The assembly of 6–12 subunits of Ca\(^{2+}\)/calmodulin-dependent kinase II (CaM kinase II) into holoenzymes is an important structural feature of the enzyme and its postulated role as a molecular detector of Ca\(^{2+}\) oscillations. Using single cell reverse transcriptase-polymerase chain reaction, we show that \(\alpha\)- and \(\beta\)-CaM kinase II mRNAs are simultaneously present in the majority of hippocampal neurons examined and that co-assembly of their protein products into heteromers is therefore possible. The subunit composition of CaM kinase II holoenzymes was analyzed by immunoprecipitation with subunit-specific monoclonal antibodies. Rat forebrain CaM kinase II consists of heteromers composed of \(\alpha\) and \(\beta\) subunits at a ratio of 2:1 and homomers composed of only \(\alpha\) subunits. We examined the functional effect of the heteromeric assembly by analyzing the calmodulin dependence of autophosphorylation. Recombinant homomers of \(\alpha\)- or \(\beta\)-CaM kinase II, as well as of alternatively spliced \(\beta\) isoforms, have distinct calmodulin dependences for autophosphorylation based on differences in their calmodulin affinities. Half-maximal autophosphorylation of \(\alpha\) is achieved at 130 nM calmodulin, while that for \(\beta\) occurs at 15 nM calmodulin. In CaM kinase II isolated from rat forebrain, however, the calmodulin dependence for autophosphorylation of the \(\beta\) subunits is shifted toward that of \(\alpha\) homomers. This suggests that Thr\(^{287}\) in \(\beta\) subunits is phosphorylated by \(\alpha\) subunits present in the same holoenzyme. Once autophosphorylated, \(\beta\)-CaM kinase II traps calmodulin by reducing the rate of calmodulin dissociation.

Multifunctional Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaM kinase II) is a major mediator of Ca\(^{2+}\) action whose activation and autophosphorylation appear to regulate numerous neuronal processes, including two forms of synaptic plasticity, long term potentiation and long term depression (1, 2). These two opposing changes in synaptic strength are differentially regulated by the frequency of stimulation of hippocampal neurons. Interestingly, CaM kinase II has been suggested to be a molecular detector of the Ca\(^{2+}\) spike frequency, based on its unique structural and regulatory properties (3) (reviewed in Refs. 4 and 5). The neuronal CaM kinase II consists of two major subunits of 52 and 60 kDa that are encoded by \(\alpha\)- and \(\beta\)-CaM kinase II genes, respectively. Additional isoforms are generated by alternative splicing of these as well as of the ubiquitous \(\gamma\) and \(\delta\)-CaM kinase II genes (6–10). All of the subunits share a common organization of functional domains and of an ultrastructural arrangement into holoenzymes (4). The C-terminal association domains of 6–12 subunits assemble into a central globular structure from which the N-terminal catalytic/regulatory domains extend radially like petals of a flower (11). This unusual structure positions the catalytic/regulatory domains for an intersubunit autophosphorylation that is dependent on the frequency of Ca\(^{2+}\) oscillations (3, 12).

Upon activation by Ca\(^{2+}\)/calmodulin, Thr\(^{286}\) in the regulatory domain of \(\alpha\) subunits (or Thr\(^{287}\) in \(\beta\) subunits) is rapidly phosphorylated. Autophosphorylation of Thr\(^{286}\) on \(\alpha\) subunits has three consequences: (a) calmodulin remains bound to the phosphorylated subunit for extended periods of time even at low Ca\(^{2+}\) concentrations (trapped state), because the autophosphorylation greatly reduces the calmodulin disassociation rate (13); (b) autophosphorylated \(\alpha\) and \(\beta\) subunits are rendered Ca\(^{2+}\)/calmodulin-independent (autonomous) (reviewed in Ref. 4); and (c) the kinase attains an enhanced affinity for NMDA receptors in postsynaptic densities, a submembranous neuronal specialization (14–16). Both autonomy and calmodulin trapping enable the phosphorylated kinase subunits to remain active beyond the limited duration of a Ca\(^{2+}\) spike. The multimeric structure of CaM kinase II is important for the regulation of the kinase by autophosphorylation, since the phosphorylation of Thr\(^{286}\)/Thr\(^{287}\) occurs by an intersubunit reaction within each holoenzyme (12, 17–19). The catalytic domain of one activated subunit in the holoenzyme phosphorylates Thr\(^{286}\)/Thr\(^{287}\) in the regulatory domain of a neighboring subunit that must also have calmodulin bound. Therefore, assembly into holoenzymes concentrates the subunits and positions them for autophosphorylation.

The multimeric structure of CaM kinase II, the intersubunit phosphorylation of Thr\(^{286}\)/Thr\(^{287}\), and the resulting calmodulin trapping and autonomous activity are crucial elements that enable the kinase to act as a Ca\(^{2+}\)-spike frequency detector (3, 5, 13). Under conditions of limited free calmodulin, each Ca\(^{2+}\) spike activates only a subset of CaM kinase II subunits in a holoenzyme, some of which become autophosphorylated and trap their bound calmodulin. If the interval between spikes is brief, subsequent Ca\(^{2+}\) spike will lead to binding of calmodulin to holoenzymes that still retain calmodulin from a previous spike, increasing the calmodulin occupancy and probability of neighboring subunits with calmodulin bound and therefore the probability of autophosphorylation. A threshold frequency is reached, beyond which autophosphorylation and calmodulin trapping become increasingly more efficient and the number of activated subunits increases. Although autophosphorylation is not necessary for activation of the kinase in vitro, it may be necessary for appropriate activation in response to brief repet-
itive stimulation in vivo. The essential nature of this autophosphorylation was nicely demonstrated with the finding that mice defective in autophosphorylation of Thr286 in α-CaM kinase II because of a point mutation that substituted an Ala286 for Thr286 do not exhibit long term potentiation, are defective in spatial learning, and have unstable hippocampal place cells (20, 21).

In light of these findings, the subunit composition of CaM kinase II holoenzymes becomes very significant. Since inter-subunit phosphorylation of Thr286/287 involves two calmodulin-bound subunits, the kinetic properties of the neighboring subunits in the holoenzyme serving as kinase and as substrate in each reaction are important variables. The frequency dependence for activation of homomers of α or β isoforms, for example, is markedly different (3). In an electron microscopic study, forebrain CaM kinase II holoenzymes were found to exist as homomeric α decamers and homomeric β octamers with no evidence of heteromers (11). However, other studies reported that α-specific monoclonal antibody (mAb) co-immunoprecipitated some β-CaM kinase II together with α-CaM kinase II from either rat forebrain (22) or rat cerebellum (23). Chicken forebrain CaM kinase II purified on an affinity column of from either rat forebrain (22) or rat cerebellum (23). Chicken CaM kinase II occurs at a 5- and 4-fold lower calmodulin level, respectively, than that reported for Thr286 in α isoforms (24).

CaM kinase II subunits differ in their calmodulin dependence, their rates of autophosphorylation, and/or their assembly of CaM kinase II subunits will have functional consequences if α and β subunits differ in their calmodulin dependence, their rates of autophosphorylation, and/or their ability to trap calmodulin. Differences in orientation of catalytic/regulatory domains between homomeric and heteromeric assembly of association domains may also alter rates of inter-subunit autophosphorylation. For example, half-maximal activation and autophosphorylation of recombinant β-CaM kinase II occurs at a 5- and 4-fold lower calmodulin level, respectively, than recombinant α-CaM kinase II (3, 25). It is not known whether α and β isoforms differ in trapping of calmodulin, since this has been demonstrated for recombinant α-CaM kinase II (13) but has not been assessed for β-CaM kinase II.

To study the functional properties of CaM kinase II holoenzymes, we analyzed the subunit composition of CaM kinase II holoenzymes from different preparations by immunoprecipitation with subunit-specific mAbs. Our results indicate that CaM kinase II can be composed of α:β heteromers with variable subunit ratios in addition to homomers of either α or β subunits. We therefore compared the autophosphorylation of homomers and heteromers composed of α, β, and alternatively spliced β subunits. Indeed, the calmodulin dependence is modified based on the subunit composition. Finally, plasmam surface resonance studies showed that autophosphorylated β subunits trap calmodulin and would contribute to prolonging the active state of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials—**γ-32P-ATP (5000 Ci/mmol) was purchased from ICN (Aurora, OH). Bovine brain calmodulin was obtained from Oceanic Biologic (Edmonds, WA). CaM kinase II substrate peptide autocamtide-3 was synthesized and purified by David King (University of California, Berkeley). Forebrain CaM kinase II was extracted and purified essentially as described (26). Recombinant CaM kinase II proteins were prepared by transient transfections of COS-7 cells with pSRa.BKS containing full-length cDNA for CaM kinase II isoforms (7, 27, 28). Protein extracts of transfected COS-7 cells were prepared, and the recombinant CaM kinase II was purified as described (29). Myosin light chain kinase (MLCK) from gizzard smooth muscle was purified as described (30).

**Single Cell RT-PCR Analysis—**In visualized rat hippocampal slice preparations, the single cell cytoplasms of CA1 pyramidal neurons, identified by morphology and electrophysiological recording, were aspirated for the recording, and then subjected to RT-PCR as described previously (31). For each CaM kinase II isoform, two sets of nested PCR primers were designed. These primers span the variable domain and can be used to distinguish between alternatively spliced isoforms based on the size of the PCR products. The outer and inner set of primers are as follows: a, base pairs 898–923 to 1342–1368 and base pairs 901–923 to 1561–1534 and 910–933 to 1338–1362; b, base pairs 901–926 to 1495–1515 and 910–933 to 1293–1317; c, base pairs 916–926 to 1444–1470 and 910–933 to 1248–1272. The first round of amplification included all four outer primer pairs, for α, β, γ, and δ isoforms. The entire cDNA produced from a single cysotol was subjected to 30 cycles of capillary PCR (Idaho Technologies, Idaho Falls, ID). The product amplified in the first round was diluted 100-fold into a second round of amplification involving separate capillary PCR reactions for each of the four isoforms using the inner primer pairs. PCR products were separated on 2% agarose gel and visualized by ethidium bromide staining.

**Immunoprecipitation Experiments—**CB2α and CB1β are subunit-specific mAbs for α- and β-CaM kinase II, respectively (35). All immunoprecipitations were carried out in radiosimmuno precipitation buffer (50 mM Tris/HCl, pH 8, 150 mM NaCl, 0.1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS containing 10 µg/ml leupeptin. Approximately 100 ng of purified CaM kinase II was incubated with the mAbs indicated in the text in a final volume of 0.5 ml for 1 h at 4 °C. Both CB2α and CB1β mAbs were diluted 1:2000. Subsequently, 40 µl of protein A beads (Repligen, Cambridge, MA) were added, and the incubation was continued for another 1 h at 4 °C. The beads were centrifuged and the supernatant was transferred to a fresh tube. A second, similar round of immunoprecipitation was initiated by the addition of mAb to the supernatant of the first round, followed by protein A beads as described above. The beads were washed three times with cold PBS containing 0.25% Nonidet P-40. After the final wash, the beads were resuspended in SDS-containing sample buffer and heated at 85 °C for 4 min, and the supernatants were loaded on 10% SDS-polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane (0.45 µm; Schleicher & Shuell), and CaM kinase II isoforms were visualized by calmodulin overlay assay using biotinylated calmodulin and enhanced chemiluminescence (8). The film was scanned and analyzed for the relative intensities of the bands using MacBas software (FUJIX, Stamford, CT).

**CaM Kinase II and Calmodulin Interaction by Surface Plasmon Resonance—**The interaction between immobilized calmodulin and several CaM kinase II isoforms was measured using a BIAcore
Subunit Composition of CaM Kinase II Holoenzymes

**Table I**

| Gene expression pattern (PCR signal) | α | αN | β | β' | βa | βb | δ | No. of single neurons |
|-------------------------------------|---|----|---|----|----|----|---|----------------------|
| A                                   | + | -  | + | -  | +  | -  | - | 7                    |
| B                                   | + | -  | - | +  | -  | +  | - | 2                    |
| C                                   | + | -  | + | -  | -  | +  | - | 1                    |
| D                                   | + | +  | + | -  | -  | -  | - | 1                    |
| E                                   | + | -  | - | +  | -  | +  | - | 1                    |
| F                                   | + | -  | - | +  | -  | +  | - | 10                   |
| G                                   | - | -  | - | +  | -  | +  | - | 1                    |
| H                                   | - | -  | - | -  | +  | -  | - | 1                    |
| Total                               | 22/24 | 1/24 | 9/24 | 2/24 | 4/24 | 0/24 | 24                   |

**Fig. 1.** Expression of α- and β-CaM kinase II mRNAs in individual hippocampal neurons. Rat brain mRNA and two different single cell cytoplasms (PP18 and PP1) served as templates for nested RT-PCR. Amplification products, corresponding to the variable domain of CaM kinase II isoforms, were separated on 2% agarose gel and stained with ethidium bromide. The identification of alternatively spliced CaM kinase II isoforms was based on their size (size marker is shown on the left).

**Results**

**Co-expression of α- and β-CaM Kinase II mRNAs in Single Neurons**—Although α and β subunits have been reported to reside on separate homomers in rat brain (11), in *vitro* co-transfections of α and β cDNAs into mammalian cells resulted in heteromeric CaM kinase II (18, 25). In light of these conflicting data, we examined the expression pattern of CaM kinase II isoforms in individual hippocampal neurons by single cell RT-PCR to determine whether CaM kinase II mRNAs are co-expressed in *vitro*. PCR primers that span the variable domain of each of the four CaM kinase II subunits were used in order to identify alternatively spliced CaM kinase II isoforms with different insertions and deletions in their variable domain. As shown in Fig. 1a, multiple CaM kinase II isoforms were detected in rat brain RNA: two α isoforms (α9 and α7), four β isoforms (β, β', βa, βb, and δ isoforms (δ, δa, δb, δc) (9)). We next studied the expression of CaM kinase II isoforms in single neurons. The expression pattern of CaM kinase II mRNAs in all the single neurons tested is summarized in Table I. In all of the individual neurons tested, mRNAs were detected for either α- or β-CaM kinase II but not for γ- or δ-CaM kinase II (Fig. 1 and Table I).

**Table I**

| Gene expression pattern (PCR signal) | α | αN | β | β' | βa | βb | δ | No. of single neurons |
|-------------------------------------|---|----|---|----|----|----|---|----------------------|
| A                                   | + | -  | + | -  | +  | -  | - | 7                    |
| B                                   | + | -  | - | +  | -  | +  | - | 2                    |
| C                                   | + | -  | + | -  | -  | +  | - | 1                    |
| D                                   | + | +  | + | -  | -  | -  | - | 1                    |
| E                                   | + | -  | - | +  | -  | +  | - | 1                    |
| F                                   | + | -  | - | +  | -  | +  | - | 10                   |
| G                                   | - | -  | - | +  | -  | +  | - | 1                    |
| H                                   | - | -  | - | -  | +  | -  | - | 1                    |
| Total                               | 22/24 | 1/24 | 9/24 | 2/24 | 4/24 | 0/24 | 24                   |

**Subunit Composition of CaM Kinase II Holoenzymes**—We analyzed the subunit composition of forebrain CaM kinase II preparation using subunit-specific mAbs in order to assess whether α and β subunits assemble as homomers or heteromers. The specificity of the mAbs under our immunoprecipitation conditions was first verified with purified recombinant α and β homomers. a-mAb (CBα2) immunoprecipitated a protein but not β protein, whereas β-mAb (CBβ1) immunoprecipitated only β protein (Fig. 2a). Furthermore, mixing the two recombinant homomeric isoforms before the addition of the mAbs did not lead to subunit exchange or nonspecific immunoprecipitation, since each antibody precipitated only its cognate subunit (Fig. 2b, lanes 1 and 3). This control experiment demonstrates that successive use of the antibodies can clearly separate the two subunits when each isoform resides on a distinct homomeric holoenzyme.

Purified rat forebrain CaM kinase II preparation was sub-
of the α protein was co-immunoprecipitated with β protein using β-mAb in the first round of immunoprecipitation (Fig. 3b, lanes 5 and 7). The addition of α-mAb to the supernatant did not precipitate a detectable amount of α protein (Fig. 3b, lane 8), suggesting that with a high β:α ratio, all of the available α subunits co-assembled with β subunits, in this case as hetero-
meric CaM kinase II with an α:β ratio of 1:3–4. The remaining β subunits assembled as β homomers. Immunoprecipitation experiments using crude protein extracts from rat forebrain and from transfected COS-7 cells gave similar results (data not shown), suggesting that the subunit composition reported above was not biased by selective purification of one or another holoenzyme type.

The CaM kinase II holoenzymes are stable even under harsh immunoprecipitation conditions. Although the enzymatic activity of kinase was diminished in the immunoprecipitation buffer (data not shown), probably due to the presence of 0.1% SDS, the interactions among the association domains in the holoenzyme persisted. The data suggest that there is little subunit exchange between holoenzymes in vitro, since mixing preparations of α and β homomers did not lead to formation of detectable levels of heteromers (Fig. 2b).

**Calmodulin Dependence of Homomorphic α- and β-CaM Kinase II**—Given the importance of the autophosphorylation state of CaM kinase II in neuronal plasticity (2, 20), it is important to characterize the calmodulin dependence for autophosphorylation, since it is likely that calmodulin is limiting in situ (37, 38) and that autophosphorylation is therefore dependent on the differential calmodulin affinity of the isoforms. We began by studying the calmodulin dependence of autophosphorylation in homomers of α or β subunits. Autophosphorylation reactions of purified, recombinant homomers of α or β isoforms were carried out in the presence of radiolabeled ATP and increasing calmodulin concentrations at saturating levels of Ca2+. The reactions were performed in the presence of high ATP concentrations and for short incubation times, conditions that have been previously shown to favor autophosphorylation of Thr286 in α-CaM kinase II and Thr287 and Thr282 in β-CaM kinase II (39, 40). The autophosphorylated kinase was visualized by autoradiography (Fig. 4a), and the relative intensity of autophosphorylation at each calmodulin concentration was quantified and plotted for each isoform (Fig. 4b). Under these experimental conditions, the recombinant Isoform reaches half-maximal autophosphorylation at approximately 15 nm calmodulin, whereas the recombinant α isomorph requires about 8-fold more calmodulin (EC50 = 130 nM).

The assessment of calmodulin sensitivity for autophosphorylation was extended to purified recombinant homomers of three alternatively spliced β isoforms: β′, β″, and β‴ (Fig. 5). Both β′ and β‴ isoforms lack exon IX, whereas β″ and β‴ isoforms lack exon VI (Fig. 5a). Interestingly, whereas the calmodulin sensitivity for autophosphorylation of β‴ is not significantly different from β‴, with an EC50 of 17 nm calmodulin, the sensitivity of the two embryonic isoforms is different (Fig. 5b). Recombinant β′ and β‴ isoforms have an EC50 of 35 and 50 nm calmodulin, respectively. The calmodulin sensitivity for substrate phosphorylation was also examined for all CaM kinase II isoforms using autogamidate-3 as exogenous substrate (data not shown). The EC50 values of the recombinant embryonic isoforms are approximately 3-fold higher than those of the adult β and β′ isoforms and 2-fold lower than that of the adult α isoform. Taken together, the results indicate that β′ and β‴, the two isoforms that lack exon IX, have a different dependence on calmodulin for either substrate phosphorylation or autophosphorylation from that of the α and β isoforms.

**Autophosphorylation in Heteromers of α- and β-CaM Kinase**

![Image](image_url)
II—If some neuronal CaM kinase II is composed of heteromeric species, and autophosphorylation requires that calmodulin be bound to both subunits involved in the intersubunit reaction (12, 41), it is possible that some autophosphorylation reactions will involve an α subunit serving as kinase to phosphorylate a β subunit serving as substrate, and vice versa. Whereas autophosphorylation in homomers involves identical subunits with either a lower (α) or higher (β) affinity for calmodulin, phosphate incorporation into either an α or β subunits resident in heteromers will be affected by the calmodulin sensitivity of their neighboring subunits in the holoenzyme, i.e. will depend on the subunit composition of the holoenzyme. Such a dependence can, in turn, be used as an alternative to co-immunoprecipitation studies in assessing the subunit composition of rat forebrain CaM kinase II.

We autophosphorylated purified forebrain CaM kinase II in the presence of increasing calmodulin concentrations and measured autophosphorylation into either the α or β subunits in comparison with the calmodulin dependence in recombinant homomers or heteromers (Fig. 6). Phosphate incorporation into the α subunit of forebrain CaM kinase II has a similar calmodulin dependence as autophosphorylation in recombinant α-CaM kinase II homomers (Fig. 6a). By contrast, the calmodulin dependence for the phosphorylation of the β subunit in the forebrain enzyme differs from that seen in recombinant β homomers; the curve is right-shifted toward that of the less sensitive α isoform (Fig. 6a). For example, at 40 nM calmodulin, homomeric β-CaM kinase II reaches 85% maximal autophosphorylation, while forebrain β-CaM kinase II shows only 55% maximal autophosphorylation. As a control, we mixed recombinant, homomeric α and β preparations before performing the autophosphorylation reactions. The calmodulin dependence of neither isoform changed significantly, indicating little or no interholoenzyme autophosphorylation (data not shown).

These results are consistent with the immunoprecipitation experiments (Fig. 3) that indicated that all forebrain β subunits are co-assembled as heteromers with an α:β ratio of 2:1. In such heteromers, the β-subunits will be largely co-assembled with α subunits (Fig. 6a, inset). The intersubunit phosphorylation of Thr287 in the β subunit would therefore be dependent on calmodulin binding properties of the neighboring, lower affinity, α subunits. This is predicted to produce a shift of the β subunit phosphorylation to higher calmodulin concentrations and, in fact, was what was observed (Fig. 6a).

In heteromers with a preponderance of β subunits, the phosphorylation of α subunits should reflect the higher calmodulin affinity of the β subunits. We therefore tested the recombinant α and β CaM kinase preparation that was used in the immunoprecipitation experiments. The calmodulin dependence for autophosphorylation of the α and β subunits for either homomers or heteromers is shown in Fig. 6b. In this case, the calmodulin dependence for phosphorylation of β subunits co-expressed with α subunits is similar to that seen for homomeric β-CaM kinase II. However, the calmodulin dependence of α subunit phosphorylation in the heteromers is strongly shifted toward that of the β subunits, suggesting that the β subunits are co-assembled with α subunits. All of the recombinant α subunits in this preparation are co-assembled with β subunits (Fig. 6b). With an α:β ratio of 1:3–4 in an average holoenzyme and assuming that there is no preference for like subunits to be positioned as neighbors in the holoenzyme, there is a high probability that most α subunits would be phosphorylated by a β subunit in the recombinant heteromer (Fig. 6b, inset).

**Consequences of Autophosphorylation of β-CaM Kinase II Isoforms**—We have previously shown that autophosphorylation of Thr287 of rat forebrain CaM kinase II and recombinant α-CaM kinase II greatly increases the affinity of the α subunit for calmodulin, trapping calmodulin for prolonged periods be-
cause of a marked increase in calmodulin dissociation time (t) (13). In those experiments, the interaction between dansyl-calmodulin and α-CaM kinase II was detected using fluorescence emission anisotropy, but the interaction of recombinant β-CaM kinase II with calmodulin was not studied. Since β subunits constitute a significant fraction of CaM kinase II in brain and even predominate in regions such as the cerebellum (23), it is important to know if phosphorylation of the β subunits also produces the high affinity calmodulin binding mode referred to as calmodulin trapping.

We used surface plasmon resonance to analyze the interaction of calmodulin with several β isoforms. Calmodulin was covalently coupled to the matrix of a sensor chip, and purified, recombinant preparations of alternatively spliced CaM kinase II isoforms. The regulatory domain shared by all β isoforms is composed of exons XI and X. The variable domain is composed of four small exons, IX–VI (54). β-CaM kinase II contains all four exons, while β', βα, and βα each lack the indicated exon(s) (dashed lines). b, purified, recombinant preparations of α (○), β (■), β' (□) (n = 3), βα (○, n = 3) and βα (○, n = 3) were autophosphorylated at increasing concentrations of calmodulin, and phosphate incorporation was quantified as described under “Experimental Procedures.”

Recombinant βα and βα-CaM kinase II were also tested for their ability to trap calmodulin. Both isoforms exhibited prolonged dissociation times in low free Ca²⁺ concentrations following the addition of ATP to the first dissociation phase, indicating calmodulin trapping (data not shown). As expected, recombinant α-CaM kinase II trapped immobilized calmodulin in this experimental system (data not shown). In addition, we looked at the effect of ATP on the interaction of another Ca²⁺/calmodulin-dependent kinase with immobilized calmodulin. The kinetics of purified turkey gizzard MLCK dissociation from calmodulin in 100 nM free Ca²⁺ did not change due to the presence of ATP in the first dissociation phase (Fig. 9b). Furthermore, even in the most favorable circumstance for detect-
Subunit Composition of CaM Kinase II Holoenzymes

FIG. 6. Calmodulin dependence of autophosphorylation in heteromeric CaM kinase II preparations. a, a forebrain CaM kinase II preparation was autophosphorylated in the presence of increasing calmodulin concentrations (n = 3). The relative intensity of the autophosphorylated bands were calculated for forebrain a (○) and β (O) isoforms. Autophosphorylation assays of homomeric α (●) and β (■) isoforms are shown for comparison. A schematic representation of forebrain CaM kinase II holoenzymes (inset), based on the immunoprecipitation results, predicts a mixture of heteromeric CaM kinase II oligomers consisting of α (white circles) and β (gray circles) subunits with a subunit ratio of 2:1 and homomeric α-CaM kinase II. b, a preparation of recombinant α/β heteromers from a cotransfection was autophosphorylated (n = 3), and the relative intensities of the α (○) and β (□) isoforms were quantified and compared with similar assays using recombinant homomers of α (●) or β (■). A schematic representation of recombinant, co-expressed CaM kinase holoenzymes (inset) is shown as a mixture of α/β heteromers with a subunit ratio of 1:3–4 and β-homomers, based on the immunoprecipitation studies above. β subunits are depicted in gray, and α subunits are shown in white.

FIG. 7. Binding of β-CaM kinase II to immobilized calmodulin is Ca2+-dependent. A sensorgram is shown for the interaction of recombinant β-CaM kinase II with immobilized calmodulin. The kinase was injected in the absence (a) or in the presence (b) of 400 μM Ca2+ in the injection buffer (buffer A). In both cases, the binding phase was followed by the injection of 2 mM EGTA buffer.

Neuronal CaM kinase II subunits assemble as large multimolecular holoenzymes. This structural property is not necessary for kinase activity per se but is critical to the proposed function of CaM kinase II as a molecular detector of Ca2+ spike frequency (3–5). The association of the kinase subunits leads to the positioning of their catalytic/regulatory domains in close proximity, and neighboring calmodulin-bound subunits functionally cooperate in rapidly phosphorylating each other (12, 18, 19). In the present study, we examined the subunit composition of CaM kinase II holoenzymes because there has been contradictory data on this matter and studied the potential effect of subunit composition on catalytic activity and intracellular targeting of the kinase. A number of biochemical studies suggested that different CaM kinase II isoforms co-assemble into heteromers (18, 22, 28, 42, 43), while the direct visualization of immunolabeled CaM kinase II holoenzymes by electron microscopy detected only homomers (11). Our immunoprecipitation experiments on brain and recombinant enzymes now indicate that α- and β-isomers form both heteromers with a variable α/β subunit ratio and homomers composed of either α or β subunits. Furthermore, we suggest that assembly of CaM kinase II into heteromers with differing subunit compositions creates functional diversity.

DISCUSSION

Neuronal CaM kinase II subunits assemble as large multimolecular holoenzymes. This structural property is not necessary for kinase activity per se but is critical to the proposed function of CaM kinase II as a molecular detector of Ca2+ spike frequency (3–5). The association of the kinase subunits leads to the positioning of their catalytic/regulatory domains in close proximity, and neighboring calmodulin-bound subunits functionally cooperate in rapidly phosphorylating each other (12, 18, 19). In the present study, we examined the subunit composition of CaM kinase II holoenzymes because there has been contradictory data on this matter and studied the potential effect of subunit composition on catalytic activity and intracellular targeting of the kinase. A number of biochemical studies suggested that different CaM kinase II isoforms co-assemble into heteromers (18, 22, 28, 42, 43), while the direct visualization of immunolabeled CaM kinase II holoenzymes by electron microscopy detected only homomers (11). Our immunoprecipitation experiments on brain and recombinant enzymes now indicate that α- and β-isomers form both heteromers with a variable α/β subunit ratio and homomers composed of either α or β subunits. Furthermore, we suggest that assembly of CaM kinase II into heteromers with differing subunit compositions creates functional diversity.
Specific mAbs for either α or β subunits were used in our immunoprecipitation experiments under stringent conditions, to avoid co-immunoprecipitation of the isoforms through weak, nonspecific interactions. Under these conditions, we found that rat forebrain CaM kinase II holoenzymes consist of both αβ heteromers and α homomers. About 45% of total α subunits associate with all of the available β subunits into heteromers with an αβ subunit ratio of 1:2 (Fig. 3a). The remaining α subunits form homomers. Co-existence of homomeric and heteromeric holoenzymes in the forebrain CaM kinase II preparation could be due to distinct expression patterns of α and β mRNAs from neuron to neuron. Our single cell PCR analysis detected α mRNA in almost all of the neurons studied, but β mRNA was only detected in about 60% of the cells. In neurons expressing mostly or exclusively α mRNA, homomorphic α-CaM kinase II would predominate. In addition, differential subcellular localization has been demonstrated for α and β mRNAs. While α mRNA is found in dendrites as well as cell bodies, β mRNA is restricted to the cell body (44). Therefore, α and β subunits could co-assemble to form heteromers in the cell body, whereas dendritic α-CaM kinase II would be largely homomers if the mRNA was translated locally in the dendrites.

When co-expressed in COS-7 cells with an αβ subunit ratio of 1:5, both αβ heteromers and β homomers were observed (Fig. 3b). The mRNAs expressed by the transfected cells and their intracellular localization are likely to be more uniform than in rat forebrain neurons. Interestingly, the αβ subunit ratio of the recombinant heteromers (1:3–4) was quite different from that found for the forebrain enzyme (2:1). These results demonstrate that CaM kinase II heteromers are not composed of α and β subunits with a fixed stoichiometry. Rather, the availability of the two subunits is a major determinant of the subunit ratio. In both the forebrain and COS-7 cell preparations, the minor isoform was found entirely in heteromeric holoenzymes, while some subunits of the major isoform, apparently in excess, also assembled into homomers. Taken together, these observations suggest that the subunit composition of CaM kinase II holoenzyme is dependent on the local and temporal availability of the two subunits.

In neurons, regulated expression of α and β subunits in response to extracellular stimuli may lead to the production of new CaM kinase II holoenzymes with altered subunit compositions. Increased expression of the mRNA for the α-CaM kinase II but not for the β-CaM kinase II isoform was observed in the dentate gyrus following long term potentiation induction in freely moving rats (45). Protein synthesis is increased locally in dendrites receiving either pharmacological or synaptic stimulation, and this may include production of dendritic α subunits (46, 47). Active neurons may therefore produce a higher ratio of α to β subunits manifest as either a higher population of homomeric α-CaM kinase II and/or a higher α subunit content of αβ heteromers. If the subunit composition of nascent CaM kinase II holoenzymes is dynamically changing in neurons, the observed αβ subunit ratio in CaM kinase II holoenzymes is dependent on the local and temporal availability of the two subunits.
ATP

The EC₅₀ for calmodulin is lower for homomeric modulin sensitivity for autophosphorylation of each subunit.

respectively); recombinant b subunits neighboring the a subunits are the low affinity a subunits (Fig. 6a).

a

Fig. 9. Calmodulin trapping by β-CaM kinase II. a, dissociation of β-CaM kinase II at 200 nM free Ca²⁺ buffer in the absence or presence of 200 μM ATP in the first dissociation phase (−ATP and +ATP, respectively); b, dissociation of MLCK at 100 nM free Ca²⁺ buffer in the absence or presence of 200 μM ATP in the first dissociation phase (−ATP and +ATP, respectively).

with nuclear and cytosolic CaM kinase II isoforms whose targeting is based on the ratio of nuclear and cytosolic subunits in the heteromers (28). Similarly, targeting of kinase subunits to the sarcoplasmic reticulum requires co-assembly with the αKAP isoform (49). In transfected cells, a greater fraction of recombinant β protein is associated with the particulate fraction than recombinant α protein (50), perhaps due to the preferential association of β-CaM kinase II with F-actin (43).

In this report, we address two regulatory properties of CaM kinase II isoforms-calmodulin sensitivity and calmodulin trapping. Co-assembly of α and β subunits can modulate the calmodulin sensitivity for autophosphorylation of each subunit. The EC₅₀ for calmodulin is lower for homomeric β than homomeric α (Fig. 4). In the forebrain CaM kinase II preparation, the calmodulin dependence of β subunit autophosphorylation is shifted toward the lower sensitivity of the α subunit, presumably because most of the subunits neighboring the β subunits in these heteromers are the low affinity α subunits (Fig. 6a).

Similarly, the calmodulin dependence of α subunit autophosphorylation in the recombinant kinase with predominantly β subunits is shifted toward the higher sensitivity of β subunits, supporting co-assembly of heteromeric subunits and functional consequences of co-assembly (Fig. 6b). Although this shift largely reflects the likelihood that the α subunits are being phosphorylated by high affinity β subunits, the shift is slightly greater than would be expected and suggests that co-assembly per se may alter kinetic properties, such as the rate of autophosphorylation. The standard assay used here does not provide an initial autophosphorylation rate, which is extremely fast, and an increased autophosphorylation rate in heteromers would be seen as an increase in the apparent calmodulin sensitivity, as found for the phosphorylation of α by β subunits in Fig. 6b. In fact, homomers of the two subunits have a different number of subunits and differ in the interactions within their respective association domains (42). It is therefore possible that co-assembly alters the orientation of the catalytic/regulatory domains, slightly increasing the rate of autophosphorylation and thereby contributing to the additional shift in apparent calmodulin affinity that is observed.

At limiting calmodulin, activation and autophosphorylation of CaM kinase II vary with the subunit composition. At 20 nM calmodulin, for example, twice as many subunits are autophosphorylated in heteromeric kinase with an α:β ratio of 2:1 than in α homomers (3–4 subunits phosphorylated per decamer) (Fig. 6a). Therefore, the association of α subunits with β subunits, which have a 6-fold higher calmodulin sensitivity, might serve to enhance the activation of CaM kinase II under limiting calmodulin concentrations or in response to brief Ca²⁺ spikes. Graded calmodulin sensitivity for kinase activation can be achieved by formation of holoenzymes with a range of subunit ratios. Recent experiments suggest that CaM kinase II holoenzymes with different subunits compositions are tuned to different Ca²⁺ spiking frequencies in vitro (3) and translocate to synaptic sites at different rates (16). In rat forebrain neurons, homomeric α-CaM kinase II would have a higher frequency threshold for activation and a steeper response to high frequency stimulation, whereas heteromers containing high sensitivity β subunits may be more sensitive to smaller, lower frequency Ca²⁺ spikes.

We have previously demonstrated that β and β' isoforms are expressed in adult brain, while the β and β' isoforms are primarily expressed at early developmental stages (7). All four β isoforms were analyzed for their calmodulin dependence by activity and autophosphorylation assays, revealing that the adult isoforms have about 3-fold higher calmodulin sensitivity than the embryonic isoforms. This is the first reported example of differences in the kinetic properties of mammalian CaM kinase II due to alternative splicing. Alternatively spliced isoforms of the Drosophila CaM kinase have also been shown to have different kinetic properties (51, 52). For mammalian kinase, deletion of exon IX from the beginning of the variable domain of β-CaM kinase II in the embryonic isoforms is correlated with reduced calmodulin sensitivity, whereas the deletion of exon VI from the end of the variable domain in β and β'₉ isoforms has no effect on this property (Fig. 5). Exon IX is immediately adjacent to the calmodulin binding domain encoded by exon X, and it is therefore conceivable that amino acids encoded by exon IX contribute to the interaction of β and β' isoforms with calmodulin, modulating the affinity. Interestingly, the α isoform has the lowest calmodulin sensitivity of all tested CaM kinase II isoforms, and its variable domain contains only the linker sequence (equivalent to exon VIII in Fig. 5a).

In the model of CaM kinase II as a frequency spike detector, all of the subunits on the holoenzyme are assumed to be capable of trapping calmodulin (5). Therefore, frequency detection of Ca²⁺ spikes by α:β heteromers would depend on the capa-
bility of autophosphorylated β subunits to trap calmodulin. We now demonstrate that the adult and embryonic β isoforms can also trap calmodulin, a feature that may be shared by all CaM kinase II isoforms. Recent results measuring the binding of calmodulin to peptides derived from the regulatory domain of kinase II isoforms. Since the corresponding sequence is identical among all CaM kinase II genes (α, β, γ, and δ) and is present in all the alternatively spliced isoforms, it is likely that γ and δ isoforms trap calmodulin as well.

Acknowledgments—We are grateful to Drs. Claude Klee and Maxine Singer for enabling the completion of this study in their laboratories and for generous advice and support. We also thank Paul DeKoninck for helpful comments on the manuscript.

REFERENCES
1. Bear, M. F., and Malenka, R. C. (1994) Curr. Opin. Neurobiol. 4, 389–399
2. Mayford, M., Wang, J., Kandel, E. R., and O’Dell, T. J. (1995) Cell 81, 891–904
3. De Koninck, P., and Schulman, H. (1998) Science 279, 227–230
4. Hanson, P. I., and Schulman, H. (1992) Annu. Rev. Biochem. 61, 599–601
5. Schulman, H., Hanson, P. I., and Meyer, T. (1992) Cell Calcium 13, 401–411
6. Bennett, M. K., and Kennedy, M. B. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1794–1798
7. Brocke, L., Srinivasan, M., and Schulman, H. (1995) J. Neurosci. 15, 6797–6808
8. Ngiem, P., Saati, S. M., Martens, C. L., Gardner, P., and Schulman, H. (1992) J. Biol. Chem. 266, 5471–5479
9. Edman, C. F., and Schulman, H. (1994) Biochim. Biophys. Acta 1221, 90–102
10. Tombes, R. M., and Krystal, G. W. (1997) Biochim. Biophys. Acta 1355, 281–292
11. Kanaseki, T., Ikeuchi, Y., Sugiyama, H., and Yamauchi, T. (1991) J. Cell Biol. 115, 1049–1060
12. Hanson, P. I., Meyer, T., Stryer, L., and Schulman, H. (1994) Neuron 12, 943–956
13. Meyer, T., Hanson, P. I., Stryer, L., and Schulman, H. (1992) Science 256, 1199–1201
14. Strack, S., and Colbran, R. J. (1998) J. Biol. Chem. 273, 20689–20692
15. Leonard, S. A., Lim, I. A., Hemsworth, D. E., Horne, M. C., and Hell, J. W. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3239–3244
16. Shen, K., and Meyer, T. (1999) Science 284, 162–167
17. Kuret, J., and Schulman, H. (1995) J. Biol. Chem. 269, 6427–6433
18. Mukherji, S., and Soderling, T. R. (1994) J. Biol. Chem. 269, 13744–13747
19. Rich, R. C., and Schulman, H. (1998) J. Biol. Chem. 273, 28434–28429
20. Giese, K. P., Fedorov, N. B., Filipkowski, R. K., and Silva, A. J. (1998) Science 279, 870–873
21. Cho, Y. H., Giese, K. P., Tanila, H., Silva, A. J., and Eichenbaum, H. (1998) Science 279, 867–869
22. Bennett, M. K., Erondou, N. E., and Kennedy, M. B. (1983) J. Biol. Chem. 258, 12735–12744
23. Miller, S. G., and Kennedy, M. B. (1985) J. Biol. Chem. 260, 9039–9046
24. Liu, N., and Cooper, N. G. F. (1995) J. Mol. Neurosci. 5, 193–206
25. Yamauchi, T., Ohsako, S., and Deguchi, T. (1989) J. Biol. Chem. 264, 1908–1911
26. Schulman, H. (1984) J. Cell Biol. 99, 11–19
27. Hanson, P. I., Kapiloff, M. S., Lou, L. L., Rosenfeld, M. G., and Schulman, H. (1989) Neuron 3, 59–70
28. Srinivasan, M., Edman, C., and Schulman, H. (1994) J. Cell Biol. 126, 839–852
29. Hanson, P. I., and Schulman, H. (1992) J. Biol. Chem. 267, 17216–17224
30. Adelstein, R. S., and Klee, C. B. (1981) J. Biol. Chem. 256, 7501–7509
31. Chiang, L. W., Schweizer, F. E., Tsien, R. W., and Schulman, H. (1994) Mol. Brain Res. 27, 183–188
32. Lin, C. R., Kapiloff, M. S., Durgerian, S., Tatemoto, K., Russo, A. F., Hanson, P., Schulman, H., and Rosenfeld, M. G. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 5962–5966
33. Tobinatsu, T., Kameshita, I., and Fujisawa, H. (1988) J. Biol. Chem. 263, 16082–16086
34. Tobinatsu, T., and Fujisawa, H. (1989) J. Biol. Chem. 264, 17907–17912
35. Baitinger, C., Alderton, J., Poenie, M., Schulman, H., and Steinhardt, R. A. (1990) J. Cell Biol. 111, 1763–1773
36. Edlund, M., Blikstad, I., and Obrien, B. (1996) J. Biol. Chem. 271, 1393–1399
37. Skene, J. H. P. (1990) Neurosci. Res. Suppl. 13, S112–S125
38. Luby-Phelps, K., Hori, M., Phelps, J. M., and Won, D. (1995) J. Biol. Chem. 270, 21532–21538
39. Lou, L. L., Lloyd, S. J., and Schulman, H. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 9437–9451
40. Miller, S. G., Patton, B. L., and Kennedy, M. B. (1988) Neuron 1, 593–604
41. Rich, D. P., Schwerer, C. M., Colbran, R. J., and Soderling, T. R. (1990) i, 107–116
42. Kim, S. J., Hudmon, A., Ginsberg, T. R., and Waxham, M. N. (1998) J. Biol. Chem. 273, 31555–31564
43. Shen, K., Teruel, M. N., Subramanian, K., and Meyer, T. (1998) Neuron 21, 593–606
44. Benson, D. L., Gall, C. M., and Isackson, P. J. (1992) Neuroscience 46, 851–857
45. Thomas, K. L., Laroche, S., Errington, M. L., Bliss, T. V. P., and Hunt, S. P. (1994) Neuron 13, 737–745
46. Feig, S., and Lipton, P. (1995) J. Neurosci. 15, 1010–1021
47. Ouyang, Y., Kantor, D., Harris, K. M., Schuman, E. M., and Kennedy, M. B. (1997) J. Neurosci. 17, 5416–5427
48. Spranger, R., and Seeburg, P. H. (1993) FEBS Lett. 325, 90–94
49. Kuret, J., and Schulman, H. (1989) EMBO J. 17, 5598–5605
50. Yamauchi, T., Sekihara, S., and Ohsako, S. (1990) FEBS Lett. 266, 55–58
51. Gypsum, B., and Griffith, L. C. (1996) J. Neurochem. 66, 1282–1288
52. Gypsum, B., Beckingham, K., and Griffith, L. C. (1996) J. Biol. Chem. 271, 19846–19851
53. Waxham, M. N., Tsai, A. L., and Putkey, J. A. (1998) J. Biol. Chem. 273, 17579–17584
54. Karls, U., Muller, U., Gilbert, D. J., Copeland, N. G., Jenkins, N. A., and Harbers, K. (1992) Mol. Cell. Biol. 12, 3644–3652