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THE EFFECT OF OXIDATIVE STRESS ON LEVELS OF CYTOSOLIC CALCIUM WITHIN AND UPTAKE OF CALCIUM BY SYNAPTOSOMES

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Abstract—The ability of synaptosomes subjected to oxidative stress, to maintain homeostasis has been evaluated using various indices of cellular integrity. These include levels of cytosolic calcium and leakiness of the plasma membrane. The status of a neural characteristic; depolarization-induced calcium entry into the cytoplasm, has also been studied. The presence of 5 μM $FeSO_4$ and 0.1 mM ascorbic acid increased peroxidative activity as judged by the rate of thiobarbituric acid reactive material production, and depressed levels of free ionic calcium $[Ca^{2+}]_i$ as determined using the calcium-sensitive flouorescent indicator dye fura-2. Depolarization-induced influx of $^{45}Ca^{2+}$ was greatly depressed under these conditions, while basal calcium uptake was inhibited to a much lesser degree. The efflux of fura-2 from synaptosomes was enhanced in the oxidizing environment, suggesting increased permeability of the synaptosomal outer limiting membrane.

The treatment of synaptosomes with 25 μM α-tocopherol succinate before and during exposure to the Fe^{2+}/ascorbate mixture prevented many of the changes otherwise induced by the oxidizing system. Similar pretreatment with β-carotene or superoxide dismutase did not have any protective effect. Ganglioside GM_1 pre-exposure did not alter the Fe^{2+}/ascorbate-induced changes in calcium-related parameters, but mitigated synaptosomal plasma membrane damage as judged by fura-2 leakage. Thus exogenous agents may be capable of reducing the severity of oxidative stress in nervous tissue.

Free radical formation can occur as a result of oxidative events leading to the formation of various active oxygen species. The central nervous system may be a tissue especially vulnerable to oxidative stress for several reasons:

(1) The high and continuous metabolic demand of this tissue is concomitant with a very high rate of oxidative phosphorylation, and aerobic mitochondrial activity is unusually large. Transient interruption of the energy and oxygen supply to the brain can lead to subsequent reperfusion injury, of which a significant component is incomplete substrate combustion and consequent formation of reactive free radicals (Rehncrona *et al.*, 1980; Cao *et al.*, 1988). Similarly, an increased formation of lipid peroxide has been found in brain tissue following temporary interruption of oxidative phosphorylation by cyanide (Johnson *et al.*, 1987).

(2) The presence of myelin provides an environment relatively rich in polyunsaturated lipids (Sun and Sun, 1972). These can be a site of sequestration of liphophilic radicals such as the hydrated superoxide radical HO^{2-} (Halliwell and Gutteridge, 1986), and can also serve as a substrate for formation of lipid peroxides, leading to a chain reaction production and propagation of free radicals.

(3) The rat brain has a relatively low content of enzymes relating to protection against oxidative damage, such as glutathione peroxidase, superoxide dismutase and catalase (Carmagnol *et al.*, 1983; Marklund *et al.*, 1982; Sinet *et al.*, 1980), and these enzymes are predominantly in glia rather than neurons (Savolainen, 1978).

(4) Certain catecholamine neurotransmitters such as norepinephrine are susceptible to autooxidation and consequent free radical formation (Cohen and Heikkila, 1974; Adam-Vizin and Seregi, 1982). Metals such as manganese and iron which can catalyze oxidative events are presented in especially high amounts in some brain regions (Halliwell and Gutteridge, 1986). It has been pro-

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posed that decompartmentalization of metal ions may be of importance in trauma-related brain injury seizure responses (Willmore *et al.*, 1983).

We have previously found that methyl mercury, a neurotoxic agent known to enhance lipid peroxidation, can elevate levels of cytosolic calcium in synaptosomes. This elevation coincided with an increased permeability of the synaptosomal plasma membrane. Both of these indices of synaptosomal damage were reversed by the antioxidant α-tocopherol (Bondy and McKee, 1990). This led us to consider whether the formation of free radicals might underlie the elevation of cytosolic calcium and leakiness of synaptosomes exposed to oxidative stress.

A role for calcium in many pathological states related to free radical formation has been postulated (Farber, 1982). Abnormal levels of intracellular calcium consequent to injury to the cell membrane or energy-dependent Ca-regulatory homeostatic mechanisms may underlie cell death (Siesjo, 1986). There is evidence for an interactive relation between oxidizing conditions and levels of intracellular calcium (Maridonneau-Parini *et al.*, 1986). Thiol groups may play a role in Ca-sequestration (Thor *et al.*, 1985). Excess oxidative activity can impair mitochondrial function and thus prevent effective sequestration of Ca^{2+} or its pumping out of the cell (Bellomo *et al.*, 1982). Conversely, calcium may potentiate free radical injury to mitochondria (Malis and Bonventre, 1986). Such interactions have not always been found to be synergistic. Omission of extracellular Ca^{2+} can elevate oxidative damage in isolated hepatocytes (Thomas and Reed, 1988). This study was conducted in order to examine the effect of free radical-forming conditions upon both the entry of calcium into synaptosomes and levels of free ionic calcium within these structures. The effect of agents known to retard oxidizing conditions upon these parameters was also evaluated.

The potentially protective compounds selected were as follows:

(a) α-tocopherol is a powerful antioxidant in a variety of systems including neuronal preparations (Noda *et al.*, 1982; Acosta *et al.*, 1987), and has been reported to protect against various neurotoxic agents including methylmercury (Chang *et al.*, 1978; Prasad and Prasad, 1982) and cadmium (Shuka *et al.*, 1988;

(b) β-carotene, another antioxidant, has been reported to be protective against hexaclorophene (Hanig *et al.*, 1977);

(c) superoxide dismutase rapidly degrades the superoxide anion to less toxic H_2O_2. A protective role against oxidative damage has been attributed to this enzyme (Saez *et al.*, 1987; Das *et al.*, 1986; DiGuiseppi and Fridovitch, 1984);

(d) although possessing no antioxidant properties, ganglioside GM_1 has been frequently reported to increase neural regenerative capacity (Leeden, 1984; Toffano *et al.*, 1984; Jonsson *et al.*, 1984; Matta *et al.*, 1986). More specifically, this ganglioside can decrease synaptosomal vulnerability to chemical injury (Bondy and Halsall, 1988a).

EXPERIMENTAL PROCEDURES

Preparation of synaptosomes

Adult male CR 1 CD rats (Charles River Breeding Laboratories Inc., Wilmington, Mass.), 4–5 months old weighing 290–330 g were used. Rats were decapitated, the brains excised quickly on ice and the whole brain except the cerebellum and pons-medulla disected out. Synaptosomes were made by the modification of Dodd *et al.* (1981) of the method of Gray and Whittaker (1962). Briefly, after homogenization in 10 vol of cold 0.32 M sucrose, the homogenate was centrifuged (1800 *g*, 8 min, 0–4°C) and the supernatant laid over 1.2 M sucrose (10 ml). After high speed centrifugation (50,000 rpm, 25 min, Beckman model L8-70, rotor Ti60), the layer at the interface was collected, diluted 2.5-fold with 0.32 M sucrose and laid over 0.8 M sucrose. After centrifugation again at high speed, the synaptosomal pellet was suspended in HEPES buffer, pH 7.4, to give a tissue concentration of 0.15 g-equivalent/ml (about 1.6 mg/ml of protein). The composition of HEPES buffer was (millimolar): NaCl, 125; KCl, 5; NaH_2PO_4, 1.2; $MgCl_2$, 0.1; $NaHCO_3$, 5; glucose, 6; $CaCl_2$, 0.1; and HEPES, 10. In order to maintain basal $[Ca^{2+}]_i$ at a relatively low level, 0.1 mM Ca^{2+} rather than 1 mM Ca^{2+} was employed (Komulainen and Bondy, 1987a).

Assay of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured using the indicator dye fura-2 AM; 1-ml aliquots of the synaptosome suspension were incubated with 5 μM fura-2 AM added in DMSO (final concentration of DMSO, 0.5%) at 37°C for 10 min. HEPES buffer (9 ml) at 37°C was then added and the incubation continued for 25 min. Synaptosomes were sedimented (12,300 *g*, 8 min), resuspended in 5 ml of HEPES buffer and kept on ice. For fluorescence measurement, 500-ml aliquots of the suspensions were centrifuged rapidly in Eppendorf-tubes in a microcentrifuge (16,000 *g*, 2 min) and the synaptosomal pellet was resuspended in 2 ml of warm (37°C) HEPES buffer. This buffer did not contain $NaHCO_3$ or NaH_2PO_4 in order to prevent precipitation of high levels of Ca^{2+} used subsequently.

The emitted fluorescence of the hydrolyzed fura-2 ester in the sample was measured in a thermostatted (37°C) cuvette using a spectrofluorometer at the excitation wavelengths of 340 and 380 nm (bandpass 3 nm) and at 510 nm for emission (bandpass 20 nm). Samples contained 140–160 μg of protein and were allowed to equilibrate for 10 min before addition of chemicals. Mixing was carried out with a magnetic stirrer 30–60 s before reading fluorescence. Extrasynaptosomal fura-2 was quenched by 4.0 μM $MnCl_2$ at the end of each

incubation. It was added 15–20 s before recording the fluorescence. This Mn^{2+} concentration corrected for extrasynaptosomal fura-2 (Jacob *et al.*, 1987; Komulainen and Bondy, 1987a). This method of determination of intrasynaptosomal fura-2 gave the same results as the more direct procedure of fluorescent assay of the supernatant after centrifuging synaptosomes down from the incubation medium (Bondy and Halsall, 1988b). However, this latter procedure has the disadvantage that no subsequent determination of the synaptosomal signal under conditions of zero or saturating Ca^{2+} is possible. The proportion of fura-2 that was found to be outside the synaptosomes is described in the results as "% leakage." In incubated but untreated synaptosomes basal leakage was 13–17%. In order to calculate $[Ca^{2+}]_i$ before addition of agents, a separate average Mn^{2+} correction was made for each batch of synaptosomes. Autofluorescence of synaptosomes was always recorded and subtracted before calculation of fluorescence ratios of fura-2. This constituted around 15% of total fluorescence. For calibration of the synaptosomal fura-2-Ca^{2+} signal, (R), R_{min} (the ratio of fluorescence at 340/380 nm in the absence of Ca^{2+}) and R_{max} (the ratio when all fura-2 of the sample was saturated with Ca^{2+}) were determined for each batch of fura-2 loaded synaptosomes. In order to determine R_{min}, synaptosomes were lysed with 0.1% sodium dodecyl sulfate, and Ca^{2+} and Mn^{2+} chelated with 5 mM alkaline EGTA. R_{max} was determined by the addition of 9 mM $CaCl_2$. $[Ca^{2+}]_i$ was calculated using the formula (Grynkiewicz *et al.*, 1985):

$$[Ca^{2+}]_i = K_d \frac{(R - R_{min})(Sf_2)}{(R_{max} - R)(Sb_2)}$$

where K_d (224 nM) is the dissociation constant of fura-2–Ca^{2+} complex and Sf_2 and Sb_2 denote fluorescence of fura-2 at zero Ca^{2+} and Ca^{2+} saturation, respectively, at the excitation wavelength 380 nm.

$^{45}Ca^{2+}$ transport

A synaptosomal suspension (0.1 ml containing 150–170 μg of protein) was mixed with 0.9 ml of calcium-free HEPES buffer and incubated 30 min at 37°C. Then 0.1 ml of $^{45}Ca^{2+}$ (0.5 μCi, 55.9 Ci/mol) in either 1.0 M KCl or 1.0 M NaCl was mixed into the suspension and incubation continued for an additional 20 s. While uptake was not completely linear over this period, the use of shorter incubation times adversely affected reproducibility. When indicated, various chemicals were added during the preincubation. α-Tocopherol succinate and β-carotene were added in DMSO (the final concentration not exceeding 1%). $^{45}Ca^{2+}$ uptake was stopped by the addition of 5 ml of ice-cold wash buffer (HEPES-buffer omitting $CaCl_2$ and containing 1 mM EGTA) followed by rapid filtration on glass fiber filters (Type A/E, Gelman Sciences Inc., Ann Arbor, Mich.) (Wu *et al.*, 1982). The filter disks were washed with 2 × 5 ml of buffer and accumulated label counted in 5 ml of Ecoscint (National Diagnostics, Somerville, N.J.) in a liquid scintillation counter. The difference in ^{45}Ca uptake between incubation in the presence of added 90 mM KCl or 90 mM NaCl was taken to represent depolarization-triggered Ca^{2+} entry. This was between 2 and 3 times the basal uptake in the presence of NaCl. Blank tubes contained synaptosomes that had been sonicated and heated to 100°C for 10 min and were thus incapable of Ca^{2+} transport.

Lipid peroxidation

The formation of thiobarbituric-reactive material (TBAR) was used as an index of peroxidative activity. The precise method used was based on the modification of Uchiyama and Mihara (1978) using 1% phosphoric acid as the protein denaturing agent. Color formation at 535 nm was determined in the supernatant with no organic solvent extraction. The molar extinction coefficient of a malondialdehyde standard (1.56×10^5) confirmed the calibration of the spectrophotometer. At the low tissue concentration used, color formation was found to be proportional to the amount of tissue present and was linear for at least 2 h at 37°C (Lambert and Bondy, 1989). A 30 min incubation was necessary to allow sufficient color development for accurate assay.

Protein determination

Protein content of synaptosomal suspensions was assayed with the method of Bradford (1976) using bovine serum albumin as a reference.

Statistics

Results were analyzed using Fisher's Least Significant Difference Test after one-way analysis of variance. Throughout results, the symbol * means $P < 0.05$, using a two-tailed t distribution, that the value differs from the corresponding control value. Each data point presented is the mean of 4–9 individual determinations.

Materials

Chemicals were obtained from Sigma Chemical Corporation (St Louis, Mo.), and $^{45}CaCl_2$ from New England Nuclear (Bedford, Mass.).

RESULTS

Effects of Fe/ascorbate on synaptosomal peroxidation, calcium levels and uptake

Exposure of synaptosomes to the Fe/ascorbate oxidizing system elevated the formation of thiobarbituric acid reactive products (TBAR) in a time-dependent manner for at least 2 h (Lambert and Bondy, 1989). Increasing ascorbate levels effected a dose-related but non-linear increase in TBAR formation (Fig. 1).

Synaptosomal ionic free calcium content $[Ca^{2+}]_i$ was depressed in the presence of 5 μM Fe^{2+} and 0.1 mM ascorbate (Fig. 2).1 We previously established that the concentration of Fe^{2+} used had no effect on the intensity of fura-2, although higher Fe^{2+} concentrations could quench this fluorescence. Leakage of the calcium-responsive dye was elevated in the presence of oxidizing conditions, suggesting damage to the synaptosomal limiting membrane (Fig. 2). The use of 0.1 mM ascorbate alone did not alter $[Ca^{2+}]_i$ or fura-2 leakage.

Since ascorbate potentiated the effect of Fe^{2+}, the standard oxidizing conditions used in subsequent

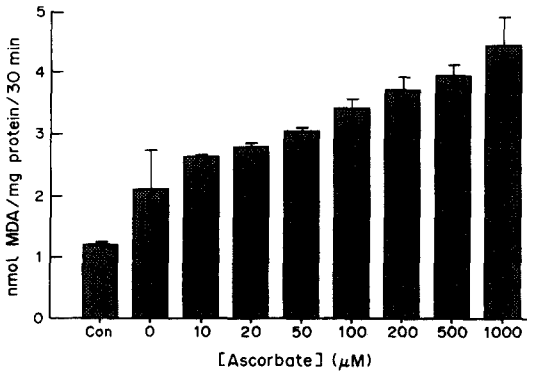

Fig. 1. Formation of thiobarbituric acid-reactive products by synaptosomes (160–180 mg protein) exposed to 5 μM $FeSO_4$ and varying concentrations of ascorbic acid for 15 min at 37°C. Control value represents formation of malondialdehyde (MDA) equivalents in absence of both Fe^{2+} and ascorbate.

Table 1. TBAR production in synaptosomes exposed to pro-oxidant conditions and to potential blockers of oxidative activity

First 15 min	Thiobarbiturate-reactive products formed: Second 15 min	(nmol/mg protein/30′)
—	—	1.43 ± 0.11
—	Fe/ascorbate	3.98 ± 0.25*
10 μM desferrioxamine	Fe/ascorbate	1.49 ± 0.10
25 μM α-tocopherol	—	1.21 ± 0.18
25 μM α-tocopherol	Fe/ascorbate	3.11 ± 0.17*
25 μM β-carotene	—	1.42 ± 0.04
25 μM β-carotene	Fe/ascorbate	3.88 ± 0.30*
300 units/ml superoxide dismutase	—	1.46 ± 0.13
	Fe/ascorbate	4.62 ± 0.16*
100 μM GM_1	—	1.58 ± 0.13
100 μM GM_1	Fe/ascorbate	4.64 ± 0.31*

*Differs significantly from basal value ($P < 0.05$).
Incubation in the presence of 5 μM $FeSo_4$–0.1 μM ascorbic acid was at 37 C for 15 min. 160–180 μg synaptosomal protein was present in each assay. Where other chemicals were added, this was for a 15-min preincubation at 37 C.

experiments consisted of a mixture of 5 μM Fe^{2+} and 0.1 mM ascorbic acid. Other oxidizing agents such as t-butyl hydroperoxide had a similar effect on both $[Ca^{2+}]_i$ and synaptosomal leakiness (data not shown). The presence of 10 μM desferrioxamine in the incubation mixture completely blocked all Fe/ascorbate-induced TBAR formation (Table 1).

This concentration of iron-chelator also totally prevented any Fe/ascorbate-induced changes in synaptosomal $[Ca^{2+}]_i$ and fura-2 leakage (data not shown). This suggested that the observed synaptosomal changes effected by Fe/ascorbate were related to oxidative damage rather than other metabolic changes.

Further evidence for altered function of the synaptosomal plasma membrane came from the study of ^{45}Ca uptake by synaptosomes (Table 2). This was reduced in the presence of 5 μM Fe^{2+} and various concentrations of ascorbate. Basal uptake was inhibited to a much lesser extent than K^+-depolarization induced uptake.

Modulation of oxidative damage by antioxidants and other chemicals

Synaptosomes were pretreated with a variety of substances potentially capable of preventing oxidative stress or of stabilization of cell membranes before being exposed to a standard Fe/ascorbate mixture (5 μM $FeSO_4$–100 μM ascorbic acid).

(a) α-Tocopherol. The preincubation of synaptosomes with 25 μM α-tocopherol succinate for 15 min partially prevented the stimulation of lipid peroxidation caused by subsequent incubation with the

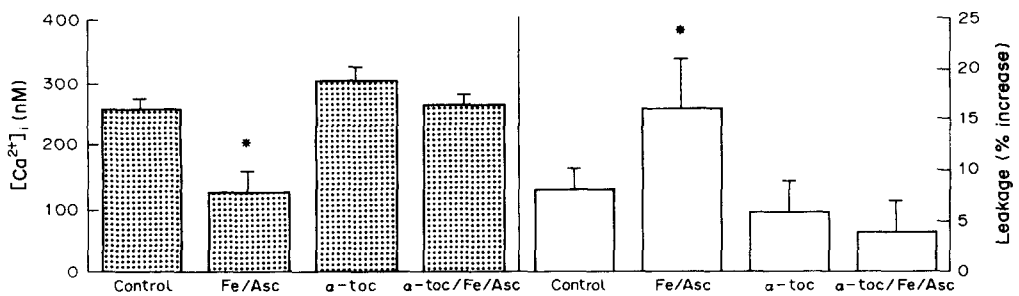

Fig. 2. Effect of α-tocopherol upon cytosolic calcium in, and leakage of fura-2 from synaptosomes exposed to oxidative conditions. Synaptosomes were incubated 5 min with 25 μM α-tocopherol prior to a 15 min incubation in the presence of 5 μM $FeSO_4$ 100 μM ascorbate. *$P < 0.05$ that value differs significantly from control value.

Fe/ascorbate (Table 1). This concentration of α-tocopherol completely prevented the reduction of ^{45}Ca uptake and of $[Ca^{2+}]_i$ caused by Fe/ascorbate. α-Tocopherol alone had no significant effect on $[Ca^{2+}]_i$ or fura leakage (Fig. 2). However, both basal and K-stimulated synaptosomal ^{45}Ca uptake were somewhat elevated (Fig. 3). The reversal of changes in Ca parameters effected by α-tocopherol was paralleled by prevention of Fe/ascorbate-induced elevations of fura-2 leakage into the extra-synaptosomal compartment (Fig. 2). This implied that plasma membrane function could be preserved by α-tocopherol.

(b) β-Carotene. Pretreatment of synaptosomes with 25 μM β-carotene for 15 min did not prevent Fe/ascorbic acid from exerting its usual effect on TBAR formation or on synaptosomal $[Ca^{2+}]_i$ (Fig. 4, Table 1). β-Carotene also did not protect the synaptosomal limiting membrane against the effects of subsequent addition of Fe/ascorbate as judged by fura-2 leakage. Furthermore, the depression in depolarization-induced ^{45}Ca uptake caused by Fe/ascorbate was not mitigated by β-carotene (data not shown).

(c) Superoxide dismutase. In view of the ability of superoxide dismutase to degrade the superoxide radical, O_2, a parallel pretreatment to those described above was carried out, by exposing synaptosomes to 300 units of superoxide dismutase/ml, for 15 min. No protective effect was found on TBAR formation (Table 1), $[Ca^{2+}]_i$ and synaptosomal leakiness changes effected by Fe/ascorbic acid (Fig. 5), or ^{45}Ca uptake (data not shown).

(d) Ganglioside GM_1. Preliminary treatment of synaptosomes with 100 μM GM_1 for 15 min at 37°C did not significantly alter their subsequent response in ^{45}Ca uptake or depression of $[Ca^{2+}]_i$ to the standard oxidizing conditions employed here (Fig. 6, data not shown). However, the permeability of synaptosomes to fura-2 was unaltered by subsequent addition of Fe/ascorbate. The effect of oxidizing conditions upon ^{45}Ca uptake and $[Ca^{2+}]_i$ was, in this case, separable from effects upon membrane integrity. Since TBAR production was not significantly altered by GM_1 (Table 1), the protective effect of this ganglioside on Fe/ascorbate-induced changes cannot be attributed to a direct antioxidant effect.

Table 2. Synaptosomal ^{45}Ca uptake under resting and depolarizing conditions, and the effect of 5 μM FeSO4 and varying concentrations of ascorbate upon such uptake

		^{45}Ca uptake (pmoles/20 s/mg protein)	
Ascorbate (μM)	$FeSO_4$ (μM)	Basal	K^+-stimulated
0	0	9.7±0.3	24.3±4.8
0	5	7.3±0.2	16.1±1.6
10	5	6.1±0.2	6.3±1.6
20	5	6.0±0.3	5.7±1.2
50	5	5.5±0.6	3.8±1.3
100	5	4.1±0.6	4.1±0.2
1000	5	5.3±0.7	1.8±0.6

Values represent mean ± SE of 5–6 individual determinations. Ascorbate and $FeSO_4$ were present for 10 min at 37°C before addition of ^{45}Ca Cl_2 for a further 20 s incubation.

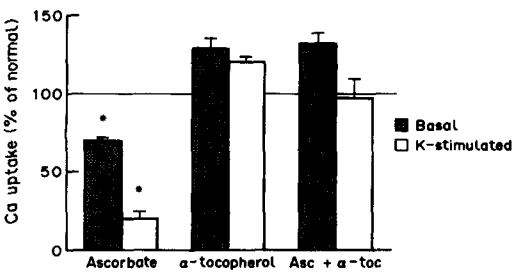

Fig. 3. Effect of α-tocopherol upon synaptosomal ^{45}Ca uptake resting and depolarizing conditions. ^{45}Ca uptake was allowed to continue for 20 s after addition with sufficient NaCl or KCl for a final concentration of 90 mM. Synaptosomes were incubated with 5 μM $FeSO_4$–0.1 μM ascorbic acid for 15 min at 37°C. Where present, 25 μM α-tocopherol was added for a 30 min preincubation at 37°C. Control samples were incubated for 30 min without any addition. $^*P < 0.05$ that value differs significantly from control value.

DISCUSSION

While α-tocopherol was able to completely prevent the effects of a subsequent addition of the Fe/ascorbate mixture upon ^{45}Ca uptake, $[Ca^{2+}]_i$ and leakiness of synaptosomes, malondialdehyde production was only partially inhibited (Table 2). Several reports indicate that α-tocopherol administration may protect against various types of damage to nervous tissue including $FeCl_2$-induced focal edema (Willmore and Rubin, 1984), penicillin-induced focal epilepsy (Nikushkin *et al.*, 1981) and hippocampal slices rendered anoxic by superfusion with oxygen free media (Acosta *et al.*, 1987). However, this protection is not always closely related to the extent of inhibition of lipid peroxidation. A limited inhibition of malondialdehyde formation afforded by α-tocopherol, to-either to the relative impermeability of synaptosomes function, has been described for hippocampal slices (Acosta *et al.*, 1987).

The reduction of $[Ca^{2+}]_i$ by oxidizing conditions is in contrast to the effect of various neurotoxic, agents including several organometals and organochlorine insecticides which elevated synaptosomal $[Ca^{2+}]_i$ (Komulainen and Bondy, 1987a,b; Bondy and

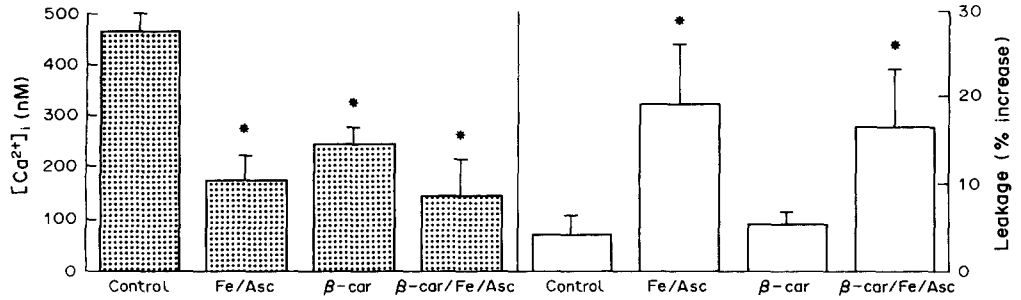

Fig. 4. Effect of 25 μM β-carotene upon cytosolic calcium in, and leakage of fura-2 synaptosomes exposed to oxidative conditions. Synaptosomes were incubated 5 min with β-carotene prior to a 15 min incubation in the presence of 5 μM $FeSO_4$–100 μM ascorbate. *$P < 0.05$ that value differs significantly from control level.

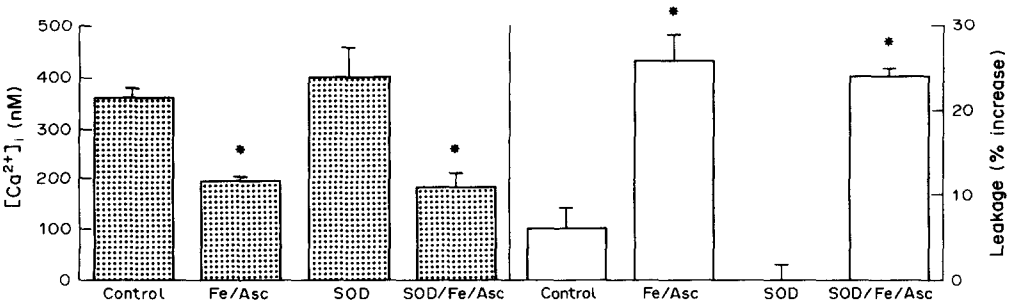

Fig. 5. Effect of 300 units/ml superoxide dismutase (SOD) upon cytosolic calcium in, and leakage of fura-2 from synaptosomes exposed to oxidative conditions. Synaptosomes were incubated 5 min with SOD prior to a 15 min incubation in the presence of 5 μM $FeSO_4$–100 μM ascorbate. *$P < 0.05$ that value differs significantly from control level.

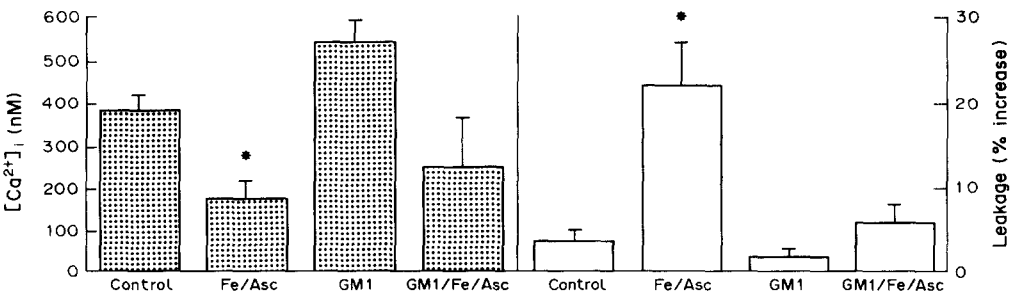

Fig. 6. Effect of 100 μM ganglioside GM_1 upon synaptosomal $[Ca^{2+}]_i$ and fura-2 leakage in the presence of oxidizing conditions. Synaptosomes were incubated 5 min with ganglioside GM_1 prior to a 15 min incubation in the presence of 5 μM $FeSO_4$–100 μM ascorbate. *$P < 0.05$ that value differs significantly from control level.

Halsall, 1988b; Joy and Burns, 1988). The increased leakiness of synaptosomes in the presence of oxidizing mixtures is, however, clear evidence of damage. The protection offered by the simultaneous presence of α-tocopherol against observed changes also suggests that oxidative activity was the cause of these changes. The data obtained using ^{45}Ca substantiate this concept. Depolarization-induced ^{45}Ca entry is known to be depressed in synaptosomes that have been exposed to a variety of neurotoxic agents such as chlordecone and lindane (Komulainen and Bondy , 1987b; Bondy and Halsall, 1988b). Basal ^{45}Ca uptake was inhibited to a much lesser extent than K^+-induced uptake by oxidizing conditions. This suggested that reduction of uptake was due to specific effects on a dynamic physiological process rather than interference with overall membrane permeability. While we have found K-stimulated Ca uptake to be reduced by peroxidative conditions, basal ^{45}Ca uptake by synaptosomes has been shown to be elevated in the presence of 200 μM Fe^{2+} (Braughler, 1988). The inhibition of depolarized-induced Ca uptake implies a more selective effect of the relatively moderate peroxidizing conditions used here (also indicated by a lower rate of formation of TBAR) upon voltage-sensitive channels. While these data form a consistent pattern, the observed depression of $[Ca^{2+}]_i$ was unexpected. Since this reduction was not due to any interference with fura fluorescence caused by the Fe/ascorbate mixture, the effect appeared related to oxidative events in that it was reversed by α-tocopherol. It may be that some of the apparent prevention of changes induced by oxidizing conditions described here are due to modification of membrane properties unrelated to antioxidant activity. This is clear in the case of ganglioside GM_1 which did not alter stimulated TBAR production (Table 2), and may also partly account for the protective effects of α-tocopherol. However, TBAR production does not give a total picture of oxidative activity and thus incomplete inhibition of TBAR formation by α-tocopherol may not completely reflect its antioxidative properties. Furthermore, the protective effect of α-tocopherol resembled that produced by desferrioxamine whose mode of action is clearly antioxidative.

The failure of superoxide dismutase to protect against synaptosomal damage (as judged by increased fura-2 leakage) or alter Ca parameters may be due either to the relative impermeability of synaptosomes to this protein or to the possibility that $O_2^{\cdot}$ is not the directly harmful active species. Since superoxide dismutase can enter intact neurons (Saez *et al.*, 1987), the latter explanation is more likely. Only in the case of ganglioside GM_1 was there dissonance between results derived from fura-2 leakage and those pertaining to calcium levels and translocations. In this case, the depression of $[Ca^{2+}]_i$ and ^{45}Ca uptake effected by Fe/ascorbate was not blocked by GM_1 pretreatment while the elevated fura-2 leakage was protected against. Further separation of leakage and $[Ca^{2+}]_i$-related indices of damage is evidenced by the fact that several neurotoxic agents increase both these parameters (Komulainen and Bondy, 1987b; Bondy and Halsall, 1988b), but Fe/ascorbate-induced oxidative stress increases leakage but decreases $[Ca^{2+}]_i$.

The relation between calcium and peroxidative activity is unclear, and seemingly conflicting results have been reported. High calcium levels in the incubation medium have been found to enhance basal and induced peroxidative damage in synaptosomes (Braughler *et al.*, 1985). Further evidence for a positive interaction between calcium and oxidative damage comes from the demonstration that hypoxia can both increase rates of peroxidation and elevate $[Ca^{2+}]_i$ in nerve tissue (Johnson *et al.*, 1986, 1987; White and Clark, 1988). However, hypoxia has also been reported to reduce both the total calcium content and ^{45}Ca uptake of synaptosomes (Peterson and Gibson, 1984). In support of an antagonism rather than synergy between peroxidative events and calcium levels is the finding that low levels of extracellular calcium are associated with increased lipid peroxidation in isolated hepatic cells, as well as increased leakiness of these cells (Smith and Sandy, 1985; Thomas and Reed, 1988). A mechanism that could account for our $[Ca^{2+}]_i$ and ^{45}Ca-derived data is the reduction of calcium influx by inhibition of voltage-sensitive and receptor-operated channels. Such an inhibition has been described for ethanol (Dildy and Leslie, 1989). The relation between intracellular calcium and the generation of free radicals is not close, since each parameter is subject to relatively independent modulation. While both calcium influx and oxidative stress can be damaging to mitochondria, these processes are clearly dissociable (Stark *et al.*, 1986).

Intracellular $[Ca^{2+}]_i$ represents an equilibrium of the sum of many processes. Calcium influx into the cytoplasm can occur (i) through specific channels or through non-specific sites in the plasma membrane; (ii) by enzymically induced release from endoplasmic reticulum, and (iii) by the mitochondrial Ca/proton exchanger. Balancing these are the energy dependent pumps and sequestration mechanisms present in many membranes and the Ca/Na exchangers of the plasma membrane. Some of these mechanisms are likely to be more sensitive to oxidative damage than

others. A critical aspect is the extent to which cellular energy-generating mechanisms which underlie all anabolic processes are compromised by excess formation of free radicals. However, even when synaptosomal ATP levels are at 50% of their normal level, $[Ca^{2+}]_i$ is not significantly elevated (Bondy and Komulainen, 1988). Calcium-removing processes may thus have a priority for available energy when ATP levels are depressed. Pro-oxidative conditions are known to increase the rate of calcium cycling across the mitochondrial outer membrane (Richter and Frei, 1988). When oxidative damage is not too severe, the final equilibrium of such increased flux may result in reduced $[Ca^{2+}]_i$. The increased cell permeability and partial collapse of mitochondrial potential provoked by oxidative stress (Lambert and Bondy, 1989) may result in an over-response of calcium homeostatic processes leading to a reduction of $[Ca^{2+}]_i$.

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