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SYNERGY BETWEEN PHORBOL ESTERS AND RETINOIC ACID IN INDUCING PROTEIN KINASE C ACTIVATION

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SUMMARY All-*trans* retinoic acid (RA) activates brain protein kinase C (PKC) in a unique fashion. Co-factors such as Ca^{2+} or PtdSer are not required for histone phosphorylation. Binding experiments have provided evidence that RA does not act as a phorbol-ester-like activator. However, phorbol esters synergistically enhance this activation in a dose-dependent manner and increase the reaction rate up to five-fold when combined with 10μM RA. Phospholipid-interacting drugs such as phenothiazines and 1-N-(6 aminohexyl) 5-chloro-1-naphtalene-sulfonamide (W7), which compete with PtdSer and inhibit phorbol ester / PtdSer-mediated activation, have potentiating effects on the RA-mediated reaction. RA elicites Ca^{2+}-dependent PKC autophosphorylation. The activation resulting from the combined treatment with PtdSer and RA is more than additive in the presence of Ca^{2+}, indicating that PtdSer-and RA-binding sites are distinct. RA shares several characteristics of activation with sodium deoxycholate and arachidonic acid. These present results suggest that the direct activation of PKC may have physiological and/or pharmacological relevance in the signaling triggered by retinoids.

Vitamin A (retinol) and many of its natural and synthetic derivatives (retinoids) evoke a large variety of effects on vertebrate development, cellular differentiation and homeostasis. The growth and differentiation properties of human tumor and normal cells in culture are strongly affected by retinoids (1). The pathways which signal the pleiotropic action of retinoids are under intensive investigation. It has been suggested that retinoids act in a steroid hormone-like fashion. Support for this idea comes from studies which have identified specific intracellular retinoid-binding proteins in tissues from embryos and adults sensitive to vitamin A (2), and the discovery of two families of nuclear retinoic acid receptors, RAR and RXR, which are members of the steroid/thyroid nuclear receptor multigene superfamily (as reviewed in Ref. 3). Recently, evidence has been provided that protein kinase C (PKC) is implicated in the signaling of retinoic-acid (RA)-induced terminal differentiation at a step occurring prior to the activation of the nuclear receptor RARβ (4). PKC is the target for tumor promoters such as phorbol esters, and it is well documented that retinoids exert dual effects, as agonists and antagonists, on phorbol ester-induced tumor promotion (5,6). Several reports have described

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cooperative effects between phorbol esters and RA with the latter reversing phorbol ester-resistance in a myeloid leukemia cell line (7-10). In addition, the treatment of promyelocytic leukemia cells with RA was shown to lead to an increase in the activity of PKC (11).

The above findings prompted us to investigate the effects of RA on purified brain PKC and examine whether the interaction of this vitamin A analog with tumor promoter 12-0-tetradecanoyl phorbol 13-acetate (TPA) occurs at the enzyme level.

MATERIALS AND METHODS

Materials. The following materials were purchased from the indicated sources (γ-^{32}P) ATP from New Engand Nuclear; histone type III-S, phosphatidylserine (PtdSer), all *trans* retinoic acid, 1-N-(6 aminohexyl) 5-chloro-1-naphtalenesulfonamide (W7); isoquinolinesulfonyl-2-methylpiperazine (H7) from Sigma; TPA from LC Laboratories; chlorpromazine (CPZ) and trifluoperazine (TFP) were kindly provided by Laboratoires Specia and Theraplix, respectively. Stock solutions of TPA were made up in dimethylsulfoxide and RA was used as a solution in ethanol; the final concentration of ethanol did not exceed 0.8%.

Enzyme preparation. PKC was purified from rat brain cytosol by a modification of the three-step procedure: ion-exchange chromatography on DEAE cellulose, followed by affinity chromatography on protamine-Sepharose and phenyl-Sepharose as modified in ref. 12. The enzyme preparation yielded a specific activity of approximately 2-3 units, with one unit transferring 1μmol of phosphate per min per mg protein in our assay conditions. The enzyme was stored at -80°C in 10% (w/v) glycerol and 0.05% (w/v) Triton X 100.

Protein kinase C assay. Enzyme activity was assayed according to a previously described technique (13) modified as follows: the reaction mixture contained in 125μl, 2.5μmol Tris HCl buffer at pH 7.5, 0.6μmol Mg acetate, 15μg histone type III-S, 2.5nmol (γ^{32}P) ATP (approximately 5 to 6 x 10^5 cpm) and I0ng of enzyme (unless otherwise indicated) diluted in distilled water. When added, PtdSer, EGTA and Ca^{2+} were present at the indicated concentrations. Incubations were carried out at 30°C for 5 min unless otherwise specified. To stop the reaction, a 50μl aliquot of the incubation mixture was loaded onto squares of Whatman P81 paper which were soaked in 1% phosphoric acid and washed. Radioactivity was quantified by Cerenkov counting. Proteins were determined by the Bradford method.

Enzyme autophosphorylation. The reaction was carried out under the conditions of the enzyme assay except that histone was omitted. After a 20-min, incubation at 30°C , the reaction was stopped in Laemmli solution and the mixture boiled for 3 min. Samples (50μl) were subjected to 7.5% SDS-polyacrylamide gel electrophoresis and autoradiography.

(^{3}H) TPA binding. In order to determine specific binding of (^{3}H) TPA to PKC, we used the cold acetone method (14), modified as follows: the reaction mixture (1ml) contained 10mmol Tris/HCl buffer (pH 7.4), 100ug phosphatidylserine, 40 ng of brain PKC, 1mg BSA, 10 pmol (^{3}H)TPA and either 5μl DMSO or 5nmol TPA in 5μl DMSO. After incubation for 2h at 4°C, the mixture was filtered on a Whatman GF/C glass filter and four washes of cold acetone were performed. Results are reported as specific (saturable) binding.

RESULTS

Retinoic acid activates PKC-mediated histone phosphorylation

Results obtained by Hidaka and coworkers showed that vitamin A acid was able to activate rabbit retina PKC in the absence of phospholipids (15). We confirmed this finding on highly purified PKC from rat brain. At variance, we report here that RA stimulated brain PKC activity in the absence of Ca^{2+} in a dose-dependent manner, using histone III-S as substrate. This activity peaked at about 30μM. At this concentration, the RA-mediated PKC activity

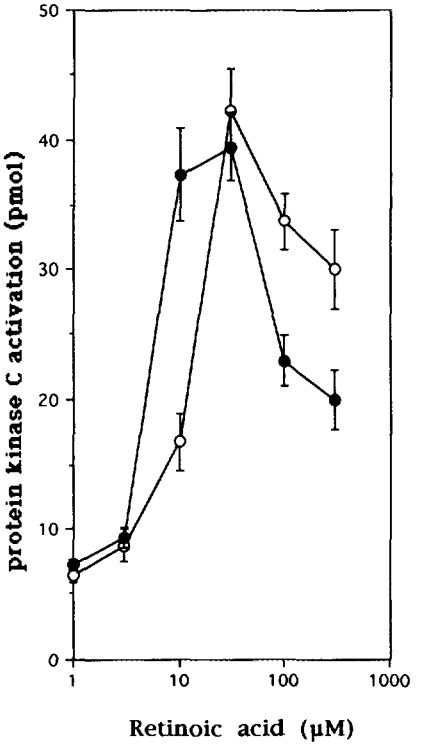

Fig. 1. Dose-response curves of PKC activation by all -*trans* RA. Enzyme was assayed as described in "Materials and Methods" in a PtdSer-free incubation mixture in the presence of either 0.5mM $CaCl_2$ (●) or 0.5mM EGTA (○). The control samples contained 0.8% ethanol. Data are mean values+/_ SD of 3 separate experiments.

reached approximately one-third of the maximal enzyme activity obtained when activators, diacylglycerol or phorbol esters, PtdSer and Ca^{2+} were present at optimal concentrations. Although Ca^{2+} was not required for PKC activation, this cation significantly increased the reaction rate at concentrations of RA lower than 30μM, as shown in fig. 1.

Retinoic acid mediates PKC autophosphorylation

It is well known that the activated enzyme undergoes autophosphorylation, this intrapeptide reaction occurs at both the regulatory and catalytic domains. After a 20-min incubation in our standard assay conditions (except that histone was omitted), the enzyme was subjected to SDS-PAGE electrophoresis. The autoradiogram showed that RA-mediated autophosphorylation of PKC occurs only in the presence of $CaCl_2$ (fig. 2). Therefore, it can be concluded that the reaction of autophosphorylation is not required for histone phosphorylation, which occurs without Ca^{2+}. In contrast, PtdSer was unable to either autophosphorylate the enzyme or activate histone phosphorylation in a Ca^{2+}-free mixture (fig. 2). This finding provides a clear distinction between the mode of action of RA and PtdSer on PKC activation, presumably associated with the formation of the histone-enzyme complex which, in the PtdSer mediated reaction is a Ca^{2+}-dependent step (16).

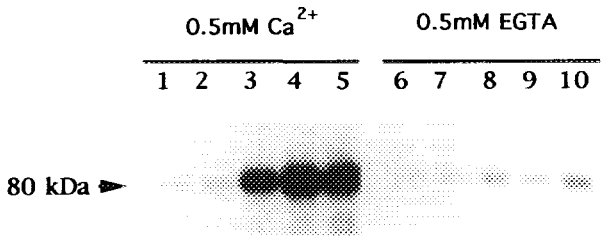

Fig. 2. Autoradiogram of autophosphorylated PKC. Samples were incubated for 20 min. without exogenous substrates after the following additions: 0.8% ethanol (lanes 1,6), RA at 10μM (lanes 2,7), RA at 30μM (lanes 3,8), RA at 100μM (lanes 4,9) and PtdSer at 20μM (lanes 5,10). The reaction was set up in 0.5mM $CaCl_2$ (lanes 1 to 5) or 0.5mM EGTA (lanes 6 to 10). The arrow indicates 80kDa.

TPA and RA interactions in PKC activation

Phorbol esters are potent PKC activators which compete with diacylglycerol on for enzyme binding site. We explored the possibility that RA acts upon this site and affects TPA binding to PKC and/or TPA-mediated enzyme activation. Using the ^{3}H-TPA binding assay we showed that RA did not compete with TPA on its binding site within the 10^{-7} to 10^{-4} molar range of concentrations, as shown in Table I. From these results, it may be concluded that RA did not act in a TPA- or diacylglycerol-like fashion. Moreover, it should be pointed out that RA did not enhance TPA binding capacity. However, in activation experiments combining RA and TPA, we showed that phorbol ester potentiated the reaction rate in a dose-dependent TPA, maximal activation occurred at concentrations of RA 3-to-5 fold lower. TPA-evoked enhancement was maximal at 10μM RA. As shown in fig. 3B, the dose-response curve of TPA levelled out at approximately 20nM. Similar results were obtained using two other phorbol esters, phorbol dibutyrate and phorbol didecanoate (data not shown).

Effect of phenothiazines and sulfonamide derivatives on RA-mediated PKC activation

To further explore the mechanism of RA-mediated activation we studied the effects of inhibitors of phorbol ester/PtdSer-mediated activation such as antipsychotic phenothiazines, namely chlorpromazine (CPZ), trifluoperazine (TFP) and triflupromazine

Table I. Effects of increasing concentrations of RA on TPA binding to PKC

Retinoic acid (μM)	Bound TPA (pmol/assay)
0	0.148+/-0.014
0.1	0.136+/-0.020
1	0.134+/-0.015
10	0.145+/-0.009
100	0.132+/-0.011

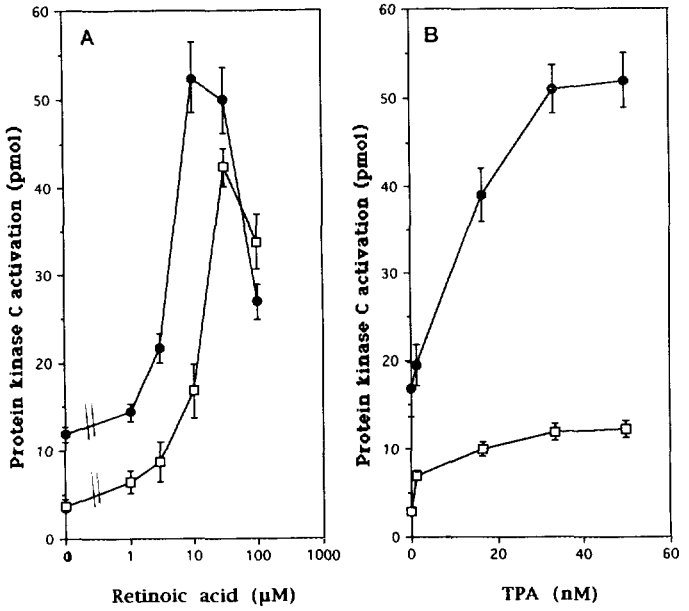

Fig. 3. Potentiation of TPA on RA-mediated PKC activation. A- Graded concentrations of RA were added in the absence (□) or in the presence of 16nM TPA (●). B- Graded concentrations of TPA were added in the absence (□) or the presence (●) of 10μM RA. Results are mean values +/- SD of at least 3 experiments.

(TFPr) as well as the N-(6 aminohexyl)-1-naphtalene sulfonamide (W7) and the 1-(5-isoquinoline sulfonyl)-2 methylpiperazine (H7). The phenothiazines and W7 are phospholipid-interacting drugs and calmodulin antagonists which indirectly inhibit PKC in a manner competitive with that of phospholipids (17). In contrast H7 acts directly on the PKC catalytic domain and competes at the ATP binding site (18). H7 behaved as expected, inhibiting the effects of both PtdSer and RA on PKC with close IC_{50} of 7 and 9μM, respectively (fig. 4A). Surprisingly, the effects of phenothiazines and W7 on activation mediated by RA and TPA/PtdSer were the opposite. As shown in fig. 4B and C, W7 and CPZ inhibited the effects of PtdSer as expected, while enhancing the effects of RA. The other two tested phenothiazines, TFP and TFPr, behaved essentially like CPZ (data not shown). Phenothiazines and W7 were devoid of activity toward the enzyme in the PtdSer-free mixture. It should be stressed that phenothiazines and W7 allow also the distinguish between PKC activation induced by PtdSer and that induced by RA since the former was inhibited while the latter was markedly enhanced.

Interactions of PtdSer and RA in PKC activation

In order to better understand the interaction of RA with the regulatory domain, we investigated the direct effects of phospholipids on the RA-mediated reaction. We set up experiments in the presence or absence of Ca^{2+}. Phospholipids were added to the incubation mixture as sonicates of PtdSer at 20 and 50μM. At the latter concentration, PtdSer in 0.5mM $CaCl_2$ maximally activated the enzyme and the addition of activators, such as diacylglycerol

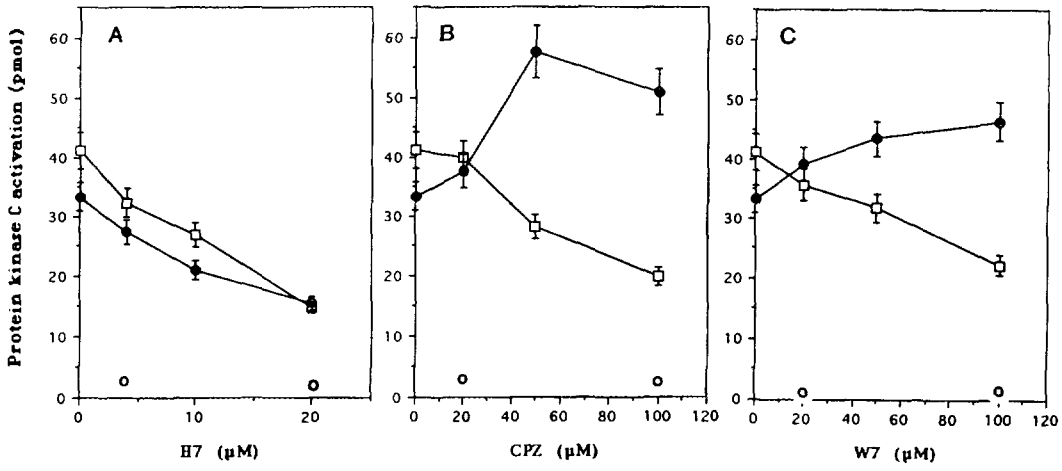

Fig. 4. Differential effects of PKC inhibitors: H7 (A), CPZ (B) and W7 (C) on RA - and PtdSer-mediated enzyme activity. PtdSer (□) and RA (●) were added at the concentrations of 20μM and 30μM, respectively. Results are mean values +/- SD of 2 independent experiments.

(19) or TPA no longer enhanced the response (fig. 5). However in these conditions, treatment with RA yielded an additional activation with even a slight potentiation. It is well documented that PtdSer, though binding to the regulatory domain in the Ca^{2+}-free mixture, is poorly effective in enzyme activation. Nevertheless, the combined treatment in 0.5mM EGTA gave rise to activation which was slightly less than additive (fig. 5). From this study, it can be

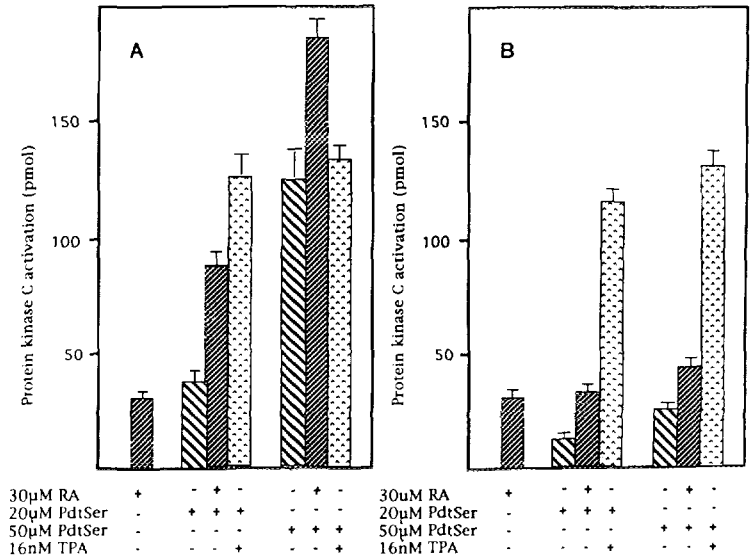

Fig. 5. Combined effects of RA and PtdSer on PKC activation. The reaction was set up as described in "Materials and Methods" and performed in 0.1mM $CaCl_2$ (A) or in 0.5mM EGTA (B). RA, PtdSer and TPA were added as stated above. Results are mean values +/- SD of 4 independent experiments.

assumed that direct or indirect interactions occur at the enzyme level between these two lipids, indicating that their binding sites are distinct, but somewhat related.

DISCUSSION

RA, like phorbol esters and diacylglycerol, mediate protein kinase C activation but in a different manner. We report herein that PtdSer, phospholipid-interacting drugs and phorbol esters, effector molecules acting directly or indirectly on PKC, impair RA-mediated activation. We had previously reported that arachidonic acid (AA) and bile salts mediated PKC activation with characteristics close to those of RA-mediated activation. We found that AA-mediated PKC activation was associated in brain with two different enzyme affinities for this unsaturated fatty acid (19). AA-low sensitive activation appeared to be quite similar to RA-mediated activation since it was not dependent on Ca^{2+} using histone as a substrate, whereas this cation was required for autophosphorylation. However, it should be stressed that RA-mediated PKC activation did not share all the characteristics of AA-low sensitive activation, i.e. the latter was not additive in 0.5mM Cl_2 to PtdSer-mediated activation suggesting that AA was closely related to the phospholipid domain. Besides, we described the characteristics of bile salt-mediated activation of PKC which turned out to be rather similar to those reported above for the RA-mediated reaction (20). However, here again some differences were noted between the two activators, i.e., Ca^{2+} markedly enhanced sodium deoxycholate-mediated histone phosphorylation without eliciting autophosphorylation, sodium deoxycholate interacted with the phospholipids binding domain (20). Phorbol ester TPA or diacylglycerol potentiated the enzyme activation evoked by the three effectors under study, i.e., sodium deoxycholate, arachidonic acid and RA.

RA is a potent inducer of differentiation in many cell lines including human promyelocytic HL60 cells, which mature along the granulocytic lineage. Various other inducers may also mature these cells towards the monocytic, eosinophilic or macrophage-like phenotypes. The activation of PKC (isoform β), which changes in expression and activity, when cells undergo macrophage-like differentiation is a critical event in this process (22-24). Likewise, PKC has been shown to be involved in HL60 cells at the early stages of granulocytic differentiation induced by RA which evoked the phosphorylation of several substrates within the first hours of treatment (25). The addition of staurosporine derivatives, which are specific inhibitors of the enzyme blocked granulocytic differentiation in these cells (manuscript in preparation).

RA is currently used in cancer therapy and its effectiveness has been shown particularly in the acute promyelocytic leukemia after treatment with 45mg/m^2 per day (26). However, the cascade signaling through which RA induces maturation of hematopoietic cells and its therapeutic effects is not precisely known. Our present results support the possibility that the action of RA *in vitro* and in patients may involve a direct interaction with some members of the PKC family.

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