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Ca^{2+} Signaling and Intracellular Ca^{2+} Binding Proteins[1]

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Changes in cytosolic Ca^{2+} concentrations evoke a wide range of cellular responses and intracellular Ca^{2+}-binding proteins are the key molecules to transduce Ca^{2+} signaling *via* enzymatic reactions or modulation of protein/protein interations (Fig. 1). The EF hand proteins, like calmodulin and S100 proteins, are considered to exert Ca^{2+}-dependent actions in the nucleus or the cytoplasm. The Ca^{2+}/phospholipid binding proteins are classified into two groups, the annexins and the C2 region proteins. These proteins, distributed mainly in the cytoplasm, translocate to the plasma membrane in response to an increase in cytosolic Ca^{2+} and function in the vicinity of the membrane. Ca^{2+} storage proteins in the endoplasmic or sarcoplasmic reticulum provide the high Ca^{2+} capacity of the Ca^{2+} store sites, which regulate intracellular Ca^{2+} distribution. The variety and complexity of Ca^{2+} signaling result from the cooperative actions of specific Ca^{2+}-binding proteins. This review describes biochemical properties of intracellular Ca^{2+}-binding proteins and their proposed roles in mediating Ca^{2+} signaling.

Key words: annexins, C2 region proteins, Ca^{2+} storage proteins, Ca^{2+} signaling, EF-hand proteins.

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Studies on Ca^{2+} signalling originated in investigations of the mechanisms of skeletal muscle contraction, but it is now accepted that Ca^{2+} plays critical roles in many cell functions as an intracellular second messenger. Increasing attention has recently been paid to Ca^{2+} signalling and intracellular Ca^{2+} binding proteins, because (i) Ca^{2+} has been demonstrated to be the key second messenger in a wide variety of biological phenomena including muscle contraction, secretory events, cell cycle, differentiation, and gene transcription; (ii) technical breakthroughs in visualization of intracellular Ca^{2+} allow resolution of local Ca^{2+} distribution with Ca^{2+}-sensitive fluorescent dyes and confocal microscopy; (iii) more and more proteins which possess Ca^{2+}-binding domains have been identified in the last decade; and (iv) information has accumulated on target proteins and related pathways downstream of Ca^{2+} binding proteins.

Most Ca^{2+}-binding proteins possess an EF hand domain, an endonexin fold or a C2 region. On the basis of the primary structures responsible for the actual binding of these domains, we propose classification of intracellular Ca^{2+} binding proteins as demonstrated in Table I.

This review is aimed at a general coverage of the Ca^{2+} binding proteins, and their possible functions in intracellular transduction of Ca^{2+} signals.

1) EF hand proteins

The EF hand is the Ca^{2+}-binding structure originally found in carp muscle parvalbumin by Kretsinger (*1*). Later studies revealed it to be conserved in many Ca^{2+} binding proteins. The EF hand domain is basically composed of 40 amino acid residues with a 12 amino acid loop structure, directly responsible for the Ca^{2+}-binding domain, sandwiched between a pair of α helix domains. Although single EF hand polypeptide have only a low affinity for Ca^{2+}, their presence of pairs is associated with high affinity binding. Phylogenetic studies suggest that the EF hand protein family may be derived from a 4 EF hand ancestor gene *via* a series of tandem gene duplication (*2*). This view is supported by a fact that calmodulin, the ubiquitous 4 EF hand protein, is conserved among all eukaryotic cell types and that incomplete EF hand structures are often found in some 3 EF hand proteins (*3*). Indeed, some of the 3EF hand proteins in our category are sometimes described as being 4 EF (*4*). As listed in Table II, calretinin (*5*), calbindin-D28K (*6*) and a few other proteins possess 6 repeats of the EF hand domain.

Theoretically, one EF hand domain binds one Ca^{2+} ion. However, there are some cases where the number of the binding Ca^{2+} ions per molecule is smaller than that of the EF hands. For example, yeast calmodulin, a 4 EF hand protein binds 3 Ca^{2+} ions per molecule (*7*), and the calpain light chain (S100 A10, see Table II), a 2 EF hand protein, has no binding activity (*8*). Members of the EF hand family are mainly located within the cytoplasm and nucleus, although a few have been demonstrated to interact with membrane lipids when myristoylated.

1-1) 4 EF hand proteins. Troponin C was, to our knowledge, the first intracellular Ca^{2+} receptor protein to be identified in skeletal muscle. It forms the 18 kDa subunit of the troponin complex and two isoforms of this Ca^{2+}-binding protein exist: one in fast skeletal muscle binds 4 Ca^{2+} ions per molecule and the other in slow cardiac muscle, binds 3 (*9*). Ca^{2+} binding to troponin C overcomes the inhibition by troponin I of actomyosin ATPase activity,

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which is a critical event for stimulus-contraction coupling in skeletal muscle.

Despite the earlier discovery and many studies on the function of troponin C, the most well-known representative of the 4 EF hand protein family is, without doubt, calmodulin. This acidic protein was first discovered as a Ca^{2+}-sensitive and heat-resistant activator of phosphodiesterase in brain from independent work by Kakiuchi *et al.* (*10, 11*) and Cheung (*12*). Whereas other EF hand proteins including troponin C appear to be distributed in a tissue-specific manner, calmodulin is present in all eukaryocyte cells examined and mammalian calmodulins share quite high homologies among all species (*3*). This Ca^{2+} binding protein plays multiple roles in a wide variety of tissues. Discovery of calmodulin was so significant that scientists other than muscle physiologists/biochemists took the plunge into research on Ca^{2+} binding proteins thereafter. One typical example is the area of Ca^{2+} signaling in the central nervous system, where calmodulin and its target proteins are highly concentrated.

Calmodulin plays versatile roles in Ca^{2+}-mediated cellular events, since its target proteins are relatively many and multipotential. It activates a number of important intracellular enzymes including cyclic nucleotide phosphodiesterase, protein kinases/phosphatase, nitric oxidase synthase, adenylate cyclase, and Ca^{2+}-ATPase. Recognition sites for calmodulin on the individual target proteins seem to be related (*13-15*) and although calmodulin is distributed ubiquitously, it has some tissue-specific actions partly because the target proteins are localized in a tissue-specific manner. The multiple roles of calmodulin (*16-18*) and Ca^{2+}/calmodulin-dependent protein kinases (CaM kinases) (*19-21*) in cell functions have been extensively studied.

Myosin light chain kinase was the first CaM kinase to be discovered, by Hartshorne and his colleagues (*22*), and subsequently, several protein kinases have been found to be Ca^{2+}/calmodulin-dependent. Glycogen phosphorylase kinase is exceptional among CaM kinases in that calmodulin is contained as a subunit of this protein kinase. Among members of the CaM kinase family, myosin light chain kinase and glycogen phosphorylase kinase, phosphorylate only myosin light chain and glycogen phosphorylase, respectively. CaM kinase III also has a narrow substrate specificity; its only known substrate is elongation factor II, suggesting a pivotal involvement in the translational control of protein synthesis. One of the reasons why calmodulin plays versatile roles in cell functions is that it activates multifunctional protein kinases such as CaM kinase I, II, IV, and V which act on a broad range of substrates, though their individual substrate specificities and tissue distributions are distinct. CaM kinase V seems to be an isoform of CaM kinase I (*23*). Some of the multifunctional CaM kinases were recently found to be phosphorylated and activated by a novel CaM kinase (*24*) which has been now cloned and suggested to be involved in the CaM kinase cascade and to amplify calmodulin-mediated Ca^{2+}-signaling (*25, 26*).

TABLE I. **Classification of calcium binding proteins.**

	Subdivision	Common domain
EF hand proteins	4 EF hand proteins	EF hand
	3 EF hand proteins	//
	2 EF hand proteins	//
	Other EF hand proteins	//
Ca^{2+}/phospholipid binding proteins	Annexins	Endonexin fold
	C2 region proteins	C2 region
Ca^{2+} storage proteins		C-domain (pairs of acidic amino acid residues)

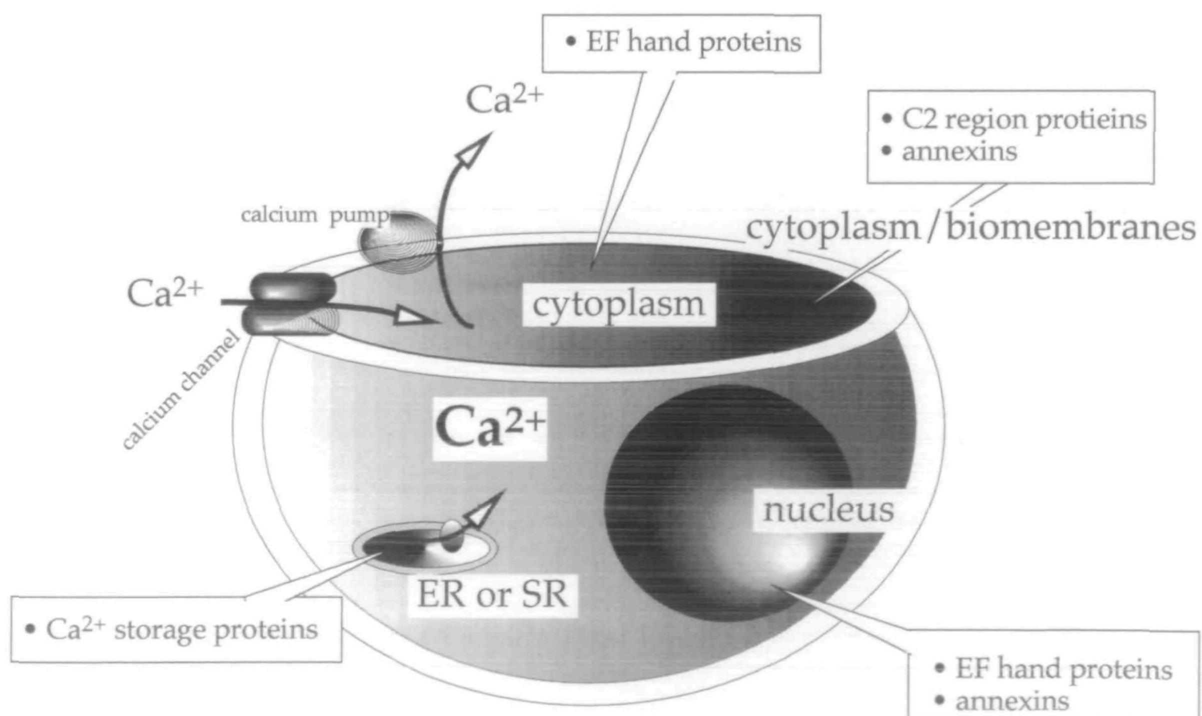

Fig. 1. **Schematic illustration for distribution of intracellular Ca^{2+} binding proteins.**

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TABLE II. **The EF hand protein family.**

	Synonyms	No of EF hand motifs	M.W.[a] (kDa)	Proposed functions[b]
Calmodulin		4	20	Multifunctional (see the text)
Troponin C		4	18	Contraction of skeletal and heart muscle
Myosin light chains		4	16-20	Muscle contraction and cellular motile events, membrane ruffling
Calpain large subunit		4	80	Cytokine processing, formation of ischemic changes
small subunit		4	30	Cell differentiation/development, platelet activation
Caltractin		4	20	Chromosomal segregation
Calcineurin B subunit	Phosphoprotein phosphatase 2B	4	19	Cytokine transcription, modulation of channel activity
Grancalcin		4	28 (24)	
Parvalbumin		3	12	
Oncomodulin		3	11	Calcinogenesis
Apoaequorin		3	20	Generation of fluorescence by Ca^{2+}-binding
Recoverin		3	23	Endogenous inhibitor of rhodopsin kinase/light adaptation
S-modulin		3	26	Endogenous inhibitor of rhodopsin kinase/light adaptation
Visinin		3	24	
VILIP		3	22	
Neurocalcin		3	22-24	Signal transduction in sensory cells
Hippocalcin		3	23	
S100A1	S100α	2	11	Cell growth
S100A2	S100L, CaN 19	2	10	
S100A3	S100E	2	12	
S100A4	Calvasculin, CAPL, p9Ka, 42A, pEL98, metastatin, mts 1, 18A2	2	11	Cell growth
S100A5	S100D	2	13	
S100A6	Calcyclin, 2A9, PRA, CaBP5B10	2	10	Cell growth, cell cycle, exoxytotic events
S100A7	Psoriasin	2	12	
S100A8	MRP-8, calgranulin A, p8, cyctic fibrosis antigen	2	11	
S100A9	MRP-14, calgranulin B p14,	2	13	
S100A10	p10, p11, 42C, calpactin light chain	2	10	Cell growth
S100B	S100β, neural extension factor	2	10	Neural extension, long term potentiation, cell proliferation
S100C	Calgizzarin	2	13	
S100P		2	10	
Calbindin-D9K	CALB3, calbindin 3, 9K ICaBP, 9K cholecalcin	2	9	Calcium transport
S100F-P	Profilaggrin	2		Aggregation of keratin intermediate filaments
S100F-T	Trichohyalin	2	248	
Calgranulin C		2	11	
CeNS		2		
CP-10		2	10	
Calsensin		2	9	Chemotaxis
Calbindin-D28K	28K ICaBP, 28K cholecalcin	6	28	Calcium transport
Calretinin		6		
Reticulocalbin		6	44	
ERC-55		6	55	

[a]M.W. on SDS-PAGE varies dependently on EGTA. [b]Buffering action of Ca^{2+} was not described as a function.

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Pharmacological approaches using specific inhibitors of calmodulin and its target proteins have provided powerful tools for investigation of the molecular mechanisms of calmodulin-mediated Ca^{2+}-signaling in living cells. We have developed W-7, a calmodulin inhibitor (*27*), and KN-62 (*28*), a CaM kinase inhibitor, from derivatives of naphthalenesulphonamide and isoquinolinesulphonamide, respectively. It must be noted that KN-62 was initially reported to be a specific inhibitor of CaM kinase II (*28*), but later studies also revealed the inhibition of CaM kinases I and IV (*29*). These inhibitors have facilitated elucidation of the biological roles of calmodulin and CaM kinases (*30*) as well as molecular analysis of the calmodulin molecule (*31*).

Although calmodulin was first reported to be localized in the cytoplasm, its presence in the nucleus has been demonstrated by both biochemical (*32*) and morphological (*33*) techniques. These studies also have indicated that the nuclear distribution of CaM is influenced by the proliferative status of the cells. Since some of its target proteins, such as CaM kinase II and IV, MLCK, calcineurin, and caldesmon, are also present in the nucleus, it has been proposed that Ca^{2+}/calmodulin may regulate DNA replication/transcription and other nuclear functions (*34*).

The question arises of why the intracellular concentra-

tion of calmodulin is so high (>10^{-5} M), since most, if not all, calmodulin-dependent enzymes do not require such high levels for activation. One possibility is that other factors such as S100 proteins may inhibit the activity of calmodulin-dependent enzymes in the cell (see below). Luby-Phelps *et al.* (*35*) recently investigated the intracellular mobility of calmodulin with a photobleaching technique using fluorescent-labeled calmodulin, and found that most was unable to diffuse in the cytoplasm presumably due to binding to anchoring protein(s). They also suggested that a small population of intracellular calmodulin is free and could be responsible for interaction with its bioactive target proteins.

Myosin is a heterohexamer protein composed of two heavy chains and two sets of regulatory and essential light chains. Both these types of light chains possess 4 EF hand domains. The heavy and regulatory light chains are phosphorylated by various protein kinases (*36*), but myosin light chain kinase only phosphorylates the regulatory light chain. Myosin hydrolyses ATP in the presence of actin and this ATPase activity is increased by phosphorylation of the regulatory light chain, resulting in smooth muscle contraction in response to elevation of the intracellular Ca^{2+} concentration. Distinct isoforms of myosin are also found in various non-muscle tissues, and their biological roles remains yet to be fully elucidated. A mechanism similar to that in smooth muscle has been proposed in endocrine tissues (*37*). Phosphorylation of non-muscle myosin light chains has been suggested to control intracellular events in endocrine tissues such as priming of catecholamine granules in chromaffin cells (*38*).

Caltractin, another 4 EF hand protein distributed in a wide variety of species, is a structural component of the basal body complex in *Chlamydomonas* or the centrosome in animal cells (*39*). Its intracellular localization and similarity to the yeast protein CDC31, which is also localized in the spindle pole body, suggest that caltractin may be involved in chromosomal segregation (*40*).

One of the features of the 4 EF hand protein family is that some members possess enzymatic activities. For example, the calpain family are Ca^{2+}-dependent proteases and calcineurin is a Ca^{2+}/CaM-dependent protein phosphatase. The latter is a heterodimer composed of A (61 kDa) and B (19 kDa) subunits. The calcineurin B subunit has 4 EF hand domains which Ca^{2+} binds, whereas calmodulin binds the A subunit where the catalytic domain of the protein phosphatase lies (*41*). Both calmodulin binding to the A subunit and Ca^{2+} binding to the B subunit are required for maximal activation of the phosphatase activity (*42*). Calcineurin dephosphorylates many phosphoproteins and has recently attracted a great deal of attention because immunosuppressants such as cyclosporin and FK-506, which form complexes with immunophilins, exhibit immunosuppression *via* its inhibition (*43, 44*).

Calpain was initially described as a Ca^{2+}-activated neutral cysteine endopeptidase abundant in the cytoplasm and later shown to have three isoforms (μ, m, and n). The μ and m isoforms are ubiquitous whereas the n isoform is rather tissue-specific (*45*). Calpain proteins are composed of 80 kDa and 30 kDa subunits, both of which possess 4 EF hand domains (*46*). Although *in vitro* experiments would suggest that calpain requires higher concentrations of Ca^{2+} than that normally reached in the cytosol for enzyme activation, proteolysis by calpain does take place in the cell, suggesting participation of some other factors in its regulation. For example, acidic phospholipids reduce the Ca^{2+}-requirement of calpain, while calpastatin inhibits its proteolytic activity (*47*). Many proteins, including cytoskeletal proteins, membrane receptors, enzymes and calpain itself, are targets of this protease. Autolysis of calpain activates its proteolytic activity and this enzyme is proposed to be involved in cell differentiation/development and processing of cytokines, as well as in the pathological state induced by ischemia (*48*).

1-2) 3 EF hand proteins. Since the EF hand domain was originally proposed on the basis of the structure of parvalbumin, many studies have been focused on the structure of this 3 EF hand protein (*49*). In comparison to what we know from structural analyses, little is known about its roles in Ca^{2+} signaling, except for a possible role as an intracellular Ca^{2+} buffer. Oncomodulin is also a 3 EF hand protein with similar exon/intron structures to parvalbumin. In contrast to parvalbumin, however, oncomodulin is known to activate phosphodiesterase activity in a Ca^{2+}-dependent manner like calmodulin (*50*). It was first found in hepatoma cells and is known to be abundant in various tumors and placental tissue (*51-53*), but its physiological/pathological functions remain to be elucidated.

Aequorin (more precisely apoaequorin, the polypeptide part of aequorin), derived from the medusa *Aequorea aequorea*, another 3 EF hand protein (*54*) exhibits the very distinct feature of generating fluorescence by binding to physiological concentrations of Ca^{2+} (*55*). It has been therefore found use as a Ca^{2+} probe to monitor intracellular Ca^{2+} concentrations in living cells (*56, 57*).

Recently, evidence has been obtained supporting participation of 3 EF hand proteins in visual transduction processes *via* regulating cGMP, the major second messenger in the phototransduction system in retina cells. Photoactivation of rhodopsin causes activation of transducin, a trimeric G-protein, which activates cGMP-specific phosphodiesterase (Type VI). Activation of phosphodiesterase hydrolyzes cGMP and close the cGMP-gated cation channel, resulting in a decrease in intracellular Ca^{2+} (*58, 59*). Recoverin, a 3 EF hand protein from bovine retina, was first reported to activate guanylate cyclase and elevate cGMP levels when Ca^{2+} was lowered (*60*). However, recoverin and S-modulin, another 3 EF hand protein from frog retina, were, in fact, found to decrease cGMP levels *via* sustained activation of retina-specific cGMP phosphodiesterase in response to Ca^{2+} increase in dark (*61, 62*). Studies have revealed that those two as well as their homologues such as p26 (Gecko), NCS-1 (rat and chick), and Ce-NCS-2 (*C. elegans*) inhibit rhodopsin kinase (*63-65*), *via* Ca^{2+}-dependent interactions (*66*). Phosphorylation of rhodopsin inhibits transducin to increase the phosphodiesterase activity, resulting in desensitization of the photosensing system. Therefore, Ca^{2+}-dependent inhibition of rhodopsin kinase by recoverin and its homologues decreases cGMP levels in photoreceptor cells, maintains the cGMP-dependent cation channel in a closed state, and eventually prevents visual desensitization by light. It should be noted that Ce-NCS-1, which has just 2 EF hand domains, is also reported to inhibit rhodopsin kinase (*65*).

Interestingly, neurocalcin, which shares common sequences with recoverin and S-modulin (*65*), is selectively

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localized in sensory neural cells like retina cells (*67*) and olfactory nerves (*68*). Furthermore, similar biochemical properties of neurocalcin and recoverin have been reported (*69*), although their distributions are distinct. cGMP-specific cation channels, originally discovered in photoreceptor cells, are also distributed in olfactory neurons and considered to play a critical role in sensory systems involving photo- and odor-receptors. Taking these findings into consideration, it is likely that these members of the 3 EF hand protein family transduce Ca^{2+}-signals in distinct sensory systems.

1-3) 2 EF hand proteins. Members of the 2 EF hand Ca^{2+}-binding protein family are called S100 proteins. The name was given originally due to the biochemical property that they remain soluble after precipitation with saturable (100%) ammonium sulphate (*70*). The earliest studies suggested that these proteins might be neuron-specific and involved in Ca^{2+} signaling in the nervous system. However, it was found later that some members of the S100 protein family are also located in non-neural tissues (*71*) and that S100A2 (originally called S100L, see Ref. *72*) seems to be preferentially expressed in non-neural cells (*73*). S100 proteins have been called various names on the basis of their biochemical properties, molecular weights, and tissue distributions. Zimmer *et al.* (*72*) proposed a comprehensive classification (Table II). All the S100A genes are clustered on human chromosome 1q21 (*71*). CalB3 (9K calbindin) is not always considered to belong to the S100 family because of its low homology with other members (*4*). Our knowledge of the functions of these proteins is still limited, but S100A1 and S100B have been relatively well studied for their biological roles and possible target proteins (*71, 74*).

Participation in cell growth has been suggested to be one of the functions of S100 proteins. This is supported by evidence that some are concentrated in tumors such as melanoma, renal carcinoma, and thyroid cancer cells (*75–77*). S100A2 is rather exceptional because it appears to be expressed to a lesser extent in tumor than in normal tissues (*78*), which implies the protein may play a role in opposition to other S100 proteins in cell growth and/or differentiation. S100B has been demonstrated to exert neurotrophic effects and cause neural extension when applied to the extracellular space (*79–81*). This Ca^{2+} binding protein may be involved in the control of memory or long term potentiation (*74*) and has been suggested to contribute to the pathology of some neural diseases, its expression in brain being enhanced in patients with Alzheimer's disease or AIDS (*82, 83*). Other S100 proteins have been also indicated to be involved in the regulation of cell growth (*72*). S100A8 and S100A9, also called migration inhibitory factor-related protein (MRP)-8 and MRP-14, respectively, are expressed in leukocytes and monocytes in early stages in their development, and are distributed predominantly in the cytoplasm (*84*), suggesting they may activate leukocytes under inflammatory conditions.

Some members of the S100 protein family participate in Ca^{2+}-dependent events in non-muscle cells. S100A4 interacts with nonmuscle tropomyosin in the presence of Ca^{2+} (*85*), and S100A6, but not S100C, enhances Ca^{2+}-induced insulin release from permeabilized pancreatic islet cells (*86*). Furthermore, S100A10 (also called calpactin light chain) may be involved in the control of Ca^{2+}-induced catecholamine release from chromaffin cells (*87*). Since Ca^{2+} plays a central role in stimulus-secretion coupling in neural, endocrine and exocrine tissues, these S100 proteins may modulate their secretory activities.

In this context it is of interest that cytoskeletal proteins such as tubulin, intermediate filament protein, microtubule-associated proteins, fibrillary acidic protein, and τ protein are reported to be functionally linked with members of the S100 family (*88–90*). In a similar way to calmodulin, S100B protein interacts with mellitin and τ protein (*91*), and the latter inhibits phosphorylation by CaM kinase II (*92*). S100 proteins have also been reported to inhibit the enhancement of brain protein phosphorylation due to calmodulin (*93*). We may propose that one of the functions of S100 proteins is to modify the versatile effects of calmodulin in Ca^{2+}-signaling. S100 proteins and calmodulin share similarities not only in their primary structures and target proteins, but also in their interactions with some synthetic compounds. Calmodulin antagonists and their derivatives may also react with other Ca^{2+}-binding proteins with 2 or 3 EF hand domains such as S100B, S100A6, or neurocalcin (*94–96*). Therefore, we may need to reevaluate the conclusions derived from pharmacological experiments using calmodulin antagonists.

It should be mentioned that several S100 proteins also interact with signal-mediating proteins other than EF hand proteins. For example, S100B inhibits phosphorylation of p53, the tumor suppressor protein, by protein kinase C (PKC) (*97*) and S100A9 interferes with casein kinase activity (*98*). Moreover, there are reports of specific binding with annexins, first described for annexin II and S100A10 (*8*). Molecular analyses have in fact been performed for such interaction (*99, 100*). We also reported specific binding between annexin XI and S100A6 (calcyclin) (*101*) and recently annexin I and S100C (calgizzarin) were found to react with each other (*102*). Although the biological significance of such binding has not yet been elucidated, S100 proteins may modify Ca^{2+}-signaling mediated not only by calmodulin, but also by annexins.

S100A1 and S100B exert an influence on glycolytic enzymes such as fructose-1,6-bisphosphate aldolase and glycogen phosphorylase, the activity of the former being increased by both (*103*), whereas S100A1, but not S100B, stimulates the glycogen phosphorylase activity (*104*).

2) Ca^{2+}/phospholipid binding proteins

There are also Ca^{2+} binding proteins which bind phospholipids in a Ca^{2+}-dependent manner. In some cases, including the annexins and conventional isozymes of protein kinase C (PKC), their affinity for Ca^{2+} and/or enzymatic activity thereby become higher. Therefore, these biochemical properties allow such proteins to translocate to cell membranes and to become active in the vicinity of the membranes in response to an increase in Ca^{2+}. Classification is into two groups, the annexins and the C2 region proteins.

2-1) Annexins. Annexins are a family of Ca^{2+}/phospholipid-binding proteins which commonly possess four repeats of the "endonexin fold" domain, the actual Ca^{2+}-binding sites. Thirteen members have been identified to date, ten in mammals (*105*). These proteins were given unrelated names such as lipocortin, calpactin, and calelectrin (see Table III), before it was suggested that they might

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TABLE III. **The annexin family.**

	Synonyms	No of endonexin folds	M.W. (kDa)	Proposed functions
Annexin I	Lipocortin I, calpactin II, chromobindin 9	4	35-40	PLA2 inhibition, ion channel activity, cell proliferation/differentiation, membrane fusion/aggregation, secretion
Annexin II	Lipocortin II, calpactin I heavy chain, protein I, chromobindin 8	4	34-39	PLA2 inhibition, cell proliferation/ differentiation, membrane fusion/aggregation
Annexin III	Lipocortin III, PAP-III 35-α-calcimedin, calphobindin III	4	36	PLA2 inhibition, membrane fusion/aggregation, hormone secretion
Annexin IV	Lipocortin IV, protein II, chromobindin 4, PAP-II, 35-β-calcimedin, endonexin I, 32.5K-calelectrin,	4	28-33	PLA2 inhibition, membrane fusion/aggregation, secretion
Annexin V	Lipocortin V, chromobindin I, PAP-I, 35-γ-calcimedin, calphobindin I, endonexin II, 35K-calelectrin, VAC α, anchorin CII	4	36	PLA2 inhibition, ion channel activity, anticoagulant, inhibition of protein kinase C
Annexin VI	Lipocortin VI, protein III, chromobindin 20, 67-calcimedin, calphobindin II, 67K-calelectrin, synhibin	8	67-73	PLA2 inhibition, ion channel activity,
Annexin VII	Synexin	4	56-57	Ion channel activity, membrane fusion/ aggregation, secretion
Annexin VIII	VACβ	4	37	
Annexin IX		4	33	
Annexin X		4	36	
Annexin XI	Calcyclin associated protein (CAP) 50	4	54	
Annexin XII		4	35	Ion channel activity
Annexin XIII		4	35	

PAP, placental anticoagulant protein; VAC, vascular anticoagulant.

have common structure(s), on the basis of their shared biochemical and immunological properties in common (*106-110*). This view was eventually proven to be right by direct cDNA sequencing, which demonstrated common amino acid sequences in their C-terminal regions (*111, 112*). Geisow and his colleagues designated these proteins to be annexins because they may function by "annexation" with biomembranes and membrane proteins in response to Ca^{2+} (*113-115*).

The K_d values of annexins for Ca^{2+} binding are 10-1,000 μM and the affinity appears to be heightened by binding to acidic phospholipids (*105*). Annexin VI has an exceptionally high affinity as its K_d is 1 μM even in the absence of phospholipids (*116*). The structure of the Ca^{2+} binding domain for annexins is completely different from those for EF hand proteins. Each member of the annexin family has a similar amino acid sequence in the C-terminal region, called the core domain, which is responsible for the Ca^{2+}/phospholipid binding (*117*). This portion in annexin V has been reported to bind to collagen as well (*118*). In the core domain, the endonexin fold with the characteristic motif GXGTDE is highly conserved (*105, 111*). The core domain contains four repeats of this sequence, with the exception of annexin VI which possesses 8 repeats (*119*). The N-terminal region, on the other hand, is variable and is proposed to be responsible for their variety of functions.

Annexins have been demonstrated in a wide variety of tissues, some being localized in the cytoplasm beneath the plasma membrane and becoming translocated to the plasma membrane in response to an increase in the intracellular Ca^{2+} concentration. A few annexins have also been identified in the nucleus (*120-122*).

Very recently, Morgan and Fernández found α-giardin from the protozoa, *Giardia lambia*, to have a similar amino acid sequence to annexins and therefore proposed that it might constitute an ancestor protein (*123*). In accordance with their argument, seven members were newly arrayed within the annexin family; annexin XIV which is the former annexin VII from *Dictiostelium*, annexins XV-XVII, three products from *Caenorhabditis elegans*, annexin XVIII from *Medicago sativa*, and two isoforms of α-giardin, as annexins XIX and XX.

Although annexins are proposed to be as multifunctional as calmodulin, direct evidence is still limited. They have been suggested to mediate membrane fusion, in line with the fact of phospholipid binding in the presence of Ca^{2+}. Since Ca^{2+} plays a central role in exocytosis, members of the annexin family could thereby control hormone/transmitter release which necessarily involves membrane fusion. Annexin IV causes Ca^{2+}-dependent aggregation of granules from the electric organ of *Torpedo marmorata* (*124*), and annexins I, III, and VII induce aggregation of granules, and membrane fusion in leukocytes (*125, 126*) and chromaffin cells (*87, 127, 128*). Morphological studies have indicated involvement of annexin I in insulin secretion from the pancreatic β-cell (*129*), and annexin-induced aggregation has also been verified using artificial liposomes (*130*). It is also interesting that annexins V and VII possess ion channel activity when reconstituted in lipid membranes (*131-133*).

Inhibition of phospholipase A_2 (PLA_2) by annexins was also demonstrated in early studies (*134*). PLA_2 releases fatty acids such as arachidonic acid from phospholipids. Since arachidonic acid is metabolized to bioactive eicosanoids, the enzyme has been considered to play a key role in activation of the immune response. A few members of the annexin family have proven potential to inhibit PLA_2 activity (*135, 136*) and since glucocorticoids, immunosuppressant hormones widely used in clinical applications, induce annexin I expression (*137, 138*), it has been suggested that annexins may play an integral role in the inhibitory actions of the hormones. Among PLA_2-inhibiting annexins, both annexins I and II exhibit this action when applied to the extracellular space (*139*). They could, therefore, find

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application as immunosuppressants (*140*). Whether inhibition of PLA_2 activity by annexins results from some direct interaction with the enzyme is argued, because excess amounts of phospholipids reduce the effect (*141*). Another extracellular influence of annexins is inhibition of blood coagulation (*142, 143*). This might be related to the demonstration that platelet membranes have a binding activity to annexins which is sensitive to phosphatidylserine (*144*).

It is noteworthy that expression of some annexins and related proteins is lowered in some autoimmune diseases. For example, production of annexin I and its binding protein is decreased in rheumatoid arthritis (*145, 146*). It has recently been reported that p56 autoantigen which appears in rheumatoid arthritis, Sjögren's syndrome and systemic lupus erythematosus (*147*), is a human homologue of annexin XI (*148*), which was originally discovered in our laboratory as a calcyclin (S100A6)-associated protein (CAP50) (*149*). This autoantigen appears also in systemic scleroderma, polymyositis, and other autoimmune diseases (*150*). Although the data still limited, annexin homologues could clearly participate in the pathology of some autoimmune diseases.

Annexins have also been suggested to be involved in the control of cell proliferation and differentiation, since some examples are known to be phosphorylated by protein kinases related to these cell functions. For instance, annexins I and II are good substrates of receptor- or non-receptor types of tyrosine kinases (*151-153*). Furthermore, annexins I, II and IV are phosphorylated by PKC (*87, 154, 155*). Phosphorylation of annexin XI changes its intracellular distribution (*156*), which might imply this annexin transduces stimuli for cell proliferation/differentiation under the control of protein kinases.

Although annexins inhibit a few enzymes as discussed above, the only report suggesting an effect on protein kinases concerned annexin V inhibition of PKC activity (*157*). This inhibition was only observed for PKC isoforms with a C2 domain, the Ca^{2+}/phospholipid binding region. It must be noted that, on the contrary to PLA_2 inhibition, high concentrations of phospholipids fail to overcome PKC inhibition by annexin V (*157*).

2-2) C2 region proteins. The C2 region, originally designated as the constant region of PKC isoforms, is responsible for Ca^{2+}/phospholipid-dependent activation of PKC (*158*). Studies have specified that the domain is conserved in phospholipase C (*159*) and phospholipase A_2 (*160*), synaptotagmin (*161*), and several other proteins (*162*, and Table IV). Some, if not all, proteins with a C2 region share biological properties such as Ca^{2+}-induced translocation from the cytosol to membrane, and interaction with common intracellular receptors (see below). The physiological functions of C2 region proteins are relatively well elucidated as compared with EF hand proteins or annexins.

PKC was discovered in late 1970s by Nishizuka and his colleagues as a Ca^{2+}-sensitive, phospholipid-dependent protein kinase in rat brain (*163*) activated by diacylglycerol, a product of phospholipid metabolism (*158, 164*). To date, eleven isoforms have been reported. They are widely distributed in a variety of tissues but have distinct specificities. Amino acid analysis of these isoforms revealed the existence of 4 constant regions (C1-C4) (*165*). C1 is cysteine-rich and binds diacylglycerol and phorbol esters. C2 is the Ca^{2+}/phospholipid binding domain composed of ~120 amino acid residues. C3 and C4 are in the catalytic domains which bind to ATP and substrates, respectively. α, $\beta1$, $\beta2$, and γ isoforms have all these four constant regions and are designated as conventional PKCs. Novel PKC isoforms (δ, ε, η, θ), which lack a C2 domain, are activated independently of Ca^{2+} (*166, 167*). Atypical PKCs (ξ, τ, λ) possess incomplete C1 but no C2 regions and are also insensitive to Ca^{2+}, diacylglycerol and TPA (*168*).

Conventional PKCs are present dominantly in the cytoplasm in a basal or non-stimulated state. Increase in the intracellular Ca^{2+} concentration causes PKC activation and translocation to plasma membranes (*170*). The C2 region is considered to be responsible for the translocation. There is, however, one report demonstrating that translocation to the plasma membranes also occurs with nPKC in the presence of a phorbol ester (*169*), suggesting PKC translocation may not simply result from binding of Ca^{2+} to the C2 region. Several proteins which bind to PKC have been identified and suggested to determine its intracellular distribution with receptors for activated PKC (RACKs) being good examples (*170*). RACKs may be anchoring proteins not only for PKC but other C2 region proteins, first because they also bind to synaptotagmin and phospholipase C (*171, 172*), and secondly because RACK binding to synaptotagmin is displaced by PKC (*171*).

A wide variety of proteins are phosphorylated by PKC and this kinase has been suggested to participate in regulation of multiple cell functions such as cell cycle/differentiation, cell growth, carcinogenesis, endocytosis/exocytosis, muscle contraction, and gene expression (for reviews, see Refs. *173* and *174*).

Synaptotagmin (or synaptotagmin I) was originally known as the 65 kDa vesicle protein (p65) purified from brain synaptosome fractions (*175*). Although, as with S100 proteins, it was first believed to be neuron-specific, later studies proved that some synaptotagmin isoforms are also distributed in non-neural tissues like the lung, heart and endocrine system (*176-178*). Eight isoforms have been reported so far. They share a peculiar structure with two C2 domains, C2A and C2B, the former binding to both phospholipids and syntaxin, in a Ca^{2+}-dependent manner.

TABLE IV. **C2 region proteins.**

	M.W. (kDa)	Proposed functions
Conventional protein kinase C (α, β, γ)	76-78	Cell cycle/ differentiation, cell growth, carcinogenesis, endocytosis/exocytosis, muscle contraction, gene expression
Synaptotagmins	43-65	Exocytotic events
Cytosolic phospholipase A_2	85	Eicosanoid production
Phospholipase C (β, γ, and δ)	150-154 (β) 145-148 (γ) 85-88 (δ)	Generation of phospholipid-derived messengers
GAP	120	Modulation of cellular functions regulated by small G-proteins
Rabphilin 3A	78	Adaptor protein for vesicle transport

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Phospholipid binding requires much less Ca^{2+} (ED 50; low μM order) than that of syntaxin (200-500 μM), except in the case of synaptotagmins III and VII which bind syntaxin at lower than 10 μM Ca^{2+} (*179*). Synaptotagmins IV, VI, and VIII, however, are also exceptional in that they do not bind phospholipids or syntaxin. Synaptotagmins also interact with clathrin-AP2, but independently of Ca^{2+}. Differential roles of C2A and C2B have been suggested on the basis of experiments using selective antibodies (*180, 181*). C2A is responsible for release of transmitter by Ca^{2+} and C2B is involved in inositol high polyphosphates-dependent release and vesicle recycling. Synaptotagmins participate in the SNARE hypothesis whereby exocytosis occurs *via* sequential interaction of vesicle proteins (*182, 183*).

PLA_2 is an enzyme which catalyzes release of fatty acids from the *sn*-positions of phospholipids (*184*). Apart from a 14 kDa form (secreted PLA_2, $sPLA_2$), which is secreted from pancreas, there is another subtype in the cytoplasm ($cPLA_2$) (*185*). $cPLA_2$ and $sPLA_2$ do not share any common sequences in their primary structures and two classes of $cPLA_2$ (85 kDa and 40 kDa) have been identified. Eighty-five kilodalton $cPLA_2$ is activated by Ca^{2+} and translocates from cytosol to membranes (*186*), whereas 40 kDa $cPLA_2$ is activated in a Ca^{2+}-independent manner (*187, 188*, for a review, see Ref. *189*). Clark *et al.* pointed out that 85 kDa $cPLA_2$ possesses a C2 domain (called the CalB domain in their paper, see Ref. *160*) and suggested that this domain is common to proteins which are activated and translocate to membranes in response to an increase in intracellular Ca^{2+}. There is, however, one report suggesting that Ca^{2+} is required for intracellular translocation, but not for the enzyme activation (*190*). It has been also reported that other divalent cations may be substituted for Ca^{2+} to obtain the same effect (*191*).

The 85 kDa $cPLA_2$ activity is also modulated by G-proteins, and the enzyme preferentially catalyzes release of arachidonic acid (*184*), which is metabolized to bioactive eicosanoids. This Ca^{2+} binding protein is, therefore, considered to be responsible for production of prostaglandins, leukotrienes and platelet-activating factor, and also the resultant immune response.

The β, γ, and δ isoforms of phospholipase C (PLC) possess a similar structure to the C2 domain of PKC (*192, 193*). The C2 domain in PLCs is the binding site for Ca^{2+}, RACKS, and also the phospholipid substrate. It is rather surprising that structural analysis revealed the presence of an EF hand structure in the N-terminal region of PLCδ (*193*), since this was, to our knowledge, the first report of one protein containing two distinct Ca^{2+}-binding domains. PLCγ is reported to translocate to the cytoskeleton on activation of the EGF receptor (*194*). GTPase activating protein (GAP) also has a C2 domain and modulates the GTPase activity of GTP-binding proteins (*195*). Rabphilin 3A which binds the small G protein, Rab 3A, has similarly been discovered to possess a C2 domain (*196*). This protein can also bind Ca^{2+} and phospholipids at its C-terminal region (*197*) and controls intracellular vesicle transport *via* inhibition of Rab3A activation by GAP (*198, 199*). It has not yet been determined whether all these C2 region proteins undergo intracellular translocation in a Ca^{2+}-dependent manner.

3) Ca^{2+} storage proteins (Table V)

Elevation of the cytosolic Ca^{2+} level is finely controlled by Ca^{2+} fluxes through the plasma membrane and Ca^{2+} uptake/mobilization from intracellular store sites like endoplasmic reticulum and sarcoplasmic reticulum, as demonstrated by Ca^{2+}-imaging using Ca^{2+}-sensitive fluorescent dyes (*200*). Ca^{2+}, bound to Ca^{2+} storage proteins in the Ca^{2+} store sites, is released by Ca^{2+}-mobilizing cytoplasmic second messengers such as inositol-1,4,5-trisphosphate (IP_3) or cyclic ADP ribose *via* IP_3- or ryanodine-receptors, respectively.

Calsequestrin, localized in the sarcoplasmic reticulum, is the major Ca^{2+} storage protein in striated muscle (*201*), and group of analogous proteins have been identified in non-muscle and smooth muscle endoplasmic reticulum (*202*). Calreticulin is one major Ca^{2+} storage protein, originally identified from sarcoplasmic reticulum (*203, 204*), which was suggested to be closely related to calsequestrin because of immunocrossreactivity (*205*). Indeed, cDNA cloning demonstrated these proteins to have highly similar primary structures (*206, 207*) with an endoplasmic retention signal (KDEL) at their N-terminals. Several other possible Ca^{2+} storage proteins have also been identified (*208*). It seems that Ca^{2+} binds pairs of carboxyl residues preferentially distributed in the tail domains called the C-domains (*209*).

One of the features of these storage proteins is their high capacity for Ca^{2+}, and such high Ca^{2+}-binding capacity of these proteins enables the store sites to hold high concentrations of total (bound and free) Ca (approx. 5 mM, see Ref. *210*). As compared with the other groups of Ca^{2+} binding proteins, their affinity for Ca^{2+} binding is relatively low (0.5-5 mM) and some also have high affinity binding sites (*211*). However, low affinity Ca^{2+}-binding sites are primarily responsible for the high Ca^{2+}-binding capacity. For example, the high and low affinity binding sites of calreticulin (K_d values; 1 and 1,000 μM), are capable of binding to 1 and 25 Ca^{2+} ions per molecule, respectively (*211*). It is notable that two proteins with 6 EF hands (reticulocalbin and ERC-55), have been identified from

TABLE V. Ca^{2+} storage proteins.

	Synonyms	M.W. (kDa)	Calcium binding capacity (mol Ca^{2+} per mol)
Calsequestrin		41-44	42
Calreticulin	Calregulin, CaBP (Ca^{2+}-binding protein) 3, CRP (calcium binding reticuloplasmin) 55, CAB (Ca^{2+}-binding protein) 63, ERp (endoplasmic reticulum protein) 60, HACBP (high affinity calcium-binding protein)	46	26
Endoplasmin	CaBP4, ERp99, hsp90	100	10
CSLP (calsequestrin-like protein)	PDI (protein disulfide isomerase)	58	23

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endoplasmic reticulum (*212, 213*). Further studies are needed to examine whether these also play roles in Ca^{2+} storage.

Ca^{2+} storage in fact may not be the only function of the so-called Ca^{2+} storage proteins. Sea urchin eggs, where fertilization occurs in response to Ca^{2+} released from intracellular stores, express a Ca^{2+} storage protein (*214*) which is very similar to the human protein disulfide isomerase and the urchin protein exhibits the enzymatic activity (*215*). Moreover, there is evidence suggesting that calreticulin may function outside the sarcoplasmic reticulum and is found in both the cytoplasm and nucleus (*216, 217*). In the latter site, it interacts with nuclear receptors for androgen, retinoic acid, and glucocorticoids (*218, 219*). Calreticulin can also bind to the cytoplasmic domains of integrin α subunits (*220*). Therefore, calreticulin may modulate signal transduction through these receptors. Endoplasmin, another Ca^{2+} storage protein identified from endoplasmic reticulum, has also been reported to be localized in the nucleus (*221*), suggesting additional functions of this protein.

Concluding remarks

There are a large number of Ca^{2+} binding proteins, and this is especially the case of some of S100 proteins, for which the functions remain to be elucidated. It would clearly be promptious to conclude that all intracellular Ca^{2+} binding proteins play some role in Ca^{2+} signalling as Ca^{2+} receptors.

Although this could not be detailed in the present paper, one approach which is making rapid progress is the structural analysis of Ca^{2+}-binding protein molecules. This method provides clues to better understanding of interactions between Ca^{2+}-binding proteins and their target proteins, as well as specific pharmacological agents. Recently it has been proposed that the EF hand structure of the Ca^{2+} receptor proteins causes a vast conformational change as a result of Ca^{2+} binding, whereas such alterations are much smaller with Ca^{2+} buffering proteins (*4*). In the near future, it may become possible to predict whether a Ca^{2+}-binding protein functions as a Ca^{2+}-receptor or just a Ca^{2+} buffer by analysing its three dimensional structure.

As overviewed in this article, specific Ca^{2+} binding proteins are key components of signal transduction pathways, in some instances *via* modulation of protein interactions and/or their enzymatic activities. In spite of recent substantial progress, however, we have to acknowledge that our understanding of the functions of Ca^{2+} binding proteins is far from complete. We rely on more and more evidence being forthcoming to elucidate the mechanisms underlying transduction of Ca^{2+} signals at the molecular level.

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