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Vanadate-induced activation of cytosolic phospholipase $A_2\alpha$ in L929 cells: Roles of tyrosine kinase, protein kinase C, and extracellular signal-regulated kinase

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ABSTRACT

Orthovanadate (Na_3VO_4), which acts as an inhibitor of protein tyrosine phosphatases, has a various pharmacological effects including the release of arachidonic acid (AA) from cells. We investigated roles of α-type cytosolic phospholipase A_2 ($cPLA_2\alpha$), Src family kinases (Src) and protein kinase C (PKC) in the release of AA induced by Na_3VO_4 from a murine fibroblast cell line, L929. C12 cells, a variant of L929 that lacks expression of $cPLA_2\alpha$, were used along with a clone of C12 cells that are stably expressing $cPLA_2\alpha$ (C12-$cPLA_2\alpha$ cells). In the presence of a Ca^{2+} ionophore (10 μM A23187), 5 and 10 mM Na_3VO_4 synergistically stimulated AA release from L929 and C12-$cPLA_2\alpha$ cells, and to a much lesser extent from control C12 cells. The release of AA by Na_3VO_4/A23187 was inhibited by a selective $cPLA_2\alpha$ inhibitor (3 μM pyrrophenone). The release of AA by Na_3VO_4/A23187 was significantly inhibited by a PKC inhibitor (10 μM GF109203X), in PKC-depleted cells, by a Src inhibitor (2 μM PP2) and by an inhibitor of extracellular signal-regulated kinase 1/2 (ERK1/2) kinase (10 μM U0126). The phosphorylation of ERK1/2 was stimulated by Na_3VO_4, and the response was significantly decreased by inhibitors of Src, PKC and ERK1/2 kinase. Our data show that Na_3VO_4 stimulates AA release largely via $cPLA_2\alpha$ activation in Ca^{2+}-dependent manner, and the cross-talk between Src and PKC and the ERK-dependent pathways are involved in Na_3VO_4-induced AA release from L929 cells.

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Abbreviations: PTP, protein tyrosine phosphatases; Src, Src family kinases; ROS, reactive oxygen species; AA, arachidonic acid; PLA_2, phospholipase A_2; $cPLA_2\alpha$, α-type cytosolic PLA_2; PMA, 4β-phorbol 12-myristate 13-acetate; PKC, protein kinase C; U0126, 1,4-diamino-2,3-dicyano-1,4-bis-(o-aminophenylmercapto)butadiene; GF109203X, 2-[1-(3-dimethylamino-propyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-*d*]pyrimidine; PP3, 4-amino-7-phenylpyrazol[3,4-*d*]pyrimidine; ERK1/2, extracellular signal-regulated kinases 1 and 2

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1. Introduction

Vanadium is a trace element found at low concentrations in mammalian tissues, although its physiological function remains unknown. A number of vanadium compounds such as vanadate and pervanadate have multiple biological and pharmacological effects [1–3]. In terms of their potential for use in the treatment of diabetes mellitus, oral drugs containing vanadium are of great interest [4]. Vanadate (VO_4^{3-}) is a phosphate analog and is generally thought to bind as a transition state analog to phosphoryl transfer enzymes such as ATPases and protein tyrosine phosphatases (PTPs) [1–3]. Orthovanadate (Na_3VO_4) is a PTP inhibitor that leads to an increase of phosphotyrosine in various proteins in cells. Tyrosine kinases, both receptor types and non-receptor types such as the Src family kinases (Src), are also phosphorylated and activated by Na_3VO_4 in various cells including L929 cells [5–8]. Src includes at least eight members (c-Src, Lyn, c-Yes, Fyn, c-Fgr, Hck, Lck, and Blk), and plays crucial roles in regulating cellular responses such as cell proliferation and metabolism. The tyrosine phosphorylation induced by Na_3VO_4 was attributed in part to the inhibition of PTP and/or activation of tyrosine kinases [1,2]. In addition, it has been demonstrated that the generation of reactive oxygen species (ROS) such as H_2O_2 plays an important role in the adverse biological effects of vanadate [9,10].

Previously, we reported that Na_3VO_4 stimulated the release of arachidonic acid (AA) and production of prostaglandin $F_2\alpha$ in two neuronal cell lines, rat PC12 cells and GH3 cells [11,12]. Na_3VO_4 stimulated and/or enhanced the release of AA from rat thyroid FRTL-5 cells [13] and murine RAW macrophages [14]. Tyrosine phosphorylation plays an important role in the regulation of vascular tone, and treatment with inhibitors for cyclooxygenase and phospholipase A_2 (PLA_2) decreased vanadate-induced contraction in gallbladder smooth muscle [15]. The liberation of AA from phospholipids induced by PLA_2 on cell activation is often the initial and rate-limiting step in eicosanoid biosynthesis. AA and its metabolites are playing important roles on many physiological and pathological functions including inflammation. The many PLA_2s identified and characterized over the past two decades are now classified as secretory PLA_2, cytosolic PLA_2 ($cPLA_2$), and Ca^{2+}-independent PLA_2 [16,17]. α-Type $cPLA_2$ ($cPLA_2\alpha$) plays a pivotal role in providing AA because of its selectivity for phospholipids containing AA at the *sn*-2 position. However, it is reported that several stimuli including H_2O_2 caused release of AA via the activation of secretory PLA_2s and Ca^{2+}-independent PLA_2s in some cases [18–20]. As described above, treatment of cells with Na_3VO_4 by itself or via other events such as the formation of ROS can regulate various cellular molecules and signaling mechanisms. However, the signaling mechanism(s) for the release of AA induced by Na_3VO_4 remain unknown.

The murine fibrosarcoma cell line L929 is widely used to study the effects of tumor necrosis factor α and ROS [21,22]. The L929 variant C12, expressing undetectable levels of $cPLA_2\alpha$, is resistant to the tumor necrosis factor α-induced release of AA, and transfection of C12 cells with $cPLA_2\alpha$ cDNA recovered the response [21]. Previously, we reported that addition of a Ca^{2+} ionophore and 4β-phorbol 12-myristate 13-acetate (PMA, an activator of protein kinase C (PKC)) stimulated the release of AA from clones of C12 cells stably expressing wild-type $cPLA_2\alpha$ (C12-$cPLA_2\alpha$ cells) [23]. In the present study, we compared the effects of Na_3VO_4 on the release of AA from L929, control C12, and C12-$cPLA_2\alpha$ cells. In addition, roles of Src and PKC pathways in the Na_3VO_4-induced activation of $cPLA_2\alpha$ and their signaling mechanism(s) are investigated.

2. Experimental procedures

2.1. Reagents

[5,6,8,9,11,12,14,15-^{3}H]AA (7.92 TBq/mmol) was purchased from Amersham (Buckinghamshire, UK). PMA (4β,9α,12β,13α,20-pentahydroxytiglia-1,6-dien-3-one 12-tetradecanoate 13-acetate), 4α-PMA, A23187, and catalase were purchased from Sigma (St. Louis, MO, USA). H_2O_2 and Na_3VO_4 were from Wako (Osaka, Japan). Pyrrophenone and indoxam were kindly provided by Dr. Hanasaki (Shionogi Pharm. Ltd., Osaka, Japan). 1,4-Diamino-2,3-dicyano-1,4-bis-(*o*-aminophenylmercapto)butadiene (U0126), 2-[1-(3-dimethylamino-propyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide (GF109203X), 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2), and 4-amino-7-phenylpyrazol[3,4-*d*]pyrimidine (PP3) were purchased from Calbiochem (San Diego, CA, USA). The concentrations of reagents were the same as those in previous reports [3,6,23,24].

2.2. Cell culture and assay for AA release

L929, C12, and C12-$cPLA_2\alpha$ cells were grown in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum, and the amount of AA released from intact cells was determined as described previously [23,24]. Briefly, cells on 12-well plates were labeled overnight with medium containing [^{3}H]AA, 10 mM HEPES (pH 7.4), and 0.1% fatty acid-free bovine serum albumin (Sigma, A-7511). The cells were washed three times with modified Tyrode HEPES buffer (137 mM NaCl, 5 mM KCl, 5 mM glucose, 2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 0.1% albumin and 10 mM HEPES, pH 7.4) and stimulated with the reagents for 30 min. In some experiments, cells were cultured with the respective inhibitors before the AA release assay. Then, the medium was collected and centrifuged at 8000 × *g* for 2 min. The radioactivity of [^{3}H]AA released into the supernatant was expressed as a percentage of all the radioactivity incorporated (20,000–40,000 dpm per well). Treatment with the respective inhibitors did not cause a change in the total amount of [^{3}H]AA incorporated in cells or the release of AA without stimuli from cells.

2.3. Phosphorylation of extracellular signal-regulated kinase (ERK1/2)

Sixteen hours prior to the Western blotting experiments, L929 cells were switched from their regular culture medium to Opti-MEM (Invitrogen). Cells were pretreated with either vehicle or the indicated inhibitors for 15 min, and then stimulated with the reagents for 10 min at 37 °C. Cells were scraped into a lysis buffer consisting of 150 mM NaCl, 50 mM Tris–HCl (pH 8.0), 5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate,

10 mM sodium fluoride, 10 mM disodium pyrophosphate, 0.1% SDS, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM Na_3VO_4, and then transferred to microcentrifuge tubes. The samples were rotated for 20 min at 4 °C and were centrifuged at 17,400 × *g* for 15 min. A similar amount of protein per lane was applied to the gel for SDS-PAGE. The blocked PVDF membranes were incubated with the respective antibody. Anti-phospho-ERK1/2 antibody (no. 9106) was purchased from Cell Signaling Tech (Beverly, MA). Anti-ERK1/2 antibodies (sc-93 and sc-153), anti-PKCα antibody (sc-208), anti-PKCδ antibody (sc-937) and anti-β-tubulin antibody (T-4026) were obtained from Santa Cruz Biotech (Santa Cruz, CA, USA). The immunoreactive bands were visualized using chemiluminescent reagents as recommended by Amersham Pharm. Biotech.

2.4. *Translocation of PKCα*

For measuring the translocation of PKC, treated cells were harvested and homogenized with an ice-cold homogenate buffer (250 mM sucrose, 10 mM EGTA, 2 mM EDTA, 1 mM benzamidine and 1 mM phenylmethanesulfonyl fluoride, 20 mM Tris–HCl, pH 7.4) at 4 °C with 20 strokes of a glass-teflon homogenizer. The soluble and crude membrane fractions were prepared as described previously [24].

2.5. *Measurements of lactate dehydrogenase leakage and intracellular free Ca^{2+} concentrations*

The viability of L929 cells was estimated from the leakage of lactate dehydrogenase, and intracellular free Ca^{2+} concentrations were determined by using fura-2 acetoxymethyl ester as described previously [25].

2.6. *Data presentation*

For measurements of AA release, values are the mean ± S.E.M. from three independent experiments performed in triplicate. Some data show the mean ± S.D. for triplicate in a typical experiment, and data are representative of two or three independent experiments. Experiments were done using different batches of cells on different days. The statistical significance for differences between two groups was assessed using Student's *t*-test. In the case of multiple comparisons, the significance of difference was determined using a one-way analysis of variance followed by the Bonferroni test. *P*-values < 0.05 were considered significant.

3. Results

3.1. *Effect of Na_3VO_4 on Ca^{2+}-stimulated release of AA via cPLA$_2$α activation*

Treatment with 10 μM A23187 alone slightly stimulated the release of AA for 30 min from L929 cells (Fig. 1, panel A), as shown previously [23]. The release induced by 10 mM Na_3VO_4 alone was also limited. The co-addition of Na_3VO_4 with A23187 markedly stimulated the release of AA, and the effect was synergistic and significant (panel B). In the $CaCl_2$-free buffer, the release of AA induced by Na_3VO_4/A23187 was 0.6–0.8%, which was almost the same as in the control without stimulation. Although 5 mM, not 1 mM, Na_3VO_4 with A23187 caused a release of AA, the response was dependent on each experiment and much less than that induced by 10 mM Na_3VO_4. Treatment with 10 mM Na_3VO_4 with or without 10 μM A23187 did not induce cell toxicity for at least 30 min; the amount of lactate dehydrogenase that leaked from the treated cells was less than 5%, which was almost the same as for the control without stimulation. In the $CaCl_2$-containing buffer, treatment with 10 mM Na_3VO_4 of L929 cells had marginal effects on basal and 10 μM A23187-stimulated levels of intracellular Ca^{2+} (data not shown).

Previously, we established a stable C12-cPLA$_2$α cell clone [23]. Addition of 10 mM Na_3VO_4 synergistically stimulated the release of AA in the presence of 10 μM A23187 from C12-cPLA$_2$α cells, but not from control C12 cells (Fig. 2, panel A). No AA was released by 10 mM Na_3VO_4 alone in the control C12 cells or in the C12-cPLA$_2$α cells. Pyrrophenone is a selective inhibitor of cPLA$_2$α and much less effective against Ca^{2+}-independent PLA_2 and diacylglycerol lipase [26]. Treatment with 3 μM pyrrophenone, which had no effect by itself (panel B), almost completely inhibited the release of AA induced by Na_3VO_4/A23187 (panel C). The inhibitory effect of pyrrophenone at 10 μM was similar to that at 3 μM. Treatment with pyrrophenone at 0.1 and 0.3 μM did not inhibit the release of AA induced by Na_3VO_4/A23187 from L929 cells, and the inhibitory effect of 1 μM pyrrophenone was depending on experiments (data not shown). These findings suggest that the

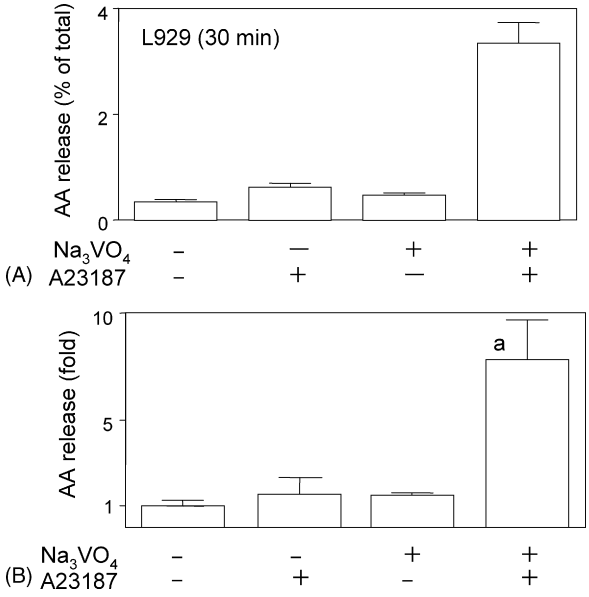

Fig. 1 – Synergistic effect of Na_3VO_4 and A23187 on the release of AA from L929 cells. Labeled cells on dishes were incubated with vehicle or 10 mM Na_3VO_4 in the presence and absence of 10 μM A23187 for 30 min. The amount of ^3H in the supernatant was calculated as a percentage of the total incorporation of [^3H]AA. In panel A, data are the mean ± S.D. of three determinations in a typical experiment. Panel B shows the fold-increase representative of three independent experiments. [a]$P < 0.05$, significantly different from the control.

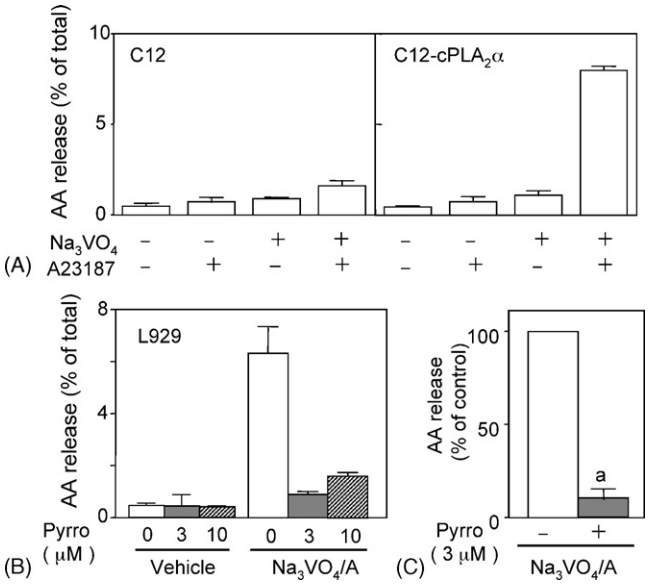

Fig. 2 – Involvement of cPLA$_2\alpha$ in the release of AA induced by Na_3VO_4/A23187. In panel A, control C12 cells and C12-cPLA$_2\alpha$ cells were labeled and incubated with vehicle, 10 mM Na_3VO_4, and/or 10 μM A23187 for 30 min. Data are the mean ± S.D. of three determinations in a typical experiment involving two representative experiments. In panels B and C, L929 cells were pretreated with the indicated concentrations of pyrrophenone for 30 min. The cells were washed and then incubated with vehicle or Na_3VO_4/A (10 mM Na_3VO_4 plus 10 μM A23187) for 30 min in the presence of same concentrations of pyrrophenone. In panel B, data are the mean ± S.D. of three determinations in a typical experiment. In panel C, the net increase in the amount of AA released by Na_3VO_4/A23187 is normalized as a percentage of the control value. Data are the mean ± S.E.M. of three independent experiments. [a]P < 0.05, significantly different from the control value without pyrrophenone.

release of AA induced by Na_3VO_4/A23187 is mainly mediated by the activation of cPLA$_2\alpha$. Dithiothreitol, a reducing reagent, decreases the activity of various secretory PLA$_2$s by the reduction of disulfide bridges in secretory PLA$_2$s [27]. Indoxam and its analog were potent inhibitors of various secretory PLA$_2$s (at least group IB, IIA, IIC, IIE, V, X) [27,28]. Treatment of L929 cells with 1 mM dithiothreitol and 10 μM indoxam had no effect on the Na_3VO_4/A23187-induced release of AA (data not shown).

3.2. *Signaling pathways for the release of AA induced by Na_3VO_4*

Next, we investigated the signaling pathways activated by Na_3VO_4. Previously we reported that PMA (an activator of PKC) and H_2O_2 stimulated the release of AA in the presence of 10 μM A23187, respectively, and that co-addition of PMA and H_2O_2 without A23187 did not cause the response in L929 cells [29]. In the presence of 10 mM Na_3VO_4 without A23187, the release of AA induced by 100 nM PMA and by 2 mM H_2O_2 was in the range of 0.4–0.6 (% of total), almost the same as for the control without stimulation. Similar results were obtained in C12-cPLA$_2\alpha$ cells; for instance, the amount of AA released by 2 mM H_2O_2 was 2.0 ± 0.2% and 2.3 ± 0.1% with and without 10 mM Na_3VO_4 in the absence of A23187 and the amount released by H_2O_2/A23187 was 8.6 ± 0.3% in a typical experiment. These findings suggest that common signaling pathway(s) are activated by Na_3VO_4, PMA, and H_2O_2, and the activation of these pathways can stimulate release of AA in the presence of Ca^{2+}.

PP2 is a potent inhibitor of Src and shown to inhibit stimuli-induced tyrosine phosphorylation of proteins at μM concentrations in fibroblasts including L929 cells [7,30]. Treatment with 2 and 10 μM PP2 for 15 min inhibited the Na_3VO_4/A23187-induced release of AA in L929 cells (Fig. 3, panel A). Treatment with PP2 at 2 μM significantly inhibited the response (panel B). Treatment with 2 μM PP3, a structural analog of PP2 which does not inhibit Src, had no such inhibitory effect (data not shown). The release of AA induced by Na_3VO_4/A23187 decreased in the PKC-depleted cells, which were cultured with 100 nM PMA for 12 h before the

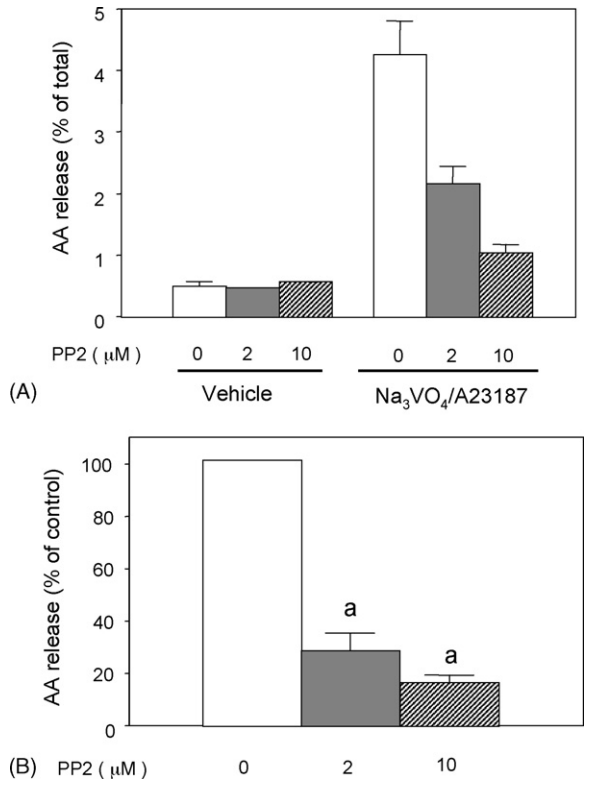

Fig. 3 – Effect of PP2 on the Na_3VO_4/A23187-induced release of AA. L929 cells were incubated with 2 and 10 μM PP2 for 15 min, and then with vehicle or 10 mM Na_3VO_4 plus 10 μM A23187 (Na_3VO_4/A23187) for 30 min in the presence of PP2. In panel A, data are the mean ± S.D. of three determinations in a typical experiment. In panel B, the net increase in the amount of AA released by Na_3VO_4/A23187 is normalized as a percentage of the control value. Data are the mean ± S.E.M. of three independent experiments. [a]P < 0.05, significantly different from the control value without PP2.

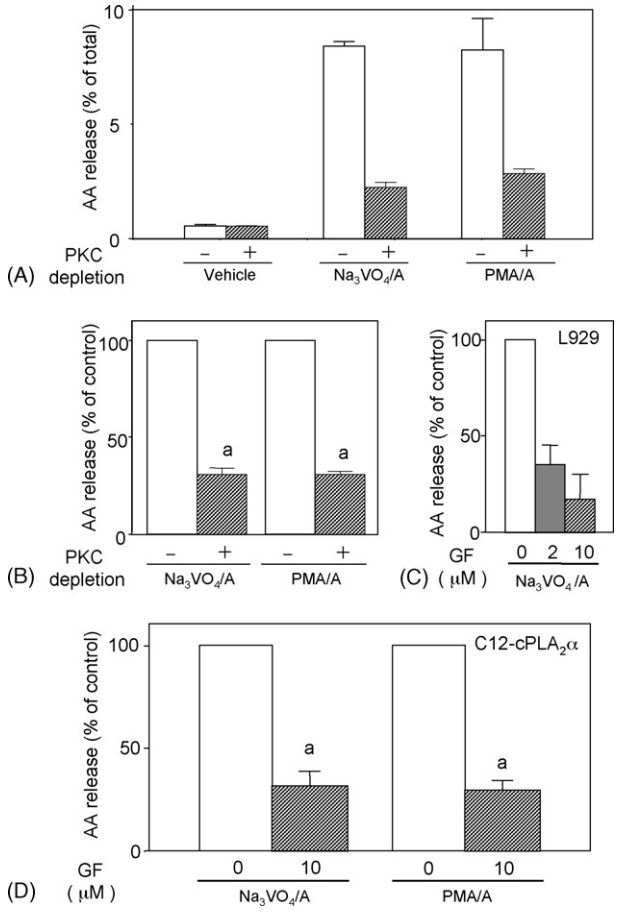

Fig. 4 – Effects of PKC depletion and GF109203X on the Na_3VO_4/A23187-induced release of AA. In panels A and B, L929 cells were cultured with vehicle or 100 nM PMA (hatched column, PKC depletion) for 12 h. The cells were incubated with 100 nM PMA plus 10 μM A23187 (PMA/A) or 10 mM Na_3VO_4 plus 10 μM A23187 (Na_3VO_4/A). In panel B, the net increase in the amount of AA released by Na_3VO_4/A23187 and PMA/A23187 is normalized as a percentage of the control value. In panel C, L929 cells were incubated with 2 and 10 μM GF109203X (GF) for 15 min, and then with Na_3VO_4/A23187 in the presence of the same concentrations of GF109203X. In Panel D, C12-cPLA$_2$α cells were incubated with 10 μM GF109203X, and then with Na_3VO_4/A23187 and PMA/A23187. In panels A and C, data are the mean ± S.D. of three determinations in a typical experiment involving two representative experiments. In panels B and D, data are the mean ± S.E.M. of three independent experiments. [a]P < 0.05, significantly different from the control value.

AA release assay (Fig. 4, panel A). Like the response to PMA/A23187, the response to Na_3VO_4/A23187 was significantly inhibited, about 60%, in the PKC-depleted cells (panel B). The release of AA induced by Na_3VO_4/A23187 was inhibited by the treatment for 15 min with 2 and 10 μM GF109203X, a selective inhibitor of PKC in L929 cells (panel C). Similar inhibition was observed in C12-cPLA$_2$α cells, and the effects of 10 μM GF109203X on the responses induced by Na_3VO_4/A23187 and PMA/A23187 were significant with about 70% inhibition achieved (panel D).

3.3. *Effect of Na_3VO_4 on the translocation of PKC*

Treatment of L929 cells with 100 nM PMA for 30 min caused the translocation of PKCα from the soluble fraction to crude membrane fraction (Fig. 5, panel A), as shown previously [29,31,32]. Treatment with 10 mM Na_3VO_4 for 30 min did not cause the translocation of PKCα (panel B), and Na_3VO_4/A23187 had no effect (data not shown). Neither Na_3VO_4 nor PMA caused the translocation of β-tubulin. Translocation of PKCδ was caused by PMA [29], but not by Na_3VO_4 in our conditions (not shown).

3.4. *Role of the ERK1/2 pathway in the Na_3VO_4-induced release of AA*

Next, we investigated the downstream signaling of Src/PKC activation induced by Na_3VO_4. The release of AA induced by Na_3VO_4/A23187 and by PMA/A23187 was significantly inhibited by 10 μM of U1026, an inhibitor of ERK1/2 kinase, in L929 cells (Fig. 6). Treatment with 10 mM Na_3VO_4 resulted in the phosphorylation of ERK1/2 in the presence of 10 μM A23187 (Fig. 7); the response began at 5 min, was maximal at 10–20 min and returned to the basal level at ~120 min after the treatment. Also, treatment with 100 nM PMA with A23187 for 10 min caused a marked phosphorylation of ERK1/2, as reported previously [29]. The responses induced by both stimulants were partially inhibited in the cells treated with 2 μM PP2 and with 10 μM GF109203X, respectively (Fig. 7, panel A). The quantified intensity of signals induced by

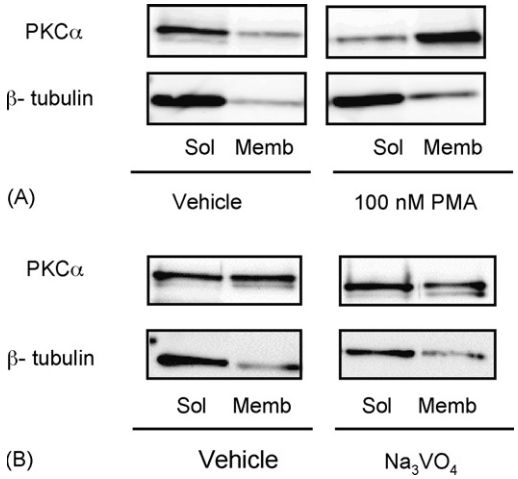

Fig. 5 – Translocation PKCα from the soluble to membrane fraction induced by PMA but not by Na_3VO_4 in L929 cells. L929 cells were incubated with vehicle, 100 nM PMA (panel A), or 10 mM Na_3VO_4 (panel B) for 30 min, and then homogenized. The levels of PKCα and β-tubulin in soluble and membrane fractions were analyzed by Western blotting with the respective antibody. Data are representative of two independent experiments, and similar data were obtained in the presence of 10 μM A23187.

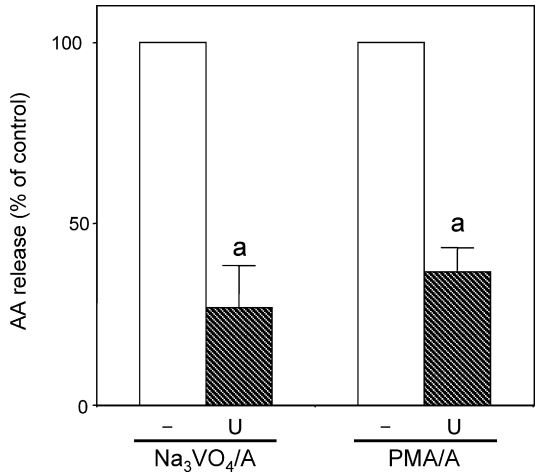

Fig. 6 – Effect of U0126 on the Na_3VO_4/A23187-induced release of AA. L929 cells were pretreated with vehicle or 10 μM U0126 (U) for 15 min, and then incubated with vehicle, 10 mM Na_3VO_4 plus 10 μM A23187 (Na_3VO_4/A), or 100 nM PMA plus 10 μM A23187 (PMA/A) in the presence of U0126. The net increase in the amount of AA released by Na_3VO_4/A23187 and PMA/A23187 is normalized as a percentage of the control value. Data are the mean ± S.E.M. of three independent experiments. [a]P < 0.05, significantly different from the control value.

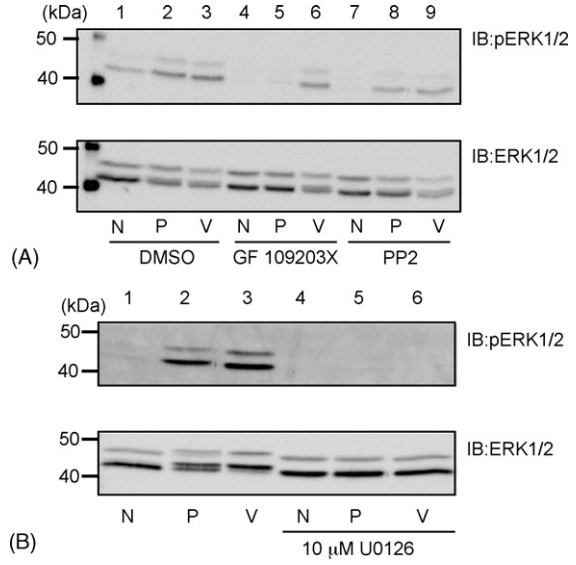

Fig. 7 – Effects of U0126, GF109203X, and PP2 on PMA- and Na_3VO_4-induced phosphorylation of ERK1/2. L929 cells were pretreated for 15 min with vehicle, 2 μM GF109203X, or 2 μM PP2 (panel A) or 10 μM U1026 (panel B) before being treated with vehicle (N), 100 nM PMA (P), or 10 mM Na_3VO_4 (V) for 10 min. A23187 (10 μM) was further added to the medium in panel A, but not in panel B. The cell lysates were analyzed by Western blotting with anti-phospho-ERK1/2 antibody (upper panel) and anti-ERK1/2 antibodies (lower panel). Data are representative of two independent experiments.

Na_3VO_4/A23187 was 100% in the control, ~50% in the GF109203X-treated cells, and ~40% in the PP2-treated cells in the representative experiments. Treatment with 10 μM U0126, almost completely decreased the response induced by Na_3VO_4/A23187 (panel B). The Na_3VO_4/A2318-induced phosphorylation of ERK1/2 at 5 and 20 min after the stimulation was inhibited in the U0126-, PP2-, and GF109203X-treated cells, and the response was not inhibited by treatment with 2 μM PP3 (data not shown). A slight retardation in the electrophoretic mobility of ERK1/2 was detected in Na_3VO_4- and PMA-treated cells, typically in panel B. The shift was inhibited by U0126 treatment. The degree of phosphorylation of ERK1/2 induced by Na_3VO_4 and PMA without A23187 was the same as that with A23187 (data not shown). Treatment with 100 nM 4α-PMA, a negative control for phorbol ester activation of PKC, did not cause the responses including the translocation of PKCα, the phosphorylation of ERK1/2 and the release of AA in L929 cells (data not shown).

3.5. *No involvement of H_2O_2 or the EGF receptor in the Na_3VO_4-induced release of AA*

Several responses induced by vanadium-related compounds including Na_3VO_4 were mediated by the cellular formation of ROS including H_2O_2 [9,10]. Loading of a catalase into the medium resulted in an increase in the intracellular catalase activity and/or blocked the intracellular accumulation of H_2O_2 [8,33]. The amount of AA released by 1 mM H_2O_2, but not by Na_3VO_4, decreased with catalase treatment (Table 1). Vanadate can cause the transactivation of the EGF receptor and downstream signaling in cells including fibroblasts [34,35]. However, treatment with 20 ng/mL of EGF did not stimulate the release of AA with or without A23187 or PMA.

Table 1 – Effects of catalase and EGF on the release of AA in L929 cells

Experiment I	Vehicle	Catalase	
		1000 U/mL	3000 U/mL
AA release (% of total)			
None	0.6 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
Na_3VO_4/A23187	4.3 ± 0.3	3.9 ± 0.4	4.4 ± 0.2
H_2O_2/A23187	2.9 ± 0.5	1.0 ± 0.1	0.7 ± 0.1

Experiment II	None	EGF (20 ng/mL)
AA release (% of total)		
None	0.6 ± 0.2	0.5 ± 0.1
10 μM A23187	1.2 ± 0.2	1.6 ± 0.2
100 nM PMA	0.7 ± 0.2	0.7 ± 0.1
PMA/A23187	4.2 ± 0.3	5.2 ± 0.3

In Experiment I, L929 cells were incubated with medium containing vehicle or 1000 or 3000 U/mL of catalase for 2 min, and then with vehicle, 10 mM Na_3VO_4 plus 10 μM A23187, or 1 mM H_2O_2 plus 10 μM A23187 for 30 min. In Experiment II, L929 cells were stimulated with the indicated reagents for 30 min. Data are the mean ± S.D. of three determinations in a typical experiment involving two representative experiments.

4. Discussion

4.1. *Involvement of cPLA$_2\alpha$ in the Na_3VO_4-induced release of AA*

The release of AA induced by 10 mM Na_3VO_4 was observed in the presence of 10 μM A23187 in the $CaCl_2$-containing buffer without a marked leakage of lactate dehydrogenase. The release of AA induced by Na_3VO_4/A23187 was inhibited by the depletion of PKC and by several of the inhibitors tested including pyrrophenone and PP2. The release was observed in L929 cells and in C12-cPLA$_2\alpha$ cells, but not in control C12 cells. These findings suggest that the responses induced by Na_3VO_4 are not likely to be due to cell toxicity, although Na_3VO_4 was used at pharmacological concentrations (5 and 10 mM). In addition to these results, the effects of inhibitors of secretory PLA$_2$s and of the deprivation of Ca^{2+} suggest that the release of AA by Na_3VO_4/A23187 is mediated via the activation of cPLA$_2\alpha$.

cPLA$_2\alpha$ activity in cells is regulated by intracellular translocation and phosphorylation of the enzyme by Ca^{2+} and kinases such as ERK1/2, respectively [16,17]. Treatment of thyroid FRTL-5 cells with Na_3VO_4 enhanced the increase in the intracellular free Ca^{2+} concentration and release of AA induced by stimulation of the ATP receptor [13]. However, Na_3VO_4 did not cause an increase in Ca^{2+} levels by itself and did not further enhance the release of AA induced by PMA that regulates phosphorylation signaling. Like PMA, Na_3VO_4-stimulated release of AA in the presence of A23187. A Ca^{2+} ionophore, but not PMA [23] or Na_3VO_4 (data not shown), stimulated the translocation of cPLA$_2\alpha$ in C12-cPLA$_2\alpha$ cells. As will be described later, the data in this study suggest that the responses induced by Na_3VO_4 were mediated by the PKC pathway. PMA and Na_3VO_4, which are regulators of various protein kinases and/or PTP, are likely to regulate phosphorylation signaling of cPLA$_2\alpha$. Since no consensus tyrosine phosphorylation site has been found in cPLA$_2\alpha$ [17], tyrosine kinases including Src are not likely to phosphorylate cPLA$_2\alpha$ directly.

4.2. *Role of PKC, Src, and ERK pathways in the activation of cPLA$_2\alpha$*

PKC is a multifunctional family of serine/threonine protein kinases, whose activities are dependent on Ca^{2+}, lipid second messengers, and/or protein regulators [36,37]. Four isoforms of PKC (α, β, δ and ε) are expressed in L929 cells [7,31,32], and we showed that PMA stimulated the translocation of PKCα from soluble to crude membrane fractions. The data concerning GF109203X and the depletion of PKC suggest that not only the PMA-induced but also the Na_3VO_4-induced release of AA in the presence of Ca^{2+} was dependent on the PKC pathway. A Ca^{2+} ionophore is able to activate PKC by itself under certain conditions [36,37]. However, Ca^{2+} appears to contribute to the translocation of cPLA$_2\alpha$ rather than activation of PKC in our conditions, since A23187 alone induced the release of much less AA than that did Na_3VO_4/A23187. Treatment with Na_3VO_4 did not have a marked effect on the translocation of PKC (α and δ) in L929 cells, as reported in other cell types [38]. Thus, Na_3VO_4 stimulated the release of AA via the activation of PKC, without inducing the translocation of PKC.

L929 cells are shown to express Src protein [6,7]. Our results demonstrated roles of Src in Na_3VO_4-induced responses (phosphorylation of ERK1/2 and AA release) in L929 cells. The PMA/A23187-induced phosphorylation of ERK1/2 was also inhibited by PP2 (Fig. 7A), as shown previously [29]. Several PKC isoforms have been shown to lie upstream of where Src is activated in cells [8,39], and can stimulate the kinase activity of Src through phosphorylation [40]. Src is known to be inactivated by phosphorylation at Tyr^{527}. Brandt et al. [41] reported that PKCδ induces Src kinase activity via the activation of α-type PTP, which dephosphorylates Src at Tyr^{527} resulting in an active Src. Thus, it seems that PKC is located upstream of Src in L929 cells. Conversely, there are reports that Src may lie upstream of where PKC is activated. Treatment with vanadium compounds induced tyrosine phosphorylation of PKCs in cells [42,43], and activated Src is able to interact with phospholipase Cγ resulting in the formation of diacylglycerol, an endogenous activator of PKCs [5,44]. Our data concerning GF109203X and PKC depletion suggest that Na_3VO_4/A23187-induced release of AA was dependent on the PKC pathway. Thus, PKCs and Src are likely to be cross-activated and regulated by Na_3VO_4, after which cPLA$_2\alpha$ is activated in L929 cells. Since treatment with a catalase inhibited the release of AA induced by H_2O_2/A23187, but not Na_3VO_4/A23187, intracellular H_2O_2 does not appear to be involved in the Na_3VO_4/A23187-induced release of AA. To characterize the molecular events upstream of the activation of Src and/or PKC induced by Na_3VO_4, the isoforms of Na_3VO_4-sensitive PKC and Src family kinases and their cross-talk mechanism remain to be elucidated.

The data obtained suggest that the Na_3VO_4-induced release of AA was at least partially dependent on the ERK pathways.

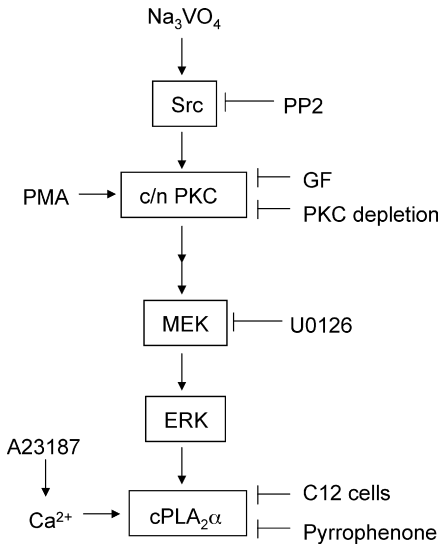

Fig. 8 – A model schema for the Na_3VO_4-induced release of AA in L929 cells. Na_3VO_4 stimulates Src, which is followed by the activation of PMA-sensitive conventional (c) and novel (n) types of PKC. U0126-sensitive MEK, an upstream enzyme of ERK1/2, is involved in the Na_3VO_4-induced phosphorylation of ERK1/2 and release of AA. Both Ca^{2+} and phosphorylation-signaling pathways including the ERK pathway are required for Na_3VO_4-induced activation of cPLA$_2\alpha$ in L929 cells.

Na_3VO_4 can directly inhibit a family of dual specificity phosphatases, for example the MAP kinase phosphatase, which negatively regulate enzymes of the ERK pathway such as Raf-1, ERK1/2 kinase, and ERK1/2 [14,45]. Since Na_3VO_4-induced responses (phosphorylation of ERK1/2 and release of AA) were almost completely inhibited by U0126, an inhibitor of an upstream enzyme of ERK1/2, the phosphorylation of ERK1/2 via inhibition of MAP kinase phosphatases by Na_3VO_4 may be limited. Since the inhibitors of Src and PKC markedly attenuated the responses to Na_3VO_4, inhibition of the phosphatases for Raf-1 and ERK1/2 kinase by Na_3VO_4 is not likely to contribute greatly to the activation of ERK1/2 by Na_3VO_4. Thus, the activation of Src and/or PKCs is likely to be a step in the response to Na_3VO_4.

4.3. *Summary*

Our model scheme for the Na_3VO_4-induced release of AA from L929 cells is shown in Fig. 8. Na_3VO_4 stimulates Src, which is followed by the activation of PMA-sensitive conventional and novel types of PKC probably because of tyrosine phosphorylation of PKC. PMA may increase the PKC activity in the presence of endogenous activity of Src in cells. Both Ca^{2+} and phosphorylation-signaling pathways including the ERK pathway are required for Na_3VO_4-induced activation of $cPLA_2\alpha$ in L929 cells. Treatment with H_2O_2, another inhibitor of PTP, stimulated release of AA through the activation of $cPLA_2\alpha$ in a similar mechanism [29]. These findings show a role of tyrosine kinases including Src on activation of $cPLA_2\alpha$ in L929 cells.

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