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## **INTRACELLULAR CALCIUM AS AN INDEX OF NEUROTOXIC DAMAGE**

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### **SUMMARY**

The availability of techniques that allow the quantitation of levels of ionized calcium within intact cells and synaptosomes, allows a new approach to understanding the events underlying neurotoxicity. By use of various pharmacological agents, it is possible to dissect out vulnerable loci within the cell that account for the increases in cytosolic calcium which accompany many neurotoxic events. The relation between calcium and cell death can now be more concisely addressed. This article gives examples of the types of questions that this new methodology can resolve.

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*Key words:* Calcium; Neurotoxicity; Synaptosomes; Fura-2; Membranes

### **INTRODUCTION**

A wide range of toxic agents has been reported to damage the first cellular component to come in contact with circulating chemicals — the plasma membrane [1,2]. Another common site of action of xenobiotic agents is upon mitochondrial function [3,4]. Disruption of mitochondria can result in reduced formation of energy transferring components such as ATP. The central nervous system has an exceptionally high basal energy demand, largely due to the maintenance of ionic gradients, and their restoration after neuronal activity. The sodium-potassium pump is the predominant consumer of ATP within nerve tissue [5]. Thus brain function is especially sensitive to chemicals that interfere with oxidative phosphorylation. Any entropy-increasing event in the cell can enhance the need for anabolic metabolism, the excess activity being necessary for tissue repair; and the maintenance of homeostasis.

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Extracellular calcium concentrations are around 5000 times as great as the free ionic content of calcium within the neuron,  $[Ca^{2+}]_e$ . The very low level of  $[Ca^{2+}]_i$  within neurons can be rapidly changed by cellular depolarization resulting in calcium influx through specific channels. Levels of calcium after such an event are rapidly reduced by sequestration of calcium within endoplasmic reticulum and mitochondria. In addition, ATP-utilizing calcium pumps within the plasma membrane can eliminate intracellular calcium. The sustaining of the calcium gradient is also critical for cell viability and elevated levels of  $[Ca^{2+}]_i$  are associated with cell death [6]. Whether calcium influx is a direct cause of, or a secondary result of cell death in the nervous system remains a matter of controversy [7–9]. Nonetheless, there is good evidence that the calcium influx following repetitive hippocampal stimulation by chemical or electrical means, is the event that leads to regional necrosis [10,11]. In cerebellar slices, the neurotoxicity of excitatory amino acid agonists can be dependent on calcium concentration [12]. Elevated neuronal  $[Ca^{2+}]_i$  can produce excessive neurotransmitter release and the ensuing hyperactivity of nerve circuits can lead to an elevated energy requirement that cannot be adequately met. This is exacerbated by the stimulation of proteolytic and peroxidative events by calcium [13].

#### DETERMINATION OF IONIC CYTOSOLIC CALCIUM

The recent development of fluorescent probes, quin-2 and fura-2, for determination of the level of free ionic calcium within the cell has allowed a convenient, relatively direct assay of responses of  $[Ca^{2+}]_i$  to differing environmental conditions [14,15]. This procedure involves the diffusion of a relatively non-toxic dye ester into the cells and its subsequent hydrolysis to a free tetracarboxylic acid, reversibly chelating calcium. The complex, when suitably excited, emits a characteristic fluorescent signal. When used in a synaptosomal preparation, such dyes do not significantly modulate some key parameters such as ATP content and membrane potential of the synaptosome [16]. Furthermore, the physiological properties of the dye-loaded synaptosome, such as neurotransmitter release and other responses to pharmacological agents appear to be appropriate. Various neurotoxic agents have been shown to elevate levels of synaptosomal  $[Ca^{2+}]_i$ , while related non-neurotoxic congeners do not have this property [17,18]. These dyes may also allow the distinction to be made between the various potential processes by which neurotoxic compounds may alter  $[Ca^{2+}]_i$ .

#### MECHANISMS OF ELEVATED CALCIUM CONTENT

There are 2 major types of mechanism by which such changes could take place.

1. Neurotoxic agents may directly damage the plasma membrane, allowing an influx of extracellular calcium  $[Ca^{2+}]_e$  into the cytoplasm. Such entry could be specifically through Ca channels or could occur due to a non-specific increase in membrane leakiness.

2. The inhibition of glycolytic or oxidative phosphorylation processes of ATP generation could cause impairment of the energy-requiring calcium pumps or calcium sequestration processes. These lie within the plasma-membrane, mitochondria and endoplasmic reticulum.

#### ROLE OF THE PLASMA MEMBRANE

Recent evidence suggests a role of the plasma membrane in accounting for increased synaptosomal  $[Ca^{2+}]_i$  following exposure to several neurotoxic agents: Chlordecone ( $20 \mu M$ ), will rapidly cause elevated  $[Ca^{2+}]_i$  in exposed synaptosomes. However, if  $[Ca^{2+}]_o$  is reduced to below  $10 \mu M$ , this increase in  $[Ca^{2+}]_i$  does not occur [17]. A similar situation is found using various organometals (Fig. 1). Furthermore, the elevation of  $[Ca^{2+}]_i$  in synaptosomes is reduced in the presence of the calcium channel blocker verapamil. A similar dependence of the level of  $[Ca^{2+}]_o$  to enable increases of  $[Ca^{2+}]_i$  has been found using  $30 \mu M$  triethyl lead (TEL),  $30 \mu M$  triethyltin (TET) and  $10 \mu M$  methyl mercuric chloride [18]. In the case of the latter 2 organometals, concurrent administration of verapamil, a calcium channel blocker, does not cause significant changes of  $[Ca^{2+}]_i$ , suggesting no influence of methyl mercuric chloride or TET on the verapamil-related calcium channel. However, the  $[Ca^{2+}]_i$  increase in response to TEL, is partly blocked by verapamil, implying selective damage to this calcium channel. The effect of TEL upon  $[Ca^{2+}]_i$  is attenuated in the presence of  $5 \mu M$  tetrodotoxin. This is in contrast to TEL and methyl mercuric chloride [18]. These data further suggest that TEL acts upon the voltage regulated calcium channel, since blockage of the sodium channel by tetrodotoxin maintains the cell in a hyperpolarized state.

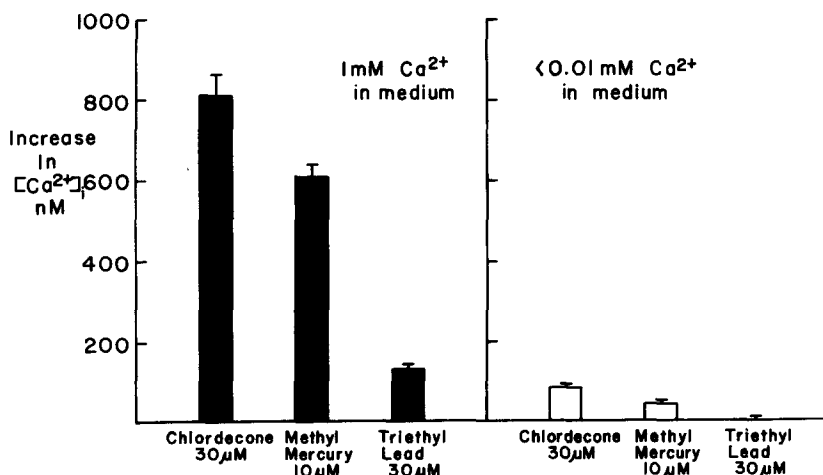


Fig. 1. Elevation of synaptosomal  $[Ca^{2+}]_i$  in high and low calcium-media, by various neurotoxic agents. Chemicals were incubated with synaptosomes for 10 min at  $37^\circ C$  prior to  $[Ca^{2+}]_i$  assay.

Organometals and  $10^{-3}$  M ouabain (a  $\text{Na}^+$ ,  $\text{K}^+$  ATPase inhibitor) together, raise  $[\text{Ca}^{2+}]_i$  in an additive manner. The lack of interaction between these agents, suggests that inhibition of the sodium pump by organometals is not the major cause of the neurotoxicity of such compounds.

#### COMPARISON WITH $^{45}\text{Ca}$ UPTAKE

The results obtained with fura-2 suggest that chlordecone would stimulate  $^{45}\text{Ca}$  uptake by synaptosomes. However, the accumulation of  $^{45}\text{Ca}$  in 10 s [19], by synaptosomes in the presence of 20  $\mu\text{M}$  chlordecone is significantly *reduced* under both depolarizing and resting conditions [17].

A similar inhibition of  $^{45}\text{Ca}$  uptake into subcellular organelles of the nervous system by organochlorine insecticides has been reported by others [20,21]. In fact, the toxicity of these compounds has been attributed to calcium deficiency as well as to excess [22,23].

The discrepancy between  $[\text{Ca}^{2+}]_i$  and  $^{45}\text{Ca}$ -based information could have several causes. A considerable proportion of synaptosomal  $^{45}\text{Ca}$  uptake is due to mitochondrial accumulation. The apparent inhibition of such uptake by chlordecone may reflect failure of mitochondrial function. Another possibility is synaptosomal destruction. The calcium signal of extracellular fura-2 can be rapidly quenched with 40  $\mu\text{M}$   $\text{Mn}^{2+}$ . This allows the estimation of synaptosomal integrity using this dye. By such means it was found that chlordecone caused significant synaptosomal leakage or lysis [17]. Thus, the loss of intact synaptosomes could account for the apparent reduction of  $^{45}\text{Ca}^{2+}$  uptake. The increase in the fura-2 calcium signal caused by chlordecone was from dye within surviving *intact* synaptosomes. The measurement of  $[\text{Ca}^{2+}]_i$  can thus provide information that cannot be attained with  $^{45}\text{Ca}$  transport studies. This may help to explain some of the apparent paradoxical data described previously.

The fact that verapamil does not completely block the elevation in  $[\text{Ca}^{2+}]_i$  induced by chlordecone, together with evidence of general damage to the plasma membrane, suggests that deleterious effects are not confined to a calcium gating area. The combined use of various techniques is illustrated using  $\gamma$ -hexachlorocyclohexane (lindane). At concentrations below 1  $\mu\text{M}$ , this pesticide has no effect on  $[\text{Ca}^{2+}]_i$  while  $^{45}\text{Ca}^{2+}$  uptake is stimulated in synaptosomes (R.M. Joy, personal communication). This may reflect an increased entry of calcium but at a rate that can be dealt with and sequestered or pumped out by a metabolically active synaptosome. This is further suggested by the observation of an increased  $^{45}\text{Ca}^{2+}$  efflux from lindane-treated synaptosomes (R.M. Joy, personal communication). At higher concentrations of lindane,  $[\text{Ca}^{2+}]_i$  is elevated while  $^{45}\text{Ca}^{2+}$  influx is inhibited. Since synaptosomal leakage is increased under such conditions (unpublished data), the results can be accounted for in terms of elevated  $[\text{Ca}^{2+}]_i$  in surviving synaptosomes but a loss of  $^{45}\text{Ca}^{2+}$  uptake ability by lysed or permeable synaptosomal fragments.

## MITOCHONDRIAL INTEGRITY

The maintenance of  $[Ca^{2+}]_i$  at a low level, does not seem to require a fully functioning mitochondrial ATP generating system. For example,  $[Ca^{2+}]_i$  within synaptosomes is not initially severely affected by uncoupling of mitochondrial oxidative phosphorylation with the  $K^+$ -ionophore valinomycin or the protonophore carbonyl cyanide *n*-chlorophenylhydrazone. Only when the concentration of ATP within the synaptosome has greatly fallen, does  $[Ca^{2+}]_i$  rise (Fig. 2).

Calcium-removing processes appear to have a priority for available energy when ATP levels are abnormally depressed. Thus the synaptosomal  $[Ca^{2+}]_i$  response to neurotoxic agents is attenuated when calcium entry from the surrounding milieu is prevented, while the effects of mitochondrial uncoupling agents upon  $[Ca^{2+}]_i$  do not generally interact with the effects of neurotoxic chemicals.

Metabolic inhibition of mitochondrial oxidative phosphorylation and glycolysis causes a major fall in cytoplasmic ATP content but has a relatively minor effect on  $[Ca^{2+}]_i$ . In contrast, several organometal and organochlorine neurotoxicants can greatly elevate  $[Ca^{2+}]_i$  while ATP levels are not very depressed (Fig. 2). This suggests that mitochondria are not the major site of damage caused by these neurotoxic agents. Another recent report also found mitochondrial ATP-generating systems not to be the primary target of triethyl lead [24].

When oxidative phosphorylation is inhibited, glycolytic activity alone can maintain synaptosomal ATP levels at around 44% of the normal level. Under

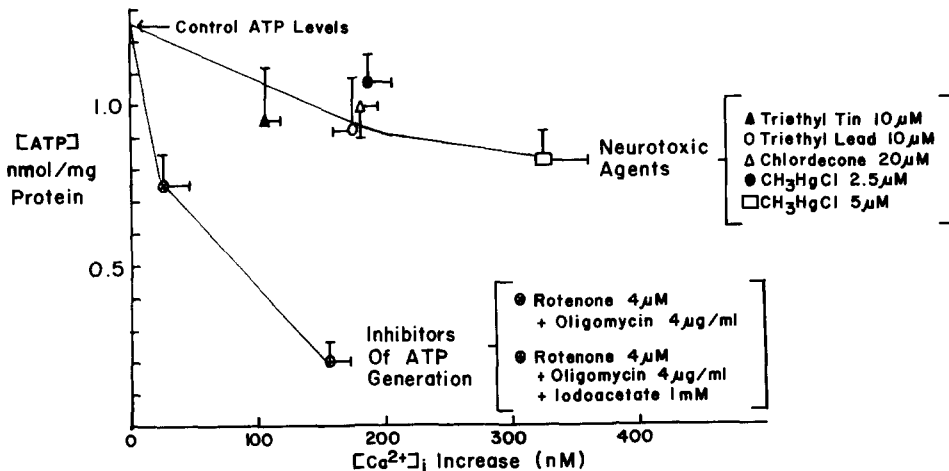


Fig. 2. Synaptosomal  $[Ca^{2+}]_i$  and ATP levels after treatment with various agents for 10 min at 37°C. Bars represent S.E. of each parameter assayed.

such circumstances the effect of TET and TEL upon  $[Ca^{2+}]_i$  is not altered, but the increase of  $[Ca^{2+}]_i$  produced by methyl mercuric chloride is actually diminished. This may be because part of the effect of this latter compound upon  $[Ca^{2+}]_i$  is due to mitochondrial impairment. A distinctive effect of methyl mercuric chloride upon mitochondrial function is also implied by the finding that this organometal, (but not TET or TEL), reduced the membrane potential of mitochondria within synaptosomes (unpublished result). These data suggest that the plasma membrane is the first and most vulnerable target of several neurotoxic agents and that mitochondrial damage must be severe before it is reflected by a failure of calcium homeostatic mechanisms.

## CONCLUSION

Neurotoxicant-induced derangement of calcium metabolism has often been inferred by alterations of  $^{45}Ca$  transport or inhibition of mitochondrial ATPase [25,26], perhaps consequent to attack on critical sulfhydryl groups [27,28]. The availability and ease of application of this new technology by which  $[Ca^{2+}]_i$  can be assayed in metabolically active cells and synaptosomes, should allow a more direct evaluation of calcium levels in intracellular compartments.

An extensive range of targets within the cell could account for an increase in  $[Ca^{2+}]_i$  effected by exposure to a neurotoxic agent. By use of appropriate pharmacological agents, the major locus of action can be dissected out. The relevance of this approach is enhanced by the finding that, while several classes of neurotoxicant elevate  $[Ca^{2+}]_i$ , related non-neurotoxic chemicals do not possess this property [17,18].

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