

Anti-ACTIVE™ Antibody for Specific Detection of Phosphorylated CaM KII Protein Kinase



By Said A. Goueli, Martha O'Brien and Kevin Hsiao
Promega Corporation

Corresponding author: email to sgoueli@promega.com

The multifunctional calcium/calmodulin-dependent protein kinase, also known as CaM kinase II (CaM KII), is present in high concentration in brain tissue and may play a role in mediating cellular functions. Many long-term potentiation phenomena involved in learning have been attributed to the autophosphorylated form of the enzyme, as it remains active even in the absence of calcium. Promega's Anti-ACTIVE™ CaM KII polyclonal antibody (T²⁸⁶) is specific for the phosphorylated form of the enzyme and can be used in studies requiring highly sensitive detection.

INTRODUCTION

Calcium/calmodulin-dependent protein kinases (CaM kinases) mediate Ca²⁺ signaling in diverse cellular functions, such as glycogen metabolism, muscle contraction, neurotransmitter synthesis and release, translation rate, transcriptional regulation and cell cycle events. The CaM kinase family includes enzymes capable of phosphorylating serine and threonine residues on multiple targets (CaM KI, CaM KII and CaM KIV), as well as kinases dedicated to the phosphorylation of a single substrate (myosin light chain kinase [MLCK], phosphorylase kinase and elongation factor 2 kinase, also called CaM KIII).

CaM KI and CaM KIV are distinguished by their dependence upon phosphorylation by distinct protein kinases (CaM kinase kinases) for maximal activity. The phosphorylation of threonine 177 (T¹⁷⁷) in CaM KI and threonine 196 (T¹⁹⁶) in CaM KIV, which are located in what is called the phosphorylation lip or activation loop, has been shown to be essential for the regulation and activity of these enzymes as well as a number of other protein kinases, including the mitogen-activated protein kinases (MAPKs), cAMP-dependent protein kinase (PKA) and cyclin-dependent protein kinases.

Most of these enzymes consist of a catalytic domain followed by a regulatory domain. The regulatory domain in many cases contains a region called the pseudosubstrate region, which in the resting state (low intracellular calcium concentration) causes the inhibition of the catalytic domain. Inhibition can be alleviated by either binding of a specific regulator, phosphorylation by a distinct protein kinase or both. The pseudosubstrate region mimics a peptide target of the enzyme and thereby blocks the substrate-binding site. Activation occurs when calcium/calmodulin binds to a site that overlaps the autoinhibitory region and strips it away from the active site. CaM KII is a representative of this class of enzymes that are autoinhibited by the regulatory domain and requires autophosphorylation of threonine 286 (T²⁸⁶) to become independent of calcium and calmodulin. When phosphorylated, the enzyme remains active even after the concentration of calcium decreases to basal level.

MULTIFUNCTIONAL Ca²⁺/CALMODULIN-DEPENDENT PROTEIN KINASE (CAM KII)

The multifunctional Ca²⁺/calmodulin-dependent protein kinase CaM KII is present in many tissues, but it is most abundant in brain. It has broad substrate specificity, suggesting that it may play a role in many cellular functions (1,2). CaM KII is a major neuronal mediator of calcium signaling that integrates multiple related functions, including neurotransmitter synthesis and release, modulation of neurotransmitter receptors and ion channels, gene expression and neurite outgrowth. It is also required for synaptic plasticity, such as long-term potentiation (LTP)--a cellular model of learning and memory (1-3).

The enzyme is composed of 8-12 subunits (alpha, beta, gamma and delta) ranging in molecular weight from 52-61kDa. The alpha and beta isoforms are predominantly and exclusively expressed in the nervous system, whereas the gamma and delta isoforms are expressed in all tissues, including brain (4,5). Autophosphorylation of the enzyme has a profound effect on its activity. The phosphorylation of T²⁸⁶ of the alpha subunit or T²⁸⁷ of the beta subunit results in a calcium-independent form (4,5), which may underlie some long-term enhancement of transient calcium signals (1-3). In fact, autophosphorylation of T²⁸⁶ converts CaM KII from an enzyme with one of the weakest affinities for calmodulin to an enzyme with one of the highest affinities for calmodulin.

Consistent with its diverse roles, CaM KII exhibits broad distribution within neurons. The alpha and beta isoforms are abundantly expressed in the brain, with alpha making up as much as 2% of total protein in certain brain regions and enriched in postsynaptic densities (PSD)--cytoskeletal specializations apposed to the postsynaptic membrane of excitatory synapses. PSDs are thought to be scaffolds for neurotransmitter receptors, ion channels and their postsynaptic modulators and effectors, and they are involved in the

regulation of synaptic functions such as synaptic plasticity, receptor function and structural modification. It was recently shown that autophosphorylation of CaM KII leads to translocation of the enzyme to the PSD fractions (6,7), and upon dephosphorylation, it dissociates back to the soluble fraction (8).

It is noteworthy that the activation of the enzyme is required for LTP, since the phosphorylation of the AMPA (alpha-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid)-type glutamate receptor, which mediates rapid excitatory synaptic transmission, is correlated with the activation and autophosphorylation of CaM KII at T²⁸⁶ of the alpha subunit (9). Recently, it was elegantly demonstrated that the absence of T²⁸⁶ in CaM KII resulted in mice with severe LTP deficiency and impaired learning ability (10).

In addition to its prominent role in neuronal functions, CaM KII is believed to be involved in myocardial hypertrophy, including increased cell size, myofilament organization and reexpression of the embryonic gene products involved in calcium transport, such as the ryanodine receptor (11) and the sarcoplasmic reticular Ca²⁺ atrial natriuretic factor (ANF) (12). The enzyme phosphorylates a wide variety of proteins and enzymes, such as neuronal proteins, phospholamban, Ca²⁺-ATPase, tyrosine hydroxylase and transcription factors, such as cAMP-responsive element binding protein (CREB) (13).

WESTERN BLOT DETECTION OF AUTOPHOSPHORYLATED CaM KII

Promega's Anti-ACTIVETM CaM KII pAb (Cat.# V1111) preferentially recognizes the phosphorylated form of CaM KII with no detectable recognition of the nonphosphorylated form of the enzyme, as illustrated in Western blot applications using brain homogenate. The results shown in [Figure 1](#) demonstrate the specificity and sensitivity of the antibody. The 52kDa band detected represents the alpha subunit of the enzyme. Comparison of the nonphosphorylated (-) with the autophosphorylated (+) forms of the purified enzyme shows that there is no signal with 100ng of the nonphosphorylated enzyme, whereas the antibody could detect as little as 10ng of autophosphorylated enzyme.

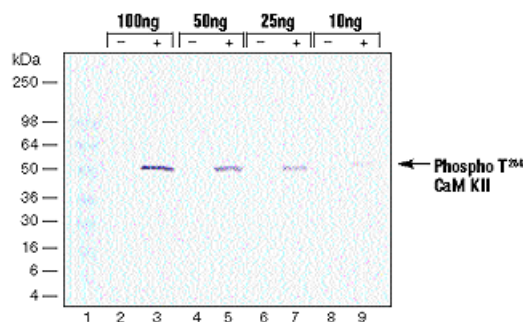


Figure 1. Western blot detection of purified CaM KII by Anti-ACTIVETM CaM KII pAb (pT²⁸⁶). Decreasing amounts of purified nonphosphorylated (-) CaM KII (lanes 2, 4, 6 and 8) and autophosphorylated (+) CaM KII (lanes 3, 5, 7 and 9) were subjected to SDS-PAGE and Western blot analysis using the Anti-ACTIVETM pT²⁸⁶ CaM KII pAb at a dilution of 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). Molecular weight markers are NOVEXTM SeeBlueTM prestained standards (Novel Experimental Technology; lane 1).

[Figure 2](#) shows that the antibody can detect autophosphorylated CaM KII in 2.5µg of brain extract protein and that there is very little nonspecific band staining. At higher concentrations of brain extract, there is indication of the presence of the beta subunit (58kDa).

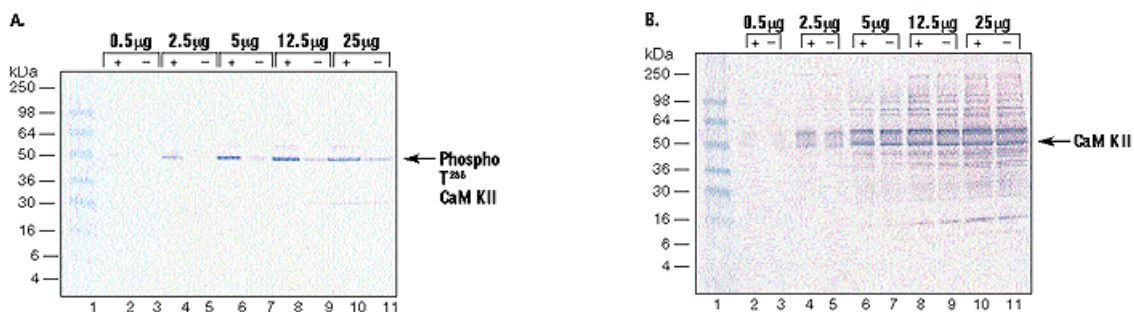


Figure 2. Detection of phophoCaM KII by Anti-ACTIVETM CaM KII pAb (pT²⁸⁶) and a total CaM KII pAb in Western analysis of brain homogenate. Rat brains were collected immediately following euthanasia and frozen in liquid nitrogen. Brains were homogenized, centrifuged at 350 x g and the supernatant collected. A sample of this extract was autophosphorylated *in vitro* (6), and aliquots of nonphosphorylated and autophosphorylated fractions were analyzed by SDS-PAGE under reducing conditions, followed by immunoblotting on 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. Markers are as indicated in [Figure 1](#). **Panel A:** The presence of the autophosphorylated CaM KII was detected using Promega's Anti-ACTIVE™ pT²⁸⁶ CaM KII pAb diluted 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 using Donkey Anti-Rabbit IgG, AP-conjugated secondary antibody (Cat.# V7971) and Western Blue® Stabilized Substrate for Alkaline Phosphatase (Cat.# S3841).

[Figure 3](#) shows that the antibody labels PC12 cells and that the labeling is blocked by preincubation of the antibody with a phosphopeptide corresponding to the T²⁸⁶ region of CaM KII. Preincubation with a nonphosphorylated form of the same peptide does not block binding.

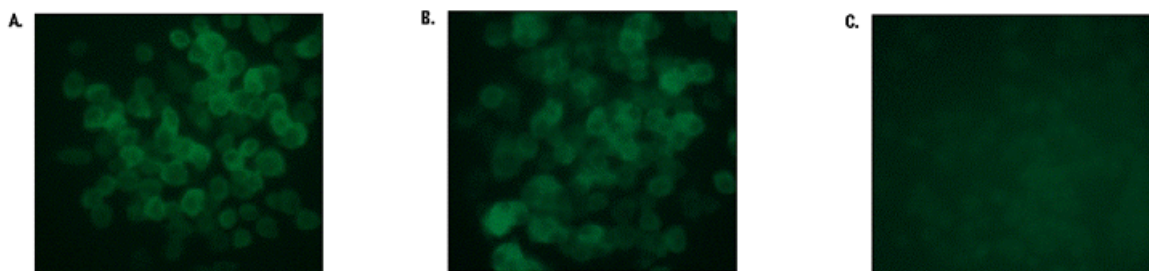


Figure 3. Immunocytochemical detection of autophosphorylated CaM KII in PC12 cells with Anti-ACTIVE™ CaM KII pAb (pT²⁸⁶). PC12 cells were adhered to slides coated with collagen, fixed in 10% paraformaldehyde for 30 minutes, rinsed in PBS, and permeabilized in methanol for 10 minutes at 20°C. The cells 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 antibody alone (**Panel A**), antibody preincubated with a nonphosphorylated CaM KII-derived peptide (1 µg/ml) (**Panel B**) or antibody preincubated with a phosphorylated CaM KII-derived peptide (1 µg/ml) (**Panel C**). The Anti-ACTIVE™ pT²⁸⁶ CaM KII pAb was used at 0.5 µg/ml (1:500 dilution) and preincubated with peptide for 8 hours at 4°C. After incubation with the primary antibody or antibody/peptide mixture, the cells were rinsed in PBS and incubated with donkey anti-rabbit FITC-conjugated secondary antibody (1:500) for 60 minutes at room temperature. Fluorescence was visualized using a fluorescence microscope (Carl Zeiss, Inc.) after treatment with DAPI (4',6-diamidino-2-phenylindole) and mounting in VECTASHIELD® mounting medium (Vector Labs).

REFERENCES

- Schulman, H. and Hanson, P.I. (1993) *Neurochem. Res.* **18**, 65.
- Colbran, R. and Soderling, T.R. (1990) *Curr. Top. Cell. Regul.* **31**, 181.
- Lisman, J. (1994) *Trends Neurosci.* **17**, 406.
- Ikeda, A., Okuno, S. and Fujisawa, H. (1991) *J. Biol. Chem.* **266**, 11582.
- Frangakis, M., Ohmstede, C.A. and Sahyoun, N. (1991) *J. Biol. Chem.* **266**, 11309.
- Yoshimura, Y. and Yamauchi, T. (1997) *J. Biol. Chem.* **272**, 26354.
- Strack, S. *et al.* (1997) *J. Biol. Chem.* **272**, 13467.
- Strack, S. *et al.* (1997) *J. Neurochem.* **68**, 2119.
- Barria, A. *et al.* (1997) *Science* **276**, 2042.
- Giese, K.P. *et al.* (1998) *Science* **279**, 870.
- Witcher, D.R., Striffler, B.A. and Jones, L.R. (1992) *J. Biol. Chem.* **267**, 4963.
- Ramirez, M.T. *et al.* (1997) *J. Biol. Chem.* **272**, 31203.
- Kapiloff, M.S. *et al.* (1991) *Proc. Natl. Acad. Sci. USA* **88**, 3710.

Ordering Information

Product	Size	Cat.#
Anti-ACTIVE™ CaM KII pAb, Rabbit, (pT ²⁸⁶)	40µl	V1111
Donkey Anti-Rabbit IgG (H+L), AP, Anti-ACTIVE™ Qualified	60µl	V7971
Donkey Anti-Rabbit IgG (H+L), HRP, Anti-ACTIVE™ Qualified	60µl	V7951
Western Blue® Stabilized Substrate for Alkaline Phosphatase	100ml	S3841

© 1998 Promega Corporation. All Rights Reserved.

Western Blue is a trademark of Promega Corporation and is registered with the U.S. Patent and Trademark Office. *Anti-ACTIVE* is a trademark of Promega Corporation.

NOVEX and *SeeBlue* are trademarks of Novel Experimental Technology.

VECTASHIELD is a registered trademark of Vector Laboratories.

Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.