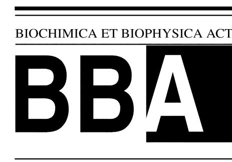
Biochimica et Biophysica Acta 1329 (1997) 269–277



cis-FFA do not alter membrane depolarization but block Ca^{2+} influx and GH secretion in KCl-stimulated somatotroph cells. Suggestion for a direct *cis*-FFA perturbation of the Ca^{2+} channel opening

Francisco R. Pérez, Jesús P. Camiña, José L. Zugaza, Mary Lage, Xesús Casabiell, Felipe F. Casanueva *

Cellular Endocrinology Laboratory, Department of Medicine, Compostela University School of Medicine and Complejo Hospitalario Universitario de Santiago, PO Box 563, 15780 Santiago de Compostela, Spain

Received 3 February 1997; revised 28 April 1997; accepted 29 April 1997

Abstract

It has been reported that *cis*-unsaturated free fatty acids (*cis*-FFA) block intracellular Ca^{2+} rise in EGFR T17 and GH_3 cells by perturbing the generation of Ins(1,4,5)P_3. In the present work, it was found that *cis*-FFA did not alter potassium-induced cell depolarization in GH_3 cells, while blocking Ca^{2+} rise and GH secretion. Interestingly enough, saturated or *trans*-unsaturated FFA exert the opposite actions, i.e., they block cell depolarization without altering Ca^{2+} rise and hormone secretion. As depolarization activates GH_3 cells via direct opening of Ca^{2+} channels with no generation of intracellular mediators, these results suggest that *cis*-FFA act by a direct perturbation of the Ca^{2+} channel opening. © 1997 Elsevier Science B.V.

Keywords: Free fatty acid; Growth hormone secretion; Voltage-operated calcium channel; Calcium influx; Depolarization; Somatotroph cell; GH_3 cell

1. Introduction

Circulating FFA regulate GH secretion both in man and in experimental animals [1]. In fact, FFA reduction by pharmacological means leads to an enhanced release of GH [2,3], while an increase in FFA blocks GH secretion [1,4]. It is now well accepted that a rise in FFA inhibits GH release by acting directly at the somatotroph cell, with no hypothalamic participation [4,5]. However, the precise mechanism of action of these metabolites on the somatotroph cell is far from being understood.

Abbreviations: FFA, free fatty acids; GH, growth hormone; TRH, thyrotropin-releasing hormone; PtdIns(4,5)P_2, phosphatidylinositol-(4,5)-bisphosphate; Ins(1,4,5)P_3, inositol-(1,4,5)-trisphosphate; DAG, diacylglycerol; PKC, protein kinase C; fura-2-A/M, fura-2-pentaacetoxy methyl ester; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; KRH, Krebs-Ringer-HEPES; EGFR, epidermal growth factor receptor; V_m, membrane potential; VOCC, voltage-operated Ca^{2+} channel; HS, horse serum; FCS, fetal calf serum

* Corresponding author. Fax: +34-81-572121; E-mail: meffcasa@uscmail.usc.es

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PII S0005-2736(97)00111-9

After their increase in plasma, FFA rapidly partition in the plasma membrane, altering the function of integral membrane proteins, as well as signal transduction events [6–9]. In GH_3 cells, *cis*-monounsaturated FFA like oleic acid, but not saturated- or *trans*-unsaturated FFA block TRH-mediated intracellular Ca^{2+} rise as well as GH secretion, by directly perturbing some integral membrane proteins [10].

In the present work, the action of both *cis*- and *trans*-unsaturated FFA has been tested in GH_3 cells activated by KCl-mediated depolarization. As depolarization triggered both Ca^{2+} rise and hormone secretion with no participation of any receptor or intracellular signalling system, like the $Ins(1,4,5)P_3/DAG/Ca^{2+}$ or the cAMP/PKA pathways, this experimental model seems to be well suited to further expand the knowledge on the point and mechanism of action of FFA. The results obtained suggest that *cis*-FFA block depolarization-induced Ca^{2+} rises via a direct perturbation of the opening of the Ca^{2+} channel.

2. Materials and methods

2.1. Chemicals

Fura-2-A/M and bis-oxonol were obtained from Molecular Probes (St. Louis, MO). FFA were purchased from Sigma (Eugene, OR) at the highest purity available, and checked by thin-layer chromatography 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 under continuous stirring. All controls received an equivalent amount of ethanol (0.1–1%. v/v), which had no effect on the measured responses. All other chemicals, including BSA and TRH, were reagent grade from Sigma.

2.2. Cell cultures

The GH_3 rat pituitary tumour cell line was obtained from the American Type Culture Collection (Rockville, MD), and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v horse serum (HS) and 2.5% v/v fetal calf serum (FCS) (both from Gibco). GH_4C_1 cells were grown in DMEM plus 10% FCS. Media were supplemented with penicillin G (sodium salt; 100 units ml^{-1}), and streptomycin sulphate (100 $\mu g\ ml^{-1}$) (all from Sigma), and cells grown under a humidified atmosphere of 95% air/5% CO_2 at 37°C. Cells were passaged once a week, and fresh medium added on alternate days. EGFR-T17 fibroblasts were cultured in DMEM + 10% FCS, as previously described [6].

2.3. Primary rat pituitary cell cultures

Pituitary glands from Sprague–Dawley rats (150–200 g) were obtained and processed essentially as described [11] Briefly, cells were dispersed by enzymatic digestion in Earle's balanced salts solution plus 1% FCS, containing collagenase (0.4%), DNAse (0.01%), dispase (0.2%) and hyaluronidase (0.1%), and cultured in 100 mm petri dishes (Ca^{2+} experiments) or 24 well multiwells (secretion experiments) in a mixture of Ham F12/DMEM/BGjb (6:3:1) [12], supplemented with BSA (2 g l^{-1}), HEPES (2.4 g l^{-1}), hydrocortisone (143 $\mu g\ l^{-1}$), IGF-1 (10 ng l^{-1}), triiodotironine (0.4 $\mu g\ l^{-1}$), glucagon (10 ng l^{-1}), EGF (0.1 $\mu g\ l^{-1}$), FGF (0.2 $\mu g\ l^{-1}$), transferrin (10 mg l^{-1}), $NaHCO_3$ (1.8 g l^{-1}), FCS (2.5% v/v) and antibiotics.

2.4. Secretion assays

Cumulative PRL and GH secretion were determined by RIA essentially as described [13,14]. Briefly, GH_3 cells were subcultured into 24-wells plates with DMEM/10% HS/2.5% FCS. After 3 days, monolayers were washed with Krebs–Ringer–Hepes (KRH), with the following composition (mM): NaCl, 125; KCl, 5; $MgSO_4$, 1.2; $CaCl_2$, 2, KH_2PO_4, 2; Glucose, 6; Hepes, 25; pH 7.4, and preincubated for 5 or 10 min at 37°C in 2 ml of serum-free DMEM supplemented with oleic acid (50 μM) or vehicle (ethanol 1% in all plates). KCl (50 mM final), or an equivalent amount of KRH, was added to the plates and incubation resumed for the indicated time. In some assays oleic acid was added after KCl. Media were collected 1 h after the addition of KCl, and stored at −80°C until assayed by RIA. RIA determinations were done with materials generously provided by the National Pituitary Hormone Distribution Program (NIAMKK, Bethesda, MD).

2.5. Measurement of the intracellular free Ca^{2+} concentration, $[Ca^{2+}]_i$

GH_3 cells were maintained in DMEM/15% HS/2.5% FCS for 4 days after confluence. They were then washed twice with Ham medium, with the following composition (mM): $CaCl_2$, 122; NaH_2PO_4, 1; KCl, 3; HEPES, 30, and treated at 37°C with the same buffer containing trypsin (0.05% w/v) and EDTA (0.9 mM). Detachment of the cells from the dish was complete within 1 min. After centrifugation at 300 × g for 5 min, cells were resuspended in KRH and loaded with 3 μM fura-2-A/M for 30 min at 37°C, under gentle continuous stirring. Cell suspensions were then diluted 1 to 4 with KRH and maintained at room temperature for 15 min. For fluorimetric measurements, 1 ml of the cell suspension was centrifuged and the pellet resuspended in 2 ml of warm KRH. Cells were then placed in a cuvette positioned in a holder, thermostatically controlled (37 ± 1°C), and the fluorescence signal measured under continuous stirring in a Perkin–Elmer LS-5B fluorimeter adjusted to $\lambda_{ex} = 345$ nm and $\lambda_{em} = 490$ nm. Absolute $[Ca^{2+}]_i$ values were calculated using the formula:

$$[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$$

where F is the fluorescence at the unknown $[Ca^{2+}]_i$, F_{max} is the fluorescence after addition of 0.02% v/v Triton X-100 and 4 mM $CaCl_2$, and F_{min} is the fluorescence after the Ca^{2+} in the solution is chelated with 10 mM EGTA and 4 mM Tris, as previously described [6] The value of K_d was 225 nM for fura-2 [15].

2.6. Measurement of membrane potential (V_m)

Membrane potential changes were monitored with the slow-response fluorescent dye bis-oxonol [16]. 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 KRH, and 750 μl was washed again by centrifugation. The resuspended pellet was transferred to the fluorimeter cuvette and 100 nM bis-oxonol added from a 1000-fold concentration solution in dimethyl sulphoxide. The cells were allowed to equilibrate with the dye for 10 min before the addition of the different test substances. Downward or upward deflections of the fluorescence signal represent hyper- or de-polarizations respectively. Data was expressed in arbitrary units, as the percentage increase from basal fluorescence values.

2.7. Statistic analysis

The statistical significance was calculated using the non-parametric Mann–Whitney test.

3. Results

The complex series of biochemical postreceptor events leading to the opening of VOCC in GH_3 cells, can be experimentally bypassed by exposing the cells to a high extracellular potassium concentration, which directly induces membrane depolarization and the ensuing opening of voltage-gated Ca^{2+} channels [17,18]. As Fig. 1A shows, the KCl-induced (50 mM)

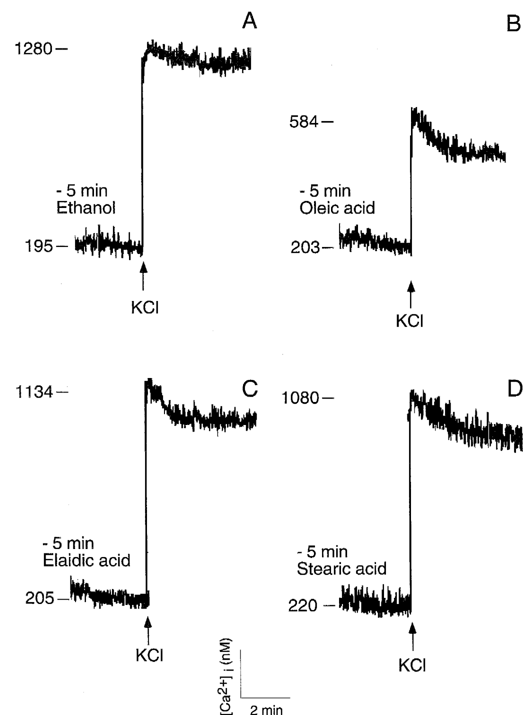

Fig. 1. Effect of free fatty acids on KCl-induced Ca^{2+} influx in GH_3 cell suspensions measured with fura-2. Cells were incubated for 5 min with either vehicle (A), 50 μM oleic acid (B), 50 μM elaidic acid (C), or 50 μM stearic acid (D), and then stimulated by a pulse of KCl (50 mM final) at time zero.

GH_3 cell depolarization determines a sharp rise in the $[Ca^{2+}]_i$, followed by a plateau phase and a very slow (up to 30 min) [19] return to resting levels. When cells were preincubated for 5 min in the presence of 50 μM oleic acid, the subsequent administration of KCl was not able to fully activate the plasma membrane Ca^{2+} channels, which resulted in a significant reduction in the Ca^{2+} influx (Fig. 1B). This effect could not be attributed to a toxic action of fatty acids, as neither elaidic acid (Fig. 1C) or stearic acid (Fig. 1D) altered the potassium-induced Ca^{2+} influx, and the oleic acid action was reversible after wash out [10].

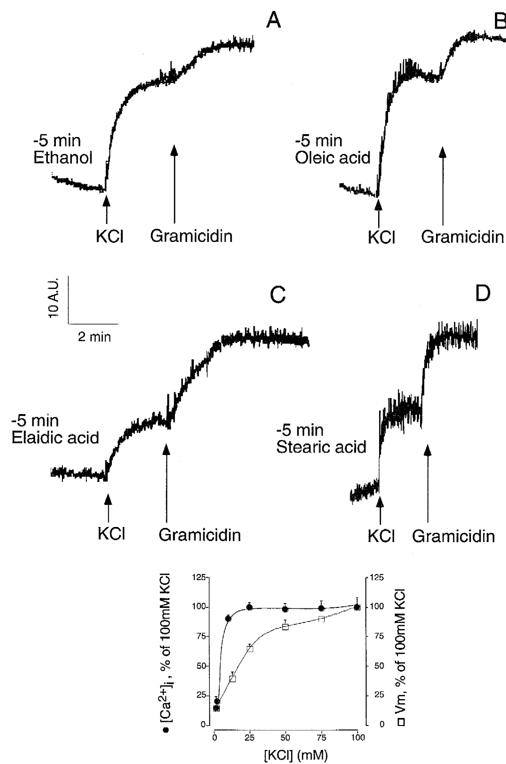

Fig. 2. Effect of different free fatty acids on the KCl-induced membrane depolarization in GH_3 cells. Cell suspensions were loaded with bis-oxonol, and the fluorescence tracings were allowed to stabilize during dye equilibration (8 min). KCl (50 mM) was added after a 5 min preincubation with either vehicle (A), 50 μM oleic acid (B), 50 μM elaidic acid (C), or 50 μM stearic acid (D). Membrane depolarization is reflected as upward deflections of the fluorescence tracing. Gramicidin was added at the end of the experiment as a control of the extent of depolarization reached after the administration of KCl. (E) dose-response curves obtained for KCl in both depolarization and Ca^{2+} influx.

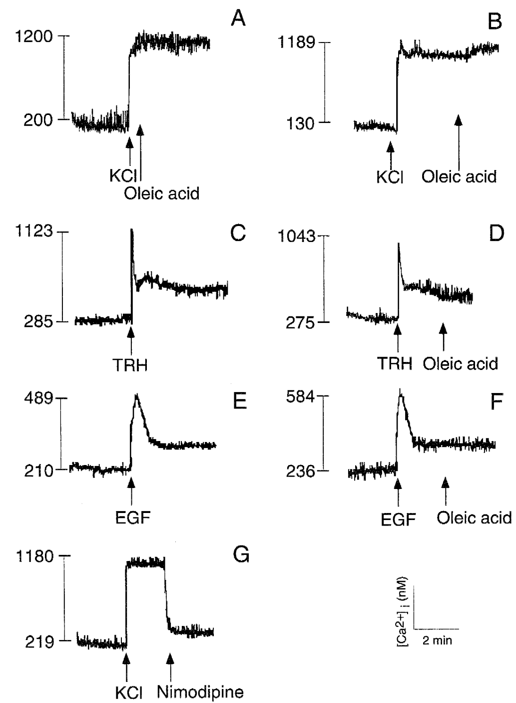

Fig. 3. Oleic acid did not alter the Ca^{2+} current through voltage-sensitive Ca^{2+} channels in GH_3 cells once they had been opened by previous depolarization with KCl. Oleic acid was administered to cells at several times ranging from 15 s (A) to 4 min (B) after the KCl pulse. Oleic acid was also ineffective when the voltage-sensitive Ca^{2+} channels were activated by TRH (C and D), or when second messenger-operated Ca^{2+} channels were opened by EGF on EGFR T17 fibroblasts (E and F). The plateau phase was not suppressed by the fatty acid in any of the cases, as when cells were treated with the Ca^{2+} channel blocker nimodipine (G).

When membrane depolarization was assessed in GH_3 cells, it was evident that the inhibition of the K^+-induced Ca^{2+} influx by oleic acid was not due to a previous inhibition of the depolarization process by the fatty acid (Fig. 2A–B). Preincubation with elaidic acid, however, resulted in a dramatic reduction of the KCl-induced membrane depolarization (Fig. 2C), a result which contrasted sharply with its lack of effect on Ca^{2+} influx (Fig. 1C). Stearic acid caused only a modest inhibition of KCl-induced membrane depolarization (Fig. 2D). The K^+-ionophore gramicidin was added at the end of the experiments to calibrate the fluorescence tracings. The lack of inhibition of the Ca^{2+} signal by elaidic acid (Fig. 1C) or stearic acid (Fig. 1D) despite the substantial inhibition of membrane depolarization induced by these two FFA, can be explained by the high sensitivity of the voltage-

gated Ca^{2+} channels to minor depolarizations. In fact, a 30% depolarization was enough to elicit a maximum $[Ca^{2+}]_i$ rise (Fig. 2E).

When oleic acid was administered to GH_3 suspensions after the stimulation by KCl (in times ranging from 15 seconds to 4 minutes, Fig. 3A–B), the fatty acid did not modify the fluorescence tracings. Similar results were obtained when oleic acid was tested on the second phase (influx) of the TRH-induced Ca^{2+} signal in GH_3 cells (Fig. 3C–D) or the EGF-induced Ca^{2+} signal in EGFR T17 fibroblasts (Fig. 3E–F). It appears that the Ca^{2+} channel is sensitive to the inhibition by oleic acid in the resting state, but not after activation. In contrast, the specific L-type VOCC

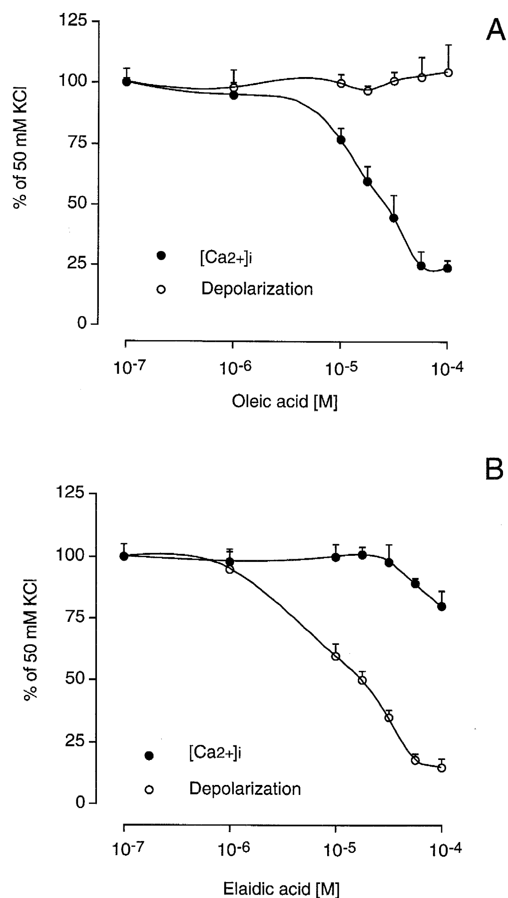

Fig. 4. The dose-response curves for both $[Ca^{2+}]_i$ and depolarization were determined for oleic acid (A) and its *trans*-isomer, elaidic acid (B). Cells were preincubated for 5 min with increasing doses of the fatty acid, and then stimulated by a pulse of KCl (50 mM final). The KCl-induced responses for $[Ca^{2+}]_i$ (●) or V_m (○) were expressed as the percentage of vehicle-preincubated control samples in quadruplicate experiments.

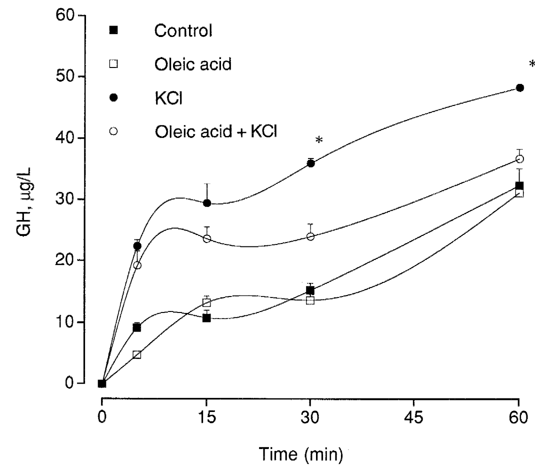

Fig. 5. Effect of oleic acid on the time-course of growth hormone secretion. GH_3 cells were preincubated for 5 min in the presence of oleic acid (50 µM final) or vehicle, and then stimulated or not by the administration of a pulse of KCl (50 mM). Results are mean ± S.D. from a representative experiment performed in quadruplicate (* $P < 0.05$, vs. oleic acid + KCl).

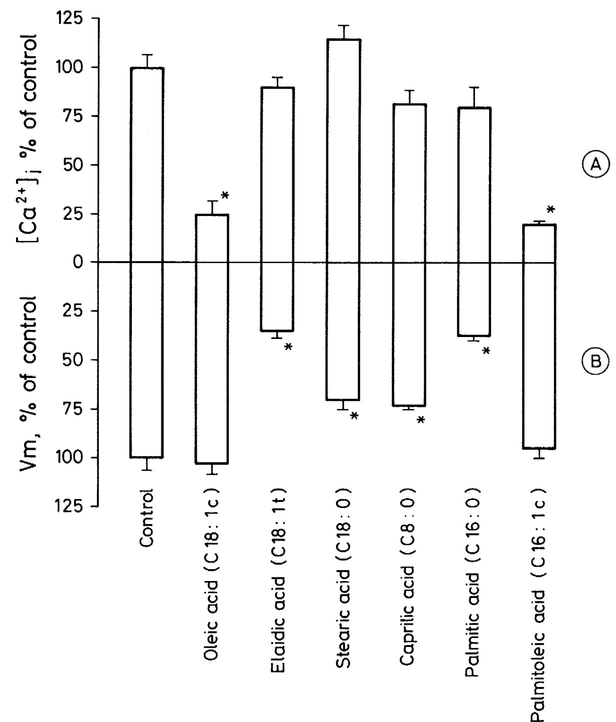

Fig. 6. The effects of FFA on membrane depolarization and Ca^{2+} influx were structure-dependent. The effects of several fatty acids (50 µM, 5 min preincubation time) on KCl-induced (50 mM) Ca^{2+} fluxes (A) and V_m modifications (B) were expressed (mean ± S.D.) as the percentage of controls in experiments performed in quadruplicate (* $P < 0.05$).

antagonist nimodipine is able to block the channel in both states (Fig. 3G).

The KCl-triggered Ca^{2+} influx was inhibited by oleic acid in a dose-dependent way, with concentrations above 10 μM resulting in a progressive inhibition of the amplitude of the Ca^{2+} current (Fig. 4A). No inhibitory effects of oleic acid were evident on KCl-induced membrane depolarization with any of the doses tested. In contrast, preincubation of the cells with elaidic acid, resulted in the opposite effects, with a sharp dose-related inhibition of KCl-induced depolarization and almost no effect on the subsequent Ca^{2+} influx (Fig. 4B).

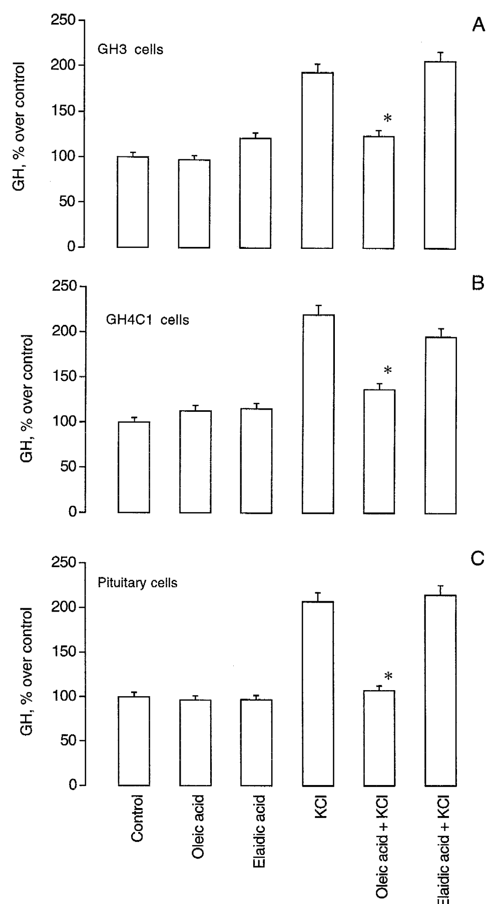

Fig. 7. Effects of different FFA on GH secretion in three different cell lines. In all experiments cells were preincubated with 50 μM FFA for 5 min and then stimulated or not by a KCl pulse (50 mM final). GH secretion was determined in GH_3 cells (A), GH_4C_1 cells (B), or in a primary dispersion culture of rat pituitary cells (C). Experiments were performed by quadruplicate and expressed as the mean ± S.D. (* $P < 0.05$).

As growth hormone (GH) and prolactin secretion in pituitary cells is dependent on the $[Ca^{2+}]_i$ signal [20], the effect of oleic acid on KCl-induced GH secretion was tested. As Fig. 5 shows, GH release was significantly inhibited in the presence of oleic acid, effectively coupling the observed inhibition of the Ca^{2+} influx current with a significant reduction of GH secretion.

The inhibitory action of FFA on the KCl-induced Ca^{2+} influx was structure-dependent. At 50 μM, only *cis*-monounsaturated FFA, like oleic and palmitoleic acids, exerted a significative inhibition. On the contrary, *trans*-FFA like elaidic acid and saturated-FFA like stearic acid, did not affect the KCl-mediated Ca^{2+} influx (Fig. 6A). Surprisingly enough, the reversed picture was observed when membrane potential changes were measured. *cis*-FFA were devoid of action, while preincubation of the cells with saturated and *trans*-unsaturated FFA resulted in a significant inhibition of KCl-induced membrane depolarization (Fig. 6B). A similar structure-dependent action was observed when KCl-induced GH secretion was assessed in the presence of different types of FFA (Fig. 7A). The results closely matched the effect of FFAs on the Ca^{2+} influx, i.e., significant inhibition of GH secretion by *cis*-FFA, and no effect by *trans*- or saturated-FFA. This action was not only observed in GH_3 cells: The same results were observed using another clonal line of pituitary lineage, as GH_4C_1 (Fig. 7B), and using rat primary pituitary cell cultures (Fig. 7C).

4. Discussion

Free fatty acids have been implicated in the regulation of GH secretion in normal subjects [1,4], and their plasmatic rise in certain pathological situations appears to be linked to the altered regulation of GH secretion observed in obesity [2]. Considerable experimental data supports a direct action of FFA on the pituitary cell, rather than an indirect one mediated by the elevation of hypothalamic somatostatin [4,5,21,22]. In agreement with this body of evidence, it has been shown that *cis*-unsaturated (but not saturated or *trans*-unsaturated) FFAs disrupt transmembrane signalling at the fibroblast EGF receptor [6–9] or the GH_3 and pituitary cells TRH receptor [10].

Such a disruption is exerted by blocking the Ins(1,4,5)P_3 and DAG generation, and is evident at a range of in vitro FFA concentrations near to the in vivo levels reached in certain pathophysiological conditions [23,24]. As a result, the receptor-activated redistribution of Ca^{2+} from intracellular Ins(1,4,5)P_3 sensitive pools is strongly inhibited after the addition of oleic acid [6]. Furthermore, the second phase of the Ca^{2+} response to TRH (an influx from the extracellular fluid that is mediated by the opening of voltage-operated Ca^{2+} channels (VOCC) [18,20,25,26] at the plasma membrane) in GH_3 cells was completely suppressed [10]. The reason for the higher sensitivity of the Ca^{2+} influx phase to the inhibition by *cis*-FFA, as compared to the sensitivity of the redistribution phase is not evident, as the transmembrane signalling appeared to be blocked at a very proximal step [10], i.e., the hydrolysis of PtdIns(4,5)P_2 by phospholipase C at the plasma membrane. To further clarify the mechanism by which *cis*-FFA inhibit pituitary cell activation, in the present work the effect of different fatty acids on the opening of voltage-sensitive Ca^{2+} channels has been studied under conditions in which no receptors or second messengers are implicated, i.e., cell depolarization induced by high extracellular potassium [17].

The results obtained show that oleic acid severely reduced the opening of the VOCC mediated by membrane depolarization. This dual mechanism of action of oleic acid, i.e., reduction of early ionic signals generated at the receptor and direct blockade of voltage-sensitive Ca^{2+} channels, can explain the considerable higher sensitivity to oleic acid inhibition showed by the influx phase, as compared to the redistribution phase of the Ca^{2+} response. This effect of oleic acid seems to be exerted directly at undetermined loci on the Ca^{2+} channels themselves, as no inhibition was observed on the KCl-induced cell depolarization. In contrast, both stearic and elaidic acid were ineffective in terms of inhibition of the Ca^{2+} influx, despite the clear-cut inhibition that they exerted over the KCl-induced membrane depolarization, a result that rules out the possibility that the inhibition observed in oleic acid-pretreated cells may be due to a toxic effect of the FFA, or to a interference of the FFA (which have an intrinsic fluorescence by themselves) with the fluorimetric measurements of Ca^{2+} with fura-2.

It is noteworthy that *cis*-fatty acids specifically block the opening of voltage-sensitive Ca^{2+} channels, being completely ineffective once the channel is open (Fig. 3A,B,D). This suggests that the mechanism of action of free fatty acids is not related to that of dihydropyridines, such as nimodipine, which are able to block the Ca^{2+} flux when administered both before or after the onset of the calcium current (Fig. 3G). It also discards a direct, non-selective mechanical blockade of the channel cavity or an unspecific unselective perturbation of the physicochemical state of the channel.

When the results for the different FFA on the cell responses studied were put together, a striking structure-related specular pattern for these molecules was observed. The effects elicited by oleic acid on KCl-activated pituitary cells, i.e., inhibition of KCl-induced Ca^{2+} influx, no action on the membrane depolarization, and blockade of GH secretion, exactly opposed the effects obtained with stearic acid and elaidic acid. The reason for this difference probably resides in the molecular structure of the fatty acids. According to Karnowsky [27], free fatty acids can be classified in two groups: Type A, or *cis*-unsaturated FFA (such as oleic and palmitoleic acids), and type B FFA, integrated by saturated and *trans*-unsaturated fatty acids (such as stearic and elaidic acids). Type B FFA have linear structures, while type A molecules, due to the spatial conformation of the *cis*-double bond, are markedly angular, with a prominent 'kink' in the molecule. This difference has important biophysical implications. After their increase in the extracellular milieu, FFA rapidly partition into the plasma membrane, where type B FFA can pack regularly causing little, if any, disorganization of the lipid bilayer. On the contrary, the incorporation of type A FFA into cellular membranes causes major structural disruptions at the hydrophobic core of the lipid bilayer, perturbing membrane fluidity, as well as lipid–lipid, and lipid–protein interactions. This consequently leads to the inhibition of cellular processes such as receptor capping, cell adhesion, signal transduction, and cell division [6–9,28,29]. As type A FFA partition into the hydrophobic core of the bilayers, one would expect that the function of proteins deeply embedded inside the plasma membrane would be strongly affected by them, whereas proteins more loosely associated with the bilayer would be less

sensitive to perturbation by these compounds. This hypothesis would adequately explain why different membrane proteins are affected to different extends by *cis*-unsaturated FFA. Membrane proteins whose functional domains lie in the extracellular space, the cytosol, or both, are not affected by type A FFA, as with the EGF receptor [8,9] and the TRH receptor [10]. Intracellular proteins associated with the plasma membrane and with enzymatic action on the lipid bilayer, as phospholipase C, would be moderately sensitive to *cis*-FFA [6,7]. Integral membrane proteins that function by switching between different conformational states in the bilayer plane, would be strongly affected. As the opening of voltage-operated Ca^{2+} channels involves voltage-driven transmembrane movements of charged amino acid residues that serve as gating charges [30], the oleic acid-induced disorganization of the lipid environment in which Ca^{2+} channels reside, may result in a lower probability of channel opening in response to changes in membrane potential.

Considering the lack of effect of *cis*-unsaturated FFA, the inhibitory effects reported here for saturated and *trans*-unsaturated FFA on membrane depolarization are intriguing. Although no direct proof is available, this inhibition is most probably due to a direct effect of type B FFA on plasma membrane sodium or potassium channels [29–34].

The main trigger for exocytosis in secretory cells is a sharp rise of the $[Ca^{2+}]_i$ [17,18,20]. When the biochemical targets affected by *cis*-FFA in cell membranes were tracked down, it was shown that these compounds effectively blunt the signalling process disrupting both the redistribution phase of the Ca^{2+} signal [6,10], and the influx phase, by inhibiting the hydrolysis of PtdIns(4,5)P_2, and ultimately the opening of voltage-sensitive Ca^{2+} channels, respectively. These findings themselves may explain the inhibition of GH secretion in situations associated with elevated plasma FFA levels. However, secretion is the result of a complex set of strictly ordered biochemical events, comprising the packing of secretory proteins into secretory vesicles that bud from the *trans*-Golgi, and their specific targeting to the plasma membrane, where they recruit a multicomponent 'fusion machine' integrated by NSF, SNAPs, and SNAREs among other factors [35,36], which in turn catalyses the fusion of the two lipid bilayers (from the secretory granule and the plasma membrane), in a process probably triggered by the activation of a Ca^{2+} sensor, much in the same way as it happens in synaptic transmission [37,38]. As *cis*-FFA distort the normal membrane architecture, a more distal role for this compounds on the secretory process cannot be ruled out at this point. Most probably FFA act by simultaneous actions at different loci in the cell machinery.

Acknowledgements

We thank Ms. V. Piñeiro and Ms. Sonia Veiga for their excellent technical assistance. This work was supported by grants from Fondo de Investigación Sanitaria, Spanish Ministry of Health, Fundación Española contra el Cáncer, Fundación Salud 2000, and from the European Union, Copernicus Program, CIPA-CT92 3014.

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