UC Irvine

UC Irvine Previously Published Works

Title

Disruption of the potential across the synaptosomal plasma membrane and mitochondria by neurotoxic agents.

Permalink

<https://escholarship.org/uc/item/5728t7pc>

Journal

Toxicology letters, 58(1)

ISSN

0378-4274

Authors

Bondy, SC

McKee, M

Publication Date

1991-09-01

DOI

10.1016/0378-4274(91)90185-9

Copyright Information

This work is made available under the terms of a Creative Commons Attribution License, available at

<https://creativecommons.org/licenses/by/4.0/>

Peer reviewed

Disruption of the potential across the synaptosomal plasma membrane and mitochondria by neurotoxic agents

Stephen C. Bondy and Monique McKee

Department of Community and Environmental Medicine, University of California, Irvine, CA (U.S.A.)

(Received 23 October 1990)

(Accepted 15 February 1991)

Key words: Electrical potential; Membrane potential; Methyl mercury; Toluene

SUMMARY

The effect of a neurotoxic organometal, methyl mercuric iodide, and an aromatic solvent, toluene, upon the transmembrane potential (ψ), across both the limiting membrane of isolated nerve terminals and their mitochondria, has been studied. Exposure of nerve endings to either of these toxicants in vitro resulted in a dose-dependent diminution of ψ that was especially pronounced in the case of mitochondria. This was not prevented by a concurrent exposure to an antioxidant (α -tocopherol), or an iron chelator (deferoxamine), or ganglioside GM₁. No significant changes were detected in synaptosomal potentials derived from cortices of rats exposed to methyl mercury or toluene at levels known to increase the rate of formation of reactive oxygen species within this region. The especial vulnerability of mitochondrial ψ to these agents may be due to the disruption of oxidative phosphorylation and may be related to the increase in intrasynaptosomal free ionic calcium that both of these chemicals can induce.

INTRODUCTION

Biological membranes present a distinct target to chemicals that are injurious to the nervous system. This may in part be due to the critical geometry of the hydrophilic and lipophilic regions within such membranes. The maintenance of this bilayered structure is essential for membrane function. Both mitochondria and the plasma membrane are characterized by a transmembrane potential generated by ion pumps. In the case of the plasma membrane, this electrical discontinuity represents an energy

reserve which can be drawn upon to allow the passage of nerve impulses. Synaptosomal membrane potential may also be directly involved in effecting neurotransmitter release [1]. The mitochondrial membrane potential is the result of uniporter-effected translocation of protons created at the inner surface of the mitochondrial membrane by operation of the respiratory chain.

A wide range of pharmacological and neurotoxic agents can cause hyperexcitability. One of the means of effecting this is by partial depolarization of the axonal limiting membrane. Since maintenance of this gradient is an energy-requiring process, any means of depletion of energy reserves (largely represented by the ATP/ADP ratio) may diminish the magnitude of the membrane potential. Xenobiotic agents having an adverse effect on mitochondrial energy generation may also impair the transmembrane potential generated by pumping of protons to the cytosol. This proton gradient is critical for the formation of ATP from ADP.

We have studied the effects of two neurotoxic agents upon the potential across the synaptosomal limiting membrane and the mitochondrial potential. One of these is an aromatic solvent (toluene), while the other is an organometal (methyl mercuric iodide). Both of these chemicals have been found to induce oxidative stress both within brain regions of exposed animals and within isolated synaptosomes [2,3]. Methyl mercury has previously been found to increase lipid peroxidation levels [4] and vitamin E can protect against the toxicity of alkyl mercurials [5,6]. The ability of various compounds with antioxidant potential, to mitigate induced changes, has also been investigated in the present work.

Reactive oxygen species have previously been reported to modulate plasma membrane potential [7]. The relevance of membrane depolarization to neurotoxic events is further illustrated by evidence that persistent depolarization may underlie longterm synaptic failure and may be an early critical step toward neuronal degeneration [8].

MATERIALS AND METHODS

Animals

Adult male CR 1 CD rats (250–300 g) were obtained from Charles River Breeding Laboratories (Wilmington, MA), and were maintained in the animal facility in clear polypropylene cages with water and food provided ad libitum.

Preparation of synaptosomes

Adult male CR 1 CD rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) 2–3 months old, weighing 175–280 g were used. Rats were decapitated, the brains excised and the whole brain except the cerebellum and pons–medulla dissected out. Synaptosomes were made by the modification of Dodd et al. [9] of the method of Gray and Whittaker [10]. After homogenization in 10 volumes of cold 0.32 M sucrose, the tissue homogenate was centrifuged ($1900 \times g$, 10 min) and the supernatant S1 laid over 10 ml 1.2 M sucrose. After high-speed centrifugation ($250\,000 \times g$, 25 min), the layer at the interface was collected, diluted 2.5-fold with 0.32 M sucrose and

laid over 8 ml 0.8 M sucrose. After further centrifugation 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 2.9 mg/ml protein). For studies of oxygen radical formation, crude synaptosomes were prepared from the S1 fraction by centrifugation at $31\,500 \times g$ for 10 min to yield the P2 fraction. This was suspended in HEPES buffer to a concentration of 0.0185 g-equivalent/ml.

Determination of electrical potential

Synaptosomes were incubated 5 min at 37°C in the presence of 1 μ M rhodamine 6G [11]. This concentration of rhodamine has a negligible effect on synaptosomal metabolism [12]. The final synaptosomal concentration was 0.015 g-equivalent tissue/ml containing 0.27–0.32 mg protein/ml, in HEPES buffer.

In order to test the effects of agents upon the potential (ψ) of mitochondria within synaptosomes, KCl was added to a final concentration of 100 mM in order to almost totally depolarize the plasma membrane while not modulating mitochondrial ψ [13,14]. The fluorescence of rhodamine was then determined at 550 nm, using an excitation wavelength of 520 nm. When toxic agents were to be studied *in vitro*, they were added after this determination, and incubation continued for a further 10 min. Any ensuing loss of mitochondrial ψ was evaluated by an increased fluorescence of rhodamine. In order to completely depolarize mitochondria, carbonyl cyanide *p*-fluoromethoxyphenylhydrazone (FCCP) was then added to a final concentration of 1 μ M [15], after which fluorescence was again determined.

Plasma membrane ψ was estimated in a parallel manner. Rhodamine fluorescence was measured 5 min after the depolarization of mitochondria by 1 μ M FCCP [16]. Plasma membrane ψ has been shown to remain unaltered for some time after such a suppression of mitochondrial ψ [16]. Any toxic agent under study was then added and, after a further 10 min incubation, fluorescence determined once more. Finally, fluorescence was determined after depolarization of the plasma membrane with 100 mM KCl. This permitted the extent of reduction of the transmembrane ψ by the toxicant to be calculated.

Values of ψ of fully polarized membranes were derived from the equation:

$$\frac{F^{-1}-1}{F_k^{-1}-1} = RT/FE[1 - \exp(-F\psi/RT)]$$

where F is fluorescence of rhodamine when synaptosomes are in HEPES buffer, F_k is that fluorescence in the presence of 100 mM KCl, and R , T and F have the usual thermodynamic significance [11]. Mitochondrial potential is similarly calculated substituting F_c (fluorescence in the presence of 1 μ M FCCP) for F_k . Synaptosomal values of ψ , derived from this calculation were -145 ± 6 mV for mitochondria, and -65 ± 5 mV for the limiting membrane. These values are in good accord with corresponding values reported by others [1,11,17–19]. Mitochondria derived from other sources also have a similar ψ [15,20,21]. The contribution of extrasynaptosomal mitochondria to

Fig. 1. Effect of varying concentrations of methyl mercuric iodide upon mitochondrial and plasma membrane potentials in isolated synaptosomes. Each point represents the mean $\pm$ SE of 4–5 individual replications. *Value differs significantly from the corresponding value for the untreated preparation.

calculated potentials is minimal [17,19]. This may be due to the deleterious effect of low potassium in the preparative medium on free mitochondria.

Protein determination

Protein content of synaptosomes was assayed by the method of Bradford [22] using bovine serum albumin as a reference.

Statistical analysis

Differences between groups were assessed by Fisher's Least Significant Difference Test after one-way analysis of variance. The acceptable level of significance was $P < 0.05$ using a two-tailed distribution.

RESULTS

The effect of various concentrations of methyl mercury upon the potentials of synaptosomes exposed *in vitro* was studied. The membrane potentials of both mitochondria and plasma membranes were depressed by methyl mercury in a dose-related manner (Fig. 1). However, mitochondrial ψ was considerably more susceptible to inhibition by this neurotoxic agent than was ψ of the plasma membrane. A variety of chemicals known to be protective against oxidative stress were tested for their ability to protect against changes in mitochondrial ψ caused by methyl mercury. These compounds were $5 \mu\text{M}$ α -tocopherol succinate, $1 \mu\text{M}$ ganglioside GM_1 , and $100 \mu\text{M}$ deferoxamine. In no instance was any mitigation effected by such chemicals (data not shown).

Methyl mercury was next administered to rats by a single intraperitoneal injection at a dose of 10 mg/kg body wt., synaptosomes being prepared after 2 days of exposure. These conditions are known to significantly elevate the rate of generation of

Fig. 2. Effect of varying concentrations of toluene upon mitochondrial and plasma membrane potentials in isolated synaptosomes. Each point represents the mean $\pm$ SE of 4–6 individual replications. *Value differs significantly from the corresponding value for the untreated preparation.

reactive oxygen species within the cerebral cortex of the rat [2]. Following such an in-vivo exposure, however, there was no significant effect on either plasma membrane or mitochondrial potential (Table I).

In a parallel series of experiments, the effect of toluene upon synaptosomal potentials was studied. This aromatic solvent also depolarized both mitochondrial and plasma membranes, and this effect was dose-related (Fig. 2). In view of the insolubility of toluene in water and uncertainty of toluene content within the tissue lipid compartment, toluene additions are given in μ l/2 ml incubation medium, rather than in concentrations. Mitochondrial ψ was again especially sensitive to inhibition by this solvent exposure in an isolated system (Fig. 2). Once more, the presence of each of several potentially protective agents (α -tocopherol succinate, ganglioside GM₁, deferoxamine), was ineffective in preventing the depolarization of mitochondria by toluene (data not shown). Isolated synaptosomes were also exposed to a related organic

TABLE I

POTENTIAL ACROSS MITOCHONDRIAL OR PLASMA MEMBRANES WITHIN SYNAPTOSOMES DERIVED FROM RATS TREATED WITH METHYL MERCURY OR TOLUENE

Treatment	Plasma membrane	Mitochondria
None	64 $\pm$ 5	147 $\pm$ 18
Methyl mercury	62 $\pm$ 6	138 $\pm$ 21
Toluene	61 $\pm$ 10	109 $\pm$ 15

Values are derived from 5 animals/group and are expressed as -mV. Rats were sacrificed 2 days after methyl mercury (10 mg/kg) or 1 h after toluene (1 g/kg) treatment.

Fig. 3. Mitochondrial potential after exposure of synaptosomes to toluene or benzene. Each point is a mean $\pm$ SE derived from 4–6 separate determinations. *Value differs significantly from the potential of the untreated control.

solvent, benzene. This chemical has not been found to induce the formation of reactive oxygen species in synaptosomes [3]. Benzene also inhibited mitochondrial ψ but to a lesser extent than toluene (Fig. 3).

One hour after treatment of rats with toluene (1 g/kg by i.p. injection using 50% corn oil, v/v, as a vehicle), animals were sacrificed and synaptosomes prepared. The mitochondrial ψ was depressed, but this did not reach significance (Table I).

DISCUSSION

Assay of synaptosomal ψ can only be carried out on fresh tissue. This is in contrast to several other procedures for evaluating synaptosomal status which can be performed on carefully frozen and subsequently re-thawed synaptosomes using the procedure of Hardy et al. [23]. These latter include assay of levels of cytosolic calcium and pH, rates of free radical generation, and high-affinity uptake of neurotransmitters. This suggests that depolarization may be an especially sensitive index of synaptosomal damage. However, adverse effects upon nerve endings incurred *in vivo* may be harder to detect. This may be related to the powerful ability of isolated nerve ending to maintain homeostasis. Synaptosomes isolated from toluene-treated rats, and subsequently incubated in toxicant-free HEPES buffer, have normal levels of $[Ca^{2+}]_i$, although the permeability of the limiting membrane is elevated [Von Euler et al., in preparation]. This differs from results obtained using synaptosomes exposed to toluene *in vitro*, where these parameters are both elevated [24]. Another possible reason for the apparent resistance of nerve endings to detectable changes following their exposure to neurotoxic agents *in vivo* is their much higher density per unit of toxicant encountered in the living animal than is the case with isolated tissue fractions. Thus, isolated synaptosomes exposed to levels of an agent, corresponding to that concentration found in the CNS of an exposed species, are present in the incuba-

tion medium at a protein concentration that is several orders of magnitude below that present in the intact brain. A critical relation between synaptosomal vulnerability to the tissue/ toxicant ratio, rather than to the absolute concentration of the toxic agent, has been previously described [25].

The concurrent presence of inhibitors of free radical formation had no effect on mitochondrial depolarization caused by either methyl mercury or toluene. Thus the susceptibility of this ψ to toluene or methyl mercury cannot be due to the known ability of these neurotoxins to stimulate the generation of oxygen free radicals. The overall neurotoxicity of the two agents under study can perhaps be ascribed to a combination of both oxidative and non-oxidative events.

The especial sensitivity of the mitochondrial membrane to depolarization by the agents under study here may be a reflection of the deleterious effects of these agents upon the electron transport processes underlying oxidative phosphorylation. One would expect the synaptosomal membrane potential to be less sensitive to disruptions in energy metabolism than the mitochondrial potential, because resting potentials across nerve terminals are largely dependent on the K^+ gradient which, in turn, is largely determined by the Donnan equilibrium, a non-energy-consuming process. Exposure to methyl mercury has been found to effect reduction of ATP-generating capacity [26,27]. The elevation of cytosolic calcium, $[Ca^{2+}]_i$, reported to be effected by both methyl mercury and toluene [19,24,27], may in part be due to the collapse of the mitochondrial potential, leading to release of calcium sequestered within mitochondrial binding sites.

The results obtained with methyl mercury parallel those reported in a previous study on synaptosomes in that the mitochondrial was much more susceptible to depolarization than was the plasma membrane [27]. However, these latter authors found inhibition of ψ to require considerably higher concentrations of methyl mercury. A similar reduction of mitochondrial ψ and elevation of $[Ca^{2+}]_i$ has also been reported for rat hepatocytes exposed to inorganic mercuric chloride [28]. In many of these studies, the omission of calcium from the external medium greatly diminishes the effect of toxicants on cytosolic calcium levels. Thus it is not clear whether collapse of the mitochondrial potential is a cause or a consequence of elevations of ionic intracellular calcium. Methyl mercury has been reported to inhibit the mitochondrial Ca/H exchanger [29], and there is a close link between $[Ca^{2+}]_i$ and mitochondrial potential. Calcium uptake by mitochondria is driven by mitochondrial ψ [18], and calcium overload can effect the collapse of the mitochondrial ψ [20]. The results of Kaupinnen et al. [27] suggest that lower concentrations of methyl mercury cause elevation of free cytosolic calcium by disruption of mitochondrial energy production, while higher levels of this toxicant disrupt the ability of the plasma membrane to exclude extracellular calcium.

The locus of action of a neurotoxicant may be either predominantly at the plasma membrane or at the mitochondrion. Damage at either of these sites has the potential of initiating a sequence of deleterious events which may include elevation of free calcium or rates of free radical generation within cells. Determination of the state of

membrane polarization is a sensitive test that can be of value in the distinction between these targets of chemically-induced damage.

ACKNOWLEDGEMENTS

This work was supported by grant no. ES 04071 from the National Institutes of Health. We would like to thank Mr. R. Vandagriff for computer generation of values for the equation used to derive membrane potentials.

REFERENCES

- 1 Sihra, T.S., Scott, I.G. and Nicholls, D.G. (1984) Verapamil, protonophores and veratridine influence the release of γ -aminobutyric acid from synaptosomes by modulation of the plasma membrane potential rather than cytosolic calcium. *J. Neurochem.* 43, 1624–1630.
- 2 Le Bel, C.P., Ali, S.F., McKee, M. and Bondy, S.C. (1990) Organometal-induced increases in oxygen reactive species: the potential of 2',7'-dichlorofluorescein diacetate as an index of neurotoxic damage. *Toxicol. Appl. Pharmacol.* 104, 17–24.
- 3 Mattia, C., Le Bel, C.P. and Bondy, S.C. (1991) Toluene increases rate of formation of reactive oxygen species in brain tissue. *Biochem. Pharmacol.* (in press).
- 4 Yonaha, M., Saito, M. and Sagai, M. (1983) Stimulation of lipid peroxidation by methyl mercury in rats. *Life Sci.* 32, 1507–1514.
- 5 Kasuya, M. (1975) The effect of vitamin E on the toxicity of alkyl mercurials on nervous tissue. *Toxicol. Appl. Pharmacol.* 32, 347–354.
- 6 Prasad, K.N. and Ramanujam, S. (1980) Vitamin E and vitamin C alter the effect of methylmercuric chloride on neuroblastoma and glioma cells in culture. *Environ. Res.* 21, 343–348.
- 7 Scott, J.A., Kham, B.A., Homey, C.J. and Rabito, C.A. (1987) Oxygen radicals alter the cell membrane potential in a renal cell line (LCC-PK1) with differentiated characteristics of proximal tubular cells. *Biophys. Biochim. Acta* 897, 25–32.
- 8 Rader, R.K. and Lanthorn, D.H. (1989) Experimental ischemia induces a persistent depolarization blocked by decreased calcium and NMDA antagonists. *Neurosci. Lett.* 99, 125–130.
- 9 Dodd, P.R., Hardy, J.A., Oakley, A.E., Edwardson, J.A., Perry, E.K. and Delaunoy, J.P. (1981) A rapid method for preparing synaptosomes: comparison with alternative procedures. *Brain Res.* 226, 107–118.
- 10 Gray, E.G. and Whittaker, V.P. (1962) The isolation of nerve endings from brain: an electron microscopic study of cell fragments derived by homogenization and centrifugation. *J. Anat. (London)* 226, 107–118.
- 11 Aiuchi, T., Matsunaga, M., Daimatsu, T., Nakaya, K. and Nakamura, Y. (1984) Effect of glucose and pyruvate metabolism on membrane potential in synaptosomes. *Biochim. Biophys. Acta* 771, 228–234.
- 12 Aiuchi, T., Matsunaga, M., Nakaya, K. and Nakamura, Y. (1985) Effect of probes of membrane potential on metabolism in synaptosomes. *Biochim. Biophys. Acta* 843, 20–24.
- 13 Aiuchi, T., Daimatsu, T., Nakaya, K. and Nakamura, Y. (1982) Fluorescence changes of rhodamine 6G associated with changes in membrane potential in synaptosomes. *Biochim. Biophys. Acta* 685, 289–296.
- 14 Davies, S., Weiss, M.J., Wong, J.R., Lampidis, T.J. and Chen, L.B. (1985) Mitochondrial and plasma membrane potentials cause unusual accumulation and retention of rhodamine 123 by human breast adenocarcinoma-derived MCF-7 cells. *J. Biol. Chem.* 260, 13844–13850.
- 15 Emaus, R.K., Grunwald, R. and Lemasters, J.J. (1986) Rhodamine 123 as a probe of transmembrane potential in isolated rat-liver mitochondria: spectral and metabolic properties. *Biochim. Biophys. Acta* 850, 436–448.

- 16 Scott, I.D. and Nicholls, D.G. (1980) Energy transduction in intact synaptosomes. *Biochem. J.* 186, 21–33.
- 17 Ramos, S., Grollman, E.F., Lazo, P., Dyer, S.A., Habig, W.H., Hardegree, M.C., Kaback, H.R. and Kohn, L.D. (1979) Effect of tetanus toxin on the accumulation of the permeant lipophilic cation tetraphosphonium by guinea pig brain synaptosomes. *Proc. Natl. Acad. Sci. USA*, 76, 4783–4787.
- 18 Akerman, K.E.O. and Nicholls, D.G. (1983) Calcium transport by synaptosomes. *Eur. J. Biochem.* 115, 67–73.
- 19 Komulainen, H. and Bondy, S.C. (1987) Increased free intrasynaptosomal Ca^{2+} by neurotoxic organometals: distinctive mechanisms. *Toxicol. Appl. Pharmacol.* 88, 77–86.
- 20 Akerman, K.E.O. and Wikstrom, M.K.F. (1976) Safranin as a probe of the mitochondrial membrane potential. *FEBS Lett.* 68, 191–197.
- 21 Kauppinen, R.A. and Hassinen, I.E. (1984) Monitoring of mitochondrial membrane potential in isolated perfused rat heart. *Am. J. Physiol.* 247, H508–H516.
- 22 Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye binding. *Anal. Biochem.* 72, 248–254.
- 23 Hardy, J.A., Dodd, P.R., Oakley, A.E., Perry, R.H., Edwardson, J.A. and Kidd, A.M. (1983) Metabolically active synaptosomes can be prepared from rat and human brain. *J. Neurochem.* 40, 608–614.
- 24 Von Euler, G., Fuxe, K. and Bondy, S.C. (1990) Ganglioside GM_1 prevents and reverses toluene induced increases in membrane fluidity and calcium levels in rat brain synaptosomes. *Brain Res.* 508, 210–214.
- 25 Komulainen, H. and Bondy, S.C. (1987) Modulation of levels of free calcium within synaptosomes by organochloride insecticides. *J. Pharmacol. Exp. Ther.* 241, 575–581.
- 26 Cheung, M. and Verity, M.A. (1981) Methyl mercury inhibition of synaptosomal protein synthesis: the role of mitochondrial dysfunction. *Environ. Res.* 24, 286–291.
- 27 Kauppinen, R.A., Komulainen, H. and Taipale, H. (1989) Cellular mechanisms underlying the increase in cytosolic free calcium concentration induced by methyl mercury in cerebrocortical synaptosomes from guinea pig. *J. Pharmacol. Exp. Ther.* 248, 1248–1254.
- 28 Nieminen, A.L., Gores, G.J., Dawson, T.L., Herman, B. and Lemasters, J.J. (1990) Toxic injury from mercuric chloride in rat hepatocytes. *J. Biol. Chem.* 265, 2399–2408.
- 29 Levesque, P.C. and Atchison, W.D. (1987) Interactions of mitochondrial inhibitors with methylmercury on spontaneous quantal release of acetylcholine. *Toxicol. Appl. Pharmacol.* 87, 315–324.