Functional Diversity of Alternatively Spliced Isoforms of Drosophila Ca\(^{2+}\)/Calmodulin-dependent Protein Kinase II

A ROLE FOR THE VARIABLE DOMAIN IN ACTIVATION*

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Isoforms of calcium/calmodulin-dependent protein kinase II (CaM kinase II) from Drosophila (R1–R6 and R3A) showed differential activation by two series of mutant calmodulins, B1K–B4K and B1Q–B4Q. These mutant calmodulins were generated by changing a glutamic acid in each of the four calcium binding sites to either glutamine or lysine, altering their calcium binding properties. All mutations produced activation defects, with the binding site 4 and B1Q mutants the most severe. Activation differed substantially between isoforms. R4, R5, and R6 were the least sensitive to mutations in calmodulin, while R1, R3, and R3A were the most sensitive. Activation of R1 and R2 by B4K and activation of R3 and R3A by B2K and B2Q produced significant (6-fold and almost 3-fold, respectively) differences in \(K_{\text{act}}\) between isoforms that differ structurally by a single amino acid. These differences could not be accounted for by differential binding, as all isoforms showed almost identical binding patterns with the mutants. High binding affinity did not always correlate with ability to increase enzyme activity, implying that activation occurs in at least two steps. The isoform-specific differences seen in this study reflect a role for the COOH-terminal variable region in activation of CaM kinase II.

Calcium/calmodulin-dependent protein kinase II (CaM kinase II)\(^{1}\) is a family of related proteins with molecular masses in the range of 50–65 kDa (for review see Ref. 1). Homologs have been found in both vertebrates and invertebrates. In rat, where CaM kinase II has been most intensively studied, diversity is generated both by multiple genes coding for separate \(\alpha\), \(\beta\), \(\gamma\), and \(\delta\) isoforms, and by alternative splicing of these genes (2–5). The major variability between isoforms and between alternatively spliced forms of a single isoform occurs between the calmodulin (CaM) binding site and the association domain, where 11–41-amino acid “inserts” are found. The function of the variable region in the rat kinase is largely unknown, although early experiments have suggested that the affinity of \(\alpha\) and \(\beta\) for CaM differ (6) and a recent report demonstrates a nuclear localization signal in the \(\alpha_9\) isoform (7).

In Drosophila, where CaM kinase II is encoded by a single gene (8), up to 18 isoforms are generated by alternative splicing to form a variable region between the CaM binding and association domains (9). Table I shows the organization of sequences found in the variable regions of the seven Drosophila CaM kinase II isoforms used in this study. The variable domain is made up of sequences from two independent exons termed Insert 1 and Insert 2. In addition to these amino acid cassettes, a glutamic acid and/or an alanine can be added to the ends of the domain by use of alternative splice acceptor sites. There is no sequence homology between the Drosophila and rat variable regions.

An examination of the catalytic, assembly, and autophosphorylation properties of Drosophila isoforms R1–R6 demonstrated that these proteins are indeed CaM kinase II homologs (10). The only catalytic properties that showed any significant difference between isoforms were the \(K_m\) for peptide substrate and the \(K_{\text{act}}\) for CaM, suggesting that the variable domain may influence the interaction between CaM and the catalytic domain. To investigate the function of CaM kinase II variable domains in activation by CaM, we have studied the interaction of this set of isoforms with a set of mutant CaMs with altered calcium binding and conformational properties. The use of the Drosophila isoforms allows unambiguous correlation of functional differences with the variable domain, since the proteins are otherwise identical. While the mammalian CaM kinase II isozymes show high homology outside this domain, they are not identical.

The production of the active Ca\(^{2+}\)/CaM complex has been studied extensively. Calcium binding to the four “EF” hands of CaM produces a conformational change required for activating target enzymes (for review, see Ref. 11). The crystal structure of calmodulin has shown that there are two calcium binding sites in both the amino and carboxyl termini of CaM, which form two globular domains separated by a central linker region (12, 13). Most of the detectable conformational changes of CaM are completed upon occupation of two of the four calcium binding sites. Several studies indicate that the carboxy-terminal sites (3 and 4) bind calcium with higher affinity than the amino-terminal sites (1 and 2) and therefore binding in the carboxyl domain is the primary contributor to calcium-induced structural changes (14, 15). Site-directed mutants of Drosophila CaM have been generated, which incapacitate each calcium binding site individually (16). In each of these mutants, a conserved glutamic acid residue, which plays a critical role in calcium binding, at position 12 of one of the calcium binding loops has been mutated to either glutamine (Q) or lysine (K).
Thus the B1Q mutant carries the glutamine mutation in binding site 1, while B1K has lysine at site 1, and so on. Calcium binding properties and conformational changes of these mutants and their ability to activate four target enzymes (skeletal myosin light chain kinase (skMLCK), smooth muscle myosin light chain kinase (smMLCK), type I adenyl cyclase, and Ca2+-ATPase) have been examined (16–18). These studies have shown that, in both series of mutants, calcium binding at the mutated site is effectively eliminated over the calcium concentration range at which CaM functions and some component of the calcium-induced conformational change is also eliminated.

We have used these CaM mutants to study the activation properties of seven isoforms of Drosophila CaM kinase II. In this report we show that, although the isoforms of CaM kinase II have similar biochemical properties (10), they exhibit differential activation by mutant CaMs. The data support a role for the COOH-terminal variable regions of CaM kinase II in the mechanism of activation of this enzyme and demonstrate that stimulation of CaM kinase II catalytic activity by calmodulin is a multistep process, with separable binding and activation steps.

EXPERIMENTAL PROCEDURES

Materials—125I-Labeled bovine brain CaM and [γ-32P]ATP were purchased from DuPont NEN.

Drosophila CaM Mutants—Mutant and WT Drosophila CaM were prepared as described (16).

Drosophila CaM Kinase II Isozymes—Isoforms of Drosophila CaM kinase II were expressed by transfecting individual cDNAs into COS cells. The method for the purification of the expressed proteins has been described previously (10).

Peptide Assay for CaM Kinase II—Assays were typically done in a volume of 50 μl in the presence of 50 mM PIPES, pH 7.0, 15 mM MgCl2, 1 mM CaCl2, 1 mg/mI bovine serum albumin, 13.8 μM peptide substrate, varying concentrations of WT or mutant CaM (as indicated), and 50 μM [γ-32P]ATP. Reactions were started by adding ATP, run for 1 min at 30°C, and stopped by adding 50 μl of 10% trichloroacetic acid. The samples were microcentrifuged for 3 min and 25 μl of the supernatant was spotted onto a strip of phosphocellulose paper. The paper strips were washed for 15–30 min with water, dried, and Cerenkov radiation measured using a Beckman LS 6500 scintillation counter.

Activation Data Analysis—Each isoform of Drosophila CaM kinase II was assayed with WT and mutant CaMs. Velocity data were plotted versus CaM concentration and fit using nonlinear least squares to a Hill equation. The Hill plots indicated a single class of binding sites for CaM (19). The Hill coefficient was determined by nonlinear least squares fit using the equation for competitive binding. Logit-log (pseudo-Hill) plots indicated a single class of binding sites for CaM (19).

RESULTS

CaM Kinase II Isoforms Exhibit Different Responses to Mutant CaMs—In general all the mutant CaMs were less effective than WT in activating all the isoforms, but the degree of effectiveness differed for each isoform. The activation defects were manifested primarily as an increase in Kact. Thus for all CaM mutants where Vmax could be calculated, it was close to that of WT CaM. Table II shows the Kact and Vmax for each isoform, and Table IV gives rank orders of the Kact values of each mutant CaM for each isoform. For all isoforms, B1Q, B4K, and B4Q appeared to be the least effective activators. In the following presentation of activity results, the isoforms have been grouped into four categories based on sequence similarity (Table I) of the variable regions.

Isolforms R3 and R3A—The R3 isoform represents the "minimal" kinase and has no insert in the variable region. The R3A isoform contains a single alanine in this region (Table I). These two isoforms are nearly identical in their response to the binding site 1, 3, and 4 CaM mutants, although they differ significantly in their response to WT CaM (Ref. 10 and Table II). They both showed poor activation by B1Q, B4Q, and B4K.

The most interesting finding for this pair of isoforms is that the minor structural difference between them leads to a significant
differences in their response to binding site 2 mutants (Fig. 1). R3A appears to be equivalently activated by B2K and B2Q, while R3 is more sensitive (by a factor of 2.5) to the B2Q. R3A appears to be equivalently activated by B2K and B2Q, while R3 is more sensitive (by a factor of 2.5) to the B2Q.

Isoforms R1 and R2—R1 and R2 both contain insert 2. They differ in that R2 contains an extra alanine (Table I). As with the R3 and R3A pair of isoforms, the presence of the additional alanine in the R2 isoform is found to affect the pattern of activation by the CaM mutants. R1 and R2 respond very differently to the B4K mutant (Fig. 2). While B4K was able to activate R2 to 75% of control levels at the highest concentration of CaM (1.4 mM), it was able to activate R1 only 25%, making R1 the isoform most sensitive to the B4K mutation. This difference between R1 and R2 corresponds to a 6-fold difference in calculated $K_{act}$.

Isoform R4—The R4 isoform contains insert 1 and an additional glutamic acid at the NH$_2$ terminus of the variable region (Table I). R4 is the least sensitive isoform for the B2K mutation, showing essentially wild type activation and having a $K_{act}$ 24-fold lower than that for R3A, the most sensitive isoform (Table II). R4 is also the least sensitive to the B4K mutation, having a $K_{act}$ 13-fold lower than that of the most sensitive isoform, R1.

Isoforms R5 and R6—R5 and R6 contain both insert 1 and insert 2. They differ in that R5 contains an extra glutamic acid at the NH$_2$ terminus of the variable domain, and an extra alanine at the COOH-terminal end. R5 and R6 show an almost identical pattern of activation for the mutant CaMs, with R6 slightly more sensitive than R5 for B1Q, B2K, B3K, and B4K. This contrasts with the R3/R3A and R1/R2 pairs, where the presence of an additional alanine resulted in significant differences in responses to some mutant CaMs. As with all other isoforms, B1Q, B4K, and B4Q are the least effective in activating R5 and R6 as judged from the $K_{act}$ values (Table I). R5 and R6 are clearly the least sensitive isoforms for activation by B1K, B2Q, B3K, B3Q, and B4Q, with $K_{act}$ values that range from 2- to 7-fold lower than other isoforms.

**Activation of CaM Kinase II by Mutant CaMs**

**Table II**

| Isoform | WT $V_{max}$ | WT $K_{act}$ | B1K $V_{max}$ | B1K $K_{act}$ | B2K $V_{max}$ | B2K $K_{act}$ | B2Q $V_{max}$ | B2Q $K_{act}$ | B3K $V_{max}$ | B3K $K_{act}$ | B3Q $V_{max}$ | B3Q $K_{act}$ | B4K $V_{max}$ | B4K $K_{act}$ | B4Q $V_{max}$ | B4Q $K_{act}$ |
|---------|--------------|--------------|---------------|---------------|--------------|--------------|--------------|--------------|---------------|---------------|--------------|--------------|---------------|--------------|--------------|--------------|
| WT      | 100          | 100          | 100           | 100           | 100          | 100          | 100          | 100          | 100           | 100           | 100          | 100          | 100           | 100           | 100          | 100          |
| R1      | 98.3         | 92.4         | 89.3          | 96.3          | 97.5         | 97.5         | 100          | 100          | 100           | 100           | 100          | 100          | 100           | 100           | 100          | 100          |
| R2      | 188.22       | 88.16        | 87.13         | 122.18        | 59.15        | 33.7         | 32.9         | 100          | 100           | 100           | 100          | 100          | 100           | 100           | 100          | 100          |
| B1Q     | 100          | 100          | 100           | 100           | 100          | 100          | 100          | 100          | 100           | 100           | 100          | 100          |
| B2Q     | 205.36       | 178.41       | 435.36        | 172.16        | 120.13       | 59.14        | 70.11        | 100          | 100           | 100           | 100          |
| B3K     | 88.3         | 96.4         | 91.8          | 97.6          | 116.8        | 94.4         | 99.4         | 99.4         | 99.4         | 99.4         | 99.4         |
| B3Q     | 348.55       | 150.34       | 379.114       | 300.59        | 178.19       | 51.6         | 65.16        | 100          | 100           | 100           | 100          |
| B4K     | 3841.968     | 641.116      | 2255.704      | 3341.575      | 294.82       | 618.162      | 1078.195     | 100          | 100           | 100           | 100          |
| B4Q     | 1717.59      | 1321.157     | 1175.65       | 1393.36       | 527.67       | 235.30       | 323.41       | 100          | 100           | 100           | 100          |

$V_{max}$ is assumed to reach 100.

Calculated $K_{act}$ assuming $V_{max}$ is 100.

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**Activation of CaM Kinase II Isoforms Bind Mutant CaMs Comparably**

To determine if the differences in activation by the mutant CaMs were simply a reflection of their ability to bind the different isoforms, the capacity of the mutant CaMs to bind to CaM kinase II was studied by testing their ability to compete with $^{125}$I-labeled bovine brain CaM in a blot overlay binding assay. Fig. 3 shows a representative blot for the R2 isoform. The same experiment was done twice for each isoform with essentially identical results obtained for all isoforms. In the absence of added cold CaM, $^{125}$I-labeled CaM bound to the CaM kinase II (Fig. 3, lane 1). In the presence of a 100-fold excess of cold WT Drosophila CaM, there was virtually no binding of labeled CaM (lane 2). The mutant CaMs exhibited differential ability to compete with $^{125}$I-labeled CaM for binding to CaM kinase II (lanes 3-10). Consistent with the data for activation, B1Q, B4Q, and B4Q (lane 10) were least able to bind all isoforms of CaM kinase II, as shown by their inability to displace labeled CaM in the assay.

Densitometry measurements of autoradiograms for the different isoforms indicates that there was no inter-isofrom variation in binding for any of the mutant CaMs, with the lone exception of R4, which appears to bind B1K, B2Q, and B3K 2-3 times better than the other isoforms (Table III). This difference does not, however, markedly affect the rank order of binding of mutant CaMs for R4 (Table IV) and is not reflected in the activation data. For example, although R4 appears to bind B1K and B4K with higher affinity than do R5 and R6, they show a lower $K_{act}$ with that CaM. In cases where R4 is the isoform least sensitive to a mutation (B2K and B4K), its binding to these CaMs is identical to that of the other isoforms.

Despite the variations among the isoforms in activation by the different mutants, the rank order binding pattern for the mutants was similar for all isoforms. Table IV summarizes the rank ordered activation and binding results. A rank of 1 was given for the CaM with the best ability to compete binding of wild type CaM; a rank of 9 was given to the CaM least able to...
compete. For binding site 1, B1K is much better than B1Q. For binding site 2, B2Q is better than B2K. At binding sites 3 and 4, the K mutants are equivalent to the Q mutants. The average rank order of ability to compete with 125I-labeled CaM is: WT > B1K = B2Q > B3K = B3Q > B2K > B4K = B4Q = B1Q. Although some isoforms show minor divergence from this ranking, these differences are not capable of explaining the isoform-specific effects seen with activation (e.g. the effect of B4K on R1 and R2 and the effect of binding site 2 mutants on R3 and R3A).

In general, the data in Table IV indicate that solid phase binding and activation rank orders are correlated; mutant CaMs that bind well also activate well. The two exceptions are B2K and B2Q, where exactly the opposite is seen. B2K activates well but binds poorly, while B2Q activates poorly, but apparently binds better than B2K in the solid phase binding assay.

The solid phase binding assay, however, can only determine relative binding of the mutant CaMs to denatured CaM kinase isoforms. To confirm that the results of the binding site 2 mutants was valid for native kinase, equilibrium solution binding was used to determine absolute Ki values for WT, B2K, and B2Q binding to the R3 isoform. WT Ki was determined to be 9.9 ± 2.3 nM, which is within the range of previously reported values for the rat α isozyme (1). The Ki values for the binding site 2 mutants were significantly larger, with B2K Ki = 186.9 ± 6.5 nM and B2Q Ki = 90.2 ± 5.5 nM. Values are expressed as mean ± S.E. These results replicate the order of binding seen in the solid phase assay with B2K having a significantly higher Ki than B2Q.

DISCUSSION

The data presented in this study provide information on the activation of CaM kinase II by calmodulin which can be interpreted at two levels. First, taken as a whole, the data are informative about the requirements of CaM kinase II for structural and functional properties of CaM, and about the mechanism of activation of CaM kinase II. This information comes...
Activation of CaM Kinase II by Mutant CaMs

The ability of CaM to compete for binding with 125I-labeled bovine brain CaM is shown. The values obtained from densitometric scanning of autoradiograms of WT and mutant CaMs were first normalized against the value for binding in the absence of any added cold CaM (this is 100% binding of labeled CaM). Data are expressed as a ratio of these values to the value for WT CaM. A value of 1 indicates binding equivalent to WT CaM; a value of 0 indicates no binding. Two complete binding experiments were done with equivalent results. Data from one set of experiments are shown.

### TABLE III

| Isoform | WT | B1K | B1Q | B2K | B2Q | B3K | B3Q | B4K | B4Q |
|---------|----|-----|-----|-----|-----|-----|-----|-----|-----|
| R1      | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| R2      | 1.0 | 0.35 | 0.10 | 0.10 | 0.35 | 0.17 | 0.17 | 0.07 | 0.09 |
| R3      | 1.0 | 0.30 | 0.06 | 0.10 | 0.30 | 0.20 | 0.13 | 0.06 | 0.07 |
| R4      | 1.0 | 0.25 | 0.11 | 0.09 | 0.39 | 0.25 | 0.23 | 0.08 | 0.13 |
| R5      | 1.0 | 0.40 | 0.06 | 0.12 | 0.30 | 0.14 | 0.14 | 0.05 | 0.10 |
| R6      | 1.0 | 1.20 | 0.05 | 0.07 | 0.80 | 0.42 | 0.12 | 0.04 | 0.06 |
| R7      | 1.0 | 0.23 | 0.05 | 0.17 | 0.40 | 0.10 | 0.22 | 0.05 | 0.09 |
| R8      | 1.0 | 0.28 | 0.05 | 0.16 | 0.33 | 0.40 | 0.22 | 0.04 | 0.10 |

### TABLE IV

Rank order of isoform activation and binding by mutant CaMs

For each CaM kinase II isoform, the ability of WT and mutant CaMs to activate and bind to a given isoform was ranked based on K_{act} and densitometric comparison with WT binding respectively. For activation, a rank of 1 is given to the CaM with the lowest K_{act}, and a rank of 9 to the CaM with the highest K_{act}. For binding, a rank of 1 is given to the CaM best able to displace binding by 125I-labeled bovine CaM and a rank of 9 to the CaM showing the least ability to compete labeled CaM. In cases where two mutants activate or bind an isoform equivalently, they are given the same ranking. Ranks are presented as "activation rank/binding rank" in the table.

| Isoform | WT | B1K | B1Q | B2K | B2Q | B3K | B3Q | B4K | B4Q |
|---------|----|-----|-----|-----|-----|-----|-----|-----|-----|
| R1      | 1/1 | 3/2 | 7/6 | 2/6 | 5/2 | 3/4 | 6/4 | 9/9 | 8/8 |
| R2      | 1/1 | 3/2 | 7/6 | 2/6 | 5/2 | 5/4 | 4/5 | 8/8 | 9/7 |
| R3      | 1/1 | 2/3 | 8/7 | 2/6 | 6/2 | 4/3 | 5/5 | 9/9 | 7/6 |
| R4      | 1/1 | 2/2 | 8/8 | 5/6 | 3/4 | 6/4 | 9/9 | 7/7 |
| R5      | 1/1 | 3/1 | 8/9 | 6/2 | 6/2 | 4/4 | 6/7 | 8/8 | 8/7 |
| R6      | 1/1 | 2/3 | 9/8 | 2/5 | 6/2 | 4/6 | 5/4 | 8/8 | 7/7 |

from studying the rank orders of activation and binding and drawing conclusions based on the general properties of the population of isoforms. Second, the data provide evidence for distinct mechanisms of activation for the alternatively spliced isoforms of Drosophila CaM kinase II. These conclusions are based on examining absolute, rather than relative levels of activation, and from analyses of cases where specific isoforms behave differently than the population as a whole.

Role of Calmodulin's Calcium Binding Sites in Activation of CaM Kinase II

Studies of the BK and BQ series of Drosophila CaM mutants have established that all of these mutants have greatly decreased calcium binding at the mutated site and some defect in calcium-induced conformational changes. In a previous study (20), three target enzymes of CaM, skMLCK, smMLCK, and type I adenyl cyclase showed similar patterns of activation by the BK and BQ series CaM mutants. With the exception of poor activation of skMLCK by B1Q, mutations at site 4 were found to affect activation most severely followed by mutations at site 2 and site 3, and finally site 1. In earlier studies of UV circular dichroism (17), gel electrophoresis, and calcium binding (16), a similar ranking of the sites emerged.

For the CaM kinase II isoforms, if one ranks the K_{act} values for each isoform for all the mutant CaMs and the binding effectiveness of each mutant for each isoform (Table IV), one finds that the same order is followed as for the other effector enzymes with two notable exceptions. First, the B1Q mutant is almost as detrimental for activation as mutations in binding site 4 (as was seen with skMLCK). B1Q also performs poorly in the binding assay. This is in contrast to B1K, which is both a good activator and able to compete effectively in the binding assay.

### Activation of CaM Kinase II Is a Multistep Process

Although for most of the mutant CaMs, there is good correspondence between their ability to bind CaM kinase II and to activate the enzyme, the site 2 mutants are interesting in that they do not show this correlation. B2K does not bind well to any of the isoforms, yet it can activate some isoforms very well. Conversely, although B2Q binds CaM kinase II better than B2K, it is unable to activate the same isoforms to a similar extent. This is clearly seen in a comparison of the rank order of activation by B2K and B2Q with the rank order of CaM binding (Table IV). Dissociation of binding and activation is also seen with the R4 isoform, which binds to B1K, B2Q, and B3K better than any of the other isoforms, yet does not show enhanced activation by these CaMs (Tables II and III). This lack of correlation between binding and increased enzyme activity implies that activation of CaM kinase II consists of at least two steps: binding of CaM to the target and subsequent stimulation of enzyme activity by the CaM/target complex. In this context, complexes formed between the CaM kinase II isoforms studied here and mutants B2K and B2Q could be very informative with regard to the mechanism of activation of this enzyme.

Role of the Variable Domain in Activation

The general properties of Drosophila CaM kinase II can be inferred from the rank orders of K_{act} and CaM binding for the mutant CaMs. While consensus emerges from such an analysis, there are indications that there are also isoform-specific effects for each of the mutant CaMs as measured by K_{act}. There are also some cases where a particular isoform diverges from the general rank order for activation.

Analysis of the Effects of Isoform Variability on Activation by Binding Site 1 Mutants—Activation of the seven isoforms of Drosophila CaM kinase II tested in this study was adversely affected by all of the mutant CaMs in the K and Q series. The CaM with the most normal activation kinetics, B1K, still showed an increase of 1.5–14-fold in K_{act}, depending on the isoform assayed. Structural modeling studies have indicated that at site 1, the mutation to lysine may be neomorphic and elicit some of the conformational changes normally induced by calcium (16). This meshes well with the activation data from four CaM target enzymes studied earlier, where it was shown to have a K_{act} only 1.5–2-fold greater than WT CaM (20). Thus the characteristics of B1K make it the mutant whose activation properties are most comparable to WT CaM.

All the isoforms of CaM kinase II studied here showed nearly maximal stimulation by this mutant CaM at micromolar concentrations. The K_{act} values, however, differed from those seen with WT CaM and showed wider variation than did the other CaM effector enzymes tested previously. Since K_{act} and V_max for WT CaM was virtually identical for all the isoforms (with the exception of R3A, which in other studies with WT bovine CaM had a significantly higher K_{act} value than other isoforms; Ref. 10), it seems likely that the variations in activation by B1K are representative of some level of variable region-specific differences among the isoforms.

The B1Q mutation has much more drastic effects on activation and binding than does the B1K mutation. K_{act} values range from 42- to 162-fold WT. Interestingly, although B1Q is one of the worst activators of Drosophila CaM kinase II, it is the least able to discriminate between isoforms. The difference between K_{act} values for the best and worst isoform is less than...
4-fold. For all of the other mutants, the difference between best and worst is on the order of 10–20-fold. Since the B1K mutant may be neomorphic (17), the role of binding site 1 is probably best understood by examining the data for the B1Q mutant. This suggests that calcium binding at site 1 and/or the conformational changes dependent on site 1 do not interact significantly with the variable domain of CaM kinase II. The defect in activation for CaM kinase II may be a direct result of the decreased ability of the B1Q mutant to bind, and may be independent of variable domain influence.

Analysis of the Effects of Isoform Variability on Activation by Binding Site 2 Mutants—For binding site 2, enzyme activity data shows clear effects of isoform variability. B2K $K_{\text{act}}$ values range from 1- to 10-fold WT, while for B2Q they range from 3- to 54-fold WT. For B2Q and B2O, three classes of activation patterns emerge. The first of these is R5 and R6 (which have both insert 1 and insert 2), which are relatively unaffected by both the mutations; the second class is R1, R2, R3, and R4 (having either insert 1 or insert 2 or no insert), which are more severely affected by B2Q and relatively unaffected by B2K; and the third is R3A (no insert; additional alanine), which is poorly activated by both the mutants. Thus mutations in binding site 2 not only separate binding and activation (see above), they can also discriminate between isoform variable regions.

Analysis of the Effects of Isoform Variability on Activation by Binding Site 3 Mutants—The binding site 4 mutants are all the worst activators of CaM kinase II activation. The only notable differences among isoforms is that R5 and R6 (which both contain insert 1 and insert 2) are relatively less affected than the other isoforms (which have single or no inserts).

Analysis of the Effects of Isoform Variability on Activation by Binding Site 4 Mutants—The binding site 4 mutants are overall the worst activators of CaM kinase II, with $K_{\text{act}}$ values ranging from 13- to 145-fold WT for B4Q and from 30- to 295-fold WT for B4K. The most dramatic discrimination between isoforms is the difference seen in activation of R1 and R2, both of which contain insert 2, with R2 having an extra alanine. R1 and R2 are activated equivalently by B4Q, but activation by B4K is quite different, with the calculated $K_{\text{act}}$ of R1 being 6-fold higher than the $K_{\text{act}}$ of R2. A difference of this magnitude implies that the addition of an alanine residue to the end of the variable domain has significant structural consequences, and that the variable domain is an active participant in stimulation of enzyme activity by the CaM target complex.

**Importance of Isoform-specific Interactions**

The interaction of CaM kinase II with CaM is unusual. Most Ca$^{2+}$/CaM effectors interact with CaM with subnanomolar-nanomolar affinities. CaM kinase II in its unphosphorylated state shows dissociation constants in the 25- to 100-nM range (1, 21). This contrasts with data for peptides based on the rat CaM kinase II CaM binding domain, which have a $K_D$ of 0.1–0.3 nM (1). For the native rat cytoskeletal CaM kinase II, the binding constant for 125I-CaM was found to be >10-fold less than $K_{\text{act}}$ (22). In addition, autophosphorylation can modulate the affinity of CaM kinase II for CaM, increasing affinity by 3 orders of magnitude (21). There is evidence that regions of skMLCK that are outside the CaM binding region interact with the first domain of CaM (23). Mutations that affect smMLCK activation but not binding have also been characterized (24). Determinants other than the core CaM binding sequence are therefore very important to the binding of Ca$^{2+}$/CaM by CaM kinase II. Using site-directed CaM mutants, we have shown here that isoforms of CaM kinase II that are structurally different show differences in their mechanisms of activation.

As indicated by the above analyses, the correlation between the structure of the isoform and its activation is not uniform for different binding site mutants or for different mutations at the same binding site. If one examines these relative sensitivities, however, several generalizations can still be made. First, R1, R3, and R3A are usually the most sensitive to CaM mutations. Second, R4, R5, and R6 are usually most insensitive. R2 is quite variable, sometimes among the most sensitive, sometimes among the least. The differential sensitivity of the isoforms to mutations in the high or low affinity calcium binding sites could be indicative of selectivity in the pattern of isoforms activated at different subsaturating intracellular calcium concentrations. This diversity in activation of the Drosophila isoforms by mutant CaMs indicates that sequence variability in this region may regulate activation of CaM kinase II. Examination of the distribution of the kinase isoforms by mRNA in situ hybridization to adult tissue has revealed no remarkable differences (9). The functional in vivo consequences of the regulatory diversity we have demonstrated remain to be determined.

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