Protein Kinase C Beta II Peptide Modulation of Superoxide Release in Rat Polymorphonuclear Leukocytes

Christina Lipscombe, Chinyere Ebo, Daphne Metellus, Rose M. Martorana, Arjun Nair, Harsh Patel, Annam Humayun, Jennifer Dang, Megan Michaels, Matthew Finnegan, Faosat Muftau-Lediju, Lucy Checchio, Anahi McIntyre, Qian Chen, Robert Barsotti, and Lindon Young

Department of Bio-Medical Sciences, Philadelphia College of Osteopathic Medicine, 4170 City Avenue, Philadelphia, PA 19131

Introduction

Activation of protein kinase C beta II (PKCβII) is known to stimulate polymorphonuclear leukocyte (PMN) NADPH oxidase (NOX-2) to produce superoxide (SO). PKCβII is dependent on diacylglycerol (DAG) and calcium for its activation. Activated PKCβII then binds to its selective receptor for activated C kinase (RACK) which enhances PKCβII translocation to the cell membrane and subsequent phosphorylation of membrane bound proteins (1,2). Of these, PKCβII phosphorylation of NOX-2 generates SO release (Figs. 1 and 2). PKCβII can be activated by both phorbol 12-myristate 13-acetate (PMA) and N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP). PMA, a lipid soluble broad-spectrum PKC agonist, is a DAG mimetic that directly activates PKCβII. fMLP is a chemotactic receptor agonist that directly activates PKCβII via the G-protein signaling cascade (see Fig. 1).

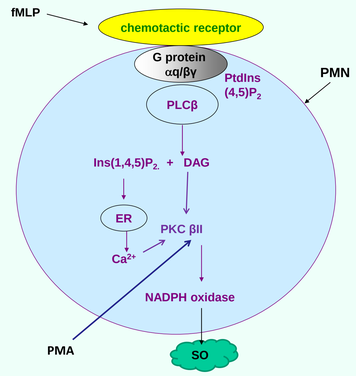
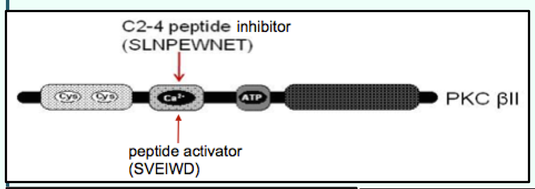


Figure 1. Schematic representation of PKCβII role in stimulating SO release in PMNs. PMN chemotactic G-protein receptors are activated by fMLP. Activated receptor stimulates phospholipase C beta (PLCβ) to produce second messengers, inositol 1,4,5 trisphosphate ($Ins(1,4,5)P_2$) and DAG respectively from phosphatidyl inositol 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 PKCβII. PMA also directly activates PKCβII. Activated PKCβII phosphorylates NADPH oxidase, which then releases SO (Adapted from 3).

Inhibition of tissue NOX-2 attenuates the inflammation mediated vascular injury seen in various diseases, including diabetes, myocardial infarction and organ transplantation (4). Previously, a myristoylated (myr-) selective PKCβII RACK peptide inhibitor (*N*-myr-SLNPEWNET; myr-PKCβII-) was found to dose dependently inhibit PMA and fMLP-induced PMN SO release and myocardial ischemia/reperfusion (MI/R) injury via the mechanism depicted in Figure 2 (3,5,6). Myristoylation of peptides is known to potentiate their entry into the cell via simple diffusion through the cell membrane to affect PKC activity (7). However, the role of myr-PKCβII RACK peptide activator (*N*-myr-SVEIWD; myr-PKCβII+) on regulation of PMN SO release has not been studied (8). The aim of the current study is to compare the effects of myr-PKCβII+/- on fMLP or PMA-induced PMN SO release.



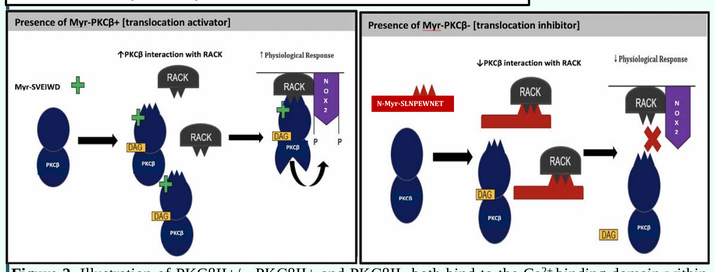


Figure 2. Illustration of PKCβII+/-. PKCβII+ and PKCβII- both bind to the Ca^{2+} binding domain within the RACK binding site (i.e., C2-4 region;) of PKCβII to regulate its translocation to the cell membrane to phosphorylate its substrate (top; Adapted from 3). PKCβII+ mechanism of action is to increase PKCβII translocation via RACK binding to interact with substrates, like NOX-2, while PKCβII- inhibits that interaction (bottom; Adapted from 2).

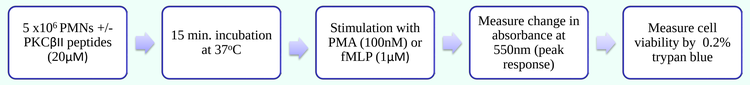
Hypothesis

We hypothesize that myr-PKCβII+ would increase fMLP or PMA -induced PMN SO release, whereas, myr-PKCβII- would decrease this response as compared to non-drug treated controls. We further predict that unconjugated, native PKCβII+/- peptide sequences would not differ from non-drug treated controls.

Research Design

Isolation of PMNs. Male Sprague-Dawley rats (350-400g, Charles River, Springfield MA) under anesthesia of 2.5% isoflurane were injected intraperitoneally (I.P.) with 16ml of 0.5% glycogen dissolved in PBS to cause accumulation of PMNs. After 16–18h, rats were re-anesthetized with isoflurane and the PMNs were harvested by peritoneal lavage as previously described (5,7).

Measurement of SO Release From Rat PMNs. The SO release from PMNs was measured spectrophotometrically by the reduction of ferricytochrome c and superoxide dismutase (SOD) 10μg/ml was used as positive control, as previously described (3,5,7). Please refer to schematic diagram below:



Statistical Analysis

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

Results

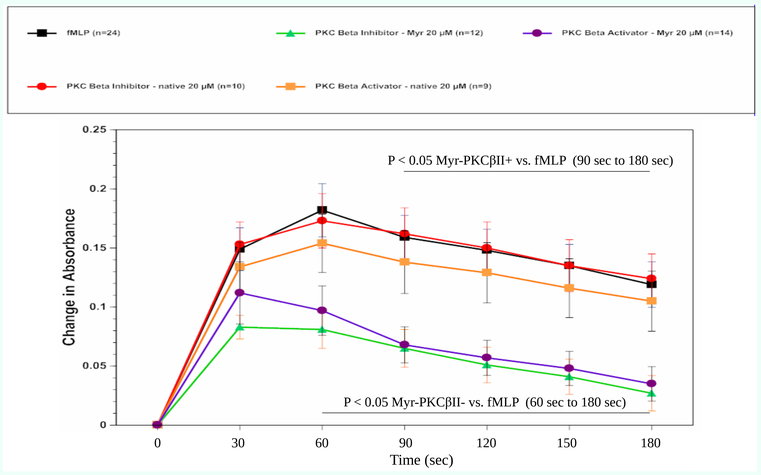


Figure 4. fMLP (1μM) induced maximal PMN SO release at 60 sec to 0.18±0.02 in non-drug controls and was significantly decreased by myr-PKCβII- (0.08±0.02) at 60 sec. By contrast, myr-PKCβII+ increased to (0.11±0.03) at 30 sec. was not different from non-drug treated controls. Native PKCβ+/- peptides were not different from non-drug controls throughout the time-course. SOD (n=8) reduced SO release >90% (not shown). Cell viability was >95% in all groups (not shown).

Results

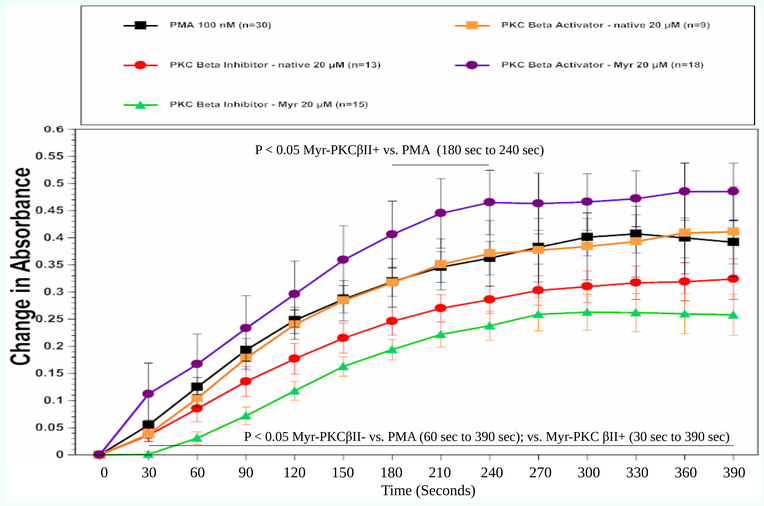


Figure 3. PMA (100nM) induced maximal PMN SO release at 330 sec to 0.41±0.04 in non-drug treated controls. PMA-induced PMN SO release was significantly increased by myr-PKCβII+ from 180 to 240 sec (P<0.05 vs. control) and generated maximal SO release at 360-390 sec.(0.49±0.05). By contrast, myr-PKCβII- significantly decreased SO release to 0.26±0.04) at 360-390sec. SOD (n=8) reduced SO release >90% (not shown). Cell viability was >95% in all groups (not shown).

Conclusions

1. **Myr-PKCβII+** significantly increased PMA-induced PMN SO release (from 180 to 240 sec.) and myr-PKCβII- significantly decreased both PMA and fMLP-induced PMN SO release (~ entire time-course). Native PKCβII+/- did not significantly attenuate PMA or fMLP induced PMN SO release as compared to non-drug treated controls. The results support our hypothesis except for the effects of PKCβII+ on fMLP-induced SO release.
2. **These results suggest that: (1)** Myristic acid conjugation is superior to native peptide in delivering the cargo sequence to augment or inhibit PKCβII translocation. **(2)** PKCβII is a principle PKC isoform that regulates PMN NOX-2 activity. **(3)** Myr-PKCβII+ may induce desensitization of the PMN chemotactic receptor in fMLP-induced SO release and may be responsible for the blunted SO release with this putative activator of PKCβII translocation. **(4)** The current data support that treatment with myr-PKCβII- would be an effective strategy to limit inflammation-induced (i.e. PMNs) tissue damage in heart attack patients or organ transplant recipients upon the restoration of blood flow.
3. **Future studies: (1a)** To test a myr-conjugated scrambled PKC βII+ or βII - to further evaluate the proposed mechanism of action (augmentation or inhibition of PKCβII translocation) of the cargo sequences (SVEIWD [PKCβII+] or SLNPEWNET [PKCβII-]). **(1b)** To conduct western blotting of native/myr-conjugated PKCβII+ or PKCβII- and their myr-scrambled peptide counterparts in treated PMN cell lysates.

References

1. Korchak HM, Kilpatrick LE. *Roles for beta II-protein kinase C and RACK1 in positive and negative signaling for superoxide anion generation in differentiated HL60 cells. J Biol Chem.* 2001 Mar 23;276(12): p. 8910-7. Epub 2000 Dec 18.
2. Csukai M, Mochly-Rosen D. *Pharmacologic modulation of protein kinase C isozymes: The role of racks and subcellular localization.* Pharmacol Res. 1999. 39(4): p. 253-259.
3. 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
4. Chen Q., et al. *Nox2ds-Tat, A Peptide Inhibitor of NADPH Oxidase, Exerts Cardioprotective Effects by Attenuating Reactive Oxygen Species During Ischemia/Reperfusion Injury.* American Journal of Biomedical Sciences 8(3): p. 208-227, 2016.
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.
6. Lipscombe C., et al. *Protein kinase C beta II (PKC βII) peptide inhibitor exerts cardioprotective effects in myocardial ischemia/reperfusion injury.* Proceedings of the 24th American Peptide Symposium Ved Srivastava, Andrei Yudin, and Michal Lebl (Editors) American Peptide Society, 24: p. 165-168, 2015.
7. 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, p. 288-289, 2011.
8. Kheifets V, Mochly-Rosen D. *Insight into intra- and inter-molecular interactions of PKC: design of specific modulators of kinase function.* Pharmacol Res. 2007 Jun;55(6): p. 467-76. Epub 2007 May 3.