Regulation of Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II catalysis by N-methyl-D-aspartate receptor subunit 2B

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INTRODUCTION

CaMKII (Ca\textsuperscript{2+}/CaM (calmodulin)-dependent protein kinase II) and NMDAR (N-methyl-D-aspartate receptor) are involved in LTP (long-term potentiation) at neuronal synapses that underlies learning and memory [1]. Upon postsynaptic Ca\textsuperscript{2+} influx, CaMKII gets activated and translocates to the PSD (postsynaptic density) [2], where it binds to the NR2B subunit of NMDAR [3,4]. This binding anchors CaMKII to the PSD and helps to maintain a separate pool of CaMKII that is located proximal to the neurotransmitter receptors [5].

NR2B binds to CaMKII at two sites, one of them being the active site, resulting in phosphorylation of Ser\textsuperscript{1303} of NR2B [6], and the other is the T-site (Thr\textsuperscript{286}-autophosphorylation site binding pocket), which lies outside the active site [7]. The voltage-gated potassium channel Drosophila, Eag, which is localized in synapses as well as in axons also interacts with the T-site of CaMKII [8]. The presence of these ligands at the T-site prevents the autoinhibitory domain from occupying the active site, thereby rendering the enzyme autonomously active [7,8].

We have previously shown that the kinetics of phosphorylation of NR2B-Ser\textsuperscript{1303} (Figure 1) are different to that of classical substrates of CaMKII such as syntide-2 [9]. We have also reported that the NR2B substrate sequence modulates the kinetic parameters of CaMKII for ATP [10]. A previous report has also suggested that the NR2B sequence motif caused catalytic modulation to a lesser extent. The kinetic parameters of ATP for the Thr\textsuperscript{286}-autophosphorylation reaction of CaMKII were also altered by NR2B in a similar manner. Interestingly, the NR2B sequence motif caused increased sensitivity of CaMKII activity to ATP, and saturation by lower concentrations of ATP, which, in effect, resulted in a constant level of activity of CaMKII over a broad range of ATP concentrations. Our findings indicate that CaMKII at the PSD may be regulated by bound NR2B in a manner that supports synaptic memories.

Key words: allosteric modulation, Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII), N-methyl-D-aspartate receptor (NMDAR), NR2B, phosphorylation.
Expression of α-CaMKII using the baculovirus/SF21 cell system

The recombinant virus containing the cDNA of α-CaMKII was prepared using the Bac-to-Bac Expression system according to the manufacturer’s protocol. For purification of the enzyme, monolayer cultures of SF21 cells in 175 cm² flasks were infected with viral stocks and were harvested 60–72 h post infection.

Preparation of crude and purified forms of α-CaMKII expressed in the baculovirus/SF21 cell system

The crude insect cell lysate expressing α-CaMKII and purified α-CaMKII were prepared as described previously [9] with modifications. The crude lysate, which had a single band of α-CaMKII on Western blot [16] (Supplementary Figure S2A at http://www.BiochemJ.org/bj/419/bj4190123add.htm), was used as the enzyme source for GST pull-down experiments.

Purification of the enzyme was carried out at 4°C throughout. The crude lysate was loaded on to a phosphocellulose column, pre-equilibrated with equilibration buffer (50 mM Pipes, pH 7.0, 100 mM NaCl, 1 mM EGTA, 0.5 mM DTT and 1 × protease inhibitor cocktail) and was incubated for 30 min. The bound proteins were eluted with 5 times the column volume (approx. 25 ml) of elution buffer (50 mM Pipes, pH 7.0, 500 mM NaCl, 1 mM EGTA, 0.5 mM DTT and 1 × protease inhibitor cocktail). The eluate containing CaMKII activity was used for affinity purification on a CaM-Sepharose column as described previously [9]. The purified enzyme preparation showed a single major band of the expected size on SDS/PAGE (Supplementary Figure S2B) and on Western blot (Supplementary Figure S2C). The yield of purified protein in a typical purification was 65%. The specific activity of purified WT α-CaMKII for phosphorylation of the GST–NR2B substrate varied from 0.014 to 0.13 μmol·min⁻¹·mg⁻¹ of protein for the different preparations used in the study. When necessary, the final purified sample was concentrated using Amicon ultrafiltration devices with a 100 kDa molecular weight cut off by spinning at 5000 g to get the required concentration.

GST pull-down assay

Glutathione–Sepharose 4B beads were washed thoroughly with PBS (10 mM disodium hydrogen phosphate, 1.8 mM potassium dihydrogen orthophosphate, 0.14 M sodium chloride, 2.7 mM potassium chloride, pH 7.4) and were mixed separately with each of the GST-fusion proteins in the presence or absence of MgCl₂. The GST–NR2B/2B sequences present in the experimental vector [9] were used in the study. When necessary, the final purified sample was concentrated using Amicon ultrafiltration devices with a 100 kDa molecular weight cut off by spinning at 5000 g to get the required concentration.

When required, GST-fusion proteins were purified by affinity chromatography using glutathione–Sepharose 4B in 50 mM Tris buffer (pH 7.5). Proteins were eluted with 50 mM Tris buffer containing 10 mM reduced glutathione (pH 7.5). The purified fusion proteins were nearly homogenous when analysed by SDS/PAGE (Supplementary Figure S1B). Yields of up to 45% could be achieved for purification of fusion proteins.

The sequence motifs of NR2A and Eag homologous to the CaMKII phosphorylation site on α-CaMKII were expressed using the respective expression vector pFastBac1-α-CaMKII [9] that was used for expression of the WT (wild-type) enzyme served as the template to generate mutants. The NR2A/2B sequences present in the experimental vector [9] were used in the study. When necessary, the final purified sample was concentrated using Amicon ultrafiltration devices with a 100 kDa molecular weight cut off by spinning at 5000 g to get the required concentration.

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assays instead of glutathione–Sepharose 4B beads. Equal volumes of fusion proteins were added to prewashed wells and were incubated for 1 h at room temperature with shaking. Unbound fusion proteins were removed and the wells were washed three times with PBS. Equal concentrations of crude lysates of insect cells expressing WT α-CaMKII in binding buffer were then added to the wells and were incubated for 1 h at 4 °C with occasional mixing. The unbound protein was removed and the wells were washed with PBS. SDS sample buffer was then added to the wells to retrieve the bound proteins which were then subjected to Western blotting.

**Fusion protein phosphorylation**

GST fusion proteins of the CaMKII phosphorylation site sequence on NR2B, NR2A and Eag (Figure 1) were used as substrates for CaMKII in an *in vitro* assay. Reactions were carried out in 20 μl volumes. Each assay tube contained 50 mM Tris/HCl, pH 8.0, 10 mM MgCl₂, 12 μM CaM, 0.2 mg/ml BSA, CaCl₂-EGTA (0.9 mM CaCl₂ in excess of EGTA), various concentrations of [γ-³²P]ATP (1000–3000 cpm/pmol) with or without 10 mM DTT and GST–NR2B, GST–NR2A or GST–Eag. In the case of ATP kinetics, the concentration of ATP was varied from 1–400 μM with saturating concentrations of GST–NR2A/NR2B/ Eag substrates. The reaction mix was incubated for 1 min at 30 °C. The reaction was initiated by adding the purified enzyme and after 1 min the phosphorylation was terminated by the addition of 5 μl of 5× SDS sample buffer. The sample was denatured, loaded on to SDS/PAGE (10 % gel), and the gel was dried and exposed to a phosphor screen. The image obtained by scanning the phosphor screen (Bio-Rad Personal Molecular Imager FX) showed a single major band corresponding to the phosphorylated fusion protein (Supplementary Figure S1C). The image was used to quantify the band intensities using QuantityOne software. The band intensity values showed a linear relationship with the amount of phosphorylated fusion protein (Supplementary Figure S4 at http://www.BiochemJ.org/bj/419/bj4190123add.htm) as well as by the phosphorimager analysis. The slope of the resulting straight line gave intensity values for radioactive spots after deducting the radioactive ATP that was used for the assay, three different volumes were spotted, after appropriate dilutions, on to Whatman paper as standards. The paper was subjected to Phosphoimager analysis along with the gel. The radioactivity of the standards was measured using the scintillation counter and those obtained from the phosphorimager analysis. The slope of the resulting straight line was used as the factor for converting band intensity to radioactivity in cpm.

The data were analysed using a Eadie–Hofstee plot and Hill plot. The plots were made in Microsoft Excel software and the values for $V_{max}$, $K_m$, $S_{0.5}$ and Hill coefficient were obtained from the equation for the best fit straight line generated by the software. The final values for $V_{max}$, $K_m$, $S_{0.5}$ (substrate concentration at half maximal velocity) and Hill coefficient are presented as the means ± S.D. of data from at least three independent experiments. The data were also fitted to the Hill equation using Origin Software which also yielded similar kinetic parameters.

**Autophosphorylation of CaMKII**

ATP kinetics of Thr²⁸⁶ autophosphorylation of CaMKII was carried out as described before [9] with different [γ-³²P]ATP (1000–5200 cpm/pmol) concentrations ranging from 0.005 to 200 μM. Each assay tube contained 50 mM Tris/HCl, pH 8.0, 10 mM MgCl₂, 0.4 mM EGTA, 1.3 mM CaCl₂, 17 μM CaM, 0.2 mg/ml BSA and 0.34 μM GST–(S1291A)-NR2A or 0.27 μM GST–(S1303A)-NR2B (both purified) or control (no GST-fusion protein) in a total volume of 20 μl. The assay was performed by first pre-incubating the purified WT α-CaMKII enzyme along with the fusion protein containing the assay mixture for 2 min at 30 °C followed by a 30 s phosphorylation reaction, initiated with the addition of [γ-³²P]ATP. The reaction was stopped by the addition of 5 × SDS sample buffer and the samples were run on SDS/PAGE. After the staining and destaining processes, the gels were dried and were subjected to autoradiography. The gel bands in the autoradiogram corresponding to phosphorylated CaMKII were quantified as described above. Autophosphorylation at Thr²⁸⁶ was confirmed by Western blotting using an anti-phospho-Thr²⁸⁶-α-CaMKII antibody (Supplementary Figure S5 at http://www.BiochemJ.org/bj/419/bj4190123add.htm) as well as by the appearance of Ca²⁺-independent activity (results not shown).

### RESULTS

**Interaction of CaMKII with ATP is influenced by the nature of the protein substrate**

The kinetic parameters of CaMKII for ATP were measured for phosphorylation of GST–NR2A and GST–NR2B, two protein substrates that are known to differ in their interaction with CaMKII. The apparent $S_{0.5}$ and $V_{max}$ values for ATP were lower when GST–NR2B was used as the protein substrate (Table 1). However, there was no significant change in the $V_{max}/S_{0.5}$ Value. More interestingly, the saturation pattern of ATP for phosphorylation of GST–NR2A was sigmoidal, whereas in the case of phosphorylation of GST–NR2B it was hyperbolic (Figure 2A). Hill coefficient values for ATP also reflected this change (Table 1 and Figure 2B). The Hill coefficient value was approaching 2.0 with GST–NR2A as the protein substrate indicating co-operative behaviour, whereas the value close to 1 with GST–NR2B as the protein substrate indicated non-co-operative behaviour. These results show that the catalytic parameters of CaMKII for ATP differ for phosphorylation of different protein substrate sequences.

<table>
<thead>
<tr>
<th>Protein substrate</th>
<th>$S_{0.5}$ (μM)</th>
<th>$V_{max}$ (μmol/min/mg of protein)</th>
<th>$V_{max}/S_{0.5}$</th>
<th>Hill coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST–NR2A</td>
<td>37 ± 5</td>
<td>0.40 ± 0.19</td>
<td>0.011 ± 0.005</td>
<td>1.82 ± 0.13</td>
</tr>
<tr>
<td>GST–NR2B</td>
<td>6.1 ± 0.8</td>
<td>0.078 ± 0.008</td>
<td>0.013 ± 0.003</td>
<td>0.96 ± 0.17</td>
</tr>
</tbody>
</table>

**Mutations that affect the T-site-mediated interaction between CaMKII and GST–NR2B also affect catalysis**

CaMKII is reported to employ an ordered substrate-binding mechanism where ATP binds first to the free catalytic site of the enzyme followed by protein substrate [17,18,19]. Hence differences in the interaction of the two protein sequences at the catalytic site are unlikely to be the cause for the observed differences in kinetic parameters for ATP. We investigated whether the binding of NR2B to the T-site of CaMKII could be causing a modulation of the
interaction of ATP with the enzyme. To test this, we measured the kinetic parameters for ATP after disrupting the T-site-mediated interaction between the CaMKII and NR2B sequence. The phosphorylation reaction was conducted with mutants of GST–NR2B or CaMKII that were defective in the T-site-mediated interaction. The mutants used were L1298A-NR2B [20] and I205K-α-CaMKII [7]. Using the GST pull-down assay, which represents binding at the T-site, we demonstrated that binding exists between WT forms of CaMKII and GST–NR2B or GST–Eag (Figure 3A), whereas it was defective in the case of GST–NR2A (Figure 3A), as well as for the mutants (Figures 3B and 3C). Both the mutants, GST–(L1298A)-NR2B (Figure 3B) and I205K-α-CaMKII (Figure 3C) exhibited significantly attenuated binding to their WT binding partner in the pull-down assay. When GST–(L1298A)-NR2B was phosphorylated by WT α-CaMKII, the kinetic parameters for ATP exhibited a shift towards those observed for phosphorylation of GST–NR2A by WT α-CaMKII. It was observed that the values of $S_{0.5}$ and $V_{\text{max}}$ were higher compared with phosphorylation of WT GST–NR2B (Figure 4). The Hill coefficient value was also higher, showing resemblance with that of GST–NR2A (Figure 4). Similar changes were observed when phosphorylation of GST–NR2B by I205K-α-CaMKII was compared with that by WT α-CaMKII (Figure 5). The apparent values of $S_{0.5}$ and $V_{\text{max}}$ showed increases along with an increase in the Hill coefficient value when phosphorylation of GST–NR2B was carried out by I205K-α-CaMKII. Interrupting the T-site-mediated stable association either by mutating the NR2B sequence or by mutating CaMKII thus seemed to result in the shifting of kinetics of phosphorylation towards that observed for GST–NR2A. This suggested that binding of NR2B at the T-site of CaMKII exerts a modulatory effect resulting in the altered kinetic parameters observed for phosphorylation of GST–NR2B.

Modulation by NR2B is mediated through ATP-binding residues

A conserved glutamate residue, Glu$^{96}$ of α-CaMKII, has been attributed a key role in binding ATP based on molecular simulation studies using the crystal structure of the catalytic domain of CaMKII [21]. We found that mutation of Glu$^{96}$ to alanine impaired the modulation of catalysis by NR2B (Table 2), even though its binding to the T-site of CaMKII is not affected by the mutation, as seen by binding in the pull-down assay (Figure 3D), indicating that Glu$^{96}$ is involved in mediating the modulation of catalysis by NR2B.

In WT CaMKII, modulation of catalysis due to the binding of NR2B sequence to the T-site results in the relatively higher value for the ratio GST–NR2A/GST–NR2B for $S_{0.5}$ and $V_{\text{max}}$ values (Table 2). However, in the case of E96A mutant, there was a clear reduction in the GST–NR2A/GST–NR2B ratios for $S_{0.5}$ and $V_{\text{max}}$ values (Table 2). The $S_{0.5}$ value for ATP with GST–NR2B was higher for the E96A mutant compared with WT α-CaMKII, although it was largely unaffected with GST–NR2A. The $V_{\text{max}}$ values for phosphorylation of GST–NR2A and GST–NR2B were similar with the E96A mutation. These changes indicate that modulation of catalysis by GST–NR2B bound at the T-site is impaired in the E96A mutant, suggesting that Glu$^{96}$ plays a role in the modulation of catalysis by the NR2B sequence.

NR2B modulates kinetics of Thr$^{286}$-autophosphorylation

We studied the effect of NR2B sequence on the Thr$^{286}$-auto-phosphorylation of α-CaMKII. Interestingly, we find that ATP saturation does not show co-operativity in the case of Thr$^{286}$ auto-phosphorylation of CaMKII (Table 3), indicating major differences between the kinetic mechanisms of the autophosphorylation and substrate phosphorylation reactions. The kinetic parameters for the autophosphorylation reaction also differed significantly from those of the substrate phosphorylation reaction. The kinetic parameters for ATP were measured in the presence of GST–(S1291A)-NR2B and were compared with the reaction carried out in the presence of GST–(S1291A)-NR2A or in the absence of any protein substrate modulators. Although the presence of NR2A sequence did not seem to affect the kinetic parameters, the presence of NR2B sequence exerted a significant influence. It could be seen that the apparent values of $K_{\text{m}}$ and $V_{\text{max}}$ were decreased in the presence of GST–(S1303A)-NR2B, whereas the $V_{\text{max}}/K_{\text{m}}$ value showed an increase (Table 3). These effects on $K_{\text{m}}$ and $V_{\text{max}}$ were similar to the observations made in the case of substrate phosphorylation (Table 1).

Activity of CaMKII is enhanced by NR2B at low ATP concentrations

Interestingly, when the ATP concentration dependence of the activity of α-CaMKII was closely examined, it was observed that at low ATP concentrations the activity of CaMKII was higher in the presence of NR2B than in the presence of NR2A. This could be observed in the case of both substrate phosphorylation
NR2B modulates CaMKII activity

Figure 3  GST pull-down of α-CaMKII

The blots were cut into two and were separately developed with anti-α-CaMKII antibody and anti-GST antibody as indicated. Molecular sizes indicated to the right are in kDa. (A) Pull-down using glutathione-coated plates with GST–NR2A, GST–NR2B, GST–Eag or GST. MW, prestained protein molecular mass marker. The results represent two separate experiments. (B) Pull-down, using glutathione-Sepharose 4B beads, of WT α-CaMKII with GST–NR2B or GST–(L1298A)-NR2B. Molecular-mass marker positions are indicated to the right in kDa. The results represent three separate experiments. (C) Pull-down, using glutathione-Sepharose 4B beads, of I205K mutant or WT of α-CaMKII with GST–NR2B. MW indicates molecular mass markers, with values in kDa. The results represent three separate experiments. (D) Pull-down, using glutathione-Sepharose 4B beads, of WT or E96A mutant of α-CaMKII with GST–NR2B. MW indicates molecular mass markers with values to the right in kDa. The results represent two separate experiments.

as well as autophosphorylation. The non-phosphorylatable GST–(S1303A)-NR2B was first allowed to bind to α-CaMKII by incubating them together in the presence of Ca²⁺/CaM and the complex was then used to phosphorylate GST–NR2A. As seen in Figure 6, the activity of α-CaMKII was higher when it was pre-treated with GST–(S1303A)-NR2B compared with the control in which the enzyme was pre-treated with GST–(S1291A)-NR2A. When phosphorylation of GST–NR2A and GST–NR2B were compared, it could be seen that the activity was higher for phosphorylation of GST–NR2B at concentrations of ATP below 5 μM (Supplementary Figure S6 at http://www.BiochemJ.org/bj/419/bj4190123add.htm). In the presence of GST–NR2B, the enzyme is saturated at relatively lower concentrations of ATP and the activity stays constant for a large range of higher ATP concentrations. In contrast, the phosphorylation of GST–NR2A continues to increase to much higher levels as the concentration of ATP is increased. The autophosphorylation activity was also higher in the presence of GST–(S1303A)-NR2B compared with the activity in the presence of GST–(S1291A)-NR2A at ATP concentrations lower than 1 μM (Supplementary Figures S7 and
Table 2  Apparent kinetic constants of E96A-α-CaMKII for ATP

Each value is the mean ± S.D. of three separate determinations. Each determination had eight or more data points in the Hill plot. The amount of α-CaMKII used was 0.0015 mg/ml (WT) or 0.0017 mg/ml (E96A) per reaction.

<table>
<thead>
<tr>
<th>Protein substrate</th>
<th>$S_{0.5}$ (μM)</th>
<th>$V_{max}$ (μmol⋅min$^{-1}$⋅mg$^{-1}$ of protein)</th>
<th>$V_{max}/S_{0.5}$</th>
<th>Hill coefficient</th>
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<tr>
<td><strong>WT</strong></td>
<td></td>
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<td></td>
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<tr>
<td>GST–NR2A</td>
<td>37 ± 3</td>
<td>0.77 ± 0.12</td>
<td>0.021 ± 0.004</td>
<td>1.92 ± 0.27</td>
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<tr>
<td>GST–NR2B</td>
<td>7.2 ± 2.8</td>
<td>0.13 ± 0.02</td>
<td>0.021 ± 0.013</td>
<td>1.15 ± 0.13</td>
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<tr>
<td>Fold difference</td>
<td></td>
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<tr>
<td>GST–NR2A/GST–NR2B</td>
<td>5.1</td>
<td>6.1</td>
<td>1.0</td>
<td>1.7</td>
</tr>
<tr>
<td><strong>E96A</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>GST–NR2A</td>
<td>38 ± 9</td>
<td>0.31 ± 0.13</td>
<td>0.008 ± 0.003</td>
<td>1.47 ± 0.13</td>
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<tr>
<td>GST–NR2B</td>
<td>18 ± 3</td>
<td>0.28 ± 0.11</td>
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<td>1.28 ± 0.14</td>
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<tr>
<td>Fold difference</td>
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<tr>
<td>GST–NR2A/GST–NR2B</td>
<td>2.1</td>
<td>1.1</td>
<td>0.53</td>
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S8 at http://www.BiochemJ.org/bj/419/bj4190123add.htm). In the presence of GST–(S1303A)-NR2B, the autophosphorylation activity also stayed constant over a broader range of ATP concentrations (Supplementary Figure S9 at http://www.BiochemJ.org/bj/419/bj4190123add.htm). These results very clearly showed that the NR2B sequence can enhance the activity of α-CaMKII at low concentrations of ATP and maintain it at a constant level over a wide range of ATP levels.

Eag potassium channel sequence exerts a lesser degree of modulation on α-CaMKII compared with NR2B

A sequence motif in the Eag potassium channel of Drosophila that is homologous to the phosphorylation site on NR2B (Figure 1) is known to bind α-CaMKII in the GST pull-down assay (Figure 3A), similar to GST–NR2B [8]. To see whether binding of Eag to the T-site of α-CaMKII also causes modulation of the activity of CaMKII, we investigated the kinetics of phosphorylation of GST-Eag by WT α-CaMKII. The kinetic parameters for phosphorylation of GST-Eag showed moderate differences compared with those of GST–NR2B (Figure 7 and Supplementary Table S1, Experiment A, at http://www.BiochemJ.org/bj/419/bj4190123add.htm). The Hill coefficient value for ATP was 1.3. The values of $S_{0.5}$, $V_{max}$ and $V_{max}/S_{0.5}$ were also marginally higher compared with GST–NR2B. The binding of Eag to the T-site of Drosophila CaMKII is known to be disrupted by mutating Ile$^{206}$ to lysine (equivalent to the I205K mutation in rat α-CaMKII) [8]. The kinetic parameters for phosphorylation of GST–Eag by I205K–α-CaMKII were higher compared with WT α-CaMKII (Supplementary Table S1). The increased $S_{0.5}$ and Hill coefficient values for I205K–α-CaMKII indicated a loss of catalytic modulation, since T-site binding was defective. This showed that binding at the T-site by the Eag sequence also can modulate catalysis by α-CaMKII.
NR2B modulates CaMKII activity

Table 3  The autophosphorylation of α-CaMKII in the presence of non-phosphorylatable mutants of GST–NR2A or GST–NR2B

<table>
<thead>
<tr>
<th>Modulator</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}/K_m$ (x 10^{-3})</th>
<th>Hill coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.22 ± 0.16</td>
<td>16 ± 6</td>
<td>0.87 ± 0.12</td>
</tr>
<tr>
<td>GST–(S1291A)-NR2A</td>
<td>1.38 ± 0.14</td>
<td>17 ± 2</td>
<td>1.07 ± 0.06</td>
</tr>
<tr>
<td>GST–(S1303A)-NR2B</td>
<td>0.27 ± 0.02</td>
<td>6.2 ± 1.7</td>
<td>1.05 ± 0.09</td>
</tr>
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</table>

Each value is the mean ± S.D. of three separate determinations. Each determination had eight or more data points in the Eadie–Hofstee or Hill plot. The concentration of WT-α-CaMKII used was 0.0061 mg/ml per reaction.

CaMKII was pre-incubated with 0.32 µM of either GST–(S1291A)-NR2A or GST–(S1303A)-NR2B in the presence of Ca^{2+}/calmodulin and the mix was used as the enzyme source to initiate the phosphorylation of GST–NR2A. Results are from a single experiment representative of two similar experiments. The autoradiogram shows phosphorylated bands of GST–NR2A in the presence of GST–(S1291A)-NR2A or GST–(S1303A)-NR2B. The specific activities calculated from the band intensities for phosphorylation of GST–NR2A by CaMKII (0.002 mg/ml) at different concentrations of ATP, in the presence of either GST–(S1291A)-NR2A or GST–(S1303A)-NR2B (C) are plotted in the graph below.

We compared the kinetic parameters of ATP for phosphorylation of GST–NR2B and GST–Eag by I205K-α-CaMKII (Figure 8 and Supplementary Table S1, Experiment B). Except for the apparent $V_{max}$ value, the Hill coefficient and $S_{0.5}$ values showed very little differences between GST–NR2B and GST–Eag. This showed that, when binding in the GST pull-down assay is defective, as in the case of I205K-α-CaMKII, the two substrates did not modulate the kinetic parameters for ATP.

The affinities of GST–NR2B and GST–Eag towards the T-site of α-CaMKII were compared by competition experiments. Binding of GST–NR2B and GST–Eag to α-CaMKII were subjected to competition by a peptide with the sequence of the binding site of NR2B (NR2B 17-mer, Figure 1). As seen in Figure 9(A), the peptide competes out GST–Eag more effectively than GST–NR2B. The $IC_{50}$ value (concentration required for 50% inhibition) of the NR2B 17-mer was measured (Figure 9B) and was found to be lower in the case of pull-down with GST–Eag.

Figure 6  Modulation of the substrate phosphorylation activity of CaMKII by the NR2B sequence motif

Figure 7  Phosphorylation of GST–NR2B and GST–Eag by WT α-CaMKII

Each bar represents the mean ± S.E.M. of three separate determinations. Each determination had six or more data points in the Hill plot. Phosphorylation was by 0.5 µg/ml WT α-CaMKII.

Figure 8  Phosphorylation of GST–NR2B and GST–Eag by I205K-α-CaMKII

Each bar represents the mean ± S.E.M. of four separate determinations. Each determination had six or more data points in the Hill plot. Phosphorylation was by 0.3 µg/ml of I205K-α-CaMKII. The mean values of $S_{0.5}$, $V_{max}$ and $V_{max}/S_{0.5}$ for phosphorylation of GST–NR2B were taken to be 100%. Phosphorylation was by 0.5 µg/ml WT α-CaMKII.
This indicated that the NR2B sequence has a higher affinity for α-CaMKII than Eag.

**DISCUSSION**

Interaction of CaMKII with NR2B has been suggested to regulate the enzymatic activity of CaMKII by making it Ca\(^2+\)/CaM independent [7]. It has also been reported that the NR2B sequence causes uncompetitive inhibition of CaMKII with respect to ATP substrate [11]. Our results bring out further novel features of the interaction between CaMKII and NR2B. We show that interaction of NR2B sequence at the T-site of CaMKII causes a shift in the saturation pattern of ATP from cooperative to non-cooperative.

Although the \( V_{\text{max}} \) and \( S_{0.5} \) values for ATP were decreased by the presence of NR2B, there were no significant changes in the observed \( V_{\text{max}}/S_{0.5} \) ratio, a parameter which could be taken as a measure of catalytic efficiency (Table 1).

Both ordered and random substrate-binding mechanisms have been proposed for CaMKII [17,18,19,22], although ordered binding is more favoured [22]. Comparing the results obtained for WT and mutant forms establishes that the kinetics of substrate phosphorylation is altered by the binding of the protein substrate to the T-site of CaMKII (Figures 4 and 5). The mutations that we used, L1298A in NR2B and I205K in α-CaMKII, might have additional influences on the catalytic parameters of phosphorylation. For example, the residue Leu\(^{1298}\) has been reported to be a specificity determinant for the catalytic site of CaMKII [23]. When this amino acid is mutated to a less hydrophobic residue, the recognition by the catalytic site could also become hampered in addition to the loss of binding at the T-site. Therefore the observed \( V_{\text{max}} \) would be a result of both of these events, leading to a decrease in the \( V_{\text{max}}/S_{0.5} \) ratio (Figure 4), whereas the \( V_{\text{max}}/S_{0.5} \) ratio of GST–NR2A was closer to GST–NR2B (Table 1). The I205K mutant of α-CaMKII exhibited a higher \( V_{\text{max}}/S_{0.5} \) ratio compared with WT α-CaMKII (Figure 5), which indicates that the mutation may have additional effects on catalysis other than disrupting the binding of NR2B at the T-site.

The effect of the E96A mutation (Table 2) shows that the catalytic modulation by NR2B is mediated in a major way through Glu\(^96\). In WT α-CaMKII, the observed lowering of \( K_{\text{m}} \) and \( V_{\text{max}} \) values consequent to binding of GST–NR2B to the T-site might require reorientation of Glu\(^96\) to a position favourable for interaction with ATP. Absence of this mechanism due to lack of Glu\(^96\) in the E96A mutant may result in a loss of regulatory control, which leads to escalation of the \( K_{\text{m}} \) and \( V_{\text{max}} \) values. The mutation causes a decrease in the \( V_{\text{max}} \) value for GST–NR2A, indicating a marginal impairment of the catalytic process of CaMKII. On the whole, the present data on the E96A mutant shows that in the CaM-activated state, binding of NR2B at the T-site further modulates catalytic parameters of ATP through Glu\(^96\). This reveals a novel structural mechanism involving Glu\(^96\), and probably helix-αD that harbours Glu\(^96\), to regulate ATP binding in CaMKII.

We find that kinetic parameters for autophosphorylation at the Thr\(^{286}\) residue of α-CaMKII differ significantly from those of endogenous substrate phosphorylation (Tables 1 and 3). The binding
of ATP to the enzyme was non-co-operative for the autophosphorylation reaction, whereas it was co-operative for substrate phosphorylation in the absence of any T-site modulators. Since in the autophosphorylation reaction two adjacent CaM-bound subunits of CaMKII form an enzyme–substrate pair, it is possible that the ATP-binding characteristics of the enzyme could differ from those of the substrate phosphorylation reaction. The autophosphorylation reaction is also modulated by the NR2B sequence, as seen by the changes in kinetic parameters (Table 3). Since CaMKII present in the PSD can be in complex with NR2B [24], it is likely that its activity for both substrate phosphorylation, as well as autophosphorylation, may be modulated, as observed in our in vitro experiments.

Although the changes in the apparent $S_{0.5}$ and $V_{\text{max}}$ values observed for substrate phosphorylation (Table 1) as well as for Thr$^{286}$ autophosphorylation (Table 3) appear to resemble uncompetitive inhibition by GST–NR2B with respect to ATP, the effect on Hill coefficient for substrate phosphorylation indicates that the observed phenomenon is not classical uncompetitive inhibition. Moreover, in uncompetitive inhibition, the enzyme activity measured in the presence of the inhibitor will always be less than that in the absence of the inhibitor [25]. However, we would like to highlight the fact that both the Thr$^{286}$-autophosphorylation activity as well as substrate phosphorylation activity were consistently higher in the presence of the NR2B sequence than in its absence for the lower range of ATP concentrations used in our study (Figure 6, Supplementary Figures S6, S7 and S8). These data suggest that, at low concentrations of ATP, binding of NR2B to the T-site of CaMKII facilitates its interaction with ATP probably by increasing its affinity for ATP. This phenomenon could result from allosteric modulation. Since the NR2B-modulated enzyme is saturated at comparatively lower concentrations of ATP (Supplementary Figure S6), the activity remains constant over a broad range of ATP concentrations compared with activity in the absence of NR2B. A mechanistic explanation of such a regulation of CaMKII would require further investigation. An earlier study reported that a peptide with the NR2B sequence causes uncompetitive inhibition of CaMKII with respect to ATP [11]. The fusion protein GST–NR2B, used in the present study, had additional sequences compared with the peptide used in the earlier study and might differ in its interaction at the T-site of CaMKII, probably due to difference in the three-dimensional structure attained by its binding motif.

Although modulation of CaMKII by NR2B at the PSD may be necessary to support synaptic plasticity, CaMKII bound to Eaq on axons may conceivably serve physiological functions of a different nature. This is probably being reflected by the difference in the extent of allosteric modulation exerted on CaMKII by NR2B and Eaq. Our experiments show that the Eaq sequence binds to CaMKII with a lower affinity compared with NR2B (Figure 9). This could explain the minor differences in kinetic parameters such as $S_{0.5}$ and Hill coefficient between NR2B and Eaq (Figure 7 and Supplementary Table S1, Experiment A). The $V_{\text{max}}$ obtained for GST–NR2B was significantly higher compared with GST–Eaq for I205K-α-CaMKII-mediated phosphorylation (Figure 8 and Supplementary Table S1, Experiment B), indicating that the NR2B sequence is a better substrate for phosphorylation at the catalytic site. This characteristic of the NR2B sequence was probably not revealed in the WT enzyme due to the unique modulation caused by the binding of NR2B at the T-site. This becomes relevant in kinetic analyses of mutated substrate sequences aimed at identifying specificity determinants for the CaMKII catalytic site. The outcome of such studies might be affected by the ability of those sequences to bind the T-site, which in turn would influence the kinetic parameters.

The crystal structure of the CaMKII catalytic domain dimer is consistent with the observed co-operative mode of binding of calmodulin [21]. The co-operative saturation by calmodulin could in turn induce co-operativity in the saturation of ATP, based on earlier reports that ATP and calmodulin reciprocally enhance the binding of each other [18]. This, however, is unlikely since we have used calmodulin at saturating concentrations for all the kinetic experiments. Interestingly, the crystal structure of CaMKII also suggests that the ATP-binding pocket has features suitable for allosteric modulation [21]. Co-operative binding of ATP [18] suggests that there could probably be additional events of structural communication between subunits of a dimer upon binding of ATP. The modulatory effects of binding of NR2B at the T-site perhaps bypass these co-operative interactions between the subunits by elevating them all to a state of higher affinity for ATP.

Dendritic spine heads, especially in the cortex, have very few mitochondria to provide ATP. Hence ATP requirement in the PSD is suggested to be met by glycolytic source endogenous to PSD which is regulated by the levels of NAD$^+$, glycer-aldehyde-3-phosphate and nitric oxide [26]. Consequently, fluctuations in ATP levels in the PSD are likely to occur. The phosphorylated state of CaMKII in the CaMKII/PPI (protein phosphatase 1) switch that has been proposed as the storehouse of synaptic memory [24,27], has to be maintained against the action of phosphatases by continuous autophosphorylation at more or less constant rates even when the concentration of ATP varies. The enhanced activity of NR2B-bound CaMKII at low concentrations of ATP and its saturation by a minimal increase in concentration of ATP ensure a constant level of activity of CaMKII over a wide range of ATP concentrations (Supplementary Figures S6 and S9). Moreover, the reduced activity of NR2B-bound CaMKII at higher ATP concentrations (Supplementary Figures S6 and S9) helps to maintain the stable state of the CaMKII/PPI switch in an energy efficient manner by consuming minimal ATP as proposed previously [24]. Thus the modulation of CaMKII activity by NR2B reported in the present study could aid in the role of CaMKII as a molecular switch in synaptic memory.

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SUPPLEMENTARY ONLINE DATA

Regulation of Ca\(^{2+}\)/calmodulin-dependent protein kinase II catalysis by N-methyl-D-aspartate receptor subunit 2B

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Table S1 Apparent kinetic parameters of \(\alpha\)-CaMKII for ATP

Kinetic parameters for ATP for phosphorylation of GST–NR2B or GST–Eag by WT or I205K mutant of \(\alpha\)-CaMKII. A and B are different sets of experiments, each of which compares phosphorylation kinetics between the two enzyme–substrate pairs. Each value is the mean ± S.D. of three (A) or four (B) separate determinations. Each determination had six or more data points in the Hill plot. Phosphorylation was carried out with 0.5 μg/ml WT-\(\alpha\)-CaMKII (A) or 0.3 μg/ml of I205K-\(\alpha\)-CaMKII (B)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Protein substrate</th>
<th>(S_0.5) (μM)</th>
<th>(V_{max}) (μmol·min(^{-1})·mg(^{-1}) of protein)</th>
<th>(V_{max}/S_0.5)</th>
<th>Hill coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>GST–NR2B</td>
<td>1.7 ± 0.61</td>
<td>0.014 ± 0.006</td>
<td>0.009 ± 0.005</td>
<td>0.88 ± 0.11</td>
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<tr>
<td>WT</td>
<td>GST–Eag</td>
<td>2.97 ± 0.59</td>
<td>0.019 ± 0.011</td>
<td>0.006 ± 0.003</td>
<td>1.3 ± 0.39</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I205K</td>
<td>GST–NR2B</td>
<td>35 ± 5.3</td>
<td>0.82 ± 0.19</td>
<td>0.023 ± 0.0068</td>
<td>2.22 ± 0.25</td>
</tr>
<tr>
<td>I205K</td>
<td>GST–Eag</td>
<td>38 ± 5.2</td>
<td>0.26 ± 0.11</td>
<td>0.007 ± 0.002</td>
<td>1.75 ± 0.11</td>
</tr>
</tbody>
</table>

Figure S1 Characterization of GST-fusion proteins

Representative results are shown. (A) Western blot of GST-fusion proteins using an anti-GST antibody. Lane 1 contains a prestained protein marker with molecular masses in kDa shown to the left. Lanes 2, 3, 4 and 5 contain 15 μg of each crude lysate of GST–NR2B, GST–NR2A, GST–Eag and GST respectively. (B) SDS/PAGE of purified GST–NR2A. Lane 1, purified GST–NR2A (5 μg). The arrow indicates the GST–NR2A band. Lane 2, molecular mass markers with values in kDa to the right. (C) Autoradiogram showing phosphorylation of 22 μg of crude GST–NR2A by 0.003 mg/ml \(\alpha\)-CaMKII. The arrow indicates the phosphorylated GST–NR2A band. Molecular masses in kDa are shown to the left.

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**Figure S2  Characterization of CaMKII**

Molecular masses of markers are indicated in kDa. Representative results are presented. (A) Western blot of crude lysate of WT-α-CaMKII. Protein (55 μg) was loaded on to a 10% gel. Molecular mass markers are shown to the right. (B) SDS/PAGE of purified WT-α-CaMKII, where 2 μg of protein was loaded on to a 10% gel. Molecular mass markers are shown to the left. (C) Western blot of purified WT-α-CaMKII, where 0.1 μg of protein was loaded on to a 10% gel. Molecular mass markers are shown to the left.

**Figure S3  GST pull-down of α-CaMKII**

The pull-down experiment was carried out as described in the Experimental section of the main paper using glutathione beads. Equal concentrations of crude lysate of WT-α-CaMKII were incubated with equal concentrations of GST–NR2A or GST–NR2B bound to glutathione-conjugated beads either in the presence or absence of Ca²⁺/CaM as indicated. The blots were cut into two and were separately developed with anti-α-CaMKII antibody and anti-GST antibody as indicated. Molecular masses (MW) are indicated to the right.
NR2B modulates CaMKII activity

Figure S4 Linearity of densitometric quantification with the amount of radioactivity

Different volumes of \(^{32}\)P-phosphorylated GST–NR2A, which was prepared in a single reaction using WT \(\alpha\)-CaMKII, were loaded onto the gel for SDS/PAGE. An autoradiogram of the gel was obtained using a Bio-Rad Personal Molecular imager FX (top panel) and densitometric analysis was carried out. A plot of the densitometric quantification values (table at the bottom) against the volume of sample loaded is presented in the middle panel. As evident from the plot, the densitometric values and the amount of radioactive fusion protein loaded show a linear relationship over a wide range.

Figure S5 Western blot using an anti-phospho-Thr\(^{286}\)-\(\alpha\)-CaMKII antibody

Lane 1, autophosphorylation reaction carried out in the absence of Ca\(^{2+}\); lane 2, autophosphorylation reaction carried out in the presence of Ca\(^{2+}\). The autophosphorylation reactions were performed as described in the Experimental section of the main paper with non-labelled ATP (100 \(\mu\)M) and with 0.2 \(\mu\)g of WT-\(\alpha\)-CaMKII in the absence of any GST-fusion proteins. Lane 3 shows molecular mass markers with values in kDa.
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Figure S6  ATP saturation profiles of WT α-CaMKII for phosphorylation of GST–NR2A and GST–NR2B

Results are from a single representative experiment out of three separate experiments. The autoradiogram shows phosphorylated bands of GST–NR2A or GST–NR2B at different concentrations of ATP. Specific activities of phosphorylation of GST–NR2A (■) and GST–NR2B (○) substrates at various concentrations of ATP are plotted. The inset, showing the data for the lower concentration points of the same graph subjected to curve-fitting separately, is presented to highlight the higher activity for phosphorylation of GST–NR2B compared with GST–NR2A at low ATP concentrations.

Figure S8  Modulation of the Thr286 autophosphorylation reaction of α-CaMKII by NR2B

The Thr286 autophosphorylation reaction of α-CaMKII (0.0061 mg/ml) was carried out at different concentrations of ATP, either in presence of GST–(S1291A)-NR2A (0.34 μM) or GST–(S1303A)-NR2B (0.27 μM). The autoradiogram shows autophosphorylated CaMKII bands. The band intensities which represent the activities in the presence of GST–(S1291A)-NR2A (■) or GST–(S1303A)-NR2B (○) are plotted in the graph below. The results corresponding to the lower concentration points alone, fitted with straight line, are shown in the inset. The results represent five separate experiments.

Figure S9  Effect of large variations in ATP concentrations on the NR2B-modulated Thr286 autophosphorylation reaction of α-CaMKII

The Thr286 autophosphorylation reaction of α-CaMKII (0.0061 mg/ml) was carried out at different concentrations of ATP, in the presence of either 0.34 μM GST–(S1291A)-NR2A (■) or 0.27 μM GST–(S1303A)-NR2B (○) (both purified) in a total volume of 20 μl as described in the Experimental section of the main paper. The results represent three separate experiments.