BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **232,** 61–64 (1997)
ARTICLE NO. RC976232

Interleukin-11 Induces Rapid PKC Activation and Cytosolic to Particulate Translocation of Alpha and Beta PKC Isoforms in Human Erythroleukemia K562 Cells

Glendora C. Spencer and Samuel E. Adunyah[1]
Biochemistry Department And Comprehensive Sickle Cell Center, Nashville, TN. 37208, USA.

Received January 24, 1997

Interleukin-11 (IL-11) is a pleiotropic cytokine which regulates the growth of hematopoietic progenitor cells and activates platelet maturation. Previous studies have shown that, IL-11 activates a set of signaling cascades involving the JAK/STAT and Raf/MAPK pathways. The purpose of the current studies was to obtain evidence about the possible involvement of PKC in the IL-11 signaling pathway. Evidence presented in this report suggests that IL-11 stimulates rapid PKC activation and markedly induces cytosolic to particulate (membrane) association of alpha and beta PKC isoforms. These findings provide preliminary evidence that PKC may be involved in the IL-11 signaling cascade.

Interleukin-11 (IL-11) is a pleiotropic cytokine which regulates the growth kinetics, of various hematopoietic progenitor cells (reviewed in Refs. 1 and 2). Originally, IL-11 was identified and molecularly cloned from a primate bone marrow derived stromal cell line, PU-34, based on its ability to stimulate the proliferation of IL-6 dependent cells (3). Recently, it has been shown that IL-11 binds to a specific receptor which requires the co-expression of the signal transducer gp130 for transmission of IL-11 signal into the cells (4). The gp130 transducer is the common component of the IL-6, leukemia inhibitory factor (LIF), oncostatin M (OSM), and ciliary neurotrophic factor (CNTF) (5). One of the early events during IL-11 signaling involves the activation of cytoplasmic protein tyrosine kinases (6). Since the IL-11 receptor chains appear not to contain an intrinsic kinase domain, attention has been focused on kinases and other intracellular proteins that may interact with the cytoplasmic domains of the IL-11 receptor. IL-11 receptor activation leads to activation of the JAK/STAT signaling pathway which is appears to be associated with the ability of IL-11 to induce platelet maturation as well as regulate adipogenesis in preadipocytes (6). In addition, receptor activation by IL-11 couples IL-11 signaling through the gp130 transducers to a variety of cytoplasmic proteins including the members of the Ras/MAPK pathway (6, 7). Recently, we have demonstrated that in human erythroleukemia K562 and monocytic leukemia U937 cells, treatment with IL-11 leads to Raf-1 and MAP kinase activation, as well as the induction of *c-jun* and *c-fos* expression (7)

The early events following receptor activation include physical interactions with a variety of cytoplasmic proteins including the protooncogene product p21ras (8, 9). These interactions are mediated by src homology 2 (SH2) domains, which are noncatalytic elements of about 100 amino acids found in a variety of proteins (10, 11). The association of target proteins containing SH2 domains with specific sequence motifs containing phosphotyrosines appears to constitute a key element in receptor tyrosine kinase signal transduction (10, 11).

Pelicci *et al* (12) have characterized a protein termed Shc that contains a SH2 domain at the C terminus. Shc (p66, p52 and p46) functions as an adaptor protein for the signal transduction through Grb2/mSos, leading to the activation of the Raf-1 signaling pathway (12). Tyrosine-phosphorylated Shc proteins bind to the SH2 domain of Grb2 (13,14) and Grb2 associates with the guanine nucleotide exchange protein, mSOS, through its SH3 domains (15,16). Based on these observations the Shc association with Grb2-mSos represents a mechanism whereby nonreceptor cytoplasmic tyrosine kinases activate p21ras (16). Activated Ras binds to the serine/threonine kinase, Raf-1, which functions as a MAP kinase kinase kinase in some cell types (17,18). MEK kinase converge with Raf-1 on a family of dual specificity kinases also known as MEKs (19). Activated MAP kinase then translocates to the nucleus where it phosphorylates several transcription factors, including

[1] To whom correspondence should be addressed. Fax: (615) 327-6442.

0006-291X/97 $25.00

c-myc and *c-jun* (20,21). Several studies have shown that activation of PKC by cytokines provide an alternative (PKC-dependent) pathway which may overlap with the Ras/MAPK pathway at Raf-1 through PKC-mediated Raf-1 phosphorylation (22, 23). And yet, it is possible for PKC to directly translocate to the nucleus upon activation leading to proliferative response (23). Therefore, it important to elucidate the possible involvement of PKC in IL-11 signaling mechanisms.

In our preliminary studies, we noted that IL-11 stimulates proliferation in both human erythroblastic K562 and monoblastic U937 cells. Since PKC plays an essential role in proliferative response mechanisms induced by mitogens including PMA, growth factors and cytokines (24, 25), it was important to investigate whether PKC is involved in IL-11R signaling. Here, we report that IL-11 stimulates rapid cytosolic to particulate (plasma and nuclear membrane) translocation and activation of PKC, detectable within 60 min. Western blot analysis revealed that the alpha and beta isoforms of PKC response markedly to IL-11 treatment as evidenced by the several fold changes in both cytosolic and membrane components of these isoforms in IL-11 treated K562 cells. This report provides the first evidence on the involvement of PKC alpha and beta and possibly delta in the signaling pathway mediated by IL-11.

MATERIALS AND METHODS

Human erythroleukemia K562 cells were purchased from the ATCC and maintained in 1640 medium containing 10% fetal bovine serum (FBS) and 50 units/mL of penicillin and streptomycin. These cultures were maintained in 5% CO_2 atmosphere at 37°C. Viability of cells were estimated to be greater than 96% by trypan blue exclusion. Recombinant IL-11 (monocyte-derived) was obtained from Genetic Institute (Cambridge, MA) and diluted into tissue culture medium. Antibodies to PKC isotypes alpha, beta, delta, epsilon gamma were obtained from Transduction Laboratories, Lexington, KY. The [gamma-^{32}P]-ATP was purchased from Dupont, Wilmington, Delaware. ECL detection reagent was purchased from Amersham Life Sciences, Arlington Heights, IL. Histone H1, pepstatin A, leupeptin, aprotnin, phenylmethanesulfonylfluoride (PMSF), and EGTA, EDTA were products of Sigma Chemicals, St. Louis, MO. All other chemicals were of high grade standard laboratory reagents.

Immunoprecipitation and Western blot detection of PKC isoforms. K562 cells (1×10^7) were untreated or treated with IL-11 (100 ng/ml) for various periods of time. Cells were resuspended in a lysis buffer (50 mM Tris-HCL, pH 7.4 containing 250 mM sucrose, 5 mM $MgSO_4$, 2.5 mM EGTA, 0.1% beta-mercaptoethanol (BME), I mM PMSF and 20 ug/ml each of leupeptin and aprotinin. The cells were disrupted by 20 passages through a needle (25 guage) attached to a 1 ml syringe whilst being maintained on ice. The fragmented cells were then centrifuged for 5 min at 1000 x g to obtain the nuclear pellet. The resulting supernatant was further centrifuged for 1 hr at 100,000 x g at 4°C to separate the supernatant or soluble (cytosolic) fraction from the plasma membrane-enriched particulate fraction. The particulate or membrane bound PKC was extracted by solubilizing the particulate fraction in the same buffer containing 0.3 % NP40 or 0.25 % Triton X-100. The mixture was incubated on ice with shaking for 45 min to extract the membrane-bound PKC, which was separated by centrifugation at 100,000 x g as indicated above. Following protein determination by Coomassie Blue analysis, the samples were either used for immunoprecipitation and Western blot analysis or for further purification and PKC activity assays. To detect PKC isoforms by immunoprecipitation and western blot analysis, equal amount of proteins (50 ug) were added to an antibody to specific PKC isoform (either alpha, beta or delta) at a protein to antibody ration of 100:1 and incubated in eppendorf tubes for 4 hr at 4°C on a rotating shaker. The pellet was obtained by microcentrifugation at 14,000 x g for 5 min and added to protein A agarose matrix followed by further incubation for 2 hr. The pellet was collected as indicated above, washed three times in the lysing buffer and solubilized in SDS-gel sample buffer for analysis by PAGE (26). The western blots (27) were probed with specific antibodies to PKC isoforms and developed with secondary antibody (IgG) followed by detected with ECL detection reagent.

Assay of PKC activity. To obtain partially purified PKC enzyme for assays, the cytosolic and particulate (membrane) fractions were applied to Hi-Trap Q DEAE-sepharose columns (Pharmacia) equilibrated in lysing buffer. The bound material was washed with 3 volumes of lysis buffer and PKC activity was eluted from the columns by batch process with lysis buffer containing 150 mM NaCl. The eluant was assayed immediately for PKC activity as detailed below. The PKC assay mixture (100 ul) contained 20 mM Tris/HCl (pH, 7.4), 5 mM $MgCI_2$, 1 mM $CaCl_2$, 50 ug/ml PS, 0.3 ug/ml diolein, 20 uM unlabeled (cold) Mg_2ATP, histone H1 (1 mg/ml) and 50 uCi [^{32}P]-gammaATP. Aliquot (10 or 20 ul) of partially purified PKC was added to initiate the reaction, which was incubated at 30°C for 20 min. To access the level of phospholipid and Ca2+-independent kinase activity, parallel assays were run in the absence of PS, diolein and Ca2+ but with EGTA (2 mM) in the reaction mixture. In order to terminate the reaction, the samples were diluted 1:2 with 40 % cold TCA containing 1 % sodium pyrophosphate and incubated on ice for 30 min. Thereafter, the radiolabeled proteins were separated by filtration on phosphocellulose (Whatman) paper. Following extensive washing with cold 10 % TCA, the filters were air dried and the bound radioactivity was then quantitated by liquid scintillation analysis.

RESULTS

Activation of PKC in response to PMA and other factors is characterized by rapid cytosolic to membrane

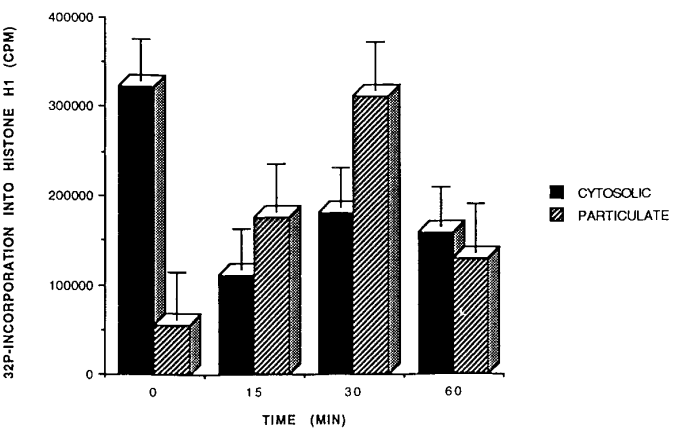

FIG. 1. IL-11-induced rapid translocation and activation of PKC. K562 cells were treated with rhIL-11 (100 ng/ml) for various periods of time. The cell lysate was separated into cytosolic and membrane (plasma and nuclear) fractions followed by partial PKC activity purification as indicated in materials and methods. PKC-induced incorporation of ^{32}P into histone H1 was measured by PKC assay using 50 ug protein/assay followed by scintillation analysis. The data represents the mean plus S.D. of three determinations.

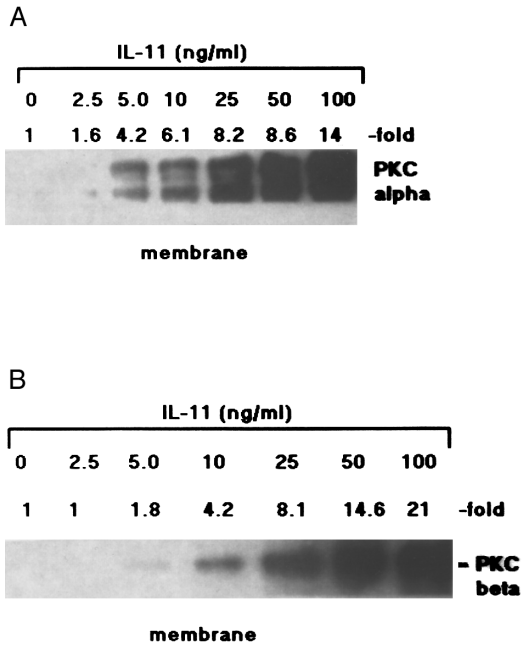

FIG. 2. Dose response of IL-11 stimulated translocation of alpha and beta PKC isoforms. K562 cells were untreated or treated with various doses of rhIL-11 for 2 hr. Cytosolic and membrane (plasma and nuclear) fractions were obtained as indicated in materials and methods. Proteins (50 ug) from the fractions were separated on 10 % polyacrylamide SDS gels and analyzed for alpha PKC (panel A) and beta PKC (panel B) proteins by Western blot hybridization to specific monoclonal antibodies.

translocation and activation of PKC activity (22-24). To determine whether IL-11 induces activation of PKC, partially purified PKC from cytosolic and membrane extracts of untreated and IL-11 treated cells were assayed for the ability of the partially purified enzyme to mediate Ca2+/PS/diolein-dependent incorporation of gamma $^{32}PO_4$ from radiolabed ATP to histone H1. The results in Fig. 1 suggest that rapid activation of PKC occurs in response to IL-11 in that transient cytosolic to particulate (membrane) translocation of total PKC activity was detected within 15 to 30 min. Specifically, the cytosolic PKC activity declined by 6-fold accompanied by a corresponding increase in the particulate fraction within 30 min. By 60 min however, PKC activity within the particulate fraction had returned to a near basal level.

In order to determine which PKC isoforms were responding to IL-11 treatment, immunoblot analysis was conducted to determine PKC protein levels in cytosolic and particulate fractions. The data in Fig. 2 indicates that IL-11 induces dose-dependent association of PKC alpha and beta isoforms with the particulate (membrane) fraction. As noted, the amount of alpha and beta PKC proteins which associated with the particulate (membrane) fractions increased by 14.6 and 21-fold respectively within 2 hr in cells treated with 100 ng/ml of rhIL-11. Similar but far less effect of IL-11 on delta PKC was detected in IL-11 treated cells (data not shown). Immunoblot analysis of time course experiments demonstrated that IL-11 (100 ng/ml) induces rapid and marked cytosolic to particulate (membrane) association of the alpha and beta PKC isoforms (Fig. 3) though the effect of IL-11 on beta PKC (Fig. 3A) was far more pronounce than was noted for the alpha PKC (Fig. 3B). Also, the cytosolic protein level of alpha PKC initially experienced a slight increase in respond to IL-11 followed by a complete translocation to the particulate fraction.

We also examined the effects of IL-11 on protein levels of two Ca2+-independent PKC isoforms. Though the PKC delta isoform was poorly detectable in K562 cells, the data in Fig. 3C suggests that IL-11 induces slight cytosolic to particulate (membrane) translocation of this isoform. A low MW band corresponding to the cleaved fragment of the delta PKC was also noted in the particulate (membrane) fraction of IL-11 treated cells (data not shown). In contrast, cytosolic and particulate epsilon PKC appear to be only moderately down-regulated by IL-11 (data not shown), indicating less response to IL-11. These findings indicate that perhaps the delta and epsilon PKC isoforms play less relevant roles in the signaling mechanisms of IL-11.

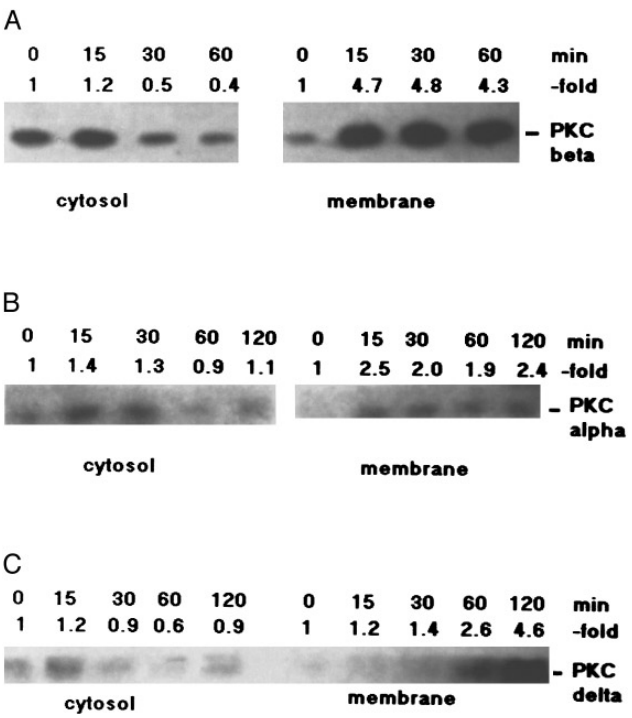

FIG. 3. Time course of IL-11-induced cytosolic to membrane redistribution of alpha, beta and delta PKC isoforms. K562 cells were untreated or treated with rhIL-11(100 ng/ml) for various periods of time. Cytosolic and membrane (plasma and nuclear) proteins (50 ug) were analyzed for PKC proteins by Western blot hybridization with specific monoclonal antibody to either beta PKC (panel A) or alpha PKC (panel B) or delta PKC (panel C).

DISCUSSION

Although PKC activation and its subsequent involvement in the signaling mechanisms of several hematopoietic growth factors has been extensively studied (22-25), information on the possible involvement of PKC in IL-11 signaling cascade is unavailable. Secondly, subcellular localization of various PKC isoforms in response to IL-11 has not been characterized. Therefore, the goal of this study was to determine the possible involvement of PKC in the signal transduction pathway of IL-11. This was accomplished by identifying specific classical and non classical PKC isoforms and their subcellular location in response to IL-11 treatment.

The data presented in this report strongly provide an evidence that PKC may be involved in the mechanism of IL-11. The evidence also suggest that the beta and alpha PKC isoforms and to a less extent the delta and epsilon PKC isoforms may be involved in mediation of the cellular responses triggered by IL-11 in K562 cells. Like the PKC activating agents PMA and GM-CSF which also regulate jun and fos expression during monocytic proliferation and differentiation (23-25), IL-11 also induces PKC activation and c-jun and c-fos mRNA expression (7). However, IL-11 is unable to induce differentiation in these cells. Rather, it induces proliferation (7).

Factor-induced PKC activation has been shown to lead to regulation of IL-11 mRNA expression (28). This observation coupled to the evidence presented in this report enable us to suggest that IL-11 may be capable of regulating its own expression through PKC activation loop. Furthermore, because of the marked membrane (plasma and nuclear) association of the alpha and beta isoforms in response to IL-11, it is likely that the beta and alpha PKC isoforms may provide a link between the signal transduction events of this cytokine and its ability to regulate nuclear events including transcriptional regulation of genes including possibly jun/fos/AP-1 and the IL-11 gene itself (7, 28). Further studies are in progress to identify which PKC isoform(s) mediate IL-11-induced erythroid progenitor cell proliferation.

ACKNOWLEDGMENTS

We appreciate the research funding provided by NIH/NHBLI through Meharry Comprehensive Sickle Cell Center and RCMI Program, the NCI for supporting Dr. S. E. Adunyah and the NIMGS for graduate fellow to Glendora C. Spencer. We are also grateful to the CTR for research support and to the Genetics Institute, Cambridge, MA, for generous supply of rhIL-11.

REFERENCES

1. Yang, Y. C. (1993) *Stem Cells* **11,** 474–486.
2. Du, X. X., and Williams, D. A. (1994) *Blood* **83,** 2023–2030.
3. Paul, S. R., Bennett, F., Calvetti, J. A., Kelleher, K., Wood, C. R., O'Hara, R. M., Jr., Leary, A. C., Sibley, B., Clark, S. C., Williams, D. A., and Yang, Y.-C. (1990) *PNAS* **87,** 7512–7516.
4. Paul, S. R., Schendel, P. (1992) *Int. J. Cell Cloning* **10,** 135–143.
5. Hilton, D. J., Hilton, A. A., Raicevic, A., Rakar, S., Smith, M., Gough, N. M., Begley, G. C., Metcalf, D., Nicola, N. A., and Wilson, T. A. (1994) *EMBO J.* **13,** 4765–4775.
6. Yin, T., and Yang, Y.-C. (1994) *J. Biol. Chem.* **269,** 3731–3738.
7. Adunyah, S. E., Spencer, G. C., Cooper, R. S., Rivero, J. A., and Ceesay, K. J. (1995) *Annals New York Acad. Sci.* **766,** 296–299.
8. Satoh, T., Nakafuku, M., and Kaziro, Y. (1992) *J. Biol. Chem.* **267,** 24149–24152.
9. Feig, L. A. (1993) *Science* **260,** 767–768.
10. Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S. (1991) *Cell* **64,** 281–302.
11. Pawson, T., and Gish, G. D. (1992) *Cell* **71,** 359–362.
12. Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Grignani, F., Pawson, T., and Pelicci, P. G. (1992) *Cell* **70,** 93–104.
13. Skolnik, E. Y., Lee, C.-H., Batzer, A., Vicentini, L. M., Zhou, M., Daly, R., and Meyer, M. G. Jr. (1993) *EMBO J.* **12,** 1929–1936.
14. Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T., and Bowtell, D. (1993) *Nature* **363,** 83–86.
15. Egan, S. E., Giddings, B. W., Brooks, M. W., Buday, L., Sizeland, A. M., and Weinberg, R. A. (1993) *Nature* **363,** 45–51.
16. Buday, L., and Downward, J. (1993) *Cell* **73,** 611–620.
17. Moodlie, S. A., Willumsen, B. M., Weber, M. J., and Wolfman, A. (1993) *Science* **260,** 1658–1660.
18. Warne, P. H., Viciana, P. R., and Downward, J. (1993) *Nature* **364,** 352–354.
19. Lange-Carter, C. A., Pleiman, C. M., Gardner, A. M., Blumer, K. J., and Johnson, G. L. (1993) *Science* **260,** 315–319.
20. Seth, A., Alvarez, E., Gupta, S., and Davis, R. J. (1991) *J. Biol. Chem.* **266,** 23521–23526.
21. Pulverer, B. J., Kyriakis, J. M., Avruch, J., Nikolakaki, E., and Woodgett, J. R. (1991) *Nature* **353,** 670–673.
22. Carroll, M. P., and May, W. S. (1994) *J. Biol. Chem.* **269,** 1249–1256.
23. Fields, A. P., Pincus, S. M., Kraft, A. S., and May, W. S. (1989) *J. Biol. Chem.* **264,** 21896–21901.
24. William, F., Wagner, F., Karin, M., and Kraft, A. S. (1990) *J. Biol. Chem.* **265,** 18166–18171.
25. Adunyah, S. E., Unlap, T. M., Wagner, F., and Kraft, A. S. (1991) *J. Biol. Chem.* **266,** 5670–5675.
26. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
27. Towbin, H., Staehelin, T., and Gordon (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76,** 4350–4354.
28. Yang, I., and Yang, Y.-C. (1993) *Proc. Am. Society Exp Biol.* **3,** 79231A.