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Regulation of the Erythrocyte Ca^{2+}-ATPase by Mutant Calmodulins with Positively Charged Amino Acid Substitutions†

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ABSTRACT: Four mutant calmodulins with site-specific charge alterations have been used to activate the human erythrocyte Ca^{2+}-ATPase. These charge alterations were accomplished either by insertion of new Lys residues or by substitution of Lys residues for Glu in two of the seven calmodulin α-helices. Two enzyme preparations, purified monomeric Ca^{2+}-ATPase and erythrocyte ghost membranes, were used with comparable results. At 100 nM Ca^{2+}, the Ca^{2+}-ATPase activity was lowered significantly by charge reversal from negative to positive in both the central α-helix and the carboxy-terminal domain. While all mutant calmodulins with charge reversal ultimately stimulated the Ca^{2+}-ATPase activity to the same extent, the concentration of mutant calmodulin required for half-maximal activation was from 36-fold (central α-helix) to 126-fold higher (α-helix in the carboxy-terminal domain) than that of the control calmodulin. There was also a significant difference in the stimulation of Ca^{2+}-ATPase activity by the different mutant calmodulins as a function of Ca^{2+} concentration, being most pronounced at submicromolar Ca^{2+} concentrations where enzyme activation by calmodulin appears to be a physiologically relevant mechanism. In contrast to the mutant calmodulins with charge reversal, mutant calmodulins in which two positive charges were added in the central α-helix activated the Ca^{2+}-ATPase in a way undistinguishable from the control calmodulin. Our results establish the relative importance of specific charged amino acids in calmodulin for the efficient activation of the enzyme and show that the interaction of the erythrocyte Ca^{2+}-ATPase with calmodulin is attenuated more by charge reversal in the α-helix of the carboxy-terminal half of calmodulin than in the central α-helix. Troponin C (TnC), another Ca^{2+}-binding regulatory protein, homologous with CaM, did not stimulate the Ca^{2+}-ATPase activity. Two experimental procedures that diminish differences in the primary structure between TnC and CaM, insertion of three amino acids into CaM or deletion of eight amino acids from TnC, did not change the function of the native molecules with respect to their ability to activate the erythrocyte Ca^{2+}-ATPase.

The human erythrocyte Ca^{2+}-ATPase is a good experimental model for plasma membrane Ca^{2+}-ATPase since in the erythrocyte there is no interference from other Ca^{2+} transporting systems, like Na^+–Ca^{2+} exchanger and gated Ca^{2+} channels or the intracellular endoplasmic/sarcoplasmic reticulum. However, regulation of this enzyme's function appears very complex. Several activation modes have been proposed, such as binding of calmodulin (CaM)[1] or acidic phospholipids, proteolytic digestion, oligomerization, and phosphorylation by kinases [for a review, see Schatzmann (1982), Eneyedi et al. (1987), Wang et al. (1989), Kosk-Kosicka and Bzdega (1988), Neyes et al. (1985), and Smallwood et al. (1988)]. We have been studying two modes of regulation, by oligomerization of Ca^{2+}-ATPase monomers and by calmodulin binding to enzyme monomers (Kosk-Kosicka & Inesi, 1985; Kosk-Kosicka et al., 1986, 1989, 1990a,b; Kosk-Kosicka & Bzdega, 1988, 1990a). Calmodulin is essential for activation of Ca^{2+}-ATPase only when the enzyme and Ca^{2+} concentrations are low: pCa below 6.25 and enzyme concentration below 15 nM (Kosk-Kosicka & Inesi, 1985; Kosk-Kosicka et al., 1989). The process of enzyme activation by CaM is complex, and believed to involve multiple substeps, including Ca^{2+} binding to calmodulin, Ca^{2+} binding to the Ca^{2+}-ATPase, binding of calmodulin to the enzyme, and finally the overall effect, enzyme activation.

In the present study, we have employed systematically perturbed, mutant calmodulins, engineered in Martin Watterson's laboratory, in which negative cluster charges were

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[1] Abbreviations: CaM, calmodulin; TnC, troponin C; MLCK, myosin light chain kinase; CaMPKII, type II calmodulin-dependent protein kinase; $C_{12}E_8$, *n*-dodecyl octaethylene glycol monoether; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane. CaM-1 has been registered in EMBL as SYNCAM. The amino acid sequences of calmodulins used in this study can be found in the EMBL Data Bank as an annotation to the accession number M11334. They were generated from the gene for SYNCAM with the following amino acid alterations: (1) CaM-8, EEE82 → KKK; (2) CaM-12A, DEE118 → 120-KKK; (3) CaM-40, E84 → K, E120 → K; (4) CaM-15, KGK insert between D80 and S81.

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selectively reversed; these alterations disrupted the localized charge properties of calmodulin while the overall calmodulin structure was preserved (Weber et al., 1989). Interaction of these mutant calmodulins with two distinct calmodulin-dependent protein kinases was impaired, showing functional importance of charge properties in calmodulin (Craig et al., 1987; Weber et al., 1989). Our goal was to establish the relative importance of these charge perturbations for the efficient activation of the plasma membrane Ca^{2+}-ATPase which does not have such a strict requirement for calmodulin as the kinases do. We report that the erythrocyte Ca^{2+}-ATPase is sensitive to the charge reversal in CaM, from negative to positive, comparably to the kinases. The effect is on the affinity of the altered CaM for the enzyme and is very much Ca^{2+} dependent. Disturbances in ionic interactions that affect CaM binding to the enzyme are implicated. Part of the data was presented in preliminary form (Kosk-Kosicka & Bzdega, 1990b).

Materials and Methods

Egg yolk phosphatidylcholine (P5763) and CNBr-activated Sepharose 4B were purchased from Sigma, $C_{12}E_8$ was from Nikkol, Japan. Coupling of bovine calmodulin to Sepharose was performed in accordance with Pharmacia instructions, as described earlier (Kosk-Kosicka & Bzdega, 1988). Mutant calmodulins were generated by Dr. T. Craig in Dr. D. M. Watterson's laboratory from the gene for CaM-1[1] (Roberts et al., 1985; Craig et al., 1987; Weber et al., 1989). TnC from rabbit skeletal muscle and its tryptic peptide TRoC (residues 9–159) were a gift of Dr. Z. Grabarek (Grease & Gergely, 1973). Bovine brain CaM was from Calbiochem.

Preparation of Ghosts. Erythrocyte membrane ghosts were prepared as described before (Kosk-Kosicka et al., 1986). They were kept frozen at −70 °C in 10 mM Tris buffer, pH 7.5, until used.

Purification of Ca^{2+}-ATPase. The Ca^{2+}-ATPase was purified from erythrocyte ghosts by calmodulin affinity column chromatography in the presence of the nonionic detergent $C_{12}E_8$ as described previously (Kosk-Kosicka & Inesi, 1985; Kosk-Kosicka et al., 1986). The enzyme was stored at −80 °C in elution after buffer containing 10 mM Tris–maleate, pH 7.5, 130 mM KCl, 0.5 mM $MgCl_2$, 5 mM EGTA, 20% glycerol, 750 μM $C_{12}E_8$, 2 mM dithiothreitol, and 0.02% of a sonicated suspension of egg yolk phosphatidylcholine.

Protein Assay. The concentration of enzyme protein in the eluate was measured by the Lowry method (Lowry et al., 1951) modified according to Bensadoun and Weinstein (1976), as well as by the Bio-Rad protein micro-assay, based on the Bradford dye-binding procedure (Bradford, 1976). Both methods gave similar values within 5%. Bovine serum albumin was used as a standard.

Ca^{2+}-ATPase Activity. Ca^{2+}-ATPase activity was determined by measurement of inorganic phosphate production, generally as described previously (Kosk-Kosicka & Bzdega, 1988). The assay was performed in a reaction mixture containing 50 mM Tris–maleate, pH 7.4, 80 mM KCl, 8 mM $MgCl_2$, 3 mM ATP, 1 mM EGTA, and $CaCl_2$ in concentrations yielding the required free [Ca^{2+}]. The reaction volume was 100 μL. Appropriate aliquots of the Ca^{2+}-ATPase in the elution buffer were added to achieve 15 nM enzyme concentration. The concentration of $C_{12}E_8$ was kept constant at 150 μM. The amounts of added calmodulins are given in the figures. The reaction was started with 3 mM ATP and carried out for up to 30 min at 37 °C. Depending on the enzyme concentration, various aliquots were withdrawn for colorimetric inorganic phosphate measurement with malachite green (Lanzetta et al., 1979). Steady-state velocities were obtained from plots of inorganic phosphate production which were linear with time.

Free Calcium. Free Ca^{2+} concentrations were calculated (Fabiato & Fabiato, 1979) from total calcium and total EGTA, based on the constants given by Schwartzenbach et al. (1957) and the pK values given for EGTA by Blinks et al. (1982), taking into account pH, ionic strength, magnesium, and ATP concentrations. Total Ca^{2+} was measured by atomic absorption.

Table I: Comparison of Mutant Calmodulins with Bovine CaM

CaM	amino acid alterations as compared to CaM-1	charge change	K_{CaM} (μM)
bovine			0.05
CaM-1			0.05
CaM-8	GluGluGlu82–84→LysLysLys	3− → 3+	1.78
CaM-12A	AspGluGlu118–120→LysLysLys	3− → 3+	6.31
CaM-40	Glu120→Lys and Glu84→Lys	2− → 2+	3.55
CaM-15	LysGlyLys insert after Glu80	+2+	0.05

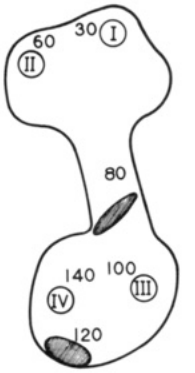

FIGURE 1: Schematic representation of the CaM molecule. The overall shape, location of calcium ions, and location of the altered amino acid residues are based on the structure of rat testes calmodulin (Babu et al., 1988) and a model of CaM-1 constructed by Weber et al. (1989). The approximate positions of the altered residues 82–84 and 118–120 are indicated as shaded areas. The four calcium-binding sites (O) are numbered I–IV, accordingly.

Results

Charge alterations were introduced in two of the seven α-helices in the calmodulin molecule (Babu et al., 1988): in the central α-helix or in the sixth α-helix (located between the third and fourth calcium-binding domains in the carboxy-terminal half of calmodulin). The specific amino acid alterations and the nomenclature used throughout the text are listed in Table I. All mutant calmodulins were generated from the gene for CaM-1: CaM-1 has an amino acid sequence that is a hybrid of the vertebrate and plant calmodulins that is isofunctional with the vertebrate calmodulin for several calmodulin-regulated enzymes [as discussed in Weber et al. (1989)]. As compared to CaM-1, CaM-8 has three lysines substituted for three glutamic acid residues in positions 82–84 in the central α-helix, which results in a charge reversal from three negative to three positive. In CaM-12A, a similar charge reversal was introduced in the carboxy-terminal domain α-helix at residues 118–120. CaM-40 combines charge reversal from negative to positive in the two α-helices in the point-mode; i.e., only one amino acid is substituted in each α-helix. Figure 1 shows the approximate location of the altered amino acids in the CaM molecule.

CaM-15 is somewhat different from the previously described group in that two extra positive charges are added to the central α-helix. The inserted amino acid sequence occurs

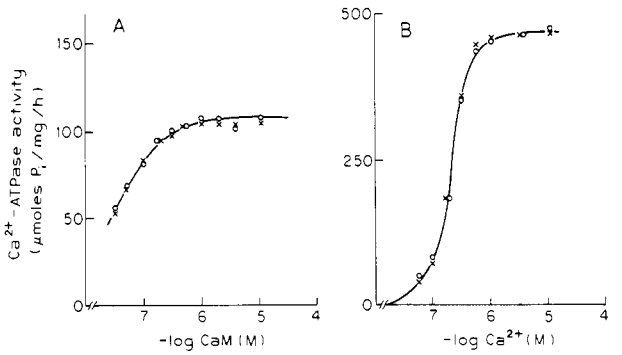

FIGURE 2: Stimulation of Ca^{2+}-ATPase activity by bovine (O) and CaM-1 (×) calmodulin as a function of calmodulin (A) or Ca^{2+} concentration (B). The Ca^{2+}-ATPase activity was measured as described under Materials and Methods. The reaction mixture contained 50 mM Tris–maleate, pH 7.4, 80 mM KCl, 8 mM $MgCl_2$, 150 μM $C_{12}E_8$, 1 mM EGTA, and 3 mM ATP. Sufficient $CaCl_2$ was added to obtain 100 nM free Ca^{2+} (A) or the calcium concentration specified on the horizontal axis (B). Ca^{2+}-ATPase concentration was 15 nM. Calmodulin concentration in (B) was 300 nM.

naturally in the TnC molecule. The effect of this insertion on the stimulation of the Ca^{2+}-ATPase activity by calmodulin was compared to the effect of TnC.

Figure 2 compares activation of the Ca^{2+}-ATPase by CaM-1 to its activation by the bovine calmodulin used routinely in our Ca^{2+}–ATPase activity assays (Kosk-Kosicka & Bzdega, 1988, 1990a). Activation of the enzyme by the two calmodulins was practically identical with respect to its dependence on both calmodulin concentration (Figure 2A) and Ca^{2+} concentration (Figure 2B). In the following studies, CaM-1 was used as a control when the effect of amino acid alterations on activation of the enzyme was studied.

Charge Reversal from Negative to Positive in the Calmodulin α-Helix: Effect on the Calmodulin Concentration Dependence of Enzyme Activation. To evaluate the effect of charge reversal from negative to positive in the α-helix of calmodulin, the Ca^{2+}-ATPase activity was determined as a function of calmodulin concentration. Figure 3A shows that all studied mutant calmodulins stimulated enzyme activity to the same extent (V_{max} not changed); however, the K_{CaM} was shifted dramatically. The concentrations of calmodulin required for half-maximal activation ranged from 36-fold (CaM-8) to 126-fold (CaM-12A) greater than the concentration of CaM-1. Comparison of the effect of the substitution of three positively charged amino acids for three negatively charged amino acids in the central α-helix (CaM-8) versus in the sixth α-helix (CaM-12A) shows that the alteration in the helix located in the carboxy-terminal domain had a more dramatic effect on enzyme activation. Concordantly, a combination of point alterations (CaM-40) increased the K_{CaM} to a lower degree than the cluster mutation in the carboxy-terminal domain helix (CaM-12A); however, again, it was more potent than the cluster mutation in the central α-helix.

Figure 3B shows the effect of charge reversal from negative to positive on the response of enzyme in the membrane; erythrocyte ghost preparation was used. These experiment were carried out to establish that the purified enzyme did not have an artifactual sensitivity to the alterations in calmodulins. The response of the enzyme in ghosts membranes was comparable to that of the purified enzyme in that all mutant calmodulins were eventually able to maximally stimulate the Ca^{2+}-ATPase activity, and the order of decreased affinity of altered calmodulins was the same as observed in experiments with the purified enzyme. The K_{CaM} values were lowered in comparison to the purified enzyme because these experiments

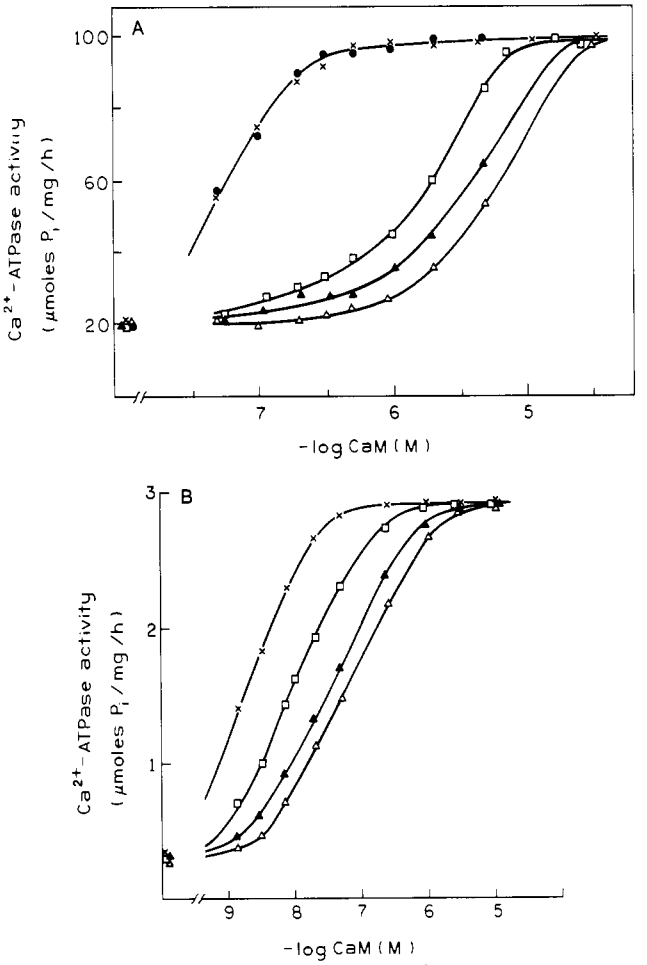

FIGURE 3: Dependence of the Ca^{2+}-ATPase activity on the concentration of the mutant calmodulins: CaM-1 (×); CaM-15 (O); CaM-8 (□); CaM-40 (▲); CaM-12 (△). The Ca^{2+}-ATPase activity was measured as described under Materials and Methods and in Figure 2. Purified enzyme concentration (A) was 15 nM (2 μg/mL), and free [Ca^{2+}] was 100 nM. With erythrocyte ghosts (B), the assay was performed at 160 μg of protein/mL and the free [Ca^{2+}] was 2 μM.

were performed at micromolar Ca^{2+} concentration as opposed to the nanomolar Ca^{2+} concentration in Figure 3A, at which the enzyme is less dependent on calmodulin (see Discussion).

Calcium Dependence of Enzyme Activation by the Mutant Calmodulins with Charge Reversal. The Ca^{2+} dependence of the Ca^{2+}-ATPase activity was studied in the presence of mutant calmodulins to assess whether their binding confers to the enzyme the ability to respond efficiently to the changes in Ca^{2+} concentration in the cell, as was observed with control calmodulin (Kosk-Kosicka et al., 1990a). As shown in Figure 4A,B, there was a significant difference in the stimulation of enzyme activity of different mutant calmodulins at up to about 300 nM Ca^{2+}, while at saturating Ca^{2+} all of them, except for CaM-12A, reached approximately the same maximal velocity. In the presence of CaM-12A, the maximal activation was decreased by 17% (Figure 3A).

Detailed analysis of the Ca^{2+}-ATPase activity at the low Ca^{2+} concentrations between pCa 7 and 6.25 [within this range, enzyme monomers are fully activated by bovine calmodulin, as shown in Kosk-Kosicka et al. (1990a)] is presented in Figure 4B. While the activation by the control CaM-1 was already observed at pCa 7, with maximal effect at pCa 6.7, among the other mutant calmodulins only CaM-8, after a slower start, was able to activate the enzyme similar to the control at these low Ca^{2+} concentrations. In comparison, the activation by CaM-40 and -12A began at about 2–3-fold

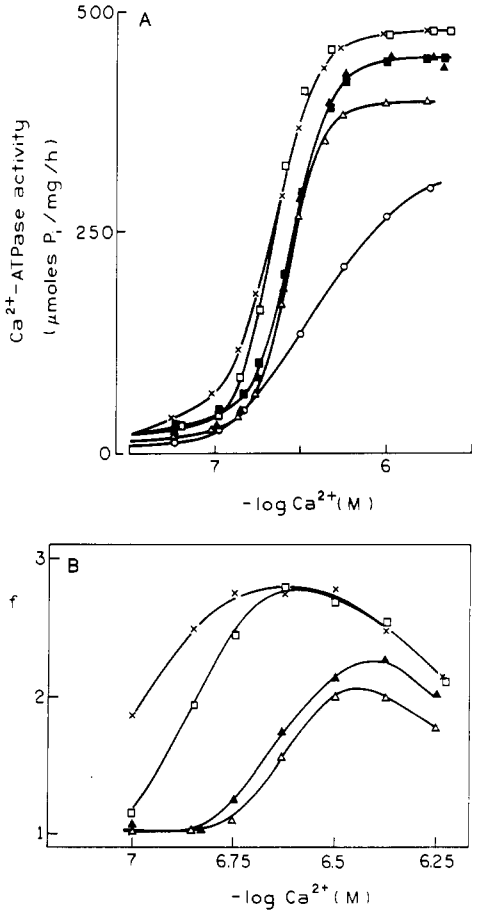

FIGURE 4: Calcium dependence of the stimulation of the Ca^{2+}-ATPase activity by mutant calmodulins. The specific activity of the Ca^{2+}-ATPase was measured at up to 2 μM free Ca^{2+} (A). In (B), the ability of different calmodulins to stimulate the Ca^{2+}-ATPase activity over the low free Ca^{2+} concentration is expressed as an activation factor (f). $f = V_c/V_0$, where V_c is the maximal activity in the presence of CaM and V_0 is the maximal activity in the absence of CaM. The Ca^{2+}-ATPase activity was measured as described under Materials and Methods and in Figure 2. Calmodulin concentration was 100 nM. In addition to symbols used for different CaM's which are as described in Figure 3, panel A shows Ca^{2+}-ATPase activity in the absence of calmodulin (O).

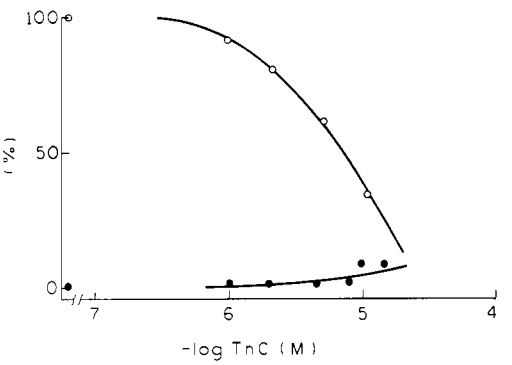

FIGURE 5: Effect of TnC on the Ca^{2+}-ATPase activity in the absence (●) and presence (O) of calmodulin. The Ca^{2+}-ATPase activity is expressed as the percent of enzyme activation in the presence of 300 nM CaM-1. The assay was performed as described under Materials and Methods. Free [Ca^{2+}] was 100 nM, and the enzyme concentration was 15 nM.

higher [Ca^{2+}]; then higher [Ca^{2+}] was required for maximal stimulation, and the maximal stimulation was lower than with CaM-1.

Activation by CaM-15 and by TnC. CaM-15 represents another type of charge alteration in calmodulin; three amino acids with two positive charges were inserted after the negatively charged aspartic acid-80 in the central α-helix. As shown in Table I and Figure 2A, this mutation did not cause any alteration in calmodulin's ability to stimulate the Ca^{2+}-ATPase activity. The amino acid sequence inserted in CaM-15 occurs naturally in TnC. We have then tested TnC for its ability to activate the Ca^{2+}-ATPase. As shown in Figure 5, TnC at concentrations up to 10 μM did not activate the enzyme. However, TnC showed an antagonistic effect with respect to calmodulin (Figure 5). In the presence of increasing concentrations of TnC, the activation of the Ca^{2+}-ATPase by CaM-1 was gradually inhibited. Half-maximal inhibition was observed at 8 μM TnC. These data suggest that TnC binds to the Ca^{2+}-ATPase but is unable to activate it.

Discussion

The aim of the present study was to assess the role of electrostatic interactions in the process of activation of the erythrocyte Ca^{2+}-ATPase by calmodulin. The approach was to use mutant calmodulins in which clusters of phylogenetically conserved acidic amino acids in α-helices have been changed to lysines. These calmodulin mutants have been previously characterized with respect to the change in the calculated stability of the folded protein, the change of the surface electrostatic potential, and the effect of these alterations on the ability to bind and activate two calmodulin-dependent enzymes, myosin light chain kinase (MLCK) and CaMPKII (Weber et al., 1989). Thus, the response of the Ca^{2+}-ATPase activity can be related to the previously established effects of charge reversal in CaM. Our experiments were performed in parallel on the purified Ca^{2+}-ATPase and on the enzyme in the erythrocyte ghost membranes to assure that purification procedure does not change the enzyme's sensitivity to calmodulin. Qualitatively, the response of the membranous enzyme in ghosts was almost identical with that of the purified enzyme, showing that we were measuring the true sensitivity of the Ca^{2+}-ATPase to the charge alterations introduced in calmodulin. The resemblance of the sensitivity of the purified and membranous enzyme to the amino acid alterations in the calmodulin molecule was also observed when calmodulins with amino acid alterations in the Ca^{2+}-binding domains were used (Bzdega & Kosk-Kosicka, 1991).

Electrostatic Effect? Comparison with Other Enzymes. It has been shown that calmodulin's calculated surface electrostatic potential is asymmetrically distributed in the molecule, and clusters of uncompensated negative charges are located near certain α-helices and Ca^{2+}-binding loops (Weber et al., 1989). The studies on interactions between mutant calmodulins with disrupted negative charges and two enzymes, MLCK and CaMPKII, showed that the charge properties are one of several properties of calmodulin that are functionally important (Weber et al., 1989). Results of the present study, performed on the plasma membrane Ca^{2+}-ATPase which is a member of the calmodulin-modulated enzyme family but one that is quite different from the two kinases, strengthen the functional importance of charge properties of CaM.

A number of structural perturbations (as judged from comparison of diverse phylogenetic CaM species, mutations, proteolysis, and chemical modifications) can be accommodated by calmodulin and the target enzyme, and a different extent of the enzymes' sensitivity to changes introduced into CaM molecule is observed [for a review, see Klee et al. (1986), Roberts et al. (1984), Craig et al. (1987), Putkey et al. (1988), Persechini et al. (1989), and Kosk-Kosicka et al. (1990a)]. The erythrocyte Ca^{2+}-ATPase tolerates relatively well chemical modifications in CaM (Jarret, 1986; Guerini et al., 1987) and

several amino acid mutations in the central α-helix, such as insertion of LysGlyLys after Asp80 (Figure 2A), substitution of Pro for Lys75 (Kosk-Kosicka and Bzdega, unpublished results; Krebs et al., 1990), or insertion of Pro-Ser-Thr-Asp between Asp76 and Pro79 (Putkey et al., 1988). By contrast, the Ca^{2+}-ATPase is quite sensitive to charge reversal in both the central helix and carboxy-terminal domain, by both point and cluster mutations (Figure 3).

The dramatically increased K_{CaM} for the activation of the Ca^{2+}-ATPase by calmodulins with charge reversal from negative to positive suggests that electrostatic interactions make a significant contribution to the interactions between the two proteins. Other supporting evidence comes from experiments in which an increase in ionic strength of the assay medium causes a decrease in the calmodulin-dependent Ca^{2+}-ATPase activity (Kosk-Kosicka and Wawrzynow, unpublished results). Interestingly, studies of calmodulin activation of MLCK, an enzyme which is also sensitive to charge reversal from negative to positive in both 80–84 and 118–120 amino acid clusters, have shown marked inhibition by increasing ionic strength (Blumenthal & Stull, 1982).

Comparison of the effect of the charge reversal from negative to positive in CaM in the central α-helix versus in the carboxy terminal shows that the mode and extent to which the alterations affect calmodulin's ability to activate the two kinases (Weber et al., 1989) and the Ca^{2+}-ATPase (present study) are different. CaMPKII is resistant to the change in the central α-helix, while MLCK and the Ca^{2+}-ATPase are sensitive to both mutations, although in various ways. MLCK is affected always in V_{max} and to a variable extent in K_{CaM}, whereas the Ca^{2+}-ATPase is not affected in V_{max} but very dramatically in K_{CaM}. CaMPKII shows a small effect on both kinetic parameters.

As CaM-8 is the best studied of all the mutant calmodulins, we compared its effect on the Ca^{2+}-ATPase with the effect on all other enzymes examined until now: the above-mentioned MLCK, CaMPKII, and additionally phosphodiesterase and NAD kinase (Craig et al., 1987). The Ca^{2+}-ATPase is in the same group as PDE and CaMPKII in that there is no change in V_{max}. However, with respect to the increase in K_{CaM}, it is definitely the most sensitive, except of course for the NAD kinase that is not activated at all. Classification in the same group could be interpreted as suggesting that the gross structure of the enzyme–calmodulin complex may be similar for these enzymes, as opposed to enzymes in the other group. The above comparison shows that such a classification may not be justified and the collected data do not yet allow for any precise conclusions. When the Ca^{2+}-ATPase activity is studied at high Ca^{2+} concentrations comparable to the assay conditions of the other enzymes, a less dramatic shift in K_{CaM} is observed, and by this criterion, the enzyme would be classified together with CaMPKII. The calmodulin–Ca^{2+}-ATPase interaction is important at low [Ca^{2+}]: calmodulin binding to the monomeric enzyme (K_d at pCa 7.04) results in Ca^{2+}-ATPase activation (K_d at 6.6). This order of events has been demonstrated by titrating enhancement in the fluorescence intensity of the MIANS–calmodulin occurring upon its interaction with the Ca^{2+}-ATPase at different Ca^{2+} concentrations, and by relating them to the Ca^{2+}-ATPase activity under the same conditions (Kosk-Kosicka et al., 1990a). At high [Ca^{2+}], the enzyme could be activated even in the absence of calmodulin (Kosk-Kosicka & Inesi, 1985; Kosk-Kosicka et al., 1990a; also visible in Figure 4A). Thus, only at low [Ca^{2+}] could calmodulin be considered a physiological regulator of the erythrocyte Ca^{2+}-ATPase.

Which Step in the Calmodulin–Ca^{2+}-ATPase Interaction is Affected? Indications for Impairment of Calmodulin Binding to the Enzyme. As with other CaM-dependent enzyme activity assays, and even more so because the studied enzyme is a Ca^{2+} pump, the Ca^{2+}-ATPase assay measures the cumulative effects of several different Ca^{2+}-dependent substeps. The process includes Ca^{2+} binding to calmodulin, Ca^{2+} binding to the Ca^{2+}-ATPase, binding of calmodulin to the enzyme, and, finally, the overall effect—enzyme activation. The Ca^{2+}-binding to CaM is not affected, at least in CaM-8, as reported by Haiech et al. (1990). The fact that with all mutant calmodulins studied the V_{max} is not changed while K_{CaM} is changed very much may mean that the alterations introduced in the calmodulin molecule affect the binding of CaM-1 to the Ca^{2+}-ATPase and not the following activtion. In line with such an interpretation, there was a very good correlation between the increase of mutant calmodulin concentration required for half-maximal activation of the MLCK and CaMPKII and the diminished binding activity of metabolically labeled calmodulins to the two enzymes in a gel overlay assay (Weber et al., 1989). Our data indicate that the Ca^{2+} dependence of CaM binding to the enzyme is affected (Figure 4A,B). While the control CaM is fully bound at pCa 6.75 under the assay conditions (Kosk-Kosicka et al., 1990a), which leads to a 100% stimulation of the Ca^{2+}-ATPase activity (Figure 4B), the impairment in mutant calmodulins, especially CaM-12A and -40, seems to destabilize their interaction with the enzyme; this results in activation at a Ca^{2+} concentrations 2–3-fold higher than in the control. The nature of the impairment needs to be explained. One possibility is that a change of electrostatic properties of these regions of the calmodulin molecule alters conformational restriction of CaM such that CaM becomes more flexible and it takes longer for the proper CaM conformer to come in contact with the Ca^{2+}-ATPase. Some energy would then be used up to bring CaM into the conformation required for its interaction with the Ca^{2+}-ATPase. This would also result in a decreased affinity of the enzyme for CaM; the effect on the binding with the enzyme would be a secondary one as the replaced residues may not be directly involved in the interaction.

CaM-15 and TnC. As opposed to the above described charge reversal alterations, the insertion of three amino acids in the central α-helix after the glutamic acid in position 80 has no effect on the activation of the Ca^{2+}-ATPase by CaM. This finding reinforces the significance of the attenuated activation of the enzyme by CaM-8, -12A, and -40. As already pointed out, the Ca^{2+}-ATPase activation is affected only by certain changes in the CaM molecule, and resistant to others. Distribution of the negative charge [see analysis by Weber et al. (1989)] rather than the overall charge of the CaM molecule appears an important factor in enzyme activation.

TnC does not activate the Ca^{2+}-ATPase (Figure 5), although the two proteins interact with each other as suggested by the competition study presented here. This is consistent with the inability of TnC to activate other calmodulin-modulated enzymes, such as adenylate cyclase, cyclic nucleotide phosphodiesterase, or calcineurin, with the only reported exception being phosphorylase kinase (Minocherhomjee et al., 1988; Sharma et al., 1988; Klee & Cohen, 1988; Cohen, 1988). In contrast, CaM binds troponin I and is able to replace TnC in the activation of reconstituted regulated actomyosin (Amplett et al., 1976). The molecular basis for the difference in specificity of TnC and CaM is not clear. The two proteins have a homologous sequence, the only substantial differences being the insertion of the tripeptide Lys-Gly-Lys in the central

α-helix and an additional helical segment at the N-terminus of TnC (Strynadka & James, 1989). The sequence in the central α-helix, studied extensively by site-specific mutagenesis, appears to have no significance with respect to the function of the two proteins. We have also tested the TRoC tryptic peptide in which the eight amino acids in the N-terminus have been removed and found that the peptide, like its parent molecule, is unable to activate the Ca^{2+}-ATPase (Grabarek and Kosk-Kosicka, unpublished results). Thus, neither the insertion of the three amino acids into CaM nor the deletion of the eight amino acids from TnC, two experimental procedures that diminish differences in the primary structure between the two Ca^{2+}-binding proteins, could change the function of the native molecules, with respect to their ability to activate the Ca^{2+}-ATPase.

In conclusion, the activation of the erythrocyte Ca^{2+}-ATPase by calmodulin is attenuated more by the amino acid substitutions resulting in charge reversal (negative to positive) in the carboxy-terminal half of calmodulin as compared to those in the central α-helix. The effect is on the affinity of the altered calmodulin for the enzyme and is very much Ca^{2+} dependent. Disturbances in ionic interactions that affect CaM's binding to the enzyme are implicated.

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Registry No. ATPase, 9000-83-3; L-Lys, 56-87-1; L-Glu, 56-86-0; Ca, 7440-70-2.

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