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Protein kinase C is crucial in signal transduction during IFN-γ induction in endothelial cells

Pirkko Mattila, Pekka Häyry and Risto Renkonen

Transplantation Laboratory, University of Helsinki, Haartmaninkatu 3, SF-00290 Helsinki, Finland

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We have demonstrated that IFN-γ, a potent peptide mediator in inflammatory responses, operates via the protein kinase C dependent transduction pathway in the induction of class II MHC antigens on rat microvascular endothelial cells. Stimulators of protein kinase C, like PMA, replaced IFN-γ in the induction of MHC class II on endothelial cells in a dose-dependent manner. Selective enzyme inhibitors of protein kinase C, H-7 as well as sphingosine down-regulated the IFN-γ induced class II expression in a dose-dependent manner. Addition of cAMP or cGMP in the culture, had no effect on the class II expression on the endothelial cells. Transient rise of cytosolic Ca^{2+} by calcium ionophore A23187, or a calmodulin antagonist W-7, had no effect on the IFN-γ induced class II expression.

Interferon-γ; Protein kinase C; Major histocompatibility complex class II induction

1. INTRODUCTION

Class II antigens are encoded by genes in the major histocompatibility complex (MHC) and function as restriction elements in immune reactions. Class II antigens are constitutively expressed on many cells, like macrophages and B cells, and can be induced onto many cells like T cells, fibroblasts and endothelial cells. Interferon-gamma (IFN-γ) has a broad variety of biological functions. Among other properties it increases class II MHC antigens of the major histocompatibility complex in many different cell types [1]. Endothelial cells are in native state negative for class II antigens, but begin to express these antigens 24 h after initiation of IFN-γ treatment [2,3]. This IFN-γ effect has been shown to trigger de novo RNA and protein synthesis [4].

Phosphatidylinositol diphosphate (PIP_2) is hydrolysed to inositoltriphosphate (IP_3) and diacylglycerol (DAG), the latter being a stimulator of protein kinase C [5,6]. Protein kinase C is an intracellular signal transduction pathway activated endogenously by diacylglycerol. Phorbol 12-myristate 13-acetate (PMA) stimulation of protein kinase C, appears to be due to the same mechanism as that of diacylglycerol, i.e., a decrease in the K_m of the enzyme for Ca^{2+} [7,8]. Thus phorbol esters mimick the endogenous stimulus of protein kinase C and can be used to study the effects of protein kinase C. The only drawback is that the phorbol esters also have many functions other than protein kinase C activation.

We analysed the intracellular second messenger pathway in the regulation of class II antigens on rat microvascular endothelial cells. Our results show that IFN-γ induced class II expression on endothelial cells can be down-regulated with specific inhibitors of protein kinase C. Direct stimulation of protein kinase C with PMA leads to similar increase in class II expression on endothelial cells as does IFN-γ. Increase in cytosolic Ca^{2+} by calcium ionophore A23187, or other intracellular second messengers like cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate

Correspondence address: P. Mattila, Transplantation Laboratory, University of Helsinki, Haartmaninkatu 3, SF-00290 Helsinki, Finland

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(cGMP), had no effect of the class II expression on the endothelial cells.

2. MATERIALS AND METHODS

4–12-day-old DA rats were used. A modification of the method of Kasten [1,9] was used. In some experiments the cells growing in T-25 flasks were stimulated with 100 U/ml recombinant rat IFN-γ for 24 h (a gift from Dr P.H. v.d. Meide, Rijswijk, The Netherlands). In other experiments endothelial cells growing in flasks were treated for 24 h with N^6-2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate (cAMP, 1 μM–1 mM), N^2-2′-*O*-dibutyrylguanosine 3′:5′-cyclic monophosphate (cGMP, 1 μM–1 mM), calcium ionophore A23187 (20 nM–1 μM) and phorbol 12-myristate 13-acetate (PMA, 0.1–1 μM) (all purchased from Sigma, St. Louis, USA).

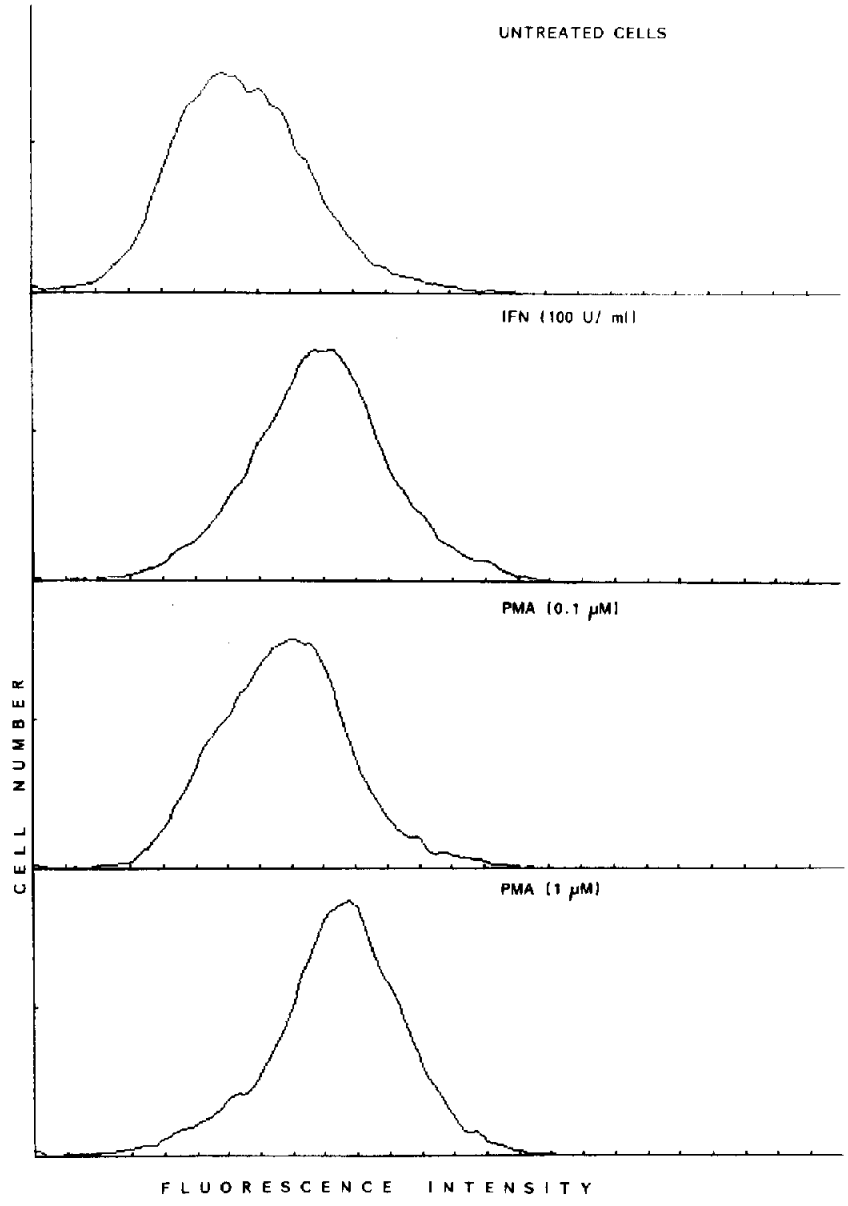

Fig.1. IFN-γ induction of class II molecules on endothelial cells. Endothelial cells were cultured in the absence or presence of IFN-γ (100 U/ml) for 24 h. In some cultures only phorbol-12-myristate 13-acetate was added to replace IFN-γ. The cells were then removed from the bottles, treated with anti-class II antibody followed by FITC-conjugated anti-mouse antibody and analysed by fluorescence activated cell sorter. The data are expressed as histograms, the relative fluorescence intensity is given on a logarithmic scale.

EC were also treated with IFN-γ in combination with protein kinase inhibitors 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7, 2–20 μM), *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride (HA 1004, 20 μM) and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7, 2–30 μM) (by Seikagaku Kogyo Co., Tokyo, Japan) as well as with D-sphingosine (0.3 μM–3 μM; Sigma).

Expression of class II antigens on endothelial cells was analysed with fluorescence activated cell sorter (FACS). The cells were first exposed to a monoclonal antibody to monomorphic determinant of rat class II diluted 1:20 (Mas 043c, Sera-Lab., Grawley Down, Sussex, England), washed twice and exposed to monoclonal fluorescein isothiocyanate conjugated anti-mouse antibody (GAM-FITC, Coulter Immunology, Hialeah, Florida, USA), washed twice, resuspended in phosphate buffered saline (PBS) and run through the FACS.

3. RESULTS

Only 3% of native endothelial cells expressed class II antigens in native state in vitro. IFN-γ at a concentration of 100 U/ml for 24 h induced class II antigen expression on 63% of rat heart endothelial cells (fig.1, table 1).

First we analysed which intracellular signals could replace the IFN-γ effect in inducing class II molecules on the endothelial cells. Cyclic-AMP and -GMP were added to the endothelial cell cultures in a dibutyryl form, which is able to penetrate into the cells. Even within a wide range of different concentrations (from 1–1000 μM), cAMP or cGMP had no effect on the class II expression, neither did the increase of cytosolic calcium with calcium ionophore A23187 have any effect (table 1).

PMA, which belongs to phorbol esters, is a direct activator of protein kinase C. PMA was able to replace IFN-γ in the class II expression in endothelial cell cultures (fig.1, table 1). With 1 μM PMA for 24 h the class II induction was half as strong as with 100 U/ml of IFN-γ. This PMA effect was dose-dependent: concentrations lower than 0.1 μM had no effect of the class II expression on endothelial cells.

Since PMA also has many other biological functions than the protein kinase C stimulation, we confirmed the role of protein kinase C in the IFN-γ response with specific inhibitors of protein kinase C. A selective enzyme inhibitor of protein kinase C, H-7 was added simultaneously with IFN-γ to the endothelial cell cultures. 24 h later the class II antigens were analysed from these endothelial cells. H-7 (20 μM) totally blocked the IFN-γ induced class II upregulation (table 2, fig.2). The inhibitory effect was dose-dependent; 10 μM H-7 inhibited the class II induction half maximally and 2 μM had no effect on the IFN-γ induced class II upregulation. Another enzyme inhibitor, HA 1004 mainly inhibits protein kinase A, but also has some inhibitory capacity against protein kinase C. With this inhibitor at a concentration of 20 μM we blocked the IFN-γ induction of class II upregulation from 63% to 22% (table 2, fig.2).

Shingosine is another inhibitor of protein kinase C, which interferes with protein kinase C activation. 3 μM sphingosine blocked the IFN-γ induced class II expression from 63% to 26% (table 2,

Table 1

Role of second messengers in regulation of class II expression in rat heart endothelial cells

Treatment		Class II positive cells (%)
None		2.8
IFN-γ	100 U/ml	60.7
PMA (μM)	1	28.6
	0.5	21.0
	0.1	6.7
c-AMP (μM)	1000	2.1
	100	2.4
	10	2.1
	1	2.1
c-GMP (μM)	1000	2.9
	100	2.5
	10	2.6
	1	2.0
Calcium ionophore (nM)	1000	2.9
	200	1.3
	20	1.0

Table 2

Inhibition of IFN-γ induced class II expression

IFN-γ (100 U/ml)	Inhibition	Class II positive cells (%)
–	–	4.6
+	–	63.2
+	H-7 (2 μM)	28.3
+	H-7 (10 μM)	14.0
+	H-7 (20 μM)	5.6
+	HA 1004 (20 μM)	21.7
+	sphingosine (3 μM)	26.4

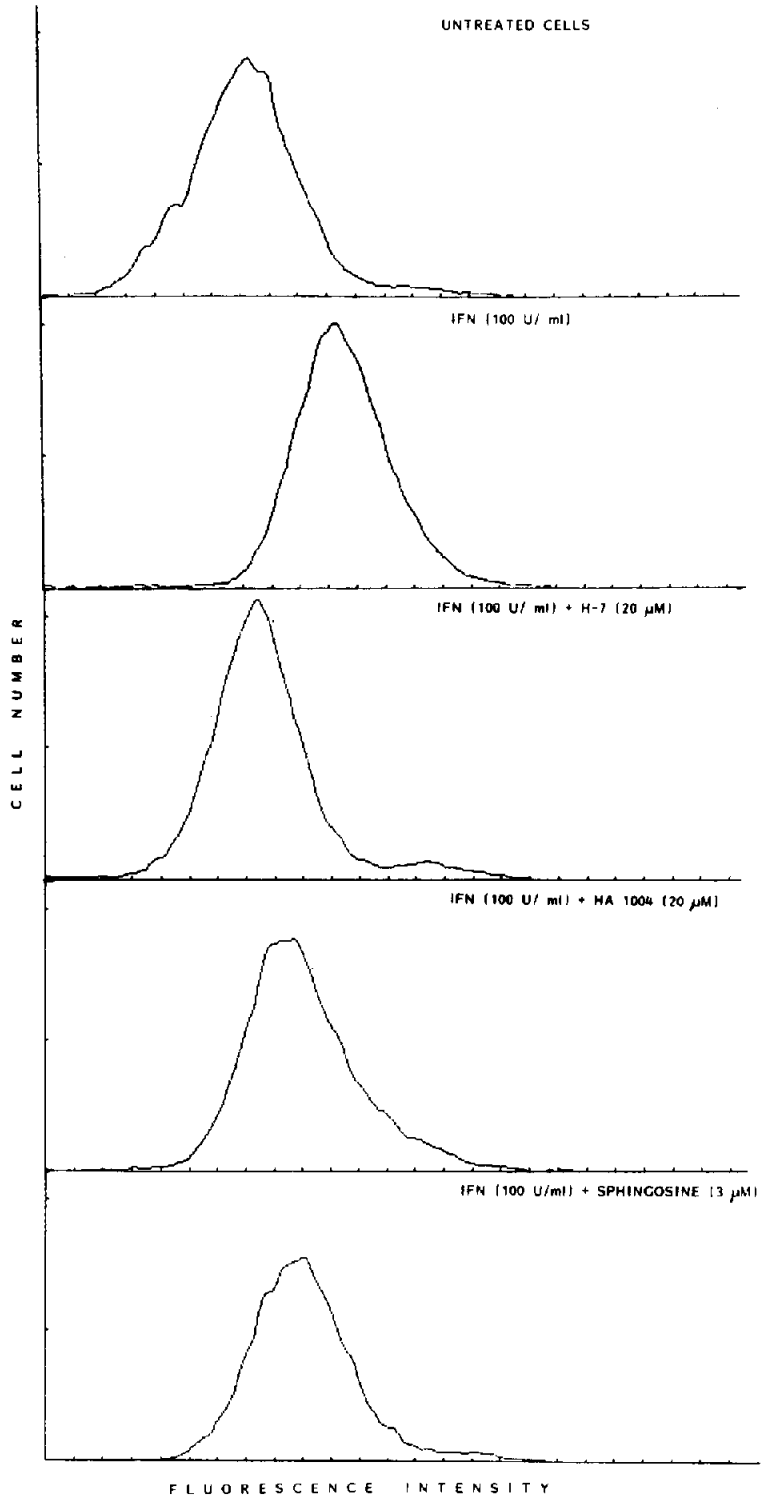

Fig.2. The effect of various protein kinase C inhibitors (H-7, 20 μM; HA 1004, 20 μM; sphingosine, 3 μM) on the IFN-γ (100 U/ml) induced class II expression on endothelial cells. The cells were cultured with the IFN-γ and the enzyme inhibitors for 24 h and analysed for class II expression. The samples were analysed as described in the legend to fig.1.

fig.2). Higher doses of sphingosine detached the EC monolayers from culture bottles. Therefore it was not possible to analyse the effect of higher sphingosine concentrations.

W-7 is a calmodulin antagonist affecting Ca^{2+}-calmodulin pathway. W-7 had no effect on the IFN-γ induced class II induction on endothelial cells (not shown).

4. DISCUSSION

Native endothelial cells do not express class II antigen on their surface in vitro [1]. The induction of class II antigens on endothelial cells in vitro by IFN-γ treatment has been shown by others and us [1,2]. After an IFN-γ (100 U/ml) stimulus the endothelial cells begin to express class II molecules on their surface within 24 h, and after 72 h nearly all endothelial cells have class II molecules on their surface [10]. Our results strongly suggests that IFN-γ upregulates the MHC class II antigens by a protein kinase C dependent pathway. This process has been shown to be dependent on both de novo mRNA and protein synthesis [11].

Very little is known so far of the postreceptor intracellular signal transduction during IFN-γ treatment. To clarify this we applied specific inhibitors of protein kinase C pathway, H-7 and sphingosine. Both inhibited the IFN-γ induced class II upregulation in a dose-dependent manner when added simultaneously with IFN-γ. These results suggested that IFN-γ works via protein kinase C in endothelial cell class II regulation. This was further confirmed by PMA, which is a direct activator of protein kinase C [12]. PMA in a micromolar dose range was able to replace IFN-γ in the induction of class II on endothelial cells. This dose is relatively high, since many other protein kinase C stimulatory functions of PMA operate already at nanomolar concentrations [13]. The need of higher doses may refer to the need of activation of a wider or a different spectrum of kinases in the protein kinase C family [14,15].

Controversial data exist concerning the signal transduction pathway after IFN-γ stimulation in transformed cell lines. IFN-γ has been shown to operate via the calcium-calmodulin pathway, but not via protein kinase C in the HL-60 monocytic cell line [16]. Yet in two other monocytic cell lines, U 937 and in a lymphoid cell line YT-1, the protein kinase C pathway but not the calcium-dependent pathway was shown to be the route of signal transduction [16]. The discrepancy might be due to the fact that both these cell lines are malignant and the transformation might have caused alterations in the intracellular signal transduction mechanisms. It is also possible that IFN-γ induces different signal pathways in different cell types, but this has not yet been demonstrated in normal cells.

Taken together, our results suggest that in cultured endothelial cells IFN-γ operates via the protein kinase C pathway in the induction of class II expression on the endothelial cell surface. Ca^{2+}, cAMP or cGMP are not involved in this regulation.

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