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ACTIVATION OF PROTEIN KINASE C DOWN-REGULATES IFN-GAMMA RECEPTORS

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SUMMARY: Treatment of mouse EL-4 cells with intracellular activators of protein kinase C, namely 4 -phorbol 12-myristate 13-acetate (PMA) and diacylglycerol, resulted in 90% reduction in cell surface interferon-gamma (IFN-gamma) receptors as judged by iodinated-IFN-gamma binding. This did not seem to be due to a decreased in the receptor affinity, since that of the remaining surface receptors appeared to be singnificantly increased as shown in Scatchard plot analysis. Kinetics experiments revealed that a PMA treatment as short as 15 min was sufficient to induce a decrease of 30% of IFN-gamma receptors, whereas the highest levels of down-regulation were observed after 60-90 min. Treatment of EL-4 cells with calcium ionophore, A23187, although ineffective by itself, dramatically increased the ability of suboptimal PMA concentrations to mediate IFN-gamma receptor down-regulation. Finally, specificity studies revealed that PMA is particularly effective in decreasing the binding of IFN-gamma to T-lymphocytes. Altogether these results suggest a possible involvement of protein kinase C in the regulation of IFN-gamma receptor expression.

Phorbol esters, tetracyclic diterpenes, are potent tumor promoting agents that exert pleiotropic effects on cells (1,2). Their effects appear to be mediated through the binding and activation of the specific intracellular receptor, Ca^{2+}/phospholipid-dependent diacylglycerol-activated, protein kinase C (3,4). Recently, it has been shown that 1,2-diacylglycerol, a product of the phosphatidylinositol metabolism, can also activate the protein kinase C (5,6). Directly related to the activation of this enzyme is the finding that PMA and 1,2-diacylglycerol can affect the expression of

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Abbreviations used in this paper: IFN-gammma, interferon-gamma; PMA, 4 β-phorbol 12-myristate 13-acetate; diC_8: 1,2-Dioctanoyl-glycerol; BSA: bovine serum albumin.

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a variety of membrane receptors by either up-regulation (7) or down-regulation (8-10).

IFN-gamma, produced by T-lymphocytes on stimulation with antigens (11), modulates cell activity by activating genes coding for a number of enzymes, as yet poorly characterized , and also by enhancing the synthesis of cell surface components (11). To achieve these effects, IFN-gamma must interact with specific membrane receptors on target cells (12,13). Although the initial physicochemical interaction between IFN-gamma and its receptor has now been characterized, nothing is known concerning the pathway of signalling transmission from the receptor to the intracellular environment.

In this study, we present evidence that activation of protein kinase C by PMA, 1,2-Dioctanoyl-glycerol (diC_8) and calcium ionophore, A23187, down-regulates the expression of the IFN-gamma receptors. These results, coupled with the finding that IFN-gamma itself down-regulates its own receptors, suggest a common transmembrane signalling apparatus involving a unique phospho-transferase system.

MATERIALS AND METHODS

Cells. The murine cell lines used, EL-4 and L1210, were maintained in RPMI-1640 medium supplemented with 10% FCS. To analyze the effects of protein kinase C activators, cells were resuspended at 5 x 10^6/ml and incubated with increasing concentrations for different times.

Chemicals. PMA, 1,2-Dioctanoyl-glycerol (diC_8) and calcium ionophore A23187 were purchased from Sigma Chemical Company (St. Louis, Mo).

Iodination of IFN-gamma. E. coli-derived recombinant MuIFN-gamma with a specific activity of 1.5 x 10^7U/mg of protein was kindly provided by Dr. E. Adolf, Boehringer, Ingelheim (Vienna, Austria). Thirty micrograms of MuIFN-gamma were reacted for 2 hours at 4°C with 1 mCi of ^{125}I-Bolton-Hunter reagent ($<$ 2000 Ci/mmol)(Amersham, England) in 0.25 ml of sodium borate buffer, pH 8.0, as previously described (12). The reaction was stopped by addition of glycine at a final concentration of 0.2M and the mixture applied to a 20 x 0.6 cm column of Sephadex G-10 equilibrated with phosphate-buffered saline, 0.5M NaCl, pH 7.4, containing 0.2% bovine serum albumin (BSA). The fractions containing ^{125}I-IFN-gamma were pooled, tested for antiviral activity and stored at 4°C. The specific radioactivity of the labeled IFN ranged from 30 to 40 uCi/ug. The overall recovery of antiviral activity was greater than 80%.

Competition binding assays. Cells from exponetially growing cultures, resuspended at 2 x 10^6 cells in 0.5 ml of RPMI-1640 medium

supplemented with 0.2% BSA, were incubated for 2 hours at 23°C with constant amounts of ^{125}I-IFN-gamma and increasing concentrations of unlabeled IFN-gamma. Nonspecific linear binding was determined by simultaneous addition of a 500-fold excess of unlabeled IFN-gamma. After 120 min incubation at 23°C the cells were washed four times and the pellets counted for cell-bound radioactivity. Nonspecific binding, never exceeding 15% of total counts at saturation, was subtracted from total counts for determining specific binding.

RESULTS AND DISCUSSION

The initial event during IFN-gamma and target cell interaction is represented by its binding to a specific membrane receptor (12,13). Although the primary structure of murine IFN-gamma has now been determined from nucleotide sequences obtained from cDNA clones (14), and that of its receptor has now partially characterized (13), nothing is known about the biochemical signal transduction in the intracellular compartment.

In order to analyze the effect of PMA on the expression of IFN-gamma receptors, EL-4 cells were initially treated with increasing concentrations of PMA for 2 h at 37°C. As shown in Fig. 1, treatment with doses as low as 10^{-9}M resulted in 25-30% loss of surface IFN-gamma receptor, as assessed by comparison with untreated control cells. At higher PMA doses (10^{-8}, 10^{-7}M) the decrease in surface

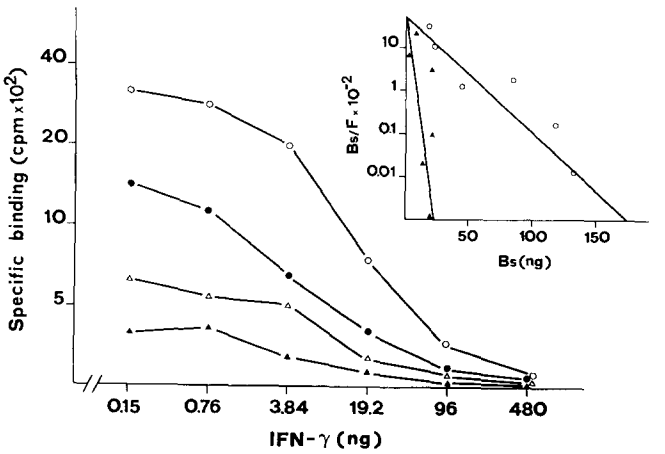

Fig. 1. Binding competition curves of ^{125}I-IFN-gamma to EL-4 cells, untreated (O----O) or treated with increasing concentrations of PMA (●----● :10^{-9}M; Δ----Δ :10^{-8}M; ▲----▲ :10^{-7}M). Each experiment has been repeated at least three times and one representative is reported in the figure.

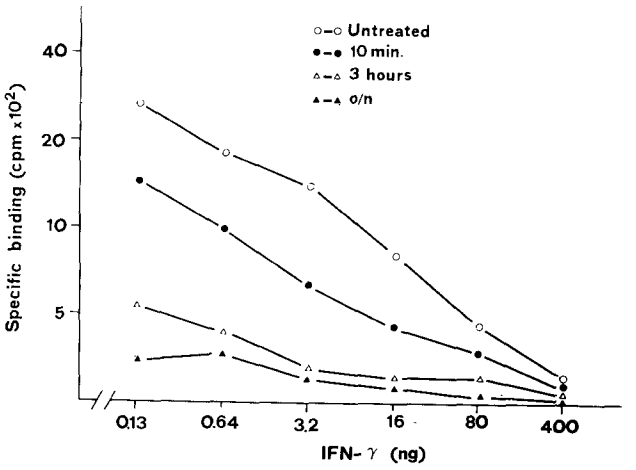

Fig. 2. Binding competition curves of ^{125}I-IFN-gamma to EL-4 cells, untreated or treated with PMA (10^{-7}M) for increasing times (●----● :10 min; Δ----Δ :3 hours; ▲----▲ :overnight). Each experiments has been repeated at least three times and one representative is reported in the figure.

receptors reached 90%, indicating that loss of IFN-gamma receptors is a function of PMA concentration. These results, therefore, suggest that the decrease of IFN-gamma binding to the EL-4 cells was mainly due to the receptor down-regulation by PMA. This hypothesis is also supported by the finding that a marked increase in the affinity of the remaining receptors (K_D 2.8 x 10^{-10}M of PMA-treated vs. 1.5 x 10^{-9}M of untreated cells), as indicated by the slope of the curve in the Scatchard analysis, was observed, ruling out the possibility that inhibition of IFN binding could be due to a decrease in the receptor affinity. At all doses tested, moreover, the viability of the EL-4 cells after PMA treatment always exceeded 95%, as judged by Trypan-bleu dye exclusion (data not shown).

The kinetics of IFN-gamma receptor down-regulation by PMA were evaluated at various times after PMA treatment. A 20-30% reduction in binding sites was already detectable at 15 min, it was maximal after 60-90 min and persisted for several hours(Fig. 2). It should be added that removal of PMA resulted in recovery of the IFN-gamma binding sites, suggesting the complete reversibility of the PMA effect (data not shown). The time course of the observed down-regulation is relatively short and closely follows the activation of proein kinase C (Fassio &

Landolfo, unpublished observations). Since the major receptor for PMA has been identified as the serine/threonine-specific Ca^{2+}/phospholipid-dependent diacylglycerol-activated protein kinase C (6), one can argue that its activation induces phosphorylation of the IFN-gamma receptor and consequently its down-regulation.

As already mentioned, diacylglycerols have been demonstrated to activate protein kinase C (5). To see whether diacylglycerol could replace PMA in the down-regulation of IFN-gamma receptors, EL-4 cells were treated with different doses of the synthetic diC_8 and used in competition binding experiments. As shown in Fig. 3, down-regulation occurred in a dose-dependent manner when increasing concentrations of diC_8 were added, with the maximal effect occurring at 12.5 ug/ml. As shown by Scatchard plot analysis, increase of affinity (K_D 8.5 x 10^{-9}M of diC_8-treated vs. 2 x 10^{-9}M of untreated cells) in the remaining receptors was observed in accord with the results obtained with PMA.

It has been recently shown that intracellular Ca^{2+} mobilization synergizes with diacylglycerol to induce transferrin receptor

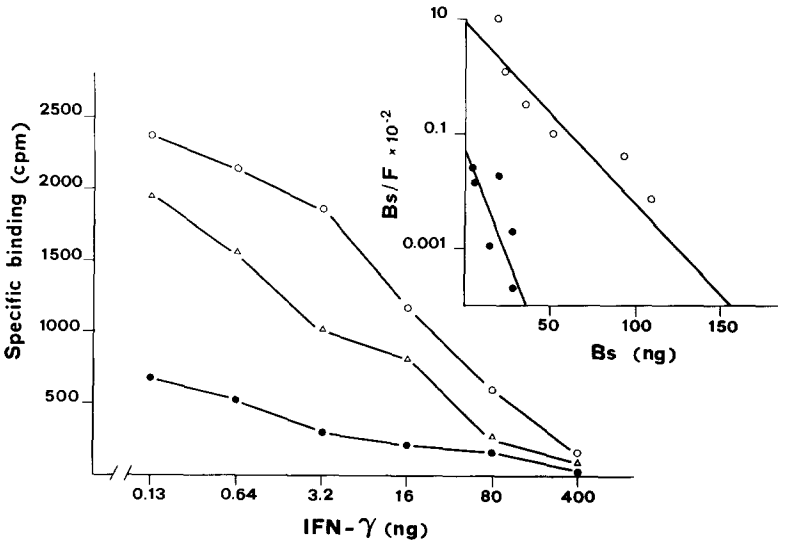

Fig. 3. Binding competition curves of ^{125}I-IFN-gamma to EL-4 cells, untreated (O----O) or treated with increasing concentrations of diC_8 (Δ----Δ :6,25 ug/ml; ●----● :12,50 ug/ml). Each experiments has been repeated at least three times and one representative is reported in the figure.

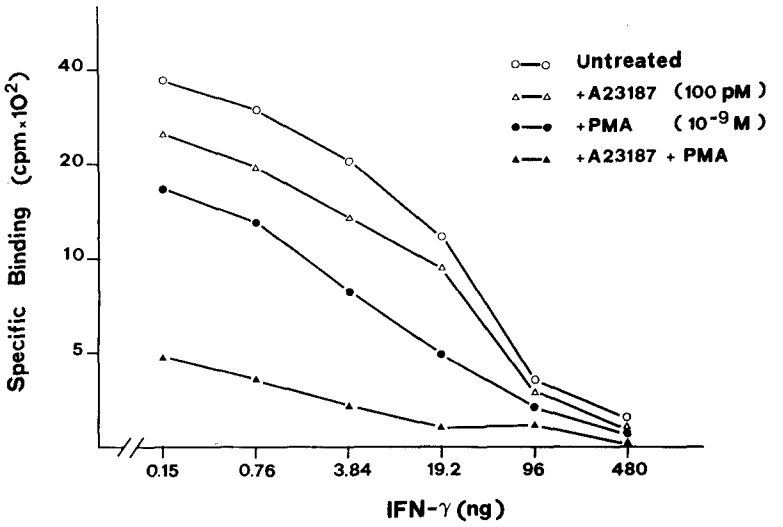

Fig. 4. Binding competition curves of ^{125}I-IFN-gamma to EL-4 cells, untreated (O----O) or treated for three hours with A23187 Ca^{2+}-ionophore (Δ----Δ : 100pM), PMA (●----● :10^{-9}M) or A23187 and PMA together. Each experiment has been repeated at least three times and one representative is reported in the figure.

down-regulation (15). To see whether Ca^{2+} mobilization could also modulate expression of the IFN-gamma receptor in our system, EL-4 cells were treated with increasing concentrations of the Ca^{2+} ionophore A23187 and used in binding experiments. As shown in Fig. 4, concentrations as low as 100 pM, although not very effective in themselves (about 23% down-regulation), increased dramatically the potency of 10^{-9}M PMA as a mediator of IFN-gamma receptor down-regulation. The results obtained with diC_8 and Ca^{2+} ionophore suggest that proein kinase C is involved in this down-regulation and decrease of affinity. Diacylglycerol, a primary product of phosphatidylinositol metabolism, has, in fact, been shown to bind and activate protein kinase C (5). Yet the use of Ca^{2+}ionophore A23187 has revealed an apparent synergism between Ca^{2+}mobilization and protein kinase C activation in the down-regulation of membrane receptors. Raising of intracellular Ca^{2+}, although ineffective by itself, increases the potency of PMA in the activation of protein kinase C, phosphorylation of transferrin receptor and its subsequent down-regulation. We would therefore suggest that IFN-gamma receptor

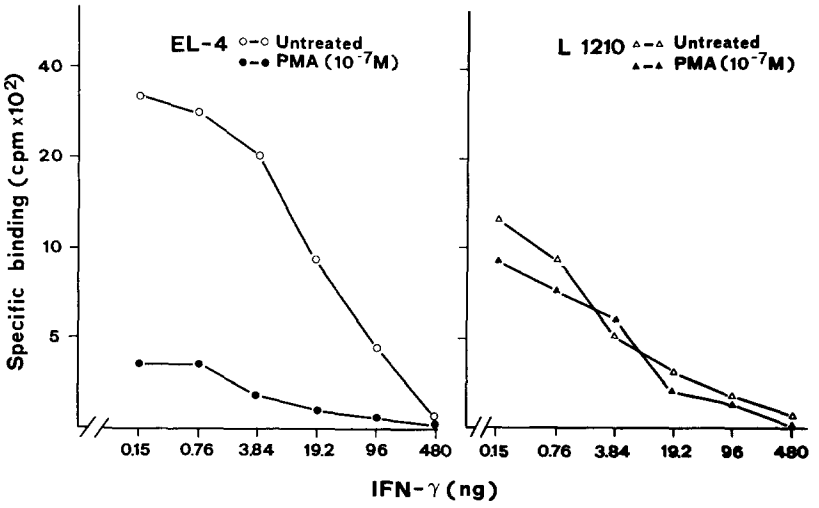

Fig. 5. Binding competition curves of ^{125}I-IFN-gamma to EL-4 and L1210 cells untreated or treated for 3 hours with PMA (10^{-7}M). Each experiment has been repeated at least three times and one representative is reported in the figure.

down-regulation also occurred via protein kinase C activation in our system.

Since the effect on surface IFN-gamma receptor expression was evaluated on EL-4 cells, a T lymphoma line, we decided to see whether cells of B-lymphocyte-, mcrophage-, fibroblast-, mastocytoma-origin were as sensitive to down-regulation by PMA. Our results indicate that these cell lines are much less sensitive. In Fig. 5, a comparison between EL-4 T-cells and L1210 B-cells is shown. Specificity studies indicate therefore that down regulation of IFN-gamma receptors by protein kinase C activators is particularly evident on T-cells. This observation can be explained by the finding that protein kinase C, although widely distributed in the tissues of many species, is particularly abundant in lymphocytes (6). In this connection, it may be mentioned that some time ago Kraft et al. (16) found high levels of protein kinase C in EL-4 cells, and demonstrated that PMA induces a translocation of protein kinase C from the cytosol to the membrane.

Activation of protein kinase C by PMA or sn-1,2-diacylglycerols causes both a decrease in the affinity of some receptors, such as

insulin and epidermal growth factor (9,10), and down-regulation of receptors such as transferrin (8). Moreover, both phenomena appear to be mediated by hyperphosphorylation of the receptors by the activated protein kinase C (8). Our demonstration that PMA and 1,2-Dioctanoyl-glycerol induce down-regulation of the IFN-gamma receptor extend those findings to the IFN-gamma system and suggest for the first time a possible involvement of protein kinase C in regulation of the IFN-gamma receptor.

ACKNOWLEDGMENTS

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