

Reactive oxygen species are involved in the activation of cellular phospholipase A₂

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Vanadate (V) potentiated (4- to 10-fold) the activation of cellular phospholipase A₂ (PLA₂) induced by H₂O₂ (H), a phorbol ester (T), a Ca²⁺-ionophore (A) and opsonized zymosan in macrophages. V+H induced in intact cells the activation and translocation of PLA₂ and protein kinase C (PKC) to the plasma membrane. V+H and V+T+A induced strong chemiluminescence (CL) which was abrogated by a specific NADPH oxidase inhibitor diphenylene iodonium (DPI). DPI markedly suppressed the stimulation of PLA₂ by V+T+A and V+OZ. The results suggest that the formation of endogenous reactive oxygen species (ROS) is important for PLA₂ activation.

Phospholipase A₂; NADPH oxidase; Reactive oxygen species; Diphenylene iodonium; Vanadate; Tyrosine protein phosphatase

1. INTRODUCTION

Phospholipase A₂ (PLA₂) which releases arachidonic acid (AA) from phospholipids is a key enzyme of the host-defence system. Cellular PLA₂ is activated by agonists such as antigen [1], opsonized-zymosan (OZ) [2], 12-*O*-tetradecanoyl phorbol 13-acetate (T) [1-3], Ca²⁺-ionophore A23187 (A) [1-3]. Since protein kinase C (PKC) and tyrosine protein kinase (TPK) [1-4] take part in agonist activation of PLA₂, protein phosphorylation on both tyrosine and serine residues is essential to PLA₂ activation.

Vanadate (V) and H₂O₂ (H) both enhance tyrosine protein phosphorylation, and together they are synergistic [5-7]. Their effect is mainly due to inhibition of tyrosine protein phosphatases (TPPase) [6,7]. V induced superoxide formation [5] and tyrosine phosphorylation were prevented by diphenylene iodonium (DPI) a specific inhibitor of NADPH oxidase [5,8]. This suggests that endogenous reactive oxygen species (ROS), the products of NADPH oxidase, may be essential for suppression of TPPase activity [5].

The objectives of the present study were to determine: (a) whether V potentiates the stimulatory activity of

T, A, OZ and H on PLA₂ activity, and (b) the possible role of endogenous ROS in the regulation of cellular PLA₂ activity in macrophages.

2. EXPERIMENTAL

2.1. Macrophages

Thioglycollate-elicited mouse (female, CD1) peritoneal macrophages (Tg-Mø) were harvested and cultivated for 96 h in 24-well Costar plates (0.8×10⁶ Tg-Mø/well) as described [9]. Bone marrow derived macrophages (BMDM) were cultured in suspension as described [10].

2.2. Enzyme assays

For determination of cellular PLA₂ activity, Tg-Mø were prelabeled for 16 h, with [³H]arachidonic acid [³H]AA; Amersham) (0.1 μCi/well) as described previously [1]. In brief, the cells were washed three times with Hank's balanced salt solution (HBSS). The amount of [³H]AA metabolites released into the medium (containing 0.5% fatty acid-free bovine serum albumin (BSA)) as a result of the specified treatments was determined and expressed as % of cell-incorporated [³H]AA which was determined on solubilized cell monolayers. PLA₂ and PKC in membrane preparations from BMDM were assayed as described [10].

2.3. Luminol-dependent chemiluminescence (CL)

Tg-Mø were gently scraped from culture dishes and suspended (3×10⁵ cells/ml) in a luminol (0.2 mM) containing HBSS supplemented with 0.5% BSA and incubated at 30°C. V (0.5 mM), the specified stimulants and inhibitors were added as indicated and CL was measured every 2 min in a Lumax apparatus, model 2080 (Lumac Instruments, Basel).

3. RESULTS

A combination of 0.5 mM ortho-vanadate (V) and 0.2 mM H₂O₂ induced a 6 to 10-fold increase in the stimulation of PLA₂ activity in Tg-Mø (Fig. 1a). Addition of V also markedly potentiated the effect of OZ, T

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Abbreviations: V, vanadate; H, H₂O₂; PKC, protein kinase C; PLA₂, phospholipase A₂; TPPase, tyrosine protein phosphatase; CL, chemiluminescence; DPI, diphenylene iodonium; ROS, reactive oxygen species; A, Ca²⁺ ionophore A23187; AA, arachidonic acid; T, 12-*O*-tetradecanoyl phorbol 13-acetate; OZ, opsonized zymosan; Sl., slaurosporine; Herb. A, herbimycin A; BMDM, bone marrow derived macrophages; Tg-Mø, thioglycollate-elicited peritoneal macrophages.

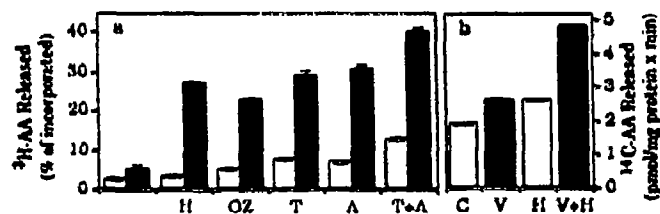


Fig. 1. Stimulation of PLA₂ activity by vanadate (V) combined with H₂O₂ (H) and various agonists. Tg-Mø (a) and BMDM (b) were incubated with (blackened bars) or without (empty bars) 0.5 mM V for 30 min at which point the various agonists were added and the cells were further incubated for 60 min. The following agonists were added: H, 0.2 mM; opsonized zymosan (OZ), 500 µg/ml; TPA (T), 100 ng/ml; Ca²⁺ ionophore A23187 (A), 2 µM; and T+A at above. C represents control cultures. The data of (a) are means of 4-6 experiments each carried out in triplicate ± S.E.M.; (b) means of 6 experiments carried out in triplicate. The S.D. did not exceed 5-10%.

and A on PLA₂ activity (Fig. 1a). PLA₂ activity was directly measured in crude membrane preparations isolated from BMDM treated with V+H. This treatment led to a sustained increase (2.5-fold) in membrane PLA₂ activity (Fig. 1b).

We next assessed the involvement of PKC and/or TPK in the activation of PLA₂ by V in combination with the various agonists.

BMDM were treated with V (0.5 mM, 30 min) and subsequently with or without H (0.2 mM, 60 min). V or H alone did not affect PKC localization or activity in crude membranes isolated from the treated cells, but in V+H treated cells the activity of PKC in the membranes was 1344 pmol/mg protein/min as compared to 189 pmol/mg protein/min in control cells.

Down-regulation of PKC by overnight incubation of Tg-Mø with T completely abolished the concerted effect of V+OZ and V+T+A and suppressed by 50% the stimulatory effect of V+H on PLA₂ activity (Fig. 2a). Treatment of Tg-Mø with PKC and TPK inhibitors; K252a, bisindolylmaleimide, GF 109203x (GF), staurosporine and herbimycin A led to a suppression (56-64%) of PLA₂ activation by the combination of V+H, V+T+A (Fig. 2b) and V + OZ (not shown).

DPI inhibited in a dose dependent manner the activation of PLA₂ by the combination of V+T+A, V+OZ and V+H (Fig. 3a). The interaction of V+H with Tg-Mø yielded a strong CL, indicating formation of ROS. This reaction was totally abolished by DPI (Fig. 3b). Incubation of Tg-Mø with V alone (0.5 mM) or H (0.2 mM) alone did not generate CL. Nor were free radicals formed and CL generated when V+H were combined in the absence of cells. The CL reaction induced by V+T+A and by V+H was suppressed by both PKC and TPK inhibitors (Fig. 3b and 3c).

4. DISCUSSION

Indirect observations suggest that activation of PKC and TPK leads to activation of PLA₂ [1,2,4]. PLA₂ acti-

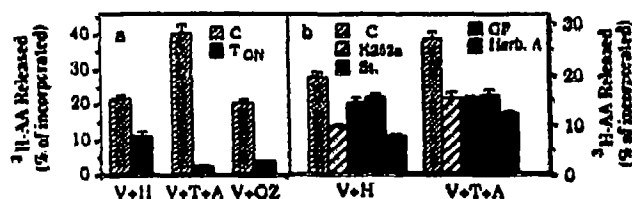


Fig. 2. Effect of down-regulation of PKC (a) and inhibitors of PKC and TPK (b) on the activation of PLA₂ by various stimulants. T_{ON} represents an overnight treatment of Tg-Mø with 100 ng/ml of the phorbol ester TPA. (a) T_{ON} treated and nontreated Tg-Mø were incubated with V and the specified additives as in Fig. 1a. (b) Tg-Mø were incubated for 30 min in buffer with or without (C) the following inhibitors: 1 µM K252a, 0.1 µM staurosporine (St.), 1 µM GF 109203x or 30 µg/ml herbimycin A (Herb. A). V (0.5 mM) was then added for 30 min, and then for additional 60 min either 0.2 mM H or 100 ng/ml T+2µM A. Means of triplicates ± S.D. of a representative experiment out of three. The basal activity (45%) was subtracted.

vation could not be achieved in a mutant devoid of TPK [4]. Purified PLA₂ was shown to undergo phosphorylation by PKC, though the phosphorylation alone did not bring about enzyme activation [11]. Activators of PKC induce the translocation of PLA₂ to the plasma membrane [1,10]. We showed that treatment of BMDM with V+H enhanced PKC activity in cell membranes and caused translocation of PLA₂ to the membranes where its activation was sustained. Moreover, inhibitors of PKC and TPK suppressed PLA₂ activation by any of the combinations tested (V+T+A, V+OZ and V+H).

Activators of PKC including the combinations of V and the various agonists used, as well as V alone [5], activate NADPH oxidase. We and others [12,13] have shown that inhibitors of PKC and TPK inhibit ROS formation. DPI, inhibits NADPH oxidase directly, by binding covalently to its flavoprotein component [8]. The novel finding that the V+H induced CL was also abolished by DPI, suggests that ROS-radical formation even with this combination is absolutely dependent on an active NADPH oxidase. V+NADPH have been shown to induce O₂⁻ formation in permeabilized HL-60 cells [6]. V⁵⁺ + O₂⁻ produce the highly reactive V⁴⁺-OO (peroxovanadate) species [14]. Within the cells, V⁵⁺ can be reduced to V⁴⁺ by any one of several reducing agents, but production of V⁴⁺-OO (probably) requires enzymatically generated O₂⁻. A complex set of oxidation-reduction reactions involving O₂⁻, H, V⁵⁺, V⁴⁺, NADPH and NADPH oxidase leads to amplification of the oxidative potential within the cell which results in inactivation of about 60% of the acid phosphatase (tyrosine protein phosphatase) activity in Tg-Mø (data not shown). It is likely that the product of V and ROS is oxidizing an essential -SH group on the TPPase molecule thereby causing the enzyme to lose its activity [15]. We suggest that inhibition of TPPase activity is responsible for sustained phosphorylation and activation of PLA₂. In various cell types TPPase specific activity is 10 to 1000-fold higher than that of TPK [16].

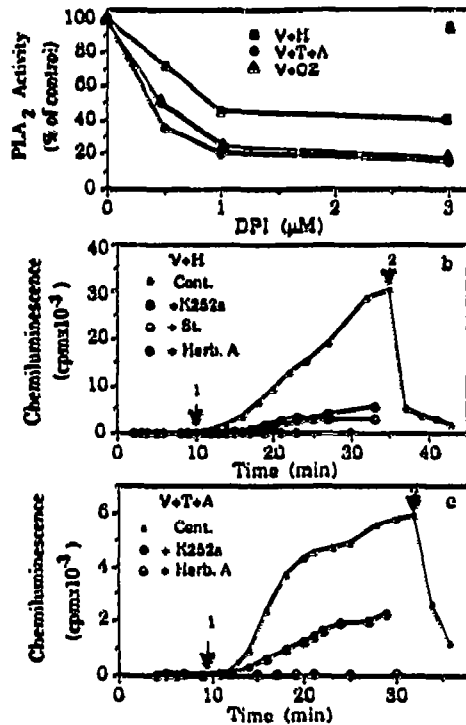


Fig. 3. The effect of DPI on PLA₂ activation (a) and ROS formation (b,c) and the effect of PKC and TPK inhibitors on ROS formation induced by V+H (b) and V+T+A (c). (a) Tg-Ms were incubated with the specified concentrations of DPI for 30 min and then treated with the stimulants as in Fig. 2b; (b and c) V (0.5 mM) with or without the specified inhibitors (as in Fig. 2b) were added (zero time) and chemiluminescence (CL) was measured every 2 min in a Lumac apparatus, model 2080 (Lumac Instruments, Basel). The stimulants H (0.2 mM) or T 100 ng/ml+A (2 μM) were added after 10 min (arrow 1) and CL was measured for an additional 20 min. DPI (4 μM) was added to control (Cont.; no inhibitor) cells at about 30 min (arrow 2) and CL was further recorded.

It should be borne in mind that V is used here as a transition metal inducing formation of potent ROS. Under physiological conditions, other transition metals such as Fe³⁺ or Cu²⁺ may play this role [17,18].

We conclude that optimal activation of PLA₂ entails at least two regulatory pathways acting in concert: (a) enzyme activation by PKC and TPK, and (b) attenuation of the protein phosphatase reaction that inactivates PLA₂. Since activators of PLA₂ (OZ, T, A, and T+A) were potentiated by V, and since their effects were markedly diminished by DPI, we suggest that PLA₂ activation was mainly via ROS formation and consequently TPPase inhibition. Stimulation of PLA₂ by V+H, a combination that could also inhibit TPPase directly, was affected to a lesser degree by inhibition of NADPH oxidase.

It is of interest that ROS were recently suggested to act as second messengers in platelets aggregation induced by collagen [19], in the metabolic effect induced

by insulin in adipocytes [20] and in the activation of transcription factors [21,22].

In various pathological conditions, such as inflammation and allergy there is a local elevation of ROS formation which both induces and exacerbates the manifestation of the pathology [17,18]. DPI-like drugs which interfere with the formation of ROS could possibly be used to alleviate these conditions.

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REFERENCES

- [1] Zor, U., Mer, E., Harel, T., Fischer, G., Naor, Z., Braquet, P., Ferber, E. and Reiss, N. (1991) *Biochim. Biophys. Acta* 1091, 385-392.
- [2] Sporn, P.H.S., Marshall, T.M. and Peters-Golden, M. (1990) *Biochim. Biophys. Acta* 1047, 187-191.
- [3] Wijkander, J. and Sundler, R. (1989) *Biochim. Biophys. Acta* 1010, 78-87.
- [4] Goldberg, H.J., Viegas, M.M., Margolis, B.L., Schlessinger, J. and Skorecki, K.L. (1990) *Biochem. J.* 267, 461-465.
- [5] Trudel, S., Paquet, M.R. and Grinstein, S. (1991) *Biochem. J.* 276, 611-619.
- [6] Zick, Y. and Sagi-Eisenberg, R. (1990) *Biochemistry* 29, 10249-10245.
- [7] Fantus, I.G., Kadota, S., Deragon, G., Foster, B. and Posner, B.I. (1989) *Biochemistry* 28, 8864-8871.
- [8] Hancock, J.T. and Jones, O.T.G. (1987) *Biochem. J.* 242, 103-107.
- [9] Goldman, R. (1990) *J. Leukoc. Biol.* 47, 79-86.
- [10] Schonhardt, T. and Ferber, E. (1987) *Biochem. Biophys. Res. Commun.* 149, 769-775.
- [11] Wijkander, J. and Sundler, R. (1991) *Eur. J. Biochem.* 202, 873-880.
- [12] Twomey, B., Muid, R.E. and Dale, M.M. (1990) *Br. J. Pharmacol.* 100, 819-825.
- [13] Naccache, P.H., Gilberg, C., Caon, A.C., Gaudry, M., Huang, C.K., Bonak, V.A., Umezawa, K. and McColl, S.R. (1990) *Blood* 76, 2098-2104.
- [14] Liochev, S.I. and Fridovich, I. (1991) *Arch. Biochem. Biophys.* 291, 379-382.
- [15] Ballou, L.M. and Fischer, E.H. (1986) *The Enzymes* 17, 311-351.
- [16] Fischer, E.H., Charbonneau, H. and Tonks, N.K. (1991) *Science* 253, 401-406.
- [17] Halliwell, B. (1987) *FASEB J.* 1, 358-364.
- [18] Halliwell, B. (1991) *Am. J. Med.* 91, 14-22.
- [19] Del Principe, D., Menichelli, A., De Matteis, W., Di Giulio, S., Giordani, M., Savini, I. and Agro, A.F. (1991) *Thrombosis Res.* 62, 365-375.
- [20] Krieger-Brauer, H.I. and Kather, H. (1992) *J. Clin. Invest.* 89, 1006-1013.
- [21] Schreck, R., Rieber, P. and Baeuerle, P.A. (1991) *EMBO J* 10, 2247-2258.
- [22] Devary, Y., Gottlieb, R.A., Lau, L.F. and Karin, M. (1991) *Mol. Cell. Biol.* 11, 2804-2811.