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Arachidonic acid release and prostaglandin $F_{2\alpha}$ formation induced by phenylarsine oxide in PC12 cells: possible involvement of secretory phospholipase A_2 activity

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Abstract

Activation of phospholipase A_2 (PLA_2) causing arachidonic acid (AA) release is involved in neuronal cell functions. Previously, we reported AA release and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) formation via activation of cytosolic PLA_2 by orthovanadate (Na_3VO_4), an inhibitor of tyrosine phosphatases, in rat pheochromocytoma PC12 cells. We investigated the effects of phenylarsine oxide (PAO), which reacts with sulfhydryl groups of proteins and thus acts as an inhibitor of tyrosine phosphatases, on AA release and $PGF_{2\alpha}$ formation in PC12 cells. PAO stimulated [^{3}H]AA release from the prelabeled cells and $PGF_{2\alpha}$ formation. The PAO responses were dependent upon the concentrations used (10 μM to 0.5 mM) and on extracellular $CaCl_2$. [^{3}H]AA release induced by PAO was decreased significantly by inhibition of secretory, but not cytosolic, PLA_2. [^{3}H]AA release by PAO was not reversed by washing the cells, but the addition of dithiol compounds such as 2,3-dimercapto-1-propanol decreased the release from the PAO-treated cells. The existence of mRNA of types I_B and II_C secretory PLA_2 in PC12 cells was detected by reverse transcriptase–polymerase chain reaction using specific primers. Addition of secretory PLA_2 from bee venom to the assay mixture stimulated [^{3}H]AA release, and PAO enhanced the response synergistically. The addition of 0.1 mM PAO directly enhanced the activity of secretory PLA_2 from bee venom. These findings suggest that PAO stimulates AA release and $PGF_{2\alpha}$ formation probably via activation of secretory PLA_2 in PC12 cells.

Keywords: Phenylarsine oxide; Arachidonic acid; Prostaglandin $F_{2\alpha}$; Secretory phospholipase A_2; PC12 cells

1. Introduction

PLA_2 catalyzes the hydrolysis of phospholipids at the *sn*-2 position to produce lysophospholipids and fatty acids such as AA [1–3]. Three types of PLA_2 have been identified: the 10- to 14-kDa secretory type, the 85- to 100-kDa cytosolic type, and the 80-kDa Ca^{2+}-independent type. Secretory PLA_2s require Ca^{2+} at millimolar concentrations for their activation, and are stabilized by disulfide linkages but inactivated by reducing agents such as dithiothreitol [1,4]. Secretory PLA_2s are known to exist in both an extracellular form in inflammatory fluids and a cell-associated form; some are involved in inflammatory responses, formation of PGs, and apoptosis [5–8]. Cytosolic Ca^{2+}-dependent PLA_2 (type IV) exhibits a preference for unsaturated fatty acids such as AA in the *sn*-2 position of phospholipids, and is regulated by Ca^{2+} at ∼micromolar concentrations and its phosphorylation.

Sulfhydryl-modifying reagents such as iodoacetamide react with the cysteine residues near the active center, and inactivate cytosolic PLA_2 activity [2,9]. Cytosolic PLA_2 plays an essential role in the production of lipid mediators in response to receptor-mediated cellular signaling events. Ca^{2+}-independent PLA_2 possibly serves as a housekeeping enzyme involved in the remodeling of membrane phospholipids [2,3].

In the brain, AA and its metabolites such as PGs are formed upon PLA_2 activation and are involved in neuronal functions including long-term potentiation, ion channel

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Abbreviations: PLA_2, phospholipase A_2; AA, arachidonic acid; PGs, prostaglandins; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PAO, phenylarsine oxide; RT–PCR, reverse transcriptase–polymerase chain reaction; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration.

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activity, and neurotransmitter release [10]. In addition, activation of cytosolic PLA_2 after ischemia was suggested to play a role in the neuronal damage [11–13]. Secretory PLA_2s have also been known to be neurotoxic and promote neuronal injury [14,15]. Secretory PLA_2 is released from rat brain synaptosomes and PC12 cells (a neuronal cell line), and inhibitors of type II secretory PLA_2 suppress the release of neurotransmitters in PC12 cells [16,17]. The addition of secretory PLA_2 to PC12 cells accelerates AA release and apoptotic cell death induced by deprivation of nerve growth factor [18], and AA stimulates apoptosis accompanied by DNA laddering in GH3 cells, a rat pituitary cell line [19]. These previous findings suggested the involvement of both cytosolic and secretory PLA_2 in the regulation of neuronal cell functions, including cell death.

PAO, an arsine oxide derivative, interacts with vicinal dithiol-containing molecules including enzymes and transcription factors, and is also known as an inhibitor of tyrosine phosphatases [20–24]. Previously, we reported that orthovanadate (Na_3VO_4), a general potent inhibitor of tyrosine phosphatases, stimulates [^{3}H]AA release and $PGF_{2\alpha}$ formation in PC12 cells probably via activation of cytosolic PLA_2 activity [25]. During this study, we screened various phosphatase inhibitors and found that PAO alone markedly stimulated AA release. Here we show that PAO stimulated AA release and $PGF_{2\alpha}$ formation in an extracellular $CaCl_2$-dependent manner in PC12 cells. Although washing the cells did not reverse the stimulatory effect of PAO, the effect of PAO was inhibited by dithiol compounds such as dithiothreitol and 2,3-dimercapto-1-propanol, but not by monothiol compounds. An inhibitor of secretory PLA_2, but not inhibitors of cytosolic and Ca^{2+}-independent PLA_2s, decreased PAO-stimulated AA release. We confirmed the existence of mRNAs of group I_B and II_C secretory PLA_2 in PC12 cells by RT–PCR. These findings suggest that sulfhydryl modification of the secretory type of PLA_2 by PAO regulated AA release and $PGF_{2\alpha}$ formation in PC12 cells.

2. Materials and methods

2.1. Materials

[5,6,8,9,11,12,14,15-^{3}H]AA (215 Ci/mmol, 7.96 TBq/mmol) was purchased from Amersham. Mastoparan and aristolochic acid were obtained from the Sigma Chemical Co. A screening kit for protein phosphatase inhibitors was purchased from Alomone Lab. PAO, 2,3-dimercapto-1-propanol, and thioglycerol were obtained from Wako. Dithiothreitol and 2-mercaptoethanol were obtained from Nacalai. Arachidonyl trifluoromethyl ketone ($AACOCF_3$) and haloenol lactone suicide substrate [HELSS, *E*-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one] were obtained from Research Biochemicals and Biomol. Research Lab. Inc., respectively. Secretory PLA_2 from bee venom was purchased from Cayman (Catalog No. 60500).

2.2. Cell culture and measurement of [^{3}H]AA release

PC12 cells were cultured on collagen-coated dishes (collagen type I-coated, IWAKI) in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, as reported previously [25,26]. [^{3}H]AA release from prelabeled PC12 cells was determined as described previously [25,26] with minor modifications. In brief, PC12 cells on dishes were incubated with Dulbecco's modified Eagle's medium (0.2% serum) and 1 μCi/mL (37 kBq/mL) of [^{3}H]AA for 24 hr. The labeled cells were detached from dishes by pipetting. The cells were washed and suspended in a modified Tyrode HEPES buffer [137 mM NaCl, 5 mM KCl, 5 mM glucose, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 20 mM HEPES (pH 7.4)]. In some experiments, the cells were suspended in the $CaCl_2$- and $MgSO_4$-free Tyrode buffer and incubated for 5 min with 0.5 mM PAO. Cell suspensions (30–50 μg protein) were incubated with the indicated agents for 30 min at 37° in the presence of 0.1% fatty acid-free bovine serum albumin (Sigma A-6003). The total volume was 200 μL, and the reaction was terminated by the addition of 500 μL of ice-cold Ca^{2+}-, Mg^{2+}-free Tyrode buffer containing 5 mM EDTA and 5 mM EGTA followed by centrifugation (8000 *g*, 30 sec) at 4°. The ^{3}H content of the supernatant was estimated by liquid scintillation spectrometry. Values were calculated as percentages relative to the total incorporation of [^{3}H]AA.

2.3. Measurement of $PGF_{2\alpha}$ formation in PC12 cells

Confluent PC12 cells on 22-mm dishes (12-well plate) were incubated with the indicated agents for 30 min at 37° in the Tyrode HEPES buffer (pH 7.4) containing 0.1% fatty acid-free albumin. The content of $PGF_{2\alpha}$ in the buffer after centrifugation (200 *g*, 30 sec, 4°) was determined using an enzyme immunoassay kit (Cayman Chemical).

2.4. RT–PCR analysis

Total RNA was prepared from PC12 cells using TRIzol reagent (Sigma, 1 mL/100 mm dish). Total RNA (1 μg) was reverse-transcribed in a mixture containing oligo(dT)12–18 primer and SuperScript™ RT (Gibco BRL). PCR was carried out in a 15 μL reaction volume containing 1.5 μL of cDNA mixture, 1× PCR buffer containing 1.5 mM $MgCl_2$, 200 μM of each dNTP, 20 μM of each primer, and 1 U of *Taq* DNA polymerase (Gibco BRL). The DNA was denatured for 10 min at 95° prior to each PCR cycle of 95° for 1 min, 50–62° (annealing) for 1 min, 72° (elongation) for 1 min. Following the last cycle, elongation was extended for an additional 7 min at 72° before refrigeration. The primers for type I_B secretory PLA_2 were: sense 5′-CGCCAAGATGAAACTC-

CTTC-3′ and antisense 5′-TAGACAGGAAGTGGGGT-GAC-3′ (expected product 471 bp); for type II_A secretory PLA_2 they were: sense 5′-AGTTTGGGCAAATGATTC-TG-3′ and antisense 5′-TCTTTCAGCAACTGGGCGTC-3′ (expected product 372 bp); for type II_C secretory PLA_2 they were: sense 5′-TGAACTGGCAGATGAAGGTG-3′ and antisense 5′-GGCTCAGACTAGAGCAGGTG-3′ (expected product 550 bp), as reported previously [27]. The number of cycles selected for each primer pair was found to produce a linear relationship between the input RNA and the resulting PCR products. The PCR products were analyzed by 6.5% non-denaturing polyacrylamide gel electrophoresis and visualized with ethidium bromide.

2.5. Statistics

Values are means ± SEM of the indicated numbers (over three) of independent experiments performed in triplicate assays. In the case of multiple comparisons, the significance of differences was determined using one-way analysis of variance followed by Dunnett's or Tukey's test. For pairwise comparisons, Student's two-tailed *t*-test was used. *P* values at <0.05 were considered to be significant.

3. Results

3.1. Extracellular Ca^{2+}-dependent [^{3}H]AA release by PAO via secretory PLA_2 activation in PC12 cells

Previously, we reported that the addition of 5 mM Na_3VO_4 alone stimulates [^{3}H]AA release for 20 min in PC12 cells [25]. Of the tyrosine phosphatase inhibitors examined, PAO, but neither sodium fluoride nor pyrophosphate, markedly stimulated [^{3}H]AA release in PC12 cells. The addition of PAO to the assay mixture markedly stimulated [^{3}H]AA release in a concentration-dependent manner (Fig. 1). In Ca^{2+}-free and 0.2 mM EGTA-containing buffer, the effect of 0.5 mM PAO was not detected (Fig. 1).

Next, we investigated the effects of inhibitors of PLA_2 on 0.5 mM PAO-stimulated [^{3}H]AA release in PC12 cells (Table 1). $AACOCF_3$ is a specific inhibitor of cytosolic PLA_2, and its inhibition of other types of PLA_2 is reduced by more than 1000-fold [28]. Previously, we reported that treatment for 10 min with 10 μM $AACOCF_3$, the concentration at which it inhibits cytosolic PLA_2 but neither secretory PLA_2 nor Ca^{2+}-independent PLA_2 [29], almost completely inhibits 20 μM mastoparan-stimulated [^{3}H]AA release induced by activation of cytosolic PLA_2 in PC12 cells [26]. The effect of 0.5 mM PAO, however, was not inhibited even by treatment with 30 μM $AACOCF_3$ for 20 min. HELSS (10 μM) is a potent and selective inhibitor of Ca^{2+}-independent PLA_2 [3]. The effect of 0.5 mM PAO was not inhibited significantly by 10 μM (data not shown) or 30 μM HELSS (Table 1). Aristolochic acid is widely

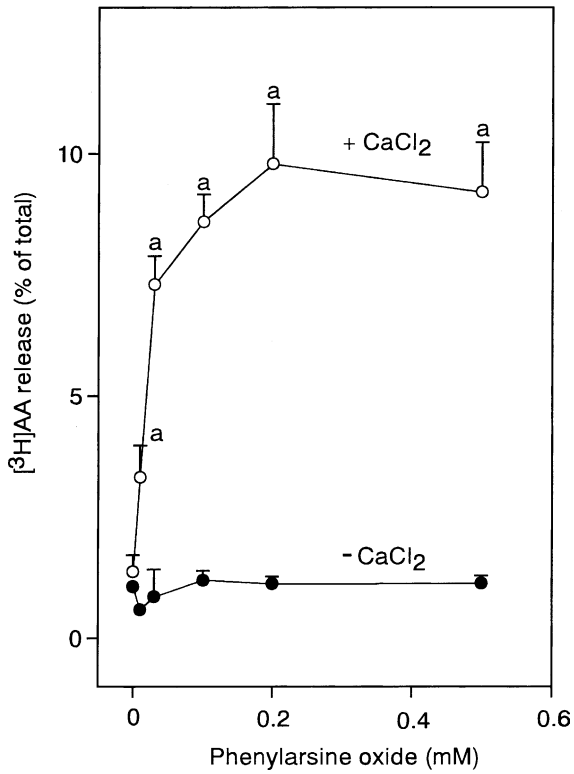

Fig. 1. Extracellular Ca^{2+}-dependent [^{3}H]AA release by PAO in PC12 cells. The PC12 cells prelabeled with [^{3}H]AA were washed three times by centrifugation with $CaCl_2$-free Tyrode HEPES buffer. For measurement of [^{3}H]AA release, the cells were incubated for 30 min with vehicle or the indicated concentrations of PAO in the absence (●) or presence (○) of 2 mM $CaCl_2$. Reactions were terminated by the addition of 500 μL of ice-cold Ca^{2+}-, Mg^{2+}-free Tyrode buffer containing 5 mM EDTA and 5 mM EGTA. In addition, EGTA (0.2 mM) was further added to the assay mixture. Values are means ± SEM of 3 independent experiments done in triplicate. Key: (a) significantly different from vehicle, $P < 0.05$.

Table 1
Effects of PLA_2 inhibitors on PAO-stimulated [^{3}H]AA release in PC12 cells

Treatment/addition	[^{3}H]AA release (% of total)	
	None	0.5 mM PAO
Experiment I		
None	1.97 ± 0.13	7.67 ± 0.24
$AACOCF_3$ (30 μM)	2.03 ± 0.16	7.21 ± 0.37
HELSS (30 μM)	1.76 ± 0.23	6.44 ± 0.45
Experiment II		
None	1.84 ± 0.32	6.42 ± 0.25 (100)
Aristolochic acid (20 μM)	1.59 ± 0.31	4.44 ± 0.53* (53.1 ± 6.5*)

Prelabeled PC12 cells were preincubated with vehicle, 30 μM $AACOCF_3$, or 30 μM HELSS for 20 min (in Experiment I) or with vehicle or 20 μM aristolochic acid for 90 min (in Experiment II). Then the washed cells were incubated with vehicle or 0.5 mM PAO for 30 min in the presence of the same concentrations of PLA_2 inhibitors. Since the inhibitory effect of aristolochic acid was partial and showed a variation among experiments, the values in parentheses are normalized as the percentage of 0.5 mM PAO-stimulated [^{3}H]AA release from control cells. Values are means ± SEM for 3 independent experiments done in triplicate.
* $P < 0.05$, significantly different from vehicle.

used as an inhibitor of secretory PLA_2 [6,30]. The effect of 0.5 mM PAO was partially, but significantly, inhibited by 20 μM aristolochic acid. Aristolochic acid did not inhibit the [^{3}H]AA release induced by 30 μM mastoparan; the values were 10.2 ± 2.2 and 10.8 ± 2.6 (% of total, N = 4) in the absence and presence of 20 μM aristolochic acid, respectively. Treatment with higher concentrations of aristolochic acid (40 μM and over) did not show a significantly higher inhibitory effect (data not shown). These findings suggest that PAO stimulates secretory PLA_2 activity in PC12 cells, which requires Ca^{2+} at millimolar concentrations, and is inhibited by aristolochic acid.

3.2. Inhibition of PAO-stimulated [^{3}H]AA release by vicinal dithiol compounds

PAO interacts with small molecular weight dithiol compounds such as dithiothreitol but hardly interacts with monothiol compounds [22,24]. Dithiol compounds such as dithiothreitol and 2,3-dimercapto-1-propanol at 1 mM, but not monothiol compounds such as thioglycerol and 2-mercaptoethanol, abolished the effect of 0.5 mM PAO on [^{3}H]AA release (Table 2). Neither 2,3-dimercapto-1-propanol nor the monothiol compounds at 1 mM inhibited [^{3}H]arachidonic acid release induced by 30 μM mastoparan via activation of cytosolic PLA_2 (Table 2). The effect of mastoparan was not inhibited by dithiothreitol (data not shown, but see Ref. [26]).

In the PAO-treated PC12 cells, which were first incubated with 0.5 mM PAO for 5 min in $CaCl_2$-free Tyrode buffer, washed with PAO-free buffer and then incubated without PAO in $CaCl_2$-containing buffer, [^{3}H]AA release was significantly higher (6.84% of total) compared with control cells (1.52% of total), which were incubated 30 min with vehicle in $CaCl_2$-containing buffer (Table 3). The addition of 0.1 mM (data not shown) and 0.5 mM PAO into the assay mixture did not show an additive effect on [^{3}H]AA release in the PAO-treated PC12 cells. Interestingly, [^{3}H]AA release from the 0.5 mM PAO-treated cells was inhibited significantly by dithiol compounds (dithiothreitol and 2,3-dimercapto-1-propanol). The effects of the monothiol compounds were limited (Table 3).

Table 2
Effects of thiol compounds on PAO-stimulated [^{3}H]AA release in PC12 cells

Addition	[^{3}H]AA release (% of total)		
	None	0.5 mM PAO	30 μM Mastoparan
None	1.52 ± 0.18	6.94 ± 0.27*	7.99 ± 0.34*
DTT	1.38 ± 0.19	1.60 ± 0.29**	Not inhibited[a]
DMP	1.49 ± 0.09	2.28 ± 0.04**	6.61 ± 0.10
ME	1.45 ± 0.19	5.79 ± 0.25	7.33 ± 0.56
TG	1.49 ± 0.33	6.61 ± 0.37	7.35 ± 0.40

Prelabeled PC12 cells were treated with vehicle, 0.5 mM PAO, or 30 μM mastoparan for 30 min. The assay mixture was supplemented further with 1 mM thiol compounds or vehicle. The thiol compounds used were dithiols [dithiothreitol (DTT) and 2,3-dimercapto-1-propanol (DMP)] and monothiols [2-mercaptoethanol (ME) and thioglycerol (TG)]. Values are means ± SEM for 3 independent experiments done in triplicate.

[a] Ref. [26].

* $P < 0.001$, significantly different from vehicle.

** $P < 0.001$, significantly different from values without thiol compounds.

Table 3
[^{3}H]AA release in the PAO-treated PC12 cells and its inhibition by dithiol compounds

Addition	[^{3}H]AA release (% of total)	
	None	0.5 mM PAO
Control cells (Table 2)	1.52 ± 0.18	6.94 ± 0.27*
0.5 mM PAO-treated cells		
None	6.84 ± 1.57*	5.24 ± 1.12
DTT	2.61 ± 0.46**	3.03 ± 0.60
DMP	2.61 ± 0.46**	3.62 ± 0.37
ME	4.99 ± 0.92	3.54 ± 1.08
TG	4.85 ± 1.01	3.70 ± 1.85

Prelabeled PC12 cells were incubated with 0.5 mM PAO in $CaCl_2$-free buffer for 5 min. Cells were washed and finally incubated with 1 mM thiol compounds or vehicle in $CaCl_2$-containing buffer for 30 min with or without the addition of 0.5 mM PAO. The thiol compounds used were dithiols [dithiothreitol (DTT) and 2,3-dimercapto-1-propanol (DMP)] and monothiols [2-mercaptoethanol (ME) and thioglycerol (TG)]. Values are means ± SEM for 4 independent experiments done in triplicate.

* $P < 0.01$, significantly different from the value without PAO in the control cells.

** $P < 0.05$, significantly different from the values without thiol compounds.

3.3. Enhancement of [^{3}H]AA release induced by exogenous addition of secretory PLA_2 from bee venom

Next we investigated the effect of the isolated secretory PLA_2 from bee venom (type III) on [^{3}H]AA release in PC12 cells (Fig. 2). The addition of the secretory PLA_2 (0.1 μg/mL) to the assay mixture markedly stimulated [^{3}H]AA release from the prelabeled PC12 cells, and the effect in the presence of 2 mM $CaCl_2$ was significantly higher than that in the Ca^{2+}-free buffer. [^{3}H]AA release induced by the addition of 0.5 μg/mL of secretory PLA_2 in the presence of $CaCl_2$ was 8.7 ± 0.7% (N = 3), which was similar to that by 0.1 μg/mL of secretory PLA_2. Interestingly, 0.5 mM PAO synergistically stimulated [^{3}H]AA release induced by secretory PLA_2 (0.1 μg/mL). The synergistic effect of PAO on [^{3}H]AA release was completely dependent upon extracellular $CaCl_2$ (Fig. 2) and was abolished by the addition of 1 mM 2,3-dimercapto-1-propanol and dithiothreitol (data not shown).

Next the effect of PAO on the activity of secretory PLA_2 was measured by using a secretory PLA_2 assay kit (Cayman). In the present conditions, an increase of absorbance units (A_{414}) by purified secretory PLA_2 from bee venom (40 ng) was linear at least for 10 min, and the value for 10 min was 0.5. The addition of 0.1 mM PAO to the assay mixture increased absorbance to 1.2 units. In the case of PC12 cell homogenates, however, we could not

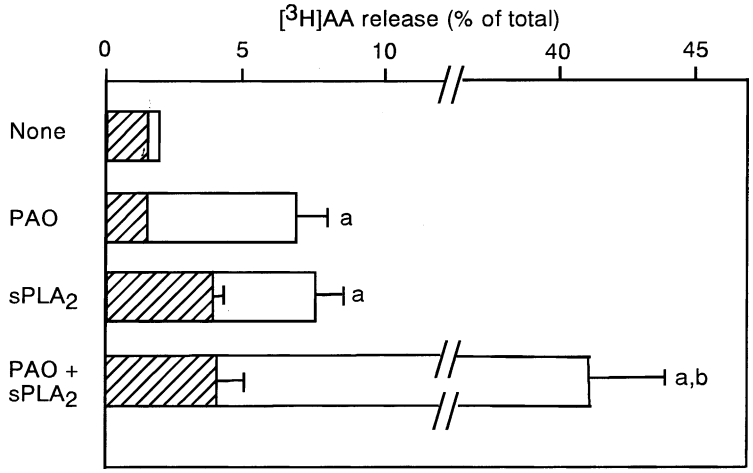

Fig. 2. [^{3}H]AA release by exogenous addition of secretory PLA_2 and its enhancement by PAO. The PC12 cells prelabeled with [^{3}H]AA were washed three times by centrifugation with $CaCl_2$-free Tyrode HEPES buffer. The cells were incubated for 30 min with vehicle, 0.5 mM PAO, 0.1 μg/mL of secretory PLA_2 from bee venom, or the combination of PAO and secretory PLA_2 in the absence (hatched bars) or presence (open bars) of 2 mM $CaCl_2$. Reactions were terminated by the addition of 500 μL of ice-cold Ca^{2+}-, Mg^{2+}-free Tyrode buffer containing 5 mM EDTA and 5 mM EGTA. In addition, EGTA (0.2 mM) was further added to the assay mixture. Values are means ± SEM of 3 independent experiments done in triplicate. Key: (a) significantly different from the value in the absence of $CaCl_2$, $P < 0.01$; and (b) significantly different from the additive value by PAO and secretory PLA_2 from bee venom, $P < 0.01$.

Table 4
Increase in $PGF_{2\alpha}$ formation by PAO in PC12 cell culture medium

Addition	Increase in $PGF_{2\alpha}$ (% of control)	
	2 mM $CaCl_2$	$CaCl_2$-free
None	100 (453 ± 87 pg/well/30 min)	100 (90 ± 26 pg/well/30 min)
PAO (0.1 mM)	987 ± 225*	232 ± 33*
PAO (0.5 mM)	2430 ± 529*	Not determined

PC12 cells on 22-mm dishes were incubated in Tyrode HEPES buffer containing 0.1% bovine serum albumin (fatty acid-free grade) for 30 min in the presence or absence of 2 mM $CaCl_2$. The indicated concentrations of PAO or vehicle were further added to the assay mixture. The content of $PGF_{2\alpha}$ in the buffer was assayed using an enzyme immunoassay kit. The content of $PGF_{2\alpha}$ in the buffer without cells was under the detection limit (<8 pg/mL). The absolute values of $PGF_{2\alpha}$ formation without PAO are shown in parentheses. Since $PGF_{2\alpha}$ formation in the buffer showed a wide variation, values are normalized as the percentage of basal $PGF_{2\alpha}$ formation without PAO. Values are means ± SEM for 3–4 independent experiments done in triplicate.

* $P < 0.05$, significantly different from vehicle.

determine the PLA_2 activity properly; factors such as glutathione in the cell homogenates probably disturbed the assay-system in the kit, and the increase of absorbance units was not linear (data not shown).

3.4. Increase in $PGF_{2\alpha}$ formation by PAO

Previously, we reported that the addition of Na_3VO_4 increases $PGF_{2\alpha}$ formation in the PC12 cell culture medium [25]. The addition of 0.1 and 0.5 mM PAO increased $PGF_{2\alpha}$ formation significantly (Table 4). In the absence of extracellular $CaCl_2$, $PGF_{2\alpha}$ formation in the medium was low; the value was 10–30% of that in the presence of 2 mM $CaCl_2$. In addition, the effect induced by 0.1 mM PAO in the absence of $CaCl_2$ was much less (200–300% of the control value without PAO) compared with that in the presence of $CaCl_2$ (Table 4). In a control experiment, the co-addition of 1 mM dithiothreitol alone had no effect on $PGF_{2\alpha}$ formation, whereas dithiothreitol inhibited 0.1 mM PAO-stimulated $PGF_{2\alpha}$ formation almost completely (80–110% of the control value without PAO) (data not shown).

3.5. Detection of type I_B and type II_C secretory PLA_2 transcripts in PC12 cells by RT–PCR

Both mRNA and protein of type II secretory PLA_2 were detected previously in PC12 cells [16,17]. Although type II secretory PLA_2 was divided into three subtypes (A, B, and C) [31], the subtype of PLA_2 in PC12 cells was not determined in their studies. We revealed the existence of

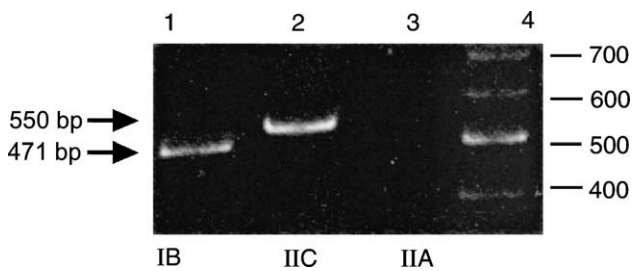

Fig. 3. Detection of secretory PLA_2 mRNA by RT–PCR in PC12 cells. Total RNA was isolated from PC12 cells and run in the presence of RT. In lanes 1, 2, and 3, the primers for type I_B, II_C, and II_A secretory PLA_2s were used, respectively. Lane 4 shows a 100 bp DNA ladder.

the transcript for type II_C, but not type II_A, secretory PLA_2 in PC12 cells by RT–PCR (Fig. 3). The transcript for type I_B secretory PLA_2 was also detected in PC12 cells.

4. Discussion

4.1. AA release via activation of secretory PLA_2 by PAO in PC12 cells

Previously, we reported that Na_3VO_4, a general inhibitor of protein tyrosine phosphatases, stimulates [^{3}H]AA release and $PGF_{2\alpha}$ formation, and enhances ionomycin- and mastoparan-stimulated [^{3}H]AA release via activation of cytosolic PLA_2 in PC12 cells [25]. In the process of that study, we examined the effects of various inhibitors of protein phosphatases, and found that PAO stimulated AA release and $PGF_{2\alpha}$ formation in PC12 cells. PAO, an arsine oxide derivative, forms stable ring structures with vicinal sulfhydryl groups of cellular proteins and enzymes [22,24], and thus PAO has been widely used as an inhibitor of protein tyrosine phosphatases [20,21,23]. However, the effects of PAO in the present study appeared to be derived from the activation of secretory PLA_2, but not cytosolic PLA_2. The reasons are as follows. First, [^{3}H]AA release stimulated by PAO was almost completely dependent upon the existence of millimolar quantities of extracellular $CaCl_2$ (Fig. 1). In addition, $PGF_{2\alpha}$ formation induced by PAO (Table 4) was induced markedly in the $CaCl_2$-containing buffer. In contrast, Na_3VO_4 markedly stimulated [^{3}H]AA release from PC12 cells treated with BAPTA-AM, a cell-permeable chelator of Ca^{2+}, in Ca^{2+}-free buffer [25]. Second, the effect of PAO on [^{3}H]AA release was inhibited significantly by aristolochic acid (20 μM, an inhibitor of secretory PLA_2), but not by $AACOCF_3$ (30 μM, an inhibitor of Ca^{2+}-dependent cytosolic PLA_2) (Table 1). Third, we confirmed the existence of secretory PLA_2 mRNA (type I_B and II_C) in PC12 cells by RT–PCR (Fig. 3). Fourth, PAO enhanced [^{3}H]AA release induced by exogenous addition of purified secretory PLA_2 from bee venom (Fig. 2). These findings suggest that PAO causes AA release and $PGF_{2\alpha}$ formation by stimulation of secretory PLA_2 activity in PC12 cells. To our knowledge, the present study is the first showing AA release and $PGF_{2\alpha}$ formation by PAO in neuronal cells.

4.2. Possible mechanism(s) of activation of secretory PLA_2 in PC12 cells by PAO

Na_3VO_4 stimulates [^{3}H]AA release probably via the tyrosine phosphorylation pathway and activation of cytosolic PLA_2 in PC12 cells [25]. In a preliminary experiment using immunoblotting with anti-phosphotyrosine antibody, incubation with 0.1 mM PAO for 10 min led to increases in phosphotyrosine accumulation in many protein bands in PC12 cells (data not shown). Yamamoto *et al.* [32] reported that PAO induced tyrosine phosphorylation of protein kinase Cδ in CHO cells. Although activation of P_{2Y2} receptors by ATP stimulates tyrosine phosphorylation of protein kinase Cδ in PC12 cells [33], the addition of ATP or phorbol 12-myristate 13-acetate (an activator of classical and novel protein kinase Cs) did not stimulate [^{3}H]AA release in PC12 cells [34]. In addition, treatment with 3 μM calphostin C, which inhibits noradrenaline release induced by protein kinase C activation in PC12 cells [35], did not inhibit [^{3}H]AA release induced by 0.5 mM PAO (data not shown).

In several cells [36,37], PAO stimulated a concentration-dependent increase in the $[Ca^{2+}]_i$. Since activity of secretory PLA_2s requires Ca^{2+} at millimolar concentrations, it is probable that PAO raises $[Ca^{2+}]_i$ levels locally and stimulates PLA_2 activity. However, the effects of ionomycin and KCl-induced depolarization on [^{3}H]AA release were limited in PC12 cells, as previously reported [25,34]. Thus, the stimulatory effect of PAO on AA release did not appear to be due to an increase in $[Ca^{2+}]_i$.

The stimulatory effect of PAO was not abolished in the cells first incubated with 0.5 mM PAO for 5 min and then washed with the PAO-free buffer (Table 3). [^{3}H]AA release from the PAO-treated cells, which was significantly higher compared with that from the control cells, was inhibited by the addition of dithiol compounds (Table 3). The addition of PAO synergistically enhanced [^{3}H]AA release induced by secretory PLA_2 from bee venom (Fig. 2), and dithiol compounds at 1 mM inhibited [^{3}H]AA release by co-addition of secretory PLA_2 and 0.5 mM PAO (data not shown). As described in Section 3, PAO interacted directly with and activated type III secretory PLA_2 from bee venom. These findings suggest that the effect of PAO on AA release is due to the sulfhydryl modification of the target molecule(s), probably secretory PLA_2s in PC12 cells. Recently, it was reported that a PAO-binding protein (p7/p23) complex is a positive regulator of NADPH oxidase activation in bovine neutrophils and that PAO diminishes the optimal concentration of AA for maximal oxidase activation [38]. It should be determined whether PAO interacts directly with secretory PLA_2 and/or interacts with another molecule(s) regulating secretory PLA_2 activity in PC12 cells.

4.3. Neuronal cell functions of secretory PLA_2

An increase in cytosolic PLA_2 activities and PG and thromboxane production has been reported in numerous brain diseases [11–13]. Secretory PLA_2s are also present in the brain, and the expression of secretory PLA_2s is stimulated in the rat brain by ischemia/reperfusion [39] and in astrocytes by cytokines [40,41]. Kudo *et al.* [16] and Matsuzawa *et al.* [17] reported that type II secretory PLA_2 and dopamine are released after exocytotic stimulation with glutamate, and that dopamine release is inhibited by an inhibitor of type II PLA_2 (thielocin A1) in PC12

cells. However, in PC12 cells first incubated with PAO and then thoroughly washed with the PAO-free buffer, the stimulatory effect of PAO on [^{3}H]AA release was still fully intact in the absence of PAO. Furthermore, we have reported that the addition of 50 mM KCl and receptor stimulation by ATP, which causes noradrenaline release [35], do not stimulate [^{3}H]AA release from PC12 cells [34]. Thus, whether secretory PLA_2 is released into the extracellular medium by receptor stimulation, and the role of secretory PLA_2 on transmitter release from PC12 cells have not been established.

Secretory PLA_2s including venom toxins are known to be neurotoxic [15,39]. In PC12 cells undergoing apoptosis induced by deprivation of nerve growth factor and serum, the addition of type II secretory PLA_2 enhances arachidonic acid release [18]. In hamster kidney cells, however, transfection of type II_A secretory PLA_2 generated anti-apoptotic survival signals [6]. Research on apoptosis and/or survival of PC12 cells induced by PAO is currently in progress in our laboratory.

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