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Phosphatidylinositol turnover is not a general regulator of neuroblastoma cell differentiation: comparison between two differentiating agents, retinoic acid and γ-interferon

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The turnover of phosphatidylinositol (PI) is believed to constitute a crucial step in the signaling pathways for stimulation of cells by a variety of bioactive substances, including differentiating agents; however decisive evidence for the idea has not been obtained. In the present paper, we investigated the involvement of PI turnover in cell differentiation using a human neuroblastoma cell line, LAN-1, which can be induced to differentiate along the neuronal pathway by both retinoic acid (RA) and γ-interferon (γ-IFN). Analysis of labelled phosphatidylinositol metabolites from prelabelled cells indicated a rapid decrease of inositol 1,4,5-trisphosphate and 1,2-diacylglycerol within 1 min of induction of LAN-1 cell differentiation by RA, while no changes were observed in γ-IFN-treated cells. These findings indicate the occurrence of decreased inositol phospholipid turnover in RA-treated LAN-1 cells and suggest that phosphoinositide-derived metabolites may not constitute general regulators of cellular differentiation.

Differentiation; Retinoic acid; Interferon-γ; Phosphatidylinositol turnover

1. INTRODUCTION

It is well established that a very early response of cells to agonist stimulation is the rapid cleavage of polyphosphoinositides by phospholipase C [1–3]. The products of this reaction, inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DG), are putative second messengers, with DG activating protein kinase C [4,5] and IP_3 triggering release of Ca^{2+} from intracellular stores [6,7]. It is also accepted that the cellular levels of PI metabolites play key roles in the control of proliferation, transformation, and differentiation [8,9]. We decided to investigate the involvement of PI turnover during differentiation of human neuroblastoma (NB) cells. The aim was to verify the hypothesis [9] that PI turnover is a general regulator of cell proliferation and differentiation, and that differentiation is induced by a decrease of PI turnover. Cultured mouse and human NB cells can be induced to differentiate by RA and γ-IFN [10–12]. Although recent studies [13,14] indicate that RA somehow affects genomic expression by its direct or indirect interaction with the cell nucleus, information is lacking with respect to the mechanism whereby the signal is transduced across the membrane. Furthermore, the mechanism by which IFN transmits its effects from the cell surface into the cell where its actions are manifested is still unclear [15,16].

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Abbreviations: PI, phosphatidylinositol; RA, retinoic acid; γ-IFN, γ-interferon; IP_3, inositol trisphosphate; DG, diacylglycerol

2. MATERIALS AND METHODS

2.1. *Reagents*

[1(3)-^{3}H]Glycerol (spec. act. 3 Ci/mmol) was from Amer-

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sham (Bucks, England). all-*trans*-RA was from Sigma (St. Louis, MO). Human recombinant interferon-γ was from Genzyme Corp. (Boston, MA). The purchase of all other materials has been described [17].

2.2. *Cell line*

Human neuroblastoma cell line LAN-1 was maintained in culture in RPMI 1640 medium containing 15% heat-inactivated FCS. Cells were grown, subcultured, and treated with RA and γ-IFN as in [18].

2.3. *Cell labelling procedure*

Inositol-depleted cells (2×10^6/point) were labelled for 24 h in inositol-free RPMI 1640 medium containing 10 μCi/ml *myo*-[1,2-^{3}H]inositol. Cells were then washed and 10 ml phenol red-free, inositol-free RPMI 1640 medium supplemented with 35 mg/ml unlabelled *myo*-inositol were added. The incubation was continued at 37°C in the presence or absence of either 10^{-5} M RA or 1000 U/ml γ-IFN and terminated as above.

DG levels were evaluated with the same protocol detailed above for inositol phosphates using [1(3)-^{3}H]glycerol.

2.4. *Extraction procedures*

Lipids were extracted as described previously [17].

2.5. *Separation of water-soluble inositol phosphates*

Anion-exchange high-performance liquid chromatography (HPLC) was used to separate [^{3}H]inositol phosphates according to Dean and Moyer [19]. The column was calibrated using authentic labelled standards obtained commercially or prepared according to published methods [19,20].

2.6. *Thin-layer chromatography of 1,2-diacylglycerol*

The pattern of distribution of the chloroform-soluble radioactivity into neutral lipids was determined by thin-layer chromatography (TLC). Lipid standards were visualized by exposure to iodine vapor. Spots corresponding to the location of authentic standards were scraped off and counted for radioactivity in a Packard Tri-Carb 4530 beta counter.

3. RESULTS

Levels of inositol phosphates in LAN-1 cells were measured over the first 15 min of treatment with RA. Cells were labelled to equilibrium with *myo*-[1,2-^{3}H]inositol to ensure that any observed changes resulted from changes in mass and not of specific activities. Direct phosphorylation of *myo*-inositol was minimized by addition of an approx. 1000-fold excess of unlabelled inositol to the medium used for the second incubation. After addition of 10^{-5} M RA to cells, the level of [^{3}H]IP_3 fell to approx. 20% of the control value within 1 min (fig.1C). This level remained 35–50% lower than control values, up to 15 min. In contrast, the level of [^{3}H]IP_3 did not change, relative to control, up to 15 min after γ-IFN induction of differentia-

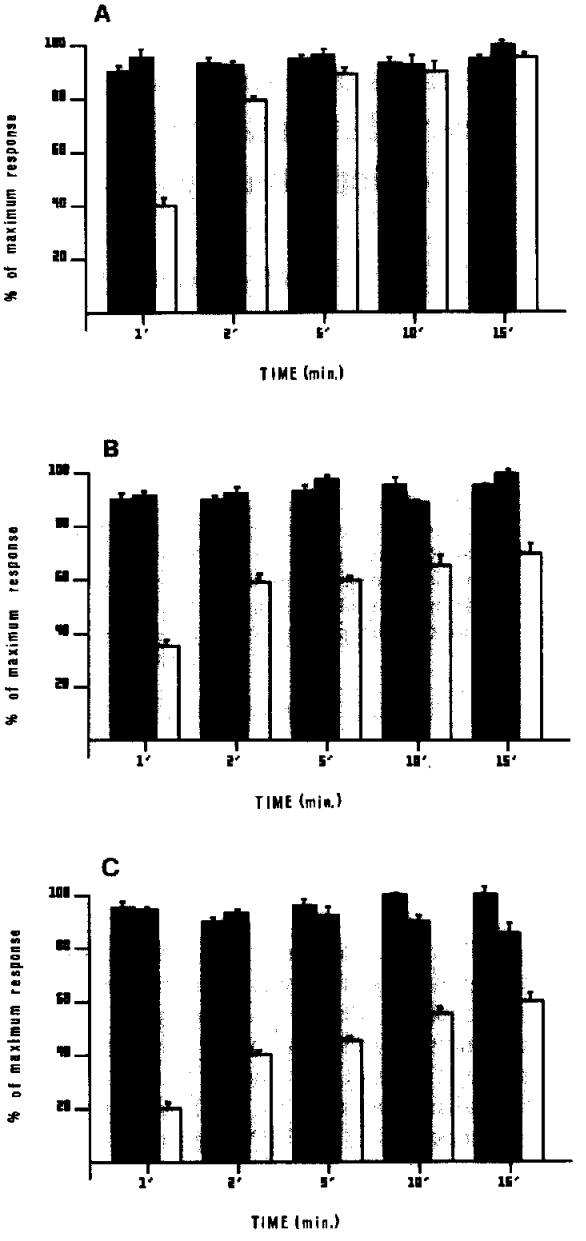

Fig.1. Effects of treatment with RA and γ-IFN on inositol phosphate levels in LAN-1 cells. LAN-1 cells were prelabelled with *myo*-[1,2-^{3}H]inositol, washed, and incubated in medium supplemented with a 1000-fold excess of unlabelled *myo*-inositol in the absence (solid bar) or presence of either 10^{-5} M RA (clear bar) or 1000 U/ml γ-IFN (stippled bar). Cells were extracted and separation of inositol phosphates was performed by anion-exchange HPLC as described [26]. Data points are single determinations from a typical experiment reproduced three times. Maximum responses were 5894 (A), 4120 (B), 977 (C) cpm/2×10^6 cells.

tion (fig.1C). Measurements of [^{3}H]inositol monophosphate (IP) and [^{3}H]inositol bisphosphate (IP_2) gave results similar to those obtained for IP_3, with lower relative response (fig.1A,B) for RA-treated cells, while γ-IFN treatment did not alter the levels of IP and IP_2 at any time point examined (fig.1A,B). Evaluation of very early points (15 and 30 s) gave the same pattern of results of 1 min treatments (not shown).

It would be expected that a decline in IP_3 would be accompanied by a decrease in DG levels. Therefore, we measured the DG levels in cells treated with RA and γ-IFN. Fig.2A shows that RA treatment of LAN-1 cells decreased the levels of DG to approx. 50% of control within 1 min of RA induction of differentiation. By 15 min, levels of DG were still 60% of control values (fig.2A). In contrast, treatment of LAN-1 cells with γ-IFN did not change the levels of DG at any experimental point observed (fig.2A). Finally, to ascertain that the decrease in DG levels was not simply an artifact of RA, we measured triacylglycerol (TG) levels. Fig.2B shows that no differences were observed between control and RA- or γ-IFN-treated cultures in the incorporation of [^{3}H]glycerol into TG.

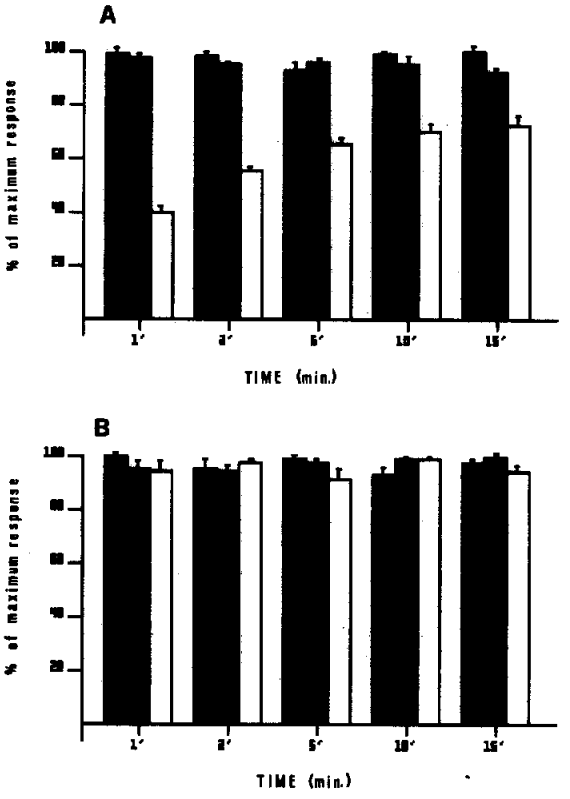

Fig.2. Effects of treatment with RA and γ-IFN on the levels of DG (A) and TG (B). LAN-1 cells were prelabelled with [1(3)-^{3}H]glycerol, washed and incubated in medium supplemented with a 1000-fold excess of unlabelled glycerol in the absence (solid bar) or presence of either 10^{-5} M RA (clear bar) or 1000 U/ml γ-IFN (stippled bar). Cells were extracted, and separation of neutral lipids was performed as described in section 2. Data points are single determinations from a typical experiment reproduced three times. Maximum responses were 20044 (A) and 23013 (B) cpm/2 × 10^6 cells.

4. DISCUSSION

The present results demonstrate that in vitro induction of differentiation of LAN-1 neuroblastoma cells by RA is associated with a rapid decrease of PI turnover, while γ-IFN treatment does not alter the levels of the inositol phospholipid-derived metabolites (i.e. IP_3 and DG).

The available evidence suggests that RA, an analog of vitamin A, has multiple effects on cells. It may constitute a natural morphogen in the developing chick limb bud [22]. In neuroblastoma tissue cultures, RA appears to be a potent compound for promoting differentiation, inhibiting cell growth, and perhaps reducing tumorigenicity [11,18,23,24]. Although the exact molecular mechanisms causing effects of RA on cellular differentiation remain to be elucidated, recent studies indicate that RA somehow affects genomic expression by its direct or indirect interaction with the cell nucleus [13,14,25]. One possibility could be that the primary site of action of RA is located on the plasma membrane. Through such interaction, 'second messengers' are formed which in turn make possible the interaction between RA and specific nuclear RA receptor(s), later affecting gene expression. Based on the hypothesis that the cellular levels of certain PI metabolites play key roles in the control of proliferation, transformation, and differentiation [3,9], we decided to test the generality of this phenomenon by studying the early events in neuroblastoma cell differentiation.

Furthermore, we decided to compare the effects of RA with those of γ-IFN, a naturally occurring differentiating agent [26], with the aim of confirming the previous hypothesis [9]. Interferon is antiviral, has immunoregulatory effects in living cells, and inhibits cell growth [27]. IFNs have been

shown to have a direct effect on the multiplication of various human tumour cells [28]. However, little is known about the mechanism by which IFN transmits its effects from the cell surface into the cell. Mills et al. [15] reported that IFNs do not signal cells by rapid alterations in phosphoinositide-metabolite levels, which apparently is contradictory to the findings of Yap et al. [16], where very rapid and transient IP_3 and DG increases are early events in the interferon-induced transmembrane signaling process. Our findings seem to confirm the data of Mills et al. and demonstrate that in vitro induction of neuronal differentiation by RA is associated with a rapid decrease of PI turnover. Decreases in inositol 1,4,5-trisphosphate and diacylglycerol were detectable within 1 min after the addition of the inducing agent RA. These decreases constitute the earliest reported events in neuroblastoma differentiation. Since no difference was observed in the pattern of inositol phosphates between RA-treated washed cells and RA-treated unwashed cells, and no radioactivity co-migrating with inositol phosphates was observed in the supernatant derived from washed cells, the possibility of a detergent-like effect of RA is unlikely (not shown). The possibility that the very early decreases in concentration of PI metabolites after induction by RA of LAN-1 cell differentiation were due to a decrease in inositol or glycerol uptake was eliminated, as no decrease was observed in either *myo*-[1,2-^{3}H]inositol or [1(3)-^{3}H]glycerol uptake in RA-treated cultures, compared to control cells (not shown). Thus, the observed changes in PI metabolites reflect real changes in mass rather than in specific activity.

These data suggest that the metabolites derived from inositol phospholipid turnover may be part of the mechanism by which certain RA signals are transduced, further indicating that PI turnover may not constitute a general regulator of cellular differentiation.

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