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Topical Review

Modulation of the Plasma Membrane Ca^{2+} Pump

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Abstract. The plasma membrane calcium pump, which ejects Ca^{2+} from the cell, is regulated by calmodulin. In the absence of calmodulin, the pump is relatively inactive; binding of calmodulin to a specific domain stimulates its activity. Phosphorylation of the pump with protein kinase C or A may modify this regulation. Most of the regulatory functions of the enzyme are concentrated in a region at the carboxyl terminus. This region varies substantially between different isoforms of the pump, causing substantial differences in regulatory properties. The pump shares some motifs of the carboxyl terminus with otherwise unrelated proteins: The calmodulin-binding domain is a modified IQ motif (a motif which is present in myosins) and the last 3 residues of isoform 4b are a PDZ target domain. The pump is ubiquitous, with isoforms 1 and 4 of the pump being more widely distributed than 2 and 3. In some kinds of cells isoform 1 or 4 is missing, and is replaced by another isoform.

Key words: Ca^{2+} pump — Calmodulin — Phosphorylation — Regulation

Introduction

The plasma membrane calcium pump plays an important role in controlling intracellular Ca^{2+}. In many kinds of cells it is the only mechanism for extruding Ca^{2+} from the cell, while in excitable cells it shares this role with the Na^{+}/Ca^{2+} exchanger. The plasma membrane determines intracellular free Ca^{2+} over the long term, since it is the only ''organelle'' that can move Ca^{2+} between the cell and the semi-infinite Ca^{2+} reservoir of the extracellular space. The short-term role of this membrane varies from cell to cell. Very large cells, with their correspondingly low plasma membrane area, must rely on endo(sarco)plasmic reticulum for short term Ca^{2+} control. Skeletal muscle is an example, and its sarcoplasmic reticulum is a very active controller of intracellular Ca^{2+}. Smaller cells rely more heavily on the plasma membrane for short term control of intracellular Ca^{2+}.

We will refer to this pump by names such as hPMCA4b, where h indicates the human species, PMCA indicates the plasma membrane Ca^{2+} pump, 4 indicates the product of the fourth gene and b indicates the alternate splice used. Of many previous reviews, several recent ones will provide keys to aspects of this pump not discussed here (Strehler, 1991; Carafoli, 1992, 1994; Penniston & Enyedi, 1994, 1998; Monteith & Roufogalis, 1995; Schatzmann, 1995).

The sarcoplasmic reticulum and the plasma membrane both contain Ca^{2+} pumps with submicromolar affinity for Ca^{2+}. These pumps perform the same basic function of ATP-energized removal of Ca^{2+} from the cytosol, but they are distinct molecules. As members of the P-type ATPase family (Pedersen & Carafoli, 1987), they have regions of strong similarity, but their overall amino acid identity when aligned is only 31.8%. The plasma membrane Ca^{2+} pump is also distinguished from that of sarcoplasmic reticulum by its possession of about 120 extra amino acids at the carboxyl terminus.

The relationship between the Ca^{2+} ligands of PMCA and the sarcoplasmic reticulum Ca^{2+} pump has been clarified by recent studies on the latter pump. These studies (Andersen & Vilsen, 1992, 1994) disclosed two types of Ca^{2+} ligands. Since the sarcoplasmic reticulum Ca^{2+} pump binds two calciums, each type of ligand probably binds one of the calciums. Of the ligands assigned

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to site I, all three are replaced in PMCA by residues which are unlikely to serve as ligands (MacLennan et al., 1997). Since PMCA is believed to bind only one Ca^{2+}, this suggests that the three conserved residues of PMCA form a site corresponding to site II of the sarcoplasmic reticulum Ca^{2+} pump which binds the single Ca^{2+}. This notion is supported by the observation that mutation of the three conserved residues of PMCA killed Ca^{2+} transport, while mutation of one of the nonconserved residues did not diminish transport (Adebayo et al., 1995; Guerini et al., 1996).

Figure 1 shows hPMCA4b unfolded into two dimensions, with some aspects of its predicted secondary structure and topology. The transmembrane domains are depicted as 10 vertically oriented helical regions near the top of the figure. The calmodulin-binding domain is highlighted in yellow, the downstream inhibitory domain in green, and the transmembrane domains which contain putative Ca^{2+} ligands in light yellow. The PDZ-binding domain is the three individual residues in yellow at the carboxyl terminus, and the approximate locations of the epitopes of well-characterized antibodies (Adamo et al., 1992; Caride et al., 1996) are highlighted in orange-brown. The site of the aspartyl-phosphate is marked by a red star, and the putative Ca^{2+} ligands are marked by red circles.

The Regulatory Carboxyl Terminus

The portion of the plasma membrane Ca^{2+} pump which is homologous with the sarcoplasmic reticulum Ca^{2+} pump (the first approximately 1100 residues) is a catalytic core which is required for Ca^{2+} pumping activity. The downstream portion of the molecule (about 120 residues in the ''b'' forms and about 85 residues in the ''a'' forms) is a regulatory region which inhibits and controls the activity of the enzyme. In Fig. 1, this region runs down the right-hand side. While the different isoforms of the pump show great similarity in their catalytic core region, their downstream regulatory regions differ substantially from one another. This diversity of the carboxyl terminal regulatory region is produced by four genes and by alternative mRNA splicing and creates about 10 different variants of the pump. The regulatory region of isoform 4b begins with a 28-residue calmodulin-binding domain which also inhibits the activity of the enzyme (Enyedi et al., 1993). This region contains 2 modified IQ motifs (*see below*). Downstream of this is an inhibitory region that contains the residues most readily phosphorylated by protein kinase C (Enyedi et al., 1996). This region does not contribute to binding of calmodulin, but does contribute an extra degree of inhibition (Verma et al., 1994), which is relieved by phosphorylation by protein kinase C. The furthest downstream region has been less well characterized, but the ultimate end of hPMCA4b (the last three residues) has recently been identified as a target for binding of PDZ domains (Kim et al., 1998). Proteins containing PDZ domains help to organize their target proteins into functional multi-enzyme complexes in the membrane. Changes in the structure of this multifunctional regulatory region would be expected to produce enzymes with different characteristics. As an example, only hPMCA4b binds strongly to PDZ domains; the other isoforms either lack that sequence or have it modified so that the binding is much weaker. Additional aspects of the consequences of the great variability within this region are summarized below.

The Calmodulin-Binding Domain

The alternate splice which creates the ''a'' and ''b'' forms of the pump has a profound effect on the structure of the downstream regulatory region. This splice occurs in the middle of the message coding for the calmodulin-binding region, and involves a frame shift which changes all of the downstream residues. Because of this, the ''a'' forms share with the ''b'' forms only 18 or 19 residues of the calmodulin-binding region, with everything downstream being different. The structure of this region has been well studied for isoforms 4a and 4b. The calmodulin-binding region of 4a is much longer than that of 4b (Fig. 2) with an overall length of about 49 residues and seems to consist of 2 binding domains separated by a short nonbinding domain (Verma et al., 1996).

Because of this difference in the calmodulin-binding region, isoform 4b has a higher affinity for calmodulin than isoform 4a, as is shown in Fig. 3. This change in the calmodulin affinity changes the effective calcium affinity, one of the most important physiological parameters of the enzyme (Enyedi et al., 1994). A similar alternative splice creates isoforms 2a and 2b, and this results in a similar, but less pronounced, difference in affinities. This is shown in Fig. 3, where it is also apparent that isoforms 2a and 2b have much higher calmodulin affinities than their isoform 4 equivalents; the concentration of calmodulin required to activate isoform 2b is nearly 100 times less than that required for isoform 4a (Hilfiker et al., 1994; Elwess et al., 1997). Since the calmodulin-binding domains of 2b and 4b (and 2a and 4a) are not very different, it is likely that the difference between 2 and 4 originates in some other region of the pump.

Beyond the affinity of calmodulin for the pump, its rates of binding to and dissociation from the pump are very important in determining its biological properties. Studies on the rate of activation of the pump have suggested that the rate of binding of calmodulin to isoform 4b of the pump is relatively slow. For example, under

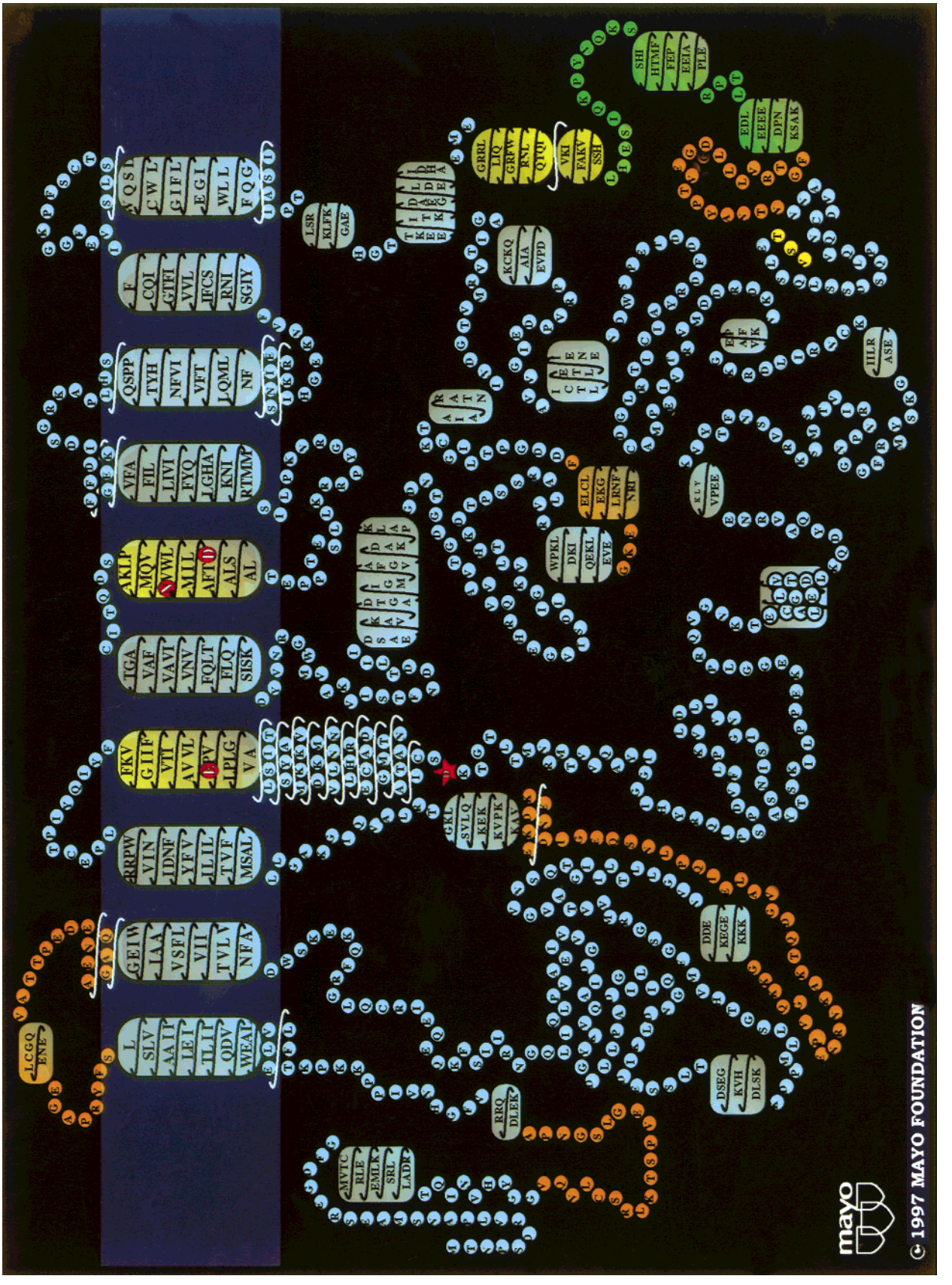

Fig. 1. Two-dimensional representation of isoform 4b of the pump, showing the predicted secondary structure. The amino terminus of the sequence is at the left edge of the figure, and the carboxyl terminus is in the lower right.

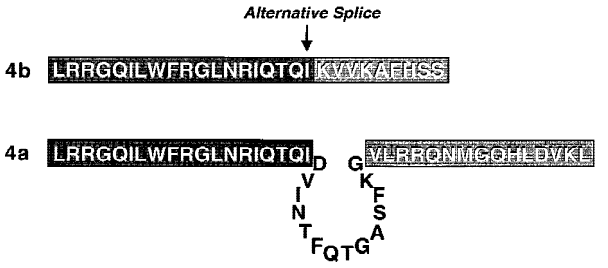

Fig. 2. A comparison of the calmodulin-binding domains of isoforms 4a and 4b. In isoform 4a, this domain is much longer, but its affinity for calmodulin is lower. The region that is shown as a hairpin in the figure has different properties than are expected for a calmodulin-binding region. It is composed of hydrophilic amino acids and is acidic, so we infer that it is excluded from the interaction of this domain with calmodulin (Verma et al., 1996). The presence of this hairpin probably accounts for the lower affinity of isoform 4a for calmodulin.

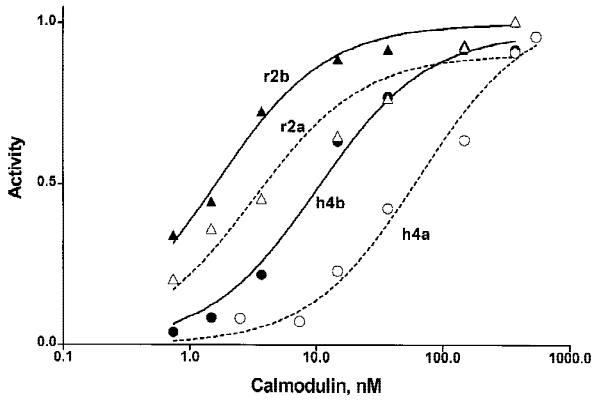

Fig. 3. Calmodulin response curves of four isoforms. The a forms have a lower affinity for calmodulin than the b forms, and isoform 2 has a higher affinity than isoform 4 (Enyedi et al., 1994; Elwess et al., 1997).

conditions approximating those believed to exist in living cells, the time required for calmodulin to occupy half of its sites was about 30 seconds, which is similar to the lengths of many calcium spikes (Scharff & Foder, 1982). The question of rates of binding of calmodulin to the pump is discussed more fully in a recent review (Penniston & Enyedi, 1998).

The Pump and Ca^{2+} Spikes

If the binding of calmodulin to the pump is slow, the dissociation of calmodulin from the pump must be even slower, because of the high affinity of calmodulin for the pump. Such slow on and off rates for calmodulin would cause activation of the pump to respond to fluctuations in the Ca^{2+} level in an integrative fashion, similar to that recently observed for calmodulin kinase II (De Koninck & Schulman, 1998). In the case of the kinase the integration was caused by its Ca^{2+}-stimulated autophosphorylation, which eventually makes the enzyme active even in the absence of Ca^{2+}. This property caused the kinase to respond to the frequency of a train of Ca^{2+} spikes, so that its response was greater at higher frequencies, even when the total amount of Ca^{2+} exposure was the same.

In the case of PMCA, the slow response to Ca^{2+}-calmodulin might be expected to cause a similar effect, except that the pump would become activated in the absence of Ca^{2+}, but would still require Ca^{2+} for actual activity, since Ca^{2+} is both activator and substrate for the pump. When the pump is the dominant means of removing Ca^{2+} from the cytosol, it is likely that its slow response to Ca^{2+}-calmodulin would have the effect of encouraging the development of spikes. In the presence of low-frequency, low-intensity fluctuation in the influx of Ca^{2+}, the slow on rate of calmodulin could intensify spikes, because the cytosolic Ca^{2+} could increase to a high level before the pump was activated. When the pump became activated, a rapid efflux of Ca^{2+} would follow. A single such spike has been demonstrated in response to a single increase in the rate of Ca^{2+} efflux (Foder & Scharff, 1992). In the presence of high-frequency, high-intensity fluctuations in the influx of Ca^{2+}, the pump would still be expected to accentuate the spikes. It would become more activated with each event of Ca^{2+} influx, so that the trailing edge of each Ca^{2+} spike would become steeper and the spikes more clearly defined. This effect would be frequency-dependent; the more rapid the spikes, the quicker the activation of the pump.

As discussed above, the different isoforms of the pump have a wide range of calmodulin affinities. The differential expression of the pump isoforms in the different cells and tissues may modulate the frequency and intensity of the spikes to satisfy the specific needs of the particular cell. The off-rate of the highest affinity forms such as 2b would be very slow: the pump would become activated quicker and would stay activated during the train of spikes. Thus, in cells where 2b is abundant, the spikes would be steeper and less intense. The low affinity forms of the pump such as 4a, on the other hand, would become activated more slowly. That might lead to longer-lasting, more intense spikes.

In the presence of the sarcoplasmic reticulum Ca^{2+} pump, the effect of the pump in accentuating a slow spike would probably not be present, since the sarcoplasmic reticulum Ca^{2+} pump would quickly remove small amounts of Ca^{2+} and prevent a spike until the Ca^{2+} influx became great enough to cause Ca^{2+}-induced Ca^{2+} release. However, the plasma membrane Ca^{2+} pump would be expected to gradually take over the removal of Ca^{2+} from the cytosol from the sarcoplasmic reticulum Ca^{2+} pump as the sarcoplasmic reticulum became filled with Ca^{2+}. In this circumstance, the gradual activation of the pump would be expected to act to keep the spikes

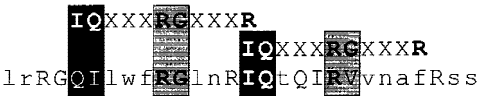

Fig. 4. Alignment of IQ motif consensus sequences (top two lines) with the calmodulin-binding domain of rPMCA1b. The partially conserved regions are highlighted, with the IQ motif in black and the RG motif in gray.

sharp after the sarcoplasmic reticulum Ca^{2+} pump was no longer able to do so.

Thus, the properties of the regulatory region of PMCA may encourage the development of Ca^{2+} spikes and enhance the digital encoding of the Ca^{2+} signal. As Putney (1998) has pointed out, such encoding may have advantages in the regulation of cells by the Ca^{2+} signal.

IQ Motifs

The protein sequence known as an IQ motif is well characterized in unconventional myosins, where domains with this sequence bind calmodulin or myosin-associated light chain (Cheney & Mooseker, 1992). The consensus amino acid sequence for such domains has been defined as IQXXXRGXXXR, which is repeated tandemly two to six times (Houdusse & Cohen, 1995). IQ motifs have also been identified in a variety of other proteins (Deloulme et al., 1997), and these motifs differ in various ways from the consensus found for the unconventional myosins.

Comparison of the consensus above with that of the ''b''-type PMCA calmodulin binding domains shows a striking resemblance. Because the alternative splice changes the downstream portion of this domain, the ''a'' type calmodulin-binding domains show much weaker resemblance to the IQ motif than the ''b.'' Figure 4 shows the calmodulin binding domain of PMCA1b aligned with two consensus IQ motifs.

While the identities (shown as bold capitals) are few, the similarities (shown as normal capitals) are many. The PMCA calmodulin binding domain may be viewed as two altered IQ motifs in tandem. The first motif of the calmodulin binding domain has the I and Q transposed, with the terminal R occurring one residue too early. In the second motif, the G is replaced by a V and the terminal R occurs one residue too late. In addition, the motifs RG and QI are inserted again, in locations where they are not normally found in IQ motifs. The alteration of the motif may confer Ca^{2+} sensitivity on the calmodulin binding. It has been inferred that complete IQ motifs bind calmodulin in its Ca^{2+}-free form, while altered IQ motifs require calmodulin with one or more bound Ca^{2+} ions (Houdusse & Cohen, 1995).

This relationship between the PMCA calmodulin binding domain and IQ motifs suggests a common ancestral sequence. Unlike the IQ motifs of unconventional myosins, the modified motif of PMCA does not interact with essential light chain (N.L. Elwess and J.T. Penniston, *unpublished results*) and does require Ca^{2+} for interaction with calmodulin. The alterations to the IQ motif found in PMCA are different from the alterations found in other non-myosin IQ motifs.

Inhibitory Domains

In isoforms 4a and 4b, the main source of inhibition of the pump is the calmodulin-binding domain, but in 4b a region farther downstream also contributes to the inhibition. About 3/4 of the inhibition of 4b is due to the calmodulin-binding domain, and 1/4 to the downstream inhibitory region (Verma et al., 1994). In isoform 4a, the total degree of inhibition is less; the inhibited state has about 40% of the activity of the activated state, compared with 10–20% for 4b. All of the inhibitory effect of the 4a carboxyl terminus is contained within its extended calmodulin-binding domain (Verma et al., 1996).

Phosphorylation by Protein Kinase C

Several studies have shown that protein kinase C stimulants can activate the pump in whole cells; aortic endothelial cells (Wang et al,. 1991*a*), erythrocytes (Wright et al., 1993) and jurkat T cells (Balasubramanyam & Gardner, 1995) have shown evidence of such effects. In isolated membranes, added protein kinase C can phosphorylate and activate the pump (Smallwood et al., 1988; Wang et al., 1991*b;* Enyedi et al., 1996). Where the phosphorylation site has been defined, it is in the carboxyl terminus, which contains a very high proportion of threonine and serine. Figure 5 shows this portion of the molecule for eight isoforms of the pump, with the threonine and serine residues highlighted in black, and the arginine and lysine residues (which may activate phosphorylation) highlighted in gray. This figure shows both the major differences between the ''a'' and ''b'' forms, caused by the alternative splice, and also the significant differences between the potential phosphorylation sites of the pumps coded by different genes.

The expectation that the different isoforms would have different phosphorylation sites is borne out by the data accumulated so far. A study on isoform 4b (Enyedi et al., 1996) showed that the primary sites of phosphorylation by protein kinase C are in the downstream inhibitory region. Subsequent experiments show that more than one serine in the upstream half of this region is phosphorylated (some possible sites are indicated by arrows in Fig. 5) (A. Enyedi et al., *unpublished results*).

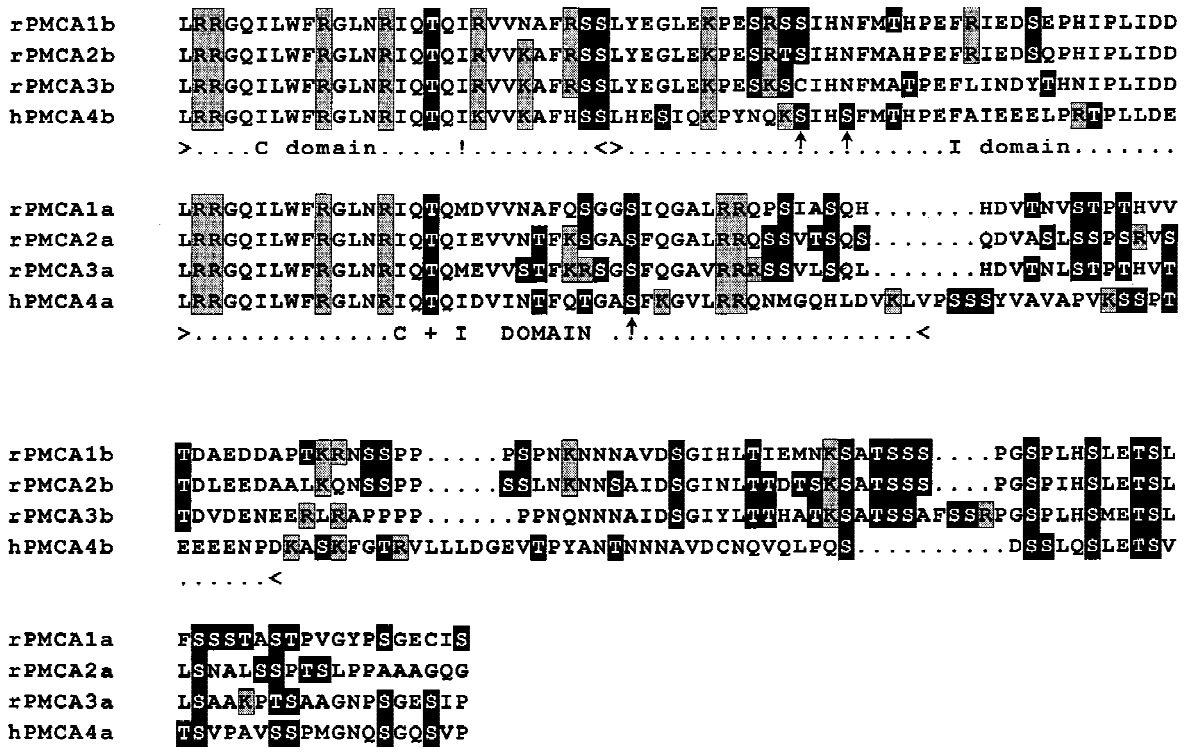

Fig. 5. Comparison of the carboxyl termini of 8 isoforms of PMCA, highlighting potential phosphorylation sites. Serines and threonines are highlighted in black, lysines and arginines in gray; the position of the alternate splice is indicated by an exclamation point. This figure illustrates the variability in potential phosphorylation sites among the PMCA isoforms.

Phosphorylation in this region activates the enzyme somewhat, relieving that portion of the inhibition caused by the downstream inhibitory region. Smaller amounts of phosphorylation occur at other sites in the carboxyl terminus; one of these sites could be the threonine of the calmodulin-binding domain.

The phosphorylation of isoforms 2 and 3 by protein kinase C has quite different effects on activity than the phosphorylation of 4b. Isoforms 2b and 3b are not phosphorylated significantly, while 2a and 3a are phosphorylated. The phosphorylation of these two isoforms does not activate them, but rather prevents calmodulin activation, so that the phosphorylated molecules will have a constant low level of activity. Thus phosphorylation of these isoforms in the living cell would raise the free intracellular Ca^{2+} levels and minimize the response of this pump to elevated Ca^{2+} (Enyedi et al., 1997).

The behavior of isoforms 2a and 3a suggests that they are phosphorylated in a part of the regulatory region that is actively involved in binding of calmodulin, and that their phosphorylation actually prevents binding of calmodulin. It is evident that the differences in regulation of the isoforms will have important consequences in the cellular regulation of the Ca^{2+} signal.

Phosphorylation by Protein Kinase A

Studies on the effect of protein kinase A on the pump in heart sarcolemmal membranes were initiated more than a decade ago (Caroni & Carafoli, 1981; Neyses et al,. 1985). These studies showed concurrent phosphorylation of the pump and increase in Ca^{2+} uptake activity. Later, the sequence KRNSS was identified as being phosphorylated with the A-kinase in the purified erythrocyte pump (James et al., 1989). Subsequent work (Strehler et al., 1990) has indicated that the isoform, hPMCA1b, which contains the sequence KRNSS, is only a minor component of the erythrocyte calcium pump. It became evident that the only isoform containing a homologous sequence in the corresponding region is 2b, which contains the sequence KQNSS, but this isoform is not expressed in human erythrocytes. The major component of the erythrocyte membrane (hPMCA4b) does not contain this sequence and in an experiment we have done recently (A. Enyedi et al., *unpublished results*) it did not show phosphorylation with the A-kinase. In heart muscle, both PMCA1b and 4b are expressed in appreciable amounts (Table), suggesting that protein kinase A could play a significant role in regulating the pump in this tissue. Whether protein kinase A phosphorylates other isoforms of the pump and how it affects the pump's activity is still unknown and needs further investigation.

Location of PMCA within Cells

In a previous review (Penniston & Enyedi, 1994), we summarized the evidence for the location of PMCA on

Table. Variants of PMCA in various tissues, estimated from a consensus of PCR and antibody measurements, primarily on rat

	1a	1b	2a	2b	3a	3b	4a	4b
Brain-cerebellum	+	0	+	+	+	+	0	?
Brain-frontal cortex	+	0	+	+	+	+	+	?
Brain-hippocampus	+	0	+	+	+	+	0	+
Skeletal muscle	0	+	0	0	0	+	0	+
Heart muscle	0	+	+	0	0	0	0	+
Stomach	0	+	0	0	+	0	+	+
Small intestine	0	+	0	0	+	0	+	+
Colon	0	+	0	0	+	0	+	+
Uterus	0	+	0	+	0	0	+	+
Liver	0	+	0	0	0	0	0	?
Kidney	0	+	0	+	+	0	0	+
Lung	0	+	0	0	0	0	0	+
Spleen	0	+	0	0	0	0	0	+

In addition to the references cited above, data from a number of other papers were considered (Greeb & Shull, 1989; Magocsi et al., 1992; Howard et al., 1994; Caride et al., 1995, 1996).

the side of epithelial cells toward which Ca^{2+} moves. Recent evidence in other types of cells shows that they also have their own specialized locations for PMCA. One specialized location found in various kinds of cells is the caveola, an invagination of the plasma membrane. In a number of different cell types that contain caveolae, the pump is highly concentrated in them (Fujimoto, 1993; Schnitzer et al., 1995; Amino et al., 1997), suggesting that these organelles have a special role in calcium metabolism. A study on cardiac muscle from dog and hamster (Iwata et al., 1994) showed that PMCA was not abundant in the T-tubule membranes, but was abundant in the surface plasma membrane. A study from another laboratory on rabbit skeletal muscle (Sacchetto et al., 1996) found PMCA present in both the T-tubules and the surface membranes.

A more unusual and specialized location of PMCA was reported by Fujii et al. (1996), who found it in the intracellular synaptic vesicles of chicken Edinger-Westphal neurons. Using the same antibody (5F10) employed in many other studies, these workers visualized the pump by several techniques, including electron microscopy, and found its distribution to be similar to that of the synaptic vesicle protein SV-2. The similar distribution of PMCA and SV-2 has been verified by another laboratory (Juhaszova et al., 1996).

These and other studies show that the abundance of PMCA in a particular kind of cell and its location within the cell is closely tied to the specific details of that cell's calcium metabolism. Rather different localizations occur in different kinds of cells, and the variety is especially great in excitable tissues such as muscle and nerve.

Isoform Distribution

It is clear that the isoform composition of cells varies greatly with their function (*see* the Table for comparison). Isoforms 1b and 4b are the most widespread, and have been considered to be ubiquitous housekeeping forms (Carafoli, 1994; Hammes et al., 1994). However, it is now becoming evident that no particular PMCA isoform is required for cell survival in mammals. In brain isoform 1b is nearly absent, and isoform 4b appears to be absent in at least some kinds of brain cells, as is discussed in the next two paragraphs.

In the case of isoform 1b, it gradually decreases during the differentiation of brain cells, as was shown by Brandt and Neve (1992). They still found some 1*b* mRNA by PCR at postnatal day 30, but it was much reduced. Other PCR studies found very low levels of 1b mRNA in mature rat brain (Keeton et al., 1993; Stauffer et al., 1993). A study with an antibody which recognizes all variants of isoform 1 found it to be present in rat and human brain (Stauffer et al., 1995), but a more detailed study with the same antibody found some cell types in rat brain that appeared to lack isoform 1 (Stauffer et al., 1997). Only recently has an antibody become available that could distinguish isoform 1b from 1a, and a study with this antibody showed negligible amounts of 1b in mature rat brain (Filoteo et al., 1997). We conclude from these studies that isoform 1b probably does not play a physiologically significant role in mature rat brain, although some may remain as a relic of embryonic development.

Neither splice of isoform 4 was detected in rat brain at any stage of development by Brandt and Neve (1992), while Stauffer et al. (1993) detected some 4b and a little 4a. On the other hand Keeton et al. (1993) found 4a, but not 4b. All of these results were obtained by PCR. A study using *in situ* hybridization found high levels of isoform 4 in some regions of rat brain, but none in other parts (Stahl et al., 1994), and concluded that isoform 4 is not a housekeeping form of the pump. This study did not distinguish the alternate splices. The antibody study of Stauffer et al. used an antibody recognizing all splices of isoform 4 and found it to be present in human brain (Stauffer et al., 1995). However, when the splices were distinguished, some regions of brain were found to have no 4a and only very low levels of 4b, while frontal cortex had larger amounts of both (Filoteo et al., 1997). While the results with isoform 4 are not as clear as those with isoform 1, the great variability in the amounts of both 4a and 4b in brain seems inconsistent with a requirement of their presence for cell survival.

While no particular isoform is always required, it still appears that some isoform of PMCA must be present for survival of normal cells. Only platelets, whose calcium metabolism is suicidal, have negligible amounts of this pump. In those brain cells that lack isoform 4b and/or 1b other isoforms are present in relatively large amounts to perform the essential function of ejection of Ca^{2+} from the cells.

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