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Enhancement of arachidonic acid release and prostaglandin $F_{2\alpha}$ formation by Na_3VO_4 in PC12 cells and GH3 cells

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Abstract

Both activation of phospholipase A_2 causing arachidonic acid release and tyrosine phosphorylation have been proposed to be involved in neuronal functions. Previously, we reported that orthovanadate (Na_3VO_4), an inhibitor of tyrosine phosphatases, stimulated tyrosine phosphorylation in proteins and enhanced Ca^{2+}-induced noradrenaline release in rat pheochromocytoma PC12 cells. However, the role of tyrosine phosphorylation on phospholipase A_2 activity and/or arachidonic acid release in neuronal cells has not been well established. The effects of Na_3VO_4 on arachidonic acid release and prostaglandin $F_{2\alpha}$ formation were investigated in two types of neuronal cell lines. In PC12 cells, addition of Na_3VO_4 stimulated [^{3}H]arachidonic acid release and prostaglandin $F_{2\alpha}$ formation in a concentration-dependent manner. Co-addition of 5 mM Na_3VO_4 enhanced ionomycin-stimulated [^{3}H]arachidonic acid release. Na_3VO_4 also enhanced ionomycin-stimulated [^{3}H]arachidonic acid release from GH3 cells, a clonal strain from rat anterior pituitary. These findings suggest that the tyrosine phosphorylation pathway regulates arachidonic acid release by phospholipase A_2 and prostaglandin $F_{2\alpha}$ formation in neuronal cells.

Keywords: Tyrosine phosphorylation; Vanadate; Arachidonic acid; Prostaglandin $F_{2\alpha}$; PC12 cell

1. Introduction

It has been established that tyrosine phosphorylation regulates the functions of various proteins and cellular responses. In the nervous system, tyrosine kinases have been implicated in signal transduction regulating neuronal differentiation, synaptic activities and survival (Walaas and Greengard, 1991; Boxall and Lancaster, 1998). Protein-tyrosine phosphatases have also been implicated in signal transduction and functions in neuronal cells (Gordon, 1991; Stone and Dixon, 1994). The overall content of protein phosphotyrosine is dependent on the balance between tyrosine kinase and protein-tyrosine phosphatase activities. Usually, the amount of protein phosphotyrosine is very low in most cells, suggesting a dominance by protein-tyrosine phosphatases. Orthovanadate (Na_3VO_4) is a protein-tyrosine phosphatase inhibitor that leads to the increase of phosphotyrosine in various proteins that are normally retained in dephosphorylated forms. Tyrosine kinases are also phosphorylated and activated by Na_3VO_4 (Stone and Dixon, 1994; Boxall and Lancaster, 1998). In the present study, we used Na_3VO_4 to investigate the role of tyrosine phosphorylation on arachidonic acid release in two types of neuronal cell line.

PC12 cells have been widely used to study the signal transduction mechanisms of neorotrophic factors, because nerve growth factor (NGF) mediates differentiation to neuron-like cells, while proliferation is induced by epidermal growth factor. These factors stimulate receptor-tyrosine kinases to trigger intracellular signal transduction pathways. It has been reported that vanadate regulates neurite formation in PC12 cells (Wu and Bradshaw, 1993; Rogers et al., 1994; Weeks et al., 1999). We reported previously that addition of 5 mM Na_3VO_4 stimulated phosphotyrosine accumulation in several proteins and enhanced Ca^{2+}-stimulated noradrenaline release in PC12 cells (Kitamura et al., 2000). GH3 cells, a clonal strain established from rat anterior pituitary, were suggested as an

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appropriate model for dopaminergic neurons (Yoshinaga et al., 1998). Recently, we reported that a dopaminergic neurotoxin (1-methyl-4-phenylpyridinium ion) induced apoptosis of GH3 cells and its protection by activation of epidermal growth factor receptor (Yoshinaga et al., 2000a). These studies and findings suggested that tyrosine phosphorylation regulates neuronal functions in PC12 cells and GH3 cells.

A variety of actions of arachidonic acid (and its metabolites) on neuronal cell functions were reported; ion channels and enzymes activities, gene expression, neurotransmitters release, the induction of long-term potentiation, differentiation, survival, etc. (for review, see Shimizu and Wolfe, 1990; Kishimoto et al., 1999). Arachidonic acid metabolism in cells is stimulated by a variety of compounds including neurotrophic factors and neurotransmitters. It is reported that NGF caused arachidonic acid release in PC12 cells (DeGeorge et al., 1988; Fink and Guroff, 1990). Previously, we reported that stimulation of ATP receptor enhanced Ca^{2+} ionophore-stimulated arachidonic acid release from PC12 cells (Murayama et al., 1995). In GH3 cells, hydrogen peroxide activated cytosolic phospholipase A_2 and arachidonic acid release, and both hydrogen peroxide and arachidonic acid induced apoptosis of the cells (Yasuda et al., 1999). However, the effects of tyrosine phosphorylation and/or Na_3VO_4 on regulation of phospholipase A_2 and arachidonic acid metabolism in these cells have not been established. In the present study, we show that Na_3VO_4 stimulated arachidonic acid release and prostaglandin $F_{2\alpha}$ formation in PC12 cells. Na_3VO_4 enhanced ionomycin-stimulated arachidonic acid release from PC12 cells and GH3 cells. The findings suggest that arachidonic acid release and prostaglandin $F_{2\alpha}$ formation in neuronal cells are activated via the tyrosine phosphorylation pathway.

2. Experimental procedures

2.1. Materials

[5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (7.92 TBq/mmol, 214 Ci/mmol) was purchased from Amersham (Buckinghamshire, UK). The enzyme immunoassay kit for prostaglandin $F_{2\alpha}$ measurement was purchased from Cayman Chemical (Ann Arbor, MI, USA). Na_3VO_4, 1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethylester (BAPTA-AM), herbimycin, tyrphostin A23 (3,4-dihydroxybenzylidenemalononitrile) and hydrogen peroxide were obtained from Wako (Osaka, Japan). Mastoparan, ionomycin and A23187 were purchased from Sigma (St. Louis, MO, USA). Protein phosphatase inhibitors screening kit was purchased from Alomone Labs (Jerusalem, Israel). Haloenol lactone suicide substrate, a selective inhibitor of Ca^{2+}-independent phospholipase A_2, and 2-(2-amino-3-methoxyphenyl)oxanaphthalen-4-one (PD98059) were obtained from Biomol (Plymouth Meeting, PA, USA). Tyrphostin AG1478 (4-(3-chloroanilino)-6,7-dimethoxyquinazoline) was purchased from Calbiochem (Baden Soden, Germany).

2.2. Culture of PC12 cells and GH3 cells

PC12 cells were cultured on collagen-coated dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, as described previously (Murayama et al., 1995; Naganuma et al., 1999). GH3 cells were cultured in Ham's F10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum, as described previously (Yoshinaga et al., 1998, 2000a,b). Forty-eight hours before the experiments, GH3 cells were plated in dishes and grown as monolayers in F10 medium.

2.3. Measurement of [^{3}H]arachidonic acid release

[^{3}H]Arachidonic acid release was assayed as reported previously (Murayama et al., 1995; Yasuda et al., 1999) with minor modifications. Briefly, PC12 cells on dishes were incubated with DMEM (0.2% fetal bovine serum) and 46 nM (10 μCi (370 kBq)/ml) of [^{3}H]arachidonic acid for 24 h. In GH3 cells, cells were incubated with F10 medium (0.2% horse serum and fetal bovine serum) and 9.2 nM (2 μCi (74 kBq)/ml) of [^{3}H]arachidonic acid for 24 h. The labeled and detached cells were washed by centrifugation ($200 \times g$, 2 min) at 4°C and resuspended in modified Tyrode HEPES buffer (137 mM NaCl, 1 mM Na_2HPO_4, 12 mM $NaHCO_3$, 3 mM KCl, 5 mM glucose, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 20 mM HEPES (pH 7.4)). Cell suspensions (40–60 μg protein) were incubated with the indicated supplements for 30 min (PC12 cells) or 60 min (GH3 cells) at 37°C in the presence of 0.1% fatty acid-free bovine serum albumin (Sigma A-6003). The total incubation volume was 200 μl. The reaction was terminated by addition of 500 μl of ice-cold, Ca^{2+}-, Mg^{2+}-free Tyrode HEPES buffer containing 5 mM EDTA and 5 mM EGTA followed by centrifugation ($8000 \times g$, 30 s) at 4°C. The ^{3}H content of the supernatant (non-esterified [^{3}H]arachidonic acid and its metabolites) was estimated by liquid scintillation spectrometry. Values were calculated as percentages relative to the total incorporation of [^{3}H]arachidonic acid. In some experiments, values are normalized as the percentage of non-stimulated (basal) [^{3}H]arachidonic acid release in order to reduce variation among experiments.

2.4. Measurement of prostaglandin $F_{2\alpha}$ formation in PC12 cells

Confluent PC12 cells on 22-mm dishes (12-well plate) were incubated with the indicated supplements for 30 min at 37°C in the Tyrode HEPES buffer (pH 7.4) containing 0.1% fatty acid-free bovine serum albumin. After centrifu-

gation ($200 \times g$, 30 s, 4°C), the content of prostaglandin $F_{2\alpha}$ in the buffer was determined using an enzyme immunoassay kit.

2.5. Measurement of [^{3}H]arachidonic acid uptake in PC12 cells

Confluent PC12 cells on 22-mm dishes (12-well plate) were incubated with vehicle or 5 mM Na_3VO_4 for 30 or 60 min at 37°C in DMEM containing 0.5% fetal bovine serum and [^{3}H]arachidonic acid (46 nM, 10 μCi/ml/dish). Cells were washed with 1 ml of the Tyrode buffer three times, and solubilized in lysis buffer (0.1 N NaOH, 0.1% Triton X100). The radioactivity was estimated by liquid scintillation spectrometry.

2.6. Measurement of lactate dehydrogenase (LDH) leakage from PC12 cell

Cell viability was estimated by the leakage of LDH as described previously (Ishikawa et al., 1999).

2.7. Statistics

Values are means ± S.E. of the indicated numbers (over three) of independent experiments performed in triplicate. In the case of multiple comparisons, the significance of difference was determined using one-way analysis of variance followed by Dunnett's or Tukey test. For pairwise comparisons, Student's two-tailed *t*-test was used. *P* values at < 0.01 were considered to be significant.

3. Results

3.1. Stimulatory effect of Na_3VO_4 on arachidonic acid release in PC12 cells and GH3 cells

First, we investigated the effects of Na_3VO_4, an inhibitor of protein tyrosine phosphatases, on [^{3}H]arachidonic acid release from PC12 cells and GH3 cells. Addition of 5 μM ionomycin slightly, but not significantly, stimulated [^{3}H]arachidonic acid release from PC12 cells and from GH3 cells (Table 1), as previously reported (Murayama et al., 1995; Yasuda et al., 1999). An addition of 5 mM Na_3VO_4 stimulated [^{3}H]arachidonic acid release by itself, and co-addition of 2 and 5 mM Na_3VO_4 enhanced ionomycin-stimulated [^{3}H]arachidonic acid release in PC12 cells (Table 1, Experiment I). The effect of 5 mM Na_3VO_4 on ionomycin-stimulated [^{3}H]arachidonic acid release was synergistic compared with the estimated effect by a combination of ionomycin and Na_3VO_4. Also in GH3 cells, co-addition of 5 mM Na_3VO_4 enhanced ionomycin-stimulated [^{3}H]arachidonic acid release synergistically (Experiment II).

Table 1
Enhancement of ionomycin-stimulated [^{3}H]arachidonic acid release by Na_3VO_4 in PC12 cells and GH3 cells

Addition	[^{3}H]Arachidonic acid release (% of total)		
	None	2 mM Na_3VO_4	5 mM Na_3VO_4
Experiment I: PC12 cells			
None	2.4 ± 0.1	3.1 ± 0.2	4.2 ± 0.6[a]
Ionomycin	3.3 ± 0.2	5.0 ± 0.5[a]	13.9 ± 1.9[a,b]
Experiment II: GH3 cells			
None	4.3 ± 0.3	3.5 ± 0.2	4.7 ± 0.4
Ionomycin	5.4 ± 0.3	7.7 ± 0.5	13.0 ± 1.0[a,b]

For measurement of [^{3}H]arachidonic acid release, PC12 cells or GH3 cells were incubated for 20 or 60 min, respectively, in the presence of vehicle (None), 5 μM ionomycin or the indicated concentrations of Na_3VO_4. Values are means ± S.E. of four to six independent experiments done in triplicate.

[a] $P < 0.01$, significantly different from vehicle (none).

[b] $P < 0.01$, significantly different from values without Na_3VO_4.

We investigated the effects of other types of protein phosphatase inhibitors on arachidonic acid release. Potent inhibitors of serine/threonine-specific protein phosphatases 1 and 2A (okadaic acid and calyculin A, 20 and 5 μM, respectively) and 2B (deltamethrin, an inhibitor of calcineurin, 100 μM) had no effect on [^{3}H]arachidonic acid release from PC12 cells. In the presence of 20 μM okadaic acid, for instance, basal and 5 μM ionomycin-stimulated [^{3}H]arachidonic acid was 2.2 ± 0.3% and 3.5 ± 0.3%, respectively. The values were almost same as the values without inhibitor shown in Table 1. Similarly, the values in the presence of 5 μM calyculin A and 100 μM deltamethrin were same as those without inhibitors (data not shown).

[^{3}H]Arachidonic acid release by Na_3VO_4 was not due to toxicity, because cell viability (measured by leakage of LDH for 60 min) was similar following 5 mM Na_3VO_4 addition. The basal (non-stimulated) and 5 mM Na_3VO_4-stimulated LDH leakage in medium was 8.0 ± 1.0 and 10.2 ± 0.4 (% of total LDH activity, $n = 3$), respectively. In addition, the intracellular free Ca^{2+} concentrations level in 5 mM Na_3VO_4-treated PC12 cells was similar to that in control cells (Kitamura et al., 2000).

It is probable that Na_3VO_4 suppresses the re-uptake of released [^{3}H]arachidonic acid (and its metabolites) into cells, and thus increases [^{3}H]arachidonic acid release as a result. However, the uptake of [^{3}H]arachidonic acid for 30 and 60 min into PC12 cells was not changed by 5 mM Na_3VO_4 (Table 2).

3.2. Enhancement of cytosolic phospholipase A_2-mediated arachidonic acid release by Na_3VO_4

Secretory phospholipase A_2 requires millimolar concentrations of Ca^{2+} for the activity, although cytosolic phospholipase A_2 is active at micromolar levels of Ca^{2+}. Dithiothreitol splits intramolecular disulfide bonds essen-

Table 2
Effect of Na_3VO_4 on [^{3}H]arachidonic acid uptake in PC12 cells

Addition	[^{3}H]Arachidonic acid uptake (pmol/well)	
	None	5 mM Na_3VO_4
Incubation time (min)		
30	2.08 ± 0.35	2.28 ± 0.37
60	4.04 ± 0.39	3.99 ± 0.45

PC12 cells on 22-mm dishes were incubated with DMEM containing 0.5% fetal bovine serum in the presence of [^{3}H]arachidonic acid (10 μCi/ml/dish) for the indicated period. Five millimolars of Na_3VO_4 was further supplemented in the assay mixture. Cells were washed with the buffer three times and solubilized in lysis buffer, and the radioactivity in cells was measured. Values are means ± S.E. of three independent experiments done in triplicate.

tial for the activity of secretory phospholipase A_2, but dithiothreitol does not inhibit cytosolic phospholipase A_2 (Leslie, 1997). We investigated the effects of $CaCl_2$ on Na_3VO_4-stimulated [^{3}H]arachidonic acid release from PC12 cells (Table 3). Addition of 5 mM Na_3VO_4 stimulated [^{3}H]arachidonic acid release in the presence or absence of extracellular 2 mM $CaCl_2$ (Experiment I). The net increase of [^{3}H]arachidonic acid release by Na_3VO_4 in Ca^{2+}-containing buffer was much higher than that in Ca^{2+}-free buffer. In PC12 cells treated with 20 μM BAPTA-AM (a cell-permeable chelator of Ca^{2+}) for 10 min in the absence of extracellular $CaCl_2$, basal (non-stimulated) [^{3}H]arachidonic acid release in the $CaCl_2$-free buffer was 0.94 ± 0.17 (% of total incorporated [^{3}H]arachidonic acid), which was significantly smaller than the value (2.05 ± 0.60%) in the buffer containing 2 mM $CaCl_2$ (Experiment II). Addition of 2 and 5 mM Na_3VO_4 stimulated [^{3}H]arachidonic acid release from BAPTA-AM-treated PC12 cells in the $CaCl_2$-free and in $CaCl_2$-containing buffer. These findings show that Na_3VO_4-stimulated [^{3}H]arachidonic acid release was not dependent on millimolar Ca^{2+}. In addition, 5 mM dithiothreitol did not inhibit [^{3}H]arachidonic acid release by 5 mM Na_3VO_4 from PC12 cells; 2.1 ± 0.2% by vehicle, 2.2 ± 0.3% by 5 mM dithiothreitol, 4.7 ± 0.4% by 5 mM Na_3VO_4 and 4.2 ± 0.1% by dithiothreitol/Na_3VO_4 ($n = 3$).

Table 3
Stimulation of [^{3}H]arachidonic acid release by Na_3VO_4 from PC12 cells in Ca^{2+}-free conditions

	2 mM $CaCl_2$	$CaCl_2$-free
Experiment I: EGTA-treated PC12 cells		
	[^{3}H]arachidonic acid release (% of total)	
None	2.35 ± 0.12	1.69 ± 0.01
Na_3VO_4 (2 mM)	3.54 ± 0.38	2.22 ± 0.48
Na_3VO_4 (5 mM)	4.09 ± 0.41[a]	3.04 ± 0.54[a]
Experiment II: BAPTA-AM-treated PC12 cells		
	[^{3}H]arachidonic acid release (% of control)	
None	100	100
Na_3VO_4 (2 mM)	131 ± 6[a]	167 ± 23[a]
Na_3VO_4 (5 mM)	206 ± 9[a]	330 ± 63[a]

The prelabeled PC12 cells were detached from dishes and washed three times by centrifugation with $CaCl_2$-free Tyrode HEPES buffer. In Experiment II, cells were further pretreated with 20 μM BAPTA-AM for 10 min in the $CaCl_2$-free buffer, and washed by centrifugation with $CaCl_2$-free buffer. For measurement of [^{3}H]arachidonic acid release, cells were incubated for 20 min with the indicated concentrations of Na_3VO_4 in the absence or presence of 2 mM $CaCl_2$. EGTA (0.2 mM) was further added to the assay mixture. Since [^{3}H]arachidonic acid release from BAPTA-AM-treated PC12 cells showed a wide variation among experiments, values are normalized as percentage of non-stimulated (basal) [^{3}H]arachidonic acid release; the absolute values of [^{3}H]arachidonic acid release from BAPTA-AM-treated PC12 cells were 2.05 ± 0.60 and 0.94 ± 0.17 (% of total incorporated [^{3}H]arachidonic acid) in the presence or absence of 2 mM $CaCl_2$, respectively. Values are means ± S.E. of three to four independent experiments done in triplicate.

[a] $P < 0.01$, significantly different from vehicle (none).

Previously, we reported that mastoparan, a wasp venom peptide, stimulated [^{3}H]arachidonic acid release from PC12 cells (Thang et al., 2000) and GH3 cells (Yasuda et al., 1999) by activation of cytosolic phospholipase A_2 in the absence of extracellular $CaCl_2$. Addition of 2 mM Na_3VO_4 to GH3 cells and 5 mM to PC12 cells enhanced 20 μM mastoparan-stimulated [^{3}H]arachidonic acid release synergistically (Table 4). These findings excluded the involvement of secretory phospholipase A_2 on arachidonic acid release caused by Na_3VO_4. In PC12 cells, the 20 μM mastoparan-stimulated [^{3}H]arachidonic acid in the presence of 20 μM okadaic acid, 5 μM calyculin A and 100 μM deltamethrin were 9.3 ± 0.7%, 8.8 ± 0.8% and 9.2 ± 0.8%, respectively. The values with inhibitors of serine/threonine-specific protein phosphatases were almost same as those without inhibitors shown in Table 4.

3.3. Increase of prostaglandin $F_{2\alpha}$ accumulation by Na_3VO_4 in PC12 cells

Arachidonic acid is metabolized to prostaglandins and other metabolites in cells, and then released to the culture medium. Next, we measured the prostaglandin $F_{2\alpha}$ accu-

Table 4
Enhancement of mastoparan-stimulated [^{3}H]arachidonic acid release by Na_3VO_4 in PC12 cells and GH3 cells

Addition	[^{3}H]Arachidonic acid release (% of total)		
	None	2 mM Na_3VO_4	5 mM Na_3VO_4
Experiment I: PC12 cells			
None	1.7 ± 0.1	2.3 ± 0.2	3.5 ± 0.1[a]
Mastoparan	8.8 ± 0.8[a]	12.1 ± 1.1[a]	15.9 ± 1.8[a,b]
Experiment II: GH3 cells			
None	3.2 ± 0.3	3.5 ± 0.1	4.5 ± 0.3
Mastoparan	10.5 ± 0.5[a]	23.0 ± 2.3[a,b]	Not determined

The labeled PC12 cells and GH3 cells were detached from dishes and washed three times by centrifugation with $CaCl_2$-free Tyrode HEPES buffer. For measurement of [^{3}H]arachidonic acid release, the cells were incubated with 0.2 mM EGTA in the absence of $CaCl_2$. Assay mixtures were further supplemented with 20 μM mastoparan or the indicated concentrations of Na_3VO_4. Values are means ± S.E. of three to five independent experiments done in triplicate.

[a] $P < 0.01$, significantly different from vehicle (none).

[b] $P < 0.01$, significantly different from values without Na_3VO_4.

Table 5
Increase of prostaglandin $F_{2\alpha}$ accumulation by Na_3VO_4 in medium of PC12 cell cultures

Addition	Accumulation of prostaglandin $F_{2\alpha}$ (% of control)	
	None	5 μM Ionomycine
None	100	973 ± 133[a]
Na_3VO_4 (1 mM)	143 ± 18	1202 ± 32[a]
Na_3VO_4 (2 mM)	291 ± 14[a]	1408 ± 117[a]
Na_3VO_4 (5 mM)	746 ± 185[a]	1365 ± 233[a]

PC12 cells were incubated with the indicated concentrations of Na_3VO_4 and/or 5 μM ionomycin. The content of prostaglandin $F_{2\alpha}$ in the buffer after centrifugation ($200 \times g$, 30 s, 4°C) was assayed using an enzyme immunoassay kit. The content of prostaglandin $F_{2\alpha}$ in the Tyrode HEPES buffer without cells was quite low and under the detection limit (< 8 pg/ml). Since prostaglandin $F_{2\alpha}$ accumulation in the buffer showed a wide variation, values are normalized as percentage of non-stimulated (basal) prostaglandin $F_{2\alpha}$ accumulation; the absolute values of prostaglandin $F_{2\alpha}$ accumulation in the buffer was 463 ± 97 pg/well/30 min ($n = 4$). Values are means ± S.E. of three to four independent experiments done in triplicate.

[a] $P < 0.01$, significantly different from vehicle (none).

mulation in medium for 30 min after stimulation with ionomycin or Na_3VO_4 in PC12 cells (Table 5). Addition of 5 μM ionomycin by itself stimulated prostaglandin $F_{2\alpha}$ accumulation tenfold, and addition of 2 and 5 mM Na_3VO_4 also stimulated the prostaglandin $F_{2\alpha}$ accumulation significantly. The effects of ionomycin and Na_3VO_4 were approximately additive. In a typical experiment, prostaglandin $F_{2\alpha}$ accumulation by 5 mM Na_3VO_4 was not inhibited by 5 mM dithiothreitol (data not shown).

3.4. Stimulatory effect of hydrogen peroxide on arachidonic acid release in PC12 cells

Previously, we reported that hydrogen peroxide stimulated [^{3}H]arachidonic acid release via cytosolic phospholipase A_2 activation in GH3 cells (Yasuda et al., 1999). In PC12 cells, addition of 0.2 mM hydrogen peroxide led to increases in phosphotyrosine accumulation in many protein bands in PC12 cells (Kitamura et al., 2000). In a similar conditions, addition of hydrogen peroxide stimulated [^{3}H]arachidonic acid release from PC12 cells; the values in the presence of 0.1 and 0.2 mM hydrogen peroxide were $4.7 \pm 0.3\%$ and $8.1 \pm 0.7\%$ ($n = 3$), respectively. The values with hydrogen peroxide were significantly higher than that with vehicle ($2.2 \pm 0.3\%$).

4. Discussion

4.1. Arachidonic acid release caused by Na_3VO_4 in PC12 cells and GH3 cells

Neuronal tissues are enriched with polyunsaturated fatty acids such as arachidonic acid and the fatty acids are believed to modulate growth cone functions and maturation of synapses (Ikemoto et al., 1999). Recently, it was reported that arachidonic acid induces apoptosis in PC12 cells (Macdonald et al., 1999) and in GH3 cells (Yasuda et al., 1999). These findings show that arachidonic acid release (and its metabolites) regulates neuronal cell functions. It has been reported that Na_3VO_4 regulates neurite outgrowth and the transcription of several response genes in PC12 cells (Wu and Bradshaw, 1993; Rogers et al., 1994). We reported that Na_3VO_4 caused phosphotyrosine accumulation in proteins and enhanced ionomycin-stimulated noradrenaline release in PC12 cells (Kitamura et al., 2000). However, the effect of tyrosine phosphorylation on arachidonic acid release from neuronal cells has not been well established. Thus, in the present study, we investigated the effect of Na_3VO_4 on arachidonic acid release from neuronal cell lines. Na_3VO_4 at 5 mM stimulated arachidonic acid release by itself, and Na_3VO_4 from 2 mM enhanced Ca^{2+}- and mastoparan-stimulated arachidonic acid release from two neuronal cell lines (PC12 and GH3 cells). Addition of Na_3VO_4 also stimulated prostaglandin $F_{2\alpha}$ formation in PC12 cells. Arachidonic acid release caused by Na_3VO_4 was neither due to a decrease of cell viability nor inhibition of arachidonic acid uptake into cells. Previous studies showed that vanadate stimulated and/or enhanced arachidonic acid release and its metabolites in several cells and tissues such as mast cells and platelets (Plass et al., 1992; Goldman et al., 1992; Koike et al., 1993; McNicol et al., 1993). We believe that the present study is the first showing the regulation of arachidonic acid release and prostaglandin $F_{2\alpha}$ formation by Na_3VO_4 in neuronal cells.

4.2. Subtype of phospholipase A_2 activated by Na_3VO_4

Phospholipase A_2s are subdivided into several classes, among which (1) cytosolic phospholipase A_2, (2) a family of secretory phospholipase A_2, and (3) Ca^{2+}-independent phospholipase A_2 have received considerable attention. The subtype of phospholipase A_2 that is activated by Na_3VO_4 in PC12 cells is not secretory phospholipase A_2. The reasons are as follows: Na_3VO_4 stimulated arachidonic acid release from BAPTA-AM-treated PC12 cells in Ca^{2+}-free buffer and the effect of Na_3VO_4 was not inhibited by dithiothreitol. Although the stimulatory effect of Na_3VO_4 on arachidonic acid release was observed in Ca^{2+}-depleated PC12 cells, Na_3VO_4 enhanced ionomycin-stimulated [^{3}H]arachidonic acid release in PC12 cells and in GH3 cells synergistically (Table 1). Previously, we showed the existence of cytosolic phospholipase A_2 protein and that [^{3}H]arachidonic acid release was inhibited by a selective inhibitor of cytosolic phospholipase A_2 in both types of cells (Yasuda et al., 1999; Thang et al., 2000). Na_3VO_4 enhanced mastoparan-induced [^{3}H]arachidonic acid release in both types of cells (Table 2). It was reported that an ionophore toxin (Pardaxin)-induced arachidonic acid release was inhibited by Ca^{2+}-indepen-

dent phospholipase A_2 inhibitor in PC12 cells (Abu-Raya et al., 1998). However, treatment with 30 μM haloenol lactone suicide substrate, a selective inhibitor of Ca^{2+}-independent phospholipase A_2, did not inhibit the effect of Na_3VO_4 on arachidonic acid release in PC12 cells (data not shown). These findings show the involvement of cytosolic and Ca^{2+}-sensitive phospholipase A_2 on arachidonic acid release by Na_3VO_4 in the neuronal cells.

4.3. Possible mechanisms of cytosolic phospholipase A_2 activation by Na_3VO_4

It has been shown that the increase in cytosolic phospholipase A_2 activity is attributed to phosphorylation of serine residues of cytosolic phospholipase A_2 by the mitogen-activated protein (MAP) kinase family such as extracellular signal-regulated kinases 1 and 2 (ERK1/2) (for review, see Leslie, 1997). The inactivation of ERK1/2 is mediated by dephosphorylation of ERK1/2 by dual specificity phosphatases called MAP kinase phosphatases, and MAP kinase phosphatases are inhibited by vanadate in PC12 cells (Misra-Press et al., 1995). Previously, we reported that Na_3VO_4 stimulated phosphotyrosine accumulation in several proteins, one of which seemed to be ERK1/2, in PC12 cells (Kitamura et al., 2000). In addition, Fujiwara et al. (1997) reported that 3,4-dephostatin, a protein-tyrosine phosphatase inhibitor, sustained for 1–3 h the tyrosine phosphrylation and activation of ERK1/2 induced by NGF and epidermal growth factor in PC12 cells. It is likely that Na_3VO_4 stimulated activation of ERK1/2 by inhibition of MAP kinase phosphatases, and thus, activation of cytosolic phospholipase A_2 in PC12 cells. Although PD98059, an inhibitor of ERK1/2 kinase, inhibited platelet-activating factor release by cytosolic phospholipase A_2 in human neutrophils (Coffer et al., 1998), it failed to inhibit arachidonic acid release caused by compound 48/80 in rat mast cells (Shefler et al., 1999) and by mastoparan in GH3 cells (Yasuda et al., 1999). In platelets, the phosphorylation of cytosolic phospholipase A_2 by ERK1/2 is not a critical factor for arachidonic acid release, and unidentified kinases for activation of cytosolic phospholipase A_2 are proposed (Kramer et al., 1996). Further studies are necessary to identify the role of ERK1/2 on Na_3VO_4-stimulated arachidonic acid release.

Tyrosine kinases are phosphorylated and activated by Na_3VO_4 (Stone and Dixon, 1994; Boxall and Lancaster, 1998). It has been demonstrated that the cells exposed to vanadium including vanadate produce reactive oxygen species including hydrogen peroxide and thus induce various biological and pharmacological effects (Stohs and Bagchi, 1995; Ye et al., 1999; Huang et al., 2000). In PC12 cells, addition of hydrogen peroxide stimulated phosphotyrosine accumulation in several proteins (Kitamura et al., 2000) and [^{3}H]arachidonic acid release as described in the current study. Accordingly, the effect of Na_3VO_4 may be due to the generation of hydrogen peroxide, which is known to stimulate tyrosine kinases in cells. Non-receptor tyrosine kinases are involved in signal events stimulated by reactive oxygen species in various cells (Devary et al., 1992; Abe et al., 1997). Cytosolic phospholipase A_2 is proposed to be phosphorylated on tyrosine residues by tyrosine kinase Jak1 that was co-immunoprecipitated with cytosolic phospholipase A_2 (Kast et al., 1993; Flati et al., 1996). Although the existence of Jak is not confirmed, there are many non-receptor tyrosine kinases such as Src family in PC12 cells (Kobayashi et al., 1997; Soltoff, 1998). Thus, the direct involvement of tyrosine kinases on cytosolic phospholipase A_2 is probable.

It was reported that NGF caused an increased release and metabolism of arachidonic acid in PC12 cells (DeGeorge et al., 1988; Fink and Guroff, 1990). However, addition of NGF did not stimulate [^{3}H]arachidonic acid release from PC12 cells in the presence or absence of 5 mM Na_3VO_4 in our conditions (data not shown). The reasons for this discrepancy are unclear at this point. In preliminary experiments, treatment for 24 h with 10 μM herbimycin, a general inhibitor of tyrosine kinase, did not inhibit the stimulatory effect of Na_3VO_4 in PC12 cells. And, treatment for 2 h with 5 μM tyrphostin AG1478 or A23 did not inhibit the effect. We are looking into the possible involvement of these inhibitors-insensitive tyrosine kinases(s) on the effect of Na_3VO_4 in the next study.

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