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Journal

Toxicology and applied pharmacology, 88(1)

ISSN

0041-008X

Authors

Komulainen, H
Bondy, SC

Publication Date

1987-03-01

DOI

10.1016/0041-008x(87)90271-7

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Increased Free Intrasyntosomal Ca^{2+} by Neurotoxic Organometals: Distinctive Mechanisms

HANNU KOMULAINEN¹ AND STEPHEN C. BONDY*

Department of Behavioral and Neurological Toxicology, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, North Carolina 27709, and *Department of Community and Environmental Medicine, University of California, Irvine, California 92717

Received August 18, 1986; accepted October 29, 1986

Increased Free Intrasyntosomal Ca^{2+} by Neurotoxic Organometals: Distinctive Mechanisms. KOMULAINEN, H., AND BONDY, S. C. (1987). *Toxicol. Appl. Pharmacol.* 88, 77-86. Effects of several alkylmetals on free intrasyntosomal Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$, were studied *in vitro* using the fluorescent Ca^{2+} indicator fura-2. Neurotoxic alkylmetals methylmercury (Met-Hg), triethyllead (TEL), triethyltin (TET), and trimethyltin (TMT) (at 2.5-30 μM) increased $[\text{Ca}^{2+}]_i$ to different degrees. Met-Hg was the most potent, elevating $[\text{Ca}^{2+}]_i$ 100-800 nM, dose dependently and significantly more than high K^+ (150 nM) or veratridine (350 nM). The effect of Met-Hg could not be inhibited with a Ca^{2+} channel blocker, verapamil, nor with a Na^+ channel blocker, tetrodotoxin. Inhibition of the mitochondrial Ca^{2+} uptake *in situ* with rotenone + oligomycin decreased the potency of Met-Hg to elevate $[\text{Ca}^{2+}]_i$ but did not change the resting $[\text{Ca}^{2+}]_i$. Met-Hg also slightly decreased syntosomal ATP. TEL and TET elevated $[\text{Ca}^{2+}]_i$ by 100-200 nM. The effect of TEL, but not that of TET, could be blocked with verapamil (36%) and veratridine (67%). TEL was less efficient in the presence of ouabain. Neither TEL nor TET had significant mitochondrial effects *in situ* contributing to $[\text{Ca}^{2+}]_i$. TMT increased $[\text{Ca}^{2+}]_i$ less than TET while dimethyltin and methyltin were inactive. These results indicate that neurotoxic derivatives of alkylmetals studied increase $[\text{Ca}^{2+}]_i$. This occurs mainly either by nonspecific increase (Met-Hg, TET) of Ca^{2+} leakage through the plasma membrane and/or specific interference with the mechanisms regulating Ca^{2+} fluxes through the plasma membrane (TEL). © 1987 Academic Press, Inc.

The organometals methylmercury (Met-Hg), triethyllead (TEL) (Seawright *et al.*, 1984; Walsh and Tilson, 1984), triethyltin (TET), and trimethyltin (TMT) (Reuhl and Cranmer, 1984; Reiter and Ruppert, 1984) are neurotoxic but the underlying biochemical mechanisms causing impaired cell function and neuronal death are not as yet fully established. Trialkyltin and trialkyllead are thought to affect primarily mitochondria and thus energy metabolism of the cell (Aldridge, 1984; Bierkamper and Bassett, 1984). They

both stimulate Cl^-/OH^- ion exchange across the inner mitochondrial membrane in isolated mitochondria and cause accumulation of Cl^- in mitochondrial matrix (Selwyn *et al.*, 1970; Aldridge, 1984). Consequently hydrolysis of ATP increases. In addition TET, but not TEL, may decrease ATP synthesis (Rose and Aldridge, 1972; Skilleter, 1975; Aldridge *et al.*, 1977). However, TEL also induces morphological alterations in mitochondria *in vivo* (Seawright *et al.*, 1984), while TMT does not (Chang *et al.*, 1982; Brown *et al.*, 1984). This indicates that mitochondria are not similarly affected by all these compounds.

Met-Hg also affects mitochondrial functions, inhibiting respiration (Verity *et al.*,

¹ Present address: National Public Health Institute, Department of Environmental Hygiene and Toxicology, P.O. Box 95, SF-70701 Kuopio, Finland.

1975; Fox *et al.*, 1975; Varnbo *et al.*, 1985), uncoupling oxidative phosphorylation, and decreasing intracellular ATP (Cheung and Verity, 1981; Sarafian *et al.*, 1984). However, nonmitochondrial processes, such as cytosolic protein synthesis, may be even more sensitive to Met-Hg (Sarafian *et al.*, 1984; Cheung and Verity, 1985).

The plasma membrane is always traversed before intracellular targets are reached and it is possible that some intracellular effects of alkylmetals are indirect due to altered characteristics of the plasma membrane. The plasma membrane maintains vital ionic gradients and is the main barrier to separate 1 mM extracellular Ca^{2+} from 0.1–0.3 μM intracellular Ca^{2+} (>3000-fold concentration gradient; Tsien *et al.*, 1982; Hesketh *et al.*, 1983; Berthon *et al.*, 1984; Sistare *et al.*, 1985; Komulainen and Bondy, 1987). Ca^{2+} is an important messenger in the cell (Rasmussen *et al.*, 1984) and by increasing free intracellular Ca^{2+} , organometals might initiate chains of events exciting and finally killing the cell. In nerve endings, Ca^{2+} triggers release of neurotransmitters and there is already evidence that Met-Hg stimulates release *in vitro* (Komulainen and Tuomisto, 1981; Atchison *et al.*, 1984). We are reporting here effects of several alkylmetals on free intrasynaptosomal Ca^{2+} ($[\text{Ca}^{2+}]_i$) *in vitro*. Synaptosomes were used because they are readily available metabolically intact fragments of isolated neural tissue. Various pharmacological, neuroactive agents were used for comparison and to assess the specificity of the effect of alkylmetals and to elucidate their sites of action.

METHODS

Preparation of synaptosomes. Male Fischer 344 rats (Charles River, Wilmington, MA) between 20 and 24 weeks of age were used. Rats were housed in standard conditions (12-hr light–dark cycle, temperature $21 \pm 2^\circ\text{C}$, and humidity $50 \pm 10\%$), four per cage. After decapitation, 0.9–1.0 g cerebrum (excluding pons, medulla, and the main part of hippocampus) was dissected out on ice and purified synaptosomes prepared as described in detail elsewhere (Komulainen and Bondy, 1987) by the modified method of Gray and Whittaker (1962) (Dodd

et al., 1981). Synaptosomes were suspended in 25 mM Hepes buffer, pH 7.4, containing NaCl 125 mM, KCl 5 mM, NaH_2PO_4 1.2 mM, MgCl_2 1.2 mM, NaHCO_3 5 mM, glucose 6 mM, and CaCl_2 1 mM (Hepes buffer A). The protein concentration of the suspension was 1.4–1.6 mg/ml.

Assay of $[\text{Ca}^{2+}]_i$. Free intrasynaptosomal Ca^{2+} was measured using the fluorescent Ca^{2+} indicator dye fura-2. Details of the method and characteristics of the dye-loaded synaptosomes are in Komulainen and Bondy (1987). Briefly, in order to load synaptosomes with fura-2 (150–200 μM concentration), 1-ml aliquots of synaptosome suspension were incubated with 5 μM acetoxy-methyl ester of fura-2 (fura-2/AM) for 20 min at 37°C . Then 9.0 ml Hepes buffer (A) at 37°C was added and incubation continued for the next 25 min (total incubation time 45 min). Synaptosomes were immediately spun down (9000g, 5 min, Sorvall RC-5B) and resuspended in 5 ml ice-cold Hepes buffer (A). An identical tissue sample incubated with 5 μl DMSO was prepared in parallel to assay autofluorescence of synaptosomes.

The fluorescence was measured with Aminco SPF-500 spectrofluorometer (Silver Spring, MA) equipped with a thermostated sample holder (37°C) and a magnetic stirrer (Instech Laboratories, Inc., Horsham, PA). The excitation wavelengths were 340 and 380 nm (bandpass 1 nm) and the emission wavelength 510 nm (bandpass 8 nm). For calculation of $[\text{Ca}^{2+}]_i$, R , the ratio of the fluorescence at 340 nm/380 nm, was determined for each synaptosomal sample as follows: 0.5 ml of fura-2-loaded synaptosome suspension (containing 0.14–0.16 mg protein) was rapidly spun down with a microcentrifuge (13,000g, 30 sec) and synaptosomes resuspended in 2 ml Hepes buffer (at 37°C) omitting NaH_2PO_4 and NaHCO_3 (Hepes buffer B). These salts were excluded because otherwise Ca^{2+} precipitated out at elevated pH during calibration of the fura-2 fluorescence. Synaptosomes were allowed to equilibrate for 10 min in a cuvette before addition of any chemical under assay. Autofluorescence of synaptosomes was separately measured and subtracted from the fluorescence of the sample. Extrasynaptosomal fura-2 was quenched by Mn^{2+} (20–40 μM). For calculations of $[\text{Ca}^{2+}]_i$, two other ratios were determined for each fura-2-loaded homogenate (Komulainen and Bondy, 1987): $R_{\min}$, the ratio of the fluorescence at zero free Ca^{2+} , and $R_{\max}$, the ratio of the fluorescence at full Ca^{2+} saturation. This calibration of fura-2 fluorescence was performed in the presence of 10 μM diethylenetriamine pentaacetic acid (DTPA), a heavy metal chelator, to ensure chelation of all added Mn^{2+} in the sample. The final $[\text{Ca}^{2+}]_i$ was calculated as described by Grynkiewicz *et al.* (1985):

$$[\text{Ca}^{2+}]_i = K_d \cdot \left(\frac{R - R_{\min}}{R_{\max} - R} \right) \cdot \left(\frac{\text{Sf}_2}{\text{Sb}_2} \right).$$

Sf_2 is fluorescence of fura-2 at zero Ca^{2+} and Sb_2 at Ca^{2+} saturation at the excitation wavelength 380 nm. K_d , the dissociation constant of the fura-2– Ca^{2+} complex, was taken to be 224 nM (Grynkiewicz *et al.*, 1985).

Assay of synaptosomal ATP. Synaptosomal ATP was measured as described by Cheung and Verity (1981) after slight modifications of the method. Synaptosomes were made and resuspended in Hepes buffer (A) at the protein concentration 1.4–1.6 mg/ml. Aliquots (0.5 ml) of this suspension were incubated in 1.5 ml Hepes buffer (B) with indicated concentrations of organometals or other agents for 10 min at 37°C. Immediately after incubation, a 0.5-ml sample of the suspension was mixed with 0.5 ml 0.6 M ice-cold perchloric acid. Then samples were rapidly microcentrifuged and 62.5 μ l 2 M K_2CO_3 was added. The precipitate formed was spun down and a 100- μ l aliquot of the supernatant diluted with 0.9 ml Tris buffer, 40 mM, pH 7.4. ATP was assayed with luciferase–luciferin mixture (Sigma No. L 1761, 40 mg/ml) which was aged overnight in a refrigerator. For each assay, 0.5 ml of Tris buffer and 30–40 μ l of luciferase–luciferin mixture were pipetted into a glass scintillation vial; 30–60 μ l of the sample was added and the mixture vortexed. The peak luminescence was immediately recorded with a liquid scintillation counter (Beckmann LS-3100) operated manually. For quantitation of ATP, standards containing known amounts of ATP (disodium salt) were measured at the same concentration range as in samples. The final ATP concentration was read graphically on a log–log paper.

$^{45}Ca^{2+}$ accumulation. In experiments intended to study effects of organometals on Ca^{2+} accumulation in synaptosomes, synaptosomes (on an average 0.16 mg/ml protein, a final concentration) were incubated in Hepes buffer (A) containing 0.5 μ Ci/ml $^{45}Ca^{2+}$ (55.9 Ci/mol) for 10 min at 37°C. Synaptosomes were filtered on glass-fiber filters (Type A/E, Gelman Sciences, Inc.), washed thrice with 5 ml cold Hepes buffer (A) omitting $CaCl_2$ and containing 1 mM EGTA, and the radioactivity remaining in the tissue was measured (Komulainen and Bondy, 1987).

Protein assay. The protein content of synaptosomal suspensions was determined according to Lowry *et al.* (1951) using bovine albumin as a reference.

Statistics. Statistical analysis of the results was done with Fisher's least significant difference test after one-way analysis of variance. $p < 0.05$, with two-tailed t -distribution, was considered to be significant.

Chemicals. The acetoxymethyl ester of fura-2 and fura-2 (tripotassium salt) were purchased from Molecular Probes, Inc. (Junction City, OR) and $^{45}CaCl_2$ was from New England Nuclear Corp. (Boston, MA). Methylmercury chloride (95% pure), methyltin trichloride, dimethyltin dichloride, trimethyltin chloride, triethyltin bromide, and triethyllead chloride were purchased from Alfa Products (Danvers, MA). Triethyllead chloride was washed with diethylether before use. Without washing, contaminating Pb^{2+} was observed to reverse the shift in the fura-2 fluorescence induced by Ca^{2+} . ($\pm$)-Verapamil hydrochloride, ouabain octahydrate, veratridine, oligomycin, rotenone, iodoacetic acid (sodium salt), tetrodotoxin, and diethylenetriamine pentaacetic acid were from Sigma Chemical Co. (St. Louis, MO).

FIG. 1. Increase of free intrasynaptosomal Ca^{2+} , $[Ca^{2+}]_i$, by methylmercury, triethyllead, and triethyltin *in vitro*. Increase of $[Ca^{2+}]_i$ is expressed as that over the resting $[Ca^{2+}]_i$, which was 354 ± 9 (SE, $n = 14$). Rat cerebral synaptosomes were incubated with alkylmetals in Hepes buffer containing 1 mM Ca^{2+} for 10 min and $[Ca^{2+}]_i$ was measured with fura-2 (about 200 μ M intrasynaptosomal concentration). Results represent 4–5 measurements using three different preparations. All points are significantly different ($p < 0.01$ or lower) from control level.

RESULTS

Effect of alkylmetals on the resting $[Ca^{2+}]_i$. Of the organometals studied only those which are known to be potentially neurotoxic elevated $[Ca^{2+}]_i$ *in vitro*. Met-Hg increased $[Ca^{2+}]_i$ from its basal level of 350 nM in a dose-dependent manner (Fig. 1). Its initial effect was rapid and the Ca^{2+} level showed a continuing increase with time (Fig. 2).

TEL and TET were much less potent at the same concentration range, elevating $[Ca^{2+}]_i$ by 50–170 nM (Fig. 1). This effect was saturable and the Ca^{2+} level changed initially more slowly than with Met-Hg (data not shown).

Trimethyltin increased $[Ca^{2+}]_i$ by 49 ± 5 nM ($n = 5$, $p < 0.01$) at 30 μ M. It was significantly less potent ($p < 0.001$) than TET at the same concentration. In contrast, dimethyltin and methyltin were inactive even at 30 μ M (results not shown).

When Ca^{2+} was omitted from the incubation medium (nominally Ca^{2+} -free buffer),

FIG. 2. Time course of the increase of free intrasynaptosomal Ca^{2+} caused by methylmercury ($10 \mu\text{M}$). Synaptosomes were incubated at 37°C with methylmercury either for 1 min ($n = 7$) or for 10 min ($n = 4$). The increase (mean $\pm$ SE) is that over the resting $[\text{Ca}^{2+}]_i$. **Significantly different from the value at 1 min ($p < 0.001$).

the $[\text{Ca}^{2+}]_i$ dropped to $230 \pm 15 \text{ nM}$ ($n = 6$). Under such circumstances neither TEL nor TET increased $[\text{Ca}^{2+}]_i$ at all and Met-Hg only marginally (Fig. 3).

$^{45}\text{Ca}^{2+}$ accumulation. Met-Hg stimulated accumulation of $^{45}\text{Ca}^{2+}$ into synaptosomes in a dose-dependent manner from the incubation medium containing 1 mM Ca^{2+} (Table 1). TEL also enhanced Ca^{2+} uptake at the highest concentration studied (Table 1).

Effect of verapamil or tetrodotoxin on the increase of $[\text{Ca}^{2+}]_i$. In order to elucidate

FIG. 3. Effect of extrasynaptosomal Ca^{2+} concentration on free intrasynaptosomal Ca^{2+} and on the increase of $[\text{Ca}^{2+}]_i$ elicited by alkylmetals. Synaptosomes were incubated with the indicated alkylmetals at 37°C for 10 min. $[\text{Ca}^{2+}]_e$, concentration of Ca^{2+} in the buffer. Mean $\pm$ SE of 4–6 measurements. * $p < 0.05$ and *** $p < 0.001$, significantly different from the respective controls.

TABLE 1

EFFECT OF METHYLMERCURY AND TRIETHYLLEAD ON Ca^{2+} ACCUMULATION IN SYNAPTOSOMES

Agent	$^{45}\text{Ca}^{2+}$ uptake (% of control)
Control	100 ± 12 (5)
Methylmercury	
$2.5 \mu\text{M}$	106 ± 5 (3) ^a
$5 \mu\text{M}$	106 ± 5 (3)
$10 \mu\text{M}$	142 ± 24 (3)
$30 \mu\text{M}$	176 ± 20 ^b (2)
Triethyllead	
$5 \mu\text{M}$	103 ± 9 (3)
$10 \mu\text{M}$	107 ± 17 (3)
$20 \mu\text{M}$	156 ± 7 ^c (3)

Note. Synaptosomes (about $0.16 \text{ mg protein/ml}$) were incubated with alkylmetals in Hepes buffer (A) containing traces of $^{45}\text{Ca}^{2+}$ (total Ca^{2+} 1 mM) at 37°C for 10 min (see details under Methods). Ca^{2+} uptake in control samples was $12.6 \pm 1.6 \text{ nmol/mg protein/10 min}$ ($n = 5$). SE of the mean are presented.

^a Number of samples in triplicate.

^b $p < 0.05$, significantly higher compared to control.

^c $p < 0.01$, significantly higher compared to control.

whether Ca^{2+} fluxed in through the voltage-sensitive Ca^{2+} channels after synaptosomal exposure to organometals, synaptosomes were preincubated for 10 min with verapamil ($30 \mu\text{M}$), a blocker of one type of Ca^{2+} channels, before the addition of alkylmetals. Verapamil inhibited 36% of the TEL-induced elevation of $[\text{Ca}^{2+}]_i$ but could not modify the increased Ca^{2+} level effected by Met-Hg or TET (Table 2). Using classical depolarization methods to elevate $[\text{Ca}^{2+}]_i$, it was found that verapamil inhibited 48% of the increase in $[\text{Ca}^{2+}]_i$ by 50 mM K^+ and 31% of that by veratridine (Table 2).

Alkylmetals appeared to resemble veratridine (causing a linear increase in $[\text{Ca}^{2+}]_i$ after an initial rise, Fig. 4) more than high K^+ (a decrease after an abrupt rise in $[\text{Ca}^{2+}]_i$, Komulainen and Bondy, 1987) in their effect on $[\text{Ca}^{2+}]_i$. This raised the possibility that such metals in part elevated $[\text{Ca}^{2+}]_i$ by increasing intrasynaptosomal Na^+ concentration. Tetrodotoxin, a blocker of Na^+ channels (Li and White, 1977), inhibited nearly completely

TABLE 2

EFFECT OF VERAPAMIL ON THE HIGH K^+ -, VERATRIDINE- OR ORGANOMETAL-INDUCED INCREASE IN SYNAPTOSOMAL $[Ca^{2+}]_i$

Addition	Increase in $[Ca^{2+}]_i$ (nM)	
	No verapamil	30 μ M Verapamil
K^+ 50 mM	143 $\pm$ 4 (4) ^a	75 $\pm$ 9 ^b (4)
Veratridine 100 μ M	362 $\pm$ 17 (3)	251 $\pm$ 16 ^c (3)
Methylmercury 2.5 μ M	188 $\pm$ 15 (6)	164 $\pm$ 23 (6)
Triethyllead 10 μ M	174 $\pm$ 15 (7)	112 $\pm$ 10 ^c (7)
Triethyltin 10 μ M	108 $\pm$ 8 (6)	97 $\pm$ 7 (7)

Note. The mean $\pm$ SE resting $[Ca^{2+}]_i$ was 352 $\pm$ 6 nM (n = 26) before any addition of chemicals. Verapamil (in 60 μ l H₂O) was added 10 min before addition of 50 mM K^+ (with which synaptosomes were incubated for 1 min), veratridine, or organometals. Synaptosomes were incubated with veratridine or organometals for 10 min.

^a Number of determinations.

^b p < 0.001, significantly lower compared to corresponding value in the absence of verapamil.

^c p < 0.01, significantly lower compared to corresponding value in the absence of verapamil.

(85%) the effect of veratridine upon $[Ca^{2+}]_i$ (Fig. 4) and inhibited 67% of the increase of free Ca^{2+} caused by TEL (Fig. 5). In contrast, it could not significantly block the effect of TET or Met-Hg (Fig. 5). Hence, TEL might specifically increase Na^+ influx through Na^+ channels. Whereas, if there was any increase in intrasynaptosomal Na^+ concentration by Met-Hg or TET, it was not through Na^+ channels.

Modulation of the effect of ouabain. Another mechanism to achieve an increase in intrasynaptosomal Na^+ concentration is an inhibition of the plasma membrane " Na^+ pump," Na^+-K^+ -ATPase. Inhibition of this enzyme with ouabain (1 mM) increased gradually $[Ca^{2+}]_i$, about 100 nM in 10 min (Table 3). Alkylmetals, added after a preincubation of synaptosomes with ouabain, had different effects on $[Ca^{2+}]_i$. TET was additive to ouabain, Met-Hg even more potent with ouabain, but TEL lost about 50% of its potency in the presence of ouabain (Table 3). Hence, TEL may have increased $[Ca^{2+}]_i$ in part by

FIG. 4. Increase of free intrasynaptosomal Ca^{2+} by veratridine and its inhibition with tetrodotoxin. Synaptosomes were incubated with veratridine (added in 10 μ l DMSO) in Hepes buffer (1 mM Ca^{2+}) for 5 or 10 min. Tetrodotoxin was added in 20 μ l H₂O 1 min before veratridine where indicated. Mean $\pm$ SE of 5–6 measurements. * p < 0.001, significantly different from control value. † p < 0.001, significantly different from veratridine value at 10 min.

inhibiting the plasma membrane Na^+-K^+ -ATPase.

Effect of alkylmetals on mitochondria in situ. Mitochondria are thought to regulate $[Ca^{2+}]_i$, particularly at elevated $[Ca^{2+}]_i$ (>0.5

FIG. 5. Effect of tetrodotoxin on the alkylmetal-induced increase in synaptosomal $[Ca^{2+}]_i$. Tetrodotoxin was added 1 min before alkylmetal and synaptosomes were incubated for a further 10 min. Mean $\pm$ SE of 5–7 measurements. * p < 0.005 and *** p < 0.001, significantly different from the corresponding value obtained without tetrodotoxin.

TABLE 3

EFFECT OF OUABAIN ON SYNAPTOSOMAL $[Ca^{2+}]_i$ AND ON THE INCREASE OF $[Ca^{2+}]_i$ BY ORGANOMETALS

Addition	Increase in $[Ca^{2+}]_i$ (nM)	
	No ouabain	1 mM ouabain
H ₂ O (control)	—	116 ± 13 (6) ^a
Methylmercury 5 μM	318 ± 35 (4)	542 ± 38 ^b (6)
Triethyllead 10 μM	174 ± 15 (7)	194 ± 21 (6)
Triethyltin 10 μM	108 ± 8 (6)	209 ± 15 ^c (6)

Note. The mean ± SE increase of $[Ca^{2+}]_i$ is over resting $[Ca^{2+}]_i$ (370 ± 6 , $n = 25$); ouabain was added in 10 μl DMSO 2 min before organometals or H₂O (20 μl) and incubation continued for 10 min.

^a Number of measurements.

^b $p < 0.01$, significantly higher compared to corresponding value without ouabain.

^c $p < 0.001$, significantly higher compared to corresponding value without ouabain.

μM; Rasmussen *et al.*, 1984), and they provide ATP for ion pumps maintaining the ionic gradients across the plasma membrane. Alkylmetals could increase $[Ca^{2+}]_i$ by inhibiting Ca^{2+} uptake in mitochondria and/or decreasing intrasynaptosomal ATP. The combination of rotenone (inhibition of respiration) + oligomycin (inhibition of mitochondrial Mg^{2+} -ATPase and subsequently both ATP synthesis and hydrolysis by this enzyme) + iodoacetic acid (inhibition of glycolysis), both inhibits mitochondrial Ca^{2+} uptake and severely depletes synaptosomal ATP (Åkerman and Nicholls, 1981a,b, Table 6). These agents in combination caused $[Ca^{2+}]_i$ to rise linearly with time by 156 ± 16 nM ($n = 5$) in 10 min (Table 4) and by 280 ± 22 nM ($n = 6$) in 20 min. In these ATP-depleted synaptosomes, TEL increased $[Ca^{2+}]_i$ in an additive manner with the increase due to ATP depletion. The effect of TET upon such ATP-depleted preparations was enhanced ($p < 0.05$) but the rise in $[Ca^{2+}]_i$ caused by Met-Hg was considerably below (42%) that effected in normal synaptosomes (Table 4). Inhibition of mitochondrial Ca^{2+} uptake by rotenone + oligomycin did not alter resting $[Ca^{2+}]_i$ (Table 5). After such treatment of syn-

aptosomes with rotenone and oligomycin the response of $[Ca^{2+}]_i$ to TET or TEL was unaltered $[Ca^{2+}]_i$ while the effect of Met-Hg was again diminished 42% (Table 5).

Only Met-Hg significantly decreased synaptosomal ATP levels at the concentrations and in the time range that $[Ca^{2+}]_i$ was increased (Table 6). Met-Hg (5 μM) decreased ATP by 35%, to a similar extent as rotenone + oligomycin (41%). Unlike iodoacetic acid, Met-Hg did not deplete synaptosomal ATP when added together with rotenone + oligomycin (Table 6), suggesting that Met-Hg did not affect mitochondrial glycolysis. These data together indicate that neither TET nor TEL had significant mitochondrial effects contributing to their elevation of $[Ca^{2+}]_i$. In contrast, Met-Hg may inhibit mitochondrial Ca^{2+} uptake. This effect cannot occur in the presence of rotenone + oligomycin which also impairs such calcium transport into mitochondria indirectly.

DISCUSSION

In the present experiments potentially neurotoxic derivatives of alkylmetals increased

TABLE 4

INCREASE OF $[Ca^{2+}]_i$ BY ORGANOMETALS IN ATP-DEPLETED SYNAPTOSOMES

Addition	Increase in $[Ca^{2+}]_i$ (nM)	
	Normal synaptosomes	ATP-depleted synaptosomes
H ₂ O (control)	—	156 ± 16 (5) ^a
Methylmercury 2.5 μM	188 ± 15 (6)	201 ± 20 (6)
Triethyllead 10 μM	174 ± 15 (7)	309 ± 20 ^b (6)
Triethyltin 10 μM	108 ± 8 (6)	335 ± 24 ^b (6)

Note. The mean ± SE increase of $[Ca^{2+}]_i$ is the elevation over the corresponding value (498 ± 8 nM, $n = 24$) obtained after incubation of synaptosomes with rotenone (4 μM) + oligomycin (4 μg/ml) + iodoacetic acid (1 mM) for 10 min. After addition of the organometal, incubation was continued for another 10 min.

^a Number of determinations.

^b $p < 0.001$, significantly higher compared to normal synaptosomes.

TABLE 5

INCREASE OF SYNAPTOSOMAL $[Ca^{2+}]_i$ EFFECTED BY ORGANOMETALS IN SYNAPTOSOMES AFTER INHIBITION OF MITOCHONDRIAL Ca^{2+} UPTAKE *IN SITU* BY ROTENONE + OLIGOMYCIN

Addition	Increase in $[Ca^{2+}]_i$ (nM)	
	No rotenone + oligomycin	Rotenone (4 μ M) + oligomycin (4 μ g/ml)
H ₂ O (control)	—	23 $\pm$ 18 (4) ^a
Methylmercury 2.5 μ M	188 $\pm$ 15 (6)	108 $\pm$ 10 ^b (3)
Triethyllead 10 μ M	174 $\pm$ 15 (7)	202 $\pm$ 11 (4)
Triethyltin 10 μ M	108 $\pm$ 8 (6)	133 $\pm$ 13 (4)

Note. The mean $\pm$ SE resting $[Ca^{2+}]_i$ was 368 $\pm$ 8 (n = 34) before addition of any indicated agent. Rotenone + oligomycin was added in 20 μ l DMSO 2 min before organometals and the incubation continued for a further 10 min.

^a Number of determinations.

^b p < 0.01, significantly lower compared to corresponding value in the absence of rotenone + oligomycin.

free intrasynaptosomal Ca^{2+} *in vitro*. Each alkylmetal appeared to have distinctive mechanisms of action. Although more work is needed to elucidate the details of the mechanisms, some sites of action can already be pinpointed.

Regulation of $[Ca^{2+}]_i$ is complex with many simultaneous reactions that balance the net free Ca^{2+} concentration (Rasmussen *et al.*, 1984). The most important barrier to Ca^{2+} appears to be the plasma membrane, across which Ca^{2+} cycles through the influx (leakage, voltage-dependent Ca^{2+} channels) and efflux (Ca^{2+} pump, Na^+/Ca^{2+} exchange) mechanisms. Inside Ca^{2+} is bound to cytoplasmic Ca^{2+} binding and membrane proteins and is sequestered in the endoplasmic reticulum. The role of mitochondria in the regulation of synaptosomal resting $[Ca^{2+}]_i$ is at present controversial (Åkerman and Nicholls, 1981b; Nachshen, 1985; present results) but they may take up Ca^{2+} at elevated $[Ca^{2+}]_i$. Hence, there are many sites in Ca^{2+} metabolism as possible targets for alkylmet-

als. The main effect of alkylmetals was in the plasma membrane. Perturbation of the mechanisms maintaining the huge Ca^{2+} gradient and/or the integrity of the plasma membrane were crucially affected.

Of the alkylmetals studied, Met-Hg was the most potent in increasing $[Ca^{2+}]_i$. It affected the plasma membrane nonspecifically, rendering it leaky to Ca^{2+} . Because Met-Hg did not cause excess release of fura-2 from synaptosomes, the plasma membrane was not lysed. The initial effect on Ca^{2+} level was so immediate that a direct binding to the plasma membrane (SH-groups), and a subsequent opening of extra "gates" to Ca^{2+} (and possibly also to Na^+), is suggested. That extrasynaptosomal Ca^{2+} was the main source of Ca^{2+} is supported by two observations. Met-Hg could not increase $[Ca^{2+}]_i$ much in nominally Ca^{2+} -free buffer, and Met-Hg stimulated accumulation of $^{45}Ca^{2+}$ into synaptosomes from the extrasynaptosomal medium.

However, the effect of Met-Hg differed clearly from the specific depolarization of the

TABLE 6
ATP LEVELS IN SYNAPTOSOMES

Addition	ATP (nmol/mg protein)
Control	1.26 $\pm$ 0.08 (10) ^a
Methylmercury 2.5 μ M	1.06 $\pm$ 0.11 (5)
5 μ M	0.82 $\pm$ 0.11 ^b (5)
Triethyllead 10 μ M	0.94 $\pm$ 0.15 (5)
Triethyltin 10 μ M	0.96 $\pm$ 0.16 (5)
Rotenone 4 μ M + oligomycin 4 μ g/ml	0.75 $\pm$ 0.11 ^b (5)
Rotenone 4 μ M + oligomycin 4 μ g/ml + iodoacetic acid (1 mM)	<0.20 (5)
Rotenone 4 μ M + oligomycin 4 μ g/ml + methylmercury 5 μ M	0.70 (2)

Note. Synaptosomes (about 0.075 mg/ml protein) were incubated with various agents in Hepes buffer (B) at 37°C for 10 min and ATP was measured as described in Methods. Results are mean $\pm$ SE.

^a Number of samples.

^b p < 0.01, significantly lower as compared to control.

plasma membrane such as that induced by high K^+ or veratridine (Krueger *et al.*, 1980). During such depolarization, different subclasses of Ca^{2+} channels open to Ca^{2+} (Nowycky *et al.*, 1985), and verapamil blocks probably one type of these channels. Consequently, it inhibits elevation of $[Ca^{2+}]_i$ by 30–50% (present results; Komulainen and Bondy, 1987). However, verapamil could not inhibit to any extent the increase of $[Ca^{2+}]_i$ by Met-Hg, suggesting that the elicited Ca^{2+} influx was not through verapamil-sensitive Ca^{2+} channels.

The pattern of the increase of $[Ca^{2+}]_i$ by Met-Hg resembled somewhat that of veratridine. After an initial rapid increase, there was a slower linear phase. In the case of veratridine the latter slower phase is likely due to increased release of Ca^{2+} from mitochondria by Na^+/Ca^{2+} exchange (Åkerman and Nicholls, 1981b).

Met-Hg increases synaptosomal Na^+ (Cheung and Verity, 1981) and if the Na^+ influx is through Na^+ channels, tetrodotoxin should block it. The effect of Met-Hg on $[Ca^{2+}]_i$ could not be blocked by tetrodotoxin, suggesting that Met-Hg did not have any specific veratridine-like effect on Na^+ channels. Met-Hg also inhibits $Na^+-K^+-ATPase$ (Henderson *et al.*, 1979) and could increase synaptosomal Na^+ also by inhibiting the extrusion of Na^+ . A specific inhibition of $Na^+-K^+-ATPase$ with ouabain increased $[Ca^{2+}]_i$ slowly, but by only about 100 nM in the time range when Met-Hg had already doubled the resting level. Because the Met-Hg effect on $[Ca^{2+}]_i$ was additive with the corresponding effects of both ouabain and veratridine (unpublished result), an increase in intrasynaptosomal Na^+ could contribute only in part to the observed elevation of $[Ca^{2+}]_i$ by Met-Hg.

Met-Hg might also elevate $[Ca^{2+}]_i$ slightly from intrasynaptosomal sources, since it marginally increased $[Ca^{2+}]_i$ in nominally Ca^{2+} -free medium. Met-Hg is known to affect mitochondrial functions *in situ* (Verity *et al.*, 1975; Fox *et al.*, 1975; Cheung and Verity, 1981), suggesting that it can reach mitochondria. Met-Hg was less effective in elevating

$[Ca^{2+}]_i$ in synaptosomes, where Ca^{2+} uptake into mitochondria was inhibited (rotenone + oligomycin) or where in addition ATP was depleted (rotenone + oligomycin + iodoacetic acid) beforehand. This clearly suggests that mitochondria are a target of Met-Hg which possibly inhibits mitochondrial Ca^{2+} uptake. Although Met-Hg decreased synaptosomal ATP, a similar change effected by rotenone and oligomycin in combination (about 40%) did not yet effect $[Ca^{2+}]_i$. Only a nearly complete depletion of ATP increased $[Ca^{2+}]_i$ by 250–300 nM as observed also by Nachshen (1985).

TEL and TET differed clearly from Met-Hg in that their effects were saturable and both induced only a 100–200 nM increase in $[Ca^{2+}]_i$. TEL had a veratridine-like effect whereas TET was completely nonspecific. The data suggest that TEL increases intrasynaptosomal Na^+ concentration by stimulating Na^+ influx through Na^+ channels and by inhibiting extrusion of Na^+ out by $Na^+-K^+-ATPase$. Such a change in intrasynaptosomal Na^+ could gradually depolarize the plasma membrane and allow the specific Ca^{2+} influx through the voltage-sensitive Ca^{2+} channels. It remains uncertain by which mechanism TET increased $[Ca^{2+}]_i$, but it did not render the plasma membrane similarly leaky to Ca^{2+} as did Met-Hg.

Neither TEL nor TET significantly decreased synaptosomal ATP content and their effects on $[Ca^{2+}]_i$ were independent of the simultaneous modulation of the function of mitochondria with other agents. Hence, the effect on the plasma membrane was far more important with respect to $[Ca^{2+}]_i$ than any possible mitochondrial effect.

The observation that neurotoxic derivatives of alkylmetals increased free intrasynaptosomal Ca^{2+} has important implications. Such an increase in free Ca^{2+} is expected to stimulate intracellular Ca^{2+} -sensitive reactions providing alkylmetals do not directly inhibit them. The spontaneous release of monoamines from synaptosomes (Komulainen and Tuomisto, 1981) and that of acetylcholine from motor nerve endings (Miya-

moto, 1983; Atchison *et al.*, 1984) by Met-Hg could be explained with increased $[Ca^{2+}]_i$. Moreover, the depression of evoked transmitter release by Met-Hg (Atchison *et al.*, 1984) and TET (Allen *et al.*, 1980; Bierkamper and Valdes, 1982) may result from permanently increased $[Ca^{2+}]_i$ (Adams *et al.*, 1985). It remains to be studied how this potential of alkylmetals to increase $[Ca^{2+}]_i$ is related to their distinctive neurotoxicity.

ACKNOWLEDGMENTS

This study was supported in part to Dr. Komulainen by the Research Council for the Environmental Sciences, Academy of Finland. We appreciate the excellent secretarial assistance of Mrs. Marjukka Turunen.

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