Polyenoic very-long-chain fatty acids mobilize intracellular calcium from a thapsigargin-insensitive pool in human neutrophils

The relationship between Ca^{2+} mobilization and superoxide production induced by long- and very-long-chain fatty acids

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Fatty acids with more than 22 carbon atoms (very-long-chain fatty acids; VLCFAs) are normal cellular components that have been implicated in the pathophysiology of a number of peroxisomal disorders. To date, however, essentially nothing is known regarding their biological activities. Ca^{2+} mobilization is an important intracellular signalling system for a variety of agonists and cell types. Given that several polyunsaturated long-chain fatty acids mobilize intracellular Ca^{2+} and that we have postulated that the VLCFAs may be involved in signal transduction, we examined whether the tetraenoic VLCFA induced Ca^{2+} mobilization in human neutrophils. We report that fatty acid-induced intracellular Ca^{2+} mobilization declined for fatty acid species of more than 20 carbon atoms, but increased again as the carbon chain length approached 30. This Ca^{2+} mobilization occurred independently of inositol 1,4,5-trisphosphate production and protein kinase C translocation and involved both the release of Ca^{2+} from the intracellular stores and changes to the influx or efflux of the ion. We further observed that triacontatetraenoic acid [30:4 ($n-6$)] mobilized Ca^{2+} from a thapsigargin-insensitive intracellular pool distinct from the thapsigargin-sensitive pools affected by arachidonic acid [20:4 ($n-6$)] or *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP). 20:4 ($n-6$) induced strong superoxide production (chemiluminescence) which was inhibited by thapsigargin pretreatment. In contrast, fatty acid-induced superoxide production progressively declined as the carbon chain length increased beyond 20–22 carbon atoms. Further studies suggested that the thapsigargin-insensitive Ca^{2+} mobilization elicited by 30:4 ($n-6$) was not related to oxy-radical formation, while the thapsigargin-sensitive Ca^{2+} mobilization induced by 20:4 ($n-6$) may be involved in the initiation but not necessarily the maintenance of superoxide production. In conclusion, this is the first report to demonstrate a biological activity for the VLCFA and indicates that 30:4 ($n-6$) influences second messenger systems in intact cells that differ from those affected by long-chain fatty acids such as 20:4 ($n-6$).

INTRODUCTION

Fatty acids with more than 22 carbon atoms (very-long-chain fatty acids, VLCFAs) are normal components of most tissues, particularly brain, retina and male reproductive tissue [1–3], and accumulate in patients with various peroxisomal diseases. Neither the normal function of these VLCFAs nor the consequences of their abnormal accumulation is known, although it is believed that they may be involved in the progression of these conditions [4–6]. In previous papers we proposed that the VLCFAs may be involved in signal transduction and that some of the pathophysiology of peroxisomal diseases might be a consequence of altered VLCFA-mediated signal transduction events [4]. In addition there is evidence that the cerebral demyelination that characterizes many peroxisomal conditions may have an immunopathogenic mechanism involving the VLCFAs [6,7]. Many fatty acids, particularly *cis*-monoenoic and polyenoic species, have been shown to alter phagocyte function. For example, long-chain fatty acid-induced activation of neutrophil oxy-radical production [8–15], aggregation [16], degranulation [17,18] and inhibition of chemotaxis [19] have all been reported. In addition, certain polyenoic VLCFAs have also been shown to stimulate neutrophil superoxide production, although they were substantially less active than the long-chain fatty acids arachidonic acid [20:4 ($n-6$)] and docosahexaenoic acid [22:6 ($n-3$)] [14,20]. Despite all these studies, little is known about the mechanisms by which the fatty acids elicit their effects or the signal transduction systems involved. What is known, however, is that many of the effects of the fatty acids on immune cells are specific and do not relate to their detergent properties, correlate with their critical micellar concentration or require the fatty acids be metabolized to eicosanoids [12,17,19,21,22]. Fatty acids which induce superoxide production in neutrophils also cause an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in a variety of cell types, including neutrophils and T-cells (for example [11,13,23–25]). While changes to $[Ca^{2+}]_i$ have been linked to a wide variety of biological responses in a number of cell types [26,27], the biological consequences of the fatty acid-induced rise in $[Ca^{2+}]_i$ in neutrophils are unknown. There is, however, evidence that increases in $[Ca^{2+}]_i$ may be important in

Abbreviations used: 18:1 ($n-9$), *cis*-9-octadecenoic acid (oleic acid); 18:4 ($n-3$), *cis*-6,9,12,15-octadecatetraenoic acid; 20:0, eicosanoic acid (arachidic acid); 20:4 ($n-6$), *cis*-5,8,11,14-eicosatetraenoic acid (arachidonic acid); 20:5 ($n-3$), *cis*-5,8,11,14,17-eicosapentaenoic acid; 22:4 ($n-6$), *cis*-7,10,13,16-docosatetraenoic acid (adrenic acid); 22:6 ($n-3$), *cis*-4,7,10,13,16,19-docosahexaenoic acid; 24:4 ($n-6$), *cis*-9,12,15,18-tetracosatetraenoic acid; 28:4 ($n-6$), *cis*-13,16,19,22-octacosatetraenoic acid; 30:4 ($n-6$), *cis*-15,18,21,24-triacontatetraenoic acid; 32:4 ($n-6$), *cis*-17,20,23,26-dotriacontatetraenoic acid; 34:6 ($n-3$), *cis*-17,20,23,26,29,31-tetratriacontahexaenoic acid; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; HBSS, Hank's balanced salt solution; lucigenin, 9,9′-bis-*N*-methylacridinium nitrate; PK-C, protein kinase C (Ca^{2+}- and phospholipid-dependent protein kinase, EC 2.7.1); PS, phosphatidylserine; PMA, phorbol 12-myristate 13-acetate; TCA, trichloroacetic acid; VLCFA, very-long-chain fatty acids (> 22 carbon atoms).

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the responses to the fatty acids, as antagonists of Ca^{2+}-binding proteins inhibit fatty acid-induced superoxide production [9,14]. This study examines the relationship between fatty acid-induced neutrophil superoxide production, increases in $[Ca^{2+}]_i$ and carbon chain length for a range of synthetically prepared tetraenoic long- and very-long-chain fatty acids. Our findings suggest that the long- and very-long-chain fatty acids have different effects on neutrophils which may be mediated, at least in part, through their ability to increase $[Ca^{2+}]_i$.

EXPERIMENTAL

Chemicals

All chemicals were purchased from the Sigma Chemical Company, St. Louis, MO, U.S.A., or as indicated, except for general laboratory reagents that were of analytical quality.

Fatty acids

1. Sources and preparation methods

Oleic [18:1, (*n*−9)], octadecatetraenoic [18:4, (*n*−3)], arachidic (20:0), arachidonic [20:4, (*n*−6)], eicosapentaenoic [20:5, (*n*−3)], adrenic [22:4, (*n*−6)] and docosahexaenoic [22:6, (*n*−3)] acids were obtained from the Sigma Chemical Co. The methyl ester of tetracosatetraenoic [24:4, (*n*−6)] acid was prepared from 22:4 (*n*−6) by sequential one-carbon homologation reaction schemes [28]. The methyl esters of octacosatetraenoic [28:4, (*n*−6)], triacontatetraenoic [30:4, (*n*−6)], dotriacontatetraenoic [32:4, (*n*−6)] and tetratriacontahexaenoic [34:6, (*n*−3)] acids were synthesized via the elongation of 20:4 (*n*−6)-, 20:4 (*n*−6)-, 20:4 (*n*−6)- and 22:6 (*n*−3)-methyl esters respectively (Nu-Chek Prep.) using the conjunctive reagent tosylmethylisocyanide (Aldrich Chemical Company) according to the method of Johnson [29] and stored in chloroform at −20 °C.

The purity of all the lipids used in these investigations was checked periodically by GC/MS and TLC.

2. Conversion of the methyl ester to the non-esterified fatty acid

On the day prior to use the non-esterified VLCFAs were prepared from their corresponding methyl esters by alkaline hydrolysis as previously described [30].

Preparation of human neutrophils

Human neutrophils were prepared from the blood of healthy volunteers by the rapid single-step method of Ferrante and Thong [31].

Preparation of agonists and fatty acid suspensions

The non-esterified fatty acid suspensions were prepared as follows. The chloroform solvent from fatty acid stocks stored in the dark at −20 °C was evaporated to dryness under a stream of nitrogen at 30 °C. The residue was resuspended in redistilled ethanol to give a final concentration of 20 mM. A working dilution in water (3.3 mM) was prepared from these solutions just prior to use, kept in the dark at 2 °C and always used within 2 h of preparation.

BSA, ovalbumin, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) and phorbol 12-*O*-myristate 13-acetate (PMA) dilutions were prepared in water or Hanks balanced salt solution (HBSS) [32] on the day of use from either powder for the BSA and ovalbumin or stock solutions made up in DMSO for fMLP and PMA and kept at −20 °C. These working solutions were kept in the dark at 2 °C until required and used within 2 h of preparation.

Stock solutions of thapsigargin (BioMol Research Laboratories; Alomone Laboratories, Jerusalem, Israel) were prepared in redistilled ethanol (2 mM) and kept at −81 °C. Working solutions were prepared from these stocks in water or buffer on the day of use.

Chemiluminescence

Superoxide release was measured by monitoring the chemiluminescence resulting from the oxidation of lucigenin (9,9′,-bis-*N*-methyl-acridinium nitrate), a reaction specific for superoxide [33]. Briefly, neutrophils in HBSS (100 μl; 1×10^6 cells) were pre-incubated for 5 or 10 min at 37 °C in a 95% air/5% CO_2 humidified atmosphere in 400 μl of pre-incubation volume. Where appropriate, test substance or the corresponding solvent control was included during this period (see below). After pre-incubation, lucigenin in HBSS (250 μM) was added, together with agonist (100 μl) and, where necessary, sufficient HBSS to bring the final assay volume to 1 ml. The cells were immediately placed in a water-jacketed (37 °C) luminometer chamber (Model 1250 or 1251 with MultiUse software; Bio-Orbit Oy, Turku, Finland) and the resulting chemiluminescence (in mV) recorded over time immediately after the addition of the lucigenin. For experiments examining the effects of albumin on established agonist-induced superoxide production, the agonist was added at $t = 0$ and BSA, ovalbumin or the corresponding solvent control added at the times indicated in Figure 4. Results were expressed as either the rate of superoxide production (in mV) or the maximum rate of superoxide production achieved (in mV) during a 10 min period. Assays under Ca^{2+}-free conditions were performed in the same way except that cells were suspended in either normal HBSS ($+Ca^{2+}$) or Ca^{2+}-free HBSS ($-Ca^{2+}$). Similarly, all other reagents were prepared in either normal HBSS ($+Ca^{2+}$), or Ca^{2+}-free HBSS supplemented with 1.5 mM EGTA ($-Ca^{2+}$).

Loading neutrophils with fura-2 AM

Neutrophils (1×10^7 cells/ml) in HBSS were incubated in the dark with fura-2 AM (1 μM) for 30 min at 37 °C in a shaking water bath. After loading, cells were washed twice (600 ***g***, 5 min at room temperature) and resuspended in HBSS (6×10^6 cells/ml). Cells loaded with fura-2 AM were kept in the dark at 2 °C until required.

Calcium mobilization

Calcium mobilization was determined using a Perkin Elmer LS50B luminescence spectrometer and Fluorescence Data Manager Software.

1. Normal protocol

Fura-2-AM-loaded neutrophils in HBSS (2 ml; 6×10^6 cells) were placed in the reaction cuvette and warmed for 5 min in the dark at 37 °C. Baseline fluorescence (excitation 340 nm, emission 510 nm, slit width 5.0 nm for both) was measured for 2 min. Under normal conditions agonist (20 μl) was added at $t = 2$ min and readings taken for 5 min. For experiments examining the effects of albumin on an established Ca^{2+} transient, BSA, ovalbumin or the corresponding solvent control was added at $t = 7$ min (20 μl) and readings continued for a further 5 min. For the albumin pre-incubation experiments (see Figures 5 and 6) the order of addition was reversed with BSA, ovalbumin or the corresponding solvent control added at $t = 2$ min and agonist at

$t = 7$ min. Maximal fluorescence ($F_{max.}$) was determined over 3 min after lysing the cells and subcellular organelles with Triton X-100 [0.15% (v/v) final concentration]. Minimum fluorescence ($F_{min.}$) was determined over a further 3 min following the addition of Tris base, pH 10.0, at 22 °C (40 mM final concentration) and EGTA (12.5 mM final concentration).

2. Protocol for Ca^{2+}-free conditions

Fura-2-AM-loaded neutrophils in HBSS (1 ml; 6×10^6 cells) were diluted with 1 ml of HBSS supplemented with 80 mM Hepes, pH 7.3, at 22 °C and warmed for 5 min at 37 °C as above. Baseline fluorescence was determined for 2 min; Ca^{2+}-free conditions were established by adding EGTA (1.5 mM final concentration at $t = 2$ min) and the Ca^{2+}-free baseline fluorescence determined for 5 min. Where appropriate thapsigargin (2 μM), or the corresponding solvent control, was added at $t = 7$ min, 10 min prior to the agonist. Agonists (20 μl) were added at $t = 7$ min or $t = 17$ min for the thapsigargin studies and readings continued for 5 min. In order to obtain meaningful $F_{max.}$ and $F_{min.}$ values and to determine the contribution of Ca^{2+} influx to the overall response, external Ca^{2+} (approximately 2 mM final concentration) was added and readings continued for a further 5 min. $F_{max.}$ and $F_{min.}$ values were determined as above.

The actual $[Ca^{2+}]_i$ was calculated by the method of Grynkiewicz et al. [34].

Preparation of neutrophil extracts for quantification of D-*myo*-inositol 1,4,5-trisphosphate ($Ins P_3$) levels

Neutrophils in HBSS (2 ml; 3×10^7 cells) were pre-incubated with LiCl (20 mM, 10 min at 37 °C) in a shaking water bath before stimulation for 30 s at 37 °C with 20:4 ($n-6$), 30:4 ($n-6$) (both 33 μM), fMLP (5×10^{-6} M) or PMA (1×10^{-7} M). Reactions were terminated with ice-cold trichloroacetic acid (TCA) (500 μl; 50%, w/v) and the suspensions incubated at 2 °C for 30 min. After centrifugation (1200 *g*, 5 min at 4 °C), the supernatants were removed and transferred to 50-ml screw-capped centrifuge tubes. TCA was removed from the supernatants by washing with 3×10 vol. (3×25 ml) of ice-cold water-saturated diethyl ether and centrifugation (1800 *g*, 5 min at 4 °C). The final aqueous phase was neutralized to pH 7.5 with $NaHCO_3$ (approximately 100 μl of a 1 M solution) and 100 μl aliquots assayed for $Ins P_3$ using an Amersham International Pty. Ltd. Biotrak assay system (Cat. No. TRK 1000) (Little Chalfont, Bucks., U.K.) in accordance with the manufacturer's instructions.

Preparation of particulate neutrophil extracts

Particulate neutrophil extracts were prepared as previously described [14].

Protein kinase assay

Protein kinase activity was determined by quantifying the incorporation of ^{32}P from [^{32}P]γ-ATP into a phosphate acceptor (see below). Reaction mixtures contained 10–30 μl of fraction or extract, 20 mM Hepes (pH 7.5) at 22 °C, 5 mM $MgCl_2$, phosphate acceptor (see below), 10 μM ATP containing [^{32}P]γ-ATP [approximately 5.261–6.773 kBq (0.142–0.183 μCi)] (Bresatec Ltd, Thebarton, South Australia, Australia), 40 μg of phosphatidylserine (PS) as self micelles (prepared by sonication) and 5 mM $CaCl_2$ in a final assay volume of 60 μl. Ca^{2+}- and PS-independent protein kinase activity was determined in the same way but by replacing the Ca^{2+} and PS with 5 mM EGTA. Diacylglycerol and PMA were not included in the assays as the concentrations of Ca^{2+} and PS used were sufficient to fully stimulate the enzymes present (results not shown). Incubations were carried out at 30 °C for 10 min and terminated by transferring an aliquot (50 μl) on to P81 paper (2.5 cm × 2.0 cm) (Whatman Ltd., Maidstone, Kent, U.K.) and immediately immersing the paper in 75 mM orthophosphoric acid. After washing with 75 mM orthophosphoric acid (5 ml/paper for 3×10 min) and 99% ethanol (2×2.5 min), the radioactivity associated with the papers was measured by liquid scintillation spectrophotometry.

Assays were performed using either histone H1 (Sigma Type III-S) (10 μg/assay) or a dodecapeptide corresponding to residues 1–12 of glycogen synthase [35] (50 μM final concentration) (Auspep Pty. Ltd.) as phosphate acceptors. Essentially identical results were obtained with both substrates (results not shown).

Statistical analysis

Results were analysed by ANOVA using the SYSTAT computer program (version 4) (SYSTAT Inc., Evanston, IL, U.S.A.) with values being considered significantly different at $P < 0.05$. Where necessary the raw data were subjected to a logarithmic transformation (base *e*) prior to analysis to ensure homogeneity of variance as required by the ANOVA test.

RESULTS

Effect of increasing carbon chain length on fatty acid-induced neutrophil superoxide production

In both the presence and absence of extracellular Ca^{2+}, the ability of the tetraenoic fatty acids to induce neutrophil superoxide production declined with increasing carbon chain length (Figure 1). Extracellular Ca^{2+} has been proposed to interact with the

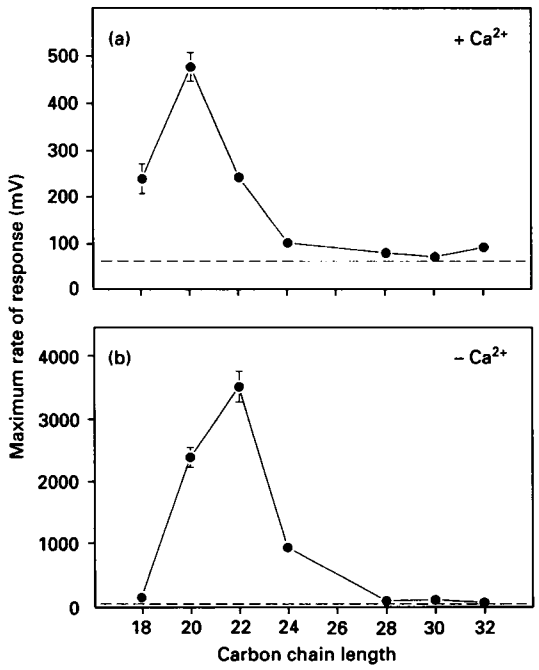

Figure 1 Neutrophil superoxide production elicited by the tetraenoic fatty acids in the presence and absence of extracellular Ca^{2+}

Neutrophils (1 ml; 1×10^6 cells) were stimulated with the indicated fatty acids (33 μM) and the superoxide production assessed by chemiluminescence. Assays were performed in HBSS (+Ca^{2+}) (**a**) or in HBSS supplemented with 1.5 mM EGTA (−Ca^{2+}) (**b**). Broken lines represent the activity of unstimulated cells. Results shown are the arithmetic means ± S.E.M. of multiple experiments ($n \geq 3$) performed using cells from different donors.

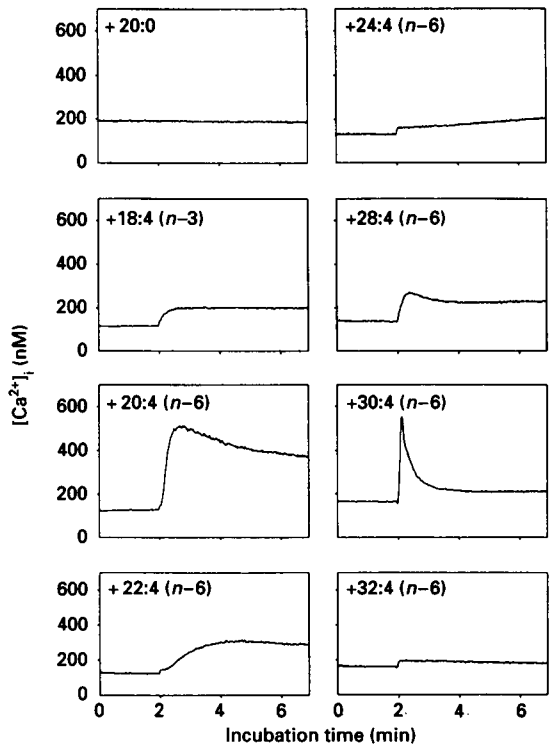

Figure 2 $[Ca^{2+}]_i$ in neutrophils treated with 20:0 or the tetraenoic fatty acids in the presence of extracellular Ca^{2+}

Neutrophils (2 ml; 6×10^6 cells) were stimulated with the indicated fatty acids (25 μM added at $t = 2$ min) and the $[Ca^{2+}]_i$ assessed fluorimetrically using fura-2 as described in the Experimental section. Assays were performed in Ca^{2+}-containing HBSS. Results shown are representative of multiple experiments ($n \geq 3$) performed using cells from different donors.

carboxyl group of the fatty acids to limit their association with the cells [10,15]. In partial support of this, all the tetraenoic fatty acids examined except 18:4 ($n-3$) and 32:4 ($n-6$) elicited greater superoxide production if extracellular Ca^{2+} was removed (Figure 1). We also observed that the most active fatty acid shifted from 20:4 ($n-6$) in the presence of extracellular Ca^{2+} to 22:4 ($n-6$) in its absence (Figure 1), although the reasons for this were not explored. Both the fully saturated eicosaenoic acid (20:0) and 20:4 ($n-6$)-methyl ester were inactive for superoxide production, irrespective of the extracellular Ca^{2+} concentration (results not shown). The effect of extracellular Ca^{2+} on the magnitude of agonist-induced superoxide production was specific for the fatty acids as fMLP-induced chemiluminescence was markedly reduced in the absence of external Ca^{2+} while responses to PMA were largely unaffected (results not shown), in agreement with previous reports [10,15,20,52].

Effect of increasing carbon chain length on fatty acid-induced Ca^{2+} mobilization

Unlike the effect on superoxide production, increasing the carbon chain length did not result in a corresponding decline in the ability of the fatty acids to mobilize Ca^{2+} (Figure 2). Instead, Ca^{2+}-mobilizing ability was maximal at 20:4 ($n-6$), declined by 24:4 ($n-6$) but increased again at 28:4 ($n-6$) to peak at 30:4 ($n-6$) (Figure 2). Note that the initial rise in $[Ca^{2+}]_i$ was more pronounced in response to 30:4 ($n-6$) than it was for the other fatty acids (Figure 2), although the extent of these differences varied considerably between donors (compare Figures 2, 3, 6 and 7). $[Ca^{2+}]_i$ was unaffected by both 20:0 and 20:4 ($n-6$)-methyl ester (Figure 2 and results not shown).

The rapid early rise in $[Ca^{2+}]_i$ seen in neutrophils from some donors in response to 30:4 ($n-6$) compared with 20:4 ($n-6$)

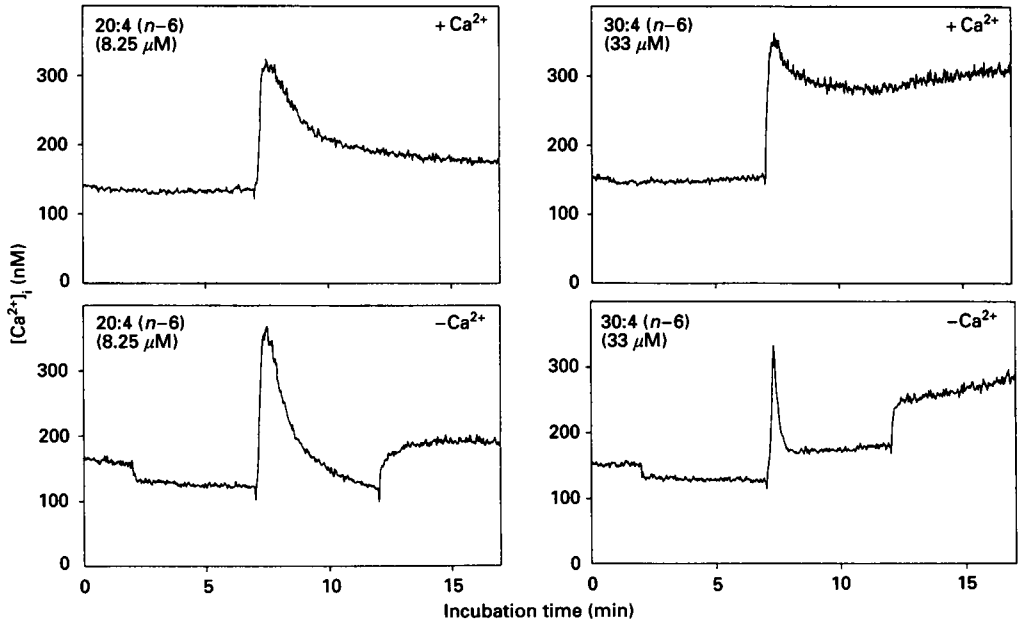

Figure 3 $[Ca^{2+}]_i$ in neutrophils treated with 20:4 ($n-6$) or 30:4 ($n-6$) in the presence or absence of extracellular Ca^{2+}

Neutrophils (2 ml; 6×10^6 cells) in 40 mM Hepes-buffered HBSS were stimulated at $t = 7$ min with 20:4 ($n-6$) (8.25 μM) (left-hand panels) or 30:4 ($n-6$) (33 μM) (right-hand panels) and the $[Ca^{2+}]_i$ assessed fluorometrically using fura-2 as described in the Experimental section. Ca^{2+}-free conditions were established at $t = 2$ min (+1.5 mM EGTA) and extracellular Ca^{2+} (2 mM final concentration) replaced at $t = 12$ min. Results shown are representative of multiple experiments ($n \geq 3$) performed using cells from different donors.

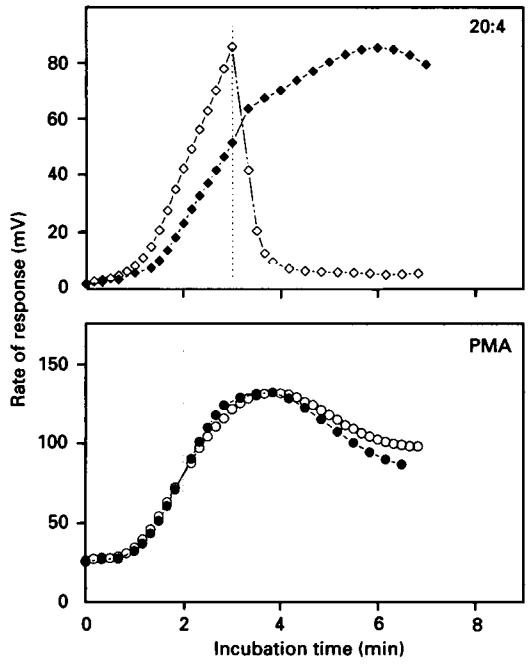

Figure 4 Effects of BSA and ovalbumin on established 20:4 ($n-6$)- and PMA-induced superoxide production

Neutrophils (1 ml; 1×10^6 cells) were stimulated with 20:4 ($n-6$) (25 μM; ◇, ◆) or PMA (1×10^{-7} M; ○, ●) at $t = 0$ and HBSS, ovalbumin or BSA (both 25 μM) added at $t =$ 3 min for 20:4 ($n-6$) or $t = 2$ min for PMA. Solid symbols, +HBSS and +ovalbumin; open symbols, +BSA. Results shown are representative of multiple experiments ($n \geqslant 3$) performed using cells from different donors.

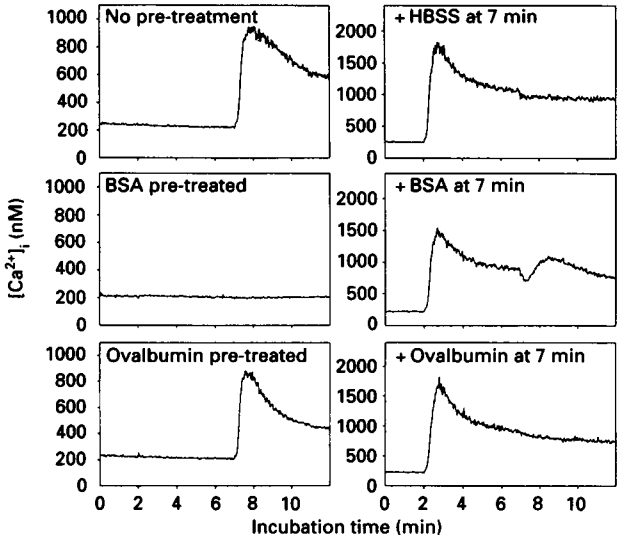

Figure 5 Effect on neutrophil $[Ca^{2+}]_i$ of adding BSA or ovalbumin either before or after stimulation with 20:4 ($n-6$)

Neutrophils (2 ml; 6×10^6 cells) were incubated with BSA or ovalbumin (25 μM) either prior to (added at $t = 2$ min, left-hand panels) or after (added at $t = 7$ min, right-hand panels) stimulation with 20:4 ($n-6$) (25 μM). Results shown are representative of multiple experiments ($n \geqslant 3$) performed using cells from different donors.

may reflect different Ca^{2+} mobilization mechanisms for these two fatty acids. 20:4 ($n-6$)- and 30:4 ($n-6$)-induced Ca^{2+} mobilization and 20:4 ($n-6$)-induced superoxide production were therefore examined under various conditions to determine whether these fatty acids mobilized Ca^{2+} by the same mechanism and whether this increased $[Ca^{2+}]_i$ was involved in superoxide production.

20:4 ($n-6$)- and 30:4 ($n-6$)-induced Ca^{2+} mobilization in the presence and absence of extracellular Ca^{2+}

Unlike superoxide production (Figure 1), removal of extracellular Ca^{2+} did not alter the magnitude of the initial $[Ca^{2+}]_i$ peak for both 20:4 ($n-6$) and 30:4 ($n-6$) (Figure 3). Furthermore, when Ca^{2+} was added back to the medium, the resultant $[Ca^{2+}]_i$ was the same as when extracellular Ca^{2+} was present throughout the assay for both 20:4 ($n-6$) and 30:4 ($n-6$) (Figure 3). A lower concentration of 20:4 ($n-6$) was used for the studies shown in Figures 3 and 7 as preliminary experiments had indicated that, in the absence of extracellular Ca^{2+}, it was not possible to obtain meaningful $F_{max.}$ and $F_{min.}$ values with higher 20:4 ($n-6$) concentrations (results not shown). While the reasons for this were not fully investigated, it is most likely a result of interference with the assay by the large amount of superoxide produced in response to 20:4 ($n-6$) under Ca^{2+}-free conditions (Figure 1).

Effect of BSA and ovalbumin on agonist-induced chemiluminescence

BSA, but not ovalbumin, binds a number of fatty acids with high affinity [36]. BSA was therefore used to remove fatty acids from the assays to further investigate the relationship between fatty acid-induced superoxide production and Ca^{2+} mobilization.

Pretreatment with BSA but not ovalbumin blocked 20:4 ($n-6$)-induced superoxide production (reduced from 215± 17 mV to 45±3 mV) while responses to fMLP and PMA were unaffected (results not shown). Likewise, when BSA but not ovalbumin was added after the agonist, 20:4 ($n-6$)-induced chemiluminescence was immediately and totally inhibited, while responses to PMA were unaffected (Figure 4). This indicates that the continuous presence of the fatty acid was required for superoxide production.

Effect of BSA and ovalbumin on agonist-induced Ca^{2+} mobilization

In contrast to the effects on chemiluminescence, the addition of BSA before but not after 20:4 ($n-6$) inhibited fatty acid-induced Ca^{2+} mobilization (Figure 5). Pre-incubation with BSA also inhibited subsequent 30:4 ($n-6$)-induced Ca^{2+} mobilization, although the inhibition was not total, as it was for 20:4 ($n-6$) (compare Figures 5 and 6, centre left-hand panels). When BSA was added to the assay system after 20:4 ($n-6$), there was a brief drop in the $[Ca^{2+}]_i$ followed by an increase that overshot the pre-BSA level (Figure 5, centre right-hand panel). In contrast, the increased $[Ca^{2+}]_i$ effected by 30:4 ($n-6$) was unaffected by the addition of BSA (Figure 6, centre right-hand panel). The effects of BSA were specific for the fatty acids as fMLP-induced increases in $[Ca^{2+}]_i$ were unaffected by BSA pretreatment (results not shown). Ovalbumin was inactive irrespective of the agonist used or time of addition (Figures 5, 6 and results not shown). These data show that, unlike superoxide production, the Ca^{2+} mobilization effected by the fatty acids does not require the continuous presence of the fatty acids.

Effect of thapsigargin on agonist-induced Ca^{2+} mobilization

Thapsigargin is a cell-permeable sesquiterpene lactone that

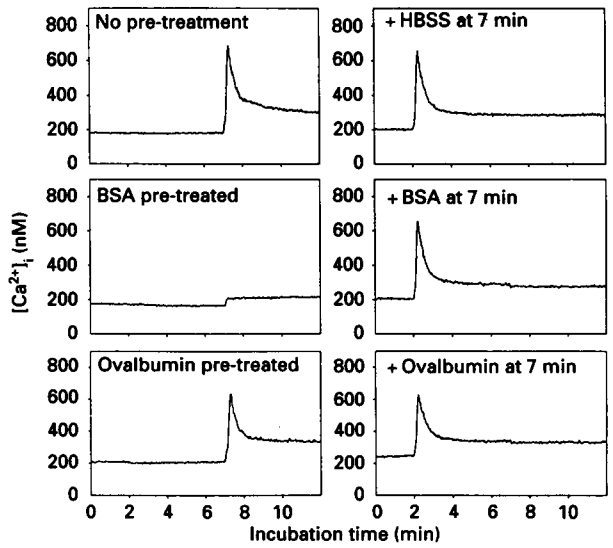

Figure 6 Effect on neutrophil $[Ca^{2+}]_i$ of adding BSA or ovalbumin either before or after stimulation with 30:4 ($n-6$)

Neutrophils (2 ml; 6×10^6 cells) were incubated with BSA or ovalbumin (25 μM) either prior to (added at $t = 2$ min, left-hand panels) or after (added at $t = 7$ min, right-hand panels) stimulation with 30:4 ($n-6$) (25 μM). Results shown are representative of multiple experiments ($n \geq 3$) performed using cells from different donors.

induces the release of intracellular Ca^{2+} stores independently of the production of $InsP_3$ by inhibiting the endoplasmic reticulum Ca^{2+}-ATPase [37,38]. By pretreating the neutrophils with thapsigargin under Ca^{2+}-free conditions it is therefore possible to deplete the thapsigargin-sensitive intracellular Ca^{2+} stores. We observed that under Ca^{2+}-free conditions and at a concentration which induced the release of intracellular Ca^{2+}, thapsigargin pretreatment blocked the increases in $[Ca^{2+}]_i$ normally elicited by both fMLP and 20:4 ($n-6$) but not that induced by 30:4 ($n-6$) (Figure 7). This demonstrates that, unlike 20:4 ($n-6$) and fMLP, 30:4 ($n-6$) induces the release of Ca^{2+} from a distinct, thapsigargin-insensitive intracellular pool.

Effect of thapsigargin on agonist-induced neutrophil chemiluminescence

20:4 ($n-6$)-induced chemiluminescence was significantly inhibited by thapsigargin pretreatment (Figure 8). The thapsigargin concentrations required to effect this inhibition have been shown to deplete the intracellular Ca^{2+} pool (Figure 7 and [37]). Responses to PMA, which do not involve an increase in $[Ca^{2+}]_i$, were largely unaffected by thapsigargin pretreatment except at high concentrations (Figure 8).

$InsP_3$ production following exposure to agonists

Previous studies have demonstrated that thapsigargin can interfere with both $InsP_3$-sensitive and -insensitive intracellular Ca^{2+} pools [39]. It was therefore important to determine whether the fatty acids induced Ca^{2+} mobilization through the production of $InsP_3$. Neither 20:4 ($n-6$) nor 30:4 ($n-6$) at concentrations that increased $[Ca^{2+}]_i$ stimulated $InsP_3$ production above baseline levels or those detected after treatment with the negative control PMA (results not shown). Increased $InsP_3$ levels were detected, as expected, after incubation with the positive control fMLP (results not shown).

Particulate-associated protein kinase activity following exposure to agonists

The particulate translocation of protein kinase C (PK-C), which is a consequence of agonist-induced phosphatidylinositol 4,5-bisphosphate hydrolysis ([40] and reviewed by Berridge [26,27]), was also examined as an independent assessment of inositol lipid hydrolysis. We observed that none of the fatty acids tested [18:1 ($n-9$), 20:0, 20:4 ($n-6$), 20:5 ($n-3$), 22:6 ($n-3$), 30:4 ($n-6$), 32:4 ($n-6$) or 34:6 ($n-3$)] increased the protein kinase activity associated with the neutrophil particulate fraction (Table 1). As expected, PMA induced a large and prolonged increase in the particulate-associated protein kinase activity at both times examined ($P < 0.001$) while fMLP induced a smaller, rapid and statistically significant rise at 0.5 min ($P < 0.01$) that returned to baseline levels after 10 min ($P > 0.05$) (Table 1). The PK-C

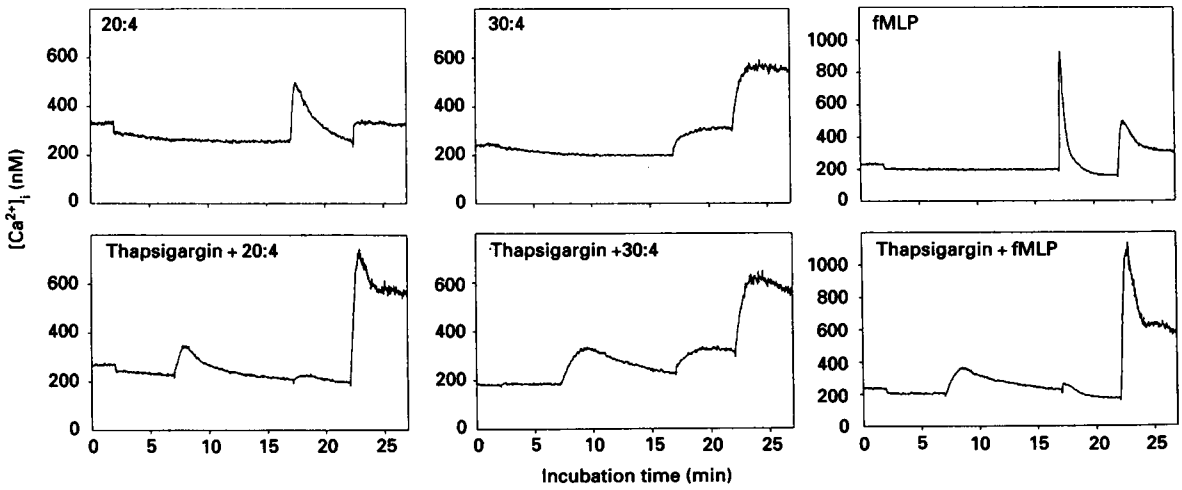

Figure 7 Effect of thapsigargin pretreatment on neutrophil $[Ca^{2+}]_i$ in response to 20:4 ($n-6$), 30:4 ($n-6$) or fMLP

Neutrophils (2 ml; 6×10^6 cells) in 40 mM Hepes-buffered HBSS were incubated with thapsigargin (2 μM, 10 min at 37 °C, added at $t = 7$ min) prior to stimulation at $t = 17$ min with 20:4 ($n-6$) (8.25 μM) (left-hand panels), 30:4 ($n-6$) (25 μM) (centre panels) or fMLP (5×10^{-6} M) (right-hand panels). Ca^{2+}-free conditions were established at $t = 2$ min (+1.5 mM EGTA) and extracellular Ca^{2+} (2 mM final concentration) added at $t = 22$ min. Results shown are representative of multiple experiments ($n \geq 3$) performed using cells from different donors.

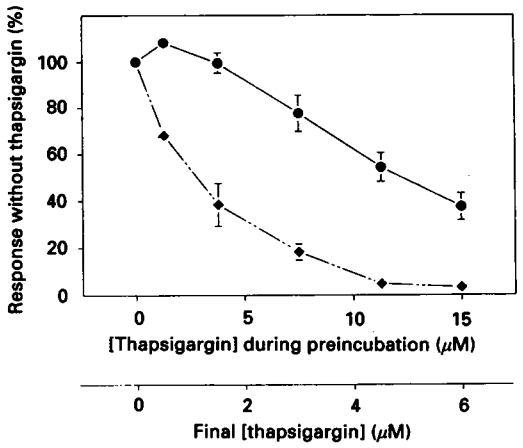

Figure 8 Effect of thapsigargin pretreatment on agonist-induced neutrophil chemiluminescence

Neutrophils (400 μl; 1×10^6 cells) were incubated (10 min at 37 °C) with either buffer (as control) or thapsigargin before stimulation with 20:4 ($n-6$) (33 μM) (◆) or PMA (1×10^{-7} M) (●) and volume adjustment to 1 ml. All incubations and assays were performed in the absence of extracellular Ca^{2+}. Results shown are the arithmetic means ± S.E.M. of multiple experiments ($n \geqslant 3$) performed using cells from different donors.

Table 1 Effect of fatty acids, fMLP and PMA on the particulate-associated protein kinase activity in neutrophils

Neutrophils (2 ml; 3×10^7 cells) were incubated with either the solvent control, fatty acid (FA) [18:1 ($n-9$), 20:0, 20:4 ($n-6$), 20:5 ($n-3$), 22:6 ($n-3$), 30:4 ($n-6$), 32:4 ($n-6$) or 34:6 ($n-3$), all 33 μM], fMLP (5×10^{-6} M) or PMA (1×10^{-7} M) at 37 °C for the indicated periods and the particulate-associated protein kinase activity determined as described in the Experimental section using the glycogen $synthase_{(1-12)}$ peptide as substrate. The particulate-associated protein kinase activity in the fatty acid-treated cells were found to be statistically homogenous between the various fatty acids and were therefore pooled. Data represent the arithmetic mean ± S.E.M. of multiple determinations ($7 \leqslant n \leqslant 56$) performed using cells from different donors. Specific activity of [^{32}P]γ-ATP: 6.773, 6.496, 5.497 and 5.261 GBq/mmol (0.183, 0.176, 0.149 and 0.142 Ci/mmol) (multiple experiments).

	Phosphate incorporation (pmol/min)			
	0.5 min		10 min	
	$+Ca^{2+}$/PS	$-Ca^{2+}$/PS	$+Ca^{2+}$/PS	$-Ca^{2+}$/PS
Control	0.44 ± 0.02	0.38 ± 0.02	0.43 ± 0.02	0.48 ± 0.01
FA	0.42 ± 0.01	0.41 ± 0.01	0.42 ± 0.01	0.43 ± 0.01
fMLP	0.54 ± 0.01	0.44 ± 0.02	0.43 ± 0.02	0.43 ± 0.02
PMA	1.80 ± 0.03	0.53 ± 0.02	1.00 ± 0.06	0.48 ± 0.03

translocations observed here for both fMLP and PMA are in agreement with previous reports [41,42].

DISCUSSION

Our results are not only the first to demonstrate that certain VLCFAs induce Ca^{2+} mobilization in intact cells but also highlight that there are both similarities and dramatic differences between 20:4 ($n-6$)- and 30:4 ($n-6$)-induced Ca^{2+} mobilization. Thus, for both 20:4 ($n-6$) and 30:4 ($n-6$), our results suggest that the initial rise in $[Ca^{2+}]_i$ was principally caused by Ca^{2+} released from intracellular stores, while the sustained increase in $[Ca^{2+}]_i$ was more a consequence of changes to the influx or efflux of the ion. The relative importance of Ca^{2+} mobilization from the intracellular stores and altered ion influx/efflux in the biological responses induced by the fatty acids is unclear. For example, although fatty acids have been reported to both inhibit Ca^{2+} inflow and induce Ca^{2+} extrusion in T-cell lines and macrophages [24,25,43–45] and activate the purified Ca^{2+}-ATPase responsible for Ca^{2+} efflux in erythrocytes [46], it is difficult to explain our results simply on the basis of Ca^{2+}-ATPase activation. Likewise, the findings that fatty acids inhibit Ca^{2+} inflow and induce Ca^{2+} extrusion against concentration gradients also suggests that these species are not acting as Ca^{2+} ionophores.

By employing BSA and thapsigargin, we have identified some important differences between 20:4 ($n-6$)- and 30:4 ($n-6$)-induced Ca^{2+} mobilization. Thus, unlike 20:4 ($n-6$), the failure of BSA pretreatment to completely block 30:4 ($n-6$)-induced Ca^{2+} mobilization or influence 30:4 ($n-6$)-induced increases in $[Ca^{2+}]_i$ suggested that BSA does not bind 30:4 ($n-6$) as well as 20:4 ($n-6$). Note also that support for our finding that BSA caused an increase in $[Ca^{2+}]_i$ if added after 20:4 ($n-6$) comes from studies using the T-cell line JURKAT in which BSA elicited a similar increase in $[Ca^{2+}]_i$ if added after either α-linolenic [25] or oleic [43] acids. These authors concluded that the fatty acids inhibited Ca^{2+} inflow and that their removal with BSA overcame this inhibition [25,43]. Our results with 20:4 ($n-6$) are consistent with this interpretation.

Our studies with thapsigargin demonstrate that there were at least two intracellular Ca^{2+} pools mobilized by fatty acids. The first was a thapsigargin-sensitive store, mobilized by the long-chain fatty acids [e.g. 20:4 ($n-6$)] and was probably the $InsP_3$-mobilizable pool in the endoplasmic reticulum. In contrast, the VLCFAs [e.g. 30:4 ($n-6$)] did not mobilize Ca^{2+} from this pool but from a thapsigargin-insensitive store which has yet to be characterized. Distinct thapsigargin-sensitive and -insensitive intracellular Ca^{2+} pools have been previously reported and partially characterized, although their identity and regulation remains unclear [39,47]. Prior studies have also shown that intracellular Ca^{2+} release in JURKAT cells in response to a number of fatty acids, including 20:4 ($n-6$), was from the $InsP_3$-mobilizable pool but independent of $InsP_3$ production [24]. While supporting a similar conclusion for 20:4 ($n-6$) in neutrophils, the present study further demonstrates that the VLCFAs, which mobilize Ca^{2+} from a different intracellular pool to 20:4 ($n-6$), also do so independently of $InsP_3$ production and PK-C translocation.

The relationship between fatty acid-induced superoxide production and Ca^{2+} mobilization is complex. Thus, the lack of correlation between the amount of superoxide produced and $[Ca^{2+}]_i$, as well as the differing effects of BSA and extracellular Ca^{2+} on fatty acid-induced Ca^{2+} mobilization and superoxide production, all suggest that the increase in $[Ca^{2+}]_i$ elicited by the fatty acids is not, by itself, a sufficient or necessary signal to induce superoxide production in the neutrophils. These findings further raise the possibility that the association of the fatty acids with the cells may be more complex than previously believed and not simply governed by the presence of Ca^{2+} in the extracellular medium [10,15]. There is, however, contrary evidence to indicate that the fatty acid-induced release of intracellular Ca^{2+} may be required for superoxide production. Accordingly, the ability of the tetraenoic fatty acids to both mobilize the thapsigargin-sensitive Ca^{2+} pool and elicit superoxide production decreased with increasing carbon chain length. Furthermore, depleting the intracellular Ca^{2+} stores by thapsigargin pretreatment inhibited subsequent 20:4 ($n-6$)-induced chemiluminescence, while responses to PMA, which does not increase $[Ca^{2+}]_i$, were unaffected by anything other than high thapsigargin concentrations. Taken

together, a possible explanation for our data is that the fatty acid-induced release of Ca^{2+} from the thapsigargin-sensitive intracellular stores may be required for the initiation but not necessarily the maintenance of neutrophil superoxide production. This is consistent both with the observation that fatty acid-induced intracellular Ca^{2+} release always preceded superoxide production and with previous studies where intracellular Ca^{2+} release was implicated in the initial stages of fMLP-induced superoxide production [48]. We furthermore believe that the thapsigargin-insensitive Ca^{2+} mobilization elicited by the VLCFAs is unrelated to superoxide production.

We have previously postulated that the VLCFA may be involved in signal transduction [4] and have demonstrated that these species can activate purified PK-C *in vitro* [30]. This study, in addition to demonstrating that certain VLCFA activate neutrophils, is the first to show that the VLCFA can influence known second messenger systems in intact cells. The effects of the VLCFA on $[Ca^{2+}]_i$ are also important as they show not only that the cell exercises selectivity in its responsiveness to these species but that 30:4 ($n-6$) liberates Ca^{2+} from an intracellular pool which is distinct from that mobilized by 20:4 ($n-6$) or fMLP. The present findings therefore raise the possibility that the pathology of the peroxisomal diseases, in which the VLCFAs accumulate, may be due, in part, to the abnormal mobilization of intracellular Ca^{2+} effected by the VLCFAs. It should be noted, however, that the *in vivo* concentrations of the long- and very-long-chain fatty acids required to elicit these effects are unknown and extremely difficult to determine. For example, in tissues rich in VLCFA or in peroxisomal diseases, the hydrolysis of phospholipid species containing these VLCFAs may result in localized intracellular concentrations far higher than those present in the body fluids. Furthermore, intracytoplasmic inclusions rich in VLCFAs are found in certain cells from patients with peroxisomal disorders [49,50] and it is possible that significant intracellular concentrations of the VLCFA may arise during the processing of these membranous structures. This is particularly relevant considering we have previously reported that the uptake and metabolism of exogenous VLCFA by neutrophils is substantially lower and slower than for 20:4 ($n-6$) [51]. Earlier reports have indicated that many of the biological effects of 20:4 ($n-6$), including superoxide production and Ca^{2+} mobilization, are due to the fatty acid itself and not a metabolite [17,21]. Consistent with this, we found that mobilization of Ca^{2+} by 20:4 (n−6) was not diminished by either indomethacin (5 μM), an inhibitor of cyclo-oxygenase, or eicosatriynoic acid (20 μM), an inhibitor of lipoxygenases (S. J. Hardy, B. S. Robinson, A. Ferrante, C. S. T. Hii, D. W. Johnson, A. Poulos and A. W. Murray, unpublished work). Likewise, our studies suggest that not only is 30:4 ($n-6$) itself, rather than a metabolite, responsible for its biological activity but also that the concentration of exogenous VLCFA required to elicit biological effects may be far higher than those required intracellularly, as only a small percentage of the added VLCFA may gain access to the cell. We have observed, for example, that superoxide production in response to 30:4 ($n-6$) was always less than 10% of that elicited by 20:4 ($n-6$) and correlated with the relative amount of these fatty acids that were associated with the cells [20]. This suggested that 20:4 ($n-6$) and 30:4 ($n-6$) may be equally potent stimuli for superoxide production and that the lower and slower responses to 30:4 ($n-6$) simply reflected the smaller amounts of this fatty acid that were associated with the cells. The effects of 30:4 ($n-6$) on $[Ca^{2+}]_i$ clearly demonstrate, however, that this fatty acid does rapidly associate with the cells and that this association has a biological activity different from that elicited by 20:4 ($n-6$). It remains to be determined not only which intracellular Ca^{2+} pool is being mobilized by 30:4 ($n-6$) but whether it is the slow or rapid association of the VLCFA with the cells that is responsible for the as yet unidentified *in vivo* function of these unique species.

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