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Retinoic acid inhibits phospholipid turnover and protein kinase C activity in RA-sensitive but not in RA-resistant cells

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Treatment with 10^{-5} M retinoic acid causes loss of anchorage-independent growth in *src*-transformed RR1022 cells but not in *ras*-transformed KNRK cells. In an effort to elucidate the mechanisms underlying this difference, we investigated the effect of RA on phospholipid turnover and PKC activity in these two cell lines. 10^{-5} M RA treatment caused a drastic inhibition of ^{32}P incorporation into PI and PA and a large increase in ^{32}P incorporation into PC in RR1022 cells. Similar treatment of KNRK cells yielded no change in PC or PA labelling and a much smaller decrease in PI labelling. Furthermore, 10^{-5} M RA treatment causes a large decrease in PKC activity in RR1022 cells (35% of control) but only a small decrease in KNRK cells (78% of control). We suggest that these effects are part of an altered signal transduction pathway which mediates the differential effects of RA on anchorage-independent growth in these two cell lines.

Retinoic acid; Phospholipid turnover; Protein kinase C; Anchorage-independent growth

1. INTRODUCTION

It has been clearly established that retinoids (vitamin A and its analogues) can cause reversible loss of anchorage-independent growth (an acknowledged marker for oncogenically transformed cells) of a large number of transformed cell lines of diverse origin, as well as intervene in the process of oncogenesis in vivo (reviewed in [1,2]). However, some transformed cells do not lose their ability to form colonies in agar medium upon treatment with retinoids [1–4]. It is not known what causes the differential response to retinoids by various transformed cells. Jetten et al. [4] have reported recently that Syrian hamster embryo cells transformed by transfection with the v-*src* oncogene lose anchorage-independent growth after treatment with RA but RA enhances anchorage-independent growth of the same cell line transformed by the v-Ha-*ras* oncogene. Our previous study [3] showed that RA inhibits phosphorylation of a group of low molecular mass plasma membrane proteins (LMPs) in RSV-transformed rat (RR1022) cells which undergo RA-induced loss of anchorage-independent growth (RA-sensitive cells). However, RA did not have any effect on the phosphorylation of LMPs in Ki-*ras* transformed normal rat kidney cells (KNRK) which also do not lose the ability to grow in an anchorage-independent manner upon RA treatment (i.e. they are RA-resistant).

In this report we provide evidence that RA causes a drastic alteration of phospholipid turnover and inhibits activation of the membrane associated, Ca^{2+}- and phospholipid-dependent protein kinase (protein kinase C). However, these RA-induced effects are either not detectable, or not as pronounced in RA-resistant KNRK cells. These observations lend further support to the hypothesis that activation of different oncogenes

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Abbreviations: RA, all-*trans*-retinoic acid; PKC, protein kinase C; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PA, phosphatidic acid

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in various transformed cells may determine their response to retinoids.

2. MATERIALS AND METHODS

2.1. *Cells and cell culture*

The Schmidt-Ruppin strain of Rous sarcoma virus (SR-RSV)-transformed rat cell line (RR1022) and Kirsten murine sarcoma virus (Ki-MSV)-transformed normal rat kidney cells (KNRK) were purchased from the American Type Culture Collection (Rockville, MD). Cells were routinely maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% newborn calf serum (Gibco). Stock solutions (10^{-2} M) of RA (all-*trans* retinoic acid; Sigma) were prepared in dimethyl sulfoxide (DMSO) and were diluted with DMEM to a final concentration of 10^{-5} M. The final concentration of DMSO was 0.1%; control experiments showed that this concentration was not toxic to the cells nor was it responsible for the effects ascribed to RA treatment (not shown).

2.2. *Phospholipid analysis*

Cells which were to be analyzed for phospholipid turnover were first starved for phosphate by incubating them in 2 ml of phosphate- and serum-free DMEM for 1 h prior to labelling. The media was changed and the cells were labelled for 2 h with 100 μCi of carrier-free [^{32}P]orthophosphate (Amersham) in 1 ml of phosphate-free DMEM containing no serum. Labelled phospholipids were extracted from cells essentially as described by Agranoff et al. [5]. Samples were spotted on silica gel G plates (Brinkman) and the phospholipids were separated by TLC using a solvent system consisting of chloroform/methanol/acetic acid/water (65:43:1:3). Radiolabelled phospholipids were identified by autoradiography on X-ray film and the spots corresponding to the various lipids were scraped from the TLC plates and quantitated by liquid scintillation counting.

2.3. *Measurement of protein kinase C activity*

Membrane vesicles were isolated from untreated and RA-treated cells according to a procedure described previously [3]. Protein kinase C (PKC) was assayed following the procedure described by Fearn and King [6]. The reaction mixture (total volume 125 μl) contained 50 μg lysine-rich histone, 20 mM Tris-HCl, pH 7.5, 0.5 mM $CaCl_2$, 75 mM $MgCl_2$, 10 μg aprotinin, 12 μg phosphatidylserine (PS), 1 μg diolein, and 25 μl membrane suspension in 0.1% NP40. The reaction was initiated by the addition of 20 mM ATP ([γ-^{32}P]ATP, Amersham) and incubated for 3 min at 30°C. The reaction was stopped by the addition of 200 μl of 25% TCA. Precipitates were collected on Whatman glass fiber filters and washed extensively with 25% TCA. Control experiments were carried out by omitting Ca^{2+}, diolein, and PS, and adding 10^{-4} M EGTA. PKC activity is expressed as the ratio of radiolabel incorporated into histone in Ca^{2+}/PS/diolein-containing reactions over those without.

3. RESULTS

3.1. *Effect of RA treatment on phospholipid turnover in RR1022 and KNRK cells*

We have previously shown [3] that RR1022 and KNRK cells can grow for extended periods of time in the presence of 10^{-5} M RA; i.e. this dose is not toxic to either cell type. However, RR1022 cells, which form large colonies in suspension in the absence of RA, show drastically reduced colony formation in soft agar in the presence of 10^{-5} M RA, both in terms of size and number of colonies. On the other hand, KNRK cells show no significant change in colony formation upon RA treatment. We have established that the mechanism of action of RA on anchorage-independent growth is membrane-related and is not related to the presence of cytosolic RA-binding proteins [7]. Therefore, we wished to examine whether phospholipid metabolism was affected by RA, and, if so, whether the effect was different for RA-sensitive and RA-resistant cells. Cells were pretreated with phosphate-free DMEM in the absence of label and with or without RA (10^{-5} M) for 1 h. Then the media was changed and the cells were labelled with $^{32}P_i$ for 2 h with or without RA. The phospholipids were then extracted and run on untreated TLC plates to separate out the major phospholipid classes. The results are shown graphically in fig.1. In RR1022 cells, the most prominent effects of RA treatment on phospholipid turnover are: (i) a large increase in the relative amount of label incorporated into PC, from 25.3% to 53.5% of the total label incorporated into phospholipid; (ii) a large decrease in the relative amount of label incorporated into PA, from 38.7% to 13.5% of the total; (iii) a significant decrease in the relative amount of label incorporated into PI, from 12.1% to 5.8% of the total label. These results are all statistically significant ($p < 0.02$ for PC and PA, $p < 0.05$ for PI; $n = 7$). In contrast, PS and PE showed no significant change. As can also be seen in fig.1, no significant changes in the relative amounts of any phospholipid occurred in KNRK cells.

3.2. *Effect of RA (10^{-5} M) on PKC activity in RR1022 and KNRK cells*

We have demonstrated here that RA causes significant alterations in phospholipid turnover in RR1022 cells but not in KNRK cells. Since PIP_2 hydrolysis (as well as that of other phospholipids) generates diacylglycerol (DG) which can then activate PKC, and since RA is known to antagonize the effects of phorbol ester tumor promotors

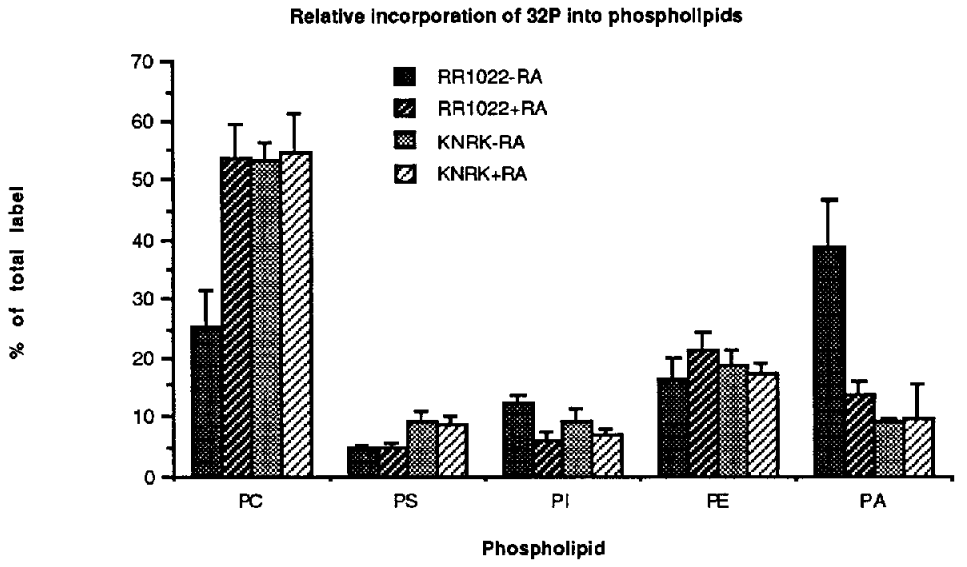

Fig.1. Effect of RA (10^{-5} M) on phospholipid turnover in RR1022 and KNRK cells. Data shown are the average ± SE of 7 replicates for RR1022 cells and 6 replicates for KNRK cells.

(which activate PKC) in many systems [2], we decided to examine PKC activity in untreated and RA-treated RR1022 and KNRK cells to see whether RA would only antagonize the enzyme's effect in RA-sensitive cells (i.e. RR1022). In table 1, the effect of 24 h treatment of 10^{-5} M RA on PKC activity in RR1022 and KNRK cells is shown. Clearly, RA inhibits PKC activity drastically in RR1022 cells (activity about 35% of untreated controls) but little or not at all in KNRK cells (activity about 78% of untreated controls). It should be mentioned that since an exogenous diacylglycerol (diolein) as well as phosphatidylserine was added to the reaction mixture, the effect of RA on PKC activity seen is presumably not mediated directly through its effect on phospholipid turnover (whose effect of activating PKC is thought to be mediated principally by the production of DG and the presence of PS). Rather, it is probable that RA inhibits phospholipid turnover and PKC activity independently.

Table 1

Effect of 10^{-5} M RA on PKC activity in RR1022 and KNRK cells, expressed as the ratio of units (μmol P_i/mg protein per min) determined with PS and DG over units determined without PS and DG

	PKC activity (+DG,PS/−DG,PS)
RR1022	2.049 ± 0.427
RR1022 + RA	0.707 ± 0.217
KNRK	2.774 ± 1.221
KNRK + RA	2.164 ± 0.592

Data shown are average of four experiments ± SE

4. DISCUSSION

The data presented here clearly show that treatment of RR1022 cells with 10^{-5} M RA, which causes a drastic inhibition of anchorage-independent growth [3], also causes a number of alterations in phospholipid metabolism. Specifically, RA treatment causes a large relative increase in the amount of $^{32}P_i$ incorporated into PC and a large decrease in the amount of $^{32}P_i$ incorporated into PA and PI. The incorporation of label into PS and PE is unaffected by RA treatment. However, KNRK cells, which do not show reduced anchorage-independent growth upon RA treatment [3], show no significant change in PC, PA, and PI turnover (fig.1); again, PS and PE are unaffected. In addition, RA treatment causes a very large decrease in the plasma membrane activity of PKC in RR1022 cells but only a small decrease in KNRK cells (table 1). This effect is not secondary to the effect of RA on phospholipid turnover, since the

phospholipids or phospholipid breakdown products (PS and DG) which are required for PKC activation were included in the in vitro reaction mixture. Thus it appears that RA inhibits the phospholiid/PKC signal transduction pathway at, at least, two separate points. The effect of RA on phospholipid turnover represents a very early effect of RA; 3 h of RA treatment produces significant changes in the phospholipid profile of RR1022 cells. This is in contrast with many effects of retinoids including most of their effects on growth and differentiation [1,2] as well as the RA-mediated induction or regulation of specific genes (e.g. [8–10]).

We do not know the mechanisms by which RA exerts these effects on phospholipid turnover and PKC activity. It has been shown that RA can inhibit phospholipase C-mediated ornithine decarboxylase induction in rat tracheal cells [11]. It is therefore possible that RA inhibits a phospholipase C activity and thereby affects phopshoinositide breakdown. Alternatively, RA may be inhibiting enzymes involved in the synthesis of phospholipids. Much work obviously remains to be done in order to establish more clearly the mechanisms of RA's effect on phospholipid turnover.

The inhibition of PKC by RA is not surprising given that in many cell types retinoids can inhibit the tumor-promoting effects of phorbol esters, which activate PKC [2]. Retinal has been shown to inhibit PKC activity in RAW264 cells [12]. RA has also been shown to inhibit PKC activity in mouse brain cells [13], which is consistent with our results.

Other reports examining the effects of retinoids on phospholipid metabolism have produced results differing from those reported here. Lochner et al. [14] have demonstrated that all-*trans*-retinol activates PI-specific phospholipase C in neutrophils and causes only a slight decrease in PKC activity. Ponec et al. [15] showed no alteration in phospholipid turnover at 3×10^{-6} M RA in squamous cell carcinoma cells. Neither report examines the relationship between the effect of RA on phospholipid turnover and its effect on anchorage-independent growth. It is evident that different cell types differ in their sensitivity to RA. It is also not surprising that neutrophils respond differently than established transformed cell lines.

The manner by which KNRK cells escape from or mitigate the effects of RA on phospholipid turnover, PKC activity and anchorage-independent growth is also not known. It is known that in NRK cells (from which KNRK cells are derived) RA can enhance the induction of anchorage-independent growth by TPA or EGF and TGF-β [16–18]. Therefore, the resistance of KNRK cells to RA's effects on phospholipid turnover and PKC activity may be partially cell-type specific. In addition, it may be that the ras protein of KNRK cells somehow confers RA resistance onto the cells. Consistent with this view are the findings of Jetten et al. [4], who reported that in a syrian hamster ovary cell line, transfection with v-*src* resulted in cells in which RA inhibited anchorage-independent growth and ODC induction by phorbol esters, whereas transfection with v-Ha-*ras* resulted in cells that showed enhanced anchorage-independent growth and phorbol ester-induced ODC activity upon RA treatment.

In conclusion, in the light of these and previous observations it can be suggested that RA's effect on anchorage-independent growth is mediated by a pathway involving phospholipid turnover (including PI turnover) and PKC activation. Furthermore, the response of a given transformed cell type to RA is critically dependent on the nature of the cell type and the transforming oncogene. It would be of interest to see whether other *ras*- and *src*-transformed cell lines show similar differential responses to RA in terms of anchorage-independent growth, phospholipid turnover, and PKC activation.

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