Effect of an Inhibitory Factor for Protein Kinase C on Cell Growth of Leukemic Cells

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A factor which inhibits protein kinase C has been detected in normal human lymphocytes and leukemia cells obtained from some untreated patients with lymphoblastic leukemia. The partially purified inhibitory factor obtained from normal human lymphocytes depressed the growth of K562 cells and HL-60 cells, while it also suppressed DNA synthesis of these cells in a dose-dependent manner. The inhibitory factor was not able to suppress the DNA synthesis, however when it was added to the medium 12 hours after the culture. The inhibitory factor also reduced the phosphorylation of endogenous proteins, mainly Mr 80,000, 40,000 and 38,000. These results suggest that the inhibitory factor is closely related to the cell proliferation of various cells through the suppression of protein kinase C activity.

Key words: Protein kinase C, An inhibitory factor, Cell proliferation, DNA synthesis, Phosphorylated protein

Protein kinase C, which was first identified by Nishizuka et al. (1, 2), is now extensively studied since it is known to be closely related to the function, growth, and differentiation of various types of cell. When in the presence of a physiological concentration of Ca$^{2+}$ protein kinase C is activated by diacylglycerol (DG), which is, in turn, produced by a phosphatidyl inositol turnover in the cell membrane. TPA (12-o-tetra decanoylphorbol-13-acetate), known to be a potent tumor promoter, is structurally similar to DG. Since it has also been shown that TPA directly activates protein kinase C (3), while the receptor of TPA on cell membrane is thought to be protein kinase C itself, it would appear that protein kinase C relates to oncogenesis in certain cells (4). We have already reported on the relationship between cell growth and the enzyme responsible for protein phosphorylation of leukemia cells (5). We have also reported on the presence of a factor which inhibits protein kinase C and was detected by us in healthy human lymphocytes as well as in acute lymphocytic leukemia cells, and we have discussed its inhibitory effects on the growth of leukemia cells (6). Since there is no difference between the inhibitory factors obtained from several cells in terms of their characteristics, we have here investigated the mechanism of the factor which inhibits protein kinase C in some leukemic cell lines, and we have detected a reduced phosphorylation of endogenous proteins that may be involved in cell growth.

MATERIALS AND METHODS

1. Reagents.

Phosphatidylserine (bovine brain), cyclic AMP,
cyclic GMP, lysine-rich histone (Histone H1, type III-S), and mixed histone (type II-S) were purchased from the Sigma Chemical Co. (St. Louis, MO). Phenylmethylsulfonyl fluoride (PMSF), diisopropylfluorophosphate (DFP), and marker proteins for molecular weight determination of SDS-polyacrylamide gels (phosphorylase b, bovine serum albumin, ovalbumin, $\alpha$-chymotrypsin, myoglobin, and cytochrome C) were purchased from Boehringer Mannheim Biochemicals (Tokyo, Japan). Carrier-free $^{32}$P phosphate in 0.01 N HCl was obtained from the Japan Atomic Energy Research Institute (Tokai-mura, Japan). Calmodulin extracted from pig brain was the generous gift of Professor Koichi Yagi, Department of Chemistry, Hokkaido University. All other reagents were of analytical grade.

2. Cell Preparation of cells taken from Patients with Leukemia as well as from Healthy Volunteers.

Venous blood (20 to 100 ml) was obtained from healthy volunteers or patients with different types of leukemia and heparinized to prevent clotting. Platelets were removed at 400 × g for 10 min. Leukocytes (lymphocytes > 95%) were purified by Ficoll density gradient centrifugation. Contaminated erythrocytes were removed by hypotonic lysis. Leukocytes resuspended in 9 volumes of Solution A [0.25 M sucrose-25 mM Tris-HCl (pH 7.5)-50 mM 2-mercaptoethanol] were treated with DIP (5 mM) for 5 min to block proteolysis. The cells were washed twice with Solution A by centrifugation at 400 × g for 10 min. The cells were resuspended in 9 volumes of solution B [0.25 M sucrose-25 mM Tris-HCl (pH 7.5)-1 mM PMSF-50 mM 2-mercaptoethanol] and then minced in a Dounce homogenizer. The homogenate was centrifuged at 105,000 × g for 60 min, and the resultant supernatant was used as the cytosolic fraction. The pellet was resuspended in 3 volumes of Solution B containing 2.5 mM EGTA and 0.1% Triton X-100. The resuspended pellet was homogenized in a glass Teflon homogenizer and allowed to stand for 60 min at 0°C, after which it was centrifuged at 105,000 × g for 60 min. The supernatant obtained was used as the (solubilized) total particulate fraction.


K562 (a human chronic myelocytic leukemia cell line) and HL-60 (a human acute promyelocytic leukemia cell line) were grown in the suspension culture of a RPMI 1640 medium containing 10% inactivated fetal calf serum, 100 IU/ml penicillin and 100 μg/ml of streptomycin. Cultures were seeded at an initial density of $2.0 \times 10^5$ cells/ml and incubated in a humidified atmosphere at 5% CO$_2$. Treated culture were maintained in a medium containing 10 μl to 100 μl of a partially purified inhibitory factor for 48 hr. The viability of the cultured cells was determined by a trypan blue dye exclusion test and more than 90% viability of the control cells was observed for 2 days after the culture.

4. Assay for Protein kinase C activity.

Protein kinase C activity was measured according to the method devised by Kuo et al. (7) with some slight modifications. Briefly, the reaction mixtures (0.2 ml) were composed of 25 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 50 μM $[\gamma-^{32}$P]ATP (containing 1 × 10$^5$ to 1 × 10$^6$ cpm), 40 μg lysine-rich histone, 0.25 mM EGTA, and appropriate amounts of cell sample as an enzyme source (5 to 70 μg protein) in the presence of 5 μg phosphatidylserine and 0.5 mM CaCl$_2$. The reaction was started by the addition of ATP and carried out for 5 min at 30°C, after which the phosphorylated histone was precipitated with 5% trichloroacetic acid containing 0.25% tungstate, with bovine serum albumin (0.65%) as a carrier protein. The activity of cyclic AMP-dependent and cyclic GMP-dependent protein kinase was assayed according to the method described by Shoji et al. (8). The activity of Calmodulin-sensitive Ca$^{2+}$-dependent protein kinase was assayed by the method described by Katoh et al. (9). In all experiments the enzyme activity was linear, depending on the incubation time and the enzyme amounts. Phosphorylation of endogenous substrates from normal lymphocytes and various types of leukemic cell, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and subsequent autoradiography of the phosphoproteins were performed according to the methods described by Katoh et al. (9). The reaction conditions were the same as those used for the protein kinase assay, except that histone was deleted from the reaction mixture and the sample protein was increased to 180 μg. In some of the experiments, partially purified protein kinase C from pig
spleen was added in order to detect substrate proteins for protein kinase C. We used a partially purified enzyme derived from pig spleen, from which all endogenous substrates of protein kinase C had been removed. Protein was determined by the method derived by Bradford (10).


The cytosolic fraction and the solubilized particulate fraction were boiled for 3 to 10 min., and then centrifuged at 10,000 x g for 10 min. at 4°C. The supernatants were applied to a DEAE-cellulose column (2 x 26cm) which had been equilibrated with 20mM Tris-HCl (pH 7.5) with 0.1mM PMSF-50mM 2-mercaptoethanol and eluted with a linear gradient of NaCl (0 to 0.6M; total 300ml). Each fraction size was 5ml and an aliquot (0.02ml) of each fraction was assayed for its ability to inhibit protein kinase C. The reaction mixture is a final volume of 0.2ml containing 25mM Tris-HCl (pH 7.5)-10mM MgCl2-0.25mM EGTA-40 fig lysine-rich Histone-5 µg phosphatidylyserine-50µM [γ-32P] ATP-2.3 fig protein kinase C from pig spleen. Protein kinase C activity was measured according to the method described above. For further purification of the inhibitory factor, fractions which showed inhibitory activity were lyophilized and dissolved in 2 ml of 1 mM NaHCO3, (pH 7.1 to 7.3); they were then applied to a Sephadex G-50 column (1.5 x 51 cm) which had been equilibrated with 1 mM NaHCO3. Each fraction size was 3 ml, and an aliquot (0.03 ml) was assayed for its inhibitory activity with protein kinase C taken from pig spleen. Conditions were the same as those described for the detection of inhibitory activity.

6. Assay for Thymidine Incorporation.

HL-60 and K562 cells were collected at various times after the culture and finally adjusted to 1 x 10^6 cells/ml. Cells (0.1ml) were cultured in triplicate in microtiter plates and a 0.1ml RPMI 1640 medium containing 1 µCi of [methyl-3H] thymidine was added. The microtiter plates were incubated for 4 hr at 37°C in 5% CO2, and the cells were harvested onto filters with a Skatron harvester. The filters were dried, placed in 2ml of a scintillation fluid, and counted in a Beckman scintillation counter.

RESULTS


As Fig. 1 shows, the growth of K562 cells was effectively inhibited by the addition of an inhibitory factor obtained from healthy human lymphocytes in a dose-dependent manner. The numbers of viable cells in the control culture and in the cultures 24 hr after the addition of the inhibitory factor (10 µl, 50 µl, and 100 µl) were 11.05 ± 0.98 x 10^5 mean ± S.E. cells/ml, 6.35 ± 0.5 x 10^5 cells/ml, 2.40 ± 0.14 x 10^5 cells/ml, and 0.40 ± 0.05 x 10^5 cells/ml M ± SE, respectively. Those found in the cultures 48 hr after the addition of inhibitory factor (0 µl, 10 µl, 50 µl, and 100 µl) were 15.2 ± 1.92 x 10^5 cells/ml, 6.65 ± 0.54 x 10^5 cells/ml, 1.80 ± 0.20 x 10^5 cells/ml, and 0.60 ± 0.02 ± 10^5 cells/ml, respectively. There was a statistically significant difference between the control culture and the culture of each group (P<0.01) in terms of the numbers of viable cells. A similar inhibitory effect on the cell growth was observed when HL-60, Raji, or Molt-4 cells were used instead of K562 cells, thus indicating that this inhibitory effect is not always specific to the cell growth of K562 cells (data not shown). When K562 cells were recultured in a fresh medium after the removal of the inhibitory factor,
they grew exponentially 14 days after the culture. However, when the inhibitory factor was added again, the cell growth was inhibited, although this secondary inhibitory effect was milder than the primary inhibitory effect. These results suggest that the inhibitory factor may act cytostatically but not cytocidally (data not shown).


In order to analyze the mechanism of the inhibitory effect on cell growth, its influence on DNA synthesis was examined by analyzing the suppression of $[^3H]$-thymidine incorporation. Fig. 2 shows that the inhibitory factor obtained from healthy human lymphocytes had suppressed $[^3H]$-thymidine incorporation of K562 cells 24 hr after the addition of the inhibitory factor in a dose-dependent manner. Namely, the addition of 10 $\mu$l, 50 $\mu$l, and 100 $\mu$l of the inhibitory factor showed 0%, 70%, and >90% inhibition, respectively, where the control showed 0% inhibition. We therefore suggest that the suppression of DNA synthesis indicates the inhibition of cell growth. In the future, however, synchronized cells need to be used to clarify the relation between inhibitory activity and the cell cycle.

3. Correlation between Time-Courses after The Addition of Inhibitory Factor and The Inhibition of DNA Synthesis.

Fig. 3 shows that when the inhibitory factor obtained from healthy human lymphocytes was added more than 12 hr after the culture, no inhibition of DNA synthesis of HL-60 cells was observed; this indicates that the inhibitor acts during an early stage of DNA synthesis. Similar findings have been obtained for K562 cells as well (data not shown).

6. De-phosphorylation of Endogenous Protein from The Cytosolic Fraction of CLL cells by The Inhibitory factor.

Many endogenous substrate proteins, which exist in animal cells, are phosphorylated by various protein kinases. In order to determine which protein-phosphorylation is inhibited by which inhibitory factor, we have used autoradiography to examine the de-phosphorylation of endogenous protein from the cytosolic fraction of CLL cells by means of the inhibitory factor obtained from healthy human lymphocytes (Fig. 4). Purified protein kinase C (2.3 $\mu$g) derived from pig spleen cells was added to lanes 1-6, and an 8. 20 $\mu$l inhibitory factor was added to lanes 3-5, and 7, but not to lanes 1, 2, 6 and 8. Observation of lanes 6 and 8 shows that no endogenous substrate proteins are found in protein kinase C itself. Furthermore, no endogenous substrate which
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Fig. 4. Dephosphorylation by the inhibitory factor in the cytosolic fraction from a CLL patient.

Fig. 4 shows the decreased phosphorylation of endogenous proteins brought about by the inhibitory factor in a cytosolic fraction from a CLL patient as seen by SDS-PAGE (10%). Each figure indicates the molecular weight of the dephosphorylated proteins. Partially purified protein kinase C (2.3 μg) from pig spleen was added to lanes 1-6 and 8. A partially purified inhibitory factor (50 μl) taken from healthy human lymphocytes was added to lanes 3-5 and 7. The cytosolic fraction (sample) was deleted from lanes 5 to 8. More details are described in Methods.

PS: phosphatidyl serine
PKC: protein kinase C
Inh.: inhibitory factor

had been auto-phosphorylated was found in the inhibitory factor (lane 7). As we can see in lanes 3 and 4, phosphorylation of 80K, 40K and 38K proteins was mainly (and most clearly of 80K protein) inhibited by the addition of inhibitory factor. Although this finding suggests that these endogenous substrates may be related to cell growth, the function of these proteins is still unknown. More precise examination is therefore needed before we can conclude that these proteins play an important role in cell growth and that the inhibitory factor blocks the phosphorylation of these proteins, thus rendering cells cytostatic.

DISCUSSION

Several inhibitory factors for protein kinase C (e.g. H-7 (11) as well as chemically synthesized peptides (12, 13)) have been reported. Naturally occurring inhibitory factors for enzymes such as palmitoylcalnitine (14), polyamines (15) and calmodulin (16) have been also reported. Furthermore, Peuch et al. (17) and Walsh et al. (18) have recently reported two kinds of inhibitory factor which are inactivated by treatment with trypsin and calcium-binding protein, respectively. As for inhibitory factors derived from blood cells, Balazovich (19) et al. have found an inhibitory factor, derived from specific granules extracted from neutrophils, which is heat-labile and can be inactivated with treatment by proteinase. However, all these inhibitory factors appear to be different from ours with regard to their molecular weight, heat-stability, resistance to proteinase treatment and so on. Although we seem to have identified some new inhibitory factors, they need further purification.

Few investigations have examined the influence of these inhibitory factors on cell growth and differentiation. Matsui et al. (20) have examined the influence of H-7 on cell growth and the differentiation of HL-60 cells by using various agents (TPA, 1,25-dihydroxyvitamin D3, all trans-retinoic acid). They report that H-7 inhibited proliferation of HL-60 cells in a dose-dependent manner, inhibited TPA-induced phenotypic differentiation and suppressed DNA synthesis. In our experiments, we have examined the correlation between cell growth and the activity of protein kinase C since we have discovered that our inhibitory factor has no influence on A-kinase, G-kinase, and calmodulin-kinase (data not shown). When the inhibitory factor had been added to several cultured human leukemia cell lines (K562, HL-60, Raji, and Molt-4), cell growth was inhibited in all these cell lines. In particular, the growth of K562 cells was inhibited in a dose-dependent manner. Although the activity of protein kinase C decreased markedly in each cell line, it was not completely abrogated (data not shown). In addition, when cells, whose growth had once been inhibited by the addition of the inhibitory factor, were cultured in a fresh medium after the removal of the inhibitory factor, more rapid growth
was observed as compared with non-treated cells, while the growth of these cells was inhibited again by the readdition of the inhibitory factor. These results clearly show that the inhibitory effect is mediated by the cytostatic action but not by the cytocidal action, although it can reduce the activity of protein kinase C.

In order to clarify the mechanism of growth inhibition brought about by this inhibitory factor, we have examined its influence on DNA synthesis. The addition of more than 50 μl inhibitory factor induced a more than 70% inhibition. When the inhibitory factor was added to the culture medium more than 12 hr after the beginning of culture, no inhibitory effect on DNA synthesis was observed. These results indicate that the inhibitory factor acts at an early stage of DNA synthesis. Since it has recently been reported that the activity of DNA methyltransferase (21), topoisomerase II (22), and DNA polymerase (23), all of which are related to DNA synthesis, is regulated by protein kinase C, we can see that the inhibitory factor suppresses DNA synthesis by modifying the activities of these enzymes.

According to the results presented in Fig. 4, the phosphorylation of 80K and 38-40K proteins was inhibited. It has already been reported that 80K protein is a useful indicator of the activation of protein kinase C, when it is analyzed by means of the phosphorylation of protein in fibroblast cells (24) and other cells (25). Feuerstein et al. (26) have reported that TPA can induce the phosphorylation of 80K protein in NIH 3T3 fibroblasts, JB-6 mouse epidermal cells, and human lymphocytes. This finding indicates the importance of 80K protein in cell growth and suggests that this protein may be a target of the inhibitory factor. Although the exact function of 80K protein is still unknown, it has been suggested that it may be related to potassium depolarization (27). It is therefore important to elucidate the role of 80K protein in the inhibition of cell growth. On the other hand, it is also known that 40K(47K) protein is related to the activation of platelets and the secretion of serotonin. Furthermore, it has been suggested that it may be an inositol phosphate 5'-phosphomonoesterase and that phosphorylation of the protein by protein kinase C is likely to increase its phosphatase activity (28).

We have already reported that the translocation of 38K protein from a cytosolic fraction to a particulate fraction can be observed during analysis of the activity of protein kinase C in myelocytic leukemia cells at the proliferative phase. The inhibition of phosphorylation of 80K protein as well as 40K and 38K proteins should therefore be examined with regard to its relation to cell differentiation and proliferation. At least 3 cDNAs for protein kinase C have recently been cloned (29, 30). Brandt et al. (31) have suggested that different mRNAs are responsible for protein kinase C in various rat organs. The Brandt result indicates that different isozymes of protein kinase C have functions specific to each organ and that several inhibitory factors may correspond to each isozyme. These speculations need to be confirmed in order to clarify the exact function of protein kinase C.

ACKNOWLEDGMENTS: We thank Dr. M. Imamura and Mr. W. Jones for their reading of the manuscript and Dr. N. Katoh and K. Kimura for their kindly cooperation during our studies.

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