Journal of Bioenergetics and Biomembranes, Vol. 24, No. 3, 1992

The Ca^{2+}-Transport ATPases from the Plasma Membrane

F. Wuytack[1] and L. Raeymaekers[1]

Received February 23, 1992; accepted February 25, 1992

The initial studies on the plasma membrane (PM) Ca^{2+}-transport ATPases were made in the erythrocyte, a structure that can not be taken as representing a typical eukaryotic cell. In other cell types however, the study of the PM Ca^{2+}-transport ATPase is complicated by the simultaneous expression of related Ca^{2+}-pumps in intracellular stores. Whereas there are as yet no known specific inhibitors for the PM Ca^{2+}-transport ATPase, a number of selective inhibitors for the endo(sarco)plasmic reticulum Ca^{2+} pumps have been described: thapsigargin, cyclopiazonic acid and 2,5-di-(tert-butyl)-1,4-benzohydroquinone. With the recent introduction of the molecular biological approach, it became quickly obvious that a family of at least 5 different PM Ca^{2+}-transport ATPase genes govern the tissue-dependent expression of PM Ca^{2+} pumps. Moreover alternative splicing of the primary gene transcripts was found to further enhance the number of pump variants. The PM Ca^{2+}-transport ATPase are subject to modulatory control by calmodulin, by acidic phospholipids, and by the known families of protein kinases. Each of the ensuing effects are mutually related and interdependent. The wide variety PM Ca^{2+} pump isoforms and their regulation by such an intricate modulatory network allows the distinct tissues to adapt most adequately to the prevailing tissue and stimulus specific requirements.

KEY WORDS: Ca^{2+}-transport ATPase; plasma membrane; calmodulin; Ca^{2+} pump.

INTRODUCTION

It is of vital importance for a cell to keep the concentration of its free cytosolic Ca^{2+} between 10^{-7} and 10^{-5}, i.e., at a concentration far below equilibrium with that of the extracellular Ca^{2+}. Most types of eukaryotic cells express in their plasma membrane (PM) a calmodulin-binding Ca^{2+}-stimulated and Mg^{2+}-dependent ATPase (Ca^{2+}-transport ATPase) that serves this goal (Penniston, 1983; Garrahan and Rega, 1986; Wuytack *et al.*, 1987), sometimes assisted by a $Na^+ : Ca^{2+}$ exchanger (see Blaustein, 1988; Missiaen *et al.*, 1991 for a review). A short overview will be presented here of some recent advances in the study of the family of plasma membrane Ca^{2+}-transport ATPases (PMCA). Special attention will be devoted to their tissue- and differentiation stage-dependent expression and on the possibilities this creates for their differential regulation by an intricate network of cellular protein and lipid components. Recently a number of reviews have appeared treating different aspects of the PM Ca^{2+}-transport ATPases, some focussing on the species- and tissue-dependent distribution of the PM Ca^{2+} pumps and on their relation to the other Ca^{2+} pumps (Penniston, 1983; Wuytack *et al.*, 1987; Carafoli, 1991), and others more on their mechanism (Schatzmann, 1989; Garrahan and Rega, 1990) or on their molecular biology (Strehler, 1991). See also the extensive work on the Ca^{2+} pumps of the plasma membranes by Garrahan and Rega (1986).

NEW TOOLS TO DISCRIMINATE PMCA AND SERCA PUMPS

The PM Ca^{2+}-transport ATPase family represents only one group of a number of Ca^{2+}-ATPases that are expressed in most cells. Among these are a heterogeneous group of poorly defined PM Ca^{2+}/Mg^{2+} ATPases not involved in transport (Lin and

[1]Laboratorium voor Fysiologie, Campus Gasthuisberg, K. U. Leuven, B-3000 Leuven, Belgium.

0145-479X/92/0600-0285$06.50/0

Russell, 1988; Magocsi and Penniston, 1991a), which have recently been reviewed by Dhalla and Zhao (1988) and also the family of endo/sarcoplasmic reticulum Ca^{2+} pumps (SERCA) which account for the accumulation of Ca^{2+} in intracellular stores. Typically the PMCA pumps represent only a minor fraction ($< 0.1\%$) of the intrinsic membrane proteins. The combination of the Ca^{2+} pump's sparsity and the simultaneous presence of other unrelated Ca^{2+}-ATPase activities demands a highly selective and sensitive method to demonstrate the PMCA present in cells. These technical problems have been extensively dealt with in a previous review (Wuytack *et al.*, 1987) but since then some new specific inhibitors of the SERCA pumps have been introduced: thapsigargin, cyclopiazonic acid, and 2,5-di(*tert*-butyl)-1,4-benzohydroquinone. These inhibitors may be helpful in defining the relative importance of PMCA versus SERCA Ca^{2+} pumps in the cellular or subcellular context. Unfortunately no potent and selective inhibitor of the PMCA pumps has been found yet.

Among the new inhibitors thapsigargin may be the most promising. Thapsigargin is a tumor-promoting sesquiterpene lactone that is a constituent of the resin of the umbelliferous plant *Thapsia garganica* L. It was first studied for its property to increase cytosolic Ca^{2+} (for a review, see Thastrup *et al.*, 1989), but it was quickly found to be an inhibitor of the endoplasmic-reticulum Ca^{2+} pumps (Thastrup *et al.*, 1990). It was initially thought that the inhibitor was selective for the SERCA2b variant (i.e., the pump isoform in most nonmuscle endoplasmic reticulum) and that it exerted little or no effect on the cardiac (SERCA2a) or fast-skeletal muscle (SERCA1) type of Ca^{2+} pumps. It has by now become clear that thapsigargin inhibits all of these SERCA pumps and also the SERCA3 Ca^{2+} pump with equal potency. Moreover, thapsigargin affects neither the PM Ca^{2+} pumps (Sagara and Inesi, 1991) nor the NaK ATPase (Lytton *et al.*, 1991). The inhibitor forms very rapidly and essentially irreversibly a complex with its target with an apparent affinity in the subnanomolar range (Sagara and Inesi, 1991). This extremely high affinity explains the almost stoichiometric reaction of the inhibitor with the SERCA pumps. It is this property that initially prompted researchers to use too low substoichiometrical amounts of the inhibitor, which led to the premature conclusion that SERCA1 and SERCA2a pumps were less sensitive to the inhibitor than SERCA2b (Lytton *et al.*, 1991).

The mechanism by which thapsigargin inhibits selectively the SERCA pumps without affecting the PMCA pumps needs further specification and will help to further define some fundamental differences between the two families of Ca^{2+} pumps. But already from the work of Sagara and Inesi (1991) it has become clear that thapsigargin binds to the Ca^{2+}-free form of the ATPase and not to the Ca^{2+}-bound form. When under conditions of enzyme cycling the enzyme assumes the Ca^{2+}-free conformation the formation of the thapsigargin–enzyme complex is favored and the enzyme is inhibited. Thapsigargin readily penetrates cells and can be used in living cells.

The mycotoxin cyclopiazonic acid was also found to make a stoichiometric complex with the SERCA1 and SERCA2a Ca^{2+} pumps, but not to affect the erythrocyte Ca^{2+} pump. (Seidler *et al.*, 1989). The fact that ATP protects the enzyme against this inhibitor in a competitive manner precludes the use of this inhibitor at physiological ATP concentrations.

2,5-Di-(*tert*-butyl)-1,4-benzohydroquinone is a potent inhibitor of the Ca^{2+} pump in hepatocyte endoplasmic reticulum but not in the plasma membrane (Moore *et al.*, 1987). It also inhibits Ca^{2+} accumulation in skeletal muscle SR. (Missiaen *et al.*, 1991). Mason *et al.* (1991) remark that both this compound and cyclopiazonic acid at higher concentrations may affect the Ca^{2+}-entry pathway in the cells.

THE PHOSPHOPROTEIN INTERMEDIATE PROVIDES AN EASY PMCA DETERMINATION CHARACTERISTIC

Along with a number of other cation-transport ATPases, also the PM Ca^{2+}-transport ATPase is characterized by the formation in its catalytic cycle of a phosphoprotein intermediate. The γ-phosphate group of the energizing ATP is thereby transferred to an aspartate residue (D) forming part of a sequence —CSDKTG—that is highly conserved in each of the members of this superfamily (Green, 1989; Green and MacLennan, 1989). This typical property clearly classifies the PM Ca^{2+} pumps in the group of P-type ATPases and distinguishes it from two other groups of cation (H^+) ATPases: the mitochondrial and vacuolar-type of ATPases (Pedersen and Carafoli, 1987). The aspartyl-phosphate bond thus formed is of the acyl-phosphate type and hence alkali- or hydroxylamine-labile. This is in contrast to the alkyl phosphates (i.e., serine-, threonine- or tyrosine-phosphates) which are formed in the protein-kinase-mediated phosphate

transfer reactions. The level of Ca^{2+}-dependent phosphoprotein intermediate of the PM Ca^{2+}-transport ATPase can be greatly (> 4 fold) increased in the presence of 50 or 100 μM of La^{3+} (Luterbacher and Schatzmann, 1983). Apparently only a fraction of the Ca^{2+} pump units remain phosphorylated during steady-state activity. This is in contrast to the situation in the endo/sarcoplasmic reticulum Ca^{2+} pumps (Kodama, 1985) where La^{3+} has either no effect or even slightly decreases the phosphoprotein level (Wuytack *et al.*, 1982). A Ca^{2+}-dependent, hydroxylamine-sensitive phosphoprotein of Mr 130,000, the level of which is increased by La^{3+}, is a clear hallmark of the PM Ca^{2+}-transport ATPase. The SERCA pumps, on the other hand, show a phosphointermediate of Mr 115,000 whose level is not increased by La^{3+}. Tracing the phosphoprotein intermediates by electrophoresis thus provides a simple and very sensitive way to demonstrate low levels of Ca^{2+} pumps even in crude membranes or homogenates and to assign them to the PMCA or SERCA family (De Smedt *et al.*, 1983; Wright and van Houten, 1990; Donnet *et al.*, 1990; Kessler *et al.*, 1990). Characterization of the tryptic fragments of the phosphoprotein intermediates provides a further way to discriminate PMCA from SERCA pumps (Wuytack *et al.*, 1984) and even allows one to distinguish SERCA1 from SERCA2 pumps (Wuytack *et al.*, 1989).

THE DISTRIBUTION OF THE PMCA-TYPE ATPASES

The ATP-driven Ca^{2+} pumps of the human erythrocyte represents the archetype of the PM Ca^{2+}-transport ATPase (see also the review by Schatzmann in this series).

The combination of observations by Dunham and Glynn (1961) who first demonstrated a Ca^{2+}-dependent ATPase activity in human erythrocytes and of Schatzmann (1966) who showed an ATP-dependent Ca^{2+} extrusion from erythrocyte ghosts, formed the basis for the research of PM Ca^{2+}-transport ATPases.

It has by now become obvious that calmodulin-stimulated Ca^{2+} pumps are ubiquitously expressed in the plasma membrane of nearly all eukaryotic cells. Representative examples from all eukaryotic Regnae are by now known. Besides the examples from the kingdom of Animalia already mentioned, the pump was also demonstrated in the kingdom of Plantae like in *Zea mays* (Dieter and Marmé, 1981; Briars *et al.*, 1988; Robinson *et al.*, 1988), of Fungi like in *Saccharomyces* (Hiraga *et al.*, 1991), and of Protoctista like in *Paramecium* (Wright and van Houten, 1990) or *Leishmania* (Benaim and Romero, 1990).

The next leap forward came with the realization that a Ca^{2+}-dependent binding of calmodulin activated the PM Ca^{2+} pump (Bond and Clough, 1972; Gopinath and Vincenzi, 1977; Jarrett and Penniston, 1977, 1978). Notwithstanding the fact that different types of cells express a number of distinct calmodulin-binding proteins, affinity chromatography on immobilized calmodulin provided an easy and powerful way to selectively purify the transport ATPase, first from erythrocytes (Niggli *et al.*, 1979; Gietzen *et al.*, 1980) and later also from cardiac muscle (Caroni and Carafoli, 1981a), smooth muscle (Wuytack *et al.*, 1981; De Schutter *et al.*, 1984), and a number of other systems including plants (Dieter and Marmé, 1981; Briars *et al.*, 1988) and yeast (Hiraga *et al.*, 1991). It is not entirely clear why in this procedure other calmodulin-binding enzymes only copurify to a limited extent, but it is a fortunate property for the purification of the PM Ca^{2+} pumps (Niggli *et al.*, 1987).

One remark is needed concerning the PM Ca^{2+} pump of hepatocytes. Recent work by Kessler *et al.*, (1990) throws some new light on this peculiar pump. This pump combines many characteristics of the classical PM Ca^{2+} pumps, e.g., it forms a La^{3+}-stimulated Ca^{2+}-dependent phosphoprotein intermediate, and it cross-reacts with antibodies against the erythrocyte Ca^{2+} pump, but at variance with the other PM Ca^{2+} pumps it is not stimulated by calmodulin. The hepatocyte PM Ca^{2+} pump does, however, bind calmodulin under denaturating conditions, and it does so even more strongly than the erythrocyte pump.

Finally, a third major breakthrough came with the advent of recombinant DNA technology. This allowed the cloning and sequencing of a number of PMCA *c*DNAs. Partial peptide sequencing of the human erythrocyte Ca^{2+} pump (Verma *et al.*, 1988) together with the cloning of other members of the P-type cation pump prepared the field for obtaining these PMCA *c*DNAs.

Shull and Greeb (1988) first identified two distinct Ca^{2+}-pump isoforms (PMCA1 and PMCA2) from a rat brain *c*DNA library using an oligonucleotide probe derived from a conserved amino acid sequence of the ATP-binding site of the P-type superfamily of transport ATPases. This was soon followed by the discovery of a third brain isoform (PMCA3)

(Greeb and Shull, 1989). All three isoforms exhibited between 81 and 85% amino acid identity and were clearly encoded by separate genes.

A PMCA1 variant (PMCA1b) was cloned from a human teratoma library (Verma *et al.*, 1988) and later also from pig (De Jaegere *et al.*, 1990) and rabbit (Khan and Grover, 1991) stomach smooth muscle. The sequence of still a fourth homologous PMCA gene product was deduced from overlapping *c*DNA clones in human small intestinal musoca and teratoma libraries (Strehler *et al.*, 1990). It showed somewhat less amino acid identity to the other PMCA pumps (75% identity to the human PMCA1b). PMCA4 represents the major Ca^{2+}-pump isoform in mature human erythrocytes as is shown by partial protein sequencing of the erythrocyte enzyme. The other isoform in mature erythrocytes is PMCA1b. Finally, a partial sequence of what appears to be a fifth PMCA isoform has been derived from bovine brain (Brandt *et al.*, 1988; Mann *et al.*, 1989).

Because only 2% of the amino acids differ between gene products of corresponding PMCA isoforms among the different mammalian species, whereas the difference between the PMCA 1-4 gene products is much larger (15–25%), the evolution of the different PMCA genes must have preceded mammalian speciation.

While the genes encoding the different Ca^{2+}-transport ATPases must have evolved from a common precursor, they are nevertheless not localized on the same chromosome. Indeed, whereas the PMCA1 gene is found on chromosome 12 q21-23, the PMCA4 gene must be assigned to chromosome 1q25-32 (Olson *et al.*, 1991). Some other P-type ATPase genes have also been assigned to these two chromosomes. The SERCA2 gene, for example, encoding the endoplasmic reticulum Ca^{2+} pump, and after alternative splicing of its primary transcript also the isoform found in cardiac/slow-twitch skeletal muscle, is also found on chromosome 12 (MacLennan *et al.*, 1987).

The genes for the α_1 and α_2 units of the NaK ATPase are together with PMCA4 found on chromosome 1, but the NaK ATPase α_1 gene maps to the short arm, whereas the NaK ATPase α_2 gene and the PMCA4 gene have their loci on the long arm albeit at least 20 mega basepairs separated from each other. The SERCA1 gene for the Ca^{2+} pump in the SR of the fast-twitch skeletal muscle is found on chromosome 16.

A comparison of the primary sequences of the PMCA pumps with other P-type ATPases, including a total of some 30 different sequences, confirmed the presumption that they are all homologously related (Green and MacLennan, 1989; Green, 1989). The organization of conserved segments of sequences of the ATP-driven cation pumps within different structural domains allowed the picturing of a tentative consensus structural model for the P-type ATPase molecule (Green and MacLennan, 1989; Stokes, 1991). Such a model then served as the basis for a site-specific mutational analysis of the structure–function relationship of the SERCA1 Ca^{2+} pumps (See Maruyama *et al.*, 1989; MacLennan, 1990 for an overview of these experiments). Most of the conclusions pertaining to the SERCA pump might also be valid for the PMCA pumps, but for the latter pumps no such site-specific mutation analysis has been reported yet. One of the underlying reasons for this lack of information is the difficulty to construct full-length clones of the PMCA *c*DNAs. Some "poisonous" sequences in the full-length PMCA *c*DNA apparently prevent its amplification in bacteria (personal observation).

The expression of the distinct PMCA genes is clearly tissue-dependent, as is shown by Northern-blot analysis (Greeb and Shull, 1989). PMCA1 mRNA, and often exclusively this member of the family, is found in most (all) tissues. PMCA1 can therefore be considered as the housekeeping form of PM Ca^{2+} pump. The other PMCA genes present a more restricted expression pattern. PMCA2 expression was detected in brain, heart, and liver, and PMCA3 in brain and skeletal muscle. Northern-blot analyses have not been reported for PMCA4, but this gene must be expressed in erythrocytes and in intestinal mucosa (Strehler *et al.*, 1990). Greeb and Shull (1989), using a mixed probe derived from PMCA1, 2 and 3 at reduced stringency, detected a reaction with a novel messenger in uterus, stomach, and intestines. Whether this represents a PMCA4, PMCA5 (Brandt *et al.*, 1988), or rather another related cation pump remains to be shown. It is remarkable that brain and muscle are the tissues that slow the widest variety of different PMCA genes expressed. Brain and muscle are also the structures where alternative processing of the messengers shows the largest variation (see below).

It has in recent years become clear that a single gene is often expressed in the form of several distinct protein phenotypes as a result of alternative processing (splicing or polyadenylation) of its primary gene transcript (Breitbart *et al.*, 1987; Smith *et al.*, 1989a). This is also the case for the SERCA and PMCA

pumps. The SERCA1 gene can give rise to a neonatal or an adult protein isoform (Brandl *et al.*, 1987; Korczak *et al.*, 1988), whereas the SERCA2 gene product shows up in a nonmuscle SERCA2b or a cardiac/slow skeletal muscle isoform SERCA2a (Lytton and MacLennan, 1988; Gunteski-Hamblin *et al.*, 1988; Eggermont *et al.*, 1989). The same is true for the PMCA gene family and here possibly even to a larger extent.

Already in the first publication of a PMCA sequence, Shull and Greeb (1988) remarked that the 3′-untranslated sequence of the PMCA1 transcript has the potential to encode also a different C-terminal peptide sequence very similar to that of PMCA2. In a similar way also the PMCA3 (Greeb and Shull, 1989) and PMCA4 (Strehler *et al.*, 1990) transcripts have at least the potential to be alternatively spliced. Both splice variants of PMCA1 are indicated as a or b. Hitherto actual full-length sequences have been published only from PMCA1a, PMCA1b, PMCA2b, PMCA3a, and PMCA4b. Later Strehler *et al.* (1989) showed by analysis of human fetal skeletal muscle and teratoma *c*DNA libraries and of human genomic sequences, that splicing of the PMCA1 gene transcript at this particular point can give rise not only to the a and b variants but to as many as four different mRNA species (a, b, c and d). Alternative exclusion, inclusion, or partial inclusion (87 or 114 basepair) of a single 154 basepair exon in the 3′-end of the PMCA1 gene explains this diversity. The Ca^{2+}-pump isoforms encoded by these different mRNAs have different C terminal regulatory regions including part of the calmodulin-binding domain. Hence the isoform diversity might well have functional significance (see below).

Interestingly, another major zone in the PMCA pumps where the distinct enzyme isoforms differ from each other by insertion of different (5 or 17 residues) amino acid sequences also forms part of a putative regulatory domain, i.e., the putative phospholipid-binding domain which is a highly charged domain, unique to the PM Ca^{2+} pumps and located upstream from the third membrane spanning region, following a glycine that is conserved in all cation transport ATPases (Andersen *et al.*, 1989). Whether the observed isoform diversity in this zone (Shull and Greeb, 1988) is the result of posttranscriptional processing or already encoded at the genomic level remains to be elucidated.

REGULATION OF THE PM Ca^{2+} PUMP BY CALMODULIN

One of the most typical properties of the PM Ca^{2+} pumps is their ability to directly interact with calmodulin, most likely in a 1 : 1 stoichiometric ratio (Hinds and Andreasen, 1981). In the absence of calmodulin or an alternative form of stimulation such as acidic phospholipids, oligomerization, proteolysis, or kinase-mediated phosphorylation (see below), the enzyme has a K_m value for Ca^{2+} of about 10^{-5} M. In the presence of calmodulin, the apparent Ca^{2+} affinity increases about 20-fold (Gietzen *et al.*, 1980; Stieger and Luterbacher, 1981; Niggli *et al.*, 1981). The effect of calmodulin on the V_{max} for Ca^{2+} is, however, more difficult to assess because no true plateau value is reached at high Ca^{2+} concentrations. At high activating Ca^{2+} the rate of catalysis decreases again (Kratje *et al.*, 1985), partially because Ca^{2+} competes with Mg^{2+} which is needed for activating the enzyme and partially because high Ca^{2+} prevents Ca^{2+} from leaving its low-affinity binding sites where it resides after translocation. Values for the increase of V_{max} ranging up to 4- or 10-fold have been reported (Garrahan and Rega, 1990).

The exact mechanisms by which calmodulin increases the apparent Ca^{2+} affinity and the turnover number of the enzyme remain at this moment unknown. Allosteric effects on Ca^{2+} binding as well as changes in the rate of formation or breakdown of the different reaction intermediates might be involved in these processes (Garrahan and Rega, 1990).

In contrast to our ignorance of the mode of action of calmodulin, considerable progress has recently been made in defining the calmodulin-binding domain of the ATPase. Proteolysis studies on the ATPase had paved the way and pinpointed the calmodulin-binding site of the ATPase to a zone within a 12-kDa fragment near the C-terminus of the enzyme (Zurini *et al.*, 1984; Sarkadi *et al.*, 1986). The calmodulin target zone was further identified and peptide sequenced using a photoactivatable, radioactive, and cleavable cross-linker conjugated to calmodulin (James *et al.*, 1988). Molecular cloning and *c*DNA sequencing confirmed the C-terminal domain as the calmodulin-binding domain. The calmodulin-binding domains of different enzymes do not usually show a strong primary sequence similarity, but they generally consist of an about 25–30 residue-long stretch which presents a basic amphiphilic α-helical structure (O'Neil and DeGrado, 1990). Such a domain can be

subdivided in an amino terminal A-subdomain and a carboxy terminal B-subdomain (Buschmeier *et al.*, 1987). This is also the case for the PM Ca^{2+} pump (Schull and Greeb, 1988).

Remarkably, whereas the A-subdomain is fully conserved between all known PM Ca^{2+} pump isoforms, irrespective of the animal species, the B-subdomain differs between the isogenes (Strehler, 1991). The isoform diversity in this particular B-subdomain is even further enhanced by the process of alternative splicing. Strehler *et al.* (1989) showed how in skeletal muscle and brain four different splice variants (a, b, c and d) could be obtained from one single PMCA transcript by exclusion, inclusion, or partial inclusion of a particular 154 basepair exon (see above). Polymerase chain reaction (PCR) analysis of the population of mRNAs confirmed that such an mRNA diversity due to alternative splicing also existed in other tissues and species (De Jaegere *et al.*, 1990; Khan and Grover, 1991).

Although in each of the four protein variants translated from these alternatively spliced mRNAs a carboxy terminal tail of a different length follows the A-subdomain, only two variants of B-subdomains would be created in this way (Fig. 1). The isoform derived from the mRNA where the 154-bp exon is fully excluded (i.e., the b isoform) would be the only one with a variant b subdomain and would differ from the three others (c, d and a). Indeed, the primary structure of the B-subdomain would be independent of the length of the insert since even the shortest insert extends beyond the end of the B-subdomain. The structure further toward the carboxy terminus of the calmodulin-binding domain is, however, affected by the splicing.

As a result of these insertions, a putative target site for *c*AMP-dependent kinase (James *et al.*, 1989c) would be positioned at increasingly longer distances from the calmodulin-binding domain in isoforms b, c and d, whereas it would be lacking from isoform a. Its absence from isoform a follows from frameshift in this particular mode of splicing resulting in a totally different carboxy terminus in isoform a that has no sequence homology with the tails of b, c, or d.

Also as a result of the extra insert which encodes a new B-subdomain, isoforms c and d now contain two B-subdomains: the newly inserted B-subdomain immediately juxtaposed to the A-subdomain and the original B-subdomain following at a distance of 29 or 38 amino acid residues.

Binding of calmodulin to the complete 28 residue-long calmodulin-binding domain (comprising the A- and B-subdomain) of the erythrocyte pump or to its first 15 or 20 amino acids but not to its last 14 amino acids could be demonstrated by a number of biophysical techniques (Vorherr *et al.*, 1990). In other tests it was investigated to what extent different variants of the peptides acted as competitive inhibitors for the activation of the ATPase by calmodulin. Apparently the peptides formed high-affinity 1:1 stoichiometric complexes with calmodulin. Within the A-subdomain a tryptophan residue that is conserved in the calmodulin-binding domains of most proteins appears to play a functional role in the calmodulin-binding process (Enyedi *et al.*, 1989b; Vorherr *et al.*, 1990). Peptides where the conserved tryptophan residue was modified showed a dramatic drop in binding affinity for calmodulin.

Also very exciting were the observations on the truncated pump that had been activated by proteolytic removal of its own endogenous calmodulin-binding domain. These results confirm the notion that the calmodulin-binding domain acts as an auto-inhibitor of the enzyme (Zurini *et al.*, 1984; Benaim *et al.*, 1984). Interestingly, after proteolytic removal of the calmodulin-binding domain, the stimulation of the calmodulin-independent Ca^{2+}-ATPase activity is also accompanied by a concomitant appearance of a Ca^{2+}-independent *p*-nitrophenyl phosphatase activity. Also this enzyme activity was found to be inhibited by the synthetic peptides (Caride *et al.*, 1991).

That the class of PM Ca^{2+} ATPases resulting from the mRNA without an insert (class b), which is the putative housekeeping form found in all cell types, might interact with calmodulin in a functionally different way from the classes with an insert (i.e., class a, c, or d) follows from experiments by Enyedi *et al.*, 1991. Here 28-residue long peptides representing the full-length (A + B) calmodulin-binding domain of the two main alternative classes were tested for their calmodulin-binding properties and for their capacity to interact with the pump from which the calmodulin-binding domain had been removed by chymotryptic proteolysis.

The peptide of class b was most effective in both capacities. These results suggest that the most widely distributed Ca^{2+} pump isoform (PMCA1b) has the highest affinity for calmodulin and an intrinsically lower activity in the absence of calmodulin. All other isoforms (a, c, or d) would then be more active in the absence of calmodulin and be activated to a lower extent by the activator.

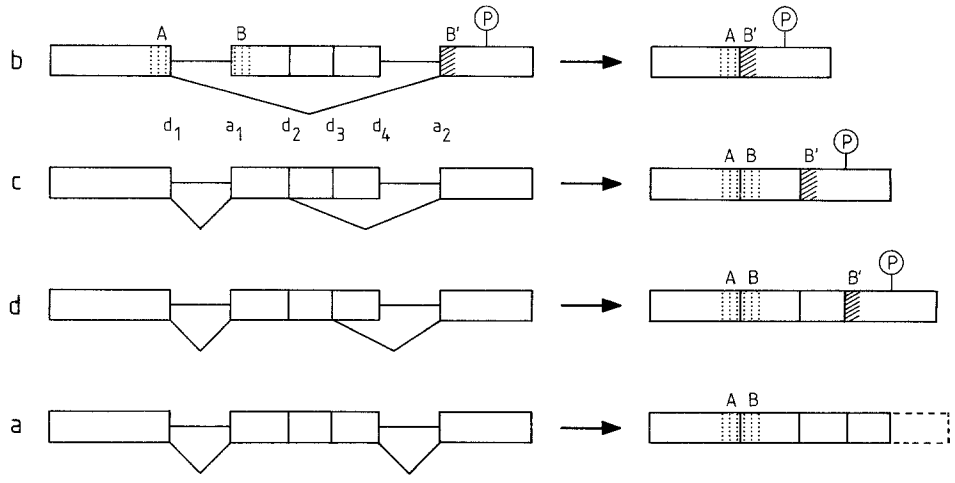

Fig. 1. The different variants of PMCA1 where alternative splicing affects the calmodulin-binding domain and the more C-terminally located target site for *c*AMP-dependent kinase. A total of four different protein isoforms results from alternative splicing of the PMCA1 pre-mRNA. PMCA 1b, c, d, and a are shown from top to bottom. Boxes represent exons, and horizontal lines in between are introns. The splicing involves four splice donor sites (d_1–d_4, of which d_2 and d_3 are internal to the same exon) and two splice acceptor sites (a_1 and a_2). The calmodulin-binding domain consists of an A and a B subdomain. Note that the A subdomain is the same in PMCA1, b, c, and d. The B subdomain presents two variants, however; it is the same in PMCA1c, d, and a but differs in PMCA1b. PMCA1c and d contain, besides the B subdomain immediately juxtaposed to the A subdomain, a second similar but distinct B′ domain located more C terminally. The target site for *c*AMP-dependent kinase (P) would be found in the translated protein at increasingly longer distances from the calmodulin-binding domain in PMCA1, b, c, and d. It is absent in PMCA1a because the use of splice donor site d_4 induces a reading frame shift resulting in the translation of different and shorter carboxy terminus in this isoform.

The calmodulin-binding domain is bracketed between two acidic domains. Initially it was thought to interact (in the absence of calmodulin) with the negatively charged stretch immediately N-terminal from it (Enyedi *et al.*, 1989b; Vorherr *et al.*, 1990). But work of Zvaritch *et al.*, (1990) excluded this possibility and pleaded in favor of a more upstream interaction. Falchetto *et al.*, (1991), using a radioactive derivative of the synthetic 28-residue long calmodulin-binding peptide carrying a photoactivatable cleavable cross-linker, could link this label to the pump at a postition located just C terminally of the phosphoenzyme-forming aspartic acid and upstream from the FITC-binding site forming part of the ATP-interaction site. These cross-linking experiments do not rule out an independent interaction of the calmodulin-binding domain with the flanking negatively charged domains. Indeed the calmodulin-binding domain appears to protect these acidic domains from proteolytic fragmentation by V8 protease. This was interpreted by Wang *et al.* (1991a) as evidence for association/interaction between the positively charged calmodulin-binding domain and both negatively charged flanking domains.

The Vitamin D-induced Ca^{2+}-binding protein calbindin 9K is a structural homologue of calmodulin that was shown to stimulate the PM Ca^{2+}-ATPase in rat duodenal basolateral membrane fractions in a way nonadditive to calmodulin (Walters, 1989). Very recently, also by conjugating calbindin 9K to the same cross-linker that had been used to identify the calmodulin-binding domain, James *et al.* (1991) were able to show that calbindin 9K also bound to the calmodulin-binding domain of the erythrocyte PM Ca^{2+} pump. Calmodulin effectively competes with calbindin 9K for the same binding domain on the erythrocyte ATPase. Calbindin shows a 5- to 10-fold lower affinity for the synthetic calmodulin-binding domain than calmodulin itself. In spite of the interaction of calbindin with the erythrocyte ATPase, an ensuing stimulation of the enzyme could not (yet) be demonstrated (James *et al.*, 1991).

Kosk-Kosicka and her coworkers have recently published a series of experiments indicating that besides by calmodulin, the Ca^{2+}-transport ATPase could also be fully activated by self-association to form oligomers (Kosk-Kosicka and Bzdega, 1988, 1990). Fluorescence energy transfer studies on purified erythrocyte ATPase demonstrated that the oligomerization is Ca^{2+} dependent (Kosk-Kosicka *et al.*, 1989).

At low Ca^{2+} concentations ($K_{Ca^{2+}} = 40$ nM), increasing the enzyme concentration in the assay revealed the gradual transformation from a calmodulin-dependent to a calmodulin-independent state of the enzyme. Half maximal oligomerization occurs at an ATPase concentration of 15 nM. Calmodulin still binds to the preformed oligomers, but whereas one calmodulin molecule binds to one ATPase monomer, the binding of calmodulin to the oligomers presents a lower stoichiometry. Presumably only one calmodulin binds per oligomer (dimer). Binding of calmodulin conserves the Ca^{2+} transport ATPase in the associated state (be it mono- or oligomeric) that it had before the addition (Vorherr *et al.*, 1991). Vorherr *et al.* also presented evidence that it is the calmodulin-binding domain proper that is involved in this oligomerization process. ATPase from which the calmodulin-binding domain was proteolytically removed failed to oligomerize with intact ATPase. Antibodies against the synthetic calmodulin-binding domain prevent the formation of oligomers. Synthetic peptides corresponding to the calmodulin-binding domain of the pump stimulate the ATPase activity presumably by forming peptide-pump hetero oligomers.

Very recently Chiesi and coworkers have proposed the unifying view that the autoinhibitory calmodulin-binding domain of the PM Ca^{2+} pump presents a remarkable structural homology to the hydrophylic cytosolic part of phospholamban (Chiesi *et al.*, 1991). Phospholamban is a small intrinsic membrane protein which interacts with the SR Ca^{2+} pumps and inhibits their Ca^{2+} transport activity. Phosphorylation of phospholamban counteracts this inhibition (Tada and Katz, 1982). Unphosphorylated phospholamban interacts with a region of the SR ATPase closely corresponding to the region in the PM Ca^{2+} pump where the calmodulin-binding autoinhibitory domain interacts (James *et al.*, 1989b). Hence, in their view, the SR ATPase could be seen as a truncated form of the PM ATPase in which a separate regulatory protein phospholamban replaced structurally and functionally the autoinhibitory domain.

REGULATION OF THE PM Ca^{2+} PUMP BY PHOSPHOLIPIDS

Like all integral membrane proteins, the Ca^{2+} pump of the plasma membrane is embedded in membrane lipids and its activity is critically dependent on the presence of surrounding phospholipids. Phospholipases abolish the ATPase activity (Roelofsen and Schatzmann, 1977). When the ATPase is purified by calmodulin-affinity chromatography in the absence of phospholipids, it becomes largely delipidated and loses its activity. If purification is done in the presence of 20% glycerol (Kosk-Kosicka and Inesi, 1985), the delipidated ATPase can be reactivated easily by the addition of 100 to 200 phospholipid molecules/ATPase monomer (Vrolix *et al.*, 1988; Missiaen *et al.*, 1989a). Acidic phospholipids carrying one or more negative charges at neutral pH are engaged in a special type of interaction with the pump. They stimulate the enzyme both by increasing its V_{max} for Ca^{2+} and by lowering its K_M for Ca^{2+}. Acidic phospholipids thus not only exert a distinct calmodulin-like activation of the pump but also render the ATPase insensitive to calmodulin (Niggli *et al.*, 1979, 1981; Gietzen *et al.*, 1980; Carafoli and Zurini, 1982; Enyedi *et al.* 1987). The phospholipids do not, however, prevent calmodulin from binding. Calmodulin-affinity chromatography works very efficiently in the presence of negatively charged phospholipids (Gietzen *et al.*, 1980; Wuytack *et al.*, 1981).

Alternatively the role of the phospholipid environment in modulating the activity of the ATPase has also been studied by solubilizing the ATPase in detergent and adding a large excess of exogenous phospholipids followed by detergent removal. This leads to reconstitution of the Ca^{2+} pump into vesicles where its Ca^{2+} pump activity can be studied (Niggli *et al.*, 1981; De Schutter *et al.*, 1984; Verbist *et al.*, 1984; Hermoni-Levine and Rahamimoff, 1990).

Missiaen *et al.*, (1989a) have made a detailed analysis of the effect of various acidic phospholipids on the V_{max}, K_m, and Hill coefficients of the Ca^{2+}-activation curves for the purified Ca^{2+} pump from erythrocytes and smooth muscle. The effect of acidic phospholipids on the V_{max} is biphasic. A stimulatory effect of small amounts of acidic phospholipids is counteracted by an opposite effect when a large fraction (> 20–50%) of acidic phospholipids surrounds the Ca^{2+} pump. The inhibitory effect of a large number of acidic phospholipids is not fully understood. It may be explained by the requirement of a minimal

number of neutral lipid molecules surrounding the Ca^{2+} pump (Nelson and Hanahan, 1985), but it is of little physiological relevance since *in vivo* the concentration of phospholipids surrounding the pump will not exceed 20–50%.

All acidic phospholipids increase the apparent affinity of the pump for Ca^{2+}. Remarkably, low concentrations of phosphatidylinositol (< 10%) have a slight decreasing effect. This was also observed by Niggli *et al.* (1981) and by Choquette *et al.* (1984). In general, a correlation was found between the number of negative charges on the phospholipid molecules ($PtdInsP_2$ > PtdInsP > PtdIns ≃ PtdA ≈ PtdS) and their relative potency in stimulating the ATPase activity. The importance of the number of negative charges for mediating the effect of phospholipids was also clearly demonstrated independently by using fluorescence energy transfer to investigate the close interaction of phosphoinositides and ATPase (Verbist *et al.*, 1991). For the pig erythrocyte ATPase, a positive correlation was observed between the number of negative charges on the phospholipids and the potency of their pyrene-labeled analogs to act as quantum acceptors in fluorescence energy transfer from tryptophan donor residues on the ATPase. As to the question how exactly the acidic phopholipids exert their action on the ATPase, only very fragmentary information is available. Circular dichroism and fluorescence measurements indicated that phosphatidylserine induces a change in the α-helical content of the ATPase (Wrzosek *et al.*, 1989).

Different species of acidic phospholipids probably affect different elementary reaction steps in the catalytic cycle. Indeed the level of phosphoprotein intermediate of the PM Ca^{2+} pump from porcine erythrocytes was not affected by phosphatidylserine (20%), but it was increased 1.6-fold by phosphatidylinositol 4-phosphate. Conversely, phosphatidylserine increased the *p*-nitrophenyl phosphatase activity of the ATPase, whereas phosphatidylinositol 4-phosphate had no significant effect. This suggests that some lipids affect mainly the phosphorylation reaction whereas others mainly accelerate dephosphorylation (Lehotsky *et al.*, 1992). Also from the work of Vrolix *et al.*, (1988) it was already clear that even in the presence of phosphatidylserine, phosphatidylinositol 4-monophosphate induced an additional stimulation of the ATPase. This observation is also compatible with a model where distinct binding sites for both types of lipids co-exist on the ATPase.

Arginine residues on the ATPase might be involved in mediating the phospholipid effect. Phenylglyoxal, a specific arginine-modifying α-carbonyl reagent (Takahashi, 1968), inhibits the erythrocyte (Raess *et al.*, 1985) and the smooth-muscle (Missiaen *et al.*, 1989c) PM Ca^{2+} transport ATPases in a time-dependent way by decreasing the V_{max} of the Ca^{2+}-activation curve. Low concentrations of phosphatidylinositol, phosphatidylinositol mono- and bisphosphate, and of phosphatidic acid partially prevent this inactivation.

Since the PM Ca^{2+} pump is activated by negatively charged phospholipids, it is not surprising to find that a number of polycationic compounds interfere with this stimulation. Among these are the polyamines. Acidic phospholipids, especially phosphatidylinositol mono- and bisphosphate, are the primary polyamine-binding sites in membranes (Tadolini and Varani, 1986). Spermine, a polyamine carrying four positive charges at pH 6.9, prevents the stimulation of the Ca^{2+} pump by phosphatidylinositol and phosphatidylinositol mono- and bisphosphate. The stimulation of the enzyme by calmodulin is, however, not affected. Spermine (three positive charges) and putrescine (two positive charges) have, respectively, a weak or no inhibitory effect (Missiaen *et al.*, 1989e). Although the total cellular concentration of polyamines ranges from 0.5 to 1 mM (Smith and Snyderman, 1988), most of these compounds are bound to other polyanions such as nucleic acids. It is therefore not clear whether *in vivo* they would affect the PM Ca^{2+} pump.

The polycationic antibiotic neomycin shows a similar inhibitory behavior toward the Ca^{2+} pump (Missiaen *et al.*, 1989e). Like for the polyamines, the inhibition of the ATPase by neomycin shows a peculiar dependence upon the concentration of phosphatidylinositol mono- or bisphosphate. the higher the concentration of the activating phospholipid, the higher the relative inhibition by neomycin. One possible explanation for this phenomenon is that the inhibitors bind cooperatively to more than one polyphosphoinositide molecule associated with the pump. One has to assume that in that case also more than one polyphosphoinositide molecule is required for the activation of the pump. Two other polycationic compounds, ruthenium red and compound 48/80, were also found to interfere with the phospholipid activation of the PM Ca^{2+} pump (Missiaen *et al.*, 1990).

Ruthenium red, a hexavalent polycation, has long been known to be an inhibitor of the PM Ca^{2+}

pump (Watson *et al.*, 1971; Sarkadi *et al.*, 1977; Raes and Vincenzi, 1980; Schuurmans-Stekhoven and Bonting, 1981). The compound, however, only selectively counteracts the stimulating effect of acidic phospholipids on the pump. It is ineffective on the ATPase in the presence of neutral phospholipids or calmodulin (Hinds *et al.*, 1981). In contrast, compound 48/80 is a potent inhibitor of the calmodulin-stimulated fraction of the PM Ca^{2+}-ATPase activity (Gietzen *et al.*, 1983; Di Julio *et al.*, 1989) but also counteracts the action of acidic phospholipids on the ATPase (Missiaen *et al.*, 1990).

MODULATION OF THE PM Ca^{2+} PUMP ACTIVITY BY PROTEIN KINASES

Stimulation of the Pump by *c*AMP-Dependent Phosphorylation

The PM Ca^{2+} pump from cardiac muscle (Caroni and Carafoli, 1981b), skeletal muscle (Michalak *et al.*, 1984), and erythrocytes (Neyses *et al.*, 1985) can all be phosphorylated *in vitro* by the catalytic subunit of *c*AMP-dependent protein kinase. Phosphorylation increases both the pump's Ca^{2+} affinity and its V_{max} for Ca^{2+} (Dixon and Haynes, 1989; James *et al.*, 1989c). To what extent this phosphorylation also occurs *in vivo* and if so, what its physiological impact is, has been learnt from studies in cultured cells. Furukawa *et al.* (1988) were unable to demonstrate effects of forskolin, dibutyryl *c*AMP, or 8-Br-*c*AMP on the Ca^{2+} extrusion by the PM Ca^{2+} pump in cultured rat aortic smooth-muscle cells. Agonists of the *c*GMP-dependent protein kinase pathway like sodium nitroprusside, atrial natriuretic factor, and 8-Br-*c*GMP, however, significantly increased the Ca^{2+} pump activity in these cells.

Later Wang *et al.* (1991c) showed, by immunoprecipitating the PM ATPase from rat aortic endothelial cells, that various agonists affecting the *c*AMP-dependent protein kinase system like 8-(4-chlorophenylthio)-*c*AMP, forskolin, and isoproterenol were indeed able to provoke phosphorylation of the pump protein and to concomitantly increase Ca^{2+} extrusion from the cells. Phosphopeptide mapping further indicated that even in unstimulated cells a basal phosphorylation occurs at, most probably, the same target sites.

One important consideration concerns the location of the putative target site(s) for *c*AMP-dependent phosphorylation. James *et al.* (1989c) assigned a serine residue in the sequence—KRNSS—of the human erythrocyte Ca^{2+} pump as the only target site for the A-kinase. This site is located in between the calmodulin-binding domain and the C-terminus of the ATPase, and its phosphorylation is inhibited by calmodulin. The position of the phosphorylation site in the regulatory carboxy terminal part of the ATPase in the vicinity of the calmodulin-binding domain is of great interest because it resembles the situation in a number of other calmodulin-binding proteins (Malencik and Anderson, 1982) including myosin light chain kinase (Conti and Adelstein, 1981) and phosphofructokinase (Buschmeier *et al.*, 1987), and it allows, in principle, synergistic/antagonistic effects of the two different regulatory mechanisms. Remarkably, however, for the erythrocyte ATPase the effect of calmodulin and that of phosphorylation on the V_{max} were nonadditive. In the presence of calmodulin, phosphorylation did not affect the V_{max} of the ATPase (James *et al.*, 1989c). This may not come as a surprise because, as mentioned above, calmodulin prevents phosphorylation of the ATPase, but is at variance with the reported 2.4-fold stimulation of the V_{max} under these conditions in cardiac muscle (Dixon and Haynes, 1989). A possible explanation for this discrepancy could be sought in the tissue-dependent Ca^{2+} pump isoform diversity. Indeed, the *c*AMP-dependent phosphorylation site is known to be present in PMCA1 and in PMCA2 and it could, although this is not yet demonstrated, theoretically also be expressed as an alternatively spliced variant (PMCA3b) in PMCA3 (Greeb and Shull, 1989). It is, however, missing in PMCA4, the major erythrocyte isoform (Strehler *et al.*, 1990). Moreover, the mechanism of alternative splicing creates, at least in the PMCA1 gene products, PMCA1b, PMCA1c, and PMCA1d variants which show a stepwise increase in the distance between the calmodulin-binding domain and the A kinase target site. Isoform PMCA1a, with the longest insert, lacks the phosphorylation site because here the insertion induces a shift in the reading frame (Strehler *et al.*, 1989). These different splice variants show a specific tissue-dependent distribution (Strehler *et al.*, 1989, De Jaegere *et al.*, 1990; Khan and Grover, 1991).

*c*AMP-dependent protein kinase could also indirectly affect the Ca^{2+} extrusion from the cells (Missiaen *et al.*, 1989b) through its effect on cellular pH (Vigne *et al.*, 1988). An increased intracellular pH following A kinase activation leads to an increased Ca^{2+} affinity of the PM Ca^{2+} pump (Villalobo *et al.*, 1986; Missiaen *et al.*, 1989b; Dixon and Haynes,

1990). Finally, it should be remarked that phosphorylation by A kinase might also help to protect the calmodulin-binding domain of the ATPase against degradation by calpain (James *et al.* 1989a; Strehler, 1991) and hence affect the activation of the ATPase by this proteolytic enzyme (Au, 1987; Wang *et al.*, 1988a, b).

Stimulation of the Pump by *c*GMP-Dependent Kinase

The role and mode of action of *c*GMP-dependent phosphorylation on the activity of the PM Ca^{2+} pump is as controversial as that of its *c*AMP-dependent counterpart. Smooth muscle has been the tissue of choice to investigate the effects of this intracellular messenger system because its function in controlling relaxation in smooth muscle by lowering cytosolic free Ca^{2+} was well documented (see Waldman and Murad, 1987 for a review). But also in other cells, like in pancreatic acinar cells, *c*GMP has recently been shown to lower free cytosolic Ca^{2+} (Pandol and Schoeffield-Payne, 1990a, b).

Ca^{2+}-flux studies on isolated smooth-muscle tissues or cells showed that the ATP-driven Ca^{2+} pump could be stimulated by agents that increase *c*GMP (Itoh *et al.*, 1985, Fujii *et al.*, 1986; Rashatwar *et al.*, 1987; Furukawa *et al.*, 1988). As for *c*AMP, also *c*GMP has been reported to increase the pump's V_{max} (Popescu *et al.*, 1985; Furukawa and Nakamura, 1987; Rashatwar *et al.*, 1987; Vrolix *et al.*, 1988; Imai *et al.*, 1990) and its affinity for Ca^{2+} (Furukawa and Nakamura, 1987; Vrolix *et al.*, 1988). Conflicting reports have been published concerning the mechanism by which G kinase stimulates the ATPase.

Furukawa and Nakamura (1987), working on the ATPase purified from bovine aorta, reported that phosphorylation of the Ca^{2+}-pump molecule mediated the stimulatory effect. In contrast, Vrolix *et al.* (1988), who studied the effect of purified *c*GMP-dependent kinase on the activity of the PM Ca^{2+}-transport ATPase from pig stomach smooth muscle, observed that the kinase only stimulated the ATPase provided phosphatidylinositol was present. In the process phosphatidylinositol was converted to phosphatidylinositol monophosphate. These findings therefore suggest that *c*GMP-dependent protein kinase activates a phosphatidylinositol kinase which had been copurified with the ATPase, and that the phosphatidylinositol thus formed stimulated the ATPase. Imai *et al.* (1990) also came to the conclusion that phosphatidylinositol could be a mediator of the effect of *c*GMP-dependent kinase on pig aortic smooth muscle. The phosphatidylinositol kinase itself could be modulated by direct phosphorylation or via phosphorylation of an associated protein. Any of the several *c*GMP-dependent protein kinase specific polypeptides could be mediating this effect.

As to the identity of the approximately 130-kDa phosphoprotein that Furukawa and Nakamura (1987) considered to be the Ca^{2+} pump, a lot of confusion remains. Vrolix *et al.* (1988) suggested that this protein was myosin light chain kinase. Baltensperger *et al.* (1988) also came to the conclusion that in pig aorta the PM Ca^{2+} pump is not a direct target of the *c*GMP-dependent protein kinase and hence that the 130-kDa phosphoprotein must be a different protein. Sarcevic *et al.* (1990), working on rat aortic cells, excluded by phosphopeptide mapping and by immunoblot analysis that the protein was myosin light chain kinase or caldesmon.

Very recently Yoshida *et al.* (1991) confirmed that *c*GMP-dependent protein kinase stimulated the PM Ca^{2+} pump ATPase from porcine aorta. However, phosphatidylinositol kinase activity was not detected in the enzyme preparation, nor was the presence of phosphatidylinositol necessary for stimulation by the G kinase. They observed phosphorylation of a 135-kDa and a 240-kDa polypeptide in association with the stimulation of the G kinase. However, they could only find a correlation between the phosphorylation level of the 240-kDa protein and the stimulation of the PM Ca^{2+} pump.

Stimulation of the Pump by Protein Kinase C

The first indirect indication that protein kinase C might stimulate the PM Ca^{2+} pump came from observations on the effect of phorbol esters. Lagast *et al.* (1984) showed that PM-enriched fractions prepared from phorbol ester-pretreated guinea-pig neutrophils exhibit a much higher rate of Ca^{2+} uptake than control membranes. Even direct addition of the phorbol ester to pig coronary artery plasma membranes was reported to stimulate the ATPase (Popescu *et al.*, 1986). Effects of phorbol esters on the extrusion of Ca^{2+} from cultured rat aortic smooth-muscle cells could also be observed. Brief pretreatment of these cells with phorbol esters partially suppressed the intracellular Ca^{2+} transients induced by ionomycin or angiotensin II and at the same time accelerated the Ca^{2+} extrusion (Furukawa *et al.*, 1989). In many of

the more recent experiments the effect of exogenous purified protein kinase C was investigated. Smallwood *et al.* (1988), measuring Ca^{2+} transport in inside-out vesicles from human erythrocytes and the activity of Ca^{2+} ATPase purified from the same source, concluded that protein kinase C increased the V_{max} for Ca^{2+} without affecting the K_m for Ca^{2+}. The effect on V_{max} was 2- to 3-fold smaller compared to that of calmodulin. Both the C kinase and calmodulin effect were additive. Others have, however, described a C kinase-induced increase of the Ca^{2+} affinity of the PM Ca^{2+} pump (Fukuda *et al.*, 1990; Ogurusu *et al.*, 1990). Although Imai *et al.* (1990) confirmed the effect of protein kinase C on the membrane-bound Ca^{2+} pump in porcine aortic membranes, no such effect was observed in the enzyme purified by calmodulin-affinity chromatography, again suggesting the removal during purification of an essential protein or lipid component.

In contrast to the situation for *c*GMP-dependent kinase however, C kinase is apparently catalyzing a direct phosphate transfer to the ATPase molecule itself, as can be shown by immunoaffinity purification of the prephosphorylated ATPase from bovine aorta smooth muscle, bovine cardiac muscle, human erythrocyte, and rat endothelial cells (Fukuda *et al.*, 1990; Ogurusu *et al.*, 1990; Wang *et al.*, 1991b; Kuo *et al.*, 1991).

Furthermore it was also shown that *in vivo* phorbol esters induced the phosphorylation of the rat endothelial cell PM Ca^{2+} pump at target sites differing from the A kinase sites. Angiotensin II, and also ATP and ionomycin, stimulated phosphorylation at the same sites as the phorbol esters (Wang *et al.*, 1991c).

In the ATPase purified from human erythrocytes, Wang *et al.*, (1991b) demonstrated a C kinase-mediated phosphorylation of the pump molecule, but also other proteins including adducin and the C-kinase itself as well as inositol lipids were phosphorylated. Both trypsin- and calpain-mediated fragmentation of the phosphorylated Ca^{2+} pump pinpointed a threonine residue located in the A-subdomain of its calmodulin-binding domain, which is common to all known isoforms of PMCA as the phosphorylation target site. An unspecified additional serine somewhere more downstream from the calmodulin-binding domain must also be phosphorylated. It remains, however, to be seen what the functional implications of these phosphorylations on the enzyme activity really are. Both inhibition and stimulation of the ATPase were observed. Different results were obained with different types of C-kinase preparations. Furthermore the results depended also on the type of lipid associated with the ATPase (Wang *et al.*, 1991b). In the absence of calmodulin the effects of C kinase on the pump were a limited (5–15%) stimulation or inhibition of its activity depending on the lipid environment. A puzzling potentiation of the simulatory effect was observed in the presence of A 23187. Maybe the most clearcut functional effects of C kinase-mediated phosphorylation are the inhibition of calmodulin activation of the ATPase and the protection against proteolysis. The physiological relevance of these two *in vitro* effects remains elusive as the antagonism exerted by C kinase on the calmodulin stimulation is only observed at a low concentration of calmodulin (150 nM) and of Ca^{2+} (0.2 μM). At higher ($> 1\ \mu$M) concentrations of Ca^{2+} (and hence at higher Ca-calmodulin complex concentrations) the effect is absent. More recently Kuo *et al.* (1991) demonstrated a small (1.3-fold) and rapid (5 min) phosphorylation of the PM Ca^{2+}-pump molecule isolated by immunoprecipitation from rat aortic endothelial cells grown in the presence of phorbol esters as compared to controls grown in their absence, indicating that endogenous C-kinase activity is sufficient for phosphorylation.

INTERACTION OF Ca^{2+} PUMPS AND GTP-BINDING PROTEINS

Heterotrimeric (α, β, γ) GTP-binding proteins (G proteins) intervene in signal transduction between receptors and a number of diverse effectors (Rodbell, 1980; Casey and Gilman, 1988). The PM Ca^{2+} pump could possibly also be subject to such a G-protein control. GTPγS inhibits the Ca^{2+} pump in cardiac sarcolemmal vesicles and in liver plasma membranes (Kuo and Tsang, 1988, Lotersztajn *et al.*, 1990). Cholera toxin increases the Ca^{2+} uptake in liver membranes and suppresses the inhibitory effect of GTPγS (Lotersztajn *et al.*, 1990). One interpretation of these data is that they are indicative of G protein–Ca^{2+} pump interaction. It should be remarked, however, that such observations can also be explained by GTPγS stimulated phospholipase C activity. This could lead to decreased levels of polyphosphoinositides and hence to a decrease in the Ca^{2+} pump's activation as has been found to be the case in cardiac muscle (Kuo and Tsang, 1988). One note of caution should be made in this respect in relation to the use of AlF_4^-. The AlF_4^- complex is a well-known activator of G proteins. But more recently it turned out to be also an inhibitor of all P-type transport ATPases

(Robinson *et al.*, 1986; Missiaen *et al.*, 1988a; Lotersztajn *et al.*, 1990). The inhibition is noncompetitive toward ATP and Ca^{2+} (Missiaen *et al.*, 1988a), but the K_i is shifted to higher values by inorganic phosphate. AlF_4^- inhibits the PM Ca^{2+}-transport ATPase from erythrocytes with a lower affinity than that of smooth muscle (Missiaen *et al.*, 1989d). This suggests an isoform-specific functional difference for both ATPases which might differ in their phosphate-binding site or its accessibility for AlF_4^-.

INHIBITION OF THE Ca^{2+}-TRANSPORT ATPase BY IP_3

The Ca^{2+}-mobilizing IP_3 has been claimed to inhibit the PM Ca^{2+} pump of coronary artery smooth-muscle cells (Popescu *et al.*, 1986) and of cardiac cells (Kuo and Tsang, 1988). These observations have recently been confirmed and elaborated. At 10^{-6}M concentration Ins(1,4,5)P_3 and Ins(4,5)P_2 inhibited the human erythrocyte ATPase for 42 or 31%. Related compounds Ins, Ins(1,3,4,5)P_4, and Ins(1,4)P_2 were ineffective. The inhibition by Ins(1,4,5)P_3 was blocked by heparin. Moreover, Ins(1,4,5)P_3 inhibited calmodulin-binding to the red cell membranes (Davies *et al.*, 1991a). How these IP_3 effects would be mediated deserves further specification.

REGULATION OF THE PM Ca^{2+} PUMP BY OTHER AGONISTS

A number of naturally occurring hormones or messengers have been reported to affect the PM Ca^{2+} pump, among which are thyroid hormone which stimulates the ATPase at a posttranscriptional level in the presence of calmodulin (Smith *et al.*, 1989b; Segal *et al.*, 1989; Pilarska *et al.*, 1991), and retinoic acid which appears to counteract the calmodulin stimulation of the Ca^{2+} pump (Davis *et al.*, 1991b). Thyroid hormone also controls the lipid composition and membrane fluidity of the skeletal-muscle sarcolemma (Pilarska *et al.*, 1991) and may elevate the levels of the PM Ca^{2+} pump (Famulski *et al.*, 1988). Oxytocin inhibited the Ca^{2+} ATPase in myometrial membranes when given immediately before preparing the membranes (Enyedi *et al.*, 1989a; Magocsi and Penniston, 1991b).

Likewise carbachol partially inhibited the PM Ca^{2+} pump in microsomes from pig stomach (Missiaen *et al.*, 1988b). Also some mating pheromones in yeast might act as inhibitors of the PM Ca^{2+} pump (Hiraga *et al.*, 1991). See Missiaen *et al.* (1991) for a more comprehensive review.

CONCLUSION

From the above, it follows that the PM Ca^{2+}-transport ATPase system appears as a intricate system where a whole spectrum of different pump isoforms is subject to a plethora of different agonists. In this manner each cell type can adjust its Ca^{2+}-extrusion system to its specific and changing needs. In a large majority of cell types apparently only one isoform, i.e., splice variant b of the PMCA1 gene product, is expressed. But the existence of at least five distinct PMCA genes in combination with the process of alternative splicing of the gene transcripts allows the expression of a potentially large number of other pump isoforms.

More specific work at the RNA level showed that brain and skeletal muscle are systems that can express the widest variety of isoforms, but as yet this has to be confirmed at the protein level. Isoform-specific antibodies would be helpful in this respect, but are currently lacking.

It is interesting to note that the isoform diversity for a large part comprises two potential regulatory domains. The C-terminal part of the PM Ca^{2+} pump where not only the calmodulin-binding domain, but also phosphorylation target sites for *c*AMP-dependent protein-kinase or for protein kinase C, are found, is one of them. The phospholipid-binding domain, which is found more toward the N-terminal part, is the other. The PM Ca^{2+} pump activity is controlled by a large number of regulators as calmodulin, phospholipids, *c*AMP-dependent protein kinase, *c*GMP-dependent protein kinase, and protein kinase C. It is, furthermore, also clear that these effectors are all interdependent components of an intricate regulatory network. A given agonist can affect the ATPase at different levels via interaction with the pump protein itself as is the case for *c*AMP-dependent protein kinase or for protein kinase C, or rather via the phospholipid or another protein interacting with the pump as for *c*GMP-dependent protein kinase, or for protein kinase C.

The phosphorylation of the Ca^{2+} pump by *c*AMP-dependent protein kinase and by protein kinase C occurs in or nearby the calmodulin-binding

domain, and hence it is not surprising that phosphorylation and calmodulin-binding might affect each other. Furthermore, phosphorylation can also negatively affect the proteolytic activation of the ATPase by calpain. *In vivo*, all these influences might therefore allow a very delicate fine-tuning of the pump's activity, but at the same time this regulatory network defies any easy description.

REFERENCES

Andersen, J. P., Vilsen, B., Leberer, E., and MacLennan, D. H. (1989). *J. Biol. Chem.* **264**, 21018–21023.

Au, K. S. (1987). *Biochim. Biophys. Acta* **905**, 273–278.

Baltensperger, K., Carafoli, E., and Chiesi, M. (1988). *Eur. J. Biochem.* **172**, 7–16.

Benaim, G., and Romero, P. J. (1990). *Biochem. J.* **1027**, 79–84.

Benaim, G., Zurini, M., and Carafoli, E. (1984). *J. Biol. Chem.* **259**, 8471–8477.

Blaustein, M. P. (1988). *J. Cardiovasc. Pharmacol.* **5**, S56–S68.

Bond, G. H., and Clough, D. L. (1972). *Biochim. Biophys. Acta* **323**, 592–599.

Brandl, C. J., de Leon, S., Martin, D. R., and MacLennan, D. H. (1987). *J. Biol. Chem.* **262**, 3768–3774.

Brandt, P., Zurini, M., Neve, R. L., Rhoads, R. E., and Vanaman, T. C. (1988). *Proc. Natl. Acad. Sci. USA* **85**, 2914–2918.

Breitbart, R. E., Andreadis, A., and Nadal-Ginard, B. (1987). *Annu. Rev. Biochem.* **56**, 467–495.

Briars, S. A., Kessler, F., and Evans, D. E. (1988). *Planta* **176**, 283–285.

Buschmeier, B., Meyer, H. E., and Mayr, G. W. (1987). *J. Biol. Chem.* **262**, 9454–9462.

Carafoli, E. (1991). *Physiol. Rev.* **71**, 129–153.

Carafoli, E., and Zurini, M. (1982). *Biochim. Biophys. Acta* **683**, 279–301.

Caride, A. J., Penniston, J. T., and Rossi, J. P. F. C. (1991). *Biochim. Biophys. Acta* **1069**, 94–98.

Caroni, P., and Carafoli, E. (1981a). *J. Biol. Chem.* **256**, 3263–3270.

Caroni, P., and Carafoli, E. (1981b). *J. Biol. Chem.* **256**, 9371–9373.

Casey, P. J., and Gilman, A. G. (1988). *J. Biol. Chem.* **263**, 2577–2580.

Chiesi, M., Vorherr, T., Falchetto, R., Waelchli, C., and Carafoli, E. (1991). *Biochemistry*. **30**, 7978–7983.

Choquette, D., Hakim, G., Filoteo, A. G., Plishker, G. A., Bostwick, J. R., and Penniston, J. T. (1984). *Biochem. Biophys. Res. Commun.* **125**, 908–915.

Conti, M. A., and Adelstein, R. S. (1981). *J. Biol. Chem.* **256**, 3178–3181.

Davis, F. B., Davis, P. J., Lawrence, W. D., and Blas, S. (1991a). *FASEB J.* **5**, 2992–2995.

Davis, F. B., Smith, T. J., Davis, P. J., and Blas, S. D. (1991b). *Biochem. J.* **277**, 603–606.

De Jaegere, S., Wuytack, F., Eggermont, J. A., Verboomen, H., and Casteels, R. (1990). *Biochem. J.* **271**, 655–660.

De Schutter, G., Wuytack, F., Verbist, J., and Casteels, R. (1984). *Biochim. Biophys. Acta* **773**, 1–10.

De Smedt, H., Parijs, J.-B., Borghgraef, R., and Wuytack, F. (1983). *Biochim. Biophys. Acta* **728**, 409–418.

Dhalla, N. S., and Zhao, D. (1988). *Prog. Biophys. Mol. Biol.* **52**, 1–37.

Di Julio, D., Hinds, T. R., and Vincenzi, F. F. (1989). *Biochim. Biophys. Acta* **981**, 337–342.

Dieter, P., and Marmé, D. (1981). *FEBS Lett.* **125**, 245–248.

Dixon, D. A., and Haynes, D. H. (1989). *J. Biol. Chem.* **264**, 13612–13622.

Dixon, D. A., and Haynes, D. H. (1990). *Biochim. Biophys. Acta* **1029**, 274–284.

Donnet, C., Medrano, S., Otero, M., Ochoa, E. L., and Rossi, J. P. (1990). *Biochim. Biophys. Acta* **1030**, 195–202.

Dunham, E. T., and Glynn, I. M. (1961). *J. Physiol.* (London) **156**, 274–293.

Eggermont, J. A., Wuytack, F., De Jaegere, S., Nelles, L., and Casteels, R. (1989). *Biochem. J.* **260**, 757–761.

Enyedi, A., Flura, M., Sarkadi, B., Gardos., and Carafoli, E. (1987). *J. Biol. Chem.* **262**, 6425–6430.

Enyedi, A., Brandt, J., Minami, J., and Penniston, J. T. (1989a). *Biochem. J.* **261**, 23–28.

Enyedi, A., Vorherr, T., James, P., McCormick, D. J., Filoteo, A. G., Carafoli, E., and Penniston, J. T. (1989b). *J. Biol. Chem.* **264**, 12313–12321.

Enyedi, A., Filoteo, A. G., Gardos, G., and Penniston, J. T. (1991). *J. Biol. Chem.* **266**, 8952–8956.

Falchetto, R., Vorherr, T., Brunner, J., and Carafoli, E. (1991). *J. Biol. Chem.* **266**, 2930–2936.

Famulski, K. S., Pilarska, M., Wrzosek, A., and Sarzala, M. G. (1988). *Eur. J. Biochem.* **171**, 363–368.

Fujii, K., Ishimatsu, T., and Kuryama, H. (1986). *J. Physiol.* (*London*). **337**, 315–332.

Fukuda, T., Ogurusu, T., Furukawa, K.-I., and Shigekawa, M. (1990). *J. Biochem.* **108**, 629–634.

Furukawa, K.-I., and Nakamura, H. (1987). *J. Biochem.* **101**, 287–290.

Furukawa, K.-I., Tawada, Y., and Shigekawa, M. (1988). *J. Biol. Chem.* **263**, 8058–8065.

Furukawa, K.-I., Tawada, Y., and Shigekawa, M. (1989). *J. Biol. Chem.* **264**, 4844–4849.

Garrahan, P. J., and Rega, A. F. (1986). *The Ca^{2+} Pump of Plasma Membranes*, CRC Press, Boca Raton, Florida.

Garrahan, P. J., and Rega, A. F. (1990). *Intracellular Calcium Regulation* (Bronner, F. ed.), Alan R. Liss, New York, pp. 271–303.

Gietzen, K., Tejcka, M., and Wolf, H. U. (1980). *Biochem. J.* **189**, 81–88.

Gietzen, K., Adamczyk-Engelmann, P., Wüthrich, A., Konstantinova, A., and Bader, H. (1983). *Biochim. Biophys. Acta* **736**, 109–118.

Gopinath, R. M., and Vincenzi, F. F. (1977). *Biochem. Biophys. Res. Commun.* **77**, 1203–1209.

Greeb, J., and Shull, G. E. (1989). *J. Biol. Chem.* **264**, 18569–18576.

Green, N. M. (1989). *Biochem. Soc. Trans.* **17**, 972.

Green, N. M., and MacLennan, D. H. (1989). *Biochem. Soc. Trans.* **17**, 819–822.

Gunteski-Hamblin, A. M., Greeb, J., and Shull, G. E. (1988). *J. Biol. Chem.* **263**, 15032–15040.

Hermoni-Levine, M., and Rahamimoff, H. (1990). *Biochemistry*. **29**, 4940–4950.

Hinds, T. R., and Andreasen, T. J. (1981). *J. Biol. Chem.* **256**, 7877–7882.

Hinds, T. R., Raess, B. U., and Vincenzi, F. F. (1981). *J. Membr. Biol.* **58**, 57–65.

Hiraga, K., Tahara, H., Taguchi, N., Tsuchiya, E., Fukui, S., and Miyakawa, T. (1991). *J. Gen. Microbiol.* **137**, 1–4.

Imai, S., Yoshida, Y., and Sun, H.-T. (1990). *J. Biochem.* **107**, 755–761.

Itoh, T., Kanmura, Y., Kuriyama, H., and Sasaguri, T. (1985). *Br. J. Pharmac.* **84**, 393–406.

James, P., Maeda, M., Fischer, R., Verma, A. K., Krebs, J., Penniston, J. T., and Carafoli, E. (1988). *J. Biol. Chem.* **263**, 2905–2910.

James, P., Vorherr, T., Krebs, J., Morelli, A., Castello, G., McCormick, D. J., Penniston, J. T., De Flora, A., and Carafoli, E. (1989a). *J. Biol. Chem.* **264**, 8289–8296.
James, P., Inui, M., Tada, M., Chiesi, M., and Carafoli, E. (1989b). *Nature (London)* **342**, 90–92.
James, P. H., Pruschy, M., Vorherr, T. E., Penniston, J. T., and Carafoli, E. (1989c). *Biochemistry* **28**, 4253–4258.
James, P., Voherr, T., Thulin, E., Forsen, S., and Carafoli, E. (1991). *FEBS Lett.* **278**, 155–159.
Jarrett, H. W., and Penniston, J. T. (1977). *Biochem. Biophys. Res. Commun.* **77**, 1210–1216.
Jarrett, H. W., and Penniston, J. T. (1978). *J. Biol. Chem.* **253**, 4676–4682.
Kessler, F., Bennardini, F., Bachs, O., Serratosa, J., James, P., Caride, A. J., Gazzotti, P., Penniston, J. T., and Carafoli, E. (1990). *J.Biol. Chem.* **265**, 16012–16019.
Khan, I., and Grover, A. K. (1991). *Biochem. J.* **277**, 345–349.
Kodama, T. (1985). *Physiol. Rev.* **65**, 467–551.
Korczak, B., Zarain-Herzberg, A., Brandl, C. J., Ingles, C. J., Green, N. M., and MacLennan, D. H. (1988). *J. Biol. Chem.* **263**, 4813–4819.
Kosk-Kosicka, D., and Bzdega, T. (1988). *J. Biol. Chem.* **263**, 18184–18189.
Kosk-Kosicka, D., and Bzdega, T. (1990). *Biochemistry*. **29**, 3772–3777.
Kosk-Kosicka, D., and Inesi, G. (1985). *FEBS Lett.* **189**, 67–71.
Kosk-Kosicka, D., Bzdega, T., and Wawrzynow, A. (1989). *J. Biol. Chem.* **264**, 19495–19499.
Kratje, R. B., Garrahan, P. J., and Rega, A. F. (1985). *Biochim. Biophys. Acta* **816**, 365–378.
Kuo, T. H., and Tsang, W. (1988). *Biochem. Biophys. Res. Commun.* **152**, 1111–1116.
Kuo, T. H., Wang, K. K. W., Carlock, L., Diglio, C., and Tsang, W. (1991). *J. Biol. Chem.* **266**, 2520–2525.
Lagast, H., Pozzan, T., Waldvogel, F. A., and Lew, P. D. (1984). *J. Clin. Invest.* **73**, 878–883.
Lehotsky, J., Raeymaekers, L., Missiaen, L., Wuytack, F., De Smedt, H. and Casteels, R. (1992). *Biochim. Biophys. Acta*, **1105**, 118–124.
Lin, S.-H., and Russel, W. E. (1988). *J. Biol. Chem.* **263**, 12253–12258.
Lotersztajn, S., Pavoine, C., Brechler, V., Roche, B., Dufour, M., Le-Nguyen, D., Bataille, D., and Pecker, F. (1990). *J. Biol. Chem.* **265**, 9876–9880.
Luterbacher, S., and Schatzmann, H. J. (1983). *Experientia* **39**, 311–312.
Lytton, J., and MacLennan, D. H. (1988). *J. Biol. Chem.* **263**, 15024–15031.
Lytton, J., Westlin, M., and Hanley, M. R. (1991). *J. Biol. Chem.* **266**, 17067–17071.
MacLennan, D. H. (1990). *Biophys. J.* **58**, 1355–1365.
MacLennan, D. H., Brandl, C. T., Chainpaneria, S., Holland, P. C., Powers, V. E., and Willard, H. F. (1987). *Somat. Cell. Mol. Genet.* **13**, 341–346.
Magocsi, M., and Penniston, J. T. (1991a). *Biochim. Biophys. Acta* **1070**, 163–172.
Magocsi, M., and Penniston, J. T. (1991b). *Biochim. Biophys. Acta* **1063**, 7–14.
Malencik, D. A., and Anderson, S. R. (1982). *Biochemistry* **21**, 3480–3486.
Mann, D. M., Brandt, P., Sisken, B., and Vanaman, T. C. (1989). *Adv. Exp. Med. Biol.* **255**, 71–78.
Maruyama, K., Clarke, D. M., Fujii, J., Loo, T. W., and MacLennan, D. H. (1989). *Cell Motility Cytoskelet.* **14**, 26–34.
Mason, M. J., Garcia-Rodriguez, C., and Grinstein, S. (1991). *J. Biol. Chem.* **266**, 20856–20862.
Michalak, M., Famulski, K., and Carafoli, E. (1984). *J. Biol. Chem.* **259**, 15540–15547.
Missiaen, L., Wuytack, F., De Smedt, H., Vrolix, M., and Casteels, R. (1988a). *Biochem. J.* **253**, 827–833.
Missiaen, L., Kanmura, Y., Wuytack, F., and Casteels, R. (1988b). *Biochem. Biophys. Res. Commun.* **150**, 681–686.
Missiaen, L., Raeymaekers, L., Wuytack, F., Vrolix, M., De Smedt, H., and Casteels, R. (1989a). *Biochem. J.* **263**, 687–694.
Missiaen, L., Droogmans, G., De Smedt, H., Wuytack, F., Raeymaekers, L., and Casteels, R. (1989b). *Biochem. J.* **262**, 361–364.
Missiaen, L., Raeymaekers, L., Droogmans, G., Wuytack, F., and Casteels, R. (1989c). *Biochem. J.* **264**, 609–612.
Missiaen, L., Wuytack, F., De Smedt, H., Amant, F., and Casteels, R. (1989d). *Biochem. J.* **261**, 655–660.
Missiaen, L., Wuytack, F., Raeymaekers, L., De Smedt, H., and Casteels, R. (1989e). *Biochem. J.* **261** 1055–1058.
Missiaen, L., De Smedt, H., Droogmans, G., Wuytack, F., Raeymaekers, L., and Casteels, R. (1990). *Biochim. Biophys. Acta* **1023**, 449–454.
Missiaen, L., Wuytack, F., Raeymaekers, L., De Smedt, H., Droogmans, G., Declerck, I. and and Casteels, R. (1991). *Pharmacol. Ther.* **50**, 191–232.
Moore, G. A., McConkey, D. J., Kass, G. E., O'Brien, P. J., and Orrenius, S. (1987). *FEBS Lett.* **224**, 331–336.
Nelson, D. R., and Hanahan, D. J. (1985). *Arch. Biochem. Biophys.* **236**, 720–730.
Neyses, L., Reinlib, L., and Carafoli, E. (1985). *J. Biol. Chem.* **260**, 10283–10287.
Niggli, V., Zurini, M., and Carafoli, E. (1987). *Methods Enzymol.* **139**, 791–808.
Niggli, V., Penniston, J. T., and Carafoli, E. (1979). *J. Biol. Chem.* **254**, 9955–9958.
Niggli, V., Adunyah, E. S., Penniston, J. T., and Carafoli, E. (1981). *J. Biol. Chem.* **256**, 395–401.
O'Niel, K. T., and DeGrado, W. F. (1990). *Trends Biol. Sci.* **15**, 59–64.
Ogurusu, T., Wakabayashi, S., Furukawa, K.-I., Tawada-Iwata, Y., Imagawa, T., and Shigekawa, M. (1990). *J. Biochem.* **108**, 222–229.
Olson, S., Wang, M. G., Carafoli, E., Strehler, E. E., and McBride, O. W. (1991). *Genomics.* **9**, 629–641.
Pandol, S. J., and Schoeffield-Payne, M. S. (1990a). *Cell Calcium* **11**, 477–486.
Pandol, S. J., and Schoeffield-Payne, M. S. (1990b). *J. Biol. Chem.* **265**, 12846–12853.
Pedersen, P. L., and Carafoli, E. (1987). *Trends Biochem. Sci.* **12**, 146–150.
Penniston, J. T. (1983). In *Calcium and Cell function* (Cheung, W. Y., ed.), Academic Press, New York, Vol. 4, pp. 99–149.
Pilarska, M., Wrzosek, A., Pikula, S., and Famulski, K. S. (1991). *Biochim. Biophys. Acta* **1068**, 167–173.
Popescu, L. M., Panoiu, C., Hinescu, M., and Nutu, O. (1985). *Eur. J. Pharmacol.* **107**, 393–394.
Popescu, L. M., Hinescu, M. E., Musat, S., Ionescu, M., and Pistritzu, F. (1986). *Eur. J. Pharmacol.* **123**, 167–169.
Raess, B. U., and Vincenzi, F. F. (1980). *Mol. Pharmacol.* **18**, 253–258.
Raess, B. U., Record, D. M., and Tunnicliff, G. (1985). *Mol. Pharmacol.* **27**, 444–450.
Rashatwar, S. S., Cornwell, T. L., and Lincoln, T. M. (1987). *Proc. Natl. Acad. Sci. USA* **84**, 5685–5689.
Robinson, C., Larsson, C., and Buckhout, T. J. (1988). *Physiol. Plantarum* **72**, 177–184.
Robinson, J. D., Davis, R. L., and Steinberg, M. J. (1986). *J. Bioenerg. Biomembr.* **18**, 521–531.
Rodbell, M. (1980). *Nature (London)* **284**, 17–22.
Roelofsen, B., and Schatzmann, H. J. (1977). *Biochim. Biophys. Acta* **464**, 17–36.
Sagara, Y., and Inesi, G. (1991). *J. Biol. Chem.* **266**, 13503–13506.

Sarcevic, B., Brookes, V., Martin, T. J., Kemp, B. E., and Robinson, P. J. (1989). *J. Biol. Chem.* **264**, 20648–20654.
Sarcevic, B., Robinson, P. J., Pearson, R. B., and Kemp, B. E. (1990). *Biochem. J.* **271**, 493–499.
Sarkadi, B., Szasz, I., Gerloczy, A., and Gardos, G. (1977). *Biochim. Biophys. Acta* **464**, 93–107.
Sarkadi, B., Enyedi, A., Foldes-Papp, Z., and Gardos, G. (1986). *J. Biol. Chem.* **261**, 9552–9557.
Schatzmann, H. J. (1966). *Experientia* **22**, 364–365.
Schatzmann, H. J. (1989). *Annu. Rev. Physiol.* **51**, 473–485.
Schuurmans-Stekhoven, F., and Bonting, S. L. (1981). *Physiol. Rev.* **61**, 1–76.
Segal, J., Hardiman, J., and Ingbar, S. M. (1989). *Biochem. J.* **261**, 749–754.
Seidler, N. W., Jona, I., Vegh, M., and Martonosi, A. (1989). *J. Biol. Chem.* **264**, 17816–17823.
Shull, G. E., and Greeb, J. (1988). *J. Biol. Chem.* **263**, 8646–8657.
Smallwood, J. I., Gugi, B., and Rasmussen, H. (1988). *J. Biol. Chem.* **263**, 2195–2202.
Smith, C. D., and Snyderman, R. (1988). *Biochem. J.* **256**, 125–130.
Smith, C. W., Patton, J. G., and Nadal-Ginard, B. (1989a). *Annu. Rev. Genet.* **23**, 527–577.
Smith, T. J., Davis, F. B., and Davis, P. J. (1989b). *J. Biol. Chem.* **264**, 687–689.
Stieger, J., and Luterbacher, S. (1981). *Biochim. Biophys. Acta* **641**, 270–275.
Stokes, D. L. (1991). *Curr. Opinion Struct. Biol.* **1**, 555–561.
Strehler, E. E. (1991). *J. Membr. Biol.* **120**, 1–15.
Strehler, E. E., Strehler-Page, M.-A., Vogel, G., and Carafoli, E. (1989). *Proc. Natl. Acad. Sci. USA* **86**, 6908–6912.
Strehler, E. E., James, P., Fischer, R., Heim, R., Vorherr, T., Filoteo, A. G., Penniston, J. T., and Carafoli, E. (1990). *J. Biol. Chem.* **265**, 2835–2842.
Tada, M., and Katz, A. M. (1982). *Annu. Rev. Physiol.* **44**, 401–423.
Tadolini, B., and Varani, E. (1986). *Biochem. Biophys. Res. Commun.* **135**, 58–64.
Takahashi, K. (1968). *J. Biol. Chem.* **243**, 6171–6179.
Thastrup, O., Dawson, A. P., Scharff, O., Foder, B., Cullen, P. J., Drobak, B. K., Bjerrum, P. J., Christensen, S. B., and Hanley, M. R. (1989). *Agents Actions* **27**, 17–23.
Thastrup, O., Cullen, P. J., Drobak, B. K., Hanley, M. R., and Dawson, A. P. (1990). *Proc. Natl. Acad. Sci. USA* **87**, 2466–2470.
Verbist, J., Wuytack, F., De Schutter, G., Raeymaekers, L., and Casteels, R. (1984). *Cell Calcium* **5**, 253–263.
Verbist, J., Gadella, T. W., Jr., Raeymaekers, L., Wuytack, F., Wirtz, K. W., and Casteels, R. (1991). *Biochim. Biophys. Acta* **1063**, 1–6.
Verma, A. K., Filoteo, A. G., Stanford, D. R., Wieben, E. D., Penniston, J. T., Strehler, E. E., Fischer, R., Heimm, R., Vogel, G., Matthews, S., Strehler-Page, M.-A., James, P., Vorherr, P., Krebs, J., and Carafoli, E. (1988). *J. Biol. Chem.* **263**, 14152–14159.
Vigne, P., Breittmayer, J.-P., Frelin, C., and Lazdunski, M. (1988). *J. Biol. Chem.* **263**, 18023–18029.
Villalobo, A., Brown, L., and Roufogalis, B. D. (1986). *Biochim. Biophys. Acta* **854**, 9–20.
Vorherr, T., James, P., Krebs, J., Enyedi, A., McCormick, D. J., Penniston, J. T., and Carafoli, E. (1990). *Biochemistry.* **29**, 355–365.
Vorherr, T., Kessler, T., Hofmann, F. and Carafoli, E. (1991). *J. Biol. Chem.* **266**, 22–27.
Vrolix, M., Raeymaekers, L., Wuytack, F., Hofmann, F., and Casteels, R. (1988). *Biochem. J.* **255**, 855–863.
Waldman, S. A., and Murad, F. (1987). *Pharmacol. Rev.* **39**, 163–196.
Walters, J. R. (1989). *Am. J. Physiol.* **256**, G124–G128.
Wang, K. K., Villalobo, A., and Roufogalis, B. D. (1988a). *Arch. Biochem. Biophys.* **260**, 696–704.
Wang, K. K., Roufogalis, B. D., and Villalobo, A. (1988b). *Arch. Biochem. Biophys.* **267**, 317–327.
Wang, K. K. W., Roufogalis, B. D., and Kuo, T. H. (1991a). *Biochem. J.* **279**, 537–544.
Wang, K. K., Wright, L. C., Machan, C. L., Allen, B. G., Conigrave, A. D., and Roufogalis, B. D. (1991b). *J. Biol. Chem.* **266**, 9078–9085.
Wang, K. K. W., Du, Y. S., Diglio, C., Tsang, W., and Kuo, T. H. (1991c). *Arch. Biochem. Biophys.*. **289**, 103–108.
Watson, E. L., Vincenzi, F. F., and Davis, P. W. (1971). *Biochim. Biophys. Acta* **249**, 606–610.
Wright, M. V., and van Houten, J. L. (1990). *Biochim. Biophys. Acta* **1029**, 241–251.
Wrzosek, A., Famulski, K. S., Lehotsky, J., and Pikula, S. (1989). *Biochim. Biophys. Acta* **986**, 263–270.
Wuytack, F., De Schutter, G., and Casteels, R. (1981). *FEBS Lett,* **129**, 297–300.
Wuytack, F., Raeymaekers, L., De Schutter, G., and Casteels, R. (1982). *Biochim. Biophys. Acta* **693**, 45–52.
Wuytack, F., Raeymaekers, L., Verbist, J., De Smedt, H., and Casteels, R. 91984). *Biochem. J.* **224**, 445–451.
Wuytack, F., Raeymaekers, L., Verbist, J., and Casteels, R. (1987). *The Role of Calcium in Biological Systems* (Anghileri, L. J. Ed.), CRC Press Bova Raton, Florida, Vol. IV, pp. 115–162.
Wuytack, F., Kanmura, Y., Eggermont, J. A., Raeymaekers, L., Verbist, J., Hartweg, D., Gietzen, K., and Casteels, R. (1989). *Biochem. J.* **257**, 117–123.
Yoshida, Y., Sun, H.-T., Cai, J.-Q., and Imai, S. (1991). *J. Biol. Chem.* **266**, 19819–19825.
Zurini, M., Krebs, J., Penniston, J. T., and Carafoli, E. (1984). *J. Biol. Chem.* **259**, 618–627.
Zvaritch, E., James, P., Vorherr, T., Falchetto, R., Modyanov, N., and Carafoli, E. (1990). *Biochemistry* **29**, 8070–8076.