Stimulus-response coupling: the search for intracellular calcium mediator proteins

Minireview

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Calcium-coupled responses to stimuli have been a topic of investigation and a scientific puzzle for over a century (Ringer, 1883). Hodgkin and Keynes (1957) were the first to demonstrate a calcium receptor, the existence of which suggested a mechanism of intracellular calcium action. The identification of such calcium receptors was achieved in both muscle and nonmuscle cells. Advances in protein purification and biochemical reconstitution led to the purification of troponin and to the definition of its role as the "calcium switch" in skeletal muscle (Ebashi and Endo, 1968; Greaser and Gergely, 1971). A related calcium-receptor protein, later termed calmodulin, was identified in nonmuscle tissue (Cheung, 1970; Kakiuchi *et al.*, 1970; Wolff and Siegel, 1972; Smoake *et al.*, 1974). Biophysical characterization of troponin C and calmodulin led to the development of a molecular paradigm for calcium action. Calcium binding to these receptor proteins causes conformational changes that lead to an alteration in the function of the respective target proteins, thereby initiating physiological cascades. These studies indicate that calcium-coupled responses consist of three major steps: 1) stimulus-specific ligands, such as hormones, growth factors and other intercellular transmitters, bind to membrane receptors, resulting in an elevation in intracellular calcium; 2) calcium binds to mediator proteins, which, in turn, modify the function of specific proteins; and 3) the altered target proteins coordinate the cellular responses, such as secretion, motility, cell division, intermediary metabolism, protein synthesis, and membrane permeability (see Campbell, 1983). While a molecular coupling of membrane depolarization and contraction in skeletal and smooth muscle can be attributed, respectively,

to troponin C and calmodulin, the coupling elements of the many other responses have resisted elucidation. An additional mechanism of calcium action was revealed by the discovery of protein kinase C, a calcium/lipid-activated enzyme (see Nishizuka, 1986). The identification and characterization of calcium-mediator proteins represents a critical step toward understanding the diverse responses evoked by calcium in the cell. This review outlines diverse approaches that have been utilized to elucidate the role of calcium in physiological processes and is illustrated by selected examples. Two complementary strategies have been utilized. One approach has been direct, that is, the identification of high-affinity calcium-binding proteins. Some calcium-binding proteins have been identified by sequence similarity with proteins having established calcium-binding properties. This approach requires efforts to define the cellular function in which the calcium-binding proteins are involved. A second approach has included biochemical or genetic dissection of calcium-dependent cellular processes, such as secretion or cell division, and subsequent identification of the essential calcium-mediator proteins.

Calcium affinity and physiological significance

A criterion for the evaluation of potential calcium-mediator proteins is their calcium affinity and ion selectivity. The use of fluorescent dyes indicates that the calcium signal can occur as a sustained elevation or as phasic oscillations in the range of $2-8 \times 10^{-7}$ M (Tsien *et al.*, 1985; Berridge and Irvine, 1989). It might be anticipated that the dissociation constant for intracellular calcium-mediator proteins would be within this range. Proteins of higher affinity would be saturated with calcium and would not be able to respond to the change in free calcium. Parvalbumin, for example, has an affinity of 10^{-8} M, which may provide the protein with calcium buffering and storage properties. An interesting

observation is that most of the purified mediator proteins have dissociation constants much lower than the peak of the intracellular calcium signal. Calmodulin displays marked increases in Ca^{2+} affinity when it is bound to its respective cellular target proteins. Dedman *et al.* (1977) noted extreme positive cooperativity for phosphodiesterase activation. Olwin and Storm (1985) demonstrated a large reduction in apparent Ca^{2+} -binding constants, from 12 to 0.4 μM , when calmodulin was complexed with its binding proteins. The affinity of troponin C for Ca^{2+} can also be altered in reconstituted systems (Potter and Gergely, 1975; Zot and Potter, 1987). Likewise, protein kinase C is an example of an enzyme whose calcium activation values can be dramatically reduced when the ligands phosphatidylserine and diacylglycerol are present (Nishizuka, 1986). The annexins also increase their affinity for calcium under a number of conditions, such as the addition of phospholipid, phosphorylation of the N-terminal region, or inclusion in complex reconstitution systems (Powell and Glenney, 1987; Schlaepfer and Haigler, 1987; Drust and Creutz, 1988; Ando *et al.*, 1989). These examples demonstrate that if calcium-binding affinities of the pure proteins were the only criterion used, doubts would have been raised regarding the physiological importance of many calcium-mediator proteins.

In summary, no strict set of criteria can yet be established to qualify a calcium-binding protein as a cellular mediator. Full knowledge of all interacting components is required to evaluate calcium-binding affinity with mediation of the intracellular calcium signal. Physiological correlations should be pursued to confirm function.

Identification of putative calcium-binding proteins through sequence similarities to the "EF-hand" motif

The most completely described family of intracellular calcium-binding proteins to date is characterized by a protein structure known as the "EF-hand." Moews and Kretsinger (1975) reported the crystal structure of parvalbumin and defined the side chain coordination of calcium within a helix-loop-helix composed of six precisely spaced amino acids. This EF-hand is found in a number of proteins as determined from the primary sequence and, in a few cases, has been confirmed by tertiary structure (Kretsinger *et al.*, 1988). The characterization of these proteins has led to a few surprises. The sequence data for the calcium-dependent pro-

tease, calpain, suggests that it may have resulted from the fusion of a gene encoding four EF-hand domains with a thiol protease gene (Ohno *et al.*, 1984; Sakihama *et al.*, 1985; Emori *et al.*, 1986). Likewise, α -actinin may have resulted from the fusion of a gene encoding an actin-binding protein with a gene for a single EF-hand domain (Noegel *et al.*, 1987; Baron *et al.*, 1987). In many cases the EF-hand is non-functional. For example, the calcium insensitivity of the muscle form of α -actinin is due to non-functional EF-hands. In addition, calcium-binding experiments suggested that the vitamin D-dependent calbindin- $\text{D}_{28\text{K}}$ would contain four calcium-binding domains; sequence data indicates that this protein has two additional non-functional domains (Hunziker, 1986).

The identification of a number of putative calcium-binding proteins has been based on sequence similarity to a member of the troponin C/calmodulin/S100 superfamily. For example, yeast that carry the temperature-sensitive allele of CDC31 are blocked in spindle-pole body duplication (Baum *et al.*, 1986). This gene has 42% sequence similarity with human calmodulin and may contain functional EF-hands. A second cell division cycle gene, CDC24, is required for bud formation. Sequence analysis indicates two potential calcium-binding EF-hands (Miyamoto *et al.*, 1987). The presumptive dorsal ectoderm of sea urchin embryo accumulates mRNAs that code for a group of small acidic proteins (Carpenter *et al.*, 1984). The Spec proteins can be divided into two groups, each with an overall 30% similarity to troponin C and calmodulin. Based on the calcium-dependent mobility shift of the recombinant fusion protein, it has been confirmed that the major Spec protein, Spec 1c, does bind calcium (Muesing *et al.*, 1984).

Several gene sequences have been shown to be similar to the S100s. These proteins are soluble in 100% saturated ammonium sulfate, and, in contrast to calmodulin, each S100 polypeptide chain has two calcium-binding regions. Calcyclin, also known as 2A9 and subsequently isolated as PRA, was identified from an mRNA induced in quiescent human fibroblasts by stimulation with serum, PDGF, or EGF (Calabretta *et al.*, 1986; Murphy *et al.*, 1988). The derived amino acid sequence of calcyclin displays 55% identity with the sequence of S100b. It has not yet been shown that calcyclin actually binds calcium, and, to date, its role in cell proliferation remains an intriguing but unsubstantiated possibility.

Four independent laboratories have identified a cDNA sequence referred to as p9Ka, 18A2,

pEL98, and 42C. It is 45% similar to S100a (Barracclough *et al.*, 1987; Jackson-Grusby *et al.*, 1987; Goto *et al.*, 1988; Masiakowski and Shooter, 1988). It was identified as a marker for a morphological change in mammary cells, as a serum inducible mRNA, as a marker distinguishing established cell lines from the parental lines, and as an mRNA induced by nerve growth factor, respectively. Another S100-like protein, leucocyte L1, is a multichain, 36.5-kDa calcium-binding protein (Dale *et al.*, 1983; Andersson *et al.*, 1988). The heavy and light chains of the L1 protein correspond directly to two macrophage proteins called MRP-14 and MRP-8, respectively (Odink *et al.*, 1987). The sequences of each chain share 28–33% sequence identity with the S100s. Bovine lung contains an additional member of the S100 family, which is 43–47% identical to the S100s and has been designated S100L (Glenney *et al.*, 1989). Calcium-binding studies indicate that S100L binds 2 mol of calcium per mole of protein.

The light chain of calpactin, also known as p11 or p10, is ~45% similar in sequence to S100a. The protein, however, does not bind calcium (Gerke and Weber, 1985; Glenney and Tack, 1985; Hexham *et al.*, 1986). The significance of the similarity between S100a and p11 is not understood. It has been suggested that, in contrast to calmodulin, which requires calcium for its interaction with target proteins, p11 may be in a permanent "on" state for its target protein (Johnsson *et al.*, 1988).

In summary, a number of proteins that contain putative EF-hand sequences have been identified. Calcium-binding studies, however, have demonstrated that not all putative EF-hands are functional. The critical spacing of amino acids that coordinate calcium is not present, thus disrupting the tertiary structure for functional EF hands. Sequence similarities should be judiciously evaluated.

Identification of Ca^{2+} -binding proteins with the use of direct binding of ^{45}Ca

Several methods based on the binding of radioactive calcium have been utilized to assess directly the presence of calcium-binding proteins in tissue samples. In fact, the original purification procedure of calmodulin utilized direct $^{45}\text{Ca}^{2+}$ -binding to monitor the protein (Wolff and Siegel, 1972). Waisman *et al.* (1985) used a competitive calcium-binding assay to demonstrate that, in addition to calmodulin and calcineurin, a number of calcium-binding proteins, designated as CAB-63 (calregulin), CAB-48, and

CAB-27 (caligulin) are present in bovine brain and heart. Similarly, Tuan *et al.* (1988) used the competition assay both to identify a 57-kDa calcium-binding protein from mouse chorioallantoic placenta and to monitor the progress of its purification.

By the use of ^{45}Ca binding to proteins immobilized on nitrocellulose sheets, both synaptophysin (Rehm *et al.*, 1986) and sorcin (Koch *et al.*, 1986) have been identified as calcium-binding proteins. Synaptophysin is a 38-kDa glycoprotein isolated from synaptic vesicle membranes. Pronase treatment has established that the calcium-binding site resides on the cytoplasmic portion of the protein (Rehm *et al.*, 1986). Examination of the derived amino acid sequence for this protein reveals no sequence similarity to any known calcium-binding proteins, suggesting that synaptophysin contains a calcium-binding structure not previously defined (Leube *et al.*, 1987; Buckley *et al.*, 1987; Sudhof *et al.*, 1987).

Sorcin was first detected in multidrug-resistant cell lines (Meyers and Biedler, 1981) and was later identified as a ^{45}Ca -binding protein (Koch *et al.*, 1986). When the cDNA sequence for sorcin was determined, similarities to calpain and calmodulin were noted (Van der Bliek *et al.*, 1986). It was first thought that sorcin might play a role in the development of the multidrug-resistant phenotype; however, examination of a number of different multidrug-resistant cell lines has shown that the amplification and overexpression of sorcin is neither sufficient nor necessary for the multidrug-resistant phenotype (Hamada *et al.*, 1988).

In summary, direct calcium-binding methods are valuable for the detection of proteins that are present at high concentrations but by themselves give little insight as to the physiological role of the proteins.

Identification of proteins that display Ca^{2+} -dependent interactions

In the presence of calcium, many proteins undergo a conformational change, thereby exposing hydrophobic sites (Gopalakrishna and Anderson, 1982). Hydrophobic resins have been used to identify and purify a number of these calcium-binding proteins. Head *et al.* (1983) used calcium-dependent binding to phenothiazine-Sepharose to isolate calmodulin and a 17-kDa calcium-binding protein from squid optic lobe. Sequence data demonstrate that this latter protein is 68% similar to bovine calmodulin and contains four EF hands (Head, 1989). Cox

(1986) purified the 18-kDa calcium vector protein from *Amphioxus* muscle. It binds 2 mol of calcium per mol of protein, suggesting that only two of the four putative EF hands are functional (Kobayashi *et al.*, 1987).

In the course of developing a rapid purification for protein kinase C based on hydrophobic chromatography, Walsh *et al.* (1984) detected a 21-kDa calcium-binding protein in bovine brain. When scaling up the purification of this protein, additional calcium-binding proteins of 18.4 kDa and 26 kDa were detected (McDonald and Walsh, 1985). ^{45}Ca -overlays and equilibrium dialysis experiments suggest that these proteins bind calcium with high affinity.

The striated flagellar roots of *Tetraselmis* are calcium-modulated contractile organelles (Salisbury and Floyd, 1978). On isolation of highly enriched striated flagellar roots fractions, a single protein, centrin, was purified by calcium-dependent hydrophobic chromatography. It was found to constitute 60% of the total protein mass of the organelle. Further analysis indicated that centrin is a phosphorylated calcium-binding protein (Salisbury *et al.*, 1984). Antibodies to *Tetraselmis* centrin show that the protein, or its immunologically related homologue, is associated with centrioles, basal bodies, and centrosomes (Baron and Salisbury, 1988). Functional studies of centrin have defined it as a novel type of elastic or contractile cytoskeletal protein. Sequence analysis of the cDNA demonstrates the presence of four putative EF-hands (Huang *et al.*, 1988).

In summary, calcium-dependent hydrophobic interactions can be exploited to identify and purify calcium-binding proteins.

Annexins: calcium-dependent phospholipid-binding proteins

Over the last 7 years, a family of calcium/phospholipid-binding proteins has been identified by a number of laboratories with distinct experimental goals. The proteins were isolated by calcium-dependent binding to chromaffin granule membranes (Creutz, 1981; Geisow and Burgoyne, 1982; Pollard and Scott, 1982), to synaptosomes prepared from *Torpedo* electric organ (Walker, 1982), and to detergent-insoluble membrane fractions (Davies *et al.*, 1981; Gerke and Weber, 1984). The p35 substrate for the epidermal growth factor receptor/kinase was also found to associate with membranes in a calcium-dependent manner (Fava and Cohen, 1984). By the use of a different approach, several proteins in addition to calmodulin were iso-

lated by calcium-dependent binding to phenothiazine resins (Moore and Dedman, 1982). More recently, several laboratories identified protein fractions that inhibited phospholipase A_2 and blood coagulation. Sequence data demonstrated identities between these protein groups (see Crompton *et al.*, 1988).

The independent identifications led to the use of different terminologies for these proteins, including synexin, calelectrins, chromobindins, calcimedins, calpactins, and lipocortins. For clarity, the term "annexin," suggested by Geisow (1986), is becoming more widely accepted. Sequence data indicate that there are seven unique proteins in the annexin family; annexins I–VI follow the numbering of Pepinsky *et al.* (1988), and annexin VII is synexin (Burns *et al.*, 1989). The sequence organization of the family is highly conserved. Annexins I–V and VII are comprised of four repeated domains, whereas annexin VI is comprised of eight domains, each domain being ~ 70 amino acids in length. The sequence conservation for each domain ranges between 40 and 60% when individual family members are compared. The amino terminus of each protein is unique, suggesting that this region may confer functional differences to the proteins.

In summary, the distinguishing features of the annexin family are the ability to bind phospholipids and the ability to bind calcium without using the classical EF-hand motif. Both calcium and phospholipids may modulate the function of these proteins and provide an independent mechanism for the mediation of intracellular calcium.

Elucidation of the multiple roles of calcium

Scientific progress is not restricted by effort but is dependent on insight and advances in technology. Advances in protein chemistry in the late 1960s and early 1970s led to the identification and characterization of the calcium receptors troponin C and calmodulin. Development of recombinant DNA technology provided precise knowledge of the molecular evolution and structural conservation of these proteins. Ca^{2+} -sensitive dyes and computer-assisted image processing have allowed for the visualization of transient, spatial changes in intracellular calcium. Detailed information is being obtained on the cellular components that are essential for maintaining these levels, including Ca^{2+} channels, $\text{Na}^+/\text{Ca}^{2+}$ exchangers, and Ca^{2+} -ATPases (Raeymaekers and Jones, 1986; Carafoli, 1987; Wuytack *et al.*, 1987; Ferguson *et al.*, 1988;

Hashimoto *et al.*, 1988; Volpe *et al.*, 1988; Lytton *et al.*, 1989). The original observations of Hokin and Hokin (1953) concerning the turnover of phosphatidylinositol were finally appreciated, leading some twenty years later to new insights in second-messenger action; inositol-1,4,5-trisphosphate and diacylglycerol coordinate, respectively, the release of intracellular Ca^{2+} from the endoplasmic reticulum and the activation of protein kinase C.

While the mechanisms involved in maintaining intracellular calcium homeostasis are being defined, the understanding of the events that couple stimuli to specific cellular responses have resisted biochemical definition. The elucidation of physiological function of specific calcium-mediator proteins is not a trivial undertaking. Initial implications of function can be explored by cellular microinjection of antibodies and antisense RNA, or by *in vitro* reconstitution. Information obtained from these inhibition experiments, however, is limited because it does not fully reflect the intact organism. For example, while our knowledge of the molecular aspects and regulatory properties of calmodulin *in vitro* is impressive, little is known of the functional role of calmodulin in the living cell or the whole animal, other than by inference. An attractive approach to obtaining this information is genetic manipulation. Fungi, such as yeast, are powerful models for the introduction and study of site-directed and temperature-sensitive mutations, and fundamental questions regarding the regulatory role of calcium in growth, division, and secretion are being addressed (Baum *et al.*, 1986; Davis *et al.*, 1986; Miyamoto *et al.*, 1987). The usefulness of such haploid systems, however, is limited to genes that are expressed in these organisms and to nonlethal mutations in these genes. The cellular function of the diverse calcium-binding proteins, which are expressed in highly differentiated cells, remains elusive, but inroads are being made with the use of homologous recombination in mouse embryonic stem cells (Frohman and Martin, 1989). Specific genes have been targeted to the genome, where they have been incorporated into the germ-line, and have been shown to function properly (Thompson *et al.*, 1989). Once the site-specific genetic manipulation of calcium-binding proteins is achieved, we will be able to evaluate specific gene products at the physiological level.

The understanding of calcium-coupled stimulus responses has advanced through the identification of calcium-binding proteins, determination of their subcellular localization and their biochemical role through reconstitution of a

cellular function, and genetic manipulation of intact cells. While many candidates for calcium-mediator proteins are currently under investigation, it is anticipated that even more calcium-binding proteins will be identified as further research continues to solve the stimulus-response coupling puzzle.

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