SUPPLEMENTAL MATERIAL

Phosphoregulation of cardiac inotropy via myosin binding protein-C during increased pacing frequency or β1-adrenergic stimulation

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Supplemental Methods

Simultaneous Force and Intracellular Calcium Measurement on Intact Papillary Muscle

We chose to perform simultaneous force and intracellular calcium concentration $[Ca^{2+}]_{in}$ measurements on intact papillary muscle to better elucidate the effects of cardiac myosin binding protein-C phosphorylation regulation of cross-bridge cycling on contractile function. The intact papillary muscle provides advantage over the previous skinned myocardium experiments by: (1) intact cardiac myocytes with working kinases, phosphatases, and organelles, (2) intact 3-dimensional myofilament lattice, and (3) triggered calcium release and re-uptake by endogenous calcium handling proteins to reflect *in vivo* environment of a beating heart. We expect the force/calcium relationships within the intact papillary muscle to be different from the skinned myocardium experiments due to both changing $[Ca^{2+}]_{in}$ and intact multi-cellular structure. Particularly, the measuring force as response to increasing and decreasing transients $[Ca^{2+}]_{in}$ will be able to detect differences caused by faster cross-cycling kinetics that cannot be detected by steady state measurements of skinned myocardium experiments. Bulk of the protocol was developed by a previous study[1].

The force measurement equipment and fluorescence optics were purchased from Scientific Instruments (Heidelberg, Germany). We used our own implementation of a data acquisition suite (National Instruments A/D and Labview™) and software to acquire and analyze the data. Papillary muscles were dissected free at 4°C in Krebs Henseleit (KH) solution with 30 mmol/L of 2,3-butadione monoxime (BDM), taking care that surgical instruments did not touch surface of papillary muscle and avoiding over-stretch. Damaging the papillary muscle during dissection causes low twitch force and negative force/frequency response. The papillary muscle was attached to the force transducer and motor by the chordae tendenae at its tip and a large mass of surrounding ventricular tissue around the base. KH solution (in mmol/L: NaCl 119, Glucose 11, KCl 4.6, $CaCl_2$ 1.8, $MgCl_2$ 1.2, $NaHCO_3$ 25, and K_2HPO_4 1.2) was continuously bubbled with 95% O_2 and 5% CO_2 gas mixture for 30 minutes to reach a stable pH of 7.4. KH solution is continuously bubbled throughout the experiment. The KH buffer is pumped through the superfusion chamber at sufficient rate to support strongest possible contraction at 3 Hz stimulation. This corresponds to > 4ml/minute in the current system.

Maximum twitch force was achieved by carefully stretching the relaxed muscle to the point beyond which a further increase in length as reflected in resting tension failed to elicit a further increase in twitch force. All measurements were obtained at a length corresponding to maximum twitch force. Stimulation voltage and pulse duration were adjusted to ensure that stimulus conditions did not limit peak force; standard conditions consisted of 7 volts with a 5 millisecond pulse duration. A Tektronix oscilloscope with Wavestar™ computer interface continuously measured, logged, and displayed trends in twitch force and resting tension to guide adjustments. Pacing at 0.5 Hz for at least 90 minutes was required for extracted papillary muscle to become stable. Papillary muscles with stable diastolic force baselines were used for experimental measurements.

FURA-2AM loading buffer consisted of KH with the additions of FURA-2AM 10 μmol/L, cremophor 5 g/L (facilitates FURA-2AM diffusion into cells), and N,N,N',N'-Tetrakis(2-pyridylmethyl) ethylenediamine (TPEN) 4.3 mg/L (chelates manganese to avoid manganese quenching of Fura2 fluorescence[2]). The KH to dimethyl sulfoxide (DMSO) volume ratio was 399. A KH to DMSO volume ratio < 200 will cause significant cellular damage. Loading FURA-2AM for 2 hours at 0.5 Hz pacing yielded a consistent fluorescence signal between twitches and allowed muscle to achieve stable twitch forces. De-esterification duration after FURA-2AM loading was 15 minutes at 1 Hz pacing. Fura2 fluorescence was captured from the entire papillary muscle. The $[Ca^{2+}]_{in}$ was estimated by[3]:

[Equation DS-1]

$$[Ca^{2+}] = \left(\frac{R - R_{\min}}{R_{\max} - R} \right) \cdot K_{apparent}; \text{where}, R = \frac{F_{Ca}}{F_{free}}$$

Calcium-bound Fura2 fluoresces with 340 nm excitation (F_{Ca}). Calcium-free Fura2 fluoresces with 380 nm excitation (F_{free}). R is calculated by F_{Ca}/F_{free}. R_{min} is measured when Fura2 is completely free of calcium. R_{max} is measured when Fura2 is saturated with calcium. $K_{apparent}$ is the apparent calcium association constant of Fura2 for the entire system. System calibrations for all configurations provided the corresponding R_{min}, R_{max}, and $K_{apparent}$ for each experiment. We used estimated $[Ca^{2+}]$ instead of R because estimating $[Ca^{2+}]$ by equation DS-1 adjusts for changes in R due to normal equipment maintenance such as replacing the mercury light bulb (Different mercury light bulbs will give different intensities at 340 nm and 380 nm to vary Fura2 fluorescence on the same $[Ca^{2+}]$ resulting in different R values).

System calibration uses no calcium with 10 mmol/L of ethylene glycol tetra acetic acid (EGTA) for Rmin, 10 mmol/L of $CaCl_2$ for Rmax, and a range of $[Ca^{2+}]$ standards with magnesium that were purchased from Molecular Probes for $K_{apparent}$. Auto-fluorescence of papillary muscles were measured before Fura-2 loading and subsequently subtracted from recorded data before calculating R.

The pacing protocol consisted of (1) increasing the pacing frequency from 1 to 2 and then to 3 Hz every 30 seconds to verify the integrity of muscle (intact muscle will show a positive force frequency response; this occurred > 90% of time), (2) returning the pacing frequency to 1 Hz for 5 minutes to allow the papillary muscle to re-equilibrate, (3) increasing pacing frequency from 1 to 2 and then to 3 Hz every 2 minutes, (4) changing the solution to KH with 1 μmol/L dobutamine with continuation of 3 Hz pacing for 5 minutes, (5) recording at a minimum of 20 contractions at the end of each pacing frequency and condition period. A wash period of 10 minutes with normal KH returned the twitch force to pre dobutamine treatment levels.

Room temperature was chosen because FURA-2AM exits the cells too quickly at 37°C. We have noticed that at room temperature of 20-21°C, the myocytes do not respond appropriately to pacing frequency above ~ 3.5 Hz without pre-treatment with a β-adrenergic agonist. Thus a maximum pacing frequency of 3 Hz was chosen. Our previous study have shown that increasing pacing frequency from 1 Hz to 3 Hz at room temperature causes similar positive force/frequency effects as increasing pacing frequency from 1 Hz to 7 Hz at 37°C.

Multiple techniques were used to ensure good signal quality. These involve optimizing the initial fluorescence to current transduction signal to noise ratio (SNR), taking advantage of the hardware, and signal averaging.

Optimization of Initial Fluorescence to Current Transduction SNR

The photomultiplier tube (PMT) converts incident fluorescence to electronically measurable current (i.e., light to current transduction). At a constant supply voltage, the PMT performs this signal transduction at a stable photon to electron gain. The amplitude of Fura2 fluorescence from the entire muscle is more than sufficient enough to be directly converted to a corresponding current by the PMT. Thus, the system operates the PMT in some literature noted as analog mode. One can estimate photomultiplier tube SNR by:

[Equation DS-2][4]

$$SNR = \frac{I_K}{(2eB \cdot (d/(d-1)) \cdot (I_K + 2I_d))^{(1/2)}}$$

I_K : cathode current (A), function of incident light power and anode radiant sensitivity
e: electron charge (C)
B: bandwidth (Hz)
d: secondary emission ratio, ~ 6, so (d/(d-1) ~ 1.2
I_d: dark current, noise independent of light source (i.e. noise produced by the equipment in total darkness), increases with supply voltage

One can increase the photon to electron gain (e.g. radiant sensitivity) by increasing the supply voltage. This allows one to detect lower intensity fluorescence signal. However, increasing the supply voltage will increase the dark current noise resulting shot noise as given by the denominator of the above equation. Thus, the supply voltage to the PMT is optimized for each experiment. The optimization is done by first recording the auto-fluorescence before Fura2 loading at range of 600-800V. This region provides good linearity between supply voltage and photon-to-electron gain. After loading, fluorescence was measured again at 600-800V. Papillary muscle contraction induced fluorescence change is than analyzed to find the optimum supply voltage. Once found, the supply voltage is set to this optimum value before start of the actual experiment. The corresponding auto-fluorescence is subtracted from the collected experimental data before further processing. This method eliminated the dark current "shot" noise associated with high PMT gain as well as producing the best possible signal to noise ratio on the raw electrical output from the optical system.

Taking Advantage of Hardware

Unlike an inverted microscope, the entire objective to PMT suite is completely enclosed in one assembly to minimize path length. This significantly reduced the long path length and multiple intervening optical parts that would cause fluorescence signal loss in an inverted microscope type of configuration.

A filter wheel passes the alternating 340nm and 380nm excitation ultraviolet (UV) light to the tissue at 250 Hz with a non-continuous time window for each wavelength. PMT than converts the resultant 510 nm fluorescence signal to an electrical signal. A phase-lock-loop controlled sample/hold with integrator circuit times averages the output of PMT to give a smoothed continuous signal. We sample this continuous analog output. Using this integrator output provide the advantage of a smoothed continuous signal.

Signal Averaging

Averaging more than 30 contractions at each frequency produced the force and $[Ca^{2+}]_{in}$ curves that were used for the analyses. An electrical pacing pulse was used as the reference starting time for averaging of traces. Averaging greater than 30 contractions increased signal to noise ratio because noise occurs randomly in time where paced contraction signal occur in stationary manner with respect to the stimulation pulse. Emissions from separate 340 nm and 380 nm excitations were examined. Only experiments showing conjugate changes in emissions in response to both 340 nm and 380 nm excitations during a contraction were used because ineffective coupling of 340 nm excitation to tissue will artifactually depress the estimated $[Ca^{2+}]_{in}$ by about 10-fold. Proper conjugate emission response consisted of simultaneous increasing emission by 340 nm excitation and decreasing emission by 380nm excitation with expected increase in $[Ca^{2+}]$ after an electrical stimulation. Sampling frequency must be fast enough to allow good approximation of the actual signal after digital to analog conversion. The Nyquist criterion states that a minimum sampling frequency of 2-times the highest frequency component

is needed. One can estimate the highest frequency component by calculating Fourier transform of the expected signal and finding the frequency where intensity in the frequency-domain Fourier spectrum has decreased by 90%. In practice, one needs about 10-times the highest frequency component to reconstruct the actual signal after digitization. The sharp rising edge of the Weibull-shaped $[Ca^{2+}]_{in}$ transient most likely has the fastest frequency components in these experiments. For these reasons, sampling frequencies of 1 KHz or faster were used.

Langendorff Perfusion

Hearts were excised, aorta was cannulated, and perfused in retrograde fashion via the cannulated aorta with KH buffer at 37^{o}C in a Langendorff apparatus (AD Instruments, Colorado)[5] to remove blood and catecholamines from the coronary circulation. The perfusion was done in the constant pressure mode ~ 90 mmHg. The pacing protocol consisted of 5-10 minutes of spontaneous rhythm, followed by pacing at 7 Hz for 15 minutes, and then pacing at 10 Hz for 15 minutes. After the initial 15 minutes at 10 Hz, hearts were either continually paced with perfusion alone, or paced with addition of 1 μmol/L dobutamine, or paced with addition of 10 mmol/L of KN-93.

Recombinant Wild-Type Cardiac Myosin Binding Protein-C

Wild-type (WT) murine MyBPC3 cDNA was spliced into pET-28 vector with His-Tag. The resultant plasmids containing MyBPC3 cDNA were cloned and maintained in DH5α competent E coli. The plasmids were extracted from DH5α competent E coli and re-cloned into BL21(DE3)pLysS competent E coli for production of full length WT murine MyBPC3. The full length WT MyBPC3 was difficult to produce in bacteria due to its length and rare-codons to bacteria (ccc, gga, cgg) that can stop translation in bacteria. We followed the increases in 600 nm absorbance to ensure the bacteria culture remained in the log-phase growth before harvest. Furthermore, reducing temperature from 37^{O}C to 25^{O}C at the point of induction increased the efficiency of full length WT MyBPC3 protein production. Finally, completing all protein extraction tasks at 4^{O}C helped to prevent protein from precipitating.

In Vitro Calcium Calmodulin Kinase

Dr. Andy Hudmon kindly provided the recombinant CaMK2d that was produced via baculovirus system. The recombinant CaMK2d was transferred from -80^{O}C freezer onto ice for slow thawing. After thawing, we activated CaMK2d in buffer consisting in mmol/L of $CaCl_2$ 0.5, calmodulin 0.02, KCl 60, $MgCl_2$ 10, HEPES 50 at pH=7.4, and ATP 1 for 5 minutes. The target recombinant MyBPC3 was placed in reaction buffer with final concentrations consisting in mmol/L of EGTA 1, KCl 60, $MgCl_2$ 10, HEPES 50 at pH=7.4, and ATP 1. The reaction was started by adding pre-activated CaMK2d into reaction buffer containing MyBPC3. We used 2.5 microgram of CaMK2d and 11.7 microgram of MyBPC3 in final reaction volume of 50 microliters. Due to EGTA, the final reaction has estimated $[Ca^{2+}]$ of 12 nmol/L. The reactions were stopped by addition of SDS sample buffer and immediately placing at 95^{O}C for 5 minutes. We chose stopping times of immediately after mixing, 15 seconds, 30 seconds, 60 seconds, 5 minutes, and 10 minutes. The negative controls consisted of adding activation buffer without CaMK2d to demonstrate there is no endogenous kinase from the recombinant protein production and inhibit CaMK2d with autocamtide-2 inhibitory peptide (which is specific inhibitor of CaMK2d, EMD-Calbiochem Catalog# 18940) at 10 μmol/L to further demonstrate it was CaMK2d that phosphorylated MyBPC3.

Phospho-protein Staining and Analyses

We treated WT mice with metoprolol (5 microgram/gram) via intra-peritoneal injection at 30 minutes prior to harvesting in order to produce hearts that were used for reference (WT+BB). Metoprolol, which

is a selective β_1-adrenergic receptor blocker, is used to reduce phosphorylation levels of myofilament proteins. We then extracted and concentrated myofibrils[6]. The myofibrils were then solubilized and electrophoresed on 4-15% gradient gel. We loaded 3-different total protein amounts on 3-different lanes for each experiment. We used Pro-Q Diamond to stain gel for phosphorylated protein[6]. After Pro-Q Diamond de-staining, we stained the same gel with Sypro-Ruby to quantify total protein. We than performed linear regression to find slopes of specific phosphorylated protein band *vs.* total protein loading (Pro-Q slope) and specific protein band *vs.* total protein loading (Sypro-Ruby slope). As described previously[6] the ratios of $\frac{[(Pro-Q\ slope\ of\ experiment)/(Pro-Qslope\ of\ WT+BB)]}{[(Sypro-Ruby\ slope\ of\ experiment)/(Sypro-Ruby\ slope\ of\ WT+BB)]}$ were used to compare phosphorylation levels.

Major Changes in the New Intact Papillary Muscle Simultaneous Force and $[Ca^{2+}]_i$ System

Small intact muscle test system (Aurora Scientific, Model 1500A) was used. This system included a very sensitive and stable force transducer (Aurora Scientific, Model 405A). The muscle is now mounted using 7-0 silk suture instead of metal clips. This system brought improved long term force transducer stability. Furthermore, the North American location of the vendor allowed easy repairs and updates instead of very difficult trans-Atlantic maintenance.

An inverted Olympus microscope is now being used with long working distance objectives that allow 340 nm and 380 nm excitation. This allowed direct visualization of the portion of papillary muscle being excited. Previously, the excitation pathway occurred in free air at 90^O angle from the upright microscope. This new configuration improved $[Ca^{2+}]_i$ imaging sensitivity and improved % success in obtaining quality Fura-2AM signals. However, one can no longer see the entire papillary muscle and measure the diameters at the experimental configuration.

An IonOptix Hyper-Switch system is now being used to (A) provide 340 nm and 380 nm excitation, (B) detect 510 nm fluorescence, and (C) record raw data, and (D) perform signal averaging. The Hyper-Switch system eliminated the 20-Hz motor wobble artifact that occurs with the older system. Furthermore, the Hyper-Switch system does not breakdown quickly like the spinning wheel of the older system. The hyper-switch system uses threshold-photon counting method of detection; therefore, we lost the ability to adjust the gain of the photomultiplier tube to optimize signal/noise ratio. Furthermore, the North American location of the vendor allowed easy repairs and updates instead of very difficult trans-Atlantic maintenance.

Cytosolic and Myofibril Preparation

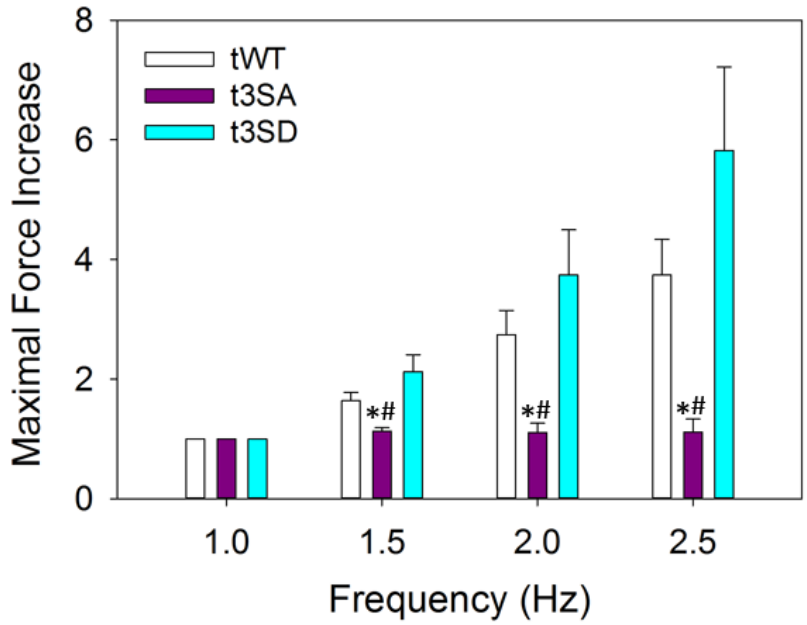
Three months old mice from all three lines (cMyBP-C(tWT), cMyBP-C(t3SA), cMyBP-C(t3SD)) were selected. Mice were anesthetized with isoflurane. Hearts were extracted and quickly rinsed free of blood in phosphate buffered saline. Afterwards, hearts were quickly immersed in cooled 4^OC solution containing KCl 60 mmol/L, MgCl2 2 mmol/L, EGTA 1 mmol/L, HEPES 20 at pH=7.4 mmol/L, DTT 2 mmol/L, phosphatase inhibitor okadaic acid 1 micromol/L, and protease inhibitor cocktail (Sigma P8340). The hearts were homogenized for 30 seconds 3-times on ice. Triton-X 100 was then added to reach final concentration of 1%. The mixture set on ice for 30 minutes with vortexing the solution at least 3-times. Centrifuging at 1200g 4^OC for 10 minutes pelleted down myofibrils without mitochondria (this is the myofibril fraction). The supernatant is then centrifuge again at 12,000g 4^OC for 20 minutes to pellet down mitochondria. The supernatant was then used for western blotting for calcium calmodulin kinase 2 delta (CaMK2d) and protein kinase-A (PKA) BioRad DC assay was used to quantify protein concentration.

The myofibril fraction was then purified using a previously developed protocol.[6]

Western Blotting for Kinases

A collection of antibodies were tested against both cytosolic fraction and myofibril fraction. Both CaMK2d and PKA occur in the cytosolic fraction. 50 microgram of cytosolic proteins were loaded for each lane. The final set of antibodies that detected kinases and its phosphorylation status are: CaMK2d (Santa Cruz, SC-5392, polyclonal goat, 1:250 dilution), Phos-CaMK2d (Santa Cruz, SC-12886, polyclonal rabbit, 1:250 dilution), PKA catalytic subunit (Santa Cruz SC-903, polyclonal rabbit, 1:500 dilution), Phos-PKA catalytic subunit (Sigma, SAB4503969, 1:500 dilution), and GAPDH (Santa Cruz, SC-166504, monoclonal mouse, 1:250 dilution). A cooled CCD camera system (BioRad)was used to capture chemiluminescence intensity for quantification.

Supplemental Figures and Figure Legends



tWT: n=9; t3SA: n=9; t3SD: n=9
Error Bar=SEM; *p<0.05 vs tWT; #p<0.05 vs t3SD

Figure S1: Force Frequency Relationships. A different set of intact papillary muscles from cMyBP-C(tWT), cMyBP-C(t3SA), and cMyBP-C(t3SD) mice were mounted on a completely new system (see material and methods supplement section). Peak twitch force was normalized to peak twitch force at 1 Hz to eliminate differences in muscle size. Both cMyBP-C(tWT) and cMyBP-C(t3SD) papillary muscles demonstrate robust positive force frequency response far exceeding cMyBP-C(t3SA) papillary muscles.

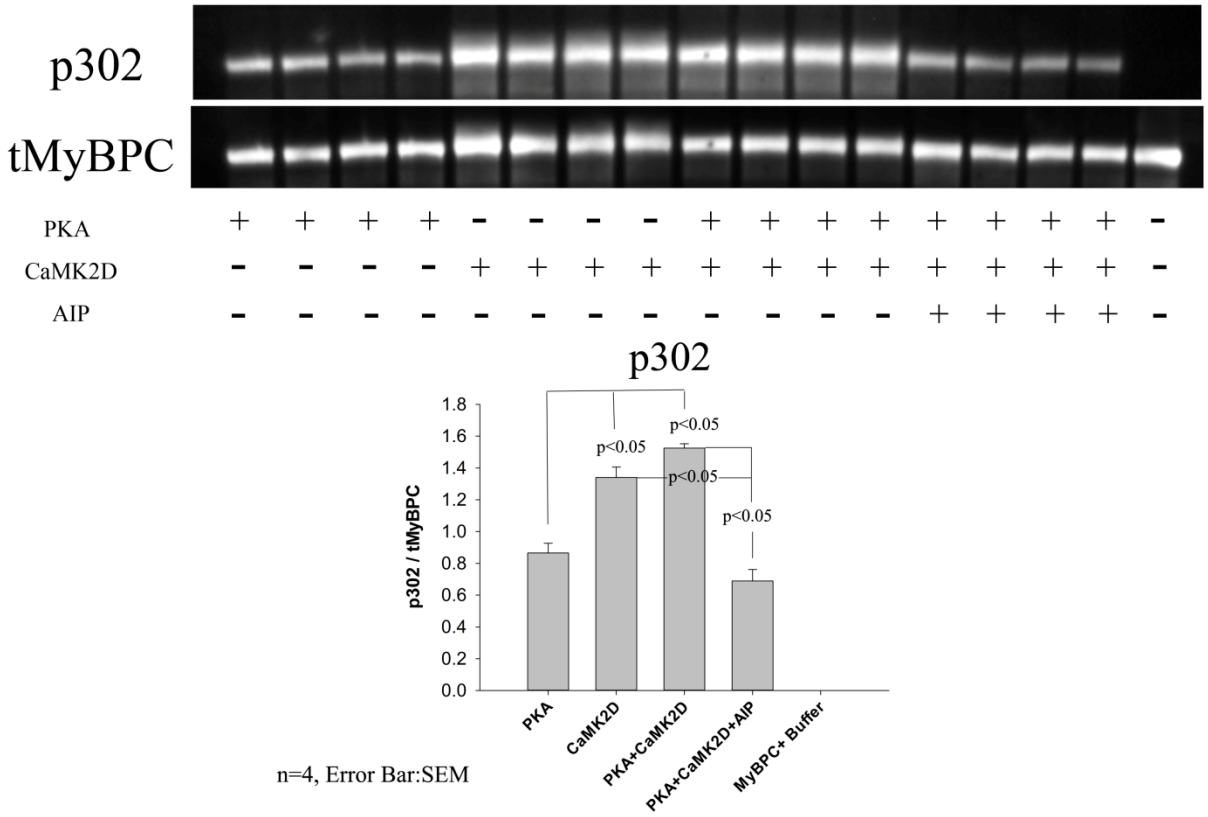


Figure S2: Effect of CaMK2δ and PKA combination. All kinase reactions were conducted at room temperature for 5 minutes. Addition of 4X SDS sample buffer at the end of 5 minutes solubilized the enzyme protein mixture to stop the reaction. Recombinant protein alone without addition of kinases was used as negative control. CaMK2δ phosphorylated Ser302 to greater extent than PKA. CaMK2δ + PKA caused greater amount of phosphorylation than either kinases alone. Inhibition of CaMK2δ with autocamtide inhibitory peptide (AIP) returned the combined kinases phosphorylation level back down to PKA phosphorylation alone. These results suggest that CaMK2δ and PKA act independently on Ser302. The net effect is additive.

Supplemental References

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