Involvement of phospholipase D in oxidative stress-induced necrosis of vascular smooth muscle cells

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Abstract Phospholipase D (PLD) has been associated with necrosis. However, it is not clear whether PLD plays a causative role in this cellular process. We investigated the role of PLD in oxidative stress-induced necrosis of vascular smooth muscle cells (VSMCs). Pervanadate (hydrogen peroxide plus *ortho*vanadate) but not hydrogen peroxide alone activated PLD in a dose- and time-dependent manner. Exposure of VSMCs to pervanadate resulted in necrosis. Pretreatment with butan-1-ol, a PLD inhibitor, attenuated both pervanadate-induced necrosis and increase of intracellular Ca^{2+}. Removal of extracellular Ca^{2+} inhibited pervanadate-induced necrosis by 50%. These results suggest that PLD activation mediates pervanadate-induced necrosis of VSMCs, which is at least partly due to Ca^{2+} toxicity.

Key words: Phospholipase D; Pervanadate; Necrosis; Vascular smooth muscle cell

1. Introduction

Phospholipase D (PLD) catalyzes the hydrolysis of phospholipids, such as phosphatidylcholine, to phosphatidic acid (PA) plus the head group. PLD is recognized as an important component of signal transduction in a variety of cells and is implicated in a wide range of pathophysiological processes that include inflammation, secretion, proliferation, differentiation, apoptosis, neuronal and cardiac stimulation, diabetes, and the respiratory burst in neutrophils [1]. PLD is also reported to be involved in cellular necrosis. Tumor necrosis factor (TNF) induces two distinct modes of cell death, namely necrosis and apoptosis [2]. During TNF-induced cell death, cytosolic phospholipase A_2 ($cPLA_2$) and PLD are activated. De Valck et al. [3] reported that PLD activation is specifically associated with TNF-induced necrosis, whereas $cPLA_2$ activation is associated with early TNF-induced apoptosis. However, it is not clear whether PLD activation is a cause or a consequence of necrosis.

It has been reported that pervanadate induces apoptosis in HeLa cells and lymphoid cell lines [4,5]. Pervanadate-elicited apoptosis involves the activation of caspases 3, 8 and 9 and the induction of the mitochondrial pathway such as the induction of mitochondrial permeability transition and the release of cytochrome *c* [4]. The activation of NF-κB showed the pro- or anti-apoptotic function depending on the nature of apoptotic stimulus. The increase in NF-κB activation by pervanadate-induced tyrosine phosphorylation also mediated the proapoptotic activity of pervanadate [5]. In contrast, little is known about the signaling pathway of necrosis, although the necrotic pathway is considered distinct from the apoptotic pathway. In a previous study, we found that H_2O_2 or vanadate alone was not an effective stimulator of PLD [6]. Preincubation of cells with higher concentrations of H_2O_2 alone mostly induced apoptotic cell death [7]. In contrast, combined treatment of H_2O_2 plus vanadate (pervanadate) resulted in a marked elevation of PLD activity in Swiss 3T3 fibroblasts [6]. These pervanadate-treated cells exhibited rapid morphological changes such as shrinkage and floating from the bottom of a culture dish less than 1 h following exposure to pervanadate. Thus, it is likely that pervanadate may induce a different type of cell death, namely necrosis. Given that, treatment of primary cultured cells such as vascular smooth muscle cells (VSMCs) with pervanadate may be a good model system to explore a relationship between cellular necrosis and PLD.

In the present study, we show that when stimulated with pervanadate, a strong activator of PLD, VSMCs underwent necrosis but not apoptosis. In addition, we, for the first time, demonstrate that the activation of PLD has a causal relation with VSMC necrosis. Our results suggest that oxidative stress-induced necrosis of VSMCs may be a critical event in the progression of several cardiovascular diseases such as ischemia–reperfusion (I/R) injury and rupture of atherosclerotic plaques.

2. Materials and methods

2.1. Materials

Hydrogen peroxide and sodium *ortho*vanadate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Thin layer chromatography plates and phosphatidylbutanol (PtdBut) standard were obtained from Whatman and Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Ca^{2+}-free medium was purchased from Gibco BRL (Rockville, MD, USA).

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2.2. Cell culture

VSMCs were obtained by collagenase digestion of aortas from 7-week-old Sprague–Dawley rats [8]. Cultured VSMCs showed the characteristic 'hills and valleys' growth pattern. VSMC purity was confirmed by immunofluorescence using smooth muscle-specific anti-actin antibody. VSMCs at six to nine passages were used for experiments.

2.3. Measurement of PLD activity in VSMC

PLD activity was assessed by measuring the formation of [^{3}H]PtdBut [6]. Briefly, VSMCs were serum-starved in Dulbecco's modified Eagle's medium for 24 h before the start of the assay. For the final 20 h of serum starvation, the cells were labeled with 1 μCi/ml [9,10-^{3}H]myristic acid. During the final 10 min of the preincubation, 0.3% butan-1-ol was included. At the end of the preincubation, cells were treated with 0.1 mM Na_3VO_4 for 20 min and then stimulated with the various concentrations of H_2O_2 for the indicated times. The radioactivity incorporated into total phospholipids was measured, and the results are presented as a percentage of the total counts per minute incorporated into PtdBut.

2.4. Detection of apoptosis and necrosis by acridine orange (AO)–ethidium bromide (EtBr) staining

Cells were cultured on poly-L-lysine-coated coverslips, and treated with either pervanadate for 30 min or 0.5 μM staurosporine for 24 h in the presence of 0.1% fetal bovine serum to control cellular apoptosis [9], followed by washes with PBS. Cells were then fixed with 4% paraformaldehyde in 0.1% Triton X-100 for 20 min and washed three times with 0.02 M glycine/PBS. Next, cells were incubated with staining solution (5 μg/ml AO, 5 μg/ml EtBr and 0.5 mg/ml RNase A) at 37°C for 1 h and then washed first, three times with 0.1% Triton X-100/PBS and then, three times with PBS. Finally, cell fluorescence was determined using laser confocal microscopy (Bio-Rad Laboratories, Richmond, CA, USA).

2.5. Flow cytometry

Cell death was assessed using the Annexin-V-FITC apoptosis detection kit (Roche Molecular Biochemicals, Germany). Following pervanadate treatment, VSMCs were incubated in labeling solution (10 mM HEPES, pH 7.4, 140 mM NaCl, and 5 mM $CaCl_2$) containing 1 μg/ml propidium iodide (PI) and 20 μl annexin-V-FITC for 15 min. Cell death was then determined by FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA).

2.6. Measurement of intracellular Ca^{2+} ($[Ca^{2+}]_i$)

Changes in $[Ca^{2+}]_i$ were monitored using laser scanning confocal microscopy (Carl Zeiss LSM 410, Jena, Germany) as described previously [10]. Briefly, cells were grown on round coverslips in multi-well culture plates and incubated with 4 mM fluo 3-AM in serum-free medium for 1 h. Each coverslip containing stained cells was placed in a perfusion chamber, and then scanned every 5 s with an argon laser with an excitation wavelength of 488 nm. The emitted fluorescence was detected at a 515-nm wavelength. All images from the scanning were processed to determine $[Ca^{2+}]_i$ changes at the level of a single cell.

2.7. Statistics

Statistical analyses were performed using the SAS System for Windows (release 6.12). $[Ca^{2+}]_i$ measurement data were subjected to Wilcoxon rank-sum test and the statistical significance was set at $P < 0.05$.

3. Result and discussion

3.1. Dose- and time-dependent activation of PLD by pervanadate

It has been reported that oxidative stress stimulates PLD. However, H_2O_2 alone did not affect PLD activity significantly. To determine whether addition of vanadate to H_2O_2 (pervanadate) stimulates PLD, growth-arrested VSMCs were labeled with [^{3}H]myristate, incubated with 0.3% butan-1-ol, and exposed to varying concentrations of H_2O_2 for 30 min following pretreatment with 0.1 mM Na_3VO_4. PLD activity was determined by measuring the formation of [^{3}H]PtdBut. Pervanadate-stimulated formation of [^{3}H]PtdBut was dose-dependent, reaching a peak at 0.5–1 mM H_2O_2 (Fig. 1A). Thus, in the following experiments, the agonist condition for PLD activation was fixed as 0.5 mM H_2O_2 plus 0.1 mM Na_3VO_4. PLD activity continued to increase for up to 50 min (Fig. 1B). These results indicate that exogenous pervanadate activates PLD in a time- and dose-dependent manner in VSMCs.

3.2. Induction of VSMC necrosis by pervanadate

Pervanadate has been shown to induce apoptosis in HeLa cells and lymphoid cell lines [4,5]. To determine whether pervanadate treatment results in necrosis or apoptosis, VSMCs were examined for morphological changes and subjected to flow cytometric (FACS) analyses after double staining with annexin-V and PI (Fig. 2). Cells were exposed to pervanadate for 30 min or to 0.5 μM staurosporine for 24 h (apoptotic control) [9]. In phase contrast microscopy, staurosporine-treated cells exhibited morphological features of apoptosis (i.e. condensation of nuclear chromatin and nuclear fragmentation), while pervanadate-treated cells frequently showed dis-

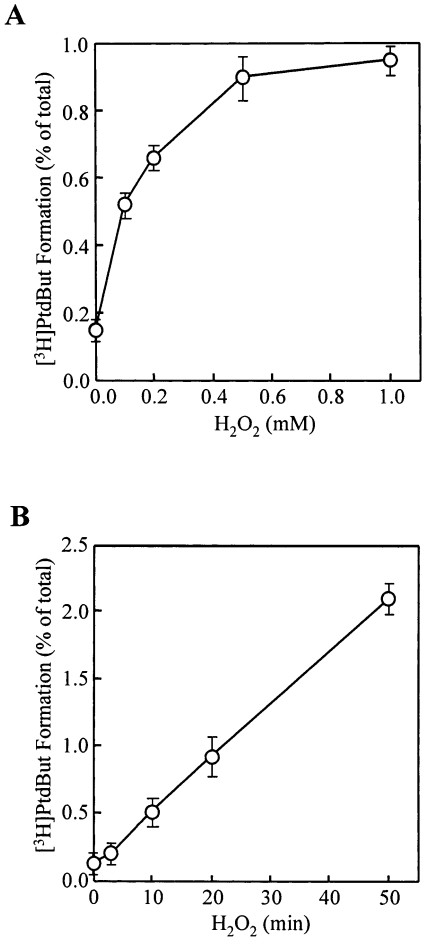


Fig. 1. Dose- and time-dependence of pervanadate-stimulated PLD activation in VSMCs. For the PLD assay, growth-arrested VSMCs were radiolabeled with [^{3}H]myristate, pretreated with 0.1 mM vanadate for 20 min, stimulated with the indicated concentrations of H_2O_2 for 30 min (A) or with 0.5 mM H_2O_2 for the indicated times (B), and then the radioactivity incorporated into PtdBut was measured. Data represent the results of three separate experiments.

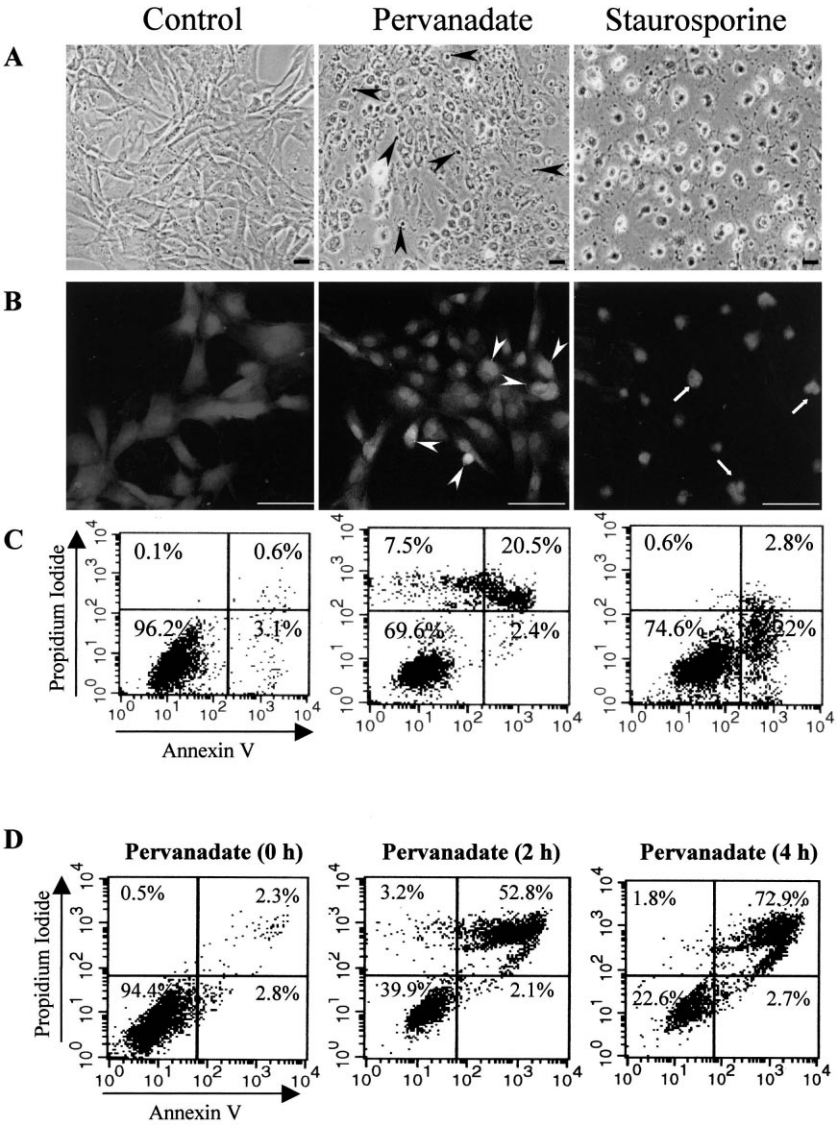
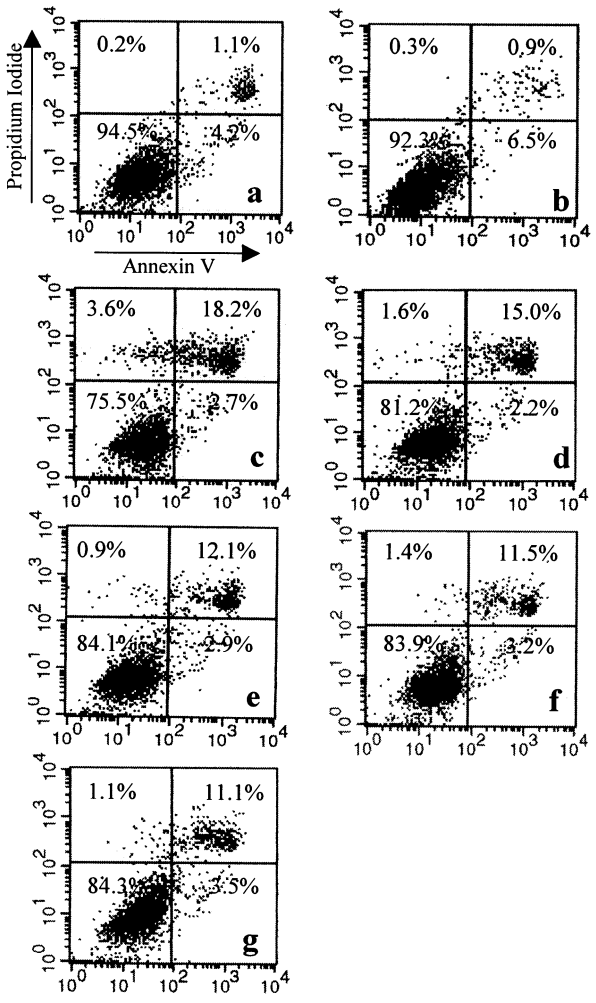


Fig. 2. Morphological features and FACS analysis of pervanadate- or staurosporine-treated VSMCs. VSMCs were either not treated (control), treated with pervanadate (0. 5 mM H_2O_2 plus 0.1 mM vanadate) as described in Fig. 1, or with 0.5 μM staurosporine for 24 h in the presence of 0.1% fetal bovine serum (staurosporine) and then visualized by phase contrast microscopy (100×) (A), or laser confocal microscopy (400×) following double staining with AO (5 μg/ml) and EtBr (5 μg/ml) (B), or they were analyzed by FACS following double staining with annexin-V-FITC and PI (C). Cells were stimulated for the indicated times and then analyzed by FACS (D). Scale bar: 50 μm.

ruption of cytoplasmic membrane and nuclear lysis (arrowheads in Fig. 2A). Changes in nuclear events were further investigated by laser confocal microscopy following staining with AO and EtBr (Fig. 2B). Untreated control cells were diffusely stained green and the size of the cells were much larger than that of pervanadate- or staurosporine-treated cells. This finding is consistent with the results from phase contrast microscopy. Some nuclei of pervanadate-treated cells were orange in color ($AO^+/EtBr^+$), typical of necrosis (arrowheads in Fig. 2B). Staurosporine-treated cells revealed a characteristic of apoptosis, fragmented nuclei of bright green (AO^+/EtBr) (arrows in Fig. 2B). Finally, FACS analysis demonstrated that 20% of the staurosporine-treated cells appeared in the lower right quadrant (annexin-V^+/PI^-), which is consistent with the nature of staurosporine as an apoptotic agent (Fig. 2C). By contrast, pervanadate treatment resulted in a different pattern. Approximately 20 and 8% of the cells were observed in the upper right quadrant (annexin-V^+/PI^+) and in the upper left quadrant (annexin-V^-/PI^+), respectively, whereas the cells in the lower right quadrant (annexin-V^+/PI^-) took an insignificant fraction of the populations. Most of the unstimulated control cells accumulated in the lower left quadrant. We further examined the time course of VSMC necrosis, since necrosis is one end of a spectrum of cell death in which apoptosis occurs rapidly. Necrotic cells markedly increased over the time course of 4 h after pervanadate treatment (Fig. 2D). No significant changes in the percentage of cells in the lower right quadrant (apoptosis) at 2 h were seen compared with that at the zero time control. Even at later time points of 4 h, proportion of early apoptotic cells was negligible. This excludes a possibility that necrosis is a passive end product of apoptosis. Taken together, these results indicate that pervanadate-treated VSMCs undergo necrosis rather than apoptosis.



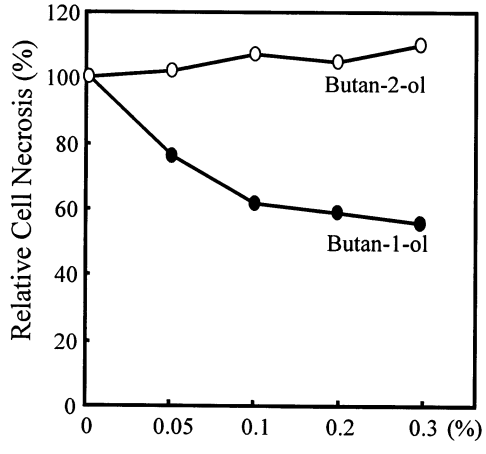


Fig. 3. Effect of butan-1-ol or butan-2-ol on pervanadate-induced necrosis of VSMCs. Quiescent VSMCs were pretreated with the indicated concentrations of butan-2-ol (open circle, bottom panel) or butan-1-ol (closed circle) for 1 h prior to stimulation with pervanadate for 30 min. Cells were then processed for FACS analysis. Cells in the upper left and the upper right quadrant were determined as necrotic ones. a: negative control without pervanadate; b: butan-1-ol (0.3%) without pervanadate; c: positive control with pervanadate; d: 0.05% butan-1-ol plus pervanadate; e: 0.1% butan-1-ol plus pervanadate; f: 0.2% butan-1-ol plus pervanadate; and g: 0.3% butan-1-ol plus pervanadate. Results represent the data from three independent experiments.

3.3. Involvement of PLD in pervanadate-induced VSMC necrosis

We next examined whether PLD is involved in pervanadate-induced necrosis of VSMCs. In recent studies, primary alcohols such as butan-1-ol or propanol have been used to inhibit PLD-induced cellular events [11,12]. In the presence of these alcohols, PLD catalyzes a transphosphatidylation reaction, thus forming phosphatidylalcohol instead of PA. No significant change was observed in cells pretreated with butan-1-ol alone compared to the untreated cells (Fig. 3a,b, top panels). Approximately 22% of VSMCs showed necrosis in response to pervanadate, whereas the proportion of early apoptotic cells (lower right quadrant) was negligible (Fig. 3c). Pretreatment with butan-1-ol rescued the pervanadate-induced necrosis in a dose-dependent manner (Fig. 3d–g and closed circle in the bottom panel), thus indicating that PLD is involved in this process. Maximal inhibition was achieved at a butan-1-ol concentration of 0.3% Butan-2-ol, which is not as effective as butan-1-ol as a PLD substrate in the formation of PtdBut, did not protect against pervanadate-induced necrosis (Fig. 3, open circle), suggesting that PLD inhibition by butan-1-ol is therefore specific. These results suggest that pervanadate-induced VSMC necrosis is mediated by PLD. One of the major effects of pervanadate on the cellular proteins is to induce tyrosine phosphorylation. PLD is tyrosine-phosphorylated by pervanadate in a time- and dose-dependent manner [6]. Tyrosine phosphorylation of PLD, however, seems to be related with necrosis. It is therefore tempting to speculate that the nature of tyrosine-phosphorylated proteins may determine the cellular fate following exposure to pervanadate. However, it remains to be further clarified how pervanadate functions as a necrotic or an apoptotic agent.

3.4. Effect of Ca^{2+} on pervanadate-induced necrosis of VSMCs

Pervanadate causes mobilization of $[Ca^{2+}]_i$ in various cell types, although it is not apparent that pervanadate is involved in Ca^{2+} influx [13–15]. PLD-catalyzed products of phosphatidylcholine are PA and choline. PA is considered a major mediator of PLD-induced biological events. Recent studies suggest a role of PA for increase in $[Ca^{2+}]_i$ concentration [16,17]. Thus, accumulation of higher levels of PA by pervanadate-induced PLD activation may exert cytotoxic effect through a persistent increase in $[Ca^{2+}]_i$. We therefore examined the possibility that pervanadate-induced VSMC necrosis may be mediated by a sustained Ca^{2+} increase. A typical tracing of $[Ca^{2+}]_i$ in single cells showed that pervanadate treatment caused a slow initial rise, followed by a sustained phase, which gradually declined to a basal level (Fig. 4A, top panel). This result is consistent with a previous study in human T cells [13]. Preincubation of cells with butan-1-ol alone did not influence $[Ca^{2+}]_i$ (data not shown). However, butan-1-ol significantly influenced the pervanadate-induced $[Ca^{2+}]_i$ response during the sustained phase, where the level gradually decreased to half that of the control at 11 min (Fig. 4A). The peak level was only slightly affected. We further examined whether the influx of Ca^{2+} is directly involved in pervanadate-induced necrosis using FACS analysis. As illustrated in Fig. 4B, in the absence of extracellular Ca^{2+}, the percentage of necrotic cells decreased by 50% compared to that from cells cultured in Ca^{2+}-containing medium. These results indicate that the major cytotoxic effect of pervanadate on VSMC is

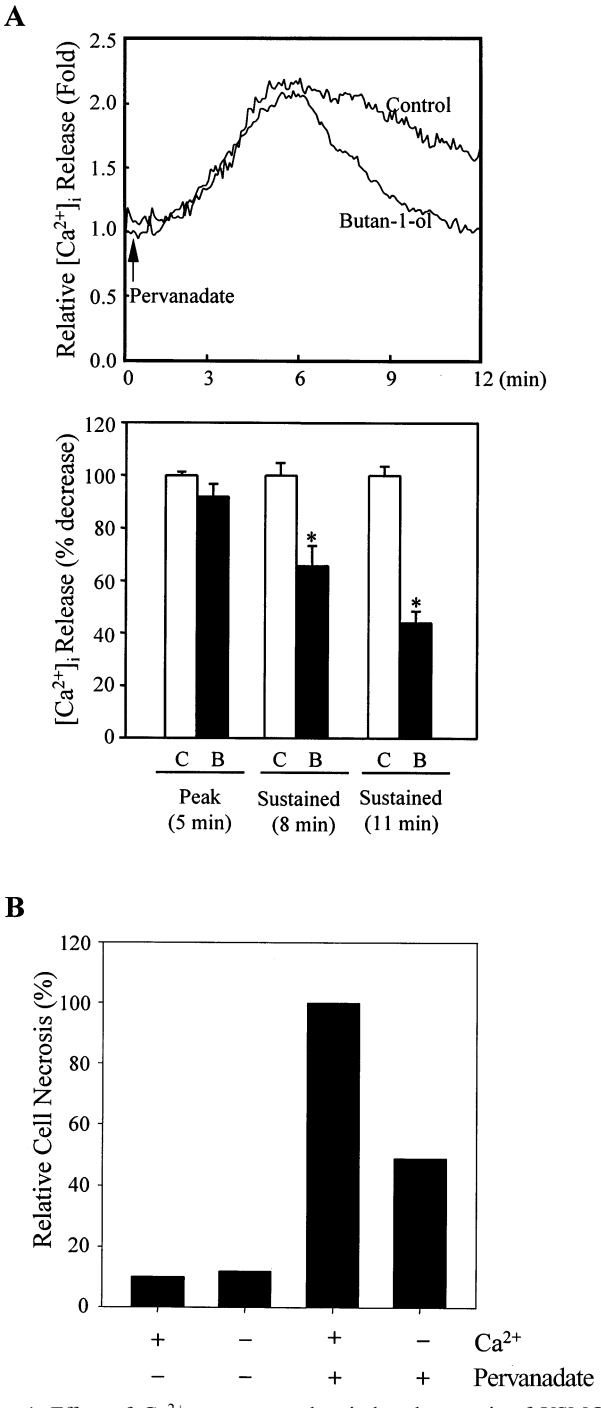


Fig. 4. Effect of Ca^{2+} on pervanadate-induced necrosis of VSMCs. A: VSMCs were preincubated with buffer alone (Control) or with 0.3% butan-1-ol (Butan-1-ol) for 30 min prior to stimulation with pervanadate. In the top panel, representative tracings of $[Ca^{2+}]_i$ in the normal medium are depicted. In the bottom, $[Ca^{2+}]_i$ during the peak (5 min after pervanadate stimulation) and the sustained phase (8 min and 11 min after pervanadate stimulation) are shown. Each value represents the mean ± S.D. ($n = 5$ cells) for three independent experiments. $^*P < 0.05$ compared to the pervanadate-treated control. Open bars (C), control; black bars (B), 0.3% butan-1-ol. B: Cells in the normal or Ca^{2+}-free medium were stimulated with pervanadate, and then analyzed by FACS as described in Fig. 2. Percentage of necrotic cells occurring in the absence of extracellular Ca^{2+} is presented relative to the control value (100%) in the presence of Ca^{2+}. Results represent the data from three independent experiments.

due at least in part to the influx of extracellular Ca^{2+}, in which PLD is involved.

Evidence indicates that cell death occurs in I/R injury and athrosclerotic plaques, which are characterized by accumulation of oxidative stress [18,19]. Lipid oxidation products such as oxidized low-density lipoprotein (LDL) have been considered prime candidates for inducing cellular necrosis [20]. Recent studies showed that oxidized LDL stimulates PLD, and this activation is downstream of tyrosine phosphorylation [21]. Pervanadate also induces tyrosine phosphorylation of cellular proteins including PLD. Taken together, it is likely that an intermittent attack of excessive oxidative stress, as shown in this study, and oxidized LDL contribute to cellular necrosis via tyrosine phosphorylation–PLD pathway, which may cause I/R injury and abrupt plaque rupturing. In conclusion, considering the recent discovery of the potential role of oxidative stress in cell signaling, we have demonstrated that pervanadate can induce cellular necrosis by modulating intracellular signal pathways. This occurs via the activation of PLD. Therefore, specific inhibition of PLD may represent a potential therapeutic modality for preventing vascular dysfunction in the aforementioned diseases.

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