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Reactive oxygen species are involved in the activation of cellular phospholipase A_2

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Vanadate (V) potentiated (4- to 10-fold) the activation of cellular phospholipase A_2 (PLA_2) induced by H2O2 (H), a phorbol ester (T), a Ca^{2+}-ionophore (A) and opsonized zymosan in macrophages. V+H induced in intact cells the activation and translocation of PLA_2 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 PLA_2 by V+T+A and V+OZ. The results suggest that the formation of endogenous reactive oxygen species (ROS) is important for $^{p}LA_2$ activation.

Phospholipase A_2; NADPH oxidase; Reactive oxygen species; Diphenylene iodonium; Vanadate; Tyrosine protein phosphatase

1. INTRODUCTION

Phospholipase A_2 (PLA_2) which releases arachidonic acid (AA) from phospholipids is a key enzyme of the host-defence system. Cellular PLA_2 is activated by agonists such as antigen [1], opsonized-zymosan (OZ) [2], 12-*O*-tetradecanoyl phorbol 13-acetate (T) [1–3], Ca^{2+} ionophore A23187 (A) [1–3]. Since protein kinase C (PKC) and tyrosine protein kinase (TPK) [1–4] take part in agonist activation of PLA_2, protein phosphorylation on both tyrosine and serine residues is essential to PLA_2 activation.

Vanadate (V) and H_2O_2 (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 PLA_2 activity, and (b) the possible role of endogenous ROS in the regulation of cellular PLA_2 activity in macrophages.

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Abbreviations: V, vanadate; H, H_2O_2; PKC, protein kinase C; PLA_2, phospholipase A_2; TPPase, tyrosine protein phosphatase; CL, chemiluminescence; DPI, diphenylene iodonium; ROS, reactive oxygen species; A, Ca^{2+} ionophore A23187; AA, arachidonic acid; T, 12-*O*-tetradecanoyl phorbol 13-acetate; OZ, opsonized zymosan; St., staurosporine; Herb. A, herbimycin A; BMDM, bone marrow derived macrophages; Tg-Mø, thioglycollate-elicited peritoneal 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 (0.8×10^6 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 [^{3}H]arachidonic acid [^{3}H]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 [^{3}H]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 [^{3}H]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 (3×10^5 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 H_2O_2 induced a 6 to 10-fold increase in the stimulation of PLA_2 activity in Tg-Mø (Fig. 1a). Addition of V also markedly potentiated the effect of OZ, T

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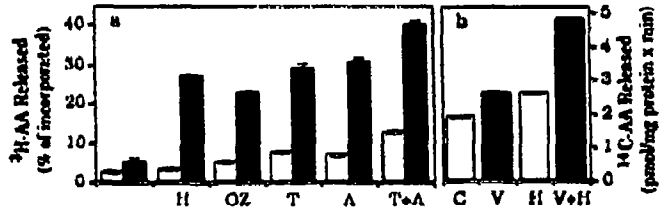

Fig. 1. Stimulation of PLA_2 activity by vanadate (V) combined with H_2O_2 (H) and various agonists. Tg-Mø (a) and BMDM (b) were incubated with (blackened bars) or without (empty bars) 0.5 mM V for 30 min at which point the various agonists were added and the cells were further incubated for 60 min. The following agonists were added: H, 0.2 mM; opsonized zymosan (OZ), 500 μg/ml; TPA (T), 100 ng/ml; Ca^{2+} 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%.

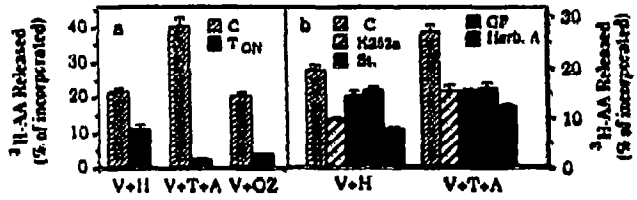

Fig. 2. Effect of down-regulation of PKC (a) and inhibitors of PKC and TPK (b) on the activation of PLA_2 by various stimulants. T_{ON} represents an overnight treatment of Tg-Mø with 100 ng/ml of the phorbol ester TPA. (a) T_{ON} treated and nontreated Tg-Mø were incubated with V and the specified additives 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.

and A on PLA2 activity (Fig. 1a). PLA_2 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 PLA_2 activity (Fig. 1b).

We next assessed the involvement of PKC and/or TPK in the activation of PLA_2 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 PLA_2 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 PLA_2 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 PLA_2 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 PLA_2 [1,2,4]. PLA_2 activation could not be achieved in a mutant devoid of TPK [4]. Purified PLA_2 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 PLA_2 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 PLA_2 to the membranes where its activation was sustained. Moreover, inhibitors of PKC and TPK suppressed PLA_2 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 O_2^- formation in permeabilized HL-60 cells [6]. V^{5+} + O_2^- produce the highly reactive V^{4+}–OO (peroxovanadate) species [14]. Within the cells, V^{5+} can be reduced to V^{4+} by any one of several reducing agents, but production of V^{4+}-OO (probably) requires enzymatically generated O_2^-. A complex set of oxidation-reduction reactions involving O_2^-, H, V^{5+}, V^{4+}, 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 PLA_2. In various cell types TPPase specific activity is 10 to 1000-fold higher than that of TPK [16].

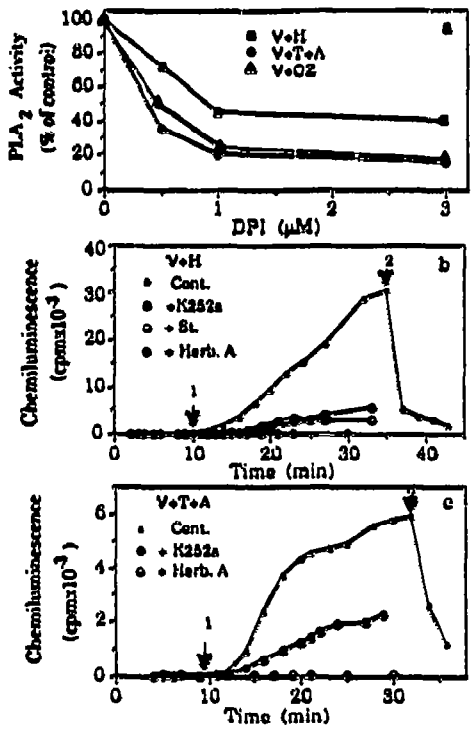

Fig. 3. The effect of DPI on PLA_2 activation (a) and ROS formation (b,c) and the effect of PKC and TPK inhibitors on ROS formation induced by V+H (b) and V+T+A (c). (a) Tg-Mø were incubated with the specified concentrations of DPI for 30 min and then treated with the stimulants as in Fig. 2b; (b and 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 shoud 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 Fe^{3+} or CU^{2+} may play this role [17,18].

We conclude that optimal activation of PLA_2 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 PLA_2. Since activators of PLA_2 (OZ, T, A, and T+A) were potentiated by V, and since their effects were markedly diminished by DPI, we suggest that PLA_2 activation was mainly via ROS formation and consequently TPPAse inhibition. Stimulation of PLA_2 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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