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# Intracellular Calcium and Neurotoxic Events

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BONDY, S. C. *Intracellular calcium and neurotoxic events*. NEUROTOXICOL TERATOL 11(6) 527-531, 1989.—Calcium is important in many intracellular regulatory processes. However, the maintenance of low levels of this cation within the cytosol is essential for maintenance of cell viability, in view of the large concentration gradient of ionic calcium across the plasma membrane. The expenditure of energy is needed to maintain intracellular calcium concentration  $[Ca^{2+}]_i$  at normal levels. In addition, the integrity of the limiting membrane is also vital for this function. Thus, any disruption of membrane characteristics or of mitochondrial anabolic processes may lead to deleterious levels of  $[Ca^{2+}]_i$ . The toxicity of a wide range of unrelated agents may, therefore, be in part due to elevation of cytosolic calcium. This general event may synergize with the more selective harmful properties of a compound, thus adversely affecting cell metabolism. The capacity now exists to measure levels of  $[Ca^{2+}]_i$  in isolated cells or organelles such as synaptosomes. The use of such in vitro models can be of value in the evaluation of the neurotoxic potential of compounds. This method, in conjunction with the use of pharmacological agents known to act at specific sites, and with the use of radioactive calcium in translocation studies, also has utility in the delineation of the biochemical mode of action of neurotoxic agents.

Intracellular calcium    Neurotoxicity    Synaptosomal metabolism    Membrane integrity    Mitochondrial function

## THE ROLE OF CALCIUM IN THE NERVOUS SYSTEM

Calcium is recognized as a key regulatory component in a wide range of cellular processes. Several classes of protein kinase have their activity modulated by calcium. These include a large number of 3'5' cyclic AMP protein kinases, as well as protein kinase C and the numerous calmodulin-regulated kinases. Superimposed on this is the characteristic of excitable tissue, of extensive depolarization-mediated calcium entry in the cell. Neurotransmitter and neuroendocrine release processes depend upon this rapid flux of  $Ca^{2+}$ . However, in order to effect cessation of release events,  $Ca^{2+}$  must quickly be restored to resting levels. These calcium oscillations essential for neuronal functioning are in the range  $10^{-7}$ – $10^{-5}$  M and, thus, involve 100-fold changes in the levels of intracellular ionic calcium,  $[Ca^{2+}]_i$ . The concentration of free calcium in extracellular fluid is about 1 mM, several orders of magnitude greater than maximal  $[Ca^{2+}]_i$  observed under normal circumstances.

### DETERMINANTS OF $[Ca^{2+}]_i$

The levels of  $[Ca^{2+}]_i$  represent the sum of many processes some of which effect increases in intracellular calcium while others act to reduce calcium levels.

#### Reduction

Reduction of  $[Ca^{2+}]_i$  is generally against a concentration gradient and, thus, requires expenditure of energy. Such energy may be in the form of ATP as in the calcium pumps found within the plasma membrane, the mitochondrion and the endoplasmic reticulum. In addition, energy may be expended by consumption of a previously established gradient of another ion. Such ion exchangers exist at the plasma membrane ( $Na^+/Ca^{2+}$  exchange) and at the mitochondrial surface ( $H^+/Ca^{2+}$  exchange).  $Ca^{2+}$  may

be extruded from the cell surface or sequestered within the mitochondrion or endoplasmic reticulum by chelation or precipitation as calcium phosphate. Since reduction of  $[Ca^{2+}]_i$  is energy-dependent, while its elevation is not, deficiencies of cellular anabolic metabolism may secondarily lead to failure to maintain relatively low  $[Ca^{2+}]_i$ .

#### Elevation

Two main sources of elevation of  $[Ca^{2+}]_i$  exist. Extracellular calcium may enter the cell through the plasma membrane. Such entry may be by way of specific calcium channels or exchangers. A number of distinctive calcium channels have been pharmacologically identified.  $Ca^{2+}$  entry may also be effected by nonspecific damage to the cell membrane, since the integrity of this membrane is essential for maintenance of the enormous gradient between  $Ca^{2+}$  of the extracellular fluid and that of the cytosol. Under normal circumstances, passive membrane permeability for Ca ions is four orders of magnitude lower than for Na or K ions (42). Eighty percent of such passive leak is through quiescent Na channels (16).

Another major means by which  $Ca^{2+}$  may be elevated within the cell is by release of ionic calcium from intracellular stores in mitochondria or endoplasmic reticulum. Less than 1% of the total cellular calcium content is in the free, soluble form; the bulk being sequestered in subcellular membranes and organelles. Such stores can be liberated by mitochondrial damage, or by specific messenger chemicals such as inositol triphosphate. The latter process is a finely regulated system whereby events at the cell surface can effect intracellular enzyme activation, with phosphoinositides acting as a primary and calcium as a secondary information carrier.  $K^+$  depolarization can both allow  $Ca^{2+}$  entry into excitable tissue and also induce release of  $Ca^{2+}$  from intracellular storage sites (28).

THE RELATION OF  $[Ca^{2+}]_i$  TO CELL DEATH

High levels of  $[Ca^{2+}]_i$  have often been correlated with irreversible damage to cells. However, whether such a correlation is causally related is not always clear. Calcium elevation may in some circumstances merely reflect breakdown of cellular integrity or failure of energy-generating metabolic events, either of which will undoubtedly lead to elevated  $[Ca^{2+}]_i$ . Many toxic agents act on mitochondrial function or on cell membranes. Cyanide, an inhibitor of oxidative phosphorylation and mitochondrial ATP generation, can elevate  $[Ca^{2+}]_i$  in a neuronal-derived cell line (27). General anesthetics which can disrupt membrane structure, also elevate axonal  $[Ca^{2+}]_i$  (56). However, calcium influx may accompany toxic effects in nerve cells without necessarily being the cause of cell death. Thus, the accumulation of insoluble calcium deposits is associated with many types of neurological damage including seizures (23), spinal cord injury (5), uremia (2), aluminum encephalopathy (20), lead poisoning (53), and kainic acid treatment (33). In any event,  $[Ca^{2+}]_i$  can often serve as an effective indicator of imminent cell death (15). A more complex question is to what extent can an abnormally high  $[Ca^{2+}]_i$  act as a toxic agent and directly contribute to cell damage.

Many potential routes for cell disruption by way of elevated  $[Ca^{2+}]_i$  have been proposed. Calcium-activation of proteases can cause excessive protein degradation (12,52). Calcium-effected activation of phospholipases elevates free fatty acid levels and this could trigger oxidative cell damage (47). Calcium-activated brain transglutaminase has been shown to cause crosslinking of neurofilament proteins into an insoluble polymer in an *in vitro* study, implying disruption of axoplasmic transport processes (46). Attempts to maintain  $[Ca^{2+}]_i$  at physiologic levels can lead to secondary deposition of excess calcium phosphate, which may itself be disruptive to the cell (54). Calcium may also potentiate oxidative cell injury (51), but conflicting data which suggest a protective role for calcium in such circumstances (19) have also been presented. The influence of oxidative processes upon calcium metabolism is less ambiguous. A variety of prooxidants have been shown to induce calcium release from mitochondria probably consequent to oxidative destruction of pyridine nucleotides (41).

Evidence for elevated  $[Ca^{2+}]_i$  being a cause rather than merely an effect of neurotoxic damage includes reports where the presence of calcium in the incubation medium can exacerbate the toxicity of excitatory amino acids (20, 21, 26, 37, 43) and a variety of toxins (45). The toxicity of excess synaptic activity, resulting in calcium reducing mechanisms being overwhelmed (23), also suggests a direct toxic role for calcium, as do the protective properties of calcium antagonists such as  $Mg^{2+}$  (57),  $Cd^{2+}$  and verapamil (18,37). Conversely, the cellular toxicity of calcium ionophores which allow calcium translocation across the plasma membrane (5,34) also substantiates a direct role for calcium in cell injury. Neuronal calcium accumulation following ischemia precedes cell death by several days (44) and such accumulation is found in dying rather than dead cells (22).

THE ASSAY OF  $[Ca^{2+}]_i$  AND THE UTILITY OF SYNAPTOSOMES

Recent techniques allow the visualization of  $[Ca^{2+}]_i$  in living cells to be followed by spectrophotometric or fluorescent means.

The development of fluorescent probes, quin-2, fura-2 and indo, allows a relatively direct assay of responses of  $[Ca^{2+}]_i$  to differing environmental conditions (24). The procedure involves the diffusion of a relatively nontoxic dye ester into the cytosol and its subsequent hydrolysis to a free anionic tetracarboxylic acid, reversibly chelating calcium. The calcium 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. Furthermore, the physiological properties of the dye-loaded synaptosome, such as neurotransmitter release and other responses to pharmacological agents, appear to be appropriate (30,31). Limitations of the use of these dyes include:

1. The generation of small quantities of toxic formaldehyde during ester hydrolysis.
2. The buffering effect of these compounds will slow the response of calcium transients to altered environmental conditions.
3. Other potential complications include the potential formation of calcium-insensitive fluorescent species from fura-2 (25) and the dependence of measured responses on the exact intracellular location of this dye (3).
4. The possibility that some neurotoxic metals will either quench dye fluorescence or form a fluorescent complex similar to the calcium-dye complex (39). The presence of several metals such as  $Fe^{2+}$ ,  $Mn^{2+}$ ,  $Zn^{2+}$ ,  $Pb^{2+}$ ,  $Hg^{2+}$  can lead to erroneous calculations of  $[Ca^{2+}]_i$  (4,30). These problems do not exist for the 5,5'-difluoro derivative of 1,2-bis(o-amino-phenoxy) ethane N,N,N',N'-tetraacetic acid (FBAPTA). This indicator can be monitored by  $^{19}F$  NMR spectroscopy (49). In this case, each metal chelate possesses a distinctive resonance profile and, thus, levels of several metals can be simultaneously determined. Another advantage of the NMR approach is its potential for study of calcium levels within nervous tissue regions in intact animals.

Aequorin has been used as a luminescent probe for calcium for almost 20 years (35). It has the disadvantages of a nonlinear response to  $[Ca^{2+}]_i$ , a responsiveness to  $Mg^{2+}$  and relatively low light emission. However, its advantages include a poor affinity for calcium so that the normal distribution of calcium in cellular compartments is not influenced and more rapid calcium transients are not slowed, and a superior signal/noise ratio (58). Similar disadvantages and advantages may exist for the dye, arsenazo III, whose absorbance increases in the presence of  $Ca^{2+}$  (1). The merits of a variety of calcium-measuring procedures are well reviewed by Cobbold and Rink (11).

NEUROTOXICITY AND  $[Ca^{2+}]_i$ 

Neurotoxic agents have the potential to influence  $[Ca^{2+}]_i$  by disruption of any of the many systems engaged in maintaining calcium homeostasis (Fig. 1). The mode of action of some agents is more understood than others. Thus, cyanide-induced elevations of  $[Ca^{2+}]_i$  are likely to be mediated by inhibition of oxidative phosphorylation, while the toxicity of the anticholinesterase diisopropylfluorophosphate (DFP) may be in part due to neuronal hyperactivity and excess calcium entry through specific voltage-operated channels at the plasma membrane (17). Anesthetic-induced elevations of  $[Ca^{2+}]_i$  may have their origins in modulation of the overall microviscosity of the plasma membrane (14). However, the toxicity of organochlorine insecticides or organometals may involve damage to both mitochondria and the plasma membrane (6, 31, 32). These chemicals with a relatively high  $LD_{50}$  are likely to be less specific than pharmacological agents or biological toxins, both of which have a relatively precise effect due to man-made or evolutionary design, respectively. Chemicals with inadvertent toxicity to man generally act at more than a single locus.

When a comparison is made between the effects of chemicals upon synaptosomal ATP levels and  $[Ca^{2+}]_i$ , agents can be divided into two broad classes (Fig. 2). Inhibitors of oxidative phosphorylation depress ATP severely, but  $[Ca^{2+}]_i$  is only relatively modestly elevated. In contrast, several organometal and organochlorine neurotoxicants can greatly elevate  $[Ca^{2+}]_i$ , while ATP

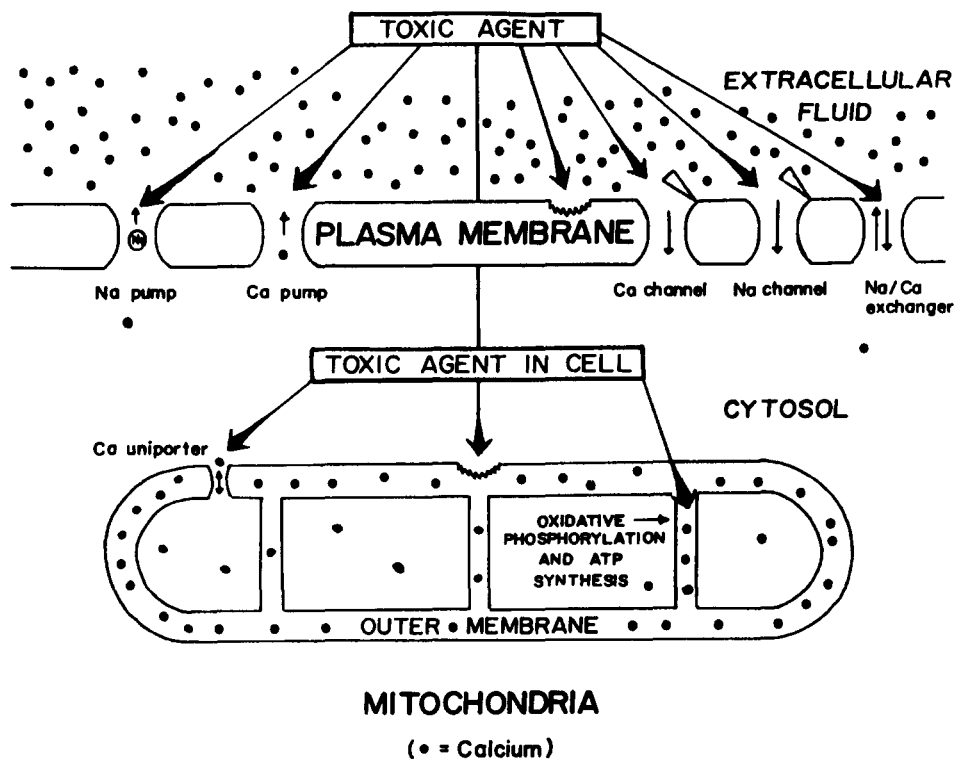


FIG. 1. Mechanisms by which neurotoxic agents may increase neuronal levels of free ionic calcium.

levels are not very depressed. Thus the maintenance of  $[Ca^{2+}]_i$  at a low level does not seem to require a fully functioning mitochondrial ATP generating system. This suggests that mitochondria are not the major site of damage caused by these neurotoxic agents. It also implies that calcium-removing processes have a priority for available energy when ATP levels are abnormally depressed (31).

A promising aspect of the use synaptosomes in this type of study is the finding that the effect upon  $[Ca^{2+}]_i$  of toxic agents within a given class is directly related to their known neurotoxicity in vivo. Thus, the neurotoxic insecticide chlordecone is much more potent than the closely related nonneurotoxic mirex in

elevating  $[Ca^{2+}]_i$  (32). Similar parallels have been found for mono, di, and trimethyl tins, of which only the latter is neurotoxic (31). Also analogous is the response of  $[Ca^{2+}]_i$  to various isomers of hexachlorocyclohexane, of which the  $\gamma$ -isomer (lindane) is the most neurotoxic (6). Such a good correlation between toxic potency and effects on  $[Ca^{2+}]_i$  suggests that these events might be causally related. Furthermore, it may be that the ability to elevate synaptosomal  $[Ca^{2+}]_i$  in vitro may be an indicator of neurotoxicity in the intact animal.

Synaptosomal studies also have the potential for a more defined study of factors influencing the effect of toxic chemicals. For example, ganglioside  $GM_1$  pretreatment appears to increase the resistance of the synaptosomal plasma membrane to damage by chlordecone (7). Also, the vulnerability of synaptosomes to chlordecone-induced lesions is increased in preparations derived from aged animals (8). The use of fura-2 in such studies can also give another index of synaptosomal integrity, namely the rate at which this dye leaks out of synaptosomes. This index of membrane permeability often confirms damage as evidenced by a rising  $[Ca^{2+}]_i$ .

Comparison of results obtained on  $[Ca^{2+}]_i$  with those derived from  $^{45}Ca$  studies of calcium movements, shows that they measure very different phenomena. Concentrations of organochlorine insecticides which elevate  $[Ca^{2+}]_i$  can also depress  $^{45}Ca$  uptake by synaptosomes. This has been attributed to synaptosomal leakiness being sufficient to prevent accumulated  $^{45}Ca$  being retained during the washout process (6,32). Similarly, while ethanol increases synaptosomal  $[Ca^{2+}]_i$  (14),  $^{45}Ca$  uptake is reduced (48,50). In this case, the inhibition has been related to blockage of specific calcium channels.

While aluminum elevates total accumulation of calcium in the nervous system (59), it also inhibits rapid voltage-dependent calcium influx into synaptosomes (29). Thus, short-term calcium-

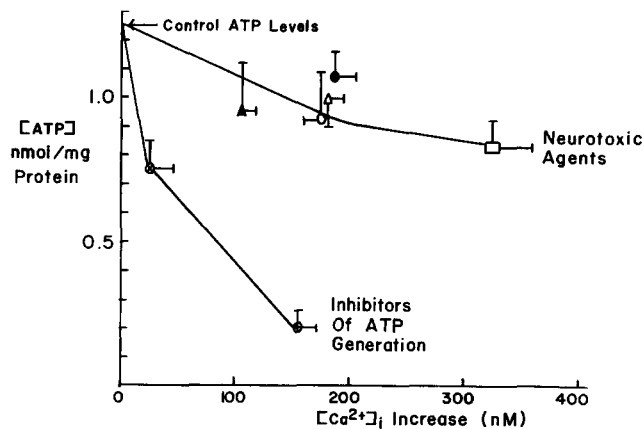


FIG. 2. Synaptosomal  $[Ca^{2+}]_i$  and ATP levels after exposure to various chemicals for 10 minutes at 37°C. Bars represent standard errors of each parameter assayed [from Bondy and Komulainen (9)].

related events may not always allow prediction of the effects of chronic exposures.

Another circumstance where  $[Ca^{2+}]_i$  can be elevated while  $^{45}Ca$  uptake is depressed, is when mitochondrial damage is the underlying lesion. Under such circumstances,  $[Ca^{2+}]_i$  can be elevated as ion pumps fail, but  $^{45}Ca$  accumulation may be depressed since 80% of  $^{45}Ca$  taken up by synaptosomes is rapidly accumulated by mitochondria. Thus, any damage at this locus could inhibit such uptake. For this reason, anoxia depresses  $^{45}Ca$  uptake by synaptosomes (38). However, some neurotoxic agents may simultaneously elevate  $[Ca^{2+}]_i$  and  $^{45}Ca$  uptake (31), so these assays clearly represent relatively unrelated parameters.

#### CONCLUSIONS

The functioning of the nervous system requires intermittent major elevations of free ionic calcium levels in the cytosol. This flux is likely to be more sensitive to disruption than maintenance of an unchanging homeostasis. Many neurotoxic agents act by increasing neuronal firing rates and causing hyperactivity within the nervous system. Virtually all insecticides possess this property. While elevated calcium levels are a result of such excess excitability, it is likely that such elevations both contribute to cellular damage and act synergistically by leading to further transmitter release and hyperactivity. Such events obviously have the potential of being propagated transneuronally.

The plasma membrane is the first cellular constituent to encounter xenobiotic chemicals and, thus, can be especially sensitive to disruption and this inevitably can imperil the maintenance of the large and critical gradient between extra- and intracellular calcium. Another potential target is the mitochondrion. This organelle contains a range of potential sites critical for optimal oxidative phosphorylation. This is a particularly active system within central nervous tissue. The exceptionally high basal energy demand of the brain is due largely to the need for maintenance of ionic gradients (36). Any entropy-increasing event in the cell can further enhance the need for anabolic, aerobic metabolism. Thus, elevations of  $[Ca^{2+}]_i$  may constitute part of a final common pathway through which the toxic effects of many agents are expressed. Harmful chemicals may act initially by various means including a) damage to energy-generating systems, b) changes in plasma membrane integrity or excitability and c)

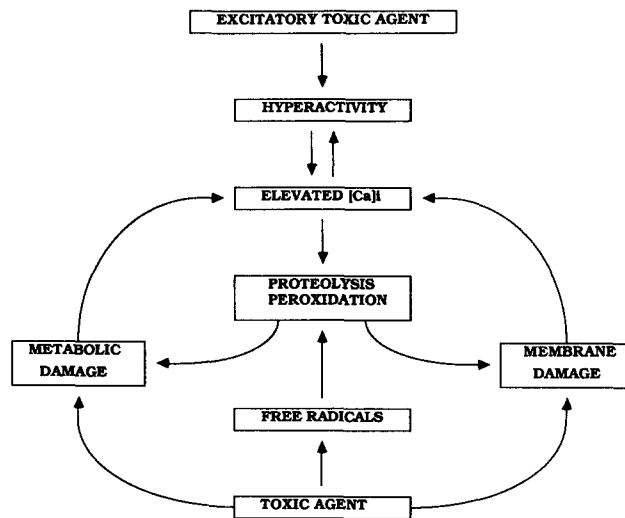


FIG. 3. Proposed means by which elevated  $[Ca^{2+}]_i$  may mediate and exacerbate various forms of neurotoxic damage.

initiation of oxidative damage (Fig. 3).

The site of toxic attack resulting in elevation of  $[Ca^{2+}]_i$  can in part be dissected out by use of a range of pharmacological agents acting at a clearly specific locus. However, this identification procedure has limitations. It is important to bear in mind that protection against chemicals acting at targets remote from direct calcium-regulating systems can often be achieved by use of selective calcium channel blockers (17).

Elevated  $[Ca^{2+}]_i$  is a feature common to many neurotoxic events, superimposed on which are the more distinctive features of each xenobiotic agent. It is likely that the adaptive capacity of nervous tissue may be challenged in a synergistic manner by such a conjunction of such specific and nonspecific events.

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