The Ras-like GTPase Rem2 is a potent endogenous inhibitor of calcium/calmodulin-dependent kinase II activity

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Supplementary Figures and Materials and Methods

Supplementary Figures

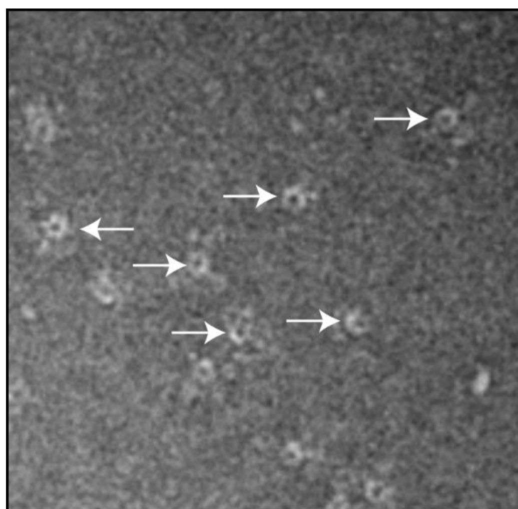

Figure S1. Purification of CaMKII holoenzyme. Negative stained EM of CaMKIIα preparation. White arrowheads indicate CaMKIIα holoenzyme.

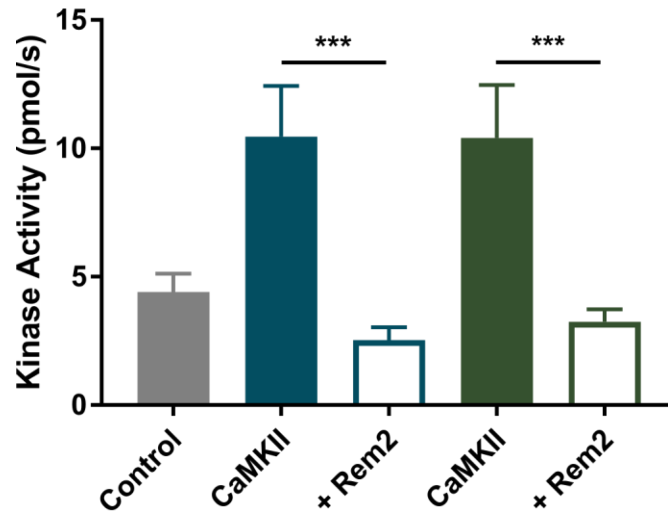

Figure S2. Quantification of CaMKIIα kinase activities shown in Figure 1. The slopes of the early kinetic phase of the Total ATP consumption shown in Figure 1C-D were used to calculate the kinase activities in pmol/s (same color code is used) (N = 6 reactions; bars indicate standard deviations; ***, p = 0.0002, using Student's t-test).

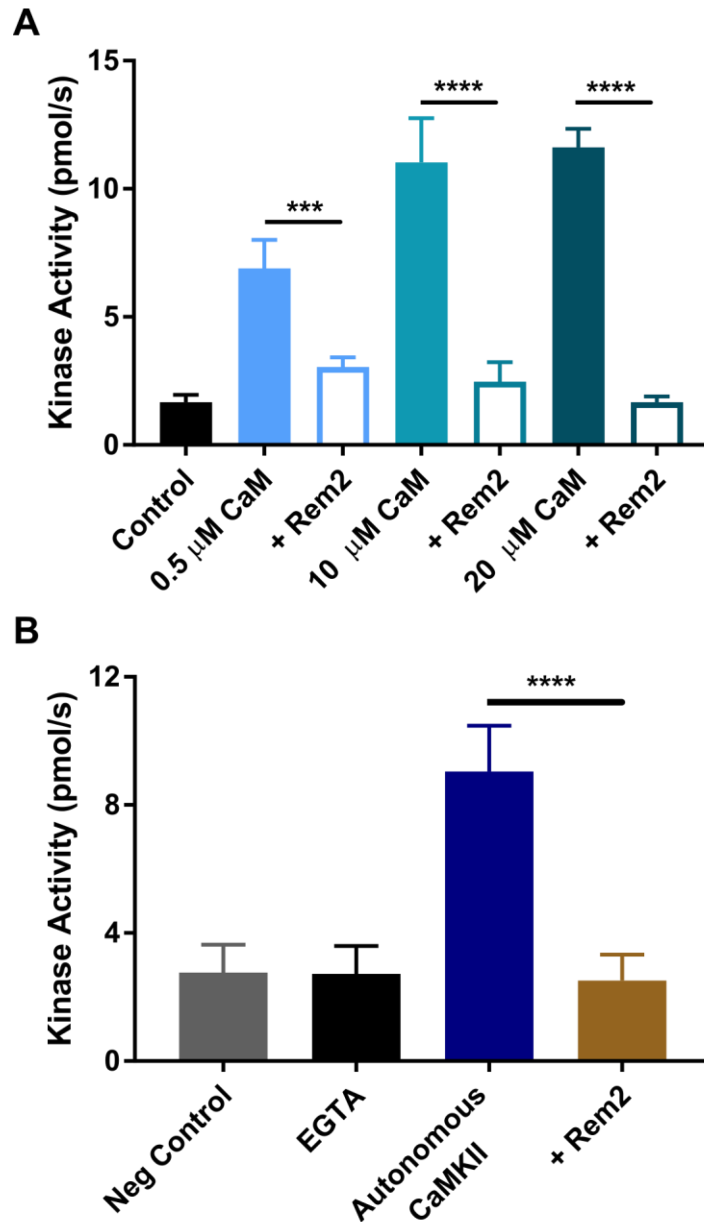

Figure S3. Quantification of CaMKIIα kinase activities shown in Figure 2. (A) The slopes of the early kinetic phase of the Total ATP consumption shown in Figure 2E were used to calculate the kinase activities in pmol/s (same color code is used) (N = 6 for all CaM only conditions; N = 4 for 0.5 μM + Rem2 condition; N = 5 for all other conditions; error bars depict standard deviations; ***, $p < 0.0002$; ****, $p < 0.0001$, using Student's t-test). (B) Similar quantification was conducted for Figure 5E (N = 6; error bars depict standard deviations; ****, $p < 0.0001$, using Student's t-test).

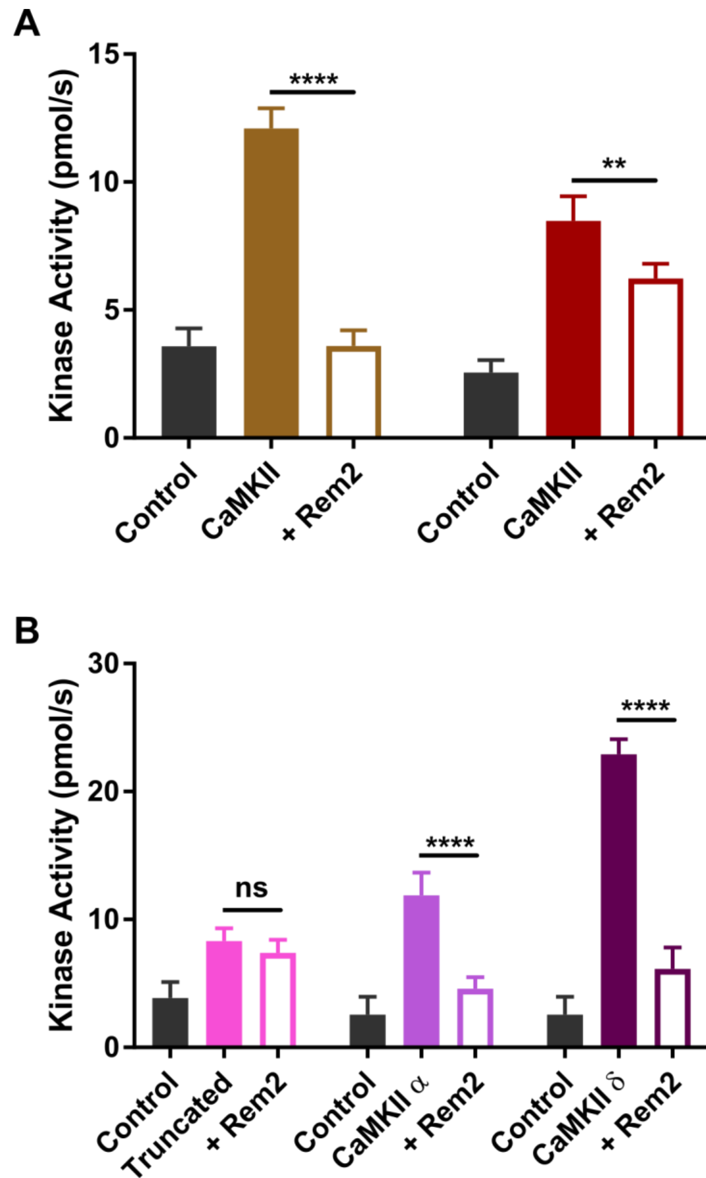

Figure S4. Quantification of CaMKIIα kinase activities shown in Figure 3. (A) The slopes of the early kinetic phase of the Total ATP consumption shown in Figure 3A were used to calculate the kinase activities in pmol/s (same color code is used) (N = 5; standard deviations shown; ****, $p< 0.0001$; **, $p = 0.0034$, using Student's t-test). (B) Similar quantification was performed for Figures 3C-E. (N = 6 for all groups; standard deviations are shown; ns, not significantly different, $p = 0.1758$; ****, $p < 0.0001$, using Student's t-test).

Materials and Methods

Molecular Biology

Plasmids containing full-length human CaMKIIα and δ cDNAs were obtained from Addgene (plasmids 23408 and 23814). The cDNAs were subcloned into the pET28-lam-ppase vector. To create pET28-lam-PPase, the λ phosphatase gene including the ribosome binding site was subcloned from the vector pPET-PKR/PPase (addgene 42934) downstream of the multiple cloning site in pET28a (Novagen, Billerica, MA). This vector contains the lambda phosphatase gene in an operon with the cloned cDNA allowing co-induction of the kinase and lambda phosphatase. Mouse Rem2 cDNA was subcloned in frame with the coding sequence of the maltose binding protein (MBP) present in the pMAL-c5x vector (New England Biolabs, Ipswich, MA) using the Gibson Assembly Cloning Kit (New England Biolabs). The vector was modified to contain a tobacco etch virus (TEV) protease recognition site followed by a 6x histidine tag between the C-terminal of MBP and the N-terminus of Rem2.
For mammalian expression, c-myc and HA epitope tags were added in frame to the N-terminal sequences of CaMKIIα and Rem2, respectively, and subcloned either into the vector pCMV (myc-CaMKIIα – Clontech, Mountain View, CA) or pcDNA3.1 (HA-Rem2 – Invitrogen, Carlsbad, CA).

Cell culture and transfection

HEK-293T cells were obtained from ATCC (Manassas, VA) and maintained at 37°C and 5% CO_2. Cells were cultured in Dulbecco's Modified Eagle's Medium (GE Healthcare Life Sciences, Pittsburgh, PA) supplemented with 10% FBS (GE Healthcare Life Sciences), Penicillin (100 Units/mL), Streptomycin (100 µg/mL), and glutamine (2 mM). Once ~70 % confluency was reached, cells were transfected with 130 ng cDNA/cm^2 for each plasmid using the calcium phosphate method (1).

Co-immunoprecipitation and Western Blotting

HEK-293T cells were harvested 24-48 hours after transfection and lysed for 20 minutes at 4°C with a gentle lysis buffer (25 mM tris(hydroxymethyl)aminomethane (Tris) pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.1% Triton X-100) containing a protease inhibitor cocktail (Complete, mini – Roche Diagnostics, Indianapolis, IN). Pierce anti-HA magnetic beads (Thermo Fisher, Carslabad, CA) were used to perform co-immunoprecipitations. Input samples were collected prior to mixing cell lysates with pre-washed magnetic beads for 30 minutes at room temperature. After mixing, the beads were washed three times with 1x Tris-buffered saline containing 0.05% Tween-20 detergent (TBS-T), 1 mM $MgCl_2$ and 1 mM $CaCl_2$. One final wash was done with deionized water also containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$ before eluting with 3x sample buffer and boiling at 95˚C for 5-10 minutes. Bound proteins were separated by electrophoresis on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Western blots were performed using anti-myc (antibody #2872, Cell Signaling Technology, Danvers, MA) and anti-HA (sc-805-G, Santa Cruz Biotechnology, Dallas, TX) primary antibodies. Western blots were developed using the Odyssey Infrared Imaging System (Li-Cor Biotechnology, Lincoln, NE). The experiment was performed four times.

Protein Expression and Purification

Human CaMKIIα or δ (tagged with 6xHis in the N-terminal) and lambda phosphatase were co-expressed in *E. coli* (BL21* (DE3) pRARE2lacI^{Q}) and protein expression induced by the addition of 0.5 mM isopropyl b-D-1-thiogalactopyranoside. Cells were grown overnight at room temperature and pellets were resuspended in buffer A (50 mM Tris, pH 8.0, 150 mM sodium chloride, 1 mM tris(2-carboxyethyl)phosphine, 5% glycerol, 0.1% Triton X-100) containing a cocktail of protease inhibitors (completeMini, Roche Diagnostics). Chicken egg white lysozyme was added to a concentration of 0.5 mg/mL and the cells incubated on ice for 20 min. Deoxycholate and sodium chloride were added to a final concentration of 0.2% and 650 mM, respectively. Cells were sonicated using a microtip sonicator to reduce viscosity. 1M imidazole was added to a final concentration of 20mM. After centrifugation at 20,000 xg at 4°C for 30 min, the supernatant was loaded onto a 0.5 mL Ni-6 Sepharose Fast Flow column (GE Healthcare). The column was washed with 40 column volumes of Buffer A containing 500 mM NaCl and 50 mM imidazole. CaMKII was eluted in buffer A containing 0.5 M imidazole. CaMKII was loaded on a HiPrep Sephacryl S-300 HR column (GE Healthcare) equilibrated in 25 mM Tris, pH 8.0, 150 mM NaCl, 1 mM 1,4-Dithio-D-threitol (DTT), 5% glycerol, 0.01% Triton X-100, 0.1mM Ethylenediaminetetraacetic acid (EDTA). Fractions containing the holoenzyme were pooled and concentrated using an Amicon Ultra-4 centrifugal filter unit (10,000 molecular weight cutoff) (EMD Millipore, Billerica, MA), frozen in liquid nitrogen and stored at -80°C. Protein purity was verified by SDS-PAGE followed by Coomassie staining.

Rat Rem2 was expressed in *E. coli* (BL21 DE3) as an N-terminal fusion to MBP. 2 mL of an overnight culture in 2xYT medium containing 0.2% glucose and 50 µg/mL kanamycin were further inoculated into 400 mL of the same media and cells grown at 37°C until the OD600 reached 0.6. Protein expression was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside and the cells cultured overnight at room temperature. The cells were pelleted by centrifugation at 4000 xg and resuspended in 10 mL of a solution containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM DTT, and 5% glycerol (buffer R) supplemented with a cocktail of protease inhibitors (completeMini – Roche Diagnostics). Chicken egg white lysozyme was added to a concentration of 0.5 mg/mL and the cells incubated at 4°C for 20 min. NaCl and Triton X-100 were added to a final concentration of 650 mM and 0.1%, respectively, and the cells lysed by sonication. After centrifugation at 20,000 xg at 4°C for 30 min, the supernatant was mixed with 500 µL of amylose-agarose resin and incubated at 4°C for 3 hours. The mix was centrifuged at 2,000 xg for 2 min and the supernatant discard. The resin was washed 3x with 10 mL of buffer R supplemented with 350 mM NaCl. At this stage the amylose-agarose resin bound to MBP-Rem2 was either used for pull-down assays or for further purification of Rem2. In the latter case, the amylose-resin was treated with TEV protease overnight at 4°C and the supernatant further purified using a nickel sepharose column (Ni Sepharose 6 Fast Flow, GE Healthcare Biosciences). Rem2 was eluted using buffer R supplemented with 500 mM imidazole and the sample dialyzed for 20 hours at 4°C against buffer R.

Calmodulin (CaM) was purified from bovine brain using a modified version of the method described previously (2). Briefly, brain tissue was homogenized in 50 mM Tris (pH 8.0), 2 mM EDTA, and 1 mM beta-mercaptoethanol using a Braun homogenizer. The homogenate was then centrifuged at 6000 xg for 30 min. Ammonium sulfate was slowly added to the supernatant with gentle stirring for 40 min until 60% saturation was reached and the sample centrifuged at 9000 xg for one hour. Sulfuric acid (10 N) was added to the supernatant to bring the pH to 4.0. After one hour incubation under stirring the sample was

centrifuged for another hour at 8000 xg. The pellet was resuspended in 10 mM Tris (pH 8.0), 200 mM NaCl, 1mM EDTA, 1 mM beta-mercaptoethanol and dialysed against the same solution for 4 hours. The dialysis bag was transferred to fresh buffer and dialysis was continued overnight. Next, the sample was loaded onto a Q sepharose FF column (GE Healthcare) and CaM was eluted using a sodium chloride gradient. Fractions containing CaM were pooled and dialyzed for 16 hours against a solution containing 10 mM Tris (pH 8.0), 500 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 1 mM beta-mercaptoethanol. The sample was further purified by separation in a HiTrap phenyl-sepharose column (GE Healthcare) and CaM eluted with 10 mM Tris (pH 8.0), 500 mM NaCl, 1 mM $MgCl_2$, 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), and 1 mM beta-mercaptoethanol. Fractions containing CaM were pooled, concentrated and stored at -20ºC. All steps were conducted at 4ºC.

Electron Microscopy

For the negative stain 3.5 μL of 0.3-1 μM CaMKII was applied to 400 mesh copper grids (Electron Microscopy Sciences) that were glow discharged for 30 s at -20 mA immediately before use. Sample was allowed to adsorb for 1 minute then blotted and then rinsed and blotted twice with ddH_20. Finally, grids were stained with 0.75% uranyl formate for 1 minute. Grids were imaged on an FEI Morgagni Transmission microscope at 80 keV at a nominal magnification of 44,000x.

Rem2 Affinity Pull-down Assay

Rem2 interactors expressed in the brain were identified using MBP-Rem2 immobilized on amylose-agarose beads. Brains from P14 rats were isolated and homogenized in 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM DTT, and 5% glycerol (buffer L) containing a cocktail of protease inhibitors (completeMini – Roche Diagnostics) and 0.1% Triton X-100. The lysate was incubated at 4ºC for 30 min and then centrifuged at 18,000 xg for 30 min. The supernatant was saved and incubated with MBP-Rem2/amylose-agarose beads for 3 hours at 4ºC. Next, the beads were washed 3x for 5 min with buffer L supplemented with 350 mM NaCl and MBP-Rem2 eluted with buffer L containing 10 mM maltose. A parallel experiment was conducted using amylose-agarose beads containing only the MBP tag to serve as control. The samples were separated by SDS-PAGE using a pre-cast 10% Bis-Tris gel (NuPage, ThermoFisher Scientific) and 50 mM 3-(N-morpholino)propanesulfonic acid, 50 mM Tris base, 0.1% SDS, 1 mM EDTA (pH 7.7) as running buffer. The gel was stained with Coomassie Brilliant Blue R-250 and bands present in the MBP-Rem2 sample only were cut and sent to further purification and identification by microcapillary liquid chromatography/tandem mass spectrometry (Taplin Mass Spectrometry Facility, Harvard Medical School – Cambridge, MA).

CaMKII Activity Assays

The kinase activity of CaMKII was assessed using a continuous spectrophotometric assay (3). The method is based on the coupling of the phosphorylation of peptides by CaMKII/MgATP to the oxidation of NADH via pyruvate kinase, phosphoenolpyruvate, and lactate dehydrogenase. Monitoring the decrease

in absorbance of NADH at 340 nm permits the continuous spectrophotometrical analysis of CaMKII kinase activity.
The standard assay (150 μL total volume) contained: 50 mM Tris (pH 7.5), 150 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 3.33 μM CaM, 400 μM sodium ATP, 200 μM phosphoenolpyruvate, 400 μM NADH, 9-15 units of pyruvate kinase, 13.5-21 units of lactate dehydrogenase, and 40 μM of either GDP-β-S (Fig. 1C, 1G, 2A-E, 3A-E) or GMP-PCP (Fig. 1C). The substrate peptides used were either syntide-2 (PLARTLSVAGLPGKK) or autocamtide-2 (AC-2 - KKALRRQETVDAL) (Genscript, Piscataway, NJ), both at 200 μM. The reaction was started by the addition of 20 nM CaMKII (monomer concentration) and the decrease in absorbance at 340 nm at room temperature monitored in a microplate spectrophotometer (Infinite 200 Pro, Tecan, Mannedorf, Switzerland). Full-length human CaMKIIα and δ were purified as described above. Rat truncated CaMKIIα was obtained from New England Biolabs (Ipswich,MA).
A calibration curve was used to convert the NADH absorbance readings at 340 nm into moles of ATP based on the 1:1 coupling of NADH:ATP consumption of the PK/LDH assay. Subsequently, the moles of ATP consumed in each step were used to construct a cumulative plot of ATP consumption. Then the kinase activity in pmols/s was calculated from the slopes of this plot. Since two kinetic distinct phases were observed in all plots, a multiple linear regression was fitted to the data points. The value of the intersection point of the two regression lines was the same for all curves for a given condition. The values obtained from the late kinetic phase were used in Figures 1, 2, and 3. Importantly, a similar relative inhibition by Rem2 was obtained if the values from the early phase were used instead (Fig. S2, S3, S4).

Autonomous CaMKII

In order to obtain autonomous CaMKII activity (i.e. Ca^{2+}-independent kinase activity), the purified enzyme (60 nM) was pre-incubated with sodium ATP (500 μM) and CaM (10 μM) in 50 mM Tris buffer (pH 7.5) containing 150 mM NaCl, 1 mM $CaCl_2$, and 2 mM $MgCl_2$ in a total volume of 50 μL. After 2 min, 6 μL of 100 mM sodium EGTA was added to stop the autophosphorylation of CaMKII. The reaction mix was immediately added to 96 μL of a solution containing 200 μM syntide-2, 400 μM NADH, 200 μM phosphoenolpyruvate, 500 μM sodium ATP, 40 μM GDP-β-S, 9-15 units of pyruvate kinase, and 13.5-21 units of lactate dehydrogenase in 50 mM Tris buffer (pH 7.5) containing 150 mM NaCl. Addition of sodium EGTA lowered the final concentration of free Ca^{2+} to < 20 nM while decreasing the free Mg^{2+} to ~ 1.2 mM (calculated with the software MaxChelator (4)). To assure that addition of EGTA allowed only the detection of autonomous CaMKII activity, samples were run with EGTA present during the pre-incubation. Accordingly, at those divalent concentrations no Ca^{2+}/ CaM -dependent activity could be detected.

Radioactive in vitro Kinase Assays

The kinase activity of CaMKIIα was monitored by measurement of the incorporation of radiolabeled phosphate from [γ-^{32}P]ATP into the substrate peptide syntide-2. The kinase assay (150 μL total volume) contained 50 mM Tris (pH 7.5), 150 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 3.33 μM CaM, 400 μM [γ-^{32}P]ATP (~0.8 Ci/mmol), 200 μM syntide-2 peptide, and 40 μM of either GDB-β-S or GMP-PCP

(SigmaAldrich, St. Louis, MO). The reaction was initiated by addition of purified CaMKII (20 nM final) and incubated at room temperature for 8 min. The reaction was stopped by addition of 600 μL 75 mM phosphoric acid and slot-blotted onto Whatman P81 phosphocellulose paper. After additional washes with 75 mM phosphoric acid, the paper was rinsed in acetone, dried and the radioactivity of bound syntide-2 measured using a phosphorimager (Typhoon FLA 7000, GE Healthcare Life Sciences). The intensities of the detected bands were quantified using the software ImageJ (NIH, Bethesda, MD).

CaMKII Autophosphorylation

CaMKII autophosphorylation was assessed by measurement of the incorporation of radiolabeled phosphate from [γ-^{32}P]ATP. The assay contained 50 mM Tris (pH 7.5), 150 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 3.33 μM CaM, 100 μM [γ-^{32}P]ATP (~16 Ci/mmol), and 40 μM GMP-PCP. The reaction was initiated by addition of CaMKII (80 nM final) and incubated at 30°C for 2 min. The reaction was stopped by addition of 4x Laemmli loading buffer (Amresco, Solon, OH) followed by incubation at 90°C for 2 min. The samples were separated by SDS-PAGE and the gel blotted onto a Whatman 1 filter paper and dried. Radioactivity of protein bands was measured using a phosphorimager (Typhoon FLA 7000, GE Healthcare Life Sciences). The intensities of the detected bands were quantified using the software ImageJ (NIH, Bethesda, MD).

Data and Statistical Analysis

Quantification of protein and peptide bands from digitized images of slot-blots, gels and membranes were performed using ImageJ (NIH). Data processing and analysis were performed using Excel (Microsoft, Redmond, WA), OriginPro 8 (OriginLab, Northampton, MA), and GraphPad Prism 7 (GraphPad Inc, La Jolla, CA). Figures were prepared using GraphPad Prism 7 and the open source software Inkscape (www.inkscape.org). Statistical calculations and analysis were done using OriginPro 8 and GraphPad Prism 7. In all experiments analyzed, the comparisons of interest were between two groups and for this reason unpaired Student's t-tests with Welch's correction were employed. Level of significance was set at $p < 0.01$.

In order to estimate the IC50 of CaMKIIα inhibition by Rem2 the data points shown in Fig. 1H were fitted using a log (inhibitor) vs response equation: $y = a + \frac{(b-a)}{(1+10^{x-IC50})}$ where a and b are constants.

References

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