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CAK β /Pyk2 Kinase Is a Signaling Link for Induction of Long-Term Potentiation in CA1 Hippocampus

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Summary

Long-term potentiation (LTP) is an activity-dependent enhancement of synaptic efficacy, considered a model of learning and memory. The biochemical cascade producing LTP requires activation of Src, which upregulates the function of NMDA receptors (NMDARs), but how Src becomes activated is unknown. Here, we show that the focal adhesion kinase CAK β /Pyk2 upregulated NMDAR function by activating Src in CA1 hippocampal neurons. Induction of LTP was prevented by blocking CAK β /Pyk2, and administering CAK β /Pyk2 intracellularly mimicked and occluded LTP. Tyrosine phosphorylation of CAK β /Pyk2 and its association with Src was increased by stimulation that produced LTP. Finally, CAK β /Pyk2-stimulated enhancement of synaptic AMPA responses was prevented by blocking NMDARs, chelating intracellular Ca²⁺, or blocking Src. Thus, activating CAK β /Pyk2 is required for inducing LTP and may depend upon downstream activation of Src to upregulate NMDA receptors.

Introduction

Excitatory transmission at central synapses is primarily mediated by the amino acid glutamate acting through postsynaptic ionotropic receptors (Edmonds et al., 1995). The NMDA receptor (NMDAR) is one type of ionotropic glutamate receptor that has central roles in development, neuroplasticity, and excitotoxicity in the central

nervous system (CNS) (McBain and Mayer, 1994; Dingledine et al., 1999). The function of NMDARs is regulated by protein phosphorylation through serine/threonine and tyrosine kinases (Raymond et al., 1994; Smart, 1997), including the nonreceptor tyrosine kinase Src (Yu et al., 1997). The upregulation of NMDARs that results from activating Src has been shown to be necessary for inducing LTP (Lu et al., 1998), a persistent enhancement of synaptic transmission proposed as a principal cellular substrate for learning and memory (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999). Numerous biochemical pathways leading to the activation of Src are known (Brown and Cooper, 1996; Della Rocca et al., 1997; Thomas and Brugge, 1997; Biscardi et al., 1999), but the identity of the pathway(s) responsible for enhancing NMDAR function and inducing LTP is unknown.

A molecular candidate for activating Src and enhancing NMDAR function is the nonreceptor tyrosine kinase, cell adhesion kinase β /proline-rich tyrosine kinase 2 (CAK β /Pyk2) (Lev et al., 1995; Sasaki et al., 1995; Husi et al., 2000). CAK β /Pyk2 is a member of the focal adhesion kinase (FAK) family (Girault et al., 1999) and is alternatively known as focal adhesion kinase 2 (FAK-2) (Herzog et al., 1996), Ca²⁺-dependent tyrosine kinase (CADTK) (Yu et al., 1996), or related adhesion focal tyrosine kinase (RAFTK) (Li et al., 1996). CAK β /Pyk2 is highly expressed in the central nervous system (Lev et al., 1995) as an unspliced isoform (Girault et al., 1999). CAK β /Pyk2 has been reported to be activated by various stimuli, including raising intracellular [Ca²⁺] ([Ca²⁺]_i), which activates CAK β /Pyk2 indirectly, possibly through stimulation of protein kinase C (PKC) (Lev et al., 1995). Upon activation, CAK β /Pyk2 autophosphorylates on Tyr-579/580 in the catalytic domain and on Tyr-402 on the linker between the catalytic domain and the band 4.1-JEF domain (Girault et al., 1999). Phosphorylation on Tyr-402 creates an SH2 ligand through which CAK β /Pyk2 binds to the SH2 domain of Src (Dikic et al., 1996) and activates this kinase by relieving autoinhibition (Thomas and Brugge, 1997; Xu et al., 1997). In the present study, we set out to determine whether CAK β /Pyk2 is the sought-after biochemical link in the pathway between tetanic stimulation and Src activation for upregulating NMDARs.

Results

CAK β /Pyk2 Potentiates NMDAR Currents and Is Physically Associated with the NMDAR Complex

We used the whole-cell patch-clamp technique to record currents from CA1 neurons acutely isolated from slices of rat hippocampus. NMDAR-mediated responses were evoked by rapid applications of NMDA from a multibarreled perfusion device. We found that including active recombinant CAK β /Pyk2 (0.05 μ g/ml) in the intracellular recording solution led to a progressive increase in the peak amplitude of NMDA currents, which stabilized at 150% \pm 12% (mean \pm SEM, n = 10 cells) of the initial level by 14 min into the recording (Figures

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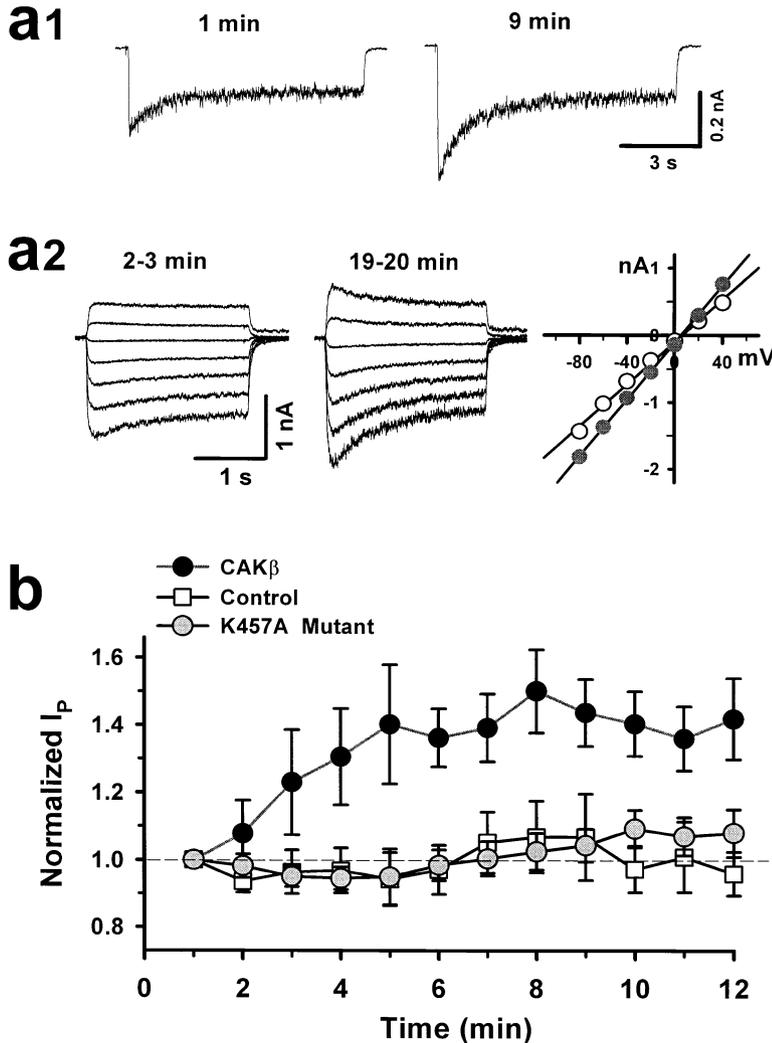


Figure 1. CAK β /Pyk2 Potentiates NMDAR Currents

(a1) Traces of the NMDA-evoked currents recorded from an acutely isolated CA1 neuron 1 and 9 min after whole-cell configuration was achieved. The intracellular solution contained purified recombinant CAK β /Pyk2 (0.5 μ g/mL).

(a2) Traces of currents recorded from another neuron at holding potentials ranging from -80 to 40 mV during the first 3 min (left) and at 20 min (right) after the start of recording with CAK β /Pyk2. The current-voltage relationship of the peak current at the start of recording (open circles) and at 20 min (closed circles) is shown in the graph.

(b) A plot of the normalized peak amplitude of NMDA-induced currents recorded with the intracellular solution containing wild-type CAK β /Pyk2 (closed circles, $n = 10$ cells), K457A CAK β /Pyk2 (gray circles, $n = 6$), or control intracellular solution (open squares, $n = 5$ cells). Peak currents are normalized to that of the first response in each cell.

1a and 1b). CAK β /Pyk2 increased the conductance of whole-cell NMDA currents without changing the driving force. In contrast, intracellularly applying a catalytically inactive mutant (Lev et al., 1995), K457A CAK β /Pyk2 (0.05 μ g/ml), had no effect on the NMDA currents (Figure 1b), suggesting that the kinase activity of CAK β /Pyk2 is required to potentiate NMDA currents. K457A CAK β /Pyk2 is also a dominant-negative mutant (Lev et al., 1995; Dikic et al., 1996), and thus, its lack of effect on NMDAR responses suggests that, while the currents are increased by exogenous CAK β /Pyk2, NMDAR currents in CA1 neurons are not tonically enhanced by endogenous CAK β /Pyk2.

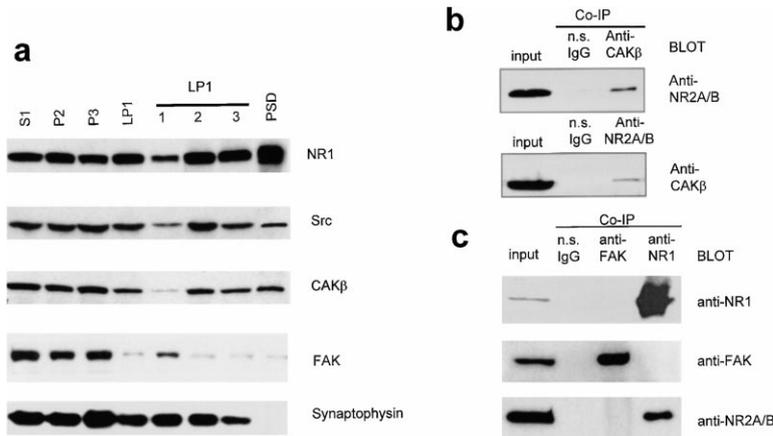
NMDARs are concentrated in postsynaptic densities (PSDs) at excitatory synapses, and hence we questioned whether CAK β /Pyk2 might also be contained in PSDs. PSD proteins were prepared by sequential fractionation, and CAK β /Pyk2 was found in the PSD fraction (Figure 2a). The subcellular fractionation pattern of CAK β /Pyk2 was similar to that of Src, which is known to be linked to the NMDAR complex (Yu et al., 1997). We also examined the subcellular fractionation pattern of FAK, the lead member of the focal adhesion kinase family, which was also present in the PSD fraction. However,

the level of FAK in the PSDs relative to other fractions was much lower than that of CAK β /Pyk2 (Figure 2a).

To determine whether CAK β /Pyk2 associates with NMDARs we immunoprecipitated membrane proteins with antibodies specifically directed against NR2A and 2B subunits (anti-NR2A/B) or CAK β /Pyk2 (anti-CAK β /Pyk2). Nondenaturing conditions were used to solubilize the membrane proteins (Blahos and Wenthold, 1996). Immunoprecipitating with anti-NR2A/B caused coprecipitation of CAK β /Pyk2 and, conversely, immunoprecipitation with anti-CAK β /Pyk2 coprecipitated NR2A/B (Figure 2b). A nonspecific IgG did not immunoprecipitate either NR2A/B or CAK β /Pyk2. On the other hand, neither FAK nor NMDARs immunoprecipitated with the other (Figure 2c). Thus, we conclude that CAK β /Pyk2, but not FAK, associates either directly or indirectly with the NMDAR complex and is thereby strategically positioned to regulate NMDAR function.

Endogenous CAK β /Pyk2 Enhances NMDAR Currents via Sequential Activation of Src

We next determined whether stimulating endogenous CAK β /Pyk2 may affect NMDARs. CAK β /Pyk2 can be stimulated by protein kinase C (PKC) (Lev et al., 1995;



as indicated to the right of each panel. Similar results were observed in each of four experiments.

(c) Immunoprecipitation of membrane proteins with anti-FAK, anti-NR1, or nonspecific IgG (n.s. IgG). In the lane marked input, 50 μ g of membrane proteins without immunoprecipitation was loaded. Blots were probed with anti-NR1, anti-FAK, or anti-NR2a/B as indicated to the right of each panel. The results are representative of four experiments.

Siciliano et al., 1996) and PKC potentiates NMDAR currents through activating Src (Lu et al., 1999), suggesting that CAK β /Pyk2 may be an intermediary between PKC and Src. We found that administering K457A CAK β /Pyk2 intracellularly attenuated the enhancement of NMDAR currents by the PKC activator, 4 β -PMA (Figures 3a and 3c): 4 β -PMA increased NMDAR currents to 146% \pm 11% in control cells ($n = 5$ cells), whereas NMDAR currents were 112% \pm 7% in cells containing K457A CAK β /Pyk2 ($n = 6$ cells; $P < 0.01$, two-way ANOVA). In other experiments, applying 4 β -PMA after NMDAR currents had been potentiated by wild-type CAK β /Pyk2 caused no further increase in current amplitude (Figures 3a and 3c). Thus, the dominant-negative CAK β /Pyk2 mutant attenuated the potentiation of NMDAR currents by PKC, and the PKC-induced potentiation was occluded by CAK β /Pyk2.

The simplest explanation for these results is that in CA1 neurons CAK β /Pyk2 is activated as a consequence of stimulating PKC. We investigated this by immunopurifying CAK β /Pyk2 from the CA1 region from hippocampal slices and used the level of tyrosine phosphorylation of CAK β /Pyk2 as a surrogate measure of activity (Lev et al., 1995; Dikic et al., 1996). Applying 4 β -PMA increased the level of tyrosine phosphorylation of CAK β /Pyk2 without increasing the amount of CAK β /Pyk2 protein immunoprecipitated (Figure 3b; average increase, 198% \pm 11%; $n = 3$). In contrast, neither the inactive isomer, 4 α -PMA (Figure 3b), nor 4 β -PMA applied in the presence of the selective PKC inhibitor chelerythrine (data not illustrated) affected the level of tyrosine phosphorylation of CAK β /Pyk2. These biochemical and electrophysiological results indicate that endogenous CAK β /Pyk2 can be activated by PKC to upregulate NMDAR function.

If the effect of CAK β /Pyk2 depends upon Src, then blocking Src function should prevent the upregulation of NMDARs by CAK β /Pyk2. Therefore, we applied CAK β /Pyk2 together with a Src inhibitory peptide (Yu et al., 1997), Src(40–58), which was found to prevent the enhancement of NMDAR currents (Figure 3d). On the other hand, administering CAK β /Pyk2 together with a control

peptide, scrambled Src(40–58) [sSrc(40–58)], increased NMDAR currents to 158% \pm 4.5% control [two-way ANOVA, $p < 0.002$ compared with Src(40–58)]. To ensure that Src(40–58) did not directly inhibit CAK β /Pyk2 activity we used a CAK β /Pyk2 immune complex assay and found that CAK β /Pyk2 activity was 102% \pm 4% of control when incubated with Src(40–58), compared with 98% \pm 9% of control with sSrc(40–58) ($n = 3$ for each). From these data together, we conclude that Src is necessary for the potentiation of NMDAR currents by CAK β /Pyk2.

Src could be downstream of CAK β /Pyk2 in a sequential cascade, or it is possible that concurrent activation of Src and CAK β /Pyk2 is required. If Src was downstream of CAK β /Pyk2, then the dominant-negative CAK β /Pyk2 mutant should not affect potentiation produced by activating Src. However, if concurrent activation of the two kinases was required, then K457A CAK β /Pyk2 should block Src-induced potentiation. To activate Src we used the high-affinity peptide EPQ(pY)EEIPIA (Yu et al., 1997), alone or together with K457A CAK β /Pyk2 (Figure 3e). Administering EPQ(pY)EEIPIA alone increased NMDAR currents to 142% \pm 5% control ($n = 5$ cells), while with EPQ(pY)EEIPIA together with K457A CAK β /Pyk2 NMDAR currents were 140% \pm 11% control ($n = 5$ cells; two-way ANOVA $P > 0.1$). EPQ(pY)EEIPIA had no effect on CAK β /Pyk2 activity (101% \pm 1% of control in immune complex kinase assay, $n = 3$). Thus, the most parsimonious explanation is that there is a sequential activation of Src by CAK β /Pyk2.

To determine whether synaptic NMDARs are affected by CAK β /Pyk2, we studied miniature excitatory postsynaptic currents (mEPSCs) in whole-cell recordings from hippocampal neurons in primary culture (Figures 4a and 4b) and pharmacologically isolated NMDAR-mediated synaptic currents in CA1 neurons in acute hippocampal slices (Figures 4c–4e). We found that intracellular application of CAK β /Pyk2 increased the amplitude of the NMDAR component of the miniature synaptic currents: after 20 min of recording, the NMDAR component of the mEPSCs was 127% \pm 5% of control ($n = 12$ cells). On

Figure 2. CAK β /Pyk2 but Not FAK Is Physically Associated with the NMDAR Complex
(a) Subcellular fractionation profiles of NR1, Src, CAK β /Pyk2, FAK, or synaptophysin. Membrane proteins from each fraction were resolved by SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting with anti-NR1, -Src, -CAK β /Pyk2, -FAK, or -synaptophysin as indicated on the right of each panel. Thirty micrograms of proteins were loaded in each lane except the PSD lane, in which only five micrograms was loaded.

(b) Immunoprecipitation of membrane proteins with anti-CAK β /Pyk2, anti-NR2A/B, or nonspecific IgG (n.s. IgG). In the lane marked input, 50 μ g of membrane proteins without immunoprecipitation was loaded. Blots were probed with anti-NR2A/B or anti-CAK β /Pyk2

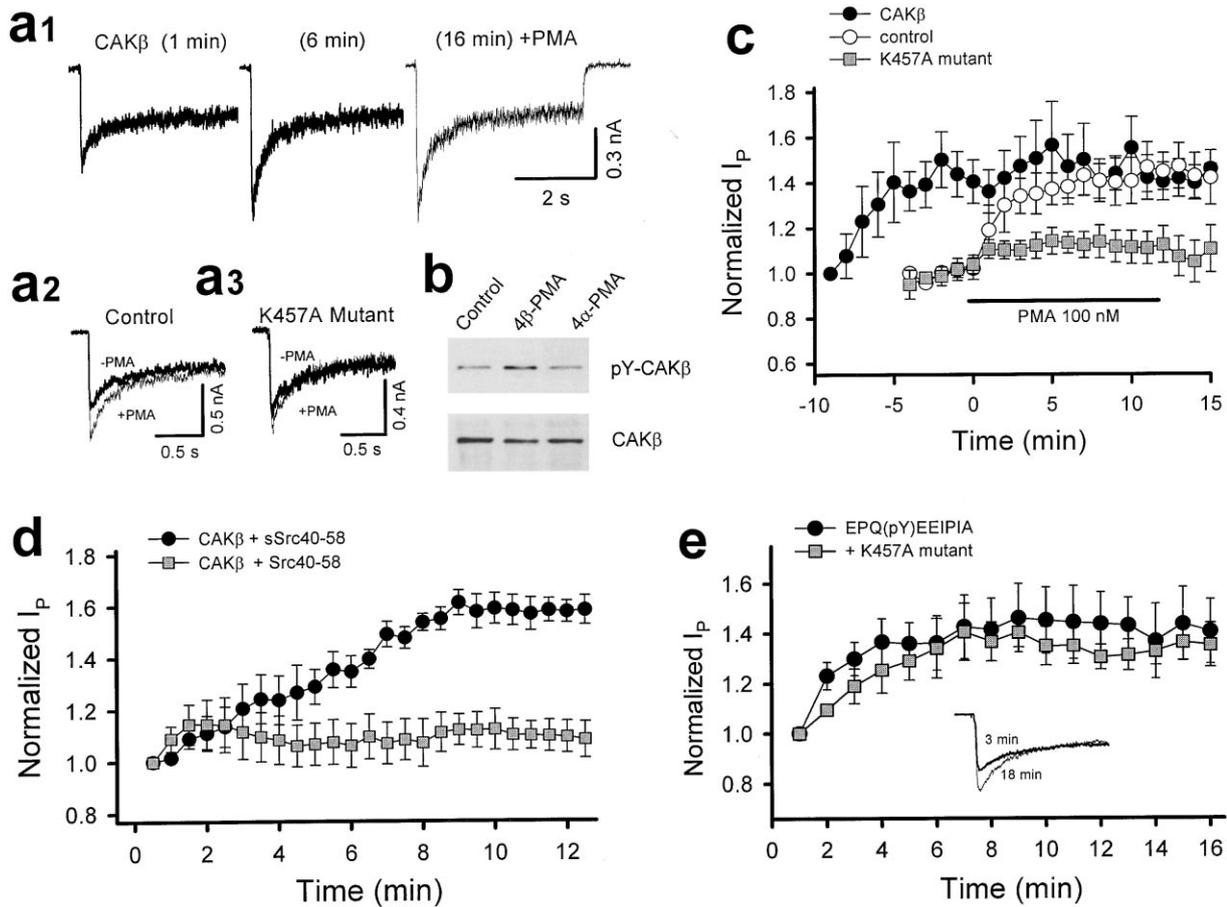


Figure 3. Endogenous CAK β /Pyk2 Enhances NMDAR Currents via Sequential Activation of Src

(a1) Responses recorded from a CA1 neuron 1, 6, and 16 min after the start of recording with intracellular solution containing CAK β /Pyk2. 4 β -PMA (100 nM) was added to the bathing solution at 10 min.
 (a2 and a3) Superimposed NMDA-evoked currents recorded before (dark traces, -PMA) and during (light traces, +4 β -PMA) bath application of 4 β -PMA (100 nM). For the neuron recorded on the left, control intracellular solution was used, and for that on the right the intracellular solution contained K457A CAK β /Pyk2.
 (b) Proteins were prepared from CA1 regions of untreated (control) hippocampal slices or slices treated with 4 β -PMA (100 nM) or the inactive analog, 4 α -PMA (100 nM), and were immunoprecipitated using anti-CAK β /Pyk2. Immunoprecipitates were separated by SDS-PAGE and probed with anti-phosphotyrosine antibody (upper panel). The blot was stripped and reprobed with anti-CAK β /Pyk2 (lower panel). The phosphoCAK β /Pyk2 after 4 β -PMA was 149% control and that after 4 α -PMA was 107% control, in the example shown.
 (c) A plot of the normalized peak amplitudes from cells recorded with control intracellular solution (open circles, $n = 5$ cells), with intracellular solution supplemented with CAK β /Pyk2 (closed circles, $n = 6$ cells), or with K457A CAK β /Pyk2 (gray squares, $n = 6$ cells). 4 β -PMA (100 nM) was applied during the period indicated by the horizontal bar. In CAK β /Pyk2 group, peak currents were normalized to the first response, whereas in the other two groups peak currents were normalized to responses recorded immediately before application of 4 β -PMA.
 (d) Plot of normalized peak NMDA-evoked currents recorded with intracellular solution containing CAK β /Pyk2 together with Src(40-58) (gray squares, $n = 6$ cells) or with CAK β /Pyk2 with scrambled Src(40-58) [sSrc(40-58), closed circles, $n = 7$ cells].
 (e) Normalized peak NMDA-evoked currents during the intracellular administration of the Src activator peptide EPQ(pY)EEIPIA alone (closed circles, $n = 5$) or together with K457A CAK β /Pyk2 (gray squares, $n = 5$) are shown. NMDAR currents increased to $142\% \pm 5\%$ EPQ(pY)EEIPIA alone and with K457A CAK β /Pyk2 currents were $140\% \pm 11\%$ control (two-way ANOVA, $p > 0.1$). The insert shows superimposed traces of NMDA-evoked currents recorded from the one cell 3 and 18 min after starting recording with the activator peptide together with K457A CAK β /Pyk2.

the other hand, NMDAR synaptic responses were $96\% \pm 5\%$ of control when CAK β /Pyk2 was applied together with Src(40-58) ($P > 0.05$ compared with CAK β /Pyk2 alone). In the CA1 neurons recorded from slices, CAK β /Pyk2 also increased the amplitude of NMDAR-mediated synaptic currents evoked by Schaffer collateral stimulation (Figures 4c-4e): by 25 min into recording, NMDAR currents were $161\% \pm 13\%$ ($n = 6$) of the initial level when CAK β /Pyk2 was administered as compared with $107\% \pm 9\%$ in controls ($n = 5$) without CAK β /Pyk2 ($P < 0.005$). The current-voltage relationship of the NMDAR

EPSCs after the potentiation by CAK β /Pyk2 was not different from control recordings (Figure 4d), indicating that CAK β /Pyk2 did not affect the voltage dependence of the blockade of NMDARs by extracellular Mg $^{2+}$. In contrast to applying CAK β /Pyk2, intracellularly applying the K457A dominant-negative mutant caused no change in NMDAR-mediated EPSCs (Figures 4c and 4e). Thus, synaptic NMDAR function can be increased by the CAK β /Pyk2-Src cascade, but these receptors are not tonically upregulated by this cascade in CA1 neurons in acute slices.

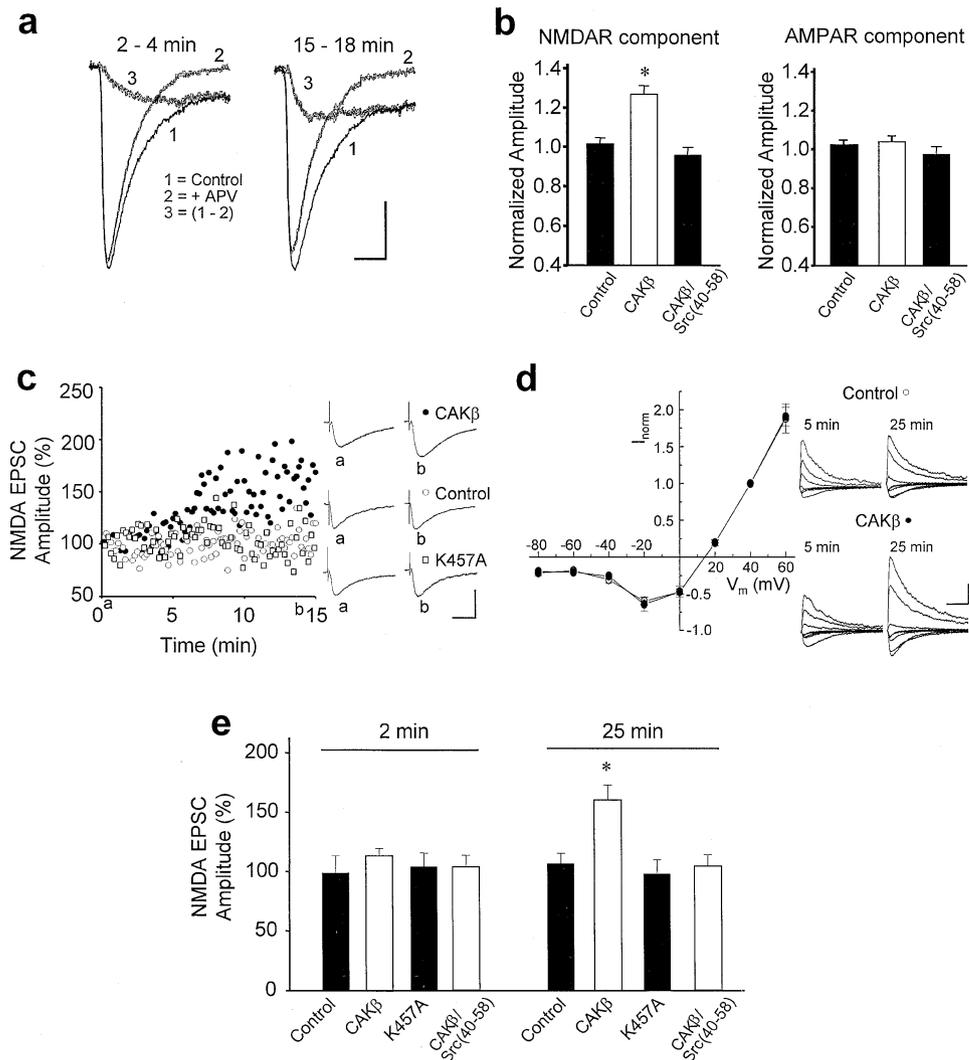


Figure 4. CAK β /Pyk2 Potentiates Synaptic NMDARs in Hippocampal Neurons but Does Not Affect Voltage Dependence of Mg²⁺ Blockade
(a) Averaged mEPSCs recorded from a neuron using a patch pipette containing CAK β /Pyk2 (1). The competitive NMDA receptor antagonist APV (20 μ M) was used to block NMDA receptor-mediated components (2), and subtraction revealed this component (3). Scale bars: 10 ms, 5 pA. For this cell, APV was applied for several minutes, starting at 2 min, and then washed out and reapplied at 15 min into the recording.
(b) Histogram of NMDAR and AMPAR components of mEPSCs recorded from cultured hippocampal neurons using patch pipettes containing the intracellular solution alone ($n = 7$ cells), with added CAK β /Pyk2 ($n = 12$ cells) with or without Src (40–58) ($n = 7$ cells). The amplitudes of the NMDAR-mediated component of the mEPSCs measured 18 to 25 min after starting whole-cell recording were normalized to those measured during the first 2 min of recording.
(c) CAK β /Pyk2-induced enhancement of pharmacologically isolated NMDAR-mediated synaptic responses from CA1 neurons in hippocampal slices. The peak amplitude of NMDA EPSC is plotted for a cell during intracellular administration of CAK β /Pyk2 (closed circles), a cell recorded with control intracellular solution (open circles), or another cell during intracellular administration of K457A CAK β /Pyk2 (open squares). Traces shown to the right were taken at the times indicated. Scale bars: 50 ms, 50 pA.
(d) Current–voltage (I - V) relationship for pharmacologically isolated NMDAR EPSCs during intracellular administration of CAK β /Pyk2 (closed circles) or control solution (open circles). The data plotted in the graph show the mean (\pm SEM) I - V relationship for control cells ($n = 8$) and cells with CAK β /Pyk2 ($n = 4$) obtained at 25 min after breakthrough. For each cell, the peak NMDAR EPSC amplitude at each holding potential is normalized to that at +40 mV. On the right, superimposed NMDAR EPSC traces, at membrane potentials from -80 to +60 mV, are shown for two individual cells at the times indicated during the recording. Scale bars: 100 ms, 200 pA.
(e) Histogram showing the effect of intracellular administration of CAK β /Pyk2 ($n = 6$ cells) or K457A CAK β /Pyk2 ($n = 5$ cells) compared with control solution without CAK β /Pyk2 ($n = 5$ cells). Average values for the isolated NMDA EPSC peak amplitude are shown at 2 and 25 min after the start of intracellular administration. For all recordings in this figure, the intracellular solution contained 10 mM BAPTA. Asterisks indicate statistically significant differences ($P < 0.05$) in all figures.

Blocking CAK β /Pyk2 Prevents Induction of LTP, and Tetanic Stimulation Activates CAK β /Pyk2

In order to examine the role of CAK β /Pyk2 in synaptic plasticity, we studied tetanus-induced LTP at Schaffer collateral-CA1 synapses (Bliss and Collingridge, 1993;

Malenka and Nicoll, 1999) using whole-cell and field recordings from CA1 pyramidal neurons. In control recordings, tetanic stimulation caused long-lasting potentiation of the intracellularly recorded excitatory postsynaptic potentials (EPSPs, Figure 5): the initial slope of the

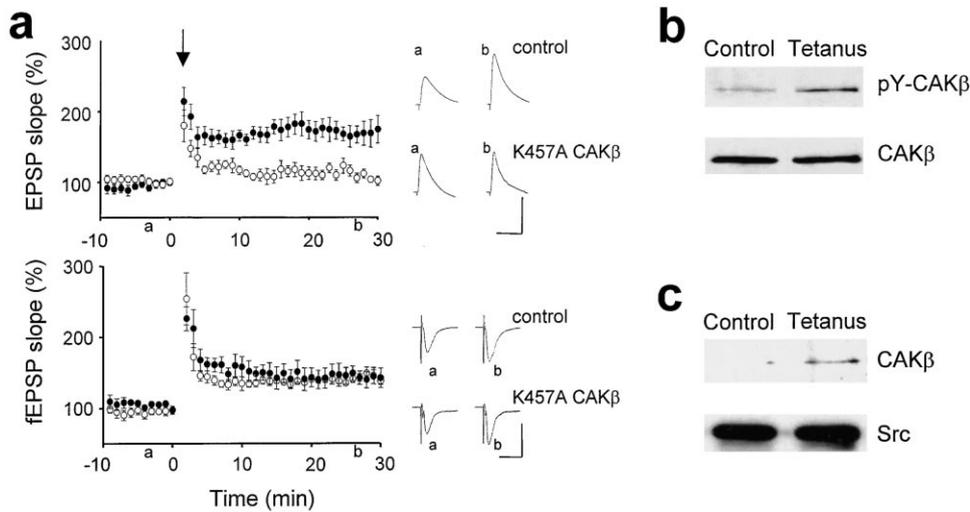


Figure 5. Blocking CAK β /Pyk2 Prevents Induction of LTP, and Tetanic Stimulation Activates CAK β /Pyk2 and Increases the Association of CAK β /Pyk2 with Src

(a) K457A CAK β /Pyk2 prevented tetanus-induced LTP. (Top graph) Averaged EPSP slope is plotted for experiments with K457A CAK β /Pyk2 (open circles, $n = 8$ cells) or without (closed circles, $n = 11$ cells). Data are normalized to baseline value. On the right are traces from individual cells with or without intracellular administration of K457A CAK β /Pyk2, taken within 5 min prior to tetanus (a) and 30 min after tetanus (b). Scale bars: 50 ms, 10 mV. (Bottom graph) Plot of averaged normalized field EPSP (fEPSP) slope with (open circles) or without (closed circles) K457A CAK β /Pyk2. Traces of field recordings taken from experiments with or without K457A CAK β /Pyk2 corresponding to those for which whole-cell EPSPs are shown above. Each field trace is the average of six individual responses taken at the times indicated. In all recordings the tetanus was delivered 20 min after the start of whole-cell recording, but only the 10 min immediately prior to tetanus is shown for clarity. Scale bars: 25 ms, 1 mV.

(b) Proteins prepared from CA1 regions of slices receiving control or tetanic stimulation were immunoprecipitated using anti-CAK β /Pyk2. Immunoprecipitates were separated by SDS-PAGE and probed with anti-phosphotyrosine antibody (upper panel). The blot was stripped and reprobed with anti-CAK β /Pyk2 (lower panel). For the example blot shown, the level of tyrosine phosphorylation signal for CAK β /Pyk2 was increased to 213% of the control level following tetanic stimulation; on average, the increase following tetanus was to 279% \pm 35% control ($n = 4$ experiments).

(c) Proteins from CA1 regions of slices receiving control or tetanic stimulation were immunoprecipitated using an anti-src antibody and probed with anti-CAK β /Pyk2 (upper panel). The blot was stripped and reprobed with anti-src (lower panel). The CAK β /Pyk2 signal that coimmunoprecipitated with Src increased 414% following tetanus. The data are representative of two experiments.

EPSPs was 172% \pm 15% of baseline by 30 min after tetanic stimulation ($n = 12$ cells). However, during intracellular application of K457A CAK β /Pyk2, EPSP slope was 105% \pm 6% of control 30 min after the tetanic stimulation ($n = 8$ cells), indicating that LTP had not been induced. On the other hand, tetanic stimulation did produce a short-lasting, posttetanic potentiation in cells recorded with K457A CAK β /Pyk2. Moreover, in these recordings tetanus caused a long-lasting increase in the slope of field EPSPs that was not different from the increase in field EPSP slope evoked in control recordings ($P > 0.05$; Figure 5a). Thus, K457A CAK β /Pyk2 prevented induction of LTP in the cells in which it was administered intracellularly but not in neighboring neurons.

Tetanic Stimulation Activates CAK β /Pyk2 and Increases Association of CAK β /Pyk2 with Src

In CA1 neurons K457A CAK β /Pyk2 had no effect on NMDAR responses, and therefore the blockade of LTP could not be attributed to removing a tonic enhancement of NMDAR function. Therefore, it may be that CAK β /Pyk2 is activated as a result of tetanic stimulation. To probe for CAK β /Pyk2 activation we measured tyrosine phosphorylation of CAK β /Pyk2 immunoprecipitated from proteins from the CA1 region around the stimulating electrode. We found that the level of tyrosine phosphory-

lation of CAK β /Pyk2 immunopurified from slices receiving tetanic stimulation was greater than that from control slices receiving only test stimulation (Figure 5b). As there was no difference in the level of CAK β /Pyk2 protein, we concluded that tetanus causes an increase in CAK β /Pyk2 activation.

Because activated CAK β /Pyk2 autophosphorylates on Tyr-402, creating a ligand for the SH2 domain of Src (Dikic et al., 1996), we examined the effect of tetanic stimulation on the association of these enzymes. Proteins from the CA1 region around the stimulating electrode were prepared using nondenaturing conditions and were immunoprecipitated using an anti-src antibody. We found that the amount of CAK β /Pyk2 that coimmunoprecipitated was greater with slices that had received tetanic stimulation as compared with control slices (Figure 5c), whereas the amount of Src immunoprecipitated was not different. Thus, tetanic stimulation increased the association of CAK β /Pyk2 and Src in CA1.

CAK β /Pyk2-Induced Enhancement of Synaptic Responses Occludes LTP and Is NMDAR and Ca²⁺ Dependent

If CAK β /Pyk2 signaling is sufficient to induce LTP, then intracellular application of CAK β /Pyk2 should mimic and occlude the effect of tetanic stimulation. We found that

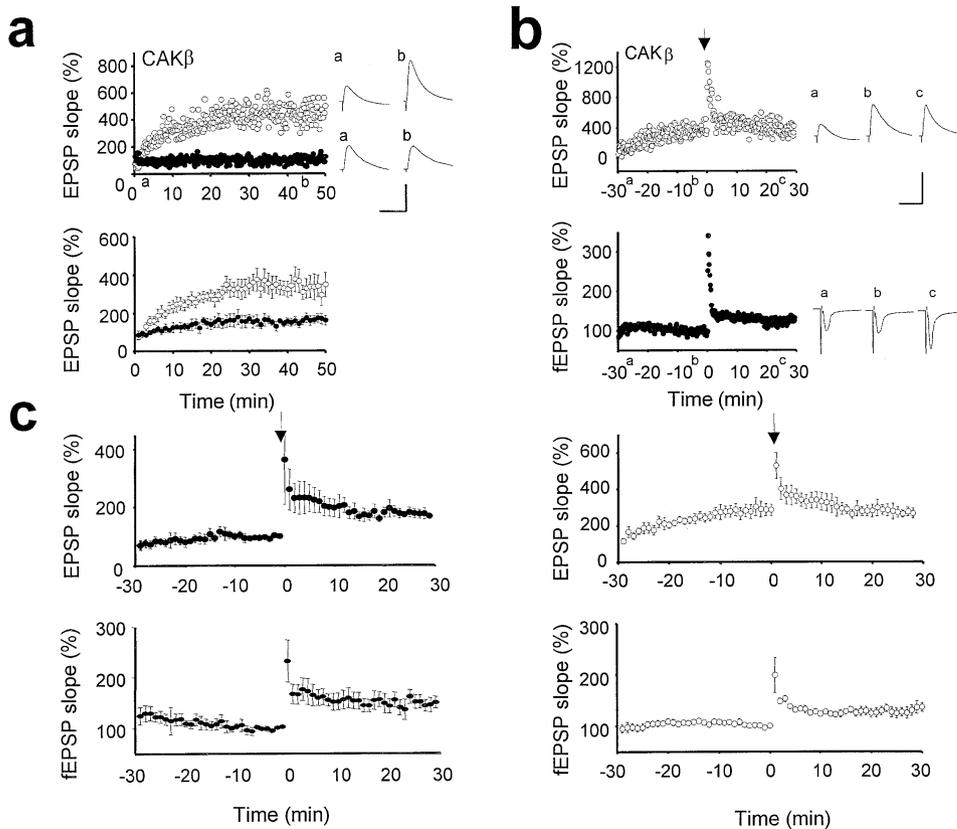


Figure 6. CAK β /Pyk2-Induced Enhancement of Synaptic Responses Occludes LTP

(a) (Upper graph) Plot of EPSP slope from individual cells during intracellular administration of CAK β /Pyk2 (open circles) or control solution (closed circles). Traces taken at the times indicated are shown to the right. Scale bars: 50 ms, 10 mV. (Lower graph) Averaged EPSP slope with (open circles, $n = 7$ cells) or without (closed circles, $n = 5$ cells) application of CAK β /Pyk2.
 (b) (Top) Upper pair of graphs show EPSP slope from one cell during intracellular administration of CAK β /Pyk2 and corresponding plot of field EPSP slope. Tetanus was delivered at the time indicated by the arrow. To the right are traces taken at the times indicated. Scale bars: 50 ms, 10 mV for EPSPs; 50 ms, 0.5 mV for field EPSPs. Lower pair of graphs show averaged EPSP slope and corresponding averaged field EPSP slope from recordings with intracellular administration of CAK β /Pyk2 ($n = 6$).
 (c) (Upper graph) Plot of EPSP slope from individual cells recorded with control solution for 30 min prior to tetanus ($n = 5$ cells). (Bottom graph) Plot of averaged normalized field EPSP slopes taken from slices in which individual cells were recorded for 30 min prior to tetanus ($n = 5$ slices).

administering CAK β /Pyk2 intracellularly caused a progressive increase in EPSP slope that reached a stable level $317\% \pm 54\%$ of the baseline level ($n = 7$ cells; Figure 6a). Once the synaptic responses had stabilized, delivering tetanic stimulation at 30 min of recording produced only a short-term but not a long-term enhancement of EPSPs ($n = 6$ cells; Figure 6b). In these cases tetanus induced a long-term potentiation of field EPSP slope not different from that produced in control slices. In control cells where CAK β /Pyk2 was not included in the intracellular solution ($n = 5$), delivering tetanus at 30 min into the recording produced a long-term enhancement of the EPSPs to $176\% \pm 15\%$ of the pretetanus control level (Figure 6c; $n = 5$ cells), which was not different from the potentiation produced when the tetanus was delivered at 10 min into whole-cell recording (Figure 5; $P > 0.20$). Taking these data together we conclude that CAK β /Pyk2 mimicked and occluded the induction of LTP.

The EPSPs recorded in CA1 neurons are mediated by AMPA receptors. As shown here, CAK β /Pyk2 associates

with and upregulates the function of NMDARs, and therefore we questioned whether NMDA receptors are required for the CAK β /Pyk2-induced enhancement of EPSPs. In experiments where NMDARs were blocked by bath applying the NMDAR antagonist, MK-801, starting just prior to whole-cell recording, we found that administering CAK β /Pyk2 produced no change in EPSP slope ($n = 5$ cells; Figure 7). Thus, CAK β /Pyk2 did not enhance AMPA receptor-mediated responses directly. Rather, NMDAR activation was necessary to induce the potentiation of AMPAR-mediated synaptic responses by CAK β /Pyk2. The increase in EPSP slope produced by CAK β /Pyk2 was prevented when the Src inhibitor peptide, Src(40–58), was included in the intracellular solution (Figure 7b; $n = 5$ cells), indicating that the enhancement of EPSPs by CAK β /Pyk2 depended upon Src.

Lastly, we determined whether the CAK β /Pyk2-induced enhancement of AMPAR EPSPs in CA1 neurons in hippocampal slices depended upon raising $[Ca^{2+}]_i$. We found that when the buffering capacity of the intracellular solution was increased by including 10 mM

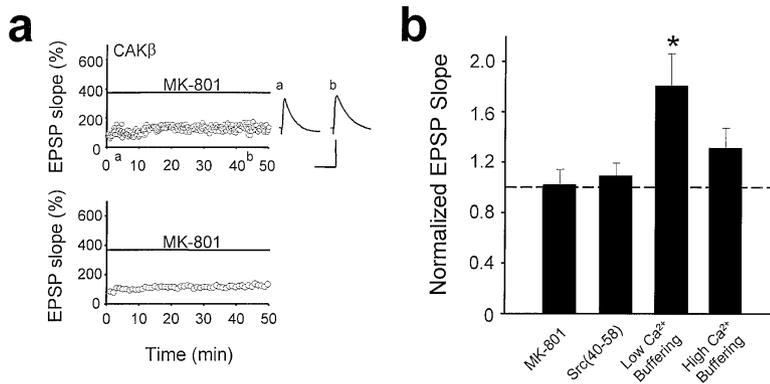


Figure 7. CAK β /Pyk2-Induced Enhancement of AMPAR Synaptic Responses Is NMDAR, Src, and Ca²⁺ Dependent

(a) Effect of bath application of MK-801 on the potentiation of EPSP slope by intracellular administration of CAK β /Pyk2. Plot of EPSP slope from one cell during application of MK-801 is shown in the upper graph. Traces to the right were taken at the times indicated. Scale bars: 50 ms, 10 mV. Averaged EPSP slope plotted for five cells treated with MK-801.

(b) Histogram showing the normalized slope of AMPAR EPSPs after 20 min of recording in individual CA1 cells from hippocampal slices with intracellular administration of CAK β /Pyk2 during extracellular administration of MK-801 (n = 5), the intracellular coadministration of Src40–58 (n = 5), of control solution (Low Ca²⁺ buffering; n = 7), or of 10 mM BAPTA solution (High Ca²⁺ buffering; n = 10).

BAPTA intracellularly, administering CAK β /Pyk2 had no effect on AMPAR EPSPs (Figure 7b). This lack of effect could not be accounted for by a lack of CAK β /Pyk2 activity in high Ca²⁺ buffering intracellular solution because CAK β /Pyk2 increased the NMDAR EPSCs evoked in slices and the NMDAR component of mEPSCs in cultured neurons, where high Ca²⁺ buffering solutions were used (Figure 4).

Discussion

The present results show that CAK β /Pyk2 is upstream of Src in the signaling cascade by which tyrosine phosphorylation enhances the function of NMDA receptors. In addition, we found that blockade of CAK β /Pyk2 prevented induction of LTP and that administering CAK β /Pyk2 produced a lasting potentiation of synaptic transmission that mimicked and occluded LTP induction. Thus, CAK β /Pyk2 fulfills the electrophysiological criteria required to consider this kinase a necessary intermediary in the direct biochemical pathway (Malenka and Nicoll, 1999) for tetanus-induced LTP at Schaffer collateral-CA1 synapses. Moreover, we showed biochemically that tetanic stimulation activated CAK β /Pyk2 and increased its association with Src (see also Lauri et al., 2000). As with LTP induced by tetanus, the potentiation of EPSPs produced by CAK β /Pyk2 depended upon NMDARs, raising [Ca²⁺]_i and Src. Taking our results together with previous work, the simplest explanation is a sequential cascade gating the induction of LTP: tetanic stimulation causes activation of CAK β /Pyk2 that associates with Src, activating this kinase, which in turn boosts the influx of Ca²⁺ through NMDARs and sets in motion the downstream cascade (Salter, 1998; Malenka and Nicoll, 1999; Soderling and Derkach, 2000) that ultimately results in potentiation of synaptic AMPAR responses.

When CAK β /Pyk2 was administered into the cells, synaptic AMPAR responses increased even though the rate of stimulation of the synaptic input was low. We have previously shown that synaptic AMPAR responses are similarly potentiated by intracellularly administering exogenous Src or the Src-activating peptide (Lu et al., 1998). The potentiation is prevented by blocking NMDARs or by strongly buffering intracellular Ca²⁺. Strongly buff-

ering Ca²⁺ does not prevent the enhancement of NMDAR currents, implying that the potentiation of AMPAR responses is indirect and dependent upon an NMDAR-mediated rise in [Ca²⁺]_i. On the surface, these observations might appear at odds with the notion that NMDARs are said to be “blocked” at the resting membrane potential. However, even with physiological concentrations of extracellular Mg²⁺, the current through NMDARs is not fully blocked at membrane potentials near rest (MacDonald et al., 1982), although this current is dramatically suppressed compared with depolarized potentials. Recent work has demonstrated that synaptically activating NMDARs from the resting membrane potential produces Ca²⁺ influx into dendritic spines (Kovalchuk et al., 2000). NMDAR-mediated rises in [Ca²⁺]_i are known to be enhanced by tyrosine phosphorylation (Wang and Salter, 1994). Therefore, the rise in [Ca²⁺]_i produced within dendritic spines by EPSPs at the resting potential would be increased by the enhancement of NMDAR function produced by CAK β /Pyk2-Src signaling. In this way, administering or directly activating CAK β /Pyk2 or Src may bypass the requirement for tetanic stimulation to trigger the downstream signaling cascade leading to LTP.

How might CAK β /Pyk2 be activated as a consequence of tetanic stimulation? Two principal mediators for activating CAK β /Pyk2 are Ca²⁺ and PKC, which may act alone or in concert (Girault et al., 1999). Although the molecular mechanisms by which Ca²⁺ and PKC activate CAK β /Pyk2 remain to be established, these mediators may be relevant for the stimulation of CAK β /Pyk2 following tetanus. For example, it is possible that influx of Ca²⁺ through NMDARs during tetanus might activate CAK β /Pyk2, which would set up a feedforward cycle to rapidly boost NMDAR function and the resultant entry of Ca²⁺. In this case, our results indicate that such a hypothetical feedforward cycle is not active prior to tetanus, as we found that NMDAR function is not tonically upregulated by CAK β /Pyk2. This could be possible if the rise in [Ca²⁺]_i produced by stimulating NMDARs at low frequency at the resting potential was not sufficient to stimulate CAK β /Pyk2 or if there was a competing mechanism to inhibit CAK β /Pyk2 and thus act as a brake.

An additional, or alternative, possibility is that tetanus-

induced activation of CAK β /Pyk2 is mediated by PKC. Our present results and those of others indicate that stimulating PKC is sufficient to activate CAK β /Pyk2 in the hippocampus. Several lines of evidence implicate PKC in LTP induction: PKC inhibitors prevent LTP (Malinow et al., 1989; Wang and Feng, 1992); LTP is diminished in mutant mice lacking PKC γ (Abeliovich et al., 1993); and intracellular administration of active PKC into CA1 neurons causes long-lasting enhancement of EPSPs (Hu et al., 1987). PKC is known to phosphorylate AMPAR subunit protein Glu-R1 on Ser-831, the same residue as is phosphorylated by CaMKII (Roche et al., 1996; Barria et al., 1997a, 1997b). Thus, PKC has been considered to have a function analogous to that of CAMKII (Malenka and Nicoll, 1999). Our present results raise the possibility of a distinct role for PKC in LTP induction as an upstream activator of CAK β /Pyk2 signaling to NMDARs.

Our demonstration that the PKC-CAK β /Pyk2-Src signaling cascade upregulates NMDAR function links the level of NMDAR activity to stimulation of G $\alpha_q/11$ -coupled G protein-coupled receptors (GPCRs) such as muscarinic receptors and LPA receptors (Lu et al., 1999). Thus, through this cascade NMDAR activity may be modulated in concert with other signaling events evoked by GPCR activation. GPCRs stimulate various intracellular signaling cascades including those involved in growth, proliferation, and differentiation (Gutkind, 1998). An emerging theme in our understanding of the nervous system is that signaling pathways used in development subserve synaptic plasticity in the adult. A main outcome of PKC-CAK β /Pyk2-Src signaling is stimulation of mitogen-activated protein kinase (MAPK) (Dikic et al., 1996; Della Rocca et al., 1999). Pharmacological inhibitors of MAPK kinase (MEK) have been shown to prevent induction of LTP in CA1 (English and Sweatt, 1997) and fear conditioning (Atkins et al., 1998). Moreover, the stimuli that cause LTP or fear conditioning produce activation of MAPK, and NMDARs have been found to be upstream of MAPK. While the downstream effector(s) remain to be determined, we expect that upstream PKC-CAK β /Pyk2-Src signaling to NMDARs may be required to engage the MAPK cascade.

In summary, CAK β /Pyk2 is abundantly expressed in the CNS (Sasaki et al., 1995), and a number of functions have been postulated for this kinase that may interact with a number of downstream signaling cascades (Girault et al., 1999). Our present results indicate that by functioning as an upstream activator of Src at excitatory synapses, CAK β /Pyk2 leads to enhanced NMDAR activity and is a necessary intermediary for the induction of LTP. Because CAK β /Pyk2 and Src (Martinez et al., 1987; Thomas and Brugge, 1997) are widely expressed in the nervous system, we expect that the signaling cascade identified here may be of general relevance to synaptic plasticity in the CNS.

Experimental Procedures

CA1 Neuron Isolation and Whole-Cell Patch-Clamp Recording

CA1 pyramidal neurons were isolated from hippocampal slices taken from postnatal rats (Wistar 10–24 days) using procedures described previously (Wang and MacDonald, 1995). The extracellular solution was composed of 140 mM NaCl, 1.3 mM CaCl $_2$, 5 mM KCl, 25 mM

HEPES, 33 mM glucose, and 0.0003 mM tetrodotoxin, with a pH of 7.4 and osmolarity of 325–335 mOsm. Whole-cell, patch-clamp recordings were made at room temperature (20°C–22°C). To maximize the rate of extracellular solution exchange, neurons were lifted into the stream of solution supplied by a multibarreled perfusion system. The intracellular solution contained 140 mM Cs methylSO $_4$ or CsF, 11 mM EGTA, 1 mM CaCl $_2$, 2 mM MgCl $_2$, 10 mM HEPES, 2 mM TEA, and 4 mM K $_2$ ATP, with a pH of 7.3 and osmolarity of 298–310 mOsm. Currents were recorded using an Axopatch 1-B amplifier and data were digitized, filtered (2 kHz), and acquired using the pClamp6 program (Axon Instruments).

Production and Purification of Recombinant Proteins

N-terminal His-tagged wild-type and K457A mutant CAK β /Pyk2 proteins were expressed in *Escherichia coli* strain BL21 and purified by metal-chelate column chromatography. Purified proteins were extensively dialyzed against Tris buffer (20 mM Tris-HCl [pH 7.6], 150 mM NaCl, 2 mM MgCl $_2$, and 0.1 mM DTT). All the CAK β /Pyk2 cDNA constructs used here originated from clone 17N, a full-length rat CAK β /Pyk2 cDNA (Sasaki et al., 1995). The K457A mutant CAK β /Pyk2 cDNA was prepared by site-directed mutagenesis (Kunkel et al., 1987) using a Mutan-K mutagenesis kit (Takara Shuzo, Ohtsu, Japan). An oligonucleotide, CTTACAGGTCGCGACGGCCA, was used to change lysine 457 to alanine. For the construction of the plasmid encoding a full-length CAK β /Pyk2 with N-terminal His tag, a rat CAK β /Pyk2 cDNA with NdeI site 15 base pairs upstream from the translational initiation codon of CAK β /Pyk2 cDNA was first prepared by polymerase chain reaction and rejoining of cDNA fragments. The NdeI-BamHI fragment, containing the whole coding region of CAK β /Pyk2 cDNA, was prepared, and this fragment was ligated into pET-15b vector (Novagen, Madison, WI) at the NdeI and BamHI sites to yield pET-His-CAK β /Pyk2 (W). The plasmid encoding full-length K457A mutant CAK β /Pyk2 with N-terminal His tag was constructed by swapping the HindIII fragment, which contains the region encoding the residue 457 of CAK β /Pyk2, of pET-His-CAK β (W) with the corresponding HindIII fragment of the K457A mutant CAK β /Pyk2 cDNA. Activity of the recombinant enzymes was measured using an in vitro kinase assay (Sasaki et al., 1995) for each batch made, and it was confirmed that the wild-type enzyme was catalytically active whereas the K457A mutant showed no activity. To preserve maximum activity, the enzymes used in the experiments were stored in single-use aliquots at -80°C , thawed immediately prior to use, and any unused enzyme discarded.

Subcellular Fractionation

Subcellular fractionation was done using methods modified from Huttner et al. (1983) and Lin et al. (1998). Briefly, rat brain Dounce homogenate was centrifuged at $1,000 \times g$ to remove nuclei and other large debris (pelleted in P1). The supernatant (S1) was collected and centrifuged at $10,000 \times g$ to obtain a crude synaptosomal fraction (P2), which was lysed hypoosmotically and centrifuged at $25,000 \times g$ to pellet a synaptosomal membrane fraction (LP1). LP1 was further fractionated by a 5%–15%–25%–35%–45% sucrose gradient. LP1-1, LP1-2 (synaptic plasma membrane), and LP1-3 designates the band between 15%–25%, 25%–35%, and 35%–45%, respectively. Concurrently, the S2 above the P2 was centrifuged at $100,000 \times g$ to pellet a light membrane fraction P3. The PSD fraction was prepared from the P2 fraction (Carlin et al., 1980) and was then subjected to detergent extraction (Cho et al., 1992). The PSD fraction was enriched in the known PSD proteins NMDAR-1 and PSD-95, but the non-PSD protein synaptophysin was not detectable. SDS-PAGE (10% gel) and immunoblotting were performed as in Huang et al. (1997). Signals were detected with enhanced chemiluminescence (ECL, Amersham) and developed on X-ray film. Sources of antibodies: anti-NR1 (mouse monoclonal 58.1), Pharmingen; anti-Src (monoclonal antibody 327), J. Bolen, DNAX, Palo Alto, CA; nonspecific mouse IgG, Calbiochem, San Diego, CA; polyclonal anti-NR2A/2B, Chemicon; anti-FAK, Transduction Labs; anti-synaptophysin, Boehringer; and anti-phosphotyrosine (monoclonal 4G10), Upstate Biotechnologies. Preparation of the anti-CAK β /Pyk2 was described in Matsuya et al. (1998).

Coimmunoprecipitation of CAK β /Pyk2 and NMDAR Subunits

Coimmunoprecipitation was done using sodium deoxycholate (DOC)-extracted membranes from rat cortex with methods modified

from Blahos et al. (1996). In brief, P2 crude synaptosomal preparation was extracted in 1% sodium deoxycholate, 50 mM Tris-HCl (pH 9.0), 10 mM EDTA at 37°C for 30 min. Equal volume of RIPA buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM orthovanadate) was added to the above extract. Solubilized proteins (500 μ g) were centrifuged at $14,000 \times g$ to remove insoluble debris and then incubated with anti-NR2A/B (2 μ g), anti-NR1 (2 μ g), anti-CAK β /Pyk2 (1:1000 dilution), or anti-FAK (2 μ g) antibody or with control, nonspecific IgG (2 μ g) overnight at 4°C. Immune complexes were isolated by the addition of 40 μ l protein G-Sepharose beads followed by incubation for 2–4 hr at 4°C. Immunoprecipitates were then washed four times with lysis buffer, resuspended in Laemmli sample buffer, and boiled for 5 min. The samples were subjected to SDS-PAGE using a 7% gel, transferred to a nitrocellulose membrane, and immunoblotted as described above. The lanes marked “input” were loaded with 20% of the starting material used for immunoprecipitation.

CAK β /Pyk2 Immune Complex Kinase Assay

CAK β /Pyk2 kinase assay was done as described elsewhere (Sasaki et al., 1995; Lu et al., 1998). CAK β /Pyk2 was immunoprecipitated from synaptosomal proteins using the anti-CAK β /Pyk2 antisera, and the phosphorylation reaction was carried out for 20 min at 24°C. After stopping the reaction, the samples were subjected to SDS-PAGE using a 7% gel, transferred to a nitrocellulose membrane, and tyrosine phosphorylation detected by immunoblotting with 4G10 anti-pY antibody. The Src activator peptide or control peptide was tested at a concentration of 1 mM, and Src(40–58) or sSrc(40–58) peptides were 0.1 mg/mL, which was 3-fold higher than that used in electrophysiological experiments. The results with peptides were normalized to controls lacking added peptide.

Tyrosine Phosphorylation of CAK β /Pyk2 and Association of CAK β /Pyk2 and Src in Hippocampal Slices

The CA1 region from acute hippocampal slices was microdissected and immediately frozen on dry ice (Lu et al., 1998). Four CA1 regions were pooled together. The tissue was homogenized in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% NP-40, 1 mM Na orthovanadate, protease inhibitors pepstatin A (20 μ g/mL), leupeptin (20 μ g/mL), and aprotinin (20 μ g/mL), and 1 mM phenylmethylsulfonyl fluoride. Insoluble material was removed by centrifugation at $14,000 \times g$ for 10 min at 4°C. The protein content of soluble material was determined by Bio-Rad Dc protein assay. Soluble proteins (100 μ g) were incubated overnight with 2 μ L of anti-CAK β /Pyk2. Immune complexes were isolated by addition of 20 μ L of protein G-Sepharose beads, followed by incubation for 1–2 hr at 4°C. Immunoprecipitates were washed four times with lysis buffer. Samples were subjected to SDS-PAGE as described above. Membranes were immunoblotted with a monoclonal antibody to phosphorylated tyrosine, 4G10 (1:1,000 dilution, Upstate Biotechnologies). The membrane was then stripped and reprobed with anti-CAK β /Pyk2 (1:3000 dilution).

For experiments on the effects of tetanic stimulation on CAK β /Pyk2 tyrosine phosphorylation or on the association of CAK β /Pyk2 and Src, hippocampal slices were prepared and stimulated and extracellular fields were recorded as described in Lu et al. (1998). After baseline synaptic responses had been stable for at least 10 min, either tetanic stimulation was delivered or the test stimulation was continued for 1 min (control). A region approximately 2×2 mm surrounding the stimulating electrode was carefully microdissected and four such regions (from control or from 1 min posttetanus slices) were pooled together. In each experiment, control and tetanized slices were taken from one animal. CAK β -Pyk2 or Src was immunoprecipitated, separated by SDS-PAGE, and immunoblotted with 4G10 or anti-CAK β /Pyk2, respectively. Band intensity on X-ray film was quantified using Scion Image software (Scion Corp., Frederick, MD). The film images were digitized and each band was identified as a region of interest. The average pixel intensity was determined and background was subtracted. For phosphoCAK β /Pyk2 the intensity of the phosphorylated signal was divided by that of the total CAK β /Pyk2 protein so as to control for small differences in gel loading. Experimental and control conditions were then compared and expressed as percentage of control. Similarly for Src-CAK β /

Pyk2 association, the intensity of the CAK β /Pyk2 band was divided by that of the Src band and the resultant value compared between experimental and control conditions.

Miniature EPSC Recording

Cultures of fetal hippocampal neurons were prepared according to previously described techniques (MacDonald et al., 1989). The cultures were used for electrophysiological recordings 12–17 days after plating. The electrodes were coated with Sylgard to improve the signal-to-noise ratio. The extracellular solution was supplemented with tetrodotoxin (0.5 μ M), strychnine (1 μ M), bicuculline methiodide (10 μ M), and glycine (1 μ M). Spontaneous miniature EPSCs were recorded for 35–60 min. Miniature EPSCs were filtered at 2 kHz and stored on tape before their off-line acquisition with an event-detection program (SCAN, Strathclyde software; courtesy of J. Dempster). For detection, the trigger level was set approximately two times higher than the baseline noise. False events were eliminated by subsequent inspection of the raw data. For averaging, the number of selected events for each group ranged from 95–250. The NMDAR component of mEPSCs was determined by subtraction after bath applying the NMDAR antagonist APV (20 μ M) (Lu et al., 1999).

Hippocampal Slices Recordings

Hippocampal slices were prepared from 4- to 6-week-old male Sprague Dawley rats and were placed in a holding chamber for at least 1 hr prior to recording (Lu et al., 1998). A single slice was transferred to a recording chamber and superfused with artificial cerebral spinal fluid (ACSF, 2 ml/min) composed of (in mM) 124 NaCl, 3 KCl, 1.25 NaH₂PO₄, 2 MgCl₂, 2 CaCl₂, 26 NaHCO₃, 10 D-glucose, and 0.002 bicuculline methiodide, saturated with 95% O₂-5% CO₂ at 30°C \pm 2°C. Synaptic responses were evoked with bipolar tungsten electrodes located about 50 μ m from the cell body layer in CA1. Test stimuli were evoked at 0.1 Hz with the stimulus intensity set to 25% of that which produced maximum synaptic responses. Tetanic stimulation consisted of 2 trains of 100 Hz stimuli lasting 500 ms, at an intertrain interval of 10 s. In controls, this stimulation caused LTP that was at a stable level by 30 min after tetanus and which persisted for greater than 1.5 hr. For clarity, we show records from only the first 30 min after tetanus. EPSP slope was calculated as the slope of the rising phase 10%–65% of the peak response. The value of EPSP slope from the 5 min period immediately before tetanus was defined as baseline (100%). Field potential recordings were made with glass micropipettes filled with ACSF placed in the stratum radiatum 60–80 μ m from the cell body layer. Data were recorded with an Axoclamp 2B amplifier and sampled at 10 kHz by computer. Field EPSP slope was calculated as the slope of the rising phase between 10% and 60% of the peak response. For current-clamp experiments, the patch pipette (4–6 M Ω) solution contained 132.5 mM K-gluconate, 17.5 mM KCl, 10 mM HEPES, 0.2 mM EGTA, 2 mM Mg-ATP, 0.3 mM GTP, and 5 mM QX 314 (pH 7.25, 290 mOsm); EGTA (0.2 mM) was replaced by BAPTA (10 mM) where indicated. For voltage-clamp experiments, the intracellular solution contained Cs-gluconate, 132 mM; CsCl, 17 mM; HEPES, 10 mM; BAPTA, 10 mM; Mg-ATP, 2 mM; GTP, 0.3 mM; and QX-314 10 mM. The intracellular solution was supplemented as required with peptides that were stored as single-use stock solutions prepared just before use. The peptides were numbered and the experimenter (DWA) was unaware of which was applied in experiments illustrated in Figures 5 and 6a. Patch recordings were done using the “blind” patch method (Blanton et al., 1989). For recording NMDAR-mediated synaptic responses, ACSF was supplemented with bicuculline (8 μ M) and DNQX (5 μ M). Raw data were amplified using an Axopatch 1-D, sampled at 5 kHz, and analyzed with Pclamp software (Axon Instruments, Foster City, CA). Series resistance ranged from 10 to 20 M Ω , as estimated from series resistance compensation of current responses to voltage steps of 5 mV, and cells were discarded if the resistance changed by more than 15%.

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