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The Role of Cell Calcium in Current Approaches to Toxicology

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All cells contain elaborate systems for the spatial and temporal regulation of the calcium ion, $[Ca^{2+}]_i$, and diverse Ca^{2+} receptor and biochemical response systems that are regulated by these changes in $[Ca^{2+}]_i$. Toxicants that perturb the mobilization or homeostasis of $[Ca^{2+}]_i$ will place the regulation of these processes outside the normal range of physiological control. Many classes of chemical toxicants, including metals, solvents, and pesticides, may have particular aspects of cell calcium as key cellular and molecular targets of toxicant action. However, experimental proof of these targets as a specific site of toxicant action is challenging and technically difficult as a result of the complexity and diversity of these processes. To fully establish and understand the target role of the calcium messenger system in toxicant action, it is necessary to distinguish between the effects of a toxicant on (*a*) the calcium mobilization and homeostatic processes, (*b*) the calcium-mediated processes, and (*c*) from those processes which co-regulate or counter-regulate these calcium-mediated processes. As our understanding of the calcium messenger system expands, these insights will be increasingly applied to understanding the mechanisms of action of toxic chemicals

Introduction

Intracellular Ca^{2+} homeostasis and the messenger role of the Ca^{2+} ion in the regulation and control of cell functions have been a very active and productive area of basic biological research during the last decade. The processes that regulate intracellular Ca^{2+} are extraordinarily intricate, numerous, and diverse. The Ca^{2+} messenger system has a central role in mediating the contraction of all forms of muscle, the secretion of exocrine, endocrine, and neurocrine products, the regulation of glycogenolysis and gluconeogenesis, the transport and secretion of fluids and electrolytes, and the growth and division of cells. Based upon the central role of the Ca^{2+} messenger system in these aspects of cell function, it is logical, if not inevitable, to examine possible disturbances in Ca^{2+} homeostasis and Ca^{2+}-mediated functions as underlying mechanisms of toxicant action.

The Ca^{2+} messenger system may be considered as the integrated function of several constitutive systems (Fig. 1). The Ca^{2+} messenger system is described in detail elsewhere and is briefly presented in this overview (*1–9*). The signal transducing system, mainly products of phosphoinositol metabolism, are the second messengers that carry the message to mobilize Ca^{2+} from the plasma membrane to the endoplasmic reticulum and other Ca^{2+} stores. The Ca^{2+} homeostatic processes are those Ca^{2+} gates, pumps, and channels that operate to keep the free cytosolic calcium ion, $[Ca^{2+}]_i$, within normal physiological range. The Ca^{2+} receptor system is a family of homologous calcium-binding proteins that act as transducers for relaying changes in $[Ca^{2+}]_i$ to the appropriate receptor enzymes that transduce the Ca^{2+} signal to the biochemical response system. The Ca^{2+} receptor system includes calmodulin, calcimedins, parvalbumins, troponin-C, and other Ca^{2+}-binding proteins. Calmodulin, perhaps the most versatile Ca^{2+} receptor, activates a multi-functional Ca^{2+} calmodulin-dependent protein kinase and Ca^{2+} calmodulin-dependent protein phosphorylases, which elicit the wide range of biochemical and physiological responses. Other second messengers, such as cyclic nucleotides, also modulate or counter regulate the calcium signal and calcium response.

Three general types of calcium-mediated functions have evolved: cell movement, including muscle contraction, ameboid movement, and cytoskeletal functions; information processing, such as nerve impulse conduction and sensory mechanisms; and the actions of hormones, including growth factors, on cell growth and differentiation. All three of these general types of Ca^{2+}-mediated functions may be important targets for toxicant action. These diverse functions are regulated and mediated by temporal and spatial changes

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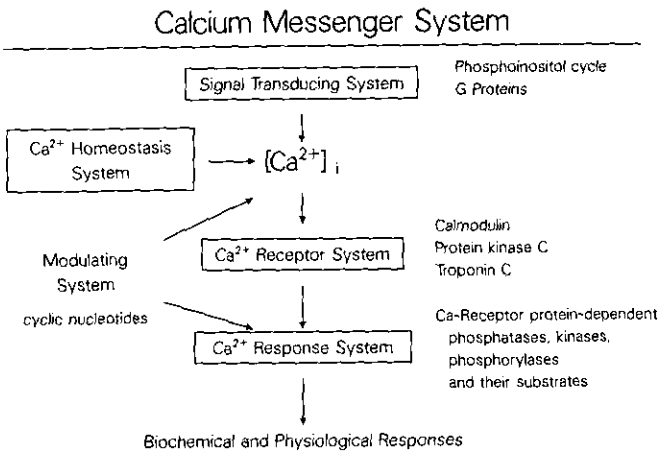

FIGURE 1. A schematic representation of the calcium messenger system illustrating the relationship of the signal transducing, receptor, Ca^{2+} homeostasis, Ca^{2+} response, and modifying ⇒ modulating systems.

in $[Ca^{2+}]_i$, each major membrane system has distinctive pathways for the uptake and release of Ca^{2+} (Fig. 2). The coordinated action of these many processes is necessary for normal Ca^{2+} function. Although specific features of this homeostatic array vary from cell to cell and tissue to tissue, the general features of Ca^{2+} homeostasis are illustrated in Figure 2. Figure 2 is highly simplified in that the involvement of the phosphatidylinositol cycle and cAMP on Ca^{2+} mobilization and homeostasis are not shown (*8*). At least

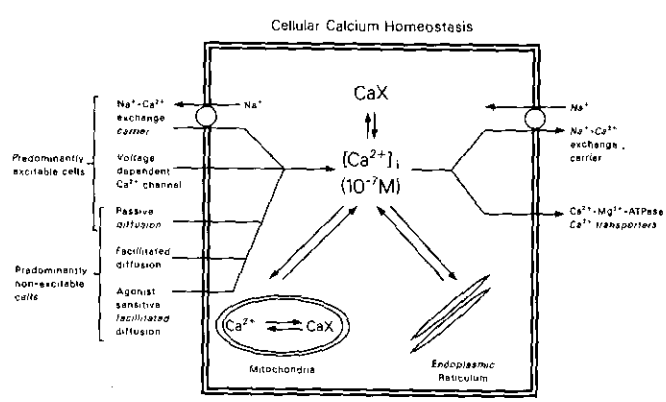

FIGURE 2. A highly condensed and simplified schematic representation of the processes involved in regulating cellular calcium homeostasis in an idealized mammalian cell. Important processes that are responsible for transducing hormonal signals and mobilizing Ca^{2+}, the Ca^{2+} receptor system, and Ca^{2+} response system, and those factors that modulate or counterregulate the calcium messenger system are not specifically shown. Figure adapted from Pounds (*16*) and Pounds and Rosen (*55*).

four processes control Ca^{2+} exchange at the plasma membrane: a passive Ca^{2+} leak dependent upon membrane potential, a passive Ca^{2+} leak not related to membrane potential, specific Ca^{2+} pumps, and Ca^{2+}-Na exchangers. In addition, there are important pump-leak, uptake-efflux systems associated with the endoplasmic reticulum, mitochondrial inner membrane, secretory vesicles, and membranes of other organelles. Ca^{2+} is also bound to a number of components within each structural compartment of a cell. Events at the plasma membrane, endoplasmic reticulum and inner mitochondrial membrane, are all involved in regulating the intracellular calcium ion concentration in concert. Perturbation of the Ca^{2+} messenger system by toxicants may place the regulation of cellular function outside the normal range of physiological control. Hence, disturbances in intracellular Ca^{2+} homeostasis and Ca-mediated functions have become attractive and frequently postulated targets for numerous pathophysiological processes involving neurosecretion, cell growth and transformation, tumor promotion and hypertension, and ischemic injury and toxicant-induced cell death.

Ca^{2+}-Mediated Processes As Targets for Toxicant Action

Experimental approaches to toxicology, toxicological concepts, and toxicologists are often classified according to their emphasis on target organ systems, chemical class of toxicants, and the level of biological organization used. The potential involvement of the calcium messenger system embraces all classifications of toxicology and toxicologists. These classifications are not inclusive or independent, but are a useful framework for considering toxicology data and concepts. The following discussion is not meant to be extensive but illustrates the pervasive and ubiquitous role of cell calcium in all aspects of toxicology (Tables 1 and 2).

Chemical Class of Toxicants

Metals

Many metals may substitute for Ca^{2+} or displace Ca^{2+} from sites of Ca^{2+} transport, binding, and storage. Lead, cadmium, mercury, and some other metals are transported across cell membranes by Ca^{2+} transporters and Ca^{2+} gates (*10–16*). Furthermore, the presence of these metals on these proteins affects Ca^{2+} movement at these sites (*10,11,13,14*). For example, the calcium channel in adrenal medullary cells is about ten times as permeable to Pb^{2+} as to Ca^{2+}, however, the channels do not inactivate when Pb^{2+} passes through them in contrast to Ca^{2+} (*14*). In addition, many of those normal hormonal signals that mobilize Ca^{2+} to increase $[Ca^{2+}]_i$ also mobilize Pb^{2+}, although the quantitative mobilization is not yet clear (*17*).

Table 1. Selected Ca^{2+}-mediated functions that are important cellular and molecular targets in toxicological processes.

Toxicological end point	Ca^{2+}-mediated process
Oncology	Regulation of cell cycle Oncogene function Cell-cell communication Tumor promotion Cell adhesion Glycolysis Growth factor secretion/action
Developmental toxicology	Cell division Regulation of cell cycle Cell-cell communication Cell adhesion Polyamine synthesis Cell movement/migration
Cell injury and cell death	Cellular energetics Ion regulation Mitochondrial function Phospholipase activation Protease activation

Table 2. Selected Ca^{2+}-mediated process that may be key cellular and molecular targets in organ system toxicity.

Organ system	Ca^{2+}-mediated process
Neurotoxicity	Neurotransmitter synthesis Neurotransmitter synthesis Axonal transport
Immunotoxicity	Chemotaxis Phagocytosis I_g E secretion Cell division Lymphocyte activation Mitogenic response
Cardiovascular toxicity	Contraction of smooth muscle Contraction of cardiac muscle Transmitter release and neuromuscular junction Renin secretion Angiotensin action Ischemic injury
Renal toxicity	Gluconeogenesis Electrolyte homeostasis Hydroosmotic regulation Cell injury/cell death
Hepatotoxicity	Gluconeogenesis/glycolysis Cell proliferation Cell injury/cell death

Metals also substitute for calcium in calmodulin and other Ca^{2+} binding proteins such as parvalbumin, troponin C, or calbindin *in vitro (18-20)*. This substitution could lead to the situation where the activity of these Ca^{2+} receptor proteins is no longer regulated by $[Ca^{2+}]_i$, with potentially far-reaching perturbation of cell and organ function. This hypothesis is attractive and is supported by many observations, but is difficult to verify *in vivo*. Metals have high affinity for many other ligands, including the sulfhydryls, phosphate, and even other carboxyl groups. It remains to be established if toxic metals are bound to calmodulin and other Ca^{2+}-binding proteins *in situ* in sufficient quantity to perturb the biochemical functions regulated by these proteins.

Pesticides

Numerous aspects of the calcium messenger system may be considered or are proposed as targets of pesticide action. Egg shell thinning in birds, caused by DDT, is an example of impaired calcium utilization. Other organochorine insecticides, including heptachlor and lindane, which is a structural analog of inositol, have been shown to alter Ca^{2+} homeostasis. The changes in nerve terminal $[Ca^{2+}]_i$ may be responsible for the neurotoxic action of these pesticides (*21-24*).

Tumor Promoters

Phorbol esters and some other tumor promoters substitute for diacylglycerol in the activation of protein kinase C. Protein kinase C is a Ca^{2+} and phospholipid-dependent enzyme that is activated by the natural activator, diacylglycerol, which occurs transiently during phosphatidylinositol turnover. This enzyme occurs in a wide range of tissues and phosphorylates a broad spectrum of proteins with a crucial role in signal transduction for activating cellular functions and proliferation. Protein kinase C also appears to be the receptor protein for tumor-promoting phorbol esters and many of the pleiotropic actions of tumor promoters, such as induction of ornithine decarboxylase, alkalinization of the cytoplasm, DNA synthesis, and expression of oncogenes may be mediated by the direct and unregulated activation of this enzyme by promoters (*25,26*).

Halogenated Hydrocarbons, Solvents, and Other Organic Toxicants

The acute toxicity of organic toxicants has been linked to altered Ca^{2+} homeostasis through several mechanisms (*27-30*). In particular, the exposure of hepatocytes to CCl_4 and certain other halocarbons, carbon disulfide, or thioacetamide results in the rapid loss of Ca^{2+}-ATPase activity and the ability of the endoplasmic reticulum to sequester Ca^{2+} (*31-33*). Other toxicants such as cisplatin may increase renal Ca^{2+}-ATPase activity (*34*).

Maintenance of normal $[Ca^{2+}]_i$ homeostasis is also dependent upon the status of other cell constituents, including sulfhydryl, glutathione, and nicotinamide-nucleotide redox levels of the cytoplasm and mitochondria. Agents such as *t*-butyl hydroperoxide that cause oxidative stress, or agents which deplete cellular glutathione through conjugation reactions will

perturb Ca^{2+} fluxes and intracellular compartmentation, resulting in deregulation of cell function and even cell death (*35*).

Toxicological End Point

Oncology

Tumor cells show various degrees of autonomy in that they do not react to the normal physiological and hormonal control over growth, cell proliferation, cell differentiation, and glycolysis and/or respiration. the calcium messenger system has important roles in the cellular and molecular process that regulate or modulate the expression of neoplasia. In contrast to normal cells, transformed cells continue to proliferate in suboptimal concentrations of extracellular Ca^{2+} and appear to have reduced Ca^{2+} requirements for other Ca^{2+}-mediated functions. Altered calcium homeostasis and cAMP metabolism and the phosphatidylinositol cycles may be related to oncogene expression. The products of several viral oncogenes affect the signal transduction mechanism involving phosphatidylinositol turnover, protein kinase C activation, and intracellular Ca^{2+} release (*25*). Future advances in tumor biology will include a more complete understanding of the differences in Ca^{2+} homeostasis between normal and transformed cells, as well as the target role of cell calcium in the initiation and promotion processes.

Developmental Toxicology

Several calcium-mediated processes may be important cellular and molecular targets for toxicants in developing systems. intercellular adhesion is mediated by a variety of molecules and intercellular junctions. In the last several years a family of adhesion proteins called cadherins has been discovered that mediates a prominent Ca^{2+}-dependent intercellular adhesion between cells in multicellular tissues. It is thought that specific recognition between like cadherin proteins mediates the sorting out of cell types during the cellular rearrangements and morphogenetic movements that occur during embryogenesis (*36*).

Gap junctions are other important cellular targets; these are dynamic cell structures that regulate communication and metabolic cooperation between adjacent cells. Gap junctions are also regulated, in part, by protein kinase C and other Ca^{2+}-dependent kinases (*37*). Inhibition of gap junction-mediated communication may interfere with embryonic development (*38*). Furthermore, the secretion and action of many growth factors is regulated by $[Ca^{2+}]_i$ so toxicants which modify the calcium messenger system could modify the availability and response of these hormones.

Cell Injury/Cell Death

The close temporal and biochemical relationship between the regulation of ions and cell injury and cell death has been of considerable interest for many years (*27,39*). Deregulation of $[Ca^{2+}]_i$ appears to be especially important in the development of irreversible cell injury. Following a biochemical injury, cells pass through a sequence of morphological and biochemical stages. The early stages, which are characterized by ion shifts including decreased $[K^+]_i$ and increased $[Na^+]_i$ and $[Ca^{2+}]_i$ are reversible. The potential contribution of lost Ca^{2+} regulation to cell injury is modified by many factors including the cellular site of injury, anoxia, and sulfhydryl status. As the role of Ca^{2+} in cell injury and cell death is elucidated, it may become possible to attenuate cell injury and abate the progression to irreversible injury with agents that modify Ca^{2+} entry or the calcium mediation of cell injury.

Target Organ

Nervous System

Calcium ions are well established as playing important roles in the signal transduction, neurotransmitter secretion, and axonal transport (*40*), and more recently in the biosynthesis neurotransmitters (*41*). At least three different calmodulin-dependent protein kinases with different substrate specificities are present in rat brain cytosol (*42*). Both the secretions and biosynthesis of monamine neurotransmitters stimulated by Ca^{2+} influx and Ca^{2+} mobilization may be regulated by the calmodulin-dependent protein kinase phosphorylation of microtubule proteins and the phosphorylation of the monooxygenases that are the rate-limiting enzymes in the biosynthesis of the neurotransmitters (*41*).

Immune System

Cell Ca is important in both cell and humoral immune responses. Phagocytosis and cell migration are dependent upon $[Ca^{2+}]_i$. Many of the processes responsible for lymphocyte activation, proliferation, and differentiation are initiated by antigen-receptor and growth-factor interactions that are mediated by the calcium messenger system. Signals induced by antigens or mitogens initiate responses including gene activation for the production of hormone-like lymphocytic growth factors and their specific receptors. The physiologic mitogenesis of lymphoscytes is then regulated by interactions of these specific growth factors with receptors that transmit the signals across the plasma membrane to induce a proliferative response. The integrated transduction of these complex signals is essential to lymphocyte immunobiology, and perturbations of the calcium

messenger system would be expressed as an immunodeficiency of the host (*43-45*).

Cardiovascular System

Calcium couples excitation to contraction in all muscle cells; however, in smooth muscle, the relationship between tension and $[Ca^{2+}]_i$ is not fixed but varies both with the nature of the stimulant and the duration of its application. At least three Ca^{2+} channels have been identified including the voltage-sensitive Ca^{2+} channel, stretch-activated Ca^{2+} channel, receptor-operated channel, and a Ca^{2+} leak channel in vascular smooth muscle. Contraction is mediated by Ca^{2+} binding with troponin-C. Other important calcium-binding proteins with high homology to calmodulin include the calcimedins in smooth muscle that are, however, virtually absent from cardiac and smooth muscles. The precise function of calcimedins is not known. Ca^{2+} has other roles in regulating including mediating response to adrenoreceptor activation, lipid metabolism, ion regulation, and the secretion of hormones that regulate cardiovascular function.

Renal System

The calcium messenger system is involved in many critical renal functions, mediating the osmotic, baro- and hormonal regulation of fluid and electrolytes and renal ischemic injury, intermediary metabolism, vitamin D activation, and the glomerular filtration rate. For example $[Ca^{2+}]_i$ may play two important roles in the regulation of the glomerular filtration rate. First, tubulo-glomerular-mediated vasoconstriction involves Ca^{2+}-mediated excitation contraction coupling of smooth muscle cells of the afferent arteriole. Second, in the juxtaglomerular cells, $[Ca^{2+}]_i$ is an inhibitory second messenger in the renin secretory process (*46-48*). Toxicant-induced alteration of renal function may also include effects on other organ systems, such as the cardiovascular system, thus altering the pharmacokinetics of the toxicant.

Effects of Toxicants on Ca^{2+} Homeostasis and Ca^{2+}-Mediated Cell Functions

Because of the complexity and diversity of Ca^{2+}-homeostatic processes and Ca^{2+}-mediated responses, it is useful to consider the many ways in which a toxicant may act on the Ca^{2+} messenger system. Perturbations of Ca^{2+} homeostasis and Ca^{2+}-mediated cell functions following toxicant exposure may each result from at least three general types of interactions: direct, indirect, and secondary. These classifications are neither mutually exclusive or absolute. The direct interactions are the most significant with respect to the mechanistic or target role of the calcium messenger system. Rather, the purpose of this classification is to give some organization to an often overwhelming number of real and potential actions of toxicants on cell calcium. This classification should also assist in defining an experimental approach, the interpretation of observations, and the formulation of new hypotheses. This range of actions of a toxicant on cell calcium is illustrated with lead, a toxicant with diverse actions on the calcium messenger system (Table 3). It is important to note that a given toxicant may conceivably, if not probably, exhibit more than one type of action depending on the dose of the toxicant, duration of toxicant exposure, time-course of the experimental observation, and level of biological organization under investigation.

Toxicant Effects on Ca^{2+} Cellular Homeostasis

Toxicants may directly alter Ca^{2+} homeostasis by substituting for Ca^{2+} at specific sites of Ca^{2+} mobilization, transport, or storage. In this sense these actions are very loosely defined as competitive with Ca^{2+} and generally reversible at the molecular level. Direct actions are most commonly observed with other divalent metals, including lead, ruthenium, cadmium, and others, which directly compete with, or displace Ca^{2+} at transport sites in the plasma membrane and inner mitochondrial membrane (*10,14,15,19*). Although the direct action of these metals on Ca^{2+} homeostasis at these sites is well documented, the explicit correlation between these effects and the manifestations of toxicity are confounded by the concurrent actions of these metals with other aspects of the Ca^{2+} messenger system as well as cell functions unrelated to Ca^{2+} homeostasis. Other examples include the direct actions of the calcium channel blockers, nifedipine or verapamil, on cardiovascular function by a blockade of calcium mobilization through membrane channels in myocardial and smooth muscle cells. It must be recognized

Table 3. Spectrum of known actions of lead on Ca^{2+} homeostasis and Ca^{2+}-mediated processes in different biological systems.[a]

Interaction	Ca^{2+} homeostasis	Ca^{2+}-mediated function
Direct	Plasma membrane Ca^{2+} channels Mitochondrial Ca^{2+} pump Ca^{2+}-ATPase	Calmodulin Calbindin Troponin C Osteocalcin Oncomodulin
Indirect	Adenylate cyclase Na^+,K^+-ATPase	Adenylate cyclase
Secondary	Hydrolysis of ATP Decreased heme Protein-SH binding	Protein-SH binding

[a]Most of these actions of lead on cell calcium have been well established in several *in vitro* studies using diverse biological systems. The difficulty is separating the direct from the less direct actions and experimentally characterizing those actions as key events in chronic lead toxicity in humans, such as altered cognitive function reproductive disorders and hypertension.

that a direct interaction does not necessarily result in an inhibition of a Ca^{2+}-mediated function but may also produce an exaggerated Ca^{2+}-mediated response if the clearance of Ca^{2+} from the cytosol and other cellular compartments is impaired (*16,49*).

Toxicants may indirectly act on specific cellular and molecular constituents of Ca^{2+}-homeostatic processes, such as Ca^{2+} pumps or gates, but at molecular sites that are independent or remote of Ca^{2+}, not at the Ca^{2+} transport or binding site. Thus the interaction is non-competitive. Adding more calcium will not alter the biochemical lesion. This type of interaction is observed with many organic toxicants. The most fully characterized example of an indirect action is the inactivation of the Ca^{2+} transporter in smooth endoplasmic reticulum by carbon tetrachloride that leads eventually to cell death (*30,50*). An indirect interaction may be irreversible at the molecular level; if the injury is sublethal, the cell and organism may be able to repair and recover—in this instance, with the biosynthesis of new endoplasmic reticulum and the associated Ca^{2+} pump. Toxicants that dissipate the Na^{+} gradient will secondarily alter Ca^{2+} homeostasis in cells where the Ca/Na exchange is a component of Ca^{2+} homeostasis. Na^{+} transport is affected by numerous drugs, thiol status, and energy metabolism. Indirect actions of lead on Ca^{2+} homeostasis would include effects on adenylate cyclase and the Na^{+}, K^{+}-ATPase (*51*).

Toxicants may secondarily alter Ca^{2+} homeostasis through non-specific effects on cell function that are biochemically and functionally remote from the processes of Ca^{2+} transport and storage. For example, ethanol, other aliphatic alcohols, and local anesthetics including dibucaine or tetracaine produce changes in nerve cell excitability and neurotransmitter release that are the result of physical-chemical changes in the cell membrane; increased fatty chain motion within the membrane bilayer, expansion of membrane volume, increased membrane fluidity resulting in altered Ca^{2+} permeability and transport properties of the membrane (*52*). Thus toxicants may alter Ca^{2+} homeostasis secondarily to the primary site of action of the toxicant; and although the effects on Ca^{2+} homeostasis may be nonspecific, the effects on Ca^{2+}-mediated processes may still be significant and mechanistically important.

Toxicant Effects on Ca^{2+}-Mediated Cell Functions

Toxicants may directly act on Ca^{2+}-mediated cell functions, including Ca^{2+}-receptor proteins or their substrates. These actions may occur independently of altered Ca^{2+} homeostasis. A well-characterized example is the inhibition of Ca-calmodulin-mediated processes by the phenothiazines that bind to the hydrophobic region of calmodulin and prevents the formation of a Ca-calmodulin-receptor enzyme complex thereby inhibiting Ca^{2+}-mediated functions. Some pharmacologic and, presumably, adverse effects of these agents are a result of this interaction with calmodulin. However, this conclusion must be tempered by observations that some calmodulin antagonists also alter membrane currents of potassium and sodium; thus, not all effects can be attributed to inhibition of calmodulin (*53*). Lead, aluminum, cadmium, and other divalent metals effectively compete with Ca^{2+} for binding sites on a variety of Ca^{2+} binding proteins including calmodulin (*18*). Replacement of Ca^{2+} in calcium receptor proteins by other metals may alter Ca^{2+}-mediated functions in unpredictable ways; the replacement metal-calmodulin complex may have different disassociation kinetics or the replacement metal-calmodulin (or other Ca^{2+} receptor) complex may have more or less affinity for the receptor enzyme(s). Because of autoregulation of Ca^{2+} homeostasis by calmodulin, direct actions of toxicants on calmodulin and related proteins may likely result in alterations in Ca^{2+} homeostasis (an indirect effect on Ca^{2+} homeostasis). Considering the importance and diverse ubiquitous nature of Ca^{2+} receptor proteins, the actions of toxicants on these receptors are of clear mechanistic interest and considerable relevance.

Toxicants may indirectly alter Ca^{2+}-mediated cell functions independently of changes in Ca^{2+} homeostasis, independent of direct interaction with the Ca^{2+} receptor or effector proteins. The response to a Ca^{2+} signal is a cascade of biochemical events that often include phosphorylation, and it is subject to feedback control by cyclic nucleotides and to sensitivity and response modulation. Because many cAMP-mediated responses are antagonistic to the Ca^{2+}-mediated response, toxicants that effect adenylate cyclase, phosphodiesterase, or other parts of the cAMP messenger system will likely alter Ca^{2+}-mediated cell functions. Examples of indirect effects include the inhibitory effects of xanthenes on phosphodiesterase leading to pharmacologic and toxic changes in muscle contraction. Although observed effects are clearly on Ca^{2+}-dependent processes, it is important to recognize that the site of the primary biochemical lesion is not directly related to Ca^{2+} homeostasis or the Ca^{2+} effectors.

Toxicants may secondarily perturb Ca^{2+}-dependent functions as a result of toxic events remote from Ca-dependent processes. That is, toxicants that grossly alter cell and organelle function may impair the ability of a cell to respond to a Ca^{2+} signal, but as a result of toxic action which has no relationship to the Ca^{2+} messenger system. For example, methyl mercury impairs neuronal migration and cell division, but the cellular target for these actions is the neurotubule and microtubules of the cells, rather than the Ca^{2+}-mediated aspects of cell movement and division. Another example might be the inhibition of ornithine decarboxylase activity by cycloheximide. This enzyme is induced by hormones that mobilize diacylglycerol

and calcium, thus activating protein kinase C. The action of cycloheximide, however, is not on any aspect of the calcium messenger system, but rather on translation of RNA. Thus, many, if not all, toxicants that alter cellular energetics, protein synthesis, etc., will eventually alter the ability of cells and tissues to maintain Ca^{2+} response systems. However, these secondary effects on Ca^{2+}-mediated function are more an expression of toxicity than the cause of toxicity. Considerable research effort must be directed at distinguishing between the mechanistically significant direct and indirect effects, and these mechanistically inconsequential (as it relates to the Ca^{2+} messenger system) secondary effects.

Challenges to Establishing the Target Role of Cell Calcium in Toxicant Action

There are several difficulties in experimentally identifying and characterizing the role of the calcium messenger system as the principal or most sensitive molecular target for toxicant action. The most important of these difficulties is the complexity an diversity of the calcium messenger system itself. First, toxicants may act on many components of the calcium messenger system in direct and less direct ways. The temporal phases of Ca^{2+} homeostasis and function may be affected independently, and the $[Ca^{2+}]_i$ signal may not be directly proportional to the Ca^{2+}-elicited function (*6,7,55*). Finally, the Ca^{2+}-mediated processes are generally under coregulation or counterregulation by other messenger systems such as cyclic nucleotides. This complexity makes it difficult to determine if changes in the calcium messenger system are a cause or a consequence of toxicity.

Component Complexity of Signal Transduction and the Ca^{2+} Homeostatic Processes

Intracellular Ca^{2+} homeostasis is an extraordinarily complex process that both integrates and requires the integration of innumerable processes occurring in all structural and functional components of cells (Fig. 2). Often it is necessary to experimentally dissect cells and tissues so that the individual components and processes of Ca^{2+} mobilization and Ca^{2+} homeostasis can be distinguished and manipulated experimentally. However, the more an experimental system is simplified, the less it will resemble the component and process at higher levels of biological control. Thus, incomplete information is obtained by investigating Ca^{2+} transport systems, or Ca^{2+}-mediated processes in an experimental system free of normal physiological controls, without attempting to demonstrate similar results at higher levels of biological organization. It is, of course, very difficult to perform and design experiments from which these comparisons can be made, yet information derived from such experiments can yield highly meaningful information.

Component Complexity of Ca^{2+}-Mediated Processes

The molecular and cellular components of the Ca^{2+} receptor and the response systems are also temporally and spatially complex and require functional and structural integrity of the cell. A number of Ca^{2+} receptor proteins, typified by calmodulin, regulates numerous response systems in different functional and structural compartments of cells, often via numerous kinases and phosphrylases. However, these Ca^{2+}-mediated processes are generally co-regulated or counter-regulated by the cAMP messenger system and the arachidonic acid cascade. This complexity of these messenger systems in constituent parts and the diversity in regulation often necessitates the investigation of a small portion or discrete components of the system, that is, isolated cells, organelles, membrane vesicles, or enzymes systems. It is important to understand the relationship of measurements made within the isolated system to the system as a whole, in order to design and interpret the appropriate experiments and to establish the target role of the calcium messenger system.

Temporal Complexity of the Calcium Messenger System

The most important point to consider for this discussion is that there are four distinct physiological phases of the calcium messenger system that must be considered as targets for toxicant action: basal or resting state, transition to the activated state, the fully activated state, and transition to the resting state. The time scale for these processes may typically range from less than a second to a few minutes, or even hours. Just as a single measure of cAMP is inadequate to define cAMP metabolism, so too are measurements of $[Ca^{2+}]_i$ that do not consider the dynamic aspects of $[Ca^{2+}]_i$. Unfortunately, when the effects of toxicants are studied during a single phase, typically the basal or activation phase, rather than all four phases, and the results extrapolated to the full cycle real or potential effects on the other phases are ignored. Thus, important effects of a toxicant on Ca^{2+} homeostasis and function may be missed completely, or the results may be misinterpreted (*49,55*).

Specific (Direct) vs. Nonspecific Effects

The experimental distinction must be made between direct and the nonspecific toxicant effects in order to establish the calcium messenger system as a

principal target of toxicant action. Unlike investigations of, for example, genetic damage, the key or target molecule(s) is generally not known. Cellular Ca^{2+} homeostasis is dependent upon the energy state and redox state of the cell; thus, alterations in energy metabolism would eventually result in perturbations of cellular Ca^{2+} homeostasis, which may not be important in understanding the mechanism of action of these agents. This distinction between direct and nonspecific effects is generally more difficult to establish with the Ca^{2+} messenger system than with many other cellular targets of toxicant action, such as DNA or receptors, because of the vast number and diversity of Ca^{2+} homeostatic and Ca^{2+}-mediated processes.

Postulates

Establishing cell calcium as a key target for the action of a toxicant is a complex and challenging process. Four general postulates or conditions for establishing the calcium messenger system as the critical molecular or cellular target are proposed.

First, perturbation of the calcium messenger system must be demonstrated in an experimental system that provides strict experimental control and manipulation of the calcium messenger system. This demonstration will often be at the cellular and subcellular level *in vitro* and will define the target cell and appropriate molecular targets to be investigated at higher levels of biological organization. Second, to be considered a key molecular or cellular target, these perturbations should precede in time and dose other signs of toxicity. This condition is to establish the specificity of the action for the calcium messenger system. Third, a toxicant must reach the molecular or cellular target process at comparable concentrations both *in vivo* and *in vitro*. That is to say the demonstration of an effect in an isolated system is of little mechanistic significance if the molecular dosimetry is not appropriate. And fourth, perturbation of Ca^{2+}-mediated function should be experimentally associated with the biochemical/physiological manifestation of toxicity *in vivo*.

Summary

Because of the central role of the calcium messenger system in diverse functions of tissues, organs, and cells, Ca^{2+} homeostasis and Ca^{2+}-mediated processes may prove to be critical cellular and molecular targets for a diverse range of toxicants. However, experimental proof of these targets as a specific site of toxicant action is challenging and technically difficult because of the complexity and diversity of these processes. Nevertheless, the investigation of the Ca^{2+} messenger system and Ca^{2+}-mediated functions will continue to be an active and productive area of basic research for several years. These insights will be increasingly applied to the understanding of the mechanisms of action of toxic agents.

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