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Interleukin 1α Stimulates Nuclear Phospholipase C in Human Osteosarcoma SaOS-2 Cells*

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Interleukin 1 (IL-1) is one of the most potent stimulators of bone resorption. However, the early biochemical events elicited by IL-1 receptor binding are not fully understood. Here we show that in human osteosarcoma SaOS-2 cells the treatment with IL-1α is able to evoke a rapid and transient increase of nuclear phospholipase C (PLC) activity. A parallel decrease of nuclear phosphatidylinositol monophosphate and phosphatidylinositol bisphosphate is observed. All these events are strictly confined to the nuclear compartment without affecting the cytoplasmatic inositol lipid pool. In addition we show that by Western blot analysis with specific monoclonal antibodies the PLC γ is located both in the cytoplasm and in the nucleus, while PLC β appears exclusively localized in the nucleus. Moreover, the increase of PLC activity in response to IL-1α is completely neutralized by monoclonal antibody against the β-form. While confirming the existence of an autonomous nuclear phosphoinositide signaling system, our data clearly indicate that in SaOS-2 cells one of the earliest events following IL-1α treatment is the breakdown of nuclear phosphatidylinositol monophosphate and phosphatidylinositol bisphosphate because of the activation of a specific nuclear PLC isoform.

Interleukin 1 (IL-1)[1] is a family of polypeptides that elicits a plethora of physiological responses in a wide spectrum of cell types, having activities that range from immunomodulation to bone resorption and fibroblast and T cell proliferation (1, 2). The most consistent property of IL-1 is to up-regulate cellular metabolism and to increase the expression of several genes coding for biologically active molecules. These responses to IL-1 are generally related to the binding to high affinity receptors present on target cells (3, 4). Two distinct forms of the IL-1 receptor have been demonstrated on T cells, and transfection of the cloned type I IL-1 receptor gene into Chinese hamster ovary cells seems to be sufficient to trigger a biological response to IL-1 (5).

However, in spite of the cloning of the receptor (4) and of the considerable effort made to elucidate the biochemical mechanism for the action of IL-1, the mechanism by which the interaction of IL-1 with its receptor is coupled to IL-1 regulation of gene transcription has remained obscure.

In fact, there is much conflicting evidence concerning the earliest intracellular events following the binding of IL-1 to its receptor; in some systems, a slight induction of both phosphatidylinositol 4,5-bisphosphate and phosphatidylcholine hydrolysis has been reported (6, 7), with subsequent generation of diacylglycerol (8), although neither elevation of intracellular calcium nor protein kinase C activation has been observed (6, 9).

Besides, the data on the involvement of the cyclic AMP-protein kinase system also appear controversial (10–14), and a clear consensus on the effector mechanism for IL-1 signal transduction is lacking.

Recently, IL-1 accumulation into the nucleus after receptor-mediated endocytosis has been observed in the murine T cell line EL-4 (15, 16), suggesting the possibility of a nuclear site for IL-1 receptor signaling, as shown for the steroid and thyroid hormone receptor superfamily (17). However, it is not clear whether a nuclear localization of the IL-1·IL-1 receptor complex is sufficient to trigger IL-1 activation of gene expression in T-cells (18).

Recently, several lines of evidence by our laboratory and others indicated the existence of an autonomous phosphoinositide signaling system located in the nucleus (19–21), capable of being modulated by extracellular stimuli (22–30). Therefore, we have investigated whether the action of IL-1 could be mediated by this nuclear pathway in a human osteosarcoma cell line, SaOS-2, which possesses high affinity receptors for human recombinant IL-1α and -β (13). We report here that the response to IL-1α in SaOS-2 is to some extent mediated by a nuclear phospholipase C, *i.e.* the β isoform.

EXPERIMENTAL PROCEDURES

Materials—Culture media and FCS were from ICN FLOW (Costa Mesa, CA), and monoclonal antibodies against PLC isoforms and IL-1 were from UBI (Lake Placid, NY). All electrophoresis reagents, as well as Trans-Blot nitrocellulose membrane, were purchased by Bio-Rad, while products for ECL and [^{32}P]orthophosphate (10 mCi/ml) were from Amersham (U.K.). Protease inhibitors were from Boehringer Mannheim GmbH (Germany). Protein A-Sepharose CL-4B was purchased from Pharmacia LKB (Uppsala, Sweden). All other products were from Sigma.

Cell Culture—Human osteosarcoma SaOS-2 cells were grown in Iscove medium supplemented with 10% FCS and 2 mM glutamine and routinely plated weekly at 2×10^4 cells/cm^2. For IL-1 stimulation, cells were used postconfluence, between 10 and 14 days after seeding, displaying at that age the highest number of receptors for IL-1 (13). Because of the presence of IL-1 in FCS, cells were starved in 0.1% FCS 24 h before treatment.

For *in vivo* labeling with [^{32}P]orthophosphate ($^{32}P_i$), cells were preincubated in phosphate-free medium containing 0.5% bovine serum

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[1] The abbreviations used are: IL-1, interleukin 1; PLC, phospholipase C; IGF, insulin-like growth factor; MoAb, monoclonal antibody; FCS, fetal calf serum; PtdInsP, phosphatidylinositol 4-phosphate; PtdInsP$_2$, phosphatidylinositol 4,5-phosphate; PAGE, polyacrylamide gel electrophoresis; MES, 4-morpholineethanesulfonic acid.

albumin for 2 h, and then $^{32}P_i$ (100 μCi/ml) was added for an additional 3 h, as previously described (31).

Isolation of Nuclei—Highly pure nuclei were obtained by hypotonic shock in combination with non-ionic detergent, as previously described (22). Briefly, cells washed in phosphate-buffered saline were resuspended in 10 mM Tris-HCl, pH 7.8, containing 1% (v/v) Nonidet P-40, 10 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and aprotinin, 10 μg/ml soybean trypsin inhibitor, 15 μg/ml calpain inhibitor I, and 7 μg/ml calpain inhibitor II (700 μl/2.5 × 10^7 cells). After 5 min at 4 °C, 700 μl of double-distilled water were added, and swelling on ice was performed for 5 more min. Cells were then sheared by 5 passages through a 22-gauge needle, and nuclei were recovered by centrifugation at 300 × *g* for 6 min at 4 °C. Nuclei were washed once in 10 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, plus protease inhibitors as above, then resuspended in 5 mM Tris-HCl, pH 8, 0.1 mM EDTA, 0.06% sodium deoxycholate. After 10 min at 4 °C, nuclei were disrupted by 40 passages through a 25-gauge hypodermic needle. The lysate, containing the PLC activity, was centrifuged for 10 min at 48,000 × *g* at 4 °C. Proteins were determined with the Bio-Rad protein assay kit. As criteria of nuclear purity, nuclei preparations were tested for glucose 6-phosphatase activity (20) that was usually less than 1% of the total cell homogenate activity and by electron microscopy (not shown).

Preparation of the Cytoplasmatic Fraction—The cytoplasmatic fraction was obtained by homogenizing cells with 20 strokes in a Dounce homogenizer in 10 mM Tris-HCl, pH 7.8, 2 mM $MgCl_2$ plus protease inhibitors as above and then pelleting the nuclei at 400 × *g*. This procedure allows the recovery of pure cytoplasmatic fraction and avoids the risk of contamination by nuclear debris that are present in the crude supernatant from nuclear purification.

PLC Assay—Essentially, the assay was carried out as described previously (22). Briefly, the assay (100 μl) contained 100 mM MES, pH 6.5, 150 mM NaCl, 0.06% sodium deoxycholate, 3 nmol of [^{3}H]PtdInsP or [^{3}H]$PtdInsP_2$ (specific activity, 30,000 dpm/nmol), and 5 μg of nuclear or cytoplasmatic protein. Incubation was at 37 °C for 30 min. Hydrolysis was stopped by adding chloroform/methanol/HCl as described (24), and the vacuum-dried inositol phosphates recovered from the aqueous phase were analyzed by anion exchange on a Whatman Partisphere 5 SAX column. After washing the column for 10 min with water, samples were loaded and eluted with a linear gradient over 60 min from 0 to 2 M ammonium formate (pH 3.7, adjusted with phosphoric acid) in water, at a flow of 1 ml/min. Fractions (1 ml) were collected and counted for radioactivity by liquid scintillation (Hionic-fluor, Packard). [^{3}H]Inositol 1,4-P_2 and [^{3}H]inositol 1,4,5-P_3 were used as standards.

The organic phase from each lipid extraction was applied to a TLC plate as previously described (20).

SDS-PAGE and Immunoblotting—Nuclear protein for electrophoresis was prepared by trichloroacetic acid precipitation as described (32). 60 μg/30 μl of loading buffer (33) were run on a 7.5% SDS-PAGE using a Bio-Rad Minigel apparatus and transferred to nitrocellulose using a Bio-Rad wet blotting system (34) overnight at 0.25 A. After blocking the nitrocellulose in phosphate-buffered saline containing 0.1% Tween 20 and 5% dried milk for 1 h at room temperature, the blots were incubated with primary antibodies (1:200 anti-β, 1:1000 anti-γ, 1:500 anti-δ) overnight at 4 °C. After appropriate washing, strips were incubated with secondary antibody conjugated to horseradish peroxidase and visualized using the ECL kit (Amersham).

Immunoprecipitation—5 μg of nuclear protein were shaken for 1 h at 4 °C with 1.25 μg of anti-β or -γ MoAb, or with an immunoglobulin of the same subclass, followed by 1 h of incubation with Protein A-Sepharose CL-4B (10% w/v). Immunocomplexes were collected by centrifugation, and the supernatant was assayed for residual PLC activity, as described above.

RESULTS

Influence of IL-1α Stimulation on PLC Activity in SaOS-2 Cells—An analysis of basal phospholipase C activities in both nuclei and cytoplasm of SaOS-2 cells, using [^{3}H]PtdInsP and [^{3}H]$PtdInsP_2$ as substrates, is shown in Fig. 1. The same figure shows that both substrates can be used by either nuclear or cytoplasmatic enzymes, but $PtdInsP_2$ is the preferred one. The assay was conducted in the presence of the optimal calcium requirement, as detected by a previous set of experiments (data not shown). Whole cells, labeled *in vivo* with [^{32}P]orthophosphate, were treated with IL-1α (1 ng/10^6 cells), and their polyphosphoinositide endogenous pool was analyzed (Table I). It is noteworthy that the percentage of radioactivity incorporated in basal conditions in phosphatidylinositol, PtdInsP, and $PtdInsP_2$ is, respectively, 49.5, 9.1, and 9.6 in cytoplasm and 30, 4.4, and 27 in nuclei, showing a 3-fold enrichment of $PtdInsP_2$ in nuclei with respect to cytoplasm. After IL-1α treatment, changes in the cytoplasmatic PtdInsP and $PtdInsP_2$ mass were marginal, whereas a very rapid, significant decrease in nuclear PtdInsP and $PtdInsP_2$ mass was observed. The rate of polyphosphoinositide hydrolysis in nuclei of SaOS-2 cells exposed to IL-1α correlates well with the acute but transient increase observed in nuclear PLC activity in that it rises by about 2-fold after 2 min of treatment and rapidly declines thereafter (Fig. 2).

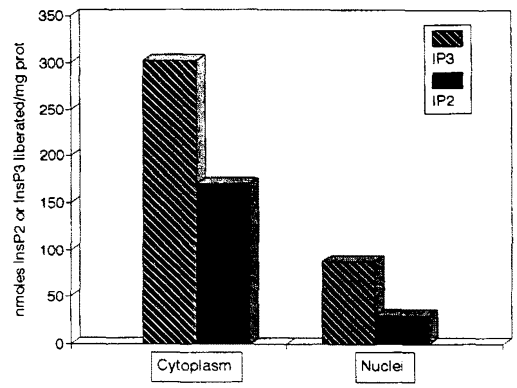

FIG. 1. **PLC activity in cytoplasm and nuclei of SaOS-2 cells.** Basal PLC activity was assayed by high pressure liquid chromatography analysis of water-soluble compounds obtained after incubating 5 μg of nuclear proteins with [^{3}H]$PtdInsP_2$ or [^{3}H]PtdInsP (90,000 dpm/3 nmol) for 30 min at 37 °C in 100 mM MES, pH 6.5, containing 100 μM or 1 mM $CaCl_2$, respectively, 0.6% NaCl, 0.06% sodium deoxycholate final concentration. Values, expressed as nanomoles/mg of protein, are the average of five separate experiments and are significantly different with *p* less than 0.05. *InsP3* (*IP3*), inositol 1,4,5-trisphosphate; *InsP2* (*IP2*), inositol 4,5-bisphosphate.

TABLE I

Recovery of inositol lipids from TLC-analyzed organic phases obtained after in vivo labeling of SaOS-2 cells stimulated with IL-1α

SaOS-2 cells, labeled *in vivo* with [^{32}P]orthophosphate (100 μCi/ml), were treated with IL-1α (1 ng/10^6 cells) for the indicated times. Then polyphosphoinositides were analyzed by TLC as described under "Experimental Procedures." Values are expressed as disintegration/min/mg of protein and are the average of five separate experiments ± S.D. The percentage of radioactivity incorporated in basal conditions in phosphatidylinositol (PtdIns), PtdInsP, and $PtdInsP_2$ is, respectively, 49.5, 9.1, and 9.6 in cytoplasm and 30, 4.4 and 27 in nuclei.

	min	$PtdInsP_2$	PtdInsP	PtdIns
Cytoplasm	0	52 ± 3.9	48 ± 2.4	268 ± 18.7
	2	44 ± 3.1	38 ± 2.28	222 ± 15.5
	30	50 ± 3.6	41 ± 2.05	233 ± 13.9
Nuclei	0	185 ± 9.3	30 ± 1.5	133 ± 9.31
	2	84 ± 5.2[a]	22 ± 1.32	128 ± 7.68
	30	128 ± 7.7	34 ± 2.38	156 ± 12.4

[a] Significant differences from controls ($p < 0.05$, Student's *t* test).

Distribution of PLC Isoforms in SaOS-2 Cells—Since the known PLC isoforms are thought to have different distribution within the cell compartments (35–38), we investigated the localization of the individual isozymes in our system by Western blotting analysis, using MoAbs whose specificity had been previously demonstrated (22, 31, 36). As shown in Fig. 3, isolated nuclei from SaOS-2 cells contain both PLC β and γ; PLC β appears to be present as a 140-kDa form, while PLC γ shows a doublet of 145 and 140 kDa. Besides the reported presence of PLC β specifically localized in the nucleus of Swiss 3T3 cells (22) and rat hepatocytes (40), the presence of PLC γ resembles the observation obtained in nuclei of PC12 cells (36) and Friend

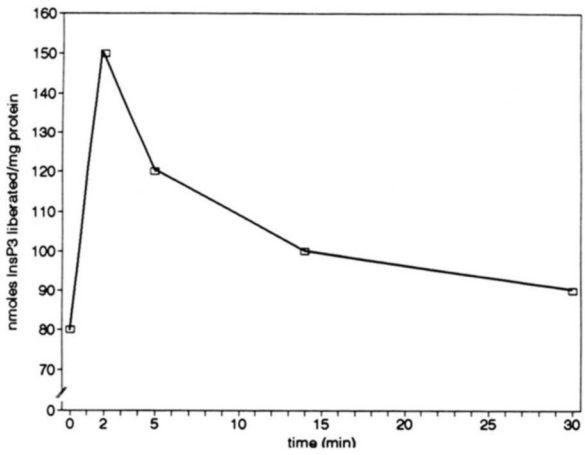

FIG. 2. **PLC activity in nuclei from SaOS-2 cells exposed to IL-1α.** Confluent SaOS-2 cells (5×10^7 cells/point) were incubated with IL-1α (1 ng/10^6 cells) for the indicated times. Nuclei were extracted as described under "Experimental Procedures." Values are expressed as nanomoles/mg of protein and are the average of five separate experiments, differing no more than 8% S.D. *InsP3*, inositol 1,4,5-trisphosphate.

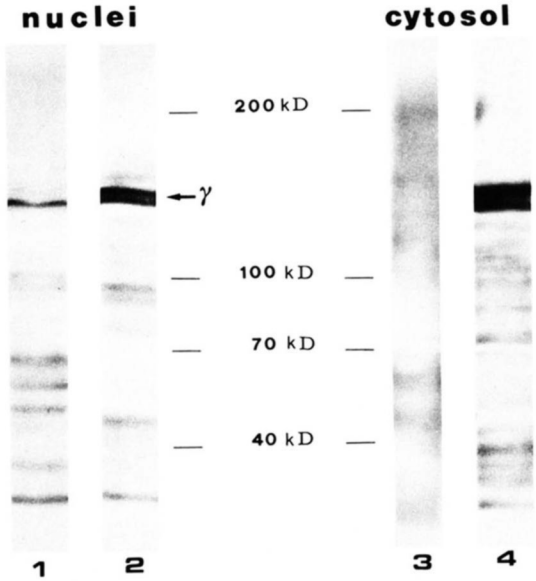

FIG. 3. **Immunochemical detection of PLC isoforms in both cytoplasm and isolated nuclei of SaOS-2 cells.** 60 μg of cytoplasmic and nuclear fractions were subjected to SDS-PAGE using 7.5% acrylamide gels and transferred to nitrocellulose overnight. Antibody dilution was 1:200 for anti-β and 1:1000 for anti-γ. *Lanes 1* and *3*, anti-PLC β MoAb; *lanes 2* and *4*, anti-PLC γ MoAb.

cells.[2] The absence of the native 150-kDa PLC β could be due to a limited protection by the calpain II inhibitor since this proteolytic enzyme, which is predominantly nuclear (41), acts on PLC β (42) as well as to a mutated form of PLC β. It is worth mentioning that the above described 140-kDa band is recognized by the mixture of MoAbs specific for PLC β (43). The cytoplasmatic fraction, on the contrary, only reacts with the γ-form. It is therefore concluded that the β-form, revealed by the MoAb we used, is specifically localized to the nucleus.

Analysis of the PLC Isoforms Involved in IL-1α Stimulation—Further experiments were carried out in order to establish which isozymes are involved in the response to IL-1α. Nuclear PLC activity, solubilized by hypotonic shock, was

[2] A. M. Martelli, A. M. Billi, R. S. Gilmour, L. M. Neri, L. Manzoli, A. Ognibene, N. M. Maraldi, and L. Cocco, manuscript in preparation.

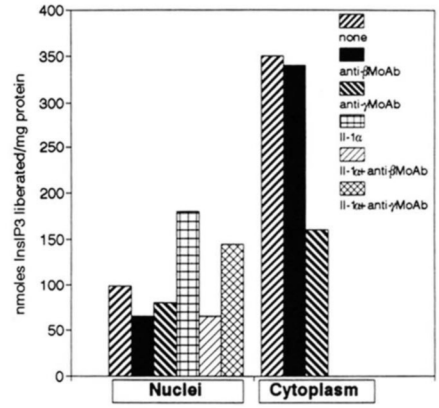

FIG. 4. **Neutralization of cytoplasmic and nuclear PLC activity by anti-PLC-β and -γ MoAbs.** Cytoplasmic or nuclear proteins from SaOS-2 cells (control or treated for 2 min with IL-1α) were incubated with anti-β or -γ MoAbs or with an IgG of the same subclass. Then, the immunocomplex was separated with Protein A-Sepharose by centrifugation, and the supernatant, containing residual isoforms, was assayed for PLC activity as described. Values, expressed as nanomoles/mg of protein, are the average of five separate experiments and are significantly different with p less than 0.05. *InsIP3*, inositol 1,4,5-trisphosphate.

tested after immunoprecipitating the β or γ isoforms with specific MoAbs.

In unstimulated cells, after immunoprecipitation with anti-PLC β MoAb, 35% of the nuclear PLC activity was neutralized while anti-PLC γ MoAb gave rise to 20% inhibition (Fig. 4). After stimulation with IL-1α, the neutralization of nuclear activity after immunoprecipitation with anti-γ MoAb was still about 20% but rose to 60% with anti-β MoAb (Fig. 4). Actually anti-β MoAb reduced stimulated PLC activity to values similar to that of basal conditions. Even if the MoAbs used possess a good neutralizing activity, they do not account for 100% inhibition (43). Finally it has to be noted that both nuclear and cytoplasmatic fractions do not show any immunoreactivity with the anti-PLC δ MoAb (not shown). This evidence strongly suggests that the activation of the nuclear PLC β can represent an early step in the IL-1α signaling pathway in SaOS-2 cells (39). Moreover, this contention is supported by the assay of the cytoplasmatic activity after immunoprecipitation with neutralizing MoAbs. Indeed Fig. 4 shows that only the anti-PLC γ MoAb is capable of removing more than 50% of the cytoplasmatic PLC activity.

DISCUSSION

Several attempts to elucidate the pathways mediating IL-1 actions in a number of selected intact cell systems (14) have failed to hint at a clear understanding of that mechanism.

The biological effects of IL-1 seem to be elicited by high affinity binding of the cytokine to specific receptors present on target cells. However, receptors are scarce, and their affinities often do not match the potency of the biological response, suggesting the presence of a very efficient amplification system. An analysis of the T cell IL-1 receptor sequence indicates that it is not related to receptor kinases nor to G-protein-coupled receptors (5).

To investigate if a different signaling pathway could account for at least some of the effects produced by IL-1, we hypothesize an involvement of the phosphoinositide cycle, since it has been clearly demonstrated by a number of reports in the last decade that many extracellular signaling molecules, on binding to their cell surface receptors, elicit intracellular responses by activating specific phospholipases C.

Recently, the existence of an autonomous, nuclear phosphoinositide cycle able to respond to extracellular stimuli has been

demonstrated by a growing number of reports (20–22).

Martelli *et al.* (22) first reported that the PLC β is specifically localized in the nucleus of Swiss 3T3 fibroblasts, where it is activated when cells are stimulated with IGF-I. The evidence of a nuclear localization of PLC β has been confirmed in other cell types, like PC12 rat pheochromocytoma cells (36), rat liver nuclei (40), and Friend erythroleukemia cells.[2]

In SaOS-2 whole homogenates, we observed a slight increase in PLC activity after treatment with IL-1α (not shown), in agreement with previous investigations on different cell systems (6, 7).

Although we cannot demonstrate that isotopic equilibrium has been reached in these experiments, the observation that the steady-state labeling of $PtdInsP_2$ showed a 3-fold enrichment in nuclei compared with the cytoplasm and that the labeling of this nuclear lipid rapidly decreases after 2 min of IL-1α treatment (Table I) led us to investigate the time course of the PLC activity in nuclei from IL-1α-treated cells.

After 2 min of treatment, IL-1α was able to evoke an acute and transient 100% increase of nuclear PLC activity that in 30 min was already back to the basal level. This resembles the effect of IGF-I on nuclear PLC β, which takes place in the same time interval (22). A parallel decrease of PtdInsP and $PtdInsP_2$ was observed. Furthermore, the assay performed after immunoprecipitation with anti-β or -γ MoAb clearly shows that the β-form is responsible for the increase of nuclear PLC activity following IL-1α stimulation of SaOS-2 cells (Fig. 4). Moreover, the anti-β antibody does not inhibit the soluble cytoplasmatic activity, in accordance with the Western blotting analysis that indicates the absence of cytoplasmatic PLC β; all together this evidence strengthens the contention that IL-1α activates a preexisting nuclear PLC β and does not induce translocation from the cytoplasm to the nucleus (40).

Taken together, our data clearly indicate that the activation of nuclear PLC β could be an early step in the mode of action of IL-1α. It is also possible to speculate that this event may mediate the effects of IL-1α on the modulation of the gene expression in various cell types including SaOS-2 cells (39).

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