Inhibition of protein synthesis in intact mammalian cells by arachidonic acid

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Optimal translation initiation in intact mammalian cells requires sequestered intracellular Ca^{2+} . Arachidonic acid, which releases sequestered Ca^{2+} from cells and isolated organelles, was studied to assess its potential role in the regulation of protein synthesis via Ca^{2+} mobilization. Unsaturated fatty acids at μM concentrations inhibited protein synthesis in intact GH_3 pituitary, C6 glial tumour and HeLa cells in a manner dependent on degree of unsaturation and cell number. Arachidonate was generally the most, and the fully saturated arachidic acid the least, potent of the fatty acids tested. At 2×10^6 GH_3 cells/ml, amino incorporation into a broad spectrum of polypeptides was inhibited by 80–90% by 10–20 μM fatty acid. Inhibition was maximal at 4–8 min and was attenuated by 1–2 h and more pronounced at lower pH. Protein synthesis was maximally inhibited when arachidonate mobilized approx. 40% of cell-associated Ca^{2+} . At lower concentrations (10 μM) arachidonate suppressed translational initiation, with the inhibition being reversed as extracellular Ca^{2+} concentrations were increased to supraphysiological values. At higher concentrations (20 μM) arachidonate inhibited peptide-chain elongation in a Ca^{2+} -independent manner. Arachidonate also blocked elongation in reticulocyte lysates. The effects of arachidonate in intact cells were reversible with time via its metabolism or by washes containing BSA. Sufficient arachidonate appears to be synthesized during ischaemic stress to inhibit translation by either mechanism.

INTRODUCTION

A growing body of literature [1] supports the hypothesis that intracellular Ca^{2+} sequestered within the endoplasmic reticulum (ER) is required for the maintenance of post-transcriptional protein synthesis in a variety of eukaryotic cell types. Amino acid incorporation is strongly inhibited within minutes by depletion of total cell Ca^{2+} with EGTA-buffered media or by exposure to agents that mobilize sequestered stores of the cation. These agents include hormones that elevate *myo*-inositol 1,4,5-trisphosphate (InsP_3), Ca^{2+} ionophores, and various benzyloxycarbonyldipeptide amides [2]. A decrease in sequestered Ca^{2+} by these agents suppresses translational initiation, on the basis of an observed disappearance of polysomes, unchanged average ribosomal transit times, and decreased formation of 43 S preinitiation complex. Inhibition of protein synthesis by each of these agents is subject to reversal by high extracellular concentrations of Ca^{2+} . These characteristics are inconsistent with elongation blockade arising from the phosphorylation of elongation factor 2 (EF2) by calmodulin-dependent protein kinase III in response to increased cytosolic free Ca^{2+} concentrations [3,4].

Arachidonic acid and various of its metabolites are putative intracellular effectors of Ca^{2+} -mediated processes [5,6]. Accumulation of unesterified arachidonate with accompanying changes in cellular Ca^{2+} homeostasis has been associated with the actions of various secretagogues in a range of cell types [7,8]. For example, GH_3 cells respond to exogenously added arachidonate with mobilization of sequestered Ca^{2+} and prolactin secretion [9]. In digitonin-permeabilized pancreatic islets, arachidonate effectively mobilizes Ca^{2+} from the ER while inducing a transient increase in insulin release [8]. Arachidonate promotes release of Ca^{2+} sequestered in isolated organelles, including vesicles of skeletal-muscle sarcoplasmic reticulum [10] and liver microsomes [11]. Both ω -3 and ω -6 polyunsaturated fatty acids were recently reported to release InsP_3 -sensitive stores of Ca^{2+} to the cytosol in

a leukaemic T-cell line [12]. Activation of the plasmalemmal Ca^{2+} pump, leading to extrusion of Ca^{2+} from the cytosol, has been proposed as an additional action of arachidonate in macrophages [13].

Although cytosolic free Ca^{2+} is widely agreed to function as an important regulatory factor, potential regulatory roles for sequestered Ca^{2+} have been largely unexplored. In the present work relationships among arachidonate and related fatty acids, intracellular Ca^{2+} and protein synthesis were sought. Exogenous arachidonate mobilized intracellular Ca^{2+} to the extracellular space and inhibited protein synthesis in a variety of mammalian cell types in a manner fully reversible with time. At lower concentrations of arachidonate (10 μM), translational initiation was inhibited. This inhibition was suppressed by supra-physiological Ca^{2+} concentrations, was not attributable to detergent action, and was not affected by indomethacin, nor mediated by cyclo-oxygenase-pathway metabolites. At higher concentrations of arachidonate, translational elongation was inhibited in a Ca^{2+} -independent manner by an undefined mechanism. The properties of arachidonate described in this paper are consistent with those expected for a physiological regulator that inhibits protein synthesis through mobilizing Ca^{2+} sequestered in the ER.

EXPERIMENTAL

Materials

Ca^{2+} ionophore A23187, dimethyl sulphoxide (Me_2SO), arachidonate and other fatty acids, and indomethacin were purchased from Sigma Chemical Co. Molecular-mass markers were obtained from Bio-Rad. L-[4,5- ^3H]Leucine, $^{45}\text{CaCl}_2$ and Tran ^{35}S -label (L-[^{35}S]methionine/L-[^{35}S]cysteine) were obtained from ICN Biomedicals. Silicone oil was purchased from Dow Corning, and dinonyl phthalate was obtained from Pfaltz and Bauer (Waterbury, CT, U.S.A.). Modified Ham's F-10 medium

Abbreviations used: ER, endoplasmic reticulum; EF2, elongation factor 2; Me_2SO , dimethyl sulphoxide.

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was prepared containing all of the concentrations of the respective components of Ham's F-10 except for omission of Ca^{2+} , leucine and methionine, and was stored frozen. Leucine and methionine, EGTA and Ca^{2+} were added before each experiment in controlled concentrations. All other reagents were of analytical grade.

General methodology

GH₃ pituitary and C6 glioma cells were obtained from the American Type Culture Collection and were maintained as described previously [14,15]. Cells were harvested and resuspended in a modified Ham's F-10 medium adjusted to contain 1.0 mM-EGTA, 1.2 mM- CaCl_2 , 100 μM -leucine and 33 μM -methionine. Amino acid incorporation into trichloroacetic acid-precipitable alkali-stable material was determined as previously described [14], by using incubations (0.5 ml) containing 2×10^6 cells/ml at 37 °C with various additives as indicated. Results are expressed as averages or as averages $\pm$ S.E.M. of values obtained for triplicate samples. Unsaturated fatty acids were stored under a N_2 atmosphere as 50 mM stocks in Me_2SO and added to incubations as discussed in the text. Arachidic acid ($\text{C}_{20:0}$) was diluted from a 50 mM stock in ethanol. Protein was determined by the Coomassie Blue dye-binding method of Bradford [16]. One-dimensional PAGE was conducted as described by Laemmli [17]. The gels were lightly stained with Coomassie Blue to locate molecular-mass marker proteins, dried, and analysed by autoradiography using Kodak X-Omat or Fuji RX 100 film. Ribosomal and polysomal size distributions were measured by density-gradient centrifugation as described previously [18].

Determination of average ribosomal transit times

Transit times were determined by a modification of the procedure of Ledford & Davis [19]. Samples (4 ml) of cells suspended at 2×10^6 cells/ml in Ham's F10 medium adjusted to contain 100 μM -leucine were equilibrated for 20 min at 37 °C in 50 ml conical polypropylene tubes with any additives to be tested. Controls (blanks) contained 2 mM-leucine and 100 μM -cycloheximide. The incubation was initiated by addition of 100 μl of [^{14}C]leucine (4 $\mu\text{Ci/ml}$). After 30 min the samples were adjusted with 100 μl of [^3H]leucine (4 $\mu\text{Ci/ml}$), and the incubation continued for 15 min. After adjustment to 125 μM -cycloheximide, the samples were placed on ice, 50 μl portions were removed for slope determination, and the cells were collected by centrifugation (1000 g for 2 min). The cells were suspended in 2.5 ml of lysis buffer, containing 50 mM-Tris buffer, pH 7.6, 25 mM-KCl, 1 % Triton X-100, 1 % sodium deoxycholate, 10 μg of cycloheximide/ml, 2 mM-leucine, 1 mg of heparin/ml and 5 mM- MgCl_2 , homogenized with a Dounce glass homogenizer equipped with a tight-fitting pestle (30 strokes), and the lysates were transferred to 15 ml Corex centrifuge tubes. The homogenizer was rinsed with 2 ml of 1 % Triton X-100 and the rinse was combined with the lysate. The lysates were centrifuged at 10000 g for 10 min, and the resulting postmitochondrial-supernatant fractions (P.M.S.) were recovered. Four 400 μl samples were removed into 5 ml vials on ice. The remainder of each fraction was centrifuged at 100000 g for 1 h, the postribosomal-supernatant fraction (P.R.S.) was decanted, and four 400 μl samples were taken as above. Both sets of samples were then precipitated with 10 % trichloroacetic acid and analysed for radioactivity by the same procedure used for conventional incorporations [14], except that the protein was redissolved with base, reprecipitated, and washed one additional time to procure added precision. Samples taken for slope determination were adjusted to contain comparable amounts of formic acid such that they exhibited ^{14}C and ^3H counting efficiencies comparable with those for incorporation samples. Samples were analysed for each radioisotope in a multichannel

scintillation counter and the data were corrected for channel crossover. Slopes were calculated from the relationship:

$$\text{Slope} = (^3\text{H}/^{14}\text{C}) (1/\text{incubation time})$$

Average ribosomal transit times were calculated by the relationship:

Transit time

$$= (2/\text{slope}) [(^3\text{H}/^{14}\text{C} \text{ of P.M.S.}) - (^3\text{H}/^{14}\text{C} \text{ of P.M.S.})]$$

The degree of experimental variation in data underpinning these calculations was such that deviations in transit time of less than 50 % were not viewed as significant.

Measurement of cell-associated $^{45}\text{Ca}^{2+}$

Washed GH₃ cells were resuspended in Ham's F-10 medium, pH 7.5, containing 50 μM - Ca^{2+} and incubated (1.5 ml) with $^{45}\text{CaCl}_2$ (4×10^6 c.p.m./ml) for 45 min. The samples were adjusted to various concentrations of arachidonate and the incubation was continued at 37 °C for 10 min. The final concentration of Me_2SO did not exceed 0.5 % (v/v). Cells were separated from the medium by centrifugation (0.4 ml) through 200 μl of dinonyl phthalate/silicone oil (1:1 v/v) for 30 s in a Beckman Microfuge. The supernatant fluid was aspirated and the centrifuge tubes were inverted to drain. The tips of the tubes containing the pellets were then severed, and the pellets were solubilized in 500 μl of 1 % SDS and analysed for radioactivity by scintillation counting. $^{45}\text{Ca}^{2+}$ in combination with 1 mM-EGTA was added to equivalent samples, and the preparations were immediately centrifuged through oil to assess the contribution of extracellular $^{45}\text{Ca}^{2+}$. These values were subtracted for experimental values. Blank values were routinely less than 10 % of control values. Results are expressed as averages or as averages $\pm$ S.E.M. of values obtained for triplicate samples.

Statistics

Statistical analysis was performed by Student's t test.

RESULTS

Effects of eicosanoic acids on amino acid incorporation into intact GH₃, C6 and HeLa cells

The reputed ability of unsaturated fatty acids to mobilize intracellular sequestered Ca^{2+} [8–12] prompted an assessment of whether these agents would inhibit protein synthesis. Polyunsaturated fatty acids proved strongly inhibitory to amino acid incorporation in GH₃, C6 and HeLa cells (Fig. 1, Table 1). Of a series of C_{20} fatty acids, only the saturated form, arachidic acid ($\text{C}_{20:0}$), failed to decrease amino acid incorporation in GH₃ cells (Fig. 1). Inhibitory potencies generally rose as a function of increasing unsaturation. Similarly, in HeLa cells optimal degrees of inhibition were achieved with fatty acids possessing three or four double bonds. As would be expected for highly hydrophobic substances, the apparent inhibitory potency varied as a function of the cell mass, with more fatty acid required to generate comparable inhibition in incubations containing higher cell numbers. Comparable inhibitory potencies were found for arachidonate for the three cell types examined; 10–20 μM routinely provided an 80–90 % decrease in incorporation. These concentrations approximate to the K_m for conversion into active metabolites [20]. Indomethacin, however, which suppresses conversion into metabolites by cyclo-oxygenase, had no significant effect on the inhibition by arachidonate (result not shown). Incorporation of arachidonate into cellular lipids did not appear to be involved, since fatty-acid-free BSA (1 mg/ml) completely

Fig. 1. Effects of eicosanoic acid analogues on leucine incorporation in GH₃ and C₆ cells

(a) GH₃ cells (2.0×10^6 /ml) in modified Ham's F-10 medium, pH 7.5, containing $33 \mu\text{M}$ -methionine, $100 \mu\text{M}$ -leucine, 1 mM -EGTA and 1.2 mM -CaCl₂ were equilibrated for 15 min at 37°C to ensure steady-state incorporation. Portions (2 ml) were adjusted to the indicated fatty acid concentrations from 5.0 mM stocks in Me₂SO (final Me₂SO concn. 0.5%) for 15 min and proteins were pulse-labelled with [³H]leucine (0.15 Ci/mmol) for 15 min. (b) Conducted with arachidonic acid as in (a), but with C₆ cells. Fatty acids were: eicosanoic (arachidic) (C_{20:0}; ■), *cis*,11-eicosanoic (C_{20:1}; ▨), eicosadienoic (C_{20:2}; ▩), eicosatrienoic (C_{20:3}; ▤) and arachidonic (C_{20:4}; □). Results are means $\pm$ S.E.M. (bars) of triplicate samples: * $P < 0.05$, ** $P < 0.005$ versus leucine incorporation for untreated controls.

reversed the inhibitory actions of fatty acids on protein synthesis within 15 min (result not shown).

A comparison of the protein populations labelled with

[³⁵S]methionine/[³⁵S]cysteine in arachidonate-treated and untreated GH₃ cells was undertaken by the technique of slab-acrylamide-gel electrophoresis with subsequent autoradiography (Fig. 2). Coomassie-Blue-staining patterns remained constant throughout the incubation (result not shown). Radioactivity was incorporated into a large number of polypeptides in all preparations. No qualitative differences in the labelling of specific polypeptide species were found, but a dramatic dose-dependent quantitative diminution in radioactive labelling of all detectable species occurred for preparations treated with arachidonate for short periods (Fig. 2, lanes a). With increasing times of exposure, the inhibition became attenuated at lower concentrations of arachidonate (Fig. 2, lanes b). For example, cells exposed to an intermediate arachidonate concentration ($6 \mu\text{M}$) recovered completely by 2 h. In contrast, cells exposed to Ca²⁺ ionophore A23187 maintained low rates of amino acid incorporation through the 2 h period.

Rates of protein synthesis partially recover ('accommodate') after chronic (2–3 h) exposure to either EGTA or A23187 in a manner that is dependent on transcription [1,21]. Accommodation, which occurs spontaneously in C₆ cells but is fostered in GH₃ cells by phorbol ester or agents that elevate cyclic AMP content [21], is associated with the induction of a resident 78 kDa protein of the ER termed BiP or GRP78 [22,23]. Even without promoters some preferential incorporation of ³⁵S label into a 78 kDa protein was detected (Fig. 2, arrow) at 2 h in the ionophore-treated preparation of GH₃ cells. This inducible 78 kDa species in ionophore-treated GH₃ cells was previously identified as authentic GRP78 by a variety of criteria [21]. Induction of GRP78 with arachidonate was insignificant under these conditions.

Differential recovery periods occurred for protein synthesis in various cell types exposed to various unsaturated fatty acids at $15 \mu\text{M}$ (Table 1). C₆ cells exhibited rapid onset (2–4 min) of inhibition of protein synthesis, followed by strong recovery ordinarily beginning within 8 min of exposure and which was complete at 60 min for each unsaturated fatty acid tested. Amino acid incorporation in GH₃ cells, in contrast, was inhibited more slowly (8 min) and remained suppressed throughout 60 min of incubation. HeLa cells recovered from inhibition more quickly than GH₃ cells, but more slowly than C₆ cells. Although the more unsaturated fatty acids appeared to act somewhat more rapidly and effectively in each cell type, the slower response of GH₃ cells accentuated this apparent differential. Over a period of 4 h GH₃ cells were found to recover progressively but completely from inhibition of protein synthesis by arachidonate (Fig. 3a).

Table 1. Amino acid incorporation by HeLa, C₆ or GH₃ cells treated with selected unsaturated fatty acids

Cells (2×10^6 /ml) in modified Ham's F-10 medium, pH 7.5, containing 1.0 mM -EGTA and 1.2 mM -CaCl₂ were equilibrated for 20 min at 37°C . The suspensions were then treated with solvent (0.1% Me₂SO) or fatty acid at $15 \mu\text{M}$. At the indicated times of incubation, 0.5 ml samples were withdrawn for measurement of leucine incorporation. Fatty acids: oleic (C_{18:1}), linoleic (C_{18:2}), linolenic (C_{18:3}) and arachidonate (C_{20:4}). Standard errors are omitted to simplify this Table; representative S.E.M. values are provided in other Tables and Figures. Abbreviation: ND, Not determined.

Incubation time (min)	Unsaturated fatty acid ... Cells...	Leucine incorporation (% of control)											
		C _{18:1}			C _{18:2}			C _{18:3}			C _{20:4}		
		HeLa	C ₆	GH ₃	HeLa	C ₆	GH ₃	HeLa	C ₆	GH ₃	HeLa	C ₆	GH ₃
2		36	36	31	48	48	23	10	14	16	18	25	7
4		34	33	19	59	59	25	10	13	12	10	20	5
8		51	51	9	80	80	11	8	8	4	13	21	4
16		ND	68	4	ND	100	11	6	17	4	32	62	5
30		80	80	5	91	91	10	22	65	5	56	82	9
60		ND	ND	3	ND	ND	ND	ND	ND	3	ND	90	5

Fig. 2. Incorporation of ^{35}S -labelled amino acids into protein populations of arachidonate-treated GH_3 cells as analysed by PAGE and autoradiography

GH_3 cells ($2 \times 10^6/\text{ml}$) in Ham's F-10 medium, pH 7.5, adjusted to $5 \mu\text{M}$ -methionine and 0.2 mM - CaCl_2 were exposed either to arachidonate [0 (Me_2SO control), 6, 9 or $12 \mu\text{M}$] or to ionophore A23187 ($0.3 \mu\text{M}$) for 15 min (lanes a) or 2 h (lanes b). Cells were then incubated with Tran^{35}S ($100 \mu\text{Ci}/\text{ml}$, $2.8 \times 10^8 \text{ c.p.m.}/\text{ml}$) for 30 min, harvested and denatured in Laemmli's sample buffer for subsequent one-dimensional slab-gel electrophoresis and autoradiography as described in the Experimental section. The following molecular-mass markers were used: myosin (200 kDa), phosphorylase b (94 kDa), BSA (66 kDa), ovalbumin (43 kDa) and carbonic anhydrase (31 kDa). The arrow indicates GRP78.

Simultaneously the cells regulated the ability to accumulate Ca^{2+} , with complete recovery of cell-associated cation observed by 4 h (Fig. 3b). At each time point incorporation was sensitive to inhibition with A23187, further corroborating that recovery centred on re-establishment of Ca^{2+} storage rather than on development of a Ca^{2+} -independent compensatory process. Incorporation was decreased to very low values as cell-associated $^{45}\text{Ca}^{2+}$ approached 40 % of that in non-treated controls. It was also established in separate experiments that incorporation in GH_3 cells that had recovered from arachidonate was strongly inhibited upon re-exposure to arachidonate and that the medium from recovered cells did not inhibit fresh cells (results not shown).

Role of Ca^{2+} in the inhibition of protein synthesis by arachidonate

The hypothesis was examined that arachidonate inhibits amino acid incorporation through mobilizing intracellular Ca^{2+} . GH_3 cells were preloaded with $^{45}\text{Ca}^{2+}$, exposed to the fatty acid, and cell-associated $^{45}\text{Ca}^{2+}$ and leucine incorporation was determined with the same preparation of cells (Fig. 4a). Both parameters

Fig. 3. Recovery of leucine incorporation and re-accumulation of Ca^{2+} by GH_3 cells after extended incubations with arachidonate

(a) Recovery of Ca^{2+} -sensitive amino acid incorporation. Cells ($2 \times 10^6/\text{ml}$) in modified Ham's F-10 medium containing 0.2 mM - CaCl_2 were exposed to 0 or $13 \mu\text{M}$ -arachidonate at 37°C . At the indicated times, 0.5 ml samples were removed for treatment without ($\square$) or with ($\blacksquare$) $1 \mu\text{M}$ -A23187 for 10 min. ^{3}H -leucine was added and incorporation determined after 15 min incubation. Results are means $\pm$ S.E.M. (bars) of triplicate determinations from a single experiment: $*P < 0.05$, $**P < 0.005$ versus leucine incorporation for untreated controls. (b) Re-accumulation of cell-associated $^{45}\text{Ca}^{2+}$. Cells ($2 \times 10^6/\text{ml}$) in modified Ham's F-10 medium containing 0.2 mM - CaCl_2 were adjusted with $^{45}\text{CaCl}_2$ ($4 \mu\text{Ci}/\text{ml}$) and incubated for 1 h at 37°C . Me_2SO or arachidonate ($12 \mu\text{M}$) was then added and samples were withdrawn for measurement of cell-associated $^{45}\text{Ca}^{2+}$ at 15, 60, 120 and 240 min. Results are means $\pm$ S.E.M. (bars) for triplicate determinations from a single experiment: $*P < 0.05$ versus cell-associated $^{45}\text{Ca}^{2+}$ for untreated controls.

were sharply decreased with increasing concentrations from 3 to $20 \mu\text{M}$ fatty acid at pH 7.5. At $20 \mu\text{M}$ -arachidonate, approx. 60 % of cell-associated $^{45}\text{Ca}^{2+}$ was mobilized and protein synthesis was almost abolished. Inhibition by arachidonate, as found previously with hormones that promote Ca^{2+} mobilization [24], was more pronounced at lower pH (Fig. 4b). For example, incorporation in a single preparation of cells equilibrated at various pH values was half-maximally inhibited by $6 \mu\text{M}$ -arachidonate at pH 6.9, as compared with $14 \mu\text{M}$ at pH 7.8. These differences are meaningful, in view of the narrow concentration range over which arachidonate inhibits. Cell cultures provided with fresh growth medium within 24 h of harvesting for an experiment required more arachidonate to generate comparable degrees of inhibition than did cells procured from more acidic growth media at similar cell densities (results not shown).

Extracellular Ca^{2+} decreased the effectiveness of lower concentrations of arachidonate as an inhibitor of amino acid incorporation (Fig. 4c). For example, at $9 \mu\text{M}$ -arachidonate, pH 7.5, Ca^{2+} from 0.05 – 0.5 mM protected modestly against inhibition, 2.0 mM - Ca^{2+} protected more strongly and 5 mM - Ca^{2+} prevented inhibition from developing. Addition of 5 mM - Ca^{2+} to non-treated controls had no effect on incorporation. At higher concentrations of arachidonate (12 or $15 \mu\text{M}$), 5 mM - Ca^{2+} only slightly suppressed the inhibition. High arachidonate concentra-

Fig. 4. Ca²⁺ mobilization by arachidonic acid and reversal of arachidonate inhibition of protein synthesis by increased extracellular Ca²⁺

(a) Ca²⁺ mobilization and leucine incorporation with increasing arachidonate concentration. GH₃ cells (6.4×10^6 /ml) were equilibrated at 37 °C in Ham's F-10 medium, pH 7.5, adjusted to 50 μM free Ca²⁺. A portion was equilibrated with ⁴⁵CaCl₂, exposed to the indicated concentrations of arachidonate for 10 min, and cell-associated ⁴⁵Ca²⁺ was measured as described in the Experimental section (○). The second portion was treated comparably with arachidonate, followed by determination of [³H]leucine incorporation during 15 min of incubation (●). (b) Inhibition of leucine incorporation by arachidonate as a function of pH. GH₃ cells (2×10^6 /ml) were equilibrated at 37 °C in Ham's F-10 medium adjusted to 0.2 mM free Ca²⁺ for 30 min at pH 6.9 (○), 7.2 (●), 7.5 (△) and 7.8 (▲), pretreated subsequently with arachidonate at various concentrations for 20 min, followed by determination of [³H]leucine incorporation during 15 min of incubation. (c) Protection by increased Ca²⁺ concentration against arachidonate inhibition of leucine incorporation. GH₃ cells (2×10^6 /ml) in modified Ham's F-10 medium were equilibrated at the indicated CaCl₂ concentrations for 30 min at 37 °C, followed by adjustment with arachidonate to 0 (○, Me₂SO control), 9 (●), 12 (△) and 15 μM (▲). After 20 min of treatment, [³H]leucine was added and incorporation determined after 15 min of incubation. (d) Inhibition of leucine incorporation by arachidonate as a function of time and extracellular Ca²⁺ concentration. GH₃ cells (2×10^6 /ml) in modified Ham's F-10 medium were equilibrated at either 0.2 mM- (○, △) or 5 mM- (●, ▲) CaCl₂ for 30 min at 37 °C. The incubation was begun with addition of [³H]leucine and sampled as a function of time for incorporation. Arachidonate (12 μM) was added at 11 min (arrow) to duplicate samples, and portions of control (○, ●) and arachidonate-treated (△, ▲) samples were taken for the following 49 min.

tions (12 μM, pH 7.2) comparably inhibited protein synthesis over a range of extracellular Ca²⁺ concentrations (Fig. 4d). After addition of the fatty acid, rates of incorporation were decreased by approx. 95 % at 0.2 mM- and 90 % at 5 mM-Ca²⁺ within 5 min. The new rates were linear for at least 45 min.

The relative effects of arachidonate on translational initiation and elongation were investigated by examining the effects of the fatty acid on cellular polysomal content, Ca²⁺ content and amino acid incorporation for the same preparation of GH₃ cells (Fig. 5, Table 2). In comparison with Ca²⁺ controls (Fig. 5a), low (10 μM) arachidonate was found to decrease polysomal content while increasing the contents of 80 s monosomes and ribosomal-subunit peaks (Fig. 5e). This pattern of changes was consistent with inhibition of translational initiation which has been shown previously to develop during extended treatment with EGTA (Fig. 5b) and during treatment with Ca²⁺ ionophore [18] (Fig. 5d). Approx. 40 % of Ca²⁺ within the cells was mobilized during treatment with 10 μM-arachidonate, in comparison with much larger degrees of mobilization with either ionophore or EGTA. Amino acid incorporation were sharply decreased by all of these conditions. At higher concentrations of arachidonate (15 or 20 μM) more than half of the Ca²⁺ was mobilized, incorporation was nearly abolished, whereas polysomal content was largely maintained (Figs. 5f and 5g). These results would be expected for

typical elongation block. This inhibition was not dependent on Ca²⁺, since a combination of 20 μM-arachidonate and EGTA extracted all of the Ca²⁺ without affecting the polysomal content of the cells (Fig. 5h). For purposes of comparison, treatment with EGTA alone for 10 min produced a comparable polysomal content with good retention of amino acid incorporation into protein and partial mobilization of Ca²⁺ (Fig. 5c). In this regard it should be noted that EGTA extracts cytosolic free Ca²⁺ within seconds, but only slowly lowers sequestered stores of the cation. A combination of EGTA and a Ca²⁺-mobilizing agent will rapidly decrease total intracellular Ca²⁺, as shown in Fig. 5(h). An analysis of EF2 phosphorylation by Western blotting (results not shown) indicated that arachidonate did not affect the phosphorylation of EF2 which is mediated by increases in cytosolic free Ca²⁺ [3,4].

Confirmation that low arachidonate inhibits translational initiation, whereas high arachidonate inhibits translational elongation, was attempted through an analysis of average ribosomal transit time (Table 3). Transit times of approx. 2 min have previously been found for cells that were either repleted or depleted of intracellular Ca²⁺ stores [18]. Cells exposed to arachidonate (20 μM) possessed average ribosomal transit times that were equivalent to those of the EGTA or Ca²⁺ controls, even though elongation blockade was apparent at the higher fatty acid

Table 2. Effects of various additives on leucine incorporation and Ca^{2+} content

Incorporation values were determined simultaneously on portions of the same cell preparations as those used for polysomal determinations in Fig. 5. Pulse incorporation (nmol/mg of protein) was conducted for 15 min. Ca^{2+} content (nmol/mg of protein) was determined from a second preparation of cells pre-equilibrated at 1 mM extracellular $\text{CaCl}_2/^{45}\text{Ca}$ and treated identically with the earlier preparation.

Additive	Time of exposure (min)	Leucine incorporation (nmol/mg)	Ca^{2+} content (nmol/mg)
None (Ca^{2+} control)	—	1.76	21.0
EGTA (4 mM)	10	1.36	8.5
EGTA	30	0.49	3.3
A23187	20	0.10	1.8
Arachidonate (10 μM)	20	0.50	11.8
Arachidonate (15 μM)	20	0.05	8.9
Arachidonate (20 μM)	20	0.02	2.4
Arachidonate (20 μM) + EGTA	20/10	0.03	0.8

concentration by polysomal analysis. Such results with high arachidonate would be predicted if ribosomal movements were arrested in an all-or-none fashion. Low concentrations of the non-ionic detergent Lubrol PX produce comparable elongation blockade in intact cells, as evidenced by high polysome content as low incorporation values, but do not mobilize Ca^{2+} (results not shown). As with arachidonate, these inhibitions increase sharply over a narrow concentration range and do not involve changes in ribosomal transit time (Table 3). In contrast, the fully reversible inhibitor cycloheximide provided typically greater extensions of elongation time with increasing concentration. Arachidonate at higher concentrations (50–200 μM) inhibited translation in reticulocyte lysates (Table 4). The ratio of arachidonate to protein in incubations wherein such inhibitions were observed, however, was comparable with that in incubations with intact GH_3 cells wherein elongation block was obtained. Addition of arachidonate at concentrations inhibitory to translation in reticulocyte lysates did not change the polysomal

Fig. 5. Effects of arachidonate on GH_3 -cell polysomal contents

Cells ($2 \times 10^6/\text{ml}$) equilibrated for 30 min in Ham's F-10 medium with 1 mM- CaCl_2 , pH 7.5, were exposed to the following treatments, followed by lysis and density-gradient centrifugation: (a) Ca^{2+} control; (b) 4 mM-EGTA for 30 min; (c) EGTA for 10 min; (d) 1 μM -A23187 for 20 min; (e) 10 μM -arachidonate for 20 min; (f) 15 μM -arachidonate for 20 min; (g) 20 μM -arachidonate for 20 min; (h) 20 μM -arachidonate for 20 min, with EGTA during the last 10 min. Corresponding leucine incorporation and Ca^{2+} -content data are presented in Table 2.

content of these lysates (results not shown), indicative of elongation blockade.

DISCUSSION

Arachidonate is the established progenitor of numerous locally active humoral substances, including various leukotrienes, prostanoids and thromboxanes, that trigger Ca^{2+} -mediated events in a wide variety of cell types. These substances are produced after generation of arachidonate from membrane phospholipids by receptor-mediated activation of phospholipase A_2 . Evidence is accumulating that arachidonate may in some circumstances be released in sufficient quantities to serve as the

Table 3. Effect of various additives on average ribosomal transit times

Cells ($2 \times 10^6/\text{ml}$) suspended in modified Ham's F-10 medium, pH 7.5, at 0.2 mM- Ca^{2+} were equilibrated for 20 min with the indicated additives. Samples were then supplemented with [^{14}C]leucine for 45 min and with [^3H]leucine for the final 15 min of this period. Average ribosomal transit times were determined as described in the Experimental section. Incorporation values tabulated below represent total incorporation for each radioisotope after correction for counting channel cross-over, and are presented as means $\pm$ S.E.M. of four replicate samples. The slope calculated for this experiment was 0.04545/min.

Condition	10 ⁻³ × Leucine incorporation (c.p.m.)						Average transit time (min)
	Postmitochondrial fraction			Postribosomal fraction			
	³ H	¹⁴ C	³ H/ ¹⁴ C	³ H	¹⁴ C	³ H/ ¹⁴ C	
Control (+ Ca ²⁺)	42.53 ± 0.98	64.07 ± 2.07	0.664	34.92 ± 0.31	56.14 ± 1.03	0.622	1.8
Arachidonate (20 μM)	3.13 ± 0.05	3.87 ± 0.06	0.807	2.45 ± 0.07	3.26 ± 0.10	0.753	2.4
EGTA (4 mM)	5.01 ± 0.23	10.35 ± 0.56	0.484	4.01 ± 0.03	9.03 ± 0.10	0.444	1.8
EGTA + arachidonate	1.41 ± 0.05	2.42 ± 0.05	0.583	1.08 ± 0.05	2.02 ± 0.08	0.535	2.1
Cycloheximide (0.3 μM)	13.57 ± 0.13	20.02 ± 0.15	0.678	9.16 ± 0.02	15.49 ± 0.14	0.591	3.8
Cycloheximide (1 μM)	7.00 ± 0.19	10.33 ± 0.35	0.678	3.73 ± 0.10	7.37 ± 0.43	0.506	7.6
Lubrol (0.0004 %)	12.07 ± 0.017	17.41 ± 0.027	0.693	9.67 ± 0.23	14.77 ± 0.54	0.655	1.7
Lubrol (0.0007 %)	5.44 ± 0.27	7.62 ± 0.37	0.714	4.18 ± 0.16	6.29 ± 0.22	0.665	2.2

Table 4. Effect of arachidonate on methionine incorporation by reticulocyte lysate

Untreated rabbit reticulocyte lysate purchased from Promega Corp. (Madison, WI, U.S.A.) was optimized and assayed according to the supplier's instructions. Incubations (30 μ l) contained 20 μ l of lysate and 10 μ l of additives, including arachidonate as indicated. After pretreatment for 10 min, pulse-labelling with [35 S]methionine was determined for 10 min.

Arachidonate concn. (μ M)	Leucine incorporation (% of control)
0	100
50	76
100	48
200	9

immediate messenger releasing intracellular sequestered Ca^{2+} to the cytoplasm. For example, Wolf *et al.* [8] found that exposure of pancreatic islets to glucose produced intracellular concentrations of unesterified arachidonate in excess of 20 μ M. Similar high concentrations are found in brain in ischaemia [25] and in amniotic fluid during labour [26], and platelets stimulated by thrombin are reported to release up to 10 nmol of arachidonate metabolites/min for 10^9 cells [7].

It is clear from the results of this report that arachidonate and other unsaturated fatty acids are highly effective, rapidly acting, reversible inhibitors of protein synthesis in intact GH₃, C6 and HeLa cells. Relatively modest concentrations of arachidonate (6–10 μ M) were found to inhibit translational initiation, whereas slightly higher concentrations (15–20 μ M) inhibited elongation as well. Multiple findings support the conclusion that arachidonic acid inhibits translational initiation indirectly through mobilizing Ca^{2+} stored in the ER in a manner akin to Ca^{2+} ionophore [18] and benzyloxycarbonyl-glycylphenylalanine amide [2]. Arachidonic acid mobilized intracellular Ca^{2+} to the extracellular space at concentrations and over time periods wherein amino acid incorporation was inhibited. As cells recovered with time from the inhibition, they also recovered the ability to store Ca^{2+} . The inhibition at moderate concentrations of the fatty acid was suppressed by addition of high concentrations of Ca^{2+} to the incubation medium. Recovery with time appeared to result from conversion of arachidonate into inactive forms either enzymically [7] or by oxidation. Fresh cells exposed to medium of recovered cells were not inhibited in amino acid incorporation. Recovered cells were susceptible to inhibition by either Ca^{2+} ionophore or fresh arachidonate. The more rapid recovery of C6 and HeLa cells as compared with GH₃ cells most likely involved differential rates of metabolic inactivation of arachidonate.

Although amino acid incorporation was inhibited almost completely by arachidonate, cellular Ca^{2+} content was decreased by only about 60 %, indicating that not all pools of sequestered Ca^{2+} were affected. Hepatocytes have been reported to extrude about 50–60 % of their total intracellular Ca^{2+} in response to InsP_3 generation caused by vasopressin or α -adrenergic agonists [27–30]. InsP_3 is generally believed to release ER-associated Ca^{2+} . Wolf *et al.* [8], using digitonin-permeabilized pancreatic islets, concluded that exogenously added InsP_3 and arachidonate were comparable in potency in directly inducing the release of ER Ca^{2+} stores. In agreement with the hypothesis that arachidonate preferentially affects the ER, Chow & Jondal [12] recently reported that Ca^{2+} released by unsaturated fatty acids from saponin-permeabilized JURKAT T-cells originated from a non-mitochondrial sequestered pool sensitive to addition of InsP_3 and

to depletion of ATP. In other studies, vesicles prepared from the sarcoplasmic reticulum have been reported to release Ca^{2+} when exposed to unsaturated fatty acids [31]. In further support of specific effects at the ER, electron micrographs of GH₃ cells treated with arachidonate displaced extensive, yet reversible, swelling of this organelle, with little modification of other organelles (results not shown). These changes were produced at arachidonate concentrations that inhibited protein synthesis by approx. 90 %.

Results of this study are consistent with the proposal that arachidonate mobilized Ca^{2+} , sustaining amino acid incorporation in a direct and specific manner. The rapid and complete reversal of arachidonate inhibition by fatty-acid-free albumin indicated that incorporation of the fatty acid into cellular lipids was not required. Indomethacin exerted little effects on the inhibition by arachidonate, indicating that the observed effects of the fatty acid were not ascribable to products of fatty acid cyclooxygenase. However, we were unable to eliminate completely the possibility that lipoxygenase metabolites mediated the Ca^{2+} mobilization or the inhibition of protein synthesis in arachidonate-treated GH₃ and C6 cells. Nordihydroguaiaretic acid, a putative lipoxygenase inhibitor, was itself strongly inhibitory (results not shown). In addition, 5-hydroxyeicosatetraenoic acid, when added at μ M concentrations, potentiated the effects of arachidonate and served as an inhibitor of protein synthesis (results not shown). The substrate specificity of the 5-lipoxygenase does, however, argue against a requirement for metabolic conversion, since a double bond at C-5 is critical [20]. Other than arachidonate, none of the inhibitory unsaturated fatty acids tested in our study possesses a C-5 double bond. We were able to ascertain that long-chain fatty acids were not generally inhibitory to protein synthesis. A requirement for unsaturated double bonds was observed, as *cis*,11-eicosanoic ($\text{C}_{20:1}$), eicosadienoic ($\text{C}_{20:2}$), eicosatrienoic ($\text{C}_{20:3}$) and arachidonic ($\text{C}_{20:4}$) acids, but not arachidic acid, inhibited amino acid incorporation.

The inhibition of peptide-chain elongation occurring at higher concentrations reflected a second, Ca^{2+} -independent, action of arachidonate at an undefined site. This inhibition was not recognized initially, since it was always imposed on translational initiation but was later readily ascertainable in polysomal distribution studies. Elongation was similarly inhibited by low concentrations of Lubrol PX which does not mobilize Ca^{2+} from cells. Inhibition by Lubrol was not influenced by high extracellular Ca^{2+} . Inhibition by either arachidonate or Lubrol occurred in a graded fashion over a very narrow concentration range, as though a critical component of elongation were being specifically inactivated by perturbant actions at a hydrophobic site(s). Both agents also inhibited elongation in reticulocyte lysates. Measurements of average ribosomal transit time, as measured by a modification of the double isotope-labelling procedure of Ledford & Davis [19], indicated that transit time was not changing even though elongation was strongly inhibited. This result would require that the effects of the inhibitor on a given ribosome be all or none. For example, a 50 % inhibition of incorporation would mean that half of the ribosomes ceased movement while the remainder read mRNA at normal rates. In contrast, cycloheximide, a readily reversible inhibitor of elongation, extended ribosomal transit times as a function of increasing concentration.

This paper illustrates that measurements of amino acid incorporation can be used as an index of Ca^{2+} mobilization from the ER to study the actions of agents such as arachidonate that act on this pool. Arachidonate has several useful properties over various other agents used more conventionally to mobilize sequestered Ca^{2+} . It does not appear to require specific receptors, and rapidly facilitates Ca^{2+} loss from a variety of intact cells. Its

effects are more readily reversible than those of Ca^{2+} ionophores and appear to be more selective than either EGTA or Ca^{2+} ionophores in terms of the Ca^{2+} pool affected. Although the physiological significance of arachidonate as inhibitor of protein synthesis remains to be established, the fatty acid can be generated in sufficient quantities in various tissues to inhibit both translational initiation and elongation.

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