Chapter 9

Intracellular Calcium-Binding Proteins

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INTRODUCTION

Calcium is one of the essential elements of eukaryotic organisms. In vertebrates, including man, over 99% of body calcium is immobilized in the bones and teeth by complexing with phosphate to form hydroxyapatite. The remaining calcium is
distributed between the extracellular fluid and intracellular space. Extracellular calcium concentration, including that of the blood plasma, is maintained at about 3 mM. This calcium level is controlled mainly by the mobilization of calcium in and out of bone deposits and the intake of dietary calcium. In addition, about 50% of the extracellular calcium exists in an ionized form (Ca\(^{2+}\)) (see Carafoli, 1987). On the other hand, the total intracellular calcium content varies. Erythrocytes contain only 20 \(\mu\)M, brain cells 1.5 mM, and heart cells 4 mM. In contrast to the extracellular pool, a very small fraction of the total intracellular calcium is ionized. Typically, the cytosolic-free Ca\(^{2+}\) concentration lies between 0.1 \(\mu\)M and 1 \(\mu\)M, which is at least three orders of magnitude lower than the extracellular level. This results in a steep electrochemical gradient of Ca\(^{2+}\) across the plasma membrane of the cell.

A cell can be considered an entity in itself, using the plasma membrane as its barrier to the outside environment. Communication between the outside and the inside of the cell is achieved by a so-called "receptor-effector coupling" mechanism. Generally, stimuli, such as hormones and growth factors, can be regarded as ligands which interact selectively with the extracellular binding site of their respective receptor proteins. Upon ligand binding, the receptor molecule is activated and exerts certain effect(s) on its effector(s). For example, phosphatidylinositol-specific phospholipase C mediates the breakdown of plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) to generate inositol-triphosphate (IP\(_3\)) and diacylglycerol (DAG). IP\(_3\) can mobilize Ca\(^{2+}\) from an intracellular calcium store viz, the endoplasmic reticulum (ER) via the IP\(_3\)-receptor-calcium channel (Berridge, 1987). In the plasma membrane of excitable cells (such as muscle cells or neurons), there are voltage-dependent Ca\(^{2+}\) channels and ligand-gated Ca\(^{2+}\) channels such as the glutamate-gated NMDA-receptor/Ca\(^{2+}\) channel (Young and Fagg, 1991). Ligand binding or depolarization will cause brief opening of these Ca\(^{2+}\) channels, and thus allow Ca\(^{2+}\) to enter the cell passively, thereby elevating the cytosolic Ca\(^{2+}\) concentration. An elevated cytosolic Ca\(^{2+}\), in turn, activates various Ca\(^{2+}\)-dependent systems. The first step is usually the binding of Ca\(^{2+}\) to intracellular target proteins (calcium-binding proteins). In the following sections, we will examine different types of calcium-binding proteins and their proposed functions in Ca\(^{2+}\) signaling.

**Properties and Classes of Calcium-Binding Proteins**

An intracellular calcium-binding protein (CaBP) can be defined as a cellular protein capable of binding Ca\(^{2+}\) (usually reversibly) under physiological conditions. Since the resting levels of cytosolic calcium are very low, most often binding of calcium occurs when there is an elevation in the intracellular Ca\(^{2+}\) level. Generally, a specific Ca\(^{2+}\)-binding site on the CaBP can be found to be made up of several Ca\(^{2+}\)-chelating atoms (usually oxygens) contributed by either the amino acid side chains or the
peptide backbone. The architecture of such a binding site is amazingly selective for Ca\textsuperscript{2+}, usually with little affinity for Mg\textsuperscript{2+}. This is an important criterion since the intracellular free Mg\textsuperscript{2+} is about 0.1–1 mM, while the free Ca\textsuperscript{2+} levels, even in excited cells, usually do not exceed 1 μM. Thus, if the calcium-binding sites do not have 1,000-fold selectivity for Ca\textsuperscript{2+} over Mg\textsuperscript{2+}, these sites would be occupied with Mg\textsuperscript{2+} most of the time.

The EF-hand CaBP and annexins are the two largest sub-family of CaBP (Crompton et al., 1988; Kreisberger et al., 1991). Proteins involved in the translocation of Ca\textsuperscript{2+} across cell or organelle membranes also appear to have sites with an affinity for Ca\textsuperscript{2+}. These include the Ca\textsuperscript{2+}-Mg\textsuperscript{2+}-ATPase (calcium pumps), various calcium channels and the Nt-Ca\textsuperscript{2+} exchanger (Coxford, 1987). There are also many CaBPs that do not readily fit into any categories, such as the Ca\textsuperscript{2+}- and phospholipid-dependent protein kinase (protein kinase C) (Nishihata, 1984), the sarcoplasmic reticulum luminal calcium-binding protein calstabin, and a list of proteins with calcium-binding sites involved in structural stability (McPhalen et al., 1991).

The calcium-binding property of a protein can sometimes be confirmed by the 45Ca overlaying technique (Schachtelé and Murao, 1988). Putative calcium-binding protein samples are resolved by denaturing electrophoresis gel; 45Ca is overlaid on the gel or the blotting membrane onto which the protein bands from the gel are first electrophoresed. A autoradiogram may then be obtained. Another technique which applies to many EF-hand CaBP is the use of the carboxyanine dye Sinteil-All which stains a normal protein bands pink on an electrophoresis gel. However, if the protein is Ca\textsuperscript{2+}-saturated, it stains blue or purple (Sharma and Balasubramaniam, 1991). Other techniques used in identifying CaBP are terbium phosphorescence, equilibrium- and flow-dialysis, and equilibrium gel filtration (Schachtelé and Murao, 1988).

**EF-HAND CALCIUM-BINDING PROTEINS**

**EF-Hand Calcium-Binding Motif**

A helix-loop-helix calcium-binding structure was first recognized in eel CaBP parvalbumin (Kreisenger and Nockolds, 1973). It is known as the EF-hand structure after the E- and F-helices identified in the crystal structure of parvalbumin. It consists of a 12-residue loop that binds calcium ions, flanked by two perpendicular alpha-helices (Figure 1). Typically, the coordination of the ion is provided by precisely spaced residues 1, 3, 5, 7, and 12, and by a water molecule hydrogen-bonded to residue 9, thus forming a 6-member octahedral structure (Figure 1).

The EF-hand protein family is now the largest superfamily of CaBP. It was believed that these were evolutionarily derived from an ancestor EF-hand gene (Kreisenger, et al., 1991). However, the overall homology between two EF-hand proteins are relatively low (as little as 27%), confirming that the primary structure
Figure 1. EF-hand Ca\textsuperscript{2+} binding site. The helix-loop-helix calcium binding site of parvalbumin is indicated by the ball-and-stick model showing the backbone carbon (left). The side chain oxygens that coordinate the Ca\textsuperscript{2+} are shown as black spheres. The first helix (f1, the calcium-binding loop and the second helix (f2) are visualized, respectively, as the index finger, the curved middle finger, and the thumb of a right hand (right). The two helices are usually perpendicular to each other. The octahedral calcium ion coordination is also illustrated with the coordinating oxygens occupying the six vertices. Reproduced with permission of Elsevier Science Publishers from Penninger et al. (1989).

is not critical (Hessmann and Hantzlke, 1991). Being conserved is the general structure—two hydrophobic alpha-helices flanking a calcium-binding hydrophilic loop of about 12 residues (Figure 2). The helices are usually about 9–12 residues long. Within the calcium-binding loop, residues 1, 3, 5, 7, and 12 are almost invariably aspartate (D), asparagine (N), glutamate (E), glutamine (Q), serine (S), threonine (T), or tyrosine (Y) which can contribute a side-chain oxygen atom for Ca\textsuperscript{2+} on coordination (Figure 2). Usually, the K\textsubscript{d} (i.e., dissociation constant) for Ca\textsuperscript{2+} is about 1–10 μM. Also, variation in the amino acids in the calcium-binding loop is thought to alter Ca\textsuperscript{2+} affinity. A variant from this theme is a family of membrane proteins, the integrins, which mediate cell adhesion to extracellular matrix proteins in a Ca\textsuperscript{2+}-Mg\textsuperscript{2+}-dependent manner. Interestingly, they have an EF-hand-like structure, with the exception that at position 12, the coordinating residue is replaced with an uncharged valine or isoleucine (Tuckwell et al., 1992).

It was proposed that the last candidate for Ca\textsuperscript{2+} is provided by an aspartate residue from a natural protein ligand of integrin, such as fibronectin. In most EF-hand
Calcium-binding proteins, there are an even number of EF-hands, e.g., two in calbindin-9K, four in calmodulin and troponin C. Two adjacent EF-hands are generally arranged to be in close proximity so that the binding of the first calcium ion enhances the binding of the second Ca\(^{2+}\) (positive cooperativity).

All of the X-ray crystallography data relating to EF-hand CaBP's have only been obtained with the Ca\(^{2+}\)-bound forms due to instability problems. On the other hand, circular dichroism and 1\(^H\) NMR studies have revealed significant conformational
changes due to calcium binding in an exposed hydrophobic region of the protein (presumably, the helical region). Since most of the EF-hand proteins do not have enzymatic activity, it is assumed that the Ca$^{2+}$-dependent exposure of hydrophobicity allows the calcium-binding protein to bind and activate its target protein, which is usually an enzyme or a dynamically regulated structural protein. This will be examined in more detail, using calmodulin as an example, in a later section.

**EF-Hand Protein Family**

The availability of the crystal structure of calbindin, calmodulin, and troponin C has clearly confirmed the EF-hand structure found by parvalbumin (see Heinemann and Humelker, 1993). Many novel proteins have already been cloned, their cDNA sequence obtained and their amino acid sequence deduced. By homology to existing EF-hand sequences, more than 200 new calcium-binding proteins of this family have been identified. They can be further divided into subfamilies based on evolutionary origin (Kreiserger et al., 1993). Table 1 lists a few of the EF-hand calcium-binding proteins with more defined functions. Calbindin-9K is the smallest with only two EF-hands. Parvalbumin and the CaBP highly expressed in tumor cells (oncornidulins) have three EF-hands but the first loop is not functional. The ubiquitous four EF-handed calmodulin (Manuel and Elee, 1984) and myocyte-specific troponin C and myosin light chain appear to be closely related. The small subunit of the protein phosphatase, calcineurin, and the Ca$^{2+}$-dependent protease, calpain, have four EF-handlike structures. The S-100 sub-family includes S-100a and S-100b which have 14 residues instead of 12 in one of their two calcium binding loops (Kligerman and HD, 1988).b Related proteins (MRP-8 and MEF-14), also called calgranulins A and B, are S-100-related proteins with two EF-hands found in monocytes and neutrophils. They are known to translocate to the cell membrane upon cell activation (and thus calcium-binding). They also are thought to be involved in inflammatory reactions (Dolink et al., 1987). Another S-100-related CaBP is calcyclin which is involved in cell cycle progression (Calabretta et al., 1986). Sorensen is a four EF-hand protein that is overexpressed in multi-drug resistant cell lines (Meyers et al., 1987). Calbindin-D28K and calcytin have six EF-hands (Table 1). More recently, two new EF-hand-like proteins present in fiber cells that form vertebbrae lam., were found to have an EF-hand structure, and to bind Ca$^{2+}$ (Balasubramanian and Sharma, 1991). In the following sections, we will look at several examples of such EF-hand proteins and discuss their proposed functions in more detail.

**Calbindin-9K and Calbindin-D28K**

Calbindin-9K is a member of the EF-hand family of calcium-binding proteins with two helix-loop-helix regions that bind two calcium ions. Calbindin-9K is
present primarily in bone and cartilage (Balmain, 1991). In cartilage, it occurs only as a cytosolic protein in mature chondrocytes while in bone, it is found in osteoblasts and mature osteocytes. It is also secreted into the extracellular matrix. Its synthesis is strictly dependent on vitamin D. It is likely that calbindin-9K plays an important role in calcio-reposition and subsequent mineral nucleation in the extracellular matrix vesicles of calcifying cartilage and bone. Calbindin-9K has attracted much attention because of its small size which is ideal for molecular structure determination using 2D-protein NMR or X-ray crystallography (Heizmann and Hamilton, 1991). Interestingly, there is also a homologous but larger calbindin-D28K which contains six EF-hand structures in nonmineralized tissues. Its synthesis is also under vitamin-D control. Calbindin-D28K is abundant in some regions of the brain and may serve as a calcium buffering system (vide infra). Calbindin-D28K is also found in intestinal absorptive cells and has been shown to activate the plasma membrane Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase which is implicated in active calcium uptake from the intestine (Wasserman et al., 1992).

### Calmodulin and Calmodulin-Binding Proteins

Calmodulin is present in virtually all eukaryotic organisms and in all cell types (Klee and Vanaman, 1982). It is a small protein (16.5 kDa) composed of four EF-hand structures that bind four calcium ions (Baba et al., 1983). The amino acid sequence from many species has been determined and an exceptionally high degree of conservation was found. Calmodulin is well known to regulate various cellular functions via its interactions with various calmodulin-binding proteins.
Cellular functions regulated by calmodulin include cell motility, mitosis, calcium transport, exocytosis, protein phosphorylation/dephosphorylation, and calcium transport (Manalan and Klee, 1984). As an exception to most of the EF-hand Ca2+ChPs, not one but many target proteins for calmodulin have been identified. The effects of calmodulin on these many cellular functions are thought to be mediated by the calmodulin-binding proteins. Calmodulin-binding proteins can be divided into three groups: (a) enzymes, (b) cytoskeletal (structural) proteins, and (c) miscellaneous. The enzyme group includes two metabolic enzymes (phosphofructokinase and phosphorylase kinase) (Wayte and Heilmeyer, 1983; Chan and Graves, 1984) and two enzyme ions in the control of calmodulin levels: adenylate cyclase in the formation of cAMP and cyclic nucleotide phosphodiesterase (PDE) in its breakdown (Cheung, 1973; Yang et al., 1985). The plasma membrane Ca2+-Mg2+-ATPase is also calmodulin-activated (Wang et al., 1992). Several calmodulin-dependent enzymes are involved in the protein phosphorylation/dephosphorylation mechanism, including: (a) phosphorylase kinase; (b) pyruvate light chain kinase (Klee, 1977); (c) calmodulin-dependent protein kinase II (which phosphorylates a large number of cellular proteins) (Kennedy et al., 1987); and (d) calmodulin-dependent phosphatase (calcineurin) (Tallant and Cheung, 1986). Inositol 1,4,5 triphosphate kinase, a key enzyme in inositol phosphate signal transduction is also calmodulin-stimulated (Johannson et al., 1988), as well as nitric oxide synthase, the enzyme that produces the highly unstable second messenger nitric oxide (Lowenstein and Snyder, 1992). In the cytoskeletal/structural protein group, erythrocyte spectrin binds calmodulin with low affinity (Sobue et al., 1981b), while the brain spectrin (S100) binds with high affinity (Carlin et al., 1983). Tubulins (α and β) also bind calmodulin with low affinity (Kumagai et al., 1982). The microtubule associated protein 2 (MAP-2) and Tau factor also appear to bind calmodulin (Sobue et al., 1981a; Lee and Wolfs, 1984). Adducin is a calmodulin-binding protein present in the plasma membrane that promotes association of spectrin and actin (Bennett et al., 1988). This activity of adducin is inhibited when it binds calmodulin. Other calmodulin-binding proteins include the calcium release channel of the sarcoplasmic reticulum (Seibert et al., 1984) and both the liver and the lens gap junction proteins (Welsh et al., 1982; Zimmerman et al., 1987). Neuronmodulin is a neuron-specific calmodulin-binding protein (also called GAP-43) that is involved in cell growth. Interestingly, neuronmodulin appears to bind the noncalcium-bound calmodulin more tightly than the calcium-saturated calmodulin (Andreason et al., 1983). In addition, calmodulin-binding proteins as a group appear to be selectively susceptible to proteolytic attack by calpain (Wang et al., 1989).

Surprisingly, the amino acid sequence of the calmodulin-binding region of these CaMBPs are not highly conserved. Instead, they share a similar three-dimensional configuration: amphiphilic α-helix, i.e., an alpha helical structure with hydrophobic amino acids (Ala, Ile, Leu, Trp, Val) on one side and basic amino acid (Arg,
Calcium-Binding Proteins

Figure 3. Amphiphilic helix as calmodulin-binding motif in calmodulin-binding protein. Residues 4–17 of myosin light chain kinase from smooth muscle is the target region for calmodulin. The sequence is predicted to form an alpha helix. The residues are fitted into the helix well (Koide and Doedel, 1983) with the backbone of three consecutive residues. The helix can be visualized as spiral away from the viewer. It is observed that four basic residues (bold letters) cluster on one side of the helix while five out of six hydrophobic residues (outlined letters) concentrate on the opposite side of the helix.

Lys, His) on the other side of the helix (Figure 3) (O’Neil and DeGrado, 1990).

Hydrophobic compounds such as phenothiazines interact with calmodulin in a calcium-dependent manner. This is consistent with the view that upon binding calcium, calmodulin exposes the hydrophobic helical region (Weiss et al., 1982). This hydrophobic region is not interacts with the calmodulin-binding helix. Presumably, electrostatic interactions between the acidic groups on the calcium-binding loop of calmodulin and the basic side chains of the amphiphilic helix of the calmodulin-binding proteins are also important.

Tropomycin C

The myosin and actin filaments of myofibrils in skeletal muscle lie in parallel arrays. As illustrated in Figure 4, the cross-bridge is the protruding head of the myosin thick filament. This component possesses ATPase activity and when muscle is not in the active state the myosin molecules are said to be in the myosin-ADP-Pi state. The thin actin filament has several proteins including troponin and three tropomysins, tropomysin T, I, and C (Leavis and Gergely, 1960). Very briefly, myosin-actin interaction is initiated in muscle by tropomyosin and troponin I during the relaxed state. Notice that during this stage the cross-bridge is not attached to actin (Figure 4a). However, during activation the myosin molecules undergo a change in state that leads to cross-bridge attachment, release of Pi, and the development of force (Figure 4b). During this phase of power stroke, the cross-bridge rotates from a 90° to a 45° angle (Figure 4c), leading to sliding of the
Figure 4. The role of tropomyosin C in skeletal muscle contraction. a) The myosin head (a mechanohexamer) projects at a 90° angle from the filament backbone in the absence of the actin filament. In muscle at rest, the cross-bridge is not attached to actin, and myosin is in the ADP state. b) Cross-bridge attachment to actin occurs at a 90° angle when myosin binds Ca²⁺ increases allowing tropomyosin C to bind Ca²⁺. This leads to a conformational change involving the movement of tropomyosin from its blocking action in the groove. Pi is released from the myosin head when attachment to actin takes place. The current view is that Pi release is the rate-limiting step in force generation. c) As the attachment increases, it does the development of the active force in the cross-bridge tension phase. The cross-bridge rotates and assumes a 45° angle relative to actin. (This is a phase of ADP ejection). Such a rotation has been demonstrated in electron microscopy of flight muscle of the giant water bug, Lethocerus. The filaments slide past each other without a change in their length. ATP then takes the place of ADP in the myosin head and when bound is eventually split by the myosin head ATPase to give ADP and Pi. The resulting conformational change leads to detachment of the cross-bridge from the actin filament and its return to the 90° angle state.
filaments past each other. ADP is then released from the myosin head. ATP takes its place which when bound is split by the myosin ATPase. The cross-bridge detaches and returns to the 90° angle state (Figure 4a).

The trigger event leading to the above sequence of changes is the rise in myoplasmic free Ca²⁺ concentration from about 10⁻⁷ M to 10⁻² M. Step one involves de-inhibition of cross-bridge attachment to the actin filament as the result of the binding by troponin C of Ca²⁺. Presumably this occurs after the movement of tropomyosin in the groove of actin (Figure 4b). The return to the relaxed state involves a fall in the myoplasmic free Ca²⁺ concentration back to 10⁻⁷ M, which is brought about primarily by the Ca²⁺ pump of the sacro-elastic reticulum (SR), along with closure of the sarcoplasmic Ca²⁺ channels and the SR calcium release channels. If, however, the myoplasmic Ca²⁺ level is still raised and Ca²⁺ remains bound to troponin C, then the above chemo-mechanical cycle is repeated cyclically.

The binding of troponin I to troponin C has been investigated using a 12-residue peptide corresponding to the troponin C binding site on troponin I (residues 104-115). Nuclear magnetic resonance spectroscopy studies reveal that the troponin C-bound form of the peptide is an amphiphilic helix with basic residues on one side and hydrophobic residues on the other (Campbell and Sykes, 1991). Such conformation is essentially identical to the pattern found in calmodulin-binding sequence in many proteins (see above).

**EF-Hand Structure as a Domain of Protein**

Thus far, the EF-hand proteins examined basically involve repeats of the helix-loop-helix. However, EF hands are found as a domain of more complex proteins. For example, the 17 kDa subunit of the calmodulin-activated protein phosphatase calcineurin is a calcium binding protein which possesses four EF-hand domains (Figure 5) (Klee et al., 1988; Guerini et al., 1989), while a phosphatase domain and a calmodulin-binding domain exist in its large subunit (Guerini and Klee, 1989) (Figure 5). Moreover, calcineurin has been identified to be the target protein for the immunosuppressive drug, cyclosporin A (Liu et al., 1991). In regard to calmodulin itself, it has also been shown to be one of the five subunits of phosphorylase kinase (Cohen, 1988). Both calcineurin and phosphorylase kinase can bind another molecule of calmodulin and thereby increase their activity. The calcium-dependent cytoskeletal protein, calpain, is another intriguing example. It has a large catalytic subunit and a small regulatory subunit (Suzuki, 1987). The 80 kDa subunit of calpain contains a catalytic cytoskeletal protease domain (similar to papain for example) and an EF-hand Ca²⁺-binding domain (Figure 5). The regulatory subunit (29 kDa) has a gycine-rich region on the N-terminal side and four more EF-hand calcium-binding sites on the C-terminal side (about 10 kDa). It is believed that this EF-hand Ca²⁺-binding domains impose strict Ca²⁺ dependence on the catalytic activity. More recently, actinin was found to contain an actin binding
Figure 5. Structural models of calcineurin and calpain. The large (80 kDa) and small (29 kDa) subunits of calpain combine as six domains. Domain II is a cysteine protease domain while domain IV and VI are both calcium-binding domains with four EF-hand structures each. Calcineurin has a larger subunit that contains a phosphatase domain and a calmodulin-binding domain (filled area). The small subunit is the calcium-binding domain with four EF-hand structures (shaded area). In both calcineurin and calpain, the EF-hand calcium-binding sites impose calcium-dependency on the enzymes.

domain as well as an EF-hand calcium binding site (Wailes et al., 1992). EF-hand structures have also been reported in Drosophila alpha-spectrin (Dubreuil et al., 1991) and the signal-transducing phosphatidylinositol-specific phospholipase C (Barroch and Cox, 1990).

Calcium Binding Proteins in the Nervous System

Calcium is widely used as a second messenger in the nervous system where it regulates axonal transport of substances, release of neurotransmitters, membrane excitability, and long-term potentiation (memory). Therefore, it is not surprising to find many CaBPs in abundant amounts in the nervous system. Parvalbumin,
calmodulin, calbindin-D28K, calciretin, and S-100b occur in high concentration in subpopulations of neurons (Heizmann and Braun, 1992). S-100b is also found in glial cells (astrocytes, microglial cells, and Schwann cells) (Hyden and McWen, 1989). Epilepsy and ischemia have been linked to overactivation of glutamate receptors which cause excessive amounts of calcium ion to enter the postsynaptic neurons (Heizmann and Braun, 1992). Parvalbumin, calbindin-D28K, and calciretin were suggested to play a role in buffering increased intracellular calcium levels and, therefore, can be considered as an endogenous neuroprotective mechanism. That parvalbumin, calbindin-D28K, and calciretin-immunoreactive neurons are relatively resistant to glutamate-induced neurotoxicity is still a moot point (Heizmann and Braun, 1992). The S-100b protein has been shown to regulate phosphorylation of a microtubule-associated protein (tau) which controls microtubule assembly and disassembly (Endo and Hidaka, 1983), and to have a role in neurite-forming activity in neuron cultures (Winningham-Majer et al., 1989). Neurotrophin receptor (Trk) is a protein tyrosine kinase receptor (related to S-100b) which is a part of the transmembrane signaling process (Styren, 1991). Upon binding calcium ion, neurotrophin receptor activates guanylyl cyclase to restore the dark state of the system. Calmodulin-dependent kinase II is a major protein component of the postsynaptic membrane. Its activity has been linked to long-term potentiation of neurons, which is believed to be the first event that leads to memory (Malinow et al., 1988). Calcium-free protein was found in high density in regions of the brain (Kucher et al., 1992) while calpain has been shown to be activated and to degrade spectrin in neurons exposed to excitotoxin glutamate or hypoxia (Suzuk and Nosaka, 1988). Its degradation activity has been suggested to play a role in the eventual neuronal death. In chronic neurodegenerative diseases, such as Alzheimer’s disease and Parkinson’s disease, abnormal levels of calbindin-D28K and S-100b have been reported (see Heizmann and Braun, 1992). In patients with Alzheimer’s disease, both calpain and S-100b along with tau have been identified as components of senile plaques—abnormal extracellular protein aggregates (Heizmann and Braun, 1992).

ANNEXINS

Annexin represents a family of calcium-binding proteins that are capable of interacting with phospholipids and cell membranes in a Ca2+-dependent manner (Crompton et al., 1988). They have been suggested to participate in membrane fusion, exocytosis, and cell signaling pathways. They serve in vivo as substrates for synaptic kinase and protein kinase C. When it is membrane bound, annexin inhibits membrane lipid degradation by phospholipases, especially phospholipase A2 and the subsequent release of arachidonic acid, which initiates inflammation. Thus, annexins may be anti-inflammatory. Through binding to the cell membrane, they inhibit binding of blood coagulation factors to the cell surface. Thus, they are also considered anticoagulants. So far, eight of the annexins have been identified in human and other animals and named annexins I, II, III, . . . VIII. Their affinity and
specificity for different phospholipids appears to vary. There are two major forms of annexin: a 36 kDa form which consists of four internal repeats and a 68 kDa form that has eight repeats. An 11 kDa S-100-like protein (p1) complex with the 36 kDa annexin II forms a tetrameric complex. Since p1 does not have functional calcium-binding loops, the complexing to annexin II is not calcium-dependent. However, the binding is known to involve the p1-binding region on annexin II, forming an amphipathic helix that interacts with p1, in a manner similar to the interaction between calmodulin and calmodulin-binding proteins.

The crystal structure of annexin V is now known (Huber et al., 1999). Each repeat is composed of five alpha-helices (α-e) connected by short loops. Three amino acids are proposed to coordinate Ca²⁺-a conserved glycine and threonine pair in the loop between helix α and β, and an aspartic acid in helix d of the repeat. When phospholipid is bound, an additional coordinate is thought to be provided by the phosphate group. This structure is generally conserved in intracellular and intercellularly. Ironically, these calcium binding sites are similar to those of phospholipase A₂, which binds and hydrolyzes phospholipids in a calcium-dependent manner. Its calcium-binding site has a pentagonal coordination of a calcium ion: two carboxylate oxygens of Asp₁₆₁, carboxyl oxygen from Tyr, Gly, and Al (Toyo et al., 1992). This five-member coordination is similar to, and yet distinct from, the six-member coordination seen in EF-hand proteins. Indeed, these findings are an indication of the emergence of a new family of calcium-binding proteins.

CA\textsuperscript{2+}L TRANSPORTING PROTEINS

A number of proteins are involved in transporting Ca\textsuperscript{2+} across biological membranes (Figure 6). These proteins have at least one calcium-binding site. The plasma membrane Ca\textsuperscript{2+}-Mg\textsuperscript{2+}-ATPase pump (130-140 kDa) (Wang et al., 1992) and the sarcoplasmic/endoplasmic reticulum Ca\textsuperscript{2+}-Mg\textsuperscript{2+}-ATPase (110 kDa) (MacLennan et al., 1985) are homologous. Both proteins actively transport Ca\textsuperscript{2+} over a membrane barrier against the chemical gradient by utilizing energy derived from ATP hydrolysis. The plasma membrane calcium pump is a high affinity (low Kₘ) system for Ca\textsuperscript{2+} and maintains low resting levels of cytosolic calcium by transporting Ca\textsuperscript{2+} out of the cell. It is especially important in cells that have no Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, such as erythrocytes. On the other hand, the SER calcium pump functions to efficiently remove excess cytosolic Ca\textsuperscript{2+} when muscle fibers contract so that they can return to the relaxed state. It has been suggested that there are two Ca\textsuperscript{2+} affinity states for the calcium pump: a high affinity state for binding Ca\textsuperscript{2+}, and one with low affinity for Ca\textsuperscript{2+}, thus enabling Ca\textsuperscript{2+} to be released to the outside. The calcium binding site was located in several transmembrane helices, presumably lining the lumen of the Ca\textsuperscript{2+} pore. In particular, two glutamic acids (residues 369 and 771) in the SER Ca\textsuperscript{2+}-Mg\textsuperscript{2+}-ATPase are implicated in the binding of calcium. The Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger is a high capacity system with low affinity (high Kₘ) for
Calcium-binding Proteins

Figure 6. Interplay of various calcium transporters in cells. Schematic of a cell showing the various membrane-bound proteins involved in transport of Ca²⁺ across a cellular membrane. The plasma membrane has both voltage-gated and ligand-gated calcium channels to allow calcium influx and two proteins to extrude calcium: the plasma membrane Ca²⁺/Mg²⁺-ATPase and the Na⁺/Ca²⁺ exchanger. Internally, calcium can be released via the IP₃, receptor-calcium channel (in the endoplasmic reticulum membrane) or the calcium release channel (in the sarcoplasmic reticulum in muscle fibres). Excess Ca²⁺ is sequestered into the intracellular Ca²⁺ store by the inward pumping ER or SR calcium pump. Note that not all of these transporters are expressed in all cell types.

calcium (Ilaustein and Nelson, 1982). However, in certain tissues, e.g., heart, it is the major system involved in the maintenance of the resting cytosolic calcium levels. These are many subtypes of voltage-gated calcium channels which allow calcium to enter the cell when the plasma membrane undergoes depolarization. In skeletal and cardiac muscle, it is primarily the L-type channel (Hoffmann et al., 1987), while in the CNS, there are also the N-type, P-type, and T-type channels (Spedding and Paoletti, 1997). Presumably, these heteromeric channels have a central hydrophilic core for Ca²⁺ to pass through and have certain acidic residues for binding Ca²⁺ at or near the entrance of such pores to facilitate the Ca²⁺ channeling process.
In skeletal muscle, the SR is interlinked via the T-tubules to the plasma membrane. When T-tubules undergo depolarization, extracellular Ca²⁺ is released into the myoplasm via the Ca²⁺ release channel (Hui et al., 1987). This 550-kDa protein has been purified and cloned (Zoratti et al., 1990) but its exact calcium binding sites have not yet been clearly defined. Similar calcium release channels have been found in other excitable cells, such as neurons. Another class of proteins involved in calcium influx are the glutamate receptors found in the central nervous system (Watts, et al., 1990). The subtype AMPA-receptor has several isoforms capable of forming functional homo- or heterotrimers. One isoform has a glutamate residue in a putative channel-forming region of the protein which appears to be important for Ca²⁺ influx. Another isoform which only conducts Na⁺ current contains an arginine residue in the same position instead of glutamine. By comparison, the subtype NMDA-receptor, which is involved in both Na⁺ and Ca²⁺ influx, contains two asparagines in a homologous region (Burnashev et al., 1992).

OTHER CALCIUM-BINDING PROTEINS

Protein kinase C exemplifies a group of more obscure calcium-binding proteins with unidentified calcium binding sites. Protein kinase C can be divided into two halves. Its C-terminal half contains an ATP-binding site and a consensus kinase domain, whereas the N-terminal half, which is the regulatory domain, has two cysteine-rich regions which chelate zinc (zinc fingers) (Nishizuka, 1984). Presumably, the phospholipid and calcium binding sites are in the vicinity of the zinc fingers. Limited proteolysis of protein kinase C by trypsin or calpain yields an enzyme that is independent of both calcium and phospholipid as a result of cleavage in the middle of the molecule. This supports the idea that the calcium-binding sites are located in the N-terminal half of the protein.

Calsequestrin (42 kDa) is a major SR membrane component which is highly enriched with acidic residues. It binds calcium with relatively low affinity (Kₐ about 1 mM) but with high capacity (up to 60-50 mol Ca²⁺/mol protein). This CaBP most likely binds Ca²⁺ through negative surface charges, and serves as a sink for Ca²⁺ in the SR during muscle relaxation (Scott et al., 1988). Triplasmamussae, which catalyzes protein cross-linking, is dependent on millimolar calcium concentrations. It apparently contains an unidentified low affinity calcium-binding site (Friedrich and Azzoud, 1993). There are also other calcium-binding sites in proteins, such as in serine proteases and in metalloendopeptidases (e.g., carboxypeptidase), which do not actively participate in regulating enzymatic activity. This is not unexpected if the binding of Ca²⁺ exerts a structure-stabilizing effect (McKeehan et al., 1991).

SUMMARY

In this chapter, we have surveyed a number of intracellular calcium-binding proteins. As calcium is such a diverse second messenger, the number of calcium-
binding proteins is equally impressive. The most well studied are the EF-hand superfamil and the annexin family. A typical calcium-binding protein uses its affinity for calcium ion as the sensor for a rise in the intracellular calcium level. Upon binding Ca$^{2+}$, the protein undergoes certain conformational changes that allow it to interact with its target protein or with another part of the same protein (the effector). Through the modified activity of the effector, the calcium signal is transduced. It is most likely that more Ca$^{2+}$-binding proteins will be found in the future.

REFERENCES


