Supplemental Materials

Molecular Biology of the Cell

Liu et al.

PKCβII ACTS DOWNSTREAM OF CHEMOATTRACTANT RECEPTORS AND mTORC2 TO REGULATE cAMP PRODUCTION AND MYOSIN II ACTIVITY IN NEUTROPHILS

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1. SUPPLEMENTAL FIGURE LEGENDS

Figure S1. PKCβII is required for fMLP-induced cAMP accumulation and chemotaxis

(A) Expression of PKCα and PKCβII is increased during PLB-985 differentiation into neutrophil-like cells. PLB-985 cells were differentiated by treatment with 1.3% DMSO for 6 days. At different days, cells were lysed and equivalent amounts of cell lysates were subjected to Western blot analysis using PKCα, PKCβII and GAPDH antibodies. Results are representative of three independent experiments.

(B) PKCα and PKCβII KD in PLB-985 cells. Cells were lysed and equivalent amounts of cell lysates were subjected to Western blot analysis using PKCα, PKCβII and GAPDH antibodies. Results are representative of three independent experiments.

(C) PKCβII KD inhibits fMLP-induced cAMP production. Differentiated cells were stimulated with 1 μM fMLP for 30 secs and intracellular cAMP levels were measured before and after chemoattractant addition. Average ± SD values are presented from six independent experiments. *$p < 0.01$ compared to the fMLP-stimulated WT group.

(D) Chemotaxis of PKCβII KD cells to a point source of fMLP. To ensure that the micropipette was properly generating a chemical gradient, WT (gray) and shRNA (green) cells were mixed and exposed to a micropipette containing 1 μM fMLP. Frames were captured every 10 secs. Representative images at the designated time are shown. The star represents the position of the tip of the micropipette. Overlay of bright field and fluorescent images representative of three independent experiments are presented.

(E) PKCβII shRNA cells show defects in RhoA-GTP activation. Differentiated cells were plated on fibronectin-coated plates for 10 min and uniformly stimulated with 1 μM fMLP. At specific time points, RhoA-GTP was pulled down and detected using RhoA antibody. Results are representative of three independent experiments.

(F) PKCβII shRNA and Rictor shRNA cells show higher P-MLC levels. Differentiated cells were treated as in (E). At specific time points, cells were lysed and equivalent amounts of cell lysates were subjected to Western blot analysis using P-MLC and GAPDH antibodies. Results are representative of three independent experiments.

(G) PKCβII shRNA cells show higher P-MLC levels. Differentiated cells were treated as in (E). At specific time points, cells were lysed and equivalent amounts of cell lysates

were subjected to Western blot analysis using P-MLC and GAPDH antibodies. Results are representative of three independent experiments.

Figure S2. PKCβII overexpression inhibits fMLP-induced cAMP accumulation and chemotaxis

(A) Expression of PKCα and PKCβII do not change in response to chemoattractant addition in the presence of cycloheximide. Differentiated cells were pretreated with cycloheximide for 1 hr and stimulated with 1 μM fMLP. At the different time point, cells were lysed and equivalent amounts of cell lysates were subjected to Western blot analysis using PKCα, PKCβII and GAPDH antibodies. Results are representative of three independent experiments.

(B) Overexpression of PKCβII inhibits chemoattractant-induced PKC activation. Differentiated cells were stimulated with 1 μM fMLP for 20 secs and PKC activity was measured before and after chemoattractant addition. Mean ± SD values are presented from four independent experiments. *p < 0.01 compared to the fMLP-stimulated Venus group.

(C) Overexpression of PKCβII inhibits chemoattractant-induced cAMP production. Differentiated cells were stimulated with 1 μM fMLP for 30 secs and intracellular cAMP levels were measured before and after chemoattractant addition. Mean ± SD values are presented from four independent experiments. #p < 0.05 compared to the fMLP-stimulated WT group.

(D) PKCβII Venus cells have a lower Chemotaxis Index. The CI of cells during the first 5 min and from 5-20 min was quantified from EZ-TAXIScan recordings. The graph represents mean ± SD from six independent experiments. *p < 0.01 compared to the Venus group.

(E) PKCβII Venus cells have decreased migration speed. Migration speed of cells during the first 5 min and from 5-20 min was quantified from EZ-TAXIScan recordings. The graph represents mean ± SD from six independent experiments. *p < 0.01 compared to the Venus group.

Figure S3. The cytosol-to-membrane translocation of PKCβII is dependent on mTORC2-mediated TM site phosphorylation

(A) PKCβII activity is required for its cytosol-to-membrane translocation. Human blood primary neutrophils were treated with or without 10 μM GO6976 for 30 min. Cells were uniformly stimulated with 1 μM fMLP. At specific time points, membrane and cytosol fractions were isolated and subjected to Western blot analysis using PKCα, PKCβII and GAPDH antibodies. A representative blot of three independent experiments is shown.

(B) The cytosol-to-membrane translocation of PKCβII is independent of actin polymerization. Human blood primary neutrophils were treated with or without 10 μM

Latrunclin A for 30 min. Cells were uniformly stimulated with 1 μM fMLP. At specific time points, membrane and cytosol fractions were isolated and subjected to Western blot analysis using PKCβII and GAPDH antibodies. A representative blot of three independent experiments is shown.
(C) The cytosol-to-membrane translocation of PKCβII is dependent on DAG and Ca^{++}. Human blood primary neutrophils were treated with or without 2 mM EGTA or 10 μM U73122 for 30 min. Cells were uniformly stimulated with 1 μM fMLP. At specific time points, membrane and cytosol fractions were isolated and subjected to Western blot analysis using PKCβII and GAPDH antibodies. A representative blot of three independent experiments is shown.
(D) The cytosol-membrane trafficking of PKCβII is independent on PI3K. Human blood primary neutrophils were treated with or without 30 μM LY294002 for 30 min. Cells were uniformly stimulated with 1 μM fMLP. At specific time points, membrane and cytosol fractions were isolated and subjected to Western blot analysis using P-Akt, PKCβII and GAPDH antibodies. A representative blot of three independent experiments is shown.
(E) WT, HM, TM and HM+TM mutants of PKCβII Venus were expressed at comparable levels in differentiated cells. Differentiated cells were lysed and subjected to Western blot analysis using antibody against GFP and PKCβII antibodies. A representative blot of three independent experiments is shown.

Figure S4. fMLP induces the phosphorylation of PKCβII on its TM and HM sites
(A) Chemoattractant-induced HM phosphorylation of PKCβII. Human blood primary neutrophils were uniformly stimulated with 1 μM fMLP. At specific time points, cell lysates were subjected to Western blot analysis using an antibody against P-PKCα (S657), PKCα, P-PKCβII (S660) and PKCβII. Quantification of three experiments is presented as the amount of P-PKC after fMLP stimulation relative to that of unstimulated cells (mean ± SD). *$p < 0.01$, compared to unstimulated cells.
(B) HM phosphorylation of PKCβII is dependent on mTORC2. Differentiated NS shRNA and Rictor shRNA cells were stimulated with 1 μM fMLP. At specific time points, cells were lysed and subjected to Western blot analysis using P-PKCα (S657), PKCα, P-PKCβII (S660) and PKCβII antibodies. Quantification of three experiments is presented as the amount of P-PKCα or P-PKCβII after fMLP stimulation relative to that of unstimulated cells (mean ± SD). *$p < 0.01$, compared to unstimulated cells.
(C) TM phosphorylation of PKCβII in PKCα shRNA cells. Differentiated PKCα shRNA cells were stimulated with 1 μM fMLP. At specific time points, cells were lysed and subjected to Western blot analysis using P-PKCα/βII (638/641) and GAPDH antibodies. A representative blot of three independent experiments is shown.

2. SUPPLEMENTAL MOVIE LEGENDS

Movie S1. EZ-Taxiscan chemotaxis of primary neutrophils in a gradient of fMLP in the presence or absence of 10 μM GO6976 or 5 μM CGP53353. Images were taken every 15 secs.

Movie S2. EZ-Taxiscan chemotaxis of differentiated NS shRNA, PKCα shRNA, and PKCβII shRNA cells in a gradient of fMLP. Images were taken every 15 secs.

Movie S3. Differentiated WT (bright field) and NS shRNA (fluorescent) cells were subjected to a micropipette filled with 1 μM fMLP and allowed to migrate directionally. Images were taken every 10 secs.

Movie S4. Differentiated WT (bright field) and PKCα shRNA (fluorescent) cells were subjected to a micropipette filled with 1 μM fMLP and allowed to migrate directionally. Images were taken every 10 secs.

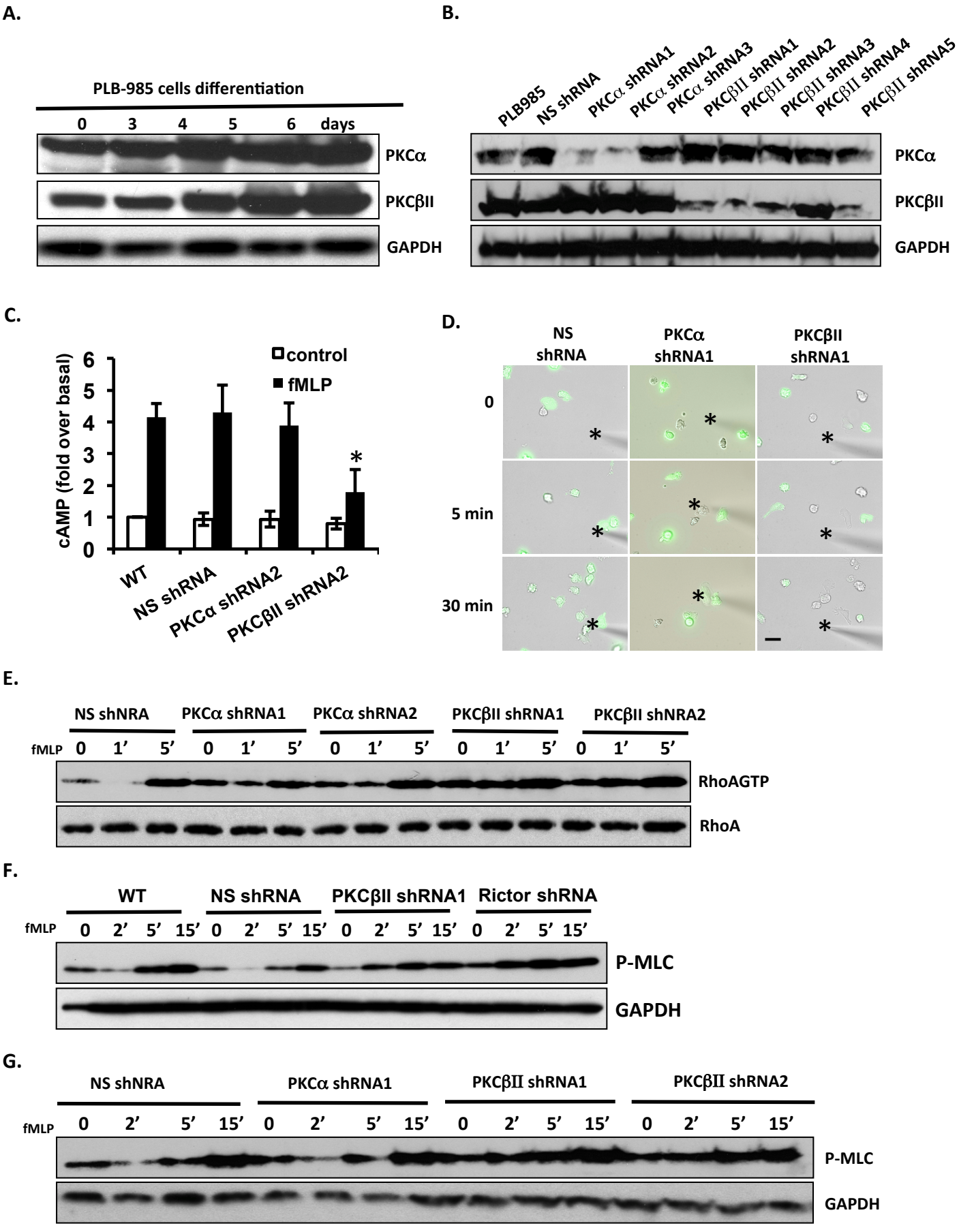
Movie S5. Differentiated WT (bright field) and PKCβII shRNA (fluorescent) cells were subjected to a micropipette filled with 1 μM fMLP and allowed to migrate directionally. Images were taken every 10 secs.

Movie S6. EZ-Taxiscan chemotaxis of differentiated Venus, PKCα Venus, and PKCβII Venus cells in a gradient of fMLP. Images were taken every 15 secs.

Movies S7. Differentiated PKCα Venus cells were uniformly stimulated with 1 μM fMLP. Images were taken every 10 secs.

Movies S8. Differentiated PKCβII Venus cells were uniformly stimulated with 1 μM fMLP. Images were taken every 10 secs.

Figure S1


A.
PLB-985 cells differentiation
0 3 4 5 6 days
PKCα
PKCβII
GAPDH
B.
PLB985
NS shRNA
PKCα shRNA1
PKCα shRNA2
PKCα shRNA3
PKCβII shRNA1
PKCβII shRNA2
PKCβII shRNA3
PKCβII shRNA4
PKCβII shRNA5
PKCα
PKCβII
GAPDH
C.
control
fMLP
cAMP (fold over basal)
WT
NS shRNA
PKCα shRNA2
PKCβII shRNA2
D.
NS shRNA
PKCα shRNA1
PKCβII shRNA1
0
5 min
30 min
E.
NS shNRA
PKCα shRNA1
PKCα shRNA2
PKCβII shRNA1
PKCβII shNRA2
fMLP 0 1' 5'
RhoAGTP
RhoA
F.
WT
NS shRNA
PKCβII shRNA1
Rictor shRNA
fMLP 0 2' 5' 15'
P-MLC
GAPDH
G.
NS shNRA
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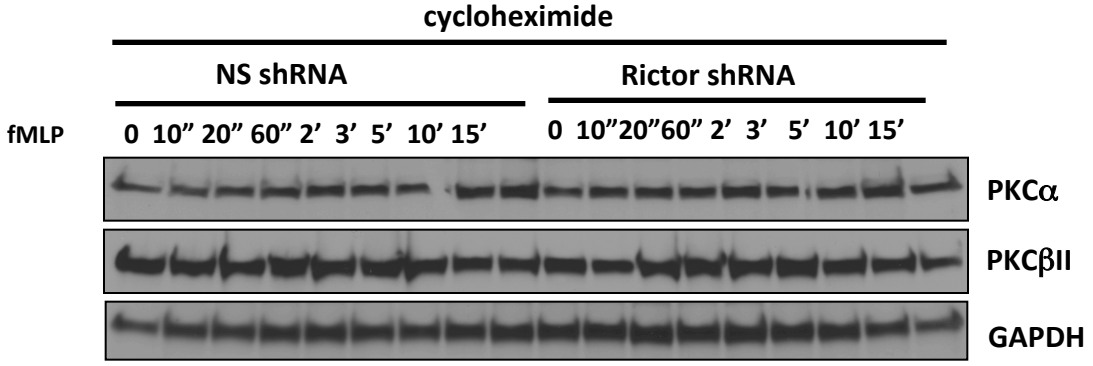
P-MLC
GAPDH

Figure S2

A.



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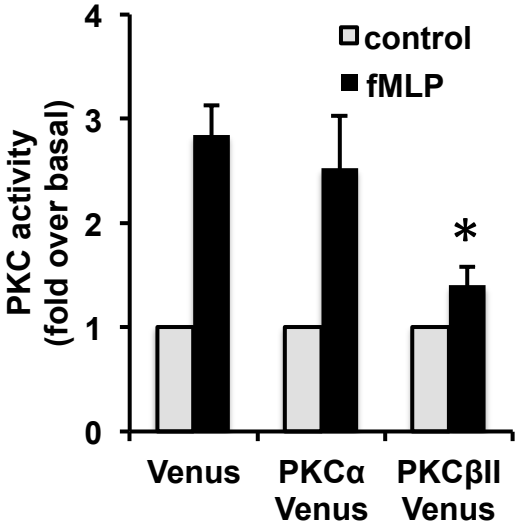

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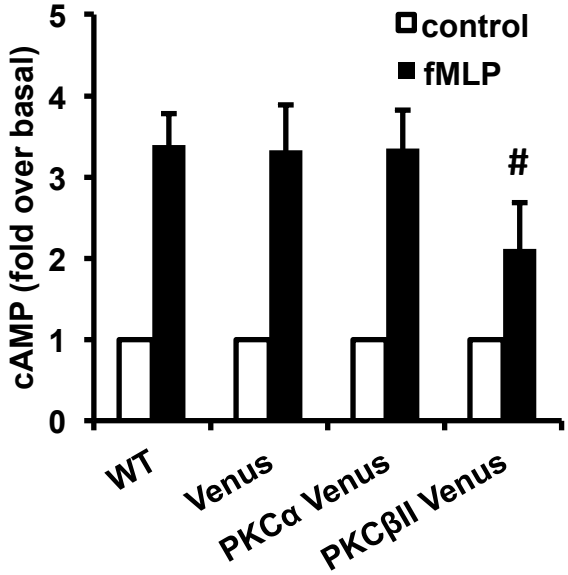

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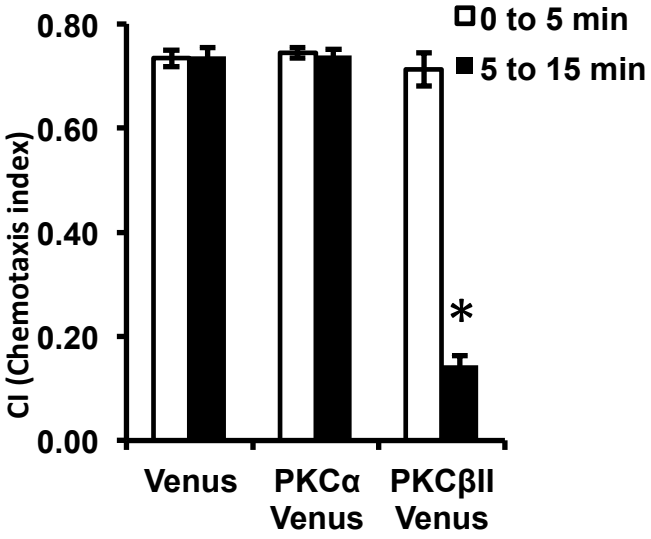


E.

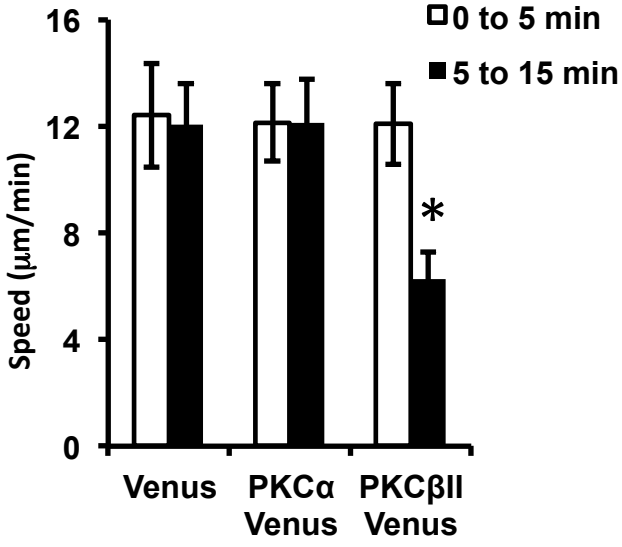
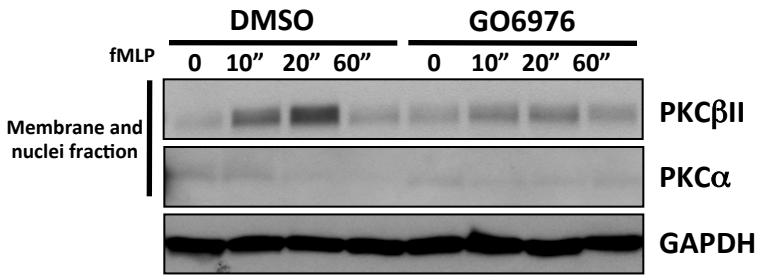


Figure S3

A.



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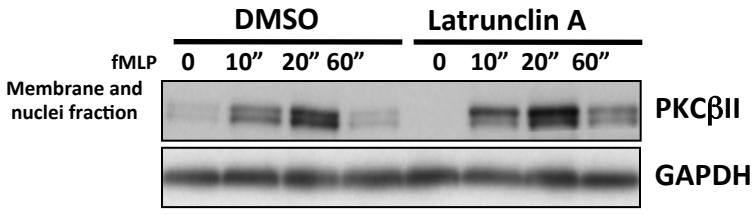

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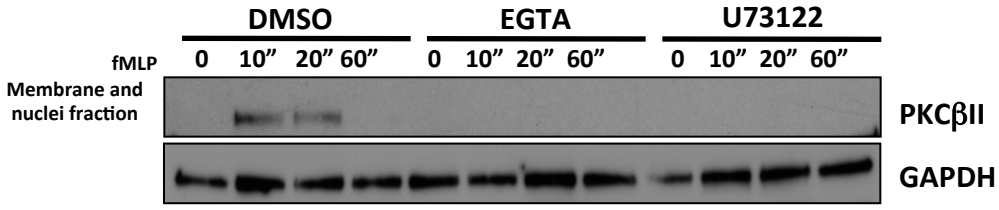

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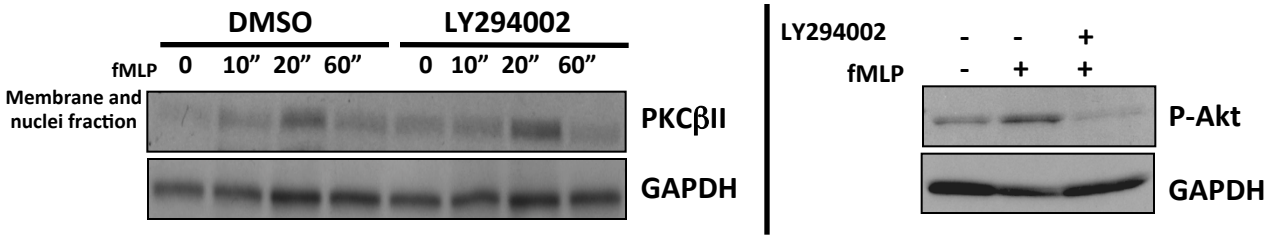

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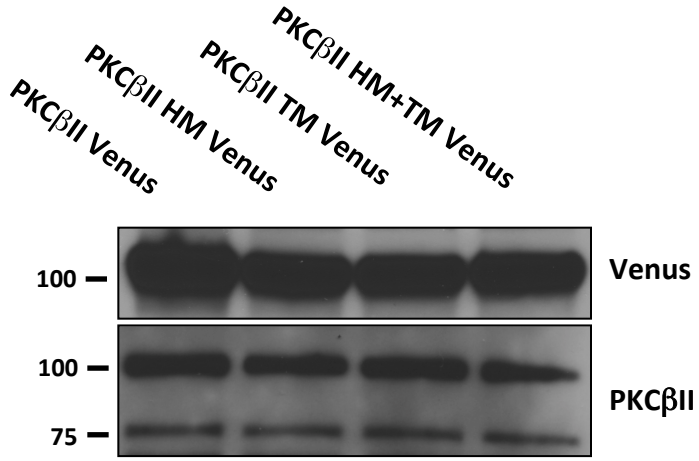
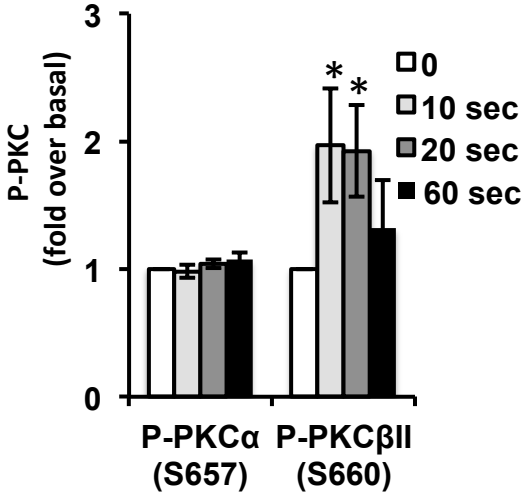


Figure S4

A.



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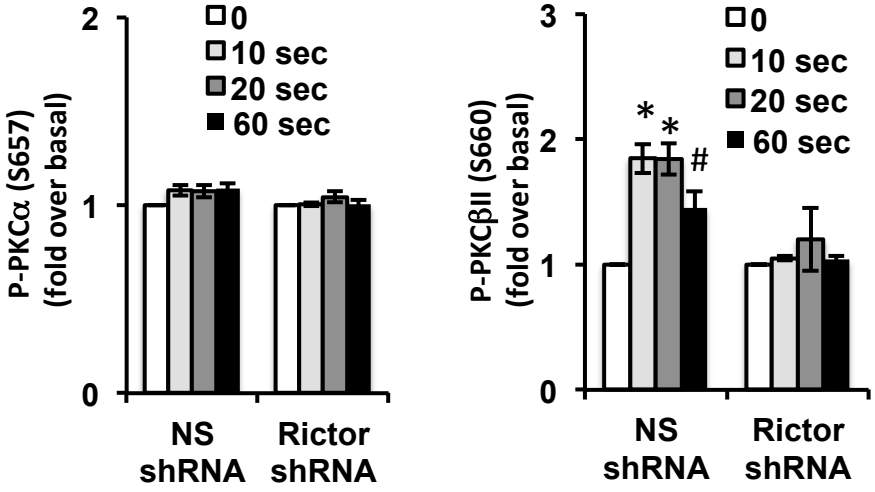

C.