A Brain-specific Ca\textsuperscript{2+}/Calmodulin-dependent Protein Kinase (CaM Kinase-Gr) Is Regulated by Autophosphorylation

RELEVANCE TO NEURONAL Ca\textsuperscript{2+} SIGNALING*

Maria V. Frangakis, Carol-Ann Ohmstede, and Naji Sahyoun
From the Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709

A neuronal Ca\textsuperscript{2+}/calmodulin-dependent protein kinase (CaM kinase-Gr) undergoes autophosphorylation on a serine residue(s) in response to Ca\textsuperscript{2+} and calmodulin. Phosphate incorporation leads to the formation of a Ca\textsuperscript{2+}-independent (autonomous) activity state, as well as potentiation of the Ca\textsuperscript{2+}/calmodulin-dependent response. The autonomous enzyme activity of the phosphorylated enzyme approximately equals the Ca\textsuperscript{2+}/calmodulin-stimulated activity of the unphosphorylated enzyme, but displays diminished affinity toward ATP and the synthetic substrate, syntide-2. The $K_{\text{m(app)}}$ for ATP and syntide-2 increased 4.3- and 1.7-fold, respectively. Further activation of the autonomous enzyme by Ca\textsuperscript{2+}/calmodulin yields a marked increase in the affinity for ATP and peptide substrate such that the $K_{\text{m(app)}}$ for ATP and syntide-2 decreased by 14- and 8-fold, respectively. Both autophosphorylation and the addition of Ca\textsuperscript{2+}/calmodulin are required to produce the maximum level of enzyme activation and to increase substrate affinity.

Unlike Ca\textsuperscript{2+}/calmodulin-dependent protein kinase type II that is dephosphorylated by the Mg\textsuperscript{2+}-independent phosphoprotein phosphatases 1 and 2A, CaM kinase-Gr is dephosphorylated by a Mg\textsuperscript{2+}-dependent phosphoprotein phosphatase that may be related to the Ca\textsuperscript{2+}/calmodulin-stimulated activity of the unphosphorylated enzyme. Dephosphorylation of CaM kinase-Gr is dephosphorylated by a Mg\textsuperscript{2+}-dependent phosphoprotein phosphatase that may be related to the Ca\textsuperscript{2+}/calmodulin-stimulated activity of the unphosphorylated enzyme. A comparison between the autophosphorylation and dephosphorylation reactions of CaM kinase-Gr and Ca\textsuperscript{2+}/calmodulin-dependent protein kinase type II provides useful insights into the operation of Ca\textsuperscript{2+}-sensitive molecular switches.

A neuron-specific Ca\textsuperscript{2+}/calmodulin-dependent protein kinase was found to be highly enriched in granule cells of the cerebellum and was, therefore, named CaM kinase-Gr\textsuperscript{1} (1). Mouse (2) and human (3) homologues are also likely to be present. A partial, deduced amino acid sequence of this protein revealed the presence of a polyglutamate-rich domain along with a regulatory domain containing a calmodulin-binding site (1). Interestingly, a testicular protein, calaparin, whose function is unknown, represents a portion of CaM kinase-Gr that contains the calmodulin-binding and polyglutamate-rich regions but lacks a protein kinase catalytic domain (4).

CaM kinase-Gr differs from a major brain calmodulin-dependent kinase, CaM kinase-II, in several aspects such as amino acid sequence, subcellular distribution, subunit molecular weights, immunohistochemical localization, and developmental profile (1). However, the observation that both enzymes phosphorylate synapsin I on the tail domain (1) and the presence of CaM kinase-Gr in the molecular layer of the cerebellum (5) imply that CaM kinase-Gr, like CaM kinase-II (6), may facilitate neurotransmitter release. In addition, the presence of a polyglutamate-rich domain suggested (1, 7), and ultrastructural examination confirmed (5, 8), the association of CaM kinase-Gr with nuclear chromatin, thereby implicating this kinase in nuclear Ca\textsuperscript{2+} signaling. Because CaM kinase-Gr seems to mediate different aspects of neuronal Ca\textsuperscript{2+} signaling, it was of interest to investigate the regulatory reactions that control its activity.

Both polypeptide components (M, 65,000 and 67,000) of CaM kinase-Gr readily undergo autophosphorylation in the presence of Ca\textsuperscript{2+} and calmodulin (1). Autophosphorylation of CaM kinase-II has been examined intensively and found to exert profound regulatory effects on enzyme activity and calmodulin binding (reviewed in Refs. 9--11). Consequently, we endeavored to characterize the autophosphorylation reaction of CaM kinase-Gr and its possible functional significance.

MATERIALS AND METHODS

\gamma\textsuperscript{32}P-Labeled ATP ($\sim$30 Ci/mmol) was purchased from Du Pont-New England Nuclear. Calmodulin, calmodulin-agarose, calcineurin, and agaorase-coupled alkaline phosphatase were obtained from Sigma. Nitrocellulose was supplied by Schleicher & Schuell. Syntide-2 was synthesized by Dr. J. McDermed from the Wellcome Research Laboratories; the C-36 antisera against CaM kinase-Gr was produced as described previously (1). Adult male Sprague-Dawley rats were used as a source of brain tissue.

CaM kinase-Gr was purified by modification of a published procedure (1). All buffers contained 2 mM DTG, 20 \mu M leupeptin, and 100 \mu g/ml phenylmethysulfonyl fluoride, and all purification procedures were performed at 4 °C. The protein kinase was detected by a dot-immunoblot assay using the C-36 antisem. Ten cerebella from male Sprague-Dawley rats were homogenized in 40 ml of buffer containing 25 mM HEPES, pH 7.5, 2 mM EDTA, 2 mM EGTA, and were centrifuged at 200,000 $g$ for 1 h. Supernatant protein was precipitated with 55% ammonium sulfate, redissolved in 20 ml of homogenization buffer, and applied to a 15-ml DEAE-cellulose column equilibrated with 25 mM HEPES, pH 7.5, and 0.5 mM EDTA. The column was washed with 75 ml of the same buffer containing 0.15 M NaCl and then eluted with 40 ml of buffer containing 0.2 M NaCl. The eluted protein was concentrated by precipitation with 80% ammonium sulfate, redissolved in 1 ml of a solution containing 25 mM HEPES, pH 7.5, 0.2 M NaCl, 0.5 mM EDTA, 10% glycerol, and 1 mg/ml polyethylene glycol 20,000, and applied to a 30-ml AcA-34 gel permutation column equilibrated with the same buffer. One-ml
fractons were collected and those containing the protein kinase were combined and applied to a 0.2-ml calmodulin-agarose column in the presence of 2 mM CaCl₂ and 2 mM MgCl₂. The resin was washed with 2 ml of 25 mM HEPES, pH 7.5, 2 mM CaCl₂, 2 mM MgCl₂, 10% glycerol, 1 mg/ml polyethylene glycol 20,000, and 0.2 M NaCl, followed by the same buffer minus CaCl₂ and NaCl, and then by the same buffer without NaCl. Specifically bound proteins were released with an elution buffer containing 25 mM HEPES, pH 7.5, 2 mM EDTA, and 2 mM EGTA followed by the same buffer with 0.2 mM NaCl. The latter fractions (total 1.0-1.5 ml) that contained the majority of the enzyme were used as a source of purified CaM kinase-Gr. Enzyme purity was routinely checked by SDS-PAGE analysis (12) followed by silver staining (13); final enzyme protein concentration was 20-50 μg/ml.

The number of picomoles of enzyme used in various assays was calculated based on a M₀ value of 65,000 determined by SDS-PAGE (1). Protein concentrations determined by the Bradford method (14) were about 50% greater than the values obtained by standard densitometric scanning of Coomassie Blue-stained gels. The values actually used in this paper were those derived at by the former method.

Enzyme autophosphorylation was carried out in duplicate, as detailed in the respective figure legends. Syntide-2 phosphorylation was measured after spotting reaction mixtures onto P-81 cellulose filters and washing in 5% phosphoric acid (15). Unless otherwise specified, syntide-2 phosphorylation assays were performed for 6 min at 23°C in 25 μl of 20 mM Tris-HCl, pH 7.7, containing 10 mM MgCl₂, 200 μM ATP, 1-2 μCi of [γ-32P]ATP, 60 μM syntide-2, 0.2 pmol of enzyme, and 2 mM CaCl₂ or EGTA, as indicated in the figure legends. Autophosphorylation was analyzed by adding sample buffer to the reaction mixture, followed by SDS-PAGE analysis and autoradiography or by the P-81 filter method; 32P-incorporation into CaM kinase-Gr polypeptides was quantitated by Cerenkov emission or by scintillation counting, respectively. Experiments were repeated independently three or more times.

Phosphoamino acid analysis and phosphopeptide maps were obtained according to standard procedures (16, 17). Hydrodynamic parameters were determined by sedimentation through sucrose density gradients and gel permeation analysis as described elsewhere (18)

As a source of phosphoprotein phosphatase, rat brain cytoplasm was prepared by homogenizing rat brain tissue in 6 volumes of a buffer containing 40 mM Tris-HCl/10 mM imidazole, pH 7.5, 5 mM EDTA, 2 mM EGTA, 1 mM DTT, 100 μg/ml phenylmethylsulfonyl fluoride, and 100 μg/ml leupeptin (19); the 250,000 x g supernatant was then obtained. For partial purification of the phosphatase, cerebella from four adult rats were homogenized as described above. 55% EDTA, 2 mM EGTA, 1 mM DTT, and 100 pg/ml phenylmethylsulfonyl fluoride were added to the homogenate.

5.5 ml were then applied to a 0.2-ml calmodulin-agarose column in the presence of 2 mM CaCl₂ and 2 mM MgCl₂. The resin was washed with 2 ml of 25 mM Tris-HCl, pH 7.7, 0.3 M NaCl, and 0.5 mM EDTA, and 1-ml fractions were collected. 50 μl of CaM kinase-Gr (1.5 μg of enzyme protein) was also applied to a 0.2-ml calmodulin-agarose column equilibrated with a buffer containing 25 mM Tris-HCl, pH 7.7, 0.3 M NaCl, and 0.5 mM EDTA, and 1-ml fractions were collected. CaM kinase-Gr was detected by substrate phosphorylation and by immunoblotting using a monospecific antibody preparation (1). Standard proteins were β-amylase, alcohol dehydrogenase, bovine serum albumin, and carbonic anhydrase; catalase was additionally used for the sucrose density gradient.

**TABLE I**

<table>
<thead>
<tr>
<th>Hydrodynamic parameters of purified CaM kinase-Gr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stokes radius (nm)</td>
</tr>
<tr>
<td>Sedimentation coefficient, M₀</td>
</tr>
<tr>
<td>Native molecular weight</td>
</tr>
<tr>
<td>Frictional ratio (f/f₀)</td>
</tr>
<tr>
<td>Subunit molecular weight (from SDS-PAGE)</td>
</tr>
</tbody>
</table>

**RESULTS**

**Hydrodynamic Analysis of CaM Kinase-Gr**—The quaternary structure of a CaM kinase may influence both the mechanism and consequences of its autophosphorylation. In fact, the oligomeric organization of CaM kinase-II seems to prevent the autophosphorylation of a few subunits to enhance the “autonomous” activity of other subunits within the same oligomer (19). It was of interest, therefore, to determine the native molecular weight and frictional ratio of CaM kinase-Gr and compare them with those of CaM kinase-II. The hydrodynamic parameters (Table I) of CaM kinase-Gr were determined by gel permeation chromatography and sucrose density sedimentation employing an enzyme preparation containing 30 μg/ml protein (500 nM). The deduced, native M₀ value was 58,000, and the frictional ratio was calculated at 1.32. The apparent M₀ value of enzyme subunits had been determined by SDS-PAGE to be 65,000 and 67,000 (1). Together, these observations indicate that CaM kinase-Gr occurs in solution as an elongated monomer whose electrophoretic migration on denaturing gels is anomalously retarded. The aberrant electrophoretic migration, as well as the elongated structure, may partly arise from the presence of a long stretch of consecutive acidic residues in the protein (1, 4, 20).

**Site and Stoichiometry of CaM Kinase-Gr Autophosphorylation—Ca²⁺/calmodulin-dependent enzyme autophosphorylation achieved apparent saturation after 20 min at 23°C at a stoichiometry of about 1.5 mol of phosphate/mol of enzyme (Fig. 1A). Further determination of the stoichiometry of autophosphorylation in eight independent experiments using the different enzyme preparations yielded a range of 0.6-1.7 mol of phosphate/mol of enzyme (1.7, 1.1, 3.1, 0.88, 0.83, 0.81, 0.73, and 0.6 mol of phosphate/mol of enzyme) with a mean value of 0.97 ± 0.12 (n = 8). Addition of excess EGTA at 3, 12, or 20 min after initiation of autophosphorylation did not generate a rapid increase or “burst” of Ca²⁺/calmodulin-independent autophosphorylation comparable with that observed with CaM kinase-II; instead, a modest 10-15% increase in phosphate incorporation was observed (data not shown).

Measurement of initial rates of ³²P incorporation disclosed a linear dependence on enzyme concentrations above 25 nM (Fig. 1B), consistent with an intramolecular autophosphorylation reaction. Significant CaM kinase-Gr autophosphorylation at enzyme concentrations below 25 nM (0.63 pmol/assay) could not be measured reliably (Fig. 1B). The data obtained at enzyme concentrations of 25 nM or higher were also analyzed as described previously by Kuret and Schulman (22). A plot of specific activity versus enzyme concentration had a linear slope of 0.05 (Fig. 1C); a logarithmic transformation of reaction velocity versus enzyme concentration yielded a slope of 1.16 (Fig. 1D). These derivative results provide additional evidence for an intramolecular autophosphorylation mechanism. However, the present observations do not allow us to determine whether the various constituents of the autophosphorylation reaction medium may cause the enzyme to undergo a transition from a monomer to a dimer or oligomer that may promote the autophosphorylation reaction itself.
Modulated Neuronal Ca²⁺ Signaling

FIG. 1. Dependence of CaM kinase-Gr autophosphorylation on duration of the assay (A) and enzyme concentration (B). A, CaM kinase-Gr (2.3 pmol) was autophosphorylated in a 25-μl reaction mixture containing 20 mM Tris·HCl, pH 7.7, 50 μM ATP, 2 μCi of [γ-32P]ATP, 10 mM MgCl₂, 1 mM DTT, 5 mM CaCl₂, and 600 nM calmodulin. Varying the concentration of CaCl₂ between 1 and 10 mM did not alter significantly the extent of CaM kinase-Gr activation in an autophosphorylation assay or in the presence of exogenous substrate (data not shown). The reaction was stopped at different time intervals and 32P incorporation was measured by the P-81 Millipore filter method. Nonspecific 32P-retention was measured by deleting CaM kinase-Gr from the reaction mixture. B, enzyme autophosphorylation was measured as described in the legend to panel A, using different enzyme concentrations, which were assayed for 5 min at 23 °C. C, a plot of specific activity versus enzyme concentration. D, log reaction velocity versus log enzyme concentration.

Phosphoamino acid analysis yielded only phosphoserine (Fig. 2A), and exhaustive trypsin digestion generated reproducibly two major, 32P-labeled phosphopeptides (Fig. 2B). Because autophosphorylation of CaM kinase-Gr generates apparently two 32P-labeled autoradiographic components, they were analyzed separately by phosphopeptide mapping. Similar "tryptic maps" were generated, indicating that the two 32P-labeled components could not be definitively distinguished from each other by this method (data not shown). Determining the actual number and site(s) of autophosphorylation and their possible bearing on enzyme structure require further investigation.

Regulation of CaM Kinase-Gr Activity by Autophosphorylation—Because CaM kinase-Gr undergoes stoichiometric autophosphorylation, it was of interest to examine the functional sequelae of this reaction. The autophosphorylated enzyme species was produced by incubation with a complete phosphorylating reaction mixture, whereas different "control" incubations were lacking either in ATP or Ca²⁺/calmodulin (Fig. 3A). Significant 32P incorporation into the enzyme required the presence of the full reaction mixture and was not observed in the presence of ATP alone.

Ca²⁺/calmodulin-independent (autonomous) enzyme activity after autophosphorylation was raised to a level which equaled or slightly exceeded that of the Ca²⁺/calmodulin-dependent activity of the unphosphorylated enzyme. The Ca²⁺/calmodulin-dependent component of enzyme activity was also apparently augmented by autophosphorylation. The degree of augmentation was calculated at 3.4-fold when compared with one control (Ca²⁺/calmodulin alone) or 1.4-fold when compared with the other control (ATP alone). The source of this apparent enhancement of enzyme sensitivity to Ca²⁺/calmodulin will be examined under "Discussion." Pretreatment of CaM kinase-Gr with Ca²⁺/calmodulin in the absence of ATP consistently produced persistent elevation of basal activity that amounted to 25% of the autonomous activity of the autophosphorylated enzyme. Thus, a minor component of the autonomous enzyme activity seems to derive from interaction with calmodulin. The formation of a high affinity complex between the protein kinase and calmodulin or a persistent change in enzyme conformation may account for the calmodulin effect. Attempts to dissociate the presumed kinase-bound calmodulin by treatment with EGTA at 4 °C did not alter the result. Pretreatment of CaM kinase-Gr with Ca²⁺/calmodulin alone also resulted in a significant reduction in subsequent enzyme activation by Ca²⁺/calmodulin. This observation remains unexplained although it appears to represent the opposite of the autophosphorylation effect on the Ca²⁺/calmodulin response of the kinase.

The role of actual CaM kinase-Gr autophosphorylation in the ensuring activity changes was corroborated by the fact that neither ADP nor App(NH)p could significantly substitute for ATP (Fig. 3B). Moreover, a positive correlation could be demonstrated between the magnitude of CaM kinase-Gr autophosphorylation and the extent of enzyme activation measured in the presence of EGTA or Ca²⁺/calmodulin (Fig. 4).

Regulation of Enzyme Dependence on ATP and Peptide
Substrate by Autophosphorylation—The effects of CaM kinase-Gr autophosphorylation on the apparent enzyme affinity for ATP and syntide-2 were examined. Figs. 5 and 6 underscore the alterations in the activity of the autophosphorylated enzyme that are brought about by the addition of Ca2+-calmodulin. At a fixed concentration of peptide substrate, the apparent $K_m$ for ATP was 19 mM when the unphosphorylated enzyme was assayed with Ca2+ and calmodulin (not shown). The autonomous activity state displayed an increase in the $K_{\text{app}}$ to 82 mM, whereas stimulation of phosphorylated CaM kinase-Gr by Ca2+-calmodulin resulted in a decrease of the value of $K_{\text{app}}$ to 5.8 mM (Fig. 5). Analogue studies performed by varying syntide-2 concentration at a fixed ATP level disclosed a similar trend in the $K_{\text{app}}$ values. Thus, the $K_{\text{app}}$ of control CaM kinase-Gr was 12 mM with Ca2+-calmodulin (not shown); the autophosphorylated enzyme yielded $K_{\text{app}}$ values of 20 and 2.4 mM in the absence and presence of Ca2+-calmodulin, respectively (Fig. 6).

Dephosphorylation of CaM Kinase-Gr by an Endogenous, Mg2+-dependent Phosphoprotein Phosphatase—Dephosphorylation of autophosphorylated CaM kinase-II represents an important limb of the regulatory cycle of this enzyme. Phosphate incorporated at threonine residues 286/305 of the $\alpha$-subunit (or 287/306 of the $\beta$-subunit) of CaM kinase-II can be rapidly removed by the action of the Mg2+-independent phosphoprotein phosphatases 1 or 2A (23–25), with concomitant abrogation of the effects of autophosphorylation on enzyme activity. In order to determine whether phosphate incorporation into CaM kinase-Gr can be reversed by an endogenous phosphatase, the 32P-labeled enzyme was incubated in the presence of a soluble brain extract. Unlike CaM kinase-II (Fig. 7A), CaM kinase-Gr was resistant to dephosphorylation in the absence of Mg2+ (Fig. 7B); however, addition of 7.5 mM MgCl2 resulted in nearly complete dephosphorylation of the enzyme (Fig. 7C). Further addition of Ca2+ and calmodulin did not enhance the dephosphorylation reaction, and the addition of excess calcineurin itself in the presence of MgCl2 or MnCl2, calmodulin, and DTT did not produce any significant dephosphorylation (data not shown).

We also endeavored to verify the reversal of the functional effects of autophosphorylation by dephosphorylation. This required the partial purification of the phosphoprotein phosphatase and its separation from endogenous Ca2+-calmodulin.
dependent protein kinase activity. We chose the cerebellum as a source of phosphoprotein phosphatase because of its enrichment in CaM kinase-Gr and because of its relatively low levels of CaM kinase-II compared to forebrain. The 250,000 × g supernatant from a cerebellar homogenate was precipitated with 55% ammonium sulfate, followed by gel permeation chromatography on an AcA-34 column and removal of calmodulin-binding proteins by adsorption to a calmodulin-agarose column in the presence of Ca\(^{2+}\) and Mg\(^{2+}\). Mg\(^{2+}\)-dependent phosphatase activity was monitored by its ability to dephosphorylate \(^{32}\)P-labeled CaM kinase-Gr (Fig. 7D). The partially purified phosphoprotein phosphatase was then employed to investigate the effects of dephosphorylation on the regulatory properties of CaM kinase-Gr. Fig. 8 clearly reveals that CaM kinase-Gr dephosphorylation (Fig. 8B) largely reverses the effects of autophosphorylation (Fig. 8, A and B) on the autonomy of the enzyme and on its response to Ca\(^{2+}\)-calmodulin but has a minimal effect on the nonautophosphorylated enzyme. Moreover, the same phosphatase preparation did not release phosphate from \(^{32}\)P-labeled syntide-2 (not shown), implying that the phosphatase does not modulate CaM kinase activity by reversing substrate phosphorylation. Commercially obtained alkaline phosphatase could also dephosphorylate \(^{32}\)P-labeled CaM kinase-Gr but resulted in nearly complete and irreversible enzyme inactivation in a manner that was unrelated to enzyme dephosphorylation (not shown).

**DISCUSSION**

The present observations suggest that, like CaM kinase-II (19, 26), CaM kinase-Gr serves as a neuronal "Ca\(^{2+}\)"-operated switch" that is modulated by autophosphorylation. However, comparison of the autophosphorylation of the two enzymes leads to a more diversified view of the consequences and reversal of the autophosphorylation reaction.

**Catalytic Autonomy—**Brain CaM kinase-II occurs as a soluble ~M, 650,000 oligomer comprised of 10–12 α- and β-subunits and can form larger molecular complexes in the postsynaptic density and the neuronal cytoskeleton (reviewed in Ref. 27). Ca\(^{2+}\)/calmodulin-dependent autophosphorylation on Thr-286 of the α-subunit or Thr-287 of the β-subunit (28–31) generates a Ca\(^{2+}\)/calmodulin-independent (autonomous) activity state that amounts to 20–80% of the activity in the presence of Ca\(^{2+}\) and calmodulin (19, 23–25). The autonomous state of CaM kinase-II is observed at a relatively low stoichiometry of phosphate incorporation such that the phosphorylation of one to three subunits can influence the activity of the other subunits within the same oligomer, presumably through cooperative subunit-subunit interactions (19, 21). In contrast, CaM kinase-Gr appears to occur as a soluble monomer in which the extent of autophosphorylation is correlated with the degree of ensuing autonomy at higher, as well as lower, degrees of autophosphorylation. Moreover, the maxi-

---

**Fig. 4. Dependence of CaM kinase-Gr activation on the extent of autophosphorylation.** 45 µl of CaM kinase-Gr (24 pmol) was incubated in a total volume of 90 µl containing 50 µM ATP, 20 µCi of [γ-\(^{32}\)P]ATP, 10 mM MgCl\(_2\), 5 mM CaCl\(_2\), 600 nM calmodulin, 1 mM DTT, and 20 mM Tris-HCl, pH 7.7. At 5-min intervals, 3- and 10-µl aliquots of this reaction mixture were removed and used for syntide-2 phosphorylation and for measurement of \(^{32}\)P-incorporation into CaM kinase-Gr (inset), respectively. Syntide-2 phosphorylation for 6 min at 23 °C revealed enzyme in the presence of 5 mM EGTA (closed bars) or 5 mM CaCl\(_2\) and 600 nM calmodulin (open bars) as indicated in the legend to Fig. 3A. Syntide-2 phosphorylation under identical conditions was also performed with CaM kinase-Gr that had been preincubated with Ca\(^{2+}\)/calmodulin but not ATP. These control values were subtracted from the activity of the autophosphorylated enzyme in order to arrive at the degree of autophosphorylation-dependent activity shown above.

**Fig. 5. Dependence of autophosphorylated CaM kinase-Gr activity on ATP concentration.** CaM kinase-Gr (6 pmol) was incubated in 60 µl of 20 mM Tris-HCl, pH 7.7, containing 1 mM DTT, 10 mM MgCl\(_2\), and 50 µM ATP with 5 mM CaCl\(_2\) and 600 nM calmodulin. After 20 min at 23 °C, 2-µl aliquot (0.2 pmol of enzyme) were removed, and the activity was measured at 60 µM syntide-2 but at different ATP concentrations with 5 mM CaCl\(_2\), 600 nM calmodulin (open circles) or with 5 mM EGTA (closed circles) for 3 min at 23 °C. Panel B shows a double reciprocal plot of the data. The conditions of syntide-2 phosphorylation had been shown to comply with linear, initial rate requirements (data not shown). Data were compiled from three different experiments.
phorylation-The autonomous phophorylation leads to the autonomous activity state of this kinase and Ca²⁺ signals appear to cooperate in modifying the enzyme activity. The experiment was performed substituting CaM kinase-II for CaM kinase-Gr; ¹⁷P-labeling of CaM kinase-II by autophosphorylation was performed under stringent conditions (19, 24) that allow phosphorylation only of Thr-286/287. ¹⁷P-Labeled CaM kinase (1 pmol) was incubated for 20 min at 23 °C with 10 μl of the soluble brain extract in the presence or absence of 7.5 mM MgCl₂. Panel A shows autophosphorylated CaM kinase-II alone (lane 1), with heat-inactivated brain extract (lane 2), and with live brain extract (lane 3), all without MgCl₂. Panel B depicts the same experiment using CaM kinase-Gr instead of CaM kinase-II. Panel C documents the effect of MgCl₂ on ¹⁷P-labeled CaM kinase-Gr dephosphorylation following incubation with heat-inactivated (lane 1) or live (lane 2) brain extract. Panel D is similar to panel C, except that partially purified phosphoprotein phosphatase was employed as detailed under “Materials and Methods.” The appearance of minor ¹⁷P-labeled polypeptides in panels C and D other than CaM kinase-Gr results from the phosphorylation of extraneous proteins in the phosphatase preparation when MgCl₂ is present. The major ¹⁷P-labeled polypeptides in panel D are identical with those in panels B and C; slight “misalignment” of the polypeptides in panel D compared with those in panels B and C is noticed because the samples were electrophoresed on separate gels.

Modification of the Ca²⁺/Calmodulin Response by Autophosphorylation—The autonomous (Ca²⁺/calmodulin-independent) states of CaM kinase-II (32) and CaM kinase-Gr both display a reduced affinity for peptide substrate. Likewise, autophosphorylated CaM kinase-Gr displays reduced affinity for ATP. In contrast, the autonomous state of CaM kinase-II does not appear to entail changes in the Ca²⁺/calmodulin response (19, 23–25), whereas autophosphorylation of CaM kinase-Gr leads concurrently to enzyme autonomy and to modulation of the Ca²⁺/calmodulin response.

Thus, addition of Ca²⁺/calmodulin to autophosphorylated CaM kinase-Gr results in a dramatic increase in the affinity for both ATP and peptide substrate, revealing that autophosphorylation and Ca²⁺ signals appear to cooperate in modifying CaM kinase-Gr activity. The enhancement of the Ca²⁺/calmodulin response following autophosphorylation, as observed in Fig. 3, may, at least in part, be ascribed to the increase in the apparent affinities of CaM kinase-Gr for ATP and syntide-2, rather than an increase in Vₘₚₙ solely. However, the relationship of the phosphorylation site(s) of CaM kinase-Gr to the enzyme domains that bind ATP, polypeptide substrate, or calmodulin remains to be elucidated.

The differences between CaM kinase-Gr and CaM kinase-II may be intrinsic to the kinase itself, or may, additionally, depend on the nature of the substrate used. A recent report (33) raises the latter possibility by showing that, upon autophosphorylation, CaM kinase-II can behave like CaM kinase-Gr when myelin basic protein, but not myosin light chain, is employed as a substrate. The role of substrate in modifying the consequences of autophosphorylation merits further consideration. Furthermore, we have not determined yet whether the autonomy of CaM kinase-Gr and the enhanced Ca²⁺/calmodulin response result from the autophosphorylation of the same site or different sites.

Phase-2 Autophosphorylation—Removal of Ca²⁺-calmodulin following the initial phase of CaM kinase-II autophosphorylation permits a second phase of Ca²⁺-independent autophosphorylation, which involves Thr-305/Ser-314 of the α-subunit and Thr-306/Ser-315 of the β-subunit (34, 35). The second phase of autophosphorylation is also accompanied by inhibition of calmodulin binding to and activation of the kinase (21, 26, 32) and inhibition of the autonomous activity (21). Recent studies utilizing synthetic regulatory peptides (34) and preferential dephosphorylation (35) indicate that phosphate incorporation at Thr-305/306 is responsible for the changes in calmodulin binding and enzyme activation. Phosphorylation of Ser-314/315 merely produces a modest reduction in enzyme activation by calmodulin. In contrast, CaM kinase-Gr does not contain Thr or Ser residues that are homologous to Thr-305/306 in its calmodulin-binding domain (1) and does not exhibit a clear, Ca²⁺-independent burst of autophosphorylation. Hence, autophosphorylation of the calmodulin-binding domain upon dissociation of the ligand

Fig. 6. Dependence of autophosphorylated CaM kinase-Gr activity on syntide-2 concentration. The experiment was performed exactly as described in the legend to Fig. 5, except that levels of syntide-2 instead of ATP were varied in the final assay, which contained 200 μM ATP.

Fig. 7. Mg²⁺-dependent dephosphorylation of ¹⁷P-labeled CaM kinase-Gr. CaM kinase-Gr was autophosphorylated under the same conditions described in the legend to Fig. 2. A similar experiment was performed substituting CaM kinase-II for CaM kinase-Gr; ¹⁷P-labeling of CaM kinase-II by autophosphorylation was performed under stringent conditions (19, 24) that allow phosphorylation only of Thr-286/287. ¹⁷P-Labeled CaM kinase (1 pmol) was incubated for 20 min at 23 °C with 10 μl of the soluble brain extract in the presence or absence of 7.5 mM MgCl₂. Panel A shows autophosphorylated CaM kinase-II alone (lane 1), with heat-inactivated brain extract (lane 2), and with live brain extract (lane 3), all without MgCl₂. Panel B depicts the same experiment using CaM kinase-Gr instead of CaM kinase-II. Panel C documents the effect of MgCl₂ on ¹⁷P-labeled CaM kinase-Gr dephosphorylation following incubation with heat-inactivated (lane 1) or live (lane 2) brain extract. Panel D is similar to panel C, except that partially purified phosphoprotein phosphatase was employed as detailed under “Materials and Methods.” The appearance of minor ¹⁷P-labeled polypeptides in panels C and D other than CaM kinase-Gr results from the phosphorylation of extraneous proteins in the phosphatase preparation when MgCl₂ is present. The major ¹⁷P-labeled polypeptides in panel D are identical with those in panels B and C; slight “misalignment” of the polypeptides in panel D compared with those in panels B and C is noticed because the samples were electrophoresed on separate gels.
seems to be a characteristic feature of CaM kinase-II but not CaM kinase-Gr.

Enzyme Dephosphorylation—The activities of CaM kinase-II and CaM kinase-Gr appear to be modulated by a phosphorylation-dephosphorylation cycle. Dephosphorylation at threonine residues 286/287 of the α-subunit and 287/306 of the β-subunit of CaM kinase-II can be catalyzed by phosphoprotein phosphatases 1 and 2A (23-25,36). In contrast, dephosphorylation of CaM kinase-Gr. Thus, the differences in the regulatory effects of dephosphorylation, as well as the sites and consequences of the autophosphorylation reactions. The strict Mg2+-dependence of the dephosphorylation reaction of CaM kinase-Gr raises the possibility that phosphoprotein phosphatases 1 and 2A do not act on this enzyme, but that phosphoprotein phosphatase 2C (37) or another Mg2+-dependent enzyme is involved. However, the actual identity of the responsible phosphatase cannot be ascertained merely on the basis of the Mg2+-effect, but requires enzyme purification.

Two Ca2+-operated Molecular Switches—The preceding observations permit the elaboration of an activity-state model for CaM kinase-II and CaM kinase-Gr as a function of the presence of Ca2+-calmodulin and phosphate incorporation (Fig. 9). Such a scheme highlights the regulatory modes of two functionally related, but distinct, neuronal, Ca2+-operated molecular switches. Operational differences between these two Ca2+-sensitive molecular targets depend on several related parameters including 1) the stoichiometry and site(s) of autophosphorylation, 2) the number of stages of autophosphorylation, 3) subunit interactions among protein kinase polypeptides, 4) interaction between the autophosphorylation and Ca2+-calmodulin signals, and 5) the mechanism of dephosphorylation. Determining the site(s) of CaM kinase-Gr phosphorylation, the mechanism by which they modulate enzyme activity, and the relative in situ rates of the phosphorylation-dephosphorylation reactions will allow a better understanding of the regulatory differences and similarities between the two neuronal Ca2+-calmodulin-dependent protein kinases.

REFERENCES
Modulated Neuronal Ca$^{2+}$ Signaling