The final concentration of DMSO and ethanol in the physiological solution were both 0·1%, which had no effects on the amplitudes of the population spikes.

Adenosine, cumene hydroperoxide, deferoxamine mesylate and hydrogen peroxide were purchased from Sigma (St Louis, MO). 6,7-Dinitroquinoxaline-2,3-dione (DNQX) was purchased from Biomol Research Laboratories (Campus Drive, Plymouth Meeting, PA).

Results

Effects of hydrogen peroxide on the evoked population spikes on the CA1 region of hippocampal slices

The amplitudes of the CA1 population spikes recorded were 3·9 ± 0·2 mV (from 3·0 to 5·9 mV, n = 13). The population spikes remained at the same level even after 200 min incubation. Hydrogen peroxide reduced the amplitudes of CA1-evoked population spikes in hippocampal slices in a concentration-dependent manner. Hydrogen peroxide at concentrations higher than 0·006% reduced the amplitudes of the population spikes. Ten minutes after administration of hydrogen peroxide (0·006%, 0·008% and 0·02%), the amplitudes of the CA1-evoked population spikes were reduced to 32·3 ± 4·9% (n = 8), 15·2 ± 2·8% (n = 3) and 8·2 ± 2·9% (n = 3), respectively, compared with pre-drug control. The effects of hydrogen peroxide (0·006% and 0·008%) on the CA1-evoked population spikes were reversible. After 40 min continuous washing with normal physiological solution, the amplitudes of the population spikes recovered to 91·4 ± 3·3 (n = 6) and 90·5 ± 4·7% (n = 3), respectively. Lower concentrations of hydrogen peroxide did not significantly alter the amplitudes of the population spikes. Ten minutes after administration of hydrogen peroxide (0·004%), the amplitudes of the CA1-evoked population spikes were 98·0 ± 1·0% (n = 3) compared with pre-drug control. Hydrogen peroxide at 0·004% did not significantly affect the amplitudes of the CA1-evoked population spikes in hippocampal slices. If, however, hydrogen peroxide was administered at a higher concentration, e.g. 0·02%, it caused irreversible damage to the population spikes. After 40 min continuous washing with normal physiological

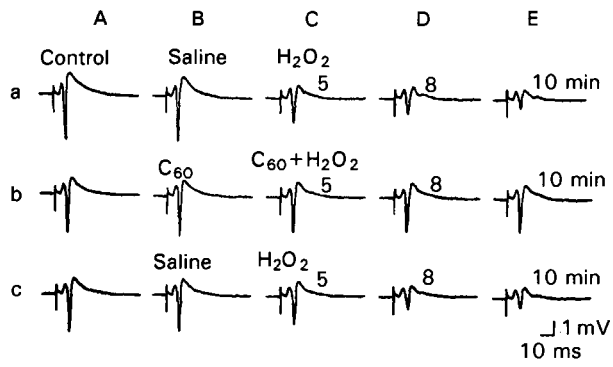

FIG. 2. Effects of polyhydroxylated C_{60} on the depressant effect of hydrogen peroxide in hippocampal slices. a, b, c were the continuous recordings of spikes from the same hippocampal slice. Aa, control, the population spike before treatment of hydrogen peroxide; Ba, saline added for 5 min; Ca, Da and Ea, the CA1-evoked population spikes in hippocampal slice after 5, 8 and 10 min perfusion of hydrogen peroxide (0·006%), respectively; Ab, the CA1 population spike after 40 min continuous washing after Ea; Bb, 5 min after addition of polyhydroxylated C_{60} (0·1 mM); Cb, Db and Eb, the CA1-evoked population spikes after further addition of hydrogen peroxide (0·006%) for 5, 8 and 10 min, respectively; Ac, the CA1 population spike after 40 min continuous washing after Eb; Bc, 5 min after addition of saline; Cc, Dc and Ec, the CA1-evoked population spikes after addition of hydrogen peroxide (0·006%) for 5, 8 and 10 min, respectively. Note that hydrogen peroxide reversibly reduced the amplitudes of population spikes. Polyhydroxylated C_{60} had no effect on the amplitudes of population spikes but prevented hydrogen peroxide-elicited depression on the amplitudes of population spikes.

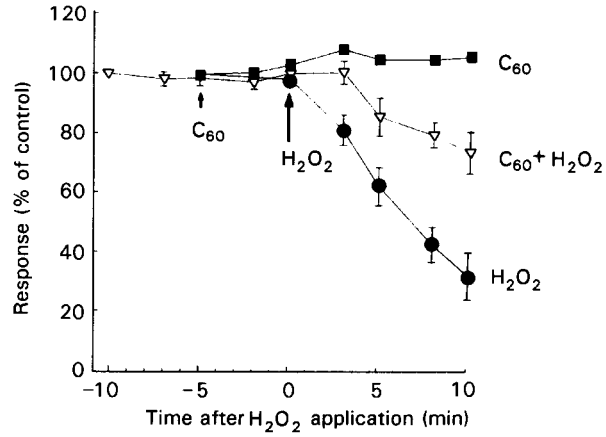

FIG. 3. Responses of population spikes to hydrogen peroxide (0·006%) (●), to polyhydroxylated C_{60} (0·1 mM) (■) and to hydrogen peroxide (0·006%) + polyhydroxylated C_{60} (0·1 mM) (▽). Amplitudes of population spikes were expressed as a percentage of control spikes measured at the start of each line. Hydrogen peroxide (0·006%) was administered at the point indicated by the long arrow and polyhydroxylated C_{60} (0·1 mM) at the point indicated by the short arrow. Each point reflects the mean ± s.e.m. of 3 to 5 preparations. Where no s.e.m. appears, the value is too small to be shown. Note that polyhydroxylated C_{60} prevented hydrogen peroxide-induced depression of population spikes in hippocampus slices and polyhydroxylated C_{60} itself had little effect on the amplitudes of population spikes.

solution, the amplitudes of the population spikes recovered to only 35·3 ± 5·2% (n = 3) compared with pre-drug control.

The effects of hydrogen peroxide on the CA1 population spikes were also dependent on the duration of hydrogen peroxide administration. Hydrogen peroxide at 0·006% caused irreversible inhibition of the CA1-evoked population spikes if it was administered for 20 min. Twenty minutes later the amplitudes of the CA1-evoked population spikes were reduced to 8·3 ± 5·3% (n = 3) compared with pre-drug control. After 40 min continuous washing with normal physiological solution the amplitudes of the population spikes recovered to only 61·3 ± 4·0% (n = 3) compared with pre-drug control. Although hydrogen peroxide at 0·006% caused irreversible inhibition of the CA1-evoked population spikes if it was perfused for 20 min or longer, the population spikes almost recovered to control level if hydrogen peroxide at 0·006% was incubated for 10 min.

Effects of polyhydroxylated C_{60} on the inhibition of population spikes in hippocampal slices by hydrogen peroxide

The effects of polyhydroxylated C_{60} (0·1 mM) on the hydrogen peroxide-elicited inhibition of population spikes in hippocampal slices is shown in Figs 2 and 3 and Table 1. Polyhydroxylated C_{60} (0·1 mM) had no significant effect on the amplitude of the CA1-evoked population spikes in hippocampal slices. The amplitudes of CA1-evoked population spikes were 103·5 ± 0·5%(n = 5), 105·5 ± 0·5% (n = 3) and 106·5 ± 1·5% (n = 3), compared with control level, after administration of polyhydroxylated C_{60} for 5, 10 and 15 min, respectively. Polyhydroxylated C_{60} significantly prevented the inhibitory effects of hydrogen peroxide (0·006%) on the population spikes of hippocampal slices, however. Ten minutes after administration of hydrogen peroxide (0·006%), the amplitude of the population spikes was reduced to 32·3 ± 4·9% (n = 8) compared with the pre-drug level. If polyhydroxylated C_{60} was incubated for 5 min before hydrogen peroxide (0·006%) administration, the amplitude of the population spikes was 103·0 ± 0·5% (n = 5) compared with pre-drug level. Ten minutes after further administration of hydrogen peroxide (0·006%), the amplitude of the population spikes was reduced to 74·6 ± 5·5% (n = 5) only. An example of the interactions between hydrogen peroxide and polyhydroxylated C_{60} are shown in Fig. 2.

Table 1. Effect of pre-treatment with polyhydroxylated C_{60} (fullerenol) on hydrogen peroxide-, cumene hydroperoxide-, adenosine- and DNQX-elicited changes of the amplitudes of evoked CA1 population spikes in hippocampal slices.

	Percentage of control*	
	Without fullerenol	With fullerenol
Hydrogen peroxide (n = 5)	32 ± 8	74 ± 6*
Cumene hydroperoxide (n = 4)	35 ± 6	74 ± 4*
Adenosine (n = 3)	25 ± 1	25 ± 2
DNQX (n = 3)	24 ± 4	22 ± 5

The values represent percentage (mean ± s.e.m.) of the amplitudes of population spikes compared with the pre-drug control. Values were measured 10 min after administration of hydrogen peroxide (0·006%), adenosine (30 mM) or DNQX (2 μM) or 15 min after administration of cumene hydroperoxide (1 μM), either alone or with fullerenol (0·1 mM). *$P < 0·05$, statistically significant by use of the paired t-test. Note that polyhydroxylated C_{60} prevented the hydrogen peroxide- and cumene hydroperoxide-induced depression of the CA1-evoked population spikes but had little effect on adenosine- and DNQX-induced depression.

Effects of hydrogen peroxide on paired-pulse facilitation of the CA1-evoked population spikes

Paired-pulse facilitation, that is the increase in amplitude of the second population spikes in response to a pair of equal size stimulation pulses (Nathan et al 1990; Kahle & Cotman 1993) was examined at an interval of 50 ms. The effects of hydrogen peroxide (0·006%) on the paired-pulse facilitation of population spikes of hippocampal slices is shown in Table 1. A value for facilitation was obtained by finding the difference in amplitude between the first and second population spikes. In the control preparation, the ratio of the amplitude of the second population spike to the first was $119{\cdot}0 \pm 2{\cdot}9\%$ ($n = 9$). Ten minutes after administration of hydrogen peroxide (0·006%), the ratio was increased to $170{\cdot}2 \pm 7{\cdot}2\%$ ($n = 3$). Hydrogen peroxide significantly increased the ratio of the paired-pulse facilitation of the spikes.

Polyhydroxylated C_{60} prevented the effects of hydrogen peroxide on paired-pulse facilitation of the CA1-evoked population spikes

Polyhydroxylated C_{60} (0·1 mM) had no significant effect on paired-pulse facilitation of the population spikes in hippocampal slices. The ratio of the facilitation of population spikes in the control and 5 min after administration of polyhydroxylated C_{60} were $120{\cdot}5 \pm 2{\cdot}7\%$ and $116{\cdot}3 \pm 2{\cdot}5\%$ ($n = 4$), respectively. If pre-treatment with polyhydroxylated C_{60} (0·1 mM) was performed for 5 min and hydrogen peroxide (0·006%) was then administered for 10 min, the ratio of paired-pulse facilitation was $138{\cdot}3 \pm 10{\cdot}7\%$ ($n = 4$). The ratio was significantly reduced compared with the ratio of $170{\cdot}2 \pm 7{\cdot}2\%$ ($n = 3$) for the preparation incubated in hydrogen peroxide for 10 min without pre-administration of polyhydroxylated C_{60}; data not shown). It seemed that hydrogen peroxide (0·006%) increased the paired-pulse facilitation of hippocampal slices and this effect was prevented by polyhydroxylated C_{60}, which had no effect on paired-pulse facilitation.

Effects of deferoxamine (1 mM) on the hydrogen peroxide-elicited reducing effect on population spikes in hippocampal slices

Deferoxamine (1 mM) had no significant effect on the amplitudes of the CA1-evoked population spikes in hippocampal slices. The amplitudes of CA1-evoked population spikes were $97{\cdot}5 \pm 0{\cdot}3\%$ (compared with control level; $n = 3$) after 30 min administration of deferoxamine. Deferoxamine, however, significantly prevented the inhibitory effects of hydrogen peroxide (0·006%) on the population spikes of hippocampal slices. Ten minutes after administration of hydrogen peroxide (0·006%), the amplitudes of the population spikes were reduced to $32{\cdot}3 \pm 4{\cdot}9\%$ ($n = 8$) compared with pre-drug level. If deferoxamine was incubated for 30 min before hydrogen peroxide (0·006%) administration, the amplitude of the population spikes was $97{\cdot}5 \pm 0{\cdot}3\%$ ($n = 3$) compared with pre-drug level. Ten minutes after further administration of hydrogen peroxide (0·006%), the amplitude of the population spikes was reduced to $85{\cdot}6 \pm 1{\cdot}0\%$ only ($n = 3$). It seemed that deferoxamine almost completely prevented the effects of hydrogen peroxide on the population spikes if deferoxamine was administered previously, that is, before hydrogen peroxide application.

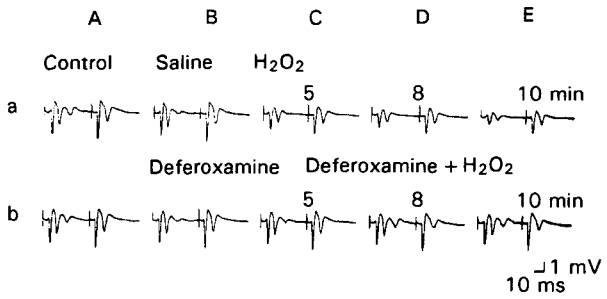

FIG. 4. Effects of deferoxamine on hydrogen peroxide-elicited changes of the amplitudes of paired-pulse facilitation of CA1-evoked population spikes in hippocampal slices. The population spikes were elicited by twin-pulse stimulation with an interval of 50 ms. Aa and Ab were the amplitudes of control and 5 min after normal physiological solution perfusion, respectively. Ca, Da and Ea were the amplitudes 5, 8 and 10 min, respectively, after hydrogen peroxide (0·006%) administration. Ab was the amplitude after 40 min washing after Ea. Bb was the amplitude 30 min after deferoxamine (1 mM) application. Cb, Db and Eb were the amplitudes 5, 8 and 10 min, respectively, after combined administration of deferoxamine (1 mM) and hydrogen peroxide (0·006%). Note that hydrogen peroxide reduced the amplitudes of CA1-evoked population spikes reversibly and also increased the paired-pulse facilitation of CA1-evoked population spikes. Deferoxamine (1 mM) prevented both the reducing effects of hydrogen peroxide on the amplitude of population spikes and the increasing of the paired-pulse facilitation of the spikes.

Deferoxamine prevented the effects of hydrogen peroxide on paired-pulse facilitation of the CA1-evoked population spikes

The effects of deferoxamine (1 mM) on the hydrogen peroxide-elicited increase of paired-pulse facilitation of population spikes in hippocampal slices is shown in Fig. 4. Deferoxamine itself had no significant effect on paired-pulse facilitation of the population spikes in hippocampal slices. The ratio of the facilitation of population spikes in control and 30 min after administration of deferoxamine were $115{\cdot}0 \pm 5{\cdot}0\%$ and $115{\cdot}0 \pm 7{\cdot}9\%$ ($n = 3$).

Hydrogen peroxide did not increase the ratio of paired-pulse facilitation in preparations pre-treated with deferoxamine. Treatment with deferoxamine for 5 min, then hydrogen peroxide (0·006%) for 10 min, gave a ratio of paired-pulse facilitation of $120{\cdot}0 \pm 6{\cdot}6\%$ ($n = 3$), not significantly different from the ratio in the presence of deferoxamine alone. It seemed that hydrogen peroxide (0·006%) increased the paired-pulse facilitation of hippocampal slices, but that this effect was prevented if the preparation was pre-treated with deferoxamine.

Effects of cumene hydroperoxide on the evoked population spikes on CA1 region in hippocampal slices

Cumene hydroperoxide reduced the amplitudes of CA1-evoked population spikes in hippocampal slices in a concentration-dependent manner. Fifteen minutes after administration of cumene hydroperoxide (0·5, 1 mM), the amplitude of the CA1-evoked population spikes was reduced to $70{\cdot}5 \pm 2{\cdot}4\%$ ($n = 3$) and $34{\cdot}5 \pm 5{\cdot}4\%$ ($n = 5$), respectively, compared with the pre-drug control. The effects of cumene hydroperoxide (0·5 and 1 mM) on the CA1-evoked population spikes were reversible. After 40 min continuous washing with normal physiological solution the amplitudes of the population spikes recovered to $94{\cdot}8 \pm 4{\cdot}0$ ($n = 3$) and $90{\cdot}5 \pm 4{\cdot}7\%$ ($n = 3$),

respectively. Cumene hydroperoxide at a concentration of 0·01 mM did not significantly affect the amplitude of the evoked population spikes 30 min after perfusion.

The effects of cumene hydroperoxide on the CA1 population spikes were also dependent on the duration of cumene hydroperoxide administration. Cumene hydroperoxide, at 0·01 mM, did not alter the amplitudes of the CA1-evoked population spikes after 30 min incubation. At 1 mM, however, cumene hydroperoxide caused irreversible inhibition of the CA1-evoked population spikes if it was incubated for 25 min.

Effects of polyhydroxylated C_{60} (0·1 mM) on the cumene hydroperoxide-elicited reducing effect on population spikes in hippocampal slices

Polyhydroxylated C_{60} (0·1 mM) significantly prevented the reducing effects of cumene hydroperoxide (1 mM) on the population spikes of hippocampal slices (Table 1). Fifteen minutes after administration of cumene hydroperoxide, the amplitude of the population spikes was reduced to $36·1 \pm 5·2\%$ ($n = 4$) compared with pre-drug level. If polyhydroxylated C_{60} was incubated for 5 min before administration of cumene hydroperoxide, the amplitude of the population spikes was $102·8 \pm 1·9\%$ ($n = 4$) compared with the pre-drug level. Fifteen minutes after further administration of cumene hydroperoxide, the amplitude of the population spikes was reduced to $75·6 \pm 3·1\%$ ($n = 4$). It seemed that cumene hydroperoxide reduced the amplitudes of the CA1-evoked population spikes and this inhibitory effect was prevented by polyhydroxylated C_{60} (Table 1).

The effects of cumene hydroperoxide on paired-pulse facilitation of the CA1-evoked population spikes

The effects of cumene hydroperoxide on paired-pulse facilitation of population spikes in hippocampal slices are shown in Fig. 5. In the control preparation, the ratio of the second and the first amplitudes of the population spikes was $121·3 \pm 4·1\%$ ($n = 3$). Fifteen minutes after administration of cumene hydroperoxide (1 mM), the ratio was increased to $177·6 \pm 6·1\%$ ($n = 3$). Cumene hydroperoxide significantly increased the ratio of the paired-pulse facilitation.

Polyhydroxylated C_{60} prevented the effects of cumene hydroperoxide on paired-pulse facilitation of the CA1-evoked population spikes

Paired-pulse facilitation was examined at an interval of 50 ms. The effects of polyhydroxylated C_{60} on the paired-pulse facilitation of the CA1-evoked population spikes elicited by cumene hydroperoxide (1 mM) are shown in Fig. 5. In the control preparation, the ratio of the second and the first amplitudes of population spikes was $122·9 \pm 2·4\%$ ($n = 7$). Fifteen minutes after further administration of cumene hydroperoxide and polyhydroxylated C_{60} (0·1 mM), the ratio was $145·9 \pm 9·1\%$ ($n = 3$), compared with $177·6 \pm 6·1\%$ ($n = 3$) in the presence of cumene hydroperoxide. Polyhydroxylated C_{60} significantly reduced the paired-pulse facilitation elicited by cumene hydroperoxide. It seemed that cumene hydroperoxide increased the paired-pulse facilitation of hippocampal slices and that this effect was prevented by polyhydroxylated C_{60}.

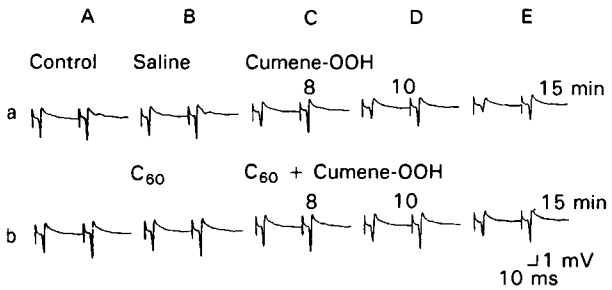

FIG. 5. Effects of polyhydroxylated C_{60} on cumene hydroperoxide-elicited changes of the amplitudes of paired-pulse facilitation of CA1-evoked population spikes in hippocampal slices. The population spikes were elicited by twin-pulse stimulation with an interval of 50 ms. Aa and Ab were the amplitudes of control and 5 min after normal physiological solution perfusion, respectively. Ca, Da and Ea were the amplitudes 5, 10 and 15 min, respectively, after cumene hydroperoxide (1 mM) administration. Ab was the amplitude after 40 min washing after Ea. Bb was the amplitude 30 min after administration of polyhydroxylated C_{60} (0·1 mM). Cb, Db and Eb were the amplitudes 8, 10 and 15 min, respectively, after administration of polyhydroxylated C_{60} (0·1 mM) and cumene hydroperoxide (1 mM). Note that cumene hydroperoxide (1 mM) reduced the amplitudes of CA1-evoked population spikes reversibly and also increased the paired-pulse facilitation of CA1-evoked population spikes. Polyhydroxylated C_{60} (0·1 mM) prevented both the reducing effects of cumene hydroperoxide on the amplitude of population spikes and the increasing of the paired-pulse facilitation of the spikes.

Effects of adenosine on evoked population spikes in hippocampal slices

The effects of adenosine, a pre-synaptic modulator (Kahle & Cotman 1993; Wu & Saggau 1994), on the evoked population spikes and the interactions between polyhydroxylated C_{60} (0·1 mM) and adenosine on the population spikes were tested. Ten minutes after adenosine (30 μM) administration, the amplitude of the population spikes was reduced to $26·7 \pm 0·4\%$ compared with pre-drug level. Adenosine significantly reduced the evoked population spikes in hippocampal slices.

Effects of polyhydroxylated C_{60} (0·1 mM) on the adenosine-elicited reducing effect of the evoked population spikes in hippocampal slices

Polyhydroxylated C_{60} alone had no significant effect on the evoked population spikes. In preparations pre-treated with polyhydroxylated C_{60} for 5 min then addition of adenosine (30 mM) to the slices for 10 min, the amplitude of the population spikes was $26·6 \pm 1·3\%$ ($n = 3$) compared with control (Table 1). It seemed that adenosine significantly reduced the amplitudes of the population spikes in the hippocampus slice at the CA1 region and polyhydroxylated C_{60} did not alter the reducing effects of adenosine on the population spikes of the hippocampal slices.

Effects of adenosine on paired-pulse facilitation of the CA1-evoked population spikes

The effects of adenosine on the paired-pulse facilitation of CA1-evoked population spikes in hippocampal slices were examined at an interval of 50 ms (Fig. 6). Adenosine (30 μM) significantly increased the paired-pulse facilitation of population spikes. The ratio of facilitation of population spikes in control was $109·0 \pm 3·5\%$ ($n = 3$). The ratio was increased to $195·2 \pm 15·0\%$ ($n = 3$) 10 min after administration of adenosine (30 mM).

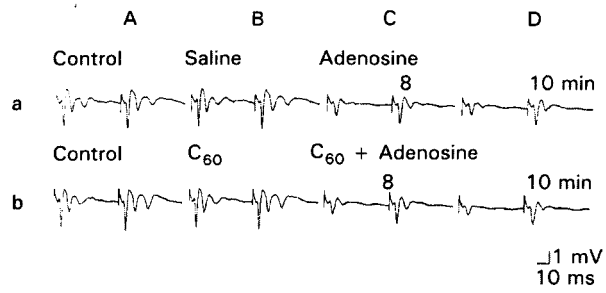

FIG. 6. Effects of adenosine (30 μM) and polyhydroxylated C_{60} (0·1 mM) on the paired-pulse facilitation of CA1-evoked population spikes in hippocampal slices. a and b were from the same hippocampal slice. Aa and Ba were the amplitudes of control and 5 min after perfusion with physiological solution, respectively. At Ca and Da, adenosine (30 μM) was applied for 8 and 10 min, respectively. At Db, adenosine (30 μM) was washed off for 15 min. At Bb, polyhydroxylated C_{60} (0·1 mM) was applied for 5 min. At Cb and Db, polyhydroxylated C_{60} (0·1 mM) and adenosine (30 μM) were added for 8 and 10 min, respectively. Note that polyhydroxylated C_{60} did not alter the effects of adenosine on the population spikes and the paired-pulse facilitation.

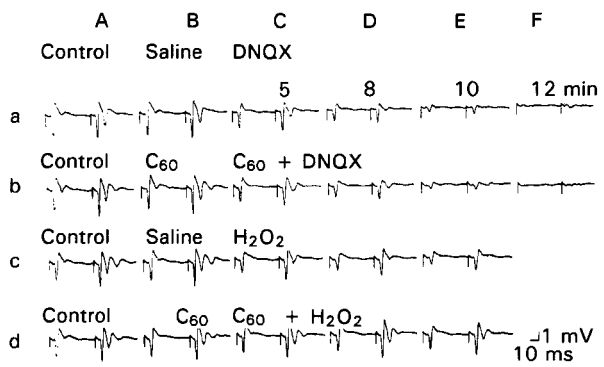

FIG. 7. Effects of DNQX, hydrogen peroxide and polyhydroxylated C_{60} on the population spikes and paired-pulse facilitation elicited of CA1-evoked population spikes. a, b, c and d were continuous recordings from the same hippocampal slice. Aa and Ba were the spikes of control and 5 min after saline administration, respectively. Ca, Da, Ea and Fa were the spikes 5, 8, 10 and 12 min, respectively, after DNQX (2 μM) administration. Ab was after 60 min continuous washing after Fa. Bb was 5 min after administration of polyhydroxylated C_{60} (0·1 mM). Cb, Db, Eb and Fb were the spikes 5, 8, 10 and 12 min, respectively, after administration of DNQX (2 μM) and polyhydroxylated C_{60} (0·1mM). Ac was the amplitude after 60 min washing after Fb. Cc, Dc and Ec were the amplitudes 5, 8 and 10 min after application of hydrogen peroxide (0·006%). Ad was after 40 min washing after Ec. Cd, Dd and Ed were 5, 8 and 10 min, respectively, after application of polyhydroxylated C_{60} (0·1 mM) and hydrogen peroxide (0·006%). Note that polyhydroxylated C_{60} did not prevent the effects of DNQX on the population spikes yet did prevent the effects of hydrogen peroxide on the spikes.

Failure of polyhydroxylated C_{60} to prevent the effects of adenosine on paired-pulse facilitation of the CA1-evoked population spikes

In the preparation pre-treated with polyhydroxylated C_{60} (0·1 mM) for 5 min then treated with adenosine (30 μM) for a further 10 min, the ratio of paired-pulse facilitation was 197·1 ± 3·0% (n = 3). Compared with the preparation treated with adenosine (30 μM) alone, it seemed that adenosine increased the paired-pulse facilitation of hippocampal slices and that polyhydroxylated C_{60} did not alter the effects of adenosine on paired-pulse facilitation (Fig. 6).

Effects of DNQX on evoked population spikes in hippocampal slices

The effects of DNQX, a non-NMDA receptor antagonist, on the evoked population spikes in hippocampal slices were tested. Ten minutes after administration of DNQX (2 μM), the amplitude of the CA1-evoked population spikes was reduced to 26·1 ± 4·5% (n = 3), compared with pre-drug control (Table 1). Twelve minutes after DNQX administration, the amplitudes of the CA1-evoked population spike was completely abolished. The effect of DNQX on the evoked population spikes was reversible. After 60 min washing with normal physiological solution to remove DNQX, the amplitudes of the population spikes recovered to control level.

Effects of polyhydroxylated C_{60} (0·1 mM) on the DNQX-elicited reducing effect of evoked population spikes in hippocampal slices

Polyhydroxylated C_{60} alone had no significant effect on the evoked population spikes. In the preparation pre-treated with polyhydroxylated C_{60} for 5 min then treated with DNQX (2 μM) for a further 10 min, the amplitudes of the population spikes were 23·4 ± 4·6% compared with pre-drug control (Table 1). Compared with the population spikes in the presence of DNQX alone, as shown previously, it seemed that polyhydroxylated C_{60} did not alter the effects of DNQX on the CA1-evoked population spikes in hippocampal slices. After 12 min administration of DNQX (2 μM) the amplitudes of the CA1-evoked population spikes were completely abolished although polyhydroxylated C_{60} was still being continuously perfused.

Effects of DNQX on paired-pulse facilitation of the CA1-evoked population spikes

The effects of DNQX on the paired-pulse facilitation of CA1-evoked population spikes in hippocampal slices were examined at an interval of 50 ms. The ratio of the paired-pulse facilitation in the control and in preparations pre-treated with DNQX (2 μM) for 10 min were 118·5 ± 2·5% (n = 3) and 118·5 ± 0·5% (n = 3), respectively. DNQX did not alter the paired-pulse facilitation of the population spikes. Examples of the effects of polyhydroxylated C_{60} (0·1 mM) on the DNQX-elicited paired-pulse facilitation of the CA1-evoked population spikes in hippocampal slices are shown in Fig. 7. In the preparations pre-treated with polyhydroxylated C_{60} (0·1 mM) for 5 min then treated with DNQX (2 μM) the ratio of paired-pulse facilitation was 117·0 ± 4·0% (n = 3). Polyhydroxylated C_{60} (0·1 mM) did not affect the effect of DNQX on paired-pulse facilitation.

Discussion

In this study hydrogen peroxide (0·006%) reversibly reduced the amplitudes of the population spikes in the hippocampus slices. Although deferoxamine alone had little effect on the amplitudes of the CA1-evoked population spikes, it almost completely prevented hydrogen peroxide-elicited damage of the population spikes.

Hydrogen peroxide had been used as a model for free-radical damage in a number of systems. Hydroxyl radicals could be generated in-vitro through the Fenton reaction; per-

oxide reacted with iron intrinsic to the tissue to produce this very reactive free radical (Mello Filho et al 1984; Pellmar 1986, 1987). Free-radical scavenger (dimethylsulphoxide, Trolox-C) and an iron chelator (deferoxamine) prevented most of the peroxide damage, suggesting that hydroxyl radicals, and not the peroxide itself, were the reactive oxygen species (Pellmar et al 1989).

Deferoxamine is a very effective iron chelator that can make the metal completely inaccessible to the Fenton reaction (Graf et al 1984). It was reported to protect against hydrogen peroxide-induced synaptic damage, impaired spike generation, and lipid peroxidation in hippocampus slices from the guinea-pig (Pellmar et al 1989). To check whether the hydrogen peroxide-elicited reducing effect on population spikes in the current study was mediated by free radicals produced from the Fenton reaction, the effects of deferoxamine on the reducing actions of hydrogen peroxide on the population spikes were tested. Our studies revealed that deferoxamine also protected against the effects of hydrogen peroxide on the population spikes in hippocampal slices. It seemed that the hydrogen peroxide-elicited damage in this study might be mainly caused by hydroxyl radicals. We also found that polyhydroxylated C_{60} (0·1 mM) reversibly prevented the hydrogen peroxide-elicited damage to the CA1-evoked population spikes.

Cumene hydroperoxide has been reported to cause lipid peroxidation by a free-radical mechanism that provokes electrophysiological alteration in isolated cardiac tissue (Nakaya et al 1987; Gill et al 1995). In the current study we also found that cumene hydroperoxide (1 mM) reversibly reduced the CA1-evoked population spikes and that polyhydroxylated C_{60} (0·1 mM) prevented the reduction in population spikes elicited by cumene hydroperoxide.

There are several compounds, such as adenosine and DNQX which reduced the population spikes by mechanisms other than free-radical-elicited damage. Adenosine inhibited the evoked synaptic transmission in region CA1 of the hippocampus primarily by reducing presynaptic calcium influx (Wu & Saggau 1994). DNQX blocked the CA1 field-evoked excitatory postsynaptic potentials and orthodromic population spikes (Andreasen et al 1989; Herreras et al 1989) and was a potent non-NMDA receptor (Daw et al 1993) antagonist (Drejer & Honore 1988; Honore et al 1988). In the current study we found that adenosine and DNQX also reduced the amplitudes of the CA1-evoked population spikes. Pre-administration of polyhydroxylated C_{60} (0·1 mM) did not, however, prevent the reducing effects of adenosine and DNQX on the amplitudes of the population spikes. The results supported the view that the blocking effects of adenosine and DNQX on the population spikes were a result of calcium influx as well as on the non-NMDA receptor, and not through free-radical damage.

Paired-pulse facilitation of population spikes was tested by twin-pulse stimulation. When synapses in the hippocampus were stimulated twice in rapid succession, there was facilitation of the second response in comparison with amplitude of the initial response (Andersen 1960a, b; Dunwiddie & Lynch 1978). Manipulations that reduced transmitter release (e.g. reduced calcium in the perfusion medium), had been shown to increase synaptic facilitation in the hippocampal CA1 region (Creager et al 1980). Adenosine was found to reduce synaptic transmission yet increase paired-pulse facilitation in the CA1 region (Dunwiddie & Haas 1985). In these studies we also found that adenosine (30 mM) reduced the CA1-evoked population spikes yet increased paired-pulse facilitation. It was notable that DNQX, which apparently acted on postsynaptic sites, reduced the CA1-evoked population spikes although it did not alter paired-pulse facilitation. It is interesting to note that polyhydroxylated C_{60} did not alter the paired-pulse facilitatory effects elicited by both adenosine and DNQX. The results also supported the view that polyhydroxylated C_{60} did not interfere with the actions of adenosine and DNQX on the amplitudes of population spikes.

In CA1 pyramidal cells of the hippocampal slice preparation, intracellular recorded excitatory postsynaptic potentials and inhibitory postsynaptic potentials were significantly reduced by exposure to peroxide, whereas responses to iontophoretically applied GABA and glutamate were unaffected. It has been suggested that peroxide has presynaptic actions on the CA1 region of hippocampus (Pellmar 1987). In the current studies, hydrogen peroxide (0·006%) reduced the amplitudes of population spikes yet increased the ratio of paired-pulse facilitation of hippocampal slices. It was possible that the process involved was interference with a presynaptic process to reduce transmitter release. Polyhydroxylated C_{60} also prevented the paired-pulse facilitation elicited by hydrogen peroxide, although paired-pulse facilitation is also modulated by recurrent inhibition. The sites of action of polyhydroxylated C_{60} on the hippocampal slices remains the subject of further studies.

Cage molecules, e.g. (60) fullerene (C_{60}) and analogues exhibited high reactivity toward organic radical addition (Krusic et al 1991; Taylor & Walton 1993). The newly synthesized polyhydroxylated C_{60} has been demonstrated to show extremely efficient superoxide radical scavenging activity (Chiang et al 1995). In the current study polyhydroxylated C_{60} prevented hydrogen peroxide- and cumene hydroperoxide-induced depression of CA1-evoked population spikes whereas it had little effect on adenosine- and DNQX-induced depression.

Free radicals are involved in many neurological diseases (Adams & Odunze 1991). Hydroxyl radicals attack membrane lipids and cellular proteins, and thus disrupt cell function. The protective mechanisms of polyhydroxylated C_{60} on peroxide-elicited damage of the neuronal membrane requires further study. The pharmaceutical and pharmacological applications of polyhydroxylated C_{60} against free-radical-elicited damage, and the therapeutic value of the material are interesting subjects for further testing.

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