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Review

Structural and functional aspects of calcium homeostasis in eukaryotic cells

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The maintenance of a low cytosolic free-Ca^{2+} concentration, ($[Ca^{2+}]_i$) is a common feature of all eukaryotic cells. For this purpose a variety of mechanisms have developed during evolution to ensure the buffering of Ca^{2+} in the cytoplasm, its extrusion from the cell and/or its accumulation within organelles. Opening of plasma membrane channels or release of Ca^{2+} from intracellular pools leads to elevation of $[Ca^{2+}]_i$; as a result, Ca^{2+} binds to cytosolic proteins which translate the changes in $[Ca^{2+}]_i$ into activation of a number of key cellular functions.

The purpose of this review is to provide a comprehensive description of the structural and functional characteristics of the various components of $[Ca^{2+}]_i$ homeostasis in eukaryotes.

It is well known how Sidney Ringer recognized the role of Ca^{2+} in muscle contraction [1] and how fortunate it was that he was working with frog heart muscle, rather than with skeletal muscle, given that contractility in the latter muscle is basically independent of extracellular Ca^{2+}. Less appreciated is the fact that it took a century before a wide-range test of the 'Ca^{2+} hypothesis' in the mediation of cellular responses became possible. So many years in fact elapsed between the publication of the seminal paper by Ringer in 1883 [1] and the report in 1982 by Tsien and co-workers [2] describing the application of quin 2 to the measurement of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) in small mammalian cells.

The exploitation of fluorescent tetracarboxylate/pentacarboxylate dyes for measuring Ca^{2+} has enabled a thorough testing of Ca^{2+} involvement in a host of cellular responses spanning fertilization to muscle contraction, proliferation to neurotransmitter release and cell motility to cell death. The fluorescent dye technique has also complemented biochemistry, electrophysiology and molecular biology, and allowed the description in real time of the actual changes in $[Ca^{2+}]_i$ following the activation of membrane Ca^{2+} influx/efflux pathways. Additional levels of complexity have emerged from the application of these techniques to the study of Ca^{2+} homeostasis and new problems have arisen, yet for the first time we are beginning to understand Ca^{2+} signaling as the integration of multiple pathways.

Ca^{2+} is maintained in the cytoplasm of mammalian cells at a concentration that is 10000-fold lower than that of the extracellular milieu. This is achieved by means of sophisticated homeostatic mechanisms given that with a negative interior-plasma membrane potential of −60 mV, a $[Ca^{2+}]_i$ of 0.1–0.2 M should be expected, if Ca^{2+} were distributed at electrochemical equilibrium [3]. The first barrier to Ca^{2+} overflow into the cytoplasm is the natural impermeability of phospholipid bilayers to charged species. However, permeability to Ca^{2+} is significant even across a pure lipid bilayer and even more so across the plasma membrane of living cells that also possess specific pathways (channels) for Ca^{2+} influx. These pathways can be opened by ligands or by depolarization and allow passive diffusion of Ca^{2+}. Ca^{2+} can also be released from intracellular depots via a complex chain of intracellular messengers, in part still unknown. Once Ca^{2+} has entered the cytoplasm, it is bound by a number of proteins that transduce the Ca^{2+} signal or simply buffer this ion. The long-term maintainance of steady and low $[Ca^{2+}]_i$, even in the absence of a massive Ca^{2+} influx through specific channels, depends on the operation of Ca^{2+}-extrusion mechanisms. Excess cytoplasmic Ca^{2+} can also be taken up by mitochondria via an electrophoretic uniport and/or accumulated into non-mitochondrial intracellular stores. The homeostasis of $[Ca^{2+}]_i$ will be the result of all these interacting mechanisms.

THE PLASMA MEMBRANE

In all eukaryotes, the primary barrier to Ca^{2+} inflow into the cytoplasm is represented by the plasma membrane, where the ultimate long-term regulators of $[Ca^{2+}]_i$ homeostasis, i.e. the Ca^{2+}-efflux mechanisms, are located. It is evident that intracellular Ca^{2+}-binding proteins or organelles capable of

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Abbreviations. $[Ca^{2+}]_i$, cytosolic free-Ca^{2+} concentration; VOC, voltage-operated channel; ROC, receptor-operated channel; SMOC, second messenger-operated channel; GOC, G-protein-operated channel; EC coupling, excitation-contraction coupling; MeDAsp, *N*-methyl D-aspartate; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; Ins(1,3,4,5)P_4, inositol 1,3,4,5-tetrakisphosphate; PtdIns(4,5)P_2, phosphatidylinositol (4,5) bisphosphate; SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; TC, terminal cisternae; E1, enzyme form 1 of ATPase; E2, enzyme form 2 of ATPase; G-protein, guanylnucleotide-binding protein.

accumulating Ca^{2+} can only transiently buffer $[Ca^{2+}]_i$ increases. The final resetting of $[Ca^{2+}]_i$ must involve the transfer of Ca^{2+} out of the cell. Ca^{2+} extrusion is accomplished by means of membranous Ca^{2+}-binding proteins that reversibly complex and translocate Ca^{2+} outside the cytosol. Two main types of Ca^{2+}-extruding systems are known to exist in the plasma membrane involving (a) Ca^{2+}-ATPases and (b) a Na^+/Ca^{2+} exchanger. These two transport systems differ in their Ca^{2+} affinity and V_{max} of Ca^{2+} transport.

Although in the steady state the net flux of Ca^{2+} across the plasma membrane is zero, there are transient situations in which large increases of Ca^{2+} influx from the extracellular milieu occur. This rapid change in the permeability of the plasma membrane to Ca^{2+} depends on the opening of the plasma membrane Ca^{2+} channels. Several types of Ca^{2+} channels have been described; they differ in ionic selectivity, conductance, tissue distribution, subcellular localization and mechanism of activation. Therefore two main systems for the regulation of $[Ca^{2+}]_i$ homeostasis are present on the plasma membrane: Ca^{2+}-extrusion mechanisms and Ca^{2+}-influx pathways.

Ca^{2+}-EXTRUSION MECHANISMS

The plasma membrane Ca^{2+}-ATPase

Ca^{2+}-pumping activity was first described in the plasma membrane of erythrocytes by Schatzmann in 1966 (see [4] for a recent review). This enzyme was subsequently found in all eukaryotic cells investigated [5] where it accounts for only 0.1% of the total membrane protein [6]. The plasma membrane Ca^{2+}-ATPases belong to the family of so called E1/E2 transport ATPases which exist in two conformational states (E1 and E2) during the reaction cycle. Energy liberated by ATP hydrolysis is stored intramolecularly as an aspartyl residue [4].

The discovery that calmodulin regulates the enzyme by directly binding to it suggested a purification procedure using a Sepharose column coupled to calmodulin. This allowed the fast and reproducible isolation of large amounts of protein [7]. Initial purification from erythrocyte plasma membrane and later isolation of complementary DNA from rat brain, gave a monomeric enzyme having a molecular mass of approximately 130 kDa (138 kDa for erythrocytes; 129.5 and 132.6 kDa for the two rat-brain isoforms) [8, 9]. Amino acid similarity and hydropathy profile comparison indicated a common transmembrane organization for Na^+/K^+-ATPase, sarcoplasmic reticulum Ca^{2+}-ATPase and the isoforms of plasma membrane Ca^{2+}-ATPase isolated from rat brain [8, 9]. Since both the N and C termini of the plasma membrane Ca^{2+}-ATPase have been shown to be on the cytoplasmic side of the membrane, it is obvious that there must be an even number of transmembrane domains. The actual number of transmembrane domains is uncertain, but there may be as many as ten; four transmembrane domains have been identified in the N-terminal half of the molecule and four to six are likely to be present in the C-terminal half [9]. Six to ten transmembrane domains have also been suggested for the membrane-spanning region of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase [10]. The transmembrane domains of the plasma membrane Ca^{2+}-ATPase also contain seven carboxylate groups that may have a role in Ca^{2+} translocation. Both isoforms cloned from rat brain possess, near the C termini, a sequence typical of calmodulin-binding domains [9]. Numerous studies suggest the presence of two ATP-binding sites with different affinities for ATP [11–13], although it is not entirely clear whether the two sites are separate or the same site is undergoing cyclic changes in affinity. The high-affinity ATP-binding site ($K_m \approx 1$ μM) is the catalytic site, while the low-affinity site (K_m 0.1–0.3 mM) has been suggested to have a regulatory role [4]. Nucleotide analysis of two cDNAs recently isolated from rat brain gave further support to this suggestion [9]. Most ATPases so far characterized possess two binding sites for ATP.

As noted above, the discovery of modulation of the plasma membrane Ca^{2+}-ATPase by calmodulin was instrumental for its purification [5, 7, 14, 15]. Calmodulin modulates this enzyme by direct interaction, not by a calmodulin-dependent phosphorylation, and shifts the enzyme to the high Ca^{2+}-affinity state [5]. Following the interaction with calmodulin, the affinity for Ca^{2+} of the transport site increases 30-fold (giving an apparent K_d for Ca^{2+} of 1 μM and 0.2 μM at pH 7.0 and 7.8, respectively) and the affinity for ATP (of the low-affinity ATP-binding site) 100-fold [16–18]. The state of phosphorylation of the enzyme remains, however, the same. At resting physiological $[Ca^{2+}]_i$ and cytosolic free-Mg^{2+} concentrations, calmodulin is dissociated from the ATPase; as $[Ca^{2+}]_i$ increases, Ca^{2+}-calmodulin–ATPase association proceeds slowly; consequently pump activity also increases slowly in response to elevations in $[Ca^{2+}]_i$ [18]. This mechanism imparts hysteretic behavior to the control of $[Ca^{2+}]_i$ homeostasis and allows overshoot of $[Ca^{2+}]_i$ before a new steady state is achieved [19]. The activity of the enzyme may also be increased, in the absence of calmodulin, by stimulation with acidic phospholipids and long-chain polyunsaturated fatty acids, or by controlled trypsin digestion. In view of the recent interest for the second-messenger functions of the metabolites of the phosphatidylinositol cycle, it is of interest that the plasma membrane Ca^{2+}-ATPase can be activated by phosphorylated metabolites of phosphatidylinositol. Phosphorylation by the cAMP-dependent kinase also activates the enzyme isolated from heart sarcolemma [20], but not all isoforms of plasma membrane Ca^{2+}-ATPases appear to contain a consensus sequence for cAMP-dependent phosphorylation. This is consistent with the observation that in numerous cell types, elevation of cAMP does not result in an increased Ca^{2+} extrusion [21]. In a reconstituted liposomal system the enzyme functions as a $Ca^{2+}/2\ H^+$ exchanger having a Ca^{2+} stoichiometry with respect to hydrolyzed ATP of 1:1 [8, 22].

Protein kinase C activation by either phorbol esters or endogenously produced diacylglycerol has been shown to activate Ca^{2+} extrusion from intact cells [23–25] (T. Pozzan, unpublished results) and also to increase Ca^{2+} accumulation by inside-out plasma membrane vesicles [23]. It is not clear yet, however, whether this positive modulation is a consequence of direct phosphorylation of the plasma membrane Ca^{2+}-ATPase, or whether it results from indirect effects.

The Na^+/Ca^{2+} exchanger

The Na^+/Ca^{2+} exchanger was discovered in the late 1960s in cardiac muscle and invertebrate nerve [26, 27]. Its presence has been well documented in the plasma membrane of a variety of excitable cells, while its presence (and importance) in non-excitable cells is debated. The coupling ratio of Na^+/Ca^{2+} is 3:1, therefore the exchanger is electrogenic and voltage sensitive. Na^+/Ca^{2+} exchanger is not energized by ATP, nonetheless it appears that the exchanger is phosphorylated under normal intracellular concentrations of ATP [28–30].

In the phosphorylated form the exchanger has high affinity for intracellular Ca^{2+} and extracellular Na^+. Furthermore, internal Ca^{2+} in the physiological range 0.1 – 1.0 μM, activates the exchanger, while internal Na^+ is ineffective. Were the Na^+/Ca^{2+} exchanger the only regulator of $[Ca^{2+}]_i$, this would drive $[Ca^{2+}]_i$ at equilibrium to a concentration given by the following equation:

$$[Ca^{2+}]_i = [Ca^{2+}]_o \frac{[Na]_i^3}{[Na]_o^3} \exp \frac{(n-2)FV_m}{RT}$$

where V_m is the transmembrane potential, F is the Faraday number, R is the gas constant, T is absolute temperature and n is the coupling factor. The electrogenicity of the system carries with it the consequence that the direction in which Ca^{2+} (and Na^+) is transported depends on the plasma membrane potential. If the transmembrane potential is positive to the reverse potential of the exchanger, Na^+ is driven out and Ca^{2+} in; the reverse happens for transmembrane potentials negative with respect to the reverse potential. The exchanger will cause Ca^{2+} influx when the plasma membrane is depolarized and Ca^{2+} efflux when it repolarizes. It will therefore work in parallel with the voltage-gated Ca^{2+} channels in increasing $[Ca^{2+}]_i$ during the action potential and with the membrane Ca^{2+}-ATPase in extruding Ca^{2+} from the cytosol during the repolarization phase. The K_m of the exchanger is ≈ 2 μM, while the V_{max} is much larger than that of the plasma membrane Ca^{2+}-ATPase [31]. This has led to the suggestion that the main role of the exchanger is in the gross resetting of $[Ca^{2+}]_i$ and not in the fine tuning; however, it cannot be excluded that in cells expressing a high level of the exchanger, and under favourable thermodynamic conditions, the antiporter could also contribute to the regulation of resting $[Ca^{2+}]_i$.

The Na^+/Ca^{2+} exchanger is a crucial point of intersection between Na^+ and Ca^{2+} homeostasis, explaining how any alteration of the Na^+/K^+ pump has immediate consequences on $[Ca^{2+}]_i$ homeostasis. For example, the positive inotropic action of cardiac glycosides can be explained in terms of an increase in $[Ca^{2+}]_i$ as follows: cardiac glycosides bind to and inhibit the Na^+/K^+ pump; the cytosolic free-Na^+ concentration is elevated; elevation of the cytosolic free-Na^+ concentration drives Ca^{2+} in via the Na^+/Ca^{2+} exchanger; $[Ca^{2+}]_i$ is increased. An elevated $[Ca^{2+}]_i$ can either directly increase cardiac muscle tension, or cause initially a larger accumulation of Ca^{2+} into the SR. The increased release of Ca^{2+} from the SR, when the muscle is activated, increases muscle tension [32].

PLASMA MEMBRANE Ca^{2+} CHANNELS

Calcium channels are membrane proteins which, in the open conformation, allow the passive flux of calcium ions across the membrane down the electrochemical gradient. Plasma membrane calcium channels can be divided into two major groups depending on the mechanism controlling the transition(s) from closed to open conformation: A) Channel in which the gating depends on voltage; B) Channels in which the gating depends on ligand binding. The calcium channels of intracellular organelles can be grouped in a different category, the so called Ca^{2+}-release channels.

Voltage-operated Ca^{2+} channels, Ca^{2+} VOCs

Ca^{2+} VOCs are found not only in excitable cells such as neurons, muscle and heart, but also in some non-excitable cells such as fibroblasts (for review see [33 – 35]). In recent years, it has become clear that several types of Ca^{2+} VOCs exist, which differ in their biophysical properties, pharmacological sensitivity and tissue distribution. Three main classes of Ca^{2+} VOCs have been clarly defined, and will be discussed separately, but there is evidence for the existence of additional types of Ca^{2+} VOCs [36, 37]. Besides the steep voltage dependence of activation, the different classes of Ca^{2+} VOCs all have in common a very high selectivity for Ca^{2+} over Na^+ in physiological solutions.

Ca^{2+} VOCs

The nomenclature adopted for Ca^{2+} VOCs in that proposed by Nowycky et al. [122].

A convenient defining property of L-type Ca^{2+} VOCs is the presence of allosterically linked, high-affinity binding sites for dihydropyridines, phenylalkylamines and benzothiazepines [38 – 40]. The functional properties of L-type Ca^{2+} VOCs in cardiac, secretory and neuronal cells are similar, but differ markedly from those of the dihydropyridine-sensitive Ca^{2+} VOCs from skeletal muscle, as will be described at the end of the next section.

The single-channel conductance of L-type Ca^{2+} VOCs is 20 – 25 pS and 7 – 8 pS with Ba^{2+} (0.1 M) and Ca^{2+} (0.1 M) serving as charge carriers, respectively [41, 42]. Activity is characterized by clusters of bursts of openings of short duration (mean open time 1 ms), separated by long periods (tens of milliseconds to several seconds) of inactivity [43, 44]. Dihydropyridine agonists greatly prolong the average opening time of the channel [43].

In the absence of divalent ions in the extracellular medium, L-type channels become permeable to monovalent ions [45, 46]; however, in the presence of micromolar concentrations of Ca^{2+} [45, 46] on the outer side of the plasma membrane, the monovalent current is blocked. The high rate of influx, despite the high affinity (micromolar) of Ca^{2+} for the channel, has been explained either with more than one intrachannel Ca^{2+}-binding site and mutual repulsion of Ca^{2+} ions [45, 46], or with a high-affinity Ca^{2+}-binding site, externally located, that controls selectivity and a single low-affinity intrachannel Ca^{2+}-binding site [47].

L-type calcium channels are potently inhibited by dihydropyridine blockers, such as nifedipine and nitrendipine. Both the binding of dihydropyridine blockers and the inhibition of Ca^{2+} current are voltage dependent, increasing with increasing (less negative) holding potentials [39, 48 – 51]. The voltage and time dependence associated with dihydropyridine blockers can be understood assuming that dihydropyridine antagonists bind more tightly to the inactivated state of the channel (which predominates at relatively depolarized holding potentials) than to the resting state (which predominates at more negative potentials) [48, 49]. The difference in binding affinities between the two states accounts for the discrepancy between binding data (obtained at 0 mV) and inhibition of biological response (obtained at negative potentials) [48]. Because of the strong voltage and time dependence of dihydropyridine blockers, these drugs are not straightforward pharmacological tools; in fact in neurons and neurosecretory cells both the relatively negative resting potential and the short duration of the action potential make dihydropyridines poor inhibitors of L-type Ca^{2+} VOCs under physiological conditions [50, 51].

Under situations close to physiological conditions, whole cell L-type calcium currents start to activate at membrane

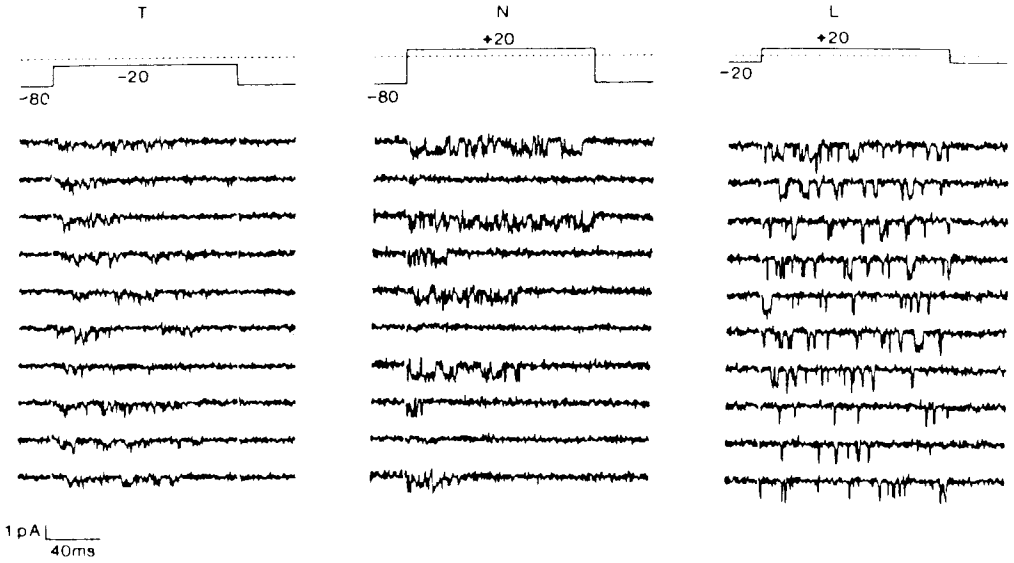

Fig. 1. *The three types of Ca^{2+} channels in chick dorsal-root ganglion neurons.* Cell-attached recordings with 110 mM Ba^{2+} in the pipette. The voltage clamp protocol is shown above the current traces. Reproduced with permission from Nowicky et al. [122]

potentials more positive than −30 mV to −20 mV, with time constants in the millisecond time range [41, 52]. At holding potentials more positive than −30 mV to −40 mV, steady-state inactivation starts to appear [49, 51]. The time course of voltage-dependent inactivation appears to be one of the most variable parameters between individual L-type Ca^{2+} VOCs. The most rapidly inactivating L-type Ca^{2+} currents are found in cardiac and vascular muscle (time constants of hundreds of milliseconds, with Ba^{2+} as the charge carrier) [53]. In neuronal and secretory cells, voltage-dependent inactivation is much slower (time constants of seconds or longer) if not totally absent [51, 52, 54]. In addition to voltage-dependent inactivation, L-type currents also show Ca^{2+}-dependent inactivation [54, 57–61]. In cardiac and smooth muscle, with Ca^{2+} as a charge carrier, L-type current results in quite rapid inactivation with time constants of 20–100 ms [53]. In secretory cells and neurones, a steady-state non-inactivatable component of Ca^{2+} current still remains when Ca^{2+} is the charge carrier [56, 57, 61].

Hormones and drugs which activate adenylate cyclase increase cardiac L-type Ca^{2+} current as a consequence of cAMP-dependent phosphorylation either of the channel or of a protein closely associated with it [62–66]. The cAMP-dependent increase in current is due to a higher opening probability of single L-type channels, primarily as a result of shorter intervals between bursts of openings and shorter periods of inactivity between clusters of bursts of openings [67, 68]. In addition to the cAMP-dependent stimulation there is evidence for a parallel direct stimulatory effect of the GTP-binding proteins on cardiac L-type Ca^{2+} VOCs [69, 70]. There are some indirect indications that protein kinase C might also have some stimulatory effects on cardiac and smooth muscle L-type Ca^{2+} channels [71–73]. cAMP-dependent phosphorylation does not seem to increase the opening probability of all L-type Ca^{2+} VOCs, since Ca^{2+} currents of some neuronal, secretory and smooth muscle cells are not increased by the elevation of intracellular cAMP [74–79].

L-type Ca^{2+} VOCs of skeletal muscle differ significantly from those in other cells in two major functional aspects; (a) they are activated more than one order of magnitude more slowly [80–82] and (b) have a smaller unitary conductance for both monovalent and divalent ions [83, 84] and a somewhat different ionic selectivity. The activity of skeletal L-type channels, as seen in their counterparts in cardiac cells, is stimulated by β-adrenergic agonists and internal cAMP [85] and also directly by the GTP-binding proteins [86]. Inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] has also been reported to increase the opening probability of single L-type Ca^{2+} VOCs of T-tubules [87], and stimulation of protein kinase C by phorbol esters to increase the number of dihydropyridine-binding sites and the ^{45}Ca influx in skeletal muscle cells [88].

Since the highest concentration by far of dihydropyridine receptors is found in the T-tubules of skeletal muscle, most of the biochemical characterization of the L-type Ca^{2+} VOCs has been performed on this tissue. The skeletal muscle dihydropyridine-sensitive Ca^{2+} VOCs have been purified by several laboratories [89–93]. The purified dihydropyridine-sensitive Ca^{2+} VOCs have then been reconstituted into phospholipid vesicles and lipid bilayers, and shown to be functional by assays for Ca^{2+} and Ba^{2+} uptake into vesicles [94, 95] and by single-channel measurements in artificial bilayers [96–99].

The purified channel from skeletal muscle is a 1:1:1:1 complex of four polypeptide chains (see [100–102] and references therein) which have been referred to as the α1 subunit (apparent molecular mass 155–200 kDa as analyzed by SDS/polyacrylamide gel electrophoresis), the α2 subunit, formerly called α (165–220 kDa in non-reducing conditions; 130–150 kDa in reducing conditions), the β subunit (52–65 kDa) and the γ subunit (30–35 kDa). Upon reduction of the α2 subunit, a fifth subunit appears, the δ subunit (24–33 kDa). The α1 and β subunits are substrates for cAMP-dependent protein kinase [103–105], for a Ca^{2+}/calmodulin-dependent protein kinase [104, 106], for protein kinase C [107], for the cGMP-dependent protein kinase [106] and for a protein kinase intrinsic to skeletal muscle triads [105].

The primary amino acid sequences of the α1, α2 and β subunits of the skeletal muscle dihydropyridine-sensitive Ca^{2+} VOCs have been deduced from their cloned cDNAs [108–110]. The α1 subunit (212 kDa), which bears a high degree

of similarity with the Na^+ channel [111] has four repeating domains of high homology and each domain contains five, presumably α-helical membrane-spanning hydrophobic segments and one positively charged segment, named S4. S4 contains five or six Arg or Lys residues at every third position, with mostly non-polar residues intervening between the basic residues and has approximately the right length to cross the membrane. The S4 sequence is highly conserved in the corresponding segment of the Na^+ [111] and K^+ channels [112, 113]. Recently, site-directed mutagenesis of the Na^+ channel expressed into oocytes has provided evidence that the S4 segment is involved in the voltage-sensing mechanism for activation of the channel [114]. The sequence of the α1 subunit contains seven potential phosphorylation sites for cAMP-dependent protein kinase and no obvious Ca^{2+}-binding sites corresponding to EF hands or similar structures present in intracellular Ca^{2+}-binding proteins.

The L-type Ca^{2+} current is practically absent in mice with muscular dysgenesis. In these animals excitation/contraction (EC) coupling fails [115] and the level of mRNA encoding the α1 subunit is greatly reduced [116]. Microinjection of an expression plasmid carrying the α1 subunit cDNA into nuclei of cultured skeletal muscle cells from dysgenic mice is able to restore functional dihydropyridine-sensitive Ca^{2+} VOCs, as well as normal EC coupling [116]. The observation that EC coupling in this system is preserved also in the absence of extracellular Ca^{2+} or in the presence of Cd^{2+}, a potent blocker of Ca^{2+} permeation through the channels, indicates that EC coupling depends on functional dihydropyridine receptors, but not on their ability to conduct an inward Ca^{2+} current [116]. This evidence strongly supports the view that the dihydropyridine receptor in the T-tubule membrane has a dual function as a Ca^{2+} channel and as the voltage sensor for EC coupling [117].

Several lines of evidence suggest that the dihydropyridine-sensitive L-type Ca^{2+} VOCs of non-skeletal muscle cells are different structural entities from those of skeletal muscle. Dysgenic mice lack dihydropyridine receptors in muscle, but have a normal density of dihydropyridine receptors and of dihydropyridine-sensitive Ca^{2+} VOCs in heart and sensory neurons [115, 116]. RNA blot analyses with probes for the α1 [109] and β [110] subunits of skeletal muscle, show very weak (α1) or no hybridization (β) to RNA from heart or to RNA from smooth muscle or brain. Specific antibodies raised against the α1 subunit of skeletal muscle are not able to recognize the analogous subunit in heart and other tissues [118, 119]. However, both RNA blot analysis and antibody crossreactivity indicate that the α2 subunits of different tissues are homologous [109, 119]. The purified α1 subunit of cardiac muscle is significantly larger (185–195 kDa) than its counterpart in skeletal muscle [118, 120] and surprisingly it could not be phosphorylated in detergent solution by cAMP-dependent protein kinase [118]. Finally, the primary amino acid sequence of the α1 subunit of the cardiac L-type channel has been recently deduced from the cloned cDNA and shown to be 66% homologous to that of skeletal muscle [121].

N-type Ca^{2+} VOCs

N-type (N-type) Ca^{2+} VOCs have been described first in chick dorsal-root ganglion neurons [122] and are probably neuron specific since they have been found only in cells of neuronal origin and their expression in PC12 cells is enhanced by nerve growth factor [35, 37, 123, 124]. N-type Ca^{2+} VOCs probably play a prevalent, if not exclusive role, in controlling transmitter release in certain neurons [123].

According to the original work of Tsien and collaborators in sensory and sympathetic neurons, N-type channels activate over a similar voltage range as L-type channels, but can be distinguished at the single channel level from L-type channels by their lower single-channel conductance (11–15 pS with 0.1 M Ba^{2+} as a charge carrier), by their insensitivity to dihydropyridine drugs and by their almost complete inactivation at holding potentials more positive than −20 mV to −30 mV [55, 122, 125, 126]. In whole cell recordings, N-type channels were previously considered to give rise to a component of high-threshold current, inactivating completely, with time constants (near 0 mV) of tens of milliseconds, in dorsal-root ganglion neurons, and hundreds of milliseconds in sympathetic neurons [55, 122, 126]. Recently, however, the view that N-type channels contribute only to the decaying component of the whole cell Ca^{2+} current, and that L-type channels underlie all of the late sustained current, has been shown to be an oyer simplification. In both sensory and sympathetic neurons in fact, the average current from single N-type channels can have a sustained component which is not inactivated at the end of test depolarizations of several hundred milliseconds [37, 127, 128]. Moreover, in some dorsal-root ganglions and sympathetic neurons, dihydropyridine blockers, even when applied at depolarized potentials, have almost no effect on currents that decay very little during long (6 s) depolarizations [129]. In general, despite large variability (that probably reflects the different sub-populations of neurons of spinal and cervical ganglia) the pharmacology of calcium currents in sensory and sympathetic neurons (in particular their irreversible block by ω-conotoxin, which has been shown to have no irreversible effect on L-type channels [37, 127]) is consistent with the view that N-type channels contribute to most (or in some cells all) of the high-voltage-activated calcium current [37, 126–131].

Several lines of evidence suggest that N-type Ca^{2+} VOCs are probably a heterogeneous group of channels [36, 37, 127, 128]. A Ca^{2+} VOC from cerebellar Purkinje cells, which is neither dihydropyridine sensitive nor ω-conotoxin sensitive, has been recently described and shown to be inhibited by a toxin from a spider venom [36]. Furthermore, another level of Ca^{2+} VOC heterogeneity could arise when comparing the channels expressed in the cell body and in the nerve terminal.

T-type Ca^{2+} VOCs

T-type Ca^{2+} VOCs with similar functional properties, originally described in vertebrate cells by Carbone and Lux [132], are found in a wide variety of excitable and non-excitable cells [34, 35]. T-type channels are also called low-threshold-activated channels since they start to activate at potentials 30–40 mV more negative than L-type and N-type Ca^{2+} VOCs. The low threshold of T-type channels renders them well suited for participating in pacemaking in many cells. During test-pulse depolarization they are inactivated quite rapidly ($t_{1/2}$ 10–40 ms at −20 mV) and completely [133–137] in a purely voltage-dependent manner (no Ca^{2+}-dependent inactivation) [138]. Steady-state inactivation is substantial at quite negative holding potentials [54, 55, 137, 139].

T-type Ca^{2+} VOCs have a single-channel conductance of 5–10 pS with either Ba^{2+} or Ca^{2+} as charge carriers (0.1 M) [122, 125, 137, 140]. They are blocked more effectively than L-type or N-type channels by Ni^{2+} [55, 135, 137, 141], they are insensitive to micromolar concentrations of dihydropyridine

antagonists [55, 122, 133, 137] and only weakly and reversibly blocked by ω-conotoxin [130, 142]. A few drugs, such as tetramethrin [137], amiloride [143], diphenylhydantoin [144] and octanol [145] have been shown to inhibit T-type channels, but none of them very specifically [34, 35].

In Fig. 1 different electrophysiological characteristics of the three types of Ca^{2+} VOCs in dorsal-root ganglion neurons are summarized.

Agonist-dependent Ca^{2+} VOC inhibition

Ca^{2+} VOCs play an essential role in a number of cellular functions, from muscle contraction to neurotransmitter and hormone secretion. Thus the discovery that Ca^{2+} VOCs can be modulated by a number of physiological agonists has been of the utmost importance. We have mentioned above the stimulatory effects of cAMP and the GTP-binding proteins on L-type Ca^{2+} VOCs. The complex kinetics and heterogeneity of N-type channels, on the other hand, makes it difficult to establish which types of Ca^{2+} VOCs are the targets for the widespread inhibitory modulation of neuronal-Ca^{2+} currents by neurotransmitters such as noradrenaline (via α2 adrenoreceptors) [146–151]; 4-aminobutyric acid (via B receptors) [146, 147]; dopamine (via D2 receptors) [149]; acetylcholine (via M1 receptors) [37, 74, 152]; adenosine (via A1 receptors) [153]; opioids (via k and δ receptors) [154–156]; somatostatin (via unknown receptors) [150, 154]. In a few cases one can safely conclude that the targets for inhibitory modulation are N-type Ca^{2+} VOCs [37, 150, 151, 153].

Most, if not all, of the reported inhibitory modulation of neuronal Ca^{2+} current seems to be independent of intracellular cAMP but dependent on the activation of a pertussis-toxin-sensitive, GTP-binding protein [37, 74, 147–156]. As previously suggested by reconstitution studies [156–158], the G-protein mediating the effect of neurotransmitters has been shown recently, using specific antibodies, to be the most abundant G-protein in neurons, denoted as the G_o-protein [159]. In sensory neurons, part of the inhibition of the Ca^{2+} current, mediated by the G_o-protein, requires protein kinase C activation [158, 160]. However, the participation of protein kinase C in inhibitory modulation of neuronal Ca^{2+} VOCs is not a general feature. Depending on the type of neuron, there is evidence for [146, 160–163] and against [74, 151] an involvement of protein kinase C. There are some indications that the targets for protein kinase C modulation in sensory neurons are L-type channels [163]. We tentatively propose, as an interesting working hypothesis, that the same neurotransmitter can modulate through a different molecular mechanism both N-type (possibly through direct G-protein/channel interaction) and L-type (possibly through protein kinase C) Ca^{2+} VOCs.

The modulation of Ca^{2+} currents in neurosecretory cells shares many features with that in neuronal cells. Thus, in some pituitary cell lines the inhibitory modulation by somatostatin of a slowly inactivating, high voltage-activated Ca^{2+} current was shown to be cAMP independent and pertussis toxin sensitive [76, 164]. Activators of protein kinase C inhibit voltage-dependent Ca^{2+} currents in many secretory cells [76, 165, 166] and in some cases they were shown to affect dihydropyridine-sensitive L-type Ca^{2+} VOCs [166].

Very few data are available on the inhibition of T-type Ca^{2+} VOCs by neurotransmitters. In sensory neurons, norepinephrine and dopamine inhibit T-type Ca^{2+} currents by an unknown mechanism [129, 149]. In GH3 cells, activation of protein kinase C has been reported to inhibit the T-type Ca^{2+} current [165] while cAMP displays no effect in several cell types [133, 141, 167]. In fibroblasts, T-type Ca^{2+} VOCs can be selectively suppressed by transforming oncogenes [136].

Receptor-activated and second-messenger-activated Ca^{2+} channels

Until a few years ago the Ca^{2+} channels with opening mechanisms not dependent on membrane-potential depolarization, but rather on the activation of specific plasma membrane receptors, were termed receptor-operated Ca^{2+} channels or Ca^{2+} ROCs. Recently [33, 168] it has been proposed to distinguish this type of Ca^{2+} channel into three major groups.

(a) ROCs are those channels in which the ligand-binding site and the channel are either on the same polypeptide or in the same molecular complex [33]. Within the group of agonist-activated Ca^{2+} channels, Ca^{2+} ROCs are by far the best characterized in molecular and electrophysiological terms and incude the nicotinic channel, the *N*-methyl D-aspartate (MeDAsp) channel and perhaps, the ATP (external) gated channel(s).

(b) The channels that are linked to the receptor via a soluble second messenger belong to a second group, and are termed SMOCs [33]. The Ca^{2+}-activated Ca^{2+} channels, found in different cell types, are the prototype of the Ca^{2+} SMOCs. Evidence has been also provided for the existence on the plasma membrane of Ins(1,4,5)P_3-regulated Ca^{2+} channels, and/or inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4]-regulated Ca^{2+} channels, but in this case a general consensus has not yet been reached.

(c) The third group may be represented by Ca^{2+} channels coupled to the receptor via a G-protein. This latter type of Ca^{2+} channel is termed a G-protein-operated channel (GOC) [168].

Common characteristics of receptor and second-messenger-activated Ca^{2+} channels are the much lower selectivity for Ca^{2+} over monovalent cations, compared to Ca^{2+} VOCs.

Ca^{2+} ROCs

ROCs are epitomized by the nicotinic receptor/channel. However, this channel, although permeable also to Ca^{2+} under physiological conditions, transports mainly Na^+ and K^+ and therefore the nicotinic channel will not be discussed here (see [169]). The only Ca^{2+} ROC extensively characterized is the glutamate-activated channel, usually indicated as the MeDAsp receptor [170], to distinguish it from the other receptors ligated by glutamate, i.e. those preferring (*S*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and kainic acid [171]. The MeDAsp channel has been extensively investigated by electrophysiological techniques, but no information is yet available about its molecular structure. The MeDAsp-channel receptor has been found so far only in vertebrate neurons mostly in the central nervous system (but see [172] for other locations). This channel is permeable to both Na^+ and Ca^{2+} and at resting (negative) plasma membrane potential, is powerfully inhibited by even micromolar Mg^{2+} concentrations [173]. Mg^{2+} blockade can be reversed by depolarization [173]. In Mg^{2+}-free solution, the MeDAsp channel exhibits a linear current-voltage relationship and a reverse potential near 0 mV. The channel conductance is 50 pS (with 140 mM CsCl as a charge carrier). When Mg^{2+} is present, MeDAsp causes fast current bursts suggesting that the channel undergoes cycles of blocking and unblocking by Mg^{2+}. The

MeDAsp channel is positively modulated by glycine [174]. The MeDAsp channel is currently the object of great interest as it is increasingly appreciated that it is involved in the long-lasting modification of neuronal excitability, known as long-term potentiation [175] and in neuronal cell death following ischemia [176].

Another member of the ROC family might be the channel(s) activated by extracellular ATP [177]. In recent years several ATP-activated Ca^{2+} fluxes have been described and in a few instances, detailed electrophysiological characterization has been carried out. In smooth muscle, exogenous ATP opens a channel with a Ca^{2+}/Na^{+} selectivity ratio of 3:1 at physiological concentrations and with a conductance of 5 pS [177]. This channel resists inhibition by classical inhibitors of voltage-gated Ca^{2+} channels and by Mg^{2+}. Besides Ca^{2+} and Na^{+}, the channel also admits glucosamine, albeit with a permeability sixfold lower in comparison to that for Na^{+} [177]. The channel is activated by ATP concentrations as low as 10 nM and without the involvement of soluble second messengers [177]. Recently, ATP-activated channels with similar features have also been described in heart and neuronal cells [178, 179] and we believe that more examples will be found as their presence will be investigated more thoroughly. Besides opening this low-conductance channel, in many cell types (i.e. macrophages, mast cells, mouse neuroblastoma cells) exogenous ATP also causes large increases in whole-cell conductance depending on the opening of large non selective channel(s) that admit aqueous solutes of molecular mass $\leqslant 0.9$ kDa [180, 181].

Ca^{2+} SMOCs

In a number, and possibly all cell types, the activation of receptors coupled to the hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] causes both release of Ca^{2+} from intracellular stores and increased Ca^{2+} influx through the plasma membrane [33]. This latter event is generally thought to involve Ca^{2+} channels, mostly because the influx of Ca^{2+} depends on the transmembrane potential, i.e. influx is increased by hyperpolarization and depressed by depolarization [182, 183]. More direct evidence for the involvement of Ca^{2+} channels in the influx, activated by receptors coupled to PtdIns(4,5)P_2 hydrolysis, is however still lacking in most systems.

Ca^{2+} ions (presumably released from intracellular stores) might serve as the second messenger that regulates the opening of plasma membrane cation channels [184]. Such channels have been described in a variety of cells, both excitable and non-excitable, but either the $[Ca^{2+}]_i$ necessary to activate them is outside the physiological range, or their permeability to Ca^{2+} is so low that their relevance for maintaining Ca^{2+} influx is doubtful. This is not the case for the cation channel activated by micromolar $[Ca^{2+}]_i$ in neutrophils [185], whose permeability to Na^{+} is approximately similar to that for Ca^{2+}. The importance of this Ca^{2+}-activated Ca^{2+} channel in the sustained increase of $[Ca^{2+}]_i$ caused by chemotactic peptides is however challenged by the observation that the Ca^{2+} (or Mn^{2+}) influx, activated by receptor stimulation in neutrophils, can persist even when $[Ca^{2+}]_i$ increases (due to Ca^{2+} redistribution) are blunted [186–188]. Thus, at best, this type of channel could contribute to the initial phase of a $[Ca^{2+}]_i$ increase after receptor triggering, but probably has no role in the sustained phase of Ca^{2+} influx.

The other major candidate for activation of Ca^{2+} SMOCs is Ins(1,4,5)P_3, alone or in combination with Ins(1,3,4,5)P_4. Kuno and Gardner [189] reported that Ins(1,4,5)P_3 can increase the opening probability of a cation channel in excised patches from lymphocyte plasma membrane; more recently, Penner et al. [183] showed that intracellular Ins(1,4,5)P_3 can induce sustained increases in the $[Ca^{2+}]_i$ in mast cells although they were unable to demonstrate the activation of an Ins(1,4,5)P_3-dependent inward current. Snyder et al. [190] showed that in sea urchin eggs intracellularly injected Ins(1,4,5)P_3 could induce a sustained increase of Ca^{2+}-dependent Cl^{-} conductance (tens of minutes), that was considered to reflect sustained Ca^{2+} influx. In this experiment, however, the effect of intracellularly injected Ins(1,4,5)P_3 was observed even several minutes after microinjection of the stimulus, when presumably all the Ins(1,4,5)P_3 had been metabolized. In the experiments reported by Snyder et al., Ins(1,3,4,5)P_4 could not mimick the Ins(1,4,5)P_3 effects on Cl^{-} current [190]. In contrast to Snyder et al., Morris et al. [191] failed to observe sustained effects of intracellular Ins(1,4,5)P_3 on Ca^{2+}-activated K^{+} permeability in rat lacrimal acinar cells, thus arguing against a role for Ins(1,4,5)P_3 as the sole mediator of the persistent Ca^{2+} influx triggered by muscarinic receptors in these cells. Rather, they showed [191] that the inclusion of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in the intracellular perifusion medium could mimick the effect of extracellularly applied acetylcholine. In a more recent paper however, Petersen and coworkers [192] showed that Ins(1,3,4,5)P_4 could affect K^{+} currents also in the absence of extracellular Ca^{2+}, thus raising doubts about the interpretation of their previous experiments. Thus at present one has to conclude that clear evidence for the existence of Ins(1,4,5)P_3-dependent Ca^{2+} SMOCs is still lacking in most systems and alternative possibilities must be taken into consideration. An attractive possibility would be a coupling of the receptor to the channel by direct interaction mediated by a G-protein, similar to the muscarinic K^{+} channel of heart myocytes [193].

Do Ca^{2+} GOCs exist? Admittedly, with one relevant exception, the demonstration that Ca^{2+} channels can be gated by G-proteins is still lacking in most systems. However, several reports in the last two years have provided indirect evidence that G-proteins may indeed modulate the activity of Ca^{2+} channels linked to receptors coupled to PtdIns(4,5)P_2 hydrolysis. Penner et al. [183] demonstrated that intracellularly injected guanosine 5′-[γ-thio]triphosphate could cause the opening of an unselective cation channel, but they were unable to correlate the opening of this channel with any sustained rise in $[Ca^{2+}]_i$, nor could they exclude the fact that guanosine 5′-[γ-thio]triphosphate was causing channel opening indirectly via generation of a second messenger. Fasolato et al. [194] reported evidence, in PC12 cells, for the opening, by the nonapeptide bradikinin, of a Ca^{2+} channel which was not regulated by any known second messenger; the authors suggested that G-protein-dependent Ca^{2+} channels could explain their data.

The only Ca^{2+} channel for which convincing evidence in favour of G-protein-mediated control has been provided, is that triggered by insulin-like growth factor II described in BALB/c 3T3 fibroblasts by Ogata and coworkers [195, 196]. The following evidence indicating that insulin-like-growth-factor-I-activated and insulin-like-growth-factor-II-activated channels are GOCs and not SMOCs is convincing [195, 196] (E. Ogata, personal communication): (a) GTP or non-hydrolyzable GTP analogues activate channel opening in excized patches; (b) the activation of the channel is inhibited by treatment with pertussis toxin; (c) in the cell-attached mode, channel opening can be triggered by insulin-like growth factor

II in the patch pipette, but not by bath application of the stimulus.

Comparable to other Ca^{2+} SMOCs or ROCs and to the influx activated by bradykinin in PC12 cells [194] the insulin-like-growth-factor-activated GOC is also an unspecific cation channel, permeable to Ca^{2+}. It remains to be demonstrated whether the Ca^{2+} GOC described in BALB/c 3T3 fibroblasts [195, 196] is specific for the receptors activated by insulin-like growth factors I and II, or whether this channel is the first example of a much larger family.

The capacitative model for Ca^{2+} influx

An alternative model for explaining receptor-activated Ca^{2+} influx has been proposed in 1986 by Putney [197] and further elaborated by Merritt and Rink [198]. In this model the signal that activates Ca^{2+} influx, after triggering receptors coupled to PtdIns(4,5)P_2 hydrolysis, depends somehow on the level of Ca^{2+} within the intracellular Ins(1,4,5)P_3-sensitive stores. According to the modification of Merritt and Rink [198] of Putney's original model [197], Ins(1,4,5)P_3 first empties the intracellular stores and, as a consequence of the low Ca^{2+} concentration in the lumen of the store, a gap-junction-like connection with the plasma membrane is formed; Ca^{2+} thus flows from the extracellular medium directly into the store through this channel and, provided the Ins(1,4,5)P_3-sensitive channel is open, into the cytoplasm. According to this model, Ca^{2+} influx into the cytoplasm will occur through the store and not through typical plasma membrane Ca^{2+} channels. The strongest argument in favour of the capacitative model is the observation that refilling of empty stores often occurs with minimal or no increase in $[Ca^{2+}]_i$ upon addition of Ca^{2+} to the medium. Recently, Putney and coworkers [199] have reformulated their hypothesis suggesting that once the Ins(1,4,5)P_3-sensitive stores are empty, a soluble messenger is released into the cytoplasm that in turn activates the opening of a traditional plasma-membrane-Ca^{2+} channel. This second model of Putney's is thus only a variation on the theme of Ca^{2+} SMOCs. The capacitative model is at present an intellectually challenging problem, though the evidence supporting it is still rather indirect.

The pharmacology of Ca^{2+} SMOCs or GOCs, unlike that of Ca^{2+} VOCs, is to date rather primitive, since the only investigative methods available are unspecific and/or not applicable to live animals. A new family of drugs, one prototype being now available from Smith, Kline and French [200], although not very specific, appears rather promising.

In conclusion, unlike in the case of Ca^{2+} ROCs and VOCs, which have become routinely amenable to investigation by molecular biology and electrophysiological techniques, Ca^{2+} SMOCs or GOCs are still in their infancy.

Ca^{2+}-SEQUESTERING ORGANELLES

All mammalian cells, except erythrocytes, are endowed with organelles capable of accumulating Ca^{2+} against the electrochemical gradient in an energy-dependent way. Under appropriate conditions, Ca^{2+} can be released from these organelles either slowly (e.g. mitochondria or secretory granules) or rapidly [e.g. sarcoplasmic reticulum (SR) or Ca^{2+} stores of non-muscle cells]. Many similarities exist between muscle SR and the rapidly exchangeable pools of non muscle cells, yet some of their characteristics are unique and these two types of organelles will therefore be discussed separately. Mitochondrial Ca^{2+} accumulation appears to be quite different from that of other organelles and they seem incapable of regulated fast Ca^{2+} release (see below). A third type of Ca^{2+}-sequestering structure are the secretory granules; they too, however, are not involved in the regulation of the metabolically responsive pool of Ca^{2+}. Scattered reports also exist in the literature about the Ca^{2+}-accumulating capacity of the Golgi network or of lysosomes [5]. Last, but not least, it has been suggested that Ca^{2+} gradients exist across the nuclear membrane, although this observation has not been confirmed by all investigators.

As will be discussed below, any Ca^{2+}-storage organelle in rapid equilibrium with the cytoplasm must be endowed with three basic characteristics: (a) a Ca^{2+}-accumulation mechanism; (b) an intravesicular Ca^{2+}-buffering system; (c) a Ca^{2+}-release channel. Each Ca^{2+}-storage organelle will be discussed according to this scheme.

SARCOPLASMIC RETICULUM

Striated muscle SR is a complex network of tubules and cisternae wrapped around the myofibrils [201] and specific for Ca^{2+} accumulation and release [202]. Anatomically, SR is distinguished in longitudinal SR, composed mainly of tubules, and small cisternae, and terminal cisternae (TC). TC comprise the large expansions of the SR facing the T-tubules, the invaginations of the plasma membrane where coupling between depolarization and the signal causing Ca^{2+} release from TC occurs [202, 203]. Longitudinal SR and TC differ not only in their morphological features, but also in their biochemical properties.

SR Ca^{2+}-ATPase

The SR Ca^{2+}-ATPase may represent up to 90% of total SR protein. Thanks to its abundance it has been purified rather early and has been the object of fruitful biochemical, physiological and molecular studies. The SR Ca^{2+}-ATPase, a member or the E1/E2 family, is a single protein of molecular mass 110 kDa that interacts with Ca^{2+} with high affinity (K_D 0.5 μM at approximately pH 7.0) and transports it with a stoichiometric ratio of 2:1 with respect to ATP [204]. Genes encoding Ca^{2+}-ATPases of fast-twitch and cardiac SR have been isolated and the amino acid sequence determined. The SR ATPase, according to most recent reconstructions, is organized with a Ca^{2+}-binding domain, a transduction domain, a phosphorylation domain, a nucleotide-binding domain, a hinge domain and six or ten membrane-spanning α-helices [10, 205]. Both the C and N termini face the cytoplasm [10, 205] and the membrane-spanning α-helices constitute the ion channel, although it is not clear whether all the helices contribute to its formation. Determination of the amino acid sequence has allowed a mechanistic hypotheses to be proposed for the transmembrane Ca^{2+} translocation, that fit previous interpretations based on biochemical and biophysical data. The Ca^{2+} ATPase of fast-twitch skeletal muscle differs significantly with regard to its primary sequence and immunological reactivity compared to that of the slow skeletal/cardiac isoforms [10, 205–207]. The Ca^{2+}-ATPase of smooth muscle SR resembles closely the cardiac isoform, but the mRNAs of cardiac and smooth muscle SR Ca^{2+}-ATPase differ in their 3′-end sequence [208].

The Ca^{2+}-ATPase from heart, slow skeletal muscle and smooth muscle SR is regulated by an acidic proteolipid called

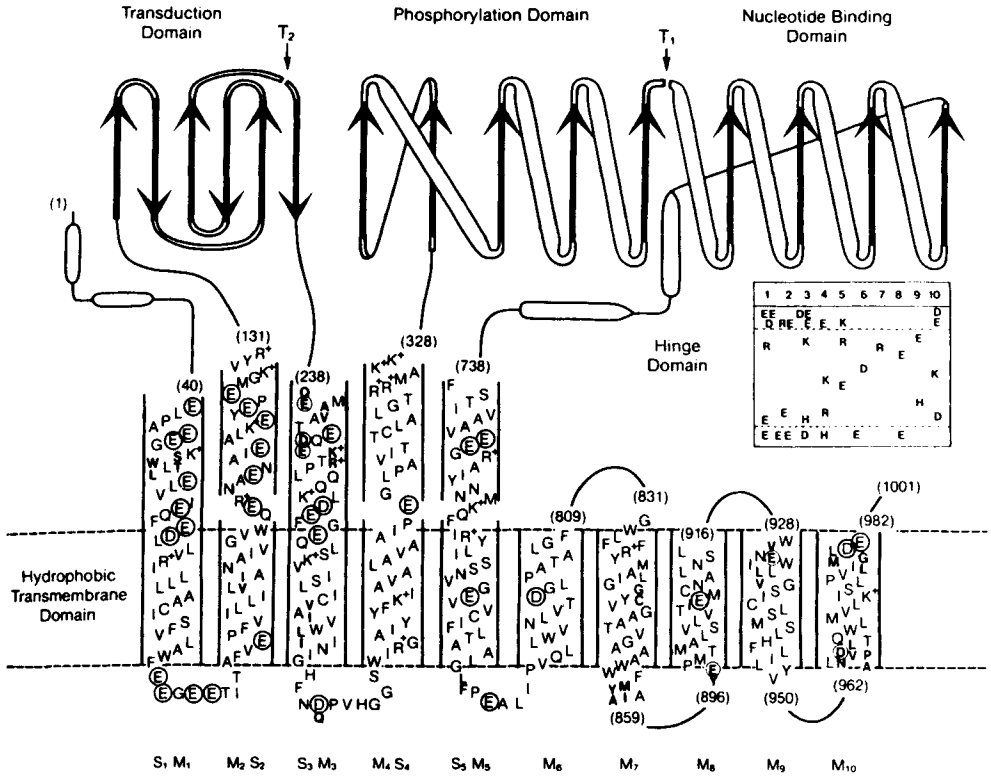

Fig. 2. *Schematic diagram of the sarcoplasmic reticulum Ca^{2+}-ATPase.* Amino acid differences between the fast-twitch and slow-twitch Ca^{2+}-ATPases are indicated by two residues, the slow above the fast. Acidic residues are encircled and basic residues are highlighted by a plus sign. In the inset the location of negative (D, E) and positive (K, R, H) charges close to or within the transmembrane helices are shown. 1–10 refer to trans-membrane helices. Reproduced with permission from Brandl et al. [10]

phospholamban [209]. This proteolipid binds ATP with a stoichiometry of 1:1 and can be phosphorylated by both cAMP and Ca^{2+}–calmodulin-dependent kinases. Recent experiments [210] have confirmed the previous suggestion [211] that phospholamban, in its dephosphorylated form, is bound to the ATPase and inhibits its activity; phosphorylation of phospholamban causes detachment from and activation of the ATPase.

The Ca^{2+}-ATPase of SR has been the object of intense investigation during the last decade and several excellent reviews have appeared dealing with most of the basic molecular and functional properties of this enzyme (for review see [20, 212–214]). Fig. 2 shows the predicted structure of SR Ca^{2+}-ATPase.

Ca^{2+} buffering within SR, calsequestrin

Among the several Ca^{2+}-binding proteins isolated from the SR, calsequestrin is the most abundant and because of its location and number of Ca^{2+}-binding sites, it is thought to represent the major Ca^{2+} buffer of the SR. Calsequestrin was initially described by MacLennan and Wong in 1971 [215] and defined as a high capacity medium/low-affinity Ca^{2+}-binding protein, specific of striated muscle. At least two isoforms of calsequestrin exist, one specific for fast-twitch skeletal muscle and the other for cardiac muscle [216]. Calsequestrin has been isolated from mammals, amphibians, birds, etc. Despite some variability in the molecular mass among the different animals and muscles, all calsequestrins share a number of properties, the most relevant being the presence of a large number of low-affinity Ca^{2+}-binding sites (K_d in the millimolar range with the number of sites from 30–50 mol/mol protein) [215–217]. Calsequestrin isolated from rabbit fast-twitch skeletal muscle is an acidic glycoprotein (P_i 4.2) with anomalous electrophoretic behaviour on SDS gels, i.e. slower mobility at alkaline pH than at neutral pH [217]. According to the predicted amino acid sequence [218], the real molecular mass of calsequestrin is 42 kDa. Calsequestrin has been very recently crystallized [219] but no structural data based on the X-ray analysis is yet available. Thus information on the structure of the protein is so far based on the hydropathy plot of the primary structure, circular dichroism measurements, fluorescence properties of the endogenous tyrosine and tryptophan, and chromatographic techniques [216–221]. Calsequestrin in striated muscles is localized exclusively in the lumen of the TC [222], in close proximity to the ryanodine-sensitive Ca^{2+} channels. This localization of calsequestrin is strategic for its function; the protein is not only essential to buffer intravesicular $[Ca^{2+}]$ (the total Ca^{2+} content of the terminal cisternae may well be >30 mM) but also to concentrate Ca^{2+} near the release sites, thus increasing the speed of Ca^{2+} release.

Although it is undisputed that calsequestrin contains many low-affinity Ca^{2+}-binding sites/mol protein [215–223], the conclusion that the protein contains only one class of site with the same affinity for the cation [223] may be an over simplification. In fact there is no repeating distribution of acidic residues along the primary sequence [218] and the putative Ca^{2+}-binding sites are often clustered, thus making it unlikely that they would not influence each other, at least electrostatically.

The sorting of calsequestrin after synthesis in the rough endoplasmic reticulum (ER) is still unresolved. Calsequestrin does not contain a KDEL *C-terminus region*, as do proteins retained in the ER [224]. Evidence has been provided in-

dicating that calsequestrin is transported via coated vesicles to the Golgi [225, 226] where further glycosylation occurs. From the Golgi (cis or intermediate portion), calsequestrin is then directed to its final destination and actually calsequestrin-containing structures may form nucleation centers for SR biogenesis [225, 226].

Calcium-release channels of the SR

Two channels for Ca^{2+} release from intracellular organelles have so far been described: the SR Ca^{2+}-release channel and the Ins(1,4,5)P_3-sensitive channel (see below).

The SR Ca^{2+}-release channel has been the object of intense investigation since its electrophysiological, biochemical and molecular features are of the utmost relevance for understanding the mechanism of EC coupling. Two alternative hypotheses have been proposed to explain EC coupling. (a) The chemical hypothesis, whereby a soluble messenger, released from the T-tubule membrane upon depolarization, opens the ryanodine sensitive Ca^{2+}-release channel of the TC. The nature of the second messenger is also a matter of discussion. Ca^{2+} itself (Ca^{2+}-induced Ca^{2+} release) or Ins(1,4,5)P_3 are the most popular candidates. (b) The electromechanical hypothesis which proposes direct physical contact between the voltage sensor on the T-tubule and the Ca^{2+}-release channel on the TC membrane. Neither of these mechanisms have, as yet, been proved conclusively by experimental evidence.

The Ca^{2+}-release mechanism from the SR has been studied using permeabilized muscle fibers (skinned fibers), isolated SR vesicles, SR membrane vesicles fused with planar lipid bilayers and purified channels inserted into black lipid films [227–229]. In both skeletal and cardiac SR vesicles, Ca^{2+} release is stimulated by extravesicular Ca^{2+} (half-maximal concentrations 0.5–1.5 μM in the absence of Mg^{2+}), by ATP (1–5 mM), caffeine, Ins(1,4,5)P_3 and by a variety of pharmacological agents [230–235], whilst Ca^{2+} release is inhibited by Ca^{2+} itself (at concentrations 2–3 orders of magnitude higher than those stimulating Ca^{2+} release), Mg^{2+} and ruthenium red [230–233, 236–239].

There are important differences between skeletal and cardiac SR vesicles in their sensitivity to activation or inhibition of Ca^{2+} release by the stimuli above mentioned. Ca^{2+} and caffeine are more effective in stimulating Ca^{2+} release from cardiac than from skeletal SR vesicles, while ATP is more effective in skeletal than in cardiac SR vesicles [231, 232, 238]. The presence of Ca^{2+} on the trans (cytoplasmic) side of the channel seems to be an essential requirement for opening the cardiac Ca^{2+}-release channel, whereas the skeletal channel can be partially activated by ATP even at nanomolar trans Ca^{2+} concentrations [238]. The strict dependence of the cardiac SR Ca^{2+}-release channel on $[Ca^{2+}]_i$ is consistent with the results obtained from skinned fibres suggesting an essential role for Ca^{2+}-induced Ca^{2+} release in cardiac muscle [240].

The other potential physiological activator of the SR Ca^{2+}-release channel is Ins(1,4,5)P_3. The first reports of Ins(1,4,5)P_3-induced Ca^{2+} release from skeletal muscle SR appeared in 1985 [234, 235]. The evidence in favour of Ins(1,4,5)P_3 may be summarized as follows: (a) Ins(1,4,5)P_3 releases Ca^{2+} both from isolated SR terminal cisternae [234] and from skinned fibre preparations [234, 235, 241–244]; (b) the entire machinery for the synthesis of the Ins(1,4,5)P_3 precursor PtdIns(4,5)P_2, Ins(1,4,5)P_3 formation (G-protein-dependent phospholipase C) and Ins(1,4,5)P_3 degradation [Ins(1,4,5)P_3 5-phosphatase] is present in the plasma membrane of striated muscles [245–248] and in a few cases, a selective enrichment of these synthetic and metabolic pathways has been found in T-tubules [245, 248]; (c) Ins(1,4,5)P_3 levels increase dramatically upon electrical stimulation of skeletal muscles [235]; (d) Ins(1,4,5)P_3, at micromolar concentrations, increases the opening probability of the Ca^{2+}-release channel of the SR after vesicle fusion with lipid bilayers [249]. A number of objections have been raised against the physiological role of Ins(1,4,5)P_3 as a mediator in EC coupling in striated muscle, the most important being: (a) the release of Ca^{2+} from skinned skeletal muscle fibers activated by photohydrolyzed Ins(1,4,5)P_3 is orders of magnitude slower than that observed under physiological conditions [250]; (b) the formation of Ins(1,4,5)P_3 in electrically stimulated cells is observed only after tetanus [235]; (c) fiber contraction is not blocked by heparin, a known inhibitor preventing Ins(1,4,5)P_3 binding to its receptor in smooth muscle and in non-muscle cells [251]. However, a high rate of contractions can be obtained by pressure injection of Ins(1,4,5)P_3 [252] and heparin has no effect on the Ins(1,4,5)P_3-induced contraction and Ca^{2+} release in skinned skeletal muscle fibers [253]. In turn, this result suggests that Ins(1,4,5)P_3-binding in skeletal muscle occurs on a receptor pharmacologically distinct from that of smooth muscle fibers and other non muscle cells. Very recently, the ryanodine receptor has been expressed in Chinese-hamster-ovary cells and shown to confer caffeine- and ryanodine-sensitive Ca^{2+} release [254]. On the other hand, the transfected channel appears to be insensitive to Ins(1,4,5)P_3. A possible interpretation of this result is that the ryanodine receptor is itself insensitive to Ins(1,4,5)P_3 and that an additional component, present in the TC membrane, is required for the expression of Ins(1,4,5)P_3 sensitivity. This hypothesis is supported by recent date by Valdivia et al. [400].

Taken together, these observations support the physiological relevance of Ins(1,4,5)P_3-induced Ca^{2+} release in skeletal muscle, yet they do not allow any conclusion as to whether Ins(1,4,5)P_3 functions as the main chemical messenger in stimulus-contraction coupling or whether it has only an ancillary role [255].

Single-channel recordings after fusion of SR vesicles with planar lipid bilayers have shown that Ca^{2+} release is mediated by a high-conductance channel ($\approx$ 100 pS with 50 mM Ca^{2+} in the lumenal side), with low selectivity for divalent over monovalent ions ($pCa^{2+}/pK^{+} \approx 5$) [238, 256, 257]. Ca^{2+}, ATP [256, 257] and Ins(1,4,5)P_3 [249] on the cytoplasmatic side affect the opening probability of the channel. In agreement with Ca^{2+}-efflux experiments from SR vesicles, the cardiac Ca^{2+}-release channel is more sensitive to activation by Ca^{2+} and less sensitive to activation by ATP than the skeletal channel [238]. No data are yet available on the effect of Ins(1,4,5)P_3 on the cardiac SR channel reconstituted in lipid bilayers.

There are conflicting reports on the effect of Mg^{2+} on single Ca^{2+}-release channels [238, 257]. There is also evidence of voltage dependence on the opening probability of the Ca^{2+}-release channel in the lipid bilayers [238, 257]. Ryanodine at concentrations close to the K_d of high-affinity binding (5–50 nM) increases the opening probability of the channel during its bursting periods [258], while at micromolar concentrations ryanodine locks the channel in a low-conductance state with an opening probability near unity [258].

Using ryanodine, the Ca^{2+}-release channel has been purified from both skeletal and cardiac muscle [259–262]. The purified ryanodine receptor has been shown to function as a Ca^{2+}-release channel when reconstituted into planar lipid bilayers with biophysical properties similar, but not identical,

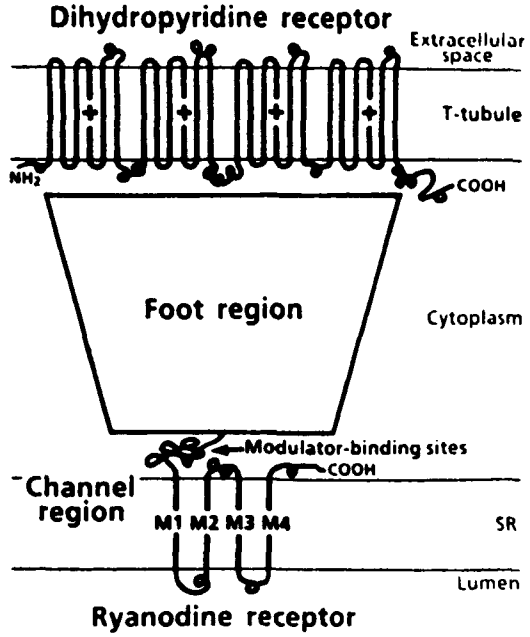

Fig. 3. *Structural model of the junctional region of the sarcoplasmic reticulum.* Plus signs within the transmembrane segments of the T-tubular dihydropyridine receptor indicate the putative voltage sensor. Reproduced with permission from Takeshima et al. [270]

to those of the channel from the SR membrane vesicles [259, 261–266]. Electron microscopy and ryanodine binding suggest that the purified ryanodine receptor forms a homotetrameric complex [261, 267, 268]. Reconstructed three-dimensional images of the purified receptor from negative-stain electron microscopy reveal a four-leaved clover (quatrefoil) structure ($27 \times 27 \times 14$ nm) similar to that previously described for the 'feet' structures that in intact muscle span the junctional gap between the T-tubule and the junctional SR [269].

Recently, the sequence of the 5037 amino acids composing the ryanodine receptor from the rabbit skeletal muscle SR has been deduced by cloning and sequencing of the complementary DNA [270]. The primary sequence of the human ryanodine receptor has also been established [271]. Only the 500 or so amino acids at the C terminus of the deduced sequence are sufficiently hydrophobic to span the membrane. The four potential transmembrane sequences identified in this region resemble the membrane-spanning segments of the subunits of the nicotinic and related receptors, thus supporting the suggestion that in the ryanodine receptor there may be a single aqueous pore formed by amino acid residues from the C-terminal domain of each of the four monomeric units. 90% of the N-terminal domain of the protein is hydrophilic and most probably forms the enormous quatrefoil foot structure shown by electron microscopy [270]. The demonstration that the ryanodine receptor, the foot structure and the SR-release channel are the same protein and that skeletal muscle dihydropyridine-receptors are voltage sensors for EC coupling, independent of their function as Ca^{2+} channels, may suggest that the large cytoplasmic region of the ryanodine receptor directly interacts with the cytoplasmic region of the dihydropyridine receptor to effect EC coupling [272] (cf. electromechanical coupling hypothesis of Schneider and Chandler) [273]. A cartoon describing the molecular organization of the triad junction of the skeletal muscle is shown in Fig. 3.

Ironically, despite the fact that the ryanodine receptor of striated muscle SR is by far the best characterized Ca^{2+}-release channel, the key question of the coupling between T-tubule depolarization and the opening of this channel still remains unanswered [255].

Ca^{2+} STORES IN NON-MUSCLE CELLS

The notion that microsomal fractions isolated from a variety of non-muscle cells accumulate Ca^{2+} in an ATP-dependent way is nearly 20 years old [274], but only in the last decade has the physiological importance of this Ca^{2+} store become obvious [275–277]. After the initial demonstration that agonist stimulation rapidly mobilizes a non-mitochondrial Ca^{2+} pool [278] (and the unraveling of the role played by Ins(1,4,5)P_3 in this phenomenon [275, 279]) the identification of an Ins(1,4,5)P_3-sensitive store with the entire endoplasmic reticulum was generally accepted. Only in the last two years has the complexity of the Ca^{2+} pools of non-muscle cells become apparent. On the one hand it has been proposed that non-muscle cells possess a specialized structure, apparently distinct from ER and related to muscle SR, named 'calciosome' [280–282]; on the other hand, several reports suggest the existence of Ca^{2+} pools insensitive to Ins(1,4,5)P_3, but sensitive to other agents (Ca^{2+} itself, caffeine etc., for review see [277, 283, 284]). Two detailed reviews on the nature, morphology, biochemistry and dynamics of the Ca^{2+} pools in non-muscle cells have been published recently and the reader should refer to these [283, 284] for a more comprehensive appraisal.

Ca^{2+} accumulation

Unlike the SR, where it is firmly established that Ca^{2+} accumulation depends on the activity of the Ca^{2+}-ATPase, in non-muscle cells the mechanism of Ca^{2+} accumulation is still a matter of discussion. Without doubt the Ca^{2+} stores in non-muscle cells possess a Ca^{2+}-ATPase similar, but not identical, to that in the SR [280–282, 285, 286]; in addition, it has also been recently suggested that the Ins(1,4,5)P_3-sensitive store may accumulate Ca^{2+} via the coordinated action of a H^+-ATPase and a 2 H^+/Ca^{2+} exchanger [287, 288].

Ca^{2+}-ATPase(s)

The activity of Ca^{2+}-ATPase in microsomal fractions enriched in ER markers is about two orders of magnitude lower than in muscle SR, suggesting that either this enzyme is scattered at low density throughout the ER or it is selectively enriched in a specific subcompartment(s). In fact, it has been demonstrated that, in a number of non-muscle cells, the SR-type Ca^{2+}-ATPase is concentrated in specialized structures, the calciosomes, distinct from the bulk of the ER, and also containing a calsequestrin-like Ca^{2+}-binding protein [280–282]. Later studies have reported the molecular cloning of cDNAs encoding ATPases that closely resemble cardiac (human) or fast- and slow-twitch (rat) Ca^{2+}-ATPases [289]. These proteins have a molecular mass of approximately 110 kDa and contain the conserved domains of E1/E2 ATPases. Like other members of this family of enzymes, these ATPases form phosphorylated intermediates [290] and are inhibited by millimolar concentrations of vanadate [291]. In one study [292]

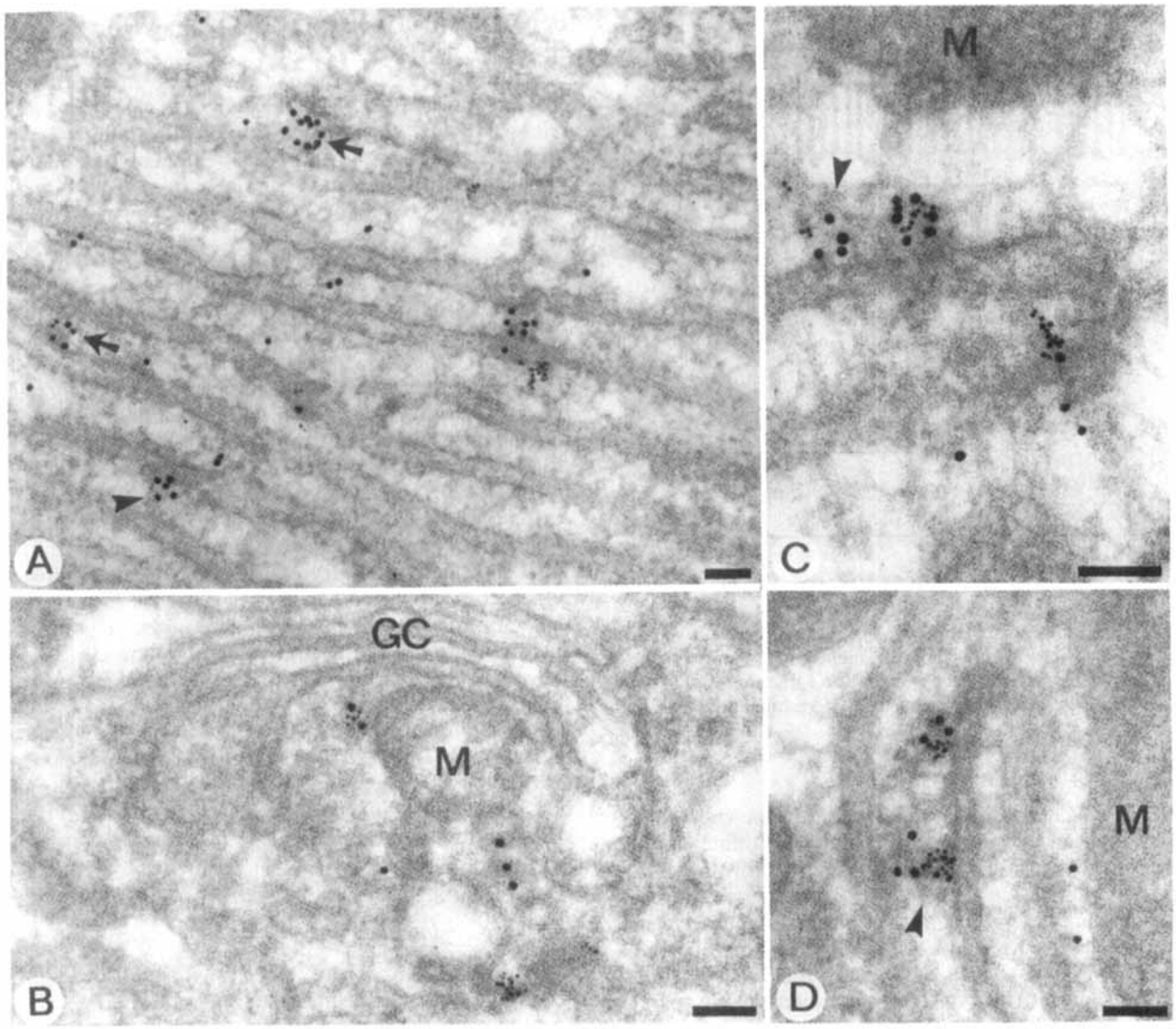

Fig. 4. *Dual labelling of ultrathin liver cryosections with anti-(ATPase) antibodies (large dots) and anti-(calsequestrin) antibodies (small dots).* The two antigens appear often, but not always, localized in the calciosomes. M, mitochondrion; GC, Golgi complex. Note that the bulk of ER is unlabelled. Bar, 0.1 μm. Reproduced with permission from Hashimoto et al. [281]

using monoclonal antibodies raised against cardiac Ca^{2+}-ATPase, a high concentration of cross reactive antigen was found in the Purkinje cells of the chicken cerebellum, where the highest concentration of Ins(1,4,5)P_3-binding sites is also found [293]. The predicted amino acid sequence from cDNA of the non-muscle Ca^{2+}-ATPase isoforms has a characteristic extended C-terminus that may either provide the binding site for cellular regulatory factors or contain a signal sequence that targets the protein to it's final subcellular location [285]. Using two distinct monoclonal antibodies raised against the Ca^{2+}-ATPase of skeletal muscle SR, it has been shown that they stain different compartments in adrenal chromaffin cells [286]. It has been suggested that the two bands recognized by these two antibodies (apparent molecular mass 110 kDa and 130 kDa) represent two different types of Ca^{2+}-ATPases that may belong to two intracellular Ca^{2+} stores with different functional properties [286]. Admittedly no evidence has been provided so far indicating that the two proteins recognized by the two antibodies are indeed Ca^{2+}-ATPases.

Ca^{2+}/2 H^+ exchanger

Schultz and coworkers [287, 288] have recently suggested that Ca^{2+} accumulation by the Ins(1,4,5)P_3-sensitive store does not depend on the activity of a Ca^{2+}-ATPase, but rather on a more complicated mechanism involving first the formation of a pH gradient (acidic interior), due to a H^+-ATPase and subsequently an electro-neutral exchange of 2 H^+/Ca^{2+}. In this model two Ca^{2+} pools are supposed to exist; one is insensitive to Ins(1,4,5)P_3 but with high vanadate sensitivity and fills via a classical Ca^{2+}-ATPase of the SR type; the other is sensitive to Ins(1,4,5)P_3 but with low vanadate sensitivity and utilizes the 2 H^+/Ca^{2+} exchanger. However, observations made in our laboratory do not support the generality of the model suggested by Shultz and coworkers. In fact, in intact cells treatment with a number of drugs capable of collapsing internal pH gradients, such as nigericin, monensin or NH_4Cl, results neither in significant depletion of Ins(1,4,5)P_3-sensitive Ca^{2+} pools, nor in inhibition of Ca^{2+} refilling into this store

[294] (T. Pozzan, unpublished results). Furthermore it can be argued that if Schultz's model were true, Ca^{2+} ionophores, such as ionomycin and A23187, should facilitate Ca^{2+} accumulation into the intracellular Ins(1,4,5)P_3-sensitive store and not counteract it. These ionophores, in fact, function essentially as 2 H^+/Ca^{2+} exchangers and therefore should act in parallel with the natural exchanger rather than counteracting it (D. G. Nicholls, personal communication). On the contrary, it is a common observation that Ca^{2+} ionophores cause complete depletion of Ins(1,4,5)P_3-mobilizable stores, both in intact cells and in isolated microsomes. In our opinion, therefore, the existence and relevance of the electro-neutral exchanger in the Ins(1,4,5)P_3-sensitive store is still dubious.

Ca^{2+} buffering within non-muscle stores, calreticulin

In the last two years proteins with properties similar to those of striated muscle calsequestrin have been described by several groups in many non-muscle tissues and they may be present in most eukaryotic cells [280–282, 295–299]. Quite surprisingly, recent data obtained in our and other laboratories [299–301], demonstrate that these calsequestrin-like proteins are in fact proteins which have already been known for some time and which have been given different names: SR high-affinity calcium-binding protein [223]; calregulin [302]; CRP55 [298]. These proteins share the same N-terminal sequence and several other biochemical and immunological characteristics and thus all these different names presumably refer to the same protein.

In two recent papers [301, 303] SR high-affinity calcium-binding protein and CRP55 have been cloned and sequenced and the common name calreticulin proposed. The reason for this confusing nomenclature and for not including calreticulin in the calsequestrin family is probably due to the fact that calreticulin possesses one high-affinity Ca^{2+}-binding site/mol protein [223, 302]. However MacLennan and coworkers, who first described calreticulin [223], had already reported its capacity to bind more than 20 mol Ca^{2+}/mol protein with low affinity. We have recently characterized calreticulin from rat liver and observed that (in addition to the high-affinity site) it also binds up to 50 mol Ca^{2+}/mol protein with low affinity, (K_d = 1 mM) [299]. Calreticulin cross-reacts with a few anti-(skeletal muscle calsequestrin) antibodies, particularly in its native form [299]. In ultrathin cryosections, anti-calreticulin antibodies, coated with gold particles, localize in vesicular structures which are morphologically indistinguishable from calciosomes [299]. Calreticulin is a glycoprotein (molecular mass from cDNA 47 kDa); the polysaccharide side chain is of the Golgi type and contains a terminal galactose suggesting that the protein has reached the trans Golgi network [304]. However, the C-terminus of the mRNA codes for KDEL [301, 303], the signal peptide believed to be characteristic of proteins retained in the ER [224]. Thus there is on the one hand, a discrepancy between the morphology and carbohydrate side chain, with both suggesting that calreticulin is not in the ER but rather contained in post Golgi structures [280–282] and, on the other hand, the primary sequence [301, 303] which suggests an ER localization of the protein. It must be pointed out however, that recently it has been shown that the C-terminus KDEL only retards, but does not block, the sorting of proteins into the Golgi apparatus [305]. The labelling of ultrathin liver cryosections with anti-calsequestrin antibodies and anti-ATPase antibodies is shown in Fig. 4.

Other proteins with low-affinity Ca^{2+}-binding sites have been described in microsomal fractions [289, 304]. None of them however appear to have the characteristics (K_d, number of binding sites, cellular concentration and ubiquitous distribution in tissues) to function as the primary Ca^{2+} buffer within Ca^{2+}-storage compartments.

The Ins(1,4,5)P_3-sensitive channel

Five years have elapsed since it was first demonstrated that Ins(1,4,5)P_3 is capable of releasing Ca^{2+} from a non-mitochondrial store [279] and the purification of an Ins(1,4,5)P_3 receptor [306]. In these five years, hundreds of papers have documented the existence of receptors coupled to Ins(1,4,5)P_3 formation, the correlation between Ins(1,4,5)P_3 production and Ca^{2+} mobilization from intracellular stores, and the presence of high-affinity Ins(1,4,5)P_3-binding sites in microsomal fractions from several tissues (see [277] for a recent review). This long gap is in large part due to the very low numbers of Ins(1,4,5)P_3-binding sites in most cells and the lack of specific, high-affinity ligands for the Ins(1,4,5)P_3 receptor. The discovery [293] that the cerebellum, in particular the Purkinje cells, contains an extrordinary high level of Ins(1,4,5)P_3-binding sites, was thus a serendipitous bonus. The other side of the coin is that the Purkinje cell Ins(1,4,5)P_3 receptor could be a specific isoform of the central nervous system and of a few types of neurons therein [307–309]. Furnichi et al. [401] have recently shown that RNA blot analysis with probes for the Ins(1,4,5)P_3 receptor of cerebellum show hybridization with in RNA extracted from several tissues. All these mRNA have a molecular mass similar to that of the cerebellum isoform.

The Purkinje cell Ins(1,4,5)P_3 receptor is a single polypeptide (molecular mass, based on the cDNA sequence, 313 kDa) [310] that was first described in 1976 [311] as a specific protein of the cerebellum Purkinje cells (named P400 from its apparent molecular mass in SDS gels). This protein is phosphorylated in a cAMP-dependent fashion [312], binds Ins(1,4,5)P_3 with high affinity [306, 313] and when reconstituted into liposomes, catalyzes Ins(1,4,5)P_3-dependent Ca^{2+} efflux [314]. The Ins(1,4,5)P_3 receptor binds strongly to heparin [293], while binding of Ins(1,4,5)P_3 to the receptor is highly sensitive to pH and $[Ca^{2+}]_i$ [308]. By scanning the sequence for hydrophobic stretches, up to six membrane-spanning regions could be predicted, all close to the C-terminus [310]. Based on structural constrains and reactivity with different monoclonal antibodies, it has been proposed that the N-terminus is exposed to the cytoplasm, while the C-terminus is localized within the vesicle lumen [310]. The protein has numerous potential glycosylation sites and at least two potential phosphorylation sites for cAMP-dependent kinase [310]. Both *in situ* hybridization and immunohistochemistry indicate that the protein is expressed at very big levels in the Purkinje cell layer [307, 310, 313]. Alignment of the amino acid sequence of the Ins(1,4,5)P_3 receptor and of the ryanodine receptor revealed strong similarities between parts of the sequences, in particular between Asp269 and Pro426 of the Ins(1,4,5)P_3 receptor and Asp4867 and Pro5023 of the ryanodine receptor (47% identity over 136 amino acids) [313].

Three different studies have been published recently concerning the subcellular localization of the Ins(1,4,5)P_3 receptor in the Purkinje cells [307, 313, 315]. In the first of these studies [307], performed using immunoperoxidase-tagged antibodies, the Ins(1,4,5)P_3 receptor has been found on a number of intracellular cysternae (smooth and rough ER, subplasmalemmal cisternae) but particularly striking was its high concentration on the nuclear membrane and its absence from

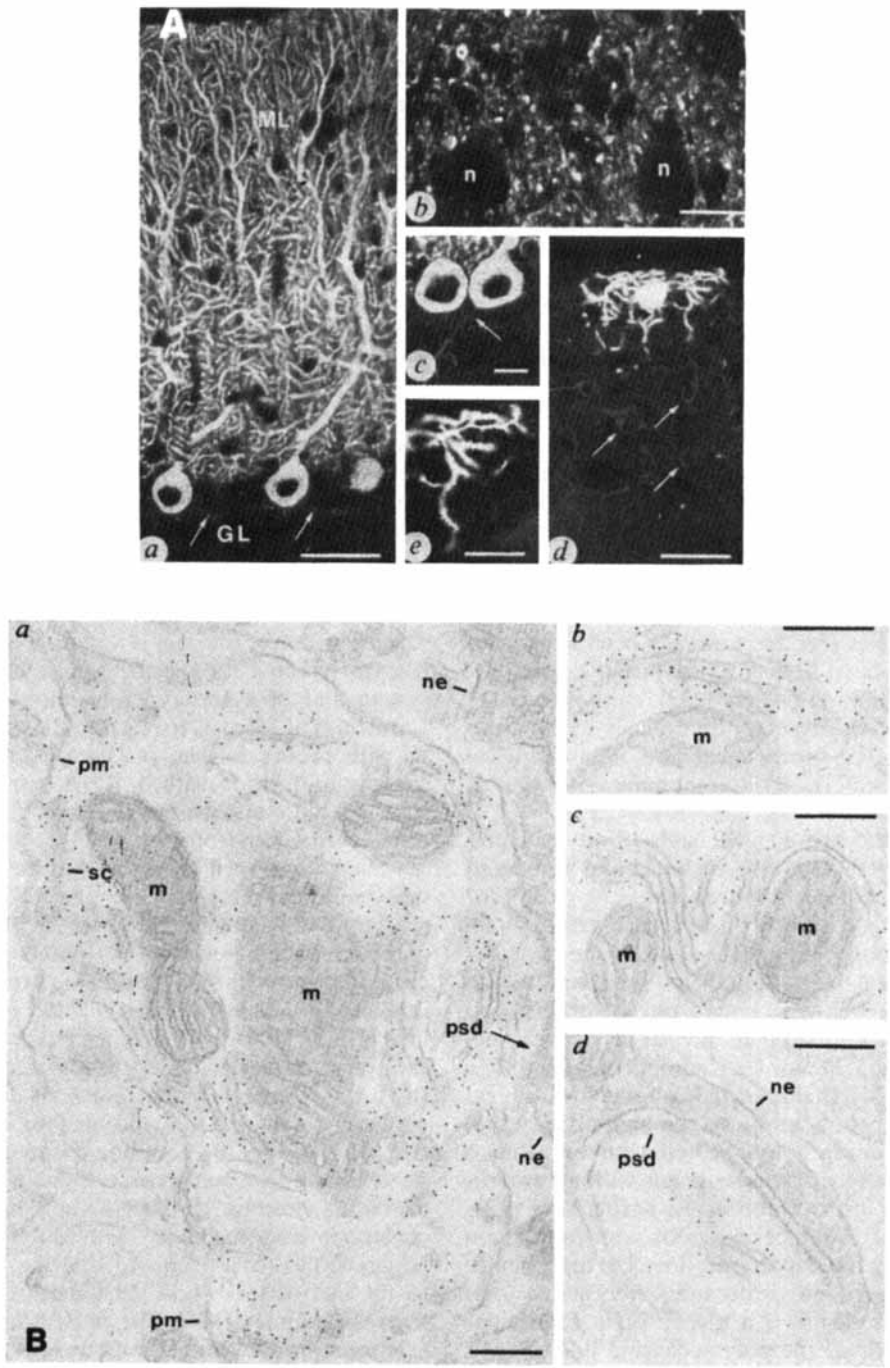

Fig. 5. *Sublocalization of the Purkinje-cell Ins(1,4,5)*P_3 *receptor visualized by immunofluorescence spectroscopy and immunoelectron microscopy.* (A) Immunofluorescence spectroscopy: GL, granule cell layer; n, neuronal perikarya (unlabelled). (B) Immunoelectron microscopy: m, mitochondria; pm, plasma membrane; sc, subplasmalemma cisterna; psd, post-synaptic density; ne, nerve ending. Note the presence of a positive cisternum in a dendritic spine (d). Reproduced with permission from Mignery et al. [313]

the plasma membrane. In a second study, using gold-labelled antibodies, Mignery et al. [313] confirmed the widespread distribution of the Ins(1,4,5)P_3 receptor on polymorphic structures described previously [307] but did not confirm the high level of labelling of the nuclear membrane. Fig. 5 shows the immunolabelling of Purkinje cells with an anti-[Ins(1,4,5)P_3 receptor] antibody.

A recent study by Meldolesi's group [316] also performed using gold-labelled antibodies, confirmed that the very high level of labelling of the nuclear membrane probably arises from an artefact of the peroxidase technique. Rather, it was shown that the highest level of labelling by far (greater than fivefold compared to the nuclear membrane) is observed in special types of smooth stacked cysternae, often continuous with rough ER. Positive cysternae were found in the cell body, in axons and dendrites, and of major physiological relevance, in the dendritic spines [316]. Using peroxidase-coupled anti-[Ins(1,4,5)P_3 receptor] antibodies, Maeda et al. [315] demonstrated the presence of antibodies on the plasma membrane and on the post-synaptic antibody-binding density, though

this result may depend again on the diffusion of the immunoprecipitate from closely apposed subplasmalemmal cisternae [316]. A recent study by Otsu et al. [402] confirms the results of Satoh et al. [316].

Since the molecular identity of the peripheral receptor is uncertain, its characterization is still mainly based on functional studies on microsomal preparations. Summarizing a wealth of data on this receptor we can conclude the following: (a) the peripheral Ins(1,4,5)P_3 receptor is present at picomolar concentrations within most cells [317]; (b) it has an affinity for Ins(1,4,5)P_3 in the nanomolar range [317, 318]; (c) it gates a channel opening within less than 20 ms upon addition of Ins(1,4,5)P_3 [319]; (d) opening of the channel by Ins(1,4,5)P_3 shows some signs of cooperativity [319]. The characteristics of a channel gated by Ins(1,4,5)P_3 have been recently described by Ehrlich and Watras [320] using microsomes from smooth muscle cells fused with black lipid films. The electrophysiological and pharmacological properties of this channel are strikingly different from those of the ryanodine receptor; the conductance is much smaller and caffeine, ryanodine and Ca^{2+} have no effect either on the conductance or on the opening probability.

Are there one or more types of Ca^{2+} stores in non-muscle cells?

This is a much debated issue and considerable evidence has been presented suggesting the existence in non-muscle cells of Ca^{2+} pools insensitive to Ins(1,4,5)P_3, but sensitive to caffeine, Ca^{2+} or GTP. Subcellular fractionation studies on a variety of tissues have given conflicting results as far as the correlation between ER markers, Ca^{2+} accumulation, Ins(1,4,5)P_3 binding and Ins(1,4,5)P_3-sensitive Ca^{2+} release. Concerning the Ins(1,4,5)P_3-sensitive store, the correlation with ER marker enzymes was poor or absent in some studies [280, 321, 322], while in others it was excellent, particularly with markers or rough ER [323, 324]. It is not known whether these discrepancies reflect the different tissues analyzed or whether they depend on the different isolation procedures. The picture emerging from these studies is that if any correlation exists between ER and the Ins(1,4,5)P_3-sensitive Ca^{2+} store, then this is probably with a subfraction of the ER and not with the whole structure. On the other hand the morphological approach followed by our group [280–282] revealed the existence of structures, named calciosomes, that are enriched with a SR-type Ca^{2+} ATPase and with calreticulin, but are devoid of typical ER markers, i.e. cytochrome *P*450. We initially proposed that the calciosomes were the Ins(1,4,5)P_3-sensitive stores; however, this tentative identification never went beyond a plausible working hypothesis, since no marker was available for the peripheral Ins(1,4,5)P_3 receptor. As more data accumulates on the newly described Ins(1,4,5)P_3-insensitive stores, the possibility that the calciosomes should be identified with this latter store must be considered and is actually suggested by some recent data [286].

With respect to GTP-induced Ca^{2+} release [324, 325] our understanding of the current interpretation of the data is that this nucleotide does not directly gate a Ca^{2+} channel, but rather permits the communication between Ins(1,4,5)P_3-sensitive and Ins(1,4,5)P_3-insensitive pools [326]. It must also be pointed out that GTP can cause fusion between vesicles, although at present it is unclear how much this phenomenon contributes to overall GTP-induced Ca^{2+} release.

Caffeine-induced Ca^{2+} redistribution in non-muscle cells has been documented in a variety of neurons or neuroendocrine cells [286, 327, 328]. The sensitivity to caffeine has been interpreted as evidence for the existence of a Ca^{2+}-induced Ca^{2+} release mechanism similar to that of striated muscle SR. It is still unclear however, whether the caffeine-sensitive pool is distinct from the Ins(1,4,5)P_3-sensitive pool, or whether the sensitivity to this drug demonstrates the existence of two different channels in the same pool or even of two gating modes of the same channel. Since these issues are discussed in much detail in two recent reviews [283, 284] the reader is referred to these contributions for further details.

MITOCHONDRIA

Had this review been written in the late seventies or early eighties, the section on mitochondrial Ca^{2+} homeostasis would be one of the lengthiest and most complex. Today the interest of the scientific community for mitochondrial Ca^{2+} homeostasis has dropped dramatically, although many aspects of Ca^{2+} handling by mitochondria remain mysterious. Here we will emphasize only the most controversial points, referring the reader to other reviews for a more detailed and exhaustive discussion [329, 330].

Mitochondrial Ca^{2+} uptake depends on the electrical gradient across the inner membrane and does not require ATP hydrolysis. Neither the nature of the transport mechanism (carrier or channel) has been established with certainty, nor the molecular components involved in Ca^{2+} uptake been purified. The only attempt to discriminate between channel- and carrier-mediated transport is, to our knowledge, that of Bragadin et al. [331] who showed that the activation energy of Ca^{2+} uptake in mitochondria is relatively low (33.6–37.8 kJ/mol), with no discontinuity in the Arrhenius plot, as in the case of typical ion carriers in mitochondria. The molecular components of the mitochondrial Ca^{2+} uptake system have been the matter of much investigation until 1982–1983 [332, 333]. Despite some claims in the past that a glycoprotein of molecular mass 30 kDa could be involved [332] the observation has neither been confirmed nor followed up by the authors themselves. A major obstacle in applying molecular biology techniques to this problem is that neither an assay for the reconstituted system is available, nor is a specific blocker of mitochondrial Ca^{2+} uptake on the market. The well-known and widely used lanthanides and ruthenium red are in fact by no means specific.

Our understanding of Ca^{2+} efflux has not improved significantly even after the discovery that because of the existence of electro-neutral $Ca^{2+}/2\ H^+$ or electrogenic $Ca^{2+}/3\ Na^+$ exchangers, mitochondrial Ca^{2+} accumulation does not reach electrochemical equilibrium (the concept of a mitochondrial set point [334–336]). It was shown that the exchange mechanism can be modulated by the membrane potential [337] and that it is sensitive to a number of drugs known to be fairly unspecific inhibitors of voltage-gated Ca^{2+} channels of the plasma membrane [338, 339]. The median effective concentration for inhibition by these drugs is however rather high (several tens of micromolar) [338, 339] thus affinity purification of the exchanger by this means appears problematic.

The intramitochondrial buffering system is also rather poorly understood. The Ca^{2+} content of mitochondria in intact cells or of carefully isolated mitochondria, [340] is much lower than previously thought, yet a major discrepancy still exists between the total Ca^{2+} content (tens of micromoles/liter of matrix water) and the free mitochondrial Ca^{2+} concentration ($< 1\ \mu M$) calculated indirectly on the basis of the

activity of intramitochondrial Ca^{2+}-activated dehydrogenases [341] and more recently, directly with fluorescent probes [342].

The significance in the intact cell of the elaborate mitochondrial Ca^{2+} homeostatic mechanism still remains the most relevant physiological question. The only point that is generally agreed upon is that mitochondrial Ca^{2+} transport plays an essential role in intramitochondrial metabolism [341]. In fact, intramitochondrial-free Ca^{2+} is a regulator of a number of key dehydrogenases and its accurate control is vital for their coordinated function. It is also well established that mitochondria are not the pool responsible for receptor-stimulated Ca^{2+} mobilization, since no physiological agent capable of triggering fast release of mitochondrial Ca^{2+} is known. Recently however, it has been reproposed that mitochondria can serve as intracellular buffering organelles, at least in some cell types. In fact, a significant proportion of the calcium released from the Ins(1,4,5)P_3-sensitive pool apparently ends up in the mitochondria [343, 344]. Therefore these organelles may be tentatively included in the elusive Ins(1,4,5)P_3-insensitive Ca^{2+} pool.

The involvement of mitochondria as the last high-capacity low-affinity Ca^{2+} store, that will serve to rescue cells when pathological increases of $[Ca^{2+}]_i$ occur, has been largely discussed in the past (see [345] for review).

CYTOPLASM

The highly complex $[Ca^{2+}]_i$ homeostatic mechanisms serve two complementary functions: (a) maintenance of a low $[Ca^{2+}]_i$, essential for cell survival; (b) allowance of controlled changes of the second-messenger level when appropriate stimuli (plasma-membrane-potential depolarization and/or triggering of specific receptors) activate Ca^{2+}-dependent pathways. Thus, the ultimate targets of $[Ca^{2+}]_i$ changes reside in the cytosol and are represented by calcium-binding proteins.

Calcium-binding proteins can be classified according to different criteria. A very comprehensive classification of the EF-hand superfamily of calcium-binding proteins based on their evolutionary relationships has been proposed recently by Kretzinger et al. [346]. In the present contribution we classify cytosolic calcium-binding proteins on the basis of their function.

Given that in all eukaryotic cells studied to date the $[Ca^{2+}]_i$, under physiological conditions, can vary at most from a tenth of a micromole to a few micromoles [347] the obvious prediction is that the useful range of Ca^{2+} affinities for a cytosolic protein must be in the submicromolar to low micromolar range. Many exceptions to this rule however exist and cells have circumvented the problem of the intrinsic low Ca^{2+} affinity of some calcium-binding proteins (i.e. protein kinase C) in various ways (see below). From a functional point of view, cytosolic high-affinity calcium-binding proteins can be divided into two major groups: (a) proteins whose only function is that of buffering Ca^{2+} and (b) proteins with modulatory activity on cellular functions. In the latter family some i.e. calmodulin, troponin C etc., regulate the function of target proteins; others, such as protein kinase C, have catalytic activity of their own.

A detailed discussion of each member of cytosolic calcium-binding proteins would require more room than is available here. Therefore we will briefly describe the major characteristics of a few of the most representative members of each group.

The Ca^{2+} buffers: parvalbumin and related proteins

Parvalbumins are a group of homologous proteins with molecular masses ranging over 9–13 kDa. These proteins were initially described by Deutike in 1934 [348] as soluble 'low-molecular mass albumins' in frog muscles, hence the term parvalbumin (*parva* being latin for small). Later, they were isolated from different fish and amphibian skeletal muscle. For some time parvalbumins were considered to be specific to white muscles of lower vertebrates, but more recently they were shown to exist in almost all mammals and not only in striated muscles (for a review see [349]). Parvalbumins have a special place in the history of calcium-binding proteins not only because they were the first to be crystallized and studied by X-ray diffraction [350], but also because some basic concepts of the structural arrangement of high-affinity Ca^{2+}-binding sites were based on the structure of carp parvalbumin. Three-dimensional structure analysis indicates that carp parvalbumin contains six helical regions (A–F) connected by loops. The A–B domain represents an aborted Ca^{2+}-binding site. The Ca^{2+}-binding sites are located in the loops between the two helices C–D and E–F. The structure of the last two helices (and loops), the EF-hand structure (see Fig. 6), has been synonymous for a long time with the high-affinity Ca^{2+}-binding site. It is now clear that this is an oversimplification and new types of cytosolic calcium-binding proteins, not belonging to the EF superfamily, have been discovered in the last few years, (the annexin family [351], gelsolin [352], protein kinase C [353, 354] etc.). Nonetheless the sequence constraints of the EF-hand structure are so rigid that a protein can be classified in the EF-hand family simply by knowing its amino acid sequence [355]. The Ca^{2+}-binding affinities of the two Ca^{2+}-binding sites of parvalbumin are very similar, i.e. no evidence for positive or negative cooperativity has been provided [356]. The reported K_d values vary over 0.1–4 μM depending on the concentration of Mg^{2+}, pH, ionic strength, source of the protein and methodology employed [356, 359]. The two high-affinity Ca^{2+}-binding sites also bind Mg^{2+} (Ca^{2+}/Mg^{2+} mixed sites) so that under resting conditions, most of the parvalbumin is in the Mg^{2+} bound form.

The only known function of parvalbumin is to bind Ca^{2+}, despite many attempts in the past to look for enzymatic or regulatory functions of the protein. The most widely accepted idea for the role of parvalbumin *in vivo* is that it functions as a soluble Ca^{2+} buffer in the cytoplasm. Consistent with this hypothesis are observations that parvalbumin in skeletal muscle is present almost exclusively in fast twitch fibers [349] and there is a good correlation between the parvalbumin content and the speed of muscle relaxation [349, 356]. According to this model, Ca^{2+} released from the SR will first bind to troponin C, initiating contraction (the dissociation of Mg^{2+} from the Ca^{2+}/Mg^{2+} mixed sites of parvalbumin is relatively slow); the muscle relaxation would be initially due to Ca^{2+} binding to parvalbumin and subsequently to Ca^{2+} uptake by the SR. This model essentially applies to fast-twitch fibers, but it is still largely debated [349, 356, 357].

Parvalbumin or parvalbumin-like proteins have more recently been found in many other tissues, in particular in the central nervous system, in which only a subpopulation of neurons appear to contain the protein and there is some correlation between the parvalbumin and the 4-aminobutyric acid content [349, 356, 358]. Parvalbumins (or related proteins) have also been found in Leydig cells of the testis, in the ovary and in mineralizing tissues [358].

Other proteins with high affinity for Ca^{2+}, whose only known function is Ca^{2+} buffering, have been described in

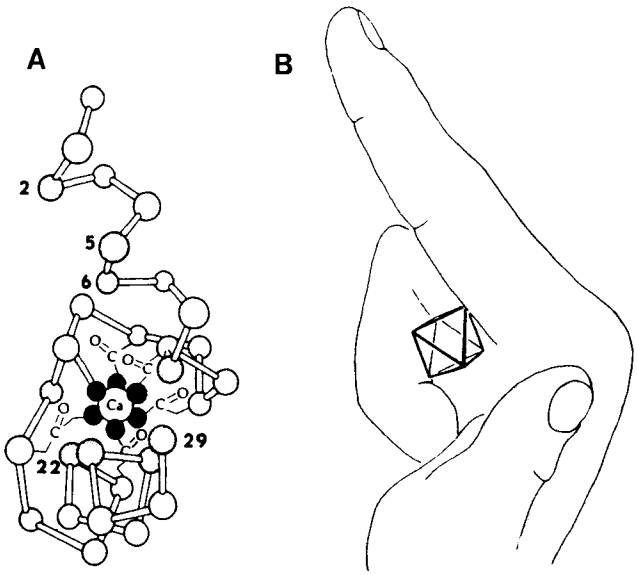

Fig. 6. *The EF-hand structure.* (A) Ball and stick model and (B) symbolic representation of the EF-hand structure. Reproduced with permission from Persechini et al. [399]

recent years. Among them the S100* intestinal Ca^{2+}-binding protein family [359], oncomodulin [360], a parvalbumin-like protein found specifically in tumor tissues, and the calbindin family [361]. All the proteins mentioned above contain two or more EF-hand motifs.

It must be stressed that energy-independent buffering of the $[Ca^{2+}]_i$ depends not only on high-affinity calcium-binding proteins, but also upon low-affinity binding sites, not exclusively provided by negative sites on proteins. Low-affinity Ca^{2+}-binding sites ($K_d > 10$ μM) in fact not only appear to be present in the cytoplasm [362], but their importance as cytoplasmic Ca^{2+} buffers cannot be neglected; it is easy to calculate that if the number of the low affinity sites is large (as it probably is) then even negligible saturation of these sites (< 1%) would provide significant Ca^{2+} buffering of cytoplasmic calcium.

The Ca^{2+} sensors: calmodulin and related proteins

Although discovered relatively late (1970) [363, 364] no other calcium-binding protein has received so much attention by the scientific community as calmodulin [365, 366]. This small acidic protein (molecular mass 16 kDa, isoelectric point 4.5) is present in large quantities in the cytoplasm of all eukaryotes (up to 1% of the total protein mass) [366]. No natural isoform of calmodulin in different tissues has been described so far (in chickens, a calmodulin pseudogene has been found [367]) and the primary structure of calmodulin has been highly conserved during evolution. The crystal structure of calmodulin (at 0.3 nm resolution) was determined in 1985 by Babu et al. [368] and the protein looks like a dumb-bell with the globular N- and C-terminal halves linked by a long α-helical portion (2.0 nm long) which contains the phenothiazine-binding domain. The N- and C-terminal globular domains are remarkably similar and two EF-hand Ca^{2+}-binding sites are localized within each half molecule. It should be emphasized that the three-dimensional structure of calmodulin was determined on crystals grown at pH 5 [368] and thus extrapolation of the data to neutral, physiological conditions may not be completely correct.

Despite the fact that Ca^{2+} binding to calmodulin has been investigated by nearly all available techniques, no consensus has been yet reached on the affinity for Ca^{2+} of the four Ca^{2+}-binding sites [366]. Two alternative models have been proposed. According to one hypothesis, the four Ca^{2+}-binding sites of calmodulin have very similar affinities with little or no cooperativity among the sites [369]; at pH 7.0 and ionic strength 150 mM, the Ca^{2+}-saturation curve fits an ideal Langmuir isotherm with four independent sites of equal affinities (K_d 10 μM). Cox [370] has however demonstrated that the experimental data can also fit a second model [371], according to which the N- and C-terminal Ca^{2+}-binding pairs differ markedly in their affinities for Ca^{2+}. This latter model is supported by kinetic and conformational studies. In fact, based on $^{43}Ca^{2+}$-NMR [372] and stopped-flow fast kinetics [373], it has been shown that the N-terminal half of calmodulin has a k_{off} of $300-500\ s^{-1}$ while the C-terminus has a k_{off} of $10-40\ s^{-1}$. Assuming a diffusion controlled on-rate constant, these data suggest the existence of high-affinity sites in the C-terminus and low-affinity sites in the N-terminus of the protein. Evidence has also been provided suggesting the existence of strong cooperativity between the two sites of each pair, particularly in the C-terminal half [374]. In conclusion, at present it is difficult to solve the controversy among the different models and the introduction of new and more sophisticated techniques should eventually solve the discrepancies.

A number of other cations, notably Mg^{2+} and H^+ can bind to calmodulin on the so called auxiliary sites [375]. Under physiological conditions part of the six auxiliary sites (distinct from the four EF-hand Ca^{2+}-specific sites) are occupied by Mg^{2+}.

Binding of the Ca^{2+}-calmodulin complex to a target enzyme increases the Ca^{2+}-binding constants and leads to the appearance of strong positive cooperativity [376]. In particular a dramatic increase in affinity occurs in the third Ca^{2+}-binding constant. Most studies indicate that the high-affinity complex Ca^{2+}-calmodulin-enzyme is only formed if at least three Ca^{2+} bind to calmodulin [377]. It goes beyond the purpose of this review to discuss in detail the complicated kinetic models for the activation of Ca^{2+} – calmodulin-dependent enzymes. It suffices here to stress three points: (a) the Ca^{2+}-bound form of calmodulin has an affinity four orders of magnitude higher for the target enzyme than the Ca^{2+}-free form [378]; (b) the complex between metal-free calmodulin and the enzyme is inactive [378]; (c) the activation of the target enzyme by Ca^{2+}-calmodulin causes derepression, rather than direct activation [379]. This last concept, initially formulated for the activation of myosin-light-chain kinase, has emerged relatively recently [379] (the pseudosubstrate hypothesis) but it might have major consequences in the design of new pharmacological strategies for specific inhibitors of Ca^{2+} – calmodulin-dependent reactions. According to this model the calmodulin-binding domain, in the absence of calmodulin, prevents access of the natural substrate to the enzyme's active site; binding of calmodulin removes this block. The discovery of this mechanism of activation explains the observation that limited proteolysis of Ca^{2+} – calmodulin-dependent enzymes removes calmodulin activation, while permanently activating the enzymatic activity [380].

The most common trigger for the activation of the Ca^{2+} – calmodulin-dependent reaction in intact cells is an increase in the $[Ca^{2+}]_i$. Thus more Ca^{2+}-calmodulin complex forms and the target enzymes are activated. There is another way to activate Ca^{2+} – calmodulin-dependent reactions, at least in theory, i.e. increasing the calmodulin concentration at fixed

$[Ca^{2+}]$. This simple observation is well known to biochemists studying calmodulin *in vitro*, but should not be underestimated *in vivo*, since the absolute concentration of calmodulin not only varies in different cells [365, 366, 369], but can also change during the cell cycle. In particular, a number of authors [381–383] have shown that the content of calmodulin abruptly increases as cells cross the G1/S boundary. In addition, the absolute level of calmodulin increases in transformed cells [384–386]. This has led to the hypothesis that calmodulin might regulate important steps in the cell cycle and in particular some crucial event in the G1 phase. Consistent with this interpretation are a number of results: (a) reversible inhibition of progression through the cell cycle by anti-calmodulin drugs [387]; (b) complete halt of vegetative growth after disruption of the calmodulin gene in the fission yeast *Schizosaccaromyces pombe* [388]; (c) selective reduction of the G1 duration in cells which overexpress calmodulin after transfection with an exogenous calmodulin gene [389]. It is obvious that increasing the calmodulin concentration is a slow and energetically expensive way to activate Ca^{2+}–calmodulin-dependent reactions, but unlike the changes in the $[Ca^{2+}]_i$, which are normally very transient, the increased content of calmodulin might have long lasting effects on cell functions.

The number of enzymatic reactions that are known to be activated by Ca^{2+}-calmodulin is impressive [365, 367, 369]. Two points will be mentioned here that are of general relevance. (a) Despite the fact that so many reactions are known to depend on Ca^{2+}-calmodulin when studied with isolated enzymes, there are much fewer physiological pathways in intact cells that can unambiguously be attributed to Ca^{2+}–calmodulin-dependent reactions. For some time the inhibition by anti-calmodulin drugs has been used by pharmacologists and cell biologists as the main criterion. Although this criterion is still used to assess the involvement of calmodulin in biological reactions, it must be stressed that all calmodulin inhibitors are hydrophobic, perturb membrane functions and are by no means specific *in vivo*. Their effects are so unpredictable, when used in intact cells, that their use should be limited to very few and well-controlled situations. (b) In many instances the amplification of the Ca^{2+} signal is mediated by the Ca^{2+}-calmodulin complex via activation of protein phosphorylation [365, 366, 369]: in this respect it is of major interest that Ca^{2+}-calmodulin kinase II, a multifunctional protein kinase [390] very abundant in the central nervous system (particularly in the hyppocampus), becomes Ca^{2+}–calmodulin-independent after autophosphorylation, i.e. the enzymatic activity becomes autonomons from the second messenger [391]. Deactivation of kinase II can occur either by dephosphorylation or by further autophosphorylation. This phenomenon represents one of the clearest example of biochemical memory whereby once the system has been activated, removal of the stimulator (Ca^{2+}) does not result in deactivation.

Troponin C [392] is closely related structurally to calmodulin, but functionally, the two proteins are quite distinct. In particular troponin C, unlike calmodulin which is a soluble cytosolic protein reversibly bound to a variety of target enzymes, is part of the troponin complex, where the three subunits (C, T and I) behave as a single unit. Furthermore, at least two isoforms (cardiac/slow skeletal and fast skeletal) of troponin C exist with different structural and functional characteristics [393]. Finally, until now troponin C has been found only in striated fibers and has one and only one cellular function, contraction (for review see [393]).

Skeletal muscle troponin C (molecular mass 18 kDa, isoelectric point 4.2) contains four EF-hand Ca^{2+}-binding sites [393]. Of these four sites, the two in the C-terminal domain have a higher affinity for Ca^{2+}, but also bind Mg^{2+}, while the two low-affinity sites of the N-terminus are Ca^{2+} specific [393]. Positive cooperativity in Ca^{2+} binding has been observed in the two high-affinity sites [394].

The basic structure of troponin C closely resembles that of calmodulin [395] in having two globular domains each containing two-EF hand Ca^{2+}-binding sites connected by a long α-helix. The rate of Ca^{2+} binding to troponin C is very fast ($k_{on} > 4 \times 10^7\ s^{-1}$) [396] thus explaining why parvalbumin does not compete with troponin C for Ca^{2+} during the onset of contraction. It must be noted that, resembling the effect of target enzymes on calmodulin, the affinity of troponin C for Ca^{2+} in the troponin complex is increased by nearly one order of magnitude [393]. It suffices here to say that troponin C is an integral part of the thin-muscle filament and that its role under resting conditions prevents the interaction of the myosin heads with actin. Upon increase in the $[Ca^{2+}]_i$, Ca^{2+} binds to troponin C and the Ca^{2+}-troponin C complex moves away from the thin filament permitting actin-myosin interaction [392–396].

The phylogenetic variability of troponin C is much higher than that of calmodulin and within the same animal species, troponin C isolated from heart is significantly different from that expressed in fast skeletal muscle, but identical to that of slow skeletal fibers. Cardiac/slow skeletal troponin C contains only three EF-hand motifs [397].

Strictly speaking several other proteins should be included in the Ca^{2+}-sensor family: myosin, α-actinin, aequorin and other photoproteins; the Ca^{2+}-ATPases; the Ca^{2+} channels and a variety of Ca^{2+}-activated enzymes such as protein kinase C; neutral proteases; calcineurin (a protein phosphatase); annexins (Ca^{2+}-dependent phospholipid-binding proteins); a number of proteins of lower organisms etc. Some of these were dealt with in the preceeding paragraphs of this review and for the others the more specialized literature should be consulted.

CONCLUSIONS

In this review we have attempted to give a brief but comprehensive description of the basic homeostatic mechanisms of $[Ca^{2+}]_i$ in eukaryotic cells. By necessity we have been selective in quotations and biased in some debated aspects. In particular we have mainly stressed the structural and molecular properties of the many components of the $[Ca^{2+}]_i$ homeostatic machinery. This is however only the starting point for understanding $[Ca^{2+}]_i$ handling in living cells. As pointed out by Berridge [398], future emphasis will also have to be put on the spatial and temporal aspects of $[Ca^{2+}]_i$ homeostasis.

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