Myristoylated PKC β II peptide inhibitor exerts dose-dependent inhibition of N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) induced leukocyte superoxide release

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Introduction

Phosphorylation of polymorphonuclear leukocyte (PMN) NADPH oxidase by protein kinase C (PKC) is essential to generate superoxide (SO) release. Inhibition of leukocyte SO release attenuates inflammation mediated vascular tissue injury (e.g. myocardial ischemia/reperfusion (MI/R)). There are 11 isoforms of PKC and the role of PKC isoforms that mediate this response has not been fully elucidated. PKC beta II (PKC βII), a classical isoform that is activated by calcium and diacylglycerol (DAG), following PMN chemotactic receptor stimulation with fMLP peptide (Fig.1) (1). Activated PKC βII will activate NADPH oxidase and stimulate SO release. Selective PKC βII peptide inhibitor has been developed based on its binding sites to receptor for C kinase (RACK) domain (Fig 2) (2). Myristoylation of peptides is known to be an effective strategy to enable simple diffusion through cell membranes to affect PKC function (3, 4).

Myristoylated (Myr) PKC βII peptide inhibitor is known to inhibit PMN SO release at doses that correlated with restoration of post-reperfused cardiac function following global MI(20min)/R(45min) in leukocyte mediated cardiac MI/R dysfunction (1,5) and more recently in prolonged MI(30min)/R(90min) in isolated rat hearts (See Poster # P 204). However, a full dose-response curve with Myr-PKC βII peptide inhibitor (0.2-20 μM) has not been indicated previously. The peptide attenuates PKC βII translocation to the cell membrane by inhibiting the interaction with the RACK domain (Fig.2). Characterizing the full dose-response effects is essential in identifying putative mechanisms responsible for attenuating vascular and tissue injury following I/R.

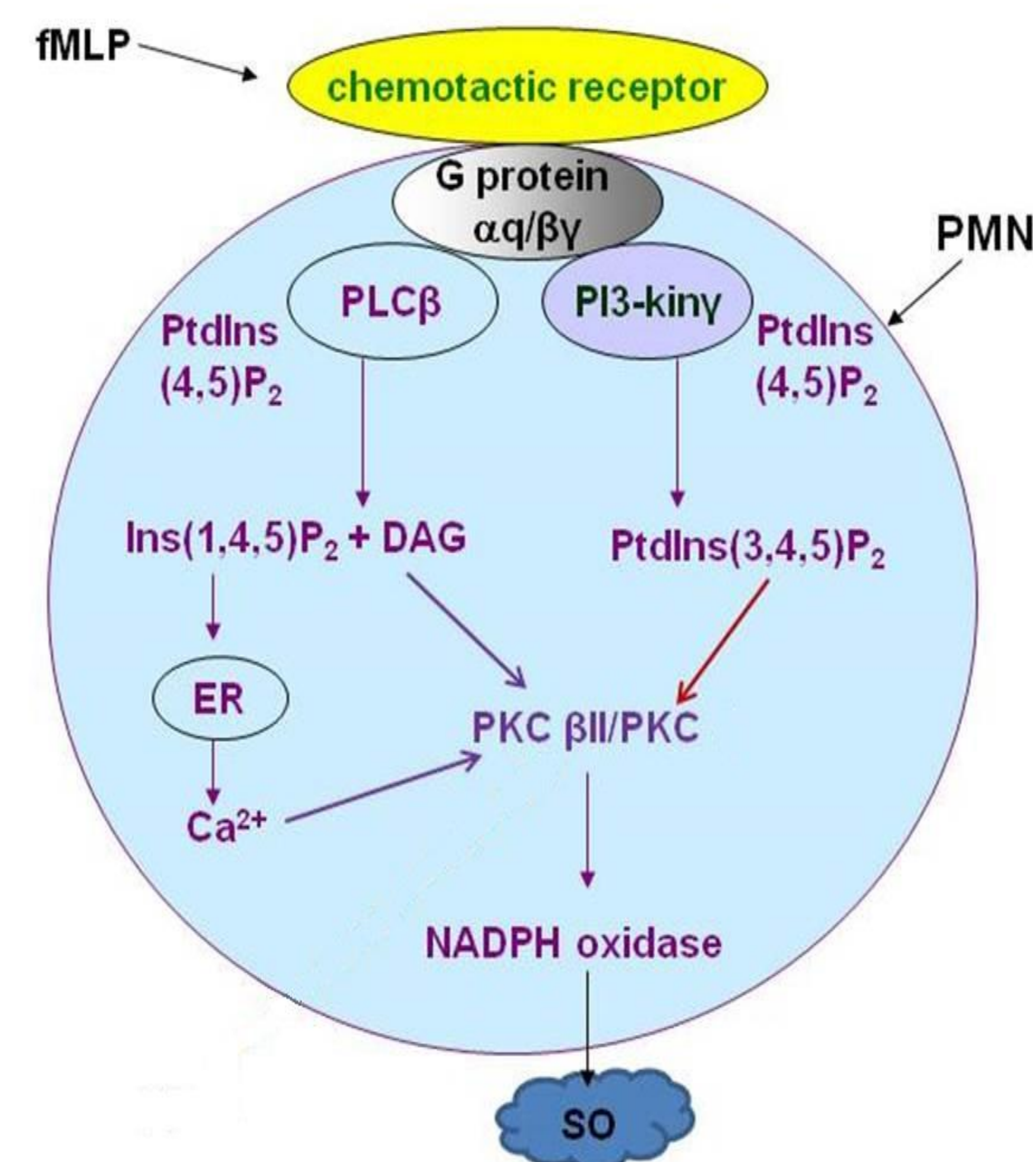

Figure 1. Schematic representation of PKC activation generating SO release in PMN. PMN chemotactic G-protein receptors are activated by fMLP. The G-protein subunits αq and βγ disassociate after stimulation and activate phospholipase C beta (PLCβ) to produce inositol 1,4,5 trisphosphate ($Ins(1,4,5)P_2$) plus DAG respectively from phospholipids phosphatidylinositol 4,5 bisphosphate ($PtdIns(4,5)P_2$). $Ins(1,4,5)P_2$ stimulates Ca^{2+} release from the endoplasmic reticulum (ER). Ca^{2+} and DAG directly activate PKC βII . Activated PKC βII phosphorylates NADPH oxidase to release SO (Adapted from Young et al.).

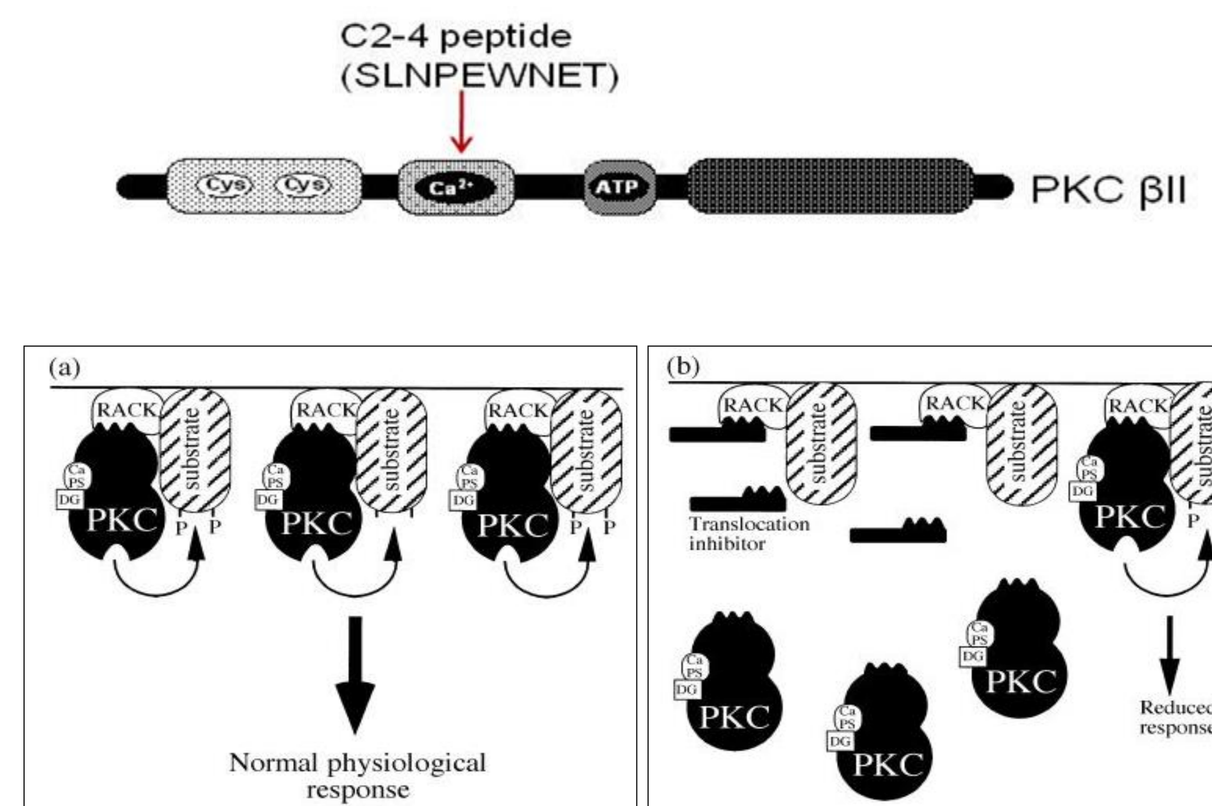

Figure 2. Illustration of PKC βII peptide inhibitor. The Ca^{2+} binding domain for PKC βII peptide inhibitor (i.e., C2-4 region) is unique for PKC βII translocation to the cell membrane when activated (Adapted from 2) (top). PKC βII peptide inhibitor mechanism of action (Adapted from 5) (bottom) is to inhibit PKC βII translocation to leukocyte substrates such as NADPH oxidase (Adapted from Csukai and Mochly-Rosen).

Hypothesis

We hypothesized that Myr-PKC βII peptide inhibitor (0.2-20 μM) would dose-dependently attenuate fMLP induced PMN SO release. We further predict that 5 to 20 μM doses would exert significant attenuation of fMLP induced PMN SO release compared to non-drug or low drug treated (0.2 and 0.5 μM) PMNs and these effects would not be associated with a decrease in cell viability.

Methods

Isolation of PMNs

Male Sprague-Dawley rats (350–400 g, Charles River), used as PMN donors, were anesthetized with 2.5 % isoflurane and given a 16 ml intraperitoneal injection of 0.5% glycogen (Sigma Chemical) dissolved in PBS. Rats were reanesthetized with isoflurane 16–18 h later, and the PMNs were harvested by peritoneal lavage in 30 ml of 0.9% NaCl, as previously described (2,3). The peritoneal lavage fluid was centrifuged at 200 g for 10 min at 4° C. The PMNs were then washed in 20 ml PBS and centrifuged at 200 g for 10 min at 4° C. Thereafter, the PMNs were resuspended in 2.5 ml PBS and density was calculated. The PMNs preparation were >90% pure and >95% viable according to microscopic analysis and exclusion of 0.3% trypan blue, respectively. Cell viability among all study groups was determined by 0.3% trypan blue exclusion.

Measurement of SO Release From Rat PMNs

The SO release from PMNs was measured spectrophotometrically (model 260 Gilford, Nova Biotech; El Cajon, CA) by the reduction of ferricytochrome c (1,3). The PMNs (5×10^6) were resuspended in 450 μl PBS and incubated with ferricytochrome c (100 μM, Sigma Chemical) in a total volume of 900 μl PBS in the presence or absence of myr PKC βII inhibitor (0.2 to 20 μM; N-myr-SLNPEWNET , 1300 g/mol) for 15 min at 37° C in spectrophotometric cells. The PMNs were stimulated with 1 μM fMLP (MW= 434 g/mol) (Calbiochem) in a final reaction volume of 1.0 ml. Absorbance at 550 nm was measured every 30 sec for up to 120sec for fMLP and the change in absorbance (SO release) from PMNs was determined relative to time 0.

Statistical Analysis

All data in the text and figures are presented as means ± S.E.M. The data were analyzed by analysis of variance using post hoc analysis with the Fisher's PLSD test. Probability values of <0.05 are considered to be statistically significant.

Results

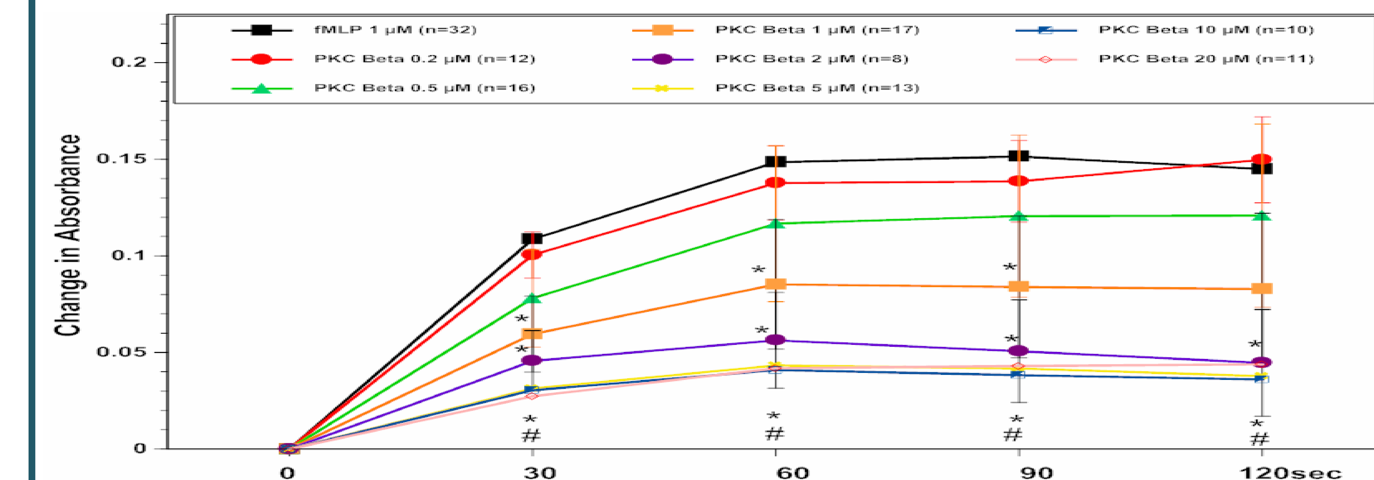

Figure 3. The time course response of fMLP (1μM)-induced SO release. Myr-PKC βII peptide inhibitor (0.5-20 μM) dose dependently inhibited SO release from untreated controls (*p<0.05) 21-26 ±12% (0.5 μM; 30-120 sec), 43-46 ±19% (1 μM; 30-120 sec), 58-69 ±9% (2 μM; 30-120 sec), 71-75 ±12% (5-20 μM; 30-120 sec). PKC βII peptide inhibitor high dose groups (5-20 μM) were significantly different from the low dose group (0.2 μM) (# p<0.05) from 30-120 sec.

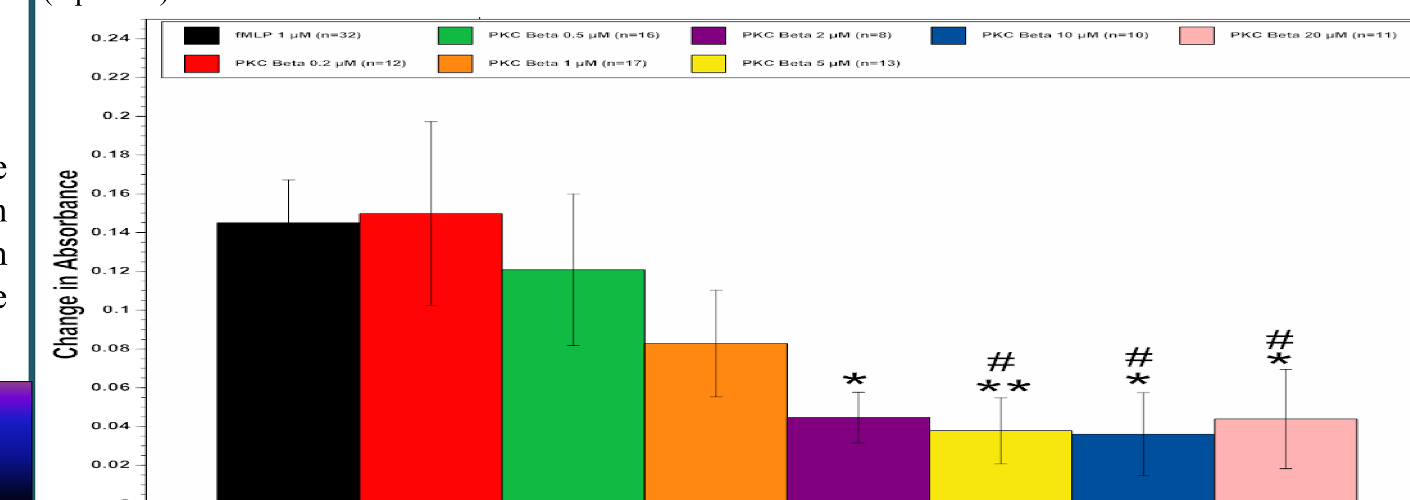

Figure 4. fMLP (1μM)-induced peak response (120 sec) SO release in PMNs. Myr-PKC βII peptide inhibitor dose dependently (0.5-20 μM) attenuated SO release compared to untreated controls (*p<0.05; **p<0.01) by 26 ±12% (0.5 μM), 43 ±19% (1 μM), and 69-75 ±12% (2-20 μM). PKC βII peptide inhibitor high dose groups (5-20 μM) were significantly different from the low dose group (0.2 μM) (# p<0.05).

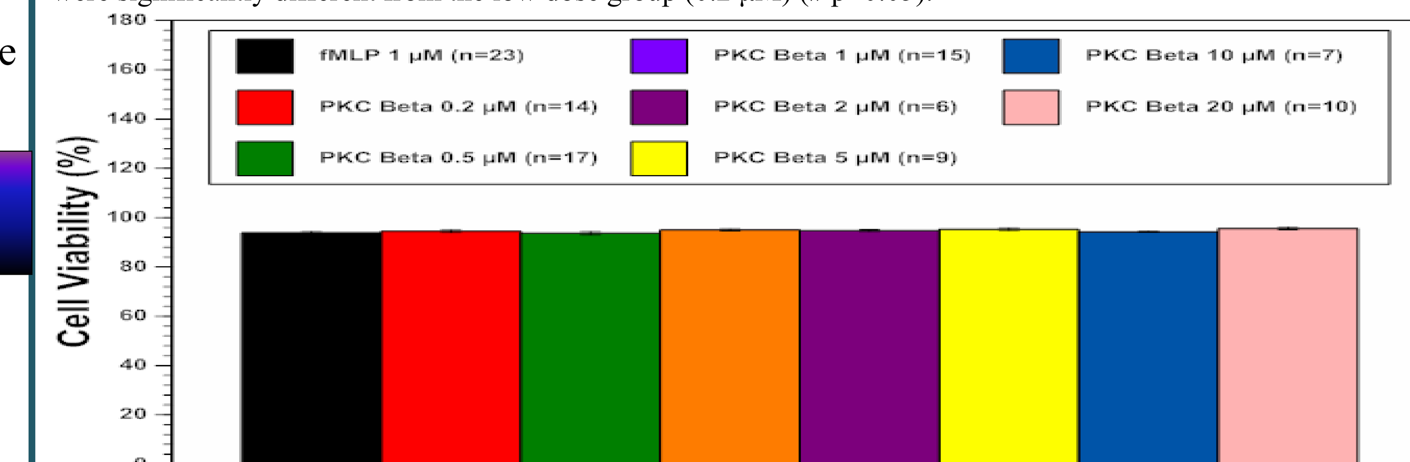

Figure 5. . The effects of Myr-PKC βII peptide inhibitor (0.2-20 μM) on cell viability in fMLP (1 μM)-induced SO release in PMNs. Cell viability ranged between 94-96% ± 1 and was not different amongst study groups, suggesting that the inhibition of leukocyte SO release was not related to cell death

Conclusions

Myr-PKC βII peptide inhibitor dose-dependently inhibited fMLP-induced SO release by about 70%; suggesting that activation of NADPH oxidase via PKC βII is the dominant pathway following stimulation of the leukocyte chemotactic receptor. This study suggests that PKC βII peptide inhibitor maybe anti-inflammatory agent that can be used in vascular complications associated with I/R.

References

1. Young, L., et al., Gö 6983: *A Fast Acting Protein Kinase C Inhibitor that Attenuates myocardial Ischemia/Reperfusion Injury*. Cardiovasc Drug Rev. 2005. 23(3): p. 255-272
2. Csukai M & Mochly-Rosen D. *Pharmacoligic modulation of protein kinase C isozymes: The role of racks and subcellular localisation* Pharmacol Res. 1999. 39(4): p. 253-259.
3. Perkins KA, et al. *Myristoylation of protein kinase C beta II/zeta peptide inhibitors, or caveolin-1 peptide facilitates rapid attenuation of phorbol 12-myristate 13-acetate (PMA) or N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) activated leukocyte superoxide release.* Proceedings of the 22nd American Peptide Symposium, Michal Lebl (Editor), American Peptide Society, 288-289, 2011
4. Eichholtz, T., et al. *A myristoylated pseudosubstrate peptide, a novel protein kinase C inhibitor.* J Biol Chem, 1993. 268(3): p. 1982-6.
5. Omiyi, D., et al. *Protein kinase C betaII peptide inhibitor exerts cardioprotective effects in rat cardiac ischemia/reperfusion injury.* J Pharmacol Exp Ther, 2005. 314(2): p. 542-51.