Identification of the Sites for CaMK-II-dependent Phosphorylation of GABA<sub>A</sub> Receptors*

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Phosphorylation can affect both the function and trafficking of GABA<sub>A</sub> receptors with significant consequences for neuronal excitability. Serine/threonine kinases can phosphorylate the intracellular loops between M3–4 of GABA<sub>A</sub> receptor β and γ subunits thereby modulating receptor function in heterologous expression systems and in neurons (1, 2). Specifically, CaMK-II has been demonstrated to phosphorylate the M3–4 loop of GABA<sub>A</sub> receptor subunits expressed as GST fusion proteins (3, 4). It also increases the amplitude of GABA<sub>A</sub> receptor-mediated currents in a number of neuronal cell types (5–7). To identify which substrate sites CaMK-II might phosphorylate and the consequent functional effects, we expressed recombinant GABA<sub>A</sub> receptors in NG108-15 cells, which have previously been shown to support CaMK-II modulation of GABA<sub>A</sub> receptors containing the β3 subunit (8). We now demonstrate that CaMK-II mediates its effects on α1β3 receptors via phosphorylation of Ser<sup>383</sup> within the M3–4 domain of the β subunit. Ablation of β3 subunit phosphorylation sites for CaMK-II revealed that for αβγ receptors, CaMK-II has a residual effect on GABA currents that is not mediated by previously identified sites of CaMK-II phosphorylation. This residual effect is abolished by mutation of tyrosine phosphorylation sites, Tyr<sup>365</sup> and Tyr<sup>367</sup>, on the γ2S subunit, and by the tyrosine kinase inhibitor genistein. These results suggested that CaMK-II is capable of directly phosphorylating GABA<sub>A</sub> receptors and activating endogenous tyrosine kinases to phosphorylate the γ2S subunit in NG108-15 cells. These findings were confirmed in a neuronal environment by expressing recombinant GABA<sub>A</sub> receptors in cerebellar granule neurons.

The γ-aminobutyric acid type A (GABA<sub>A</sub>)<sup>2</sup> receptor is a pentameric ligand-gated ion channel responsible for fast synaptic and tonic inhibition in the brain. The function of GABA<sub>A</sub> receptors can be modulated by phosphorylation, which affects inhibitory synaptic plasticity and thus has significant consequences for the control of neuronal network excitability (9, 10). Phosphorylation of the intracellular domains between M3–4 of the β and γ subunits by serine (Ser)/threonine (Thr) and tyrosine (Tyr) kinases has been shown to modulate receptor function either through a direct effect on receptor properties, such as the probability of channel opening or desensitization, or by regulating trafficking of the receptor to and from the cell surface (1, 2, 9).

The use of glutathione-based fusion proteins and site-directed mutagenesis has enabled the sites of phosphorylation within β and γ subunits to be identified. The β2 subunit has one main site for phosphorylation at Ser<sup>410</sup> (the equivalent of Ser<sup>409</sup> in β1), which can be phosphorylated by PKG, PKA, PKC, and CaMK-II (3). Similarly, Ser<sup>409</sup> has also been identified as the major site of phosphorylation in the β3 subunit for PKC, PKA, PKG, and CaMK-II with neighboring Ser<sup>408</sup> additionally phosphorylated by PKC. Furthermore, Ser<sup>383</sup> can also be phosphorylated, but only by CaMK-II. In comparison, the γ2S subunit can be phosphorylated by PKC at Ser<sup>327</sup>, and at Ser<sup>338</sup> and Thr<sup>350</sup> by CaMK-II (4, 11).

In addition to phosphorylation by Ser/Thr kinases both the β and γ subunits are substrates for tyrosine kinases (12, 13). Mutation of the tyrosine residues at 365 and 367 on the γ2S subunit (corresponding to Tyr<sup>372</sup> and Tyr<sup>375</sup> on γ2L) removed the functional modulation of GABA<sub>A</sub> receptors expressed in HEK293 cells by the tyrosine kinase, Src (12).

CaMK-II has been demonstrated to potentiate GABA<sub>A</sub> receptor function by increasing the fraction of receptors (measured as a B<sub>max</sub>) in synaptosomal membranes (14, 15). CaMK-II can also increase the amplitudes of GABA whole cell currents and IPSCs measured in hippocampal, spinal cord dorsal horn, and cortical neurons, in addition to cerebellar Purkinje and granule neurons (5–8, 16). Furthermore, we have recently reported that NG108-15 cells, which lack endogenous GABA<sub>A</sub> receptors, form a suitable environment for CaMK-II modulation of transiently expressed α1β3 and α1β3γ2S GABA<sub>A</sub> receptors (10). Using this heterologous expression system, and by expressing recombinant GABA<sub>A</sub> receptors in cerebellar granule neurons, we were able to identify which CaMK-II phosphorylation sites were important for functional modulation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—In brief, NG108-15 cells were maintained in Dulbecco’s modified Eagle’s medium (4 g/liter glucose) supple-
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mented with 10% v/v fetal calf serum (Invitrogen), 2 mM glutamine (Sigma), 100 μM hypoxanthine, 0.4 mM aminopterin, 16 mM thymidine (Sigma), and 20 units/ml penicillin G and 20 μg/ml streptomycin (Sigma) and incubated at 37 °C, in 90% air, 10% CO<sub>2</sub>. Subculturing was performed 2–3 times/week. Cells used for transfection were plated onto poly-l-ornithine-coated coverslips (500 μg/ml) and used for electrophysiology 2–3 days later (8).

Cerebellar granule cell cultures were prepared from postnatal day (P) 0–1 Sprague-Dawley rats as follows: cerebella were enzymatically dissociated in trypsin (0.1%, 10 min at 37 °C) followed by trituration in DNase I with glass pipettes of decreasing diameter (×3), and plated onto poly-l-ornithine (500 μg/ml) coated coverslips and maintained in BME (Invitrogen) supplemented with 0.5% w/v glucose, 5 mg/liter insulin, 5 mg/liter transferrin, 5 mg/liter selenium (Sigma), 20 units/ml penicillin G, and 20 μg/ml streptomycin, 0.14 mM glutamate, 1.4 mM NaCl, 0.01 mM bovine serum albumin (Sigma), and 20 mM KCl for 7–10 days before use (8). All animals were used in accordance with UK home office guidelines.

cDNA Constructs and Transfection—Murine GABA<sub>A</sub> receptor subunit α1, β3, and γ2S cDNAs were cloned into the plasmid vector pRK5 (17). The 9E10 (Myc) epitope was inserted between the fourth and fifth residue of the mature protein by site-directed mutagenesis (18, 19). Wild-type rat α2CaMK-II and mutant α2-CaMK-II T286A cDNAs were cloned into the plasmid vector pcDNA3. NG108-15 cells and cerebellar granule cells were transfected using the reagent, Effectene (Qiagen Ltd, West Sussex, UK) in the presence of 0.3–0.5 μg of total cDNA/dish, with cDNAs encoding for GABA<sub>A</sub> receptor subunits and enhanced GFP (for cell identification) present in equal ratio.

Patch Clamp Electrophysiology—Whole cell membrane currents were recorded from single cells with an Axopatch 1-C amplifier (Molecular Devices, Union City, CA). Patch pipettes (resistance 5–6 MΩ for NG108-15 cells; 8–9 MΩ for granule neurons) were filled with the following solution containing (mM): 150 CsCl, 1 MgCl<sub>2</sub>, 10 HEPES, 4 Na<sub>2</sub>ATP, 0.1 CaCl<sub>2</sub>, and 1.1 mM EGTA, pH adjusted to 7.2 with CsOH (290–310 mOsm). The cells were perfused with the following Krebs solution containing (mM): 140 NaCl, 4.7 KCl, 1.2 MgCl<sub>2</sub>, 2.52 CaCl<sub>2</sub>, 11 n-glucose, 5 HEPES adjusted to pH 7.4 with 1 mM NaOH (290–310 mOsm). Currents were filtered at 3 kHz (8-Pole Bessel filter) and analyzed using Clampex 8.2. Cells were not used for analysis if their access or series resistances changed by more than 15%. GABA (dissolved in Kreb’s solution) was applied using a modified Y-tube rapid perfusion system as described previously (20). All experiments were carried out at room temperature (25 °C).

CaMK-II—A purified truncated recombinant form of α-CaMK-II (New England Biolabs, Beverly, MA) with the same substrate specificity as the full-length form (21) was pre-activated (40–50 ng/μl; specific activity ~200–250 units/μl) by incubation in a reaction buffer containing: 50 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, 0.1 mM Na<sub>2</sub>EDTA, supplemented with 1.2 μM calmodulin (CalM), 1.5 mM CaCl<sub>2</sub>, and 0.4 μM ATPγS for 15 min at 25 °C (8). Pre-activated α-CaMK-II was diluted within the patch pipette solution to give a final α-CaMK-II concentration of 60 nm and then maintained on ice throughout the recording period (5, 22, 23). Control whole cell recordings were made with a patch pipette solution containing an appropriate dilution of the pre-activation buffer, which omitted the α-CaMK-II.

Analysis—The peak amplitudes of membrane currents activated by GABA (10 μM) were determined at ~50 mV (NG108-15) or ~60 mV holding potential (granule cells). Statistical analyses were performed using either one-way analyses of variance (ANOVA) with a Bonferroni post-test to compare selected groups, or the nonparametric Kruskal-Wallis test with Dunn’s post-test if the group variances were significantly different. The ANOVA post-test compared GABA-activated currents in the presence and absence of CaMK-II (+), as well as the effect of receptor mutations on the modulation by CaMK-II (+). In all cases, p < 0.05 was considered significant. All time points were tested for statistical significance, but for clarity only a selected few are shown for illustrative purposes.

Biochemistry—NG108-15 cells were transfected using electroporation and cultured for 48 h (17). Expressing cells were then labeled with 0.5–1.0 mCi/ml [32P]orthophosphoric acid (12,000 Ci/mmol) for 4 h at 37 °C, before treatment with specific kinase/phosphatase inhibitors and/or 50 mM KCl (24–27). Cells were then lysed in 2% boiling SDS, immunoprecipitated in a buffer supplemented with 2% Triton X-100 using antibodies against GABA<sub>A</sub> receptor subunits, followed by SDS-PAGE. The levels of incorporated radioactivity were measured using a phosphorimager (26, 27). Immunoprecipitated material was subject to SDS-PAGE and 32P-labeled. Isolated GABA<sub>A</sub> receptor subunits were also subject to phosphopeptide mapping and phosphoamino acid analysis (24). To specifically measure tyrosine phosphorylation of the γ2 subunit, SDS-soluble lysates were prepared from transfected NG108-15 cells. These were subject to SDS-PAGE prior to immunoblotting with antibodies specific for phosphorylated Tyr<sup>365</sup> and Tyr<sup>367</sup> (anti-pY365/7) in the γ2 subunit and with antibodies against total γ2 subunits (anti-γ2) coupled to 125I-protein A (12, 25, 26). The ratios of pY365/7 to γ2 signals were then determined with levels measured under control conditions assigned a value of 100%.

Immunocytochemistry—Cerebellar granule cell cultures were fixed in 4% paraformaldehyde for 10 min and quenched with 50 mM NH₄Cl (in phosphate-buffered saline (PBS); two washes) followed by three washes in PBS and then one wash with 10% v/v FCS and 0.5% w/v bovine serum albumin in PBS. The cells were then incubated with the primary antibody (45 min, rabbit anti-Myc, 1:200, Santa Cruz Biotechnology) followed by three washes in 10% v/v fetal calf serum and 0.5% w/v bovine serum albumin in PBS, prior to incubation with the secondary antibody (45 min, anti-rabbit TRITC conjugate 1:250, Sigma). Immunofluorescent images were acquired using a Zeiss confocal microscope (LSM 510 Meta), equipped with Argon (488 nm) and Helium Neon lasers (543 nm), with a ×63 objective.

RESULTS

Modulation of αβ GABA<sub>A</sub> Receptors by CaMK-II: Role of M3-4 Serine Residues—Previously, we demonstrated that the internal application of pre-activated (8, 22, 23) α2-CaMK-II to α1β3/γ2S GABA<sub>A</sub> receptors, expressed in NG108-15 cells,
To determine if this potentiation resulted from GABA receptor phosphorylation by CaMK-II, we mutated consensus sites for phosphorylation by CaMK-II. We presumed that one or more of the three phosphorylation sites identified in previous studies, Ser(383),408,409 in β3 subunits (3, 28) were responsible. Although the α1 subunit has been reported to be phosphorylated by CaMK-II (15), no specific residues were identified by fusion protein analysis (4, 29). Moreover, in our previous study, α1β2 receptors were not modulated by CaMK-II (8). Therefore, we initially assumed that the β3 subunit was the primary site of CaMK-II phosphorylation. Accordingly, all three serines in the β3 subunit were converted to non-phosphorylated alanines. Using NG108-15 cells expressing α1β3Ser(383),408,409A GABA_A receptors, the repeated application of 10 μM GABA elicited currents that were quite stable over 20 min when recording with the control patch pipette solution, which always contained the pre-activation buffer without α-CaMK-II (60 nM), the GABA current amplitudes retained their stability (93.2 ± 7.6%) compared with the controls (101.3 ± 4.6% at t = 6 min, Fig. 1A). This strongly indicated that phosphorylation of at least one or more of these three serine residues mediated the α-CaMK-II modulation of the α1β3 receptor.

PKA phosphorylation of β3 subunits has been reported to increase the amplitude of GABA-activated currents by phosphorylating Ser(408,409) (28). However, the application of α-CaMK-II to α1β3Ser(408,409)A receptors still potentiated current amplitudes to 120.6 ± 6.2% compared with the controls (93.9 ± 3.8% at t = 6 min). This was not significantly different from the effect of α-CaMK-II on α1β3 wild-type receptors (118 ± 4.8%, Fig. 1B (8)). The time courses for the potentiating effects of α-CaMK-II were also similar for both the Ser(408,409)A mutant and wild-type receptors, attaining a steady-state within 6 min. By contrast to Ser(408,409)A, application of α-CaMK-II to α1β3Ser(383)A receptors prevented the GABA current potentiation (94 ± 6.8%) compared with control (101.7 ± 1.5% at t = 6 min, Fig. 1C). This indicated that the α-CaMK-II modulation of α1β3 receptor function is mediated solely through phosphorylation of Ser(383).

Serine 383 Is Phosphorylated by CaMK-II—To confirm whether Ser(383) is the main site of CaMK-II phosphorylation, NG108-15 cells expressing α1β3γ2S receptors were exposed to...
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[32P]orthophosphoric acid prior to activating CaMK-II by depolarizing the cells with 50 mM KCl-based external solution. Receptor β3 subunits were isolated by immunoprecipitation using subunit-selective antisera linked to protein A-Sepharose. Precipitated subunits were resolved using SDS-PAGE and visualized with autoradiography. The β3 subunits exhibited basal phosphorylation, which was enhanced by the KCl external solution and inhibited back to control levels by exposure to the CaMK-II inhibitor, KN-62 (Fig. 2, A and B). To identify those residues that are phosphorylated we subjected gel slices to phosphoamino acid analysis. All the phosphorylation was located to serine residues (Fig. 2C). By mutating Ser<sup>383</sup> to alanine, basal phosphorylation remained associated with the serine residues, but the increase caused by KCl was ablated (Fig. 2C). Quantitative analysis revealed that KCl increased β3 phosphorylation in wild-type α1β3γ2 receptors by 50 ± 15% (Fig. 3, A and B), which was reduced to basal levels by 1 μM KN-62. In addition, phosphorylation remained unaltered by KCl if only a mutant form of CaMK-II<sup>T286A</sup> was present which lacked the ability to autophosphorylate and become Ca<sup>2+</sup> independent. Although β3<sup>S408A,409A</sup> prevented phosphorylation of β3 subunits by CaMK-II, the mutation β3<sup>S408A,409A</sup> did not, with phosphorylation levels in the presence of CaMK-II increasing by over 50% (Fig. 3B). Together these experiments strongly suggest that Ser<sup>383</sup> is the principal site of CaMK-II phosphorylation in the receptor β3 subunit.

CaMK-II Regulation of αβγ Receptors—For α1β3γ2S receptors expressed in NG108-15 cells, CaMK-II induced a significantly larger potentiation of GABA currents when compared with its modulation at α1β3 receptors (8). This suggested that α-CaMK-II modulation may be partly mediated through the γ2S subunit. Indeed, in parallel experiments, applying α-CaMK-II (60 nM) to α1β3<sup>3S383A,408,409A</sup>γ2S receptors still resulted in a significant potentiation of 10 μM GABA current amplitudes to 117.8 ± 5% as compared with the controls (93.4 ± 4%; at t = 8 min; Fig. 4A). The potentiation required longer to peak compared with the time course for wild-type receptors. The effect was also transient with current amplitudes returning to baseline levels within 20 min (Fig. 4A). This residual potentiation was significantly smaller than that observed with α-CaMK-II on α1β3γ2S wild-type receptors (α1β3<sup>3S383,408,409A,γ2S</sup>, 107.5 ± 3.5; α1β3γ2S, 145.8 ± 10.8%; at t = 4 min (8)). These results confirmed that α-CaMK-II is mediating part of its potentiating effect via the γ2S subunit.

Prior analysis of GST fusion proteins in vitro identified the potential substrate sites for CaMK-II phosphorylation on the γ2S subunit as Ser<sup>348</sup> and Thr<sup>350</sup>. By applying α-CaMK-II to α1β3γ2S<sup>S348A,T350A</sup> receptors, GABA currents were still potentiated up to 142.3 ± 9.1% compared with controls (99 ± 6.5%; Fig. 4B; at t = 6 min). This level of potentiation was the same as observed with CaMK-II and wild-type α1β3γ2S receptors (8), indicating that Ser<sup>348</sup> and Thr<sup>350</sup> are not involved in CaMK-II modulation of these receptors.

The application of α-CaMK-II to the mutant receptor, α1β3<sup>3S383,408,409A,γ2S</sup>, where all potential CaMK-II sites of phosphorylation were now removed, also allowed a
significant potentiation of GABA currents by α-CaMK-II. In accord with our previous observations, the potentiation was transient and after 10 min the effect of α-CaMK on α1β2γ3S383,408,409A γ2S5S348A,T350A receptors was significantly different (114.2 ± 6.4%) from the effect on wild-type receptors (148.5 ± 12.2%) but not significantly different from control (102.7 ± 5.8%, Fig. 4C).

To ensure that α1β3γ2S5S348A,T350A receptors were correctly assembled, we applied 10 μM diazepam which potentiated approximately EC50 GABA-activated currents, indicating that the γ2S5S348A,T350A subunit was indeed expressed at the cell surface (Fig. 4D). Furthermore, if the γ2S subunit had been absent, the resultant receptor, α1β3γ3S383,408,409A, should not have been modulated by α-CaMK-II (Fig. 1A). Thus the increased GABA current in the presence of α-CaMK-II confirmed that αβγ heteromers were correctly assembled and expressed at the cell surface (Fig. 4C).

**CaMK-II and Tyrosine Residues on γ2S**—Although Ser348 and Thr350 are the main sites of CaMK-II phosphorylation on the γ2S subunit, their mutation clearly had no effect on α-CaMK-II modulation of α1β3γ2S receptors. This raised the possibility that CaMK-II was either phosphorylating new sites on the γ2S subunit, or that another kinase, driven by CaMK-II activating downstream signaling pathways, was phosphorylating the GABA_2A receptor or an associated protein. The γ2S subunit contains two sites for tyrosine kinase phosphorylation, Tyr365 and Tyr367, which after phosphorylation, can potentiate GABA_2A receptor function in a similar manner to that observed in the present study (12). To assess whether tyrosine kinases were involved in CaMK-II modulation of α1β3γ2S GABA_2A receptors, the tyrosine kinase inhibitor genistein (100 μM) was co-applied in the patch pipette solution with α-CaMK-II (60 nM). A significant increase in the amplitude of GABA currents to 126.3 ± 6.3% as compared with control, 98.6 ± 2.7% (Fig. 4C, at t = 8 min). Once again, this was not significantly different from that seen with α1β3γ3S383,408,409A-γ2S receptors, further indicating that phosphorylation of Ser348 and Thr350 does not contribute to the potentiation of GABA currents by α-CaMK-II.
Furthermore, application of /H9251 amplitude of GABA currents recorded over 20 min (Fig. 5 (8)). The application of genistein alone, had no effect on the response of the mutant 8–10 min of recording, there was no significant difference in current amplitudes to 143.8 (94.5 (30). As expected, the current density recorded from NG108-15 cells expressing αβ heteromers (20.2 ± 11.5 pa/pF) was significantly increased for αβ3y2 receptors (94.1 ± 19 pa/pF (8)). By contrast, there were no significant differences in current densities between αβ3y2, αβ3y2S338A,T350A (87.1 ± 21.3 pa/pF) and αβ3y2Y365F,Y367F (82.1 ± 15.6 pa/pF) receptors. Thus site-specific mutation of phosphorylation sites in the y2 subunit did not disrupt the surface expression of GABAα receptor subunits in NG108-15 cells.

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**FIGURE 5. α-CaMK-II modulation of α1β3γ2S subunit-containing GABAα receptors requires tyrosine kinase phosphorylation.** Peak amplitude GABA-activated currents recorded from NG108-15 cells expressing: α1β3y2S (A) or α1β3S338A,y2S (B) GABAα receptors in the presence of α-CaMK-II (60 nm) and/or 100 µM genistein applied via the patch pipette. Control recordings were obtained with a normal patch pipette solution supplemented with the pre-activation buffer without α-CaMK-II and 100 µM genistein (n = 4–7). Data obtained from parallel experiments with wild-type receptors are shown for comparison (8). Representative 10 GABA-activated currents are shown at different time points in the presence of genistein or genistein and α-CaMK-II. The symbol (*) indicates significant differences between wild-type receptors in the presence of genistein and the presence or absence of genistein, and (†) denotes significance between wild-type receptors in the presence of genistein and the presence or absence of α-CaMK-II.

By mutating the y2 subunit to prevent tyrosine phosphorylation, the application of α-CaMK-II to α1β3y2Sβ γ2S receptors resulted in a significant potentiation of the GABA current amplitudes to 143.8 ± 4.5% as compared with control (Fig. 6A) at t = 16 min. However, as before, this potentiation was significantly less than that induced by α-CaMK-II on α1β3γ2S receptors (wild-type: 145.7 ± 10.2%; Y365F,Y367F: 119.4 ± 3.5%) at t = 6 min (8). Notably, after 8–10 min of recording, there was no significant difference in the response of the mutant y2Sβ γ2S subunit is transient and is no longer evident after 10–15 min.

Finally, application of α-CaMK-II to α1β3S338A,y2SY365F,Y367F receptors abolished the response to α-CaMK-II on GABA current amplitudes over 20 min (103.3 ± 3.4% at t = 6 min) as compared with control (108.4 ± 4.3% at t = 6 min, Fig. 6B). This indicates that the functional effects of α-CaMK-II-dependent phosphorylation on α1β3γ2S receptors are mediated entirely via β3 Ser383 and γ2S Tyr365,367. Moreover, α-CaMK-II is likely to directly phosphorylate the receptor via the β subunit and cause an indirect activation of a tyrosine kinase leading to phosphorylation of the γ2S subunit.

Application of 10 µM diazepam to α1β3γ2SY365F,Y367F receptors resulted in a potentiation of the GABA current indicating that the γ2Sβ subunit was expressed and correctly assembled at the cell surface (Fig. 6C). It has been noted that the addition of the γ subunit to αβ heteromers increases the amplitudes of the GABA currents (30). As expected, the current density recorded from NG108-15 cells expressing αβ heteromers (20.2 ± 11.5 pa/pF) was significantly larger compared with that observed with α-CaMK-II alone (wild-type: 145.7 ± 10.2%, + genistein 116.1 ± 6.4 at t = 6 min (8)). The application of genistein alone, had no effect on the amplitude of GABA currents recorded over 20 min (Fig. 5A). Furthermore, application of α-CaMK-II in the presence of genistein to α1β3S338A,y2S receptors failed to affect GABA current amplitude (103.6 ± 2.8%) over 20 min when compared with control (97 ± 7.1% at t = 6 min, Fig. 5B). Taken together, these results suggested that phosphorylation at Ser383 in the β3 subunit and α-CaMK-II-dependent activation of tyrosine kinase activity is necessary for the modulation of GABAα receptors by α-CaMK-II.

By mutating the γ2 subunit to prevent tyrosine phosphorylation, the application of α-CaMK-II to α1β3y2SY365F,Y367F receptors resulted in a significant potentiation of the GABA current amplitudes at 143.8 ± 4.5% as compared with control (94.5 ± 6.3% Fig. 5A) at t = 16 min. However, as before, this potentiation was significantly less than that induced by α-CaMK-II on α1β3γ2S receptors (wild-type: 145.7 ± 10.2%; Y365F,Y367F: 119.4 ± 3.5%) at t = 6 min (8). Notably, after 8–10 min of recording, there was no significant difference in the response of the mutant y2Sβ γ2S subunit compared with wild-type. This correlated with the observation that the response to α-CaMK-II mediated by the γ2S subunit is transient and is no longer evident after 10–15 min.

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**CaMKII Induces Phosphorylation of Tyrosine Residues in γ2 Subunits**—Whether the γ2 subunits can be phosphorylated by tyrosine kinases after activation of CaMK-II was assessed using our phosphospecific antibodies directed against Tyr365 and Tyr367 (anti-pY365/7) in the γ2 subunit (25), the principal sites of tyrosine phosphorylation within the GABAα receptor (12). Using transfected NG108-15 cells expressing α1β3γ2 subunits and α-CaMK-II, phosphorylation was activated by exposing the cells to a high K+ external solution. SDS-soluble cell extracts were then immunoblotted with anti-pY365/7 and with anti-γ2 directed against phosphoindependent epitopes. The γ2 subunit was detected as a band of ~48 kDa in cells transfected with GABAα receptor cDNAs but not in cells expressing just GFP (Fig. 7A). A doublet of identical mass was also observed with anti-pY365/7 in cells expressing γ2 subunits but not GFP. Significantly the detection of these bands was blocked by preabsorption with the phosphorylated antigen (not shown) used to...
produce this sera (25). The intensity of this band doubled after exposure to high K\(^+\) external solution while the level of total \(\gamma_2\) subunits remained unaltered (Fig. 7B). Overall, these data indicate that the \(\gamma_2\) subunits are phosphorylated at positions 365 and 367 after CaMK-II activation.

**Recombinant GABA\(_A\) Receptors Are Modulated by CaMKII in Cerebellar Granule Neurons**—Previously, we reported that recombinant GABA\(_A\) receptors expressed in cerebellar granule neurons modified the effect of CaMK-II suggesting that the recombinant receptors functionally dominated the native receptor population (8). Thus, to assess the importance of the CaMK-II phosphorylation sites in a native environment, we expressed GABA\(_A\) receptor subunits containing single mutated sites for phosphorylation, in granule neurons.

To ensure robust cell surface expression of recombinant \(\beta\) subunits in cerebellar granule neurons we co-expressed them with recombinant \(\alpha\) subunits (8). Co-expression of \(\alpha\) with \(\beta_3^{S408A,S409A}\) or with \(\beta_3^{S383A}\) revealed that despite the disrupted phosphorylation sites, these recombinant subunits were capable of efficient expression at the cell surface. Co-expression of \(\alpha\) and \(\beta_3\) with Myc-tagged \(\gamma_2\)\(^{Y365F,Y367F}\) also resulted in clear expression of surface \(\alpha_1\beta_3\gamma_2\)\(^{Y365F,Y367F}\) receptors (Fig. 8C). As a control, EGFP only expressing neurons had similar resting membrane potentials to untransfected cells (43.9 \(\pm\) 2.8 mV; \(n = 8\) and \(-39.2 \pm 2.5\) mV; \(n = 6\), respectively) and displayed stable GABA current amplitudes over time with little evidence of rundown (93.7 \(\pm\) 8.7\% at \(t = 16\) min) (8).

Application of 60 nM \(\alpha\)-CaMK-II to \(\alpha_1\beta_3^{S408A,S409A}\)-expressing granule cells resulted in a significant increase in the GABA current amplitude to 127.1 \(\pm\) 5.1\% at \(t = 6\) min as compared with the application of \(\alpha\)-CaMK-II to \(\alpha_1\beta_3^{S383A}\)-expressing granule cells (103.1 \(\pm\) 3\% at \(t = 6\) min, Fig. 8A). The level of potentiation for \(\alpha_1\beta_3^{S408A,S409A}\) was not significantly different from that observed in parallel experiments after applying \(\alpha\)-CaMK-II to wild-type \(\alpha_1\beta_3\) heteromers (8). These results demonstrated that recombinant GABA\(_A\) receptors responded similarly to \(\alpha\)-CaMK-II whether they were expressed in granule neurons or NG108-15 cells. The mutation of Ser\(^{383}\) on the \(\beta_3\) subunit still ablated the \(\alpha\)-CaMK-II-dependent effect on \(\alpha_1\beta_3\) heteromers in a neuronal environment.

Application of 60 nM \(\alpha\)-CaMK-II to \(\alpha_1\beta_3^{S408A,S409A}\)-expressing granule cells increased whole cell GABA current amplitudes to 116.7 \(\pm\) 3.6\% at \(t = 6\) min. This increase, however, was significantly smaller than the increase observed after application, in parallel experiments, of \(\alpha\)-CaMK-II to \(\alpha_1\beta_3\gamma_2\)-expressing granule cells (167.8 \(\pm\) 13.7\%, at \(t = 6\) min,
The slower time course of the GABA current potentiation mediated by phosphorylation of the γ2 subunit is in accord with downstream tyrosine kinase activity.

Serine 383 on the β3 subunit would appear to be a unique site for α-CaMK-II-mediated phosphorylation because no other Ser/Thr kinase phosphorylated this residue when the β3 subunit M3–4 intracellular loop was expressed as a GST fusion protein (3). The homologous residue in a β1 subunit fusion protein, Ser383, can also be phosphorylated by CaMK-II (4); however, the β2 subunit does not contain this site. This accorded with our previous work on recombinant GABA\(_A\) receptors expressed in NG108-15 cells demonstrating that β2 subunit-containing receptors are not apparently modulated by CaMK-II (8).

As α1β3γ2S \(Y^{365F,Y^{367F}}\) mutants were less sensitive to α-CaMK-II modulation, it appears that α-CaMK-II is capable of directly activating a tyrosine kinase or acting indirectly, to allow increased tyrosine phosphorylation of the GABA\(_A\) receptor γ2S subunit. Phosphorylation of these tyrosine residues would be expected to result in an increase in the whole cell GABA current amplitude due to an increase in the single GABA channel mean open time and open probability (12).

The results of our study suggest that the tyrosine kinase phosphorylation of the γ2S subunit must also be dependent on the β subunit present in the receptor complex, as α1β2γ2S receptors expressed in NG108-15 cells are insensitive to CaMK-II-dependent modulation (8). For tyrosine kinases such as Src, binding to the NMDA receptor is thought to occur via association with PSD-95 or possibly via another adaptor protein rather than by directly interacting with the receptor itself (31, 32). If a similar situation occurs with GABA\(_A\) receptors, the binding of CaMK-II to the receptors, either directly or through an adaptor protein, may be β3 subunit-dependent and unable to occur if β2 subunits are present within the receptor complex. This would explain the lack of tyrosine kinase-dependent modulation of α1β2γ2S receptors after applying CaMK-II (8).

Modulation of GABA\(_A\) receptors by PKA and PKC has also been suggested to be dependent on the subtypes of β and γ subunits present in the receptor complex, with both being required for the full modulatory effect to be observed (33). Serine 409 on the β1 subunit together with Ser377/343 on the γ2L subunit, were found to be important for PKC modulation in HEK cells and L929 fibroblasts (33, 34). Both subunits were also found to be important for PKC modulation of α1β2γ2S receptors in Xenopus oocytes (35). However, although the β and γ subunits are the main targets for phosphorylation of GABA\(_A\) receptors, little is known about how the intracellular loops of different subunits might interact during phosphorylation. An interaction seems likely and possibly crucial, given the insensitivity of β2 subunit-containing receptors to α-CaMK-II. We can also conclude from our data that the presence of phosphorylation sites on the γ2 subunits alone is insufficient to ensure maximum CaMK-II dependent modulation. It is possible that phosphorylation of the β subunit is critical in allowing effective phosphorylation of the γ2S subunit.

Overall, the most parsimonious explanation of our results is that CaMK-II activates a tyrosine kinase. Although it is also possible CaMK-II may act to inhibit a tyrosine phosphatase and...
so promote tyrosine phosphorylation of the \( \gamma_2 \) subunit indirectly. Interestingly, there is much more evidence that \( \text{Ca}^{2+} \)-sensitive tyrosine kinases exist (36, 37) and that PKC can influence the activity of the non-receptor tyrosine kinase Src, implying that Ser/Thr kinases and the Src family of tyrosine kinases could interact (38, 39). It is generally considered that activation of Src through PKC is mediated by activation of PYK2 (40), a member of the focal adhesion kinase family highly expressed in the nervous system (41, 42), which responds to \( \text{Ca}^{2+} \) (41). Activation of PYK2 leads to autophosphorylation which creates an SH2 ligand that can bind the SH2 domain of Src, thereby activating it (39, 40).

Activation of PYK2, and subsequently Src, has been demonstrated in some cases to be \( \text{Ca}^{2+} / \text{calmodulin} \)-dependent (43, 44). In this regard there is evidence for CaMK-II-dependent activation of Src in glomerular mesangial cells (45) and HEK293 cells (46), whereas, CaMK-II-dependent activation of PYK2 and Src has been demonstrated in vascular smooth muscle (47, 48), PC12 cells (49), and in hippocampal neurons following ischemia (50). There is also evidence for CaMK-II activating the related non-receptor tyrosine kinase, focal adhesion kinase (FAK) (51). It is unknown if NG108-15 cells express PYK2, but given that this protein is abundant in the CNS (41, 42) and the neuronal origin of the secondary cell line, it seems plausible.

Src activation has been implicated in the regulation of excitatory synaptic transmission by phosphorylating NMDA receptors (38, 52, 53). Moreover, PYK2-induced Src activation is thought to be necessary for certain forms of long-term potentiation (54). At the NMDA receptor, Src acts as a point of convergence for a number of different signaling pathways (52). There is also evidence that tyrosine phosphorylation of NR2B can modulate CaMK-II binding to the NMDA receptor (55). In comparison,
CaMK-II phosphorylation of GABA<sub>A</sub> receptor β3 subunits could be a prerequisite for tyrosine kinase targeting to the γ2 subunit, for example, by allowing binding of another kinase or regulatory protein. Such a phenomenon of metaplasticity of kinase function has been demonstrated in the regulation of a nonspecific cation channel in Aplysia bag cell neurons where inhibition of Src promotes the interaction of this ion channel with PKC. Src phosphorylation of the channel was proposed to either prevent association of PKC with the channel, or Src may act as a scaffolding/marshalling protein (56).

There is also evidence that tyrosine kinases play a role in the modulation of inhibitory synaptic transmission. Src has been shown to mediate an increase in the amplitude of GABA<sub>A</sub> modulation of inhibitory synaptic transmission. Src has been shown to either prevent association of PKC with the channel, or Src may act as a scaffolding/marshalling protein (56).

In conclusion, tyrosine kinase and CaMK-II signaling pathways may interact to modulate the function of GABA<sub>A</sub> receptors. Given the important role for β3 subunits early in neuronal development (59, 60) this could have important consequences for synaptogenesis. It is early on in development when GABA has an excitatory role that CaMK-II and other Ca<sup>2+</sup>-sensitive signaling pathways may also play an important role, possibly in activity-dependent strengthening of synaptic connections.

The evidence presented in this study suggests that α-CaMK-II mediates its effects on α1β3γ2S receptors in NG108-15 cells and cerebellar granule neurons through direct phosphorylation of the β3 subunit at Ser<sup>383</sup> and via CaMK-II activation of a tyrosine kinase that phosphorylates γ2S subunits at Tyr<sup>365,367</sup>.

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Identification of the Sites for CaMK-II-dependent Phosphorylation of GABA$_A$ Receptors
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