Biochem. J. (1988) 253, 425–433 (Printed in Great Britain)

Menadione-treated synaptosomes as a model for post-ischaemic neuronal damage

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Menadione bisulphite increased endogenous oxygen-radical production by rat brain synaptosomes, as indicated by H_2O_2 generation. Increased oxygen-radical production was also demonstrated in synaptosomes prepared from menadione-treated rats and synaptosomes reoxygenated after an anoxic insult. Acetylcholine synthesis *de novo* was inhibited in synaptosomes incubated with menadione *in vitro*, in synaptosomes prepared from menadione-treated animals *in vivo*, and in depolarized post-anoxic synaptosomes. Intrasynaptosomal free Ca^{2+} was increased by menadione *in vitro* (50 μM), but this increase was not due to stimulation of Ca^{2+} entry into the nerve terminals. Acetylcholine release was stimulated by menadione *in vitro*, possibly as a consequence of the elevated intrasynaptosomal Ca^{2+} content. The Ca^{2+} contents of synaptosomes prepared from menadione (10 mg/kg)-treated animals *in vivo* and synaptosomes reoxygenated after anoxia were unchanged. In synaptosomes prepared from menadione-treated animals, acetylcholine release was no longer significantly stimulated by K^+, whereas it was unchanged from control (normoxic) values in synaptosomes reoxygenated after anoxia. None of these treatments caused any measurable damage to the synaptic plasma membrane (as judged by the release of lactate dehydrogenase), or to synaptosomal phospholipases (as judged by choline release from membrane phospholipids). Synaptosomes prepared from menadione-treated rats were found to be a good model for the study of post-anoxic damage to nerve-terminal function.

INTRODUCTION

The brain is particularly susceptible to free-radical damage, since the concentrations of antioxidant enzymes [superoxide dismutase (EC 1.15.1.1), catalase (EC 1.11.1.6) and glutathione peroxidase (EC 1.11.1.9)] are relatively low (Cohen, 1985), whereas the concentrations of polyunsaturated fatty acids and iron are relatively high (Halliwell & Gutteridge, 1986). It has been proposed that, during reoxygenation/reperfusion after cerebral anoxia/ischaemia, neuronal damage may be incurred by the generation of oxygen radicals and subsequent lipid peroxidation (Rehncrona *et al.*, 1980). A possible role for perturbed Ca^{2+} homoeostasis in the evolution of brain injury has also been suggested (Siesjo, 1981; Siesjo & Wieloch, 1985). However, the pathogenic events that give rise to irreversible brain damage during recirculation are not yet clearly defined.

Harvey *et al.* (1982) have shown that depolarized synaptosomes are a useful model for assessing anoxic damage to the nerve ending. Post-anoxic recovery of the respiratory rate and acetylcholine synthesis *de novo* were decreased by stimulation of the synaptosomes with veratridine. These parameters were also decreased in veratridine-depolarized synaptosomes from rats made hypoxic *in vivo* (Booth *et al.*, 1983). A similar decline in incorporation of [U-^{14}C]glucose into acetylcholine in anoxic synaptosomes was reported by Ksiezak & Gibson (1981).

K^+-stimulated acetylcholine release was decreased in anoxic synaptosomes, but this returned to control values on reoxygenation at pH 7.4 and in the presence of Ca^{2+} (Sanchez-Prieto *et al.*, 1987). Peterson & Gibson (1984) demonstrated a decrease in K^+-stimulated Ca^{2+} uptake into synaptosomes under hypoxic and anoxic conditions. Therefore the decline in Ca^{2+}-dependent release of acetylcholine during anoxia may be due to altered Ca^{2+} homoeostasis and/or the decreased availability of acetylcholine because of the inhibition of acetylcholine synthesis.

In the present experiments menadione was employed to increase the endogenous superoxide-radical formation from coenzyme Q (Cadenas *et al.*, 1977). The effect of the free radicals thus formed should be more physiologically relevant than the use of an exogenous free-radical-generating system, such as hypoxanthine/xanthine oxidase (Hillered & Ernster, 1983; Braughler *et al.*, 1985*a*,*b*). Superoxide dismutase catalyses the dismutation of the superoxide radical to give H_2O_2, which is able freely to permeate cell membranes (Halliwell & Gutteridge, 1986). Superoxide dismutase is active within all areas of the rat brain (Loomis *et al.*, 1976; Mizuno & Ohta, 1986) and is located in the cytosol (the Cu^{2+}- and Zn^{2+}-containing form) and the mitochondrial matrix (the Mn^{2+}-containing form). Synaptosomes were exposed to menadione, anoxic insult and reoxygenation, and the concentration of H_2O_2 produced was taken as an indication of the generation of oxygen radicals. The effect of these treatments on neurological competence was assessed by the ability of nerve terminals to synthesize and release acetylcholine and to maintain Ca^{2+} homoeostasis. Some of the results reported here have been presented in a preliminary form (White & Clark, 1987).

MATERIALS AND METHODS

Materials

Chemicals required for chemiluminescence assays were as described in Willoughby *et al.* (1986). $^{45}CaCl_2$

(1.87 mCi/ml) was purchased from Amersham International, Amersham, Bucks., U.K. Veratrine hydrochloride and menadione sodium bisulphite salt were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Quin-2 acetoxymethyl ester was purchased from Lancaster Synthesis, Morecombe, Lancs, U.K. All other chemicals were of analytical grade and were made up in double-glass-distilled deionized water.

Animals

Male Wistar rats (50–70 days old) were provided with food and water *ad libitum*. In some experiments rats were injected subcutaneously with menadione bisulphite dissolved in water (10 mg/kg body wt. per day) for 5 weeks, or with an equivalent concentration (360 mM) of $Na_2S_2O_5$ (6.9 mg/kg body wt.).

Preparation of synaptosomes

Synaptosomes prepared by the method of Booth & Clark (1978) were suspended in a modified Krebs–Henseleit buffer of the following composition for all experiments employing menadione [final concns. (mM)]: NaCl 136, KCl 5.6, $MgCl_2$ 1.2, $NaHCO_3$ 16.2, NaH_2PO_4 1.2, $CaCl_2$ 2.2, glucose 10, Hepes 10 (pH 7.4). When synaptosomes were to be exposed to anoxic insult, they were resuspended in Krebs phosphate buffer (NaCl 141, KCl 5.0, $MgSO_4$ 1.3, Na_2HPO_4 10, glucose 10, $CaCl_2$ 1.2, pH 7.4) rather than the Krebs–Henseleit buffer. For each of the parameters measured there was no difference between control experiments where the synaptosomes were resuspended in Krebs phosphate buffer (for subsequent anoxic insult) compared with those where the synaptosomes were resuspended in Krebs–Henseleit buffer (for subsequent menadione treatments), except when measuring intrasynaptosomal Ca^{2+} content (see below).

When acetylcholine metabolism was to be determined, the synaptosomes were preincubated before resuspension at 37 °C, for 15 min at 4 °C in the appropriate buffer (see the legends to Tables 1 and 4) containing phospholine (12.5 μM) and then washed by centrifugation and resuspension three times in buffer without phospholine to remove the excess phospholine.

Synaptosomes were made anoxic by resuspension in Krebs phosphate buffer (pregassed with pure N_2 at 37 °C for 2 h) and 30 min preincubation in glass conical flasks capped with Subaseals carrying a gassing line (delivering N_2 gas) suspended above each incubation. Post-anoxic synaptosomes were exposed to N_2 atmosphere for 25 min as above, and for 5 min to pure O_2 atmosphere. Normoxic control conditions were carried out similarly, but pure O_2 was supplied for the whole experiment. Over 60 min, less than 2% evaporation occurred in experiments using this protocol. The rate of gas flow was 50 ml/min. Experiments were carried out at 37 °C in a shaking water bath.

O_2 consumption by synaptosomes was measured with a Clark-type oxygen electrode. Lactate dehydrogenase was measured by the method of Bergmeyer (1963). Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard. Statistical analyses were carried out by Student's *t* test.

H_2O_2 production

Samples (100 μl) of the final synaptosome suspension (about 0.5 mg of protein) were added to the appropriate buffer (see the legends to Figs. 1 and 2 and Table 4) in micro-centrifuge tubes so that the final volume of incubation was 1 ml after additions. After 15 min preincubation at 37 °C, the appropriate additions were made (see the Results section) in 40 μl of the appropriate buffer. The incubations were terminated after 2 min by centrifugation at 10000 *g* for 1 min. Samples (50 μl) of the supernatant obtained were added to 950 μl of chemiluminescence buffer cocktail, which contained 0.2 M-NaH_2PO_4 (pH 8.6) and 16 μM-luminol. The samples were loaded into a LKB 1251 luminometer (LKB Wallac, Turku, Finland) controlled by an Apple IIe microcomputer. Peroxidase (0.7 Sigma unit/ml final concn.) was automatically injected into each sample. H_2O_2 was determined by peak integration and quantified by comparison with a standard curve (linear) constructed by using 0–1 nmol of H_2O_2. The time-integrated voltage signal was used rather than the peak voltage signal as a measure of H_2O_2 concentration in the sample (see Willoughby *et al.*, 1986).

Choline and acetylcholine release from synaptosomes

Phospholine-washed synaptosomes were added to the appropriate buffer (see the legends to Tables 2 and 5) in micro-centrifuge tubes containing the appropriate additions, so that the final volume of incubation was 1 ml and the final protein content was about 0.5 mg/ml. After 30 min incubation at 37 °C, the incubations were terminated by centrifugation at 10000 *g* for 1 min. Choline and acetylcholine were assayed in the supernatant by the chemiluminescence method described by Israel & Lesbats (1981, 1982), as modified by Willoughby *et al.* (1986).

Acetylcholine metabolism

Total acetylcholine content, by the chemiluminescence method to detect acetylcholine concentration, and acetylcholine synthesis *de novo* from [U-^{14}C]glucose were measured as described by Willoughby *et al.* (1986).

Measurement of $^{45}Ca^{2+}$ uptake

Portions (250 μl) of the final synaptosome suspension were added to the appropriate buffer (see the legends to Tables 3 and 6) in 5 ml plastic vial inserts so that the final volume of incubation was 1 ml after additions. After 15 min preincubation, the appropriate additions and $^{45}CaCl_2$ (final sp. radioactivity 0.18 Ci/mol) were added in a 60 μl volume of Krebs phosphate or Hepes buffer as appropriate. After a total period of incubation with $^{45}Ca^{2+}$ of 10 min, the incubations were terminated by placing a 250 μl portion into 4 ml of ice-cold Krebs buffer. The synaptosomes were then separated from the incubation medium by rapid filtration through Whatman GF/C filters (2.5 cm diameter) on a filtration manifold. The filters were washed with 3 × 4 ml of Krebs buffer and subsequently dried and placed in 5 ml of liquid-scintillation cocktail [0.1 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene, 5.0 g of 2,5-diphenyloxazole in 1 litre of toluene] for radioactivity counting in a Philips PW 4700 liquid-scintillation counter.

Intrasynaptosomal Ca^{2+} concentration

The procedure of Ashley *et al.* (1984) was followed, with modifications, to load synaptosomes with quin-2. The crude synaptosomal pellet was resuspended in the appropriate buffer (see the legends to Tables 3 and 6) at

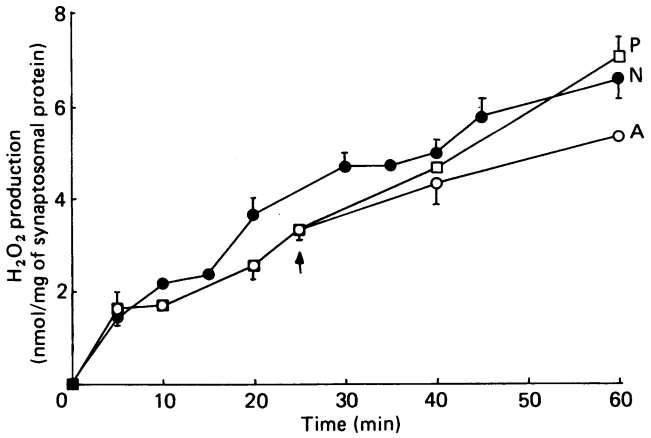

Fig. 1. Time course of H_2O_2 production in synaptosomes under various conditions

Data are expressed as means ± S.E.M. (bars) from three experiments, where the S.E.M. lies outside the dimension of the symbol. Normoxic synaptosomes (N, ●) were initially resuspended in pre-oxygenated Krebs phosphate buffer and incubated in an atmosphere of pure O_2 (see the Materials and methods section). Anoxic synaptosomes (A, ○) were initially resuspended in Krebs phosphate that was pre-equilibrated with N_2 and then incubated in an atmosphere of pure N_2 throughout the experiment. Post-anoxic synaptosomes (P, □) were initially resuspended in a similar manner to anoxic synaptosomes and incubated in an atmosphere of pure N_2 for the first 25 min of the incubation and then in pure O_2 (indicated by arrow).

approx. 100 mg of protein/ml, and incubated at 37 °C for 10–15 min. After a 10-fold dilution of the incubation with the appropriate buffer, samples were placed in glass vials and quin-2 acetoxymethyl ester (50 μM) in dimethyl sulphoxide was added, or dimethyl sulphoxide alone (1 %, v/v, initially) for control incubations. After 30 min at 37 °C, during which the vials were shielded from the light, a further 10-fold dilution was made with the appropriate buffer, and the incubations were divided into 1.5 ml portions. The diluted suspension was centrifuged at 10000 *g* for 2 min and washed with the appropriate buffer at 37 °C; finally the pellets were resuspended in 1.5 ml of the appropriate buffer just before being placed in a 1 cm-square quartz cuvette. Fluorescence was measured with a Perkin–Elmer LS-3 fluorescence spectrometer. Excitation and emission wavelengths of 339 nm and 492 nm were employed. Calibration was performed as described by Tsien *et al.* (1982).

There was an increase in Ca^{2+} content of synaptosomes incubated in Krebs–Henseleit medium (which contained 2.2 mM-$CaCl_2$) compared with those incubated in Krebs phosphate (which contained 1.2 mM-$CaCl_2$); this compares well with the dependence of intrasynaptosomal Ca^{2+} content on the extrasynaptosomal Ca^{2+} concentration found by Nachshen (1985), using quin-2-loaded synaptosomes.

RESULTS

Free oxygen-radical generation by anoxic synaptosomes

When synaptosomes were resuspended in Krebs phosphate buffer (that had been pre-equilibrated with pure O_2 at 37 °C, so that the O_2 concentration in the medium was 450 μM initially) and incubated for 30 min in an atmosphere of pure O_2, the rate of O_2 consumption by the synaptosomes was 6.7 ± 0.2 ($n = 15$) nmol of O/min per mg of protein. Krebs phosphate buffer pre-equilibrated with pure N_2 at 37 °C contained 47 μM-O_2, and the rate of respiration of synaptosomes resuspended in this medium and incubated for 30 min in an atmosphere of pure N_2 was 1.8 ± 0.4 ($n = 11$) nmol of O/min per mg of protein. Over a further 30 min the respiration rate remained constant; however, when synaptosomes were incubated in this manner for 25 min and then reoxygenated for 5 min, there was a partial recovery in the rate of oxygen consumption, to 4.1 ± 0.4 ($n = 4$) nmol of O/min per mg of protein. Harvey *et al.* (1982) obtained a full recovery of synaptosomal respiration in post-anoxic conditions; however, this was after a shorter period of anoxic insult (10 min).

The production of H_2O_2 by synaptosomes after initial resuspension in the appropriately pregassed Krebs phosphate medium is illustrated in Fig. 1. After 60 min under the appropriate gassing conditions (see the legend), H_2O_2 generation was significantly less in anoxic synaptosomes (A) compared with those in normoxic (N) ($P < 0.05$) and post-anoxic (P) ($P < 0.005$) conditions. H_2O_2 production from post-anoxic synaptosomes was not significantly different from that of normoxic synaptosomes. In the absence of O_2, there was a greater H_2O_2 generation than might be expected. This may be because under this experimental protocol the synaptosomes were not truly anoxic initially. The medium in which these synaptosomes were resuspended contains 47 μM-O_2, which is consumed by the synaptosomes over the initial 25 min, before becoming anoxic. Therefore under these conditions there is sufficient O_2 present for the generation of free radicals. This period of hypoxia before complete anoxia must also occur *in vivo* on commencement of an anoxic/ischaemic insult as endogenous O_2 concentrations are consumed. The stimulated H_2O_2 generation by reoxygenation of anoxic synaptosomes is comparable with the increased free oxygen-radical generation occurring on recirculation after ischaemia (Siesjo, 1981). In the presence of veratrine (80 μM) similar trends are seen for H_2O_2 production under each of these conditions, but the actual values are slightly elevated (approx. 30 %; results not shown).

Acetylcholine metabolism and Ca^{2+} turnover in anoxic synaptosomes

Acetylcholine synthesis *de novo* was stimulated by K^+ (Table 1), probably to replace the acetylcholine that has been released from the nerve terminal. The inhibition of acetylcholine synthesis *de novo* by veratrine may be due to a decrease in the availability of acetyl-CoA. Acetylcholine synthesis *de novo* and the acetylcholine content were significantly decreased under anoxic conditions in resting and stimulated synaptosomes. In resting synaptosomes, acetylcholine synthesis *de novo* and acetylcholine content fully recovered on reoxygenation, whereas there was only partial recovery in stimulated synaptosomes as compared with their respective normoxic values. This compares well with the results obtained by Harvey *et al.* (1982), and is an indication that depolarized synaptosomes are more vulnerable to post-anoxic damage than are resting synaptosomes, and therefore represent a better model on which to investigate the irreversible

Table 1. Effect of anoxia on acetylcholine metabolism in synaptosomes

The values are means ± S.E.M. and are expressed per mg of synaptosomal protein, and are for at least three values obtained from two or more separate experiments. Phospholine-washed synaptosomes were resuspended in the appropriately gassed Krebs phosphate medium. Acetylcholine content was measured after 30 min incubation. At zero time the acetylcholine content was 1.14 ± 0.11 ($n = 12$) nmol/mg of synaptosomal protein. Acetylcholine synthesis *de novo* was measured in the same incubations after 30 min. Statistical significances calculated by Student's t test: *$0.05 > P < 0.01$; **$P < 0.01$; ND, not detectable; NS, not significant (i.e. $P > 0.05$). Significances on the left, below each column, are with respect to the resting values for each treatment (i.e. anoxic or post-anoxic). Significances on the right are with respect to the normoxic condition for resting or stimulated (i.e. with veratrine or K^+) synaptosomes.

Condition	Total acetylcholine content (nmol/mg)	Acetylcholine synthesis *de novo* (pmol/min per mg)
Normoxic synaptosomes	1.55 ± 0.02	15.1 ± 0.9
+ 80 μM-Veratrine	1.37 ± 0.03 **	7.1 ± 0.6 **
+ 50 mM-K^+	1.80 ± 0.17 NS	22.9 ± 0.6 **
Anoxic synaptosomes	1.27 ± 0.03 **	4.3 ± 0.2 **
+ 80 μM-Veratrine	1.12 ± 0.07 NS *	ND
+ 50 mM-K^+	1.39 ± 0.05 NS NS	6.8 ± 1.0 NS **
Post-anoxic synaposomes	1.47 ± 0.08 NS	13.3 ± 2.2 NS
+ 80 μM-Veratrine	1.28 ± 0.01 ** *	3.9 ± 0.9 ** *
+ 50 mM-K^+	1.53 ± 0.11 NS NS	15.6 ± 0.3 NS **

damage caused by anoxic insult *in vivo* (Harvey *et al.*, 1982).

Acetylcholine release from synaptosomes was significantly stimulated by 50 mM-K^+ under normoxic and post-anoxic conditions, whereas in anoxic conditions there was no significant stimulation of acetylcholine release (Table 2). This was also observed by Sanchez-Prieto *et al.* (1987), using a crude synaptosomal preparation, and may be due to the decrease in acetylcholine synthesis *de novo* in synaptosomes exposed to anoxic insult, since newly synthesized acetylcholine is preferentially released (Tucek, 1978). Alternatively, the decrease in stimulated acetylcholine release from anoxic synaptosomes may have been due to the concomitant decrease in Ca^{2+} uptake and intrasynaptosomal Ca^{2+} concentration (Table 3).

Anoxic insult significantly decreased both veratrine- and K^+-stimulated uptake of $^{45}Ca^{2+}$ by synaptosomes (Table 3) (cf. Peterson & Gibson, 1984). However, on reoxygenation, K^+-stimulated Ca^{2+} uptake was completely restored, whereas veratrine-stimulated uptake only partially recovered. In addition, anoxic insult significantly decreased the intrasynaptosomal Ca^{2+} concentration in both resting and stimulated synaptosomes (Table 3). However, on reoxygenation the intrasynaptosomal Ca^{2+} concentrations in stimulated preparations returned to those found in normoxic preparations, but those in resting synaptosomes did not recover. The recovery of intracellular Ca^{2+} concentrations in depolarized reoxygenated synaptosomes correlates well with the recovery of both the stimulated acetylcholine release and the Ca^{2+}-uptake rates seen on reoxygenation of the preparations.

Free-radical generation by menadione-treated synaptosomes

Fig. 2 illustrates the enhancement of H_2O_2 production in resting and depolarized synaptosomes by menadione. Incubation *in vitro* in the presence of menadione bisulphite (50 μM) significantly increased endogenous H_2O_2 production by synaptosomes compared with untreated and $Na_2S_2O_5$-treated control synaptosomes (Table 4). The bisulphite form of menadione was employed because it is water-soluble, and the sodium metabisulphite salt was employed as a control since it produces sodium bisulphite when dissolved in aqueous media. The bisulphite moiety is oxidizing, and significantly increased H_2O_2 production by synaptosomes compared with the untreated control conditions.

In each condition where the depolarizing agents (veratrine and increased K^+) were employed, there was generally a slight increase in H_2O_2 generation compared with the unstimulated controls (Table 4), but this was considerably masked by the increase in H_2O_2 production by menadione and bisulphite. In synaptosomes prepared from rats that were treated with bisulphite or menadione *in vivo*, there were small changes in the production of H_2O_2 compared with synaptosomes from untreated rats; however, the overall effect of menadione was negligible compared with its effect *in vitro*.

Acetylcholine metabolism and Ca^{2+} turnover in menadione-treated synaptosomes

Incubation of synaptosomes *in vitro* in the presence of bisulphite (50 μM) had no significant effect on acetylcholine content or acetylcholine synthesis *de novo* in

Table 2. Effect of anoxia on choline and acetylcholine release from synaptosomes

Phospholine-washed synaptosomes were resuspended in the appropriately gassed Krebs phosphate medium and incubated for 30 min in the absence or presence of 50 mM-K^+ while the appropriate gassing conditions were maintained, as described in the Materials and methods section. Choline and acetylcholine were measured by the chemiluminescence method. The results are expressed as pmol/min per mg of synaptosomal protein, and the values represent means ± S.E.M. of at least three values obtained from two or more separate experiments. Further details are as in the legend to Table 1.

Condition	Release (pmol/min per mg) Choline	Acetylcholine
Normoxic synaptosomes	40.4±5.6	2.9±0.5
+50 mM-K^+	48.4±4.6 NS	6.6±0.7 **
Anoxic synaptosomes	44.4±1.6 NS	3.9±0.2 NS
+50 mM-K^+	48.4±5.2 NS NS	4.5±0.3 NS *
Post-anoxic synaptosomes	48.2±2.8 NS	3.3±0.1 NS
+50 mM K^+	59.0±5.0 NS NS	7.2±1.2 * NS

Table 3. Effect of anoxia on Ca^{2+} turnover in synaptosomes

Synaptopsomes were resuspended in the appropriately gassed Krebs phosphate medium and incubated with $^{45}Ca^{2+}$, or loaded with quin-2 as described in the Materials and methods section. The results are expressed as means ± S.E.M. as detailed in the legend to Table 1.

Condition	Ca^{2+} uptake (nmol/10 min per mg)	Intrasynaptosomal Ca^{2+} concn. (nM)
Normoxic synaptosomes	3.2±0.4	163±9
+80 μM-Veratrine	9.3±0.4 **	328±26 **
+50 mM-K^+	5.5±0.6 **	365±25 **
Anoxic synaptosomes	2.6±0.6 NS	128±12 *
+80 μM-Veratrine	5.5±0.7 * **	183±18 ** **
+50 mM-K^+	3.6±0.3 NS *	197±20 ** **
Post-anoxic synaptosomes	3.0±0.7 NS	105±8 **
+80 μM-Veratrine	6.2±0.8 * *	291±22 ** NS
+50 mM-K^+	5.0±0.7 * NS	334±24 ** **

resting or stimulated conditions (Table 4). When menadione was used in place of bisulphite, there was a significant decrease in acetylcholine synthesis *de novo* in resting and stimulated synaptosomes and a significant

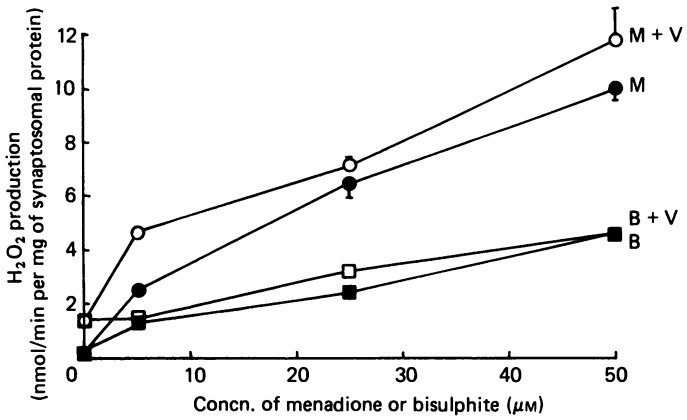

Fig. 2. Dose/response curves of menadione and bisulphite on H_2O_2 production

Data are expressed as means ± S.E.M. (bars) from three experiments, where the S.E.M. lies outside the dimension of the symbol. The synaptosomes were initially resuspended in Krebs–Henseleit medium. After 15 min preincubation, menadione (M, ●) or bisulphite (B, ■) was added to the resting incubations, and menadione + veratrine (M + V, ○) or bisulphite + veratrine (B + V, □) were added to the stimulated incubations. Veratrine was employed at 80 μM. After 2 min the incubations were terminated and H_2O_2 was measured as described in the Materials and methods section.

decrease in the acetylcholine content of resting synaptosomes. However, there was no significant effect on the acetylcholine content of stimulated synaptosomes (Table 4).

Depolarized synaptosomes from rats treated *in vivo* with menadione showed a significant decrease in acetylcholine synthesis *de novo* compared with synaptosomes from untreated rats; however, there was no apparent effect in resting synaptosomes from the menadione-treated rats (Table 4). When compared with synaptosomes from rats treated *in vivo* with bisulphite, there was an overall decrease in acetylcholine synthesis *de novo* and acetylcholine content in resting and stimulated synaptosomes. The effect of menadione *in vivo* on acetylcholine content was similar to its effect *in vitro*.

There was no significant alteration in basal or stimulated acetylcholine release by synaptosomes exposed to bisulphite *in vitro*, but menadione significantly increased both of these parameters (Table 5). The effect of menadione on acetylcholine release was greater when applied *in vitro* than *in vivo*, which was similar to the effect obtained by menadione on H_2O_2 production. The resting and stimulated uptake of $^{45}Ca^{2+}$ into synaptosomes was not significantly perturbed by menadione or bisulphite applied *in vitro* (Table 6). Synaptosomes that were prepared from rats treated *in vivo* with menadione had slightly increased resting and K^+-stimulated Ca^{2+} uptake. The actual difference between basal and stimulated Ca^{2+} uptake was very similar in each of these conditions; therefore it appears that exposure *in vivo* to menadione only affected the basal rate of apparent Ca^{2+} uptake.

The addition of bisulphite to synaptosomes had no significant effect on the resting or depolarized intrasynaptosomal Ca^{2+} concentration (Table 6). Menadione *in vitro* produced a significant increase in resting

Table 4. Effect of menadione on H_2O_2 production and acetylcholine metabolism in synaptosomes

The values are means ± S.E.M. and are expressed per mg of synaptosomal protein, and were for at least three values from two or more separate experiments. Synaptosomes were resuspended in Krebs–Henseleit medium and incubated for 2 min for H_2O_2 production, as described in the Materials and methods section. Acetylcholine metabolism was measured after 30 min incubation of phospholine-washed synaptosomes, resuspended in Krebs–Henseleit medium (see also the legend to Table 1). Significances on left, below each column, are with respect to the resting values for each treatment (i.e. with bisulphite or menadione). Significances on the right are with respect to the control 'untreated' condition for resting or stimulated (i.e. with veratrine or K^+) synaptosomes. Statistical significances were calculated by Student's t test: $*0.05 > P < 0.01$; $**P < 0.01$. Menadione and bisulphite were employed *in vitro* at final concentrations of 50 μM.

Condition	H_2O_2 production (nmol/min per mg)	Total acetylcholine content (nmol/mg)	Acetylcholine synthesis *de novo* (pmol/min per mg)
Control	0.13±0.01	1.57±0.02	11.0±0.5
+80 μM-Veratrine	0.79±0.03 **	1.37±0.04 **	6.4±0.5 **
+50 mM-K^+	1.55±0.07 **	1.82±0.04 **	25.6±1.7 **
Bisulphite *in vitro*	3.06±0.55 **	1.61±0.04 NS	10.6±1.0 NS
+80 μM-Veratrine	4.25±0.14 NS **	1.39±0.01 ** NS	6.4±0.2 ** NS
+50 mM-K^+	4.89±0.25 * **	1.76±0.11 NS NS	25.6±0.9 ** NS
Menadione *in vitro*	12.6±0.34 **	1.43±0.03 **	5.5±0.7 **
+80 μM-Veratrine	12.09±0.81 NS **	1.30±0.03 ** NS	2.7±1.0 ** **
+50 mM-K^+	14.97±0.73 ** **	1.87±0.03 ** NS	11.7±1.1 ** **
Bisulphite *in vivo*	0.44±0.02 **	1.76±0.07 *	18.7±1.6 **
+80 μM-Veratrine	0.42±0.02 NS **	1.42±0.03 ** NS	7.5±0.7 ** NS
+50 mM-K^+	1.77±0.04 ** *	1.80±0.17 NS NS	21.4±1.3 ** *
Menadione *in vivo*	0.56±0.03 **	1.42±0.03 **	11.7±1.5 NS
+80 μM-Veratine	1.05±0.06 ** **	1.30±0.04 * NS	2.7±0.4 ** **
+50 mM-K^+	0.77±0.04 ** **	1.70±0.07 ** NS	9.2±1.3 NS **

intrasynaptosomal Ca^{2+}. Neither menadione nor bisulphite *in vivo* had any effect on the intrasynaptosomal Ca^{2+} concentration.

Synaptosomal integrity

Over 60 min there was less than 10% release of lactate dehydrogenase from synaptosomes under normoxic, anoxic or post-anoxic conditions. Furthermore there was no significant difference between the release of lactate dehydrogenase under any of the gassing conditions employed (results not shown). Similarly, over 30 min there was less than release of 4% lactate dehydrogenase from synaptosomes treated with menadione or bisulphite *in vivo* or *in vitro*, and no significant difference between the lactate dehydrogenase release under any of these conditions and the untreated control values (results not shown). There was no significant loss of choline from membrane lipids (Tables 2 and 5). These observations suggest that the reduced oxygen species generated under these conditions did not cause any measurable damage to the synaptic plasma membrane.

DISCUSSION

Reduced oxygen species in the synaptosome

Menadione can undergo enzymic one-electron reduction to a semiquinone radical, which, in the presence of O_2, can transfer an electron to form the superoxide radical (Cadenas *et al.*, 1977). However, the generation of this radical or any other reactive radical (e.g. hydroxyl) in synaptosomes cannot be readily measured directly, since the reactivity of radicals shows an inverse relationship with their diffusion radius (Weiss & LoBuglio, 1982) and they would be unlikely to cross the synaptic membrane (Halliwell & Gutteridge, 1986) and hence gain access to the assay system in the extra-synaptosomal environment. However, the superoxide radical is likely to be dismuted to H_2O_2 by superoxide dismutase, which is relatively abundant within the synaptosome (Loomis *et al.*, 1976), and the H_2O_2 can readily cross the synaptic plasma membrane and be detected by the luminol–peroxidase chemiluminescence method (Sietz, 1978).

Table 5. Effect of menadione on choline and acetylcholine release from synaptosomes

Phospholine-washed synaptosomes were resuspended in Krebs–Henseleit medium and incubated for 30 min in the absence or presence of 50 mM-K^+ as described in the Materials and methods section. Choline and acetylcholine were measured by the chemiluminescence method. Results are expressed as pmol/min per mg of synaptosomal protein, and the values represent means ± S.E.M. Further details are as indicated in the legend to Table 1.

Condition	Release (pmol/min per mg)	
	Choline	Acetylcholine
Control	40.9 ± 2.2	2.5 ± 0.2
+ 50 mM-K^+	36.4 ± 1.2 NS	4.3 ± 0.3 **
Bisulphite *in vitro*	41.6 ± 4.4 NS	2.4 ± 0.3 NS
+ 50 mM-K^+	35.5 ± 2.0 NS NS	3.6 ± 0.8 NS NS
Menadione *in vitro*	47.4 ± 4.9 NS	4.8 ± 0.5 **
+ 50 mM-K^+	42.2 ± 2.5 NS NS	9.6 ± 0.8 ** **
Bisulphite *in vivo*	—	3.2 ± 0.6 NS
+ 50 mM-K^+	—	4.0 ± 0.3 NS NS
Menadione *in vivo*	—	3.9 ± 0.3 **
+ 50 mM-K^+	—	4.8 ± 0.5 NS NS

Patole *et al.* (1986) have reported rates of H_2O_2 generation by a rat brain crude mitochondrial preparation of 350 pmol/min per mg, which compares well with the values obtained here in resting synaptosomes (130 pmol/min per mg), considering that their crude mitochondrial preparation would contain 'free' mitochondria in addition to synaptosomes.

When synaptosomes are depolarized, oxidative metabolism is stimulated (Harvey *et al.*, 1982), thereby leading to an increased flux through the mitochondrial electron-transport chain. It is likely therefore that veratrine and high concentrations of K^+ stimulated the production of H_2O_2 from synaptosomes (Table 4) by increasing the rate of superoxide-radical formation at this site.

There was no measurable damage to the synaptic plasma membrane when synaptosomes were exposed to menadione or subjected to reoxygenation after anoxia. This may be because superoxide dismutase is able to scavenge the superoxide radicals before they reach the plasma membrane. However, Ca^{2+} chelation within the synaptosomes was altered (Table 6), which indicates that the site of superoxide production may be the mitochondria and/or the endoplasmic reticulum.

When an endogenous free-radical-generating system of Fe^{2+}/ADP/H_2O_2 (Braughler *et al.*, 1985*a*) was employed, synaptosomal Ca^{2+} uptake was stimulated. This influx was mediated by an Fe^{2+}-dependent mechanism, as desferrioxamine (500 μM) completely blocked it.

Table 6. Effect of menadione on Ca^{2+} turnover in synaptosomes

Synaptosomes were resuspended in Krebs–Henseleit medium and incubated with $^{45}Ca^{2+}$, or loaded with quin-2 as described in the Materials and methods section. The results are expressed as means ± S.E.M. as indicated in the legend to Table 1.

Condition	Ca^{2+} uptake (nmol/10 min per mg)	Intrasynaptosomal Ca^{2+} concn. (nM)
Control	3.4 ± 0.1	192 ± 8
+ 80 μM-Veratrine	5.8 ± 0.4 **	437 ± 33 **
+ 50 mM-K^+	5.0 ± 0.3 **	350 ± 33 **
Bisulphite *in vitro*	3.6 ± 0.5 NS	187 ± 15 NS
+ 80 μM-Veratrine	7.1 ± 0.8 ** NS	364 ± 23 ** NS
+ 50 mM-K^+	3.6 ± 0.3 NS **	292 ± 17 ** NS
Menadione *in vitro*	3.1 ± 0.3 NS	313 ± 24 **
+ 80 μM-Veratrine	6.4 ± 0.6 ** NS	522 ± 28 ** NS
+ 50 mM-K^+	5.3 ± 0.3 ** NS	321 ± 16 NS NS
Bisulphite *in vivo*	5.5 ± 0.6 **	182 ± 11 NS
+ 80 μM-Veratrine	6.5 ± 0.4 NS NS	389 ± 36 ** NS
+ 50 mM-K^+	6.1 ± 0.7 NS NS	341 ± 23 ** NS
Menadione *in vivo*	4.1 ± 0.3 *	191 ± 16 NS
+ 80 μM-Veratrine	7.3 ± 1.2 ** NS	362 ± 43 ** NS
+ 50 mM-K^+	6.1 ± 0.4 ** *	302 ± 30 ** NS

The Ca^{2+}-channel blockers verapamil, nifedipine and cinnarizine did not prevent Fe^{2+}-induced Ca^{2+} uptake. This contrasts with the lack of effect of menadione on synaptosomal Ca^{2+} uptake. However, this may be a further indication that menadione is acting at an intracellular site(s) and that the damage so caused is confined to the locality of free-radical generation.

Menadione and Ca^{2+} homoeostasis

It has been proposed from studies on isolated hepatocytes that menadione causes oxidative stress, which results in the depletion of soluble and protein thiol groups, and that the latter perturbs Ca^{2+} homoeostasis and causes loss of cell viability (DiMonte *et al.*, 1984). This effect was not observed until 200 μM-menadione was employed. Menadione (at 50 μM) had no effect on the plasma-membrane Ca^{2+} pump, but caused efflux of Ca^{2+} from the mitochondria and endoplasmic reticulum (Thor *et al.*, 1982), leading to an increase in intracellular Ca^{2+}. At 200 μM menadione also perturbed the plasma-membrane Ca^{2+}-ATPase, causing a more pronounced effect on intracellular Ca^{2+}. In our experiments an increase in intrasynaptosomal Ca^{2+} was observed in the

presence of menadione *in vitro* (50 μM) (Table 6). This was not due to an increased entry of Ca^{2+} into the nerve terminals; therefore it appears that menadione (50 μM) acts in a similar manner on Ca^{2+} homoeostasis in synaptosomes as in isolated hepatocytes. It is possible that menadione induces release of an internal pool of Ca^{2+} in depolarized synaptosomes too, but that the depolarization-induced increase in intrasynaptosomal Ca^{2+} masks any additional effect of menadione.

The decrease in intrasynaptosomal Ca^{2+} concentration in unstimulated synaptosomes under anoxic and post-anoxic conditions may have been due to increased sequestration of Ca^{2+} by synaptic mitochondria, as observed under reperfused conditions in ischaemic rat brain (Meldrum *et al.*, 1985). However, in depolarized synaptosomal preparations, the decrease in intrasynaptosomal Ca^{2+} observed during anoxic insult almost completely recovers during the reoxygenation period and may be associated with similar trends seen in the Ca^{2+}-uptake rates in these preparations.

Exposure of nerve terminals to menadione *in vitro*

Incubation of synaptosomes *in vitro* in the presence of menadione (50 μM) significantly decreased acetylcholine synthesis *de novo* in stimulated synaptosomes (Table 4). A similar effect on acetylcholine synthesis is observed in synaptosomes reoxygenated after a period of anoxia (Table 1). However, menadione also inhibited acetylcholine synthesis in resting synaptosomes, in contrast with the effect of reoxygenation on resting anoxic synaptosomes. This suggests that the incubation with menadione *in vitro* causes more damage than reoxygenation, a conclusion that correlates with the amounts of H_2O_2 produced under these conditions. The K^+-stimulated release of acetylcholine from synaptosomes treated with menadione *in vitro* was significantly increased compared with synaptosomes exposed to bisulphite (Table 5). However, this effect, coupled with the decrease in acetylcholine synthesis, was not extensive enough to cause a decrease in acetylcholine content. The increase in acetylcholine release obtained in menadione-treated synaptosomes does not compare directly with the results obtained from post-anoxic synaptosomes, where there was no significant effect (Table 2). Thus exposure of synaptosomes *in vitro* to menadione is of limited value as a model for post-ischaemic damage; however, it may prove to be a useful model in which to study the effects of agents that scavenge reduced oxygen species, in order to clarify the role of these species in the disruption of the metabolism of nerve terminals.

Exposure of nerve terminals to menadione *in vivo*

When menadione was used *in vivo*, there was no overall effect on synaptosomal H_2O_2 production when compared with bisulphite treatment *in vivo*, and, although the basal concentration of H_2O_2 and acetylcholine release were higher than those from untreated rats, they were not as great as when menadione was added *in vitro* (Tables 4 and 5). Menadione treatment *in vivo* caused a significant decrease in acetylcholine synthesis *de novo* in stimulated synaptosomes (Table 4). This suggests that menadione does enter the brain, increase oxygen-radical production and alter acetylcholine metabolism when injected subcutaneously. If menadione was distributed uniformly throughout the body, the actual concentration in the brain would be approx. 50 μM (assuming that 1 kg body wt. contained 700 ml of water); however, menadione may preferentially accumulate in the lipid environment of the brain. Menadione is metabolized by DT-diaphorase [NAD(P)H:quinone oxidoreductase, EC 1.6.99.2] in the liver (Thor *et al.*, 1982); therefore the precise menadione concentration at the nerve terminals is unknown. The amounts of superoxide radical generated *in vivo*, although sufficient to inhibit acetylcholine synthesis, may be insufficient to cause extensive tissue damage. This may be due to dismutation of the superoxide radical by superoxide dismutase, followed by diffusion of the H_2O_2 so produced out of the nerve terminal into the blood circulation (Halliwell & Gutteridge, 1986). Synaptosomes from rats treated *in vivo* with menadione also showed no significant changes in intrasynaptosomal Ca^{2+} content, and in this respect these preparations were more comparable with depolarized post-anoxic synaptosomes than with synaptosomes treated with menadione *in vitro*. However, synaptosomes prepared from either bisulphite- or menadione-treated animals no longer show stimulation of acetylcholine release with K^+. This may be solely an effect of the bisulphite moiety, and differs from the situation in post-anoxic synaptosomes. Thus exposure of nerve terminals to menadione *in vivo* may prove to be a useful model for post-ischaemic neuronal damage.

In summary, acetylcholine synthesis may be particularly vulnerable to attack by a free-radical mechanism. Menadione *in vitro* and *in vivo*, directly or indirectly, through the production of reduced oxygen species, inhibited acetylcholine synthesis. The dose of menadione employed *in vivo* was sufficiently high to stimulate the production of reduced oxygen species and to damage acetylcholine metabolism, but apparently lower than the concentration used *in vitro*, which additionally altered Ca^{2+} homoeostasis and acetylcholine release.

This work was supported by a grant from the Medical Research Council.

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Received 14 December 1987/3 March 1988; accepted 22 March 1988