Site-specific amino acid alterations in Ca^{2+} binding domains in calmodulin impair activation of RBC Ca^{2+}-ATPase

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INTRODUCTION

We have demonstrated that the RBC Ca^{2+}-ATPase, which is regulated by calmodulin (CaM) can also be activated by oligomerization in the absence of CaM (1–4). Activation of the enzyme by either of the two pathways, interaction with calmodulin or enzyme self-association, could depend on several factors, such as the level of cytosolic free Ca^{2+}, enzyme concentration, and the availability of CaM to the enzyme monomers. To characterize the interactions between Ca^{2+}-ATPase and CaM we have employed various mutant CaMs created with a synthetic calmodulin gene (5). We first examined CaMs in which negative charges were selectively reversed; this study provided strong evidence for a functional role of charged residues in the helical regions of CaM in the activation of the RBC Ca^{2+}-ATPase similar to effects seen with protein kinase (6). In the present study we have selected CaM mutants to determine the importance of specific amino acid residues in the Ca^{2+} binding domains of CaM for activation of the Ca^{2+}-ATPase.

RESULTS AND DISCUSSION

We have compared activation of the Ca-ATPase by four CaM mutants in which Ala has been substituted for the invariant Glu at the 12th position in each of the four Ca^{2+} binding domains (Glu 31, 67, 104, or 140). Two of these mutants (CaM-E67 and CaM-E140) have been reported to have significantly altered Ca^{2+} binding properties as compared to the control, wild-type CaM or CaM-1, such that mutation of a single site appears to

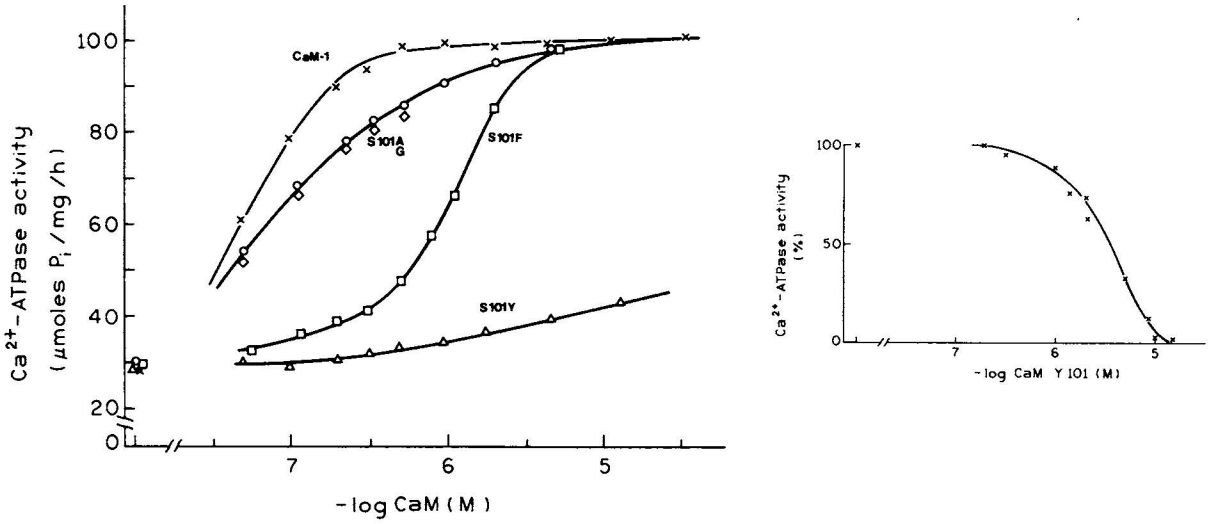

FIGURE 1 Dependence of the Ca^{2+}-ATPase activity on the concentration of the mutant CaM with substituted Ser 101: CaM-1(X), CaM-S101A(○), CaM-S101G(◇), CaM-S101F(□), CaM-S101Y(△). Ca^{2+}-ATPase activity was determined by measurement of P_i production, as described previously (6). The enzyme was 15 nM (monomeric), free Ca^{2+} was 100 nM. (*Inset*) Inhibition of the Ca^{2+}-ATPase activity by CaM-S101Y. The Ca^{2+}-ATPase activity is expressed as % of enzyme activation in the presence of 300 nM CaM-1.

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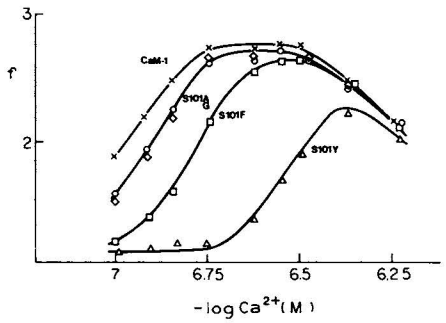

FIGURE 2 Calcium dependence of the Ca^{2+}-ATPase activity by mutant calmodulins with substituted Ser 101. The ability of different mutant CaMs to activate the enzyme over the low free Ca^{2+} is expressed as an activation factor (f). $f = V_c/V_o$, where V_c is the maximal enzyme activity in the presence of the CaM, and V_o is the maximal activity in the absence of CaM.

influence the Ca^{2+} binding properties of the other sites (7). Our experiments demonstrate that each of the Glu mutations significantly increases the concentrations of both CaM and Ca^{2+} required for full activation of the Ca^{2+}-ATPase (8). All of the mutants, however, do stimulate the enzyme to the maximum observed with control CaM. The decrease in CaM and Ca^{2+} sensitivity is strikingly dependent on the position of the mutation, being most dramatic for CaM-E140 and CaM-E104 (from 2- to 71-fold increase in K_{CaM} from CaM-E31, and -E67 to CaM-E140). Thus, like the CaM charge mutants, the most pronounced effects on RBC Ca^{2+}-ATPase activation are found with CaM Glu-mutants with modifications in the carboxyl-terminal half of the molecule. For activation of the Ca^{2+}-ATPase by the CaM dependent pathway at physiologically relevant Ca^{2+} concentrations ($\sim$100 nM) the invariant Glu in the COOH-terminal half, and especially Glu 140, is crucial. The on-going physical measurements will allow to determine which step in the enzyme activation by CaM is affected by the specific mutations. Our preliminary results show that binding of CaM-E140 to the Ca^{2+}-ATPase is reduced two-fold as compared to the wild-type CaM.

Investigation of the importance of other amino acid residues in the Ca^{2+} binding domains in CaM for Ca^{2+}-ATPase activation is in progress. CaM mutants with single substitutions for Ser 101 in the third Ca^{2+} binding domain have been selected as Ser 101 has several functions related to the stability of the loop in the Ca^{2+} bound conformation (9). Interestingly, a Ser 101 to Phe mutation occurs in CaM in a mutant Paramecium strain, which leads to a loss of Ca^{2+} dependent K^+ efflux (10). We anticipated that replacement of Ser 101 with a bulky amino acid such as Phe, which may distort the geometry of the loop or cause loss of some stabilizing interactions, could have detrimental effects on Ca^{2+} binding to CaM and/or interaction with enzyme. Four different mutations of Ser 101 (Gly, Ala, Phe, and Tyr) were tested for functional consequences with respect to activation of the Ca^{2+}-ATPase by CaM. As shown in Figs. 1 and 2 Phe causes substantial increase in the concentration of CaM required for half-maximal activation (23-fold increase in K_{CaM}) as well as a decrease in Ca^{2+} sensitivity (activation begins at about twice the Ca^{2+} concentration required for CaM-1), and Tyr totally eliminates activation of the enzyme at the physiologically relevant Ca^{2+} concentrations. The smaller Gly and Ala cause very little perturbation in CaM-enzyme interaction (Figs. 1 and 2). Competition experiments show that the CaM-S101Y mutant is a mixed agonist/antagonist and gradually inhibits enzyme activation by wild-type CaM at low Ca^{2+} concentration (Fig. 1, *inset*). These results are consistent with the proposal that the presence of Tyr or Phe at residue 101 in CaM impairs an activation step which follows binding of CaM to the enzyme.

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