Suppression of superoxide anion and elastase release by C_{18} unsaturated fatty acids in human neutrophils

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Abstract The structure-activity relationship of 18-carbon fatty acids (C_{18} FAs) on human neutrophil functions and their underlying mechanism were investigated. C_{18} unsaturated (U)FAs potently inhibited superoxide anion production, elastase release, and Ca^{2+} mobilization at concentrations of <10 μM in formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)-activated human neutrophils. However, neither saturated FA nor esterified UFAs inhibited these neutrophil functions. The inhibitory potencies of C_{18} UFAs decreased in the following order: C_{18}:1 > C_{18}:2 > C_{18}:3 > C_{18}:4. Notably, the potency of attenuating Ca^{2+} mobilization was closely correlated with decreasing cellular responses. The inhibitions of Ca^{2+} mobilization by C_{18} UFAs were not altered in a Ca^{2+}-containing Na^{+}-deprived medium. Significantly, C_{18} UFAs increased the activities of plasma membrane Ca^{2+}-ATPase (PMCA) in neutrophils and isolated cell membranes. In contrast, C_{18} UFAs failed to alter either the cAMP level or phosphodiesterase activity. Moreover, C_{18} UFAs did not reduce extracellular Ba^{2+} entry in FMLP- and thapsigargin-activated neutrophils. In summary, the inhibition of neutrophil functions by C_{18} UFAs is attributed to the blockade of Ca^{2+} mobilization through modulation of PMCA. We also suggest that both the free carboxy group and the number of double bonds of the C_{18} UFA structure are critical to providing the potent anti-inflammatory properties in human neutrophils.—Hwang, T-L., Y-C. Su, H-L. Chang, Y-L. Leu, P-J. Chung, L-M. Kuo, and Y-J. Chang. **Suppression of superoxide anion and elastase release by C_{18} unsaturated fatty acids in human neutrophils. *J. Lipid Res.* 2009.** 50: **1395–1408.**

Supplementary key words calcium • cAMP • structure-activity relationship • plasma membrane Ca^{2+}-ATPase

FAs have been reported to exert their effects by modulating immune cell functions, resulting in stimulation and/or inhibition of the production of cytokines, chemokines, growth factors, reactive oxygen species (ROS), lipid mediators, and antibodies (1–3). PUFAs are thought to play an important function in the prevention and treatment of cardiovascular, inflammatory, and autoimmune diseases (4–7). Alterations in dietary levels of linear-chain 18-carbon (C_{18}) unsaturated (U)FAs have significant benefits in cardiovascular diseases and inflammatory syndromes (8–12). However, not all studies support the anti-inflammatory role of C_{18} FAs in neutrophils. Depending on the experimental conditions, C_{18} FAs can either inhibit or enhance neutrophil activation. For example, the saturated FA, stearic acid (SA; C_{18}:0), and the UFAs, oleic acid (OA; C_{18}:1n-9), linoleic acid (LA; C_{18}:2n-6), and γ-linolenic acid (γLA; C_{18}:3n-6), have been shown to elicit ROS generation, CD11b expression, leukotriene B_4 production, and/or β-glucuronidase release (13–17). In contrast, some studies reported that OA, LA, and γLA inhibit ROS generation, myeloperoxidase release, and/or phagocytosis in neutrophils (18, 19). The controversial results of those studies may be due to different concentrations used. For example, OA and LA at high concentrations (100 and 1,000 μM) induced ROS production by themselves, whereas both C_{18} UFAs at low concentrations (1–10 μM) inhibited formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)-activated ROS release in human neutrophils (20). OA at high (40 and 80 μM) but not low concentrations (2.5, 5, and 10 μM) elicited CD11b expression in human neutrophils (15). Obviously, further research is needed to clarify the effects and action mechanisms of C_{18} FAs in neutrophil functions.

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Abbreviations: Ba^{2+}, barium; C18, 18-carbon; CB, cytochalasin B; CVA, *cis*-vaccenic acid; ER, endoplasmic reticulum; ERCA, endoplasmic reticulum Ca^{2+}-ATPase; FMLP, formyl-L-methionyl-L-leucyl-L-phenylalanine; IC_{50}, 50% inhibitory concentration; IP_3, 1,4,5-triphosphate; LDH, lactate dehydrogenase; LA, linoleic acid; αLA, α-linoleic acid; γLA, γ-linoleic acid; NCX, Na^{+}-Ca^{2+} exchanger; OA, oleic acid; $O_2^{\bullet -}$, superoxide anion; PDE, phosphodiesterase; PKC, protein kinase C; PMA, phorbol myristate acetate; PMCA, plasma membrane Ca^{2+}-ATPase; ROS, reactive oxygen species; SA, stearic acid; SOCE, store-operated Ca^{2+} entry; SOD, superoxide dismutase; UFA, unsaturated fatty acid.

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Neutrophils play a pivotal role in the defense of the human body against infections. However, overwhelming activation of neutrophils is known to elicit tissue damage. Human neutrophils are known to play important roles in the pathogenesis of various diseases, such as ischemic heart disease, acute myocardial infarction, sepsis, and atherogenesis (21–24). In response to diverse stimuli, activated neutrophils secrete a series of cytotoxins, such as the superoxide anion ($O_2^{\bullet -}$), a precursor of other ROS, granule proteases, and bioactive lipids (25, 26). $O_2^{\bullet -}$ production is linked to the killing of invading microorganisms, but it can also directly or indirectly cause damage by destroying surrounding tissues. Neutrophil granules contain many antimicrobial and potentially cytotoxic substances. Neutrophil elastase is a major secreted product of stimulated neutrophils and a major contributor to the destruction of tissue in chronic inflammatory disease (27). Therefore, it is crucial to restrain respiratory burst and degranulation in physiological conditions while potentiating these functions in infected tissues and organs.

In this study, the effects of a series of linear-chain C_{18} FAs on respiratory burst and degranulation were studied in human neutrophils. We found that C_{18} UFAs significantly inhibited the generation of $O_2^{\bullet -}$ and release of elastase at concentrations of <10 μM in FMLP-activated human neutrophils. However, C_{18} saturated FA and esterified UFAs caused no inhibition. Furthermore, our results reveal a close correlation of the inhibition of $O_2^{\bullet -}$ and elastase release with attenuation of the intracellular calcium concentration ($[Ca^{2+}]_i$). FMLP, via a G-protein-coupled receptor, mobilizes rapid Ca^{2+} release from inositol 1,4,5-triphosphate (IP_3)-sensitive endoplasmic reticulum (ER) Ca^{2+} stores. Such Ca^{2+} release depletes ER Ca^{2+} stores and subsequently activates extracellular Ca^{2+} influx across the plasma membrane (28). The magnitude and duration of $[Ca^{2+}]_i$ signal responses to G-protein-coupled chemoattractants are obviously important. Herein, we also discuss possible mechanisms of C_{18} UFAs that can account for their modulation of $[Ca^{2+}]_i$ in human neutrophils.

MATERIALS AND METHODS

Materials

C_{18} FAs (obtained from Sigma-Aldrich, St. Louis, MO) were dissolved in absolute ethanol and stored under a nitrogen atmosphere at −20°C before use. The final concentration of ethanol in the cell experiments did not exceed 0.1% and did not affect the parameters measured. HBSS was purchased from Gibco BRL (Grand Island, NY). A23187, aprotinin, H89 (*N*-(2-((*p*-bromocinnamyl)amino) ethyl)-5-isoquinolinesulfonamide), leupeptin, PMSF, and Ro318220 (3-(1-(3-(amidinothio)propyl-1H-indol-3-yl))-3-(1-methyl-1H-indol-3-yl)maleimide) were obtained from Calbiochem (La Jolla, CA). Fluo 3-AM and fura 2-AM were purchased from Molecular Probes (Eugene, OR). All other chemicals were obtained from Sigma-Aldrich.

Preparation of human neutrophils

Blood was taken from healthy human donors (20–32 years old) by venipuncture, using a protocol approved by the Institutional Review Board at Chang Gung Memorial Hospital. Neutrophils were isolated with a standard method of dextran sedimentation prior to centrifugation in a Ficoll Hypaque gradient and the hypotonic lysis of erythrocytes. Purified neutrophils that contained >98% viable cells, as determined by the trypan blue exclusion method, were resuspended in Ca^{2+}-free HBSS buffer at pH 7.4 and maintained at 4°C before use.

Measurement of $O_2^{\bullet -}$ generation

The $O_2^{\bullet -}$ generation assay was based on the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c*. In brief, after supplementation with 0.5 mg/ml ferricytochrome *c* and 1 mM Ca^{2+}, neutrophils (6×10^5/ml) were equilibrated at 37°C for 2 min and incubated with FAs for 5 min. Cells were activated with 100 nM FMLP, 0.5 μM thapsigargin, or 100 nM phorbol myristate acetate (PMA) for 10 min. When FMLP and thapsigargin was used as a stimulant, 1 μg/ml cytochalasin B (CB) was incubated for 3 min before activation. Changes in absorbance with the reduction of ferricytochrome *c* at 550 nm were continually monitored in a double-beam, six-cell positioner spectrophotometer with constant stirring (Hitachi U-3010; Tokyo, Japan). Calculations were based on differences in the reactions with and without SOD (100 U/ml) divided by the extinction coefficient for the reduction of ferricytochrome *c* (ε = 21.1/mM/10 mm).

The $O_2^{\bullet -}$-scavenging ability of FAs was determined using xanthine/xanthine oxidase in a cell-free system, based on a previously described method (29). After 0.1 mM xanthine was added to the assay buffer (50 mM Tris, pH 7.4, 0.3 mM WST-1, and 0.02 U/ml xanthine oxidase) for 15 min at 30°C, the absorbance associated with the $O_2^{\bullet -}$-induced WST-1 reduction was measured at 450 nm.

Measurement of elastase release

Degranulation of azurophilic granules was determined by elastase release. Experiments were performed using MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide as the elastase substrate. Briefly, after supplementation with MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide (100 μM), neutrophils (6×10^5/ml) were equilibrated at 37°C for 2 min and incubated with FAs for 5 min. Cells were activated by FMLP (100 nM) in the presence of CB (0.5 μg/ml), and changes in absorbance at 405 nm were continuously monitored to determine elastase release. The results are expressed as a percentage of the initial rate of elastase release in the FMLP/CB-activated, drug-free control system.

To assay whether FAs exhibit an inhibitory ability toward elastase activity, a direct elastase activity assay was performed in a cell-free system. Neutrophils (6×10^5/ml) were incubated for 20 min in the presence of FMLP (100 nM)/CB (2.5 μg/ml) at 37°C. Cells were then centrifuged at 1,000 *g* for 5 min at 4°C to collect the elastase from the supernatant. The supernatant was equilibrated at 37°C for 2 min and incubated with or without FAs for 5 min. After incubation, the elastase substrate, MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide (100 μM), was added to the reaction mixtures. Changes in absorbance at 405 nm were continuously monitored for 10 min to assay the elastase activity.

Lactate dehydrogenase release

Lactate dehydrogenase (LDH) release was determined by a commercially available method (Promega, Madison, WI). Neutrophils (6×10^5/ml) were equilibrated at 37°C for 2 min and incubated with FAs for 15 min. Cytotoxicity was represented by LDH release in a cell-free medium as a percentage of the total LDH released. The total LDH released was determined by lysing cells with 0.1% Triton X-100 for 30 min at 37°C.

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Determination of cAMP concentration

The cAMP level was assayed using an enzyme immunoassay kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). The reaction of neutrophils was terminated by adding 0.5% dodecytrimethylammonium bromide. Samples were then centrifuged at 3,000 *g* for 5 min at 4°C. The supernatants were used as a source for the cAMP samples. The assay was performed according to the manufacturer's instructions.

Assay of phosphodiesterase activity

Neutrophils (5×10^7 cells/ml) were sonicated in ice-cold buffer containing 25 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 2 mM EDTA, 5 mM $MgCl_2$, 10 μM leupeptin, 100 μM PMSF, and 10 μM pepstatin. Unbroken cells were removed by centrifugation at 300 *g* for 5 min, and then the supernatant was centrifuged at 100,000 *g* for 40 min at 4°C. The cytosolic fraction was used as the source for the phosphodiesterase (PDE) enzymes. PDE activity was analyzed using a tritium scintillation proximity assay system, and the assay was performed according to the manufacturer's instructions (Amersham Pharmacia Biotech). Briefly, assays were performed at 30°C for 10 min in the presence of 50 mM Tris HCl (pH 7.5) containing 8.3 mM $MgCl_2$, 1.7 mM EGTA, and 0.3 mg/ml BSA. Each assay was performed in a 100-μl reaction volume containing the above buffer, the neutrophil supernatant fraction, and around 0.05 μCi [^{3}H]cAMP. The reaction was terminated by the addition of 50 μl PDE scintillation proximity assay beads (1 mg) suspended in 18 mM zinc sulfate. Assays were performed in 96-well microtiter plates. The reaction mixture was allowed to settle for 1 h before counting in a microtiter plate counter.

Measurement of $[Ca^{2+}]_i$

Neutrophils were loaded with 2 μM fluo 3-AM at 37°C for 45 min. After being washed, cells were resuspended in Ca^{2+}-free HBSS to 3×10^6 cells/ml. In some experiments, neutrophils were suspended in sodium (Na^+)-deprived HEPES buffer (124 mM *N*-methyl-D-glucamine, 4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.64 mM K_2HPO_4, 0.66 mM KH_2PO_4, 5.56 mM dextrose, 10 mM HEPES, and 15.2 mM $KHCO_3$, pH 7.4) (30). The change in fluorescence was monitored using a Hitachi F-4500 spectrofluorometer (Tokyo, Japan) in a quartz cuvette with a thermostat (37°C) and continuous stirring. The excitation wavelength was 488 nm, and the emission wavelength was 520 nm. FMLP, thapsigargin, and A23187 were used to increase $[Ca^{2+}]_i$ in the presence or absence of 1 mM Ca^{2+}. $[Ca^{2+}]_i$ was calibrated by the fluorescence intensity as follows: $[Ca^{2+}]_i = K_d \times [(F - F_{min})/(F_{max} - F)]$, where F is the observed fluorescence intensity, F_{max} and F_{min} were respectively obtained by the addition of 0.05% Triton X-100 and 20 mM EGTA, and K_d was taken to be 400 nM.

Measurement of barium influx

Neutrophils were loaded with 4 μM fura 2-AM at 37°C for 45 min. After being washed, cells were resuspended in Ca^{2+}-free HBSS to 3×10^6 cells/ml. The entry of barium (Ba^{2+}) into FMLP- and thapsigargin-stimulated neutrophils was measured, followed by the addition of 1 mM Ba^{2+} to Ca^{2+}-free medium. Ba^{2+} uptake was monitored from the increase in fura 2 fluorescence at 510 nm with excitation at 360 nm, which is insensitive to variations in $[Ca^{2+}]_i$ (31).

Assay of plasma membrane Ca^{2+}-ATPase activity

Neutrophils were sonicated in ice-cold Tris buffer (pH 7.4), and then cells were centrifuged at 20,000 *g* for 40 min at 4°C. The pellet fraction was collected and used as the source of Ca^{2+}-ATPase enzymes. Ca^{2+}-ATPase activity was measured using an assay in which ATP hydrolysis is coupled to NADH oxidation by pyruvate kinase with phosphoenolpyruvate and LDH (32, 33). ATP hydrolysis was measured in 800 μl of relaxation buffer containing 8×10^6 cell equivalents of membrane extract, 20 mM HEPES (pH 7.4), 130 mM NaCl, 4 mM $MgCl_2$, 3 mM ATP, 0.2 mM $CaCl_2$, 0.8 mM NADH, 1.68 mM phosphoenolpyruvate, 24 units of pyruvate kinase, 57.6 units of LDH, 10 μM ouabain, and 10 μM thapsigargin at 25°C. Changes in absorbance at 340 nm were continually monitored in a double-beam, six-cell positioner spectrophotometer with constant stirring. After incubation for 5 min, FAs (5 μM) and calmodulin (10 μg/ml) were added to the assay buffer, and the ATPase activity was measured for 50 min. The mmol extinction coefficient for NADH was 6.22. Plasma membrane Ca^{2+}-ATPase (PMCA) activity was calculated from the slope of the tracing curve between 20 and 35 min and expressed as μmol Pi/1×10^7 cells/h.

Statistical analysis

The 50% inhibitory concentration (IC_{50}) values were calculated from concentration response curves obtained from several independent experiments using Sigmaplot (Jandel Scientific, San Rafael, CA). Data are presented as the mean ± SEM, and comparisons were made using Student's *t*-test. A probability of <0.05 was considered significant.

RESULTS

C_{18} UFAs inhibit $O_2^{\bullet-}$ generation and elastase release in FMLP/CB-induced human neutrophils but not in cell-free systems

To investigate whether C_{18} FAs reduced respiratory burst and degranulation by human neutrophils in response to FMLP/CB, the amounts of $O_2^{\bullet-}$ and elastase were determined. UFAs significantly inhibited $O_2^{\bullet-}$ production and elastase release in FMLP/CB-activated human neutrophils in a concentration-dependent manner with IC_{50} values of <10 μM (**Table 1**, **Fig. 1A**). The inhibitory potencies of C_{18} UFAs were in the order of *cis*-vaccenic acid (CVA; C_{18}:1n-7) > OA > LA > α-linolenic acid (αLA; C_{18}:3n-3) = γLA > stearidonic acid (C_{18}:4n-3), indicating that the inhibitory actions decreased in the following order: C_{18}:1 > C_{18}:2 > C_{18}:3 > C_{18}:4. Nevertheless, the comparison of data shows that there are no preferences for the double bound position of C_{18} UFAs on the anti-inflammatory activity. In addition, neither OA methyl ester (OA-me) nor LA-me inhibited these neutrophil functions. The saturated SA (C_{18}:0) at a concentration of 10 μM showed minor effects in human neutrophils. On the other hand, none of these C_{18} FAs altered the basal $O_2^{\bullet-}$ generation or elastase release under resting conditions. Moreover, none of these C_{18} FAs (up to 30 μM) scavenged $O_2^{\bullet-}$ formation or inhibited elastase activity in cell-free systems (data not shown). These data rule out the possibility that the inhibitory effects of C_{18} UFAs on $O_2^{\bullet-}$ release occur through scavenging of $O_2^{\bullet-}$ and elastase. Culturing with C_{18} FAs (up to 10 μM) did not affect cell viability, as assayed by LDH release (data not shown). Moreover, $O_2^{\bullet-}$ release induced by PMA (100 nM), a protein kinase C (PKC) activator, was not inhibited by OA, CVA, and LA (5 μM) (Fig. 1C), suggesting that C_{18} UFAs exert their inhibitory influence by interfering with specific cellular signaling pathways.

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TABLE 1. Effects of C_{18} FAs on superoxide generation, elastase release, peak intracellular calcium concentrations $[Ca^{2+}]_i$, and the time taken for this concentration to decline to half of its peak values ($t_{1/2}$) in FMLP-activated neutrophils

C_{18} FA	IC$_{50}$ of C_{18} FAs		Effects of C_{18} FAs at 5 μM	
	$O_2^{\bullet -}$ generation	Elastase Release	Peak $[Ca^{2+}]$i	$t_{1/2}$
	μM		nM	s
Control	–	–	313.19 ± 14.60	24.23 ± 1.79
SA	>10	>10	304.68 ± 11.33	24.02 ± 1.93
OA (*cis*, 1n-9)	2.56 ± 0.13	1.40 ± 0.07	288.79 ± 15.21	8.99 ± 0.86***
CVA (*cis*, 1n-7)	2.31 ± 0.20	1.36 ± 0.07	291.46 ± 22.67	10.34 ± 1.24***
LA (*cis*, 2n-6)	2.64 ± 0.17	1.80 ± 0.12	303.39 ± 16.88	10.00 ± 0.58***
αLA (*cis*, 3n-3)	4.13 ± 0.24	3.19 ± 0.25	313.22 ± 25.96	14.71 ± 1.80**
γLA (*cis*, 3n-6)	4.57 ± 0.25	3.17 ± 0.18	303.99 ± 32.34	12.30 ± 0.47***
SDA (*cis*, 4n-3)	7.98 ± 0.40	8.06 ± 0.29	311.63 ± 20.59	19.28 ± 2.68
OA-me (*cis*, 1n-9)	>10	>10	309.91 ± 20.67	25.62 ± 2.82
LA-me (*cis*, 2n-6)	>10	>10	301.83 ± 11.02	24.55 ± 1.84

For all data, values are the mean ± SEM (n = 3–7). *$P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the control. SDA, stearidonic acid; OA-me, OA-methyl ester; LA-me, LA-methyl ester.

The effect of C_{18} UFAs on $O_2^{\bullet -}$ generation and elastase release in human neutrophil in the presence of BSA was also investigated to determine whether these FAs could inhibit human neutrophil functions under more physiological condition. As shown in Fig. 1B, FMLP/CB-induced $O_2^{\bullet -}$ generation and elastase release were inhibited by higher concentrations (1–10 mM) of OA and CVA in the presence of 0.1% BSA. In addition, OA-me even at higher concentrations (1–10 mM) did not alter FMLP/CB-induced $O_2^{\bullet -}$ generation and elastase release in the presence of 0.1% BSA (data not shown). These results indicate that C_{18} UFAs, essentially when the concentrations exceed the FA binding capacity of albumin, are likely to reduce FMLP/CB-induced $O_2^{\bullet -}$ generation and elastase release in human neutrophils.

Effects of C_{18} FAs on $[Ca^{2+}]_i$

Many cellular functions of neutrophils, such as respiratory burst and degranulation, are regulated by Ca^{2+} signals (34). None of these C_{18} FAs (5 μM) affected peak $[Ca^{2+}]_i$ values in FMLP-induced cells, but the time it took for $[Ca^{2+}]_i$ to return to half of the peak value ($t_{1/2}$) was significantly shortened by C_{18} UFAs in a concentration-dependent manner (Table 1, **Fig. 2A**). Among them, OA, CVA, and LA showed the most potent inhibition of Ca^{2+} mobilization. In contrast, neither OA-me, LA-me, nor SA affected $t_{1/2}$ values in FMLP-activated human neutrophils. Additionally, when C_{18} UFAs were incubated before FMLP in Ca^{2+}-free medium, similar actions on the kinetics of FMLP-induced $[Ca^{2+}]_i$ mobilization were obtained. Meanwhile, changes in $[Ca^{2+}]_i$ caused by the subsequent addition of 1 mM Ca^{2+} were inhibited by C_{18} UFAs (**Table 2**, Fig. 2B). The latter responses were also confirmed when OA, CVA, and LA were added before or after the reintroduction of Ca^{2+} (Fig. 2C). On the other hand, OA, CVA, and LA (5 μM) significantly inhibited $O_2^{\bullet -}$ production in FMLP/CB-activated human neutrophils in Ca^{2+}-free medium (Fig. 2D).

Thapsigargin, a specific and potent inhibitor of ER Ca^{2+}-ATPases (ERCAs), is able to induce the influx of Ca^{2+} through store-operated Ca^{2+} entry (SOCE) by blocking ER Ca^{2+} reuptake, thus elevating $[Ca^{2+}]_i$ and depleting ER Ca^{2+} stores (35). The increase in $[Ca^{2+}]_i$ induced by thapsigargin (0.1 μM) was initiated by the slow release of Ca^{2+} from intracellular Ca^{2+} stores, which caused considerable and sustained Ca^{2+} entry. Neither OA, CVA, nor LA (5 μM) affected the initial $[Ca^{2+}]_i$ increases but suppressed the sustained $[Ca^{2+}]_i$ changes in thapsigargin-activated human neutrophils (**Fig. 3A**). In line with these data, thapsigargin/CB-induced $O_2^{\bullet -}$ generation was moderately reduced by OA, CVA, and LA (5 μM) (Fig. 1D). On the other hand, A23187 is a Ca^{2+} ionophore that equilibrates Ca^{2+} gradients across plasma membranes and can cause a rapid rise in $[Ca^{2+}]_i$ levels. C_{18} UFAs showed inhibitory responses toward $[Ca^{2+}]_i$ levels induced by lower (20 nM) but not higher concentrations (200 nM) of A23187 (Fig. 3B, C).

The cAMP pathway is not involved in the inhibitory effects of C_{18} UFAs

Elevation of intracellular cAMP concentrations has been demonstrated to inhibit the generation of $O_2^{\bullet -}$ and the release of elastase, as well as to shorten the value of $t_{1/2}$ in FMLP-activated human neutrophils (36, 37). To examine whether cAMP pathways are involved in the inhibitory effects of C_{18} UFAs, cAMP concentrations and PDE activities were assayed. PGE_1 (an adenylate cyclase activator) and rolipram (a PDE4 inhibitor) were used as positive controls. Neither cAMP concentrations nor cAMP PDE activities were altered by OA, CVA, or LA (**Fig. 4A, B**). Moreover, the protein kinase (PK)A inhibitor, H89, did not restore the UFA-induced inhibition of $O_2^{\bullet -}$ release (Fig. 4C).

Effects of C_{18} UFAs and PKC on SOCE

FMLP, via a G-protein-coupled receptor, mobilizes rapid Ca^{2+} release from IP_3-sensitive ER Ca^{2+} stores. Such Ca^{2+} release depletes ER Ca^{2+} stores and subsequently initiates Ca^{2+} entry via SOCE (38, 39). It has been reported that PKC is able to inhibit SOCE in human neutrophils (31). In agreement with this finding, low concentrations of PMA (1 and 3 nM) concentration-dependently inhibited extracellular Ca^{2+} entry in FMLP-activated human neutrophils, which was completely abolished by the PKC inhibitor, Ro318220

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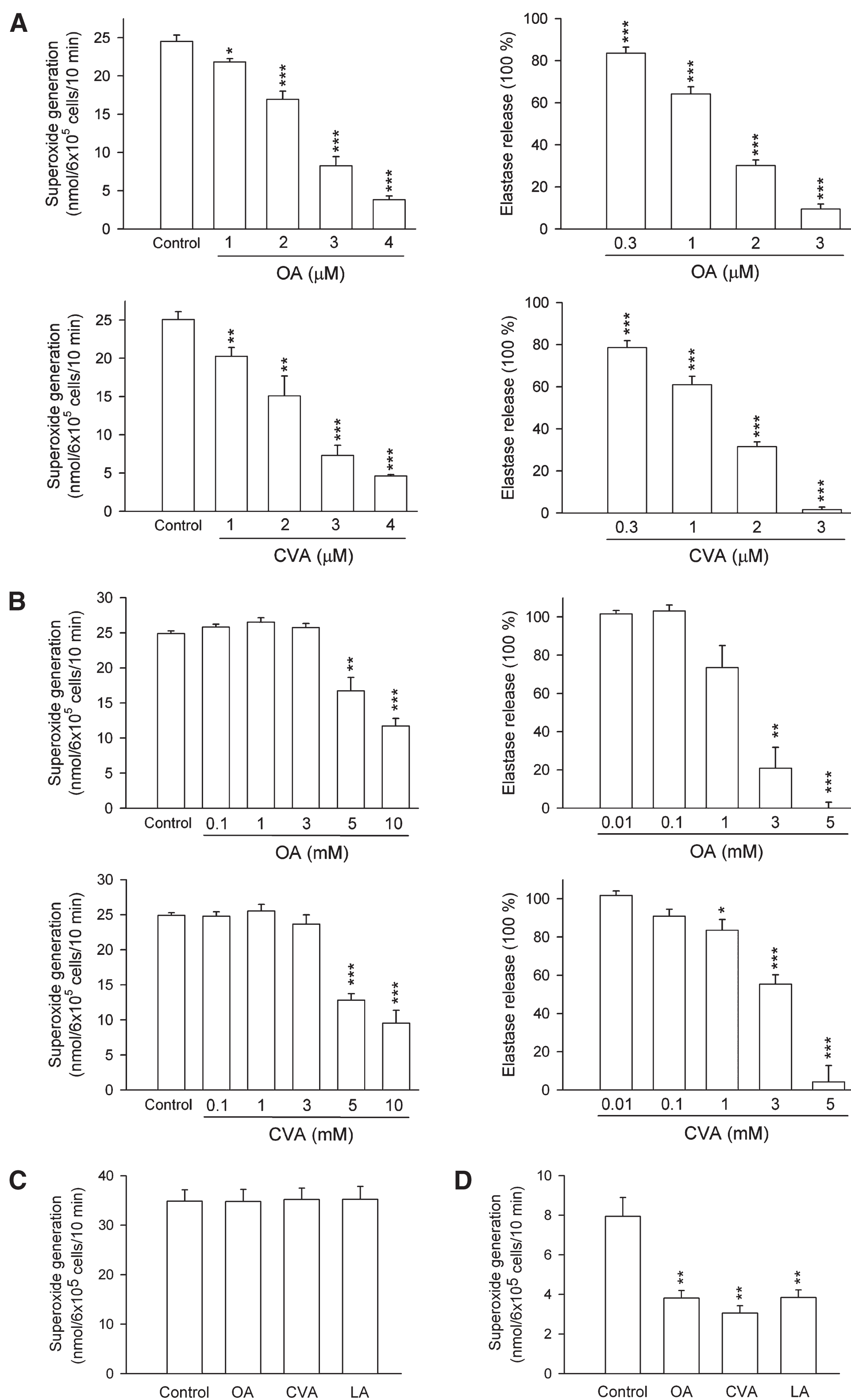

Fig. 1. Effect of C_{18} UFAs on $O_2^{\bullet -}$ generation and elastase release in activated human neutrophils. Human neutrophils were incubated with different concentrations of OA and CVA for 5 min and then activated by FMLP/CB in the absence (A) or presence (B) of 0.1% BSA. C: Human neutrophils were incubated with ethanol (0.1%, control), OA, CVA, or LA (5 μM) for 5 min and then activated by PMA or thapsigargin/CB (D). $O_2^{\bullet -}$ generation and elastase release were induced by FMLP/CB and respectively measured using SOD-inhibitable cytochrome *c* reduction and by monitoring *p*-nitroanilide release, as described in Materials and Methods. All data are expressed as the mean ± SEM (n = 3–7). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the control.

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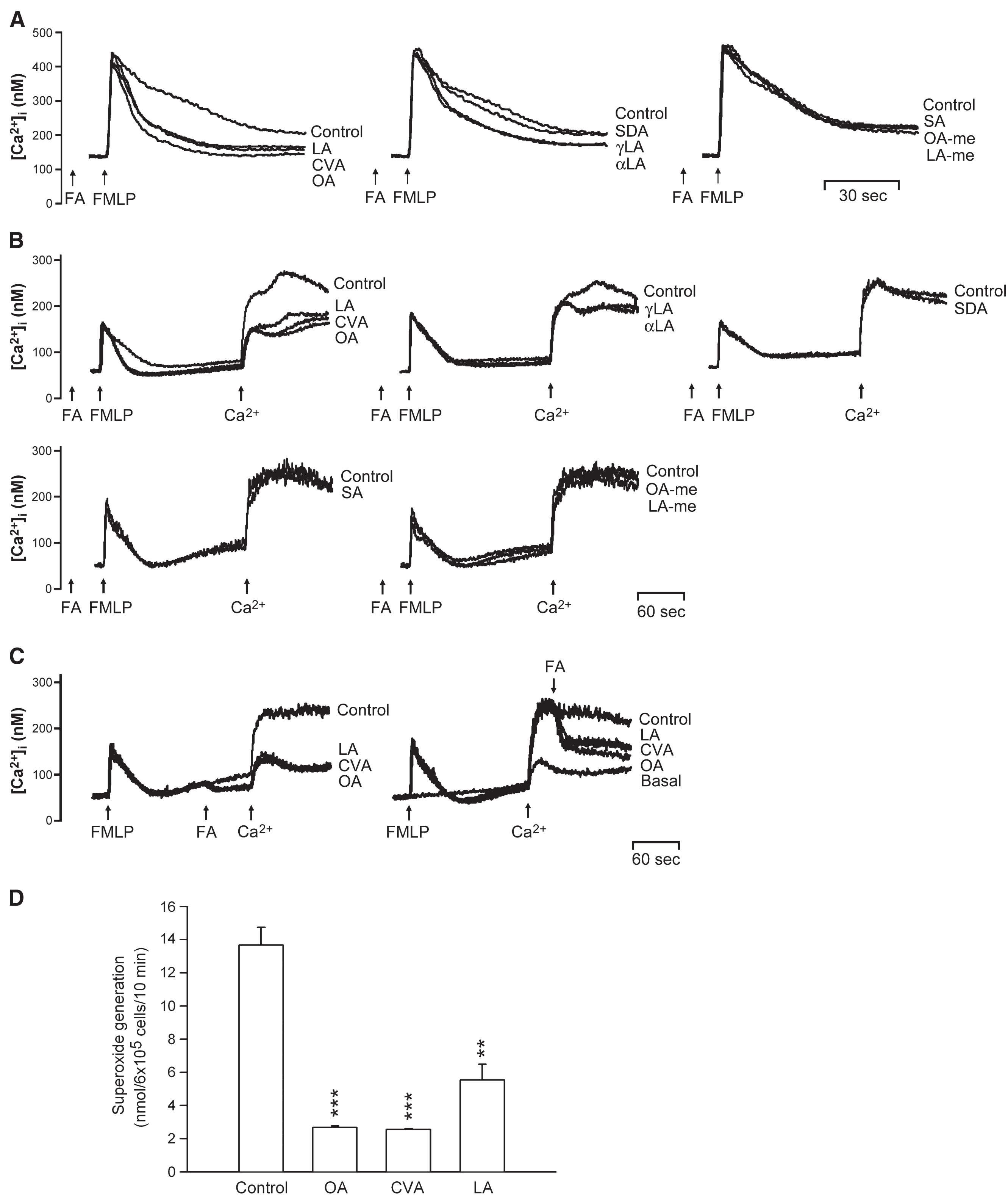

Fig. 2. Effect of C_{18} FAs on Ca^{2+} mobilization and $O_2^{\bullet -}$ generation in FMLP-activated human neutrophils. Fluo 3-loaded neutrophils were incubated with ethanol (0.1%, control) or FAs (5 μM) for 5 min and then activated by 0.1 μM FMLP in 1 mM Ca^{2+}-containing HBSS (A) or in Ca^{2+}-free HBSS followed by the addition of 1 mM Ca^{2+} (B). C: FAs (5 μM) were treated before or after the reintroduction of 1 mM Ca^{2+}. The traces shown are from four or five different experiments. D: Neutrophils were incubated with ethanol (0.1%, control), OA, CVA, or LA (5 μM) for 5 min and then activated by FMLP/CB in Ca^{2+}-free HBSS. $O_2^{\bullet -}$ generation was measured using SOD-inhibitable cytochrome *c* reduction. All data are expressed as the mean ± SEM (n = 3). ** $P < 0.01$ and *** $P < 0.001$ compared with the control.

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TABLE 2. Effects of C_{18} FAs on peak $[Ca^{2+}]_i$ and the time taken for this concentration to decline to half of its peak values ($t_{1/2}$) in FMLP-activated neutrophils in Ca^{2+}-free HBSS

C_{18} FA	FMLP		Ca^{2+}
	Peak $[Ca^{2+}]$i	$t_{1/2}$	Peak $[Ca^{2+}]$i
	nM	s	nM
Control	127.84 ± 15.01	30.60 ± 0.91	199.42 ± 11.23
SA	115.27 ± 25.06	32.08 ± 0.36	191.42 ± 17.48
OA (*cis*, 1n-9)	124.01 ± 2.22	16.71 ± 1.38 ***	81.40 ± 6.22 ***
CVA (*cis*, 1n-7)	118.55 ± 13.96	15.23 ± 1.44 ***	86.33 ± 8.70 ***
LA (*cis*, 2n-6)	121.57 ± 13.23	17.88 ± 2.21 ***	97.13 ± 13.20 ***
αLA (*cis*, 3n-3)	117.17 ± 13.21	23.52 ± 1.17 **	130.84 ± 18.50 *
γLA (*cis*, 3n-6)	115.71 ± 14.26	25.38 ± 0.78 **	133.17 ± 13.88 **
SDA (*cis*, 4n-3)	123.13 ± 17.65	30.64 ± 1.54	189.25 ± 15.76
OA-me (*cis*, 1n-9)	110.50 ± 7.74	30.84 ± 0.36	221.47 ± 6.08
LA-me (*cis*, 2n-6)	111.61 ± 19.49	30.07 ± 1.43	199.23 ± 12.57

Fluo 3-loaded neutrophils were incubated with ethanol (0.1%, control) or FAs (5 μM) for 5 min and then activated by 0.1 μM FMLP in Ca^{2+}-free HBSS followed by the addition of 1 mM Ca^{2+}. For all data, values are the mean ± SEM (n = 4–5). *$P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the control.

(**Fig. 5A**). In contrast, Ro318220 failed to alter either CVA- or LA-produced inhibition of extracellular Ca^{2+} entry (Fig. 5B).

Effects of C_{18} UFAs and PKC on extracellular Ba^{2+} entry

Ba^{2+}, which is not pumped by Ca^{2+}-ATPase either into internal stores or out of the cell, has an affinity for fura-2 (31). Therefore, Ba^{2+} was used as a Ca^{2+} surrogate for the Ca^{2+} entry pathway to trace unidirectional divalent cation movements. FMLP and thapsigargin pretreatment in a Ca^{2+}-free medium and the subsequent addition of 1 mM Ba^{2+} resulted in an increase in Ba^{2+} entry. PMA (3 nM) completely inhibited Ba^{2+} entry in FMLP- and thapsigargin-activated human neutrophils, which was abolished by Ro318220 (**Fig. 6**). These data suggest Ba^{2+} entry via SOCE in FMLP- and thapsigargin-activated human neutrophils. In contrast, OA, CVA, and LA (5 μM) failed to alter the

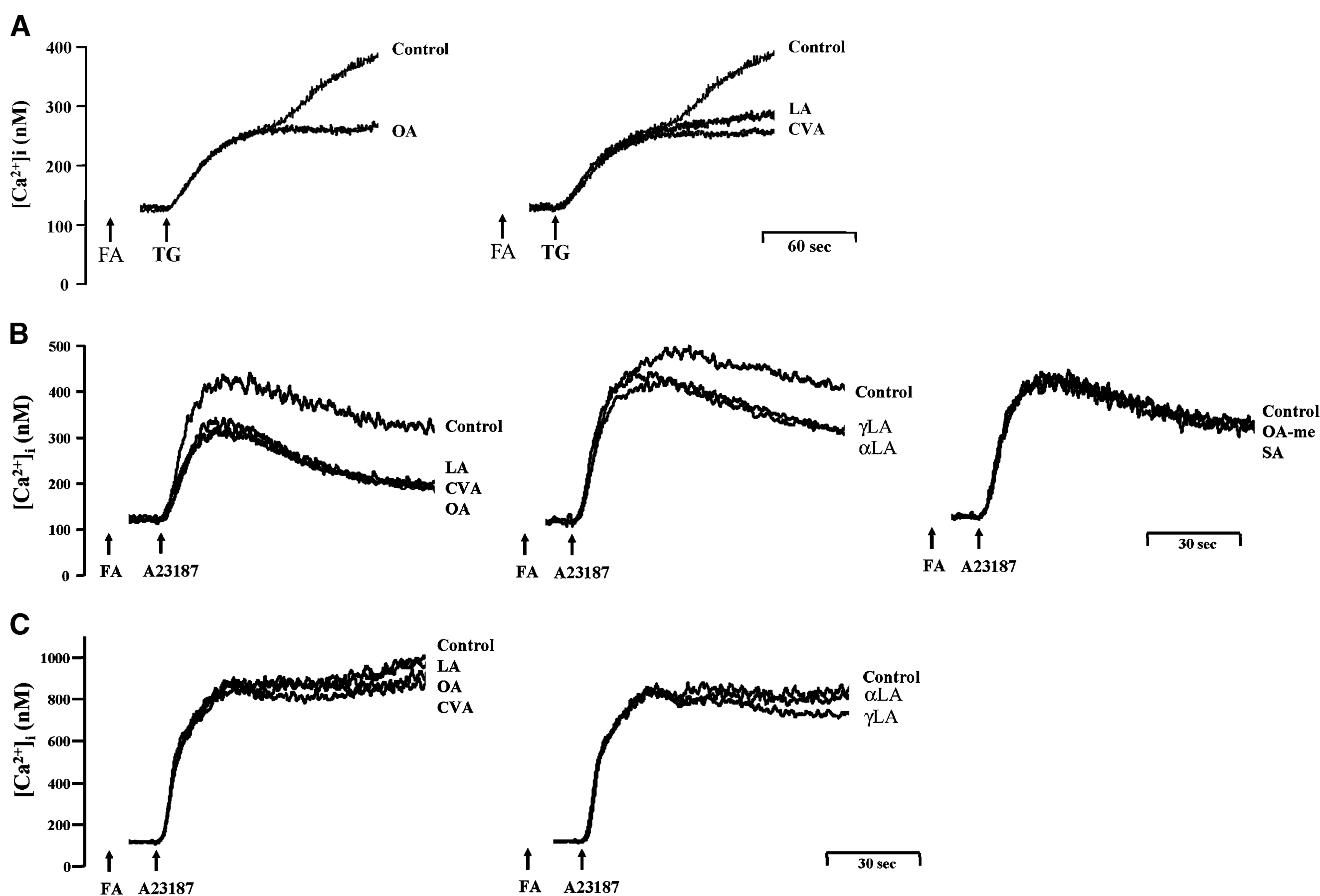

Fig. 3. Effect of C_{18} UFAs on Ca^{2+} mobilization in thapsigargin- or A23187-activated human neutrophils. Fluo 3-loaded neutrophils were incubated with ethanol (0.1%, control) or FAs (5 μM) for 5 min and then activated by 0.1 μM thapsigargin (TG) (A) or 20 (B) or 200 (C) nM A23187 in Ca^{2+}-containing HBSS. The traces shown are from four to six different experiments.

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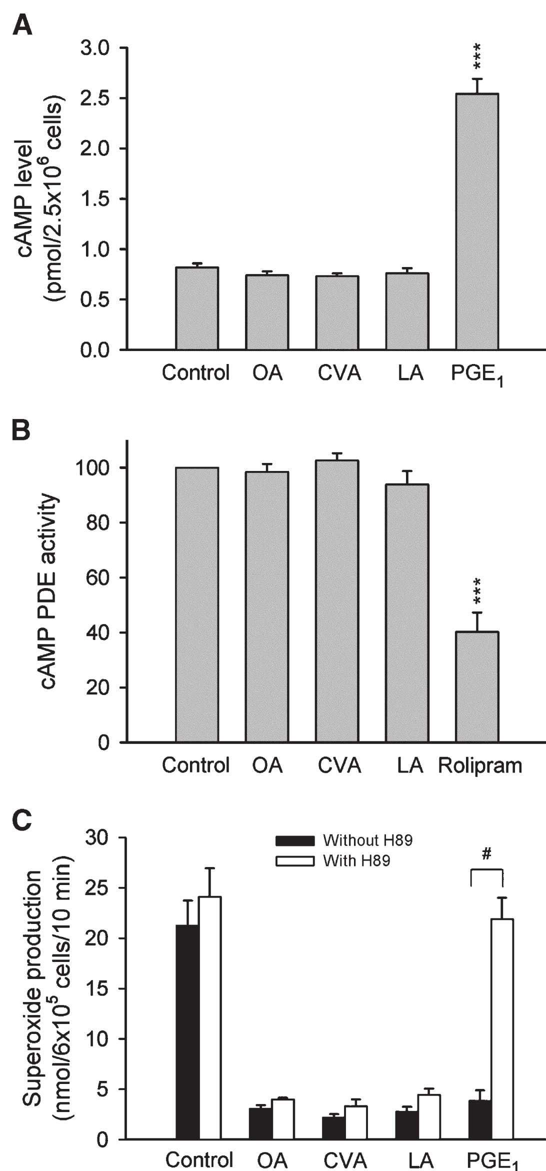

Fig. 4. Effect of C_{18} UFAs on the cAMP pathway in human neutrophils. A: Neutrophils were incubated with ethanol (0.1%, control), FAs (5 μM), or PGE_1 (1 μM) for 10 min, and cAMP was assayed using an enzyme immunoassay kit. B: Neutrophil homogenates were incubated with ethanol (0.1%, control), FAs (5 μM), or rolipram (3 μM), and then 0.05 μCi [^{3}H] cAMP was added to the reaction mixture at 30°C for 10 min. PDE activity was measured as described in Materials and Methods. C: $O_2^{\bullet -}$ generation was induced by FMLP/CB and measured using SOD-inhibitable cytochrome *c* reduction. H89 (3 μM) was preincubated for 5 min before the addition of ethanol, FAs, and PGE_1. Data are shown as the mean ± SEM (n = 3–4). ***$P < 0.001$ compared with the control. $^{\#}P < 0.001$ compared with the corresponding PGE_1.

entry of Ba^{2+} in FMLP- and thapsigargin-activated human neutrophils (Fig. 6).

Effects of Na^+ deprivation on the inhibition of C_{18} UFAs

The existence of a Na^+-Ca^{2+} exchange mechanism on the plasma membrane of neutrophils was previously described (40). It is known that the plasma membrane Na^+-Ca^{2+} exchanger (NCX) plays a role in removing Ca^{2+} from the cytosol. To examine whether the NCX is involved in the inhibitory effects of C_{18} UFAs, experiments were carried out in Na^+-free solutions to avoid a possible contribution of the NCX. As shown in **Fig. 7**, OA, CVA, and LA (5 μM) significantly inhibited $O_2^{\bullet -}$ production and shortened the value of $t_{1/2}$ in FMLP-activated human neutrophils in Ca^{2+}-containing Na^+-deprived medium, suggesting that the role of the NCX can be excluded.

Effects of C_{18} FAs on PMCA activity

Calcium homeostasis is maintained through a balance between cell membrane permeability and the energy-dependent transport of Ca^{2+} by Ca^{2+}-ATPase at the level of the cell membrane and at the level of ER. To address the effects of C_{18} UFAs on PMCA pumps, PMCA activity was assayed in human neutrophils as previously described (30) with some modifications. Neutrophils were preincubated with thapsigargin (0.5 μM) and A23187 (0.2 μM) to inhibit ERCA and induce Ca^{2+} entry in Na^+-deprived medium containing carbonyl cyanide 3-chlorophenylhydrazone (1 μM) and oligomycin A (1 μM) to prevent mitochondrial Ca^{2+} uptake and to inhibit ATP-utilizing and -generating systems. After this treatment, an extensive increase in $[Ca^{2+}]_i$ was observed, and then EDTA (1 mM) was added to remove the external Ca^{2+} evoking a decline in $[Ca^{2+}]_i$ due to Ca^{2+} clearance by PMCA, which was inhibited by calmidazolium (5 μM), a calmodulin inhibitor (data not shown). Under these conditions, the clearance rate of $[Ca^{2+}]_i$ was enhanced by OA and LA from 626.49 ± 29.96 (control) to 758.56 ± 13.67 ($P < 0.01$) and 725.80 ± 27.64 nM/min ($P < 0.01$), respectively. Additionally, OA-me and PMA failed to modify the clearance rate of $[Ca^{2+}]_i$ in this assay (**Fig. 8A**).

The effects of C_{18} FAs on PMCA activities were further tested by determining ATPase activities in neutrophil membrane fractions in the presence of thapsigargin and ouabain to inhibit ERCA and Na^+/K^+ ATPase. The removal of Ca^{2+} from the cytosol is maintained through an energy-dependent process. Since no ATP hydrolysis was observed when the plasma membrane was subtracted, the results suggest that the difference in the rate of ATP hydrolysis in the presence or absence of C_{18} FAs results through modulation of ATPase activity in the plasma membrane. As shown in Fig. 8B, the activities of PMCA were significantly enhanced by C_{18} mono- and bi-UFAs and by calmodulin. OA, CVA, LA, and calmodulin enhanced the activities of ATPase from 0.39 ± 0.03 (control) to 0.65 ± 0.02 ($P < 0.001$), 0.62 ± 0.06 ($P < 0.01$), 0.58 ± 0.05 ($P < 0.01$), and 0.58 ± 0.02 μmol Pi/1 × 10^7 cells/h ($P < 0.001$), respectively. In contrast, the C_{18} saturated FA and esterified UFAs had no effect on PMCA activities (Fig. 8B).

DISCUSSION

In this study, a cellular model of isolated human neutrophils was established to elucidate the anti-inflammatory functions of linear-chain C_{18} FAs. Our data show that C_{18} UFAs potently inhibited the generation of $O_2^{\bullet -}$ and the release of elastase in FMLP-activated human neutrophils

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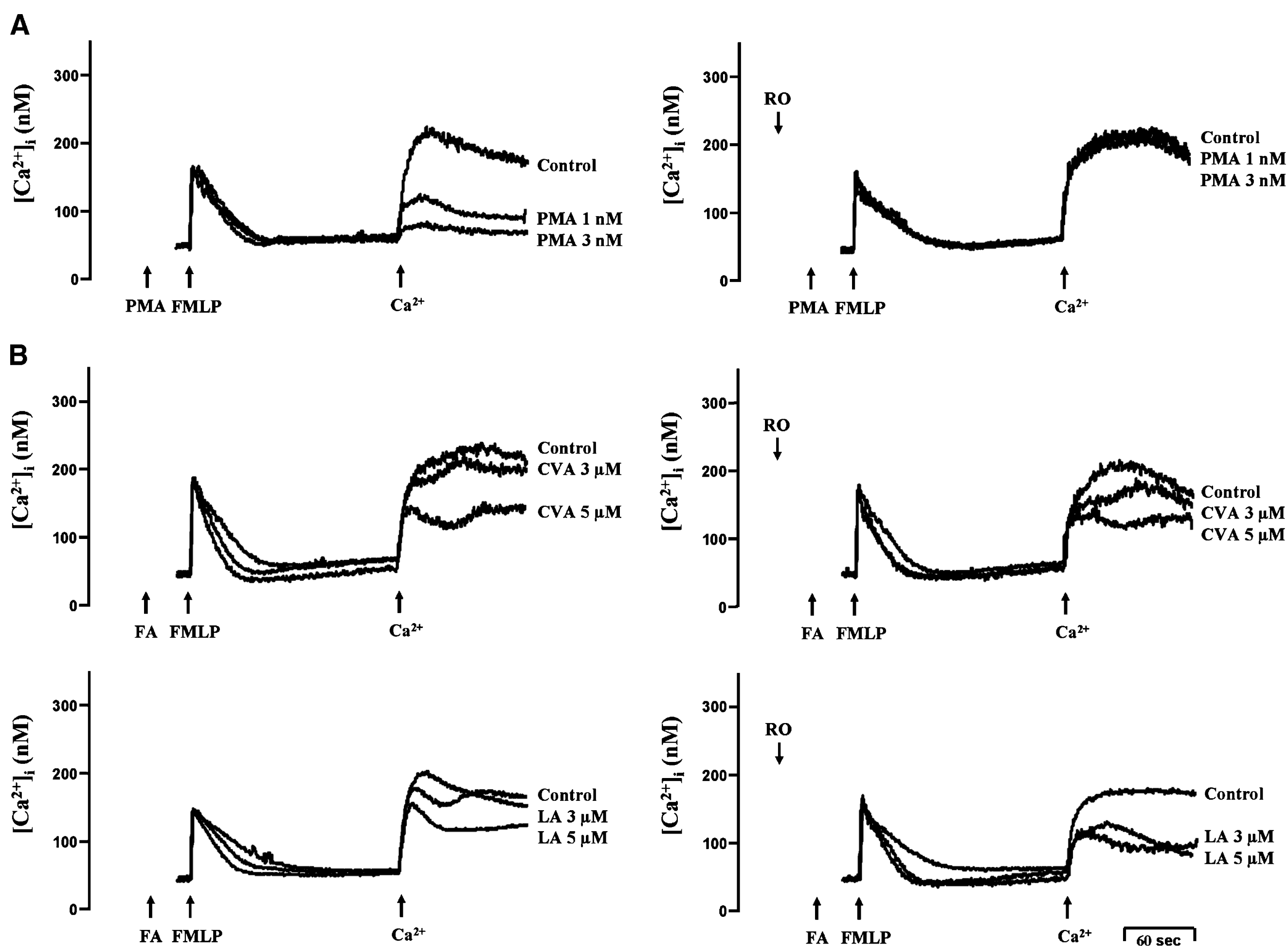

Fig. 5. Effect of PMA and C_{18} UFAs on Ca^{2+} mobilization in FMLP-activated human neutrophils. Fluo 3-loaded neutrophils were incubated with ethanol (0.1%, control), PMA (1 and 3 nM) (A), CVA, or LA (3 and 5 µM) (B) for 5 min and then activated by 0.1 µM FMLP in Ca^{2+}-free HBSS followed by the addition of 1 mM Ca^{2+}. Ro318220 (Ro; 1 µM) was preincubated for 3 min before the addition of PMA and FAs. The traces shown are from three or four different experiments.

in a concentration-dependent fashion with IC_{50} values of <10 µM. The inhibitory potency decreased with an increasing number of double bonds in C_{18} UFAs. When the carboxy group of C_{18} mono- or bi-UFA was chemically modified by adding a methyl ester, however, these C_{18} UFAs did not exhibit inhibition. In addition, the C_{18} saturated FA caused no effect on human neutrophil responses. Multiple observations made in the study suggest that C_{18} UFAs suppress respiratory burst and degranulation of human neutrophils through an increase in the clearance of cytosolic Ca^{2+}.

It is well established that Ca^{2+} signaling is a key second messenger regulating neutrophil functions (34). Consistent with this, BAPTA-AM, a cell-permeable Ca^{2+} chelator, inhibited the generation of $O_2^{\bullet -}$ and the release of elastase in FMLP-activated human neutrophils (data not shown). Interestingly, C_{18} UFAs did not alter FMLP-induced peak $[Ca^{2+}]_i$ values but accelerated the rate of decline in $[Ca^{2+}]_i$. Clearly, the potency of C_{18} FAs of attenuating $[Ca^{2+}]_i$ is correlated with decreasing $O_2^{\bullet -}$ generation and elastase release. These results suggest that the inhibitory effects of C_{18} UFAs are mediated through a blockade of Ca^{2+} signaling pathways. Furthermore, C_{18} UFAs with a free carboxyl end, added before or after FMLP, suppressed changes in $[Ca^{2+}]_i$ caused by the subsequent addition of Ca^{2+}. These data indicate that the inhibition of $[Ca^{2+}]_i$ by C_{18} UFAs is not through alteration of the IP_3 signaling pathway. In line with this, C_{18} UFAs, OA, CVA, and LA, suppressed the sustained $[Ca^{2+}]_i$ changes in thapsigargin-activated human neutrophils. Moreover, thapsigargin-induced $O_2^{\bullet -}$ generation was reduced by OA, CVA, and LA.

Calcium homeostasis is maintained through a complex set of mechanisms that controls cellular Ca^{2+} influx and efflux as well as intracellular Ca^{2+} stores. Because of this critical dependence of activation of the proinflammatory activities of neutrophils on Ca^{2+}, the mechanisms used by these cells to both mobilize and dispose of Ca^{2+} have been identified as potential targets for anti-inflammatory agents (34). We previously reported that cAMP-elevating agents can accelerate Ca^{2+} clearance from the cytosol as well as inhibit the generation of $O_2^{\bullet -}$ and the release of elastase in FMLP-activated human neutrophils (36, 37). This contention is supported by a previous study, which reported that cAMP/protein kinase A increases the Ca^{2+} sequestering/

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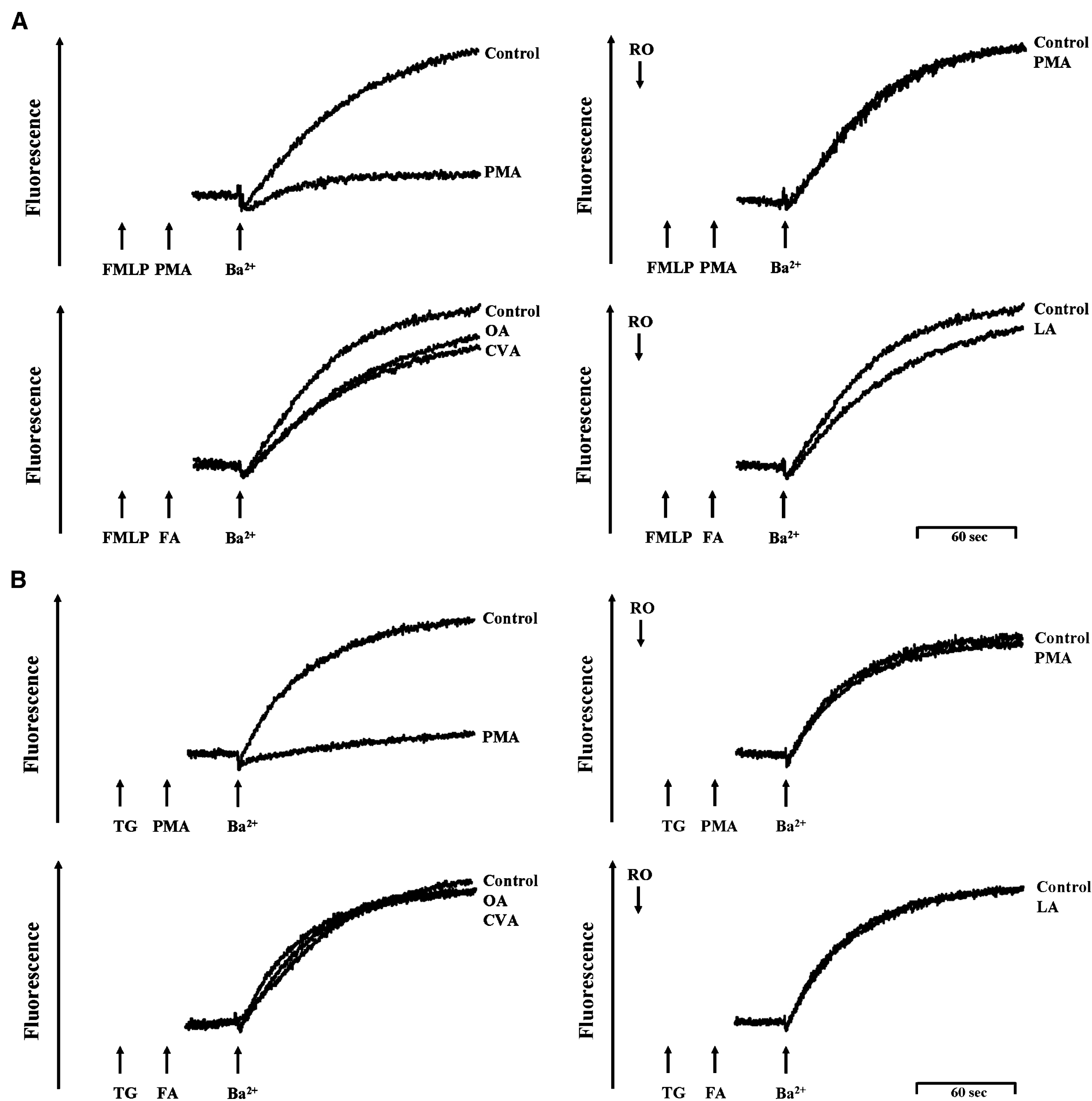

Fig. 6. Effect of C_{18} UFAs on extracellular Ba^{2+} entry in FMLP- and thapsigargin-activated human neutrophils. Fura 2-loaded neutrophils were stimulated with 0.1 μM FMLP (A) or 0.5 μM thapsigargin (TG) (B) for 3 min in Ca^{2+}-free HBSS followed by supplementation with 1 mM Ba^{2+}, and the fluorescence was monitored at 37°C with stirring. PMA (3 nM) or C_{18} UFAs (5 μM) were administered 1 min prior to the addition of Ba^{2+}. Ro318220 (Ro; 1 μM) was preincubated for 3 min before activation. The traces shown are from five different experiments.

resequestering activity of ERCA by phosphorylation of the regulatory polypeptide, phospholamban (41). In this study, however, we show that pretreatment with a protein kinase A inhibitor did not restore the UFA-induced inhibition of $O_2^{\bullet-}$ release, suggesting that a cAMP-dependent pathway is not involved in the inhibition of C_{18} UFAs. Consistent with this result, C_{18} mono-, bi-, and tri-UFAs failed to elevate the concentrations of cAMP or to alter the activities of cAMP-specific PDEs.

Murakami, Chan, and Routtenberg (42) reported that OA and LA, but not SA, activate PKC independently of Ca^{2+} and phospholipids. Moreover, PKC has been shown to inhibit SOCE in human neutrophils (31). Interestingly, a similar effect of regulating FMLP-induced $[Ca^{2+}]_i$ changes in the presence of extracellular Ca^{2+} was obtained with C_{18} UFAs and PMA. PMA, at low concentrations of 1 and 3 nM, did not significantly affect the release of Ca^{2+} from intracellular stores, but this agent hastened the rate of decline in $[Ca^{2+}]_i$ of FMLP-activated human neutrophils (data not shown). On the basis of these results, it is possible to postulate that the C_{18} UFA-induced $[Ca^{2+}]_i$ decrease was mediated by activation of PKC and inhibition of SOCE.

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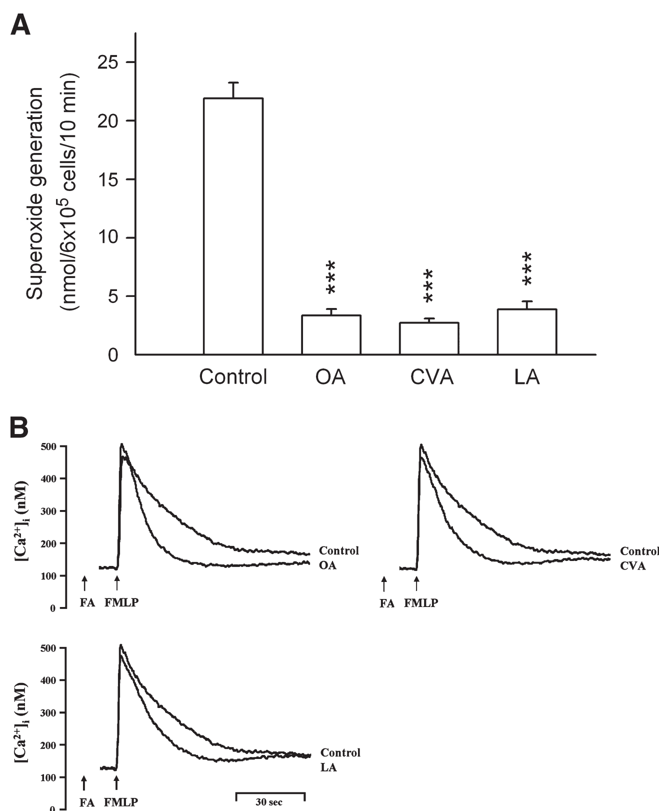

Fig. 7. Effect of C_{18} UFAs on $O_2^{\bullet-}$ production and Ca^{2+} mobilization in FMLP-activated human neutrophils in Ca^{2+}-containing Na^+-deprived medium. A: Human neutrophils were incubated with ethanol (0.1%, control), OA, CVA, or LA (5 μM) for 5 min and then activated by FMLP/CB. $O_2^{\bullet-}$ generation was measured using SOD-inhibitable cytochrome *c* reduction. All data are expressed as the mean ± SEM (n = 4). *** $P < 0.001$ compared with the control. B: Fluo 3-loaded neutrophils were incubated with ethanol (0.1%, control), OA, CVA, or LA (5 μM) for 5 min and then activated by 0.1 μM FMLP in Ca^{2+}-containing Na^+-deprived medium. The traces shown are from four or five different experiments.

Our data confirmed that PMA concentration-dependently inhibited extracellular Ca^{2+} and Ba^{2+} entry in FMLP- and thapsigargin-activated human neutrophils, which was completely abolished by Ro318220, a PKC inhibitor. Nevertheless, a role for PKC in C_{18} UFA-mediated inhibition was ruled out because the C_{18} UFA-inhibited extracellular Ca^{2+} entry in FMLP-activated neutrophils was not reversed by a PKC inhibitor. Indeed, C_{18} UFAs failed to alter extracellular Ba^{2+} entry in FMLP- and thapsigargin-activated human neutrophils, indicating that C_{18} UFAs are unable to inhibit the SOCE pathway.

The rate of decline in $[Ca^{2+}]_i$ is governed by the efficiency of clearance of Ca^{2+} from the cytosol and by the regulation of the time of onset, rate, and magnitude of the influx of extracellular cations (34). The C_{18} UFA-induced $[Ca^{2+}]_i$ decrease was not due to inhibition of Ca^{2+} entry; thus, we propose that this effect should be due to stimulation of Ca^{2+} efflux. A23187 is a Ca^{2+} ionophore that equilibrates Ca^{2+} gradients across plasma membranes and can cause a rapid rise in $[Ca^{2+}]_i$ levels. Interestingly, C_{18} UFAs caused inhibitory responses in $[Ca^{2+}]_i$ levels induced by lower but not higher concentrations of A23187, suggesting that the inhibitory actions of C_{18} UFAs are attenuated by high rates of extracellular Ca^{2+} influx. The efflux of Ca^{2+} from the cytosol is mediated by the NCX and PMCA in activated human neutrophils. Inhibition of FMLP-activated $O_2^{\bullet-}$ production and Ca^{2+} entry by C_{18} UFAs was obtained in both normal and Na^+-deprived media; therefore, there is no indication that C_{18} UFAs act via promoting NCX activity on the plasma membrane. PMCA is known to be important for Ca^{2+} homeostasis in most cells. PMCA represents a high-affinity system for the expulsion of Ca^{2+} from cells and is responsible for long-term setting and maintenance of $[Ca^{2+}]_i$ levels (43, 44). PMCA pumps are stimulated by calmodulin in the presence of Ca^{2+} (45). Moreover, PMCA activity is also influenced by the membrane lipid composition and structure (33). Adamo et al. (46) suggested that acidic phospholipids activate PMCA by interacting with an independent acidic lipid-responsive region. This study showed that the activities of PMCA were significantly enhanced by C_{18} mono- and bi-UFAs activated human neutrophils and in isolated neutrophil membranes. Consistent with these results, it was reported that purified PMCA of erythrocyte membranes can be activated by the C_{18} UFAs, OA and LA (47). Our results show that the increasing potency of C_{18} FAs on PMCA activities appears to be correlated with decreases in $O_2^{\bullet-}$ generation and elastase release as well as the accelerated clearance of $[Ca^{2+}]_i$ in human neutrophils. Similarly, Oda et al. (48) reported that both the free carboxy group and the number of double bonds of the C_{18} UFA structure are important in providing a potent telomerase-inhibitory effect. The three-dimensional structure of the telomerase active site appears to have a pocket that could bind OA (48). It is obvious that an analysis of the binding sites of C_{18} UFAs on PMCA and telomerase will be critical in clarifying the molecular mechanism of C_{18} UFAs.

Although the effect of C_{18} FAs on human neutrophil functions has been studied extensively, several points remain to be fully established, as some authors have found an increase (13–17), whereas others have found a decrease (18, 19), in ROS generation, CD11b expression, leukotriene B_4 production, enzyme release, and/or phagocytosis by neutrophils in response to C_{18} FA treatment. Contradictory observations reported in previous studies may in part be the result of different concentrations and methods used to determined neutrophil functions. For example, OA and LA at high concentrations (100 and 1,000 μM) induced ROS production by themselves, whereas both C_{18} UFAs at low concentrations (1–10 μM) inhibited FMLP-activated ROS release in human neutrophils (20). Furthermore, we found that exposure of human neutrophils to high concentrations (30 and 50 μM) of C_{18} FAs caused a concentration-dependent increase in LDH release (data not shown). Additionally, Hatanaka et al. (14) showed that OA, LA, and γLA at high concentrations (50 and 100 μM) per se lead to cytochrome *c* reduction. Our data indicate that C_{18} UFAs significantly suppressed $O_2^{\bullet-}$ generation and elastase release in activated human neutrophils in a concentration-dependent manner with IC_{50} values of <10 μM. Activated neutrophils that produce ROS and granule proteases are

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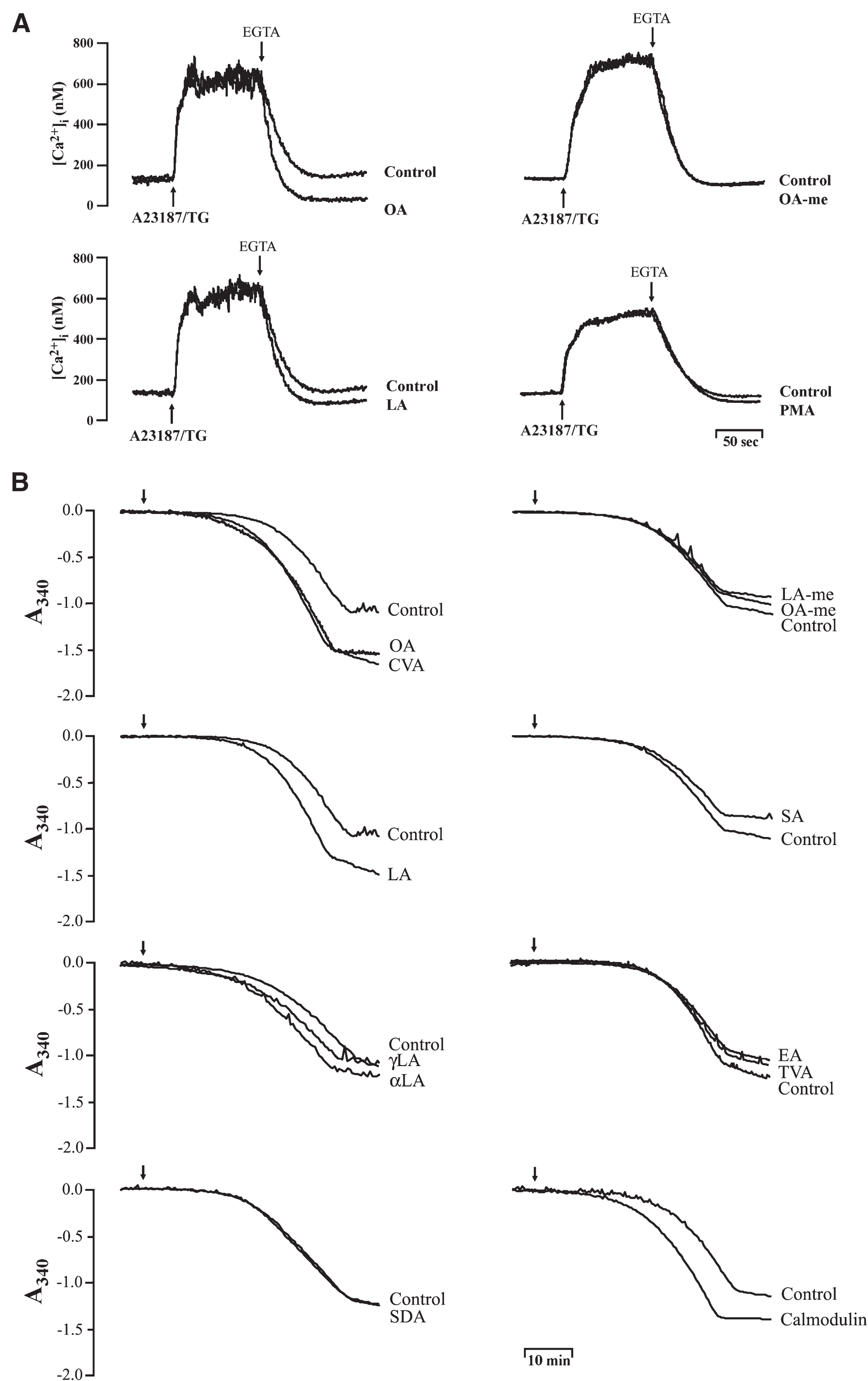

Fig. 8. Effect of C_{18} FAs on PMCA activity in human neutrophils and in isolated neutrophil membranes. A: Fluo 3-loaded neutrophils were stimulated with thapsigargin (TG; 0.5 μM) plus A23187 (0.2 μM), and subsequently EGTA (1 mM) was added in combination with FAs (5 μM) or PMA (3 nM) in Ca^{2+}-containing Na^{+}-deprived medium supplemented with oligomycin A (1 μM) and CCCP (1 μM). B: PMCA activity of neutrophil membranes was measured using a coupled enzyme assay in the presence of ouabain (10 μM) and thapsigargin (10 μM) as described in Materials and Methods. The indicated FAs (5 μM) and calmodulin (10 μg/ml) were added to the assay buffer, and the ATPase activity was measured spectrophotometrically at 340 nm for 50 min at 25°C with stirring. The traces shown are from four or seven different experiments.

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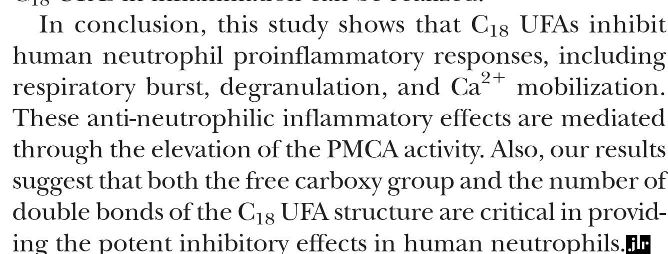
involved in oxidative stress and inflammation. Alterations in dietary levels of C_{18} UFAs have significant clinical benefits in cardiovascular diseases and inflammatory syndromes (8–12). LA, αLA, and γLA are thought to have important functions in preventing and treating coronary artery disease and rheumatoid arthritis (8, 12). However, Juttner et al. (49) showed that parenteral nutrition containing OA and LA can induce $O_2^{\bullet -}$ generation of neutrophils. Obviously, further research is needed to clarify the effects of C_{18} UFAs in neutrophil functions before the therapeutic potential of C_{18} UFAs in inflammation can be realized.



In conclusion, this study shows that C_{18} UFAs inhibit human neutrophil proinflammatory responses, including respiratory burst, degranulation, and Ca^{2+} mobilization. These anti-neutrophilic inflammatory effects are mediated through the elevation of the PMCA activity. Also, our results suggest that both the free carboxy group and the number of double bonds of the C_{18} UFA structure are critical in providing the potent inhibitory effects in human neutrophils.

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