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Stimulation of intracellular Ca^{2+} elevation in neutrophils by thiol-oxidizing phenylarsine oxide

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Abstract

Phenylarsine oxide (PAO), a trivalent arsenical compound, stimulated $[Ca^{2+}]_i$ elevation in rat neutrophils in a Ca^{2+}-containing medium but caused no appreciable response in a Ca^{2+}-free medium. PAO also induced external Mn^{2+} entry, which was inhibited by *N*-acetyl-L-cysteine (NAC), but failed to elicit any appreciable Ba^{2+} and Sr^{2+} entry. Pretreatment of neutrophils with thiol-reducing agents including dithiothreitol (DTT), NAC, 2,3-dimercapto-1-propanol (DMP), 2,3-dimercaptopropane-1-sulfonic acid (DMPS) and tris-(2-carboxyethyl)phosphine (TCEP), all greatly inhibited PAO-induced $[Ca^{2+}]_i$ elevation. Addition of Ni^{2+} or La^{3+} followed by PAO stimulation also attenuated the Ca^{2+} signals in a concentration-dependent manner. PAO had no significant effect on the production of reactive oxygen intermediates (ROI) and nitric oxide (NO) nor did it decrease cellular low molecular weight thiols levels. PAO-induced $[Ca^{2+}]_i$ elevation was significantly inhibited by 1-[6-[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U-73122), the inhibitor of phospholipase C-coupled processes, genistein, a general tyrosine kinase inhibitor, phorbol 12-myristate 13-acetate (PMA), a protein kinase C (PKC) activator, calyculin A, a cortical actin stabilizer, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY 294002), a phosphoinositide 3-kinase inhibitor, 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1*H*-imidazole (SKF-96365), and *cis*-*N*-(2-phenylcyclopentyl)azacyclotridec-1-en-2-amine (MDL-12,330A), the blockers of receptor-gated and store-operated Ca^{2+} channels, whereas there was no appreciable effect exerted by aristolochic acid, a phospholipase A_2 inhibitor, 7-nitroindazole and *N*-(3-aminomethyl)benzylacetamidine (1400W), the blockers of NO synthase, and by suspension in a Na^+-deprived medium. In contrast, 2-aminoethoxydiphenyl borane (2-APB), the blocker of IP_3 receptor and Ca^{2+} influx, enhanced the PAO-induced response. PAO had no effect on the plasma membrane Ca^{2+}-ATPase (PMCA) activity in the pharmacological isolated neutrophil preparation and the neutrophil membrane fractions. These results indicate that PAO stimulates $[Ca^{2+}]_i$ rise in rat neutrophils mainly through the oxidation of vicinal thiol groups on the cell surface membrane to activation of a non-store operated Ca^{2+} entry (non-SOCE) without affecting the activity of PMCA and the plasmalemmal Na^+/Ca^{2+} exchanger.

Keywords: Phenylarsine oxide; Cation entry; Intracellular free-Ca^{2+}; Signal transduction; Non-store operated Ca^{2+} entry; Neutrophils

Abbreviations: 1400W, *N*-(3-aminomethyl)benzylacetamidine; AA, arachidonic acid; 2-APB, 2-aminoethoxydiphenyl borane; CPA, cyclopiazonic acid; DAF-2/DA, 4,5-diaminofluorescein diacetate; DMP, 2,3-dimercapto-1-propanol; DMPS, 2,3-dimercaptopropane-1-sulfonic acid; DTT, dithiothreitol; GEA3162, 5-amino-3-(3,4-dichlorophenyl)1,2,3,4-oxatriazolium; HBSS, Hanks' balanced salt solution; H_2DCF/DA, 2′,7′-dichlorodihydrofluorescein diacetate; IP_3, D-*myo*-inositol 1,4,5-trisphosphate; LY 294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; mBBr, monobromobimane; MDL-12,330A, *cis*-*N*-(2-phenylcyclopentyl)azacyclotridec-1-en-2-amine; NAC, *N*-acetyl-L-cysteine; NO, nitric oxide; PAO, phenylarsine oxide; PIK3, phosphoinositde-3-kinase; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; PMCA, plasma membrane Ca^{2+}-ATPase; ROI, reactive oxygen intermediates; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; SKF-96365, 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1*H*-imidazole; SOCE, store-operated Ca^{2+} entry; TCEP, tris-(2-carboxyethyl)phosphine; U-73122, 1-[6-[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione

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1. Introduction

Ca^{2+} signals have been implicated in many cellular functions of neutrophils. The increase in $[Ca^{2+}]_i$ involves two mechanisms: Ca^{2+} release from internal stores and Ca^{2+} entry across the plasma membrane. It is well established that the receptor-mediated initial Ca^{2+} spike is mediated by the activation of phosphoinositide-specific phospholipase C (PLC) that hydrolyzes membrane phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) to generate the second messenger, D-*myo*-inositol 1,4,5-trisphosphate (IP_3), which interacts with IP_3 receptor on the internal stores for the release of Ca^{2+} [1]. However, the mechanism regulating Ca^{2+} influx across the plasma membrane, which accounts for the sustained increase in $[Ca^{2+}]_i$, is still unclear. In non-excitable cells, including neutrophils, depletion of the intracellular Ca^{2+} stores induces entry of Ca^{2+} across the plasma membrane, referred to as SOCE (capacitative Ca^{2+} entry) [2]. Several hypotheses have been considered for the mechanism of SOCE. Recently, a secretion-like coupling model based on a physical and reversible trafficking of portions of the endoplasmic reticulum toward the plasma membrane has been proposed [3], which is supported by the dynamic cytoskeletal structure. It is far from certain that this mechanism is the only one involved in the increase in Ca^{2+} entry in non-excitable cells. A non-store operated Ca^{2+} entry (non-SOCE) mechanism that involves protein kinase C (PKC) has been reported in human platelets [4]. The $PI(3,4,5)P_3$-sensitive Ca^{2+} entry that is independent of the filling state of internal Ca^{2+} stores was observed in FcεRI-stimulated mast cells [5]. In addition, arachidonic acid (AA) activates the non-SOCE in smooth muscle cells [6]. The thiol modification of a number of important membrane proteins or channels induced Ca^{2+} entry in neutrophils through a non-SOCE mechanism [7,8].

Phenylarsine oxide (PAO) is a membrane-permeable trivalent arsenical compound, covalently binding the vicinal thiol groups of proteins that are in suitable proximity to form stable ring structures [9], and has been reported to inhibit phosphotyrosine phospha tases [10], the superoxide anion generation [11], and to induce L-selectin shedding [12] in neutrophils. PAO increased in $[Ca^{2+}]_i$ in a number of cell types including endothelial cells [13], platelets [14], T cells [15], and macrophages [16], and inhibited Ca^{2+} entry via N-type Ca^{2+} channel in neuromuscular junctions [17]. Our recent reports indicated that *N*-ethylmaleimide and 5-amino-3-(3,4-dichlorophenyl)1,2,3,4-oxatriazolium (GEA3162) induced Ca^{2+} entry in neutrophils through thiol-modification [7,8]. The aim of this study was to examine the effect of PAO on Ca^{2+} signaling in rat neutrophils.

2. Materials and methods

2.1. Materials

Dextran T-500 was purchased from Amersham Pharmacia Biotech. Hanks' balanced salt solution was obtained from Invitrogen. Fluo-3/AM, fura-2/AM, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF/DA), tris-(2-carboxyethyl)-phosphine (TCEP) and monobromobimane (mBBr) were purchased from Molecular Probes. 2-(4-Morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY 294002) was obtained from Biomol Research Laboratories. Cyclopiazonic acid (CPA), 1-[6-[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U-73122), 4,5-diaminofluorescein diacetate (DAF-2/DA), 2-aminoethoxydiphenyl borane (2-APB), 7-nitroindazole, 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1*H*-imidazole (SKF-96365), 1400W, calyculin A and oligomycin A were obtained from Calbiochem-Novabiochem. *cis*-*N*-(2-Phenylcyclopentyl)azacyclotridec-1-en-2-amine (MDL-12,330A) and GEA3162 were purchased from Alexis. All other reagents and chemicals were purchased from Sigma–Aldrich. The final volume of DMSO in the reaction mixture was $\leq$0.5% (v/v).

2.2. Preparation of rat neutrophils

Blood was collected from the abdominal aorta of male Sprague–Dawley rats (3 months old) and the neutrophils were purified by dextran sedimentation, centrifugation through Ficoll–Paque, and hypotonic lysis of erythrocytes [7]. Purified neutrophils containing >95% viable cells were normally resuspended in Hanks' balanced salt solution (HBSS) containing 10 mM HEPES, pH 7.4, and 4 mM $NaHCO_3$, and kept in an ice-bath before use. All experiments in the present study were performed under the guidelines of the Institutional Experimental Laboratory Animal Committee and were in strict accordance with the principles and guidelines contained in the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health.

2.3. Measurement of intracellular free Ca^{2+}

Neutrophils (5×10^7 cells/ml) were incubated with 5 μM fluo-3/AM for 45 min at 37 °C. After being washed, the cells were resuspended in HBSS to 5×10^6 cells/ml. In some experiments, cells were suspended in Na^+-deprived HEPES buffer (124 mM *N*-methyl-D-glucamine, 4 mM KCl, 0.64 mM K_2HPO_4, 0.66 mM KH_2PO_4, 10 mM HEPES, pH 7.4, 5.56 mM dextrose, and 15.2 mM $KHCO_3$). Fluorescence changes were monitored with a

fluorescence spectrophotometer at 535 nm with excitation at 488 nm. $[Ca^{2+}]_i$ was calibrated from the fluorescence intensity as follows: $[Ca^{2+}]_i = K_d \times [(F - F_{min})/(F_{max} - F)]$, where F is the observed fluorescence intensity. The values F_{max} and F_{min} were obtained at the end of experiments by the sequential addition of 0.33% Triton X-100 and 50 mM EGTA. The K_d was taken as 400 nM. In some experiments, neutrophils (5×10^7 cells/ml) were incubated with 5 μM fura-2/AM at 37°C for 45 min. After being washed, the cells were resuspended in HBSS to 5×10^6 cells/ml [7]. Fluorescence was monitored with a double-wavelength fluorescence spectrophotometer (PTI, Deltascan 4000) at 510 nm with excitation at 340 and 380 nm in the ratio mode, and calibration of the excitation ratio in terms of $[Ca^{2+}]_i$ was performed.

2.4. Measurement of Mn^{2+}, Sr^{2+}, and Ba^{2+} influx

The entry of Mn^{2+} into fura-2-loaded neutrophils (5×10^6 cells/ml) was measured with the fura-2 fluorescence quenching technique [7]. Fluorescence changes were monitored at 510 nm with excitation at 360 nm, and fluorescence intensity declined as Mn^{2+} was added to the Ca^{2+} (1 mM)-containing medium. Diethylenetriamine pentaacetic acid (2 mM) was added at the end of the experiments, and indicated that <5% of the total fluorescence quenched by Mn^{2+} was due to leakage of fura-2. In the measurement of Sr^{2+} or Ba^{2+} influx, fura-2-loaded neutrophils were activated in a Ca^{2+}-free medium, and then supplemented with 1 mM Sr^{2+} or Ba^{2+}. Fluorescence changes were monitored at 510 nm with excitation at 340 and 380 nm in a ratio mode.

2.5. Measurement of intracellular reactive oxygen intermediates (ROI) and nitric oxide (NO) production

Neutrophils (2.5×10^7 cells/ml) were incubated with 4 μM H_2DCF/DA at 37 °C for 1 h to detect $[ROI]_i$ [18]. After being washed, aliquots of cell suspension were then dispensed into 96-well plates at 5×10^6 cells/well. Fluorescence changes, termed as relative fluorescence unit (RFU), were monitored with a fluorescence microplate reader (Fluoroskan Ascent, Labsystems) at 530 nm with excitation at 485 nm. For the measurement of $[NO]_i$, neutrophils (5×10^7 cells/ml) were incubated with 15 μM DAF-2/DA [19] at room temperature for 1 h. After being washed, the cells were resuspended in HBSS to 5×10^6 cells/ml. Fluorescence changes were monitored at 515 nm with excitation at 495 nm in the presence of 100 μM L-arginine methylester.

2.6. Measurement of the total small molecule thiol contents

Neutrophil suspensions were dispensed into 96-well plates at 5×10^6 cells/well. After reaction with the tested drugs, cells were washed and then loaded with 0.2 mM mBBr for 15 min in the dark [20]. After being washed, cells were resuspended in 2% paraformaldehyde for 10 min. Fluorescence changes were monitored with a fluorescence microplate reader at 510 nm with excitation at 405 nm.

2.7. Measurement of Ca^{2+}-ATPase activity in the membrane fraction

Neutrophils (5×10^7 cells) were disrupted in Tris buffer by sonication. After centrifugation, pellets were collected and resuspended in Tris buffer as the membrane fraction. The membrane fraction (about 20 μg) was incubated in the reaction mixture containing 20 mM HEPES (pH 7.2), 100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 μM ouabain, 5 mM NaN_3, 10 μM CPA and test drugs for 3 min at 37°C before the addition of 3 mM ATP for another 30 min. The membrane Ca^{2+}-ATPase activity was determined colorimetrically by measuring the inorganic phosphate liberated from ATP hydrolysis [21].

2.8. Statistical analysis

Statistical analyses were performed using the Bonferroni *t*-test method after ANOVA for multigroup comparison and the Student's *t*-test method for two groups comparison; $p < 0.05$ was considered statistically significant. Values are expressed as mean ± S.D.

3. Results and discussion

3.1. PAO increases the external Ca^{2+} and Mn^{2+} entry

Previous studies have demonstrated that PAO induced $[Ca^{2+}]_i$ increase mainly from internal stores and partly from extracellular medium in RBL-2H3 cells [22], by mobilizing Ca^{2+} from the internal Ca^{2+} pool in rat pancreatic acinar cells [23], and independent of extracellular Ca^{2+} in thymocytes [24]. In contrast, the PAO-induced $[Ca^{2+}]_i$ increase in rat peritoneal macrophages and human foreskin fibroblasts [16] is not due to depletion of intracellular Ca^{2+} stores but mainly to a stimulation of Ca^{2+} entry form the extracellular medium. Consistent with the latter result, PAO showed a concentration-dependent increase in $[Ca^{2+}]_i$ preceded by a lag in fluo-3-loaded neutrophils in a Ca^{2+}-containing medium (Fig. 1A), whereas there was no appreciable effect in a Ca^{2+}-free medium (Fig. 1B). These results imply that PAO-stimulated external Ca^{2+} entry is primarily responsible for the increase in $[Ca^{2+}]_i$ in rat neutrophils. The viability was >90% when cells were treated with 100 μM PAO for 10 min at 37 °C in a Ca^{2+}-containing or Ca^{2+}-free medium as assessed by trypan blue exclusion. The Ca^{2+} entry activity of PAO was then further confirmed using the

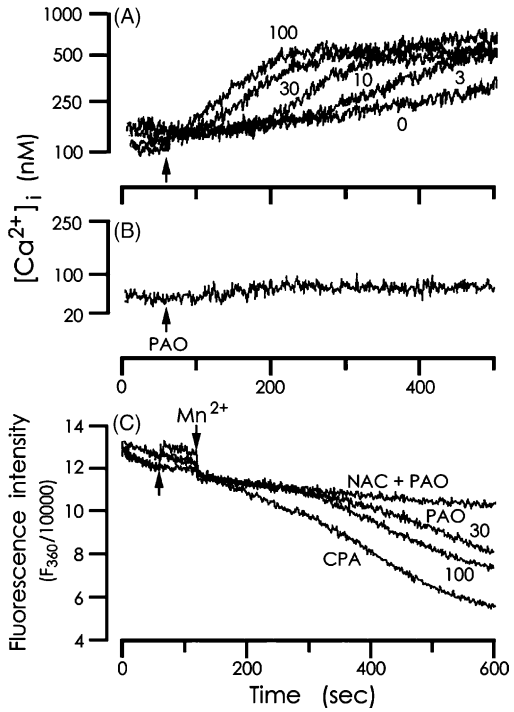

Fig. 1. PAO-stimulated $[Ca^{2+}]_i$ elevation and external Mn^{2+} influx. Fluo-3-loaded cells were stimulated with (A) the indicated concentrations (μM) of PAO (arrow) in a Ca^{2+} (1 mM)-containing medium or (B) 100 μM PAO in a Ca^{2+}-free medium. (C) Fura-2-loaded cells were activated (upward arrow) with 10 μM CPA or the indicated concentrations (μM) of PAO followed by addition of 0.5 mM Mn^{2+}. In some experiments, cells were pretreated with 1 mM NAC for 10 min before stimulation with 100 μM PAO. Similar results were obtained from three to four independent experiments.

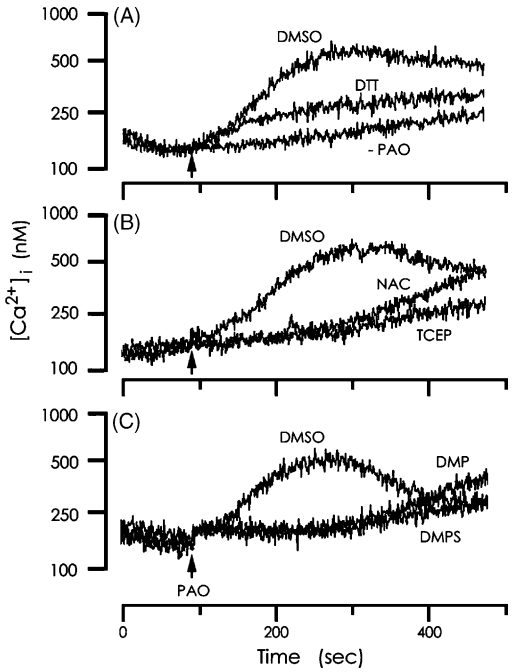

Fig. 2. Effect of thiol-reducing agents on PAO-induced $[Ca^{2+}]_i$ changes. Fluo-3-loaded cells were incubated with DMSO (as vehicle control): (A) 1 mM DTT for 1 min; (B) 1 mM NAC or 2 mM TCEP for 10 min; and (C) 0.3 mM DMP or DMPS for 1 min at 37 °C before stimulation (or without stimulation) with 100 μM PAO (arrow) in a Ca^{2+} (1 mM)-containing medium. Similar results were obtained from three to four independent experiments.

Mn^{2+} influx assay. Mn^{2+} has been shown to permeate through the neutrophils via the Ca^{2+} influx pathway activated by CPA [25], the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) blocker, and subsequent quenching of the fluorescence signal by its high-affinity binding activity to fura-2. Mn^{2+} is not a substrate for the Ca^{2+} pump and, hence, a surrogate of Ca^{2+} influx. As with 10 μM CPA, PAO resulted in a concentration-dependent decrease in fluorescence signal in fura-2-loaded neutrophils in the presence of 0.5 mM Mn^{2+} in medium (Fig. 1C), whereas 1 mM *N*-acetyl-L-cysteine (NAC), a cell permeant mono-thiol-reducing agent, fully eliminated the PAO-induced response. It is likely that PAO-induced the Mn^{2+}/Ca^{2+} influx through the thiol oxidation. Our previous report also demonstrated that GEA3162, a lipophilic NO-releasing agent, activates Ca^{2+} entry in neutrophils through direct protein thiol oxidation [8]. Therefore, we next examined the influence of thiol-reducing agents on PAO-induced $[Ca^{2+}]_i$ changes.

3.2. Effect of thiol-reducing agents on the PAO-induced Ca^{2+} signal

2,3-Dimercapto-1-propanol (DMP) and 2,3-dimercapto-propane-1-sulfonic acid (DMPS), respectively, cell permeant and impermeant vicinal thiol-reducing agents, compete for PAO on the PAO-protein complex by stripping PAO from its target protein, and forming a stable, soluble chelate. PAO-stimulated phospholipase D activation is prevented by DMP and dithiothreitol (DTT), a cell permeant dithiol-reducing agent, in RBL-2H3 cells [22], and DMP prevents PAO-induced $[Ca^{2+}]_i$ increase in T cells [15]. Pretreatment of neutrophils with 1 mM DTT effectively inhibited ($75.3 \pm 5.1\%$ inhibition of maximal $[Ca^{2+}]_i$, $p < 0.01$) (Fig. 2A), whereas 1 mM NAC, 2 mM TCEP (Fig. 2B), a cell impermeant reductant without sulfhydryl group, 0.3 mM DMP or 0.3 mM DMPS (Fig. 2C) nearly abolished the PAO-induced $[Ca^{2+}]_i$ elevation. We reasoned that the PAO-induced Ca^{2+} entry is mediated by the modification of the vicinal thiol groups on the cell surface membrane by the evidence that the inhibition of Ca^{2+} signals: (1) by cell permeant thiol-reducing agents as well as by impermeant thiol-reducing agents; and (2) by thiol-containing reductants as well as by the reductant without the sulfhydryl group. Our previous reports demonstrated that the thiol-modifying agents activate Ca^{2+} entry in neutrophils through the non-SOCE mechanism [7,8]. We were interested in whether the non-SOCE mechanism is responsible for the observed Ca^{2+} entry by PAO.

3.3. Characteristics of PAO-mediated cation permeability

The effectiveness of the specific Ca^{2+} channel blockers, La^{3+} and Ni^{2+}, in blocking passage of Ca^{2+} has been widely

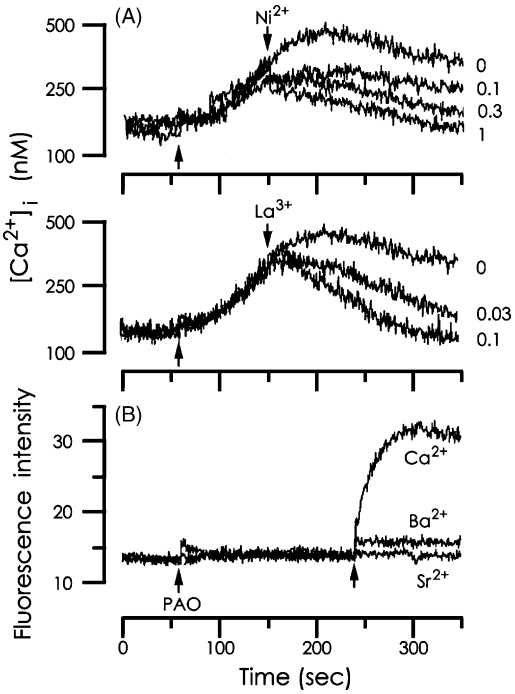

Fig. 3. Effects of Ni^{2+} and La^{3+} on PAO-induced $[Ca^{2+}]_i$ changes and the permeability of Ba^{2+} and Sr^{2+} in response to PAO. (A) Fluo-3-loaded cells were stimulated with 100 μM PAO (upward arrow) followed by addition of the indicated concentrations (mM) of Ni^{2+} or La^{3+} in a Ca^{2+} (1 mM)-containing medium. (B) Fura-2-loaded neutrophils were stimulated with 100 μM PAO in a Ca^{2+}-free medium then supplemented (second arrow) with 1 mM each of Ca^{2+}, Sr^{2+} or Ba^{2+}. Fluorescence changes were measured in a ratio mode. Similar results were obtained from three to four independent experiments.

used as a criterion for defining differences between putative entry mechanisms. A previous report demonstrated that Ni^{2+} inhibits PAO-activated Ca^{2+} entry in rat peritoneal macrophages and human foreskin fibroblasts [16]. Varying concentrations of La^{3+} or Ni^{2+} were added after the activation of external Ca^{2+} entry by PAO, and both channel blockers attenuated the PAO-induced Ca^{2+} signals in a concentration-dependent manner (full inhibition by 1 mM Ni^{2+} and 0.1 mM La^{3+}) (Fig. 3A). The inhibitory activities of La^{3+} and Ni^{2+} were within the same range of potency as that in the CPA-induced SOCE pathway [7], which implies the similar characteristics of these two Ca^{2+} influx pathways.

In neutrophils, store emptying stimulated entry of Ca^{2+}, Ba^{2+} and Sr^{2+} has been reported [7]. Ba^{2+} is not pumped by Ca^{2+}-ATPase either into internal stores or out of the cell [26] and hence can be used as a surrogate for Ca^{2+} to trace channel activity. Entry of these divalent cations in the SOCE pathway is concentration-dependent with permeability in the following order: $Ca^{2+} > Ba^{2+} \geq Sr^{2+}$ in rat neutrophils [7]. After PAO addition to a Ca^{2+}-free medium, the subsequent addition of Ca^{2+} to the medium was followed by a massive Ca^{2+} influx into neutrophils by fura-2 ratio-fluorimetry (Fig. 3B). Under the same conditions, however, no appreciable entry of Ba^{2+} and Sr^{2+}, respectively, occurred when Ca^{2+} was replaced with Ba^{2+} or Sr^{2+}. These results revealed a difference in the apparent selectivity for passage of cations activated by SOCE as opposed to the PAO-induced Ca^{2+} entry pathway in neutrophils.

3.4. Effects of PAO on the intracellular contents of ROI and total thiols

Thiols play a principal role in maintaining the appropriate oxidation-reduction state of cells. Thus, the thiol oxidation by PAO may increase the intracellular oxidative stress. It has been reported that ROI may affect mitochondrial function by increasing the open probability of the mitochondrial permeability transition pore [27]. Mitochondria are well known participants in the regulation of $[Ca^{2+}]_i$ homeostasis, capable of modulating cytosolic Ca^{2+} signals. PAO caused mitochondrial permeability transition and was also associated with increased $[Ca^{2+}]_i$ in isolated ventricular myocytes [28]. Cell-permeant H_2DCFDA, commonly used to detect the generation of ROI in phagocytes [29], is oxidized to highly fluorescent dichlorofluorescin by phagocytosis during oxidative burst. Exposure to phorbol 12-myristate 13-acetate (PMA), a PKC activator, but not to PAO, effectively increased dichlorofluorescin oxidation in H_2DCF-loaded neutrophils (Fig. 4A), thus obviating the requirement for ROI production

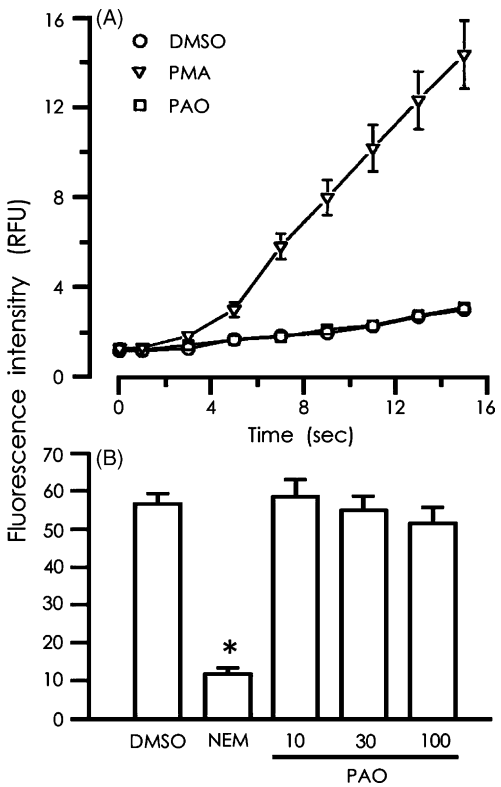

Fig. 4. Effects of PAO on the ROI production and the thiols content. (A) H_2DCFDA-loaded neutrophils were exposed to DMSO (as vehicle control), 10 nM PMA or 100 μM PAO in a Ca^{2+} (1 mM)-containing medium. (B) Neutrophils were treated with DMSO (as vehicle control), 100 μM NEM, or the indicated concentrations (μM) of PAO in a Ca^{2+} (1 mM)-containing medium, and then loaded with mBBr. The fluorescence changes were determined. Values are mean ± S.D. of three to five independent experiments. $^{*}p < 0.01$, compared with the control value.

in PAO-evoked $[Ca^{2+}]_i$ elevation. In line with our observation, PAO suppressed the superoxide production in human and porcine neutrophils [11]. The changes in low molecular weight thiols were performed by quantitative measurement of the fluorescence of mBBr, which is essentially non-fluorescent until conjugated with thiols. Exposure to *N*-ethylmaleimide, a thiol-alkylating agent, resulted in a loss of detectable thiol, whereas PAO had no significant effect on the fluorescence of mBBr (Fig. 4B). These results give further support to the notion that the PAO-induced $[Ca^{2+}]_i$ rise is not mediated by the general oxidative stress.

3.5. Effect of Ca^{2+} signal blockers on PAO-induced $[Ca^{2+}]_i$ changes

Neutrophil surface adhesion molecular L-selectin plays a major role in neutrophil adherence and transmigration through endothelial cells. The ligation of L-selectin induced significant changes in $[Ca^{2+}]_i$ in canine neutrophils [30] with a small delay. However, PAO does not cause cell activation at the low concentrations (<1 μM) that induce L-selectin shedding in human neutrophils [12].

IP_3, the product of PLC activation, mediates rapid Ca^{2+} store release by activating the IP_3 receptor in the internal stores. After this initial Ca^{2+} release phase, external Ca^{2+} entry is through plasma membrane channels, providing a secondary and more prolonged Ca^{2+} signal [31]. Previous reports demonstrated that PAO induced $[Ca^{2+}]_i$ rise without production of IP_3 in RBL-2H3 cells and T cells [15,22]. It would be reasonable to assume that IP_3 does not play an important role in PAO-induced $[Ca^{2+}]_i$ elevation in rat neutrophils because of the lack of appreciable Ca^{2+} signal in a Ca^{2+}-free medium (Fig. 1B). However, pretreatment of neutrophils with 1 μM U-73122, the inhibitor of PLC-coupled processes, attenuated the PAO-induced Ca^{2+} signal (61.4 ± 6.1% inhibition of maximal $[Ca^{2+}]_i$, $p < 0.01$) (Fig. 5A). This result is unlikely attributable to the blockade of the external Ca^{2+} entry, which appears prominent at higher concentrations of U-73122 (≥3 μM) [32]. A useful tool in elucidating the coupling mechanism for SOC channel activation has been the cell-permeant IP_3 receptor blocker, 2-APB. 2-APB (100 μM) abolished the CPA-induced $[Ca^{2+}]_i$ changes [7,8], and, interestingly, enhanced the PAO-induced response (Fig. 5B). This result is consistent with our previous reports that 2-APB enhanced the non-SOCE pathways induced by two thiol-modifying agents, *N*-ethylmaleimide [7] and GEA3162 [8]. It has been reported that 2-APB activated a nonselective cation channels in the plasma membrane of RBL-2H3 m1 cells [33] and enhanced the thapsigargin-induced SOCE of murine immature B lymphoma WEHI-231 cells [34]. In addition, 2-APB exerts an inhibitory effect on SERCA pumps (this effects occur at >200 μM 2-APB) [35]. These effects do not, however, explain the present results since the inhibition of SERCA by 2-APB occurs at higher concentrations than used in this study and 2-APB treatment nearly abolished the CPA- and formyl-Met-Leu-Phe (fMLP)-induced $[Ca^{2+}]_i$ changes in rat neutrophils [8,36]. The mechanisms implicated in the enhancement of PAO-induced Ca^{2+} response remain to be clarified. Recent evidence also indicates that the principal antagonistic effect of 2-APB is on Ca^{2+} entry rather than Ca^{2+} release [37] and 2-APB has been used as a means to distinguish between putative non-SOCE and SOCE pathways [38]. The opposite effect to 2-APB between CPA- and PAO-induced Ca^{2+} responses suggests that PAO mainly activated a non-SOCE pathway in neutrophils. It has been reported that the activity of transient receptor potential (TRP) channel is dependent on the activation of PLC and independent of the filling state of internal Ca^{2+} stores [39], which offers a possible explanation that PLC activation might also exert a direct action in the PAO-induced external Ca^{2+} entry. Further study will be required to clarify this hypothesis. The involvement of ryanodine-sensitive store in PAO-induced $[Ca^{2+}]_i$ elevation is not supported based on the observations that: (1) the lack of appreciable $[Ca^{2+}]_i$ changes by PAO in a Ca^{2+}-free medium (Fig. 1B); and (2) ruthenium red (10 μM), a ryanodine receptor inhibitor, did not modify PAO-induced Ca^{2+} influx (data not shown).

Protein tyrosine kinase activity has been suggested to control Ca^{2+} entry induced by G protein-coupled receptor in numerous cells. In human platelets, the regulation of

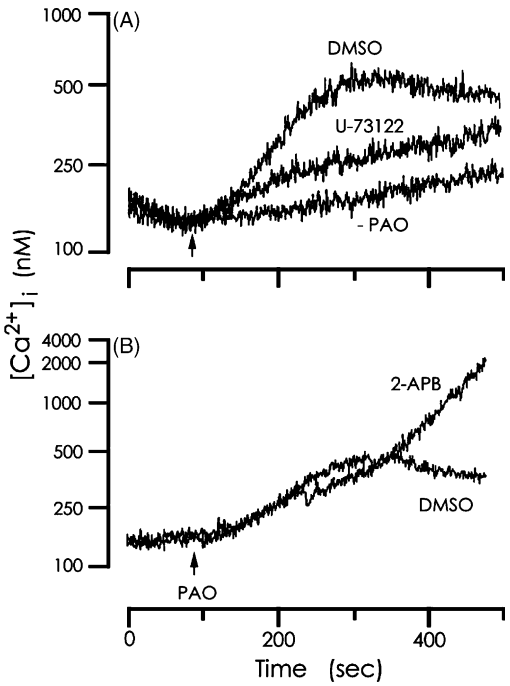

Fig. 5. Effects of U-73122 and 2-APB on PAO-induced $[Ca^{2+}]_i$ changes. Fluo-3-loaded cells were incubated with DMSO (as vehicle control): (A) 1 μM U-73122 or (B) 100 μM 2-APB for 1 min at 37 °C before stimulation (or without stimulation) with 100 μM PAO (arrow) in a Ca^{2+} (1 mM)-containing medium. Similar results were obtained from three to four independent experiments.

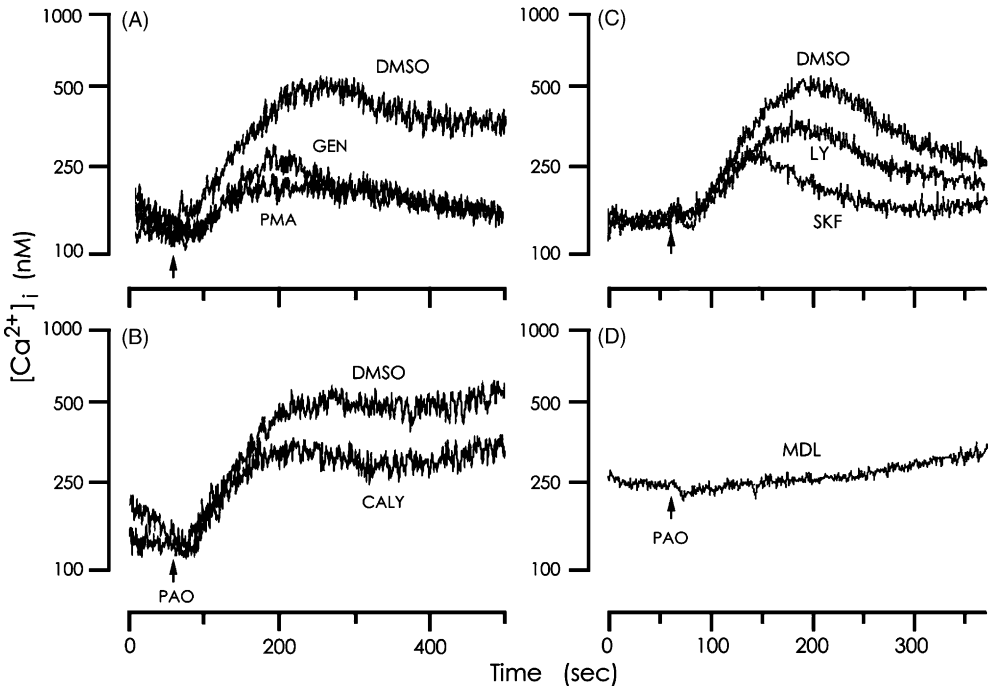

Fig. 6. Effects of genistein, PMA, calyculin A, LY 294002, SKF-96365, and MDL-12,330A on PAO-induced $[Ca^{2+}]_i$ changes. Fluo-3-loaded cells were incubated with DMSO (as vehicle control): (A) 100 μM genistein (GEN) or 0.1 μM PMA for 1 min; (B) 0.1 μM calyculin A (CALY) for 10 min; (C) 50 μM LY 294002 (LY) or SKF-96365 (SKF) for 1 min; or with (D) 30 μM MDL-12,330A (MDL) for 10 min at 37 °C before stimulation with 100 μM PAO (arrow) in a Ca^{2+} (1 mM)-containing medium. Similar results were obtained from three to four independent experiments.

SOCE by tyrosine kinase is entirely mediated by the tyrosine phosphorylation-dependent actin reorganization and polymerization, rather than through phosphorylation of a Ca^{2+} entry channel [40]. Treatment of T cells and human endothelial cells with PAO resulted in increased tyrosine phosphorylation of a number of intracellular substrates [13,15]. In human neutrophils, PAO inhibited tyrosine phosphatases [10]. PAO-activated Ca^{2+} entry is attenuated by the tyrosine kinase inhibitors, genistein and methyl-2,5-dihydroxycinnamate, in rat peritoneal macrophages and human foreskin fibroblasts [16]. The result that 100 μM genistein attenuated the PAO-induced Ca^{2+} signal (65.9 ± 5.2% inhibition of maximal $[Ca^{2+}]_i$, $p < 0.01$) (Fig. 6A) implies that the protein tyrosine phosphorylation might play a role in PAO-induced $[Ca^{2+}]_i$ elevation. It has been reported that PKC regulates SOCE and non-SOCE [41,42] in HL60 cells. Direct activation of PKC by phorbol ester has been shown to stimulate actin polymerization [43], which may prevent Ca^{2+} entry by acting as a barrier in neutrophils. Pretreatment with 0.1 μM PMA attenuated the PAO-induced Ca^{2+} signal (73.0 ± 4.9% inhibition of maximal $[Ca^{2+}]_i$, $p < 0.01$) (Fig. 6A). Moreover, calyculin A redistributed actin filaments to the cell periphery [44], which prevented SOCE activation by acting as a barrier to block the coupling process, and also attenuated the action of PAO (46.0 ± 6.2% inhibition of maximal $[Ca^{2+}]_i$ at 0.1 μM calyculin A, $p < 0.05$) (Fig. 6B). The findings suggest that the reorganization of actin cytoskeleton and the stabilization of the cortical F-actin network might also prevent $[Ca^{2+}]_i$ rise in the PAO-mediated process.

Phosphoinositde-3-kinase (PIK3) and its product phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$) are involved in the recruitment and activation of PLC-γ in many different cell types including neutrophils [45]. The existence of a $PI(3,4,5)P_3$-dependent Ca^{2+} entry system on platelet and T cell membranes has been reported [46,47], and $PI(3,4,5)P_3$ directly stimulates a Ca^{2+} transport system in the plasma membrane independent of PLC activity in RBL-2H3 mast cells [5]. We, therefore, used LY 294002, a selective inhibitor of PIK3, to examine the implication of the PIK3 pathway in the PAO-induced $[Ca^{2+}]_i$ rise. Pretreatment with 50 μM LY 294002 significantly attenuated PAO-induced $[Ca^{2+}]_i$ elevation (54.3 ± 5.6% inhibition of maximal $[Ca^{2+}]_i$, $p < 0.01$) (Fig. 6C), implying an important role of PIK3 signal in PAO-induced response. This result is inconsistent with our recent report that indicated only a slight inhibition (about 25%) by LY 294002 of CPA-induced SOCE [7]. It is plausible that the SOCE and the PAO-mediated Ca^{2+} signal process utilize different mechanisms in rat neutrophils. The mechanisms implicated in the PIK3 regulation of PAO-induced Ca^{2+} entry in rat neutrophils remain to be clarified.

MDL-12,330A blocked the Ca^{2+} entry following store emptying [48]. Our recent reports indicated that MDL-12,330A and SKF-96365, a blocker of receptor-gated and SOC channels [49], greatly reduced the $[Ca^{2+}]_i$ elevation by CPA and the Ca^{2+} entry through the non-SOCE pathway caused by thiol-modification in neutrophils [7,8]. In the present study, SKF-96365 attenuated (70.6 ± 5.3% inhibition of maximal $[Ca^{2+}]_i$, $p < 0.01$) and MDL-12,330A

nearly abrogated the PAO-induced Ca^{2+} signal (Fig. 6C and D). Thus, besides the existence of certain distinct features, there are several similar characteristics displayed between the SOCE pathway and the non-SOCE pathway caused by thiol-modification.

3.6. The role of AA and NO on the PAO-induced Ca^{2+} signal

The activation of phospholipase A_2 (PLA_2) is followed by the release of AA, which is involved in the opening of the non-SOCE pathway [50]. PAO stimulated AA release in PC12 cells [51]. Treatment of neutrophils with 50 μM aristolochic acid, a PLA_2 inhibitor, had no appreciable inhibitory effect on the PAO-induced $[Ca^{2+}]_i$ rise ($26.2 \pm 5.7\%$ inhibition of maximal $[Ca^{2+}]_i$) (Fig. 7A), implying a minor role for AA generation. NO has been proposed to control Ca^{2+} transport via cGMP-dependent and independent pathways. Recent report demonstrated that the NO donors stimulated Ca^{2+} influx through the non-SOCE pathway independently of cGMP in rat neutrophils [8], and PAO induced a slow increase in intracellular NO levels in a Ca^{2+}-dependent manner in endothelium [52]. A functional neuronal NO synthase (nNOS) system was found, while no evidence exists for inducible NO synthase (iNOS) in normal rat neutrophils. However, iNOS expression is upregulated in neutrophils after administration of endotoxin [53]. Pretreatment with 7-nitroindazole (150 μM), a relatively selective inhibitor of nNOS, and 1400W (100 μM), a potent and selective inhibitor of iNOS, failed to exert appreciable inhibitory effect on the PAO-induced Ca^{2+} response ($23.4 \pm 5.5\%$ and $9.7 \pm 4.3\%$ inhibition of maximal $[Ca^{2+}]_i$, respectively) (Fig. 7B). In addition, the changes in $[NO]_i$ were performed by quantitative measurement of the fluorescence of DAF-2, which is relatively non-fluorescent until it reacts rapidly with NO in an oxygen-containing solution to yield its highly fluorescent product triazolofluorescein. Thus, the lack of NO production upon PAO stimulation is further confirmed by the results that GEA3162 increased green-fluorescent intensity, whereas PAO had no effect in DAF-2-loaded neutrophils (Fig. 7C).

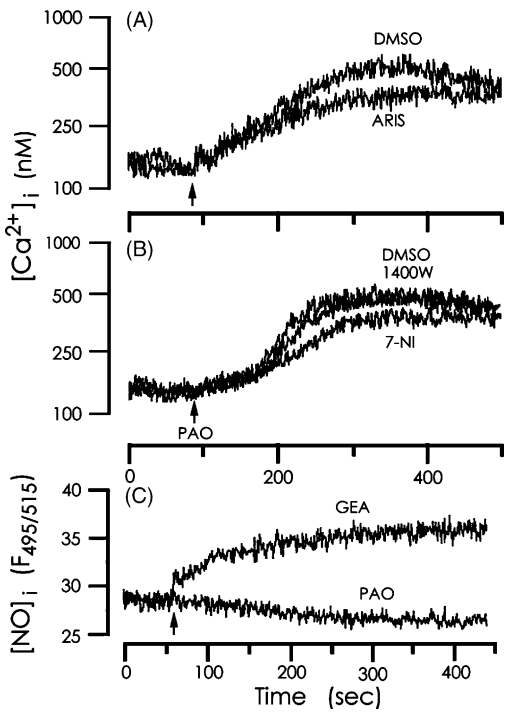

Fig. 7. Effects of aristolochic acid, 1400W, and 7-nitroindazole on PAO-induced $[Ca^{2+}]_i$ changes and the effect of PAO on NO production. Fluo-3-loaded cells were incubated with DMSO (as vehicle control): (A) 50 μM aristolochic acid (ARIS) for 1 min; (B) 100 μM 1400W or 150 μM 7-nitroindazole (7-NI) for 10 min at 37 °C before stimulation with 100 μM PAO (arrow) in a Ca^{2+} (1 mM)-containing medium; and (C) DAF-loaded neutrophils were exposed (arrow) to 100 μM GEA3162 (GEA) or 100 μM PAO in a Ca^{2+} (1 mM)-containing medium supplemented with 100 μM L-arginine methylester. Similar results were obtained from three to four independent experiments.

3.7. Effect of PAO on membrane Ca^{2+}-ATPase activity

After cell activation, removal of Ca^{2+} from the cytosol occurs via extrusion by the plasma membrane Ca^{2+}-ATPase (PMCA) and the plasmalemmal Na^+/Ca^{2+} exchanger as well as sequestration into intracellular stores by the SERCA. It is unlikely that the PAO stimulation of $[Ca^{2+}]_i$ rise occurs through the blockade of SERCA, because this would result in depletion of the internal store followed by the activation of the SOCE pathway. To address the effect of PAO on PMCA, we attempted to isolate the PMCA activity pharmacologically. Neutrophils were stimulated with fMLP and CPA to empty the internal Ca^{2+} store maximally and inhibit SERCA, and to induce Ca^{2+} entry in a Na^+-deprived medium containing 1 μM carbonyl cyanide *m*-chlorophenylhydrazone, an uncoupler of mitochondria function, to prevent mitochondrial Ca^{2+} uptake and 1 μM oligomycin A to inhibit the mitochondrial ATP synthase and prevent ATP consumption. This treatment caused a large increase in $[Ca^{2+}]_i$. Subsequent removal of external Ca^{2+} with 1 mM EDTA evoked a decline in $[Ca^{2+}]_i$ possibly due to Ca^{2+} clearance by PMCA. Under these conditions, the Ca^{2+} clearance rate was inhibited by La^{3+} and calmidazolium (data not shown) but not inhibited by PAO (Fig. 8A). The lack of effect on PMCA activity by PAO is further strengthened by determination of PMCA activity in neutrophil membrane fractions in the presence of CPA and sodium azide, to inhibit the ATPase activity in the ER and mitochondria contamination, respectively, and ouabain, a Na^+/K^+ ATPase inhibitor (Fig. 8B). In line with our observation, PAO at a concentration that increased $[Ca^{2+}]_i$ had no effect on the activity of PMCA in platelets [14]. Moreover, there is no indication that PAO affected Na^+–Ca^{2+} exchange activity on the plasma membrane because similar responses of PAO-activated Ca^{2+} signals were obtained in normal as well as in Na^+-deprived HEPES buffer (Fig. 8C).

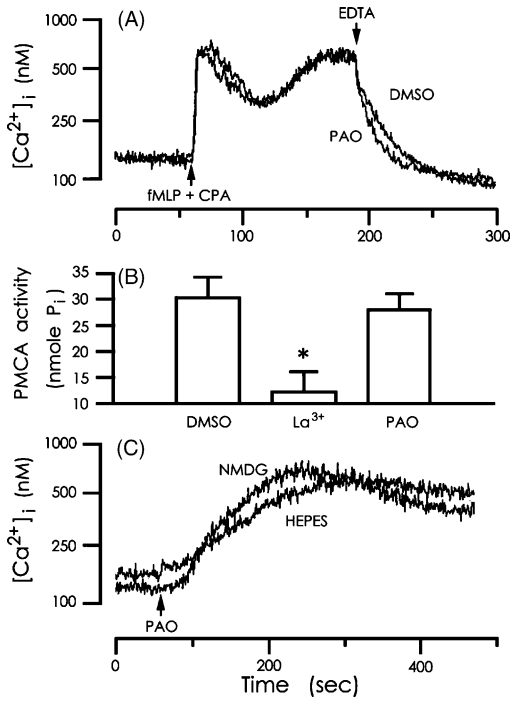

Fig. 8. Effects of PAO on PMCA activity and the effect of Na^+-deprivation on PAO-induced $[Ca^{2+}]_i$ changes. (A) Fluo-3-loaded cells were stimulated with 0.3 μM fMLP plus 10 μM CPA followed by addition of DMSO (as vehicle control) or 100 μM PAO together with 1 mM EDTA (downward arrow) in a Ca^{2+} (0.3 mM)-containing Na^+-deprived medium supplemented with 1 μM oligomycin A and 1 μM carbonyl cyanide *m*-chlorophenylhydrazone. (B) Neutrophils membrane fractions were incubated with DMSO (as vehicle control), 3 mM La^{3+} or 100 μM PAO for 3 min at 37°C in the presence of 10 μM ouabain, 5 mM NaN_3 and 10 μM CPA before the addition of 3 mM ATP. (C) Cells were stimulated with 100 μM PAO in a normal HEPES medium or a Na^+-deprived medium (NMDG). Similar results were obtained from three to four independent experiments.

In conclusion, PAO stimulates $[Ca^{2+}]_i$ rise in rat neutrophils mainly through the oxidation of vicinal thiol groups on the cell surface membrane to activation of Ca^{2+} entry through the non-SOCE pathway without affecting the activity of PMCA and the plasmalemmal Na^+/Ca^{2+} exchanger. Protein tyrosine phosphorylation and PIK3 signaling pathways are probably involved in the PAO-activated non-SOCE.

Acknowledgements

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