Generation of Reactive Oxygen Species (ROS) is a key factor for stimulation of macrophage proliferation by ceramide 1-phosphate

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Abbreviations: BMDM, bone marrow-derived macrophages; C1P, ceramide 1-phosphate; M-CSF, macrophage-colony stimulating factor; PBS, phosphate-buffered saline; FBS, fetal bovine serum; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; ROS, reactive oxygen species; BHNB, 4-bromo-5-hydroxy-2-nitrobenzhydryl.

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Abstract

We previously demonstrated that ceramide 1-phosphate (C1P) is mitogenic for fibroblasts and macrophages. However, the mechanisms involved in this action were only partially described. Here, we demonstrate that C1P stimulates reactive oxygen species (ROS) formation in primary bone marrow-derived macrophages, and that ROS are required for the mitogenic effect of C1P. ROS production was dependent upon prior activation of NADPH oxidase by C1P, which was determined by measuring phosphorylation of the p40phox subunit and translocation of p47phox from the cytosol to the plasma membrane. In addition, C1P activated cytosolic calcium-dependent phospholipase A_2 and protein kinase C-α, and NADPH oxidase activation was blocked by selective inhibitors of these enzymes. These inhibitors, and inhibitors of ROS production, blocked the mitogenic effect of C1P. By using BHNB-C1P (a photolabile caged-C1P analog), we demonstrate that all of these C1P actions are caused by intracellular C1P. It can be concluded that the enzyme responsible for C1P-stimulated ROS generation in bone marrow-derived macrophages is NADPH oxidase, and that this enzyme is downstream of PKC-α and cPLA$_2$-α in this pathway.

Keywords:

Ceramides

ceramide 1-phosphate

NADPH oxidase

ROS

Proliferation

sphingolipids.

Introduction

The bioactive sphingolipid ceramide 1-phosphate (C1P) has emerged as a crucial regulator of cell homeostasis. First, we found that C1P had mitogenic properties as it stimulated DNA synthesis and cell division in rat fibroblasts [1] and macrophages [2]. Subsequently, we demonstrated that C1P plays a key role in cell survival, an action involving inhibition of apoptosis [3-5]. The latter action involved inhibition of acid sphingomyelinase [5] or serine palmitoyltransferase [6] activities. Other groups have reported that C1P can control inflammatory responses [7-9], phagocytosis [10,11], and neutrophil degranulation [12]. Another relevant feature of C1P is its ability to stimulate cell motility [13]. In this connection, we first found that stimulation of macrophage migration required the interaction of exogenous C1P with cell membranes, while elevation of intracellular levels of C1P did not cause cell migration. These observations led us to identify a specific receptor that seems to be essential for the regulation of macrophage migration by C1P [13]. This action may be associated with the proinflammatory effects of C1P that were previously described [7,14,15].

Our previous data on cell growth stimulation by C1P indicate that protein kinase B (PKB, also known as Akt), extracellularly regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK), and the mammalian target of rapamycin (mTOR) are important kinases implicated in this process [16]. However, the mechanisms by which C1P stimulates cell proliferation are not fully understood. Of interest, reactive oxygen species (ROS) have been shown to also play important roles in the stimulation of cell growth as well as in the induction of apoptosis, depending on the cell type. In particular, ROS can be generated in response to a variety of growth factors [17-19] to induce DNA synthesis and cell division, but growth factor independent stimulation of cell proliferation by ROS has also been demonstrated [20,21].

Given the importance of macrophages in the inflammatory response and their well-established role in the development and progression of atherosclerosis [22], the present work was undertaken to examine whether ROS generation is a relevant factor in the stimulation of macrophage proliferation by C1P, and to define the mechanism by which C1P exerts this action.

Materials and Methods

Materials

N-Hexadecanoyl-D-erythro-sphingosine-1-phosphate (N-palmitoyl-Ceramide 1-phosphate) (C1P) was supplied by Matreya. Culture medium RPMI 1640, allopurinol, 1-aminobenzotriazole (ABT), pertussis toxin (Ptx), apocynin, N-acetylcysteine (NAC), NG-nitro-L-arginine methyl ester (L-NAME), diphenyleneiodonium chloride (DPI), rotenone, and rottlerin were from Sigma-Aldrich. Fetal bovine serum (FBS) was from Gibco. The fluorescent probe 5-(and 6-)-chloromethyl-2, 7-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) was from Molecular Probes. [^{3}H-Methyl]-thymidine (2.0 Ci/mmol) was from Perkin Elmer. Go6976 was from Tocris Bioscience. Antibodies to phospho-p40phox (Thr154), p47phox, and the p85 subunit of PI3-K were purchased from Cell Signaling, and those to total p40-phox (D-8) and β-actin (H-196) were from Santa Cruz Biotechnology. Phorbol-12-myristate-13-acetate (PMA) and N-{(2S,4R)-4-(biphenyl-2-ylmethyl-isobutyl-amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl}-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl]acrylamide (pyrrolidine-2) were from Calbiochem. The $cPLA_2$ assay kit was supplied by Cayman Chemical. BHNB-C1P was synthesized as described previously [23]. Nitrocellulose membranes, protein markers, and BCA assay reagents were purchased from Bio-Rad. All of the other chemicals and reagents were of the highest grade available. The transilluminator (Darkreader DR-45M) used to expose the cells to light was from Clare Chemical.

Cell culture

Bone marrow-derived macrophages (BMDM) were isolated from femurs of 6-8-week old female CD-1 mice as described [24]. Cells were plated for 24 h in RPMI 1640

medium containing 10% fetal bovine serum (FBS) and 10% L-cell conditioned medium as the source of macrophage-colony stimulating factor (M-CSF) [25]. The non-adherent cells were removed and cultured for 4-6 days in the same medium until about 80% confluence was reached. The cells were then incubated for 24 h in RPMI 1640 medium containing 10% FBS and 1.5% L-cell conditioned medium, as previously described and used in experiments.

Delivery of C1P to cells in culture

An aqueous dispersion (in the form of liposomes) of C1P was added to cultured macrophages as previously described [2,4,5]. Specifically, stock solutions were prepared by sonicating C1P (1 mg) in sterile nanopure water (1 ml) on ice using a probe sonicator until a clear dispersion was obtained. The final concentration of C1P in the stock solution was approximately 1.62 mM. This procedure is considered preferable to dispersions prepared by adding C1P in organic solvents because droplet formation is minimized and there are no organic solvent effects on the cells. We also delivered C1P to cells by using the photolabile caged C1P analog, BHNB-C1P [23], which was dissolved in ethanol at 1.62 mM. The final ethanol concentration was < 0.16 %. The cells were exposed to 400-500 nm light in a transilluminator equipped with a 9 W lamp for 60 min at a distance of 1.5 cm at 37 °C, so as to release the C1P into the cytosol.

Measurement of intracellular reactive oxygen species

ROS production was determined using CM-H2DCFDA, which is de-esterified upon entering cells, and then oxidized to fluorescent DCF by intracellular ROS. BMDM were incubated in 35-cm diameter dishes at 500,000 cells/dish and were grown in RPMI 1640 medium containing 10% FBS and 10% L-cell conditioned medium (as the source

of M-CSF) for 4-6 days until they were about 80% confluent. The cells were then incubated for 24 h in RPMI 1640 medium containing 10% FBS and 1.5% L-cell conditioned medium as reported previously [2]. The macrophages were incubated in the presence or absence of agonist, without refreshing the medium, and CM-H2DCFDA was added at 5 μM during the last 30 min of incubation, at 37 ºC in the dark. The cells were then washed twice with phosphate-buffered saline (PBS) and lysed with 10 mM Tris-HCl buffer, pH 7.4, containing 0.5% Tween 20. The homogenates were centrifuged at 10,000 x g for 10 min to remove cell debris. DCF fluorescence in the supernatant was measured using a spectrofluorimeter with excitation and emission wavelengths of 490 nm and 522 nm, respectively. Data were processed and normalized to values obtained from untreated controls.

Determination of p47phox translocation

Macrophages were harvested and lysed in ice-cold homogenization buffer as described [26]. Nuclei were removed by centrifugation at 500 x g for 5 min, and the membrane and soluble fractions were separated by centrifugation at 100,000 x g at 4 ºC for 30 min. Then 20-30 μg of protein from each sample was loaded and separated by SDS–PAGE, using 12% separating gels, and analyzed by western blotting

Determination of cPLA$_2$ activity

BMDM were incubated in 60-mm diameter dishes at 3.5 x 10^6 cells/dish and were grown in RPMI 1640 medium containing 10% FBS and 10% L-cell conditioned medium until they reached about 80% confluence. The macrophages were then incubated as described above, and preincubated with PMA (100 nM) for 16 h, or with Go6976 (50 nM) for 30 min prior to stimulation with C1P (40 μM) for 4 h. The cells

were washed and harvested in cold buffer (1 mM EDTA, 50 mM HEPES, pH 7.4). Samples were sonicated and centrifuged at 10,000 x g for 15 min at 4ºC. The activity of $cPLA_2$ in the supernatant was determined using a commercial kit (see the Materials section) according to the manufacturer's instructions.

Determination of DNA synthesis

BMDM were seeded at 500,000 cells/well in 12-well plates and were grown in RPMI 1640 medium as previously reported [2]. The cells were incubated in the presence or absence of agonists for 24 h. [^{3}H]Thymidine (0.5 μCi/ml) was added for the last 2 h of incubation. The medium was then removed and cells were washed twice with PBS. [^{3}H]Thymidine incorporation into DNA was measured as previously described [1,2].

Cell viability and proliferation assay

Macrophages were seeded at 50,000 cells/well in 96-well plates and incubated overnight in RPMI 1640 with 10% FBS and 10% L-cell conditioned medium as the source of M-CSF. The medium was then replaced with fresh RPMI 1640 medium with 10% FBS and 1.5% L-cell conditioned medium in the presence or absence of agonists and/or inhibitors as appropriate. Cell growth was estimated by measuring the rate of reduction of the tetrazolium dye MTS as described [2, 25].

Western blotting

Macrophages were harvested and lysed in ice-cold homogenization buffer as described [26]. Protein (20-40 μg) from each sample was loaded and separated by SDS-PAGE, using 12% separating gels. Proteins were transferred onto nitrocellulose paper

and blocked for 1 h with 5% skim milk in Tris-buffered saline (TBS) containing 0.01% NaN_3 and 0.1% Tween 20, pH 7.6, and then incubated overnight with the primary antibody in TBS/0.1% Tween at 4ºC. After three washes with TBS/0.1% Tween 20, membranes were incubated with horseradish peroxidase-conjugated secondary antibody at 1:5000 dilution for 1 h. Bands were visualized by enhanced chemiluminescence.

Statistical analyses

Results are expressed as means ± SEM of three independent experiments performed in triplicate, unless indicated otherwise. Statistical analyses were performed using one-way analysis of variance analyses (ANOVAs), or Student's t-test as appropriate, with the level of significance set at $P < 0.05$.

Results and Discussion

We have previously demonstrated that C1P promotes macrophage proliferation potently [1, 27]. This action involved stimulation of kinases that are usually implicated in the regulation of cell growth including phosphatidylinositol 3-kinase (PI3-K), ERK1/2, JNK, PKC-α, and mTOR [22]. We now demonstrate that activation of NADPH oxidase and the subsequent generation of ROS are essential processes required for the mitogenic effect of C1P. Fig. 1 shows that C1P stimulated ROS production in a concentration- (panel A) and time-(panel B) dependent manner at concentrations previously found to stimulate macrophage growth (20-40 μM). NADPH oxidase is an enzyme complex composed of two membrane subunits, p22phox and gp91phox, and four cytosolic subunits, p47phox, p67phox, p40phox, and a Rho guanosine triphosphate protein, usually Rac 1 or Rac 2 [28]. Key events for activating NADPH oxidase are phosphorylation of the p40phox subunit and translocation of p47phox from the cytosol

to the plasma membrane. Therefore, we examined whether these two actions were elicited by C1P. Fig. 2A shows that C1P caused phosphorylation of p40phox as well as translocation of the p47phox subunit to the membrane fraction (Fig. 2C), suggesting activation of NADPH oxidase. Although phosphorylation of p40phox took place within minutes (the maximal effect was attained after 10 min of treatment with C1P), translocation of p47phox occurred at 2-6 h, which is consistent with the time frame for ROS generation by C1P (Fig. 1).

It is known that although ROS can be generated by different cell types through different pathways, the major mechanism for the production of ROS by phagocytes is activation of NADPH oxidase [29]. In agreement with this hypothesis, we found that inhibition of NADPH oxidase with the selective inhibitors DPI or apocynin [30, 31] completely blocked C1P-stimulated ROS production (Fig. 2E). Although primary cultures are considered to be an appropriate model to study the mechanisms involved in macrophage proliferation, transfection of these cells is highly inefficient [32]. Therefore, we used chemical inhibitors to study the contribution of NADPH oxidase-derived ROS to macrophage growth. Figs. 3A and 3B show that the NADPH oxidase inhibitors DPI and apocynin, as well as the general antioxidant NAC, completely blocked C1P-stimulated DNA synthesis, as determined by the incorporation of [^{3}H]-thymidine into DNA. Furthermore, macrophage growth, as measured by using the MTS-formazan assay [2, 16], was also inhibited. However, inhibitors of other ROS generating pathways, such as allopurinol (an inhibitor of xanthine oxidase), ABT (an inhibitor of cytochrome p450 enzymes), L-NAME (an inhibitor of nitric oxide synthase), and rotenone (an inhibitor of the mitochondrial complex I respiratory chain), did not affect C1P-stimulated ROS production (data not shown). Therefore, these

results suggest that the NADPH oxidase/ROS axis is essential for the stimulation of cell proliferation by C1P in primary macrophages.

It was reported previously that C1P activates cPLA$_2$-α potently in A549 lung adenocarcinoma cells [9] and NR8383 alveolar macrophages [33], and that this phospholipase is important for endothelial cell cycle progression and proliferation. Therefore, we next investigated whether C1P can also stimulate cPLA$_2$-α significantly in primary BMDM; stimulation was about 40 ± 5% higher than the control value; mean ± SEM of three independent experiments performed in triplicate, $p < 0.05$. Activation of cPLA$_2$-α was also studied by measuring its phosphorylation state after treatment of the macrophages with C1P. Phosphorylation of cPLA$_2$ was detected after 1 h of incubation of the macrophages with C1P, and it was maintained up to at least 5 h (Fig. 4A,B). To study whether cPLA$_2$-α activation was important for the mitogenic effect of C1P, we used the selective cPLA$_2$-α inhibitor, pyrrolidine-2. Fig. 4 C,D shows that this inhibitor completely blocked C1P-stimulated DNA synthesis and cell growth, indicating that cPLA$_2$-α is critical for stimulation of macrophage proliferation by C1P.

Since both ROS generation and cPLA$_2$-α are involved in the mitogenic effect of C1P, we sought to determine if there is a link between these two effectors. Fig. 4D shows that the selective cPLA$_2$-α inhibitor (pyrrolidine-2) completely blocked C1P-induced ROS generation, suggesting that cPLA$_2$-α activation is also a relevant factor in this process. This observation also indicates that cPLA$_2$ activation is upstream of ROS production in the cascade of events triggered by C1P in BMDM.

Recently, we demonstrated that C1P stimulates PKC-α and that this kinase is also implicated in the mitogenic effect of C1P in BMDM [34]. Therefore, studies were conducted to determine whether PKC-α might be implicated in the generation of ROS by C1P. This was achieved by using the PKC-α inhibitor Go6976, as well as by a

prolonged incubation with the phorbol ester PMA, which is known to downregulate conventional PKC isoforms [35-38]. Fig. 5A shows that C1P-stimulated ROS generation was completely inhibited by Go6976; this was also the case when the macrophages were incubated for 24 h in the presence of 100 nM PMA to downregulate PKC (Fig. 5B).

The intervention of $cPLA_2$-α and PKC-α may be critical factors for the production of ROS by C1P. This was further studied by using the selective inhibitors of these enzymes on p47phox translocation and p40phox phosphorylation. Fig. 6 (panels A-D) shows that inhibition of $cPLA_2$ or PKC-α blocked C1P-stimulated p47phox translocation as well as phosphorylation of p40phox (panel E). Most likely, activation of PKC-α is upstream of $cPLA_2$-α stimulation, as the PKC-α inhibitor Go6976 was able to completely block cPLA2-α (Fig. 7) whereas the $cPLA_2$-α inhibitor did not affect PKC-α activity (data not shown).

Our previous work demonstrated that C1P can act both intracellularly to stimulate cell growth [16,23] and inhibit apoptosis [4,5], or through interaction with a specific, Gi protein-coupled Ptx-sensitive plasma membrane receptor to stimulate cell migration [13]. Therefore, studies were aimed at defining whether C1P-stimulated ROS production was dependent or independent of receptor interaction. We found that Ptx, at concentrations known to inhibit G_i proteins (50 – 100 ng/ml) in the same cells [39], did not affect C1P-stimulated ROS generation (Fig. 8 A), suggesting that C1P was acting intracellularly rather than through interaction with its receptor. This observation was confirmed by using a novel caged C1P analog, 4-bromo-5-hydroxy-2-nitrobenzhydryl C1P (BHNB-C1P), which was previously found to be plasma membrane permeable and bypass surface receptors of BMDM [23]. The bioactive lipid C1P is released in the cytosol upon photolysis using visible light that does not damage cellular components (>

360 nm) [23]. The caging/uncaging strategy allowed us to conclude that only intracellular C1P was responsible for stimulation of cell growth in BMDM [23]. In addition, we found that concentrations as low as 1-2.5 μM μM BHNB-C1P were almost as effective as 50 μM of exogenous C1P in stimulation of BMDM proliferation, which is consistent with our previous observation that exogenously added C1P (in the form of liposomes) is only poorly taken up by cells [27]. Fig. 8B shows that treatment of BMDM with BHNB-C1P in the dark (conditions in which the C1P remains in the biologically inactive caged form) failed to stimulate ROS production, whereas BHNB-C1P-treated cells (at 1-2.5 μM) that were exposed to 400-500 nm light for 60 min (to release C1P into the cytosol) stimulated ROS generation as potently as 40 μM of exogenous C1P.

In conclusion, we have demonstrated that ROS production is a key factor for stimulation of macrophage proliferation by C1P. The enzyme responsible for C1P-stimulated ROS generation in BMDM is NADPH oxidase. This enzyme lies downstream of $cPLA_2$-α, which in turn, is activated by phosphorylation by PKC-α in this pathway.

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Figure legends

Fig.1. Intracellular ROS generation by C1P. BMDM were pre-incubated for 16 h in 1.5% L-cell conditioned medium. **A.** BMDM were incubated with C1P at the indicated concentrations for 8 h. After incubation, ROS production was determined by measuring the fluorescence intensity of CM-H2DCFDA. Data are expressed as the fluorescence intensity relative to the control value and are the mean ± SEM of 6 replicate experiments. **B.** Cells were incubated with 40 μM C1P for the indicated time periods. ROS generation was measured as above. The results are shown as fluorescence intensity relative to control value and are the mean ± SEM of 4 duplicate experiments (*p<0,05; **p < 0.01; ***p< 0,001).

Fig.2. C1P induces NADPH oxidase activation as determined by translocation and phosphorylation of its p47phox and p40phox subunits, respectively. Involvement of NADPH oxidase in C1P-stimulated ROS generation. **A.** BMDM were incubated as above and treated with 40 μM C1P for the times that are indicated. The presence of p47phox in the microsomal fraction was determined by western blotting using an antibody specific to this subunit. Equal loading of protein was monitored using a specific antibody to p85. **B.** Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units of intensity and are the mean ± SEM of 3 replicate experiments, except for the 4 h time point, which is the mean ± SEM of 4 replicate experiments. **C**. Phosphorylation of p40phox was analysed using an antibody specific to the phosphorylated form of this subunit. Equal loading of protein was monitored using a specific antibody to total p40phox. Similar results were obtained in each of 4 replicate experiments. **D.** Results of scanning densitometry of the exposed film. Data are

expressed as arbitrary units of intensity and are the mean ± SEM of 4 replicate experiments. **E**. BMDM were preincubated with 0.5 mM apocynin or 0.5 μM DPI for 30 min. Then C1P (40 μM) was added, and ROS production was determined after 8 h of incubation by measuring the fluorescence intensity of CM-H2DCFDA. The results are expressed as fold stimulation over the control value and are the mean ± SEM of 3 independent experiments performed in duplicate (*$p<0.05$; **$p < 0.01$).

Fig.3. ROS are implicated in the stimulation of macrophage proliferation by C1P. **A**. Cells were preincubated with 0.5 mM NAC, 0.5 mM apocynin, or 0.5 μM DPI for 30 min prior to treatment with C1P (40 μM) for 24 h. DNA synthesis was determined by measuring the incorporation of [^{3}H]-thymidine into DNA as described in the Methods section. Results are expressed as fold stimulation relative to the control value without inhibitors, and show the mean ± SEM of 3 independent experiments performed in triplicate. **B.** BMDMs were preincubated as in A. Cell growth was determined using the MTS-formazan assay. Results are expressed as fold stimulation relative to the control value, and are the mean ± SEM of 6 independent experiments performed in triplicate (*$p<0.05$; **$p < 0.01$).

Fig.4. C1P-stimulated macrophage proliferation is inhibited by the selective cPLA$_2$-α inhibitor pyrrolidin-2. **A**. Cells were treated with 40 μM C1P for the indicated time points and harvested in lysis buffer. The presence of phosphorylated cPLA$_2$-α in cell homogenates was determined using an antibody specific to phospho-cPLA$_2$-α. Equal loading of protein was monitored using a specific antibody to total cPLA$_2$-α. Similar results were obtained in each of 4 replicate experiments. **B**. Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units of intensity and

are the mean ± SEM of 4 replicate experiments (*p<0.05). **C.** Cells were preincubated with 2.5 μM of the selective cPLA$_2$-α inhibitor for 30 min and then treated with C1P (40 μM). ROS generation was determined after 8 h of incubation. Results are indicated as the fluorescence intensity relative to the control value and are the mean ± SEM of 3 experiments performed in duplicate. **D.** BMDM were preincubated with 2.5 μM of the selective inhibitor of cPLA$_2$-α for 30 min and then stimulated with 40 μM C1P for 24 h. Cell growth was determined using the MTS-formazan assay. Results are expressed as fold stimulation relative to the control value and are the mean ± SEM of 6 independent experiments performed in triplicate (*p<0.05; **p < 0.01).

Fig.5. C1P-induced ROS generation is blocked by PKC-α inhibitors. **A.** Cells were preincubated with 100 nM of PMA for 16 h. C1P (40 μM) was then added and ROS generation was determined as indicated in the Methods section, after 8 h of incubation. Results are indicated as the intensity of fluorescence relative to the control value and are the mean ± SEM of 3 independent experiments performed in duplicate. **B.** Cells were preincubated with 50 nM Go6976 for 30 min, and ROS generation was measured as in A. Results are indicated as the intensity of fluorescence relative to the control value and are the mean ± SEM of 5 independent experiments performed in duplicate (*p<0.05).

Fig.6. cPLA$_2$-α and PKC-α are critical enzymes for stimulation of NAPDH oxidase by C1P. **A**. Cells were preincubated with 100 nM PMA for 16 h, or with 2.5 μM of the selective cPLA$_2$-α inhibitor (pyrrolidine-2) for 30 min prior to treatment with C1P (40 μM) for 4 h. The presence of p47phox was assessed by western blotting, as indicated in the Methods section. Equal loading of protein was monitored using a specific antibody to p85. Similar results were obtained in each of 3 replicate experiments. **B.** Results of

scanning densitometry of the exposed film. Data are expressed as arbitrary units of intensity and are the mean ± SEM of 3 independent experiments. **C.** Cells were preincubated with the selective PKC-α inhibitor Go6976 (50 nM) for 30 min, and then C1P (40 μM) was added for 4 h. The presence of p47phox in the microsomal fraction was analysed by western blotting. Equal loading of protein was monitored using a specific antibody to p85. **D.** Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units of intensity and are the mean ± SEM of 3 replicate experiments. **E.** Cells were preincubated with 50 nM Go6976 for 30 min and then incubated for 10 min with 40 μM C1P. Phosphorylation of p40phox was determined by western blotting using an antibody specific to phospho-p40phox. Equal loading of protein was monitored using specific antibodies to total p40phox and p85. **F.** Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units of intensity and are the mean ± SEM of 4 replicate experiments (*$p<0.05$).

Fig.7. C1P-stimulated $cPLA_2$ activity is blocked by PKC inhibitors. Cells were pre-incubated with 100 nM PMA for 16 h, or with 50 nM Go6976 for 30 min prior to treatment with C1P (40 μM) for 4 h. $cPLA_2$ activity was measured as indicated in the Methods section. Basal $cPLA_2$ activity was 5.12 ± 0.49 μmol/min/mg protein (mean ± SEM, n=4). Results are expressed as fold stimulation relative to the control value and are the mean ± SEM of 4 independent experiments (*$p<0.05$ versus control value; #$p<0.05$ versus C1P-stimulated value).

Fig.8. ROS are generated by C1P acting intracellularly. **A.** Ptx does not impair C1P-stimulated ROS generation. Cells were preincubated overnight with 0.05 or 0.10 μg/ml Ptx and then stimulated with 40 μM C1P for 8 h. ROS generation was determined as

described above. Results are expressed as the intensity of fluorescence relative to the control value, and they are the mean ± SEM of 3 independent experiments performed in duplicate. **B.** Macrophages were incubated with 1 or 2.5 μM BHNB-C1P, or with 40 μM C1P. The cells were then exposed to 400-500 nm light for 1 h using a transilluminator (open bars) or incubated in the dark (filled bars). Intracellular ROS generation was measured after 8 h of incubation. The results are expressed as mean ± SEM of 3 independent experiments performed in duplicate, except the point at 1μM BHNB-C1P, which is the mean ± range of two experiments.

Figure
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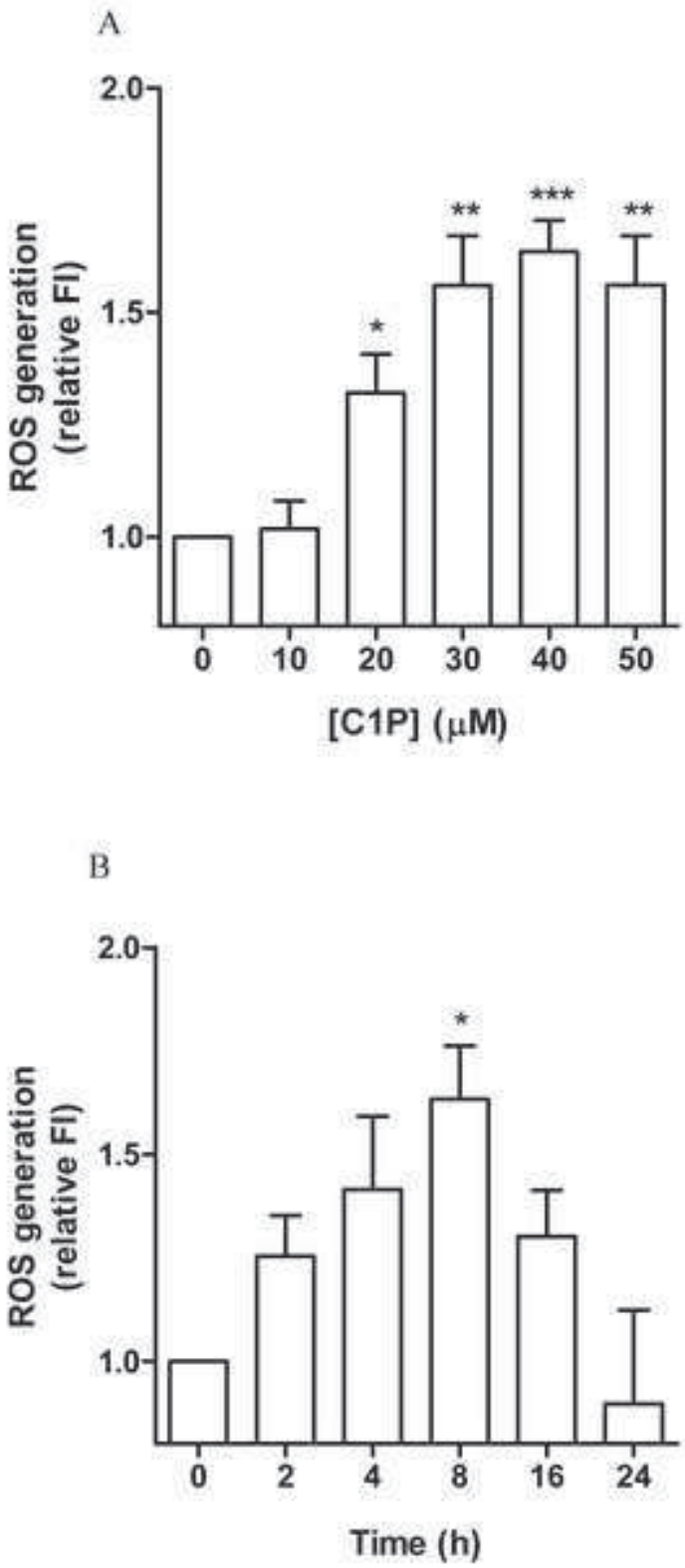

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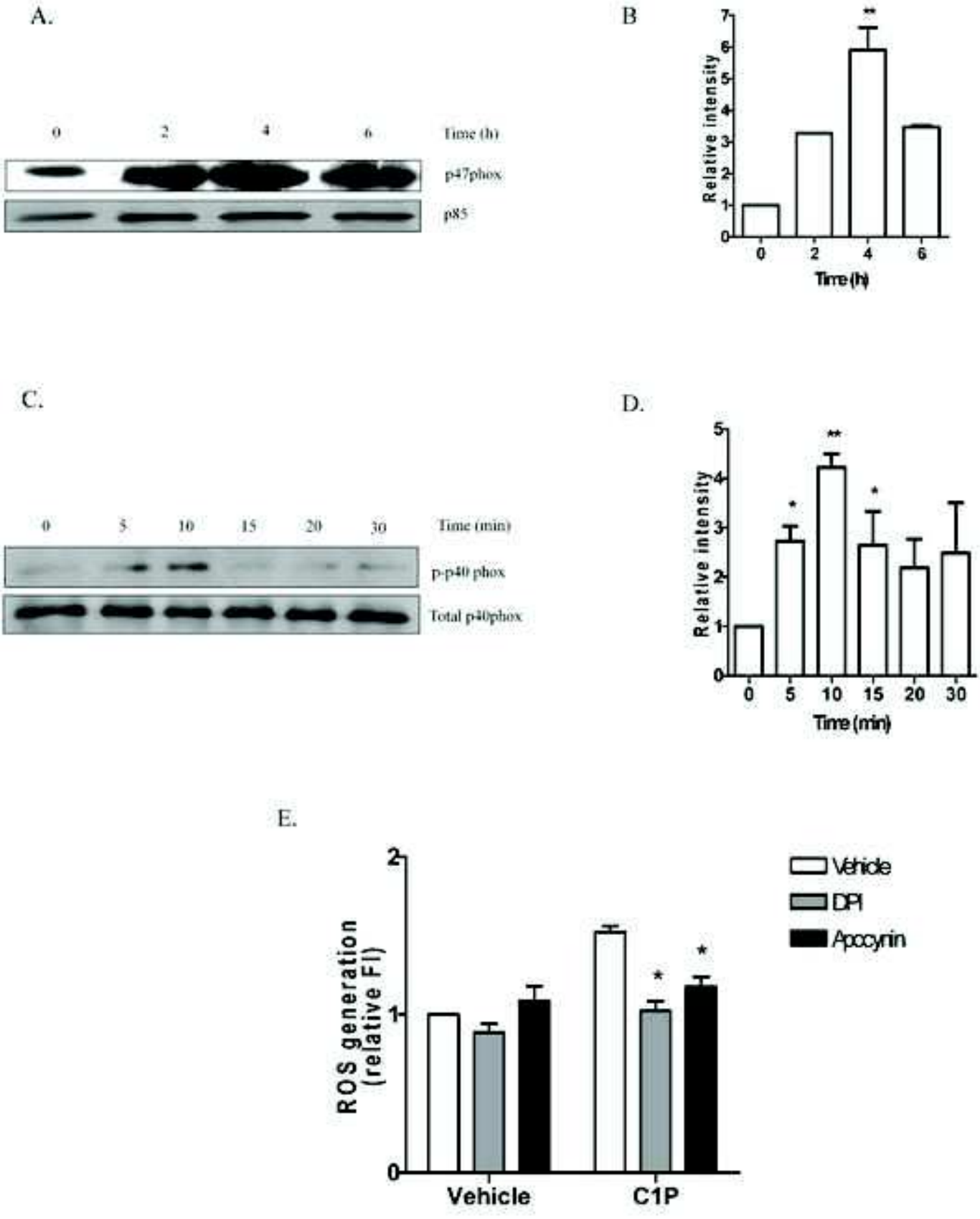

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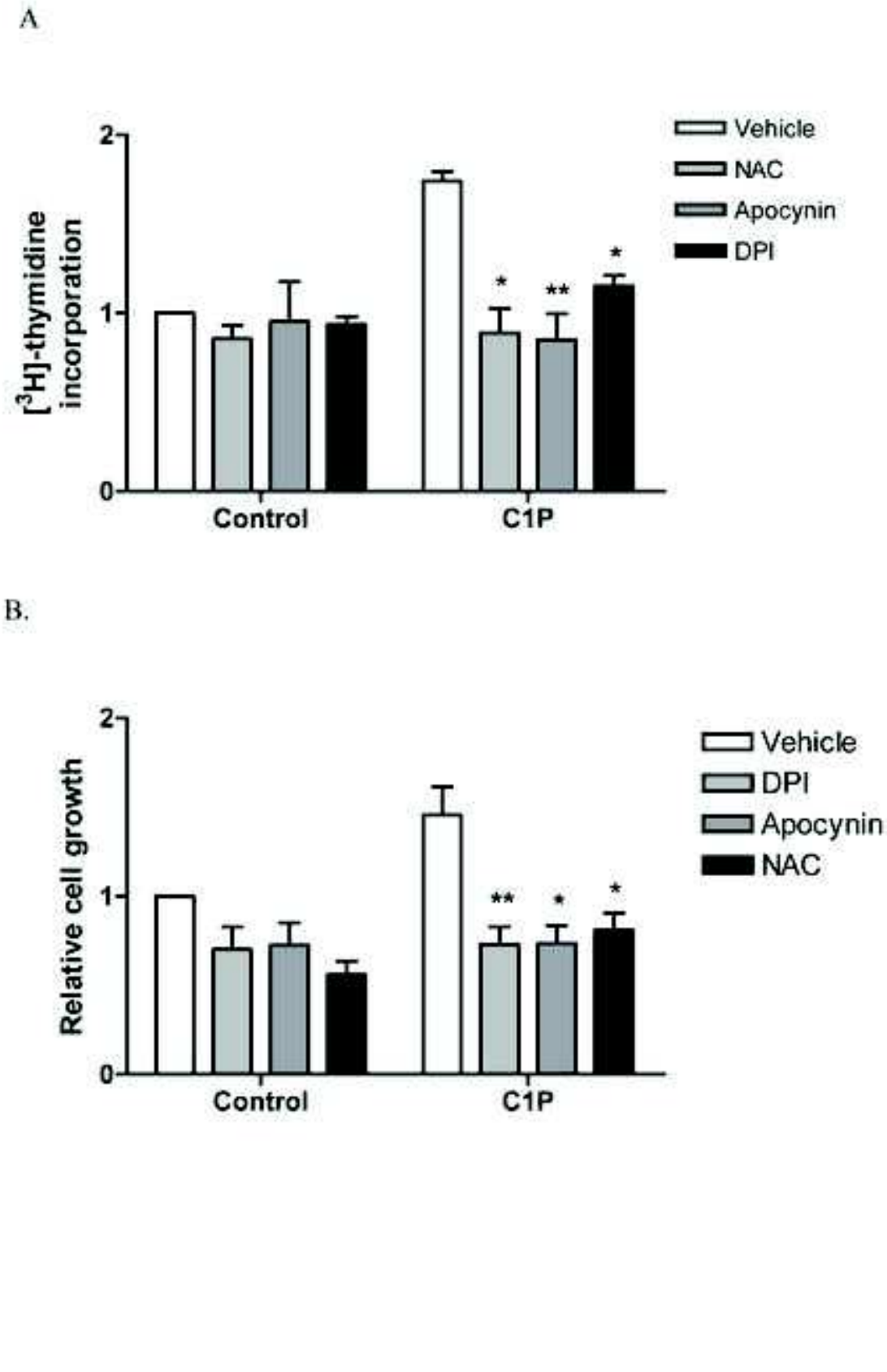

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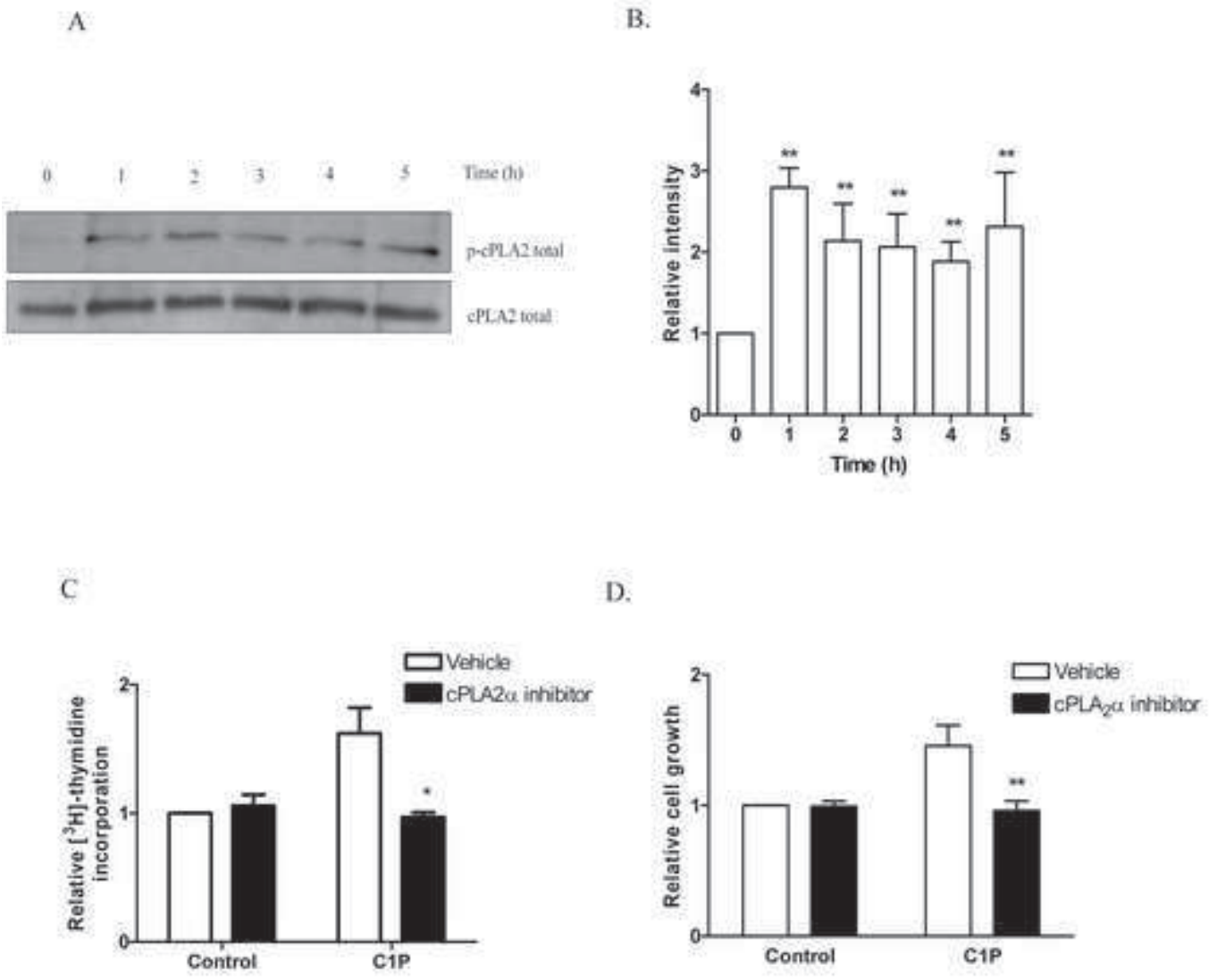

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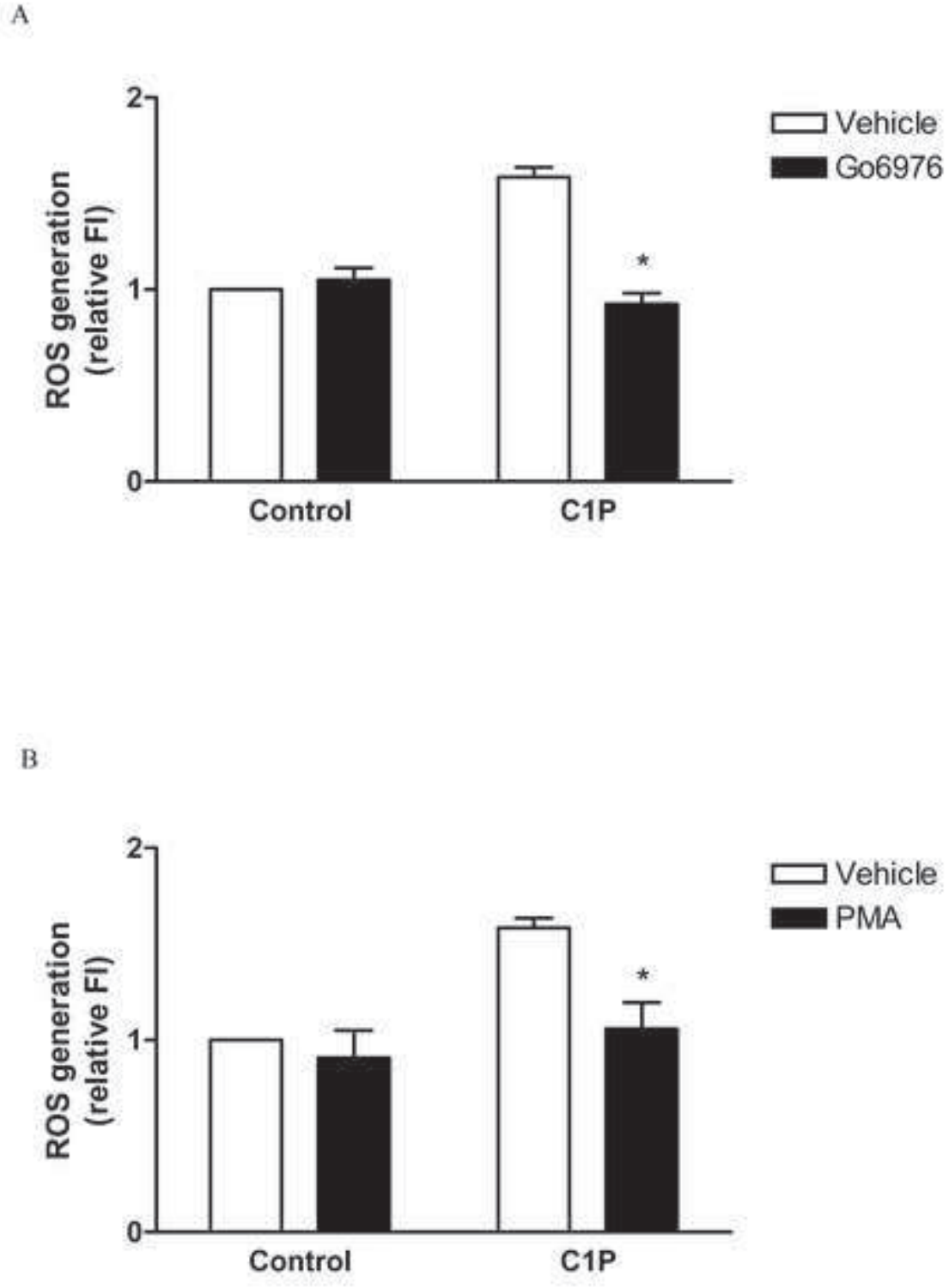

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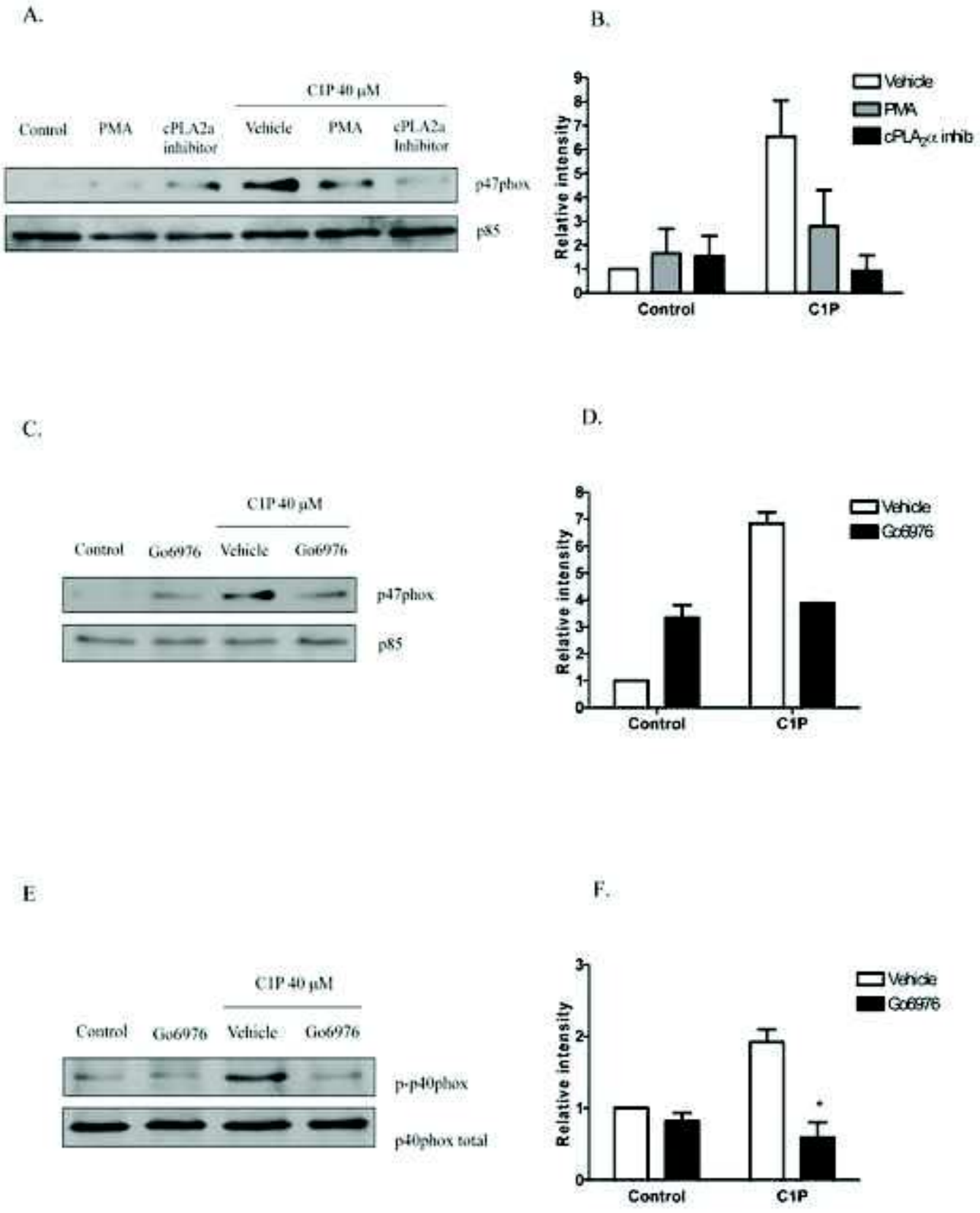

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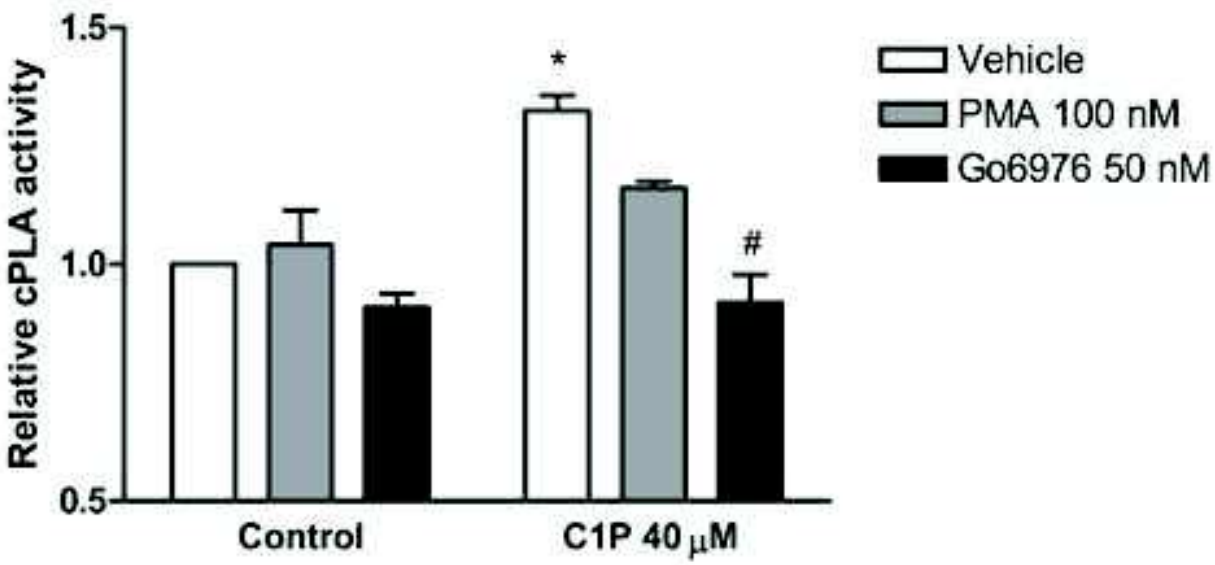

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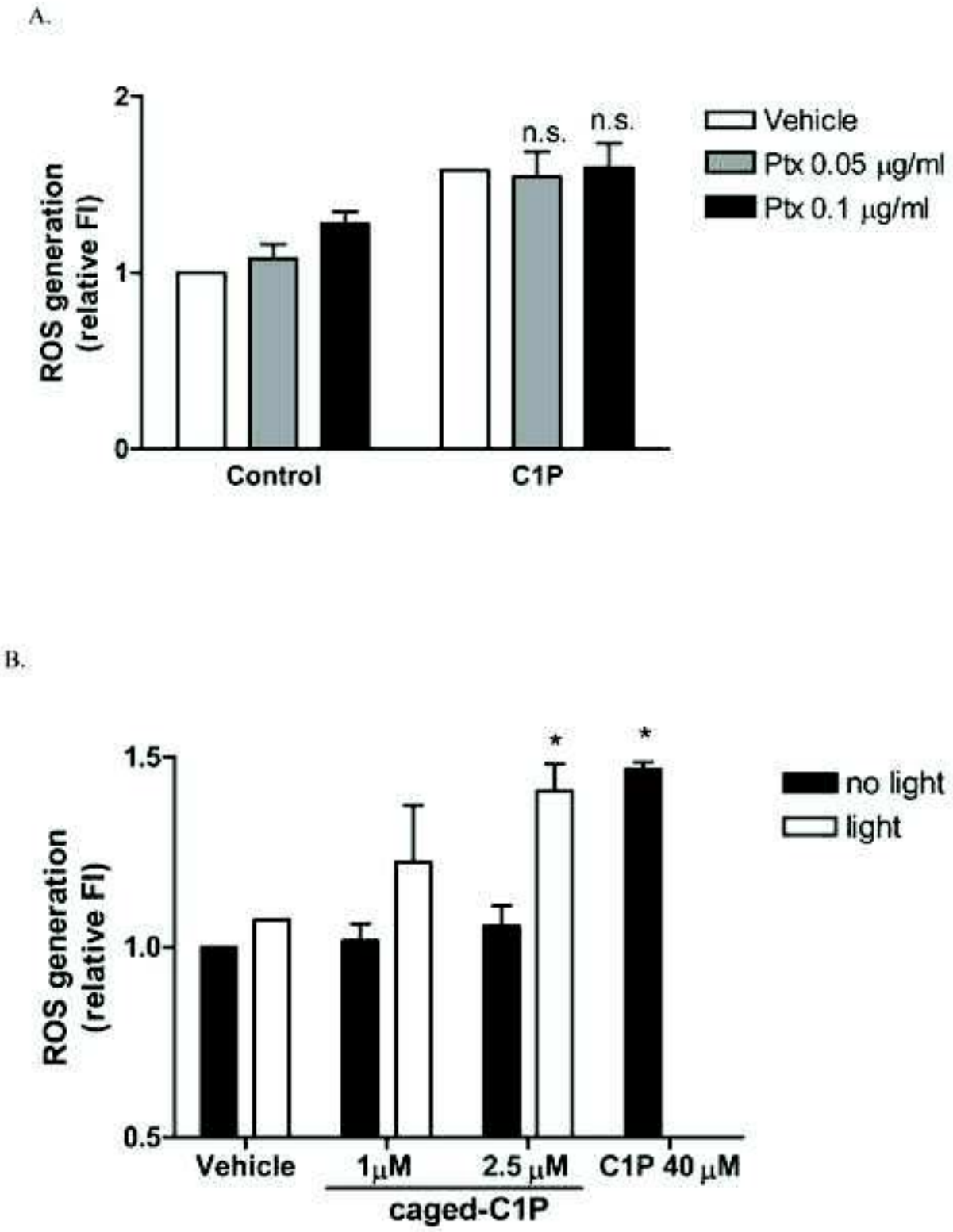

Figure 8.