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ORIGINAL RESEARCH PAPER

Crosstalk between PKC and MAPK pathway activation in cardiac fibroblasts in a rat model of atrial fibrillation

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Abstract
Objective Atrial fibrillation (AF) is the most frequent form of cardiac arrhythmia and major cause of cardiac ischemia. Defective calcium homeostasis due to anomalous expression of ryanodine receptor type 2 (RyR2) or its hyperactivation by phosphorylation by serine threonine kinases has been implicated as a central mechanism of AF pathogenesis. Given the role of protein kinase C (PKC) isoforms in cardiac function we investigated role of PKC in AF using a rat model.
Results PMA induced global increase in protein synthesis in cardiac fibroblasts isolated from AF rats, but not healthy controls, and the increase was inhibited by PKC inhibition. PMA mediated activation of both PKC and ERK and either inhibition of PKC by Go6983 or ERK by the MEK inhibitor Trametinib attenuated both P-ERK and P-PKC in both cardiac fibroblasts isolated from AF rats or from healthy rats but transduced with PKC-delta. The PKC and ERK mediated induction of global protein synthesis was found to be mediated by increased phosphorylation of the ribosomal protein S6.
Conclusion Our findings provide a foundation for future testing of PKC and MEK inhibitors to treat AF in pre-clinical models. It also needs to be determined if PKC and MAPK pathway activation is functioning via RyR2 or some yet undefined substrates.

Keywords Atrial fibrillation · PKC · MAPK · Protein synthesis

Introduction

One of the most promiscuous forms of cardiac arrhythmia is atrial fibrillation (AF), which coincidentally is the major etiology for cardiac ischemia (Kopecky et al. 1987; Greer et al. 1989; Allessie et al. 1995; Ohkusa et al. 1999;). Incidental loss of atrial refractoriness is the central pathological mechanism of AF (Allessie et al. 1995); however, the signaling pathways involved in such changes are not completely defined (Guo et al. 2015; Scott et al. 2019).

One important mediator of AF has been characterized as the ryanodine receptor (RyR) that is essentially a calcium (Ca2+) release channel functioning in the

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myocardial sarcoplasmic reticulum. Hence, compromised calcium uptake or release due to anomalous RyR hyperactivity will hamper homeostatic regulation of ventricular fibrillation, which can be additionally aided by impaired activity of the ATPase ATP2A2 (Koretsune and Marban 1989; Kihara and Morgan 1991; Kojima et al. 1994; Zaugg et al. 1995). Indeed, it has been clearly shown that the RyR type 2 receptor (RyR2) is a central node in both origin and maintenance of AF (Voigt et al. 2013; Chiang et al. 2014a; Li et al. 2014). In paroxysmal AF, the non-phosphorylated RyR2 mediates the process whereas the hyperphosphorylated RyR2 mediates pathogenesis in chronic AF (Chelu et al. 2009; Beavers et al. 2013; Voigt et al. 2013; Chiang et al. 2014a, b; Li et al. 2014). In both cases increased expression of RyR2 or its activity results in increased Ca2+ mobilization into the sarcoplasmic reticulum concurrent with changes associated with AF (Lab and Lee 1990; Chelu et al. 2009; Beavers et al. 2013; Voigt et al. 2013; Chiang et al. 2014b).

RyR2 is normally phosphorylated at a basal level; however, during AF Ca2+/calmodulin-dependent protein kinase II (CaMKII) and protein kinase A (PKA)-mediated hyperphosphorylation of RyR2 ensues (Wehrens et al. 2006). Furthermore, RyR2 hyperphosphorylation has been shown to be sensitive to inhibitors of protein kinase C (PKC) (Carter et al. 2011), indicating that other kinases might be involved as well. Indeed, RyR2 is known to be phosphorylated at serine (S) 2808, S2814, and S2030 residues and given that PKC is a serine/threonine kinase it might be possible that it alone or other serine/threonine kinases are also involved in the hyperphosphorylation of RyR2. However, the role of PKC activation in general on AF is unknown. Hence, the objective of the current study was to study the role of PKC activation on downstream signaling pathways in a rat model of AF.

Materials and methods

Establishment of rat AF model, tissue harvest, isolation and culture of cardiac fibroblasts

AF rat models were generated in 12 healthy male Sprague-Dawley rats as previously described (Sharifov et al. 2004; Li et al. 2019). Briefly, rats were injected via tail vein with acetylcholine (66 μg/ml; Selleckchem, Shanghai, China) and calcium chloride (10 mg/ml; Sigma-Aldrich, Shanghai, China) once per day for 7 days. Appearance of typical f-waves in absence of P-waves in ECG was used to confirm successful indication of establishment of AF (Xu et al. 2010). After 7 days, rats were sacrificed, and heart was removed after removing the thoracic cavity. The heart was rinsed in ice-cold saline before the atira were excised out and immersed in 75% alcohol for 10 min. The atrial tissue was dissected to small pieces and digested with trypsin (2.5 g/l) at 37 °C for 30 min. The tissue was subsequently rinsed with PBS before being centrifuge at 3300×*g* for 10 min. The supernatant was discarded, and cells were washed thrice with DMEM/F12 media (Sigma-Aldrich) containing 10% FBS (Gibco, Shanghai, China). Cardiac fibroblasts were further isolated by differential adhesion method and cultured in DMEM/F12 media containing 20% FBS for indicated time points. Similarly cardiac fibroblasts were generated from 12 healthy control age-matched rats.

Transduction

HA-PKC-delta lentivirus was obtained from Origene (USA) and transduced in cardiac fibroblasts using polybrene. Transduced cells were assayed after 72 h.

Treatment

As indicated, cultured cardiac fibroblasts were treated with the following reagents: puromycin (100 μg/ml) for 15 min, PKC inhibitor Go6983 (10 μM for 5 min; Selleckchem), PMA (30 μM for 5 min; Sigma-Aldrich), Bis-9 (10 μM for 5 min; Sigma-Aldrich), MEK inhibitor Trametinib (MEKi) (20 μM for 10 min; Selleckchem), and PI3K inhibitor LY294002 (5 μM for 10 min; Selleckchem).

Immunoblot and immunofluorescence analysis

Cardiac fibroblasts were pelleted and lysed using IP Lysis buffer (ThermoFisher, Shanghai, China) and quantified using BCA kit (ThermoFisher). Twenty-five micrograms of lysates were resolved by SDS-PAGE and immunoblotted using antibodies (all antibodies were used at 1:2000 dilution and obtained from Abcam). Shown are representative blots. Relative differences in protein expression were quantified from

blots obtained from all representative animals by densitometry analysis using NIH ImageJ software (https://imagej.nih.gov/ij/). Immunofluorescence was performed using routine methodologies as has been used before (Yu et al. 2014).

Statistical analysis

All data was represented as mean ± standard deviation (SD) of at least three independent replicates. Statistical significance between groups was analyzed using the Student's t-test. A p-value < 0.05 was considered statistically significant.

Results

We initially determined if PMA stimulation of cardiac fibroblasts resulted in increase in total protein synthesis. PMA stimulation resulted in robust induction of protein synthesis as assessed by puromycin labeling (Fig. 1a). To confirm if the induction in global protein synthesis observed was due to PKC activity induced by PMA treatment, we treated the cardiac fibroblasts with the PKC inhibitor Go6983. Treatment with Go6983 drastically reduced the total protein synthesis induced by PMA treatment (Fig. 1b), confirming that PCK activity induces global translational upregulation in cardia fibroblasts obtained from AF rats. PMA stimulation of cardiac fibroblasts isolated from non-AF rats, as determined by puromycin labeling, did not result in induction of protein synthesis (data not shown), indicating that this is an AF-specific observation. Role of PKC activity in normal heart has not been extensively studied (Singh et al. 2017), even though there are studies indicating its role in proliferation of cardiac fibroblasts (Gray et al. 1998; Brown et al. 2005; Khan et al. 2006).

Activated PKC can mediate its effect on global translation through multiple mechanisms including MAPK pathway (Singh et all. 2017). PMA treatment of cardiac fibroblasts isolated from AF rats activated PKC as assessed by P-PKC (Y311) (Fig. 2a, b). Importantly, PMA treatment also induced P-ERK activation (Fig. 2a, b). Co-treatment with the MEK inhibitor Trametinib inhibited induction of P-PKC as well as P-ERK (Fig. 2a, b), indicating that there is a feedback loop between P-ERK and PKC activation.

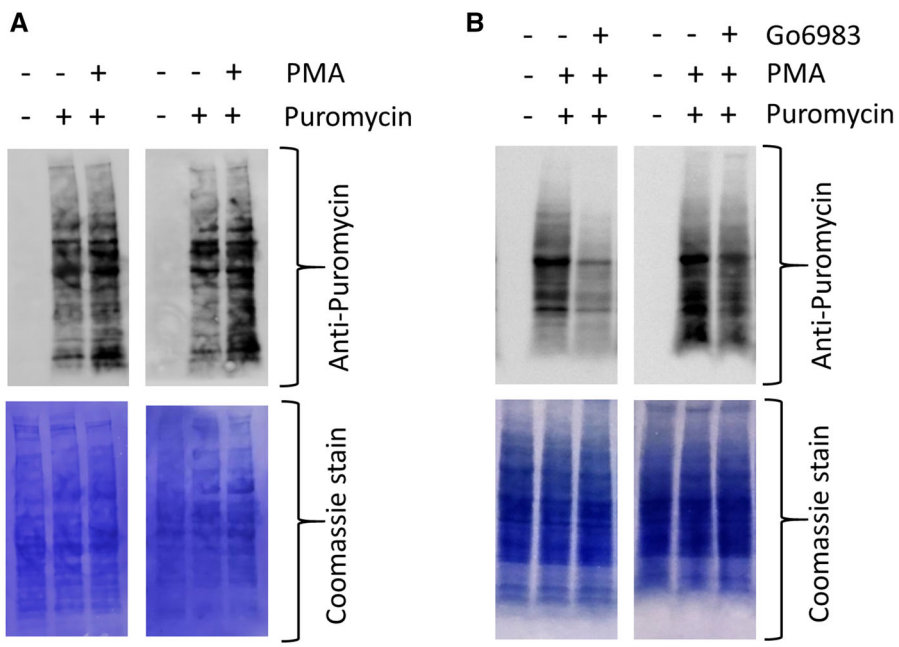

Fig. 1 PKC is activated in cardiac fibroblasts isolated from AF rats and results in increase in protein synthesis. Shown are representative western blot analysis of puromycin labeling in cardiac fibroblasts isolated from two representative AF rats treated with PMA alone (**a**), or PMA and the PKC inhibitor, Go6983 (**b**). Blots in each case were proved with Coomassie stain to confirm equal protein was loaded between different sample points

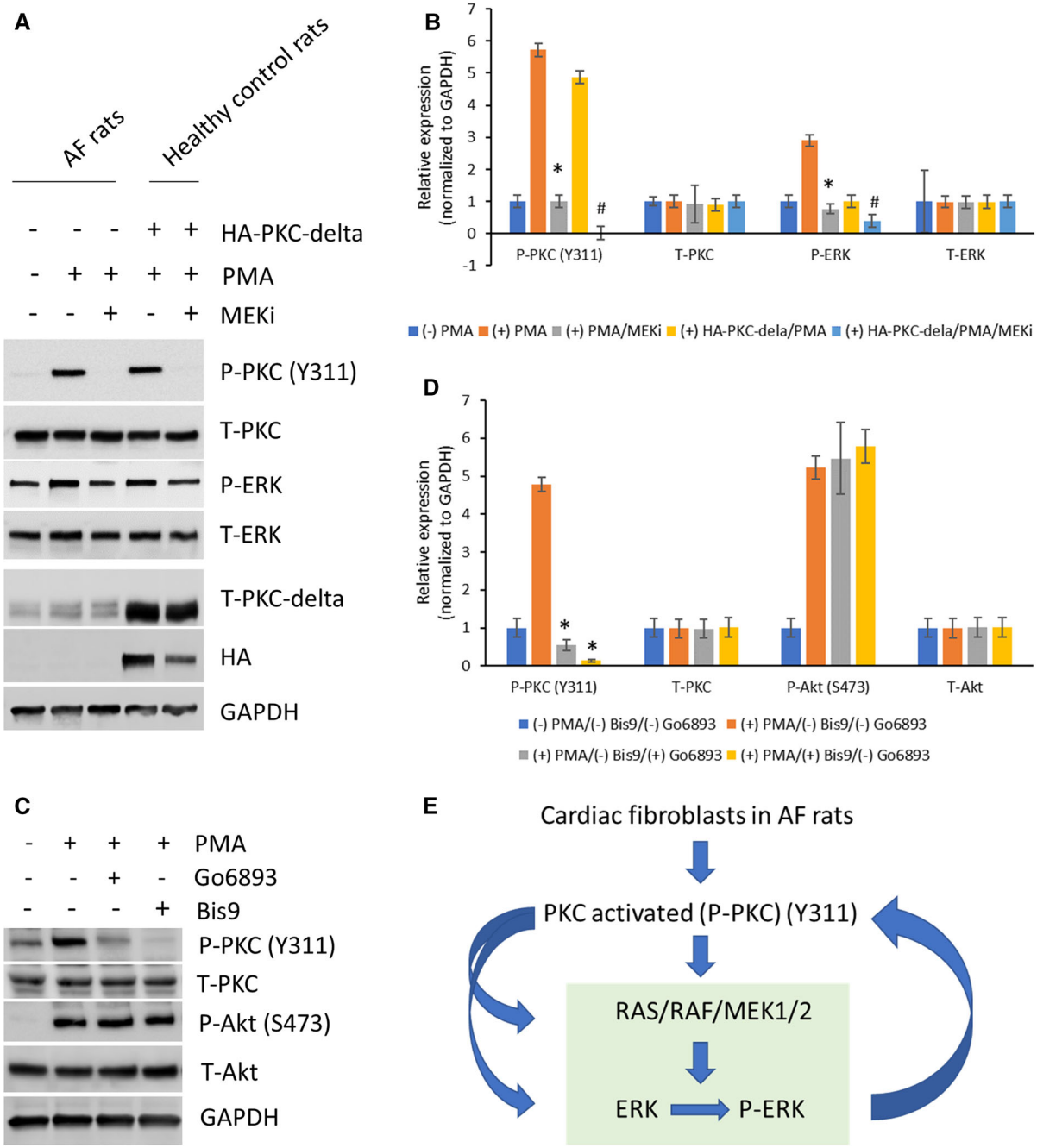

Fig. 2 Crosstalk between PKC and MAPK pathway activation in cardiac fibroblasts isolated from AF or healthy rats (transduced with PKC-delta). Lysates were probed with indicated antibodies (**a**, **c**). Graphs in **b** and **d** are densitometry analysis of blots shown in **a**, **c**, respectively (n = 12 animals each). *$P < 0.05$, compared to (+) PMA in **b** and (+) PMA/(−) Bis9/(−)Go6893 in **d**. #$P < 0.05$, compared to PKC-delta/PMA in **a**. **e** Schematic representation summarizing results of panels **a–d** indicating feedback regulation between PKC and MAPK pathway (*green box*) activation in cardiac fibroblasts of AF rats. Our results do indicate crosstalk between PKC and ERK; however, whether PKC is activating ERK directly or its upstream RAS/RAF/MEK1/2 is currently unknown—hence the two arrows from activated PKC to RAS/RAF/MEK1/2 and ERK

Given that PMA treatment alone in cardiac fibroblasts isolated from normal rats failed to induce PKC activity, we transduced them with HA-tagged PKC-delta. Transduction of PKC-delta induced P-PKC and P-ERK (Fig. 2a, b) and both were inhibited by MEKi. To confirm that the observation in Figs. 1 and 2a were due to PKC activation and not off-target activation of other kinases, we treated the cardiac fibroblasts with Go6983 and another pan-PKC inhibitor Bis-9 post-PMA treatment. Both inhibitors robustly suppressed PKC activation as assayed by P-PKC (Y311), without any effect on P-Akt (S473) (Fig. 2c, d). Taken together, these results confirmed that there is a feedback loop between MAPK and PKC pathways activation (Fig. 2e).

We next performed immunofluorescence assay to determine ERK activation in the cardiac fibroblasts following PMA treatment. PMA treatment induced robust amount of P-ERK (Fig. 3b) compared to untreated cells (Fig. 3a). Both Go6983 (Fig. 3c) and MEKi (Fig. 3d) inhibited P-ERK induction in the cardiac fibroblasts isolated from AF rats. Whereas the observation following MEKi was expected given that it will inhibit the MAPK activation, effect of Go6983 on P-ERK confirms the feedback loop between MAPK and PKC pathway activation in cardiac fibroblasts of AF rats.

PKC and MAPK signaling affect global translation by inducing the activation of p70 S6 kinase and subsequent activating phosphorylation of the S6 ribosomal protein (Peterson and Schreiber 1998;

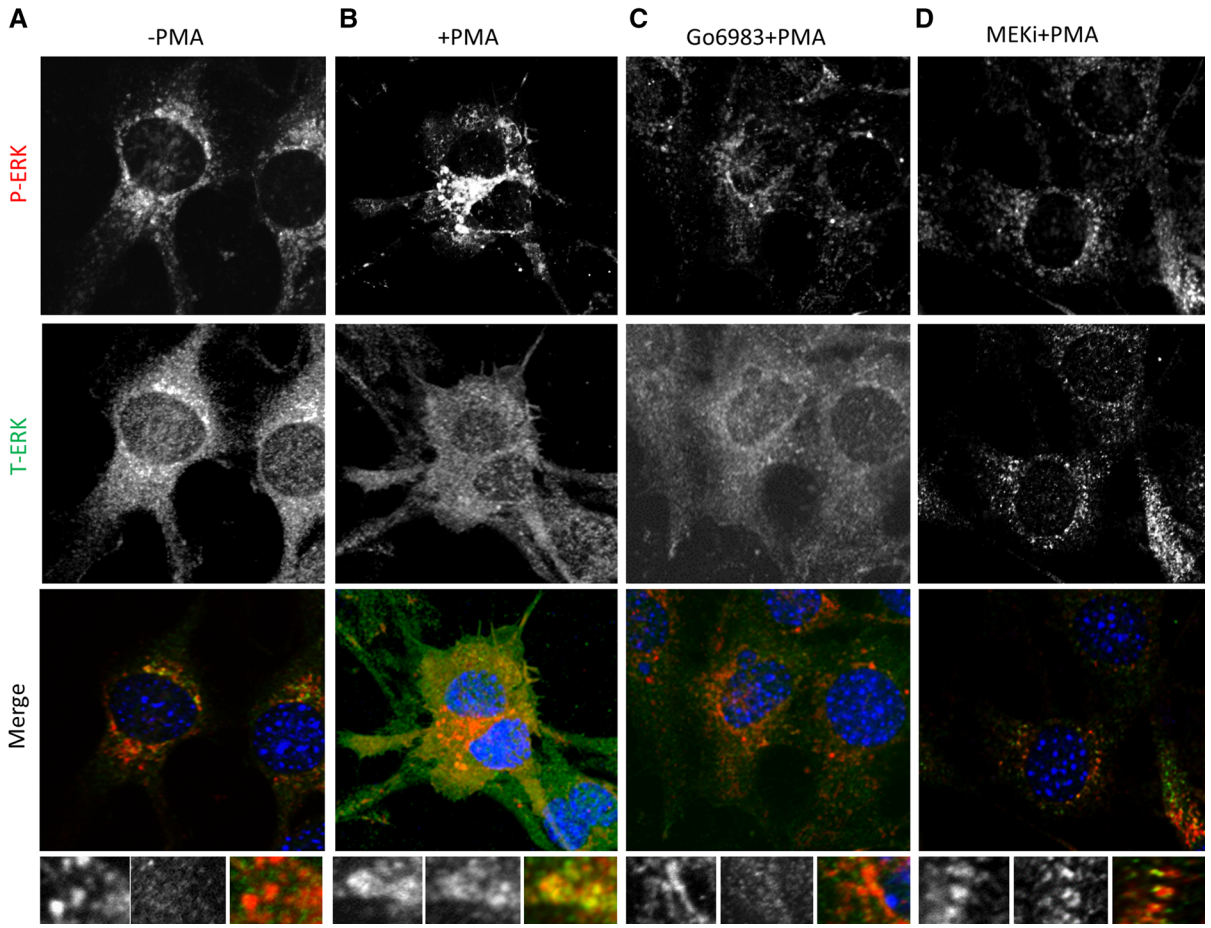

Fig. 3 Immunofluorescence analysis of P-EEK and T-ERK in AF cardiac fibroblasts induced with PMA alone or co-treated with PKC inhibitor Go6983 and the MAPK pathway inhibitor MEKi. Shown are representative images and merged figures obtained at × 20 and × 400 (bottom panels). Scale bar: 40 μm

Dufner and Thomas 1999). S6 ribosomal protein gets phosphorylated at S235, S236, S240, and S244—all located within the *C*-terminal region of S6 protein (Ferrari et al. 1991; Flotow and Thomas 1992). Hence, we next determined if PKC and MAPK pathway activation is also impinging on ribosomal protein S6. Indeed, PMA treatment of normal cardiac fibroblasts transduced with PKC-delta or cardiac fibroblasts from AF rats showed robust induction of P-S6 (S240/S244) (Fig. 4a). Induction of P-S6 (S240/S244) was inhibited by treatment with MEKi (Fig. 4a) or Go6983 (Fig. 4b) confirming effect of PKC and MAPK pathway activation in cardiac fibroblasts of AF rats is mediated via activation of S6.

Similar results were obtained when P-S6 (S235/S236) was evaluated (Fig. 4b). However, this was independent of PI3K/Akt pathway activation as the PI3K inhibitor LY294002 did not impact PKC and MAPK pathway activation. Overall these results indicated that activated PKC and MAPK signaling pathway is involved in global increase in translation of cardiac fibroblasts during AF.

Discussion

PKCs play a major role in signal transduction events in the heart. Activation of PKC can regulate gene expression changes associated with cell growth and differentiation, stimulating other kinases, substrate phosphorylation, ion channels (Farago and Nishizuka 1990; Kwiatkowska-Patzer and Domanska-Janik 1991; MacLeod and Hrding 1991; Harrington and Ware 1995; Mellor and Parker 1998). Indeed, PKC activation is involved in delayed preconditioning often by crosstalk with MAPK pathway (Singh et al. 2017).

Activation of angiotensin II type 1 receptors (AT-1) induces cascade of phosphorylation events including activation of MAPK pathway (Goette et al. 2002). AT-1 activation can also result in stimulation of phospholipase C and subsequent DAG (diacylglycerol)-mediated activation of PKC, ultimately leading release of calcium from intracellular stores (Urata et al. 1990; Berk 1998). PKC can also phosphorylate L-type calcium channels resulting in further influx of calcium (Goette et al. 2002).

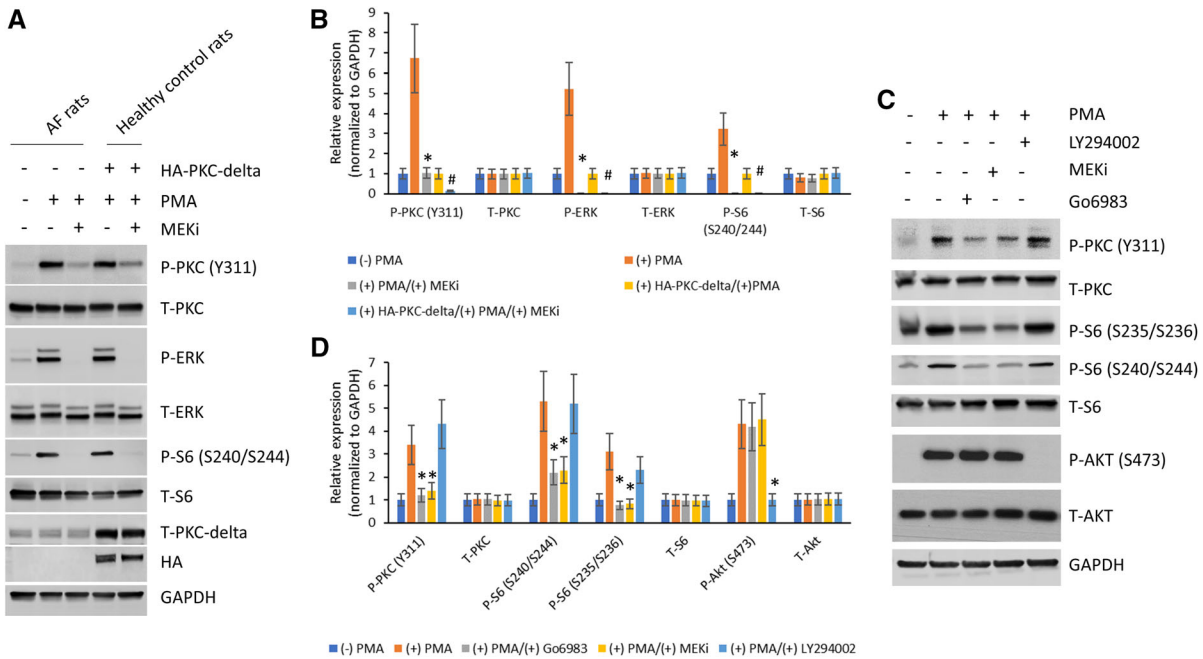

Fig. 4 PKC and MAPK activation in cardiac fibroblasts of AF rats induces activation of ribosomal protein S6 (**a**) and is independent of PI3K/Akt pathway(**c**). Shown are representative blots. Graphs in **b** and **d** are densitometry analysis of blots shown in **a** and **c**, respectively (n = 12 animals each). *P < 0.05, compared to (+) PMA in **b** and **d**. #P < 0.05, compared to PKC-delta/(+)PMA in **a**

It has recently been shown that PKC is hyperactive in patients with AF (Zhu et al. 2019). also It was speculated that PKC activation might function by maintaining Ca2 + homeostasis in cardiac sarcoplasmic reticulum (Zhu et al. 2019). Our results raise the possibility of it being regulated by PKC and MAPK pathway activation. PKC have been indicated to regulate different cardiac pathways, including acute reperfusion injury, hypertrophy and vascular anomaly, and myocardial infarction (Harper and Poole 2011; Palaniyandi et al. 2009; Newton et al. 2016). Thus it will be important to determine if PKC and/or MEKi will potentially be able to delay or abate AF pathogenesis in animal models, and by extension in patients with AF.

It will be important to identify whether RyR2 is a direct substrate of PKC or if there are other substrate(s) of PKC and MAPK that are equally or more important players in AF pathogenesis. PKC family has 15 isoforms (Newton et al. 2016; Newton 2003). It has been shown that PKCα is expressed the most among the different PKC isozymes in human heart, even though β2, δ, ε and γ isoforms have also been detected (Ping et al. 1997; Simonis et al. 2007; Geraldes and King 2010; Boghini et al. 1994). Even though we could replicate the observations in cardiac fibroblasts isolated from normal rats by transducing with PKC-delta, it remains to be determined if additional PKC isoforms are also involved in AF pathogenesis, and if they are involved whether they have specific or redundant functions.

It will also be important to determine if AF can be induced in animal models genetically modified to express kinase inactive mutant PKC. That will perhaps be the most convincing pre-clinical proof of involvement of PKC in AF. Also, gene expression analysis in these models will help define the specific alterations happening due to PKC and MAPK pathway activation during AF pathogenesis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All animal studies were approved by the Institutional Animal Care and Use Committee of the Beijing Tongren Hospital, Capital Medical University (Approval Number TRECKY 2018-056).

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