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CaMKII δ subtypes differentially regulate infarct formation following *ex vivo* myocardial ischemia/reperfusion through NF- κ B and TNF- α



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ABSTRACT

Deletion of Ca²⁺/calmodulin-dependent protein kinase II delta (CaMKII δ) has been shown to protect against *in vivo* ischemia/reperfusion (I/R) injury. It remains unclear which CaMKII isoforms and downstream mechanisms are responsible for the salutary effects of CaMKII δ gene deletion. In this study we sought to compare the roles of the CaMKII δ_B and CaMKII δ_C subtypes and the mechanisms by which they contribute to *ex vivo* I/R damage. WT, CaMKII δ KO, and mice expressing only CaMKII δ_B or δ_C were subjected to *ex vivo* global ischemia for 25 min followed by reperfusion. Infarct formation was assessed at 60 min reperfusion by triphenyl tetrazolium chloride (TTC) staining. Deletion of CaMKII δ conferred significant protection from *ex vivo* I/R. Re-expression of CaMKII δ_C in the CaMKII δ KO background reversed this effect and exacerbated myocardial damage and dysfunction following I/R, while re-expression of CaMKII δ_B was protective. Selective activation of CaMKII δ_C in response to I/R was evident in a subcellular fraction enriched for cytosolic/membrane proteins. Further studies demonstrated differential regulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signaling and tumor necrosis factor alpha (TNF- α) expression by CaMKII δ_B and CaMKII δ_C . Selective activation of CaMKII δ_C was also observed and associated with NF- κ B activation in neonatal rat ventricular myocytes (NRVMs) subjected to oxidative stress. Pharmacological inhibition of NF- κ B or TNF- α significantly ameliorated infarct formation in WT mice and those that re-express CaMKII δ_C , demonstrating distinct roles for CaMKII δ subtypes in I/R and implicating acute activation of CaMKII δ_C and NF- κ B in the pathogenesis of reperfusion injury.

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1. Introduction

The calcium/calmodulin dependent protein kinase II (CaMKII) is a dodecameric enzyme consisting of subunits encoded by four different genes known as CaMKII α , β , γ , and δ . The predominant cardiac isoform is CaMKII δ and it is alternatively spliced in the heart to generate

Abbreviations: Animals expressing only CaMKII δ_B , (δ_B TG/ δ KO); Animals expressing only CaMKII δ_C , (δ_C TG/ δ KO); BMS-345541, (BMS); Ca²⁺/calmodulin-dependent protein kinase II delta, (CaMKII δ); Cyclosporine-A, (CsA); Fractional shortening, (FS); Inhibitor of kappa B kinase, (IKK); Interleukin 6, (IL-6); Ischemia/reperfusion, (I/R); Left ventricular developed pressure, (LVDP); Neonatal rat ventricular myocytes, (NRVMs); Nuclear factor kappa-light-chain-enhancer of activated B cells, (NF- κ B); Reactive oxygen species, (ROS); Receptor-interacting protein kinase 3, (RIP3); Sarcoplasmic reticulum, (SR); Triphenyl tetrazolium chloride, (TTC); Tumor necrosis factor alpha, (TNF- α).

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CaMKII δ_B and CaMKII δ_C as well as other minor subtypes [1–5]. We have generated global and cardiac specific knockouts of CaMKII δ and demonstrated that deletion of this protein ameliorates heart failure development in response to pressure overload, $G\alpha_q$ expression, and isoproterenol infusion [6–8]. We further reported that deletion of CaMKII δ diminishes infarct development in response to *in vivo* ischemia/reperfusion [9]. Other studies using CaMKII inhibitory peptides or knock-ins of activation-deficient CaMKII have similarly concluded that CaMKII activation by a range of cardiac insults, including myocardial infarction, is deleterious [10–13]. In all of the aforementioned studies both the CaMKII δ_B and CaMKII δ_C subtypes of CaMKII δ were genetically deleted or inhibited. Accordingly it is not yet known which subtype is responsible for the protective effect of ablating CaMKII δ activity in the heart.

The studies reported here use mice in which CaMKII δ deletion has been restored by crossing CaMKII δ KO mice with transgenic lines expressing either the CaMKII δ_B (δ_B TG/ δ KO) or CaMKII δ_C (δ_C TG/ δ KO) subtype [14]. This approach allowed us to examine the unique roles of

CaMKII δ_B and CaMKII δ_C in cardiomyocyte survival and infarct formation in response to I/R. We demonstrate that CaMKII δ_C expression reverses and exacerbates the diminished I/R damage observed in CaMKII δ_KO mouse hearts whereas CaMKII δ_B expression further attenuates I/R damage. The difference in infarct development observed in δ_B TG/ δ_KO and δ_C TG/ δ_KO mice is associated with greater I/R-induced inhibitor of kappa B kinase (IKK) and NF- κ B activation in δ_C TG/ δ_KO mice. CaMKII δ_C -mediated NF- κ B activation is recapitulated in NRVMs exposed to oxidative stress, and selective activation of CaMKII δ_C in a cytosol/membrane fraction is observed in NRVMs exposed to oxidative stress and in hearts exposed to I/R. TNF- α expression is also selectively increased in hearts from δ_C TG/ δ_KO mice following I/R. Blocking either IKK activation or TNF- α signaling diminished infarct development in δ_C TG/ δ_KO as well as in WT mice. These data suggest that selective activation of the CaMKII δ_C subtype in cardiomyocytes regulates cardiac-autonomous pro-inflammatory signaling events that contribute to ischemia/reperfusion injury.

2. Methods

2.1. Transgenic animals

Transgenic Black Swiss mice in which the predominant cardiac subtypes of CaMK, CaMKII δ_B and CaMKII δ_C , are over expressed were generated in our laboratory and characterized as described [15,16]. Conventional CaMKII δ_KO mice were generated and characterized as previously described [6]. CaMKII δ_B and CaMKII δ_C transgenic mice were crossed with conventional CaMKII δ_KO mice to generate mice that express only CaMKII δ_B or CaMKII δ_C . We refer to these animals as CaMKII δ_B TG/ δ_KO and CaMKII δ_C TG/ δ_KO . All mice used in the present study were male at 8 weeks of age, unless otherwise noted. Animal studies were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committees of University of California at San Diego.

2.2. CaMKII activity assay

CaMKII activity was measured in ventricular homogenate using Syntide-2, a synthetic CaMKII-specific substrate peptide. Hearts were isolated and ventricles homogenized in lysis buffer (50 mmol/L HEPES, 10% ethylene glycol, 2 mg/mL BSA, 5 mmol/L EDTA, pH 7.5), and assayed immediately without freezing. The assay buffer contained 50 mmol/L HEPES, 10 mmol/L magnesium acetate, 1 mg/mL BSA, 20 μ mol/L Syntide-2, 1 mmol/L DTT, 400 nM [γ - 32 P]ATP, pH 7.5 and either 1 mmol/L EGTA (for autonomous activity) or 500 μ mol/L CaCl $_2$, plus 1 μ mol/L calmodulin (for maximal activity). The reaction was carried out at 30 °C for 10 min and blotted onto Whatman P81 phosphocellulose paper.

2.3. Heart tissue fractionation procedure

Mice were killed by cervical dislocation, and hearts were quickly removed and placed in ice-cold PBS. Following cannulation, hearts were perfused with PBS to remove blood, frozen in liquid N $_2$, and pulverized. Tissue was then homogenized in isolation buffer (70 mmol/L sucrose, 190 mmol/L mannitol, 20 mmol/L HEPES, 0.2 mmol/L EDTA) that was supplemented with various inhibitors: sodium vanadate, leupeptin, aprotinin, *p*-nitrophenyl phosphate, and phenylmethylsulfonyl fluoride. Homogenate was centrifuged at 600g for 10 m at 4 °C, and the supernatant transferred to another tube. The supernatant was centrifuged at 5000g for 15 m at 4 °C to yield a cytosol/membrane fraction. The pellet from the initial 600 g centrifugation was resuspended in nuclear lysis buffer (20 mmol/L NaCl, 1.5 mmol/L MgCl $_2$, 20 mmol/L HEPES, 200 nmol EDTA, 25% glycerol) and centrifuged at 600g for 10 m at 4 °C to yield a nuclear fraction.

2.4. Neonatal rat ventricular myocyte (NRVM) isolation and adenoviral infection

Neonatal rat ventricular myocytes (NRVMs) were isolated from 1 to 2-day-old Sprague-Dawley rat pups, digested with collagenase, plated at density of 3.5×10^4 /cm 2 and maintained overnight in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and antibiotics (100 units/mL penicillin and 100 μ g/mL streptomycin). Prior to adenoviral infection, isolated NRVMs were transferred to serum-free medium and infected with CaMKII δ_B or δ_C adenovirus at 10, 30, 50, 100, 300 multiplicity of infection (MOI) for 3 h. Cells were washed and maintained in serum-free medium for an additional 21 h prior to treatment with H $_2$ O $_2$ or vehicle. To assess changes in nuclear and cytosolic/membrane fractions, NRVMs were fractionated according to a previously reported protocol [17]. Purity was determined using the cytosolic marker Rho GDP dissociation inhibitor (RhoGDI) and the nuclear marker Lamin A/C.

2.5. Transthoracic echocardiography

Echocardiography was performed using the VisualSonics VeVo 770 Imaging System (VisualSonics, Toronto, Canada) equipped with high-frequency 30 MHz probe, as described [18]. Body temperatures were maintained between narrow ranges (37.0 ± 1.0 °C) to avoid confounding effects of hypothermia.

2.6. Isolated perfused (ex vivo) I/R

Mice were killed by cervical dislocation, and hearts were quickly removed and placed in ice-cold Ca $^{2+}$ -free Krebs-Henseleit buffer. Aortas were cannulated and hearts perfused by gravity flow on a Langendorff perfusion system (Radnoti LLC) at 37 °C and a constant pressure of 80 mm Hg with a modified Krebs-Henseleit buffer solution containing (in mmol/L): 2.0 CaCl $_2$, 130 NaCl, 5.4 KCl, 11 dextrose, 2 pyruvate, 0.5 MgCl $_2$, 0.5 NaH $_2$ PO $_4$, and 25 NaHCO $_3$ and aerated with 95% oxygen and 5% carbon dioxide, pH 7.4. To measure infarct size, hearts were subjected to 25-minute global ischemia and 1-hour reperfusion; the ventricles were then frozen and cut transversely into 5 slices of equal thickness. The slices were then incubated in 1% TTC/PBS and fixed in 10% formalin-PBS for 24 h. Fixed slices were then scanned, and ImageJ was used to measure and calculate the size of the infarct area and the total area. For experiments utilizing BMS-345541 (Sigma-Aldrich) the drug was dissolved in Krebs-Henseleit buffer solution at a concentration of 5 μ mol/L and was present throughout the I/R protocol. For those using etanercept, the drug was present for the entire I/R procedure at a concentration of 5 μ g/mL. To assess cardiac function, a water-filled balloon connected to a pressure transducer (Gould Statham P23 ID) was inserted into the left ventricle through the left atrium to monitor left ventricular developed pressure (LVDP); data collected using Powerlab, were processed with AD Instruments Chart 4 software (v4.12). Hearts were submerged in warm KHB (37 °C) throughout the perfusion. Functional recovery was expressed as a percentage of pre-ischemic LVDP.

2.7. Immunoblotting

Ventricular tissue was homogenized in RIPA buffer (10 mmol/L Tris-Cl (pH 8.0), 1 mmol/L EDTA, 0.5 EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mmol/L NaCl) that was supplemented with various inhibitors: sodium vanadate, leupeptin, aprotinin, *p*-nitrophenyl phosphate, and phenylmethylsulfonyl fluoride. Western blot analysis was performed according to protocols described previously [9]. The antibodies for immunoblotting were as follows: CaMKII δ (D.M. Bers, UC Davis); GAPDH (CST); phospho-IKK α / β at its autophosphorylation site (Ser176/180) (CST); phospho-CaMKII at its autophosphorylation site (Thr286) (Thermo); NF- κ B p65 (CST); α -actinin (CST); RhoGDI (CST); Lamin A/C (CST); I κ B α (CST)

2.8. RT-PCR

RNA extraction for real time analysis was performed using the solid-phase RNeasy purification kit from Qiagen (Venlo, Netherlands). First strand cDNA synthesis for Real time PCR was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY). Gene expression was determined using Taqman® Universal PCR master mix, Cy5-labeled Taqman® probe for TNF- α and IL-6 and FAM-labeled Taqman® probe for GAPDH (Applied Biosystems).

3. Statistical analysis

Data are presented as mean \pm SEM as indicated and were analyzed by 2-tailed Student *t*-test between 2 groups or by ANOVA when 3 or more groups were compared. *P* values <0.05 were considered statistically significant.

4. Results

To examine the independent roles of the CaMKII δ_B and δ_C subtypes in the heart we restored either δ_B or δ_C expression in a CaMKII δ -null (KO) background. The resulting animals express only δ_B (δ_B TG/ δ KO) or only δ_C (δ_C TG/ δ KO). Survival of WT, KO, δ_B TG/ δ KO, and δ_C TG/ δ KO mice was assessed. Deletion of CaMKII δ produced no overt phenotypic changes and did not affect survival relative to WT mice as shown previously [6]. δ_B TG/ δ KO animals also survived normally for at least 6 months. In contrast, the δ_C TG/ δ KO animals, like the previously studied δ_C TG [16], exhibited premature death with <20% survival by 21 weeks (Fig. 1A). Expression of the CaMKII δ_B and δ_C subtypes also had markedly different effects on *in vivo* cardiac function. Echocardiography on 6–8 week old mice revealed that fractional shortening (FS) was decreased by 63% in δ_C TG/ δ KO mice compared to WT mice (Fig. 1B) while δ_B TG/ δ KO animals did not display cardiac dysfunction.

We previously demonstrated that CaMKII δ deletion attenuates I/R injury in response to *in vivo* left anterior descending coronary artery occlusion and subsequent reperfusion [9]. To examine the cardiac-intrinsic role of CaMKII δ in I/R we performed *ex vivo* I/R experiments on isolated perfused hearts from 8-week-old mice. Infarct formation following 25 min ischemia and 1 h reperfusion was determined by TTC staining of heart sections. In WT animals, *ex vivo* I/R induced infarcts comprising $36.2 \pm 2.5\%$ of the cross-sectional area. Infarcts were significantly smaller, only $24.1 \pm 1.4\%$, in CaMKII δ KO mouse hearts (Fig. 2A, B). Thus regulation of infarct development by CaMKII is evident not only *in vivo* but also in an *ex vivo* I/R model.

Hearts from δ_B TG/ δ KO mice were then examined and found to be protected against *ex vivo* I/R damage, with infarcts measuring $12.2 \pm 1.9\%$ of cross-sectional area. Conversely, in δ_C TG/ δ KO mouse hearts, the protective effect of CaMKII δ gene deletion was lost with infarcts measuring $45.2 \pm 1.8\%$ of the cross-sectional area, significantly larger than those of WT, KO, and δ_B TG/ δ KO (Fig. 2A, B). Assessment of left ventricular developed pressure (LVDP) recovery during reperfusion confirmed that expression of CaMKII δ_C in CaMKII δ KO mice exacerbates I/R damage while expression of CaMKII δ_B does not (Supplemental Fig. 1).

CaMKII is known to be activated during *ex vivo* I/R [19,20], but previous experiments have not assessed subtype-specific CaMKII activation. Although we previously demonstrated that δ_B and δ_C can be equivalently activated by several pharmacological interventions [14], we wondered if I/R might lead to differential activation of the two subtypes. To assess I/R-induced activation of CaMKII we analyzed phosphorylation of CaMKII at its autophosphorylation site threonine-286. The increases in CaMKII autophosphorylation observed in whole cell lysates from hearts of δ_B TG/ δ KO and δ_C TG/ δ KO mice subjected to I/R were equivalent (Fig. 3A).

Many substrates of CaMKII δ_C (e.g. phospholamban, RyR2, and histone deacetylase 4) localize to the cytosol or the cytosolic face of the

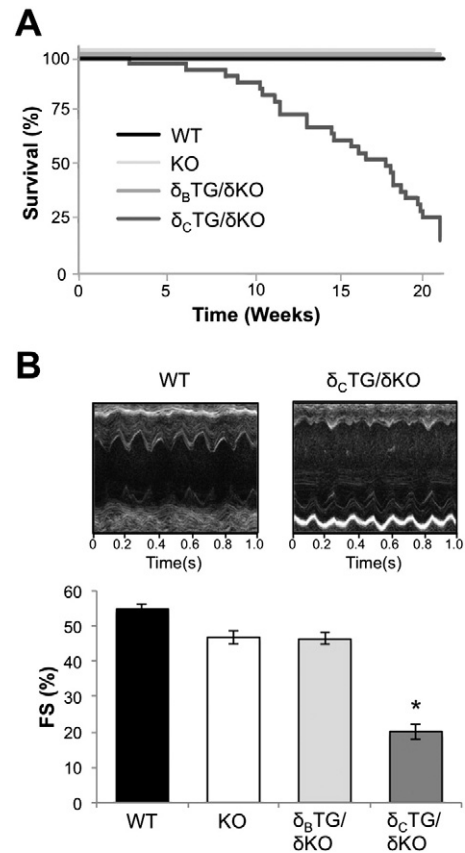


Fig. 1. CaMKII δ_C TG/ δ KO but not CaMKII δ_B TG/ δ KO mice display diminished survival and cardiac function. A, Kaplan-Meier analysis of survival of WT, CaMKII δ KO, δ_B TG/ δ KO, and δ_C TG/ δ KO mice. B, Representative echocardiographic recordings from WT and δ_C TG/ δ KO mice and quantification of fractional shortening (FS) measured in 6–8-week-old WT, KO, δ_B TG/ δ KO, and δ_C TG/ δ KO mice. Data are mean \pm SEM values from 4 to 6 mice. **P* < 0.05 vs WT.

cardiac sarcoplasmic reticular (SR) membrane. Thus we carried out further studies using a subcellular fraction enriched for cytosolic and membrane proteins. Strikingly in this fraction we observed differential activation of the δ_B and δ_C subtypes in response to I/R. Indeed whereas no increases in CaMKII autophosphorylation were observed in cytosolic/membrane fractions from δ_B TG/ δ KO mouse hearts, there was a >3-fold increase in autophosphorylated CaMKII in cytosolic/membrane-enriched fractions from δ_C TG/ δ KO hearts (Fig. 3B).

Our earlier studies examining *in vivo* I/R damage linked the deleterious effects of CaMKII to activation of IKK and subsequent NF- κ B nuclear accumulation [9]. To determine which CaMKII δ subtype was responsible, and also to determine if CaMKII δ -mediated NF- κ B activation can occur in the absence of systemic factors (e.g. leukocyte infiltration), we examined regulation of IKK and NF- κ B by I/R in the *ex vivo* heart. The phosphorylation of IKK was found to be elevated during *ex vivo* reperfusion in δ_C TG/ δ KO mice but not in δ_B TG/ δ KO mice (Fig. 4A). Furthermore I/R-mediated activation of IKK in δ_C TG/ δ KO mice was associated with an increased nuclear localization of the p65 subunit of NF- κ B, which was not observed in δ_B TG/ δ KO animals (Fig. 4B).

Nuclear p65 translocation would be expected to result in transcriptional activation of NF- κ B target genes. Thus as further evidence that differences in NF- κ B activation in δ_C TG/ δ KO and δ_B TG/ δ KO are functionally significant, we measured mRNA levels of genes regulated by NF- κ B. Ischemia/reperfusion increased interleukin 6 (IL-6) mRNA in hearts from δ_C TG/ δ KO mice to a greater extent than in those from δ_B TG/ δ KO mice (Fig. 5A). Even more striking was the robust increase in TNF- α expression in δ_C TG/ δ KO mice and the absence of upregulation of this gene in δ_B TG/ δ KO animals (Fig. 5B).

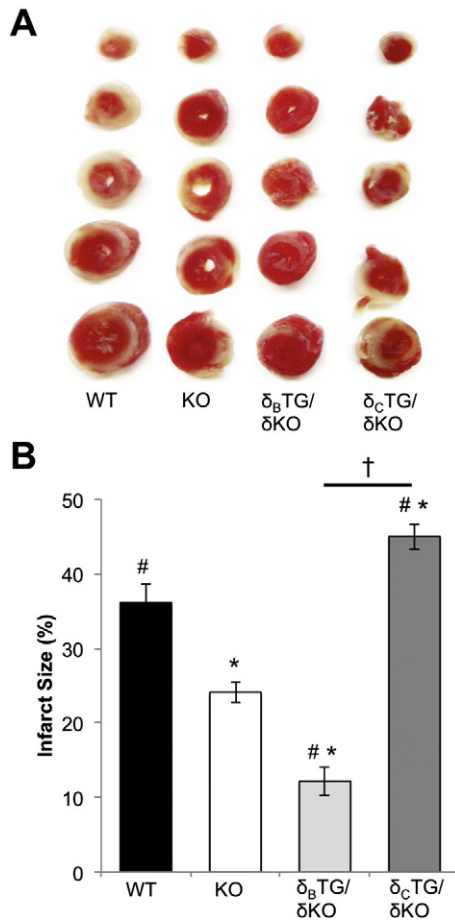


Fig. 2. Differential effects of CaMKII subtypes on I/R injury and oxidative stress. **A**, Representative images hearts from WT, CaMKII δ KO, CaMKII δ_B TG/ δ KO, and CaMKII δ_C TG/ δ KO mice subjected to 25 min ischemia and 1 h of reperfusion in the Langendroff mode. Hearts were sectioned and stained with TTC to reveal infarcted tissue. **B**, Infarct size was quantified from TTC stained heart sections. Data are mean \pm SEM values from 14 to 16 mice. * $P < 0.05$ vs WT. # $P < 0.05$ vs KO. † $P < 0.05$ vs δ_C TG/ δ KO.

To confirm that oxidative stress can lead to selective activation of CaMKII δ_C and of NF- κ B in a cardiomyocyte-autonomous fashion, we infected NRVMs with adenovirus expressing CaMKII δ_B or CaMKII δ_C . Cells were infected with a range of MOIs of CaMKII δ_B or δ_C virus and subsequently treated with 50 μ M H₂O₂ for 30 min to elicit CaMKII and NF- κ B activation. In NRVM whole cell lysates we observed equivalent activation of CaMKII δ_B and CaMKII δ_C in response to H₂O₂ at all MOIs (Fig. 6A, top panel), as quantitated using an MOI of 50 (Fig. 6A lower panel). In NRVM lysates that were fractionated to enrich for cytosolic and membrane proteins, however, there was significantly greater activation of CaMKII δ_C following H₂O₂ treatment (Fig. 6B). We further determined that expression of CaMKII δ_C , but not CaMKII δ_B , enhanced H₂O₂-mediated I kappa B alpha (I κ B α) degradation (Fig. 6C) and nuclear p65 accumulation (Fig. 6D). These data, like those obtained in the isolated perfused heart (Figs. 3 and 4) suggest that oxidative stress-induced CaMKII δ_C activation in the cytosolic/membrane compartment is associated with activation of NF- κ B.

To demonstrate that the deleterious effects of CaMKII δ_C activation seen in the isolated perfused heart were mediated by IKK/NF- κ B signaling we blocked IKK activation with the pharmacological inhibitor BMS-345541 (BMS) [21]. Since this drug had not, to our knowledge, been used in the *ex vivo* perfused heart we evaluated its efficacy and determined a dose (5 μ mol/L) that sequestered p65 in the cytosol/membrane fraction (Supplemental Fig. 2). Hearts from δ_B TG/ δ KO, δ_C TG/ δ KO, WT, and KO mice were then exposed to 5 μ mol/L BMS or vehicle prior to and throughout the I/R protocol. Infarct size was significantly reduced

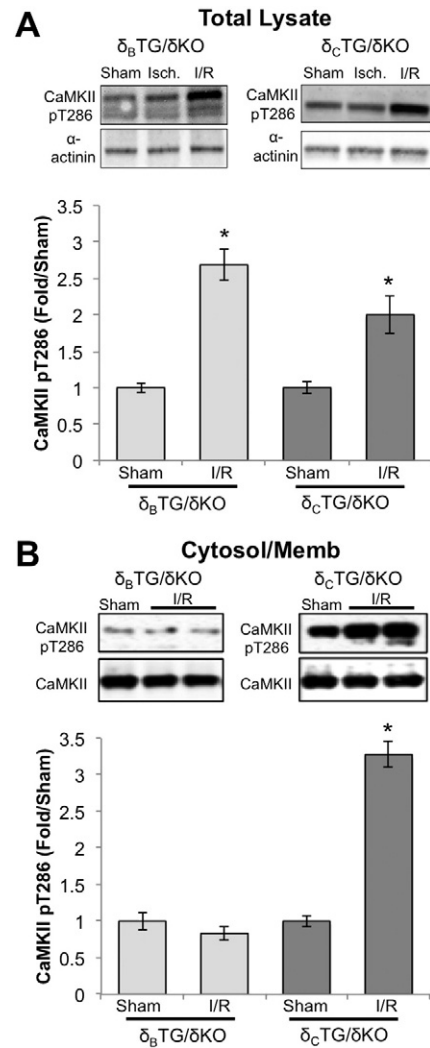


Fig. 3. CaMKII δ_C is selectively activated in a subcellular fraction containing cellular membranes and cytosol. **A**, Lysates from the hearts of δ_B TG/ δ KO and δ_C TG/ δ KO mice were subjected to western blot analysis using an antibody specific for autophosphorylated CaMKII. Data are mean \pm SEM values from 4 to 6 mice. **B**, Subcellular fractionation of hearts from δ_B TG/ δ KO and δ_C TG/ δ KO mice was carried out as described in methods yielding a fraction that includes cytosol and cellular membranes. Data are mean \pm SEM values from 4 to 6 mice. * $P < 0.05$ vs sham.

by BMS administration in WT and δ_C TG/ δ KO mice, while the already diminished infarct formation that was observed in δ_B TG/ δ KO animals was not affected (Fig. 7). CaMKII δ KO animals showed a modest but significant further reduction in infarct size.

In light of the selective increase in TNF- α mRNA in δ_C TG/ δ KO mice, and evidence that active TNF- α can be produced in and secreted from the isolated perfused heart [22], we asked whether TNF- α mediated the deleterious effects of CaMKII δ_C . For these studies we used etanercept, which blocks the effects of TNF- α by preventing its interaction with TNF- α receptors [23,24]. Etanercept was perfused at 5 μ g/mL prior to and throughout the I/R protocol. The results were similar to those obtained with IKK/NF- κ B inhibition *i.e.* infarct size was significantly reduced in WT and δ_C TG/ δ KO mice and was not reduced further in δ_B TG/ δ KO animals. CaMKII δ KO animals showed modest but significant further reductions in infarct size in the presence of etanercept (Fig. 8). These findings reveal a surprisingly important role for autocrine and/or paracrine TNF- α signaling in the *ex vivo* isolated perfused heart during I/R, demonstrate that this process is regulated by CaMKII δ_C , and establish an essential cardiac-intrinsic role for NF- κ B in I/R injury (Fig. 9).

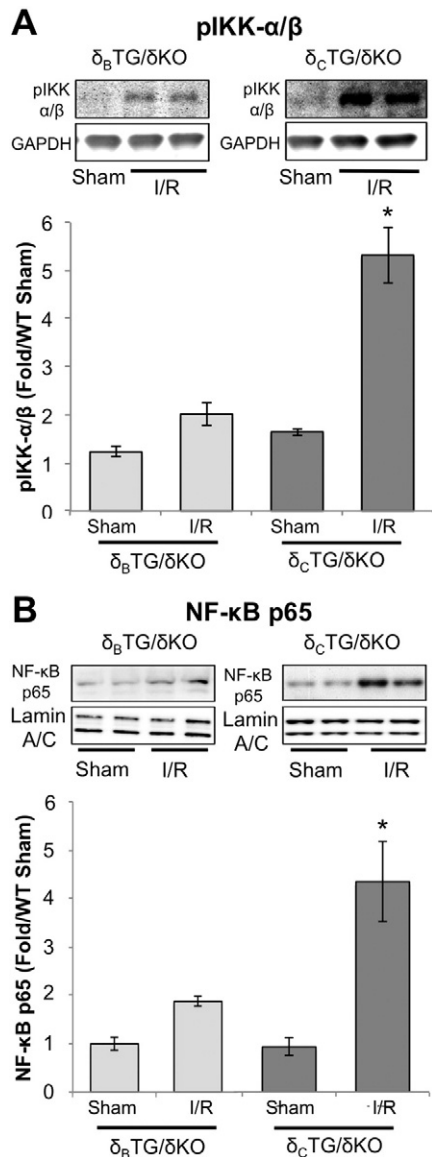


Fig. 4. The NF- κ B pathway is activated in CaMKII δ_C TG/ δ KO mice following reperfusion. *A*, Activation of IKK was assessed in δ_B TG/ δ KO and δ_C TG/ δ KO mice using an antibody specific for auto-phosphorylated IKK α/β . *B*, NF- κ B p65 accumulation was assessed in nuclear extracts made from hearts of δ_B TG/ δ KO and δ_C TG/ δ KO mice subjected to *ex vivo* I/R. Data are mean \pm SEM values from 6 mice. $P < 0.05$ vs sham.

5. Discussion

Our laboratory previously demonstrated that mice in which CaMKII δ was selectively deleted from cardiomyocytes exhibited diminished infarct formation in response to *in vivo* I/R [9]. These experiments provided evidence that CaMKII δ activation in cardiomyocytes mediates the deleterious effects of I/R injury but did not address the question of which subtype(s) of CaMKII δ were responsible for cardiomyocyte death in response to I/R. Here we characterize and utilize mice in which the CaMKII δ_B or CaMKII δ_C isoforms are expressed in the CaMKII δ KO animals to independently determine the role of each of these isoforms in cardiomyocyte CaMKII signaling. In addition we analyzed I/R damage in the *ex vivo* isolated perfused heart where the role of systemic inflammatory factors is eliminated. Our studies demonstrate that signals that are rapidly and locally generated regulate infarct development in the *ex vivo* heart and are significantly attenuated in the absence of CaMKII δ . We further show that CaMKII δ_C re-expression in cardiomyocytes reverses the attenuation of infarct formation seen

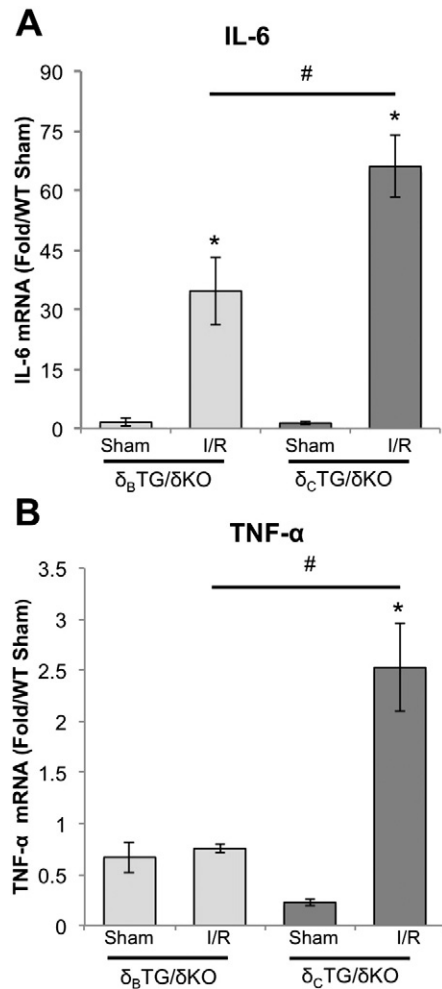


Fig. 5. Genes downstream of NF- κ B are upregulated in CaMKII δ_C TG/ δ KO mice after *ex vivo* I/R. NF- κ B activation was assessed by measuring mRNA transcripts of genes regulated by NF- κ B. *A*, IL-6 *B*, TNF- α . Data are mean \pm SEM values from 4 mice. * $P < 0.05$ vs sham # $P < 0.05$ vs δ_C TG/ δ KO.

in CaMKII δ KO mice. In contrast CaMKII δ_B re-expression further limits infarct development compared to that observed in KO mice.

While total CaMKII δ expression and maximal activity are somewhat higher in δ_C TG/ δ KO mice (Supplemental Fig. 3 B) the amount of activated (Ca^{2+} -autonomous) CaMKII is similar in δ_C TG/ δ KO and δ_B TG/ δ KO animals (Supplemental Fig. 3 A). Thus it is unlikely that differential baseline levels of active CaMKII δ explain the response of δ_C TG/ δ KO versus δ_B TG/ δ KO animals to I/R. There may, however, be a greater propensity for activation of inflammatory responses when CaMKII δ_C is activated thus we determined whether I/R could lead to selective increases in NF- κ B activation in δ_C TG/ δ KO mice. NF- κ B has been implicated in *in vivo* I/R damage and myocardial infarction and our earlier studies demonstrated that CaMKII δ activates NF- κ B during *in vivo* I/R [9,25,26]. We previously assumed that *in vivo* I/R triggered systemic responses such as infiltration of non-cardiac inflammatory cells into the heart during the 24–48 h of reperfusion, and that this was a significant contributor to infarct development. Here, however, we demonstrate that CaMKII δ_C and subsequent NF- κ B-mediated responses occur in the isolated perfused heart implicating cardiac-intrinsic signaling in the effects of CaMKII δ_C on infarct formation.

It is possible that the deleterious effect of CaMKII δ_C is initiated by induction of cardiomyocyte cell death and release of factors from necrotic cells that induce inflammatory signaling [27,28]. Our previous work showed, however, that cyclosporine-A (CsA) treatment did not block NF- κ B activation in response to *in vivo* I/R, indicating that this response

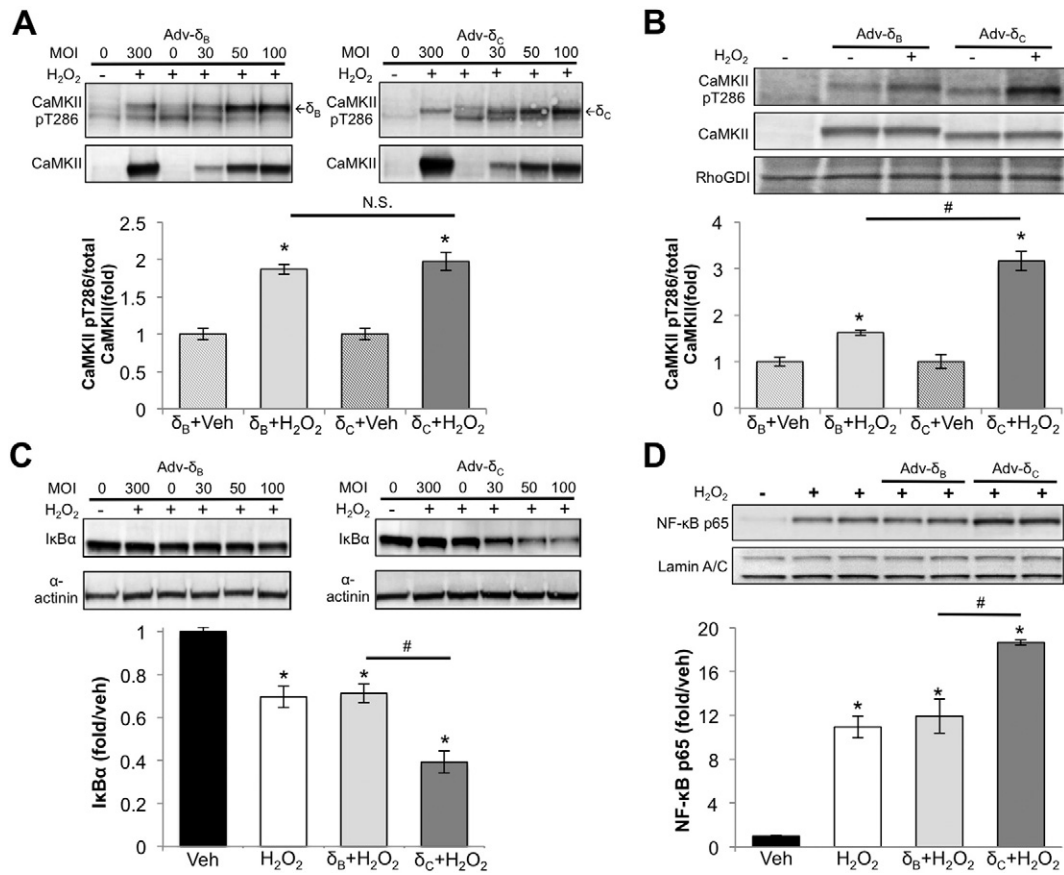


Fig. 6. Selective CaMKII δ_C and NF- κ B activation in NRVM treated with 50 μ M H₂O₂ for 30 min. **A**) CaMKII activation measured in NRVM infected with adenovirus, MOI of 50 was quantified. **B**) CaMKII activation measured in a cytosolic/membrane fraction isolated from NRVM expressing δ_B or δ_C (MOI 50). **C**) I κ B α degradation assessed in NRVM infected with adenovirus. MOI of 50 was quantified. **D**) NF- κ B p65 measured in nuclear extracts from NRVM expressing δ_B or δ_C (MOI 50). All data are mean \pm SEM $n = 3$ * $P < 0.05$ vs Veh # $P < 0.05$ vs δ_C .

was independent of necrosis [9] and we confirmed this in the current *ex vivo* study (Supplemental Fig. 4). Accordingly we suggest that there is a direct effect of CaMKII on NF- κ B activation. This conclusion is further supported by our current data showing that acute activation of CaMKII δ_C with H₂O₂ elicits NF- κ B activation in NRVMs and by our previous demonstration that adenoviral expression of activated CaMKII δ_C in NRVMs induces IKK activation [9].

Strikingly, we show here that NF- κ B activation in response to I/R is enhanced by cardiac expression of CaMKII δ_C but not CaMKII δ_B . This is consistent with the finding that phosphorylation of the upstream kinase IKK is increased by I/R in δ_C TG/ δ KO but not in δ_B TG/ δ KO animals. Furthermore, we demonstrate that I/R increases activation of CaMKII δ_C but not of CaMKII δ_B in the cytosol/membrane, where IKK is localized. We previously reported that despite its nuclear localization sequence [29] CaMKII δ_B is not strictly confined to the nucleus [14] but is in fact present in the cytosol/membrane compartment (Fig. 3B). It remains unclear why there is selective activation of CaMKII δ_C and not CaMKII δ_B in this location. Nevertheless our observations in NRVMs confirm that there is differential activation of equivalently expressed levels of CaMKII δ_B and δ_C by oxidative stress and further indicate an association between cytosolic CaMKII δ_C autophosphorylation and subsequent I κ B α degradation and nuclear accumulation of p65. Notably another recent report confirmed the ability of CaMKII δ subtypes to undergo differential posttranslational modification during reperfusion [30].

Previous studies using pharmacological inhibitors to demonstrate involvement of CaMKII δ in *ex vivo* I/R damage linked the effects of CaMKII to phosphorylation of PLN and RyR2 and subsequent Ca²⁺ dysregulation [31], supporting the hypothesis that rapid phosphorylation of CaMKII targets was a driver of myocardial injury. Indeed, we originally considered it unlikely that transcriptional regulation by

CaMKII δ_C -mediated activation of NF- κ B could play a significant role in determining cell viability over the course of only one hour of reperfusion. Surprisingly, however, we found that inhibition of either IKK or TNF- α had a profound effect on infarct formation not only in δ_C TG/ δ KO but also in WT mouse hearts subjected to 25 min *ex vivo* ischemia and 60 min reperfusion.

Recent studies have shown that receptor-interacting protein kinase 3 (RIP3) plays a role in TNF- α -mediated cell death through formation of the necrosome [32]. RIP3 phosphorylates and activates CaMKII during I/R [33] accordingly TNF- α could participate in a deleterious positive feedback loop leading to sustained CaMKII δ_C and NF- κ B activation. The experiments presented in this study clearly indicate that TNF- α signaling and myocardial NF- κ B activation are mechanisms by which CaMKII δ_C elicits infarct formation in the isolated perfused heart. This signaling would likely be further enhanced and sustained by Ca²⁺ dysregulation resulting from CaMKII-mediated phosphorylation of SR substrates and increases in reactive oxygen species (ROS) during I/R [19,34,35].

Paradoxically, some reports indicate that NF- κ B may be protective in I/R injury [36,37] while others demonstrate a deleterious role for NF- κ B activation [25,38]. Importantly, these studies differ in how I/R is elicited and how NF- κ B is inhibited, thus resolution of these conflicting conclusions remains elusive. In the current study we have demonstrated that CaMKII δ_C expressed in cardiomyocytes mediates NF- κ B activation, TNF- α induction and infarct development during I/R and that these events occur rapidly and in the absence of systemic inflammatory factors. Our data contribute to the understanding of the dichotomous effects of NF- κ B by clearly demonstrating an adverse cardiomyocyte-autonomous role of NF- κ B activation in I/R injury.

While we demonstrate that cardiac TNF- α expression during I/R is mediated by CaMKII δ_C activation in cardiomyocytes, we do not know

the extent to which TNF- α is formed in and secreted from these cells. Cardiac-resident macrophages and other non-myocytes are potential sources of TNF- α and could thus act in an autocrine or paracrine fashion to affect cardiomyocyte survival. The extent to which CaMKII δ_c activation in myocytes sends signals to other cells is not clear, but our studies demonstrate that the cardiomyocyte initiates signals through CaMKII δ that lead to upregulation of TNF- α . TNF- α inhibitors such as etanercept have been used to inhibit TNF- α signaling in patients with various autoimmune disorders [39]. Effects of TNF- α inhibitors on cardiovascular disease outcomes have also been evaluated with uncertain results that could in part be due to systemic responses to sustained antibody administration [28]. Such detrimental effects would not be expected to occur during short-term treatment thus use of TNF- α inhibitors could be of value if employed at the onset of reperfusion following primary percutaneous intervention for myocardial infarction.

In summary, we demonstrate that the δ_c isoform of CaMKII contributes significantly to myocardial damage following *ex vivo* I/R. Signaling occurs through NF- κ B and TNF- α and acute inhibition of the generation or function of these molecules has a very robust protective effect in WT animals and in those expressing CaMKII δ_c . Importantly, we show that these events occur during a much shorter timeframe than would have been predicted by previous studies of CaMKII δ and NF- κ B signaling in *in vivo* I/R, and that these events occur in the absence of systemic factors such as infiltration of cells originating outside of the heart. CaMKII inhibition is predicted to be of therapeutic benefit in a number of contexts. Our results suggest that selective CaMKII δ_c inhibition would confer the

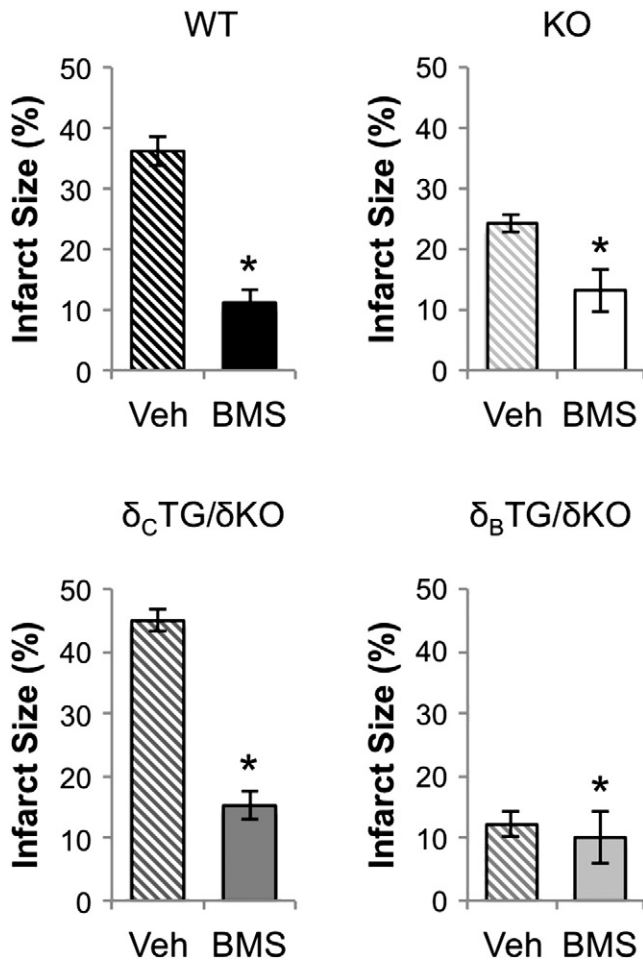


Fig. 7. Inhibition of IKK ameliorates I/R damage. Vehicle and BMS-345541 (5 μ mol/L) were perfused into hearts from WT, KO, δ_β TG/ δ KO, and δ_c TG/ δ KO animals. Infarct size was measured via TTC staining following 1hr of reperfusion. Data are mean \pm SEM values from 4 to 8 mice. * P < 0.05 vs veh.

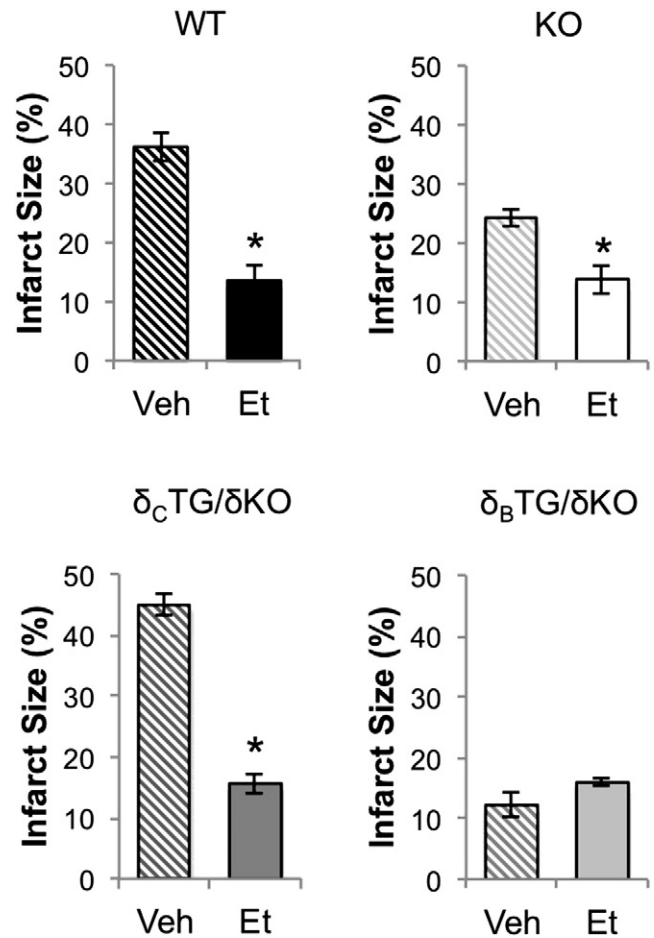


Fig. 8. Inhibition of TNF- α ameliorates I/R damage. Vehicle or etanercept (5 μ g/mL) were perfused into hearts from WT, KO, δ_β TG/ δ KO, and δ_c TG/ δ KO animals. Infarct size was measured via TTC staining following 1hr of reperfusion. Data are mean \pm SEM values from 4 to 8 mice. * P < 0.05 vs veh.

most benefit over blockade of all cardiac CaMKII isoforms although specific means of locally inhibiting the δ_c isoform do not yet exist. An alternative approach would be to acutely block events that occur downstream of CaMKII δ_c activation during I/R such as IKK/NF- κ B activation or TNF- α signaling.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jmcc.2017.01.002>.

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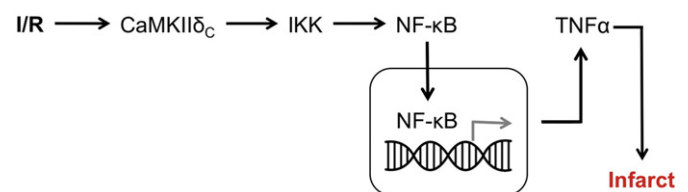


Fig. 9. Summary of findings. NF- κ B nuclear translocation and subsequent TNF- α upregulation during I/R is mediated by cytosolic CaMKII δ_c activation.

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Disclosures

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