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Calcium Ion As Intracellular Messenger and Cellular Toxin

by Howard Rasmussen,* Paula Barrett,* Joan Smallwood,* Wendy Bollag,* and Carlos Isales*

Ca^{2+} serves a nearly universal intracellular messenger function in cell activation, but excess Ca^{2+} is also a cellular toxin. The possibility of Ca^{2+} intoxication is minimized by an elaborate autoregulatory system in which changes in Ca^{2+} influx rate across the plasma membrane are rapidly compensated for by parallel changes in Ca^{2+} efflux rate. By this mean, cellular Ca^{2+} homestasis is maintained so that minimal changes in total cell calcium and cytosolic Ca^{2+} concentration occur during sustained Ca^{2+}-mediated responses. Rather than a sustained increase in cytosolic Ca^{2+} concentration, it is the localized cycling of Ca^{2+} across the plasma membrane that is the critically important Ca^{2+} messenger during the sustained phase of cellular responses mediated via surface receptors linked to the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2). PIP_2 hydrolysis gives rise to inositol(1,4,5)trisphosphate (IP_3) and diacylglycerol (DAG). The IP_3 acts to release Ca^{2+} from an intracellular pool, thereby causing a transient rise in cytosolic Ca^{2+} concentration. This transient Ca^{2+} signal activates calmodulin-dependent protein kinases transiently, and hence, causes the transient phosphorylation of a subset of cellular proteins that mediate the initial phase of the response. The DAG brings about the association of protein kinase C (PKC) with the plasma membrane where a receptor-mediated increase in Ca^{2+} cycling across the membrane regulates PKC activity. The sustained phosphorylation of a second subset of proteins by PKC mediates the sustained phase of the response. Hence, Ca^{2+} serves as a messenger during both phases of the cellular response, but its cellular sites of action, its mechanisms of generation, and its molecular targets differ during the initial and sustained phases of the response. It is likely that this Ca^{2+} messenger system is a target for many cellular toxins.

Introduction

When Prometheus stole fire from the gods and presented it to man, it was a gift of immense value (*1*). It provided heat against the cold, light against the dark, and a means of communication. Yet, with this gift came also the destructive power of uncontrolled fire with its ability to imperil man's very existence.

This myth of the gift of fire to man can serve as the life motif for the evolutionary gift of Ca^{2+} to the cell. This ion has become the means of regulating heat production and of facilitating intracellular communication. Yet, with this gift has also come a destructive potential: excess calcium ions are cellular toxins (*2*). Because of the potential for toxicity, an elaborate system has been developed to ensure the maintenance of cellular Ca^{2+} homeostasis over a wide range of circumstances (*3*). This creates a seeming paradox: changes in Ca^{2+} ion concentration are thought to serve a nearly universal messenger function in cells that possess very effective mechanisms by which to minimize any change in intracellular Ca^{2+} ion concentration. A resolution of this seeming paradox has come from the recognition that the messenger function of Ca^{2+} is expressed in quite subtle ways within both a spatial and temporal context (*4*). The purpose of the present article is to review our present views of the mechanisms by which cellular Ca^{2+} homeostasis is achieved and then to consider the messenger function of Ca^{2+} within this context.

*Departments of Internal Medicine and Cell Biology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510.

Address reprint requests to H. Rasmussen, Yale School of Medicine, Dept. of Internal Medicine, P.O. Box 3333, New Haven, CT 06510.

Evolutionary Relationship between Ca^{2+} and Cell Function

Before considering the mechanisms by which cellular Ca^{2+} homeostasis is maintained, it is worth considering the evolutionary aspects of cellular Ca^{2+} metabolism. In so doing, it is instructive to begin by considering how mammalian cells have come to maintain such an enormous (10,000-fold) Ca^{2+} concentration gradient across their plasma membranes. Recent

studies of the nature of sedimentary rocks formed during different geologic eras have led to new insights into the nature and composition of the primordial sea (*5*). It appears likely that when life first evolved, the pH of the sea was high (circa pH 8) and the concentration of Ca^{2+}, low (10^{-7} M-10^{-6} M). In this low Ca^{2+} environment, the basic life processes involving nucleic acids and, particularly, nucleotides (for example, ATP) could develop. These nucleotides are phosphorylated organic anions, and as such, their Ca^{2+} salts are sparingly soluble. On the other hand, a crucial aspect of geologic evolution was the gradual increase in the Ca^{2+} content of the sea (*6,7*). This increase posed a threat to the most fundamental of life processes: those involving the participation of phosphorylated compounds, e.g., DNA and RNA synthesis and the basic energy-transducing systems, because these compounds would precipitate or form stable Ca^{2+} complexes in a higher Ca^{2+} environment (*8*). This threat was met by the development of a surface membrane having a low Ca^{2+} permeability and possessing energy-dependent mechanisms (pumps) by which Ca^{2+} could be expelled from the cell interior. By these means, it was possible to maintain a low intracellular Ca^{2+} (circa 0.1 μM). By the time multicellular life evolved, the $[Ca^{2+}]$ in the sea was close to 1 mM (*9*). This later precambrian sea was incorporated into the multicellular organism as the extracellular fluid. The heritage of this evolutionary sequence was a cell with a plasma membrane displaying a limited permeability to Ca^{2+} and possessing very efficient mechanisms for pumping out any Ca^{2+} that did leak into the cell. Thus, the challenge of Ca^{2+} intoxication was met by excluding environmental Ca^{2+} from the cell interior. As a consequence, a large Ca^{2+} gradient developed across the surface membrane. This gradient was then exploited as a means to communicate surface events to the cell interior by using controlled and limited increases in Ca^{2+} influx rate across the surface membrane: Ca^{2+} became an intracellular messenger. As long as these pulses of Ca^{2+} were brief and infrequent, any Ca^{2+} that entered the cell during a period of activity could be pumped out during a subsequent period of inactivity. Thus, even if there was a brief temporal dissociation between Ca^{2+} influx and efflux, Ca^{2+} homeostasis could be maintained. Therefore, in its most primitive mode Ca^{2+} homeostasis is maintained by events taking place at the plasma membrane. These are responsible for both generating and terminating the Ca^{2+} messenger.

Cellular Calcium Metabolism

Even though a 10,000-fold Ca^{2+} concentration gradient normally exists across the plasma membrane of most mammalian cells, there is a continuous, slow leak or influx of Ca^{2+} across this membrane (*10*). Nonetheless, total cell calcium content does not increase because there is an energy-dependent efflux of Ca^{2+} out of the cell against this concentration gradient. Even though maintenance of this gradient requires metabolic energy, the cost of this activity is minimized by the very marked impermeability of the plasma membrane to Ca^{2+}. Ca^{2+} can only enter the cell across the plasma membrane through specific channels that allow for a limited rate of Ca^{2+} entry. The maintenance of a fixed cellular Ca^{2+} content in the face of a continued influx of Ca^{2+} indicates that the rate of efflux is coupled to the influx rate. The primary bases for this coupling is the regulation of Ca^{2+} efflux pathways by changes in the Ca^{2+} concentration within the cell $[Ca^{2+}]_c$ or within a specific subcellular domain, $[Ca^{2+}]_{sm}$, a submembrane domain of the plasma membrane.

Two such pathways have been characterized (*11,12*): a $Ca^{2+}/2H^+$-ATPase or Ca^{2+} pump driven by the hydrolysis of ATP and a $3Na^+/Ca^{2+}$ exchanger driven by the Na^+ electrochemical gradient across the plasma membrane. As very little is known about the regulation of the exchanger *in situ*, the ensuing discussion will focus on the crucial role of the Ca^{2+} pump in maintaining cellular Ca^{2+} homeostasis even though it is clear that the $3Na^+/Ca^{2+}$ exchanger plays an equally important role in maintaining cellular Ca^{2+} homeostasis in cells such as the cardiac myocyte.

Unlike the primitive cell in which the plasma membrane is the major regulator of Ca^{2+} metabolism, the highly developed mammalian cell has a considerably more complex system for regulating cellular Ca^{2+} metabolism and for employing Ca^{2+} as an intracellular messenger. In addition to the plasma membrane, there are two other membrane-limited intracellular organelles which play important roles in Ca^{2+} homeostasis: the mitochondria, and the sarcoplasmic reticulum or calciosome. Both of these intracellular membranes possess pump-leak pathways that are directionally oriented, so there is a net leak of Ca^{2+} from the matrix space of the particular organelle to the cytosol and an energy-dependent uptake that actively removes Ca^{2+} from the cytosol and translocates it into the matrix space of that organelle.

In cellular Ca^{2+} metabolism, these two intracellular organelles play quite different roles. These roles appear to be based on *a*) differences in the affinities of the respective active transport systems for Ca^{2+}, *b*) the difference in the mechanisms by which they store Ca^{2+}, and *c*) the mechanism by which Ca^{2+} is released from these storage sites.

The Ca^{2+} pump in the sarcoplasmic reticulum (SR) has a K_m for Ca^{2+} transport that is similar to that of the plasma membrane Ca^{2+} pump (in the range of 0.3-0.9 μM). Because Ca^{2+} is stored in the SR by binding to a specific calcium-binding protein, calsequestrin, the size of the storage Ca^{2+} pool is determined by the extent of the specialized reticulum containing this protein and the quantity of this protein within this compartment (*13*). In contrast, the K_m for active Ca^{2+}

uptake by the mitochondria is in the range of 0.9 to 1.6 μM, and the Ca^{2+} stored in the mitochondrial matrix space is in the form of a nonionic complex with phosphate and ATP. There is also a difference in the mechanisms by which events at the plasma membrane can lead to the rapid release of Ca^{2+} from the calciosome (*14,15*) or sarcoplasmic reticulum, but not from the mitochondria.

From a functional point of view, the pool in the calciosome (or SR) serves as a source of trigger Ca^{2+} for stimulus-response coupling. This pool is most highly developed in skeletal muscle where it serves both as the source and sink for the Ca^{2+} involved in the regulation of contraction. The Ca^{2+} in this pool is released during the initiation of the response and reaccumulated during the termination of the response. Because of the geometry of these large multinucleated cells and the organization of the SR that is in close proximity to the contractile elements, this pool is little influenced by the flux of Ca^{2+} across the plasma membrane. As a consequence, when it is released from the sarcoplasmic reticulum, it has very little influence on the Ca^{2+} concentration just beneath the plasma membrane. Hence, during a normal contraction-relaxation cycle, very little of the Ca^{2+} released from the SR is pumped out of the cell (*10,16*).

In nonmuscle cells or in the small uninuclear cells found in mammalian smooth muscle, this Ca^{2+} trigger pool serves a similar function but is relatively small; also it appears to be located close to the plasma membrane. Once Ca^{2+} is released from this pool in response to an appropriate stimulus, its location near the plasma membrane allows it to induce an increase in $[Ca^{2+}]_{sm}$, thereby activating the Ca^{2+} pump in this membrane. Thus, significant amounts of the released Ca^{2+} are pumped out of the cell when the cell is activated (*10*).

Two possible functions have been attributed to the intramitochondrial Ca^{2+} pool. On the one hand, this ion pool has been proposed to have a regulatory function in the control of mitochondrial metabolism. Increases in the $[Ca^{2+}]$ in the matrix space do regulate the activities of certain Ca^{2+}-sensitive enzymes within this compartment, and it seems likely that the Ca^{2+} concentration in this compartment increases—at least transiently—during cell activation. Hence, it has been proposed that changes in $[Ca^{2+}]$ in the mitochondrial matrix space that occur as a consequence of changes in $[Ca^{2+}]$ in the cell cytosol serve to couple metabolic events in these two compartments (*17*). On the other hand, it has been proposed that the nonionic pool of calcium within the matrix space serves as a sink for excess Ca^{2+} when the plasma membrane is unable to maintain Ca^{2+} homeostasis. This latter role provides a means whereby the cell can sequester excessive Ca^{2+} in times of a temporary imbalance between Ca^{2+} influx and efflux across the plasma membrane. However, the Ca^{2+} sequestering capacity of the mitochondria is finite.

Ca^{2+}-Activated Response Elements

Before discussing the mechanisms by which Ca^{2+} serves its intracellular messenger functions, an additional feature in the evolution of this system needs to be considered. Once Ca^{2+} began to be used as an intracellular messenger, there was a need for specific intracellular receptors (proteins) that would receive this message and then couple it to specific cellular responses. There are two broad classes of Ca^{2+} receptor proteins that arose early in evolution and are nearly universally expressed in animal cells. The first class are protein molecules that have no intrinsic enzymatic activity, but interact with other protein molecules which do. The second class are molecules which possess both Ca^{2+}-binding sites and intrinsic enzymatic activity. The best characterized of the first class are calmodulin and troponin C (*18*); they are structurally related and possess four Ca^{2+}-binding sites. In addition, they possess other sites that interact specifically with other proteins (response elements), thereby modifying their functions. Troponin C has a restricted cellular distribution being confined to skeletal and cardiac muscle where it interacts with and serves to couple the Ca^{2+} message to the contractile proteins by facilitating a sequence of protein-protein interaction. Calmodulin (CaM), on the other hand, is nearly universally distributed throughout plant and animal cells. It participates in a wide variety of Ca^{2+}-regulated intracellular processes. It brings about its effects in one of two ways. In both, the Ca^{2+}-CaM complex interacts with another protein to modify its activity. In one case this is a protein which possesses an enzymatic activity other than protein kinase activity. In the other case the response element is one of a class of Ca^{2+}-CaM-activated protein kinases. An example of the former class is the Ca^{2+} pump or $Ca^{2+}/2H^{+}$-ATPase in the plasma membrane (*19*). The Ca^{2+}-CaM complex binds directly to this enzyme to alter its activity by direct protein-protein interaction such that its K_m for Ca^{2+} is reduced and its V_{max} for Ca^{2+} transport increased. The latter class contains an increasing number of protein kinases with either very restricted or more universal specificity as regards their substrate proteins. Their importance lies in the increasing evidence that the regulation of the state of phosphorylation of intracellular proteins is a major mechanism by which changes in cell functions are brought about. In these cases, the Ca^{2+}-CaM complex interacts with the protein kinase and serves as the regulatory subunit of this aggregate to bring about the activation of the particular kinase.

The protein kinase C (C-kinase) (*20*) is most important member of the class of enzymes that possess intrinsic enzymatic activity and are activated directly by the Ca^{2+} ion without the particpation of CaM or a calmodulinlike protein. This enzyme is activated directly by Ca^{2+}, but its sensitivity to Ca^{2+} activation

depends on its association with lipids. In the nonactivated cell, C-kinase is confined largely to the cytosol. In this state it is a relatively inactive Ca^{2+}-insensitive enzyme. Upon activation of the cell, the C-kinase becomes associated with the plasma membrane as a consequence of both the increase in the DG content of this membrane and the Ca^{2+} transient (see below). In this membrane-associated form, the C-kinase is a very active, Ca^{2+}-sensitive protein kinase (*21*).

Classic Views of Ca^{2+} As Intracellular Messenger

Having discussed cellular Ca^{2+} metabolism and Ca^{2+}-activated response elements, it is now possible to consider the classic views of Ca^{2+} messenger function. These views were largely elaborated by an analysis of the events involved in neurosecretion at synapses, contraction of skeletal muscle, and contraction of heart muscle.

The first site where the messenger function of Ca^{2+} was defined was the presynaptic plasma membrane whose depolarization, as a result of an action potential, induces a brief opening of a particular class of voltage-dependent Ca^{2+} channel (N channels). The resultant brief influx of Ca^{2+} is thought to act in the cellular domain just beneath the plasma membrane to trigger the association of synaptic vesicles with the plasma membrane, resulting in the release of neurotransmitter (*22*). Only a very transient increase in Ca^{2+} influx is required. This brief increase in Ca^{2+} influx is followed by a quiescent period during which the Ca^{2+} that entered the cell during its transient depolarization is pumped back out. Hence, Ca^{2+} homeostasis is maintained.

The second system where the messenger function of Ca^{2+} was defined was that of the skeletal muscle cell. The depolarization of the plasma membrane in this cell is linked to the release of Ca^{2+} from the SR pool at a specialized junction between the two membrane systems (the so-called T system). The precise manner in which the information is conveyed from one membrane to another remains a matter of discussion, but the consequence is clear: a sudden and transient release of Ca^{2+} from the SR pool leads to a rise in $[Ca^{2+}]_c$ in the cell cytosol sufficient to activate contraction. This rise in $[Ca^{2+}]_c$ is transient because the Ca^{2+} is rapidly reaccumulated by the SR. As a result, the contractile response is also transient (*23*).

The third system, the mammalian heart, combines features of the other two. During each electrical systole, depolarization of the plasma membrane leads to an increase in Ca^{2+} influx via another type of voltage-dependent Ca^{2+} channel (L channel). The bolus of Ca^{2+} which enters the cell is not sufficient by itself to trigger a contractile response. Rather, it acts as a signal to trigger the release of Ca^{2+} from the SR that causes a sufficient increase in $[Ca^{2+}]_c$ to initiate a contractile response. Because the Ca^{2+} released is rapidly reaccumulated by the SR, the contractile response is a transient one because the rise in $[Ca^{2+}]_c$ is transient. In addition, the Ca^{2+} that entered the cell during systole is pumped out of the cell during diastole so that cellular Ca^{2+} homeostasis is maintained (*24*).

In spite of the different mechanisms by which the Ca^{2+} signal is generated, all three systems possess a common attribute: the magnitude and duration of the cellular response is a direct function of the magnitude and duration of the Ca^{2+} message.

Calcium Toxicity due to Disorders of the Classic Pathways

These homeostatic systems can break down and lead to serious and even lethal consequences. The pathophysiologic realty of cellular Ca^{2+} toxicity can be illustrated in skeletal and cardiac muscle, respectively, by considering the condition of malignant hyperthermia and Isoproterenol-induced cardiac myotoxicity.

Malignant Hyperthermia

Malignant hyperthermia is a disease that occurs in susceptible humans when these individuals are exposed to certain anesthetic agents such as halothane. This susceptibility is inherited in humans as also in an inbred strain of pigs (*25*). In these animals, halothane exposure first leads to an increase in metabolic rate and heat production in skeletal muscle that is followed, eventually, by irreversible muscle contracture and death. The intracellular site of halothane action is the SR. Under resting conditions the rate of Ca^{2+} efflux from this organelle in these pigs' muscles is greater than that from the SR of normal pigs. This increase in Ca^{2+} efflux rate leads to a slight increase (0.2-0.4 μM) in the $[Ca^{2+}]_c$ of the muscle cell, as measured by a Ca^{2+}-sensitive microelectrode. This rise is not sufficient to cause a contraction of the muscle, but it is sufficient to stimulate the ATP-dependent reuptake of Ca^{2+} by the SR. Hence, there is an increased rate of Ca^{2+} cycling across the SR which, because of the increased rate of ATP hydrolysis, is associated with an increase in metabolic rate. When the animal is exposed to low doses of halothane, the Ca^{2+} efflux pathway is stimulated further. As a consequence, the $[Ca^{2+}]_c$ rises more and further stimulates the reuptake of Ca^{2+}, i.e., there is a further increase in Ca^{2+} cycling and an attendant increase in heat production. Initially, however, this rise in $[Ca^{2+}]_c$ is still insufficient to induce a contraction. Hence, in this early phase of malignant hyperthermia, the major observable effect is an increased rate of heat production and hyperthermia. However, as the rate of Ca^{2+} efflux increases further, it eventually reaches a point where it exceeds the capacity of the reaccumulation pathway. Under these

circumstances, the $[Ca^{2+}]_c$ rises further, and an irreversible contracture and death ensues.

Isoproterenol-induced Cardiac Muscle Toxicity

When rats are treated with large doses of Isoproterenol for a period of hours, they develop irreversible cardiac muscle dysfunction and die. Death is due to Ca^{2+} intoxication of the myocytes that develops as a consequence of an imbalance between the influx and efflux of Ca^{2+} across the plasma membrane of these cells (*26*). Isoproterenol is a beta agonist that interacts with catecholamine receptors on the heart muscle cells causing, thereby, an activation of adenylate cyclase and a rise in the cAMP content of these cells. As a consequence, both the heart rate and the force of contraction of each heart beat increase. The major result of cAMP action is the activation of the cAMP-dependent protein kinase. This enzyme catalyzes the phosphorylation of Ca^{2+} channels in the plasma membrane (L channels). Consequently, during each electrical systole, there is an increased rate of Ca^{2+} influx into the cell, and hence a larger Ca^{2+} signal to trigger Ca^{2+} release from the SR. Consequently, the intracellular Ca^{2+} transient is higher and the force of contraction greater. The rise in cAMP also leads to a greater rate of Ca^{2+} efflux across the plasma membrane via both the Ca^{2+} pump and the $3Na^+/Ca^{2+}$ exchanger. Hence, during diastole there is a greater efflux of Ca^{2+} from the cell. However, as the heart rate increases, the period of diastole is shortened considerably more than the period of systole. This means that at high rates of beating not all of the Ca^{2+} that has entered during a particular systole can be pumped back out of the cell during the subsequent diastole. A net accumulation of Ca^{2+} occurs during each cardiac cycle. Nonetheless, because this extra Ca^{2+} is sequestered within intracellular organelles, the heart continues to beat effectively, using transient rises and falls of $[Ca^{2+}]_c$ to regulate its function.

At first, this extra Ca^{2+} is stored in the SR and provides for a larger pool to be released at each systole. However, the storage capacity of this SR pool is quite limited and is soon saturated. The mitochondrial matrix compartment then becomes the major storage site for the excessive Ca^{2+}. Because the capacity of this storage site is relatively large, the heart can be steadily gaining total cell calcium and yet continue to display a normal Ca^{2+}-dependent contraction and relaxation cycle. Eventually, however, the capacity of the mitochondrial storage site is exceeded, and the $[Ca^{2+}]_c$ then rises sufficiently to interfere with relaxation.

Also, the increase in $[Ca^{2+}]_c$ activates proteases and phospholipases so that cell destruction and death ensue. However, if Verapamil, a Ca^{2+} channel blocker that prevents Ca^{2+} influx, is given simultaneously with Isoproterenol, the net accumulation of Ca^{2+} by the myocytes is blocked. As a consequence, the sequence of events outlined above is prevented. These results indicate that maintenance of cellular Ca^{2+} homeostasis is essential for the maintenance of normal cell function when cells employ extracellular Ca^{2+} as an intracellular messenger.

The Phosphoinositide-linked Ca^{2+} Messenger System

In contrast to these three classic systems where there is a direct relationship between the amplitude and duration of the Ca^{2+} message and the strength and duration of the response, there is a large number of cellular responses for which this direct relationship appears not to hold (*10*). These responses have in common the following characteristics: *a*) they are usually linked to the turnover of polyphosphoinositides; *b*) they are often continued responses to the sustained presence of an agonist; *c*) they are associated with a transient increase in $[Ca^{2+}]_c$, lasting only 1 to 2 min even in the continued presence of the agonist; *d*) they display a sustained increase in the rate of Ca^{2+} influx across the plasma membrane; *e*) they are not associated with an increase in total cellular Ca^{2+} content, despite the sustained increase in Ca^{2+} influx; and *f*) they are mediated by the temporal and spatial activation of more than one type of Ca^{2+}-dependent protein kinase.

Phosphoinositide Metabolism and Ca^{2+}

For our purposes, the important facts are that agonist-receptor interaction leads, via a G protein (*27*), to the activation of a specific phospholipase C that catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate two intracellular messengers, 1,2-diacylglycerol and inositol 1,4,5-trisphosphate (*28*). The former is lipid soluble and remains within the plasma membrane to bring about the protein kinase C activation. Inositol 1,4,5-trisphosphate is water soluble and functions in the cytosolic domain to initiate the release of Ca^{2+} from the specialized component of the endoplasmic reticulum, the calciosome. Whether there are other receptor-linked pathways by which 1,2-diacylglycerol is generated, or whether additional inositol phosphates such as inositol 1,3,4,5-tetrakisphosphate and inositol 1,3,4-trisphosphate have messenger functions remain matters of debate.

Changes in Ca^{2+} Metabolism during Agonist-induced Responses

Methods available to measure changes in total cell Ca^{2+}, Ca^{2+} influx rate and intracellular free Ca^{2+} concentration have made is possible to understand the subtleties of the Ca^{2+} messenger function. Such studies have led to an appreciation of the fact that there

are temporally and spatially distinct phases of these Ca^{2+}-mediated responses.

Using the photoprotein aequorin as an intracellular indicator, it is possible to measure transient changes in intracellular free Ca^{2+} in response to appropriate agonists (*29*). Thus, for example, when angiotensin II acts on adrenal glomerulosa cells, it stimulates the turnover of the inositol phospholipids; the generation of inositol 1,4,5-triphosphate and 1,2-diacylglycerol; and a transient rise in $[Ca^{2+}]_c$ from approximately 0.15 to 0.8 μM, which lasts only 1 to 2 min (*30*). Following this, the $[Ca^{2+}]_c$ returns to a value only slightly above the original basal value. Nevertheless, the increase in aldosterone secretion (the specific cellular response) occurs gradually over a period of 10 to 15 min, reaching a sustained plateau value that is maintained for an hour or more, as long as angiotensin II is present. The removal of angiotensin II leads to a prompt reversal of the enhanced rate of aldosterone secretion with little change in $[Ca^{2+}]_c$.

Even though there is a dissociation between the magnitude and duration of the Ca^{2+} transient and the magnitude and duration of the cellular response, there is, nevertheless, a continued need for Ca^{2+} during the sustained phase of the cellular response. The removal of extracellular Ca^{2+} or an inhibition of Ca^{2+} influx across the plasma membrane by the addition of drugs that block Ca^{2+} channels leads to a prompt inhibition of the cellular response (*31*). Furthermore, in order for the sustained response to occur, the agonist must induce a sustained increase in Ca^{2+} influx rate.

During cell activation, total cell calcium falls (at the time when the Ca^{2+} transient appears), reaches a plateau, and during the sustained phase of the response is maintained at a level below that found in the resting cell (*31*). Hence, during the sustained phase of the response, total cell Ca^{2+} is changing very little in spite of the fact that the Ca^{2+} influx rate is increased. Hence, the Ca^{2+} efflux rate across the plasma membrane must increase sufficiently to balance the increase in Ca^{2+} influx rate. Thus, during the sustained phase of the response, there is an increase in Ca^{2+} cycling across the plasma membrane even though $[Ca^{2+}]_c$ has changed very little from its basal value. These findings raise three important questions: Why is the rise in $[Ca^{2+}]_c$ a transient event? What cellular mechanisms determine the rise and fall of $[Ca^{2+}]_c$? What is the role of Ca^{2+} during the sustained phase of the response?

Factors Determining the Transient Nature of the Change in $[Ca^{2+}]_c$ Transient

There are two major factors that determine the transient nature of the change in $[Ca^{2+}]_c$: depletion of the trigger pool of Ca^{2+} and activation of the plasma membrane Ca^{2+} pump. The Ca^{2+} trigger pool contains a finite amount of calcium. This calcium is rapidly mobilized and soon depleted, so that once activated, the pool does not refill in the continued presence of the agonist (*32*).

Release of Ca^{2+} from the calciosomes leads to a rise in $[Ca^{2+}]$ in the submembrane domain of the plasma membrane, as well as in the bulk cytosol. This increase in $[Ca^{2+}]_{sm}$ leads to a stimulation of the Ca^{2+} pump (*33*). As a consequence, much of the Ca^{2+} released from the trigger pool is pumped out of the cell, thereby accounting for the net loss of total cell calcium. This mechanism is so efficient that a bolus equivalent to 20 μmole or more of Ca^{2+} (per liter of cell H_2O) must be released in order to raise the cytosolic Ca^{2+} concentration 1 μM (*4*).

Cellular and Molecular Mechanisms of Autoregulation

There are at lease two feedback mechanisms operating to maintain Ca^{2+} homeostasis during the period when there is a sustained increase in the Ca^{2+} influx rate. It is intuitively obvious that maintenance of homeostasis must involve, predominantly, the mechanisms by which the Ca^{2+} efflux across the plasma membrane is controlled. As noted previously, there are two such efflux mechanisms: Na^+/Ca^{2+} countertransport via a $3Na^+/Ca^{2+}$ exchanger and a $2H^+/Ca^{2+}$ countertransport driven by the hydrolysis of ATP, i.e., a Ca^{2+} pump.

Our discussion will be confined to considering the factors that regulate the pump. The most fundamental means for regulating the activity of this plasma membrane Ca^{2+} pump is via Ca^{2+}-calmodulin. Any increase in $[Ca^{2+}]_{sm}$, whether due to an influx of Ca^{2+} across the plasma membrane or the release of Ca^{2+} from an intracellular pool, leads to an increase in the association of Ca^{2+} CaM with the Ca^{2+} pump protein. This increase results in an increase in V_{max} of the Ca^{2+} transport rate and a decrease in the K_m for Ca^{2+} (*34*). Hence, any physiologically induced change in $[Ca^{2+}]_c$ leads to a prompt change in the activity of the pump. By this device, changes in both the amplitude and duration of the change in $[Ca^{2+}]_c$ are minimized. Additionally, the Ca^{2+}-dependent activation of protein kinase C leads to the phosphorylation of the Ca^{2+} pump protein that causes an increase in V_{max} of the transport system without altering its K_m for Ca^{2+} (*35*). Thus, during a sustained cellular response, two negative feedback loops link the activity of the Ca^{2+} pump to changes in the Ca^{2+} influx rate.

Role of Ca^{2+} during the Sustained Phase of the Cellular Response

Given the fact that there are two distinct temporal phases of altered cellular Ca^{2+} metabolism during agonist action, it is not surprising that distinct phases of agonist-induced changes exist in protein kinase function. Evidence for such time-dependent activations of distinct protein kinases was found by

analyzing the temporal patterns of agonist-induced changes in protein phosphorylation in angiotensin II-mediated aldosterone secretion (*36*) and carbacholamine-induced smooth muscle contraction (*37*). The phosphoproteins that appear in conjunction with the $[Ca^{2+}]_c$ transient are thought to be substrates for CaM-dependent kinases; those which appear during the sustained phase in conjunction with the increased cycling of Ca^{2+} across the plasma membrane are believe to arise as a consequence of protein kinase C activation.

From these data a two-phase model of cell activation has been developed. During the first phase the important initiating event (occurring as a consequence of agonist-receptor interactions) is the IP_3-induced release of Ca^{2+}; the result is a transient rise in $[Ca^{2+}]_c$ and the activation of CaM-dependent enzymes, including CaM-dependent protein kinases that phosphorylate a specific subset of cellular proteins responsible for initiating the cellular response.

During the second phase, the two important changes are the association of protein kinase C with the plasma membrane (which occurred during the initial phase as a consequence of the rise in the DG content of the plasma membrane and the $[Ca^{2+}]_c$ transient), and a sustained cycling of Ca^{2+} across the plasma membrane. These two components are linked. The rate of Ca^{2+} cycling, or the submembrane Ca^{2+} concentration $[Ca^{2+}]_{sm}$ determined by this cycling, controls the activity of the membrane-associated Ca^{2+}-sensitive form of protein kinase C. The phosphorylation of a second subset of cell proteins by this kinase is the mechanism by which the response is sustained.

In this view, Ca^{2+} is a critical messenger during both the initial and the sustained phase of the cellular response; however, the source of the messenger Ca^{2+}, its site of action within the cell, and its molecular targets differ. During the initial phase, the Ca^{2+} messenger arises from the intracellular trigger pool and acts within the total cytosolic domain to control the activities of CaM-regulated enzymes. During the sustained phase, the Ca^{2+} messenger arises from the extracellular pool, acts in a restricted subcellular domain at the endoplasmic face of the plasma membrane, and along with receptor-generated diacylglycerol regulates the activity of protein kinase C.

Properties of the C-Kinase Transducer

In effect, during the process of cell activation, a new plasma membrane transducer has appeared. This transducer consists of two components: the membrane-associated, calcium-sensitive form of protein C kinase and the cycling of Ca^{2+} across the cell membrane. This transducer has several interesting properties. First, under physiologic circumstances both components are necessary. During the initial phase of cell activation, there is a transient rise in $[Ca^{2+}]_c$ and a sustained or biphasic rise in the DG content of the plasma membrane. As a consequence, a percentage of the protein kinase C in the cytosol is translocated to the plasma membrane where the sustained increase in the DG content of the membrane assures its continued association (*38*). However, because $[Ca^{2+}]_c$ returns nearly to its basal value during this phase of the response, no additional C-kinase becomes associated with the membrane during the sustained phase of an agonist-induced response. Thus, it is postulated that the maximal capacity of the sustained response depends upon the amount of protein C kinase that becomes associated with the plasma membrane during the initial phase of the response. The rate of Ca^{2+} flux or Ca^{2+} cycling, on the other hand, determines what percentage of this maximum is actually expressed in a given circumstance. Under normal physiologic conditions, the magnitude of the sustained response can be enhanced by increasing the rate of Ca^{2+} influx. Hence, the membrane-associated protein C kinase is not normally maximally activated. Drugs or physiologic agents such as BAY K 8644, which do nothing more than increase or decrease Ca^{2+} flux across the membrane, can increase or decrease the magnitude of the response up to the limit imposed by the ability of the membrane-associated C-kinase molecules to respond to the Ca^{2+} signal.

The C-Kinase Transducer and Cellular Memory

One unexpected feature of the sustained cellular responses mediated by this dual Ca^{2+}-regulated information flow is the discovery that this control system displays a type of short-term memory (*39*). For example, when adrenal glomerulosa cells are exposed to angiotensin II for 30 min, the rate of aldosterone secretion reaches a plateau that will be maintained for several hours during continuous exposure to the angiotensin II. However, if after 30 min, the angiotensin II is removed, the rate of aldosterone secretion returns to the prestimulation value within 10 to 15 min. Likewise, the angiotensin II-induced increase in Ca^{2+} influx rate is rapidly (within minutes) reversed. However, if the same concentration of angiotensin II is applied 15 to 20 min after cessation of the previous exposure to angiotensin II, then the glomerulosa cells respond with a prompt increase in aldosterone secretory rate to a sustained plateau that is significantly higher than the rate seen in cells exposed continuously to the same concentration of angiotensin II. If this cycle is repeated, a third exposure to angiotensin II leads to an even greater sustained rate of aldosterone secretion, i.e., there is a cumulative memory. A similar phenomenon has been observed in tracheal and vascular smooth muscles when exposed intermittently to an appropriate agonist. Similarly, an exposure of islets of Langerhans to intermittent glucose

pulses induces a similar kind of cumulative memory in terms of glucose-induced insulin secretion (*40*). Hence, memory appears to be a common property of this control system.

The basis for this short-term memory in endocrine target cells remains to be fully defined. Nonetheless, it appears that protein kinase C plays a central role in this phenomenon. Our data are consistent with a model in which there are three states in which C-kinase may exist. In the nonactivated cell, this enzyme is located largely in the cytosol and exists in its Ca^{2+}-insensitive state. Upon activation of the cell, a percentage of this cytosolic enzyme is translocated to the plasma membrane. The amount of enzyme translocated in this step depends upon the magnitude of the DG signal in the plasma membrane and the size of the $[Ca^{2+}]_c$ transient. Once the enzyme has associated with the membrane, it is transformed into its Ca^{2+}-sensitive form; in this form its activity is regulated by the rate of Ca^{2+} flux or cycling across the plasma membrane. It is postulated that with time this membrane-associated protein C-kinase undergoes some type of covalent modification that anchors the enzyme to the membrane. In this form the enzyme remains Ca^{2+} sensitive; however, when agonist action is terminated and receptor activation ends, the anchored C-kinase leaves the membrane only slowly. In contrast, the agonist-induced Ca^{2+} influx rate reverses rapidly so that information flow through the C-kinase branch of the calcium messenger system ceases. Upon readdition of agonist, another bolus of protein C-kinase is translocated to the membrane. Hence, during the second exposure to agonist, the total amount of protein C-kinase associated with the membrane is greater than during the first exposure. As a consequence, the agonist-mediated sustained response is greater during the second exposure. This model appears to account for the short-term memory in endocrine systems and may also account for the phenomenon of associative learning in the marine snail Hermissenda (4) and in short memory in the mammalian nervous system.

The Protein C-Kinase Transducer and Tumor Promoters

Of particular importance to a consideration of toxicology is a group of drugs first identified as tumor promoters. These drugs, such as phorbol myristic acid, can replace DG as activators of protein kinase C (*41*). However, this class of compounds does more than simply activate protein kinase C. They act somewhat differently than does DG. First, their addition causes an association of protein C-kinase with the plasma membrane in the absence of a $[Ca^{2+}]_c$ transient; second, because of this relatively Ca^{2+}-independent membrane association of protein C-kinase, a progressive accumulation of protein C-kinase within the membrane occurs; and third, the C-kinase that is associated with phorbol ester in the membrane is considerably more sensitive to activation by Ca^{2+} than is the enzyme associated with DG in the membrane (*42*). Hence, this class of drugs can turn on the transducing system under circumstances where there is little or no change in cellular Ca^{2+} metabolism. Because activation of the inositol phospholipid-calcium messenger system often serves as one of the inputs that mediate cell proliferation (*21*), these drugs can promote cell growth either appropriately, or more often inappropriately, in conjunction with other physiologic and/or environmental signals.

Conclusion

The highly sophisticated organization of the calcium messenger system attests to the fact that during evolution cells have devised a system by which Ca^{2+} can serve as an intracellular messenger to mediate sustained cellular responses under conditions in which cellular Ca^{2+} homeostasis is maintained. Nevertheless, the potential of cellular calcium intoxication persists. Circumstances that alter the properties of this calcium messenger system and, in particular, interfere with the autoregulatory system in the plasma membrane have the potential to lead to serious cellular dysfunction and death.

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REFERENCES

1. Jayne, W. A. The Greek Deities in the Healing Gods of Ancient Civilization. Yale University Press, New Haven, CT, 1925, pp. 344-345.
2. Cheung, J. Y., Bonaventure, J. V., Malis, C. D., and Leaf, A. Calcium and ischemic injury. N. Engl. J. Med. 314: 1670-1676, (1986).
3. Schanne, F. A. X., Kanne, A. B., Young, E. E., and Farber, J. L. Calcium dependence of toxic cell death. Science 206: 700-702 (1979).
4. Alkin, K. L., and Rasmussen, H. A spatial-temporal model of cell activation. Science 239: 998-1005 (1988).
5. Kazmerczak, J., Degens, W., and Degens, E. T. Calcium and the early eukaryotes. MiMellungen aus dem Geologish-Palaontologischen Institut der Universitat Hamburg. Heft 61: 1-20 (1986).
6. Burton, F., Neuman, M. W., and Neuman, W. F. On the possible role of crystals in the origins of life. I. The absorption of nucleosides, nucleotides, and pyrophosphate by apatite crystals. Curr. Mod. Biol. 3: 20-26 (1969).
7. Neuman, W. F., and Neuman, M. W. On the possible role of crystals in the origins of life. IV. The phosphorylation of nucleotides. Curr. Mod. Biol. 3: 277-282 (1970).
8. Kretsinger, R. H. A comparison of the roles of calcium in biomineralization and in cytosolic signalling. In: Biomineralization and Biological Metal Accumulation (P. Westbroek and E. W. de Jong, Eds.), D. Dordrecht Reidel Publishing Co., 1983, pp. 123-131.
9. Kempe, S., and Degens, E. T. An early soda ocean? Chem. Geol. 53: 95-108 (1985).
10. Rasmussen, H. The calcium messenger system. N. Engl. J. Med. 314: 1094-1101 (1986).
11. Carafoli, E. Plasma membrane Ca^{2+} transport and Ca^{2+} handling by intracellular stores: an integrated picture with

emphasis on regulation. In: Mechanism of Intestinal Electrolyte Transport and Regulation by Calcium (M. Donowitz and D. W. Sharp, Eds.), Alan R. Liss, New York, 1984, pp. 121-134.
12. Schatzman, H. J. Calcium extrusion across the plasma membrane by the calcium-pump. In: Calcium and Cell Physiology (D. Marusic, Ed.), Springer-Verlag, Berlin, pp. 18-52, 1985.
13. Humes, D. Regulation of intracellular calcium. Semin. Nephrology 4: 117-133 (1984).
14. Denton, R. M., and McCormach, J. G. Ca^{2+} transport by mammalian mitochondria and its role in hormone action. Am. J. Physiol. 249: E543-E554 (1985).
15. Volpe, P., Krause, K. H., Hashimoto, S., Zorzato, F., Pozzan, T., Meldolesi, J., and Lew, D. P. "Calciosome," a cytoplasmic organelle: the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} store of nonmuscle cells. Proc. Natl. Acad. Sci. (U.S.) 85: 1091-1095 (1988).
16. Exton, J. H. Role of calcium and phosphoinositides in the actions of certain hormones and neurotransmitters. J. Clin. Invest. 75: 1753-1757 (1985).
17. Denton, R. M., McCormack, J. G., and Edgell, N. J. Role of calcium ions in the regulation of intramitochondrial metabolism: effects of Na^+, Mg^{2+} and ruthenium red on the Ca^{2+}-stimulated oxidation of oxoglutarate and on pyruvate dehydrogenase activity in intact rat heart mitochondria. Biochem. J. 107-117 (1980).
18. Rasmussen, H., and Barrett, P. Q. Calcium messenger system: an integrated view. Physiol. Rev. 64: 938-984 (1984).
19. Smallwood, J. I., Waisman, D. M., Lafreniere, D., and Rasmussen, H. Evidence that the erythrocyte calcium pump catalyzes a Ca^{2+}:nH^+ exchange. J. Biol. Chem. 258: 11092-11097 (1983).
20. Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., and Nishizuka, Y. Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids. J. Biol. Chem. 254: 3692-3697 (1979).
21. Nishizuka, Y. The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature 308: 693-698 (1984).
22. Zucher, R. S., and Lando, L. Mechanism of transmitter release: voltage hypothesis and calcium hypothesis. Science 231: 574-579 (1986).
23. Ebashi, S., Mikawa, T., Hirata, M., and Noromura, Y. The regulatory role of calcium in muscle. Ann. N. Y. Acad. Sci. 307: 451-461 (1978).
24. Chapman, R. A. Excitation-contraction coupling in cardiac muscle. Prog. Biophys. Mol. Biol. 35: 1-52 (1979).
25. Lopez, J. R., Allen, P., Alama, L., Ryan, J. F., Jones, D. E., and Streter, F. Dantrolene prevents the malignant hyperthermic syndrom by reducing free intracellular calcium concentration in skeletal muscle of susceptible swine. Cell Calcium 8: 385-396 (1987).
26. Bloom, S., and Cancilla, P. A. Myocytolysis and mitochondrial calcification in rate myocardium after low doses of isoproterenol. Am. J. Physiol. 54: 373-391 (1969).
27. Wallace, M. A., and Fain, J. N. Guanosine 5′-0-thiotriphosphate stimulates phospholipase C activity in plasma membranes of rat hepatocytes. J. Biol. Chem. 260: 9527-9530 (1985).
28. Berridge, M. J. Inositol triphosphate and diacylglycerol: two interacting second messengers. Ann. Rev. Biochem. 56: 159-193 (1987).
29. Snowdowne, K., and Borle, A. B. Measurement of cytosolic free calcium in mammalian cells with aequorin. Am. J. Physiol. C396-C408 (1984).
30. Apfeldorf, W. J., and Rasmussen, H. Simultaneous determination of intracellular free calcium and aldosterone production in bovine adrenal zona glomerulosa. Cell Calcium 9: 71-80 (1988).
31. Kojima, I., Kojima, K., and Rasmussen, H. Role of calcium fluxes in the sustained phase of angiotensin II-mediated aldosterone secretion from adrenal glomerulosa cells. J. Biol. Chem. 260: 9177-9184 (1985).
32. Streb, H., Irvine, R. F., Berridge, M. J., and Schulz, I. Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. Nature 306: 67-69 (1983).
33. Irvine, R. F. Calcium transients: normalization of intracellular Ca^{2+}. British Med. Bull. 42: 269-374 (1986).
34. Carafoli, E. Intracellular calcium homeostasis. Ann. Rev. Biochem. 56: 395-433 (1987).
35. Smallwood, J. I., Gugi, B., and Rasmussen, H. Regulation of erythrocyte Ca^{2+} pump activity by protein kinase C. J. Biol. Chem. 263: 2195-2202 (1988).
36. Barrett, P. Q., Kojima, I., Kojima, K., Isales, C. M., and Rasmussen, H. Temporal patterns of protein phosphorylation after angiotensin II, A23187 and/or 12-*O*-tetradecanoyl phorbol-13-acetate in adrenal glomerulosa cells. Biochem. J. 238: 893-903 (1986).
37. Rasmussen, H., Takuwa, Y., and Park, S. Protein kinase C in the regulation of smooth muscle contraction. FASEB J. 1: 177-185 (1987).
38. Griendling, K. K., Rittenhouse, S. E., Brock, T. A., Ekstein, L. S., Gimbrone, M. A., and Alexander, R. M. Sustained diacylglycerol formation from inositol phospholipids in angiotensin II-stimulated vascular smooth muscle cells. J. Biol. Chem. 261: 5901-5906 (1986).
39. Barrett, P. Q., Kojima, I., Kojima, K., Isales, C. M., and Rasmussen, H. Short term memory in the calcium messenger system. Biochem. J. 238: 905-912 (1986).
40. Rasmussen, H., Isales, C. M., Takuwa, Y., Takuwa, N., Barrett, P. Q., and Zawalich, W. The cellular basis for short-term memory in endocrine systems. In: Cellular Mechanisms of Conditioning and Behavioral Plasticity (C. D. Woody, D. L. Alkon, and J. L. McGaugh, Eds.), Plenum Publishing Co., New York, 1988, pp. 3-10.
41. Castagna, M., Takai, Y., Kaiuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor promoting phorbol esters. J. Biol. Chem. 257: 7847-7851 (1982).
42. Hannun, Y. A., Loomis, C. R., and Bell, R. M. Protein kinase C activation in mixed micelles. J. Biol. Chem. 261: 7184-7190 (1986).