Vol. 199, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1994 Pages 334-338

ACTIVATION OF MACROPHAGE PtdIns-PLC BY PHORBOL ESTER AND VANADATE: INVOLVEMENT OF REACTIVE OXYGEN SPECIES AND TYROSINE PHOSPHORYLATION

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Received December 7, 1993

Phorbol ester (TPA) is generally considered to be a negative regulator of PtdIns-PLC activity. Here we show, for the first time, that the combination of TPA+ vanadate is a positive regulator (activator) of PtdIns-PLC in mouse elicited peritoneal macrophages. Vanadate or TPA on their own had no effect on PtdIns-PLC activity. In addition, TPA+ vanadate enhanced reactive oxygen species formation and protein tyrosine phosphorylation. PtdIns-PLC activation was suppressed by down regulation or inhibition of PKC, by inhibition of NADPH oxidase activity and scavenging of its product, and by inhibitors of protein tyrosine kinase activity. We conclude that PKC activation by TPA in the presence of vanadate activates the formation of reactive oxygen species, which are essential for the enhancement of protein tyrosine phosphorylation and eventually to PtdIns-PLC activation.

PtdIns-PLC activity represents a family of subtypes of enzymes ($\alpha,\beta,\gamma,\delta$) differing in their structure as well as in their regulation (1-4). The β- subtype is activated via a GTP-binding protein, while the γ-subtypes are activated by tyrosine phosphorylation (1-4). Growth factors, hormones and antigen binding to the respective receptors induces protein tyrosine phosphorylation and activation of γ - type PtdIns-PLC (1-8). In various cell types, PKC activation was shown to negatively control PtdIns-PLC activity (1,4,5,7,8). It was suggested that phosphorylation of a serine residue diminishes the phosphorylation on tyrosine and thereby PtdIns-PLC activation (7,8).

Vanadate+H_2O_2 (V+H) was shown in several cell types to activate PtdIns-PLC (9). This is probably due to its inactivating potential of protein tyrosine phosphatase (PTP) and enhancement of protein tyrosine phosphorylation (9,10). PKC is a prime positive regulator of reactive oxygen species (ROS) formation in phagocytes; TPA invokes membranal PKC activation, and together with vanadate it stimulates luminol-dependent chemiluminescence that reflects superoxide anion formation by NADPH oxidase (10). We have previously shown that vanadate+TPA (V+T) inhibit PTP activity, enhance tyrosine phosphorylation and protein tyrosine kinase (PTK) activity

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Abbreviations: Tg-Mø, thioglycollate elicited macrophages; ROS, reactive oxygen species; TPA, 12-O-tetradecanoylphorbol 13-acetate, DPI, diphenyleneiodonium; DTT, dithiotreitol; NAC, N-acetylcysteine; GSH, reduced glutathione; HBSS, Hank's balanced salt solution; V, vanadate; PtdIns-PLC, phosphatidylinositol specific phospholipase C; bovine serum albumin, BSA; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase.

0006-291X/94 $5.00

and enhance PLA_2 activity (10,11). Thus V+T mimic the effect of V+H on tyrosine phosphorylation. In the present report we explored the possibility that V+T have a positive regulatory effect on PtdIns-PLC. Indeed, V+T activated PtdIns-PLC and this activation stemmed from its potential to induce the production ROS.

MATERIALS AND METHODS

Materials - Sodium ortho-vanadate (Na_3VO_4) was from Fluka. GF109203X, was a gift from Dr. H. Coste, Lab Glaxo, France. Genistein was purchased from Biomol, Penn. ST638 was a gift from G. Lawton, Roche Products, England. DPI was synthesized in the laboratory. All other materials were from Sigma.

Peritoneal macrophages - Peritoneal exudate cells were harvested from female CD1 mice, 4 days post i.p. injection of thioglycollate broth. Exudate cells (about 90% Tg-Mø) were seeded unto 24 well Nunc plates at $8x10^5$ macrophages/well. The adherent cells (1 h adherence) were washed, and cultured in Dulbecco's modified Eagle's medium containing 10% heat inactivated-fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) for 96 hr, at 37°C in a humidified atmosphere containing 5 % CO_2 in air .

Luminol-dependent chemiluminescence (CL) - Freshly harvested Tg-Mø were suspended ($1x10^6$ cells/ml) in Hank's balanced salt solution (HBSS) supplemented with 0.1 % BSA and 0.2 mM luminol, and incubated with or without the specified reagents at 30 °C. CL was measured every 5 min in a Lumac apparatus, model 2080 (Lumac Instruments, Basel) as described (10,11).

Protein tyrosine phosphorylation in intact cells - Tg-Mø were treated as specified, washed, and lysed at 4 °C as described (10). The lysates were mixed with a concentrated (x3) sample buffer containing β-mercaptoethanol (25%), boiled for 5 min, and run on 10% SDS-gels. The proteins were transferred to nitrocellulose papers (NC) for Western blot analysis essentially as described (10). The blots were then incubated overnight at 4°C with 10 µg/ml of a poly clonal rabbit antiphosphotyrosine antibody (Zymed, Cal.), washed and incubated with Protein A-horseradish peroxidase (Amersham) for 1 h at room temperature (10). Following x3 washes, the blots were immersed in an Amersham ECL kit for 1 min, dried and chemiluminescence recorded for 5 sec by exposure to a sensitive film (Medical C62, Fugi Medical Company).

PtdIns-PLC assay - The assay is based on (12) with slight modifications. Tg-Mø cultures were metabolically labeled for 20 hours in low inositol medium (α-MEM) containing 0.5 % heat inactivated-fetal calf serum and 1 µCi [^{3}H]myo-inositol (NEN, Dupont). The cells were washed with HBSS and incubated in HBSS containing 10 mM LiCl and 0.2 % BSA with or without the specified additives. Inositol phosphates and free inositol were extracted into cold 10 % trichloroacetic acid. The extraction solution was treated with ether, neutralized and loaded onto Dowex-1 (formate form) columns (12). The [^{3}H]inositol phosphates were eluted sequentially using 0.2 M ammonium formate/ 0.1 M formic acid (for $InsP_1$), 0.4 M ammonium formate/ 0.1 M formic acid (for $InsP_2$) and with 1 M ammonium formate/ 0.1 M formic acid (for $InsP_3$). Residual cell [^{3}H]phosphoinositides were assessed after solubilization with SDS. Results are expressed as % [^{3}H]Inositol phosphates formed of total [^{3}H]Inositol incorporated. Representative experiments out of three carried in duplicate.

RESULTS

V+T invoke a pronounced ROS formation which is suppressed by diphenyleneiodonium (DPI) an inhibitor of NADPH oxidase, and antioxidants such as dithiotreitol (DTT), N-acetylcysteine (NAC) and reduced glutathione (GSH) (Fig. 1). Thus, DPI by inhibiting NADPH oxidase and the antioxidants by reducing/ scavenging the superoxide anions generated, abolish the availability of *de novo* generated ROS.

Down regulation of PKC by long-term treatment with TPA or inhibition of PKC by GF109203X (Fig. 2) suppressed tyrosine phosphorylation invoked by V+T. Protein tyrosine phosphorylation

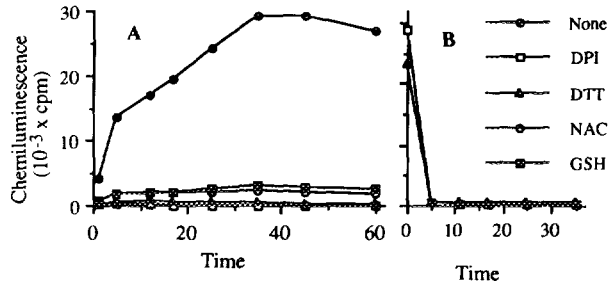


Fig. 1. Activation of ROS formation by vanadate+TPA and its inhibition by DPI and antioxidants. Tg-Mø were incubated with A- 10 mM DTT, 10 mM GSH, 10 mM NAC, 4 μM DPI or without additives (None) for 20 min. Vanadate (0.25 mM) and TPA (160 nM) were then added and the chemiluminescence was assessed at the specified times. B- vanadate+TPA (as above) were added for 30 min (zero time) followed by addition of DPI (4 μM) or DTT (10 mM).

induced by V+T (Fig. 2) was also suppressed by DPI and the antioxidants, a further confirmation of our previous evidence suggesting the involvement of ROS in the process (10).

V+T activated PtdIns-PLC in a dose dependent manner (Fig. 3A). On their own, V at 0.25 mM and T at 160 nM had a rather small effect. In the presence of V (0.25 mM) , the optimal concentration of TPA was around 8 nM whereas in the presence of 160 nM TPA, optimal activation required about 50 μM V (Fig. 3A). The production of inositol phosphates was prevented by suppression of PKC (long-term TPA and GF109203X). In addition, the formation of inositol phosphates was inhibited by suppression of ROS formation (DPI) and by scavenging of ROS with reducing agents, or by the putative inhibitors of protein tyrosine kinase (genistein and ST 638) (Fig. 3 B).

DISCUSSION

We have demonstrated that the combination of V+T leads to activation of PtdIns-PLC in mouse macrophages. The data suggest that this activity depends on the ability of V+T to invoke

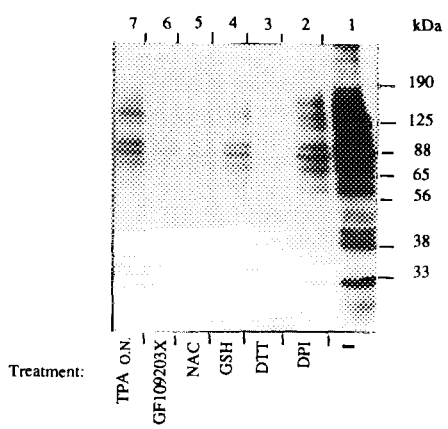


Fig. 2. Protein tyrosine phosphorylation invoked by vanadate+TPA : effect of DPI, antioxidants, and modulators of PKC activity. Tg-Mø were incubated for 10 min with or without the specified agents; 4 μM DPI, 10 mM DTT, 10 mM GSH, 10 mM NAC, 10 μM GF109203X. TPA O.N. (lane 7) denotes cells incubated with 160 nM TPA for 16 h. Vanadate (0.25 mM) and TPA (160 nM) were added for further 60 min. Control cultures and cultures treated with vanadate or TPA alone showed no phosphorylation under the given exposure conditions.

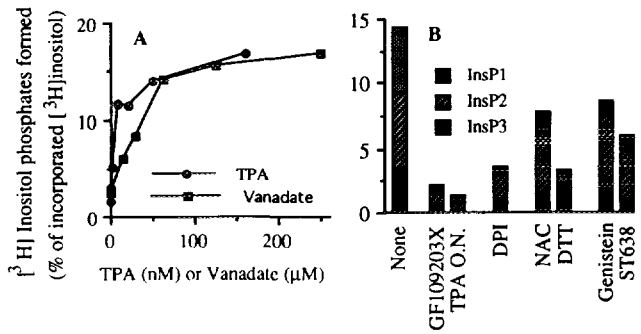


Fig. 3. Activation of PtdIns-PLC by vanadate+TPA: effect of suppression of PKC activity, inhibition of NADPH oxidase, antioxidants and protein tyrosine kinase inhibitors. A-Dose dependence. The circles denote incubation of Tg-Mø (45 min) with the specified concentrations of TPA and vanadate (0.25 mM). The squares denote incubation with a constant TPA concentration (160 nM) and the specified concentrations of vanadate. B- Tg-Mø were incubated with the specified agents for 20 min and for additional 45 min with vanadate (0.25 mM)+ TPA (160 nM). Sequential elution of $InsP_1$, $InsP_2$ and $InsP_3$ (see Materials and Methods). GF109203X, 10 μM; TPA O.N., 20 h preincubation with 160 nM TPA; DPI, 4 μM; NAC, 30 mM; DTT, 10 mM; genistein, 100 μM; and ST638, 100 μM. (None, no additive at the first stage of incubation and vanadate+TPA in the second). Basal PtdIns hydrolysis (3 %) was subtracted. A representative experiment (out of three) was carried out in duplicate.

ROS formation and protein tyrosine phosphorylation (Scheme 1). This is based on the following observations:

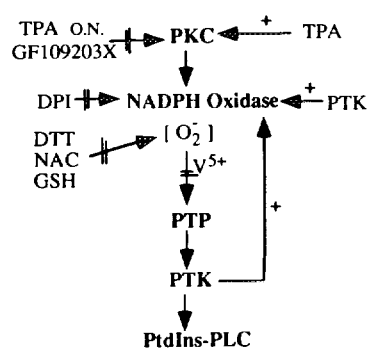
a- activation of PtdIns-PLC by V+T was suppressed by DPI and by reducing agents.

b- V+T suppress PTP activity (10) and enhance tyrosine phosphorylation . These effects were prevented by DPI and the reducing agents.

c- V+T invoke chemiluminescence, an activity of NADPH oxidase. Chemiluminescence was inhibited by DPI and reducing agents.

Moreover, PKC down regulation by long-term TPA treatment or PKC inhibition by GF109203X, as well as inhibition of protein tyrosine phosphorylation by the PTK inhibitors, genistein and ST638, suppressed the activation of PtdIns-PLC, of ROS formation and of tyrosine phosphorylation (10,11).

PtdIns-PLC activation by V+T in macrophages thus shows a similar dependence on ROS formation as that observed in PLA_2 (10,11) and PLD activation (13).



Scheme 1. A Working hypothesis for the steps involved in the activation of PtdIns-PLC by TPA+vanadate. In the scheme the plus sign on the arrows means that activation of the enzyme at the source of the arrow leads to activation of the enzyme at its head; an arrow with a break sign means inhibition or neutralization of the effector. For abbreviations see footnote.

TPA was shown to be a negative regulator of PtdIns-PLC activity in many cell types (1,4,5,7,8). However, in the same cell, bombesin and vasopressin activate PtdIns-PLC via a G-protein dependent mechanism that is negatively regulated by TPA, whereas platelet derived growth factor (PDGF) mediated enzyme activation involves tyrosine phosphorylation and is not affected by TPA (5). TPA inhibits tyrosine phosphorylation of several proteins including PtdIns-PLCγ (7,8,14), while enhancing the tyrosine phosphorylation of mitogen-activated protein kinases (14).

The mechanism by which TPA inhibits protein tyrosine phosphorylation and activation of PtdIns-PLC probably entails activation of PKC, that phosphorylates the enzyme on serine residues, diminishing its ability to undergo tyrosine phosphorylation (7,8).

In phagocytes, V+T activates NADPH oxidase, which is responsible for the formation of relatively large amounts of ROS (10,11). ROS form together with vanadate a very active oxidizing species, peroxovanadate (10), that permeates the cell and causes via the inhibition of protein tyrosine phosphatases, an increased/ sustained tyrosine phosphorylation (10). This activity might counteract the suppressing activity that PKC may exert as a result of serine phosphorylation of the enzyme.

It is of interest that other agonist combinations such as, vanadate+zymosan activate PtdIns-PLC and that this activation is also dominated by PKC activation and ROS formation, and follows essentially the same pattern as described for V+T (not shown).

Under basal conditions (absence of stimuli) the intracellular milieu is in a reduced state. Under these conditions, PKC and NADPH oxidase are low, PTP is high, PTK is low and the phospholipases are essentially inactive (10,11). Increasing the oxidative stress, by exogenous ROS or by activation of NADPH oxidase, leads to the activation of the three phospholipases, the regulation of which occurs by disparate routes (10,11,13).

ACKNOWLEDGMENTS

Thanks are due to R. Meller, T. Harel and O. Asher for devoted technical assistance. U. Z. is an incumbent of the W.B. Graham Professorial Chair in Pharmacology. The study was partially supported (U.Z.) by the German- Israel Foundation (GIF) for scientific research and development (Grant 1-156-040.0219).

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