Vol. 191, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 26, 1993 Pages 240-246

A NEW PROTEIN KINASE C, nPKCη', AND nPKCθ ARE EXPRESSED IN HUMAN PLATELETS: INVOLVEMENT OF nPKCη' AND nPKCθ IN SIGNAL TRANSDUCTION STIMULATED BY PAF

Fusheng Wang, Ulhas P. Naik, Yigal H. Ehrlich^^, Zachary Freyberg, Shin-ichi Osada*, Shigeo Ohno*, Toshio Kuroki, Koichi Suzuki^, AND Elizabeth Kornecki[1]**

Dept. of Anatomy/Cell Biology, SUNY Health Science Center at Brooklyn, Brooklyn, N.Y. 11203

***Dept. of Molecular Biology, Yokohama City University School of Medicine, Yokohama 236, Japan**

****Dept. of Cancer Cell Res., Institute of Medical Science, University of Tokyo, Tokyo 108, Japan**

^Institute of Applied Microbiology, University of Tokyo, Tokyo 113, Japan

^^CSI/IBR Ctr. Dev. Neurosci., CUNY at Staten Island, N.Y.10301

Received January 15, 1993

SUMMARY: We have detected in human platelets two protein kinase C isozymes that have not been reported previously. Using an anti-nPKCθ antibody and Western blotting, we calculated the molecular weight of platelet nPKCθ as 79K. This molecular weight is identical to that described for nPKCθ in skeletal muscle and in COS cells transfected with the nPKCθ-cDNA. Using an anti-nPKCη antibody, we determined the molecular weight of an immunoreactive protein, which we called nPKCη', to be 95K. This molecular weight is higher than that of nPKCη found in lung and skin tissue of 82K and 78K, and it is higher than nPKCη of COS cells transfected with the nPKCη-cDNA expression plasmid. Together with previous reports, these findings make the total number of PKC isozymes in human platelets equal to six. These are the PKC isozymes: α, β, δ and ζ,which have been previously described, and η' and θ which we describe here. To assess the functionality of these new PKC isoforms, we stimulated platelets with PAF. We found a 200% and 175% increase in the levels of membrane-bound nPKCη' and nPKCθ, respectively, in human platelets stimulated by PAF. A concomitant decrease in the level of these isoforms in the cytoplasm was observed. This PAF-induced translocation was time-dependent, and it reached its peak after a 1 minute incubation of human platelets with PAF for nPKCθ and 30 seconds for nPKCη'.

[1]To whom correspondence should be addressed at Box 5, Dept. Anatomy/Cell Biology, SUNY HSC at Brooklyn, 450 Clarkson Ave., Brooklyn, NY 11203.

Abbreviations: PKC, protein kinase C; PAF, platelet-activating factor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetra acetate; EGTA, [ethylene bis(oxyethylenenitrilo)]tetraacetic acid; PGE_1, prostaglandin E_1, PMA, phorbol 12-myristate 13-acetate.

0006-291X/93 $4.00

Introduction: Protein kinase C (PKC) plays an important role in platelet secretion by phosphorylating a substrate protein of 47K (1). PKC is a family of multiple isozymes. To date, ten isozymes of PKC have been identified in various tissues and cells. They have been classified into three groups (2): the conventional calcium dependent cPKC (α, βI, βII, and γ), the calcium-independent isozymes nPKC (δ, ε, η and θ), and the atypical aPKC (ζ and λ). PKC plays an important role in signal transduction in human platelets stimulated by agonists such as PAF, collagen, thrombin and phorbol esters (3-6). PKC has been shown to translocate from the cytosol to the plasma membrane in human platelets. Crabos et al. (6) identified the presence and translocation of the PKC isozymes α, β, and ζ in human platelets stimulated by PMA. Baldassare et al. (5) described the presence and translocation of PKC isozymes α, β, δ, and ζ in platelets stimulated by thrombin. PAF (1-o-alkyl-2 acetyl, 3-phosphorylcholine) is a phospholipid that acts as a potent agonist inducing platelet aggregation and secretion (7-8). We previously have shown that the interactions of PAF with platelets are altered during pregnancy (9) and also that PAF interacts with neural cells (10). In present study, we have used PAF as a primary agonist to determine the functional involvement of nPKCη' and nPKCθ in platelet activation. Our study has determined the presence of two previously undetected PKC isozymes in human platelets namely nPKCη' and nPKCθ. One appears to be an isoform which has not been described before. We show here functional involvement of these two isozymes in platelets stimulated by PAF.

MATERIALS AND METHODS

Collection of Blood -- Blood was collected from healthy individuals free of any form of medication for at least 2 weeks prior to blood collection. All volunteers signed an informed consent form approved by the State University of New York, Health Science Center at Brooklyn, New York Committee on Human Research.

Preparation and Washing of Platelets -- Platelets were collected in acid citrate dextrose(ACD) (11) and washed by differential centrifugation and resuspended in a Tyrode-Hepes solution: 0.35% bovine serum albumin, 2.68mM KCl, 2mM $CaCl_2$, 136mM NaCl, 0.23mM $MgCl_2$, 0.36mM NaH_2PO_4, 5.5mM D-glucose, 20mM Hepes, pH 7.4. The solution contained: heparin (2units/ml), potato apyrase(320μg/ml), and PGE_1(1μM). Washed platelets were suspended in a Tyrode-Hepes solution free of bovine serum albumin and aggregatory inhibitors. Platelet counts were obtained and the aggregation of platelets by PAF was tested as previously described (9, 12).

Preparation of Cytosol and Membrane Fractions --Washed platelets ($2x10^9$/ml) were incubated with 500nM PAF at different time points (10 seconds to 5 minutes) at 37° C. The incubation was stopped by the addition of cold double concentrated sonication buffer: 20mM Tris-HCl (pH 7.5), 2mM EDTA, 50mM β-mercaptoethanol, 10mM EGTA, 1mM PMSF, 5μg/ml leupeptin, 5μg/ml trypsin inhibitor, 25mM benzamidine (6). The platelet suspension was sonicated 3 times at 10 second bursts by a sonic disruptor (Tekmar Co.) whose sonication level was set to 20. The sonicated samples were centrifuged at 100,000 x g for 30 minutes at 4°C by a Beckman TLA 100 ultracentrifuge (Beckman Corp.). The cytosol fractions were collected and the pelleted membrane fractions were resuspended in cold sonication buffer containing 1% Triton-X 100 (extraction buffer). The pellets were dispersed and sonicated as described above, and centrifuged at 100,000 x g for 30 minutes at 4°C. The proteins extracted into the supernatants were removed as the membrane fraction.

Antibodies Used --Anti-cPKCα (polyclonal antibody against peptide sequence: ^{313}AGNKVISPSEDRRQ^{326}, [5μg/ml], Gibco), anti-cPKCβ (monoclonal, [5μg/ml], Seikagaku America Co.), anti-cPKCγ (polyclonal antibody against peptide sequence: ^{306}NYPLELYERVRTG^{318}, [5μg/ml], Gibco), anti-nPKCδ (polyclonal, [1:100 dilution], donated by Dr. Peter J. Parker, Imperial Cancer Research Fund, London), anti-nPKCε (polyclonal antibody against peptide sequence: ^{313}KGFSYFGEDLMP^{326}, [5μg/ml], Gibco), anti-aPKCζ (polyclonal, directed against peptide sequence: ^{577}GFEYINPLLLSAEESV^{592}, [5μg/ml], Gibco), anti-nPKCη (polyclonal, directed against the peptide: ^{56}QTSTKQKTNKPTYNEEFC^{73}, [3μg/ml] (13), and anti-nPKCθ (polyclonal, against the peptide sequence: ^{332}CVPTPGKREPQGISWDSPLDGSNK^{355}, [3 μg/ml]) (14).

SDS-PAGE and Immunoblotting -- SDS-PAGE was performed using a 3% stacking gel and an 8% running gel as described by Laemmli (15). Equal aliquots of 0.5ml of each cytosolic fraction and membrane fraction from every time point were mixed with 100μl of 6x Laemmli sample buffer consisting of: 62.5mM Tris, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.001% Bromophenol blue. Samples were vortexed and boiled for 5 minutes. The PKC isozymes η and θ extracted from COS cells which were transfected with the nPKCθ and η-cDNA expression plasmids were used as positive controls. Proteins were transferred onto the nitrocellulose membranes (Schleicher & Schuell Co., 0.45μm). The membranes were rinsed 3 times and incubated with 1% BSA in TBST [50mM Tris-HCl (pH 7.5), 0.15M NaCl, and 0.05% Tween-20] for at least 2 hours to block non-specific binding of proteins to the membranes. The membranes were then cut into 3mm strips and incubated with specific anti-PKC isozyme antibodies. After overnight incubation with anti-PKC antibodies at 4°C, the nitrocellulose membranes were washed 3 times for 10 minutes with 1% BSA in TBST. The membranes were incubated with alkaline phosphatase conjugated anti-IgG antibody (Sigma Chemical Co.) for 2 hours at 22°C. The membranes were then washed five times in 1% BSA in TBST, and immunoreactive bands were visualized by color development using 0.41M nitro blue tetrazolium and 0.38M 5-bromo4-chloro-3-indoyl phosphate dissolved in 0.1M Tris, 0.1M NaCl, and 5mM $MgCl_2$.

Quantitation of Immunoblots -- Protein determination was followed the method of Bradford et al. (16). The density of PKC bands shown in the immunoblots was quantitated using the XRS Omnimedia and Sharp scanners(XRS Omnimedia Inc., and Sharp Co.). The images were numerically quantitated accoding to band density by **Image 1.43**, a computer program using Macintosh IIci computer.The raw numerical data was divided by the protein concentration of each sample and expressed as density units per μg protein.

RESULTS

Identification of PKC isozymes in human platelets: By immunoblot analysis, we have detected the PKC isozyme α, β,δ, ζ, and θ PKC in human platelets (Fig. 1A). The specific anti-cPKCγ and anti-nPKCε antibodies did not recongnize their respective PKC isozymes in the same platelet preparations. The molecular weight of an immunoreactive protein recognized by the anti-nPKCη antibody was calculated to be 95K (Fig. 1A and B). This molecular weight differs from the 82K molecular weight of nPKCη present in murine cells and COS cells transfected with nPKCη-cDNA. To prove the specificity of the anti-nPKCη antibody, we incubated the immunoblot strips with a mixture of the anti-nPKCη antibody and its corresponding peptides. As a result, the 95K band disappeared, and the same was true for the 82K nPKCη band in COS cells transfected with nPKCη (Fig. 1B). Due to the differences in molecular weight between the nPKCη in murine cells and the

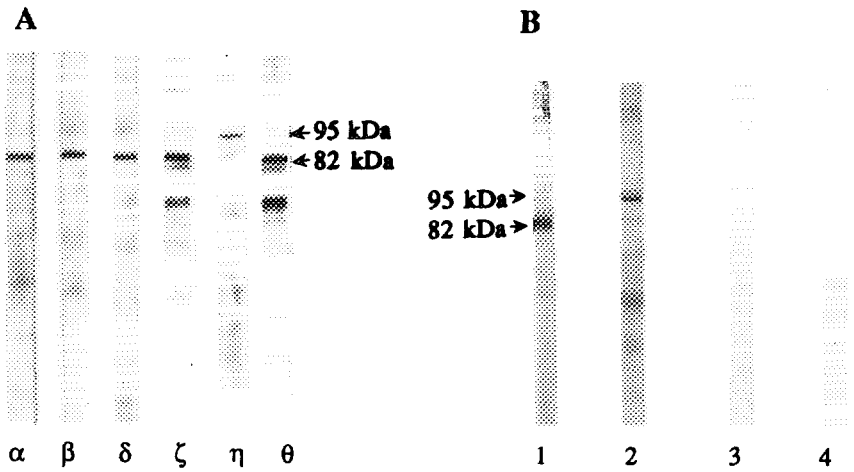

Fig.1. Western blot analysis of PKC isozymes in human platelets. Total proteins from washed human platelets (50μg/strip, Fig. 1A, and strip 2 and 4 in Fig. 1B) and proteins from COS cells transfected with nPKCη-cDNA (5μg/strip, strip 1 and 3 in Fig. 1B) were dissolved in SDS-Laemmli buffer and analyzed by Western blots using the specific anti-PKC antibodies listed under the immunoblot A, above. Fig. 1B: strips were incubated with anti-nPKCη antibody (3μg/ml, in strip 1 and 2) and with a mixture of anti-nPKCη antibody + nPKCη peptides (10μg/ml, in strip 3 and 4).

immunoreactive protein in human platelets found by this study, we have named this PKC isozyme as nPKCη' (prime).

Translocation of PKC η' and θ induced by PAF: In order to further characterize the identity of nPKCη' and θ, we conducted translocation studies using PAF. Washed human platelets were incubated with 500nM PAF at 37°C. The distribution of PKC isozymes between the cytosol and membrane fraction, following the incubation of washed human platelets with 500nM PAF, was determined by Western blot analysis (Fig. 2). The controls used were washed platelets incubated with Tyrode-Hepes buffer free of PAF. Quantitation of protein amount by densitometric analysis showed an initial rapid increase of PKCη' in the plasma membrane at 10 seconds (Fig.3). Maximum increase of nPKCη' was observed at 30 seconds. In parallel, the cytosol level of nPKCη' decreased to 50% in 30 seconds. The maximum translocation of nPKCθ was reached at 1 minute (Fig. 4).

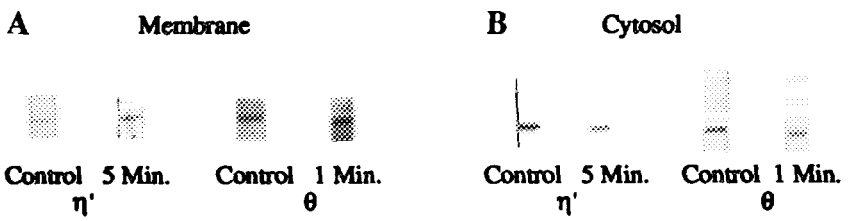

Fig. 2. nPKCη' and nPKCθ in membrane and cytosol fractions from human platelets stimulated by PAF. Washed human platelets were incubated with 500nM PAF at 37°C. Controls were incubated with Tyrode-Hepes buffer free of PAF. Immunoblotting of membrane (Fig. 2A) and cytosol (Fig. 2B) fractions was performed as described under "Materials and Methods" by using specific anti-nPKCη and anti-nPKCθ antibodies.

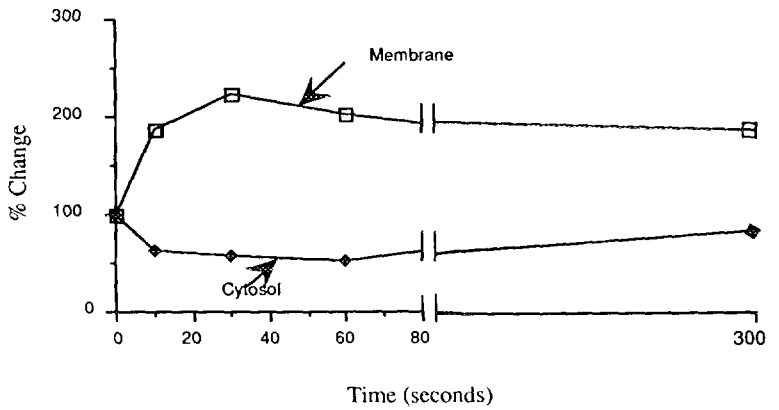

Fig.3. Time-course of the translocation of nPKCη' from the cytosol to the plasma membrane in human platelets stimulated by PAF. Washed human platelets were incubated with 500nM PAF for the different time periods, indicated above, and immunoblots of membrane and cytosol fractions were performed as described in "Materials and Methods" by using a specific anti-nPKCη antibody. The y-axis indicates the % change in the nPKCη' levels in membrane and cytosol compared with unstimulated platelets (control). Each point indicates the average value from two separate experiments.

DISCUSSION

Relatively little is known about the identity and function of different PKC isozymes in human platelets. Recently, Baldassare et al. (5) have reported that human platelets contain PKCα, β, δ and ζ. Their study also showed differential translocation of these isoforms in platelets stimulated by thrombin. Crabos et al. (6) have shown the expression of PKCα, β and ζ and reported the differential translocation of these isoforms in platelets stimulated with the phorbol ester; PMA. In the present study we have detected two additional PKC isoforms, namely, nPKCη' and nPKCθ in human platelets. The molecular weight of

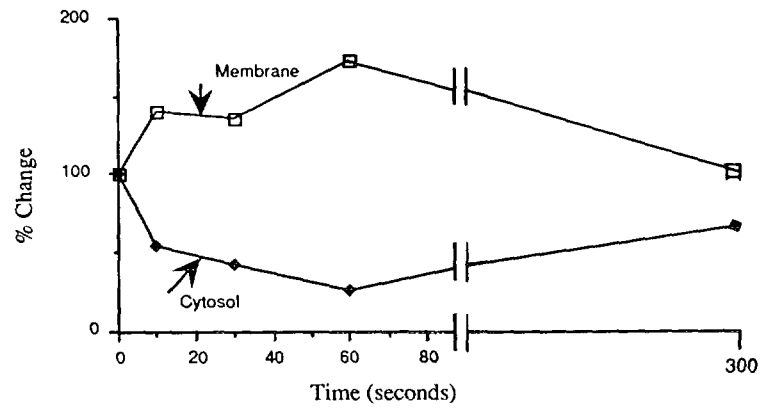

Fig. 4. The translocation of nPKCθ from the cytosol to the membrane as a function of time in human platelets stimulated by PAF. Immunoblots of membrane and cytosol fraction from washed human platelets were performed by using a specific anti-nPKCθ antibody. The y-axis indicates the % change in the nPKCθ levels in membrane and cytosol compared with unstimulated platelets. Each point indicates the average value for two separate experiments.

nPKCη' was 95K, which is higher than nPKCη found in lung, skin and COS cells transfected with nPKCη–cDNA. A protein with high molecular weight recognized by an antibody directed against the C-terminus of nPKCη has been recently reported in rat hippocampus (17). In human platelets, we have found that nPKCη' was translocated from the cytosol to the membrane by PAF, suggesting that it plays a functional role in cellular activation by PAF. It is possible, therefore, that nPKCη' is the PKC isozyme translocated in the hippocampus during long-term potentiation (LTP) (17), when LTP is induced by PAF (18). PAF is a potent phospholipid mediator with a diversity of biological actions, including vesicular secretion from human platelets (19) and neural cells (10). The mechanisms of post-receptor signaling by PAF are not fully delineated. In human platelets, a 47K protein, which has been cloned and sequenced (20), is a major protein substrate for PKC. This 47K protein is rapidly phosphorylated when human platelets are stimulated by PAF, implying the involvement of PKC in platelet activation by this agonist (21). The translocation of nPKCη' and nPKCθ from the cytosol to the membrane indicates that nPKCη' and nPKCθ respond to PAF. PAF induced translocation of nPKCη' and nPKCθ from the cytosol to the membrane was seen as early as 10 seconds after platelet stimulation. The discovery of nPKCη' and nPKCθ and their functionality in human platelets will aid in the elucidating the differential roles of various PKC isozymes in the signal transduction system of human platelets and other tissues following cellular activation by agonists.

Acknowledgments: We are grateful to Dr. Todd C. Sacktor for helpful discussions. We thank Debbie Sherman and Meghna U. Naik for their technical assistance. This study was supported in part by a grant from the American Heart Association, New York Affiliate (#: 19007 and 90083). E. K. is the recipient of an RCDA (#: 5K04HL0241203) from the NHLBI.

REFERENCES

1. Sanno,K., Takai,Y., Yamanishi, J., and Nishizuka, Y. (1983) J. Biol. Chem. 258, 2010-2013.
2. Nishizuka, Y. (1992) Science 258, 607-614.
3. Block, L.M., Abraham, W.M., Groscurth, P., Qiao, B.Y., and Perruchoud,A.P. (1989) Am. J. Respir. Cell Mol. Biol. 1, 277-278.
4. Walker, G., Biurguignon, L.Y.W. (1990) FASEB J. 4, 2924-2933.
5. Baldassare, J.J., Henderson, P. A., Burns, D., Loomis, C., and Fisher, G. (1992) J. Biol. Chem. 267, 15585-15590.
6. Crabos, M., Imber, R., Woodtli, T., Fabbro, D., and Erme. P. (1991) Biochem.Biophys.Res. Commun.178,878-883.
7. Benveniste, T., Le Couedie, J. P., and Kamoun, P. (1975) Lancet 1, 344-345.
8. Kornecki, E., Ehrlich, Y.H., and Lenox, R.H. (1984) Science 226, 1454-1456.
9. Kornecki, E. and Ehrlich, Y.H. (1990) Am. J. Physiol. 259, H766-H771.
10. Kornecki, E. and Ehrlich, Y.H. (1988) Science 240, 1792-1794.
11. Mustard, J.F., Perry, D. W., Ardlie, N. G., and Packman, M.A. (1972) Br. J. Harmatiol. 22, 193-204.
12. Kornecki, E., Walkowiak, B., Naik, U.P., and Ehrlich, Y.H. (1990) J. Biol. Chem. 265, 10042-10048.
13. Osada, S-I., Mizuno, K., Saido, T. C., Akita, Y., Suzuki, K., Kuroki, T., and Ohno, S. (1990) J. Bio. Chem. 265, 22434-22440.

14. Osada, S-I., Mizuno, K., Saido, T. C., Suzuki, K., Kuroki, T., and Ohno, S. (1992) Molecular and Cellular Bio. 12, 3930-3938.
15. Laemmli, U.K (1970) Nature (London) 227, 680-685.
16. Bradford, M. M. (1976) Analytical Biochem. 72, 248-254.
17. Sublette, E., Naik, M., Jiang, X., and Sacktor, T. C. (1992) Soc. Neurosci. Abstr. 18, 282.
18. Wieraszko, A., Li, G., Kornecki, E., Hogan, M.V., and Ehrlich, Y.H. (1993) Neuron. In Press.
19. Barens, P. J., Page, C. P., and Henson, P. M.(eds) (1989) Platelet Activating Factor and Human Disease. Blackwell, Sci. Pub., London.
20. Tyers, M., Rachubinski, R.R., Stewart, M.I., Varrichio, A.M., Shorr, R.G.L., Haslam, R.J., and Harley, C.B. (1988) Nature 333, 470-473.
21. Ieyasu, H., Takai, Y., Kaibuchi, K., Sawamura, M., and Nishizuka, Y. (1982) Biochem. Biophys. Res. Commun. 108, 1701-1708.