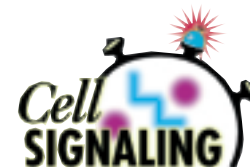


Tools to Study the Activation of CaM KII in Neuronal Functions



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Protein kinases play an important role in cell signaling and in various cellular functions such as neurotransmitter release, long-term potentiation, the mediation of hormonal actions and in regulation of ion channel responses to extracellular signals. The availability of the appropriate tools to study protein kinases in neuronal signaling will certainly hasten the pace of discoveries and understanding of the mechanisms involved in neuronal growth, signaling and neurodegeneration. We have developed two such reagents, the SignaTECT® CaM KII Kinase Assay System and Anti-ACTIVE® CaM KII pAb, Rabbit, (pT²⁸⁶). The SignaTECT® System can be used to detect the kinase activity and the activation of CaM KII with high sensitivity (i.e., nanogram level of enzyme). Anti-ACTIVE® CaM KII pAb, Rabbit, (pT²⁸⁶) can detect the phosphorylation of a threonine residue that is critical to CaM KII regulation as a result of enzyme autophosphorylation under increased calcium signaling. These reagents will allow researchers to study CaM KII signaling in various model systems including neuronal cultures, PC12 cells and brain tissue.

INTRODUCTION

The multifunctional Ca²⁺/calmodulin-dependent protein kinase is present in many tissues but most abundant in brain and has broad substrate specificity, suggesting that it may play a role in many cellular functions (1–3). It is a major neuronal mediator of calcium signaling that integrates multiple related functions, neurotransmitter synthesis and release, modulation of neurotransmitter receptors and ion channels, gene expression, neurite outgrowth and is also required for synaptic plasticity such as long-term potentiation (LTP), a cellular model of learning and memory (4,5).

The enzyme consists of 8–12 subunits in a combination of various isoforms (α , β , γ and δ) ranging in molecular weight from 51kDa (α) to 58–61kDa (β , γ and δ). The α and β isoforms are predominantly and exclusively expressed in the nervous system, whereas the γ and δ isoforms are expressed in all tissues including brain (2,3). Autophosphorylation of the enzyme has a profound effect on its activity. The phosphorylation of T²⁸⁶ in the α subunit (T²⁸⁷ of the β subunit) results in a calcium-independent form (6,7); the generation of this autonomous kinase may underlie some long-term enhancement of transient calcium signals (4,5). In fact, autophosphorylation of T²⁸⁶ converts CaM KII from an enzyme with one of the weakest affinities for calmodulin (CaM) to an enzyme with one of the highest affinities for CaM.

Consistent with its diverse roles, CaM KII exhibits broad distribution within neurons, and thus discrete localization of the enzyme may play an important role in its physiological functions. The α and β isoforms are abundantly expressed in the brain, making up as much as 2% of total protein in certain regions enriched in postsynaptic densities (PSD), cytoskeletal specializations apposed to the postsynaptic membrane of excitatory synapses. It is believed that PSD serve as scaffolds for neurotransmitter receptors, ion channels, and their postsynaptic modulators and effectors and are involved in the regulation of synaptic functions such as

Table 1. Advantages of the SignaTECT® CaM KII Assay System.

1. Quantification of the kinase activity of CaM KII in either pure form or in crude tissue or cellular extracts.
2. Very sensitive, detecting CaM KII enzyme activity with as little as 2.5ng of enzyme per reaction or in a few (5–10) micrograms of protein in crude extracts.
3. Detection can be completed in a very short time (10–15 minutes) after reaction completion.
4. Only the phosphorylated peptide (and not other phosphorylated proteins present in the nuclear extract) will bind to the SAM²® Membrane, thus giving a true estimate of enzymatic activity. In other assays such as P81 phosphocellulose, any positively charged protein(s) that undergoes phosphorylation may bind to the filters, and thus the data obtained will give an overestimate of enzyme activity (12).
5. The substrate in the SignaTECT® CaM KII Kinase Assay System is biotinylated, which does not influence the efficiency of phosphorylation by CaM KII. The P81 method requires that the substrate be positively charged; therefore, basic residues must be added to the peptide substrate. Naturally, the added basic amino acids can greatly alter the specificity of the substrate.
6. The binding capacity of the membrane for the biotinylated peptide substrate is high (a minimum of 2.0nmol/cm²), allowing for the use of high concentrations (up to 1mM) of peptide substrates in the kinase reaction, which in turn maximizes the signal-to-noise ratio and allows kinetic studies to be performed.
7. The binding of biotin to streptavidin is strong and rapid, and once formed, it is unaffected by extremes in pH (2.0–10.0), temperature, organic solvents, ionic and nonionic detergents (SDS, CHAPS, Triton® X-100, Tween® 20 or Tween® 80), and other denaturing agents (5M guanidine HCl and 2M urea) (13). Therefore, the peptide is not likely to be washed off during the washing procedure as is the case when the peptide substrate is bound to filters via weak electrostatic interactions (e.g., P81 filter assay).
8. As a result of the high binding capacity of the disks, optimal kinetic conditions for the enzyme under study can be used, resulting in linear time response and enzyme response curves (i.e., a concentration of peptide substrate that is greater than 10 times the K_m value can be used).

synaptic plasticity, receptor function and structural modification (8,9). The enzyme phosphorylates multiple proteins and enzymes such as neuronal proteins, phospholamban, Ca²⁺-ATPase, tyrosine hydroxylase (1–4), and transcription factors such as cAMP-responsive element binding protein (CREB) (10). Thus, it is of interest to develop an assay system that can selectively detect the activity of individual protein kinases in crude cellular or tissue extract or in column fractions during enzyme purification. The assay should be accurate and simple enough to be performed successfully by a novice as well as an expert in the field. In addition, since autophosphorylation of the enzyme results in its activation, it will be important to develop tools that recognize autophosphory-

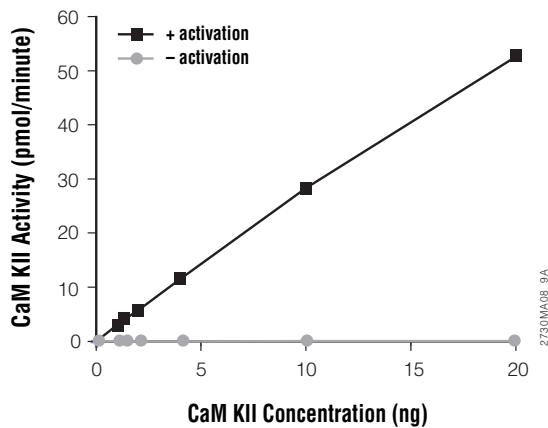


Figure 1. Sensitivity and linearity of CaM KII detection using a commercially available, purified CaM KII enzyme and the SignaTECT® Calcium/Calmodulin-Dependent Protein Kinase (CaM KII) Assay System (Cat.# V8161).

lated forms and be able to quantify the activation of the enzyme under various experimental conditions. Promega has successfully developed two separate tools for the selective determination of CaM KII activity: SignaTECT® CaM KII Kinase Assay System^(a) and Anti-ACTIVE® CaM KII pAb, Rabbit, (pT²⁸⁶), which recognizes only the autophosphorylated form of the enzyme.

SIGNATECT® CAM KII KINASE ASSAY SYSTEM

We have developed a novel assay system that determines the enzyme activity of a specific protein kinase in cellular or tissue extracts (11). The assay takes advantage of the high affinity (10^{-15} M) and selective binding of biotin to avidin and streptavidin. Thus, when a biotinylated derivative of a selective peptide substrate for CaM KII is phosphorylated by the enzyme, the biotinylated phosphopeptide can be separated from free ATP and endogenously phosphorylated proteins using the streptavidin-coated SAM²® Membrane^(a). The excess free [γ -³²P]ATP and endogenous phosphoproteins are readily removed by a simple washing procedure (i.e., 5–7 washes for 1–4 minutes each). The membrane is dried and the ³²P incorporated into the peptide substrate is quantified using a liquid scintillation counter or analyzed by a phosphorimager.

RESULTS AND DISCUSSION

The SignaTECT® CaM KII Assay System described here allows for the determination and quantification of the enzyme activity of CaM KII in a purified enzyme sample as well as in brain tissue and cellular extracts. Since the enzyme is activated by calcium and calmodulin, enzyme activity is measured in the absence and presence of these activators. An increase in the activity of the enzyme of at least 10-fold was observed in the presence of activators when compared with its activity in their absence. In addition, due to the very low nonspecific binding, the background radioactivity incorporated is <0.2% of input radioactivity counts. The combination of higher fold activation by the activators and the extremely low background results in a very high signal-to-noise ratio and an increase in the sensitivity of the assay system.

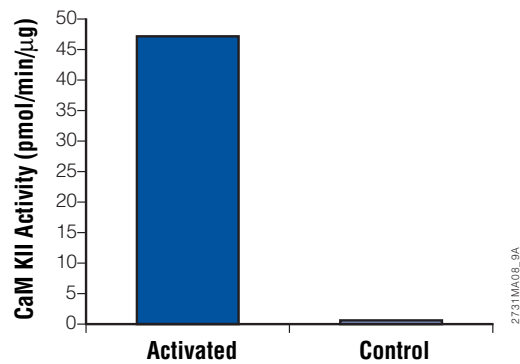


Figure 2. Detection of CaM KII activity using the SAM²® Biotin Capture Membrane (Cat.# V7861, membrane only). Extract (40μg) was isolated from rat brain as described in the SignaTECT® Calcium/Calmodulin-Dependent Protein Kinase (CaM KII) Assay System Technical Bulletin (#TB279), and CaM KII activity was measured using this system.

To establish optimum peptide substrate concentration, enzyme activity was determined at 30°C with a two-minute incubation using various concentrations of peptide substrate. The optimal enzyme activity was obtained at a substrate concentration of 50μM, thus we have chosen this concentration for determining enzyme activity in the current assay format. It is noteworthy that the two-minute reaction time was selected to ensure that the linearity of enzyme activity is maintained. However, if longer incubation time is desired, enzyme dilutions should be made to ensure the linearity of enzyme activity at the selected reaction time is maintained with the enzyme concentration in the reaction. As shown in Figure 1, the activity of the enzyme can be measured with as little as 1ng of purified enzyme, and the activity is linear over one order of magnitude range of enzyme concentration (1–10ng). In addition, enzyme activity can be determined in as little as a few (5–10) micrograms of brain extract protein (Figure 2). Similar results were obtained using PC12 cell extract as the source of CaM KII (results not shown). It is important to note that the enzyme activity in the absence of activators was very low and in fact was close to the background levels obtained in the absence of enzyme. Finally, the activity measured represents a true estimate of enzyme activity, since no phosphorylated products other than the biotinylated peptide substrate are bound to the matrix as is evident by the very low background counts in the absence of the peptide substrate and enzyme.

Table 2. Features of Anti-ACTIVE® CaM KII pAb.

Specific: The antibody preferentially detects the monophosphorylated form of CaM KII, phosphorylated on threonine-286 (pT²⁸⁶) of the α subunit or on threonine-287 (pT²⁸⁶) of the β subunit.

Sensitive: It can detect as little as 5μg of in vitro-phosphorylated brain cytosol CaM KII; it can detect as little as 10ng of purified, autophosphorylated enzyme.

Pure: The antibody is purified by first immunodepleting with the nonphosphorylated peptide followed by positive immunoaffinity selection with the phosphorylated peptide.

Versatile: It can be used in a wide range of applications, such as Western blotting, with common systems of detection, including chemiluminescent and colorimetric immunostaining.

Value: High-titer antibody will generate up to 200ml of blotting solution—sufficient for 20 immunoblots of 10ml each.

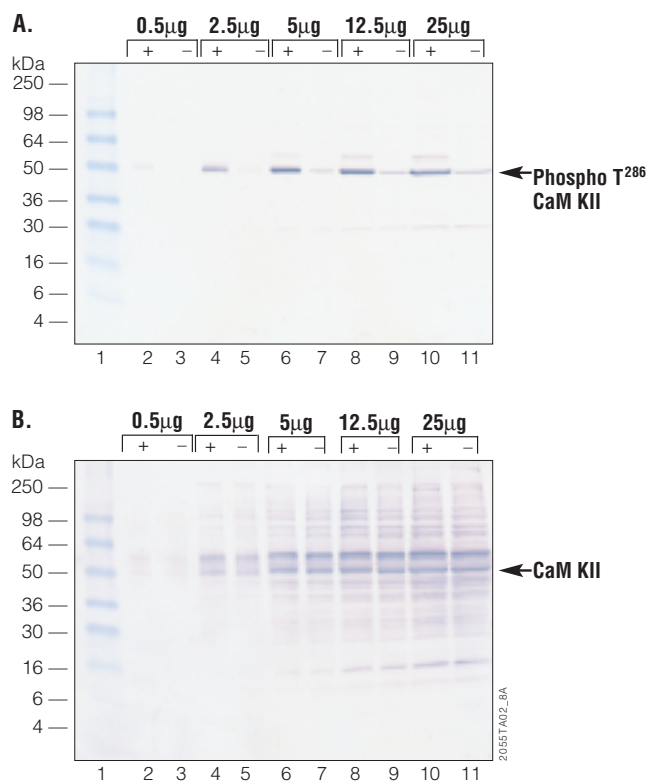


Figure 3. Detection of phospho CaM KII by Anti-ACTIVE® CaM KII pAb (Cat.# V1111) and a pan CaM KII pAb in Western analysis of brain homogenate. Rat brains were collected immediately following euthanasia and frozen in liquid nitrogen. Brains were homogenized, centrifuged at 2,000 x g and the cytosolic fraction collected. A sample of this extract was autophosphorylated in vitro (8), and aliquots of nonphosphorylated and autophosphorylated fractions were analyzed by SDS-PAGE under reducing conditions followed by immunoblotting onto nitrocellulose membrane. **Panels A and B:** lanes 2, 4, 6, 8 and 10 contain autophosphorylated (+) brain extract protein in the amounts shown; lanes 3, 5, 7, 9 and 11 contain nonphosphorylated (-) brain extract protein in the amounts shown. **Panel A:** The presence of the autophosphorylated form was detected using Anti-ACTIVE® CaM KII pAb (1:5,000). **Panel B:** The presence of total CaM KII was detected using a commercially available anti-CaM KII antibody that binds both phosphorylated and nonphosphorylated CaM KII. Detection was performed for both panels using Donkey Anti-Rabbit IgG, AP-conjugated secondary antibody (Cat.# V7971) and Western Blue® Stabilized Substrate for Alkaline Phosphatase (Cat.# S3841).

ANTI-ACTIVE® CaM KII pAb, RABBIT, (pT²⁸⁶)

It is well documented that subcellular localization and compartmentalization of enzymes and their substrates play a significant role in mediating specific cellular responses to external signaling molecules. Consistent with its diverse roles, CaM KII exhibits broad distribution within neurons, and thus discrete localization of the enzyme may play an important role in its physiological functions. It is believed that autophosphorylation of T²⁸⁶ not only converts CaM KII from an enzyme with one of the weakest affinities for calmodulin (CaM) to an enzyme with one of the highest affinities for CaM but also leads to translocation of the enzyme to the PSD fractions (8,9), and upon dephosphorylation, dissociates back to the soluble fraction (14). It is noteworthy that the activation of the enzyme is required for LTP, since the phosphorylation of the AMPA-

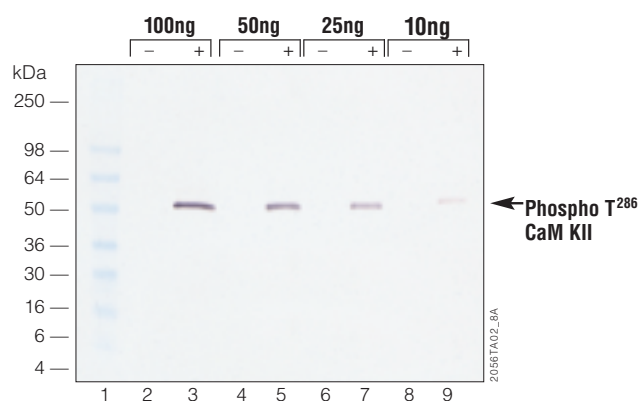


Figure 4. Sensitivity level of the Anti-ACTIVE® CaM KII pAb (Cat.# V1111) in detecting autophosphorylated CaM KII. Decreasing amounts of purified nonphosphorylated (-) CaM KII (lanes 2, 4, 6 and 8) and autophosphorylated (+) CaM KII (lanes 3, 5, 7 and 9) were subjected to SDS-PAGE and Western blot analysis using the Anti-ACTIVE® CaM KII pAb (1:5,000). Detection was performed using Donkey Anti-Rabbit IgG, AP-conjugated secondary antibody (Cat.# V7971) and Western Blue® Stabilized Substrate for Alkaline Phosphatase (Cat.# S3841).

type glutamate receptor, which mediates rapid excitatory synaptic transmission, is correlated with the activation and autophosphorylation of CaM KII (15).

Most recently, it was elegantly demonstrated that a T²⁸⁶→A²⁸⁶ substitution in CaM KII resulted in mice with severe LTP deficiency and impaired learning ability (5). Promega's Anti-ACTIVE® CaM KII pAb, Rabbit, (pT²⁸⁶), preferentially recognizes the phosphorylated form of CaM KII with no detectable recognition of the nonphosphorylated form of the enzyme. Table 2 lists the features of this antibody. In Western blot applications using brain homogenate and the PSD fraction, the antibody recognizes mainly the phosphoform of the enzyme. Figure 3 shows that the antibody can detect as little as 2.5 µg of in vitro-phosphorylated brain cytosol CaM KII. Figure 4 shows that the antibody can detect as little as 10ng of purified, autophosphorylated enzyme.

WESTERN BLOT DETECTION AND IN SITU LOCALIZATION OF AUTOPHOSPHORYLATED CaM KII

The results shown in Figure 3 demonstrate the specificity of Anti-ACTIVE® CaM KII pAb, Rabbit, (pT²⁸⁶) at detecting in vitro-phosphorylated CaM KII in brain homogenate. It is apparent that the signal-to-noise ratio is remarkably high (compare lanes 2, 4, 6 and 8 with lanes 3, 5, 7 and 9 in Figure 3, Panel A). The 52kDa band represents the α subunit of the enzyme whereas the 58kDa band represents the β subunit, albeit at lower intensity, which corresponds well with the distribution of the two isoforms in brain. The blot also indicates that the amount of brain cytosolic protein required for detection is as low as 5 µg. The sensitivity of detection of the autophosphorylated pure enzyme is remarkably high (Figure 4) so that the antibodies can detect as little as 5ng of enzyme (compare the various levels of enzyme tested). The results of in situ localization of the phospho CaM KII using the Anti-ACTIVE® antibodies (Figure 5) show that the antibody binds to PC12 cells and that this binding is inhibited by a phosphorylated CaM KII-derived peptide. A nonphospho CaM KII-derived peptide does not block the binding, suggesting that the antibody is binding to the phosphorylated form of CaM KII in these cells (Figure 5).

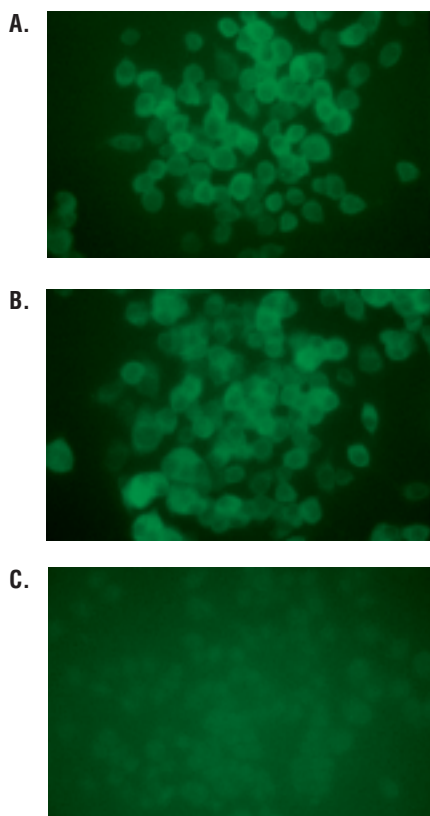


Figure 5. Immunocytochemical detection of autophosphorylated CaM KII in PC12 cells with Anti-ACTIVE® CaM KII pAb (Cat.# V1111). PC12 cells were adhered to slides coated with collagen, fixed in 10% paraformaldehyde for 30 minutes, rinsed in PBS, and permeabilized in methanol for 10 minutes at -20°C . The cells were then blocked in 1% BSA in PBS for 45 minutes, followed by 2% horse serum in PBS for 60 minutes. Cells were incubated overnight at 4°C with Anti-ACTIVE® CaM KII pAb only (**Panel A**), pAb pre-incubated with a nonphosphorylated CaM KII-derived peptide ($1\mu\text{g}/\text{ml}$) (**Panel B**), or pAb pre-incubated with a phosphorylated CaM KII-derived peptide ($1\mu\text{g}/\text{ml}$) (**Panel C**). The Anti-ACTIVE® CaM KII pAb was used at $0.5\mu\text{g}/\text{ml}$ (1:500 dilution) and pre-incubated with peptide for 8 hours at 4°C . After incubation with the primary pAb or pAb/peptide mixture, the cells were rinsed in PBS and incubated with donkey anti-rabbit FITC-conjugated secondary pAb (1:500) for 60 minutes at room temperature. Staining was visualized with a Zeiss® fluorescent microscope after mounting in Vectashield® and DAPI. The results demonstrate that the pre-incubation of the Anti-ACTIVE® CaM KII pAb with phosphorylated CaM KII-derived peptide completely abolished immunostaining (Panel C), but pre-incubation with the nonphosphorylated CaM KII-derived peptide has no effect on immunostaining (compare Panels B and C).

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SAID GOUELI

Ordering Information

Product	Size	Cat.#
SignaTECT® Calcium/Calmodulin-Dependent Protein Kinase (CaM KII) Assay System ^(a)	96 reactions	V8161
Anti-ACTIVE® CaM KII pAb, Rabbit, (pT ²⁸⁶)	40ml	V1111
SAM ² ® Biotin Capture Membrane	96 sample	V2861
Donkey Anti-Rabbit IgG (H+L), AP	60ml	V7971
Western Blue® Stabilized Substrate for Alkaline Phosphatase	100ml	S3841

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^(a)U.S. Pat. No. 6,066,462 has been issued to Promega Corporation for quantitation of protein kinase activity.