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Regulation of epidermal-growth-factor-receptor signal transduction by *cis*-unsaturated fatty acids

Evidence for a protein kinase C-independent mechanism

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The effect of acute treatment with non-esterified fatty acids (NEFA) on transmembrane signalling has been investigated in three different cell lines. In EGFR T17 cells, pretreatment with *cis*-unsaturated (oleic and palmitoleic acids) NEFA, but not with saturated or *trans*-unsaturated NEFA, inhibited the epidermal-growth-factor (EGF)-induced increases in cytosolic [Ca^{2+}], membrane potential and Ins(1,4,5)P_3 generation. The blocking effect was found to be time- and dose-dependent and rapidly reversible after washout. However, oleic acid treatment did not block either binding of ^{125}I-EGF to its receptor or EGF-induced autophosphorylation of the EGF receptor. The mechanism of action of NEFA could not be attributed to protein kinase C activation, since (i) down-regulation of the enzyme by long-term treatment with phorbol esters did not prevent blockade by oleic acid, and (ii) the effects of acutely administered phorbol ester and oleic acid were additive. In this cell line, signalling at bradykinin and bombesin receptors was also impaired by oleic acid. In A431 cells, oleic acid also blocked signal transduction at the EGF and B_2 bradykinin receptors. Finally, in PC12 cells, oleic acid blocked the Ca^{2+} influx mediated by the activation of B_2 bradykinin receptors. In conclusion: (1) NEFA block signal transduction by interfering with receptor–phospholipase C or phospholipase C–substrate interaction without preventing ligand binding; (2) NEFA do not act by a protein kinase C-mediated mechanism; (3) the effect of NEFA is dependent on their configuration rather than hydrophobicity or chain length; (4) this effect is evident in several different cell lines and receptor systems.

INTRODUCTION

Non-esterified fatty acids (NEFA) are normal constituents of biological membranes, in levels ranging from 1 to 40 mol% of total lipids [1]. They are common metabolites carried by the bloodstream, and their levels oscillate widely during the day or in pathological states [2,3]. It is not known at present whether the abnormally high levels of NEFA present acutely in hypoxia or ketoacidosis, or chronically in obesity or pregnancy, could account for some of the morbid complications associated with such states. After their increase in plasma, NEFA rapidly partition into biological membranes, thus influencing the physicochemical state of lipid domains [4]. Recent evidence indicates that the activity of many integral membrane proteins is modulated by the lipid composition of the membrane domain in which the protein resides [5]. The accepted view is that, after incorporation, NEFA perturb the bilayer structure of the plasma membrane in a manner similar to some anaesthetics, leading to alterations in membrane–cytoskeleton interactions and altering the physical state of transmembrane receptors and regulatory proteins [6,7]. Consistent with this hypothesis are reports demonstrating that NEFA are able to alter platelet aggregation, lymphocyte mitogenesis, surface receptor capping and cell-to-cell substrate adhesion [8–11]. It has been reported that NEFA can inhibit cellular secretion within minutes in a dose-related manner, suggesting a new, direct, mechanism of action of these molecules [12]. Though their effects are relevant and widespread, the precise point of action of NEFA is at present unknown.

In order to identify the level at which NEFA perturb cellular activity, in the present study we have employed the EGFR T17 cloned cell line, a mouse fibroblast line derived from NIH 3T3, which expresses high levels of the human epidermal growth factor (EGF) receptor [13]. This cell line provides an ideal system for the study of the mechanism of action of NEFA, because of the growing understanding of EGF-receptor (EGFR) function achieved in recent years. Upon binding, EGF induces EGFR oligomerization, with the ensuing activation of protein tyrosine kinase activity and EGFR autophosphorylation as the earliest signal [14]. The activated EGFR induces subsequent tyrosine phosphorylation of cellular substrates, including the γ isoform of phospholipase C (PLC), through a close physical association which leads to its activation [14]. PLC-mediated hydrolysis of PtdIns(4,5)P_2 produces two second messengers. One is diacylglycerol (DAG), the physiological activator of the enzymes of protein kinase C (PKC) family [15]. The second mediator, Ins(1,4,5)P_3, is a small, highly hydrophilic, molecule that freely diffuses throughout the cell cytosol, leading to Ca^{2+} release from microsomal stores and a transient rise in cytosolic free Ca^{2+} concentration ([Ca^{2+}]$_i$) [15,16]. Concomitantly, Ca^{2+} and K^+ conductances across the plasma membrane are stimulated [16–18]. How the two hydrolysis products, DAG and Ins(1,4,5)P_3, may be linked to the delayed responses which lead the cell to enter the mitotic cycle is not known.

The results of this study demonstrate that *cis*-unsaturated NEFA inhibit EGFR-activated PtdIns(4,5)P_2 hydrolysis, the rise in [Ca^{2+}]$_i$ and ionic fluxes without affecting EGF-induced EGFR

Abbreviations used: NEFA, non-esterified fatty acids; EGF, epidermal growth factor; [Ca^{2+}]$_i$, cytosolic free Ca^{2+} concentration; EGFR, EGF receptor; PLC, phospholipase C; DAG, diacylglycerol; PMA, 4-phorbol 12-myristate 13-acetate; FCS, fetal-calf serum; DMEM, Dulbecco's modified Eagle's medium; KRH, Krebs–Ringer Hepes buffer; fura-2/AM, fura-2 penta-acetoxymethyl ester; V_m, membrane potential; PKC, protein kinase C; Ins*P*s, inositol phosphates.

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autophosphorylation. These NEFA effects are mediated by a PKC-independent mechanism, a significant result considering the recent reports that PKC could be an important target for NEFA [19–21].

MATERIALS AND METHODS

Cell culture

EGFR T17 and human epidermoid carcinoma A431 cells were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% (v/v) fetal-calf serum (FCS) supplemented with antibiotics (penicillin 100 units/ml, streptomycin 100 μg/ml). Subculture (1:5 split ratio) was done when cells approached 95% confluence.

Rat pheochromocytoma PC12 cells were cultured in RPMI 1640 medium supplemented with antibiotics, insulin and transferrin (each 10 μg/ml), putrescine (6 mg/l), progesterone (62 μg/l), selenite (40 μg/l) and BSA (0.5 mg/ml). PC12 cells were subcultured weekly in a split ratio of 1:4. In order to attach PC12 cells, the bottom of the Petri dish was overlayed with FCS during 30 min. The serum was then removed and the cells were plated. All cell cultures were maintained at 37 °C under a humidified atmosphere of air/CO_2 (19:1).

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

Measurement of $[Ca^{2+}]_i$ in cell suspensions was performed by using the fluorescent Ca^{2+} indicator fura-2 [22]. Monolayers from five to seven 100 mm-diameter dishes (EGFR T17 and A431 cells) were washed twice with phosphate-buffered saline [18] and then treated at 37 °C with 2 ml of the same buffer containing trypsin (0.05%) and EDTA (0.9 mM). Detachment of the cells from the dish was complete within 1 min (EGFR T17 cells) or 10 min (A431 cells). Trypsin was neutralized by washing the cells in the final incubation medium, Krebs–Ringer Hepes (KRH), with the following composition (mM): NaCl, 125; KCl, 5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2; glucose, 6; Hepes, 25 (pH 7.4). Cells were resuspended in KRH at 5×10^6 cells/ml and loaded with fura-2 by a 30 min incubation period at 37 °C with 3 μM fura-2 penta-acetoxymethyl ester (fura-2/AM). The cell suspensions were then adjusted to 10 ml with KRH and maintained at room temperature until processed. For fluorimetric measurements, 1 ml of the cell suspension was centrifuged at 10^4 *g* for 3 s and the pellet resuspended in 2 ml of warm KRH. The cells were then transferred to a thermostatically controlled (37 ± 1 °C) holder, and fluorescence intensity was measured under continuous stirring, at an excitation wavelength of 345 nm and emission at 490 nm, with slit width of 5 nm. The fluorescence signals were calibrated in terms of $[Ca^{2+}]_i$ by using a K_d for fura 2–Ca^{2+} interaction of 225 nM [22]. In all the experiments, data were expressed as means ± S.E.M., and compared by Student's *t* test (unpaired). Statistical significance was established at $P < 0.05$.

$[Ca^{2+}]_i$ was measured in PC12 cells as described elsewhere [23]. In brief, PC12 cells were detached from the dish by gently streaming culture medium over the monolayer with a Pasteur pipette. Detached cells were washed twice and resuspended in KRH at a final cell concentration of 10^7 cells/ml. After loading the cells with fura-2 by incubating them with 3 μM fura-2/AM, cells were processed for fluorimetric measurements.

Measurement of membrane potential (V_m)

This was done in cell suspensions with the slow-response fluorescent dye bis-oxonol [18]. For these experiments, cell suspensions were prepared as described for $[Ca^{2+}]_i$ measurements. After washing once with KRH, cells from four dishes were resuspended in 10 ml of the incubation buffer, and 750 μl was washed again by centrifugation at 10^4 *g*. The resuspended pellet was transferred to the fluorimeter cuvette and 100 nM-bis-oxonol added from a 1000-fold-concentrated solution in dimethyl sulphoxide. The cells were allowed to equilibrate with the dye, and after approx. 10 min different test substances were added. Downward or upward deflections of the fluorescence signal represent hyper- or de-polarizations respectively, and the data were expressed as the percentage decrease from basal fluorescence values.

Measurement of generation of inositol phosphates (Ins*P*s)

For measurement of Ins*P*s release [24], EGFR T17 cells were plated in 35 mm-diam. dishes and grown until 70% confluence in complete DMEM. Labelling with *myo*-[2-^{3}H(n)]inositol was done by incubating cell monolayers with the radioisotope (2 μCi/ml) in a 1:4 mixture of DMEM and Eagle Basal Medium (inositol-free) plus 10% dialysed (M_r cut-off = 12000) FCS (final inositol concn. approx. 10 μM). Then 24 h later the incubation medium was removed and the monolayers were washed three times in cold KRH, in which 10 mM-LiCl replaced equimolar quantities of NaCl (Li-KRH). Cells were preincubated for 5 min at 37 °C in Li-KRH, and incubated for 10 min at 37 °C with shaking (120 rev./min) in 2 ml of prewarmed Li-KRH with or without oleic acid. At 20 min after EGF (10 nM) addition, media were discarded and the reactions stopped immediately by addition of 2 ml of ice-cold 10% (w/v) trichloroacetic acid. Dishes were placed on ice, and water-soluble radioactivity was extracted during 30 min. The whole supernatant was transferred to glass tubes and washed with 5 × 1 vol. of diethyl ether. Samples were adjusted to alkaline pH (approx. 8.5) by adding a small (50 μl) volume of 3 M-Tris. Anion-exchange resin (Dowex 1-X8, formate form) was added to each sample and washed with 8 × 3 ml of 60 mM-ammonium formate/5 mM-$Na_2B_4O_7$. Ins*P*s were eluted with 3 ml of 1.7 M-ammonium formate adjusted to pH 3.7 with 85% (w/v) H_3PO_4. Portions (2.5 ml) of the supernatant were transferred to scintillation vials and counted for radioactivity after mixing with scintillation fluid.

The levels of Ins(1,4,5)P_3 were specifically measured by anion-exchange h.p.l.c. [25]. In brief, cells were grown until 70% confluence in 60 mm-diam. Petri dishes, and labelled for 24 h with *myo*-[^{3}H]inositol (10 μCi/ml) as for the Ins*P*s experiments. The monolayers were washed three times in cold KRH, preincubated for 5 min at 37 °C in KRH, and incubated for 10 min at 37 °C with shaking (120 rev./min) in 2 ml of prewarmed KRH with or without 0.1 mM-oleic acid. After incubation, the medium was removed and 1 ml of prewarmed KRH with or without the fatty acid, supplemented with EGF (50 nM), was added to the dishes. At the stated times, media were aspirated and reactions stopped by addition of 2 ml of ice-cold 10% trichloroacetic acid. Acid-soluble radioactivity was extracted (30 min on ice) with occasional rocking. Supernatants were prepared as described for Ins*P*s, diluted to 2.5 ml with ultrapure water, and injected on an anion-exchange h.p.l.c. column (Partisil 10 SAX, 250 mm × 4.6 mm). Inositol trisphosphates were resolved by using a stepwise gradient of ammonium formate at 2 ml/min essentially as described [25], and detected by liquid-scintillation counting after mixing 0.4 min fractions with 5.5 ml of scintillation fluid. Experiments were performed in triplicate, and the results are expressed as means ± S.E.M. of peak areas obtained by the trapezoidal method. The identity of Ins(1,4,5)P_3 and Ins(1,3,4)P_3 peaks was determined by comparison with chromatograms from tritiated standards.

Binding experiments

For binding experiments, EGFR T17 cells were plated at 7×10^4 cells/well in 24-well dishes and grown for 24 h. Cultured

cells were washed three times with KRH incubation buffer, preincubated for 15 min with the stated oleic acid concentration (or the equivalent volume of ethanol), and then ^{125}I-EGF was added to wells at 60 pM final concentration, in a total volume of 250 μl. Non-specific binding was calculated in replicate wells preincubated with a 1000-fold excess of unlabelled EGF 5 min before ^{125}I-EGF addition, and was always lower than 50 c.p.m. After 30 min at 37 °C, monolayers were washed with 5 × 0.5 ml of ice-cold KRH, and cell-associated radioactivity was solubilized overnight with 500 μl of 0.5 M-NaOH. Samples (400 μl) of the latter were introduced into plastic tubes and counted in a γ-radiation counter.

Autophosphorylation of the EGFR

For these experiments, EGFR T17 cells were collected by trypsin treatment, washed once in KRH, and resuspended at 10^7 cells/ml. Samples (1 ml) were transferred to Eppendorf tubes and preincubated for 5 min at 37 °C. Thereafter, test substances were added and suspensions incubated for 15 min with periodic shaking. Reactions were terminated by a 10 s centrifugation at 10^4 *g*, followed by resuspension of cell pellets in 300 μl of electrophoresis sample buffer. SDS/PAGE was performed overnight at 40 V constant voltage on 7.5%-acrylamide gels. Gels were then blotted on to Whatman nitrocellulose sheets that were immunostained with a rabbit polyclonal anti-phosphotyrosine antibody by the ^{125}I-Protein A method, as described elsewhere [26]. In cells metabolically labelled with [^{35}S]methionine, this antibody immunoprecipitated a M_r-170000 band that co-migrated with a band of identical M_r immunoprecipitated with monoclonal anti-EGFR antibodies.

Materials

Culture media and sera were from GIBCO and Flow. Plastic culture dishes were purchased from Falcon and Costar. Recombinant EGF was from Boehringer. Insulin, transferrin, sodium selenite, putrescine, progesterone, BSA, bradykinin, bombesin and 4-phorbol 12-myristate 13-acetate (PMA) were from Sigma. Fura 2/AM was from Calbiochem. Bis-oxonol was from Molecular Probes. *myo*-[2-^{3}H(n)]Inositol, D-[1-^{3}H(n)]Ins(1,4,5)P_3, D-[1-^{3}H(n)]Ins(1,3,4)P_3 and ^{125}I-EGF were from New England Nuclear. Monoclonal anti-EGFR antibody and ^{125}I-Protein A were from Amersham. Reagents, and resins used in the InsP_s assays were from Merck and Fluka. The Partisil SAX h.p.l.c. column was from Whatman. NEFA were purchased from Sigma at the highest purity available, and checked by t.l.c. in our laboratory. They were then stored at −20 °C as 100–1000-fold-concentrated ethanolic solutions under an inert atmosphere, and administered to cells with continuous stirring. All controls received an equivalent amount of ethanol (0.1–1%, v/v), which had no effect on the measured responses.

RESULTS

Effect of oleic acid on ionic fluxes and Ins*P*s generation

To investigate the possibility that NEFA may affect EGFR transmembrane signalling, suspensions of EGFR T17 cells were loaded with fura-2 and $[Ca^{2+}]_i$ was analysed under different conditions. Addition of a saturating EGF dose (10 nM) (Fig. 1*a*) induced a transient rise in $[Ca^{2+}]_i$, characterized by an initial rapid $[Ca^{2+}]_i$ peak, followed by a second phase or plateau and then a slower but progressive return toward resting values as described previously [17,25]. Pretreatment with oleic acid induced only a slight increase in basal $[Ca^{2+}]_i$, but at 10–100 μM it largely prevented the initial $[Ca^{2+}]_i$ peak and completely eliminated the subsequent plateau phase obtained in response to EGF (Table 1, Figs. 1*b* and 1*c*). A toxic effect of oleic acid on the cells was excluded by a Trypan Blue exclusion test and cell counting after both short (5 min) and long-term (24 h) exposures to the NEFA (results not shown).

Addition of EGF to EGFR T17 cells incubated with the fluorescent membrane-potential indicator bis-oxonol induced a downward deflection of the fluorescence tracing, reflecting membrane hyperpolarization [18] (Fig. 1*d*). As Fig. 1(*e*) shows, oleic acid was able to inhibit membrane hyperpolarization in response to the growth factor (control cells, 23.9 ± 3.3 arbitrary units, $n = 6$; 10 μM-oleic acid-pretreated cells, 12.7 ± 1.4 arbitrary units, $n = 7$; $P < 0.01$).

The blocking effect of oleic acid on the EGF-induced $[Ca^{2+}]_i$ rise was found to be time- and dose-dependent. An appreciable inhibition was observed with concentrations of oleic acid as low as 1 μM, with half-maximal blocking effect occurring at 5 μM (Fig. 2*a*). At concentrations of the fatty acid of 10 μM or above,

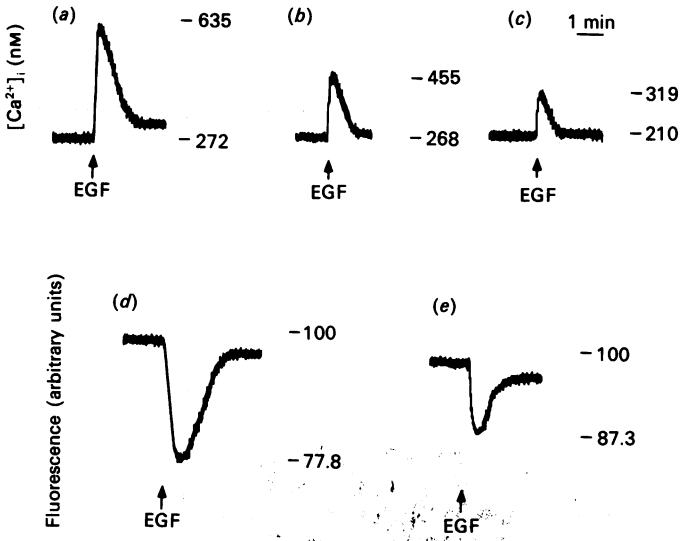

Fig. 1. Effect of oleic acid on EGF-induced $[Ca^{2+}]_i$ and V_m increases in EGFR T17 cells

Cell suspensions were loaded with fura-2/AM (*a–c*) or bis-oxonol (*d*, *e*) as described in the Materials and methods section. Fluorescence changes in response to EGF addition (10 nM) were continuously monitored both in controls (*a*) and in cells preincubated for 5 min with 10 μM- (*b*) or 100 μM-oleic acid (*c*). Downward deflections of fluorescence traces in cells loaded with bis-oxonol represent membrane hyperpolarization induced by EGF (10 nM) (*d*). In these experiments, preincubation (5 min) with 10 μM-oleic acid caused a significant decrease in the magnitude of the response (*e*).

Table 1. Effect of oleic acid on EGF-induced $[Ca^{2+}]_i$ rise

EGFR T17 cells were loaded with fura-2, and $[Ca^{2+}]_i$ (nM; mean ± S.E.M.) in response to EGF (10 nM) stimulation was measured as described in the Materials and methods section, after preincubating the suspensions for 5 min at 37 °C with or without oleic acid: *$P < 0.05$ versus basal control; **$P < 0.01$ versus control peak).

	$[Ca^{2+}]_i$ (nM)		
	Basal	Peak	*n*
Control	228 ± 6.5	647 ± 40.9	31
Oleic acid (10 μM)	233 ± 11.7	337 ± 37.8**	9
Oleic acid (100 μM)	260 ± 34.8*	369 ± 47.7**	7

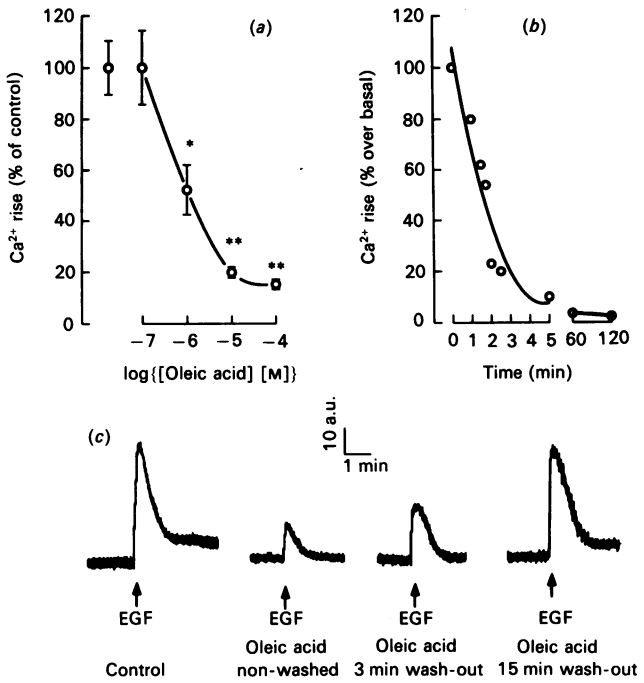

Fig. 2. Time- and dose-dependence and reversibility of oleic acid inhibition of EGF-triggered $[Ca^{2+}]_i$ rise

(*a*) Effect of different concentrations of oleic acid (5 min pretreatment) on EGF-induced $[Ca^{2+}]_i$ rise (*$P < 0.05$; **$P < 0.01$). (*b*) Time-course analysis of oleic acid (10 μM) inhibition of EGF-induced $(Ca^{2+})_i$ rise. (*c*) Recovery of responsiveness in EGFR T17 cells after long-term pretreatment with oleic acid. In these experiments EGFR T17 cells were preincubated overnight in complete culture medium supplemented with oleic acid (10 μM), loaded with fura-2/AM (in the presence of the same amount of oleic acid), washed once with KRH and treated with EGF at the times indicated after the wash-out procedure [the results are expressed as arbitrary fluorescence units (a.u.)]

up to 80% of the EGF-induced $[Ca^{2+}]_i$ rise was blocked. Time-course analysis of the NEFA effect showed that a brief 5 min preincubation period was sufficient to achieve a near-maximal effect (Fig. 2*b*). Half-maximal blockade of EGF-induced $[Ca^{2+}]_i$ rise was observed within 1.5 min of preincubation with 10 μM-oleic acid.

To determine whether the interference of NEFA with membrane physiology might be reversible, EGFR T17 cells were pretreated overnight with 10 μM-oleic acid and resuspended as described above, the same concentration of the fatty acid being used in all buffers and solutions. Cells were spun down quickly (10^4 *g*, 3 s), resuspended in KRH without oleic acid, and $[Ca^{2+}]_i$ was measured after different time periods. Cells regained the Ca^{2+} response to EGF stimulation after a brief (3 min, maximum at 15 min) wash-out in KRH (Fig. 2*c*). The results were essentially the same in short-term experiments; in cell suspensions pretreated for 5 min with 10 μM-oleic acid, the response to EGF challenge was restored within 15 min after washing the cells (results not shown). This reversibility of the cell response to EGF stimulation after wash-out ruled out any toxic action of oleic acid at the dose employed.

Generation of Ins(1,4,5)P_3 upon EGFR activation is by far the major factor responsible for the initial $[Ca^{2+}]_i$ rise and the ensuing increase in V_m [15,18]. Since NEFA are lipophilic substances, able to interact even with intracellular membranes, they may block intracellular Ca^{2+} release either at the Ins(1,4,5)P_3 receptor or alternatively by blocking Ins(1,4,5)P_3 generation. In order to analyse this aspect, EGFR T17 cells were isotopically labelled with *myo*-[^{3}H]inositol, and both the InsP_2 and the inositol trisphosphate isomers released were analysed. Preincubation of the cells with 10 μM-oleic acid inhibited the EGF-induced generation of Ins*P*s (EGF: 2.51-fold increase; oleic acid + EGF: 1.54-fold increase) (Table 2). Comparison of Ins(1,4,5)P_3 levels with high-velocity $[Ca^{2+}]_i$ measurements (Fig. 3) revealed parallel Ins(1,4,5)P_3 and $[Ca^{2+}]_i$ time courses in both control and oleic acid-pretreated cells, showing that oleic acid prevented the EGF-induced Ins(1,4,5)P_3 rise.

Table 2. Effect of oleic acid on EGF-induced generation of Ins*P*s

EGFR T17 cells plated on 35 mm-diam. dishes were labelled overnight with *myo*-[^{3}H]inositol, washed, and preincubated 10 min at 37 °C in KRH containing 10 mM-LiCl, with or without oleic acid. EGF was then added and incubations were quenched 20 min later by addition of ice-cold trichloroacetic acid. Ins*P*s were separated from other compounds by anion-exchange chromatography. Results are from one experiment that was repeated twice, and are means ± S.E.M. ($n = 3$): **$P < 0.01$.

	Ins*P*s (c.p.m.)
Control	2070 ± 79
EGF (10 nM)	5201 ± 35
Oleic acid (10 μM)	2007 ± 122
Oleic acid (10 μM) + EGF (10 nM)	3195 ± 323**

Effect of oleic acid on ^{125}I-EGF binding

To exclude the possibility that the blocking action of oleic acid on both the $[Ca^{2+}]_i$ rise and Ins(1,4,5)P_3 generation might be due to the prevention of EGF binding to its receptor, the effect of oleic acid on ^{125}I-EGF binding was analysed. As shown in Table 3, oleic acid, at 10 or 100 μM, did not block ^{125}I-EGF binding. PMA, a treatment that has been shown to decrease EGF binding by blocking high-affinity sites at the EGFR [27,28], significantly ($P < 0.01$) decreased ^{125}I-EGF binding.

Effect of oleic acid on autophosphorylation of the EGFR

As previously reported [29,30], activation of the receptor tyrosine kinase is essential for the generation of early signals at the EGFR. As the *C*-terminal tail of the EGFR is by far the major substrate of tyrosine kinase, the effect of oleic acid on autophosphorylation of the EGFR was investigated. Fig. 4 (lane A) shows that in resting EGFR T17 cells the phosphotyrosine content of the EGF receptor was very low compared with that present on EGF-stimulated cells (Fig. 4, lane B). Preincubation for 5 min with 100 μM-oleic acid did not block autophosphorylation of the EGFR (Fig. 4, lane C). As an internal control, preincubation (5 min, 1 μM) with PMA, the well-known activator of PKC, efficiently blocked EGFR autophosphorylation (Fig. 4, lane D). Thus the kinase activity of the EGFR seems to be unaltered in cells treated with levels of oleic acid that efficiently block early components of the EGFR signal-transduction pathway. Taken together, the results reported here, i.e. normal binding of EGF to EGFR, unimpaired tyrosine kinase activity, and blockade of both ionic fluxes and Ins(1,4,5)P_3 generation, strongly suggested that oleic acid blocked EGF-induced early signals at the level of receptor–effector coupling.

PKC participation in fatty acid action

Activation of enzymes of the PKC family induces marked inhibition of various early signals generated at the EGFR, in

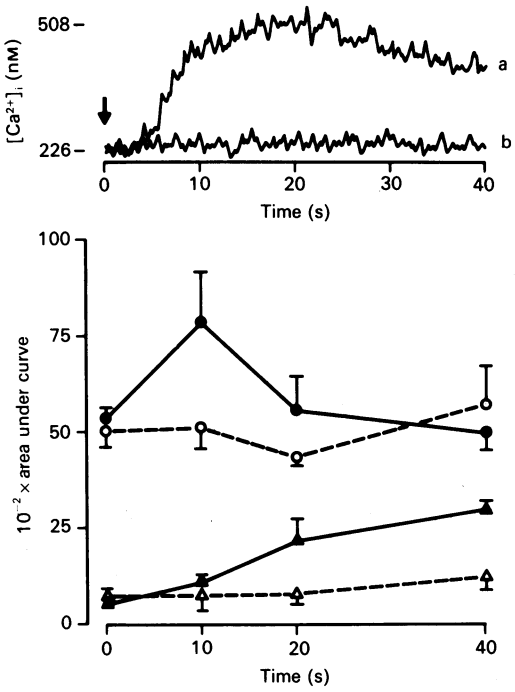

Fig. 3. Effect of oleic acid on EGF-induced generation of Ins(1,4,5)P_3

The time course of EGF-induced $[Ca^{2+}]_i$ rises and Ins(1,4,5)P_3 generation were compared in control and oleic acid-pretreated (100 μM, 10 min) EGFR T17 cells. Parallel subconfluent 60 mm Petri dishes were (i) resuspended and loaded with fura-2, and $[Ca^{2+}]_i$ rises in response to EGF (50 nM, arrow) were monitored at high velocity (upper panel) or (ii) labelled with *myo*-[^{3}H]inositol and EGF-induced (50 nM) generation of Ins(1,4,5)P_3 was determined by anion-exchange h.p.l.c. (lower panel; see the Materials and methods section). In control cells (trace a: ●, ▲), the peak of Ins(1,4,5)P_3 (●, ○) was found at 10 s, and preceded the $[Ca^{2+}]_i$ peak. Ins(1,3,4)P_3 levels (▲, △) rose slowly after Ins(1,4,5)P_3. In oleic acid-pretreated cells (trace b: ○, △), levels of Ins(1,4,5)P_3, $[Ca^{2+}]_i$ or Ins(1,3,4)P_3 were not modified by the EGF treatment.

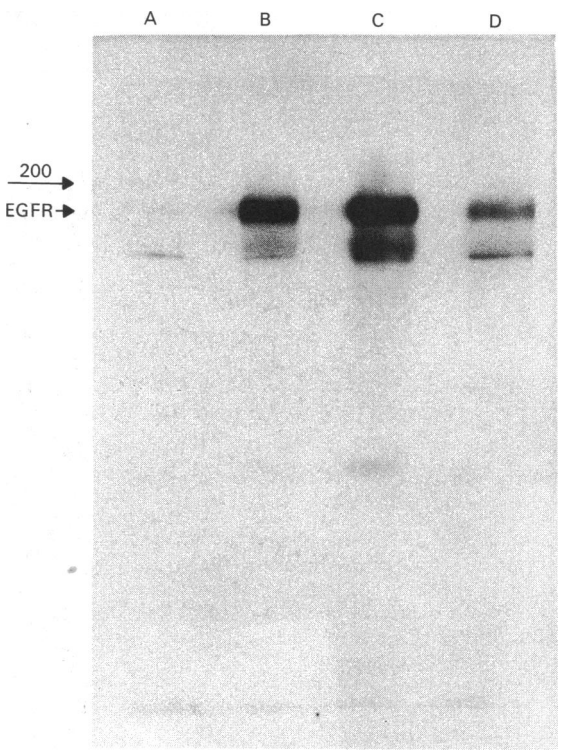

Fig. 4. Effect of oleic acid and phorbol ester on EGFR autophosphorylation

Suspensions of EGFR T17 cells were prepared as described for $[Ca^{2+}]_i$ measurements, and phosphotyrosine content of cell lysates was analysed by immunoblotting with rabbit polyclonal anti-phosphotyrosine antibodies. Lanes: A, control cells; B, cells treated with 10 nM-EGF (15 min at 37 °C); C, cells pretreated with oleic acid (100 μM, 5 min) and challenged with EGF for another 15 min period; D, cells preincubated with PMA (1 μM, 5 min) and then treated with EGF. The upper arrow shows the position of the 200000-M_r standard.

Table 3. Effect of oleic acid on ^{125}I-EGF binding

EGFR T17 cells were grown to confluency on 24-well dishes. ^{125}I-EGF (60 pM; 7×10^3 c.p.m./well; 250 μl final volume) was added to control, oleic acid-pretreated (10 or 100 μM, 15 min) or PMA-pretreated (1 μM; 15 min) cells. After 30 min at 37 °C, specific binding (mean ± S.E.M., $n = 6$) was calculated as described in the Materials and methods section (*$P < 0.05$; **$P < 0.01$).

	^{125}I-EGF binding (c.p.m.)
Control	1116 ± 88
Oleic acid (10 μM)	1354 ± 31
Oleic acid (100 μM)	1403 ± 48*
PMA (1 μM)	622 ± 14**

particular autophosphorylation of the receptor, PtdIns(4,5)P_2 hydrolysis and $[Ca^{2+}]_i$ increase [14,25,28]. Since oleic acid has been shown to increase PKC activity in some cellular systems [19–21], this effect could account for the inhibition by NEFA of the Ca^{2+} increase previously mentioned. Fig. 5 shows the effect of phorbol ester on the capability of oleic acid to affect the EGF-induced $[Ca^{2+}]_i$ increase, both before (Figs. 5*a*–5*d*) and after (Figs. 5*e*–5*h*) PKC down-regulation by chronic PMA treatment. In untreated cells, sequential addition of PMA and oleic acid blocked more efficiently (100% blockade) than did the addition of either agent alone. Chronic treatment of NIH 3T3 cells with phorbol esters (PMA 1 μM, 24 h) induced a prolonged down-regulation of PKC activity [31]. Under these conditions, the rise in $[Ca^{2+}]_i$ in response to EGF was unaffected by a new exposure to PMA, but oleic acid inhibition was still evident. These data, together with those from the autophosphorylation experiments, exclude a major role of PKC as the mediator of the inhibitory effects of oleic acid.

Blocking effect of NEFA is dependent on the presence of a *cis*-double bond in the molecule

In order to analyse the influence of the hydrocarbon chain length and the conformation of the molecule on blocking effects of NEFA, different NEFA molecules were used. Of the unsaturated FFA studied, only *cis*-9-octadecenoic acid (oleic acid) and *cis*-9-hexadecenoic (palmitoleic acid), but not *trans*-9-octadecenoic acid (elaidic acid), were able to block the EGF-induced $[Ca^{2+}]_i$ rise (Fig. 6) or plasma-membrane hyperpolarization (results not shown). In contrast, saturated NEFA of different chain lengths, such as octanoic, hexadecanoic (palmitic) and octadecanoic (stearic) acids, were unable to block EGF-induced $[Ca^{2+}]_i$ (Fig. 6) and membrane-potential increases. These

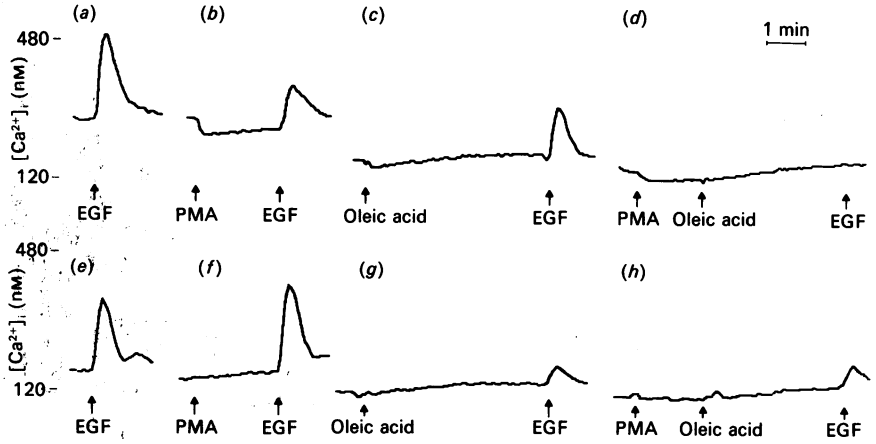

Fig. 5. Additive blocking effects of phorbol ester and oleic acid on EGF-induced $[Ca^{2+}]_i$ rise

The $[Ca^{2+}]_i$ rise in response to 10 nM-EGF was measured in EGFR T17 cells before (traces *a–d*) and after (traces *e–h*) chronic PMA treatment (1 μM, 24 h, in order to down-regulate PKC activity). In untreated cells, the inhibitory effects of acutely administered PMA (1 μM) (trace *b*) and oleic acid (10 μM) (trace *c*) were additive (trace *d*). When PKC activity was down-regulated in cells, acute PMA administration had no effect on the EGF-induced $[Ca^{2+}]_i$ rise (trace *f*), whereas oleic acid was still able to inhibit the EGF-induced $[Ca^{2+}]_i$ rise (trace *g*). Furthermore, in PKC-depleted cells, the sequential addition of PMA and oleic acid showed no additive effects (trace *h*). Conditions for $[Ca^{2+}]_i$ measurements were identical with those in Fig. 1. In these experiments, fluorescence signal was calibrated in terms of $[Ca^{2+}]_i$ values by calculating the ratio F_{345}/F_{380}, as described by Grynkiewicz *et al.* [22].

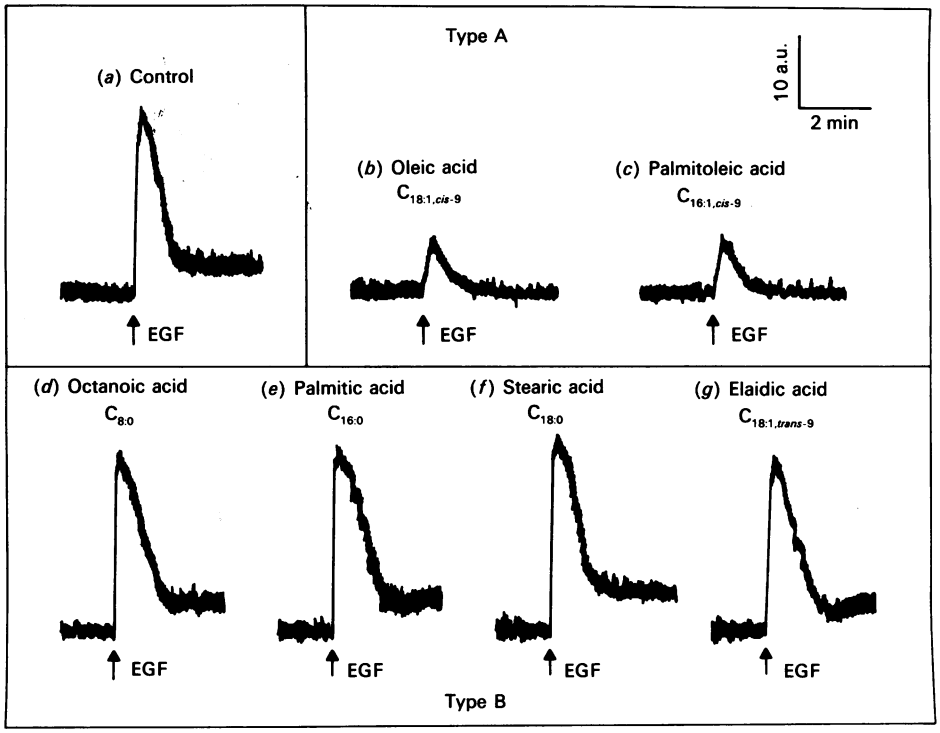

Fig. 6. Effect of different NEFA on EGF-induced $[Ca^{2+}]_i$ rise

The $[Ca^{2+}]_i$ in response to EGF (10 nM) was monitored on EGFR T17 cells preincubated (5 min) with 10 μM-*cis* unsaturated (*b*, *c*), -saturated (*d–f*) and -*trans*-unsaturated (*g*) NEFA. The results are expressed as arbitrary fluorescence units (a.u.).

results lead to the conclusion that the blocking effect of NEFA on EGF-induced early signals depends on the spatial conformation of the hydrocarbon chain rather than on the presence of double bonds or on chain length.

Effect of NEFA on other receptor and cell types

$[Ca^{2+}]_i$ homoeostasis was analysed in other cell lines and with a number of different agonists in order to determine whether the blocking effect of NEFA was restricted to the EGFR or was a widespread phenomenon. In the EGFR T17 cell line, the $[Ca^{2+}]_i$ rise activated by both bradykinin and bombesin was blocked by oleic acid (Figs. 7*a–7d*). As with EGFR T17 cells, pretreatment of PC12 cells with oleic acid (100 μM) clearly blocked bradykinin-induced (1 μM, Figs. 7*e* and 7*f*) $[Ca^{2+}]_i$ rises. In this cell line, oleic acid treatment also induces a small increase in the basal $[Ca^{2+}]_i$. Similar results to those obtained in EGFR T17 and PC12 cells

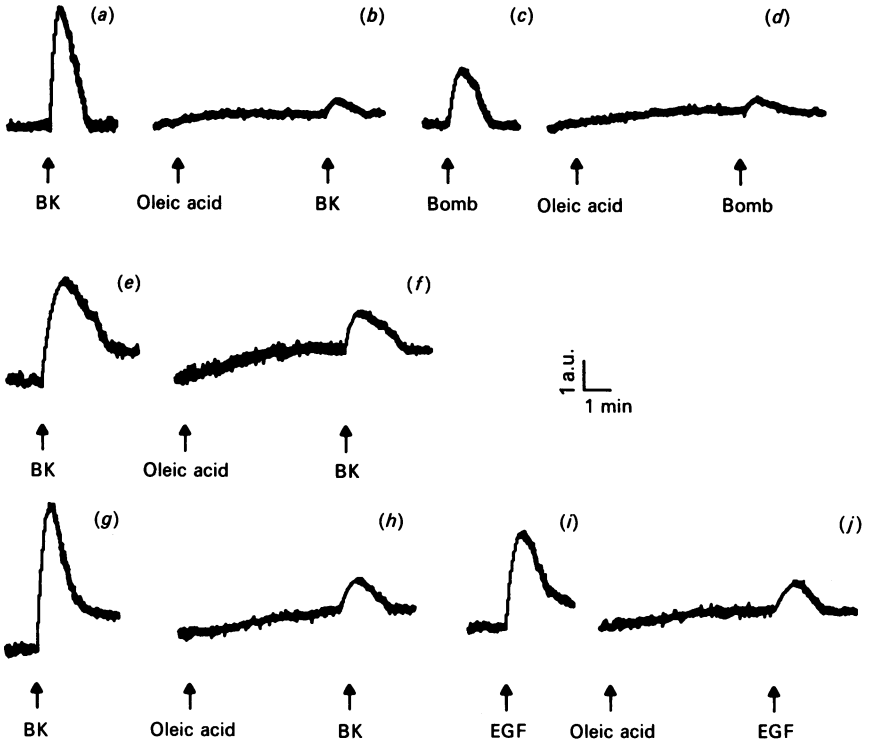

Fig. 7. Effect of oleic acid on other receptor systems and cell types

$[Ca^{2+}]_i$ was measured on EGFR T17 (*a–d*), PC12 (*e*, *f*) and A431 (*g–j*) cells as described in the Materials and methods section. In EGFR T17 cells, oleic acid (10 μM, 5 min) also inhibited bradykinin (BK, 100 nM) (compare *a* and *b*) and bombesin (Bomb, 100 nM) (compare *c* and *d*)-induced $[Ca^{2+}]_i$ transients. In PC12 cells, preincubation with oleic acid (100 μM, 5 min) inhibited BK (1 μM) (*e*, *f*)-induced Ca^{2+} responses. In A431 cells, both EGFR and BK B_2 receptors showed decreased responses to 100 nM-BK (compare *g* and *h*) and 10 nM-EGF (compare *i* and *j*) after preincubation with oleic acid (10 μM, 5 min). The results are expressed as arbitrary fluorescence units (a.u.) and are parallel determinations carried out on different samples of the same batch of cells.

were found in the human epidermoid carcinoma cell line A431. Preincubation of these cells with oleic acid had a similar blocking effect on the $[Ca^{2+}]_i$ rise generated upon B_2 bradykinin-receptor activation (Figs. 7*g* and 7*h*) and EGFR activation (Figs. 7*i* and 7*j*).

DISCUSSION

$[Ca^{2+}]_i$ functions as an important intracellular signalling mechanism whereby hormones and growth factors regulate different cellular processes, such as secretion, cell division or differentiation [32]. The results in the present study clearly demonstrate that *cis*-NEFA block the rise in $[Ca^{2+}]_i$ in response to different stimuli and in diverse cell lines.

Long-chain fatty acids are moderately hydrophobic and strongly amphiphilic molecules, which after local administration rapidly adsorb to lipid bilayers and biological membranes [6,7]. The action of NEFA could be attributed to a physical effect on the membrane rather than a metabolic transformation such as oxidation or chemical incorporation into membrane phospholipids [7]. In fact, oleic acid-induced blockade of $[Ca^{2+}]_i$ rise after EGF administration was dose-dependent, extremely rapid ($t_{\frac{1}{2}}$ approx. 1.5 min), and fully reversible after a few minutes of washout. The interesting finding that, after washing, the oleic acid-pretreated cell recovers its normal physiology implies that the NEFA is not chemically incorporated into the membrane, and indicates the necessity of maintaining a NEFA chemical gradient from the extracellular medium toward the cell, in order to maintain the long-lasting blocking properties of oleic acid. It could not be determined whether, after wash-out, NEFA pass from the membrane to the incubation medium because of their strong amphiphilic properties with low hydrophobicity. Another possibility is that, since the plasma membrane possesses a high-velocity lipid traffic toward the cytoplasm [33], NEFA might be cleared from the plasmalemma after elimination of the gradient. In any case, the reversibility of the oleic acid effect, after both short-term and 24 h incubation, rules out a toxic action or permanent damage upon the cell by such compounds.

The $[Ca^{2+}]_i$ rise that follows EGFR activation is generated by two components: release of Ca^{2+} from microsomal stores [15,16], accompanied by a more persistent Ca^{2+} influx across the plasma membrane through a voltage-independent Ca^{2+} channel [17,34,35]. The first such process is mediated by Ins(1,4,5)P_3, while considerable controversy remains with regard to the mediator of the second one [36,37]. Though less striking than the blockade of the transient EGF-induced Ca^{2+} release, oleic acid was able to inhibit more clearly the second or Ca^{2+}-plateau phase mediated by Ca^{2+} influx across the membrane.

EGF-induced cell hyperpolarization has been identified as a member of the group of early signals triggered by the activation of the EGFR [18]. This EGF-stimulated hyperpolarization, owing to an outward current mediated by a Ca^{2+}-activated K^+ channel, was also inhibited by pretreatment with oleic acid. These three early ionic signals activated by EGF, i.e. Ca^{2+} redistribution from microsomal stores, Ca^{2+} influx, and membrane hyperpolarization, are mediated by different final acceptors, and the capability of oleic acid to block all of them suggests a multimodal mechanism of action for NEFA. Alternatively, NEFA may block the common signals which generate them, i.e. PtdIns(4,5)P_2 hydrolysis products. In fact,

oleic acid at similar doses blocked the EGF-induced generation of Ins(1,4,5)P_3 from PtdIns(4,5)P_2, pointing toward PLC γ as the target of NEFA.

A simpler explanation for the observed effects of oleic acid on EGF-generated early ionic signals might be that NEFA prevented the binding of EGF to its receptor. The studies performed on ^{125}I-EGF binding and EGF-stimulated EGFR autophosphorylation argue against such a possibility. Though the precise steps are not known, the prevailing view is that after EGF binding the EGFR undergoes oligomerization, leading to an activated state of the catalytic or tyrosine kinase domain [38,39]. The first step after EGFR activation is autophosphorylation at tyrosine residues [38]. The finding that oleic acid at effective doses did not alter EGFR autophosphorylation after the addition of EGF strongly suggests that NEFA did not interfere with these basic steps, nor did they alter the tyrosine kinase activity of the EGFR.

On theoretical grounds, a strong candidate for the mediation of the inhibitory effects of NEFA was PKC. Evidence was accumulated during the last several years suggesting that NEFA might directly stimulate PKC [19–21]. Activation of the latter is known to induce a decrease in PtdIns(4,5)P_2 hydrolysis and ionic fluxes in several different cell types [40,41]. In particular, phosphorylation of the EGFR at Thr-654 and other residues largely decreases the high-affinity state of the receptor and decreases its tyrosine kinase activity. However, data from the present study exclude PKC as a mediator of NEFA inhibition. In fact, (i) NEFA, in contrast with PMA, did not block EGF binding and EGFR autophosphorylation, and (ii) down-regulation of PKC by prolonged treatment with phorbol esters did not affect the ability of NEFA to block EGFR-triggered $[Ca^{2+}]_i$ rises.

In the chain of events initiated after EGF binding to the cognate receptor, oleic acid did not alter the first steps, i.e. binding, receptor oligomerization, activation of protein tyrosine kinase and EGFR autophosphorylation, whereas it powerfully inhibited the successive ones: Ins(1,4,5)P_3 generation, $[Ca^{2+}]_i$ transients and ionic fluxes. These data point toward PLC as the target of oleic acid. Though NEFA have been demonstrated to alter plasma-membrane-bound enzymes, and particularly the transmembrane-signalling adenylate cyclase [42], this is the first report of NEFA modulation of the PtdIns–PLC signalling system.

The NEFA effects observed were not specific for the EGFR T17 cell line; they were also evident in other cell lines, suggesting that the actions described are of a general significance. Most important were the results obtained with different NEFA types, suggesting that the disrupting effects of NEFA are structure-dependent. In fact only *cis*-unsaturated NEFA were able to block the EGF-induced early signals, whereas *trans*-unsaturated or saturated NEFA were devoid of action. Oleic and elaidic acids share the same number of carbon atoms and double bonds and amphipathic properties, oleic being a *cis*- and elaidic a *trans*-unsaturated fatty acid. The lack of activity of elaidic acid ruled out any non-specific or toxic mechanism in the actions observed. According to Karnovsky's studies [8], NEFA belong to two groups: group A, composed of *cis*-unsaturated fatty acids such as oleic acid ($C_{18:1,cis\text{-}9}$), and group B, composed of saturated fatty acids such as octanoic ($C_{8:0}$) or *trans*-unsaturated fatty acids such as elaidic acid ($C_{18:1,trans\text{-}9}$). Group A and group B fatty acids have completely different physical structures: the *cis*-conformation establishes a kink in the molecule, whereas the *trans*-conformation results in a rather straight molecule. Also, group B NEFA partition into the gel phase of lipid membranes, i.e. more externally than group A, which partition into the fluid phase, powerfully disturbing the lipid arrangement because of their configuration [6,8,9]. This disturbance would be the one responsible for the alteration of PLC, inhibiting its activity. Another plausible explanation could arise from the fact that, after its generation by PLC, DAG is catabolized by membrane phospholipase A, releasing NEFA, particularly oleic acid [20,43]. Supposing that the released NEFA acted as a feedback inhibitor on PLC, one might wonder whether after oleic acid administration we were just increasing the levels of a physiological PLC inhibitory factor at the internal leaflet of the membrane. Both hypotheses need further investigation.

It is difficult to determine the extent to which the NEFA actions described in this study are responsible for the physiopathological complications found in clinical situations (ketoacidosis, pregnancy, obesity etc.) associated with high NEFA levels. Though plasma proteins would complex with NEFA to determine NEFA levels outside the cells which are lower by one order of magnitude than in this study, their large partition coefficients can result in significant mole fractions of NEFA in membranes [6]. In addition to their putative physiopathological relevance, *cis*-NEFA could be a useful tool for blocking some early signals elicited by growth factors while preserving others intact.

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