

Calcium in Biological Systems

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I. INTRODUCTION

Calcium, like many other “inorganic elements” in biological systems, has during the last decade become the subject of much attention both by scientists and by the general public.¹ The presence and central role of calcium in mammalian bones and other mineralized tissues were recognized soon after its discovery as an element by Davy in 1808. Much later, the insight arrived that Ca^{2+} ions could play an important role in other tissues as well. Experiments of great historical influence were performed by the British physiologist Sidney Ringer a little over a century ago.² He was interested in the effects of various cations on frog-heart muscle and somewhat serendipitously discovered that Ca^{2+} ions, ever-present in the tap water distributed in central London, in millimolar concentrations were necessary for muscle contraction and tissue survival.

Today it is widely recognized that Ca^{2+} ions are central to a complex intracellular messenger system that is mediating a wide range of biological processes: muscle contraction, secretion, **glycolysis** and **gluconeogenesis**, ion transport, cell division and growth (for definitions of terms in boldface, see Appendix A in Section IX). The detailed organization of this messenger system is presently the subject of considerable scientific activity, and some details are already known. One of the links in the system is a class of highly homologous Ca^{2+} -binding proteins, to be discussed later on in this chapter, that undergo Ca^{2+} -dependent conformational changes and respond to transitory increases in intracellular Ca^{2+} -ion concentrations. A prerequisite for the proper function of the calcium messenger system in higher organisms is that the **cytosolic** Ca^{2+} concentration in a “resting” cell be kept very low, on the order of 100 to 200 nM. Transitory increases in the Ca^{2+} concentration that may result from hormonal action on a membrane receptor must rapidly be reduced. Several transport proteins, driven either by ATP hydrolysis or by gradients of some other ion like Na^+ , are involved in this activity.

Ca^{2+} ions are also known to play various roles outside cells. In the plant kingdom Ca^{2+} ions often form links between individual cells and are required for maintaining the rigidity of whole plants; some seaweeds are typical examples. In the blood plasma of mammals, in which the Ca^{2+} concentration exceeds the intracellular by a factor of about 10^4 , Ca^{2+} ions are instrumental in joining certain proteins in the blood-clotting system with membrane surfaces of circulating cells. Many extracellular enzymes also contain Ca^{2+} ions, sometimes at the active site but most often at other locations. It is generally believed that Ca^{2+} ions confer on proteins an increased thermal stability, and indeed proteins in heat-tolerant microorganisms often hold many such ions.

Vertebrates require much calcium in their food; in the USA the recommended daily allowance (RDA) for adult humans is 800 mg, and most other countries have comparable recommendations. During gestation in mammals, calcium must be transported across the placenta into the fetus, in particular during those phases of pregnancy when bone formation is most rapid. Interestingly, there appear to be some parallels between intestinal and placental transport that will be discussed further below. The role of calcium in biominerals is a vast subject that we can treat only superficially in this chapter.

To provide a background to the more biologically oriented sections that follow, we begin with a brief recapitulation of some basic facts about calcium. Then we continue with an outline of calcium distribution in biological tissues and organelles, and of the methods that can be used to obtain this information. After this follows a brief section on Ca^{2+} transport, and an account of the mechanism of intracellular Ca^{2+} release as it is presently understood. A discussion of some selected Ca^{2+} -binding proteins of general interest, both intracellular and extracellular, then follows. Before we conclude the chapter, we will summarize some recent observations on Ca^{2+} -binding proteins in prokaryotes.

II. BASIC FACTS ABOUT CALCIUM: ITS COMPOUNDS AND REACTIONS

A. Basic Facts

Calcium was first recognized as an element in 1808 by Humphry Davy, and the name was given after the Latin for lime: *calx*. Several isotopes of calcium are known. The stable isotopes are, in order of decreasing natural abundance, ^{40}Ca (96.94%), ^{44}Ca (2.1%), ^{42}Ca (0.64%), and ^{43}Ca (0.145%). ^{43}Ca is the only isotope with a nuclear spin ($I = \frac{7}{2}$) different from zero, which makes it amenable to NMR studies. ^{45}Ca is a radioactive isotope of some importance (β^- decay; 8.8 min half life).³ It has been used in studies of calcium localization and transport in biological systems.

Calcium constitutes about 3 percent by weight of the Earth's crust, mostly in the form of sedimentary rocks of biological origin dating back some three

Table 3.1
 Ca^{2+} concentrations in fluids and tissues.⁶⁻⁹

Specimen	Units are mM if not otherwise stated
Sea water	10
Fresh water	0.02–2
Rain water	0.002–0.02
“Hard” tap water	1.5
“Good” beer	4
Adult human serum	2.45 ± 0.05
Serum of other vertebrates	1.5–5
Nematode body fluids	6
Molluscan serum—marine	9–15
—fresh water	1.5–7.8
—land	3.3–12.3
Milk	70
Bone	0.8–1.0
Mitochondria from rat liver	0.8 ± 0.1 mmol/kg
Endoplasmatic reticulum	8–10 mmol/kg
Cytoplasm of a resting mammalian cell	0.0001
Cytoplasm of <i>E. coli</i>	0.0001

billion years. In sea water the total concentration of calcium ranges from 5 to 50 times higher than in fresh water, which, in turn, has a calcium concentration ten times that of rain water (see Table 3.1). This explains the pleasant feeling when ordinary soaps are used in rain water. The calcium concentration in ordinary tap water varies with location; calcium is usually added to water in distributing networks in order to prevent corrosion of iron pipes. Tap water with a calcium concentration above 1.5 mM is usually classified as “hard.” Interestingly, the taste of beer seems related to the calcium concentration, and it is claimed that “good” beer should have a concentration higher than that of “hard” tap water.

In the body fluids of higher organisms the total calcium concentration is usually on the order of a few millimolar (see Table 3.1). In adult human serum, the concentration is observed to be, within narrow limits, 2.45 mM.

B. Essentials of Ca^{2+} Chemistry

Since the Ca^{2+} ion accomplishes its biological tasks in an environment with 1 to 3 mM Mg^{2+} , it is of particular interest to compare the properties of these two ions in order to understand how a discrimination is made in biological systems. In addition, the coordination chemistry of Ca^{2+} is closely related to that of Mg^{2+} (as well as Cd^{2+}), though there are several obvious differences. First of all, the ionic radius of a Ca^{2+} ion with a given coordination number (CN) is always higher than that of an Mg^{2+} or Cd^{2+} ion with the same CN.

At $CN = 6$, the ionic radii of Ca^{2+} , Cd^{2+} , and Mg^{2+} are 1.00, 0.95, and 0.72 Å, respectively, whereas at $CN = 8$ they are 1.12, 1.10, and 0.89 Å, respectively.⁴

Ligand preferences of Ca^{2+} depend on the fact that it is a hard metal ion. Thus Ca^{2+} strongly prefers oxygen ligands over nitrogen or sulfur ligands; $Ca^{2+} \cdots N$ bonds are about 0.25–0.3 Å longer than $Ca^{2+} \cdots O$ bonds.^{5,6,10} Large differences in coordination number and geometry have been observed for Ca^{2+} complexes. In a study of 170 x-ray structures of Ca^{2+} complexes involving carboxylate groups,¹¹ binding was found to be either (i) unidentate, in which the Ca^{2+} ion interacts with only one of the two carboxylate oxygens, (ii) bidentate, in which the Ca^{2+} ion is chelated by both carboxylate oxygens, or (iii) mixed (“ α -mode”) in which the Ca^{2+} ion is chelated by one of the carboxylate oxygens and another ligand attached to the α -carbon (see Figure 3.1). The Ca^{2+} -oxygen distances span a range from 2.30 to 2.50 Å, with the average distance being 2.38 Å in the unidentate and 2.53 Å in the bidentate mode, respectively.¹¹ Observed coordination numbers follow the order $8 > 7 > 6 > 9$. By contrast, Mg^{2+} nearly always occupies the center of an octahedron of oxygen atoms ($CN = 6$) at a fixed Mg^{2+} -oxygen distance of 2.05 ± 0.05 Å.

In Table 3.2, stability constants for the binding of Ca^{2+} and Mg^{2+} to various ligands are collected. We may note that selectivity of Ca^{2+} over Mg^{2+} is not very great for simple carboxylate ligands, but that it tends to increase for large multidentate ligands, such as EDTA and in particular EGTA. The Ca^{2+} sites in many intracellular proteins with “EF-hand” binding sites (see Section V. C) bind Ca^{2+} about 10^4 times more strongly than Mg^{2+} .

Another difference in ligand-binding properties of Mg^{2+} and Ca^{2+} can be seen by comparing the rates of substitution of water molecules in the inner

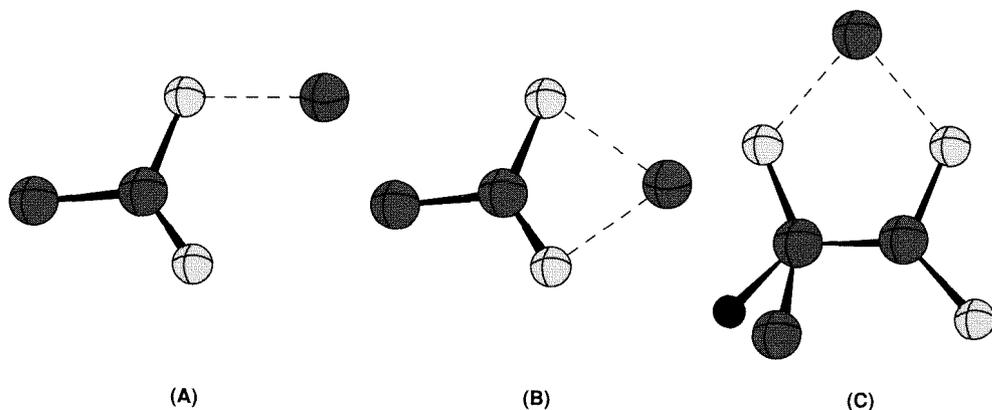


Figure 3.1

The three commonly observed modes of calcium carboxylate ligation. (A) The unidentate mode, in which the calcium ion interacts with only one of the two carboxylate oxygens. (B) The bidentate mode, in which the calcium ion is chelated by both oxygen atoms. (C) The α -mode, in which the calcium ion is chelated by one carboxylate oxygen, and another ligand is attached to the α -carbon. Adapted from Reference 11.

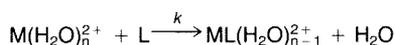
Table 3.2
 Ca^{2+} and Mg^{2+} (where available) stability constants ($\log K$) for different organic and biochemical ligands. Most values are at ionic strength 0.1 and 25°C .^{5,6,12-15}

Ligand	Ca^{2+}	Mg^{2+}
Acetate	0.5	0.5
Lactate	1.1	0.9
Malonate	1.5	2.1
Aspartate	1.6	2.4
Citrate	3.5	3.4
Nitrilotriacetate	6.4	5.5
EGTA ^a	10.9	5.3
EDTA ^b	10.6	8.8
Glycine (Gly)	1.4	3.4
γ -Carboxyglutamic acid (Gla)	1.3	
Gly-Gly dipeptide	1.2	
Gla-Gla dipeptide	3.2	
Macrobicyclic amino cryptate [2.2.2]	4.5	
Fluo-3	6.2	2.0
Fura-2	6.9	2.0
BAPTA	7.0	1.8
Quin-2	7.1	2.7
Phospholipase A2	3.6	
Thrombin fragment 1	3.7	3.0
Trypsinogen	3.8	
Chymotrypsinogen	3.9	
Chymotrypsin	4.1	
Calmodulin, N-terminal	4.5	3.3
Trypsin	4.6	
Calmodulin, C-terminal	5.3	
Protein kinase C	~7	
α -Lactalbumin	~7	
Rabbit skeletal muscle		
Troponin C, $\text{Ca}^{2+}/\text{Mg}^{2+}$ sites	7.3	3.6
Carp parvalbumin	~8.5	4.2
Bovine calbindin D_{9k}	8.8	~4.3

^a EGTA: ethylenebis(oxyethylenenitrilo)tetraacetate

^b EDTA: ethylenedinitrilotetraacetate

hydration sphere by simple ligands, according to



This rate ($\log k$, with k in s^{-1}) has been determined to be 8.4 for Ca^{2+} and 5.2 for Mg^{2+} .¹⁶

The formation of biominerals is a complex phenomenon. In order to obtain a feeling for the conditions under which inorganic solid phases in biological systems are stable, it is of some interest to look at solubility products. Solubility products, K_{sp}° , have a meaning only if the composition of the solid phase is specified. For a solid compound with the general composition $(A)_k(B)_l(C)_m$ the solubility product is defined as

$$K_{sp}^{\circ} = [A]^k[B]^l[C]^m, \quad (3.1)$$

where $[A]$, $[B]$, etc., denote activities of the respective species, usually ionic, in equilibrium with the solid. Activities are concentrations multiplied by an activity coefficient, γ , nearly always less than unity. Activity coefficients for ions in real solutions can be estimated from Debye-Hückel theory¹⁷ if the ionic strength of the solution is known. In human blood plasma, the ionic strength, I , is about 0.16, and the activity coefficient for Ca^{2+} at 37°C is 0.34. In many discussions it may be sufficient to equate concentrations with activities.

The solid phase involved is essentially assumed to be an infinitely large, defect- and impurity-free crystal with a well-defined structure. Microscopic crystals have higher solubilities than large crystals, a well-known phenomenon that leads to "aging" of precipitates, in which larger crystals grow at the expense of smaller ones.

Many anionic species appearing in the solubility products may also be involved in protonation equilibria in solution, such as those of phosphoric acid: $\text{H}_2\text{PO}_4^- \rightleftharpoons \text{H}^+ + \text{HPO}_4^{2-}$; $\text{HPO}_4^{2-} \rightleftharpoons \text{PO}_4^{3-} + \text{H}^+$; etc. When the prospects for the formation of a solid phase under certain solution conditions are investigated, the activity, or concentration, of the particular anionic species specified in the solubility product must be known, not only "total phosphate" or "total calcium," etc. The data in Table 3.3 show that, at $\text{pH} > 5$, the most stable (i.e., insoluble) solid calcium phosphate is hydroxyapatite.

Table 3.3
Solubility products, at $\text{pH} 5$ and 25°C , for solid calcium phosphates.

Solid phase	$-\log K_{sp}^{\circ}$	$-\log K_{sp}^{\circ}$ of corresponding Mg^{2+} compound where applicable
$\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ (sulfate, "gypsum")	5.1	< 1.0
$\text{Ca}(\text{OH})_2$ (hydroxide)	5.3	10.7
$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ (hydrogen phosphate)	6.6	—
CaCO_3 (carbonate, "calcite," "aragonite")	8.5	7.5
$\text{CaC}_2\text{O}_4 \cdot \text{H}_2\text{O}$ (oxalate, "whewellite")	10.5	5.0
$\beta\text{-Ca}_3(\text{PO}_4)_2$ (β -phosphate)	29	—
$\text{Ca}_5(\text{PO}_4)_3\text{OH}$ (hydroxyapatite)	58	—

III. CALCIUM IN LIVING CELLS: METHODS FOR DETERMINING CONCENTRATIONS AND SPATIAL DISTRIBUTIONS

Much of our present knowledge of the biological role of Ca^{2+} ions in the regulation and modulation of cellular activities rests on the development of analytical techniques in three different areas: our ability to measure the low concentration levels in the cytoplasm of resting cells, follow the concentration changes, both temporally and spatially, that may occur as a result of an external stimulus, and measure the distribution of Ca^{2+} in various compartments of a cell. The last decade has seen the emergence of many such new techniques, and the improvement of old ones, which has had a major impact on our understanding of the detailed molecular mechanisms and dynamics of the Ca^{2+} messenger system. In this section, we will survey some of the most important techniques and results obtained using these. Broadly speaking there are two main groups of experimental techniques: those that aim at measuring the concentration of “free” (or uncomplexed) Ca^{2+} -ion concentrations (or activities), and those that measure *total calcium*.

A. Measurements of “Free” Calcium Concentrations

1. Ca^{2+} -selective microelectrodes

Ion-selective electrodes can be made from a micropipette (external diameter 0.1–1 μm) with an ion-selective membrane at the tip.^{18,19} For Ca^{2+} the membrane can be made of a polyvinyl chloride gel containing a suitable Ca^{2+} -selective complexing agent soluble in the polymer gel. A commonly used complexing agent is “ETH 1001” (see Figure 3.2A). An additional “indifferent” reference electrode is needed. For measurements inside cells, the reference electrode can also be made from a micropipette filled with an electrolyte gel. Often the ion-selective and reference electrodes are connected in a double-barrelled combination microelectrode.²¹ The whole assembly can then be inserted, using a micromanipulator, into a single cell typically 30–50 μm across. The arrangement is depicted in Figure 3.2B. With proper care, Ca^{2+} microelectrodes can be used to measure Ca^{2+} -ion concentrations down to 10^{-8} M.^{19, 21} One limitation of the technique is that the response time is usually in seconds or even minutes, making rapid concentration transients difficult to follow.

2. Bioluminescence

Several living organisms are able to emit light. The light-emitting system in the jellyfish (*Aequorea*) is a protein called aequorin ($M_r \approx 20$ kDa). The light is emitted when a high-energy state involving a prosthetic group (coelenterazine) returns to the ground state in a chemical reaction that is promoted by Ca^{2+}

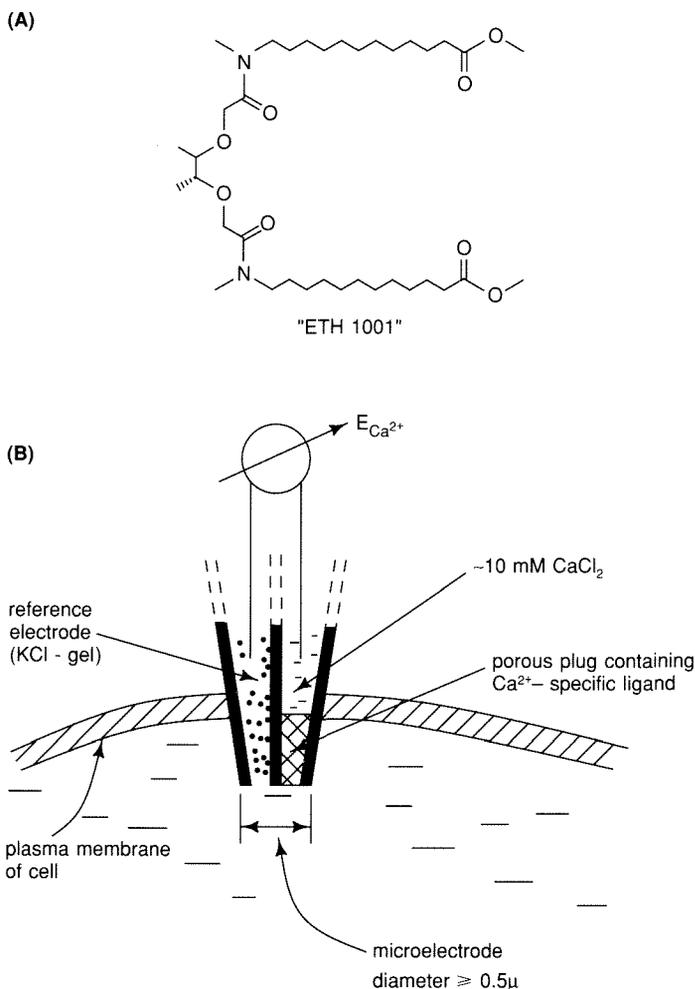


Figure 3.2

(A) Structure of a commonly used neutral Ca^{2+} chelator in Ca^{2+} -selective electrodes, "ETH 1001" (N,N'-di[11-(ethoxycarbonyl) undecyl]-N,N'-4,5-tetramethyl-3,6-dioxaoctan-1,8-diamide).²⁰ (B) Schematic arrangement for the measurement of the activity (or concentration) of Ca^{2+} ions in cells using a Ca^{2+} -selective double-barreled microelectrode. Frequently the microelectrode is supplemented by a third, indifferent, electrode inserted into the bathing medium surrounding the cell.²¹

ions. At Ca^{2+} concentrations below $\sim 0.3\ \mu\text{M}$ the emission is weak, but in the range $0.5\text{--}10\ \mu\text{M}$ the emission is a very steep function of the concentration (roughly as $[Ca^{2+}]^{2.5}$).^{18,19,22} The response to a Ca^{2+} -concentration transient is rapid ($\tau_{1/2} \approx 10\ \text{ms}$ at room temperature), and the light emitted can be accurately measured even at very low light levels by means of image intensifiers and/or photon counting. For measurements of Ca^{2+} concentrations inside cells, aequorin has usually been introduced either through microinjection or through some other means. A novel idea, however, is to utilize recombinant aequorin reconstituted within the cells of interest, thus circumventing the often difficult injection step.¹⁷⁴

3. Complexing agents with Ca^{2+} -dependent light absorption or fluorescence

An important advance in the field of Ca^{2+} -ion determination was made by R. Y. Tsien, who in 1980 described²³ the synthesis and spectroscopic properties of several new tetracarboxylate indicator dyes that had high affinity and reasonable selectivity for Ca^{2+} . All these dye molecules have a high UV absorbance that is dependent on whether Ca^{2+} is bound or not; a few also show a Ca^{2+} -dependent fluorescence. Tsien has also demonstrated that these anionic chelators can be taken up by cells as tetraesters, which, once inside the cells, are rapidly enzymatically hydrolyzed to give back the Ca^{2+} -binding anionic forms. Fluorescent tetracarboxylate chelators with somewhat improved Ca^{2+} selectivity such as "BAPTA," "Quin-2," and "Fura-2" (Figure 3.3) were later described.²⁴ These chelators are very suitable for measurement of Ca^{2+} -ion concentrations in the range 1 μM to 10 nM in the presence of 1 mM Mg^{2+} and 100 mM Na^+ and/or K^+ —i.e., conditions typically prevailing in animal cells. Recently a new set of chelators that are more suitable for measurements of calcium concentrations above 1 μM was presented.²⁵ The most interesting of these is "Fluo-3," with a calcium-binding constant of 1.7×10^6 .

Whereas the emission spectrum for Fura-2 (Figure 3.3B), which peaks at 505–510 nm, hardly shifts wavelength when Ca^{2+} is bound, the absorption spectrum shifts toward shorter wavelengths. In studies of free Ca^{2+} concentrations where internal referencing is necessary, for example, in studies of single cells, it is therefore advantageous to excite alternately at ~ 350 and 385 nm, and to measure the ratio of fluorescence intensity at ~ 510 nm.

The use of fluorescent chelators has recently permitted studies in single cells of rapid fluctuations or oscillations of free Ca^{2+} and the formation of Ca^{2+} concentration gradients. Using a fluorescence microscope coupled to a low-light-level television camera feeding a digital image processor, Tsien *et al.*²⁶ have been able to reach a time resolution of about 1 s in single-cell studies. The results of some highly informative studies made using this instrument are shown in Figure 3.4. (See color plate section, page C-7.) The concentration of free Ca^{2+} is presented in pseudocolor, and the Fura-2 concentration inside cells is 50–200 μM , as indicated in the figures. We see a Ca^{2+} gradient diffusing through an entire sea-urchin egg ($\sim 120 \mu$ across) in 30 s. The free Ca^{2+} concentration of the resting egg (~ 100 nM) is increased to about 2 μM as Ca^{2+} diffuses through the egg. The mechanism of propagation is believed to be a positive feedback loop with inositol trisphosphate releasing Ca^{2+} and vice versa (see Section V).

A pertinent question concerning the uses of intracellular Ca^{2+} chelators is whether or not the chelator significantly perturbs the cell. The chelator will obviously act as a Ca^{2+} buffer in addition to all other Ca^{2+} -binding biomolecules in the cell. The buffer effect is probably not of any major consequence, since the cell may adjust to the new situation by an increase in total Ca^{2+} , especially if the chelator concentration is in the μM range. The chelators could, however, interact with and inhibit intracellular enzymes or other molecules, an

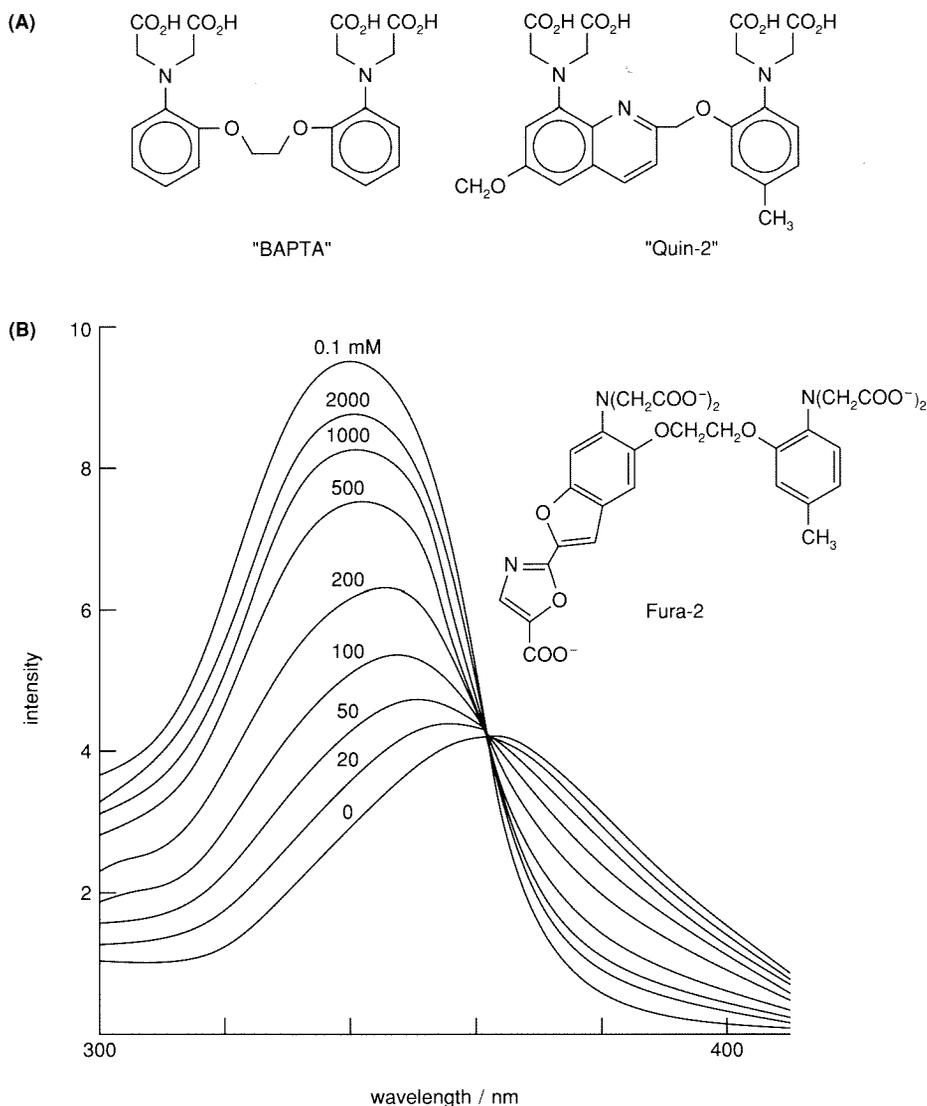


Figure 3.3

Molecular structure of three chelators frequently used in measurements of "free" Ca^{2+} -ion concentrations. They may all be regarded as aromatic analogues of the classical chelator "EDTA"; their optical spectroscopic properties change upon binding Ca^{2+} ions. (A) For "BAPTA" the spectral changes are confined to the absorption spectrum, whereas "Quin-2" and the "Fura-2" in (B) show Ca^{2+} -dependent changes in their fluorescence spectra.^{23, 24} (B) The relative fluorescence intensity of "Fura-2" at 505 nm as a function of the wavelength of the excitation light at different Ca^{2+} concentrations. The data²⁶ refer to a solution containing 115 mM KCl, 20 mM NaCl, and 1 mM Mg^{2+} at 37°C and pH 7.05. At increasing Ca^{2+} concentration, the excitation efficiency at ~ 350 nm is increased, but that at ~ 385 nm is decreased. In order to eliminate (as much as possible) variations in fluorescence intensity in biological samples due to slight variations in dye concentrations and/or cell thickness, it is often advantageous to measure the intensity ratio at 345 and 385 nm excitation wavelengths.

effect that could result in aberrant cellular behavior. It is not unlikely that BAPTA will bind to certain proteins.²⁷

4. Complexing agents with Ca^{2+} -dependent NMR spectra

A series of symmetrically substituted fluorine derivatives of BAPTA (see Figure 3.3A) has been synthesized.^{28,29} One of these chelators is 5F-BAPTA (Figure 3.5A), which has a binding constant for Ca^{2+} , K_B^{Ca} , of $1.4 \times 10^6 \text{ M}^{-1}$ and a ^{19}F NMR chemical shift, δ , that in the free ligand is different from that in the complex with Ca^{2+} ($\Delta\delta_{\text{Ca}^{2+}} \approx 6 \text{ ppm}$). The rate of Ca^{2+} dissociation, k_{off} , is $5.7 \times 10^2 \text{ s}^{-1}$, which gives the rate of association, k_{on} , as $8 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ according to

$$K_B = k_{\text{on}}/k_{\text{off}}. \quad (3.2)$$

This exchange rate means that we are approaching the slow exchange limit in ^{19}F NMR, and in subsaturating concentrations of Ca^{2+} two ^{19}F signals are seen (see Figure 3.5B).

Since the areas of the NMR signals from the bound (B) and free (F) forms of the ligand are proportional to their concentration, the free Ca^{2+} concentration

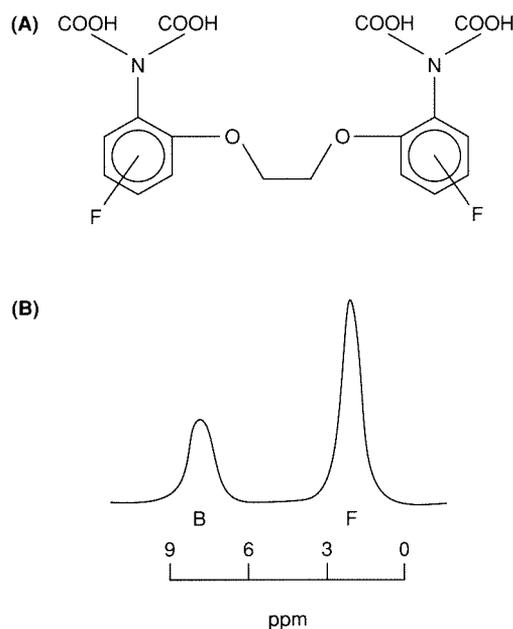


Figure 3.5

(A) Molecular structure of the calcium chelator 5F-BAPTA whose ^{19}F chemical shift changes upon calcium binding. (B) ^{19}F NMR spectrum of a solution containing 5F-BAPTA and Ca^{2+} in a molar ratio of 3:1. Signal B originates from the Ca^{2+} -5F-BAPTA complex, and F from free 5F-BAPTA. Adapted from Reference 29.

is obtained simply as

$$[\text{Ca}^{2+}]_{\text{free}} = \frac{B}{F} \cdot \frac{1}{K_B}. \quad (3.3)$$

An additional beneficial property of 5F-BAPTA and other fluorinated analogues of BAPTA is that they will also bind other metal ions with a ^{19}F chemical shift of the complex that is characteristic of the metal ion.²⁹ Under favorable circumstances, it is thus possible to measure simultaneously the concentrations of several cations.

For 5F-BAPTA the selectivity for Ca^{2+} over Mg^{2+} is very good ($K_B^{\text{Mg}^{2+}} \approx 1 \text{ M}^{-1}$). In applications of 5F-BAPTA to intracellular studies, the same protocol is used as with the parent compound and its fluorescent derivatives: some esterified derivative, e.g., the acetoxymethyl ester, is taken up by the cells and allowed to hydrolyze in the cytoplasm. The intracellular concentrations of 5F-BAPTA needed to get good ^{19}F NMR signals depend on the density of cells in the sample tube and the number of spectra accumulated. With accumulation times on the order of ten minutes (thus precluding the observation of concentration transients shorter than this time), Ca^{2+} concentrations of the order of $1 \mu\text{M}$ have been studied in perfused rat hearts using 5F-BAPTA concentrations of about $20 \mu\text{M}$.³⁴

B. Measurements of Total Calcium Concentrations

The measurement of total calcium in a biological sample can be made by any method sensitive only to the element and not to its particular chemical form. Atomic absorption spectroscopy is excellent as such a method. Obviously, the spatial resolution that can be obtained with this method is limited, and it is hard to imagine its application to elemental mapping of single cells. The techniques discussed in this subsection have been limited to those that permit a spatial resolution of at least $1 \mu\text{m}$ on samples usually prepared by sectioning the frozen biological specimens.

1. Electron probe and electron energy-loss techniques

When the electron beam in an electron microscope hits a thin sample, some atoms in the sample will be excited or ionized, and returning to their ground state will emit characteristic x-rays. The x-ray emission at different wavelengths may then be measured by a photon-energy-sensitive detector. This is the basis of *electron probe x-ray microanalysis* (EPMA). The electrons that pass through the sample, and that give the transmission image in electron microscopy, will suffer energy losses that depend on the nature (to some extent also, the chemical state) and distribution of different elements. The outcome of these phenomena forms the basis of *electron energy-loss spectroscopy* (EELS; see Figure 3.6).

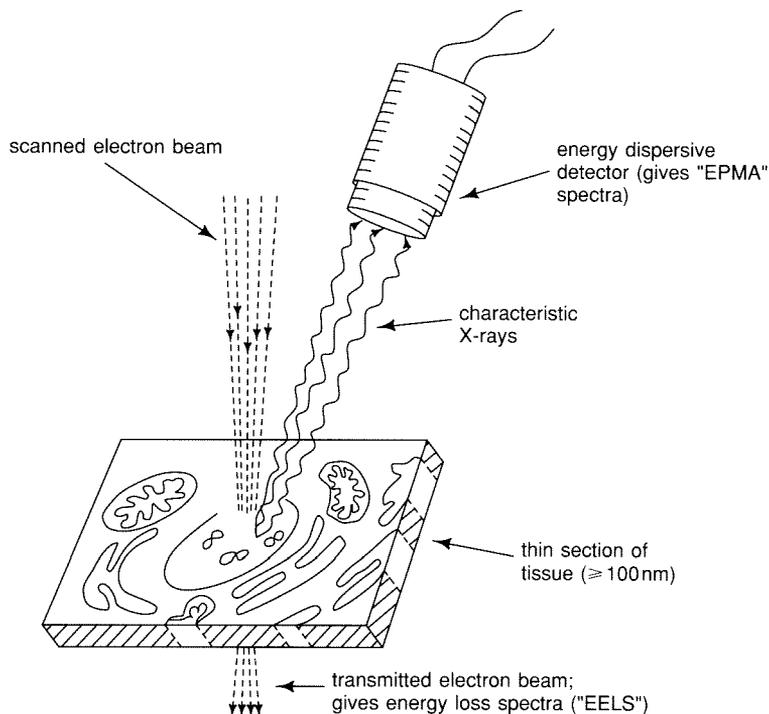


Figure 3.6
Principles of *electron probe microanalysis* ("EPMA") and *electron energy loss spectroscopy* ("EELS").

A thin slice of a freeze-dried sample is exposed to a collimated beam of electrons that may be scanned across the sample. Atoms ionized by electron collisions will emit x-radiation at wavelengths characteristic of their nuclear charge (i.e., characteristic of each element). With the use of an energy dispersive x-ray detector, signals from different elements can be differentiated. Apart from the characteristic x-rays, a broad-spectrum background x-ray emission is also present because of inelastic scattering of the incident electrons. Some of the electrons that pass through the sample will have lost energy because of ionization of atoms in the sample. The energy loss is again characteristic for each element, and if the energy distribution of the transmitted electrons is analyzed, this will have "peaks" at certain characteristic energies. If the energy resolution is pushed far enough (<1 eV), the loss peaks even become sensitive to the chemical state of the element.

The EPMA technique as applied to calcium has been improved by Somlyo in particular.³⁰ Typically samples are rapidly frozen and sectioned at low temperatures (-130°C) to preserve the *in vivo* localization of diffusible ions and molecules. Spatial resolutions of 10 nm or better have been attained on ≥ 100 nm thick freeze-dried cryosections. The minimal detectable concentration, which requires some signal averaging, is approximately 0.3 mmol Ca per kg dry specimen (i.e., 10 ppm). The calcium content of **mitochondria** and **endoplasmic reticulum** in rat liver cells has been studied by EPMA (see Table 3.1).⁸

The high calcium content of endoplasmic reticulum (ER) is consistent with the view that this organelle is the major source of intracellular Ca^{2+} released

through the messenger inositol trisphosphate (see Section IV.C). Other EPMA studies have shown mitochondria to have a large capacity for massive calcium accumulation in cells where cytoplasmic Ca^{2+} concentrations have been abnormally high, for example, as a result of damage of the cell membrane.³⁰

EELS is presently less well-developed than EPMA. Two of the major difficulties in the use of EELS for quantitative analysis of calcium and other elements are (i) large background, since it is a difference technique, and (ii) sensitivity to specimen thickness. The major advantage of EELS is that the spatial resolution is potentially much better than in EPMA, and can be 1 to 2.5 nm in favorable specimens.

2. Proton-induced x-ray emission (PIXE)

A specimen exposed to a beam of high-energy (1 to 4 MeV) protons will also emit characteristic x-rays just as in EPMA. The advantage of using protons instead of electrons is that protons are more likely to collide with an atom, thus producing excited atoms emitting x-rays. The sensitivity in detecting a particular element is therefore much higher in PIXE than in EPMA or EELS. The PIXE technique, which was developed at the University of Lund, Sweden, in the late 1960s, was originally used mainly for studies of fairly large objects.⁹

In 1980 a group at Oxford University succeeded in focusing the proton beam to a diameter of 1 μm with sufficient energy (4 MeV) and beam intensity (100 $\text{pA}/\mu\text{m}^2$) to allow elemental mapping at ppm concentrations.³¹ Similar beam performances ($\sim 0.5\mu\text{m}$ diameter) are now also available at the University of Lund and other laboratories. Beam diameters of 0.1 μm are likely to be achieved in the near future. Like EPMA, the PIXE method allows the simultaneous observation of several elements in the same sample. The biological applications of the microbeam PIXE technique are limited, but it is clear that its potential is great. Some representative results obtained with the Oxford microbeam are shown in Figure 3.7. (*See color plate section, page C-8.*)

3. Ion microscopy

Ion microscopy is another technique capable of detecting all elements at the ppm level. The basic idea is to expose a freeze-fixed, cryofractured, and freeze-dried sample, which has been put onto a conducting substrate in a vacuum chamber, to a beam of ions (e.g., D_2^+ or Ar^+). These ions will remove the top two or three atomic layers of the sample surface by sputtering. A certain fraction of the removed atoms will leave as ions. This secondary ion beam is accelerated into a double-focusing mass spectrometer, where the ions are separated according to their mass-to-charge ratio. The ion optics are designed to preserve the spatial distribution of the emitted secondary ions, and an element image of the sample can thus be produced with a spatial resolution of $\sim 0.5\mu\text{m}$.³² The ion-microscope technique can form images of a particular isotope of an element. In principle, then, one could perform isotope labeling or "isotope chase" studies

and follow, say, the fate of isotope-enriched ^{43}Ca externally applied to a cell. The ion-microscope technique has not yet come into widespread use, but the quality of element (or ion) images obtained on single cells is impressive.³³

C. Summary

Much of our present knowledge about the biological role of Ca^{2+} rests on detailed measurements of the concentration, distribution, and chemical nature of Ca^{2+} and its complexes. Concentrations of uncomplexed, or "free," Ca^{2+} can be measured by Ca^{2+} -selective microelectrodes, bioluminescence and complexing agents with Ca^{2+} -dependent light absorption, fluorescence, or NMR spectra. An outcome of such studies is that the "free" Ca^{2+} concentration in resting **eukaryotic** cells generally is very low, on the order of 100 to 200 nM. Total Ca^{2+} concentrations, uncomplexed and complexed, can be measured by a variety of physical techniques. Some techniques, like atomic absorption, are sensitive but give poor spatial resolution. Others involve the bombardment of the sample with electrons or charged atoms, and can yield spatial resolutions of the order of a few nm; however, there is a trade-off between detectability and resolution.

IV. THE TRANSPORT AND REGULATION OF Ca^{2+} IONS IN HIGHER ORGANISMS

All living organisms need calcium, which must be taken up from the environment. Thus, Ca^{2+} ions have to be distributed throughout the organism and made available where needed. In higher organisms, such as humans, the blood-plasma level of total calcium is kept constant (≈ 2.45 mM) within narrow limits, and there must be a mechanism for regulating this concentration. On a cellular level we have already seen in the preceding section that the basal cytoplasmic Ca^{2+} concentration, at least in eucaryotic cells, is very low, on the order of 100 nM. At the same time the concentrations of Ca^{2+} in certain organelles, such as endoplasmic (or sarcoplasmic) reticulum or mitochondria, may be considerably higher. If Ca^{2+} ions are to be useful as intracellular "messengers," as all present evidence has it, Ca^{2+} levels in the cytoplasm would have to be raised transiently as a result of some stimulus. Ca^{2+} ions may enter the cytoplasm either from the extracellular pool or from the Ca^{2+} -rich organelles inside the cell (or both). We could imagine Ca^{2+} channels being regulated by chemical signaling, perhaps by a hormone acting directly on the channel, or by a small molecule released intracellularly when a hormone is attached to a membrane-bound receptor. Some channels may be switched on by voltage gradients, and both these mechanisms may operate concurrently.

Increased intracellular Ca^{2+} levels must eventually be brought back to the basal levels, in some cells very quickly. The ions could be transported out of the cell or back into the Ca^{2+} -rich organelles. This transport will be against an

electrochemical potential gradient, and thus requires energy. There are many possibilities for different forms of Ca^{2+} transport and regulation in living systems, and we still know fairly little about the whole picture. Detailed studies are also complicated by the fact that, in higher organisms, cells are differentiated. Nature is multifarious, and what is valid for one type of cell may not be relevant for another. With these words of caution we will start out on a macroscopic level and continue on toward molecular levels.

A. Ca^{2+} Uptake and Secretion

The uptake of Ca^{2+} from food has mostly been studied in typical laboratory animals, such as rats, hamsters, chickens, and humans. In humans, uptake occurs in the small intestine, and transport is regulated by a metabolite of vitamin D, calcitriol (1,25-dihydroxy vitamin D_3).³⁴ The uptake process is not without loss; roughly 50 percent of the calcium content in an average diet is not absorbed. To maintain homeostasis and keep the calcium level in blood plasma constant, excess Ca^{2+} is excreted through the kidney. The main factor controlling this phenomenon in vertebrates is the level of the parathyroid hormone that acts on kidney (increases Ca^{2+} resorption), on bone, and, indirectly, via stimulated production of calcitriol, on the intestinal tract (increases Ca^{2+} uptake). Calcium enters the cells from the outside world, i.e., the intestinal lumen, by traveling through the brush-border membrane of the intestinal **epithelial cells**, through the cytosolic interior of these cells, and into the body fluids through the **basal lateral membranes** of the same cells. The molecular events involved need to be studied further. Figure 3.8 outlines the Ca^{2+} transport processes known or thought to occur.

Transfer through the brush-border membrane is assumed to be “passive” although indirectly facilitated by calcitriol. The calcitriol effect may be due to synthesis of a carrier protein,³⁵ but could also be an effect of altered membrane lipid composition.³⁶ The fate of Ca^{2+} ions, once inside the epithelial cell, is a much-debated subject. What appears clear is that the Ca^{2+} ions entering through the brush-border membrane do not cause an increase of the low cytosolic Ca^{2+} concentration. It is thus quite likely that the Ca^{2+} ions are carried through the cell but the means of transportation is unknown. One plausible carrier is the intracellular low-molecular-weight Ca^{2+} -binding protein calbindin $\text{D}_{9\text{K}}$ ($M_r \approx 9$ kDa) formerly known as ICaBP (see Section V.C).³⁵ Its synthesis is induced by vitamin D, and it is mainly found in mammalian intestines. The porcine and bovine calbindin $\text{D}_{9\text{K}}$ has a Ca^{2+} binding constant of $K_B \approx 3 \times 10^8 \text{ M}^{-1}$ in low ionic strength media³⁷ and $K_B \approx 2 \times 10^6 \text{ M}^{-1}$ in the presence of 1 mM Mg^{2+} and 150 mM K^+ .³⁸ The concentration of calbindin $\text{D}_{9\text{K}}$ in epithelial cells can reach millimolar levels,³⁵ which could allow it to facilitate Ca^{2+} diffusion across the cytosol. This was first suggested by Williams, subsequently elaborated by Kretsinger *et al.* in 1982,³⁹ and later demonstrated in a model cell by Feher.⁴⁰ The basic idea is that, although the diffusion rate of Ca^{2+} ions ($\sim 10^{-5} \text{ cm}^2 \text{ s}^{-1}$) is higher than for the $(\text{Ca}^{2+})_2$ calbindin complex

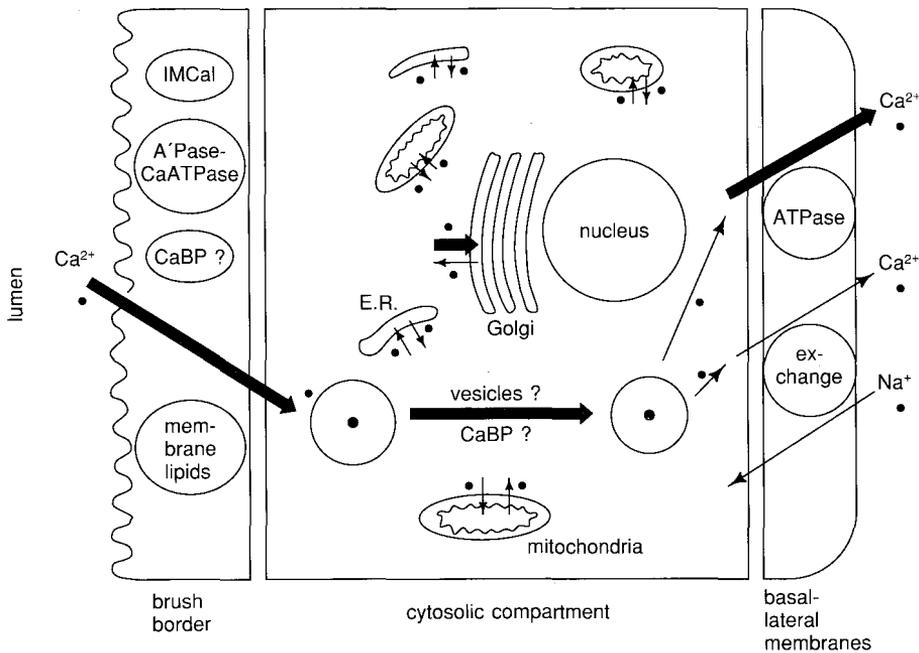


Figure 3.8

A scheme representing some of the known and hypothetical molecular participants in the transport of Ca^{2+} across intestinal epithelial cells. Transport across the brush-border membrane is generally assumed to be passive or to be facilitated by a carrier (IM Cal), and is also influenced by vitamin D. Transport through the cell may be in vesicles and/or in association with Ca^{2+} -binding proteins (CaBP), notably calbindins $\text{D}_{9\text{k}}$ (mammals) or $\text{D}_{28\text{k}}$ (avians). Temporary storage or buffering of Ca^{2+} may be through cytosolic CaBPs, mitochondria, endoplasmic reticula (ER), or other organelles. Transport of Ca^{2+} out of the cell through the basal-lateral membranes is energetically uphill, and appears primarily accomplished by a Ca^{2+} -ATPase and possibly to some extent by a Na^{2+} - Ca^{2+} antiport. Adapted from Reference 35.

($\sim 0.2 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$), the fact that the concentration of the latter complex may be about 10^3 times higher than that of free Ca^{2+} will result in an increased net calcium transport rate. Calbindin would, in fact, act very much like myoglobin in facilitating oxygen transport through muscle tissue.

Plausible as the above mechanism may seem, it may, however, not be the whole truth. An alternative mechanism is vesicular transport. In chicken intestine it has been shown that the only epithelial organelles that increased in Ca^{2+} content as a result of calcitriol treatment were the lysosomes.⁴¹ The result lends support to a transport mechanism involving Ca^{2+} uptake across the brush-border membrane by **endocytic vesicles**, fusion of these vesicles with lysosomes, and possibly also delivery of Ca^{2+} to the basal lateral membrane of the epithelial cell by **exocytosis**. This process would also explain the vitamin-D-induced alterations in brush-border-membrane lipid compositions as a consequences of preferential incorporation of certain types of lipids into the vesicles. Interestingly, the lysosomes in the chicken studies also contained high levels of calbin-

din D_{28k} —a type of vitamin-D-induced Ca^{2+} -binding protein found in avian intestines—making it conceivable that this protein acts as a “receptor” for Ca^{2+} at the brush-border membrane and upon Ca^{2+} binding could become internalized in endocytic vesicles.⁴¹

The basal lateral plasma membrane contains at least two types of Ca^{2+} pumps that also may play a role in Ca^{2+} uptake, one ATP-driven, one driven by a concurrent flow of Na^+ ions into the cytoplasm (i.e., a Na^+ - Ca^{2+} **antiport**; see Figure 3.8). We discuss these types of transporting proteins in the next subsection.

There are some apparent analogies between intestinal Ca^{2+} transport and that occurring in the placenta. Transplacental movements of Ca^{2+} increase dramatically during the last trimester of gestation.⁴² In mammalian placental **trophoblasts**, high concentrations of calbindin D_{9K} are found.^{43,44} The protein synthesis also in this tissue appears to be under calcitriol regulation. Ca^{2+} ions have to be supplied by mammalian females, not only to the fetus during pregnancy, but also to the newborn child through the mother’s milk. The molecular details of Ca^{2+} transport in the mammalian glands have not been extensively studied. In milk, Ca^{2+} is bound mainly to micelles of casein, and the average Ca^{2+} content is reported to be 2.5 g/liter (see Table 3.1).

B. Intracellular Ca^{2+} Transport

In order to provide a better understanding of the role of Ca^{2+} as an almost universal regulator of cellular function, we need to take a brief look at the many ways by which Ca^{2+} ions can be transported in or out of eukaryotic cells. Although various transport pathways have been elucidated, the present picture is probably not complete, since the molecular structures and properties of the transport proteins are only partially known. The major pathways for Ca^{2+} transport across cellular membranes involve three membrane systems: the plasma membrane, the inner mitochondrial membrane, and the membrane of the endoplasmic reticulum (ER) (or, in striated muscle cells, a specialized form of ER called the sarcoplasmic reticulum (SR): (Figure 3.9). Two of the membrane-bound transport systems are Ca^{2+} -ATPases, since they derive their main energy from the hydrolysis of ATP (1 and 2 in Figure 3.9). Their properties do, however, differ in many other respects, as we will see.

1. The Ca^{2+} -ATPases

The plasma membrane Ca^{2+} -ATPase (PM Ca^{2+} -ATPase) of **erythrocytes**—first recognized by Schatzmann in 1966⁴⁵—was isolated in pure form by Niggli *et al.* in 1979, using an affinity column with an ATP-ase binding protein, calmodulin (see Section V.A), coupled to the gel.⁴⁶ Ca^{2+} -ATPases purified from other types of plasma membranes appear to be very similar. The schematic structure of the erythrocyte membrane Ca^{2+} -ATPase is presented in

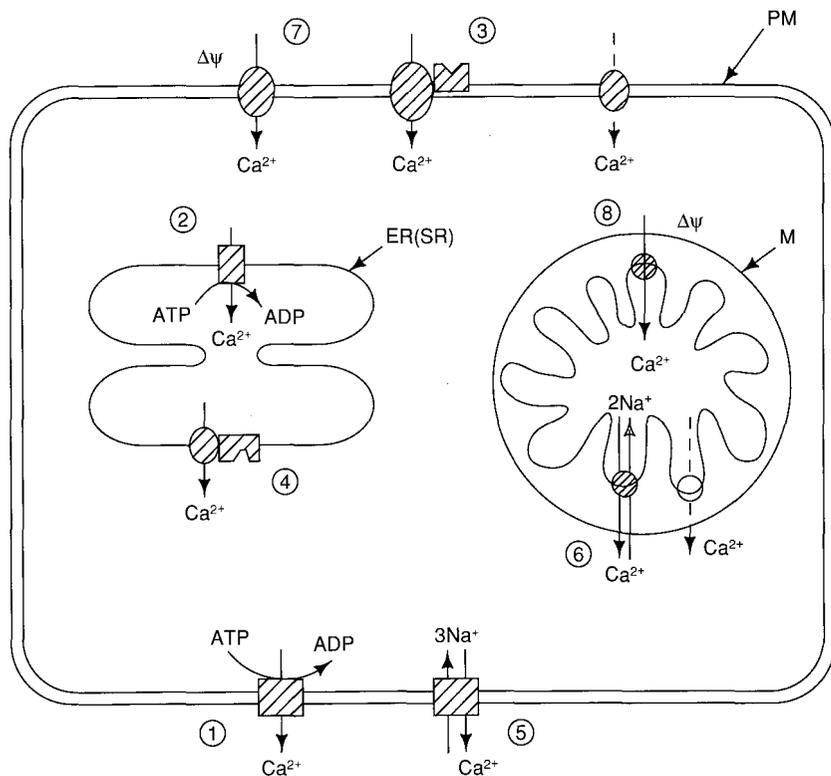


Figure 3.9

Schematic representation of the major pathways for the transport of Ca^{2+} across cellular membranes. PM, plasma membrane; ER(SR), endoplasmic reticulum (sarcoplasmic reticulum); M, mitochondria; $\Delta\psi$, difference in membrane potential. The transport proteins shown are: 1 and 2, PM and ER(SR) Ca^{2+} -ATPases; 3 and 4, PM and ER(SR) receptor-mediated Ca^{2+} channels; 5 and 6, PM and M (inner-membrane) $\text{Na}^+/\text{Ca}^{2+}$ exchangers; 7 and 8, PM and M voltage-sensitive Ca^{2+} channels. In addition, some not-well-defined "passive" transport pathways are indicated by dashed arrows.

Figure 3.10.⁴⁷ The sarcoplasmic reticulum in muscle cells is abundant in Ca^{2+} -ATPase. It is estimated that this protein constitutes more than 80 percent of the integral membrane proteins, and covers a third of the surface area.

The sarcoplasmic reticulum Ca^{2+} -ATPase (SR Ca^{2+} -ATPase) was first purified by MacLennan in 1970.⁴⁸ Presently it is the best characterized Ca^{2+} -ATPase. A schematic model and a summary of some properties are given in Figure 3.11.⁴⁹ Ten hydrophobic segments of about 20 amino-acid residues each are revealed by **hydropathy** plots, and these segments are assumed to span the membrane as α -helices. (For the one-letter codes for amino acids, see Appendix B in Section IX.) The phosphorylation site has been identified as Asp-351, and the nucleotide binding domain is following the phosphorylation domain. The Ca^{2+} -binding sites are located within the predicted trans-membrane domains

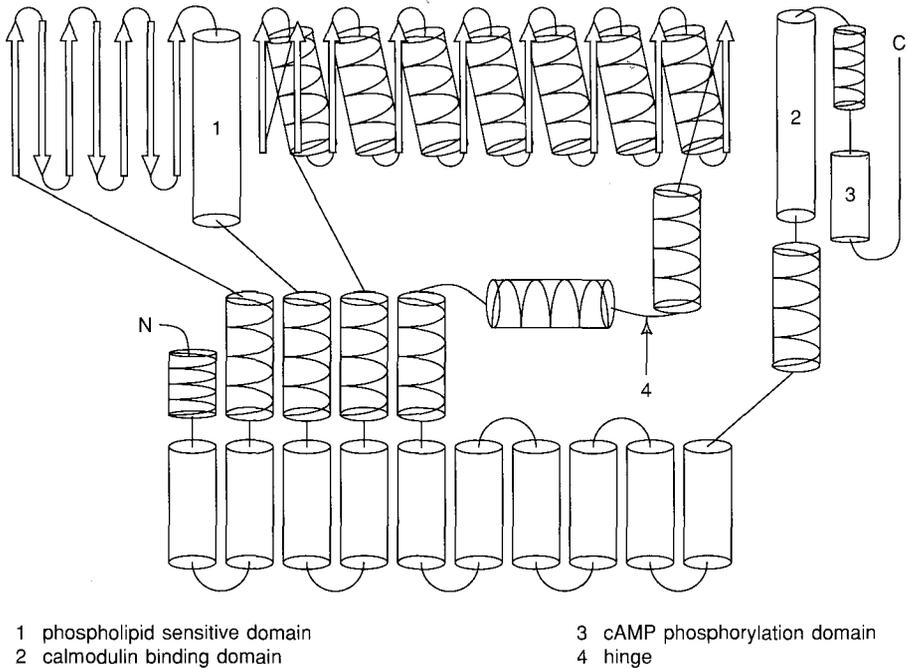


Figure 3.10

Schematic structure of the calmodulin (CaM)-activated plasma membrane Ca^{2+} -ATPase of erythrocytes. Some molecular characteristics are: $M_r = 138,000$; transport rate (30°C), 20–70 Ca^{2+} ions per protein molecule per second; $K_M(\text{Ca}^{2+}) \approx 0.5 \mu\text{M}$ (cytoplasmic side in high-affinity form); $\text{Ca}^{2+}/\text{ATP}$ ratio, 1(?); activated not only by CaM but also by acidic phospholipids and unsaturated fatty acids. Figure kindly provided by R. Moser and E. Carafoli.

(see Figure 3.11). This was shown through a series of site-directed mutations in which likely Ca^{2+} -liganding residues like Asp, Glu, and Thr were mutated into residues lacking possible side-chain ligands (e.g., Asn, Gln, and Ala).⁵⁰

The presently accepted reaction cycle involves two main alternative conformations, E_1 and E_2 , the former with two high-affinity sites ($K_m \approx 1 \mu\text{M}$)⁴ on the cytoplasmic side, which in E_2 are open to the luminal side with $K_m \sim 1 \text{ mM}$.^{49,51} The mechanism suggested for Ca^{2+} transport (Figure 3.12) has many features similar to that suggested by Williams for H^+ translocation in the mitochondrial ATPase.⁵²

It is instructive to consider briefly the thermodynamic limits of the transport. (The discussions about the thermodynamics behind $\text{Ca}^{2+}/\text{Na}^+$ transport pertain to Na^+/K^+ gradients in excitable tissues as well). Let us define an “inside” and an “outside” separated by a membrane, as shown in Figure 3.13, where $[\text{Ca}^{2+}]$ and ψ denote activities and membrane potentials, respectively. The difference in electrochemical potential, $\Delta\mu$, across the membrane for a Ca^{2+} ion is given by

$$\Delta\mu_{\text{Ca}^{2+}} = +RT \ln \frac{[\text{Ca}^{2+}]_o}{[\text{Ca}^{2+}]_i} + 2F\Delta\psi, \quad (3.4)$$

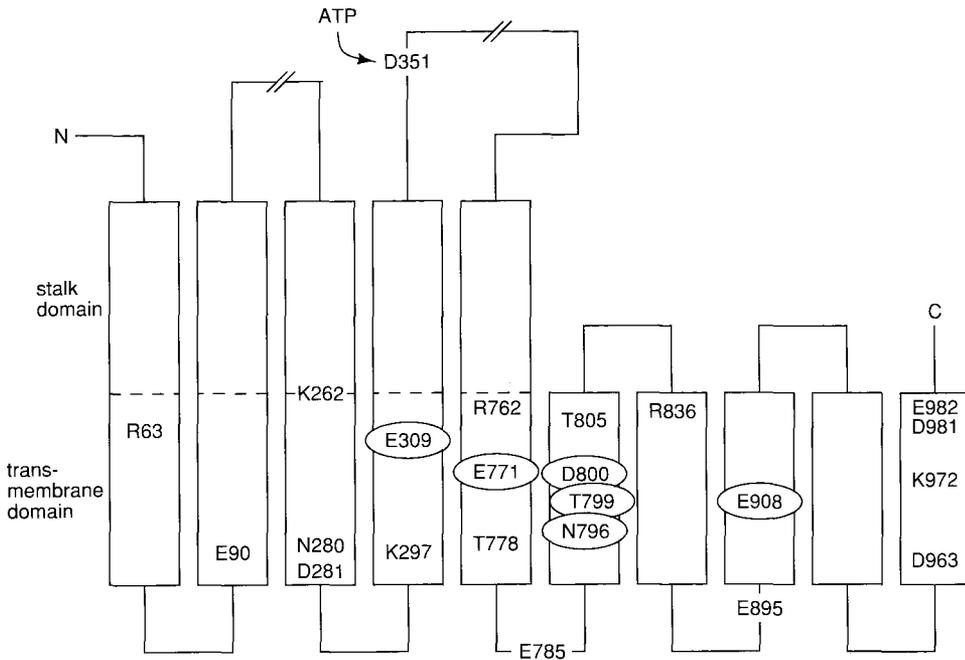


Figure 3.11

Schematic structure of the Ca^{2+} -ATPase of sarcoplasmic reticulum. Some molecular characteristics are: $M_r = 110,000$; $K_m < 1\mu\text{M}$ (two Ca^{2+} sites on cytoplasmic side in high-affinity form); $\text{Ca}^{2+}/\text{ATP}$ ratio, 2; Mg^{2+} required for activity. The amino-acid residues labeled were mutated to a residue lacking side chains capable of binding Ca^{2+} . Mutations at the circled residues resulted in complete loss of Ca^{2+} transport activity, suggesting that the circled residues participate in Ca^{2+} binding. Adapted from Reference 50.

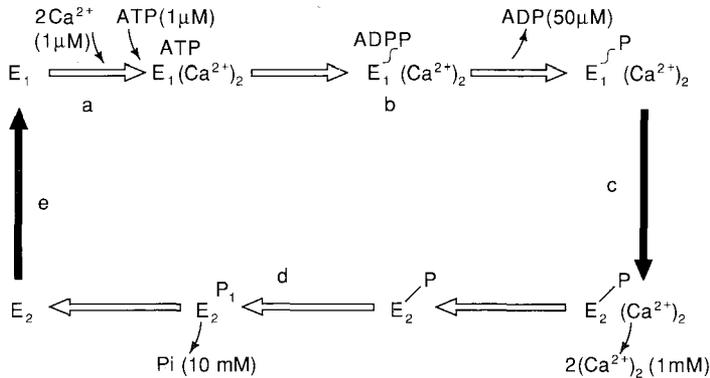


Figure 3.12

Simplified schematic reaction cycle of the Ca^{2+} -ATPase of sarcoplasmic reticulum (SR).^{49,51} The transport protein is assumed to be in either of two states, E_1 on the cytoplasmic side, or E_2 on the side of the SR lumen. Starting from E_1 in the upper left corner, the reactions steps shown are: (a) binding of Ca^{2+} and ATP (approximate dissociation constants within parentheses); (b) rapid phosphorylation of Asp-351 of the protein (E_1^{-P}) and release of ADP; (c) transformation from an energy-rich, high- Ca^{2+} -affinity conformation ($E_1^{-P}(\text{Ca}^{2+})_2$) to a low-energy, low-affinity conformation ($E_2^{-P}(\text{Ca}^{2+})_2$); (d) hydrolysis of the phosphorylated protein and release of the phosphate into the lumen; (e) return to the original state.

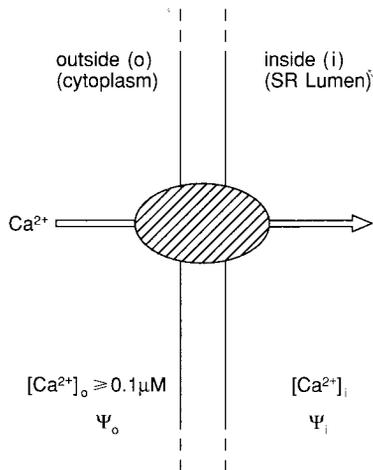


Figure 3.13

Schematic representation of Ca^{2+} transport through a membrane by a Ca^{2+} -ATPase molecule. Ψ denotes membrane potentials.

where F is Faraday's constant, T the temperature, and R the gas constant. If we assume $\Delta\Psi = 0$, which appears reasonable for the SR membrane according to experimental evidence, we may calculate the free-energy change, ΔG , at 25°C for transferring Δn moles of Ca^{2+} across the membrane. This becomes $\Delta G = -\Delta n \times \Delta\mu_{\text{Ca}^{2+}} = \Delta n \times 4.1 \text{ kcal/mol}$ if $[\text{Ca}^{2+}]_o/[\text{Ca}^{2+}]_i = 10^{-3}$ and $\Delta G = \Delta n \times 5.4 \text{ kcal/mol}$ if $[\text{Ca}^{2+}]_o/[\text{Ca}^{2+}]_i = 10^{-4}$. Under the pertinent cellular conditions, the free-energy change associated with ATP hydrolysis to ADP and P_i has been calculated by Tanford to be $\Delta G = -13$ to -14 kcal/mol .⁵³ In the absence of a membrane potential, it is thus possible to transport two Ca^{2+} ions for every ATP molecule hydrolyzed against a concentration (or activity) gradient of 10^4 or more. This treatment says nothing, of course, about the molecular details of this transport. A more detailed model for the transport cycle has been proposed by Tanford.⁵³

In the specialized cells of muscle tissue, the sarcoplasmic reticulum may contain much calcium, and if all were "free" Ca^{2+} , the concentration could be as high as 30 mM .⁵⁴ This value would cause an osmotic pressure difference across the membrane, as well as put a high demand on the SR Ca^{2+} -ATPase. A lowering of the free Ca^{2+} concentration inside the SR would clearly be beneficial. In the presence of oxalate or phosphate ions in the external medium, calcium oxalate or phosphate may precipitate inside the sarcoplasmic reticulum, but under normal circumstances it appears that Ca^{2+} ions inside the SR are bound to a very acidic protein, *calsequestrin*.⁵⁴ Each molecule ($M_r \approx 40 \text{ kDa}$) is able to bind 40 to 50 Ca^{2+} ions with an effective dissociation constant of about 1 mM (at $I = 0.1$). The protein has a low cation specificity and behaves in many respects like a negatively charged polyelectrolyte. It has been crystallized⁵⁵ and we may soon have access to its x-ray structure.

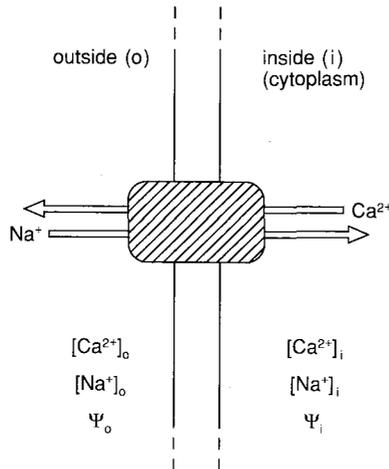


Figure 3.14
Schematic representation of the $\text{Ca}^{2+}/\text{Na}^{+}$ exchanger of the plasma membrane. Ψ denotes membrane potentials.

2. The $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger of the plasma membrane

Presently available information on the $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger has mainly been obtained from studies of the large cells of the giant squid axon and of plasma-membrane vesicles from various other tissues.^{56,57} In heart plasma-membrane vesicles, the exchanger has the following characteristics: $K_m = 1.5\text{--}5\ \mu\text{M}$ for Ca^{2+} and $\sim 20\ \text{nM}$ for Na^{+} ; $V_{\max} \approx 20\ \text{nmol}\ \text{Ca}^{2+}/\text{mg}\ \text{protein}$.⁵⁸ The stoichiometry is at least 3:1 $\text{Na}^{+}/\text{Ca}^{2+}$. Very few molecular details of the exchanger are available at present. We may again briefly consider the thermodynamic framework for an $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger (Figure 3.14). The difference in electrochemical potential for Na^{+} and Ca^{2+} across the membrane is:

$$\Delta\mu_{\text{Ca}^{2+}} = RT \ln \frac{[\text{Ca}^{2+}]_o}{[\text{Ca}^{2+}]_i} + 2F\Delta\Psi, \quad (3.5)$$

$$\Delta\mu_{\text{Na}^{+}} = RT \ln \frac{[\text{Na}^{+}]_o}{[\text{Na}^{+}]_i} + F\Delta\Psi. \quad (3.6)$$

The free-energy change, $\Delta G_t^{\text{Ca}^{2+}}$, associated with a transfer of $\Delta n_{\text{Ca}^{2+}}$ moles of Ca^{2+} from the inside to the outside is $\Delta G_t^{\text{Ca}^{2+}} = \Delta n_{\text{Ca}^{2+}} \times \Delta\mu_{\text{Ca}^{2+}}$, and the corresponding change associated with the movement of $\Delta n_{\text{Na}^{+}}$ moles of Na^{+} from the outside in is $\Delta G_t^{\text{Na}^{+}} = -\Delta n_{\text{Na}^{+}} \times \Delta\mu_{\text{Na}^{+}}$. If these free-energy changes are coupled via the exchanger, there will be a net flux of Ca^{2+} as long as the free-energy difference,

$$\Delta\Delta G = \Delta G_t^{\text{Ca}^{2+}} - \Delta G_t^{\text{Na}^{+}} = \Delta n_{\text{Ca}^{2+}} \times \Delta\mu_{\text{Ca}^{2+}} - \Delta n_{\text{Na}^{+}} \times \Delta\mu_{\text{Na}^{+}}, \quad (3.7)$$

is less than zero. We can write $\Delta\Delta G$ for the transport of 1 mol Ca^{2+} as

$$\Delta\Delta G = 2.303 RT \left[\log \frac{[\text{Ca}^{2+}]_o}{[\text{Ca}^{2+}]_i} - \Delta n_{\text{Na}^+} \times \log \frac{[\text{Na}^+]_o}{[\text{Na}^+]_i} \right] + (2 - \Delta n_{\text{Na}^+}) \times F\Delta\Psi. \quad (3.8)$$

Equating ion activities with concentrations, we note that in a typical mammalian cell $[\text{Na}^+]_o \approx 110\text{--}145$ mM, and $[\text{Na}^+]_i \approx 7\text{--}15$ mM, or $[\text{Na}^+]_o/[\text{Na}^+]_i \approx 10$. In the absence of a membrane potential difference ($\Delta\Psi = 0$), Equation (3.8) can thus be simplified to

$$\Delta\Delta G = 2.3 RT \left[\log \frac{[\text{Ca}^{2+}]_o}{[\text{Ca}^{2+}]_i} - \Delta n_{\text{Na}^+} \right]. \quad (3.9)$$

To pump one Ca^{2+} ion out of a cell against a concentration gradient of about 10^3 ($1 \mu\text{M} \rightarrow 1$ mM) requires that at least 3 Na^+ ions pass in the opposite direction, thus maintaining $\Delta\Delta G < 0$. What then will be the effect of a membrane potential difference? Most animal cells, particularly excitable cells such as nerve and muscle cells, have resting potential differences, $\Delta\Psi$, over the plasma membrane of 30 to 90 mV (cytoplasm negative). For this value we find the change in free energy, $\Delta\Delta G$, for the transport of one mol Ca^{2+} to be

$$\Delta\Delta G = 2.3 RT \left[\log \frac{[\text{Ca}^{2+}]_o}{[\text{Ca}^{2+}]_i} - \Delta n_{\text{Na}^+} \right] + (2 - \Delta n_{\text{Na}^+}) 0.1 F. \quad (3.10)$$

Thus for $\Delta n_{\text{Na}^+} > 2$, we have $\Delta\Delta G < 0$, and the transport of Ca^{2+} against a concentration gradient of about 10^3 will be promoted. This is another good reason for having a $\text{Na}^+/\text{Ca}^{2+}$ exchange stoichiometry of 3:1.

3. Mitochondrial Ca^{2+} transport: influx

Mitochondria isolated from various types of animal cells—but, interestingly, not those from plant cells—can rapidly accumulate exogenous Ca^{2+} .⁵⁹ The transporter is located in the inner membrane and the driving force behind the Ca^{2+} transport appears to be merely the high potential difference across this membrane ($\Delta\Psi \approx 150$ to 180 mV, negative in the inner matrix). This potential difference is fairly closely maintained by the pumping out of H^+ from the matrix by cell respiration. For the transport of 1 mol Ca^{2+} from the “outside” (= cytoplasm) to the “inside” (= inner mitochondrial matrix), we may deduce from Equation (3.4) that the free-energy change ΔG may be written ($\Delta n_{\text{Ca}^{2+}} = -1$)

$$\Delta G = - RT \cdot \ln \frac{[\text{Ca}^{2+}]_o}{[\text{Ca}^{2+}]_i} - 2F \Delta\Psi. \quad (3.11)$$

From this analysis it may be inferred that the limiting Ca^{2+} concentration (or activity) ratio that can be achieved by this **electrogenic** pump (i.e., $\Delta G = 0$) is

$$\frac{[\text{Ca}^{2+}]_o}{[\text{Ca}^{2+}]_i} = e^{-2F\Delta\Psi/RT} \quad (3.12)$$

With $\Delta\Psi = 150$ mV, this ratio is calculated to be 8.4×10^{-6} at 25°C . It is evident that, as long as the Ca^{2+} influx would not lower the membrane potential difference, the Ca^{2+} **uniporter** has a very high pumping potential. Measured values of the pumping rate, V_{\max} , are indeed high (>10 nmol/mg protein⁵⁹) and probably limited only by the rate of electron transport and H^+ extrusion in the mitochondria.

Mitochondria may accumulate large quantities of Ca^{2+} , probably to maintain electroneutrality. To prevent the buildup of high concentrations of free Ca^{2+} (and of osmotic pressure), phosphate ions are also transported into the inner matrix, where an amorphous calcium phosphate—or possibly a phosphocitrate⁶⁰—is formed. The equilibrium concentration of free Ca^{2+} in the mitochondrial matrix may as a result be comparatively low, on the order of $1 \mu\text{M}$.

The molecular nature of the mitochondrial Ca^{2+} uniporter continues to be elusive, and needs to be studied further.

4. Mitochondrial Ca^{2+} transport: efflux

Mitochondria, as well as SR, release Ca^{2+} ions by mechanisms other than “back leakage” through the pumps. In mitochondria from excitable cells, the efflux occurs mainly through an antiport, where 2Na^+ ions are transported inward for every Ca^{2+} ion departing for the cytosolic compartment.⁶¹ In other cells there is evidence for the dominance of a $2\text{H}^+ - \text{Ca}^{2+}$ antiport.⁵⁹ In all likelihood the Ca^{2+} efflux is regulated, possibly by the redox state of pyridine nucleotides in the mitochondria. As with the Ca^{2+} uniporter, few details on the molecular nature of the antiporters are presently available.

5. Ca^{2+} efflux from non-mitochondrial stores

Release of Ca^{2+} from ER and SR presently appears to be the prime effect of the new intracellular messenger 1,4,5-triphosphoinositol (1,4,5-IP₃) released into the cytoplasm as a result of an external hormonal stimulus (see Section IV.C). It seems that receptors for 1,4,5-IP₃ have been established on ER, and that the binding of 1,4,5-IP₃ causes a release of Ca^{2+} stored in this organelle.^{62,63,170,171} In addition to the receptor-controlled Ca^{2+} efflux, there may be other pathways for Ca^{2+} release, and Ca^{2+} mobilization may be regulated by other intracellular entities, the Ca^{2+} ions themselves included.

6. Other voltage-gated or receptor-activated Ca^{2+} channels

In addition to the transport pathways already discussed, some cells seem to have Ca^{2+} channels in the plasma membrane that can be opened by the action of an agonist on a receptor or that are gated in response to changes in membrane potential.⁶⁴ For example, Ca^{2+} channels can be opened by nicotinic cholinergic agonists⁶⁵ or by the excitatory amino acid N-methyl-D-aspartate (NMDA).⁶⁶ Endocrine cells and also some muscle and neuronal cells have voltage-sensitive Ca^{2+} channels.^{67,68} We will not discuss these further, but merely point to their existence. We finally note that during the last few years knowledge about the mechanisms of Ca^{2+} entry and release to and from extracellular and intracellular pools has increased dramatically, and we refer the reader to recent reviews of the field.^{175,176}

C. Inositol Trisphosphate and the Ca^{2+} Messenger System

A “second” messenger is an entity that inside a cell mediates the action of some hormone at the plasma membrane, the hormone being considered the “first” messenger. The first such second messenger to be discovered—in fact, the very molecule that led to the formulation of the whole concept—was cyclic AMP.⁶⁹ During the decade following the discovery of cAMP, it was gradually realized that intracellular release of Ca^{2+} ions also accompanied hormonal stimuli, and the Ca^{2+} ion slowly became regarded as a second messenger. This idea was first clearly enunciated by Rasmussen⁷⁰ as early as 1970, and gained general acceptance when the ubiquitous intracellular Ca^{2+} -binding protein calmodulin (see Section V.A) was discovered. In the mid-1970s this protein was shown to be a Ca^{2+} -dependent regulator of a large number of Ca^{2+} -dependent enzymes, transport proteins, etc., establishing a molecular basis for Ca^{2+} action in cells.

There were some puzzling facts, however. Although a transitory increase in intracellular Ca^{2+} concentration in response to the binding of a hormone or transmitter substance to a surface receptor could result from extracellular Ca^{2+} being released into the cytoplasm, there was compelling evidence for muscle cells that the main Ca^{2+} source was the sarcoplasmic reticulum (SR). This result led to the hypothesis of “ Ca^{2+} -induced Ca^{2+} release,” i.e., that upon stimulation of the cell, a small amount of Ca^{2+} entered into the cytoplasm and triggered the release of greater amounts of Ca^{2+} from the SR. For some cell types it could, however, be shown that transient increases in intracellular Ca^{2+} could occur even when extracellular Ca^{2+} was removed, although *prolonged* responses required the presence of extracellular Ca^{2+} . Although some specialized cells have gated plasma-membrane Ca^{2+} channels, release of Ca^{2+} into the cytoplasm from intracellular stores appears to be of at least equal importance. Furthermore, there is now overwhelming evidence^{63,70-72} that intracellular Ca^{2+} is released in response to the formation of a new type of intracellular messenger: 1,4,5- IP_3 . Receptors for this messenger have recently been found in the membranes of intracellular organelles, and binding of 1,4,5- IP_3 to these receptors results in the release of Ca^{2+} ions.⁷³

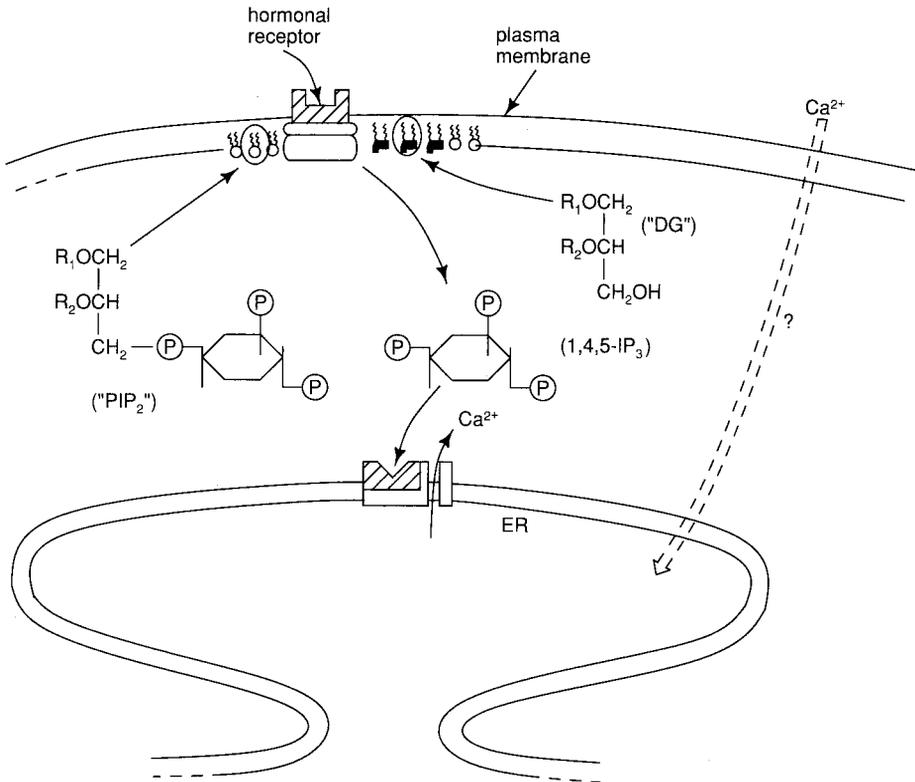


Figure 3.15

Outline of the presumed role of inositol phosphates in the intracellular mobilization of Ca^{2+} . Upon binding of an agonist to a plasma-membrane receptor, phosphatidylinositol 4,5-bisphosphate (PIP₂) membrane lipids are hydrolyzed to give diacylglycerol (DG) and inositol 1,4,5-trisphosphate (1,4,5-IP₃). The latter interacts with specific receptors on the endoplasmic reticulum (ER) membrane that trigger the release of Ca^{2+} into the cytosol. Ca^{2+} may be returned to the ER through the Ca^{2+} -ATPase of the ER membrane (see Figure 3.9) and also by a direct influx of Ca^{2+} from the extracellular medium.^{70,81}

1,4,5-IP₃ is formed as a product in the hydrolysis of a special phospholipid present in the cell membrane: phosphatidylinositol-4,5-bisphosphate. This reaction, then, is the initial receptor-stimulated event. The newly formed 1,4,5-IP₃ is assumed to diffuse into the cytoplasm, and eventually reach intracellular 1,4,5-IP₃ receptors on the ER, thereby triggering the release of Ca^{2+} . A simplified reaction scheme is shown in Figure 3.15. A diacylglycerol (DG) is also formed in the hydrolysis step. DG can also act as an intracellular messenger, and stimulates the activity of a membrane-bound protein kinase, known as *protein kinase C* (PKC). As a result, PKC may phosphorylate certain key proteins and influence their activity. Protein kinase C is also activated by Ca^{2+} ions, a fact that illustrates Nature's knack in designing regulatory networks! 1,4,5-IP₃ is either directly degraded in a series of enzymatic steps back to inositol, which is then used to resynthesize the phospholipid, or it may be further phosphorylated to inositol-1,3,4,5,-tetraphosphate (1,3,4,5-IP₄), which may undergo de-

phosphorylation to form inositol-1,3,4-trisphosphate (1,3,4-IP₃). The biological functions of the latter compounds are now being investigated.

The intracellular levels of Ca²⁺ are restored back to the normal low resting values (100 to 200 nM) via transport back into the SR, and/or into mitochondria, or out through the plasma membrane by the pumping mechanisms discussed in Section IV.B. As was briefly mentioned above, depriving a cell of extracellular Ca²⁺ will eventually make the cell incapable of prolonged responses to external stimuli. It appears that the intracellular Ca²⁺ stores may become depleted if not replenished. It has been suggested that the intracellular ER Ca²⁺ pool has a direct route of access to the extracellular pool, a route that is closed when the ER pool is full.⁷⁴

In a sense, then, Ca²⁺ seems to have been downgraded by the inositolphosphates from a "second" to a "third" messenger; however, the pivotal role of Ca²⁺ as a regulator of cellular activities remains undisputed.

D. Summary

The fluxes of Ca²⁺ ions and their regulation in higher organisms, as well as in microorganisms, depend on several transport proteins in addition to vesicular and gated processes. An important class of transport proteins are the Ca²⁺-ATPases, which are particularly abundant in muscle cells. These proteins translocate Ca²⁺ ions against large activity (or concentration) gradients through the expenditure of ATP. Transport of Ca²⁺ ions against activity gradients across membranes may also be accomplished by coupled transport of other ions, like Na⁺, with a gradient in the opposite direction.

As a result of some external stimulus—the action of a hormone, for example—the "free" Ca²⁺-ion concentrations in the cytoplasm of many cell types may transiently increase several orders of magnitude. This increase largely results from the release of Ca²⁺ from intracellular stores (ER, SR) in response to the initial formation of a new type of messenger, 1,4,5-IP₃. The activity of Ca²⁺-transport proteins eventually restores the Ca²⁺ concentration levels to resting levels. This sequence of events forms the basis for Ca²⁺'s role in the regulation of a wide variety of cellular activities (see Section V).

V. MOLECULAR ASPECTS OF Ca²⁺-REGULATED INTRACELLULAR PROCESSES

So far we have mainly discussed the routes and means by which the concentration of Ca²⁺ ions in the cytoplasm can be transiently increased and brought back to resting levels. But changing the cytoplasmic Ca²⁺ concentration is not enough. In order to influence the cellular machinery, the Ca²⁺ ions must interact with different proteins, *intracellular Ca²⁺ receptors* if you like. These intracellular Ca²⁺-receptor proteins must have certain properties in order to function.

(i) Their Ca^{2+} -affinity must be such that their Ca^{2+} -binding sites are essentially unoccupied at resting levels of free Ca^{2+} ($\sim 10^{-7}$ M) and occupied at levels reached upon stimulus (generally assumed to be 10^{-5} to 10^{-6} M). This means that the binding constants $K_B^{\text{Ca}^{2+}}$ should be $\sim 10^6 \text{ M}^{-1}$.

(ii) We should also remember that Ca^{2+} must exert its function in the presence of a number of other ions; in mammalian cells the intracellular concentration of “free” Mg^{2+} ions is around 1 mM, and that of K^+ ions around 100 to 150 mM. The receptors must therefore have an adequate selectivity for Ca^{2+} .

(iii) In response to Ca^{2+} binding, a Ca^{2+} receptor must undergo some kind of conformation change that either alters its interaction with other molecules or changes its activity if it is an enzyme.

(iv) Finally, there are *kinetic* considerations. In many cells a rapid response is essential, and therefore the receptors must be able to interact swiftly—within milliseconds—with incoming Ca^{2+} ions, and the ions must also be able to depart almost as rapidly.

A few proteins have been discovered that qualify as intracellular Ca^{2+} receptors. The best known of these is *calmodulin* (CaM), which appears to be present in all eukaryotic cells. Most of the cellular responses elicited by Ca^{2+} appear to result from interactions between the Ca^{2+} -calmodulin complex and various other target enzymes and proteins.⁷⁵ Another important Ca^{2+} -receptor protein is *troponin C* (TnC), which occurs in muscle cells and is instrumental in mediating muscle contraction.⁷⁶ These two types of proteins are highly homologous, as we shall see, and may be considered members of a superfamily of closely related intracellular Ca^{2+} -binding proteins. This superfamily has been given the name “the calmodulin superfamily,” and close to 200 distinct family members are presently known.⁷⁷ Not all members of the superfamily may qualify as Ca^{2+} receptors; some like *parvalbumins* and *calbindins* (see Section IV.A) appear to have a role in intracellular transport and/or Ca^{2+} -buffering. For others, such as the *S-100* proteins⁷⁸ found predominantly in brain tissue, and *calcimedins*,⁷⁹ isolated from smooth muscle, the biological function is still unclear.

One Ca^{2+} receptor with enzymatic activity is *protein kinase C*. Its activity is markedly increased in the presence of Ca^{2+} , and it has a high calcium-binding constant (see Table 3.2) in the presence of diacylglycerol or **phorbol esters**.⁸⁰

During recent years, groups interested in the role of Ca^{2+} in secretion and in the control of membrane cytoskeleton have identified some intracellular Ca^{2+} /phospholipid-binding proteins that appear to be distinct from the calmodulin superfamily; these include *lipocortin*, *endonexin*, *calelectrin*, *p36*, and *calpactin*.^{81–83} These membrane-binding proteins are collectively called *annexins*,⁸⁴ and contain repeated domains distinct from EF-hands. The Ca^{2+} sites are very similar to that observed in phospholipase A_2 , as shown by the recently determined x-ray structure of annexin V.¹⁷² A condensed overview of the interaction of Ca^{2+} with intracellular proteins is shown in Figure 3.16. We will now go on to discuss the molecular properties of some of the proteins mentioned above, starting with calmodulin.

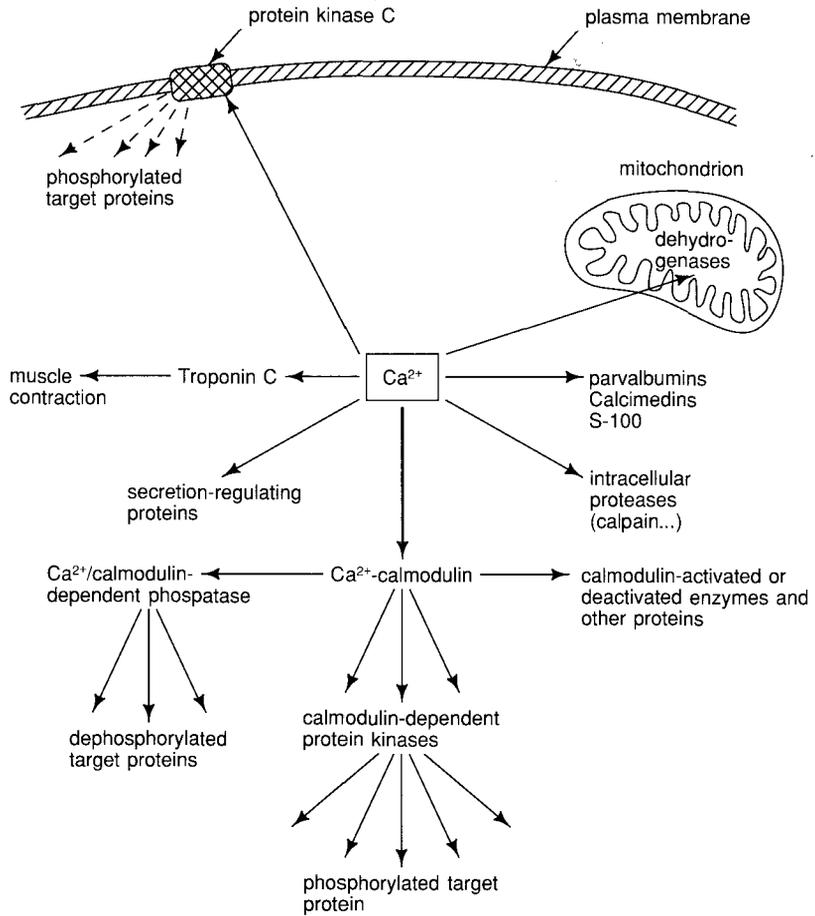


Figure 3.16
Condensed overview of the interaction of Ca^{2+} with intracellular proteins.

A. Calmodulin

Calmodulin is a small acidic protein ($M_r \approx 16,700$), the amino-acid sequence of which has been remarkably preserved during evolution. Early on, an analysis of its amino-acid sequence indicated that it should have four Ca^{2+} -binding sites, a deduction that proved to be correct. The three-dimensional x-ray structure of bovine brain calmodulin⁸⁵ has been solved to a resolution of 2.2 Å. A space-filling model is shown in Figure 3.17. (See color plate section, page C-9.) The molecule has a dumbbell-like shape, with two globular domains connected by an eight-turn α -helix—an unusual structural feature. In the crystal structure, there are no direct contacts between the two globular domains, each of which contains two Ca^{2+} -binding sites. The Ca^{2+} sites are all constructed in the same way: two α -helices separated by a calcium-binding loop, 12 amino acids long, and wrapped around the Ca^{2+} ion. This structural arrangement is nearly iden-

tical with that first observed in the x-ray structure of carp parvalbumin, and is colloquially termed “the *EF-hand*.”⁸⁶ This structural unit is also observed in all available x-ray structures of proteins of the calmodulin superfamily (see Sections V.B and V.C). The Ca^{2+} ligands are all oxygen atoms, located approximately at the vertices of a pentagonal bipyramid.

The binding of Ca^{2+} and other cations to CaM has been extensively investigated.⁸⁷ The first two Ca^{2+} ions are bound in a cooperative manner, with an average binding constant of about $2 \times 10^5 \text{ M}^{-1}$ in 150 mM KCl and 1 mM Mg^{2+} . The third and fourth Ca^{2+} ions are bound with binding constants of about $3 \times 10^4 \text{ M}^{-1}$ under the same conditions. Spectroscopic evidence has shown that the first two Ca^{2+} -ions are bound in the C-terminal domain. Mg^{2+} has been shown to bind primarily to the N-terminal domain (see Table 3.2).⁸⁸

The rates of dissociation of Ca^{2+} from the $(\text{Ca}^{2+})_4$ CaM complex have been studied by both stopped-flow and NMR techniques.^{89,90} Fast and slow processes are observed, both corresponding to the release of two Ca^{2+} ions. At an ionic strength $I = 0.1$ and 25°C , the rates for the two processes differ by a factor of 30 (see Table 3.4).

A body of biophysical measurements, mostly made before the advent of x-ray structures, indicated that CaM is constructed from two largely independent domains.⁸⁷ This conclusion emanated from studies of the two tryptic fragments, TR_1C and TR_2C . The major site of cleavage is between Lys-77 and Asp-78 of the central helix, and results in N-terminal and C-terminal fragments of nearly equal size. To a good approximation, the biophysical properties of the intact CaM molecule—NMR, UV and CD spectra, kinetic properties, thermochemical data, etc.—are the sum of the same properties of the fragments TR_1C and TR_2C . This means that we may assign the slow dissociation process, k_{off}^s , to the C-terminal domain, and the fast, k_{off}^f , to the N-terminal domain of CaM. Combining binding constants and off-rates, we may calculate that the rates of Ca^{2+} binding to CaM are on the order of $10^7 \text{ M}^{-1} \text{ s}^{-1}$ at high ionic strength, and

Table 3.4
Rates of Ca^{2+} -dissociation and -association of some enzymes and proenzymes.

	$k_{\text{off}} [\text{s}^{-1}]$	$k_{\text{on}} [\text{M}^{-1} \text{ s}^{-1}]$
Macrobicyclic amino cryptate [2.2.2]	0.3	10^4
Phospholipase A2	1.1×10^3	4×10^6
sTroponin C: Ca^{2+} sites	300	
Ca^{2+} - Mg^{2+} sites	5	
Trypsin	3	1.1×10^5
Trypsinogen	≤ 10	6×10^4
Chymotrypsin	70	$\sim 10^6$
Chymotrypsinogen	350	2.8×10^5
Calmodulin: N-terminal	300–500	10^7
Calmodulin: C-terminal	10–20	

$10^8 \text{ M}^{-1} \text{ s}^{-1}$ or higher at low ionic strength. Recently the x-ray structure of the C-terminal fragment TR₂C was solved, and indeed showed a structure nearly identical with C-terminal domain of intact CaM.⁹¹

The structural changes occurring in CaM as Ca²⁺ ions are bound are associated with pronounced changes in ¹H NMR, UV, fluorescence, and CD spectra.⁸⁷ The observed changes in CD and fluorescence spectra in the presence of Mg²⁺ are only about 20 to 25 percent of those induced by Ca²⁺. A comparison of the CD spectra of CaM and its **tryptic** fragments indicates that the structural changes induced by Ca²⁺ are substantially greater in the C-terminal than in the N-terminal half.⁹² By and large, few structural details of the conformation changes have as yet been obtained. However, one aspect of the Ca²⁺-induced conformation change is that *hydrophobic sites*, probably one on each domain of the molecule, become exposed. In the presence of excess Ca²⁺, CaM will bind to other hydrophobic molecules, e.g., phenyl-Sepharose, a variety of drugs, many small peptides, and—last but not least—its target proteins. This brings us to the question of how CaM recognizes and interacts with the latter. We may suspect that the hydrophobic sites on each domain are somehow involved, but the role played by the central helix is still not clear. To explain small-angle x-ray scattering data, the interconnecting helix needs to be kinked, bringing the intact globular domains closer.⁹³

A putative CaM-binding segment (27 amino acids long) of myosin light-chain kinase (MLCK), an enzyme activated by CaM, has been identified.⁹⁴ The interaction between the segment peptide (“M13”) and CaM has been studied⁹⁵ by CD spectroscopy and ¹H NMR. From these studies it appears that a unique 1:1 complex is formed, and that secondary and tertiary structural changes occur not only in the peptide M13 but also in both halves of the CaM molecule. Further NMR studies^{96,97} of the interaction between CaM and naturally occurring peptides (mellitin and mastoparan) that share some structural features of M13—clusters of basic residues, hydrophobic residues adjacent to the basic residues, and a predicted high α -helical content—show very much the same results. Based on these results, a model, shown in Figure 3.18, for the interaction between CaM and M13 has been proposed. In this model the central helix is kinked at position 81, allowing the two domains to wrap around the assumed α -helical M13. Preliminary structure calculations of calcium-loaded CaM, based on NMR data, indicate that the central helix in solution indeed is kinked and very flexible,⁹⁹ and comparisons¹⁰⁰ of chemical shifts in calmodulin with and without M13 complexed supports the model in Figure 3.18. Recent structural studies using NMR spectroscopy and x-ray diffraction have essentially confirmed the general features of this model, although the orientation of the peptide is found to be reversed.¹⁷³

In conclusion, two important features of the protein should be recognized.

(i) The binding of Ca²⁺ to CaM (and to its complex with the target protein) is quite likely *cooperative*, meaning that the switch from inactive to active conformation may occur over a much more narrow Ca²⁺-concentration interval than otherwise.

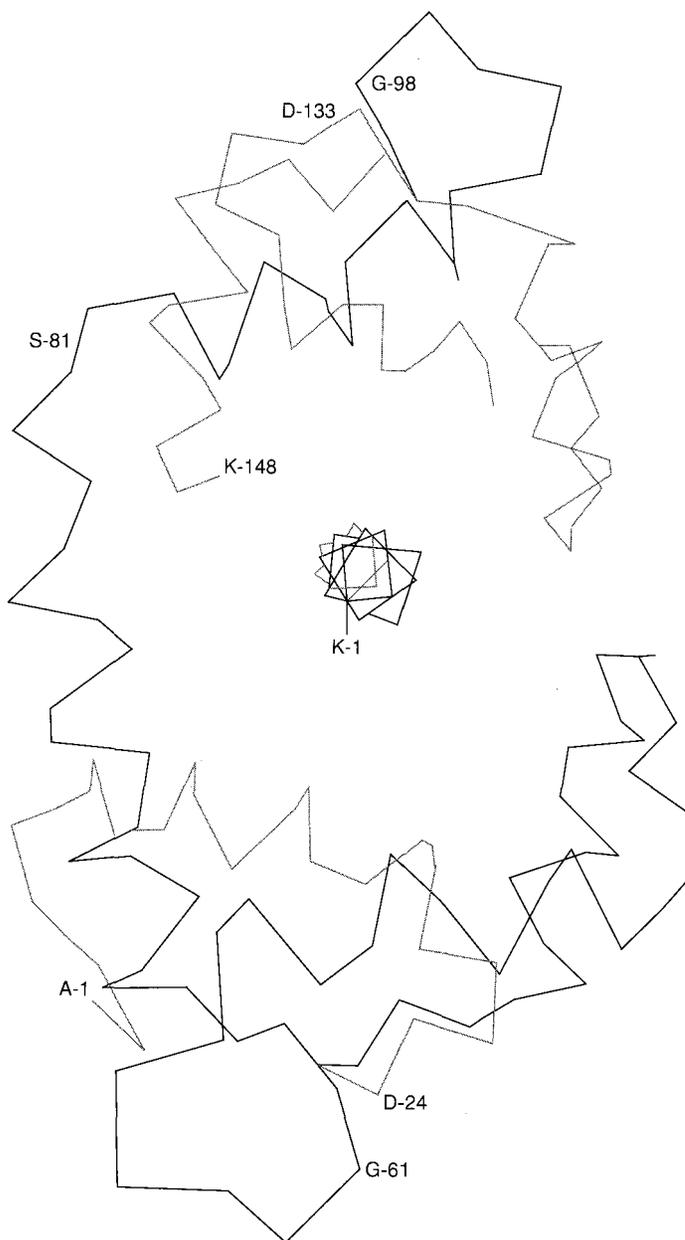


Figure 3.18

A model for the interaction between calmodulin (CaM) and the assumed α -helical ($\phi = -57$, $\psi = -47$) peptide M13. To produce this model, the backbone dihedral angles of Ser-81 in the central α -helix of CaM have been changed (to $\phi = -54$, $\psi = +98$), allowing the hydrophobic patches of both globular domains (green in Figure 3.17) of CaM to interact with the peptide simultaneously. Figure kindly provided by R. Kretsinger; see also Reference 98.

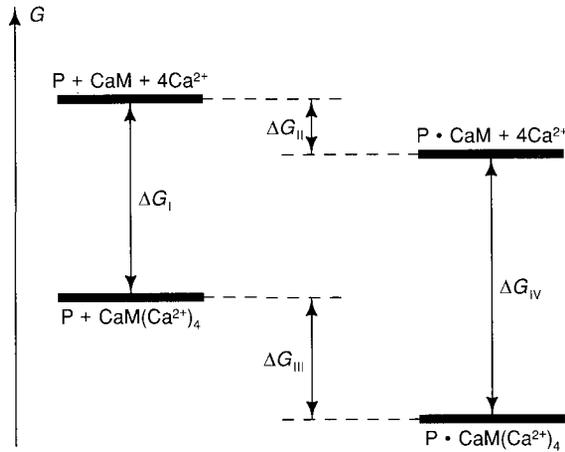


Figure 3.19

Scheme depicting the standard free energies of different states in a system consisting of Ca^{2+} , calmodulin (CaM), and a target protein (P). $\text{P} \cdot \text{CaM}$ denotes a complex between calcium-free CaM and P, $\text{P} \cdot \text{CaM}(\text{Ca}^{2+})_4$ denotes a complex with Ca^{2+} -loaded CaM. If the affinity of the Ca^{2+} -loaded CaM with the target protein P is higher than that of the Ca^{2+} -free form—i.e., $|\Delta G_{\text{III}}| > |\Delta G_{\text{II}}|$ —it follows that the Ca^{2+} affinity of the complex $\text{P} \cdot \text{CaM}$ is higher than that of CaM itself.

(ii) The effective Ca^{2+} affinity will be different in the presence of the target proteins. To illustrate this second point, consider the standard free energies in the minimum scheme depicted in Figure 3.19. If the affinity of the Ca^{2+} -calmodulin complex ($\text{CaM}(\text{Ca})_4$) for the target protein (P) is greater than that of Ca^{2+} -free calmodulin (CaM)—i.e., $|\Delta G_{\text{III}}| > |\Delta G_{\text{II}}|$ —it follows that the Ca^{2+} affinity of the complex between P and CaM ($\text{P} \cdot \text{CaM}$) must be higher than in CaM itself. This effect is also found experimentally in model systems.¹⁰¹

B. Troponin C

The contraction of striated muscle is triggered by Ca^{2+} ions. Muscle cells are highly specialized, and contain two types of filaments that may slide past each other in an energy-consuming process. One of the filaments, the thin filament, is built up by actin molecules ($M_r \approx 42$ kDa) polymerized end-to-end in a double helix. In the grooves of this helix runs a long rod-like molecule, tropomyosin; and located on this molecule at every seventh actin, is a complex of three proteins, *troponin*. The three proteins in the troponin complex are *troponin I* (TnI), *troponin T* (TnT), and *troponin C* (TnC). A schematic picture of the organization of the thin filament is shown in Figure 3.20.

Troponin C is the Ca^{2+} -binding subunit of troponin, and it is structurally highly homologous to calmodulin. Skeletal-muscle troponin C (sTnC; $M_r \approx 18$ kDa) can bind four Ca^{2+} ions, but cardiac-muscle troponin C (cTnC) has one of the four calcium sites modified, so that it binds only three Ca^{2+} ions. The

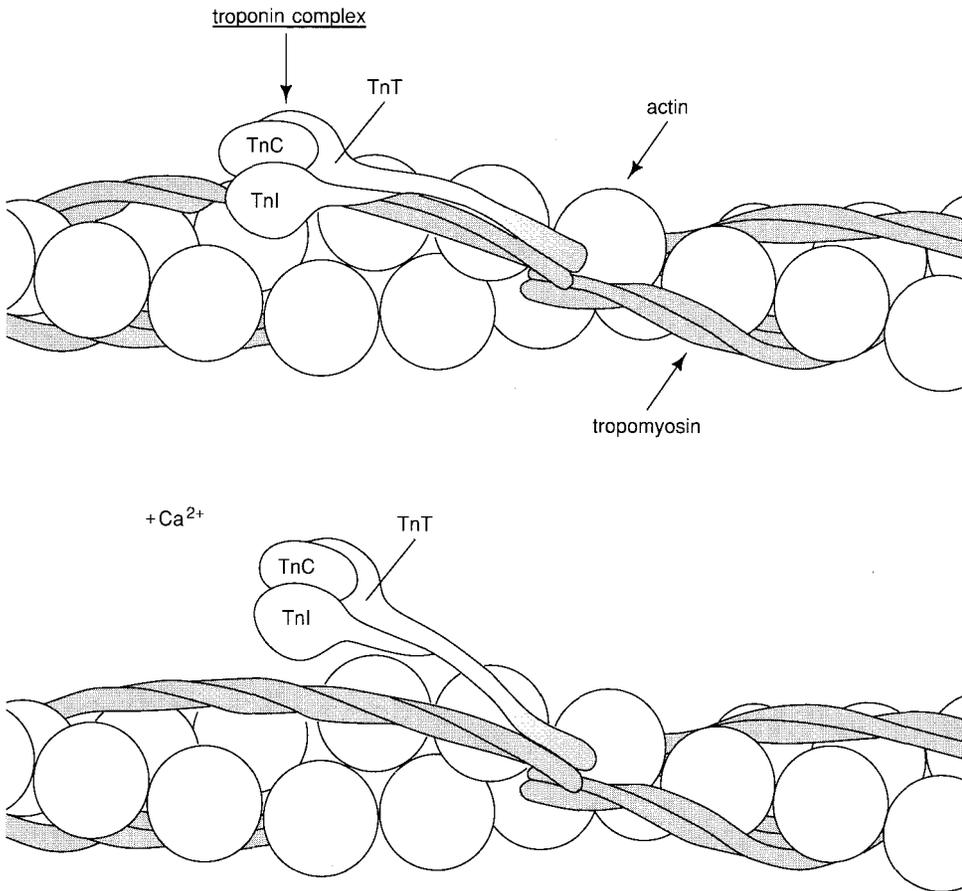


Figure 3.20

Schematic diagram of the organization of skeletal muscle thin filament, showing the position of tropo-myosin and the troponin complex on the actin filament. The binding of Ca^{2+} to TnC, the calcium-binding subunit of the troponin complex, removes TnI, the inhibitory subunit, from actin and thus permits an interaction with a specialized protein, myosin, on neighboring thick muscle filaments (not shown). An ATP-driven conformation change in the myosin head group makes the thick and thin filaments move relative to one another, so that muscle contraction occurs.

x-ray structures of sTnC from turkey and chicken skeletal muscle have been determined to resolutions of 2.8 and 3.0 Å, respectively.^{102,103} The structure of turkey sTnC is shown in Figure 3.21. The similarity between the structures of CaM (Figure 3.17) and sTnC is obvious. In sTnC we again find two domains, each with two potential Ca^{2+} sites, separated by a 9-turn α -helix. The crystals were grown in the presence of Ca^{2+} at a low pH (pH \approx 5), and only two Ca^{2+} ions are found in the C-terminal domain. The two Ca^{2+} -binding sites in this domain have the same helix-loop-helix motif that is found in CaM, and they both conform to the archetypal EF-hand structure. The interhelix angles between helices E and F and between G and H are close to 110° . By contrast, the helices in the N-terminal domain, where no Ca^{2+} ions are bound, are closer to being

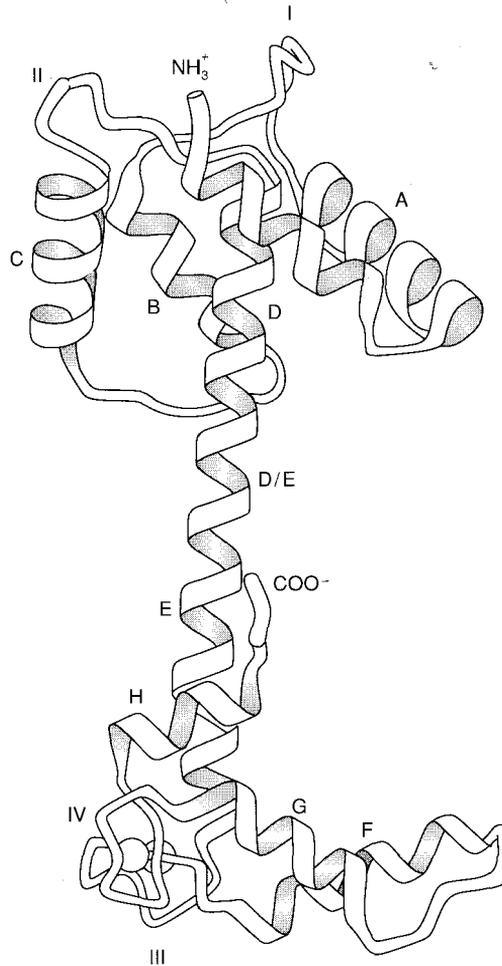


Figure 3.21

A ribbon backbone representation of the three-dimensional structure of turkey-skeletal-muscle troponin C according to Herzberg and James.¹⁰² The crystals were grown at pH 5 in the presence of excess Ca^{2+} , and at this low pH only Ca^{2+} ions bound to the high-affinity domain (the C-terminal domain) are observed. Note the high structural homology with calmodulin (Figure 3.17).

antiparallel, with interhelix angles of 133° (helices A and B) and 151° (helices C and D).

Both sTnC and cTnC have two high-affinity Ca^{2+} -binding sites (see Table 3.2) that also bind Mg^{2+} ions competitively, although with a much lower affinity. These two sites are usually called “*the Ca^{2+} - Mg^{2+} sites.*”^{76,104} In sTnC there are also two (in cTnC, only one) Ca^{2+} -binding sites of lower affinity ($K_B^{\text{Ca}^{2+}} \approx 10^5 \text{ M}^{-1}$) that bind Mg^{2+} weakly and therefore have been called “*the Ca^{2+} -specific sites.*” Since Ca^{2+} binding to the latter sites is assumed to be

the crucial step in the contractile event, they are often referred to as “*the regulatory sites*” (see below). The existence of additional weak Mg^{2+} sites ($K_B \approx 300 \text{ M}^{-1}$) on sTnC, not in direct competition with Ca^{2+} , has also been inferred.^{76,104,105} Spectroscopic studies have shown that the two strong Ca^{2+} - Mg^{2+} sites are located in the C-terminal domain, and the weaker Ca^{2+} -specific sites in the N-terminal domain of sTnC.¹⁰⁶ This pattern is similar to that observed with CaM. NMR spectroscopic studies strongly suggest that binding of Ca^{2+} to both sTnC and cTnC is cooperative.¹⁰⁷ In sTnC the C-terminal domain binds Mg^{2+} much more strongly than the N-terminal domain, by contrast to CaM, where the reverse is true.

The rates of dissociation of Ca^{2+} and Mg^{2+} from sTnC have been measured by both stopped-flow and ^{43}Ca NMR techniques.^{76,108} As with CaM, the actual numbers depend on the solution conditions, ionic strength, presence of Mg^{2+} , etc. (see Table 3.4). On the rate of Mg^{2+} dissociation from the Ca^{2+} - Mg^{2+} sites, quite different results have been obtained by stopped-flow studies⁷⁶ of fluorescence-labeled sTnC ($k_{\text{off}}^{\text{Mg}^{2+}} \approx 8 \text{ s}^{-1}$) and by ^{25}Mg NMR ($k_{\text{off}}^{\text{Mg}^{2+}} \approx 800\text{--}1000 \text{ s}^{-1}$).¹⁰⁹ This apparent discrepancy seems to have been resolved by the observation that both binding and release of Mg^{2+} ions to the Ca^{2+} - Mg^{2+} sites occur *stepwise*, with $k_{\text{off}}^{\text{Mg}^{2+}} < 20 \text{ s}^{-1}$ for one of the ions, and $k_{\text{off}}^{\text{Mg}^{2+}} \geq 800 \text{ s}^{-1}$ for the other.¹¹⁰ The rates of dissociation of the Mg^{2+} ions are important, since under physiological conditions the Ca^{2+} - Mg^{2+} sites of sTnC are likely to be predominantly occupied by Mg^{2+} ions, release of which determines the rate at which Ca^{2+} can enter into these sites.

Spectroscopic and biochemical data¹¹¹ indicate that upon binding Ca^{2+} , sTnC and cTnC undergo significant conformation changes. Comparisons of NMR spectroscopic changes on Ca^{2+} binding to intact sTnC, as well as to the two fragments produced by tryptic cleavage (essentially the N-terminal and C-terminal halves of the molecule, just as was the case with CaM), have shown that the conformation changes induced are mainly localized within the domain that is binding added ions.^{110,112} Thus the central α -helix connecting the domains seems unable to propagate structural changes from one domain to the other. It has been suggested that the structural differences found in the x-ray structure of turkey sTnC between the C-terminal domain, which in the crystal contains two bound Ca^{2+} ions, and the N-terminal domain, in which no Ca^{2+} ions were found, may represent these conformational changes.¹¹³ This rather substantial conformational change is schematically depicted in Figure 3.22. However, preliminary structure calculations¹¹⁴ of the calcium-saturated and calcium-free forms of calbindin D_{9k} indicate that much more subtle conformational changes take place upon binding Ca^{2+} in calbindin D_{9k} . Interestingly, ^1H NMR spectroscopy has provided evidence for the concept that the structural change induced by Mg^{2+} binding to the C-terminal domain of sTnC must be very similar to that induced by Ca^{2+} ions. Another result obtained by ^{113}Cd NMR studies¹⁰⁸ is that the cadmium-loaded N-terminal domain of sTnC in solution undergoes a rapid interchange between two or more conformations, with an exchange rate on the order of $10^3\text{--}10^4 \text{ s}^{-1}$.

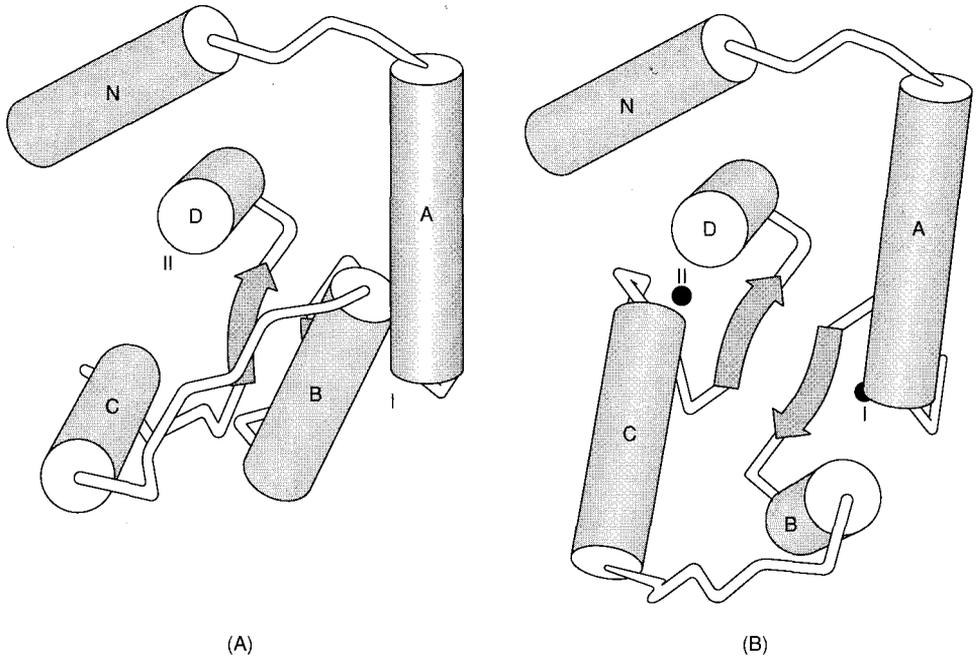


Figure 3.22

Diagrammatic representation of the proposed conformational changes in the N-terminal domain of troponin C upon Ca^{2+} binding.¹¹³ Helices are depicted as cylinders, and I, II denote the Ca^{2+} -binding sites. Helices N, A, and D retain their relative positions, and the relative disposition of helices B and C are also kept constant. (A) Ca^{2+} -free conformation as determined by x-ray crystallography.¹⁰² (B) Proposed Ca^{2+} -saturated conformation based on the structure of the highly sequence-homologous Ca^{2+} -loaded C-terminal. Figure kindly provided by N. C. J. Strynadka and M. N. G. James.

Just as CaM exerts its biological function in complexes with other proteins, TnC participates in the three-protein troponin complex. It presently appears that TnC and TnI form a primary complex that is anchored by TnT to a binding site on tropomyosin.¹¹⁵ In the troponin complex the Ca^{2+} affinity is increased by a factor of about ten over that in isolated sTnC, both at the Ca^{2+} - Mg^{2+} sites and at the Ca^{2+} -specific sites. A similar increase in affinity is found for Mg^{2+} . Given the amounts of “free” Mg^{2+} inside muscle cells (1 to 3 mM), it seems likely that the Ca^{2+} - Mg^{2+} sites in the resting state of troponin are filled with Mg^{2+} , so that a transitory release of Ca^{2+} leads primarily to rapid Ca^{2+} binding to the Ca^{2+} -specific sites, and subsequently to conformation change and contraction.

C. Parvalbumin and Calbindins D_{9K} and D_{28K}

A few intracellular Ca^{2+} -binding proteins have been discovered that by sequence homology clearly belong to the CaM-TnC family with Ca^{2+} sites of the “EF-hand”-type, but that do not appear to exert a direct regulatory function. Parvalbumins ($M_r \approx 12$ kDa), calbindin D_{9K} ($M_r \approx 8.7$ kDa) and calbindin D_{28K}

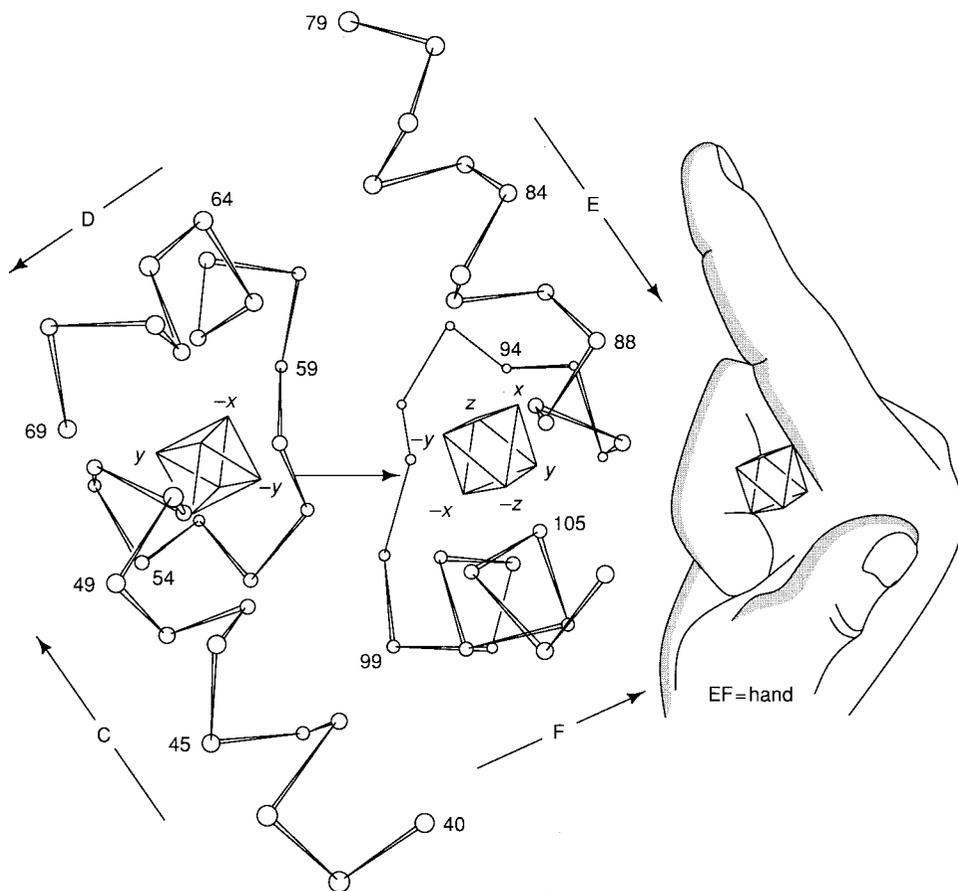


Figure 3.23

Structure of the Ca^{2+} -binding sites of carp parvalbumin. The Ca^{2+} ions are depicted as regular octahedra making six ligand contacts with oxygen atoms at each vertex, labeled x , y , z , $-x$, $-y$, $-z$. The helix-loop-helix structure that forms a Ca^{2+} -binding site can be regarded as a hand with the forefinger representing one helix (e.g., the E-helix) in the plane of the figure, the thumb oriented perpendicular to the plane representing the second helix (the F-helix), and the remaining fingers make up the Ca^{2+} -binding loop. After Kretsinger and Barry.¹¹⁸

($M_r \approx 28$ kDa) belong to this group. *Parvalbumin(s)* exist in two main types, α and β , found in large quantities in the white muscle of fish, amphibia, and reptiles, but also in different mammalian tissues,^{116,117} including neurons of the central and peripheral nervous system. The molecule has two fairly strong Ca^{2+} -binding sites (see Table 3.2). The x-ray structure of carp parvalbumin was solved in 1973 by Kretsinger *et al.*,¹¹⁸ and for a decade provided the basis for all discussions on intracellular Ca^{2+} -binding proteins. The concept of the canonical “EF-hand” Ca^{2+} -binding site originated from the parvalbumin work, and the name “EF” derives from the labeling of the two helices that flank the second of the two Ca^{2+} sites in parvalbumin, as shown in Figure 3.23. If the first Ca^{2+}

D_{9k} is also available.¹²⁴ The average solution structure calculated from NMR data is shown in Figure 3.25 (See color plate section, page C-10.)

The protein has four main α -helices and two Ca^{2+} -binding loops (I and II). The interior of the molecule shows a loose clustering of several hydrophobic side chains; in particular, three phenylalanine rings come very close in space. The Ca^{2+} -binding loops constitute the least-mobile parts of the molecule. The crystallographic temperature factors have pronounced minima in these regions, with the lowest overall B-factor observed in loop II. Both Ca^{2+} ions are roughly octahedrally coordinated with protein oxygen atoms. There are some striking differences between the two sites, however. Whereas the C-terminal site (II) has a general structure very similar to the archetypal "EF-hand," as observed in CaM, sTnC, and parvalbumin, the N-terminal site (I) has an extra amino-acid residue inserted between vertices x and y , and z and $-y$ (see Figure 3.24). As a consequence, the peptide fold in site I is different from that in site II. Three carboxylate groups are ligands in site II, but in site I there is only one.

Despite this marked difference in charge and peptide fold, the Ca^{2+} affinity of both Ca^{2+} sites is remarkably similar, as has been shown in a study in which site-directed mutagenesis was combined with different biophysical measurements.³⁷ Cooperative Ca^{2+} binding in the native calbindin D_{9k} (the "wild type") was first demonstrated at low ionic strength by means of the values of the two stoichiometric Ca^{2+} -binding constants, K_1 and K_2 , which could be measured with good accuracy ($K_1 = 4.4 \times 10^8 \text{ M}^{-1}$ and $K_2 = 7.4 \times 10^8 \text{ M}^{-1}$). The effects of amino-acid substitutions in Ca^{2+} site I were primarily localized to this site, with virtually no effects on the structure or other biophysical properties pertinent to site II. The appearance of sequential Ca^{2+} binding in some of the calbindin mutants did allow the identification of ^1H NMR resonances that respond primarily to binding of Ca^{2+} to either one of the sites. This result in turn permitted an estimate of the ratio between the site-binding constants (K_A and K_B) in the wild-type protein and in one of the mutant proteins (Tyr-13 \rightarrow Phe). In this way the researchers¹²⁵ could assess, to within narrow limits, the free energy of interaction, $\Delta\Delta G$, between the two Ca^{2+} sites as 7.7 kJ/mol at low ionic strength and 4.6 kJ/mol in the presence of 0.15 M KCl. How this site-site interaction is transmitted on a molecular level is still unknown.

Through a combination of site-specific mutations and biophysical measurements, it has recently been demonstrated that carboxylate groups at the surface of the protein, but not directly ligated to the bound Ca^{2+} ions, have a profound effect on the Ca^{2+} affinity.¹²⁶ Neutralization of the surface charges reduces affinity and increases the stability of the protein toward unfolding by urea.¹²⁷

A surprising discovery about the structure of bovine calbindin D_{9k} in solution has also been made recently.¹²⁸ Detailed analysis of the 2D ^1H NMR spectrum of wild-type calbindin has revealed that it exists as a 3:1 equilibrium mixture of two forms, corresponding to a *trans* and *cis* conformation around the Gly-42-Pro-43 peptide bond. The global fold appears essentially the same in the two forms, and structural differences are primarily located in the inter-domain loop in which Pro-43 is located.

D. Sarcoplasmic Calcium-Binding Protein from *Nereis diversicolor*

The calmodulin superfamily of proteins also includes *sarcoplasmic Ca²⁺-binding proteins* (SCPs) that can be found in both vertebrate and invertebrate muscle.¹²⁹ The function of SCPs is not yet known, but their sequence homology with Ca²⁺-binding proteins of known tertiary structure suggests that they originally contained four helix-loop-helix Ca²⁺-binding domains. Ca²⁺ binding has been preserved in the first and third domains of all known SCPs, but only one, if any, of domains II and IV is functional. The three-dimensional crystal structure of an SCP from the sandworm *Nereis diversicolor* analyzed at 3.0 Å resolution¹³⁰ can be seen in Figure 3.26. (See color plate section, page C-11.) The C-terminal half (domains III and IV) of the molecule contains two Ca²⁺-binding EF-hands (green and red in Figure 3.26) similar to calbindin D_{9k} and the globular domains of troponin C and calmodulin. The N-terminal half is, on the contrary, markedly different from the normal helix-loop-helix geometry. Domain I binds Ca²⁺ with a novel helix-loop-helix conformation, whereas domain II lacks calcium-binding capacity. The two halves are packed closely together, and are not, as in troponin C or calmodulin, connected by a solvent-exposed α -helix.

E. Membrane Cytoskeleton and Phospholipid Binding Proteins

It has long been suspected that Ca²⁺ ions are somehow involved in exocytosis. Recently several groups¹³¹ have isolated intracellular proteins that associate with membranes, and/or membrane cytoskeleton proteins, in a Ca²⁺-dependent manner, and that seem able to mediate vesicle fusion or aggregation at Ca²⁺ concentrations above 200 μ M. These proteins—endonexin, calelectrin, p36, and pII—have stretches of consensus amino-acid sequences that are also found in a phospholipase A₂ inhibitor protein, lipocortin.¹³² It appears that further studies of this new class of proteins, known as annexins, will lead to new insights into cell-signaling pathways. Multiple functions have been proposed for the annexins, but no cellular role has yet been defined.¹³³ The first crystal structure of an annexin, human annexin V—which *in vitro* will form voltage-gated Ca²⁺ channels—has been determined recently.¹⁷² In annexin, the three Ca²⁺-binding sites are located on the side of the molecule that is involved in membrane binding.

F. Ca²⁺-Dependent Proteases

An interesting Ca²⁺-activated intracellular protease, sometimes called *calpain*, was discovered during the last decade.¹³⁴ The ending -pain refers to its relation with other proteolytic enzymes like papain. It may seem dangerous to have a proteolytic enzyme loose inside a cell, and it must have rather specialized functions and be under strict control. The complete primary structure of the calcium protease ($M_r \approx 80,000$) in chicken tissues has recently been deduced from the nucleotide sequence of cloned DNA.^{135,136} The findings are quite unexpected.

The protein contains four distinct domains. The first and third domains have no clear sequence homologies with known protein sequences, but the second domain has a high homology with the proteolytic enzyme papain, and the fourth domain is highly homologous to calmodulin. This fourth domain thus has four EF-hand-type Ca^{2+} -binding sites, although the third site has a somewhat unusual loop sequence. Here we apparently are faced with an unusual invention by Nature: by fusing the gene for a protease with that of the canonical Ca^{2+} receptor, she has created a molecule in which a regulatory protein is covalently linked to its target enzyme!

G. Protein Kinase C

Before we leave our brief survey of intracellular Ca^{2+} -binding proteins, we must write a few lines about an important Ca^{2+} -regulated kinase (a phosphorylating enzyme), i.e., *protein kinase C* (PKC). The activity of this enzyme, or rather family of enzymes,¹³⁷ appears to be regulated by three factors: phospholipids, in particular phosphatidylserine; diacyl-glycerols, one of the products of inositol lipid breakdown; and Ca^{2+} ions. The high-activity form of PKC, which appears responsible for much of the phosphorylation activity of many cells, is presumably membrane-bound, whereas the low-activity form may be partly cytosolic (Figure 3.27). The schematic structure of rabbit PKC ($M_r \approx 77$ kDa)

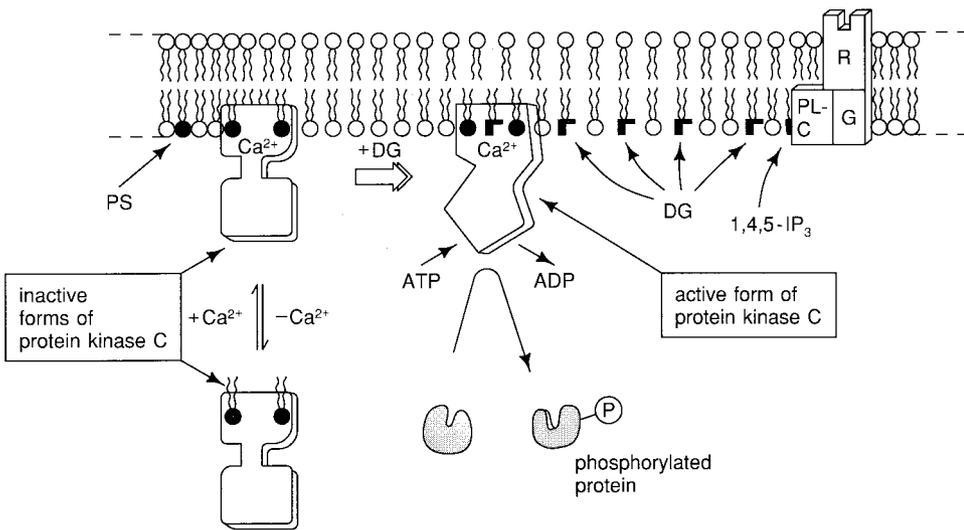


Figure 3.27

Outline of the cellular events that result in the activation of protein kinase C (PKC). The enzyme apparently exists in at least two states. Recent sequence work indicates that it has a Ca^{2+} -binding site of the EF-hand type. When no Ca^{2+} ion is bound, and when the "concentration" of diacylglycerol (DG) in the inner layer of the plasma membrane is low, the kinase exists in a low-activity form, possibly dissociated from the membrane. When a hormone binds to a plasma-membrane receptor (R), cleavage of phosphoinositide into 1,4,5- IP_3 and DG is induced. The latter lipid may bind to and activate the calcium-loaded form of PKC. The active form of protein kinase C will now phosphorylate other cytoplasmic proteins, and in this way modify their biochemical properties. R = receptor; PL-C = phospholipase C; G = a GTP-binding protein that is assumed to act as an intermediary between the receptor and the membrane bound PL-C.

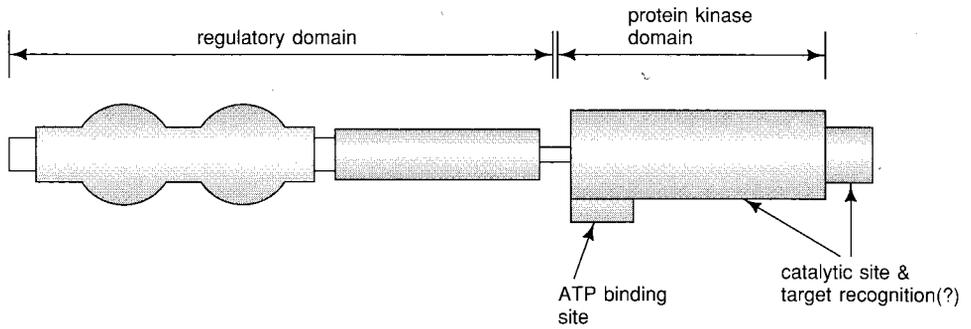


Figure 3.28

Schematic representation of the structure of rabbit protein kinase C.¹³⁸ Three highly homologous protein kinases C were actually identified with $M_r \approx 76,800$. The kinase region shows clear similarity with other kinases. The regulatory domain should contain binding sites for Ca^{2+} , phosphatidyl serine (PS), and diacylglycerol (DG).

according to Ohno *et al.*¹³⁸ is shown in Figure 3.28. The Ca^{2+} site(s) are presumably in the regulatory domain. No typical “EF-hand” pattern has been found in the amino-acid sequence. A protein kinase that requires Ca^{2+} but not phospholipids nor calmodulin for activity has been purified from soybean. From the amino-acid sequence the protein appears to have a calmodulin-like Ca^{2+} -binding domain, very much as in calpain.¹³⁹

H. Summary

Many different biological processes in eukaryotic cells are regulated by intracellular Ca^{2+} concentration levels. Examples of such processes are muscle contraction, transport processes, cell division and growth, enzyme activities, and metabolic processes. A link in this regulatory chain is a number of intracellular Ca^{2+} receptors with Ca^{2+} affinities such that their binding sites are largely unoccupied at resting Ca^{2+} concentration levels, but are occupied at Ca^{2+} levels reached as a result of some external stimulus. This class of Ca^{2+} receptors is often called the “calmodulin superfamily” and includes the well-known members troponin C (regulating muscle contraction in striated muscle) and calmodulin (playing an important role in the regulation of many cellular processes). Amino-acid sequence determinations as well as x-ray and 2D ^1H NMR studies have revealed a strong homology between the regulatory Ca^{2+} -binding proteins. The Ca^{2+} -binding sites are located in a loop flanked by two helices, and the Ca^{2+} ions are ligated with approximately octahedral or pentagonal bipyramidal symmetry. The ligands are six or seven oxygen atoms that are furnished by side-chain carboxylate or hydroxyl groups, backbone carbonyls, and water molecules. Pairs of these Ca^{2+} sites, rather than individual sites, appear to be the functional unit, and a common consequence of their arrangement is cooperative Ca^{2+} binding. Ca^{2+} binding to the intracellular receptor proteins is accompa-

nied by structural changes that expose hydrophobic patches on their surfaces, thereby enabling them to bind to their target proteins.

VI. EXTRACELLULAR Ca^{2+} -BINDING PROTEINS

The Ca^{2+} concentration in extracellular fluids is usually orders of magnitude higher than intracellular concentrations. In mammalian body fluids, the “free” Ca^{2+} concentration is estimated to be 1.25 mM (total Ca^{2+} is ~ 2.45 mM) with only minor variations.¹⁴⁰ We would thus expect that Ca^{2+} ions in extracellular fluids play a very different role from that inside cells. To ensure Ca^{2+} binding the macromolecular binding sites need have only a modest Ca^{2+} affinity ($K_B^{\text{Ca}^{2+}} \approx 10^3$ to 10^4 M^{-1}), and since extracellular Ca^{2+} does not seem to have a signaling function, the rates of Ca^{2+} association or dissociation in protein-binding sites need not be very high.

One particularly important aspect of Ca^{2+} in mammals is its role in the blood coagulation system. Here we will meet a new type of amino acid, γ -carboxyglutamic acid (“Gla”)—see Figure 3.29, that seems to have been de-

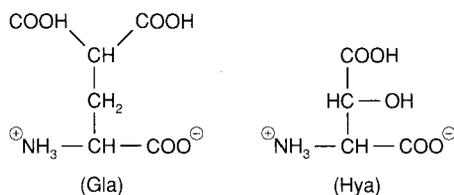


Figure 3.29

Chemical structures of two novel amino acids believed to bind calcium in, e.g., blood-clotting proteins.

signed by Nature as a Ca^{2+} ligand with rather special functions. Gla-containing proteins are also encountered in some mineralized tissues. The formation of bone, teeth, and other calcified hard structures is an intriguingly complicated phenomenon that will be dealt with in Section VII. We start, however, with a brief discussion of the role of Ca^{2+} in some extracellular enzymes.

A. Ca^{2+} -Binding in Some Extracellular Enzymes

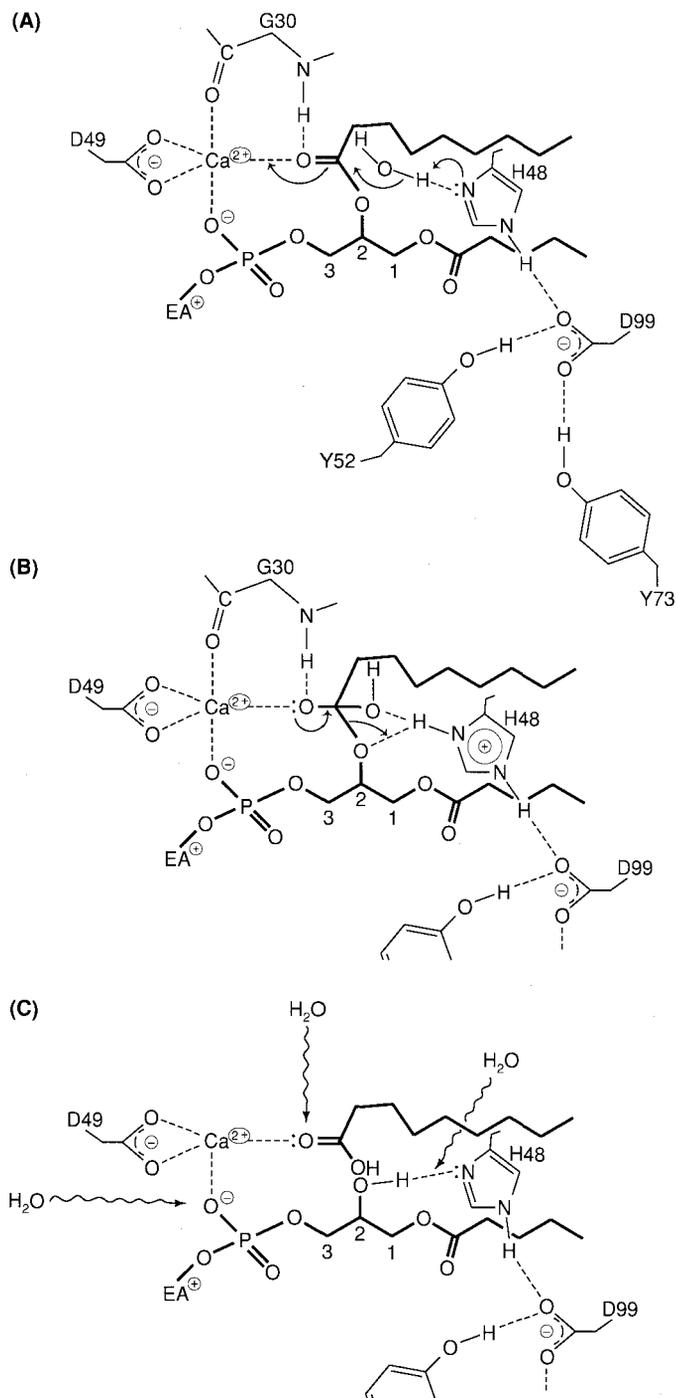
Several extracellular enzymes have one or more Ca^{2+} ions as integral parts of their structure. In a very few of them the Ca^{2+} ion is bound at or near the active cleft, and appears necessary for maintaining the catalytic activity (phospholipase A₂, α -amylase, nucleases), whereas other enzymes show catalytic activity even in the absence of Ca^{2+} (trypsin and other serine proteases). In the latter proteins, the Ca^{2+} ion is usually ascribed a “structural” role, although its function may be rather more related to “dynamics” and so be more subtle and complex.

Trypsin has one Ca^{2+} -binding site with four ligands (two side-chain and two backbone oxygens) donated by the protein (Glu-70, Asn-72, Val-75, and Glu-80) and two ligating water molecules, making the site roughly octahedral.¹⁴¹ The binding constant of Ca^{2+} to trypsin and its inactive precursor "proenzyme," *trypsinogen*, has been measured (see Table 3.2). The binding constant is slightly smaller for the precursor, as is also true for *chymotrypsin* and *chymotrypsinogen*.¹⁴² The Ca^{2+} affinities of the serine proteases and their proenzymes are such that their Ca^{2+} sites will be largely occupied in extracellular fluids, but would be unoccupied inside a cell. It has been suggested that this phenomenon constitutes a safeguard against unwanted conversion of the proenzymes into the active enzymes as long as they still are inside the cells where they are synthesized.

The rates of Ca^{2+} dissociation of the above enzymes and proenzymes have been measured by ^{43}Ca NMR and stopped-flow techniques,¹⁴² and are collected in Table 3.4. We note that the values of k_{on} and k_{off} are generally much smaller than in the intracellular regulatory EF-hand proteins discussed in Section VI. Whereas the latter have dynamic and equilibrium properties similar to those of flexible low-molecular-weight chelators such as EDTA and EGTA, the serine proteases are more similar to the more-rigid cryptates, such as the macrobicyclic amino cryptate [2.2.2] (see Tables 3.2 and 3.4).

As mentioned above, there are a few enzymes in which a Ca^{2+} ion is present in the active cleft and essential for activity. Pancreatic *phospholipase A₂* ($M_r \approx 14$ kDa) is an enzyme of this type. The x-ray structure is known to high resolution, and a single Ca^{2+} ion is found to be surrounded by six ligands, four presented by the protein (Tyr-28, Glu-30, Glu-32, and Asp-49) and two water molecules.¹⁴³ A mechanism for the action of phospholipase A_2 has been proposed¹⁴⁴ and is shown in Figure 3.30. This mechanism is based on three high-resolution x-ray crystal structures of phospholipase A_2 with and without transition-state analogues bound. The binding constant for Ca^{2+} together with the rate of dissociation found from variable-temperature ^{43}Ca NMR studies¹⁴⁵ can be used to calculate $k_{\text{on}} \approx 4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, again lower than in EF-hand proteins. Recent ^1H NMR studies indicate that the global structure of the lipase is very much the same in the Ca^{2+} -free and the Ca^{2+} -bound forms. Structural changes upon Ca^{2+} binding appear primarily located in the region of the binding site.^{112,146}

The mammary glands produce, among other substances, a Ca^{2+} -binding enzyme activator, α -lactalbumin, that has about 40 percent sequence identity with lysozyme. This protein, which is involved in the conversion of glucose into lactose, is secreted in large quantities, and in human milk constitutes some 15 percent of total protein. The Ca^{2+} -binding constant of bovine or human α -lactalbumin is on the order of 10^7 M^{-1} under physiological conditions. In addition to Ca^{2+} , the enzyme also binds Zn^{2+} . It appears that Ca^{2+} -ion binding affects enzymatic activity, and somehow controls the secretion process, but the biological role of metal-ion binding to α -lactalbumin needs to be studied further. The x-ray structure of α -lactalbumin from baboon milk ($M_r \approx 15$ kDa) has been



determined¹⁴⁷ to a high resolution (~ 1.7 Å). The Ca^{2+} -binding site has an interesting structure. The ion is surrounded by seven oxygen ligands, three from the carboxylate groups of aspartyl residues (82, 87, and 88), two carbonyl oxygens (79 and 84), and two water molecules. The spatial arrangement is that of a slightly distorted pentagonal bipyramid with the carbonyl oxygens at the apices, and the five ligands donated by the proteins are part of a tight "elbow"-like turn. The α -lactalbumin site has a superficial structural similarity to an "EF-hand," although the enzyme presumably has no evolutionary relationship with the intracellular Ca^{2+} -binding regulatory proteins.

Blood clotting proceeds in a complicated cascade of linked events involving many enzymes and proenzymes. About a decade ago it was shown that several of these proteins contained a previously unknown amino acid, γ -carboxyglutamic acid (Gla), and more recently yet another new amino acid, β -hydroxyaspartic acid (Hya), has been discovered (see Figure 3.29). The former is formed postribosomally by a vitamin-K-dependent process in the liver.¹⁴⁸ Presently the most-studied Gla protein in the blood-clotting system is *prothrombin* ($M_r \approx 66$ kDa). Ten Gla residues are clustered pairwise in the N-terminal region, essentially lining one edge of the molecule, forming a highly negatively charged region.¹⁴⁹ A small (48 residues) proteolytic fragment (F1) that contains all ten Gla amino acids can be prepared. Prothrombin can bind about 10 Ca^{2+} ions, but F1 binds only 7. Binding studies to F1 show that the Ca^{2+} ions bind at three high-affinity cooperative sites and four noninteracting sites,¹⁵⁰ and that this binding takes place in conjunction with a spectroscopically detectable conformational change (see Table 3.1).

In the presence of Ca^{2+} ions, prothrombin and other vitamin-K-dependent proteins in the blood-coagulation system will bind to cell membranes containing acidic phospholipids, in particular, the platelet membrane, which is rich in phosphatidylserine. A proposed model for the prothrombin-membrane interaction is shown in Figure 3.31.

It has long been known that calcium ions are involved in cell-to-cell and cell-to-extracellular matrix interactions, but the molecular details largely remain to be unraveled. In the late 1980s a large, adhesive, calcium-binding matrix glycoprotein ($M_r \sim 420$ kDa) named thrombospondin was characterized. This multifunctional adhesion molecule is composed of three polypeptide chains, each with 38 amino-acid-long repeats that are homologous with the calcium-binding helix-loop-helix sites of the calmodulin superfamily.¹⁵² Each thrombospondin molecule is reported to bind 12 calcium ions with an affinity of about 10^4 M^{-1} , and the removal of calcium is accompanied by a conformational change.^{153,154}

B. Summary

In higher organisms, the Ca^{2+} concentration in extracellular fluids generally is considerably higher than the intracellular concentrations. In mammalian body fluids, the Ca^{2+} concentration is typically on the order of a few mM. The

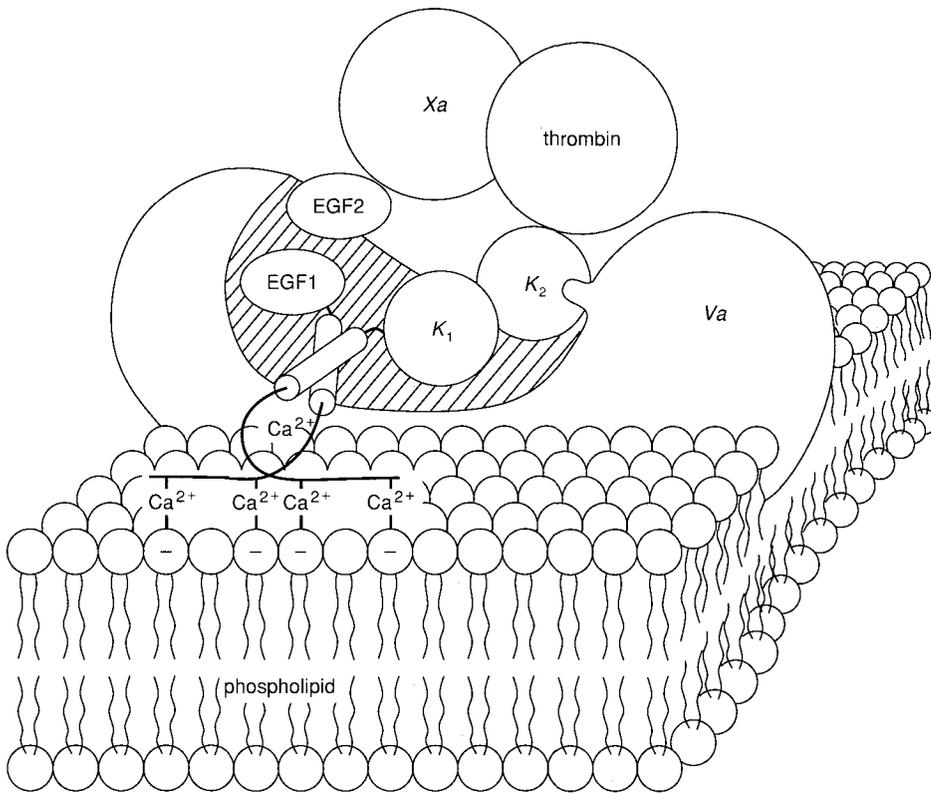


Figure 3.31

Proposed features of the interaction between the prothrombinase complex and a membrane lipid bilayer.¹⁵¹ K_1 and K_2 are the kringle domains of prothrombin, and EGF1 and EGF2 are the two epidermal growth factor units of factor X_a . Prothrombin and factor X_a form a heterodimer complex harbored within the membrane protein factor V_a . The proposed interaction between prothrombin and factor X_a involves hydrophobic interactions between two helices and bridging by a Ca^{2+} ion between two Glu residues. The N-terminal Glu residues attach the heterodimer complex to the phospholipid surface. Figure kindly provided by C. C. F. Blake.

extracellular concentration levels are highly regulated and undergo only minor variations. A consequence of these high levels of Ca^{2+} in extracellular fluids is that the binding constant need be only 10^3 to 10^4 M^{-1} in order for a protein site to be highly occupied by Ca^{2+} . Several extracellular enzymes and enzyme activators have one or more Ca^{2+} ions as integral parts of their structures. Some Ca^{2+} ions are bound at, or near, the active cleft and may take part in the enzymatic reactions (e.g., phospholipase A_2 , α -amylase). In other molecules, for example, serine proteases like trypsin and chymotrypsin, the Ca^{2+} ion is not essential for enzymatic activity, and may play more of a structural role. Ca^{2+} ions are involved in the cascade of enzymatic events that results in blood clotting in mammals. Several of the proteins in this system contain two new amino acids, γ -carboxyglutamic acid (Gla) and β -hydroxyaspartic acid (Hya), which

are strongly suspected to be involved as ligands in Ca^{2+} binding. In the presence of Ca^{2+} ions, prothrombin and other Gla-containing proteins will bind to cell membranes containing acidic phospholipids, in particular, the platelet membrane. It appears likely that Ca^{2+} ions form a link between the protein and the membrane surface.

VII. CALCIUM IN MINERALIZED TISSUES

The formation of calcified tissue—shells, bone, and teeth—is a very complex process that is under strict regulatory control. Despite the obvious importance of this field, relatively little research has been directed toward elucidation of the underlying mechanisms, perhaps because the field spans a broad range of subjects, from inorganic solution and solid-state chemistry to cellular physiology.¹⁵⁵

Historically, it was long held that formation of biological minerals such as bone was simply the nucleation and growth of calcium hydroxyapatite within an extracellular matrix of collagen. Many proteins other than collagen have now been discovered in appreciable quantities in bone and other biological minerals. It is also apparent that the pattern of calcification differs in shells, bone, teeth, and other mineralized tissues; so it is not likely that there is only one underlying mechanism. Considering the immensity of the subject, we will here only make a few brief comments, mainly about bone and teeth.

As was briefly mentioned earlier in this chapter, the inorganic matter of bone and teeth in many ways resembles apatite minerals ($\text{Ca}_5(\text{OH})(\text{PO}_4)_3$). Table 3.5 summarizes inorganic solid components of other biominerals. A detailed

Table 3.5
A summary of the main inorganic solid component^a of the most-common biominerals in living systems.¹⁴⁸

Anion	Formula	Crystal form	Occurrence	Main function
Carbonate	CaCO_3	Calcite Aragonite Valerite	Sea corals, molluscs, and many animals and plants	Exoskeleton; Ca-store; eye lens
Oxalate	$\text{Ca}(\text{COO})_2 \cdot \text{H}_2\text{O}$ $\text{Ca}(\text{COO})_2 \cdot 2\text{H}_2\text{O}$	Whewellite Weddellite	Insect eggs; verte- brate stones	Deterrent; cytoskeleton; Ca store
Phosphate	$(\text{Ca})_{10}(\text{PO}_4)_6(\text{OH})_2$ (unit cell comp.)	Hydroxyapatite	Bones; teeth; shells; intracellular in some bacteria	Skeletal; Ca storage; pres- sure-transducer (piezo-elec- tric)
Sulfate	$\text{CaSO}_4 \cdot \text{H}_2\text{O}$	Gypsum	Jelly fish; plants	Gravity device; S and Ca store

^a Most real biominerals are actually nonstoichiometric, and contain a number of additional cations (e.g., Mg^{2+}) or anions (e.g., F^-). In addition, the inorganic phase may be interpenetrated by a biopolymer.

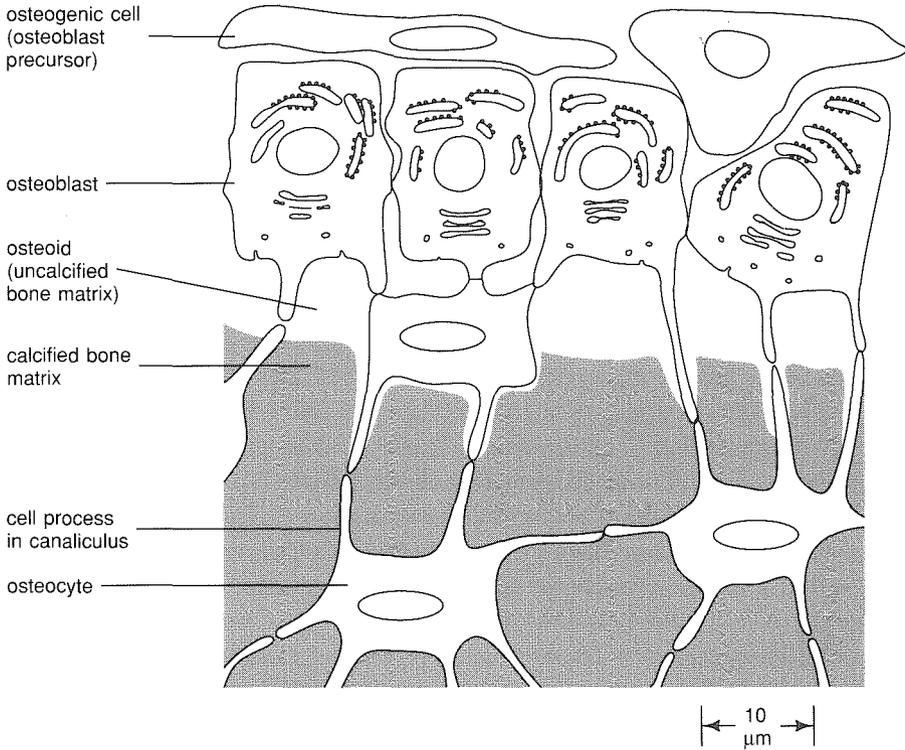
analysis¹⁵⁶ shows that, apart from Ca^{2+} and PO_4^{3-} , many other cations and anions occur in bone, e.g., Mg^{2+} , Na^+ , K^+ , Sr^{2+} , CO_3^{2-} , F^- , Cl^- , and citrate. X-ray diffraction patterns and electron-microscope pictures of bone show that the inorganic phase is made up of many very small and imperfect crystals. By contrast, dental enamel is made up of much larger and uniform thin crystals. Although the solubility product of calcium hydroxyapatite (see Section II) is such that the equilibrium Ca^{2+} concentration should be in the low micromolar range, bone mineral appears to be in equilibrium with much higher Ca^{2+} concentrations (0.8–1.0 mM).¹⁵⁷ This discussion brings us to the question of how the inorganic crystallites are formed. Obviously both Ca^{2+} and PO_4^{3-} ions must be concentrated in cells or organelles bordering on the regions where mineralization is to take place. Fresh layers of bone matrix are formed by a continuously replenished layer of cells called *osteoblasts* (Figure 3.32A), which, in addition to apatite crystallites, also secrete collagen, and large specific proteins called *osteonectin*, *osteocalcin* (a Gla protein), proteoglycans, and phosphoproteins. In tissues undergoing rapid mineral deposition, the crystallites appear to be formed in vesicles that may have peeled off from the adjacent cell layers. These vesicles seem able to concentrate calcium and phosphate in a manner not well understood.

Bone, unlike diamond, is not forever. It can be remodeled and dissolved. A serious medical problem, which affects some women after menopause, is **osteoporosis**, i.e., the decalcification of bone. This loss of bone mass, which occurs with increasing age, makes bones more susceptible to breaking under stress. About 50 percent of American women, and 25 percent of American men, over 45 years of age are affected by osteoporosis.¹⁵⁸ Whereas osteoblast cells handle bone formation, another type of cells, *osteoclasts*, can erode it (Figure 3.32B). These macrophage-like cells can form deep tunnels in a bone matrix, and the cavities left behind are rapidly invaded by other cells forming blood vessels and new layers of osteoblasts. The *modus operandi* of osteoclast cells is not well understood at present. They may secrete calcium-chelating organic anions, such as citrate, to assist in the solubilization of the bones, as well as extracellular proteases that degrade the organic part of the matrix.

Summary

Calcium is, along with iron, silicon, and the alkaline earth metals, an important constituent of mineralized biological tissues. Some Ca^{2+} -based biominerals, like bone or mother-of-pearl, can be regarded as complex composites with microscopic crystallites embedded in a protein matrix. The formation of calcified biominerals is a highly regulated process, and human bone, for instance, is constantly being dissolved and rebuilt. When the rates of these two counteracting processes are not in balance, the result may be decalcification, or *osteoporosis*, which seriously reduces the strength of the bone.

(A)



(B)

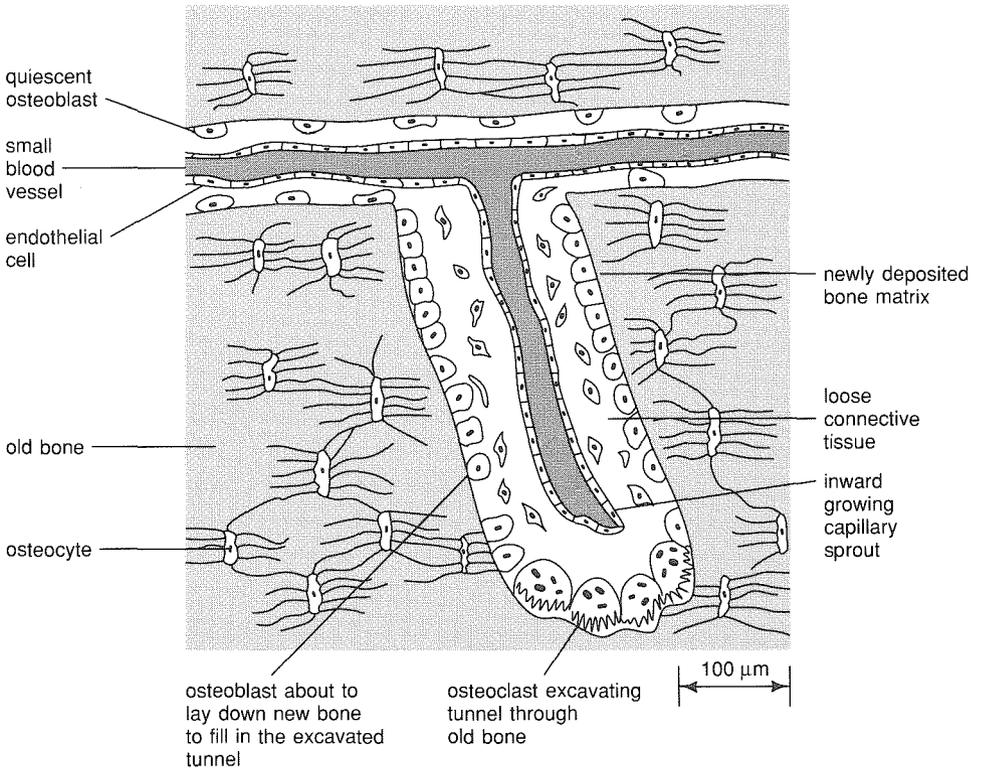


Figure 3.32 (facing page)

Schematic diagram depicting the roles of the most important cell types in bone formation. (A) The *osteoblast* cells line the bone surface and secrete the inorganic and organic components (collagen, etc.) that will form new bone. Some osteoblast cells gradually become embedded in their own secretion. A particular secreted bone-specific protein, osteonectin, forms strong links between calcium hydroxyapatite and collagen. The bone-forming cells that become trapped in the bone matrix are now called *osteocytes*. (B) The osteoclast cells function to remodel compact bone. A group of cells acting together excavate a tunnel through old bone at a rate of about 50 μm per day. Behind the advancing osteoclasts follow a contingent of osteoblasts that line the wall of the tunnel and start to form new bone. Concurrently a capillary vessel is formed along the center of the tunnel and provides the cells with nutrients. Eventually the tunnel will become filled with concentric layers of new bone with only a narrow canal remaining. It is apparent that bone is far from a dull inorganic deposit, and very much a site of continuous activity. It is estimated that 5 to 10 percent of the bone in an adult mammal is replaced per year. Adapted from Reference 159.

VIII. Ca^{2+} -BINDING PROTEINS IN MICROORGANISMS: THE SEARCH FOR A PROKARYOTIC CALMODULIN

Since Ca^{2+} ions evidently play an important role in regulating a variety of cellular responses in animals and higher organisms, one may ask whether this use of Ca^{2+} is a recent discovery of Nature, or if it was invented early in evolution. It now appears well-established that the key intracellular “ Ca^{2+} -receptor” protein calmodulin (CaM; see Section V.A) is present in all eukaryotic cells. Even in a unicellular eukaryote like common yeast (*Saccharomyces cerevisiae*), Ca^{2+} has an important regulatory role, and recently yeast CaM, as well as the single-gene encoding for it, was isolated.¹⁶⁰

The amino-acid sequence of the yeast CaM (147 a.a.; $M_r = 16.1$ kDa) is 60 percent identical with the sequences of all other CaMs known. In fact, if generally accepted conservative amino-acid replacements are allowed, the homology increases to 80 percent or more, the most highly conserved portions being the four putative Ca^{2+} -binding sites. Sites I and III match the EF-hand test sequence (see Figure 3.24) very well; in site II, a His occurs after the “z”-ligand instead of the archetypal Gly; and in site IV there is no amino acid between the residues that usually make up ligands “x” and “y.” The effect of these alterations on the Ca^{2+} affinity of yeast CaM is not yet known.

That CaM is essential for the growth of yeast cells was shown by deletion or disruption of the gene. This constitutes, in fact, the first demonstration in any organism that CaM is an essential protein. (Deletions of genes in mammals are ethically questionable research procedures!)

In the biochemically less sophisticated (than eukaryotes) **prokaryotic** cells, a regulatory role of Ca^{2+} is not well-established. What is known is that calcium is massively accumulated during sporulation in many bacteria, for example, in strains of *Bacillus*, *Streptomyces*, and *Myxococcus*. In *Myxococcus xanthus* a development-specific protein called protein S assembles at the surface of myxospores in the presence of Ca^{2+} . The DNA sequence of the gene that encodes this

protein has been deciphered.¹⁶¹ The primary sequence of protein S (175 a.a., $M_r = 19.2$ kDa) turns out to closely resemble mammalian CaM. It has four internally homologous regions with putative Ca^{2+} sites. At least two of these are partly similar to the typical EF-hand, but uncharacteristically there are many more prolines in the *M. xanthus* protein than in bovine CaM (12 versus 2); so it is questionable if the bacterial protein really has the repeated helix-loop-helix structure found in mammalian CaM.¹⁶²

One candidate for a prokaryotic CaM was reported by Leadlay *et al.*¹⁶³ in *Streptomyces erythraeus*, the bacterium that produces the well-known antibiotic "erythromycin." The amino-acid sequence of a low-molecular-weight Ca^{2+} -binding protein, as determined from the gene encoding it, revealed a high homology with mammalian CaM. The protein is made up of 177 amino acids ($M_r = 20.1$ kDa), and has four regions that are predicted to have the helix-loop-helix secondary structure typical of EF-hand proteins. The aligned sequences of the 12 residues in each of the four potential calcium-binding loops in the *S. erythraeus* protein are compared with those of human calmodulin in Table 3.6. The pattern of residues in the *S. erythraeus* protein is typical of an EF-hand at least in sites I, III, and IV. Site II is unusual in having Gly at both positions 1 and 3. ¹¹³Cd NMR studies show that the bacterial protein binds three metal ions strongly ($K \geq 10^5 \text{ M}^{-1}$) with chemical shifts close to those expected for EF-hands, and ¹H NMR studies show that it undergoes a Ca^{2+} -dependent conformational change.¹⁶⁴

Although the *S. erythraeus* protein has a homology with eukaryotic CaM, it has been pointed out that the protein has an even higher homology with a group of eukaryotic sarcoplasmic Ca^{2+} -binding proteins¹⁶⁵ (see Section V.D). The search for a prokaryotic CaM analogue continues, and the prospect of success has been improved after recent reports of a 21-amino-acid-long polypeptide from an *E. coli* heat-shock protein¹⁶⁶ that shows the typical structural features of CaM-binding domains in other eukaryotic proteins.¹⁶⁷

Table 3.6
Aligned EF-hand sequences for the prokaryotic and human calmodulins.

Ligands		1	3	5	7	9	12						
<i>S. erythraeus</i> protein	I	D	F	D	G	N	G	A	L	E	R	A	D
	II	G	V	G	S	D	G	S	L	T	E	E	Q
	III	D	K	N	A	D	G	Q	I	N	A	D	E
	IV	D	T	N	G	N	G	E	L	S	L	D	E
Human calmodulin	I	D	K	D	G	D	G	T	I	T	T	K	E
	II	D	A	D	G	N	G	T	I	D	F	P	E
	III	D	K	D	G	N	G	Y	I	S	A	A	E
	IV	D	I	D	G	D	G	Q	V	N	Y	E	E

Summary

The role of Ca^{2+} ions in the regulation of biological activities of procaryotic organisms is still largely unsettled. Over the last decade, however, evidence has gradually accumulated that calcium ions are involved in diverse bacterial activities, such as chemotaxis and substrate transport, sporulation, initiation of DNA replication, phospholipid synthesis, and protein phosphorylation.¹⁶⁸ An important landmark is the recent demonstration that the intracellular Ca^{2+} concentration in *E. coli* is tightly regulated to about 100 nM, a level similar to that typical of resting eukaryotic cells.¹⁶⁹ Furthermore, increasing numbers of calcium-binding proteins, some of which also have putative EF-hand Ca^{2+} sites characteristic of the calmodulin superfamily of intracellular regulatory proteins, have been isolated in bacteria.¹⁶⁸

IX. APPENDIXES

A. Definition of Biochemical Terms

Antiport	A transport protein that carries two ions or molecules in opposite directions across a membrane.
Basal lateral membrane	The membrane in intestinal epithelial cells that is located on the base of the cells, opposite the microvilli that face the intestinal lumen.
Cytosol	The unstructured portion of the interior of a cell—the cell nucleus excluded—in which the organelles are bathed.
Electrogenic	A biological process driven by electric field gradients.
Endocytosis	The process by which eukaryotic cells take up solutes and/or particles by enclosure in a portion of the plasma membrane to (temporarily) form cytoplasmic vesicles.
Endoplasmic reticulum (ER)	Sheets of folded membranes, within the cytoplasm of eukaryotic cells, that are the sites for protein synthesis and transport.
Epithelial cells	Cells that form the surface layer of most, if not all, body cavities (blood vessels, intestine, urinary bladder, mouth, etc.).
Erythrocytes	Red-blood corpuscles.
Eukaryotic cells	Cells with a well-defined nucleus.

Exocytosis	The process by which eukaryotic cells release packets of molecules (e.g., neurotransmitters) to the environment by fusing vesicles formed in the cytoplasm with the plasma membrane.
Gluconeogenesis	Metabolic synthesis of glucose.
Glycolysis	Metabolic degradation of glucose.
Hydrophathy	A measure of the relative hydrophobic or hydrophilic character of an amino acid or amino-acid side chain.
Lamina propria mucosae	The layer of connective tissue underlying the epithelium of a mucous membrane.
Mitochondrion	A double-membrane organelle in eukaryotic cells that is the center for aerobic oxidation processes leading to the formation of energy-rich ATP.
Organelle	A structurally distinct region of the cell that contains specific enzymes or other proteins that perform particular biological functions.
Osteoporosis	Brittle-bone disease.
Phorbol esters	Polycyclic organic molecules that act as analogues to diacylglycerol and therefore are strong activators of protein kinase C.
Prokaryotic cells	Cells lacking a well-defined nucleus.
Sarcoplasmic reticulum	The ER of muscle cells.
Trophoblasts	The cells between the maternal and fetal circulation systems.
Tryptic digest	Fragmentation of proteins as a result of treatment with the proteolytic enzyme trypsin.
Uniporter	A transport protein that carries a particular ion or molecule in one direction across a membrane.

B. One-Letter Code for Amino-Acid Residues

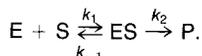
A—alanine, C—cysteine, D—aspartate, E—glutamate, F—phenylalanine, G—glycine, H—histidine, I—isoleucine, K—lysine, L—leucine, M—methionine, N—asparagine, P—proline, Q—glutamine, R—arginine, S—serine, T—threonine, V—valine, W—tryptophan, Y—tyrosine.

C. The Activity of a Transport Protein

This is usually described in terms of the classical Michaelis-Menten scheme:

$$V (= \text{transport rate}) = V_{\max} \cdot \frac{[S]}{[S] + K_m},$$

where [S] is the concentration of the solute to be transported and $K_m = (k_{-1} + k_2)/k_1$ is the Michaelis constant (dimension “concentration”) for the reaction



Approximated as the reciprocal ratio between on- and off-rate constants relevant to the solute-protein complex, $1/K_m = k_1/k_{-1}$ may be taken as a lower limit of the affinity of the protein for the solute.

X. REFERENCES

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