Alternative splicing modulates the frequency-dependent response of CaMKII to Ca²⁺ oscillations

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Ca²⁺ oscillations are required in various signal transduction pathways, and contain information both in their amplitude and frequency. Remarkably, the Ca²⁺/calmodulin(CaM)-dependent protein kinase II (CaMKII) can decode such frequencies. A Ca²⁺/CaM-stimulated autophosphorylation leads to Ca²⁺/CaM-independent (autonomous) activity of the kinase that outlasts the initial stimulation. This autonomous activity increases exponentially with the frequency of Ca²⁺ oscillations. Here we show that three β -CaMKII splice variants (β_M , β and β_e) have very similar specific activity and maximal autonomy. However, their autonomy generated by Ca²⁺ oscillations differs significantly. A mechanistic basis was found in alterations of the CaM activation constant and of the initial rate of autophosphorylation. Structurally, the splice variants differ only in a variable 'linker' region between the kinase and association domains. Therefore, we propose that differences in relative positioning of kinase domains within multimeric holoenzymes are responsible for the observed effects. Notably, the β -CaMKII splice variants are differentially expressed, even among individual hippocampal neurons. Taken together, our results suggest that alternative splicing provides cells with a mechanism to modulate their sensitivity to Ca²⁺ oscillations.

Keywords: alternative splicing/calcium oscillation/CaMKII/frequency

Introduction

A variety of cellular stimuli such as action potentials, neurotransmitters, hormones and antigens lead to transient changes in cytosolic free Ca²⁺, in the form of Ca²⁺ spikes, sparks, puffs and waves, which give rise to localized Ca²⁺ oscillations (for a review, see Berridge *et al.*, 2000; Bootman *et al.*, 2001). Such Ca²⁺ signals can encode information in their kinetics, spike numbers and frequencies. Thus, cells must locally express appropriate molecular decoders capable of translating such Ca²⁺ signals into specific downstream signaling events.

Ca²⁺/calmodulin(CaM)-dependent protein kinase II (CaMKII) is a major mediator of Ca²⁺-based signals and regulates a large variety of cellular functions, including carbohydrate metabolism, gene expression, cell cycle,

neurotransmitter synthesis and release, membrane excitability, and different forms of synaptic plasticity that may underlie learning and memory (for a review, see Malenka and Nicoll, 1999; Schulman and Braun, 1999; Soderling *et al.*, 2001; Hudmon and Schulman, 2002). CaMKII possesses key attributes to differentially decode localized Ca²⁺ oscillations. (i) It specifically targets to distinct cellular compartments, such as the nucleus (Srinivasan *et al.*, 1994; Brocke *et al.*, 1995), actin filaments (Ohta *et al.*, 1986; Shen *et al.*, 1998), post-synaptic densities (PSD) (Kennedy *et al.*, 1983; Shen and Meyer, 1999; Bayer *et al.*, 2001; Dosemeci *et al.*, 2001) and intracellular membranes (Bayer *et al.*, 1998). (ii) It can, remarkably, translate the number and frequencies of Ca²⁺ oscillations into different levels of activity (De Koninck and Schulman, 1998).

The ability of CaMKII to extract spatial and temporal information from Ca²⁺-mediated signals is largely based on its unique structural arrangement, with 12 kinase subunits symmetrically assembled into hub- and spoke-like holoenzymes (Kanaseki *et al.*, 1991; Kolodziej *et al.*, 2000) (see also Figure 1A). Each subunit contains a C-terminal association domain and an N-terminal kinase domain that includes the regulatory region with the Ca²⁺/CaM binding site. This topology is shared by the highly homologous α , β , γ and δ isoforms, which are encoded by four different genes. Additionally, each CaMKII gene gives rise to multiple splice variants that differ in a variable region, which is thought to comprise the linker between the kinase and association domains seen in the holoenzyme structure (Figure 1A).

It is important to note that CaMKII can form both homo- and hetero-multimeric holoenzymes. Differential targeting of the large holoenzymes is actually controlled by specific insertion of subunits that contain unique targeting domains, such as in the CaMKII isoforms α_B and δ_B (nucleus), α (dendritic spines), β (F-actin), and the non-kinase variant α KAP (intracellular membranes) (for a review, see Bayer and Schulman, 2001). Differential decoding of Ca²⁺ oscillation frequencies is based on a Ca²⁺/CaM-stimulated inter-subunit autophosphorylation within holoenzymes (at T286 for α and at T287 for the other isoforms). Such a regulated autophosphorylation leads to Ca²⁺/CaM-independent (autonomous) activity of the kinase that outlasts the initial stimulation (for a review, see Schulman and Braun, 1999; Soderling *et al.*, 2001). This autophosphorylation requires simultaneous binding of at least two Ca²⁺/CaM molecules per holoenzyme: one for activation of a kinase subunit and another to expose T286 (α) or T287 (β , γ and δ) for phosphorylation on a neighboring subunit (Hanson *et al.*, 1994; Rich and Schulman, 1998). This dual Ca²⁺/CaM requirement, both kinase and substrate directed, has led to the proposal that generation of autonomous CaMKII activity is sensitive to

Fig. 1. Structure of β -CaMKII splice variants. **(A)** Primary structure of the β -CaMKII splice variants in a schematic representation (left), and CaMKII holoenzyme structure (Kolodziej *et al.*, 2000) (right). **(B)** The variable region of β -CaMKII. Amino acid sequences missing in β_e' are boxed. The lines above the sequence indicate the three β_M -specific repeats. **(C)** Intron/exon borders of the three β_M -specific exons (black boxes) as determined by further sequence analysis of the murine β -CaMKII locus (for complete β_M exon sequences see Bayer *et al.*, 1999; more intron sequences at DDBJ/EMBL/GenBank accession Nos AF416336 and AF416337).

the frequency of Ca²⁺ oscillations (Hanson *et al.*, 1994; see also Dosemeci and Albers, 1996). To test this model, we recently developed an approach that allows the exposure of isolated CaMKII to brief (e.g. 200 ms) Ca²⁺ spikes under precisely controlled kinetics and frequencies (De Koninck and Schulman, 1998). This highly sensitive approach indeed revealed that autonomous CaMKII activity increases exponentially with the frequency of Ca²⁺ oscillations (De Koninck and Schulman, 1998).

While the immense diversity of possible CaMKII holoenzyme species, generated by the dodecameric co-assembly of four gene products with >20 splice variants, has been shown to differentially control targeting of the kinase (for a review, see Bayer and Schulman, 2001), we asked here whether this diversity can also provide for differential sensitivities to Ca²⁺ oscillations. We compared, under steady and pulsatile Ca²⁺ stimulation, the activation and generation of autonomy for three splice variants of the β -CaMKII isoform that contain identical kinase domains. We show here that these three splice variants, β , β_e' and β_M , share very similar catalytic features under steady stimulation, yet can differentially decode Ca²⁺ oscillation frequencies. Furthermore, we describe differential expression of the splice variants, even among individual hippocampal neurons, suggesting that alternative splicing of CaMKII provides cells with a mechanism to regulate their frequency sensitivity to Ca²⁺-based signals. Our study demonstrates that very subtle functional differences between kinase subtypes can lead to significant changes in output signals of the distinct isoforms when exposed to specific oscillatory input signals.

Results

β_M -CaMKII is a splice variant of the β isoform

β_M -CaMKII is closely related to the β isoform, but contains a 12 kDa insert at the C-terminus of the variable region (Bayer *et al.*, 1998). Further sequence analysis of the murine β -CaMKII gene (Karls *et al.*, 1992) revealed that β_M is, indeed, a splice variant of the β isoform (Figure 1). These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession Nos AF416336 and AF416337. The β_M -specific insert consists of three proline-rich repeats, which are homologous to each other (Bayer *et al.*, 1998, 1999) (Figure 1A and B). Each repeat is encoded by a separate exon, and the conserved intron borders contain the consensus sequences for splice sites (Figure 1C; for a review, see Smith and Valcarcel, 2000). Three previously described β -CaMKII splice variants (β' , β_e and β_e') lack one or two exons in the variable region 5' proximal to the β_M -specific insert (Karls *et al.*, 1992; Brocke *et al.*, 1995) (Figure 1A and B); β_3 -CaMKII resembles the β_e' variant, but additionally contains the two β_M repeats b and c (Urquidí and Ashcroft, 1995). Therefore, all known β -CaMKII variants differ only in their variable region, which is thought to comprise the 'linker' between the N-terminal kinase domain and the C-terminal association domain (Figure 1A).

Specific activity and maximal autonomy of β -, β_M - and β_e' -CaMKII are similar

To compare the biochemical properties of β -CaMKII splice variants under steady-state and pulsatile Ca²⁺ stimulation, we tagged each of them with a hemagglutinin A epitope (HA) at the C-terminus, which allows their immobilization onto anti-HA antibody-coated PVC microtiter plates or tubing. Such tagging and immobilization do not interfere with normal activation by Ca²⁺/CaM, autophosphorylation and generation of autonomous activity, or oligomerization into holoenzymes (De Koninck and Schulman, 1998). First, we assessed the autophosphorylation status of the kinase variants overexpressed in COS-7 cells (Figure 2A). Prolonged Ca²⁺/CaM stimulation induced essentially the same T287 autophosphorylation for all β variants (and similar to the autophosphorylation of the homologous T286 for the α isoform); no phospho-T287 (or T286) was detected without such stimulation (Figure 2A). We then adsorbed the tagged kinase variants onto microtiter plates, determined the amount of bound kinase by quantitative immunoblotting (Figure 2B), and tested the kinase activity of the splice variants towards the exogenous peptide substrate autocalcine-3 (AC-3) (Figure 2C). We found that the Ca²⁺/CaM-stimulated specific activities of the three β -CaMKII splice variants (β , β_M and β_e') are very similar (Figure 2B and C). Furthermore, the variants are equally capable of becoming highly Ca²⁺ independent (autonomous) after T287 autophosphorylation induced by prolonged Ca²⁺/CaM stimulation (1.5 min) (Figure 2C). The basal activity without Ca²⁺/CaM stimulation or autophosphorylation was <1.5% for all variants ($\beta_M = 0.71 \pm 0.42\%$, $\beta = 0.99 \pm 0.40\%$ and $\beta_e' = 0.80 \pm 0.51\%$). In initial experiments, we used extracts of COS-7 cells overexpressing untagged β_M -, β - or β_e' -CaMKII and observed essentially the same results, with similar specific activities of the splice variants also

Fig. 2. (A) Autophosphorylation at the autonomy site T287 can be induced by Ca^{2+} /CaM for all three β variants, as detected by immunoblotting using a phospho-T287-specific antibody (top). Similar phosphorylation was observed for α (at the homologous T286). No T287 phosphorylation was detected before stimulation with Ca^{2+} /CaM (bottom). (B) Immunodetection of total immobilized β -CaMKII splice variants with the same specific kinase activity (3000 fmol of ATP/min), using the CB- β 1 antibody. Below: purified β -CaMKII as standard. (C) Specific activities of β splice variants (means $\pm$ SEM) in a peptide substrate phosphorylation assay. Stimulated activity was induced by 300 nM CaM; autonomous activity was measured in the absence of Ca^{2+} /CaM, but after autophosphorylation. Autonomy is indicated relative to the stimulated activity. Basal activity (not stimulated and without autophosphorylation) for all variants was $<1.5\%$, as indicated. The experiments were performed with HA-tagged kinase isoforms immobilized on anti-HA antibody-coated microtiter plates.

when measured in solution (data not shown). These results suggest that alternative splicing within the linker region has little effect on the steady-state activity of the kinase.

β -CaMKII splice variants with differential response to Ca^{2+} oscillations

We reasoned that differences in the variable linker region could influence the relative positioning of the kinase domains within the holoenzyme structure (Figure 1A), which could lead to subtle differences in generation of autonomous activity among the splice variants. Autophosphorylation at the autonomy site (T287 in β -CaMKII) requires interaction between two activated kinase subunits within a holoenzyme (Hanson *et al.*, 1994; Rich and Schulman, 1998). Thus, under very brief stimulation or non-equilibrium conditions, such as during Ca^{2+} oscillations, any subtle difference in kinase-domain interaction may have more detectable functional effects. To test for this possibility, we exposed immobilized β -CaMKII splice variants to Ca^{2+} oscillations inside anti-HA antibody-coated PVC tubing connected to a computer-controlled pulse-flow device that allows rapid exchange of solutions by use of two pressurized chambers. Immobilized kinase

variants were exposed to brief (200 ms) activating pulses (Ca^{2+} spikes) alternating with de-activating pulses of various duration (spike intervals, containing 1 mM EGTA). Each stimulation protocol exposed the kinase to 30 Ca^{2+} spikes delivered at various frequencies by adjusting the duration of spike intervals. Following each protocol, the evoked autonomous kinase activity was measured inside the tubing by AC-3 phosphorylation in the absence of Ca^{2+} (De Koninck and Schulman, 1998). Figure 3 shows that the autonomous activity of β -CaMKII increases exponentially with the frequency of Ca^{2+} spikes. Each β splice variant shows such frequency sensitivity. However, they differ significantly from each other in the degree of autonomy evoked by any given stimulation frequency. While β_M is consistently more autonomous than β , autonomy of β_e' is always lower than that of β . Furthermore, β_M has the broadest frequency response with the lowest factor of exponential increase, followed by β and β_e' . These results demonstrate that alternative splicing modulates the response of β -CaMKII to the frequencies of Ca^{2+} oscillations, but not to sustained maximal Ca^{2+} stimulation.

Two biochemical parameters that can influence frequency decoding by CaMKII

How can this alternative splicing of β -CaMKII lead to isoform-specific frequency responses without affecting the steady-state catalytic properties? Although β - and β_e' -CaMKII have an identical CaM binding site within their regulatory regions, we found that β_e' has a higher activation constant for CaM than β (Figure 4A; K_a for CaM of $\beta = 22.4 \pm 2.5$ nM, and of $\beta_e' = 47.5 \pm 6.9$ nM). A higher activation constant (or lower affinity) for CaM should, in fact, result in reduced activation by submaximal stimuli, such as the brief Ca^{2+} spikes in the above stimulation protocol. For β_e' , the CaM affinity might be reduced by the shorter length of the variable 'linker' between the kinase/regulatory domain and the association domain. In contrast, the K_{as} for CaM of β and β_M are nearly identical (22.4 ± 2.5 and 21.4 ± 1.7 nM, respectively) and their CaM concentration-response curves are almost undistinguishable (Figure 4A). As shown above, the β and β_M isoforms also have essentially the same specific activities towards a peptide substrate (Figure 2), as well as apparent rates of autophosphorylation when measured after 12 s or longer (Figures 2C and 4B).

How, then, can these two splice variants, β and β_M , respond differently to Ca^{2+} oscillations? One possibility could be a difference in their initial rate of autophosphorylation, even though the phosphorylation rates are the same for an exogenous peptide substrate (Figure 2). Thus, we monitored T287 autophosphorylation evoked by a single brief Ca^{2+} stimulus (0.5 mM CaCl_2 , 300 nM CaM), using the controlled pulse-flow device for the stimulation and autonomous kinase activity as readout. Autonomous activity is dependent on T287 autophosphorylation, and autonomy actually provides a more sensitive and more accurate quantitative readout for T287 autophosphorylation than phosphate incorporation (Bradshaw *et al.*, 2002). Indeed, the initial rate of autophosphorylation, as measured by generation of autonomy within 0.2–1 s of stimulation, is significantly higher for β_M than for β -CaMKII (Figure 4B). Our results indicate that the

Fig. 3. Differential response of β -CaMKII splice variants to Ca^{2+} oscillations. Different frequencies of Ca^{2+} oscillations evoke different levels of autonomous activity for β , β_M and β_e' . Curves were fitted to the single-exponential function $y = ae^{bx} + c$. The factor of exponential increase, b , for β_e' is 10% greater than that for β , and 50% greater than that for β_M . All data points are means $\pm$ SEM.

Fig. 4. Calmodulin activation constant and initial rate of autophosphorylation as parameters that can modulate the response of CaMKII to Ca^{2+} oscillations. (A) Calmodulin concentration–response curves reveal a higher K_a for β_e' than for β and β_M (47.5 ± 6.9 , 22.4 ± 2.5 and 21.4 ± 1.7 nM, respectively). (B) The initial rate of autophosphorylation is higher for β_M than for β , as measured by the resulting autonomous activity. All data points are means $\pm$ SEM.

probability of inter-subunit autophosphorylation during a brief Ca^{2+} spike is higher for β_M than for β (Figure 4B). This should indeed lead to a higher degree of autonomy for β_M during Ca^{2+} oscillations (Figure 3), even if the activation kinetics by Ca^{2+} /CaM are the same for β and β_M . We speculate that the longer linker in β_M increases the spatial mobility of kinase domains, thereby facilitating interactions necessary for autophosphorylation. Our results show that β_M - and β_e' -CaMKII differ from the β isoform in their initial rate of autophosphorylation and CaM affinity, respectively, two parameters that can significantly modulate the response to Ca^{2+} oscillations.

β -CaMKII variants are differentially expressed in brain

Alternative splicing may modulate the sensitivity of CaMKII-mediated signaling pathways to the various

Fig. 5. β_M -CaMKII is expressed in neurons. (A) RT-PCR with β_M -specific primers at increasing cycle numbers from oligo-dT-primed cDNA prepared from mRNA from different rat tissues. The positions of 300 and 400 bp marker bands are indicated. (B) *In situ* hybridization of cultured hippocampal neurons with a digoxigenin-labeled β_M -specific RNA probe (left) (nucleotides 133–354 in Bayer *et al.*, 1999) and the corresponding sense probe control (right). Color coding represents different expression levels, as indicated. (C) Immunocytochemistry for β -CaMKII and MAP-2 in the cultured neurons. Nearly all neurons (MAP-2-positive cells) are positively stained with an antibody specific for all splice variants of β -CaMKII. Scale bars = 40 μm .

forms of Ca^{2+} transients and oscillations in cell type- and development-specific manners. Indeed, β_e and β_e' are the predominant CaMKII isoforms expressed in the postnatally developing brain (Brocke *et al.*, 1995), largely replaced in adult rats by β -CaMKII. Such a change likely increases the sensitivity of the neuronal CaMKII to low-frequency stimulation. Interestingly, the β -CaMKII splice variants are also differentially expressed among individual adult hippocampal–pyramidal neurons, as revealed by single-cell RT-PCR (Brocke *et al.*, 1999). The β_M variant was not known at the time of the latter study, but a band corresponding in length to β_M was detected in the PCR reactions from 2 of 24 neurons (L. Chiang and H. Schulman, unpublished data). By RT-PCR with β_M -specific primers, we found significant expression of β_M -CaMKII in brain, although its expression level is likely to be 10- to 100-fold lower than in skeletal muscle (Figure 5A), the tissue with highest expression of β_M (Bayer *et al.*, 1998, 1999). In comparison, β_M expression in heart was only barely detectable, even with the highly sensitive RT-PCR. To examine whether brain β_M -CaMKII is expressed in neurons or other cell types, we performed *in situ* hybridization of dissociated rat hippocampal cultures, using a digoxigenin-labeled RNA probe derived

from the β_M -specific insert essentially as described previously (Bayer *et al.*, 1999). β_M -CaMKII transcripts were indeed detected in neurons, with significant expression restricted to a subset of them (Figure 5B). In contrast, essentially every cultured hippocampal neuron contains at least one β variant at the protein level, as shown by immunocytochemistry with a β -specific antibody that does not discriminate between the splice variants (Figure 5C). Neurons were distinguished from glia by co-staining for the neuronal marker MAP-2 (Figure 5C); the almost complete overlap of the β stain with the MAP-2 stain indicates that significant expression of β -CaMKII splice variants is restricted to neurons.

Discussion

Transient rises in intracellular Ca^{2+} concentration contain information not only in their amplitude and duration, but also in their frequency; however, CaMKII is presently the only identified molecular decoder for such oscillation frequencies (De Koninck and Schulman, 1998). We have demonstrated here that alternative splicing provides an array of β -CaMKII variants (Figure 1) with different sensitivities to Ca^{2+} oscillations despite their catalytic similarities observed under steady stimulation (Figures 2 and 3). Two biochemical parameters that can account for the differential frequency decoding by β_M -, β - or β_e '-CaMKII were identified, e.g. the initial rate of autophosphorylation (for β_M) and CaM activation constant (for β_e ') (Figure 4). Remarkably, the β -CaMKII variants are differentially expressed in different tissues, during development, and even among individual adult hippocampal neurons (Figure 5; Brocke *et al.*, 1995, 1999; Bayer *et al.*, 1998), suggesting that nature indeed utilizes alternative splicing of CaMKII as a mechanism to regulate cellular sensitivity to Ca^{2+} oscillation frequencies.

The β -CaMKII splice variants contain identical kinase domains, including the regulatory region with the binding site for Ca^{2+} /CaM. Thus, our findings that β_M -, β - and β_e '-CaMKII have very similar specific activities and degrees of maximal autonomy were not too surprising. We argued that the most sensitive readout for any possible subtle difference would be the frequency-dependent response of the kinase to Ca^{2+} oscillations, as small effects during short pulses with submaximal, non-equilibrium stimulation would be enhanced by the multiple repetitions of an oscillation. To expose CaMKII to such short pulses (200 ms) and oscillations (0.5–4 Hz), we utilized a computer-controlled pulse-flow device developed recently (De Koninck and Schulman, 1998). Indeed, significant differences of β_M -, β - and β_e '-CaMKII were revealed in their generation of Ca^{2+} -independent (autonomous) activity during Ca^{2+} oscillations at different frequencies.

Generation of autonomous CaMKII activity depends on an inter-subunit autophosphorylation within a multimeric holoenzyme. Structurally, β -CaMKII splice variants differ only in a variable region, which is thought to comprise the linker connecting the foot-like kinase domains with the central hub formed by the association domains (Kolodziej *et al.*, 2000; Figure 1A). We argued that the length of this linker could influence the kinetics of autophosphorylation by modulating the accessibility of the kinase subunits to each other or of CaM to an individual kinase subunit.

Although alternative splicing does not directly affect the CaM binding site, the variable region is immediately adjacent. Indeed, compared with β - and β_M -CaMKII, the CaM activation constant for the smallest variant, β_e '-CaMKII, is higher, corresponding to a lower affinity. Similar effects of alternative splicing on CaMKII activation by CaM have been reported previously for CaMKII isoforms from *Drosophila* and mammals (GuptaRoy *et al.*, 1996; Brocke *et al.*, 1999). Compared with any β variant, α -CaMKII has both a shorter variable linker and a lower CaM affinity (Brocke *et al.*, 1999), further suggesting a causal link. In contrast, the CaM activation curves of β and β_M are indistinguishable, indicating that additional elongation of the β variable region, even by the 12 kDa β_M insert, does not further increase CaM affinity. However, compared with β -CaMKII, the longer β_M variant shows a marked increase in the initial rate of autophosphorylation, as measured by autonomous activity generated during brief (0.2–1 s) stimulation. The β_M insert may achieve this accelerated autophosphorylation within a holoenzyme by facilitating the interaction between two kinase subunits, or by increasing the number of kinase subunits that can access each other. Alternatively, the autonomy site at T287 might be a better substrate sequence for β_M -CaMKII than for β -CaMKII. In fact, alternative splicing was recently found to modulate the substrate preference of *Drosophila* CaMKII (GuptaRoy *et al.*, 2000). However, the peptide substrate AC-3 that we used to determine the specific activities of the β splice variants is actually derived from the region surrounding T287, and the activity of β_M -CaMKII is essentially the same as for the β isoform; if anything, the β_M activity appeared slightly lower. Thus, the β_M insert has most likely a steric effect that allows the kinase subunits to access each other more readily. In fact, the structure of CaMKII holoenzymes (Kolodziej *et al.*, 2000) does suggest a significant steric restriction for the interaction between α -CaMKII subunits, which contain a shorter variable region.

Both CaM affinity and the initial rate of autophosphorylation should indeed affect the response to Ca^{2+} oscillations even in our most simple model for frequency detection by CaMKII. During Ca^{2+} spikes, CaM binds to the kinase, and dissociates during the spike intervals. When the spike intervals are long (low frequency), each Ca^{2+} pulse elicits the same kinase response. However, when the spike intervals are short (high frequencies) and within the range of the CaM dissociation constant (Meyer *et al.*, 1992), CaM accumulates on the kinase with each pulse. A lower CaM affinity would reduce the activation of the kinase at low frequencies, shift the exponential rise to higher frequencies, and make this exponential function steeper, as was observed for β_e '-CaMKII. A faster rate of autophosphorylation would increase the generation of autonomous activity with each pulse, even at low frequencies, as seen for β_M -CaMKII. As autophosphorylation also increases the affinity of CaMKII to CaM (Meyer *et al.*, 1992), a higher degree of autophosphorylation per Ca^{2+} spike would also shift the exponential rise to lower frequencies and make this rise appear less steep. As a consequence, significant autonomous activity of β_M -CaMKII is generated even by low-frequency Ca^{2+} oscillations, whereas β_e '-CaMKII requires high-frequency stimulation for appreciable autonomy.

It should be noted that the frequency response of CaMKII is considerably shaped by the pulse duration and amplitude: pulses of short duration and/or low amplitude activate autophosphorylation at a higher threshold frequency, whereas pulses of longer duration and/or higher amplitude can be sensed by the kinase over a broader range of frequencies (De Koninck and Schulman, 1998). These effects are directly related to the probability of CaM binding during the pulses. The frequency range of Ca²⁺ oscillation that CaMKII can sense is thus expected to vary considerably depending on the properties of the Ca²⁺ spikes and the local factors that bind, chelate or release Ca²⁺ and CaM. The length and frequencies of Ca²⁺ spikes in cells vary tremendously, from a few milliseconds to several seconds and from 10⁻³ to at least 10 Hz, depending on the stimuli or the subcellular compartments (Koester and Sakmann, 2000; for a review, see Berridge *et al.*, 2000; Bootman *et al.*, 2001). Thus, alternative splicing of CaMKII should differentially impact the kinase output signal, depending on the cellular localization and the input stimulus.

What are the functional implications of the differential Ca²⁺ oscillation response detected for the different CaMKII isoforms? In skeletal muscle, β_M -CaMKII is one of the most prominent CaMKII isoforms and the only β variant expressed (Bayer *et al.*, 1998). There, β_M is targeted by a CaMKII-related anchoring protein, α KAP, to the membrane of the sarcoplasmic reticulum (Bayer *et al.*, 1998), where CaMKII controls Ca²⁺ release from intracellular stores in a negative feedback loop by phosphorylating the ryanodine receptor (the Ca²⁺ release channel) (Witcher *et al.*, 1991; Wang and Best, 1992; Hain *et al.*, 1995), phospholamban (a regulator of the Ca²⁺ pump protein) (Wegener *et al.*, 1989) and the Ca²⁺ pump protein itself (Xu *et al.*, 1993; Hawkins *et al.*, 1994). Thus, CaMKII can control Ca²⁺ oscillations, and its own sensitivity to such oscillations, modulated by kinase isoform and splice variant expression, would influence this feedback regulation. Expression of both β - and β_e '-CaMKII is restricted to the central nervous system. However, α - and β -CaMKII are by far the most abundant isoforms in the mature brain. In contrast, β_e - and β_e '-CaMKII are the predominant β variants during early postnatal development, a time when there is virtually no α - or β -CaMKII present. Like β_e '-CaMKII, the β_e variant has reduced CaM affinity (Brocke *et al.*, 1999). Thus, neonatal neurons mostly express CaMKII isoforms that can, in contrast to α -CaMKII, associate with the actin cytoskeleton (Shen *et al.*, 1998), but have a high frequency threshold, more similar to the α isoform. A requirement for distinct frequencies of spontaneous Ca²⁺ spikes and waves in neural development has been demonstrated (Gu and Spitzer, 1995), and Ca²⁺ and CaMKII have actin-based morphological effects on dendritic arbors and spines (Wu and Cline, 1998; Fischer *et al.*, 2000), probably with distinct functions in differentiating versus mature neurons. High frequencies of neuronal stimulation can trigger long-term potentiation of synaptic strength, whereas low-frequency stimulation leads to long-term depression. The autophosphorylation state of CaMKII is thought to be involved in setting the frequency threshold for the induction of long-term potentiation (for a review, see Deisseroth *et al.*, 1995). Thus, this form of metaplasticity

would be modulated by differential expression of CaMKII isoforms with distinct Ca²⁺ oscillation sensitivities for autophosphorylation. Indeed, such metaplasticity is known to change during development (Molloy and Kennedy, 1991; Deisseroth *et al.*, 1995; Mayford *et al.*, 1995).

Remarkably, β_M -, β - and β_e '-CaMKII are differentially expressed even among individual mature hippocampal neurons, as shown here for β_M and previously for the other β variants (Brocke *et al.*, 1999). Thus, regulated alternative splicing may tune the sensitivity to Ca²⁺ oscillations not only during development and for different tissues, but also for individual cells of the same type. Thereby, differential expression of CaMKII variants can provide a novel level of neuronal plasticity, and it will be interesting to elucidate the mechanisms and cellular stimuli that regulate the alternative splice events. The ubiquitous γ and δ isoforms of CaMKII show a high degree of structural homology to α - and β -CaMKII and are subject to even more extensive alternative splicing. Thus, alternative splicing and co-assembly into heteromeric holoenzymes with mixed properties (De Koninck and Schulman, 1998; Brocke *et al.*, 1999) can give rise to finely tuned arrays of molecular decoders of Ca²⁺ oscillations in a large variety of different cell types.

Materials and methods

Molecular cloning and sequencing

Genomic clones of the murine β -CaMKII locus have been initially derived from a λ -library screen (Karls *et al.*, 1992). Subclones in pBKS mapping the region 5' of the first exon coding for the association domain and 3' of the last exon coding for the conventional variable region (exons V and IV in Karls *et al.*, 1992) were sequenced using the ABI system. The following sequencing primers were derived from both ends of each β_M -CaMKII-specific repeat, allowing generation of sequence information in both directions, with coding sequences known from cDNA analysis preceding the unknown intron border sequences: bM-1.3, CAG-GGAGAGTCGGAGAT; bM-2.3, TAGGAGACCCGGAGACAA; bM-3.3, CTAATGGGAAGTGGAGAT; bM-4.5, TCCTCAACTCAGTGAGGC; bM-5.5, TCCTGAAGTCAAGTGAAGAA; bM-6.5, TGAGCT-TGGTGAGGA.

Mammalian expression vectors in SR α for β -, β_e '- and β_M -CaMKII have been described previously (Brocke *et al.*, 1995; Bayer *et al.*, 1998). A C-terminal HA-epitope tag was introduced in the β_e ' and β_M sequences essentially as carried out previously for the β isoform (De Koninck and Schulman, 1998).

Protein expression, immunodetection and activity assay

COS-7 cells were transfected with expression vectors by the CaPO₄ method and harvested 68–76 h after transfection, as described previously (Srinivasan *et al.*, 1994; Bayer *et al.*, 1998). CaMKII was detected by western blotting and subsequent immunostaining with a β -CaMKII-specific antibody (CB- β 1; Gibco/Invitrogen, Carlsbad, CA) or a phospho-T286/T287-CaMKII-specific antibody (Upstate Biotech, Charlottesville, VA) using the Western Lightning detection system (NEN/PerkinElmer, Boston, MA), essentially as described (Srinivasan *et al.*, 1994; Bayer *et al.*, 1998). For detection after immobilization (see below), the kinase was re-solubilized for 10 min in SDS loading buffer at 95°C. Stimulated kinase activity was tested by phosphorylation of the peptide substrate AC-3 during 1 min reactions at 30°C [50 mM PIPES pH 7.2, 10 mM MgCl₂, 0.1% BSA, 25 μ M AC-3, 0.5 mM CaCl₂, 300 nM CaM or as indicated, and 250 μ M [γ -³²P]ATP (1 Ci/mmol)]. For assessing Ca²⁺-independent (autonomous) kinase activity, the reaction mix contained 1 mM EGTA and no Ca²⁺ or CaM. Reactions were stopped by precipitation with 10% TCA on ice, and the supernatants containing the peptide substrate were spotted onto Whatman P81 paper squares. ATP was removed by washing the filters for 45 min, first in 0.5% phosphoric acid and then in water, and ³²P incorporation of the bound peptide was measured in a scintillation counter.

Immobilized kinase assay, autophosphorylation and oscillatory stimulation

HA-tagged CaMKII was immobilized on PVC microtiter plates or tubing coated with anti-HA antibody (BabCO/Covance, Princeton, NY) (De Koninck and Schulman, 1998). Stimulated and autonomous activity of the immobilized kinase were assessed as described above, however without the TCA precipitation step. Maximal autonomy was generated by 1.5 min autophosphorylation on ice in activating solution (non-radioactive assay buffer with 30 nM CaM but without peptide substrate). For measuring autonomy after brief (0.2–12 s) Ca^{2+} stimulation, tubing with immobilized CaMKII was connected to a custom-made device with computer-controlled valves allowing alternate flow from two pressurized chambers containing either activating solution or de-activating solution (without CaCl_2 , CaM or ATP, but with 1 mM EGTA) at 30°C (De Koninck and Schulman, 1998). Autophosphorylation was induced by activating solution, and stopped after various times by a pulse of de-activating solution. To assess the response of CaMKII to Ca^{2+} oscillations, 30 brief activating pulses (0.2 s) were alternated with de-activating pulses. The oscillation frequency was controlled by the duration of the de-activating pulses.

RT-PCR, in situ hybridization and immunocytochemistry

RT-PCR was performed essentially as described previously (Karls *et al.*, 1992; Bayer *et al.*, 1996). RNAs from different tissues (Clontech, Palo Alto, CA) were used as templates for oligo-dT-primed cDNA synthesis with Superscript reverse transcriptase (Invitrogen, Carlsbad, CA). The PCR primers are described above [bM-6.5 and bM-1.3; nucleotides 22–36 and complement of nucleotides 348–354 in Bayer *et al.* (1999)].

In situ hybridization of hippocampal neurons cultured for 3 weeks at high density (Bayer *et al.*, 2001) with digoxigenin-labeled RNA probes was performed as described previously (Bayer *et al.*, 1999). The antisense probe corresponds to nucleotides 133–354 of the β_{M} -specific insert (Bayer *et al.*, 1999), and a sense-strand probe was used as control. For immunocytochemistry with antibodies against β -CaMKII (monoclonal CB- β 1; Gibco; 1:200 dilution) and MAP-2 (polyclonal; Chemicon, Temecula, CA; 1:300 dilution), neurons were fixed with 4% paraformaldehyde (in 0.1 M phosphate buffer pH 7.4, 4% sucrose) for 10 min, permeabilized and pre-blocked for 60 min in PBS containing 0.05% Triton X-100, 5% normal goat serum. Primary antibodies were incubated for 1 h at room temperature in blocking solution. Secondary antibodies (goat anti-mouse–Alexa-488 and goat anti-rabbit–Alexa-594; Molecular Probes, Eugene, OR; 1:200 dilution) were incubated for 45 min at room temperature. Following multiple washes, coverslips were mounted on glass slides with Immuno-Fluore mounting media (ICN, Costa Mesa, CA). Fluorescent images were acquired with a Coolsnap-HQ CCD camera (Roper Instruments, Roper Scientific, Tucson, AZ), through a 10 $\times$ achroplan water immersion objective (0.3 numerical aperture) on a Zeiss Axioskop FS-2 microscope.

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