

CaMKII T287 and T305 regulate history-dependent increases in α agonist-induced vascular tone

S. Munevar^{a, c}, S. S. Gangopadhyay^{a, d}, C. Gallant^{a, d}, B. Colombo^{a, b},
F. W. Sellke^c, K. G. Morgan^{a, d}

^a Boston Biomedical Research Institute, Watertown, MA, USA

^b Bowdoin College, Brunswick, ME, USA

^c Division of Cardiothoracic Surgery, Harvard Medical School, Beth Israel Deaconess Medical Center, Boston, MA, USA

^d Health Sciences Department, Boston University, Boston, MA, USA

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Abstract

CaMKII is a calcium and calmodulin-activated kinase that has been shown to regulate learning and memory in the brain, and contractility in blood vessels. Following Ca activation, CaMKII autophosphorylates, gaining a calcium-independent autonomous activity that reflects a molecular memory of having previously come into contact with calcium. The present study addresses whether the molecular memory properties of CaMKII are involved in the modulation of sustained vascular tone. We demonstrate a history-dependence of α agonist-induced vascular tone and show that CaMKII activation in vascular cells is also history dependent. Autophosphorylation of Thr287, which is classically associated with autonomous activity, does not persist during tone maintenance after transient increases in intracellular calcium levels. However, we have found that another site, Thr305, known from *in vitro* studies to be inhibitory, is regulated by α agonists in that the inhibitory action is removed, thus leading to a delayed reactivation of CaMKII as measured by Thr287 phosphorylation. By the use of a small molecule CaMKII inhibitor (KN93) as well as a decoy peptide (autoinhibitory peptide; AIP) we show a cause and effect relationship between CaMKII reactivation and sustained vascular tone maintenance. Thus, it appears that a complex interplay between the regulation of Thr305 and Thr287 provides a novel mechanism by which a history-dependence is developed and contributes to a new facet of molecular memory for CaMKII of relevance to vascular tone maintenance.

Keywords: CaMKII • differentiated vascular smooth muscle • contractility • molecular memory • protein kinase • vasospasm

Introduction

Calmodulin-dependent protein kinase II (CaMKII) is a Serine/Threonine kinase expressed as four different

isoforms [1, 2], each encoded by a separate gene. The kinase is a large multimer of 12–14 subunits ranging in molecular mass from 50–65 kD. The α and β isoforms are largely restricted to neural tissues whereas the α and β isoforms are more widely distributed. It is thought that the γ isoform predominates in differentiated smooth muscle and that the δ isoform predominates in cultured smooth muscle

*Correspondence to: K. G. MORGAN,
Department of Health Sciences, Boston University, 635
Commonwealth Ave, Boston MA, 02215, USA.
Tel.: +1 617-353-7464
Fax: +1 617-353-7567
E-mail: kmorgan@BU.edu

cells [3–5]. Recent studies ([6–8]) utilizing both pharmacological and molecular knock down approaches have made it clear that CaMKII is a significant regulator of contractility in differentiated vascular smooth muscle (dVSM).

Binding of Ca/CaM activates the individual CaMKII subunits and also results in inter-subunit activation by an autophosphorylation on T287 (CaMKII γ isoform, T286 in the CaMKII isoform) that causes both an increase in the calmodulin (CaM) binding affinity and the generation of Ca/CaM-independent activity. The ability of CaMKII to continue to have activity in the absence of Ca/CaM or in the presence of low concentrations of Ca/CaM has been referred to as ‘autonomous activity’ or, a ‘*molecular memory*’, of having previously come into contact with Ca/CaM [9] and, for the brain isoforms (α and β), has been linked to processes such as learning, synaptic plasticity and cognitive processes in the brain [10].

In the current study, we tested the hypothesis that the potential molecular memory function of CaMKII contributes to vascular tone maintenance. The results indicate that classical autonomous activity of CaMKII through T287 phosphorylation alone does not contribute to tone maintenance directly, but that a novel type of regulation at the less studied CaMKII inhibitory phosphorylation site, T305, may indirectly regulate vascular tone through inhibitory effects on CaMKII activation.

Materials and methods

Tissue preparation and contraction measurements

All procedures were approved by the Boston Biomedical Research Institute and Boston University Institutional Animal Care and Use Committees. As previously described [8], the aorta was excised from euthanized ferrets and immersed in oxygenated (95% O₂ to 5% CO₂) physiological saline (PSS) consisting of 120 mmol/l NaCl, 5.9 mmol/l KCl, 1.2 mmol/l NaH₂PO₄, 25 mmol/l NaHCO₃, 11.5 mmol/l dextrose, 1 mmol/l CaCl₂ and 1.4 mmol/l MgCl₂ (pH = 7.4). The aorta was carefully dissected to remove all connective tissue and the endothelium. The aorta was cut into circular rings (~3 mm wide), which were then attached to a force transducer and stretched to ~1.3 times their resting length to approximate the optimal length for force production (a resting tension of ~2.5 g). The tissue was allowed to equilibrate at 37°C for 1 hr in oxygenated PSS and then were

stimulated for up to 10 min with 51 mmol/l KCl for purposes of normalization. At the end of the experiment the tissues were quick-frozen by immersion into a dry ice-acetone slurry containing 10% trichloroacetic acid (TCA) and 10 mM dithiothreitol (DTT).

Peptide loading

An inhibitory CaMKII decoy peptide (H₂N-FITC- β A-GYGRKKRRQRRR-KKKLRRQEAFDAL-COOH) was synthesized by the Boston Biomedical Research Institute peptide synthesis core using sequence from the autoinhibitory domain of CaMKII (KKKLRRQEAFDAL). An N-terminal FITC tag was added to monitor loading efficiency and a TAT sequence (GYGRKKRRQRRR) was added to facilitate loading. This peptide has been previously characterized [11]. A control peptide (FITC- β A—GYGRKKRRQRRR-EAK-FRLKRAQLKD) was synthesized by randomizing the peptide sequence. Tissues were loaded with the peptides by incubation for three hours at 50 μ mol/l (diluted in PSS), with one solution change midway. Uniform cellular loading was confirmed by confocal microscopy of the FITC signal.

Immunoblots

Quick-frozen tissue samples were homogenized and analysed *via* western blot as previously described [8]. Briefly, the tissue samples were acetone washed and homogenized in a buffer containing 20 mmol/l 3-(N-morpholino) propane sulfonic acid (MOPS), 4% SDS, 10% glycerol, 10 mmol/l DTT, 20 mmol/l (β -glycerophosphate, 5.5 μ mol/l leupeptin, 5.5 μ mol/l pepstatin, 20 kallikrein inhibiting units (KIU) aprotinin, 2 mmol/l Na₃VO₄, 1 mmol/l NaF, 100 μ mol/l ZnCl₂, 20 μ mol/l 4-(2-Aminoethyl) benzenesulfonylfluoride hydrochloride (AEBSF) and 5 mmol/l ethylene glycol tetraacetic acid (EGTA). The samples were protein matched using a modified Lowry assay (DC Protein Assay Kit, BioRad) and then boiled in Laemmli sample buffer. The samples were separated using SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). Protein matching between samples was confirmed by staining the membrane with naphthol blue black and measuring the densitometry of the actin bands. Immunoblotting was performed using enhanced chemiluminescence (Pierce) or the odyssey infrared imaging system (LI-COR Biosciences, Lincoln, Nebraska).

Materials and antibodies

The phosphoT286 (1:500, Millipore, Charlottesville, VA), phosphoT305 (1:10,000; PhosphoSolutions, Aurora, CO)

-specific antibodies were used as primary antibodies. Secondary antibodies conjugated with HRP or infrared dyes (1:1000) were purchased from Molecular Probes (Eugene, Oregon). KN93 and KN92 were purchased from Calbiochem, San Diego, CA.

Statistical analysis

Data were analysed and expressed as means (S.E. unless it is specified otherwise). Data were compared by ANOVA where multiple comparisons were made and an unpaired, two-tailed t-test for force data where only two values were compared.

Results

CaMKII T287 phosphorylation levels are time dependent during α agonist- induced tonic contractions

Ferret aorta tissues display a sustained tonic increase in contractile force in response to α adrenergic agents, such as phenylephrine (PE) (Fig. 1A). In contrast, we have previously published that, for the same cell type, $[Ca^{2+}]_i$ levels peak by 30 sec. after the addition of a maximally effective concentration of PE and then to fall to near baseline levels by 30 min. in the continued presence of PE [12]. We tested the hypothesis that CaMKII activation, as measured by autophosphorylation at T287, persists after $[Ca^{2+}]_i$ falls to near baseline levels by virtue of the generation of autonomous activity characteristic of CaMKII *in vitro*. Thus, tissues were exposed to 10^{-5} M PE for increasing times from 0 to 60 min., quick frozen and probed in immunoblots for phospho-T287 levels.

We have previously published that this tissue expresses 6 different γ CaMKII variants [5]. As expected, staining whole cell homogenates quick frozen after contractile stimulation with an anti-phospho-T287 antibody gave multiple bands (Fig. 1B). We were able to resolve three groups of bands on a consistent basis with both KCl PSS and PE stimulation, groups at approximately 60kD, 55kD and 51kD, probably representing variants G1&G2, B & J and C1 & C2 variants, respectively, based on gels with purified recombinant proteins [5]. The top band was difficult to quantitate because of interference from the pronounced 55kD band, but the quantitative time course

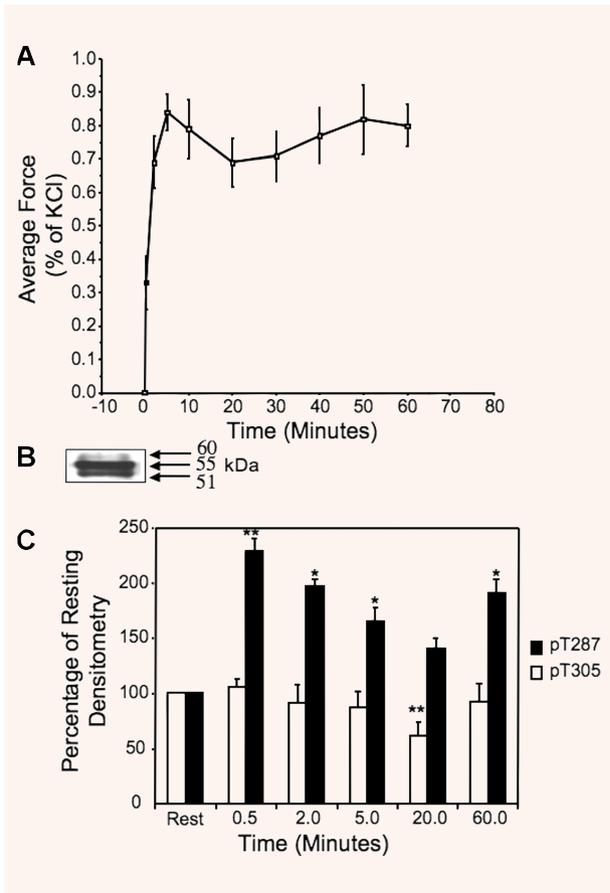


Fig. 1 Time course of contractile response and CaMKII phosphorylation after addition of phenylephrine (10^{-5} M) to aortic rings. **(A)** Isometric force, normalized as a percentage of the force response to a 51 mM KCl PSS challenge performed at the beginning of each experiment $n = 5$ separate animals. **(B)** Typical immunostain of aortic homogenate for T287 phosphorylation illustrating the three groups of bands described in text as paralleling known CaMKII γ variants. The sample was quick frozen after 10 min. exposure to 51mM KCl PSS. **(C)** Time course of phosphorylation of CaMKII γ , 55kD band, at the T287 site (dark bars) and at the T305 site (clear bars). *, $P < 0.05$ versus rest samples; **, $P < 0.01$ versus rest samples, (data analysed before normalization to resting). $n = 5-12$ separate animals.

for the 51 kD and 55 kD bands were indistinguishable in response to PE and only data for the 55kD band are shown here.

The average data for the time course of T287 phosphorylation of the 55kD band are shown in Fig. 1C, dark bars. Phosphorylation reaches a peak at 30 sec.,

then falls to a minimum by 20 min., mimicking transient increases in $[Ca^{2+}]_i$ levels in this cell type [12]. Unexpected, however, was a subsequent increase in T287 phosphorylation at 60 min. (Fig. 1C).

CaMKII T305 is regulated in dVSM in response to α agonists

CaMKII T305 phosphorylation is reported, from *in vitro* studies, to be inhibitory with respect to catalytic activity of CaMKII since it blocks the binding of Ca/CaM [2]. *In vitro* phosphorylation at T305 has been described as increasing only when Ca is removed from solutions after CaMKII is initially activated and T287 has undergone autophosphorylation in the presence of Ca/CaM [13–16]. Whether this site is phosphorylated *in vivo* in dVSMCs has not been described. We monitored T305 phosphorylation by immunoblot of aortic samples quick frozen during activation with 10^{-5} M PE. From 0.5 to 20 min., a time when $[Ca^{2+}]_i$ [12] and T287 phosphorylation (Fig. 1C) decrease, T305 also decreases but in this case the levels fall below baseline, indicating that this site is phosphorylated under resting conditions (Fig. 1C). This decrease removes an inhibitory influence from the kinase and may explain the subsequent increase in autophosphorylation of T287.

α agonist-induced contractility is inhibited by KN93

The question arises as to whether the increased activation of CaMKII, as monitored by the increased T287 phosphorylation and decreased T305 phosphorylation contributes to the simultaneous sustained increase in contractility. Thus, the effect of a small molecule inhibitor of CaMKII, KN93, on tissues was compared to sham treated tissues or tissues treated with an inactive congener of KN93, KN92. As is shown in Figure 2, KN93 causes a significant decrease in T287 (Fig. 2A) as well as T305 (Fig. 2B) phosphorylation and steady-state contractile force (Fig. 2C) at the 60 min. time-point, compared to the PE treated tissues. In contrast, the KN92 treated tissues were not different from the PE treated tissues in any of these parameters (Fig. 2). No effect on contractility was seen at 20 min. (data not shown).

CaMKII T287 phosphorylation is history dependent

We have made the observation that the amplitude of the force response to a 20 min. exposure to 10^{-5} M PE is history dependent. When given as a single 20 min. challenge, the contraction to a maximally effective (10^{-5} M) concentration of PE averages about 65% of the response to steady state challenges to KCl PSS performed at the beginning of the experiment. However, if the same 20 min. exposure to 10^{-5} M PE occurs as part of a concentration-response protocol, force increases to about 96% of the response to the KCl PSS challenge (Fig. 3C).

The increase in contractility could be due to additivity with other mechanisms, such as myosin light chain kinase activation or caldesmon phosphorylation which might occur at lower concentrations during the concentration-response protocol. However, when CaMKII T287 phosphorylation was measured in tissues quick frozen during the same protocol, T287 phosphorylation levels were found to be significantly increased by the concentration response protocol even though both samples were frozen after 20 min. of exposure to 10^{-5} M PE (Fig. 3A). T305 phosphorylation levels were also measured in this protocol (Fig. 3B). In this case no significant difference was detectable between the single exposure and the concentration response protocol for this phosphorylation site, suggesting a difference in the kinetics of phosphorylation/de-phosphorylation T287 *versus* T305.

Time and history-dependent CaMKII activation contributes to increased contractility in dVSM

To determine if the history-dependent increases in CaMKII T287 phosphorylation and contractile force are related in a cause-and-effect manner, we determined the effect of the small molecule CaMKII inhibitor, KN93 in the concentration-response protocol. As is shown in Fig. 4A, KN93, but not the inactive congener KN92, significantly decreased both T287 phosphorylation (left panel) and contractile force (right panel) relative to the vehicle-treated control.

Because of the possibility of non-specific actions of KN93, we further used a second approach to inhibit CaMKII. We synthesized a previously

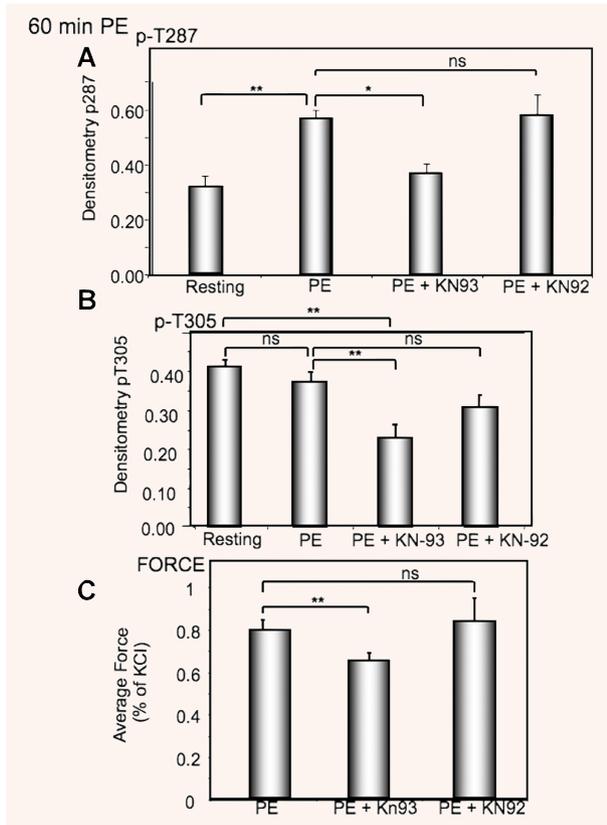


Fig. 2 CaMKII inhibitor, KN93, inhibits CaMKII phosphorylation and tonic force in response to PE. **(A)** Effect of KN93 versus an inactive congener, KN92 on PE(10^{-5} M)-induced increases in T287 phosphorylation at the late, 60 min. time-point. **(B)** Effect of KN93 versus that of an inactive congener, KN92 on PE-induced changes in T305 phosphorylation. **(C)** Effect of KN93 versus that of an inactive congener, KN92 on PE-induced increases in contractile force at the late, 60min time point. In all cases, $n = 6-10$ separate animals. *, $P < 0.05$; ** $P < 0.01$ compared to rest; NS, not significantly different.

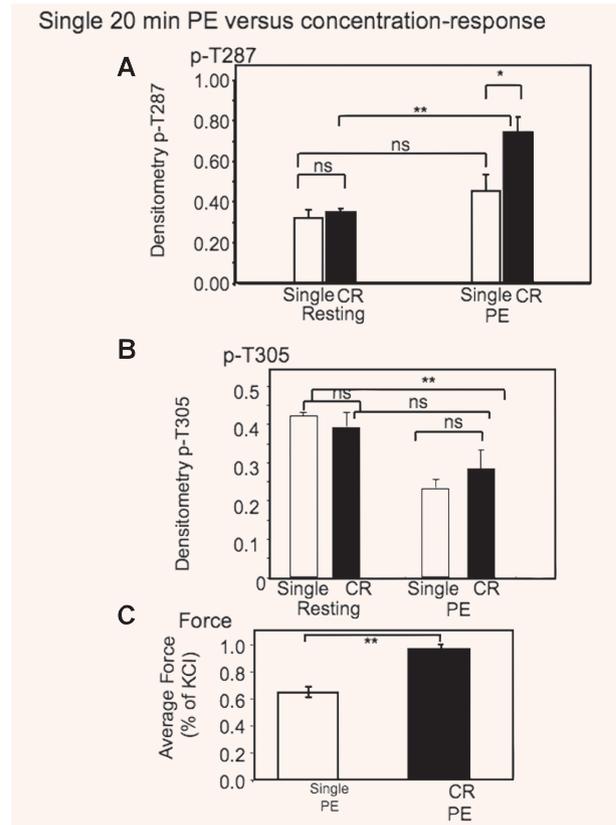


Fig. 3 History dependence of steady state phosphorylation and force responses to a maximally effective concentration of PE. **(A)** Comparison of T287 phosphorylation levels after 20 min. exposure to 10^{-5} M PE either as a single challenge or after exposure to increasing concentrations of PE (CR) from 10^{-8} to 10^{-5} M. **(B)** Comparison of T305 phosphorylation after 20 min exposure to 10^{-5} M PE either as a single challenge or after exposure to increasing concentrations of PE (CR) from 10^{-8} to 10^{-5} M. **(C)** Comparison of contractile force levels after a 20 min. exposure to PE either as a single challenge or after exposure to increasing concentrations of PE (CR) from 10^{-8} to 10^{-5} M. *, $P < 0.05$; **, $P < 0.01$. NS, not significantly different. In all cases $n = 5-21$ animals.

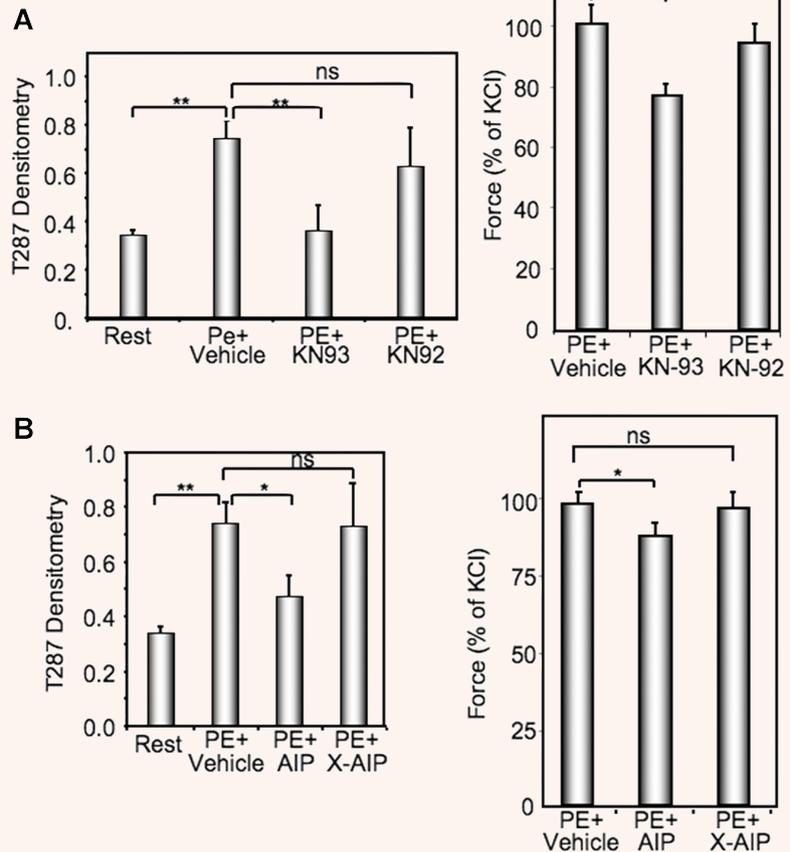
described [17] decoy peptide containing the autoinhibitory sequence from the CaMKII regulatory domain, commonly called AIP. As can be seen in Fig. 4B, this peptide similarly inhibited both PE-induced increases in CaMKII T287 phosphorylation (left panel) and contractile force (right panel), further supporting a cause-and-effect relationship between the history-dependent increases in CaMKII activity and contractility.

Discussion

The present study is the first to report that T305 phosphorylation of CaMKII γ is regulated in dVSM. This site has been studied almost exclusively in purified proteins *in vitro* where it has been shown that T305 is phosphorylated only after: (1) T286 (CaMKII α site corresponding to T287 in CaMKII γ) phosphorylation renders the kinase autonomously active and then (2)

Fig. 4 History dependent increases in CaMKII phosphorylation and force are inhibited by two different CaMKII inhibitors. **(A)** Comparison of the effect of KN93 versus the inactive congener KN92 on T287 CaMKII phosphorylation (left panel) or on contractile force (right panel) in resting aortic tissues or those exposed to a concentration response protocol ending with 20 min in 10^{-5} M PE. **(B)**, Comparison of the effect of AIP peptide versus a scrambled version of AIP (X-AIP) on T287 CaMKII phosphorylation (left panel) or on contractile force (right panel) in resting aortic tissues or those exposed to a concentration response protocol ending with 20 min. in 10^{-5} M PE. *, $P < 0.05$; ** $P < 0.01$; NS, not significantly different. $n = 3-10$.

Concentration-response protocol



the removal of Ca/CaM with EGTA, since bound Ca/CaM protects the phosphorylation site [2]. Very recently T305 phosphorylation of CaMKII α has been shown to be at high levels in postsynaptic densities fractionated from brain slices [18]. Also, in drosophila, a protein, dCASK or Camguk, has been identified and shown to stimulate CaMKII to phosphorylate T306 (drosophila α CaMKII site corresponding to T305 in mammals) under conditions of low-synapse activity and, presumably low $[Ca^{2+}]_i$ [19]. Since T305 blocks Ca/CaM binding it will also block activation of the kinase and consequent further T287 phosphorylation. It is said to decrease the 'gain' of the system in the synapse. We found that T305 levels are significantly elevated in unstimulated, 'resting' tissues. This is expected from the low $[Ca^{2+}]_i$ levels in unstimulated tissues and may produce a decreased 'gain' with respect to contractility in un-stimulated tissues.

The fact that the resting levels of T305 phosphorylation are significantly higher than those at the 20 min. time point after PE addition suggests that some factor is promoting T305 phosphorylation in the absence of stimulation and also in the absence of detectable T287 phosphorylation and activation of CaMKII activity. Similarly, from theory, based on *in vitro* work with purified proteins, the falling $[Ca^{2+}]_i$ level after the initial $[Ca^{2+}]_i$ transient would be expected to promote increasing T305 levels but this is not seen. It is not known if there is a mammalian equivalent of Camguk, but our data suggest that a similar regulatory protein or a kinase other than CaMKII may cause the observed but unexpected pattern of regulation of inhibitory T305 in dVSM.

In parallel, there is also a significant decrease in T287 phosphorylation. Presumably, the occurrence of the autonomous, Ca-independent CaMKII catalytic

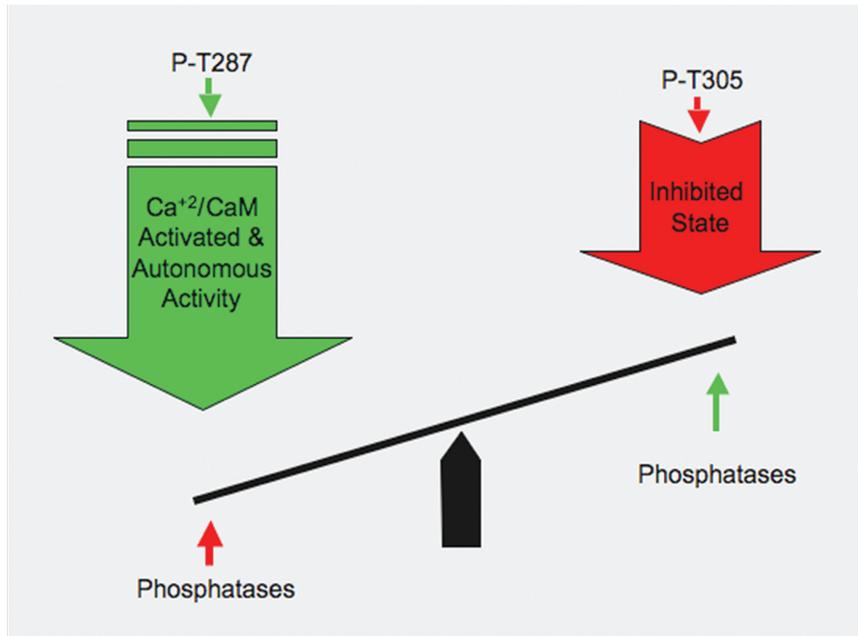


Fig. 5 Model of complex interactions between activating phosphorylation at T287, inhibitory phosphorylation at T305 and dephosphorylation by phosphatases leading to a net level of CaMKII phosphorylation.

activity is prevented *in vivo* because of the presence of phosphatases in the cell that dephosphorylate T287 and terminate catalytic activity. The action of these phosphatases presumably also explains the fall in T305 autophosphorylation levels at this time. This would remove an inhibitory influence on the kinase by un-blocking the Ca/CaM binding site. It is of interest that apparently this site is blocked in the resting state, keeping the kinase in the off state. During the late time-points of the PE response at a time when $[Ca^{2+}]_i$ is at near basal levels, the change in the ratio of the T287 to T305 phosphorylation could tend to activate CaMKII again and perhaps explain the subsequent increase seen at late time points in T287 phosphorylation.

We had expected to see evidence for autonomous CaMKII γ activity in dVSM after Ca transients, but we did not. After an agonist-induced $[Ca^{2+}]_i$ transient, our results demonstrate that T287 is phosphorylated but that as $[Ca^{2+}]_i$ falls, T287 phosphorylation also falls. Presumably this is because cellular phosphatases dephosphorylate T287 before autonomous activity can occur. Rokolya and Singer [6] have demonstrated the presence of autonomous CaMKII activity in homogenates of carotid artery frozen under conditions of high $[Ca^{2+}]_i$, and subsequently treated with EGTA to remove Ca during the assay. However, this assay is performed with the use of

phosphatase inhibitors, consistent with the concept that different results are obtained *in vivo* because of intracellular phosphatase activity.

Thus, a classic example of autonomous activity of CaMKII was not observed; however, *in vivo* there seems to be a delicate balance between activating *versus* inhibiting factors (Fig. 5) that allow the unexpected late increase in T287 phosphorylation and, presumably, CaMKII activity. The decrease in the phosphorylation level of the inhibitory T305 site seen in the presence of PE, which must involve a phosphatase as well, appears to allow the subsequent increase in T287 phosphorylation and CaMKII activity at the 60 min. time-point, and could also be considered a reflection of the 'memory' properties of CaMKII. It is of note that if PE is removed at the 60 min. time point the muscle does relax. Thus, the cells require the presence of PE to 'constantly remind' CaMKII to stay active. This presumably reflects the requirement for PE-triggered signalling pathways that regulate the kinase/phosphatase balance. This late surge in T287 activity is relevant functionally as well since, as we have shown here, it contributes to tone maintenance in the presence of an α agonist. This potential memory property may play a significant role in sustaining vascular tone and could potentially represent a novel mechanism by which vasospasm is maintained.

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