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CaMKIIδ interacts directly with IKKβ and modulates NF-κB signalling in adult cardiac fibroblasts
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ABSTRACT
Calcium/calmodulin dependent protein kinase IIδ (CaMKIIδ) acts as a molecular switch regulating cardiovascular Ca^{2+} handling and contractility in health and disease. Activation of CaMKIIδ is also known to regulate cardiovascular inflammation and is reported to be required for pro-inflammatory NF-κB signalling. In this study the aim was to characterise how CaMKIIδ interacts with and modulates NF-κB signalling and whether this interaction exists in non-contractile cells of the heart. Recombinant or purified CaMKIIδ and the individual inhibitory -κB kinase (IKK) proteins of the NF-κB signalling pathway were used in autoradiography and Surface Plasmon Resonance (SPR) to explore potential interactions between both components. Primary adult rat cardiac fibroblasts were then used to study the effects of selective CaMKII inhibition on pharmacologically-induced NF-κB activation as well as interaction between CaMKII and specific IKK isoforms in a cardiac cellular setting. Autoradiography analysis suggested that CaMKIIδ phosphorylated IKKβ but not IKKα. SPR analysis further supported a direct interaction between CaMKIIδ and IKKβ but not between CaMKIIδ and IKKα or IKKγ. CaMKIIδ regulation of IκBα degradation was explored in adult cardiac fibroblasts exposed to pharmacological stimulation. Cells were stimulated with agonist in the presence or absence of a CaMKII inhibitor, autocamtide inhibitory peptide (AIP). Selective inhibition of CaMKII resulted in reduced NF-κB activation, as measured by agonist-stimulated IκBα degradation. Importantly, and in agreement with the recombinant protein work, an interaction between CaMKII and IKKβ was evident following Proximity Ligation Assays in adult cardiac fibroblasts. This study provides new evidence supporting direct interaction between CaMKIIδ and IKKβ in pro-inflammatory signalling in cardiac fibroblasts and could represent a feature that may be exploited for therapeutic benefit.

Key words: Cardiovascular; Calcium/calmodulin dependent protein kinase II; Nuclear Factor kappa B; Inhibitory kappa B kinase; Inflammation

Abbreviations: CaMKII, Calcium/calmodulin dependent protein kinase; IKK, Inhibitory kappa B kinase; NF-κB, Nuclear Factor kappa B; IκB, Inhibitor Factor kappa B; CFs, cardiac fibroblasts; ECs, endothelial cells; SPR, Surface Plasmon Resonance; PLA, proximity ligation assay
1. Introduction

CaMKII is a multi-functional serine/threonine kinase which is now well recognised as being constitutively expressed in the heart (the δ isoform being predominant), where it serves to regulate normal calcium (Ca\(^{2+}\)) homeostasis, contractility, metabolism and gene expression. Crucially, CaMKIIδ becomes hyper-activated in pathophysiological conditions and this sustained activation is known to contribute to heart failure, arrhythmia and cell death [1]. Cardiovascular disease is a very complex and dynamic process involving not only the cardiac muscle, but also the vasculature and different cell types within both. Non-contractile cells of the heart, predominantly cardiac fibroblasts (CFs), play a key role in normal cardiac function through their fundamental role in extracellular matrix deposition as well as their ability to modulate contractile cell activity via secretion of bioactive molecules. Under conditions of stress however, these cells become hyperproliferative and contribute to fibrotic remodelling of the heart and contractile dysfunction [2]. The possibility that CaMKIIδ may have an overarching role in non-contractile cell types of the cardiovascular system in health and disease has yet to be established.

A key component in the progression of cardiovascular disease is chronic inflammation. Levels of inflammatory mediators increase as cardiac function deteriorates and these are significantly elevated in the failing heart [5]. This chronic inflammatory phenotype is also evident in vascular disease, which subsequently leads to endothelial dysfunction underpinning pathologies such as atherosclerosis and hypertension [6,7]. The transcription factor Nuclear Factor kappa B (NF-κB) has been identified as an important mediator of cardiovascular inflammatory status, where activation of the NF-κB signalling pathway has been implicated as a causal event in cardiac hypertrophic progression [8] and vascular endothelial dysfunction [9]. Activation of NF-κB requires phosphorylation and release of an associated inhibitory protein IκB. The IκB protein is subsequently degraded and NF-κB can translocate to the nucleus and induce gene transcription. IκB degradation can therefore be used as a marker of activation of NF-κB signalling. The enzymes that cause phosphorylation and release of IκB are known as IκB kinases (IKKs) and these form the IKK complex, composed of IKKα, β and γ. Both α and β are active kinases whereas IKKγ (also known as NEMO-NF-κB essential modulator) functions solely as a scaffold component of the complex [10]. Although the IKKs are well defined as key modulators of IκB degradation, the potential
exists that other kinases may mediate this pathway either via direct phosphorylation and modulation of IKK activity or via direct phosphorylation of IκB. Observations that inhibitors of calmodulin led to prevention of IκB phosphorylation prompted speculation that CaMKII (a calmodulin-dependent kinase) may be involved in NF-κB modulation [11]. Several studies have suggested that CaMKII may regulate NF-κB and that this may occur at the level of the IκB kinase [12,13]. A link between these pathways in the cardiovascular system has also been made. Studies performed in neonatal cardiac myocytes have shown that overexpression of CaMKIIδ increases NF-κB activity while inhibition of CaMKII (using KN-93) attenuates activity [14]. Increased NF-κB activity in response to activated CaMKIIδ leads to hypertrophic responses in these cells. More recent work has shown that CaMKII inhibition with AC3-I (an inhibitory peptide) markedly reduces upregulation of pro-inflammatory genes induced by myocardial infarction [15]. One of these genes (complement factor B (Cfb)) was induced through activation of the NF-κB pathway by CaMKII. Suppression of NF-κB activity by AC3-1-mediated inhibition of CaMKII led to reduced Cfb expression and sarcolemmal injury following infarction. A separate in vivo study in wild type and cardiac-specific CaMKIIδ knockout mice has demonstrated that CaMKIIδ mediates NF-κB activation in cardiac myocytes and the development of apoptosis and inflammation following ischaemia-reperfusion [16]. Evidence strongly suggests that CaMKIIδ modulates pro-inflammatory NF-κB signalling in the contractile cells of the heart. Could this relationship also exist in the non-contractile cardiac fibroblasts and, if so, how is it mediated?

Here for the first time, we investigate the potential for CaMKIIδ to modulate pro-inflammatory NF-κB signalling in adult cardiac fibroblasts. Using recombinant proteins for Surface Plasmon Resonance (SPR) along with autoradiography analysis, we have investigated whether CaMKIIδ may modulate NF-κB signalling by targeting specific components of the IKK complex. Building upon this work, we have shown that CaMKII modulates NFκB signalling in primary adult rat cardiac fibroblasts. We have provided novel evidence for interaction of CaMKIIδ specifically with IKKβ in these cells and for the first time, have highlighted the potential for CaMKIIδ modulation of NF-κB signalling at the level of IKKβ in these non-contractile cells of the heart.
2. Methods

2.1 Isolation of adult rat cardiac fibroblasts

Adult rats (male Sprague Dawley, ~350-400g) were used to isolate CFs under sterile conditions. Procedures complied with the ARRIVE guidelines and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and Directive 2010/63/EU of the European Parliament. Hearts were dissected immediately following termination by intravenous injection with pentobarbital sodium (10µl/g weight of animal; Euthatal) and heparin (0.1µl/g weight of animal; 5000 units/ml). For CF isolation, a chunk dissociation protocol using collagenase type II was followed as previously described [17]. Cells were subsequently collected by centrifugation and re-suspended in Dulbecco’s modified Eagle medium (DMEM; GIBCO 21969) supplemented with 20% foetal calf serum (heat-inactivated; GIBCO), 1% (v/v) L-glutamine (GIBCO) and 4% (v/v) Pen/Strep. Cells were grown to 60-80% confluence in T75 flasks at 37°C in an atmosphere of 5% CO₂ with media changed every second day. Cells were only used for experimental work up to passage 3.

2.2 Preparation of cardiac homogenates

Hearts were isolated, finely minced on ice and whole tissue homogenates prepared as previously described [18].

2.3 Immunoblotting

Cells grown on a 12-well plate 48h prior to assay were solubilised with addition of 150 µl 1X Laemmli sample buffer containing 75mM DTT. Cells were scraped from the bottom of wells and DNA disrupted by dispersion through a 28G needle. Samples were then heated to 70°C for 10 min prior to loading onto either 7% (v/v) Tris-acetate or 10% (v/v) Bis-Tris polyacrylamide gels (Invitrogen) and analysed by SDS-PAGE using the Invitrogen NuPAGE® system. Following transfer of proteins to nitrocellulose, membranes were blocked in 5% (w/v) nonfat dry milk diluted in TBST buffer (20 mM Tris-base, 137 mM NaCl and 0.1 % (v/v) Tween 20, pH 7.6) for 1h at room temperature, then incubated overnight at 4°C with antibodies against IKKα, IKKβ (both mouse monoclonal antibody 1:500 (Calbiochem (#OP133 and OP134 respectively), IκBα (rabbit polyclonal 1:1000 (Cell Signalling Technology #4814)) or CaMKIIδ (rabbit polyclonal antibody 1:1000, custom-made against the C-terminus of CaMKIIδ (Eurogentec)) prepared in 5% (w/v) BSA in TBST. All primary
antibodies were chosen based on their ability to react specifically with rat. This was followed by incubation for 2 h at room temperature with either goat anti-mouse IgG-HRP (Jackson Immunoresearch #715-035-150) or goat anti-rabbit IgG-HRP (Sigma-Aldrich #A6154) diluted 1:5,000 and 1:10,000 respectively and development using the ECL detection system (Thermo Fisher #32106).

For quantitative analysis of IκBα, GAPDH was also measured as an internal loading control for these samples (mouse monoclonal 1:40,000 (Abcam #ab8245) followed by anti-mouse IgG-HRP 1:5,000). Both IκBα and GAPDH were probed on the same blot. After incubation in ECL reagent and development of films, signals from the scanned immunoblots were quantified by densitometry using a GS-800 densitometer and Quantity One Image software (version 4.5.2, BioRad) and signals expressed as ratios (IκBα:GAPDH).

2.4 Immunofluorescence

CFs, aortic endothelial cells and colonic vascular smooth muscle cells were grown on coverslips until confluent and fixed by aspirating the culture medium and applying 4% (v/v) paraformaldehyde for 10 min. Cells were then exposed to cold methanol for 10 min. This was followed by washing 3 times with PBS and permeabilisation with Triton X-100 (0.01% (v/v)) for 10 min. Non-specific binding was blocked using 1% (w/v) Bovine Serum Albumin (BSA) in Phosphate Buffered Saline (PBS) for 1h at room temperature followed by direct addition of primary antibody overnight at 4°C. Primary antibodies (vimentin (abcam #Vim3B4), α-smooth muscle actin (Sigma-Aldrich #A5228), CD31 (Sigma-Aldrich #P8590) and von Willebrand Factor (abcam #ab194405)) were prepared with 1% (w/v) BSA in sterile PBS at a 1:100 dilution. Following washes in PBS anti-mouse IgG-FITC (Sigma-Aldrich#FO257) (1:1,000) and anti-rabbit IgG- Alexa Fluor 594 (Thermo Fisher #A11032) (1:400) respectively were then applied for 1h at room temperature. After washing, coverslips were mounted on to slides using Mowiol® (Sigma-Aldrich) mounting medium containing DAPI (Vecta laboratory) and stored at 4°C in the dark until they were viewed and photographed. The DAPI counter-stain in the mounting medium stained the cell nuclei blue. Slides were then viewed using a Zeiss Axio Imager microscope (Carl Zeiss MicroImaging GmbH, Germany) under a 20x and 10x lens (NA 0.50). Fluorescence was stable allowing digital images to be captured using AxioVision v.4.6 (Zeiss, Germany).
2.5 Autoradiography

Assay components consisted of 30 ng CaMKIIδ and 100 ng IKKα (GST-tagged) or IKKβ (His-tagged) (all Human recombinant, Millipore). Autocamtide II (50 μM) (Millipore), the CaMKII peptide substrate, was included as a positive control for CaMKII phosphorylation. In some samples, Autocamtide Inhibitory Peptide (AIP) (Calbiochem) was included to inhibit CaMKII activity. Components were mixed with 2 μg calmodulin (Calbiochem), 1.5 mM CaCl₂ (final concentration) and 50 μM ATP/2 μCi [γ-³²P]ATP (final concentration (PerkinElmer)) and incubated at 30°C in assay dilution buffer (ADB) for 30 min. ADB consisted of 20 mM HEPES (pH 7.6), 25 mM MgCl₂, 2 mM DTT, 20 mM β-Glycerophosphate and 0.5 mM sodium orthovanadate. Reactions were terminated on ice by addition of Laemmli sample buffer containing 32 mg/ml DTT. Samples were loaded onto a 10% (v/v) acrylamide gel and analysed by SDS-PAGE using the BioRad system. Gels were subjected to electrophoresis at a constant voltage of 140 mV for 1.5 h and were then left overnight in 20 ml fixer solution (40% (v/v) methanol and 10% (v/v) acetic acid). After 24 h gels were rehydrated in dH₂O for ~30 min before the drying of gels at a medium heat for ~1 h. Protein phosphorylation was then examined by autoradiography.

2.6 Surface Plasmon Resonance

Studies were performed using a Biacore 3000 instrument and were based on an SPR protocol described previously [19]. Briefly, CaMKIIδ (Human recombinant, Millipore) was immobilised on to a CM5 sensor chip at ~ 7 pg/mm². Immobilisation was performed at 25°C at a flow rate of 5 μl/min using SPR running buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 0.005% (v/v) surfactant P20). All experiments used BSA as a negative control with equivalent amounts of protein immobilised under exactly the same conditions as for CaMKIIδ. Prior to starting any experiments, the system was primed with binding buffer (running buffer + 5 mM MgCl₂, 1.5 mM CaCl₂ and 2 mM DTT). Calmodulin (CaM), IKKα (GST-tagged), IKKβ (His-tagged) and IKKγ (GST-tagged) (all Human recombinant, Millipore) were prepared in binding buffer at final concentrations of 0.0125 - 0.2 μM in running buffer and were injected randomly for 3 min (association phase) on to the flow cell at a rate of 30 μl/min followed by a 15 min dissociation period. In experiments investigating the effect of Ca²⁺, imidazole and ATP on protein-protein interactions, SPR running buffer had no added CaCl₂ or contained 100 mM imidazole or 1 mM ATP respectively. Interactions were followed in real time on a sensorgram. After each measuring cycle, the sensor chip surface was regenerated with 2 x 30
s pulse of regeneration buffer containing 2 mM EDTA and 1 M NaCl, followed by a 2 min stabilisation period before another injection. Data analysis was carried out using the BIAevaluation version 3.2 (Biacore).

2.7 IkBα degradation

Prior to stimulation, CFs (passage 1-2) at 80-100% confluency were serum starved for 24 h. CFs were treated with lipopolysaccharide (LPS, 5μg/ml) over a time course up to 120 min in the presence or absence of AIP (5μM) (Merck). Where appropriate, CFs were pre-treated with AIP for 1h prior to stimulation. After specified times, reactions were terminated on ice via aspiration of media and addition of 150 μl 1x Laemmli sample buffer containing 75mM DTT. Cells were scraped and DNA disrupted by aspiration using a 28G needle. Samples were analysed by quantitative immunoblotting.

2.8 Proximity Ligation Assay

The Duolink® in situ Orange Proximity Ligation Assay (PLA) was used to detect CaMKII-IKKβ interactions in CFs following manufacturers’ protocol (Sigma-Aldrich) and as previously described [20]. Optimisation of antibodies was performed initially by immunofluorescence (IKKβ, 1:200, mouse monoclonal (Abcam); CaMKIIδ, 1:50, rabbit polyclonal (Eurogentec custom-made); ox-CaMKII, 1:50, rabbit polyclonal (GeneTex). For subsequent PLA experiments, IKKβ and ox-CaMKII primary antibodies were used prior to Duolink® probe incubation. Image analysis (images captured by a confocal microscope equipped with LSM510 software) was performed using Image J software [https://imagej.nih.gov/ij/] as previously described [20]. The best threshold was set and images converted to black and white. Images were analysed by applying the region of interest (ROI) and fluorescent dots measured with minimum and maximum pixel area size set and cells on the edge of the image excluded. Background (ROI using exactly the same settings in areas where there were no cells) was subtracted for each of the experimental conditions and comparisons made across controls (no primary antibody), IKKβ alone, IKKβ plus CaMKIIδ and IKKβ plus ox-CaMKII.

2.9 Statistical analysis

Where appropriate, data were expressed as mean values ± SEM of n observations, where n represents the number of samples. Comparisons were assessed using the student’s t-test or
one- and two-way ANOVA with Tukey’s post-test as indicated. Differences were considered statistically significant when p < 0.05. Where no p value is shown, p > 0.05.

3. Results

3.1 CaMKII phosphorylation of specific IKK components of the NF-κB signalling pathway

Initial experiments set out to determine whether CaMKIIδ interacted with and/or phosphorylated selected IKK components of the NF-κB cascade. The use of recombinant or purified proteins was employed to explore the potential for interaction. To assess whether CaMKIIδ interacts with and phosphorylates specific IKK components of the NF-κB signalling pathway, CaMKIIδ (30ng) was incubated with either IKKα or IKKβ (each at 100ng) in the presence of [{^{32}P}]ATP (Fig. 1A). The CaMKII peptide substrate autocamtide II (50μM) has previously been used as a positive substrate control to demonstrate CaMKII activity using the same amounts/activities of CaMKIIδ and [{^{32}P}]ATP [19]. Incubation of CaMKIIδ with IKKα and β in the presence of ATP results in phosphorylation of all three of these protein components (Fig. 1A, lanes 3 and 4). It is important to note that both CaMKIIδ and the IKKs are capable of autophosphorylation therefore the intensity of phospho signal could reflect differences in CaMKIIδ-mediated phosphorylation (of CaMKIIδ itself (autophosphorylation) and of the IKKs) and/or could show evidence for IKK autophosphorylation. These reactions were therefore repeated in the presence of the CaMKII inhibitor peptide AIP (5μM). AIP will inhibit CaMKII and therefore, can potentially inhibit CaMKIIδ-mediated phosphorylation of both CaMKIIδ and the IKKs but will not affect autophosphorylation of IKKs, so any decrease in signal intensity following AIP treatment will only be due to CaMKIIδ-mediated effects. As expected, inclusion of AIP markedly reduced any autophosphorylation of CaMKIIδ (Fig. 1A, lane 2). Interestingly, the addition of AIP also significantly inhibited the phosphorylation signal associated with IKKβ (Fig. 1A, lane 6) but not that associated with IKKα (Fig. 1A, lane 5). Importantly, all samples were run on the same gel (Fig. 1A shows relevant sections taken from the same gel) and therefore were exposed for the same length of time. This data highlights the possibility that CaMKIIδ may phosphorylate/interact selectively with IKKβ. In order to discount any contamination of the protein preparations used, purity of these preparations is shown in an accompanying coomassie stained gel (Fig. 1B).
3.2 Surface Plasmon Resonance analysis of CaMKIIδ interaction with IKKs

In order to assess the potential for direct interaction between CaMKIIδ and individual components of the IKK complex (IKKa, β and γ), Surface Plasmon Resonance (SPR) studies were performed. Previous work by our group using SPR has already established the immobilisation and buffer conditions required for analysis of CaMKIIδ-protein interactions [19]. Using the same established conditions for immobilisation of CaMKIIδ onto the sensor chip, the IKK complex components were individually tested as analytes and any binding between IKK and CaMKIIδ measured as a change in refractive index as previously explained [19]. All experiments with the experimental protein (CaMKIIδ) were performed in parallel with a control protein (albumin) which was immobilised to the same final density on a second flow cell of the sensor chip. Any binding of the analyte of interest to the control protein was automatically subtracted from the experimental response to take into account any non-specific binding.

Initial control experiments using calmodulin as the analyte were performed to check that CaMKIIδ was properly immobilised to the sensor chip surface and capable of binding. Increasing concentrations of calmodulin (ranging from 0.0125 – 0.2μM) applied to the sensor chip surface, resulted in a concentration-dependent increase in binding, reflected in an increased response difference (ranging from ~25RU for 0.0125μM to ~70 RU for 0.2μM calmodulin) (Fig. 2A). This was consistent with our previous work [19] and confirmed that CaMKIIδ was immobilised sufficiently to the chip surface and capable of interaction. IKKa, β and γ were then individually prepared as analytes and each protein was injected over the sensor chip at a range of concentrations (0.0125 – 0.2μM) in a random manner. This concentration range was chosen based on previous SPR studies which have investigated protein-protein interactions with components of the NF-κB signalling pathway [22]. Interestingly, injection of IKKβ (Fig. 2B) showed a very clear positive response difference, which increased in a concentration-dependent manner (increasing from a difference of ~10RU for 0.0125μM IKKβ to ~ 170RU for 0.2μM IKKβ). This response is much greater than that observed for the equivalent concentrations of calmodulin and reflects a strong capacity for direct interaction between the two proteins (CaMKIIδ and IKKβ).

Neither IKKa nor IKKγ (Fig. 2C and 2D respectively) showed any positive response difference when injected across the chip surface at any of the concentrations tested, suggesting there is no direct interaction with CaMKIIδ. IKKγ at higher concentrations (0.1
and 0.2μM) actually showed a negative change in the response which probably reflects differential refractive index changes at flow cells 1 and 2. It is worth noting that this effect was only observed for the two highest concentrations of the analyte.

3.3 Assessment of conditions for CaMKIIδ – IKKβ interaction

Having established that there is a direct interaction between CaMKIIδ and IKKβ, we then explored some key regulatory parameters that might affect this. The IKKβ used in these experiments was HIS-tagged. To verify that the observed interaction was not via the HIS-tag of IKKβ, additional experiments were performed in the presence of 100mM imidazole. Imidazole is an analogue of histidine and traditionally used to modulate non-specific binding when purifying HIS-tagged proteins with affinity gels. As shown in Figure 3A, treatment with imidazole had virtually no effect on IKKβ binding to the CaMKIIδ sensor chip (~10RU for 0.0125μM IKKβ to ~140RU for 0.2μM IKKβ) and showed a similar concentration-dependent profile when compared with binding in the absence of imidazole (Fig. 2B). If IKKβ was interacting with CaMKIIδ via the HIS-tag, then treatment with imidazole should displace bound IKKβ from the sensor chip, leading to a dramatic reduction in the observed response. The lack of effect of imidazole suggests CaMKIIδ is interacting with IKKβ at a site distinct from the HIS-tag.

Since both CaMKIIδ and IKKβ are kinases and capable of autophosphorylation, the effect of adding ATP to the running buffer was tested (Fig. 3B). It could be that phosphorylation of either or both proteins may affect the interaction. Although there was some reduction in absolute response in the presence of ATP (120RU (+ATP) v’s 170RU (-ATP) for 0.2μM IKKβ), binding was still observed and in a concentration-dependent manner as previously (Fig. 2B). This suggests addition of ATP and possible subsequent phosphorylation does not prevent interaction but could affect the extent of interaction. Since both proteins are capable of phosphorylation, this effect could be at the level of CaMKIIδ and/or IKKβ.

Finally, the possibility that the interaction may be Ca²⁺-dependent was examined. Removal of Ca²⁺ from the running buffer had no effect on the CaMKIIδ – IKKβ interaction (Fig. 3C) with concentration-dependent increases in binding occurring as before and a maximum response difference of ~160RU for 0.2μM IKKβ.

3.4 Characterisation of CaMKIIδ and NF-κB signalling components in cardiac fibroblasts
Following on from the use recombinant proteins, follow up experiments were conducted in CFs. CFs were isolated under sterile conditions as described in the methods section. Typical CF preparations are shown in Figure 4A(i). Lack of contamination of CFs by smooth muscle cells or myofibroblasts was demonstrated by immunostaining with alpha-smooth muscle actin which did not stain fibroblasts (Fig. 4A (ii)) but did stain colonic smooth muscle cells (Fig. 4A (iii)). Cells were also stained for vimentin which is a recognised non-selective marker for fibroblasts (Fig. 4B (iv)). To determine that CFs were appropriate for studying potential interaction between CaMKIIδ and NF-κB signalling components, it was important to demonstrate that the relevant proteins were expressed in these cells. Whole cardiac homogenised tissue (WH) was used as a positive control since we know these proteins are expressed in the cardiomyocyte component of homogenates. Immunoblotting shows that CaMKIIδ, the active kinases IKKα and IKKβ as well as IκBα proteins are all clearly expressed in adult CFs (Fig. 4B).

3.5 A functional role for CaMKII in NF-κB signalling – effect of CaMKII inhibition on IκB degradation in cardiac fibroblasts

In order to establish whether CaMKII plays a role in the regulation of NF-κB signalling, the effect of CaMKII inhibition upon IκBα degradation was assessed. LPS is a potent activator of the NF-κB pathway and was therefore used in this study [22,23]. The selective CaMKII inhibitor peptide AIP was used (5μM for 1h) prior to LPS (5μg/ml) stimulation of CFs for the indicated periods and degradation of IκBα monitored by immunoblotting. Treatment with LPS resulted in significant IκBα degradation in CFs over the time course tested (0-60 mins). When cells were pre-treated with AIP (5μM), IκBα degradation in response to LPS was significantly reversed (Fig. 4C(i)) and this was sustained at 30 minutes, 45 minutes, and in some cases 60 minutes of stimulation (mean densitometric data from four separate experiments is shown in Figure 4C(ii)). We have previously shown that the concentration of AIP used here results in 95-100% inhibition of CaMKII activity in rabbit cardiac preparations [19]. This data highlights for the first time the potential for CaMKIIδ in regulating agonist-stimulated IκBα degradation and corresponding activation of NF-κB signalling in adult CFs.

3.6 CaMKIIδ interacts with IKKβ in cardiac fibroblasts

Given the results obtained with SPR showing that CaMKIIδ can bind selectively to IKKβ, and in light of the fact that CaMKIIδ influences IκBα degradation in CFs, the possibility that a
CaMKIIδ-IKKβ interaction may exist in these cells was explored. In order for interaction to be detected natively in CFs, Proximity Ligation Assays (PLAs) were employed using primary antibodies against the two proteins of interest (CaMKII and IKKβ).

Initial work was conducted to optimise the primary antibodies using indirect immunofluorescence prior to conducting PLA experiments. Signals for IKKβ were clearly detected (Fig. 5A) however signals obtained using the custom-made CaMKIIδ antibody were very weak (Fig. 5B). Whilst both IKKβ and CaMKIIδ antibodies used here had previously been shown to work well for immunoblotting (Fig. 4) it was clear that their utility for immunofluorescent-based imaging studies was limited. While the IKKβ antibody proved suitable for PLA-based experiments, the CaMKIIδ antibody did not. The approach was therefore taken to use a CaMKII antibody directed against the active (oxidised) form of the protein (ox-CaMKII). Although it is likely that there will be some ox-CaMKII present in cells under basal resting conditions, it is much more likely that levels will rise following activation, particularly with a stimulus that drives oxidation. In order to promote oxidation, H2O2 was used to treat CFs and the ox-CaMKII signal is shown in immunoblots from CFs following treatment with H2O2 (1-10μM) (Fig. 5C). Cells were subsequently exposed to 10μM H2O2 prior to PLA. The fluorescence signal for ox-CaMKII from CFs proved to be much greater than that obtained using the CaMKIIδ antibody (Fig. 5D).

Based on these findings, PLAs were conducted using antibodies directed against ox-CaMKII and IKKβ. In CFs treated with H2O2, the fluorescence intensity following incubation with both antibodies was significantly greater than incubation with either IKKβ alone or both antibodies in unstimulated cells (Fig 6A and 6B). This suggests interaction between the antibodies (and hence proteins) of interest when cells had been stimulated with H2O2 signifying greater levels of interaction in activated cells (Fig 6B).

4. Discussion

There is increasing evidence for a link between CaMKII and NF-κB signalling, however our understanding of this link in the heart remains limited [15, 16, 24]. This is the first study to present evidence for a direct interaction between a specific component of the pathway (IKKβ) and CaMKIIδ and to demonstrate a functional interaction in CFs. These cells contribute to contractile and non-contractile function in normal and diseased heart [25]. Crucially, there is strong evidence that CaMKII plays a key role in pathophysiological events in CFs [25, 26] yet nothing is known of the potential for CaMKII modulation of pro-inflammatory signalling in
these cells. Given that inflammation is a common component of the onset and progression of cardiovascular disease, and that both CaMKII and NF-κB signalling are central to the inflammatory process in cardiac and vascular cells [27,28], it is pertinent to understand (i) whether these pathways are inter-dependent in different cells of the heart and (ii) how any potential interaction between the two may occur.

To identify a specific target or targets for CaMKII activity within the NF-κB pathway, our initial work used recombinant or purified proteins. Autoradiography analysis of IKK phosphorylation by CaMKIIδ was performed in the presence and absence of AIP. Interestingly, only the phosphorylation associated with IKKβ (and not IKKα) was significantly reduced in the presence of AIP. This suggests that the phosphorylation signal associated with IKKα is due primarily to autophosphorylation but the phosphorylation associated with IKKβ is at least partly due to CaMKIIδ-mediated phosphorylation (Fig. 1). This indicates that CaMKIIδ may preferentially associate with and phosphorylate IKKβ as a means of modulating NF-κB signalling. This interpretation was strengthened by SPR data which clearly showed a selective interaction between IKKβ and CaMKIIδ, but not between either IKKα or IKKγ and CaMKIIδ (Fig. 2). The kinetics of IKKβ binding were rapid with a slower dissociation rate, similar to that observed with calmodulin. Importantly, the extent of binding of IKKβ to CaMKIIδ was greater than that observed for calmodulin binding to CaMKIIδ (~160RU c.f. ~70RU for 0.2μM IKKβ and 0.2μM calmodulin respectively). This is the first evidence for a direct, selective and high affinity interaction between CaMKIIδ and IKKβ. Although autoradiographic analysis suggests CaMKII can phosphorylate IKKβ, the interaction between these proteins does not appear to be ATP-dependent, at least when analysed by SPR (Fig. 3).

Previous work has demonstrated the prevalence of IKKβ in (i) induction of NF-κB activity and (ii) modulation of cardiac function. Importantly, for the majority of cellular agonists canonical NF-κB cannot be activated by agonist stimulation in IKKβ deficient cells and disruption of the IKKβ locus is lethal. In contrast, IKKα disruption does not abolish activation of the classical IKK complex that mediates canonical NF-κB [29], suggesting that IKKβ plays a more important role in canonical NF-κB activation than IKKα. In the heart, inhibition of IκB phosphorylation has been shown to reduce reperfusion injury [30] and specific targeting and inhibition of IKKβ has provided both acute and delayed cardioprotection [31, 32]. This has highlighted IKKβ as a potential therapeutic target that
could be used to modulate inflammation and cardiac damage following injury. The possibility that CaMKIIδ may modulate cardiac NF-κB pro-inflammatory signalling via interaction with IKKβ indicates an important link that could be used for more selective modulation of this inflammatory route. The interaction does not appear to be Ca\(^{2+}\)-dependent but could be affected to some extent by phosphorylation. Further work would be essential to map the specific site or sites of interaction between the two proteins to assess this in more detail.

The present study has shown that adult CFs express key components of NF-κB signalling, including the IKK complex. These cells also express CaMKIIδ (Fig. 4), which to our knowledge, although having been widely examined in the heart, has only received limited examination in adult CFs. Initial experiments using CFs examined the potential for interaction between CaMKII and NFκB signalling by measuring degradation of IκBα as a functional read-out of NF-κB activation. IκBα degradation was assessed in the presence and absence of CaMKII inhibition and using a potent agonist of NF-κB activation (LPS). For the first time we have demonstrated that inhibition of CaMKII (using AIP pre-treatment) prevents agonist-stimulated IκBα degradation/ NF-κB activation in these cells (Fig. 5). How CaMKII may target NF-κB signalling in CFs has not previously been explored however, based on our SPR data, the possibility that interaction at the level of IKKβ may occur was investigated. Using PLA technology (Duolink, Sigma), interaction between CaMKII and IKKβ was evident following treatment with H\(_2\)O\(_2\). This is a well-known driver of local and systemic inflammation in vivo and therefore an appropriate treatment to use in the context of the present study [33,34] (Fig. 6). That said, H\(_2\)O\(_2\) may cause aggregation of CaMKII through formation of disulphide and non-disulphide bridges so there is the possibility that the presence of dimers could affect the PLA signal and this was something we did not explore further in the present study. Taking these cell-based experiments as a whole though, this study provides crucial evidence for the first time for CaMKII modulation of NF-κB in adult CFs.

Strengthened by recombinant protein work, the present study identifies IKKβ as a potential novel substrate for modulation by CaMKII and a source through which CaMKII may exert its effects on NFκB signalling across the cardiovascular system.

Given the central importance of CaMKIIδ in cardiac function and dysfunction, it is surprising that studies examining its potential role in mediating cardiac inflammation are still relatively limited, particularly in relation to non-contractile cells of the heart. Similarly, despite strong evidence for altered NF-κB signalling in cardiac dysfunction, details of the underlying regulatory mechanisms are unclear. This study provides evidence for the first time that
CaMKIIδ may modulate NF-κB signalling in adult CFs via direct interaction with IKKβ. The possibility of targeting this interaction to modulate cardiac inflammation therapeutically could improve specificity on previous approaches for targeting NF-κB [35, 36]. The argument exists that more selective therapeutic strategies may be less damaging due to lower incidence of off-target and wider-ranging effects. A key strength of the present study has been the investigation in adult CFs, a cardiac cell that, although non-contractile, plays a crucial role in modulation of structural integrity as well as indirectly modulating contractile function. Activation of NF-κB in CFs will induce gene programs that promote inflammation and this could potentially be halted across contractile and non-contractile cell types via targeting of the CaMKIIδ-IKKβ interaction. Further examination of the site(s) of interaction should prove informative and will indicate whether CaMKII may phosphorylate unique sites on IKKβ. This could form the basis for disruptive intervention strategies in cardiovascular disease where both CaMKIIδ and NF-κB signalling are significantly elevated.

5. Conclusion
In conclusion, we have provided evidence that CaMKII modulates NFκB signalling in adult CFs and that this occurs, at least in part, via direct interaction with IKKβ. There is no evidence from SPR experiments for interaction of CaMKIIδ with IKKα or IKKγ. Interaction between CaMKII and IKKβ is elevated under conditions of oxidative stress in CFs, providing evidence that CaMKII is an important conduit for pro-inflammatory signalling in non-contractile myocardial cells and a molecule that could be pivotal to the role these cells play in inflammatory cardiac dysfunction.

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Disclosures
The authors declare no conflicts of interest.
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Figure legends

Fig. 1. Phosphorylation profiles of CaMKIIδ, IKKα and IKKβ following co-incubation with ATP. (A) CaMKIIδ (30ng) was incubated with either IKKα or IKKβ (both at 100ng) at 30°C for 30 minutes in the presence of [32P]ATP and in the presence (lanes 5 and 6) or absence (lanes 3 and 4) of AIP (5μM). Equivalent loads of sample were subjected to electrophoresis and incorporation of phosphate into CaMKIIδ and IKKs was detected using autoradiography. Representative autoradiogram data is shown as relevant sections from one autoradiogram (representative of two others) where the same batch of CaMKIIδ was incubated with IKKs and the exposure time consistent across all lanes. (B) Purity of individual proteins CaMKIIδ, IKKα and IKKβ (100ng each) was shown by coomassie staining of a gel following electrophoresis.

Fig. 2. SPR analysis reveals that IKKβ interacts directly with CaMKIIδ in a concentration dependent manner but IKKα and IKKγ do not. The potential for CaMKIIδ interaction with components of the IKK complex was analysed by SPR. Calmodulin (A), IKKβ (B), IKKα (C) and IKKγ (D) proteins (0.0125-0.2μM) were injected across a CaMKIIδ surface which had ~7000RU immobilised protein (~7pg/mm²). Analytes were injected in duplicate and in random order with respect to concentration. Association was monitored for 3 min and dissociation for 15 min. Binding surfaces were regenerated following 2 x 30s pulses with regeneration reagent (100mM HCl, 0.5M NaCl). A control flow cell contained BSA immobilised at equivalent levels to CaMKIIδ and responses from this flow cell together with the response to zero analyte were subtracted from the experimental responses. Data is representative of 3 independent experiments, performed in duplicate. For clarity only a single response at each analyte concentration is shown.

Fig. 3. The CaMKIIδ-IKKβ interaction does not occur via the HIS-tag on IKKβ nor is it affected by the presence of ATP or removal of Ca²⁺. The CaMKIIδ-IKKβ interaction was explored in more detail using SPR analysis performed under different running conditions. IKKβ was injected over a range of concentrations (0.0125-0.2μM) across a CaMKIIδ-immobilised sensor chip as previously. (A) To assess possible binding via the HIS-tag of IKKβ, 100mM imidazole was included in the running buffer. (B) To assess whether phosphorylation (inclusion of ATP) may be an important requirement for the interaction,
1 mM ATP was included in the running buffer. (C) To determine whether the interaction may be Ca\(^{2+}\)-dependent, analysis was performed in the absence of CaCl\(_2\). Data for each condition is representative of two independent experiments.

**Fig. 4.** Agonist-induced \(\text{IκB}_{\alpha}\) degradation in cardiac fibroblasts is reversed following CaMKII inhibition. (A(i)) A typical population of adult rat cardiac fibroblasts (p1) in culture (x20 magnification). (B) CFs were double-stained for (i) vimentin/DAPI (ii) von-Willebrand factor(vWF)/DAPI (iii) CD31/DAPI and (iv) \(\alpha\)-smooth muscle actin/DAPI to check for any contamination with endothelial cells, smooth muscle cells or myofibroblasts. Positive controls included endothelial cells stained with (v) vWF/DAPI and (vi) CD31/DAPI and smooth muscle cells stained with \(\alpha\)-SMA. Nuclei are stained blue with DAPI and antibody staining is shown in green and red from FITC or Alexa-Fluor 594. Panels i-iv were obtained using 20x objective and panels v-vi used the 10x objective. (C) Cardiac fibroblasts or whole homogenate preparations were solubilised in Laemmli sample buffer as described in the methods section and protein expression was examined by immunoblotting. Total protein from either 2x10\(^4\) cells (CF) or 20\(\mu\)g whole cardiac homogenate used as a positive control (WH) was probed for expression of CaMKII\(\delta\), IKK\(\alpha\), IKK\(\beta\) and IκB\(\alpha\). This data is from an individual experiment, typical of two others. (D(i)) Representative immunoblot showing LPS-induced IκB\(\alpha\) degradation in the presence or absence of CaMKII inhibitor AIP (5\(\mu\)M). (D(ii)) Densitometric analysis was performed and all data normalised to GAPDH. All data are expressed as mean ± S.E.M, n=3, *p<0.05.

**Fig. 5.** IKK\(\beta\) and oxidised CaMKII can both be detected in cardiac fibroblasts using immunofluorescence. (A) Images showing positive staining for IKK\(\beta\) by immunofluorescence in CFs. Cells were fixed, permeabilised and incubated with primary anti-IKK\(\beta\) antibody (1:200) (Abcam) overnight at 4°C and anti-mouse FITC conjugated secondary antibody (1:200) under basal conditions. Cells stained with secondary antibody alone served as a negative control. Nuclei were counterstained with DAPI. (B) Similar experiments were performed to assess CaMKII\(\delta\) expression (1:50 (Eurogentec, custom-made) followed by anti-rabbit TRITC conjugated secondary antibody (1:200) incubation. Secondary antibody alone was again used as a negative control. (C) Representative immunoblots showing sensitivity of ox-CaMKII antibody (1:1000) (GeneTex) to treatment of CFs with increasing concentrations of H\(_2\)O\(_2\) (1h). All data was normalised to GAPDH and 10\(\mu\)M H\(_2\)O\(_2\).
selected as the optimal concentration. (D) Images showing ox-CaMKII expression as detected by immunofluorescence (1:50) following under basal conditions and following treatment with 10µM H₂O₂. Anti-rabbit TRITC conjugated secondary antibody (1:200) alone was used as a negative control. All images are from one experiment, representative of two others.

**Figure 6. PLA analysis of oxidised CaMKII-IKKβ binding in cardiac fibroblasts.** (A) Proximity Ligation Assay (PLA) data showing ox-CaMKII-IKKβ interaction in CFs following H₂O₂ (10µM) stimulation (bottom right panel). The interaction was also assessed under basal conditions (bottom left panel). Controls were included where no primary antibody incubation was used (control, top left panel) or IKKβ antibody alone (top right panel) was used. (B) Quantification of signals was performed using four images per treatment group and counting number of fluorescent dots/cell as shown in histogram. Statistical analysis was performed using a one-way ANOVA followed by Tukey’s post-test. All data is presented as mean ± S.E.M of fluorescent dots/cell, n=3, *p<0.05, ***p<0.001.
Highlights

- Surface Plasmon Resonance using recombinant or purified proteins demonstrates that CaMKIIδ interacts directly with IKKβ but does not interact with IKKα or IKKγ.
- Pharmacologically-induced NFκB activation in adult cardiac fibroblasts is inhibited following selective CaMKII inhibition.
- Interaction between CaMKII and IKKβ in adult cardiac fibroblasts is evident under conditions of oxidative stress.
- CaMKII is an important modulator of NFκB signalling in adult cardiac fibroblasts.
Figure 1
A  Calmodulin

B  IKKβ

C  IKKα

D  IKKγ

Figure 2
Figure 3

A. Imidazole-containing

B. 1mM ATP added

C. CaCl$_2$ removed
Figure 4
A. 
IKKβ

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Ox-CaMKII → [image] 56kDa
GAPDH → [image] 37kDa

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ox-CaMKII

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Figure 6

(A) Control vs. IKKβ only

(B) CaMKII-IKKβ interaction in situ PLA signal

- Control
- IKK only
- ox-CaMKII & IKK (basal)
- ox-CaMKII & IKK (stim)

***, * indicate statistical significance.