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Development of a medium throughput whole-cell microtiter plate Thr286 autophosphorylation assay for CaMKIIα using ELISA

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ABSTRACT

Ca^{2+}/calmodulin-dependent protein kinase II alpha (CaMKIIα) is a multifunctional Ser/Thr kinase involved in several neuronal signaling pathways including synaptic plasticity. CaMKIIα autonomous activity is highly dependent on Thr286 autophosphorylation (pThr286), which is widely used as a readout for its enzymatic activity. To readily characterise compounds and potential drug candidates targeting CaMKIIα, a simple, generic cell-based assay for quantification of pThr286 levels is needed. In this study, we present a cell-based assay using an adapted ELISA as a suitable and higher throughput alternative to Western blotting. In this 96-well plate-based assay, we use whole HEK293T cells recombinantly expressing CaMKIIα and apply a phospho-specific antibody to detect pThr286 levels by chemiluminescence. In parallel, total CaMKIIα expression levels are detected by fluorescence using an Alexa488-conjugated anti-myc antibody targeting a C-terminal myc-tag. By multiplexing chemiluminescence and fluorescence, phosphorylation levels are normalised to CaMKIIα total expression within each well. The specificity of the assay was confirmed using a phosphodead mutant (T286A) of CaMKIIα. By applying Ca^{2+} or known CaMKIIα inhibitors (KN93, tatCN21 and AS100105) and obtaining concentration-response curves, we demonstrate high sensitivity and validity of the assay. Lastly, we demonstrate the versatility of the assay by determining autophosphorylation levels in CaMKIIα patient-related mutations, known to possess altered pThr286 responses (E109D, E183V and H282R). The established assay for CaMKIIα is a reproducible, easily implemented, and facile ELISA-based assay that allows for reliable quantification of pThr286 levels.

1. Introduction

Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII) is a multifunctional and ubiquitously expressed Ser/Thr kinase (Tobimatsu & Fujisawa, 1989). The kinase works as a Ca^{2+}-detector in several Ca^{2+}-related activities including neurotransmitter release, cell cycle progression and synaptic plasticity (Backs et al., 2010; Coultrap et al., 2014; Hinds, Goussakov, Nakazawa, Tonegawa, & Bolshakov, 2003). Four distinct genes encode CaMKII α, β, δ and γ proteins that display high sequence homology (Tobimatsu & Fujisawa, 1989). In the mouse forebrain, predominant subtypes include heteromers (holoenzymes) consisting of α (CaMKIIα) and β (CaMKIIβ) in a presumed 3:1 ratio (Chao et al., 2011; Cook et al., 2018) and homomeric complexes. Upon activation with Ca^{2+}/CaM, CaMKIIα translocates to the postsynaptic density (PSD) and thus gets closer to its substrates, e.g. the glutamatergic NMDA (N-Methyl-D-aspartate) receptors and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors (Barria, Muller, Derkach, Griffith, & Soderling, 1997; Shen & Meyer, 1999). Combined with its high expression (up to 2% in cortex and hippocampus), this mode of action makes CaMKIIα a major regulator in synaptic plasticity underlying learning and memory (Giese, Fedorov, Filipkowski, & Silva, 1998; Sanhueza et al., 2011).

Abbreviations: (AMPA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; (BSA), Bovine serum albumin; (CaMKII), Ca^{2+}/calmodulin-dependent protein kinase II; (CaM), Calmodulin; (DMEM), Dulbecco's Modified Eagle Medium; (DPBS), Dulbecco's Phosphate-Buffered Saline; (ECL), Enhanced Chemiluminescence; (ELISA), Enzyme-linked immunosorbent assay; (HBSS), Hank's Balanced Salt Solution; (HRP), Horseradish peroxidase; (HEK293T), Human embryonic kidney 293 T; (NMDA), N-Methyl-D-aspartate; (PDL), Poly-D-lysine; (PEI), Polyethyleneimine; (PSD), Post synaptic density; (pThr286), Thr286 phosphorylation; (TBS), Tris-Buffered Saline; (TBS-T), Tris-Buffered Saline + Tween; (Thr286), Threonine 286; (Thr305/306), Threonine 305/306.

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Typically, the CaMKIIα holoenzymes are composed of 12 or 14 subunits (Hoelz, Nairn, & Kuriyan, 2003; Kolodziej, Hudmon, Waxham, & Stoops, 2000). A single CaMKIIα subunit consists of an N-terminal kinase domain, a regulatory segment, a variable linker region and a C-terminal hub/association domain. In the autoinhibited state, the kinase domain is tightly bound to the regulatory segment (Colbran, Fong, Schworer, & Soderling, 1988). Activation of CaMKIIα is initiated by increasing levels of intracellular Ca^{2+} and subsequent binding of Ca^{2+}/Calmodulin (Ca^{2+}/CaM) to the regulatory segment (Lai, Nairn, Gorelick, & Greengard, 1987; Schworer, Colbran, Keefer, & Soderling, 1988). This will release the autoinhibitory interaction between the regulatory segment and the kinase domain and expose the central autophosphorylation sites, threonine 286 (Thr286) and threonine 305/306 (Thr305/306) (Colbran et al., 1988; Colbran & Soderling, 1990; Lai et al., 1987; Schworer et al., 1988). Upon autophosphorylation of Thr286, the kinase will remain activated, even when Ca^{2+} levels decrease, and Ca^{2+}/CaM dissociates (Colbran et al., 1988; Lai, Nairn, & Greengard, 1986; Saitoh & Schwartz, 1985). This Ca^{2+}/CaM-independent state, also known as the autonomous state, requires activation of two adjacent subunits by Ca^{2+}/CaM, since Thr286 autophosphorylation occurs in an intersubunit manner within the holoenzyme (Hanson, Meyer, Stryer, & Schulman, 1994; Rich & Schulman, 1998). Besides autonomous activity, Thr286 autophosphorylation also leads to increased subcellular translocation to the PSD and enhanced binding to CaMKIIα substrates, e.g. the NR2B subtype of the NMDA receptor (Barria et al., 1997; Shen & Meyer, 1999; Strack & Colbran, 1998). Taken together, this underscores Thr286 autophosphorylation as a central regulation of CaMKIIα activity and the downstream effects. Additionally, increased levels of autophosphorylation is shown to be involved in several disease states, e.g. cerebral ischaemia (Coultrap, Vest, Ashpole, Hudmon, & Bayer, 2011; Leurs et al., 2021) and some types of cancer (Chi et al., 2016). Thus, any compound able to regulate Thr286 autophosphorylation will be of great interest for future treatment of CaMKIIα malfunction. Consequently, reliable and effective assays targeting Thr286 autophosphorylation are needed for assessment of compound effects in drug discovery.

Traditional approaches to investigate CaMKIIα autophosphorylation include radiolabelled ATP assays and immunodetection by western blotting (Barria et al., 1997; Saitoh & Schwartz, 1985). However, both these methods include several undesirable components such as the hazard related to the radioisotope and low throughput. In this study, we present an autophosphorylation assay using ELISA (enzyme-linked immunosorbent assay) as an alternative to the existing methods, providing a higher throughput and higher sensitivity than western blotting. Inspired by the commercially available CaMKIIα phosphorylation ELISA kit from LSBio (Lifespan Biosciences Inc., Seattle, WA, USA), we have modified the assay to conveniently be carried out in 96-well plates using adherent whole cells for compound stimulation and washing. Here, we present the implementation and validation of multiplex autophosphorylation assay for use in characterisation of pharmacological compounds and patient-related CaMKIIα mutations. Due to its high flexibility, the assay can easily be implemented in other laboratories as a semi-quantitative, yet sensitive, alternative to western blotting for investigations of CaMKIIα Thr286 levels or adapted to other CaMKII subtypes.

2. Materials and methods

2.1. Compounds and DNA constructs

KN93 phosphate (*N*-[2-[[[3-(4-Chlorophenyl)-2-propenyl]methylamino]methyl]phenyl]-*N*-(2-hydroxy-ethyl)-4-methoxybenzenesulphonamide phosphate) was purchased from Tocris (Bristol, UK). The tat-fused version of CN21 (CaMKII peptide inhibitor), tatCN21 (GRKKRRQRRRKRPP-KLGQIGRSKRVVIEDDR), was purchased from GenScript Biotech (Leiden, The Netherlands). AS100105 was a kind gift from Dr. Howard Schulman. Ionomycin calcium and BAPTA-AM (1,2-*bis*(*o*-aminophenoxy)ethane-*N*,*N*,*N*,*N*-tetraacetic acid tetra(acetoxymethyl) ester) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA) was purchased from VWR International (Radnor, PA, USA). For transfection, we employed plasmid pCMV6-CaMKIIa-Myc-DDK (#RR201121, Origene) encoding myc-tagged rat CaMKIIα. The mutations T286A, E109D, E183V and H282R were generated and sequence-verified by GenScript in the same vector. For mock transfections, a pUNIV plasmid containing the human $GABA_A$ α_4 subunit was used (Falk-Petersen et al., 2017).

2.2. Cell culture and transfections

Human embryonic kidney 293 T (HEK293T) cells were purchased from ATCC (293 T-ATCC, #CRL-3216; authenticated to be mycoplasma-free) and maintained in Dulbecco's Modified Eagle Medium (DMEM) with GlutaMax medium (#61965026, Gibco, Thermo Fisher Scientific, West Palm Beach, FL, USA) supplemented with 10% (*v*/v) fetal bovine serum (Gibco) and 1% (v/v) penicillin/streptomycin (#15140122, Invitrogen) at 37 °C and 5% CO_2. The cells were transfected using polyethyleneimine (PEI, #23966 from Polysciences Inc., Warrington, PA, USA) with a DNA:PEI ratio of 1:3 similar to (Longo, Kavran, Kim, & Leahy, 2013). On the day before transfection, 2×10^6 cells were plated in 10 cm cell culture dishes. For transfection, 8 μg DNA was mixed with 24 μg PEI and diluted in 1 mL DMEM +1% (*v*/v) penicillin/streptomycin. After 15 min incubation at room temperature, the DNA/PEI mixture was added to the cells.

2.3. Antibodies

The following primary antibodies were used: c-myc Alexa488 (c-Myc Monoclonal Antibody (9E10), Alexa Fluor 488, #MA1–980-A488, Invitrogen) and phospho-CaMKII (Thr286) (#AP12716b, RRID:AB_10820669; rabbit monoclonal IgG, clone D21E4, Cell Signaling Technology). The secondary antibody was Goat Anti-Rabbit Immunoglobulins/HRP (#P0448, RRID:AB_2617138; polyclonal, Agilent). The anti-pThr286 antibody was validated for use in ELISA in this study.

2.4. ELISA-based autophosphorylation assay

One day after transfection, transfected HEK293T cells were plated into white 96-well polystyrene CulturPlate™ microplates (PerkinElmer, Waltham, MA, USA) coated with poly-D-lysine (PDL; Sigma-Aldrich) at a density of 5×10^4 cells/well and left overnight (37 °C and 5% CO_2). As assay buffer, we used Hank's Balanced Salt Solution (HBSS, Gibco, #14170120) supplemented with 20 mM HEPES, pH 7.4. Prior to compound stimulation, cells were washed in 100 μL assay buffer per well. Compound stimulation (careful addition of 100 μL of solution per well) was performed at 37 °C and 5% CO_2 for 1 h, except for ionomycin (2–3 min stimulation) and Ca^2 (5 min stimulation for concentration response curves for maximum sensitivity). To examine concentration-dependent responses, the following ranges were used: Ca^{2+} (0.01 to 10 mM), KN93 (0.1 to 100 μM), AS100105 (0.003 to 10 μM) and tatCN21 (1 to 30 μM). All stimulations were performed in technical triplicates and included two sets of buffer controls (one for normalisation and one for comparison). We terminated the stimulation by washing twice with ice-cold Tris-Buffered Saline (TBS, pH 7.4). All described washing procedures were conducted using an electronic 8-Channel ViaFlo, 50–1250 μL, (Integra Biosciences AG, Zizers, Switzerland) set at dispensing speed 3 followed by manual ejection and a final tapping of the plate for complete removal of the liquid. Cells were fixated for 5 min using 50 μL/well 4% (*w*/*v*) paraformaldehyde dissolved in Dulbecco's Phosphate-Buffered Saline (DPBS, Gibco, #12037539) followed by two times washing with TBS. Cells were permeabilised for 5 min using 50 μL/well 0.05% (*w*/*v*) Triton-X in TBS followed by blocking for 30 min in 100 μL/well 3% (w/v) BSA dissolved in TBS. Each antibody was probed for 45 min at room temperature (1:2000 dilution of each), followed by three and four

washes with 100 μL 3% BSA and TBS, respectively. The fluorescence signals (excitation/emission at 490/525 nm) were detected in 100 μL TBS using EnSpire Multimode Plate Reader (PerkinElmer) after incubation with the anti-myc Alexa488 antibody. Following the incubations first with anti-pThr286 and subsequently the anti-rabbit HRP antibody, 10 μL of SuperSignal ELISA Femto Substrate mixture (ThermoFisher Scientific, #37075, 1:1 of luminol enhance solution and stable peroxidase solution mixed right before use) was added to 100 μL TBS. The chemiluminescence signals at 425 nm were detected after 1 min incubation with the ECL substrate.

2.5. Western blot analysis of Thr286 autophosphorylation

The day before the assay, transfected HEK293T cells were seeded into 48-well clear culture plates at a density of 1×10^5 cells/well. Prior to stimulation with compounds, cells were washed with 200 μL assay buffer. Stimulation was performed with 500 μL/well compound solution for 1 h at 37 °C and 5% CO_2. Cells were harvested and lysed using 30 μL/well RIPA buffer (radioimmunoprecipitation assay buffer, Sigma-Aldrich) supplemented with 1% (v/v) phosphatase inhibitors (Phosphatase Inhibitor Cocktail 2 and 3, Sigma-Aldrich) and 1× protease inhibitors (cOmplete™, EDTA-free Protease Inhibitor Cocktail, Sigma-Aldrich). Cell lysates were mixed with 4× Sample Buffer (NuPAGE® LDS Sample buffer 4×, ThermoFisher Scientific) and 100 mM DL-dithiothreitol (Sigma-Aldrich) before loaded into western blot gels. Proteins were transferred from the gel to a polyvinylidene difluoride membrane (Bio-Rad Laboratories) using semi-dry TransBlot® Turbo™ Transfer System (Bio-Rad Laboratories) following the manufacturer's instructions. Membranes were blocked in 3% BSA dissolved in TBS-T (TBS + 0.05% Tween-20 from Sigma-Aldrich) for minimum 1 h. Immunolabelling was done as follows: anti-myc Alexa488 for 1 h at room temperature (1:1000 dilution, stored at 4 °C, re-used up to three times), anti-pThr286 CaMKIIα overnight at 4 °C (1:1000 dilution, stored at −20 °C, re-used up to four times) and polyclonal goat anti-rabbit HRP for 1 h at room temperature (1:2000 dilution, stored at 4 °C, re-used up to four times). All antibody dilutions were made in 1% BSA in TBS-T. Following each antibody incubation, membranes were washed in TBS-T for either 5 min (following anti-pThr286 antibody) or 10 min (following anti-myc and anti-rabbit HRP antibodies). The fluorescence and luminescence signals were captured using iBright™ FL1500 Imaging System (Invitrogen) with excitation/emission filter 490/525 nm for the fluorescent bands. Prior to the luminescence detection at 525 nm, membranes were probed for 4 min with Amersham ECL™ Prime detection reagent (1:1 mixture of luminol and peroxide solution mixed right before use).

2.6. Data analysis and statistics

All total expression results using the myc antibody were corrected for the background fluorescence from empty wells at each plate reading. The autophosphorylation levels (pThr286) were normalised to the total protein expression levels (myc) by calculating the ratio and are reported as pThr286/myc ratio:

$$pThr286 \Big/ myc\ ratio = \frac{pThr286}{myc_{total} - myc_{background}}$$

All conditions in each experiment were performed as technical triplicates. For pooling of data, we normalised the pThr286/myc responses to basal stimulation (buffer) within each experiment and performed the experiments with 3–6 independent transfections. Here, the autophosphorylation responses are stated as "normalised pThr286/myc". For the concentration-response curves of Ca^{2+}, the responses were normalised to 0.01 mM Ca^{2+}, otherwise the basal was used for normalisation. Furthermore, particularly for the Ca^{2+} curve, the data were constrained to the max concentration (10 mM) for enabling the curve-fitting.

Concentration-response curves were fitted, and potencies determined, using non-linear regression and the four-parameter model with a variable slope,

$$Normalised\ pThr286 \Big/ myc = Bottom + \frac{Top - Bottom}{1 + 10^{((log(EC_{50}/IC_{50}) - [compound])*n_h)}}$$

where top and bottom represent the upper and lower plateaus, respectively. EC_{50} or IC_{50} is the concentration of the stimulant or inhibitor that respectively produces or inhibits 50% of the response interval between basal and maximum response, whereas [compound] is the logarithmic concentration of the ligand, and n_h is the Hill slope representing the steepness of the curve.

All statistical and data analyses were performed on pooled, normalised data using GraphPad Prism 8 (Graphpad Software Inc., San Diego, CA, USA). For statistical analysis, we used one-way ANOVA followed by Dunnett's multiple comparison test. We set the level of significance at $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$. All data were found to follow a normal (Gaussian) distribution, according to a normality test (D'Agostino-Pearson omnibus normality test, significance level = 0.05).

3. Results

3.1. Development of an in-well ELISA-based CaMKIIα pThr286 assay

With the aim of developing a pharmacologically relevant and versatile assay to monitor CaMKIIα autophosphorylation, we chose a heterologous expression system, in this case the HEK293T cell line. This cell line allows for the measurement of cellular responses upon stimulation with pharmacological compounds in the presence of intrinsic biological membranes. Additionally, HEK293T cells do not express CaMKIIα endogenously (nor any of the other CaMKII isozymes) and are easily transfected, creating a versatile system to test both wild-type as well as different genetic variants of CaMKIIα in parallel.

To monitor total expression, we utilized a commercially available CaMKIIα construct fused with a C-terminal myc-tag. Fig. 1A illustrates the principle of the adapted ELISA exemplified with Ca^{2+} stimulation. Step 1 is the stimulation with Ca^{2+} and subsequent change in autophosphorylation. Step 2 is the actual ELISA resulting from binding of the antibodies for total expression (primary anti-myc Alexa488) and autophosphorylation (primary anti-pThr286 followed by secondary anti-rabbit HRP). By using these antibodies, we successfully performed multiplex recordings by combining chemiluminescence and fluorescence measurements through the application of HRP and Alexa488, respectively. As seen in Fig. 1B, the antibodies were successfully employed in western blot analysis of phosphorylation and expression showing an increase in pThr286 level after Ca^{2+} stimulation compared to buffer (basal). However, employing simultaneous chemiluminescence and fluorescence recordings in a plate-based setup lead to considerations regarding the choice of cell culture plate. Normally, fluorescence recordings use black culture plates and chemiluminescence recordings use white plates to minimize the amount background arising from the plates. In this assay, the main signal of interest was the autophosphorylation, and thus white culture plates were used to ensure optimal chemiluminescence signal. To correct for the background fluorescence signal from the white plates, we conducted control fluorescence recordings of empty wells at each plate reading. For all three antibodies, we optimised the dilutions to achieve a high signal with limited amount of background (see *Materials and Methods*). Moreover, we used BSA as the preferred blocking agent over skim milk to limit non-specific binding of the phospho-specific antibody (data not shown).

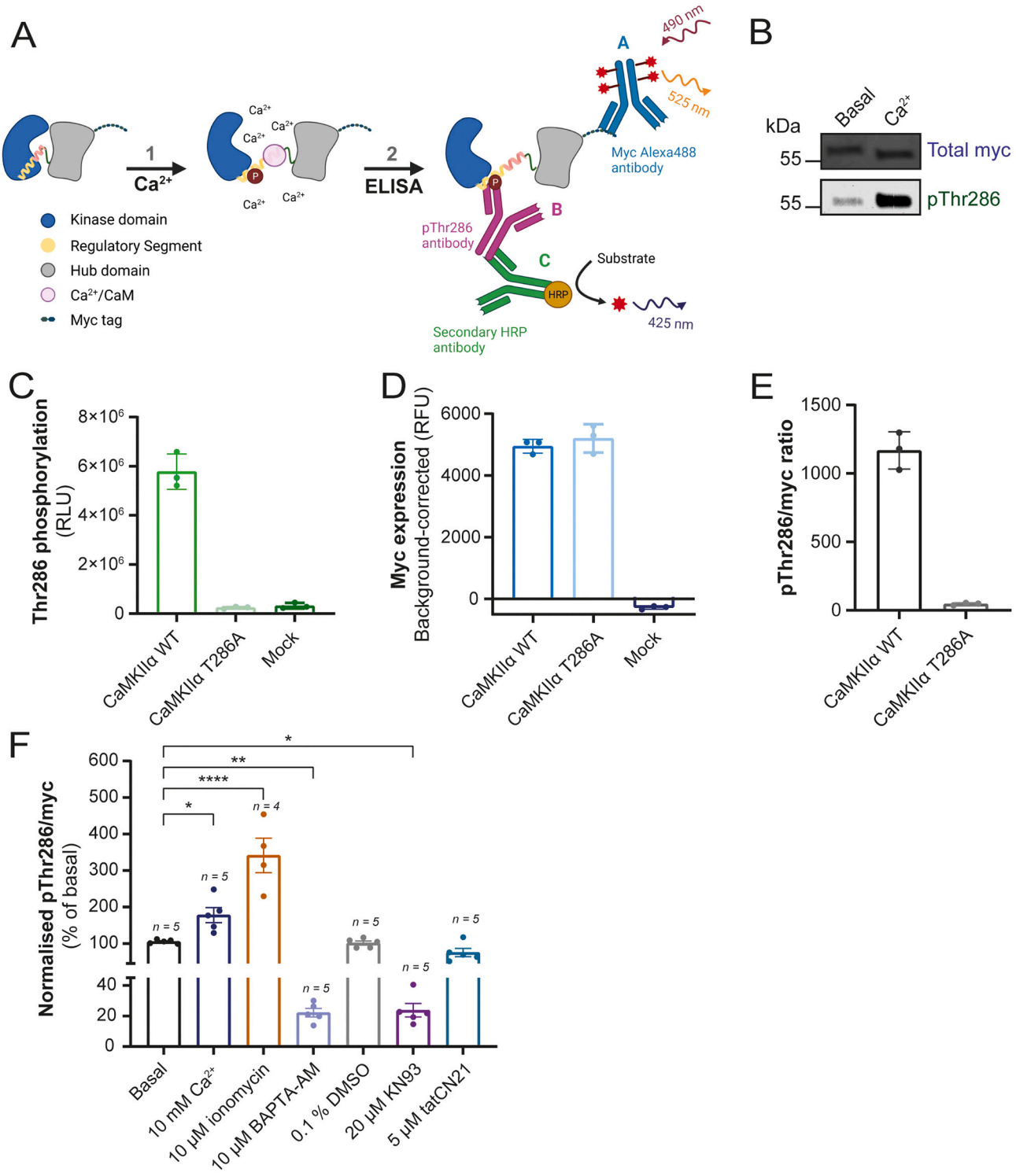

Fig. 1. ELISA-based autophosphorylation assay for CaMKIIα detects changes in Thr286 phosphorylation. (A) Principle behind the adapted ELISA using the multiplex technique (created with BioRender.com). After Ca^{2+} stimulation, the following ELISA results in binding of the primary anti-myc Alexa488 antibody (antibody A), the primary anti-pThr286 CaMKIIα antibody (antibody B) and the HRP-conjugated secondary anti-rabbit antibody (antibody C). Thus, multiplex detection can measure the expression levels by fluorescence and phosphorylation levels by chemiluminescence. HRP; horseradish peroxidase. (B) Representative western blot analysis of myc expression (fluorescence signal) and pThr286 level (chemiluminescence signal) in transfected HEK cells stimulated 1 h with buffer (basal) and Ca^{2+}. (C) Levels of pThr286 phosphorylation and (D) total expression levels in HEK cells transiently expressing wild type (WT) CaMKIIα, T286A CaMKIIα and mock-transfected cells using ELISA-based autophosphorylation assay after 1 h stimulation with buffer. RLU; Relative Luminescence Units, RFU; Relative Fluorescence Units. (E) pThr286/myc ratio for WT and T286A CaMKIIα expressing cells. Data are representative from three independent experiment performed in technical triplicates and shown as mean ± SD. (F) Validation of the ELISA-based autophosphorylation assay using Ca^{2+}, Ca^{2+}-regulating compounds (ionomycin and BAPTA-AM) and known inhibitors (KN93 and tatCN21). All autophosphorylation levels were first normalised to expression levels and then to basal and obtained after 1 h stimulation (except for ionomycin). Data are shown as mean percentage of basal ± S.E.M. pooled from four to five independent experiments (One-way ANOVA followed by Dunnett's multiple comparison to basal; $*P < 0.05$, $***P < 0.001$, $****P < 0.0001$); note the broken y-axis.

3.2. Validation of antibodies for ELISA

To validate the anti-Thr286 phospho-antibody in ELISA, we aimed at testing the specificity by examining Thr286 phosphorylation produced by CaMKIIα WT compared with the phosphodead CaMKIIα T286A mutant as previous validation using western blotting (Barria et al., 1997; Waxham, Aronowski, Westgate, & Kelly, 1990). As seen in Fig. 1C, cells transfected with WT CaMKIIα T286A produced a higher pThr286 response than cells transfected with CaMKIIα T286A mutant indicating a high specificity of the phospho-antibody. Additionally, the pThr286 response from CaMKIIα T286A cells was equal to the response from mock-transfected cells. The results in Fig. 1D confirmed equal expression of myc-tagged CaMKIIα in both WT and T286A CaMKIIα transfected cells. Moreover, the mock-transfected cells confirmed the absence of myc-tagged protein in HEK293T cells. In Fig. 1E, the pThr286/myc ratio showed 25 times higher pThr286 levels for WT compared with T286A CaMKIIα (ratios of 1168 and 47, respectively).

3.3. Assay validation by Ca^{2+} stimulation and pharmacological inhibitors

Next, we validated the autophosphorylation assay with known reference compounds. We observed similar total expression levels between the wells used to assess the effects of the different compounds in ELISA (data not shown). For pThr286, we found that increasing intracellular Ca^{2+} levels significantly increased the pThr286 levels: stimulation with 10 mM Ca^{2+} for 1 h increased pThr286 level to 178% of basal level, whereas ionomycin stimulation (10 μM for 2–3 min) increased pThr286 level to 341% of basal (Fig. 1F). Ionomycin is a ionophore used to increase intracellular Ca^{2+}-levels and to initiate CaMKIIα activation (Fukunaga, Rich, & Soderling, 1989; Li, Hidaka, & Wollheim, 1992). On the contrary, chelation of Ca^{2+} with the cell-permeable BAPTA-AM reduced the phosphorylation level to 21% of basal. Previous studies used EGTA for the chelation of Ca^{2+}-ions, but we preferentially used BAPTA-AM due to its faster reaction time and selectivity for Ca^{2+} over Mg^{2+} (Miller & Kennedy, 1986; Tsien, 1980). For the pharmacological inhibitors, 20 μM KN93 decreased pThr286 levels significantly to 27% of basal (Fig. 1F), whereas the cell-permeable, tat-fused version of the peptide CN21 (tatCN21), did not significantly affect pThr286 levels (Fig. 1F). Since BAPTA-AM and ionomycin were dissolved in DMSO, a control (0.1% DMSO) was included, which did not affect pThr286 levels (Fig. 1F).

3.4. Concentration-response curves for Ca^{2+} and pharmacological inhibitors

To demonstrate further the usefulness of the assay, we conducted concentration-response curves of selected CaMKIIα ligands. We assessed the Ca^{2+} concentration-dependence using 5 min stimulation (Fig. 2A) based on initial testing as we observed the highest pThr286 levels at this time point (data not shown). Due to inconsistent phosphorylation responses for the lowest Ca^{2+} concentration (0.01 mM Ca^{2+}), we normalised the remaining responses in the Ca^{2+} curve to this concentration instead of basal. From this curve, we obtained an EC_{50} value of 0.981 mM (Table 1). For the pharmacological compounds, we stimulated for 1 h to ensure cellular uptake of the inhibitor and normalised to the basal phosphorylation response. As expected, KN93 and AS100105 both inhibited pThr286 levels in a concentration-dependent manner (Fig. 2B). AS100105 is a relatively new ATP-competitive inhibitor of CaMKII (Neef et al., 2018). KN93 had an IC_{50} of 25 μM, whereas AS100105 was more potent with an IC_{50} 1.0 μM (Table 1). For tatCN21, we did not observe any inhibitory nor stimulating effect at the applied concentrations.

3.5. Autophosphorylation of patient-related mutations

Finally, to demonstrate the versatility of the developed assay we assessed pThr286 levels in CaMKIIα mutants identified in humans and known to cause intellectual disabilities (Küry et al., 2017; Stephenson et al., 2017). These point mutations (E183V, H282R and E109D) are reported to produce differential pThr286 responses, and therefore, are selected to display the sensitivity and detection range of the assay (Küry et al., 2017). Hereby, we confirmed the altered pThr286 response of the mutants, as we observed that the loss of function mutation, E183V, produced a significant decrease in pThr286 compared to WT CaMKIIα (Fig. 3A). For the two known gain of function mutants, H282R and E109D, the pThr286 levels were up to 5- and 9-times higher than WT, respectively. As depicted in Fig. 3B, two of the residues are located in the kinase domain with the E183 residue at the top and the E109 at the bottom, and therefore, they are most likely affecting the catalytic activity of the kinase. The last residue, H282 is located in the bottom of the

Table 1
Stimulating effect of Ca^{2+}, and inhibiting effect of KN93 and AS100105 determined by ELISA-based autophosphorylation assay. EC_{50} and IC_{50} values were calculated from concentration-response curves.

Compound	EC_{50} (μM) [95% CI] (pEC_{50} ± S.E.M.)	IC_{50} (μM) [95% CI] (pIC_{50} ± S.E.M.)	n
Ca^{2+}	981 [581–1460] (3.0 ± 0.086)[a]	–	6
KN93	–	19 [9.8–29] (4.7 ± 0.17)	4
AS100105	–	0.54 [0.28–2.1] (6.3 ± 0.15)	3

[a] Ca^{2+} data were fitted to the value of 10 mM which was assumed to be the maximum response.

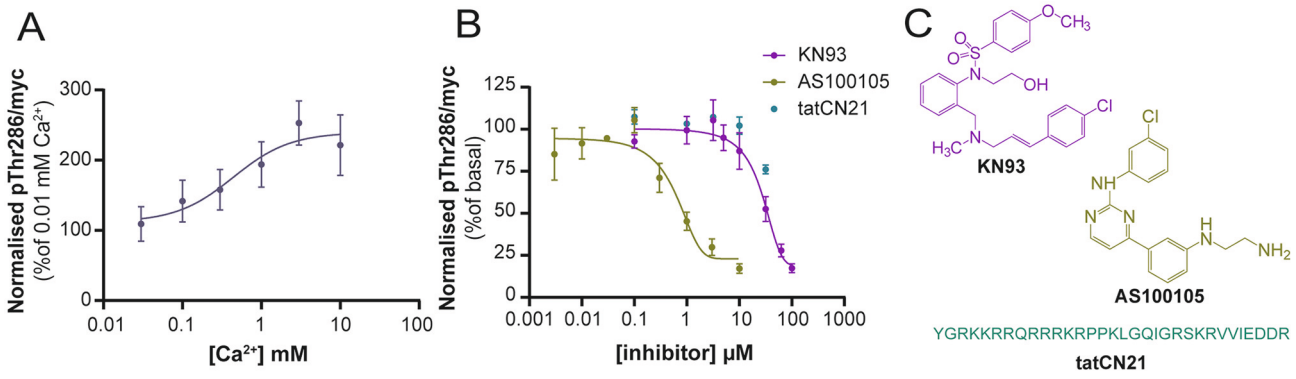

Fig. 2. Concentration-dependent response curves of Ca^{2+}, KN93, AS100105 and tatCN21 at CaMKIIα pThr286 levels. CaMKIIα was stimulated with increasing concentrations of (A) Ca^{2+}, (B) KN93, AS100105 and tatCN21 (*note:* inhibitors in the absence of added Ca^{2+}). Responses are normalised to cells treated with buffer (basal) or 0.1 mM Ca^{2+} for the Ca^{2+} curve (*see Materials and Methods*). Data are representative of three or six independent experiments performed in technical triplicates and shown as mean values ± SD. (C) Chemical structures of the CaMKII inhibitors, KN93 and AS100105 and the peptide sequence of tatCN21.

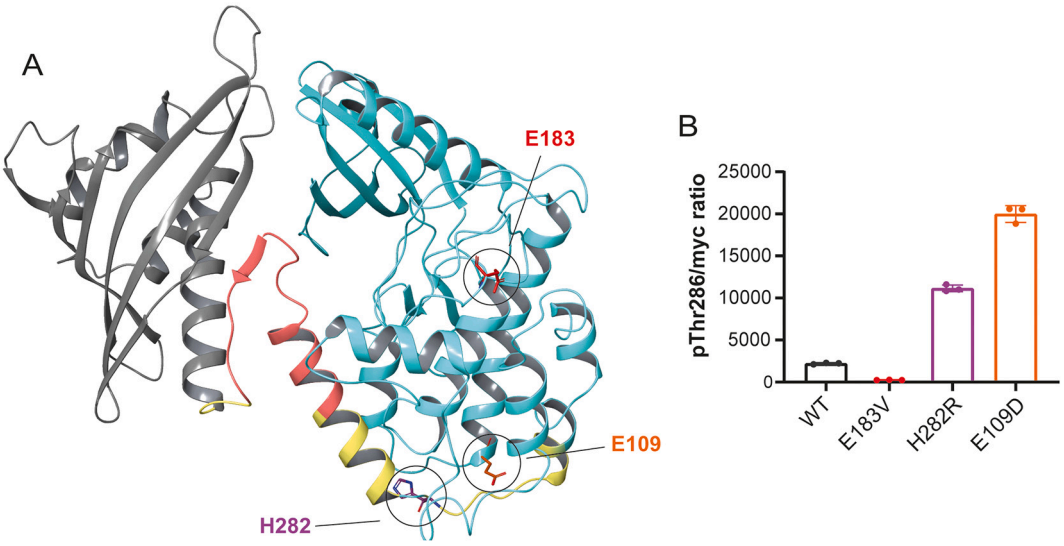

Fig. 3. Altered Thr286 autophosphorylation responses in patient-related CaMKIIα mutants. (B) Levels of Thr286 autophosphorylation produced by wild type CaMKIIα (WT, black), and the CaMKIIα mutants, Glu183Val (E183V, red), His282Arg (H282R, purple) and Glu109Asp (E109D, orange) after 1 h stimulation with buffer (basal) using the ELISA-based assay. Data are representative from three independent experiments performed in technical triplicates and shown as mean bar graph ± SD. (B) Protein structure of CaMKIIα showing the location of the mutations. The structure includes the kinase domain (in turquoise), the regulatory domain (in yellow) including the calmodulin binding site (in red) and the assembly/hub domain (in grey) (PDB ID: 3SOA). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

regulatory segment, close to the phosphorylation site at Thr286.

4. Discussion

Autophosphorylation of Thr286 is crucial for CaMKIIα activity and is a key point of regulation for downstream effects in several physiological and pathophysiological conditions (Coultrap et al., 2011; Küry et al., 2017; Lisman, Schulman, & Cline, 2002). Thus, compounds able to regulate CaMKII activity have great potential for treatment of CaMKIIα malfunction. An example of this is the anti-ischemic action of the peptide tatCN21 in cerebral ischemia (Vest, O'Leary, Coultrap, Kindy, & Bayer, 2010). In the present study, we present a 96-well based assay suitable for pharmacological identification and characterisation of compounds that affect CaMKIIα Thr286 autophosphorylation using overexpressing HEK293T cells. To our knowledge, this is the first assay reported to use whole-cell stimulation and in-well ELISA multiplex detection of CaMKIIα autophosphorylation. Previous studies have used purified protein or cell lysate followed by western blotting or radiolabelled ATP (Barria et al., 1997; Bradshaw, Kubota, Meyer, & Schulman, 2003; Hudmon, Aronowski, Kolb, & Waxham, 1996; Sumi et al., 1991; Urakubo, Sato, Ishii, & Kuroda, 2014; Vest, Davies, O'Leary, Port, & Bayer, 2007). Using the developed assay, we enable medium-throughput testing of CaMKIIα ligands and concentration-response determinations. The assay was based on a commercially available, immunochemical kit from LSBio; however, improved in terms of usefulness of the assay by targeting a c-terminal myc tag with a fluorophore-conjugated antibody. Thereby, we present a simple and convenient way to correct for well-to-well variations.

An ELISA-based assay requires antibodies with a high specificity. The phospho-specific antibody we used was previously demonstrated to bind to the denatured, unfolded protein in western blotting (Lu et al., 2020; Shrestha, Sultana, Lee, & Ogundele, 2019). Our results indicate that the antibody also recognises and binds the protein in its native, folded form thereby expanding its applicability. Moreover, the antibody detected the same phosphorylation pattern for the patient-related mutations (E109D, E183V and H282R) and the phosphodead mutant (T286A) in ELISA as previous studies using western blotting (Küry et al., 2017). As these mutants have different phosphorylation responses compared with wild type, this indicates a different conformation or assembly of the protein that is still recognised by the antibody. This high specificity underscores the suitability of the presented ELISA as a potential biochemical tool for addressing clinical questions. Besides identifying malfunctional CaMKIIα mutants, the assay method may be further used to test for potential drug effects in specific patient variants. Also, the assay could be adapted to identify inhibitors that work under pathophysiological conditions such as ischemia or excitotoxicity, characterized by Ca^{2+} overload and autonomous levels of Thr286 autophosphorylation. 22,23].

Using Ca^{2+} and Ca^{2+}-regulating compounds (ionomycin and BAPTA-AM), we successfully validated the assay and its applicability. Moreover, the assay provided high enough sensitivity to conduct concentration-response curves of Ca^{2+} and determine the stimulating effect of the ion despite some variations. These variations may arise due to differences in intracellular Ca^{2+} and fluctuating endogenous levels of ATP and CaM. Moreover, we were able to use the assay to characterise the effect of three different CaMKIIα ligands (KN93, AS100105 and tatCN21) on Thr286 autophosphorylation. To our knowledge, this study is the first to characterise the inhibitors in living whole-cells using an ELISA-based CaMKIIα autophosphorylation assay. Previously, the effect of KN93 on Thr286 phosphorylation was characterized using purified protein (Sumi et al., 1991). Our results confirm the inhibitory effect of KN93, however with a higher IC_{50} value (19 μM cf. the reported of 3 μM), which was expected due to the cellular system employed here (Sumi et al., 1991). Although KN93 was originally believed to bind the calmodulin binding site of CaMKII in the regulatory domain, a recent study suggests that KN93 is more likely binding to the Ca^{2+}/CaM complex (Sumi et al., 1991; Wong et al., 2019). Thereby, KN93 prevents the Ca^{2+}/CaM complex from binding to the regulatory segment and activating the kinase. This indirect inhibition of KN93 will inevitably result in decreased Thr286 autophosphorylation. The other known inhibitor of CaMKII, tatCN21, is reported to mainly inhibit substrate phosphorylation and only mildly affect Thr286 phosphorylation (Vest et al., 2007). This is explained by the site of interaction, i.e. the kinase domain T-site. This occupies the binding site for the regulatory domain but keeps the Thr286 residue accessible for phosphorylation. In correlation with this, we did not see an inhibitory effect of tatCN21 on Thr286 autophosphorylation. For the third inhibitor, AS100105, which binds to the ATP site in the kinase domain and hereby inhibiting the catalytic activity, we observed

an expected inhibition of Thr286 phosphorylation with an IC_{50} of 0.5 μM (Neef et al., 2018). Although this potency does not compare well with previously reported data, where AS100105 was tested in substrate phosphorylation assays (IC_{50} of 8 nM) (Neef et al., 2018), this decreased potency was expected due to higher levels of ATP in our experimental setup, the cell line used and the differences in assay read-out. Of interest, AS100105 inhibited Thr286 phosphorylation with a 38 times higher potency than KN93 (0.5 μM vs. 19 μM) and thus constitutes a suitable reference inhibitory tool compound or assay control. In general, our setup is highly relevant as it is in a cellular context and therefore resembles in vivo conditions more, which makes it relevant for drug development.

As described, the presented assay is widely applicable and can be used for screening of patient-related variants and/or for compound characterisation in a medium throughput fashion suitable e.g. for drug discovery. Because of extensive washing procedures, we ensured strong adherence of the HEK293T cells using PDL-coated assay plates. The heterologous expression system allows for flexibility in regards to testing different CaMKII isoforms and variants. However, the applied homomeric assembly of CaMKIIα does not resemble all subunit compositions, especially not the common assembly in hippocampal neurons of 9:3 with CaMKIIβ (Brocke, Srinivasan, & Schulman, 1999). As the subunit composition of the holoenzyme can affect the autophosphorylation response, it would be highly relevant to test other assemblies as well, e.g. the 9:3 assembly found in hippocampal neurons (Brocke et al., 1999). The whole-cell system requires that the applied compounds are able to permeate the cell membrane to reach the target. This can potentially limit the identification of potent and efficacious pharmacological compounds and influence the absolute pharmacological values. Om the other hand, it also presents a potential advantage for further drug development as the assay will pick up only cell-permeable compounds and thus mimic in vivo conditions better. Overall, we have set up a versatile and facile ELISA-based autophosphorylation assay for studying Thr286 phosphorylation of CaMKIIα as an improved alternative to western blotting.

Data availability

The data generated and analysed during the present study are available upon request.

CRediT authorship contribution statement

Line B. Palmelund: Conceptualization, Methodology, Investigation, Validation, Writing – original draft. **Geeske M. van Woerden:** Supervision, Writing – review & editing. **Hans Bräuner-Osborne:** Supervision, Writing – review & editing. **Petrine Wellendorph:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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