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INTRACELLULAR CALCIUM AND CELL FUNCTION

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INTRODUCTION

For many years scientists have investigated the mechanism by which agents (hormones, growth factors, and drugs) interacting with cell surface receptors control intracellular events. This process involves transmission of information from surface receptors to those molecules (second messengers) in the membrane, cytoplasm, or nucleus that control important subsequent physiological events. It is well known that occupation of membrane receptors leads to the

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production of several classes of second messengers (e.g. cAMP, Ca, and diacylglycerol) that in turn initiate distinct biochemical pathways. The interaction of cAMP with cAMP-dependent protein kinase was the first second messenger pathway to be described in detail (126), but a role for calcium as a regulator of cellular function was in fact proposed much earlier (57, 93, 125). Although technical problems in studying the role of intracellular Ca significantly delayed the development of the Ca second messenger story, in the last decade tremendous progress has been made toward understanding the complicated mechanism by which intracellular Ca is regulated and, in turn, the mechanism by which Ca acts through a variety of Ca-binding proteins to control numerous intracellular processes. In this article we review the pathways for controlling intracellular Ca, some of the processes thought to be regulated by an elevation of intracellular Ca, and the Ca-binding proteins that are involved in mediating the actions of Ca on cellular events. Because the literature in this area is so vast, we have necessarily restricted citations to key papers or recent reviews of specific topics.

MEASUREMENT OF INTRACELLULAR Ca

Measurement of Total Cell Ca and Ca Fluxes

The field of intracellular Ca regulation was slow to develop because of the difficulty in measuring the intracellular free Ca concentration, $[Ca]_i$. Early attempts to measure $[Ca]_i$ involved extracting cells and measuring the entire cellular Ca content. Even with such crude methods, there were indications that activation of sea urchin eggs led to an increase in total cell Ca (57). Although these values undoubtedly were reflections of the amount of Ca stored in intracellular compartments, rather than $[Ca]_i$, the increase in these compartments' sizes probably did reflect an increase in $[Ca]_i$ and an attempt by the cell to buffer the rise in $[Ca]_i$. With the introduction of ^{45}Ca and the ability to perform Ca influx and efflux experiments in steady-state cells, it soon became obvious that much of the Ca in cells was sequestered in several different compartments, and that the earlier Ca content measurements were not accurate estimates of free $[Ca]_i$. These findings suggested that the regulation of intracellular Ca was very complex. Careful mathematical analysis of Ca influx and efflux curves indicated that interaction of agents with surface receptors probably led to an increase in Ca influx and to the mobilization of Ca from intracellular Ca stores (110, 154); however, these techniques could not provide any information about the resting level of $[Ca]_i$ or the level to which hormonal stimulation would drive $[Ca]_i$. Since $[Ca]_i$ is the most important variable in terms of Ca interaction with Ca-binding proteins and regulation of Ca-dependent processes, the inability to measure this value directly was a critical barrier to our understanding the role of Ca as a second messenger.

Ca-Sensitive Optical Probes and Microelectrodes

Initial estimates of $[Ca]_i$ of 0.1–1μM were obtained from indirect measurements of the Ca concentration needed to contract glycerinated muscle fibers (51, 119). These led investigators to surmise that resting Ca levels must be approximately 0.1 μM. However, only with the introduction of Ca-sensitive optical probes did information began to appear concerning the true level of $[Ca]_i$. In the late 1960s and early 1970s, $[Ca]_i$ was successfully measured in large invertebrate cells utilizing the two Ca-sensitive photoprobes aequorin and arsenazo III. Aequorin, with its Ca-sensitive chemiluminescence, has been utilized successfully in cells that were microinjected with this photoprotein (15). Although aequorin provides some useful information on $[Ca]_i$, there are disadvantages to its use. Each photoprotein molecule can emit only one photon; thus the indicator is constantly consumed. In addition, initial use of aequorin necessitated the microinjection of the photoprotein into cells. In more recent studies, aequorin has been loaded into substrate-attached cells by a technique called "scrape-loading" (94), and into suspension cells by "centrifuge loading" (17), which enables the probe to be loaded into a large number of cells without the use of microelectrodes.

Other studies on $[Ca]_i$ utilizing optical techniques were performed with the Ca-sensitive dye arsenazo III, which shows changes in absorption spectrum on binding Ca. This optical probe was useful in early studies measuring $[Ca]_i$ (15, 128), but also has significant limitations. The dye had to be microinjected into cells, and there were problems in the estimation of $[Ca]_i$ created by the complex binding characteristics of Ca to this probe.

Microelectrode measurements in large cells such as neurons and muscles supported findings from the optical studies that in resting cells $[Ca]_i$ lies in the range of 50 to 100 nM, and that upon depolarization $[Ca]_i$ could rise to values > 1 μM. Therefore, it became apparent that for a cell with resting $[Ca]_i$ in the 100-nM range, sitting in a bath containing 1–2 mM of Ca, the opening of a small number of Ca channels could have profound effects on $[Ca]_i$. Likewise, there is tremendous potential for elevating $[Ca]_i$ by mobilizing Ca stored in intracellular compartments. For example, if the stored Ca was uniformly distributed over the cell water, the $[Ca]_i$, in most cell types, would be in the millimolar range. Thus, release of intracellular Ca could have profound effects on $[Ca]_i$.

The introduction of Ca-sensitive optical probes and Ca-sensitive microelectrodes had profound effects upon the study of Ca metabolism in large cells, but for most cells, because of their small size, these techniques were not applicable.

Ca-Sensitive Fluorescent Probes

With the introduction of Ca-sensitive fluorescent probes, the $[Ca]_i$ in cells of all sizes could be measured. Quin-2 was the first widely used Ca-sensitive

fluorescent probe (150). Tsien designed this Ca probe (*a*) to have an active site which mimicked that of ethyleneglycol-bis(β-amino ethyl ether) N,N,N',N'-tetraacetic acid (EGTA), (*b*) to provide high Ca selectivity, and (*c*) to have a ring structure that when binding Ca would increase its emisson intensity. Therefore, one could selectively monitor [Ca] in a solution by the level of fluorescence emission from quin-2 without concern for effects of Mg, Na, K, or protons. To allow easy loading of the dye into small cells, the four carboxyl groups, which make up the heart of the active binding site for Ca, were masked with acetoxymethylester (AM) groups. Quin-2–AM is lipid soluble and easily crosses the plasma membrane; inside the cell, cytoplasmic esterases cleave the AM groups to produce free quin-2 (151). Therefore, these dyes can be loaded into any cell, no matter how small, provided that the cell has sufficient esterase activity to cleave the ester bond.

Although the introduction of quin-2 was a tremendous boon to the study of $[Ca]_i$ in cells such as lymphocytes, fibroblasts, neutrophils, and platelets, some features of quin-2 are not optimal. The quantum efficiency of quin-2 is low; thus high concentrations of the dye must be loaded to get measurable fluorescent signals from the cell. Such high concentrations tend to buffer $[Ca]_i$ during rapid transients. Although transients were likely to be reduced in amplitude, modeling of the mechanism for regulation of $[Ca]_i$ suggested that the resting levels of Ca would not be changed by introduction of quin-2. Thus, quin-2 provided much essential information on the resting levels of intracellular Ca, but likely perturbed the hormonally stimulated Ca transients reported in a number of different cell types. Another problem with quin-2 was the difficulty in calibrating $[Ca]_i$ from the changes in cellular fluorescence.

Many of the difficulties experienced with quin-2 were resolved with the introduction of fura-2 (50). This probe is similar in design to quin-2 but has two distinct advantages over the original dye. First, fura-2 has a much higher quantum efficiency and therefore can be loaded into cells at much lower concentrations; this reduces the possibility that the presence of the indicator will perturb the Ca responses that it is monitoring. Second, when fura-2 binds Ca there is a dramatic shift in the excitation spectrum of the probe, such that Ca binding increases the fluorescence of dye excited at 340 nm and decreases the fluorescence of dye excited at 380 nm. Thus, by plotting the ratio of the 340-nm fluorescence to the 380-nm fluorescence one can obtain a very accurate calibration curve for Ca. One can take this ratio of the two fluorescences from the cell and relate it directly to a Ca concentration. By using a ratio, one also eliminates the complications arising from differences in optical path length, differential dye loading between cells, or fluctuations in the lamp intensity.

Recently, other Ca-sensitive fluorescence probes (indo-1 and flo-3) have been synthesized to aid in the study of $[Ca]_i$ (152). In addition, "caged" Ca

compounds (nitro-5 and 1-(2-nitro-4,5-dimethoxyphenyl)-N,N,N',N'tetrakis [(oxycarbonyl) methyl]-1,2 ethanediamine (DM)-nitrophen) have been produced to allow manipulation of intracellular Ca (67, 152).

REGULATION OF INTRACELLULAR Ca LEVELS

A low $[Ca]_i$ is maintained by a complex interaction of several biochemical processes. These include the regulation of Ca channel opening (both voltage-sensitive and receptor-coupled), the binding of Ca to membrane surfaces and Ca-binding proteins, the extrusion of Ca from the cytoplasm either out of the cell across the plasma membrane or into multiple intracellular storage sites, and the regulation of release of Ca from these intracellular compartments. In different cell types the $[Ca]_i$ can be regulated via different combinations of these regulatory pathways.

Ca Channels

Since the Ca concentration is 10^4 times higher outside the cell than in the cell cytoplasm, and since entering Ca moves down an electrical gradient, the opening of Ca channels, even transiently, is a very effective mechanism for elevating $[Ca]_i$, as well as for carrying a depolarizing Ca current into the cell. In some cells Ca channels appear to open strictly for the control of $[Ca]_i$ while in others, such as cardiac cells, the Ca current is an important component of their electrophysiological response. Depending on the cell type, there are several mechanisms by which Ca channels can be opened. In excitable cells there exist voltage-sensitive Ca channels (VSCCs) that open in response to a membrane depolarization. Work from the laboratory of Richard Tsien (42, 43) has contributed greatly to the classification of various subsets of VSCCs. Based on biophysical and pharmacological criteria, the VSCCs in chick sensory neurons have been characterized as either T, N, or L type. Since the characterization of VSCCs in excitable cells, as well as their regulation by cAMP, Ca/CaM, protein kinase C, and G proteins, was recently reviewed (153), we do not discuss those apects of VSCCs here.

Recent studies suggest that VSCCs exist in nonexcitable cells such as fibroblasts (22) and may be regulated by factors other than membrane depolarization. For example, evidence from our laboratory suggests that an L-type VSCC is present in cultured human fibroblasts and may be regulated by epidermal growth factor (EGF). Fura-2 studies reveal that EGF increases $[Ca]_i$ in these fibroblasts and that this increase depends on the presence of external Ca (112). EGF can also induce an influx of ^{45}Ca about three times greater than basal levels, an influx inhibited by the dihydropyridine antagonist nitrendipine (155). ^{45}Ca influx can also be stimulated in these cells by

membrane depolarization in high-potassium medium, an effect enhanced with the dihydropyridine agonist Bay K 8644 and inhibited by nitrendipine (155).

In addition to VSCCs, whose activity can be regulated by second messengers produced by receptor stimulation, there are Ca channels that appear to be directly associated with the receptor protein structure. The classic example of a receptor molecule that functions as a channel, although it does not carry much in the way of Ca current, is the acetylcholine receptor. Isolation and purification of this receptor and its reconstitution in membranes indicate that the receptor and the channel are built from the same multisubunit association. The best example of a receptor-operated Ca channel is the N-methyl-D-aspartate (NMDA) type of glutamate receptor, which appears to gate an ion channel that is highly permeable to Ca but also to Na and K. These channels are blocked by Mg in a voltage-dependent manner, opened by NMDA and other agonists, and modulated by agents such as glycine and phencyclidine (4).

Release from Intracellular Ca Stores

In many cells the response to hormonal stimulation is mediated by a release of Ca from intracellular stores. For years, investigators had hypothesized receptor stimulation of Ca release based upon ^{45}Ca efflux studies in fibroblasts, kidney, pancreas, and liver cells. Although it was known that Ca could be elevated in skeletal muscle cells via Ca release from the specialized network of intracellular membranes, called the sarcoplasmic reticulum, it was not clear how Ca mobilization occurred in nonmuscle cells. This process has been elucidated in recent years, principally based on work initiated in Michael Berridge's laboratory on the phosphatidylinositol cycle. These studies, in turn, were a major extension of earlier investigations that suggested a link between the turnover of phosphatidylinositol and Ca metabolism (59, 96), although the earlier hypothesis (96) proposed a linkage to Ca influx rather than intracellular Ca mobilization. The work in the Berridge laboratory described a new pool of phosphatidylinositol, namely phosphatidylinositol bisphosphate (PIP_2), which, when acted on by phospholipase C, could release inositol triphosphate ($InsP_3$) and diacylglycerol (13). This work suggested that $InsP_3$ could serve as a second messenger of some sort. Streb et al (139) then demonstrated that the addition of $InsP_3$, but not InsP or $InsP_2$, to permeabilized pancreatic acinar cells leads to the release of Ca from intracellular pools. This opened a whole new field of investigation in terms of how $[Ca]_i$ is controlled. Subsequent studies in numerous other cell types have confirmed that the release of $InsP_3$ leads to the mobilization of intracellular Ca in response to many hormones, and even though it is somewhat controversial, has led to the proposal that $InsP_3$ release is important in the Ca release in skeletal muscle. Since several recent reviews have thoroughly described the

mechanism for metabolizing PIP_2, we focus here on some of the recent developments in this story relating directly to the complexities of the metabolism of $InsP_3$ and the implications of these findings for the control of $[Ca]_i$.

When Berridge's laboratory first described the products arising from the breakdown of PIP_2, methods for separating the water-soluble inositol phosphates could only resolve InsP, $InsP_2$, and $InsP_3$. With improved HPLC separation methods it soon became clear that futher inositol phosphates were present in cell extracts. The elution peak thought to contain $InsP_3$ was actually a combination of Ins1,4,5P_3 and Ins1,3,4,P_3, and the peak thought to contain $InsP_2$ contained Ins1,4P_2 and Ins3,4P_2. Studies were rapidly undertaken to determine which of these newly described inositol phosphates were active in releasing Ca from intracellular stores. Thus far only for Ins1,4,5P_3 is there substantial evidence for a Ca mobilizing activity.

Recently, more highly phosphorylated metabolites of inositol have been detected, e.g. Ins1,3,4,5P_4 (7). It was soon learned that Ins1,3,4,5P_4 was produced when Ins1,4,5P_3 was phosphorylated by a Ins1,4,5P_3 kinase. This kinase was first discovered as a soluble enzyme present in brain (61), but has subsequently been identified in liver (52), parotid (55), lymphocytes (138), and insulinoma cells (14). This kinase is specific for the 3-position of Ins1,4,5P_3 and produces Ins1,3,4,5P_4 from only this isomer of $InsP_3$.

The demonstration of a kinase that phosphorylates the active Ca-mobilizing agent Ins1,4,5P_3 suggested several hypotheses concerning the importance of this phosphorylation. First, this pathway could represent an alternative pathway for metabolizing and inactivating Ins1,4,5P_3. It was already known that Ins1,4,5P_3 was dephosphorylated by a Mg-dependent 5-phosphatase to produce the inactive product Ins1,4,P_2. The phosphorylation could be a parallel pathway for rapidly removing Ins1,4,5P_3 and thereby terminating the Ca signal. This seemed reasonable since the Ins1,3,4,5P_4 was found to be dephosphorylated by a 5-phosphatase to produce the apparently inactive isomer Ins1,3,4P_3. However, an alternative hypothesis is that Ins1,3,4,5P_4 could be an active product that performs further messenger functions. While it was clear that Ins1,3,4,5P_4 did not mobilize Ca from intracellular stores, it was possible that there were other actions of this molecule.

Current speculation is that Ins1,3,4,5P_4 may be involved in regulation of Ca influx channels. This hypothesis arises from recent work in which Ins-1,3,4,5P_4 microinjection into sea urchin eggs caused the immediate elevation of the fertilization envelope for eggs in the presence, but not in the absence, of external Ca (63). This did not appear to be a simple process. In order to see the effect of Ins1,3,4,5P_4, one had to coinject Ins2,4,5P_3, an isomer that cannot be converted to Ins1,3,4,5P_4 but is known to produce mobilization of intracellular Ca. Ins2,4,5P_3 does not cause elevation of the fertilization envelope when added alone.

Some investigators have speculated that the synergism between the Ca-mobilizing agent Ins2,4,5P_3 and Ins1,3,4,5P_4 is related to an effect of Ins-1,3,4,5P_4 on the reloading of the Ins1,4,5P_3-sensitive Ca compartment via a Ca influx across the plasma membrane. This represents an extension of the theory that vesicles reload with Ca by a specialized network to bring external Ca directly into the Ca storage vesicles without changing cytoplasmic Ca levels (124). Recent studies in mouse lacrimal acinar cells support a form of synergism between Ca-mobilizing inositol phosphates and Ins1,3,4,5P_4. For cells in whole-cell perfusion via patch clamp methods, both Ins1,4,5P_3 and Ins1,3,4,5P_4 are necessary to see Ca-activated K currents (98). Recently, Irvine and his colleagues (62) suggested that Ins1,3,4,5P_4 may serve to control Ca fluxes between Ins1,4,5P_3-sensitive and Ins1,4,5P_3-insensitive compartments. This model is supported by the observation that Ins1,4,5P_3-induced Ca release from microsomal fractions from pituitary cells can be enhanced by the presence of Ins1,3,4,5P_4 (137). The hypothesis that Ins-1,3,4,5P_4 production is important for the extension of the short burst of Ca from internal stores into a prolonged Ca response is consistent with the observation that the activity of Ins1,4,5P_3 kinase is stimulated by Ca via a calmodulin-dependent mechanism (99, 127). Therefore, the short burst of Ca can trigger increased production of the Ins1,3,4,5P_4 proposed to be necessary for the prolonged Ca response.

Initial studies on Ca mobilizaton from permeabilized cells by inositol trisphosphate demonstrated that Ca was released from a pool that was nonmitochondrial. Although the Ca pool was subsequently referred to as being the endoplasmic reticulum, until recently there was no detailed information concerning the true structural identity of this intracellular Ca pool. However, recent studies suggested that there is a specific structure that stores Ca and releases it in response to Ins1,4,5P_3. Volpe et al (156) report the existence of a protein immunologically related to calsequestrin, the protein responsible for high-capacity Ca binding in sarcoplasmic reticulum, in nonmuscle cells (see also 87). Immunogold procedures localize this protein to discrete organelles, termed calciosomes (156). Subcellular fractionation suggested copurification of the calsequestrin-like protein with markers for the Ins1,4,5P_3-sensitive calcium store, but not with markers for endoplasmic reticulum, Golgi, mitochondria, and endosomes.

The observation that Ins1,4,5P_3 mobilizes Ca from intracellular stores whereas very similar inositol phosphates do not, argues for a high degree of specificity in the mechanism for Ca release. This suggests the existence of specific receptors that recognize Ins1,4,5P_3 on the surface of calciosomes. Recent studies, using binding of ^{3}H-Ins1,4,5P_3 as an assay, reported the solubilization of the Ins1,4,5P_3 receptor from rat cerebellum and its purification to apparent homogeneity (144). The purified receptor shows the same

selectivity for Ins1,4,5P_3 seen in crude membranes. Electrophoretic analysis of the purified receptor indicates a single protein band with a molecular weight of 26 kd. Recently, work has been reported on attempts to purify the receptor for Ins1,3,4,5P_4 from brain tissue (148) and from HL-60 cells (18).

Alternative Pathways for Mobilizing Intracellular Ca

Although the Ins1,4,5P_3 pathway is certainly the best described for mobilizing intracellular Ca, other intracellular products may also be messengers for mobilization of Ca. For example, arachidonic acid has been proposed to mobilize Ca in pancreatic islets (162), and cyclic InsP_3 effectively stimulates a response when microinjected into the *Limulus* photoreceptor (161). However, both arachidonic acid and cyclic InsP_3 can be produced as the result of PIP_2 breakdown and are therefore part of this whole regulatory mechanism.

Recent reports from Lee's laboratory (23, 24) describe a new mechanism for mobilizing intracellular Ca, totally distinct from the PI turnover pathway. This laboratory group, while screening for Ca-releasing agents in a homogenate of sea urchin eggs, identified a metabolic product of NAD that had potent Ca-releasing ability. The metabolite, originally called E-NAD (for enzyme-activated NAD), could be produced by incubating high-speed supernates of sea urchin eggs with NAD. Microinjection of HPLC-purified E-NAD into sea urchin eggs resulted in a transient Ca release and subsequent cortical reactions. Lee et al (78) recently identified the structure of E-NAD as cyclic ADP-ribose. Although the early work on cyclic ADP-ribose suggests that it may be an important alternate pathway for mobilizing intracellular Ca, much work needs to be done in the sea urchin egg system to describe how the production of this compound is regulated in vivo and in other cell systems to determine the universality of this mechanism. Studies with extracts of liver and heart tissues indicate that the enzyme for modifying NAD to cyclic ADP-ribose is present and suggest that this may indeed be a mechanism present in other cell systems.

Extrusion of Ca from the Cytoplasm

To maintain the resting $[Ca]_i$ at 100 nM, the cell must actively extrude Ca from the cytoplasm. This Ca is both extruded across the plasma membrane and sequestered into the intracellular Ca compartments, from which it can be released upon receptor stimulation. The two major transport systems for pumping Ca are the Ca-ATPase and the Na/Ca exchanger. The Ca-ATPase system couples the energy released in the hydrolysis of ATP to the movement of Ca against large concentration gradients; the Na/Ca exchanger utilizes the energy stored in the Na electrochemical gradient to move Ca against a concentration gradient. There are two distinct types of Ca-ATPases, one present in the plasma membrane and the other present in the structures that

sequester intracellular Ca (21, 135). Since the characteristics of these Ca transport systems were reviewed in detail recently (21), we discuss only the aspects of the Ca extrusion mechanisms that deal with regulation of these systems by Ca-binding proteins (see below).

EFFECTOR SYSTEMS REGULATED BY INTRACELLULAR Ca

The physiological effects of Ca are mediated by a multiplicity of Ca-binding proteins (Ca-BPs). In a few cases the binding protein is closely linked to, or is a part of, the effector molecule, e.g. this appears to be the case with Ca-activated K channels. In many other instances the Ca-binding protein has no biological activity of its own but must interact with other proteins to produce the final physiological effect. Thus Ca-binding proteins can be thought of as "third messengers" of the original extracellular signal. The best-studied class of Ca-BPs is that characterized by the "E-F hand," an acronym introduced by Kretsinger (75) based on his crystallographic studies with parvalbumin, for those proteins exhibiting characteristic α-helix-coil-α-helix domains that provide "pockets" in which Ca can bind. A second major class of Ca-BP that does not contain the E-F hand structure is the "annexins." [Other general names such as calcimedins (35), calelectrins (142), chromobindins (30), lipocortin/calpactin family (32) have been used to describe various members of this group.] Other intracellular Ca-BPs such as calsequestrin, protein kinase C, and certain actin-binding proteins such as villin and gelsolin fall outside these two major groups. These three categories of Ca-BPs are reviewed below. Extracellular Ca-BPs (which generally have a very low affinity for Ca) are not covered here.

The E-F hand Ca-BPs include several members with specialized functions that are not considered in this review. Instead, we cover calmodulin (CaM) and calmodulin-binding proteins (CaM-BPs) in detail. The interested reader is directed to appropriate reviews on parvalbumins (58), troponin C (120), S100 proteins (38), and calbindins (or vitamin D–dependent Ca-BP, 146). The calcium-activated neutral proteases ("calpains") are discussed here because these enzymes appear to be of general significance.

Calmodulin

By far the most important and best-characterized Ca-BP of the E-F hand class is CaM. The structure and properties of CaM have been well reviewed elsewhere (74, 89). In brief, CaM is a highly conserved, acidic, 16.7-kd protein that is ubiquitous in eukaryotic cells. The cytoplasmic concentration of CaM lies between 2 and 30 μM in various mammalian tissues, making it an important intracellular Ca buffer. CaM has four binding sites for Ca (two of

high affinity and two of lower affinity) and two low-affinity metal-binding sites. The binding of each Ca ion leads to stepwise conformational changes (the largest being with the binding of the third Ca) that result in the exposure of hydrophobic domains in the protein. These domains are thought to contribute to the interaction of CaM with various CaM-BPs and CaM antagonists such as trifluoperazine. The exact kinetics and mechanisms determining metal ion binding to CaM have been controversial from the outset and remain so (for review, see 29). Suffice it to say that uncertainty still exists as to whether individual metal ions bind to CaM independently of each other or whether two pairs of sites exist with marked positive cooperativity between the sites in each pair. The evidence for cooperative Ca binding to the two highest affinity sites in the C-terminal domain is good (74). More recently, cooperativity among all four Ca sites has been proposed on the basis of work with a mutant CaM molecule (72). Whatever the precise mechanism, it is generally agreed that 3–4 Ca ions must associate with CaM in order for the complex to activate (though perhaps not simply to bind) various CaM-BPs. A rare exception of a brain protein that binds CaM preferentially in the absence of Ca (and releases CaM in the presence of Ca) was also recently described ["neuromodulin," a 57-kd protein that appears to be similar to the developmentally regulated protein kinase C substrate GAP-43 (1)].

Both the crystal structure (5) and the primary sequence (159) of CaM have been determined; modeling shows that CaM has a "dumbbell" structure with pairs of globular Ca-binding domains connected by a central α-helical stalk (5). Much recent work has been focussed on analysis of the function of different regions of the CaM molecule. Earlier studies using calmodulins from widely divergent species revealed significant differences in behavior, but a more rational approach has been ushered in with the application of mutagenesis, chemical modification, and protein engineering techniques. Ablation of the CaM gene in yeast is lethal (34), but subtle changes in CaM structure can have selective nonlethal effects. A striking example of a CaM altered by mutagenesis occurs in *Paramecium* (see 129 for references). A particular mutant (pantophobiac) was selected for an exaggerated backward-swimming response to aversive stimuli. The mutation was traced to aberrant regulation of a Ca-activated K channel by CaM and could be corrected by microinjection of wild-type CaM into mutant cells. Analysis of the mutant CaM revealed a single amino acid substitution (ser $_{101}$ to phe) occurring in Ca-binding domain III (129).

A totally synthetic CaM and several variants with different amino acid substitutions have been produced in *E. coli* (85, 122, 123). These different molecules have strikingly different abilities to activate various CaM-BPs. For example, changing ala$_{88}$ to pro results in a molecule that can activate phosphodiesterase, myosin light-chain kinase, and CaM kinase II but fails to

activate phosphorylase kinase (85). Such data indicate that different regions of CaM may interact with different target proteins and confirm other results using calmodulin fragments as well as a variety of chemically modified calmodulins (e.g. 76, 90). Together with the vastly different affinities that CaM exhibits for various binding proteins (see below), these results underscore the complexity of CaM action in intact cells.

Calmodulin-Binding Proteins

The final effectors of many Ca-regulated processes are the CaM-BPs, which are numerous. The best studied can be loosely grouped into four classes: (*a*) protein kinases and phosphatases, (*b*) phosphodiesterases and adenylate cyclases, (*c*) cytoskeletal proteins, and (*d*) Ca-ATPases. Other CaM-binding proteins that fall outside of this classification are not considered here (e.g. phosphofructokinase; see 19). An important distinguishing feature of CaM-BPs is their widely differing affinities for CaM: for example myosin light-chain kinase (MLCK) binds CaM with a $K_d \sim 1$ nM whereas human erythrocyte β-spectrin has a much lower affinity ($K_d \sim 10$ μM). From this diversity it might be predicted that CaM-binding sequences in different proteins would show considerable diversity, and this appears to be the case (for review, see 19, 53).

Nevertheless, common features of CaM-binding domains have emerged from identification of the appropriate amino acid sequences in CaM-BPs and from comparisons with CaM binding to model peptides (3). The first CaM-binding domain to be identified was that in MLCK (16). In the primary structure, the characteristic features of CaM-binding domains are a relatively high number of amino acids with hydrophobic and basic side chains, a conserved tryptophan in the first half of the domain, and conserved serine or threonines in the second half of the domain (19). Modeling of such CaM-binding domains suggests that the secondary structure involved is an amphipathic α-helix with hydrophobic residues on one side and basic residues on the other. Presumably these interact, respectively, with hydrophobic residues on CaM, that become exposed after Ca binding, and acidic amino acid side chains. CaM recognition sites in different proteins can also be distinguished immunologically. For example, a monoclonal antibody directed against the known CaM-binding sequence of skeletal muscle MLCK, which acts as a potent inhibitor of CaM binding to this enzyme, had no effect on CaM interactions with smooth muscle MLCK (105).

In an intact cell the effects of CaM are modulated not only by the affinity but also by the abundance of various CaM-BPs. This implies that there will be differences in the kinetics and degree of occupancy of different CaM receptors during the Ca response. More abundant proteins with a lower affinity for CaM (e.g. those with a K_d in the range of intracellular [CaM]) are only partially saturated under these conditions.

CaM-DEPENDENT PROTEIN KINASES AND CALCINEURIN CaM has been implicated in the regulation of protein kinase activity since the discovery of the CaM dependence of MLCK activation (see 141 for review). Simultaneously, a dramatic effect of CaM on the phosphorylation of numerous proteins in the brain was found (131). This result suggested that other kinases beside MLCK were stimulated by CaM, as MLCK is very substrate specific. In 1978, the δ-subunit of phosphorylase kinase was found to be identical to CaM (27), and addition of exogenous CaM stimulated the enzyme further. At present five CaM-dependent protein kinases (CaM-PKs) have been identified; these enzymes can be divided into two groups: (*a*) those with narrow substrate specificity (CaM-PKs I and III, MLCK, and phosphorylase kinase), and (*b*) those with wide substrate specificity (CaM-PK II, often called the "multifunctional" CaM-PK).

The two classical members of the CaM-PK family, namely MLCK and phosphorylase kinase, have relatively well-defined functions. MLCK is responsible for phosphorylating the P light chains of myosin (141). This event initiates contraction in smooth muscle and possibly in platelets and other nonmuscle cells. In striated muscle, the situation is not clear but it has been proposed that phosphorylation regulates the degree of tension produced during contraction. The skeletal and smooth muscle MLCKs differ in several properties and are antigenically distinct (141). Both enzymes are activated on binding a single CaM with K_d 1–5 nM. Recent work suggests that the mode of activation of the skeletal muscle enzyme involves binding of CaM to a 17-amino-acid stretch of the protein close to the C-terminus of the protein (16); a similar sequence is present in the smooth muscle enzyme (70). The sequence in the smooth muscle MLCK region corresponds well to a sequence around the phosphorylation site in the substrate for the enzyme (70). This similarity led to a hypothesis that the CaM-binding region on the enzyme constitutes a "pseudosubstrate" domain that occupies the enzyme active site in the resting state. CaM binding to this domain, leading to MLCK activation, can thus be thought of as relief of a tonic inhibition (70; similar theories have been proposed for the activation of protein kinase C and CaM-PK II).

Phosphorylase kinase was the first protein kinase to be studied intensively. Indeed, phosphorylase kinase was central to the original concept of second messenger cascades as this enzyme can be activated by cAMP-dependent phosphorylation as well as by Ca (for review, see 26). As is well known, phosphorylation of glycogen phosphorylase by phosphorylase kinase initiates glycogen breakdown in skeletal muscle, liver, heart, and other mammalian tissues. The enzyme from all sources so far examined is a giant 16-mer with four units, each of α, β, γ, and δ subunits. The δ subunit is an intrinsically bound CaM, and the γ subunit contains the catalytic domain. The function of the α and β subunits is less certain, but these subunits contain cAMP-kinase phosphorylation site and a site for binding of a second CaM

molecule. The α subunit is phosphorylated by cAMP-kinase subsequent to β-subunit phosphorylation. The β subunit can be "autophosphorylated" in an intramolecular reaction carried out by the γ subunit and this also leads to activation. Reassociation experiments starting with isolated subunit complexes suggest that α and/or β subunits inhibit the catalytic activity of the γ subunit (115). Thus activation of the enzyme by either Ca binding to the δ subunit or phosphorylation of the β subunit may involve removal of an inhibitory influence, as described above for MLCK.

CaM-PK I was initially discovered in rat brain on the basis of its ability to phosphorylate a specific site on the brain-specific protein synapsin I; the enzyme has been purified to homogeneity (100) and shown to exist in other tissues. Little information is available regarding the possible functions of this kinase.

CaM-PK II was also initially discovered in rat brain, where it comprises about 0.2% of the total brain protein. Subsequently, similar enzymes were found in a variety of tissues (e.g. 44) and it seems likely that the enzyme is ubiquitous. The intense interest in this enzyme since its initial discovery in 1978 (most of the CaM-dependent phosphorylation seen in rat brain is mediated by this enzyme) can be traced to its broad substrate specificity and its abundance in brain. Much work has been carried out on the brain enzyme, and several reviews have recently been published (e.g. 25, 71). Both α (50 kd) and β (60 kd) subunits from brain have been sequenced and show a high degree of similarity (10, 82). The cerebral cortical enzyme subunits are arranged in a tight complex of nine α and three β subunits. During activation each subunit binds CaM (the affinity is variously reported as 10 to >100 nM; see 71) and the enzyme rapidly autophosphorylates in an intramolecular reaction. The first site of autophosphorylation in the α subunit is at residue thr-286 (thr-287 in the β subunit) (149). Phosphorylation of this residue in only a few subunits renders the entire holoenzyme Ca and CaM independent (71, 77) i.e. activity in vitro can continue in the absence of Ca and further phosphorylation of exogenous substrates or of the enzyme itself occurs. Thr-286 lies in a region having similarity with sequences found in typical substrates for CaM-PK II and overlaps the presumed CaM-binding domain of the protein (116). Inhibition of the enzyme by synthetic peptides consisting of appropriate sequences within this region has led to a proposed "pseudosubstrate" hypothesis (see above) for CaM-PK II (116). That is, the enzyme active site may be occupied at rest by a sequence of the polypeptide chain that resembles the substrate; first CaM binding and then subsequent phosphorylation of thr-286 relieve this tonic inhibition by making the active site available for exogenous substrates.

The Ca-independent behavior of CaM-PK II suggests that it could play a role in certain higher-order phenomena in the nervous system such as long-

term potentiation and, by extension, memory (71, 83). While the high concentration of CaM-PK II in both pre- and especially postsynaptic regions of the brain certainly hints at important functions for the enzyme in synaptic processes, there is little empirical evidence that either autophosphorylation or enzyme activity outlasts the Ca signal in nervous tissue. In synaptosomes (presynaptic compartment) phosphorylation of the CaM-PK II substrate synapsin I and of thr-286 in CaM-PK II rapidly reverses during continued depolarization, which suggests that phosphatase(s) rapidly dephosphorylate both proteins (49). A maximum level of 22% Ca-independent kinase was estimated in these experiments. Recent data show that autophosphorylation also occurs in CaM-PK II from nonneuronal sources (107).

CaM-PK II efficiently phosphorylates a wide variety of proteins in vitro (25). The best substrate is synapsin I, and evidence has been presented that this phosphorylation alters the properties of synapsin with respect to synaptic transmission (84). Glycogen synthase, microtubule-associated protein-2 (MAP-2), tau proteins, and tyrosine hydroxylase are also readily phosphorylated by CaM-PK II, but in these cases the consequences of phosphorylation are not completely understood.

The initial observations with respect to CaM-PK III were made in 1983, when a common 100-kd substrate for a CaM-PK was found in a number of mammalian tissues (111). Subsequently, CaM-PK III was shown to be distinct from other CaM-PKs and to exhibit extreme substrate specificity, phosphorylating the 100-kd substrate exclusively (102). The substrate was identified as protein synthesis elongation factor-2 (EF-2) (101). Phosphorylation of the factor at a single threonine residue abolishes its ability to support protein synthesis in vitro; thus first messengers that generate Ca signals may affect protein synthetic rate (101). Experiments in mitogen-stimulated fibroblasts (112), histamine- and thrombin-stimulated endothelial cells (88), and acetylcholine-stimulated adrenal chromaffin cells (56) all show that EF-2 is phosphorylated in intact cells in response to Ca signals. In particular, there is a good correlation in fibroblasts between $[Ca]_i$ and EF-2 phosphorylation after addition of the mitogen bradykinin; $[Ca]_i$ rises to a peak within 15 seconds of mitogen addition and then rapidly returns to baseline within one minute, while EF-2 phosphorylation peaks at about 30 seconds and returns to baseline by five minutes (112). The precise role of EF-2 phosphorylation in cell function remains to be determined.

Calcineurin is a CaM-activated protein phosphatase (protein phosphatase 2B) found at high concentrations in brain (mostly in neurons) and in lower amounts in many other tissues (for recent reviews, see 73, 114). The protein is a tight complex of one A (61 kd) and one B (19 kd) subunit, the former containing the catalytic and CaM-binding domains while the latter is a Ca-binding protein of the E-F hand class in its own right. Activation of

calcineurin by Ca alone (presumably mediated by Ca binding to the B subunit) is very modest, but addition of CaM, which binds directly to the A subunit, leads to a further >10-fold increase in activity. Binding of CaM to calcineurin is 1:1 and of very high affinity ($K_d \sim 0.1$ nM) (60). Calcineurin is quite substrate specific, more so than protein phosphatases 1 and 2A, which constitute the major dephosphorylating enzymes found in most mammalian tissues (for review, see 26).

Several proteins, such as protein phosphatase inhibitor-1, MAP-2, and tau protein, have K_m and K_{cat} values in the physiological range for calcineurin-mediated dephosphorylation. Others such as the regulatory subunit of cAMP-PK have a higher K_m. Though calcineurin can dephosphorylate proteins containing phosphotyrosine in vitro, the activity may not be physiologically relevant as it requires ~1 mM of Ni or Mn (114). Indeed, studies to determine the activity of calcineurin or to elucidate substrate dephosphorylation in intact cells have not yet been performed. Calcineurin can itself be phosphorylated by at least three enzymes: casein kinase, protein kinase C, and CaM-PK II (the latter in its CaM-independent form, see above). Only CaM-PK II–mediated phosphorylation of the A subunit appears to lead to a partial inactivation of phosphatase activity (54). Conversion of calcineurin to a CaM-independent form by calpain I, involving degradation of the 61-kd subunit to 45 kd, has been demonstrated (147). The physiological significance of these observations remains to be determined.

PHOSPHODIESTERASES AND ADENYLYL CYCLASE Cyclic nucleotide phosphodiesterases (PDEs) have been particularly well studied with respect to CaM activation, as it was through studies on this enzyme that CaM was first discovered. The current picture with respect to these enzymes is complex. At least three distinct families of cyclic nucleotide PDEs have been identified that share a conserved catalytic domain but have different substrate specificities and regulatory behavior. The CaM-regulated PDEs are one member of this family and comprise closely related enzymes from different tissues. In brain, the best-studied tissue, two isozymes of CaM-PDE exist with subunits of 63 kd (Type I) and 61 kd (Type II) (132). As both enzymes have low K_m values for cAMP, they are probably involved in the Ca-dependent attenuation of the cAMP signal, one of the several points at which these two second messengers interact within the cell. Aside from regulation by CaM binding, both enzymes seem to be regulated by phosphorylation; e.g. the Type II enzyme is phosphorylated by CaM-PK II and this results in an increase in the Ca concentration required for activation. Moreover, calcineurin appears to be involved in the dephosphorylation of both enzymes (132).

A particular subset of adenylyl cyclases, identified in brain and several other tissues, is activated by CaM (for review, see 133). In brain as much as

60% of the total cyclase activity could be CaM sensitive. The enzyme from bovine brain has been purified and consists of subunits of 150 (catalytic), 47 (α subunit of G_s) and 35 (β subunit of G_s) kilodaltons (164). The CaM-binding site is assumed to reside on the catalytic subunit, though the recent demonstration of direct binding of $\beta\gamma$ subunit complexes of G proteins to CaM, with consequent inhibition of cyclase activity (68), has raised the issue of whether CaM effects might not also arise from some interaction at this level. Certain bacteria, particularly *Bordetella pertussis,* secrete a CaM-activated adenylyl cyclase that is thought to play a major role in the pathology of infection by these organisms (133). This enzyme is not closely related to its mammalian counterparts.

CYTOSKELETAL PROTEINS CaM binds to several cytoskeletal proteins in a Ca-dependent manner. In general the affinity of proteins such as caldesmon, spectrin, and adducin for Ca/CaM is lower (i.e. ~0.2–2 μM range) than that of the enzymes discussed above. Additionally, these proteins are usually present in much higher amounts than CaM-regulated enzymes. As their intracellular concentration may approach that of CaM itself, it is likely that during a Ca pulse some cytoskeletal proteins may exhibit only partial saturation with CaM. In addition to being CaM receptors, the three proteins discussed below are also actin-binding proteins. Together with other Ca-sensitive actin-binding proteins, such as calpactin and gelsolin (see below), this indicates a high degree of regulation of the actin-based cytoskeleton, and by inference contractility and motility, by Ca signals in cells other than striated muscle (where troponin C is the major Ca receptor controlling contractility). In addition, microtubule-based structures are also regulated by Ca-sensitive accessory proteins such as tau and MAP-2, which are both CaM-binding proteins and CaM-PK II substrates. It is clear that much work still remains in understanding the effects of Ca on cytoskeletal architecture.

Caldesmon was first purified by Sobue et al in 1981 (136). In smooth muscle, such as avian gizzard, it exists as a monomeric protein of 130–155 kd, but in other tissues (e.g. platelets, chromaffin cells) a 70–80-kd form is prevalent (for review, see 157). The molecule is highly asymmetric (flexible rod) and tends to oligomerize readily. Each monomer binds a single CaM with a K_d of 1.4–1.7 μM. The concentration of caldesmon in smooth muscle is very high and it has been proposed as a Ca-sensitive modulator of contractility in this and other tissues (157). Consonant with this idea, caldesmon binds to F-actin in vitro and almost certainly in vivo, where the molecule can interact with the actin-tropomyosin complex in smooth muscle. Such binding inhibits the ability of the thin filament to activate myosin ATPase. CaM interaction lowers the affinity of caldesmon for actin and relieves this inhibition. Recalling that CaM activation of MLCK appears to initiate smooth muscle contrac-

tion (see above), it is likely that caldesmon must have another function in smooth muscle and nonmuscle contractility. It has been suggested that caldesmon may be involved in the maintainance of the contracted state, because on prolonged contraction the correlation between MLC phosphorylation and state of contraction no longer holds. In other cell types caldesmon might perform other functions; for example, in chromaffin cells caldesmon appears to bind to secretory granules in a Ca-dependent manner and may have a role in exocytosis (20). Further complexity may be introduced by phosphorylation of caldesmon, which may be by PK-C or by a CaM-dependent kinase (103). Phosphorylation seems to reduce the affinity of caldesmon for actin and increase it for CaM, thus potentially shifting the equilibrium of the system.

Spectrin was first characterized as a major component of the subplasmalemmal membrane skeleton in human erythrocytes. In its native form the protein is a tetramer comprising two α (240 kd) plus one β (220 kd) subunit complexes, linked head to head (11, 163). Recently, it has been realized that homologues of spectrin occur in other cell types (11; the alternate name "fodrin" is also used for these species, 80), where the protein also seems to underlie the plasma membrane (81). Short- and long-term rearrangements of fodrin distribution have been noted after ligand binding to cells (81) and during development (e.g. in brain), which suggests plasticity in the organization of this protein. Human erythrocyte spectrin is a CaM-BP with a weak affinity for CaM (K_d ~7–25 μM), but brain fodrin has a higher affinity (K_d ~0.2 μM). Two binding sites for CaM have been proposed for human erythroid spectrin (one on the α and one on the β subunit), but fodrin seems to retain only the high-affinity binding to the α subunit (2). The CaM binding site in brain α-fodrin lies toward the center of the molecule and its sequence shows only weak similarity with CaM-binding sequences from other proteins (53). Spectrin is an actin-binding protein, and CaM binding to spectrin decreases the ability of spectrin both to seed actin polymerization and to cross-link actin filaments in vitro (140). While it is difficult to extrapolate such observations to intact cells, it is possible that a weakening of the spectrin-actin lattice might provide additional flexibility to the membrane skeleton. However, the physiological significance of this effect in the human erythrocyte is dubious because of the weak association between spectrin and CaM. It may well play an important role in regulating cytoskeletal behavior in other cell types, but the function of fodrin in non-erythroid cells is poorly defined. A role for fodrin in secretion has been proposed, based on partial inhibition by anti-α-fodrin antibodies of catecholamine release from permeabilized adrenal chromaffin cells (117). CaM binding to non-erythroid α fodrin (e.g. from brain and platelets) appears to increase its susceptibility to proteolysis by calpain I [which cleaves at a site within the CaM-binding domain (53)]. Proteolytic cleavage of fodrin by calpain may play a role in synaptic remodelling in the central nervous system (86).

Adducin is another CaM-binding protein first discovered in the human erythrocyte membrane skeleton (45). The protein consists of α (120 kd) and β (110 kd) subunits in a tight 1:1 dimeric complex. The subunits show only limited overall similarity as seen by peptide mapping methods, but may have a higher degree of similarity around their phosphorylation sites (158). CaM binds to the whole complex, as demonstrated by affinity chromatography (158), and to the β subunit, as demonstrated by affinity labelling (45). The stoichiometry was not estimated but the apparent affinity was 0.23 μM, much higher than that of human erythrocyte spectrin. Adducin is an actin-binding protein (97; but cf. 46) and increases the interaction between spectrin and actin in the membrane skeleton. Both the actin-bundling activity and the stimulation of further spectrin binding to spectrin-actin complexes of adducin are inhibited by CaM (46, 97). Again, it is not clear what function this may have in intact cells but, if operative, it would tend to weaken protein-protein interactions within the membrane skeleton and could increase flexibility in this structure. Proteins that cross-react with anti-adducin antibodies have been found in other red cells, platelets, fibroblasts (113), and brain (12, 13). Adducin is a target for PK-C–mediated protein phosphorylation in erythrocytes and fibroblasts, but the function of such phosphorylation is not known.

Tau and MAP-2: Early experiments with crude tubulin preparations revealed that Ca inhibited microtubule assembly (160; see also 130). The Ca-sensitive factor(s) in such preparations has never been defined, but CaM involvement has been suggested repeatedly, based on biochemical experiments (e.g. 66, 69, 91). Moreover, immunofluorescence experiments using anti-CaM antibodies with a variety of interphase and mitotic cells have shown localization of CaM to the microtubule network (37) and spindle apparatus (36), respectively. This has led to a search for CaM-binding elements in microtubules. The microtubule-associated proteins MAP-2 and tau (for review, see 108) directly bind CaM (79). MAP-2 is a protein of ~280 kd that forms the arms linking adjacent tubulin filaments in mictrotubule structures. CaM binding to MAP-2 has been demonstrated by several techniques; however, the affinity is low (K_d ~7 μM) and there is no indication as yet that CaM binding to MAP-2 affects microtubule function. Tau consists of a series of four closely related proteins of 55–68 kd that are important for microtubule assembly. Although tau will bind CaM on blots (79), the affinity of the interaction has not been defined. The tau proteins are highly phosphorylated both in vitro and in vivo (both CaM-PK II and PK-C appear to be involved) and phosphorylation inhibits the ability of tau to facilitate microtubule polymerization. Both CaM and S100b (another Ca-binding protein of the E-F hand class) are able to inhibit phosphorylation and may play a role in regulating the state of phosphorylation of tau (8). (Note that the proposed roles for CaM appear to be mutually exclusive, i.e. CaM binding to the protein inhibiting CaM-PK II action.) Whether CaM binding to MAP-2 and

tau, or the phosphorylation of these proteins by CaM-PK II, will explain the effects of Ca on microtubules remains to be defined.

CA-ATPASES The plasma membrane Ca pump of many mammalian cell types is regulated by CaM. As this topic was reviewed recently (21), we simply summarize recent experiments defining the structure of these proteins and their CaM-binding sites. The complete amino acid sequence of two isoforms of the enzyme from rat brain has been deduced from cDNA cloning (134). The enzymes (both about 130 kd, with 82% identical amino acids) are related to other transporting ATPases. The Ca-ATPases have an even number of transmembrane domains with a cytoplasmically located C-terminus that binds CaM. Presumptive catalytic and regulatory ATP-binding sites are located on other cytoplasmic loops. The well-studied erythrocyte enzyme is similar to isoform 1 from rat brain. A potential CaM-binding domain with characteristics similar to those in other CaM-BPs (i.e. two ~15-amino-acid regions that come together to form an amphipathic α-helix; see above) has been identified (64). CaM activates these enzymes with a 1 : 1 stoichiometry and a high affinity ($K_d \sim 1$ nM).

Calpains

The calcium-activated neutral proteases have received considerable attention recently from both a structural and functional point of view (for reviews, see 95, 118, 145). They may be important regulators of cell function and have been proposed to be involved, e.g. in remodeling and synaptic plasticity in the central nervous system and as participants in platelet activation. Two types of enzyme have been described: calpain I (active at micromolar Ca) and calpain II (active at millimolar Ca). Both enzymes consist of two subunits: an 80-kd subunit that contains the thiol protease-like catalytic domain, and a 30-kd subunit that contains a glycine-rich hydrophobic domain (145). Both subunits also contain Ca-binding domains, i.e. four E-F hand-like structures. The 30-kd subunits of calpain I and II are identical, which implies that the difference in Ca sensitivity of the two enzymes must lie in the distinct 80-kd subunits. Potentially, both enzymes could bind eight calcium ions, but this has not been rigorously shown. Autoproteolysis has been proposed as a mechanism of activation of both calpains. Thus, in the presence of Ca, both the large and small subunits undergo cleavage at their N-termini, and thereby produce enzymes that appear to be more sensitive to Ca. Whether or not the 30-kd subunit dissociates from the 80-kd subunit during activation remains controversial (95, 118, 145). Some workers suggest that cleavage of the small subunit is the key event in enzyme activation (28), but others feel that cleavage of the large subunit is crucial (145). Although the requirement of calpain II for millimolar Ca would appear to be unphysiological, the Ca

requirement is reduced in the presence of phosphatidylinositol-containing vesicles and on proteolysis. There is some evidence for an association between calpains and membranes (95), and many good substrates for these enzymes are cytoskeletal or membrane-associated proteins (e.g. fodrin in brain, ankyrin and band 4.1 in red cells, and various contractile proteins in striated muscle). Recent studies on the immunofluorescent localization of calpain II heavy chain in cultured cells indicate an association with adhesion plaques [cell-substrate attachment sites (9)].

Calpains are likely to have many functions. However, assignment of a specific function in intact cells based on the use of permeant protease inhibitors such as leupeptin is fraught with ambiguity because these compounds also inhibit other proteases. The discovery of a specific endogenous protein inhibitor of calpains, calpastatin, has considerably complicated the area. The inhibitor has a widespread distribution and is specific for calpain. Erythrocyte calpastatin is a tetrameric 240-kd protein made up of identical ~68-kd subunits. Each subunit in turn has four internally homologous inhibitory domains (40). The myocardial and liver subunits are larger (110 kd), having an extended N-terminal segment that is absent in the erythrocyte enzyme (109). In some tissues, there appears to be more of the inhibitor than the enzyme, which suggests that regulation of the calpain system may be complex.

Annexins

Several parallel lines of investigation led to the identification of a new family of proteins, the annexins (32). A common property of the group is that of Ca-dependent interaction with hydrophobic surfaces such as phospholipids, membranes, or artificial apolar matrices, the latter being frequently used as a purification tool. The number of well-defined members now stands at 5–6, and the overall degree of sequence similarity between them is 40–60%. All contain repeated domain structures, each 70–80 amino acids long, that probably comprise the binding sites for Ca and phospholipid, connected by regions showing more heterogeneity. For example, calpactins I and II have four such domains and bind four calcium ions in a phospholipid-dependent manner (47). The 67–68-kd "calelectrin" has eight repeated domains and thus may bind eight calcium ions (31, 143).

Calpactin I is a well-characterized member of this group (47, 48). The holoprotein is a tetramer consisting of two 36-kd "heavy-chain" subunits having the annexin-like structure and two 11-kd "light-chain" subunits that have similarity with S100 proteins. Calpactin I localizes in the subplasma membrane region of a number of cells and may be a cytoskeletal element that possibly interacts with spectrin and actin (47, 48). The membrane-associated form of the protein contains both heavy and light chains and it has been

suggested that a soluble pool of heavy chains alone also exists (166). The association of calpactin I with the membrane is assumed to occur via Ca-dependent binding to negatively charged phospholipids (primarily phosphatidylserine). Thus, it is conceivable that calpactin and other members of the annexin family provide Ca-sensitive associations between the cytoskeleton and membrane surfaces. This potentially important function remains to be fully documented but could have consequences for events such as exocytosis that are Ca-dependent and involve the coordination of cytoskeletal and membrane phenomena. Indeed, calpactin has been shown to aggregate chromaffin granules in a Ca-dependent manner (39).

The dissociation constants reported for Ca binding to this family of proteins vary (stretching over several orders of magnitude), perhaps dependent on the specific assay employed. For example, the K_d for Ca-dependent calpactin aggregation of chromaffin granules was estimated at 1.8 μM (39), whereas phospholipid vesicle binding and aggregation occurred at 10 nM Ca (121). These data suggest that physiologically relevant concentrations of Ca are capable of modulating calpactin function. Calpactin I heavy chain can be phosphorylated on tyrosine residues lying in the N-terminal 3-kd region (i.e. outside the repeated domains) by growth factor–dependent or viral protein kinases (e.g. 121; this prominent characteristic first drew attention to this protein). Phosphorylation may regulate the Ca- and phospholipid-binding properties of the protein (121). Lipocortin II (which is identical to calpactin I) was earlier thought to be a physiological inhibitor of phospholipase A_2, but it appears that this function of the molecule may be entirely due to its ability to bind phospholipid, thus shielding the lipase from its substrate (33). An interesting series of recent observations suggests that proteins similar to calpactin I light chain are induced by growth factors in different cell types (e.g. 92), but whether these proteins are in fact the light chain and whether the heavy chain is similarly induced remain to be determined. Calpactin II is related to calpactin I; it exists only as a "heavy-chain" monomer, but it also binds phospholipids in a Ca-dependent manner (48). This protein was also first identified as a tyrosine kinase substrate.

Though the function of the annexins is still unclear, it seems certain that they will prove to be important transducers of the Ca signal in mammalian cells.

Other Calcium-Binding Proteins

A number of Ca-BPs fall outside of the categories indicated above or have not yet been fully characterized. A couple of examples are given here. One such protein not covered is protein kinase C, which has been extensively reviewed in the last few years (e.g. 104).

CA-DEPENDENT ACTIN-SEVERING PROTEINS Proteins such as gelsolin, villin, and fragmin all require Ca to sever F-actin. Severing is presumed to have a function in remodeling of actin fibers during processes such as shape change and motility in mammalian cells. Some of these proteins also bind to G-actin, and the complex then can act as a nucleus for further polymerization; they may also cap actin filaments after severing them and thus prevent further polymerization. Gelsolin (87 kd) is the most widespread of this group and also occurs in a secreted form ("brevin" or plasma gelsolin may be derived from smooth muscle) with a small N-terminal extension (165). Villin (95 kd) has a much narrower distribution (primarily brush borders of epithelial cells). These two proteins are ~50% identical and are comprised of six homologous domains (an additional "headpiece," which confers actin-bundling activity, is present in villin). The proteins are not identical in function (65). For example, gelsolin can perform its severing function at low micromolar Ca whereas villin requires much higher levels of Ca. Differing levels of Ca apparently support different functions of gelsolin, e.g. blocking of the "barbed" ends of actin filaments by gelsolin can occur at lower [Ca] than severing activity. Gelsolin binds two, and villin three, moles of Ca per mole of protein, with a K_d of ~1 μM. The actin-severing domain lies in the N-terminal region whereas the Ca-binding sites are in the C-terminal half. Structural studies (65) have not revealed E-F hand structures, so the nature of the Ca-binding domains is still unknown. Physiological activation of cells results in an increase in the amount of gelsolin associated with actin, possibly because of Ca binding to gelsolin. Understanding the multiple functions of gelsolin is further complicated by the discovery of the effects of polyphosphoinositides; PIP_2 may reverse some of the effects of gelsolin on actin (165).

Recent DNA sequencing data indicate that another actin-binding protein, α-actinin, has calmodulin-like domains (6). However, there is no evidence that Ca binds to α-actinin or modifies its function.

CALSEQUESTRIN This protein is found at very high concentrations in the sarcoplasmic reticulum of striated muscle, but recent immunological studies suggest that it may have a more widespread distribution (156). Calsequestrin from rabbit skeletal muscle is a 367-amino-acid protein with a calculated molecular weight of ~42 kd (41). Because of its extremely elongated structure in the absence of Ca, the protein migrates anomalously, for example, on SDS gels (apparent 55 kd). Binding of Ca induces a marked conformational change increasing the α-helical content and rendering the protein less asymmetric. The preponderance of acidic amino acids presumably contributes to the high Ca-binding capacity of ~1 μmol/mg protein (40–50 Ca/molecule) with a very low affinity (K_d ~1 mM), but the exact nature of Ca binding to calsequestrin remains undefined. The low-affinity, high-capacity characteris-

tic is ideal for the Ca storage function of calsequestrin in the sarcoplasmic reticulum. Analogous Ca-binding proteins, including those related to calsequestrin, appear to exist in endoplasmic reticulum from nonmuscle cells (87, 156); this finding has contributed to the concept of the "calciosome" (156).

SUMMARY AND PROSPECT

The second messenger functions of Ca are much more diverse than those of other second messengers such as cAMP and diacylglycerol. While the latter messengers exert most of their effects via interaction with a single class of binding protein, many such targets exist for Ca. In addition, the pathway for regulation of intracellular Ca is complex and includes mechanisms for control of Ca influx, Ca mobilization from intracellular stores, and Ca extrusion from the cytoplasm. The elucidation of the mechanisms for regulating $[Ca]_i$ and of the structure and function of many binding proteins has progressed rapidly in the last several years. Individual pathways for Ca action have been identified, but the integration of the "Ca response," which must involve many coordinated events, will take many years to define.

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