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Docosahexaenoic Acid and Signaling Pathways in Rabbit Colon

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SUMMARY

The effects of one of the main components of fish oil, docosahexaenoic acid (DHA), on prostaglandin (PG) and Ca^{2+} signaling pathways were examined in intact mucosa and freshly isolated crypt cells of rabbit descending colon. Preincubation of serosal mucosa for 20 min with 1 μM DHA fully suppressed the short-circuit and transepithelial conductance increase induced by serosal addition of 10 μM arachidonic acid (AA). DHA at 1 μM also prevented the Cl^- secretion promoted by 10 μM AA, as estimated by unidirectional ^{36}Cl flux measurements (net flux = 0.68 ± 0.30 versus −1.91 ± 0.20 μEq/hr/cm^2, four experiments, $p < 0.001$), whereas it did not affect the electrophysiological and ion flux responses to PGE_2. Addition of 1 μM DHA to the serosal side of the mucosa also inhibited the PG cascade activation elicited by AA (PG synthesis and second messenger cAMP increase). *In vitro* assays of colonic cyclooxygenase activity showed that 1 μM DHA inhibited (with a 20-min lag) cyclooxygenase activity to the same extent as 5 μM indomethacin (~82% and 80%, respectively). DHA also affected the Ca^{2+} signaling pathway; in isolated crypt cells, the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) dropped by 49 ± 7.6% (mean ± standard error, six experiments) after incubation with 1 μM DHA. The sustained phase of the $[Ca^{2+}]_i$ response to 500 nM concentrations of the intracellular Ca^{2+}-ATPase inhibitor thapsigargin was also inhibited within 150 sec upon 1 μM DHA addition (141 ± 5.8 versus 243 ± 8.2 nM $[Ca^{2+}]_i$, mean ± standard error, eight experiments, $p < 0.01$). The $[Ca^{2+}]_i$-lowering effect of DHA, which was not achieved by incubation with other free fatty acids, was not prevented by removal of Na^+ from the incubation medium (−46 ± 4.3% versus −47 ± 3.8%, mean ± standard error, four experiments), nor it was mediated by cAMP-, protein kinase C-, or calmodulin-dependent mechanisms. The incubation of highly purified basolateral membranes of crypt cells with 1 μM DHA for 1 min produced a 5-fold increase (IC_{50} = 0.25 μM) in the plasma membrane Ca^{2+}-ATPase activity (34.3 ± 2.73 versus 6.02 ± 0.50 nmol/mg of protein/min, mean ± standard error, four experiments, $p < 0.0001$), thus indicating that the DHA effects on the Ca^{2+} pathway were mediated mainly by an increase in plasma membrane Ca^{2+} pump activity. These findings suggest that DHA is a powerful modulator of the cellular response to activation of PG and Ca^{2+} signaling pathways.

A large array of epidemiological and clinical observations have suggested that feeding with marine ω−3 fatty acids produces favorable effects on a broad spectrum of factors implicated in the pathogenesis of atherosclerosis and inflammatory and proliferative disorders (1–4). Moreover, there is now increasing evidence that fish oil-enriched diets attenuate the progression of several types of human and experimental renal and intestinal diseases (5–8). In addition, diets containing high proportions of ω−3 fatty acids diminished tumor development in animal models (9) and inhibited colon cancer in mice and rat (10–12). Nevertheless, the molecular mechanisms underlying the protective effects of fish oil are still unclear. In recent years, it has become increasingly evident that a variety of physiological processes are mediated by the various types of UFAs esterified in membrane lipids. As pointed out by others (13), these processes may be broadly categorized as being either eicosanoid dependent or eicosanoid independent. According to current views, many of the biological effects of fish oil depend on eicosanoid production changes elicited by EPA (20:5), an analog of AA that reduces the amount of AA available for eicosanoid production upon phospholipases activation. Because EPA is a substrate for cyclooxygenase and 5-lipoxygenase, there is a concomitant increase in EPA-derived eicosanoids, albeit of poor biological activity (14). Several observations suggest, however, that fish oil effects on cell functions are more complex. The changes in cell membrane fluidity and composition, which in turn may influence membrane-related processes (15), are eicosanoid-independent effects of UFAs. It is now well established that many physiological and pathological conditions leading to changes in membrane fluidity are associated with predictable alterations in the activity of several membrane transport systems (16). UFAs inhibit K^+ and Cl^- channels in different cell types (17–21) and Na^+-dependent amino acid transport in rat synaptosomes (22). UFAs also regulate dihy-

ABBREVIATIONS: UFA, unsaturated fatty acid; EPA, eicosapentaenoic acid; AA, arachidonic acid; DHA, docosahexaenoic acid; PM, plasma membrane; PG, prostaglandin; TG, thapsigargin; CPA, cyclopiazonic acid; I_{sc}, short-circuit current; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide HCl; IBMX, isobutylmethylxanthine; MEM, minimum essential medium; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; RIA, radioimmunoassay; Tx, thromboxane; AM, acetoxymethyl ester; DMSO, dimethylsulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NDGA, nordihydroguiaretic acid.

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dropiridine-sensitive calcium channels in myocardium (23), as well as the protein kinase C (24–27) and phosphoinositides branches of the calcium messenger system (28, 29). Furthermore, DHA and other free fatty acids reduce the agonist-induced Ca^{2+} increase in vascular smooth muscle cells, monocytic U937 cells, and platelets (30–32). On these bases, it is conceivable that some protective effects of fish oil are related to changes in cell functions not triggered by the eicosanoid system. In an attempt to address this issue, we examined the role of the other main component of fish oil, DHA (22:6) on PGs and Ca^{2+} signaling pathways. Very little is known about the cell biology and biochemistry of DHA (33); it accumulates in most tissues, especially the retina and brain, and is the most prevalent linolenic acid product in cells (34). Although it can be retroconverted to EPA (35, 36), DHA is most unlikely to be a storage form for excess EPA (37).

Electrophysiological and ion flux studies revealed that DHA prevented the increase in I_{SC}, net chloride secretion, and PGE_2 and second messenger cAMP synthesis promoted by AA; these effects were mediated by cyclooxygenase inhibition, as estimated in colonic microsomal fractions. DHA was also found to affect the Ca^{2+} signaling pathway; at low medium concentrations (1–10 μM), it reduced basal $[Ca^{2+}]_i$ in isolated crypt cells and prevented the sustained phase of the Ca^{2+} response triggered by TG or CPA. The assessment of PM Ca^{2+}-ATPase activity of highly purified basolateral membranes of crypt cells suggested that DHA effects on the Ca^{2+} signaling pathway were mediated primarily by changes in PM Ca^{2+} pump activity.

Materials and Methods

Isolation and mounting of mucosa. New Zealand White male rabbits (2.5–3.5 kg) were fed standard rabbit chow and water *ad libitum* and were killed by cervical dislocation. Colonic mucosa was prepared as described previously (38). Briefly, a segment of distal colon, about 10 cm in length, was rapidly removed and placed on a plastic rod. The tissue was then opened along its mesenteric border and placed under a dissecting microscope (8×) as a flat sheet, facing up. A glass microscope slide was used to hold the tissue and a second glass slide was used to produce an incision across the mucosa. Then, the mucosa was gently pushed away from the muscularis mucosa and submucosa and mounted between silicon rubber seals, to avoid edge damage, in Ussing-type chambers (1.12-cm^2 exposed surface). Tissues were bathed on both sides with 5 ml of standard Ringer solution containing (in mM) 113.6 NaCl, 5.4 KCl, 0.2 HCl, 0.6 NaH_2PO_4, 2.4 Na_2HPO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 21 $NaHCO_3$, and 10 glucose, pH 7.4 when gassed with 95% O_2/5% CO_2. Solutions were recirculated by gas lifting and were maintained at 37° in water-jacketed reservoirs.

Electrophysiology and ion flux measurements. Transepithelial electrical potential difference and I_{SC} (μEq/hr/cm^2) were measured with Ringer-agar bridges 1 mm from each side of the tissue and referenced to mucosal solution. Ag-AgCl electrodes were placed in the rear of each half-chamber to deliver a square current pulse and to monitor I_{SC}. Electrodes were connected to an automatic device allowing the tissues to be clamped to measure I_{SC}, and all measurements were corrected in series for the solution resistances. Transepithelial conductance, G_T (mS/cm^2), was calculated by Ohm's law. Test substances were added to the mucosal or serosal compartment, according to the experimental protocol, after a 40–50-min equilibration period.

Unidirectional mucosal-to-serosal (J_{ms}), serosal-to-mucosal (J_{sm}), and net ($J_{net} = J_{ms} - J_{sm}$) ion fluxes were calculated in short-circuited paired tissues by matching the resistances (differences of <20%). Tracer quantities of ^{36}Cl were added after a 1-hr equilibration period. Steady state fluxes (control period) were calculated from four samples taken at 15-min intervals starting 30 min after the addition of the tracer. Unless otherwise specified, test agents were added immediately after this period and, following another equilibration period, four samples were taken every 15 min (test period).

PG assay. Colonic mucosa was obtained as described previously and was divided into several 1-cm pieces (39). Each piece was rapidly weighed, transferred to polyethylene test tubes (100 × 15 mm) containing 3 ml of incubation medium with 0.5% dithiothreitol, gassed with 5% CO_2/95% O_2, and maintained at 37° in a shaking incubator. After a 30-min incubation, each piece was transferred to fresh Ringer solution and was handled according to the experimental protocol.

At the end of each experiment, indomethacin (10 μM) was immediately added, the tissues were homogenized in 4 ml of 0.1 N HCl, the suspension was centrifuged at 30,000 × *g* for 15 min, and the supernatant was loaded onto a C-18 Sep-Pak cartridge (Waters) that had been previously washed with methanol and water. After the sample loading, the cartridge was washed three times with 5 ml of water, 10% ethanol in water, and *n*-hexane; finally, the sample was eluted with 5 ml of ethyl acetate. The eluate was evaporated under vacuum and stored. The samples were processed for RIA by using standard techniques. The amounts of PGE_2, 6-keto-$PGF_{1\alpha}$, and TxB_2, the stable metabolites of PGI_2 and TxA_2, respectively, were measured by RIA (Amersham International), with standard methods, in tissues processed as described above. The cross-reactivity of anti-TxB_2 antiserum with other PGs was <3%. Cross-reactivity of anti-6-keto-$PGF_{1\alpha}$ antiserum was also <3%. All RIA determinations were done in triplicate and are expressed as ng/mg of wet weight.

cAMP assay. For cAMP measurements in intact mucosa, tissues were immediately added to 200 μl of 10% trichloroacetic acid and homogenized in a glass-glass Dounce homogenizer for 30 strokes, after addition of 1 ml of 50 mM Tris·HCl, pH 7.5. The homogenate was centrifuged for 10 min in an Eppendorf centrifuge and the supernatant was washed three times by addition of 3 volumes of diethyl ether. Finally, cAMP was measured by an RIA (Amersham) whose sensitivity ranged from 0.05 to 5 pmol. Determinations were done in triplicate and are expressed as pmol/mg of wet weight.

Cyclooxygenase activity assay. The preparation of colonic microsomes was based on the method of Hassid and Dunn (40). In short, colonic mucosa (about 2.3 g), stripped as described for cAMP assays, was minced and homogenized in a Potter homogenizer in 3 volumes of 0.1 M Tris buffer, pH 8.0. The homogenate was centrifuged for 30 min at 10,000 × *g*. The resultant supernatant was centrifuged for 1 hr at 100,000 × *g*. The precipitate was suspended in 0.1 M Tris buffer, pH 8.0, and recentrifuged for 1 hr at 100,000 × *g*. The microsomial pellet was immediately used for enzyme assay.

Cyclooxygenase activity was assayed by measuring the rate of conversion of AA to PGE_2, as described previously (41). Briefly, microsomal fractions (50 μl) were incubated with test agents for 3 min at 37° in 30 μl of Tris-HCl, pH 8.0, containing 2 mM reduced glutathione, 5 mM L-tryptophan, and 1 μM hematin. The substrate, 20 μM AA with tracer amounts of [1-^{14}C]AA (~220,000 cpm), was then added and the reaction proceeded for 3 min at 37°. The reaction was stopped by the addition of 0.2 ml of ethyl ether/methanol/0.2 M citric acid (30:4:1), which had been precooled to −25°. PGE_2 was extracted twice into the same mixture. The solvent was evaporated under a N_2 stream and radiolabeled AA was separated from radiolabeled PGE_2 by reverse phase high performance liquid chromatography. High performance liquid chromatographic analysis was performed on an Hitachi spectrophotometer (model 100-40) equipped with a flow cell; the sample was injected onto an Ultrasphere column (ODS, 5 mm, 4.6 mm × 25 cm; Beckman), with 2 nmol of unlabeled PGE_2 as internal standard. The PG chromatographic profile was obtained by isocratic elution with 150 mM H_3PO_4 in water, pH 3.5, containing 30% acetonitrile, at a flow rate of 1 ml/min, with monitoring of the UV absorption at 214 nm. Radioactivity that coeluted with authentic PGE_2 was quantified by liquid scintillation counting.

Isolation of crypt cells. Isolation of colonic crypt cells was carried out as described previously (42). Briefly, distal colon was washed with

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ice-cold Hanks' solution containing 1 mM dithiothreitol and the mucosa, obtained as described, was incubated for 20 min in 100 ml of ice-cold buffer containing 27 mM trisodium citrate, 5 mM Na_2HPO_4, 96 mM NaCl, 8 mM KH_2PO_4, 1.5 mM KCl, 0.5 mM dithiothreitol, 55 mM D-sorbitol, and 44 mM sucrose. During this period, the mucosa was gently shaken by hand. At the end of this step, microscopic inspection of the pellet obtained by low-speed centrifugation of the incubation medium demonstrated the absence of crypts and the presence of villus cells and large sheets of epithelium. The mucosa was then transferred to ice-cold MEM buffered with 20 mM HEPES, pH 7.4, oxygenated with 95% O_2/5% N_2, pH 7.4, and containing 0.8 mg/ml collagenase type 1S (Sigma); the mucosa was incubated for not more than 10 min, to avoid complete digestion of the tissue, and then discarded. This step allowed separation by gravity of the crypts, removed by enzymic digestion, from the remaining cells. After standing, the supernatant was aspirated and the pellet containing crypts was resuspended in ice-cold MEM. This phase, repeated twice, allowed removal of isolated cells and crypt fragments and enriched for whole crypts with a high degree of structural preservation. The pellet was centrifuged at 100 × *g* for 5 min and resuspended in an appropriate volume of MEM. To facilitate counting and fluorescent dye loading, the crypts were disaggregated and broken up by gentle refluxing with a Pasteur pipette. The entire procedure yielded 15–20 × 10^6 isolated crypt cells whose viability was >90%, as assessed by 0.2% trypan blue cytoplasmic exclusion test. The cells were then sedimented, resuspended in the appropriate medium, and kept at room temperature until used. Cells were counted immediately after their last manipulation, before addition to the cuvette, to ensure that the appropriate cell number was added.

Crypt cell basolateral membrane preparation. The preparation of basolateral membranes was carried out using the procedure described by Wiener *et al.* (43), with minor modifications. Briefly, distal colon crypt cells harvested from three rabbits as described were homogenized at 4° by 40 strokes in 4 volumes of HEPES-MEM containing 0.2 M sucrose, 100 μg/ml soybean trypsin inhibitor, and 100 μg/ml leupeptin, in a Potter apparatus at 1000 rpm using a Teflon pestle (tube volume, 10–50 ml).

The homogenate was subjected to low-speed differential centrifugation at 800 × *g* for 15 min. Centrifugation (at 70,000 × *g* for 35 min) of the supernatant containing the crude membrane fraction allowed a soluble fraction and the pellet containing the crude membrane fraction, which was further subfractionated by isopycnic centrifugation, to be obtained. The crude membranes were resuspended at 5–10 mg of protein/ml in 250 mM sucrose, 5 mM HEPES-Tris, pH 7.4, by 40 strokes in a Dounce homogenizer. Preparative subfractionations were obtained with a step gradient of 8 ml of 16% sucrose, 25 ml of 38% sucrose containing the crude membrane fraction, and 6 ml of 43% sucrose cushion, centrifuged for 14 hr at 75,000 × *g*. The fraction collected at the 38/43% interface was further purified by centrifugation at 70,000 × *g* in Ficoll 400 for 20 min. Each tube contained a 3-ml sample layer, 2 ml of 6% (w/v) Ficoll 400 in 250 mM sucrose, 5 mM HEPES-Tris, pH 7.4, and a 5-ml 25% sucrose cushion. After centrifugation, the 3-ml sample and a small band at the sample/Ficoll interface (containing endoplasmic reticulum and Golgi membranes, as assessed by marker enzyme analysis) were discarded. The higher purified basolateral membrane fraction trapped within the Ficoll barrier was collected, diluted 5-fold with medium, and centrifuged at 70,000 × *g* for 35 min. The pellet, resuspended in medium to 12–15 mg of protein/ml by repeated passage through a 25-gauge needle, was frozen in liquid nitrogen and stored at −80° until used. Protein concentration was measured by the method of Bradford (44), using bovine serum albumin as the standard.

Galactosyl transferase activity was measured by the method described by Bergeron *et al.* (45), to evaluate the presence of Golgi apparatus, and the extent of contamination with endoplasmic reticulum was judged on the basis of NADPH-cytochrome *c* reductase activity measured in freshly prepared fractions by the method of Beaufay *et al.* (46). The identification of the basolateral membrane domain was performed by assaying the Na^+/K^+-ATPase activity as the rate of inorganic phosphate release in the medium as reported by Mircheff and Wright (47). Table 1 summarizes the specific activities of marker enzymes in basolateral membrane vesicles, compared with those measured in whole-cell homogenates.

PM Ca^{2+}-ATPase assay. The Ca^{2+}-dependent ATPase activity of basolateral membranes of crypt cells was assayed following the procedure described by Niggli *et al.*(48), with modifications. Briefly, basolateral membrane vesicles (150 μg of protein/ml) were incubated at 25° for 4 min in medium (final volume, 1 ml) containing 100 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM MgATP, 20 mM HEPES, pH 7.5, 0.2 mM NADH, 1.1 mM EGTA, $CaCl_2$ in appropriate amounts (~0.8 mM total $CaCl_2$) to yield a free Ca^{2+} concentration of 12.6 μM (49), 2 μM A23187, 1 mg/ml digitonin, 10 mM phosphoenolpyruvate, 18 IU/ml purified pyruvate kinase, and 18 IU/ml lactate dehydrogenase (Sigma). Assays were started by addition of 0.5 mM MgATP, and the rate of ATP hydrolysis was calculated from spectrophometric recordings of the oxidation of NADH at 340 nm, using the molar absorption coefficient of NADH of 6.22 × 10^3 M^{-1} cm^{-1} (50). Ca^{2+}-ATPase activity was expressed as nmol/mg of protein/min. Basal ATPase (or Mg^{2+}-ATPase) activity was measured in assay medium containing 4 mM EGTA without added Ca^{2+}; the Ca^{2+}-dependent ATPase was determined from the difference between total ATPase (12.6 μM Ca^{2+} in the assay medium) and basal ATPase activities. Pilot experiments showed that under these conditions the addition of Triton X-100 (0.005%) did not affect Ca^{2+}-ATPase activity.

Measurement of $[Ca^{2+}]_i$. Freshly dissociated cells (1–1.5 × 10^6/ml cells) resuspended in HEPES-MEM were loaded with 2 μM fura-2/AM, which was cleaved to the free acid inside the cell. Loading was for 30 min at room temperature, with gentle shaking. The cells were then washed twice and resuspended in HEPES-MEM at a concentration of 1 × 10^6/ml for use. In Ca^{2+}-free studies, cells were incubated in HEPES-MEM free of Ca^{2+} and containing 1.2 mM EGTA. Stock solutions of fura-2/AM were made up in DMSO (Aldrich) and were premixed in a 2:1 (v/v) ratio with 25% (w/w) Pluronic F-127, a nonionic dispersing agent, in DMSO. Fluorescence changes of a 1.2-ml stirred crypt cell suspension kept at 37° were monitored with a MFP-66 Perkin Elmer fluorimeter, using 340- and 380-nm excitation wavelengths and 510-nm emission wavelength. $[Ca^{2+}]_i$ concentrations were calculated as described by Grynkiewicz *et al.* (51). A dissociation constant of 224 nM for the fura-2/Ca^{2+} complex was used to calculate $[Ca^{2+}]_i$. Autofluorescence of unloaded cells was subtracted before measurement of loaded cells.

Reagents. PGE_2, ionomycin, W-7, IBMX, digitonin, CPA, TG, free fatty acids, and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Fura-2/AM and Pluronic F-127/AM were purchased from Molecular Probes (Eugene, OR). Because UFAs are susceptible to oxidation, they were stored in aliquots at −20° in an N_2 atmosphere.

Statistics. Student's paired *t* test was used for pair-matched controls

TABLE 1

Marker enzyme activities of rabbit distal colon crypt cell basolateral membranes

Marker enzyme activities were measured in cell homogenates and basolateral membranes purified as described in Materials and Methods. Numbers in parentheses indicate enrichment factors. Values are mean ± standard error of three separate experiments, from different animals.

Marker	Whole-cell homogenate	Basolateral membrane
Na^+/K^+-ATPase (μmol of P_i/mg of protein/min)	0.81 ± 0.1	9.4 ± 0.3 (11)
UDP-galactose:*N*-acetylglucosamine galactosyltransferase activity (nmol of galactose transferred/hr/mg of protein)	1.3 ± 0.4	0.2 ± 0.1 (0.15)
NADPH cytochrome *c* oxidase (μmol/mg of protein/min)	18 ± 3.2	1.6 ± 3.8 (0.08)

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in the same animal; otherwise, Student's unpaired t test was used. A p value of <0.05 was assumed to be significant. All values are expressed as mean ± standard error unless otherwise specified.

Results

Effects on PG synthesis. The effects of DHA on the time course of AA-induced I_{SC} increase were first investigated in whole mucosa. As shown in Fig. 1, the serosal addition of 10 μM AA brought about an immediate increase in I_{SC} that peaked at 5 min (ΔI_{SC} = 4.58 ± 0.22 μEq/hr/cm², mean ± standard error) and decreased thereafter. However, serosal incubation with 1 μM DHA for 20 min before stimulation with AA fully suppressed the AA-induced I_{SC} increase.[1] The DHA-mediated I_{SC} inhibition depended on the incubation time, because pretreatment of the mucosa for 1–15 min did not prevent the increase in I_{SC} elicited by AA. Incubation for 30 min with fresh medium after removal of DHA-containing medium did not restore the responsiveness to AA, suggesting that DHA effects were irreversible, at least within the narrow limits of this observation period (data not shown). As reported in Fig. 2, the extent of the DHA inhibition of AA-induced I_{SC} increase was dose dependent; incubation with 0.5 μM DHA was associated with complete inhibition of AA-stimulated I_{SC} rise, and further increases in DHA concentrations above 5 μM did not produce additional I_{SC} inhibition. However, this effect was distinctive for DHA, because it was not reproduced by serosal or mucosal incubation with equimolar or higher concentrations (up to 30 μM) of palmitic or oleic acids (data not shown).

Parallel studies revealed that the pretreatment with 1 μM DHA inhibited net chloride secretion (J_{net}) promoted by AA but not that associated with PGE_2. As summarized in Table 2, the Cl^- secretion associated with the serosal addition of 10 μM AA or PGE_2 was mainly due to an increase in the serosal-to-mucosal Cl^- transport rate; the serosal exposure of colonic mucosa to 1 μM DHA brought about complete inhibition of the increase in transepithelial net Cl^- flux elicited by AA, whereas it failed to prevent the Cl^- secretion promoted by 10 μM PGE_2.

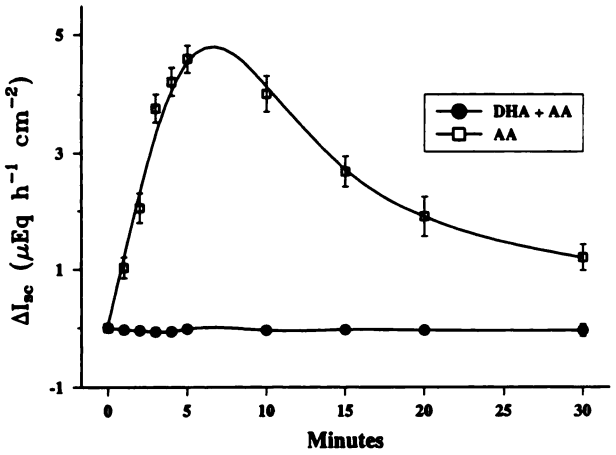

Fig. 1. The I_{SC} time course in tissues stimulated with AA after preincubation with DHA. Paired tissues were prepared from the same animal, as described in Materials and Methods. After a 40–60-min equilibration period in standard Ringer's solution, tissues were allowed to incubate with DHA (1 μM) for 20 min before stimulation with 10 μM AA. Each *point* represents the mean ± standard error of eight experiments.

[1] Serosal exposure of colonic mucosa to 1 μM DHA for up to 60 min did not affect basal I_{SC}.

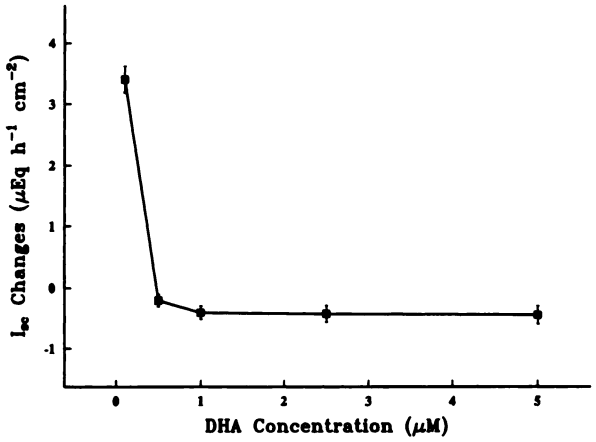

Fig. 2. Concentration-dependent inhibition of AA-stimulated I_{SC} by DHA. Appropriate amounts of DHA were added to the serosal side of paired tissues from the same animal after an equilibration period of 40–60 min. After a 20-min incubation with DHA, tissues were stimulated with 10 μM AA. Each *point* represents the mean ± standard error of the values measured after a 5-min incubation with AA in four experiments.

The DHA inhibition of AA-mediated Cl^- secretion was not prevented by the substitution of serosal solution containing DHA with fresh medium (data not shown).

Additional experiments showed that the inhibition of electrophysiological and ion flux patterns produced by DHA was associated with a reduction in PGE_2 and second messenger cAMP synthesis. As shown in Table 3, incubation with 10 μM AA caused a rise in intracellular PGE_2 and cAMP levels. In contrast, 20-min incubation of mucosa with 1 μM DHA inhibited the cAMP and PGE_2 increase stimulated by 10 μM AA with the same strength as did 10 μM indomethacin. Subsequent experiments indicated that DHA pretreatment did not affect the rate of cAMP generation in tissues stimulated with 10 μM PGE_2 (0.97 ± 0.02 pmol/mg of wet weight in DHA-pretreated tissues versus 1.03 ± 0.03 pmol/mg of wet weight in paired controls, four experiments, $p > 0.05$). All of these findings clearly suggest that DHA does not affect PGE_2 binding and the biochemical events underlying DHA inhibition of the AA response do not involve cAMP regulatory steps.

All of these experiments focused on the possibility that DHA affects the cyclooxygenase activity in intact mucosa. To this end, we assayed the effects of DHA on colonic cyclooxygenase activity. As shown in Fig. 3, DHA inhibited the *in vitro* cyclooxygenase activity in a concentration-dependent manner. The addition of 1 μM DHA to assay medium inhibited cyclooxygenase activity by 82%, whereas indomethacin inhibited the enzyme to a comparable extent (~80%) only at a 5 μM concentration. In accordance with electrophysiological studies in intact mucosa, cyclooxygenase inhibition was critically dependent on the incubation time, because it occurred only 20 min after the addition of DHA to assay medium. This behavior implies that cyclooxygenase inhibition by DHA is mediated not by DHA itself but by some of its transformation products.

Effects on $[Ca^{2+}]_i$. DHA had a dual effect on basal $[Ca^{2+}]_i$, in a concentration-dependent manner. Medium DHA concentrations of 1 and 10 μM induced a drop in basal $[Ca^{2+}]_i$; the lowering effect of DHA on $[Ca^{2+}]_i$ was maximal at 1 μM (−49 ± 7.6%, mean ± standard error, six experiments, $p < 0.001$), decreased at 10 μM (−21 ± 6.9%, mean ± standard error, six

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TABLE 2

Effects of DHA on AA- and PGE_2-mediated Cl^- secretion

Measurements of unidirectional ^{36}Cl fluxes and I_{sc} were performed in paired tissues as described in Materials and Methods. Amiloride (10^{-4} M) was added to the mucosal side in all groups. At the end of the control period tissues were stimulated with 10 μM AA or PGE_2. All values represent the mean ± standard error of *n* paired experiments.

	J_{ms}	J_{sm}	J_{net}	I_{sc}	G_T
	μEq/hr/cm²	μEq/hr/cm²	μEq/hr/cm²	μEq/hr/cm²	mS/cm²
AA					
Control period	4.77 ± 0.15	4.20 ± 0.11	0.57 ± 0.20	0.0 ± 0.1	4.35 ± 0.09
+AA (10 μM) (*n* = 4)	5.11 ± 0.13	7.02 ± 0.24[a]	−1.91 ± 0.20[a]	3.15 ± 0.30[a]	7.22 ± 0.18[a]
DHA + AA					
Control period	4.05 ± 0.09	3.22 ± 0.15	0.83 ± 0.22	0.0 ± 0.1	4.03 ± 0.09
+DHA + AA (*n* = 4)	4.44 ± 0.13	3.76 ± 0.16	0.68 ± 0.30	0.0 ± 0.1	4.11 ± 0.16
PGE_2					
Control period	3.89 ± 0.15	3.38 ± 0.09	0.51 ± 0.06	0.0 ± 0.1	4.02 ± 0.18
+PGE_2 (10 μM) (*n* = 4)	4.14 ± 0.12	6.14 ± 0.12[a]	−2.0 ± 0.20[a]	3.12 ± 0.22[a]	4.52 ± 0.06[a]
DHA + PGE_2					
Control period	4.27 ± 0.11	3.69 ± 0.10	0.58 ± 0.18	0.0 ± 0.1	3.53 ± 0.14
+DHA + PGE_2 (*n* = 4)	4.49 ± 0.05	6.16 ± 0.10[a]	−1.67 ± 0.16[a]	2.41 ± 0.25[a]	4.55 ± 0.14[a]

[a] Significantly different ($p < 0.01$ or less) from control period.

TABLE 3

Effects of DHA pretreatment on PGE_2 and cAMP levels after incubation with AA or PGE_2

In each experiment, paired mucosal preparations from the same animal were processed as described in Materials and Methods and divided into three groups, i.e., the basal group, the control group incubated for 10 min with 10 μM AA (in the absence or presence of indomethacin) or PGE_2, and a group stimulated for 10 min with 10 μM AA or PGE_2 after 20-min pretreatment with 1 μM DHA. The overall incubation times were identical in all samples. Values represent the mean ± standard error of *n* experiments.

	PGE_2	cAMP
	ng/mg of wet weight	pmol/mg of wet weight
Basal (*n* = 4)	0.61 ± 0.04	0.12 ± 0.01
+AA (*n* = 4)	5.43 ± 0.12[a]	0.66 ± 0.02[a]
+Indomethacin + AA (*n* = 4)	0.55 ± 0.10[b]	0.13 ± 0.03[b]
+DHA + AA (*n* = 4)	0.69 ± 0.14[b]	0.16 ± 0.01[b]
+PGE_2 (*n* = 4)		1.03 ± 0.03[a]
+DHA + PGE_2 (*n* = 4)		0.97 ± 0.02[a]

[a] Significantly different ($p < 0.01$ or less) from basal group.
[b] Significantly different ($p < 0.01$ or less) from control group.

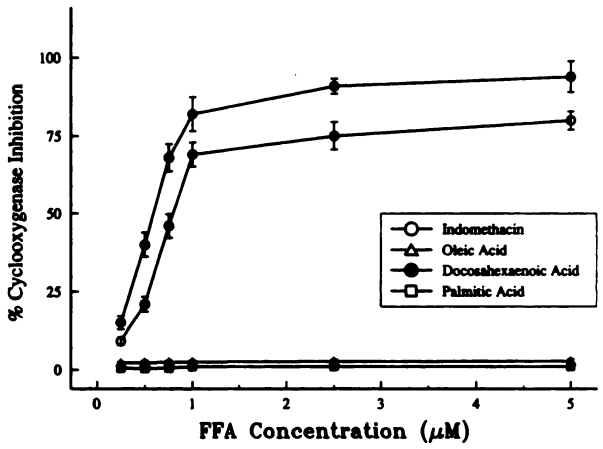

Fig. 3. Effects of free fatty acids (*FFA*) on *in vitro* colonic cyclooxygenase activity. Free fatty acids were incubated with colonic microsomes and buffer for 20 min at 37° before addition of AA to initiate the reaction, which ran for 3 min at 37° (see Materials and Methods). Test samples were compared with paired control incubations containing the same amount of DMSO. The percentage of inhibition was calculated as follows: (cpm control − cpm test)/cpm control × 100. Each *point* represents the mean ± standard error of three experiments.

experiments, $p < 0.001$), and disappeared at concentration of 25 μM.

We next investigated whether the $[Ca^{2+}]_i$ response to other free fatty acids shared the same pattern. Saturated fatty acids, such as palmitic acid, did not have significant effects on $[Ca^{2+}]_i$ at concentrations ranging from 0.5 to 100 μM (data not shown); in contrast, the $[Ca^{2+}]_i$ response to UFAs appeared quite diversified. At medium concentrations ranging from 0.5 to 100 μM oleic acid never induced a decrease in $[Ca^{2+}]_i$, but it brought about a dose-dependent rise in $[Ca^{2+}]_i$ from 10 to 100 μM that was fully prevented by removal of extracellular Ca^{2+}. However, the Ca^{2+} pattern response to EPA, the other main component of fish oil, differed greatly from that elicited by DHA; in fact, the addition of 1–20 μM EPA to fura-2-loaded cells preincubated with 10 μM indomethacin and NDGA did not change basal $[Ca^{2+}]_i$ (110 ± 8.6 versus 104 ± 6.2 nM after exposure to 1 μM EPA, mean ± standard error, six experiments, $p > 0.05$). EPA affected $[Ca^{2+}]_i$ only at doses higher than those attainable *in vivo* (36, 58); at medium concentrations between 30 and 100 μM, it induced a dose dependent $[Ca^{2+}]_i$ rise that was prevented by extracellular Ca^{2+} withdrawal.

These findings led us to examine the effects of DHA in cells where intracellular Ca^{2+} had been mobilized by TG or CPA. The tumor promoter sesquiterpene lactone TG and CPA have been used to study the mechanisms of intracellular Ca^{2+} release. They inhibit microsomal Ca^{2+}-ATPase, thus increasing $[Ca^{2+}]_i$ and promoting Ca^{2+} influx with no activity on the inositol lipid pathway (52). This feature renders them particularly suitable for our purposes, keeping in mind the well recognized effects of DHA and other polyunsaturated fatty acids on the phosphoinositide branch of the Ca^{2+} messenger system (28, 29).

$[Ca^{2+}]_i$ responses to 500 nM TG were seen in ~90% of the cell suspensions (18 experiments). In Ca^{2+}-containing medium, 500 nM TG produced a biphasic response; cytosolic Ca^{2+} peaked with a 20–30-sec lag, and then declined to a plateau of elevated $[Ca^{2+}]_i$ that remained above basal levels (Fig. 4A). However, it was found that DHA did not influence the $[Ca^{2+}]_i$ peak response to TG, but it markedly affected the subsequent sustained phase. In fact, 1 μM DHA addition to TG-treated cells led to a fast decline in $[Ca^{2+}]_i$ within 150 sec of DHA exposure (from 251 ± 8.1 to 141 ± 5.8 nM, mean ± standard error, eight experiments) (Fig. 4C). Equimolar amounts of DHA also inhibited to the

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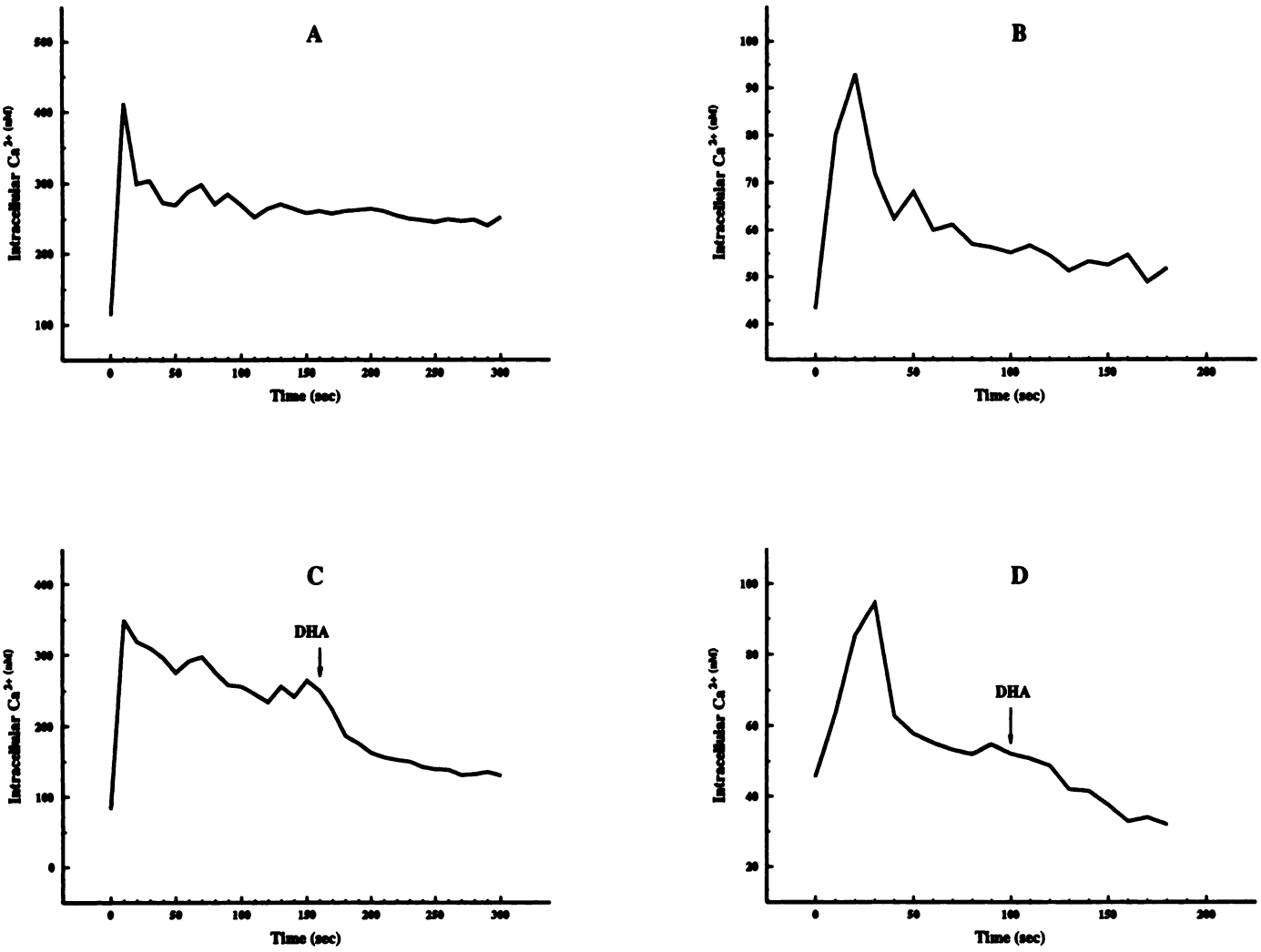

Fig. 4. Effects of DHA on TG-induced $[Ca^{2+}]_i$ increase. Colonic crypt cells (1×10^6/ml) were suspended in MEM buffered with 25 mM HEPES containing 1.25 mM Ca^{2+} (A and C) or 0 mM Ca^{2+} plus 1.2 mM EGTA (B and D) and were loaded with fluorescent dye as described in Materials and Methods. A, Typical $[Ca^{2+}]_i$ increase induced by 500 nM TG under control conditions; B, in Ca^{2+}-free medium, the $[Ca^{2+}]_i$ sustained phase was reduced; C, application of 1 μM DHA (*arrowhead*) resulted in a $[Ca^{2+}]_i$ decrease in the presence of 500 nM TG in 1.25 mM Ca^{2+}-containing medium; D, this effect was not suppressed by the removal of extracellular. Ca^{2+}. TG was added at time 0 in all experiments. *Traces* in A–D are representative of at least eight experiments.

same extent the sustained phase of intracellular Ca^{2+} mobilization induced by 50 μM CPA (data not shown).

To closely examine whether the decrease in Ca^{2+} levels after DHA application ensued from Ca^{2+} influx inhibition or stimulation of Ca^{2+} extrusion mechanisms, we looked at the effects of 1 μM DHA under conditions where no significant Ca^{2+} influx occurred. As shown in Fig. 4B, the magnitude of the plateau phase was reduced in cells stimulated with 500 nM TG in Ca^{2+}-free medium; $[Ca^{2+}]_i$ was 61 ± 4.4 nM after 90 sec and dropped close to basal levels within 180 sec of TG addition (55 ± 5.3 nM, mean ± standard error, eight experiments). However, incubation in the absence of extracellular Ca^{2+} did not prevent the DHA-induced $[Ca^{2+}]_i$ fall (from 58 ± 4.9 nM after 90-sec TG addition to 29 ± 3.7 nM, mean ± standard error, eight experiments, $p < 0.01$) (Fig. 4D). On these bases, it seemed that the $[Ca^{2+}]_i$ decrease after DHA addition was mainly mediated by activation of Ca^{2+} efflux.

Ca^{2+} export from the cell occurs via stimulation of the Na^+/Ca^{2+} exchanger or plasma membrane Ca^{2+}-ATPase. To ascertain whether DHA incubation affects Na^+/Ca^{2+} exchanger activity, we examined the $[Ca^{2+}]_i$ response in cells incubated in Na^+-free medium. As shown in Fig. 5, exposure of colonic cells to DHA decreased $[Ca^{2+}]_i$ also when extracellular Na^+-dependent Ca^{2+} efflux was expected to be inhibited (53–55).

Therefore, DHA is likely to affect the Ca^{2+}efflux pathway by triggering PM Ca^{2+}-ATPase. To address this question, we examined the effects of DHA on PM Ca^{2+}-ATPase activity in highly purified basolateral membranes of crypt cells (Fig. 6). Preincubation with 1 μM DHA for 1 min produced a 470% PM Ca^{2+} pump activity increase (34.31 ± 2.73 versus 6.02 ± 0.50 nmol/mg of protein/min, mean ± standard error, four experiments, $p < 0.0001$), which was substantially unaffected by longer incubations or DHA medium concentrations up to 10 μM (data not shown). However, the stimulatory effects of DHA on PM Ca^{2+}-dependent ATPase activity were more relevant than those exerted by equimolar amounts of oleic acid and EPA, which increased Ca^{2+}-ATPase activity by 17 and 53%, respectively. In contrast, incubations with 1–10 μM palmitic

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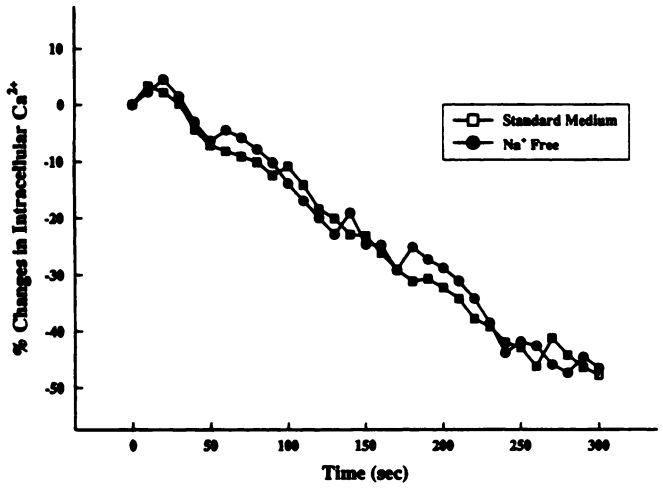

Fig. 5. Effects of isotonic substitution of extracellular Na^+ with choline on $[Ca^{2+}]_i$ upon stimulation with DHA. Isolated cells (1×10^6/ml) were loaded with 2 μM fura-2/AM in standard medium, as described in Materials and Methods, and were then transferred to Na^+-free medium. DHA (1 μM) was added after a 10-min incubation in Na^+-free medium. Data points represent the mean of four experiments; the standard error ranged from 1.6 to 2.7.

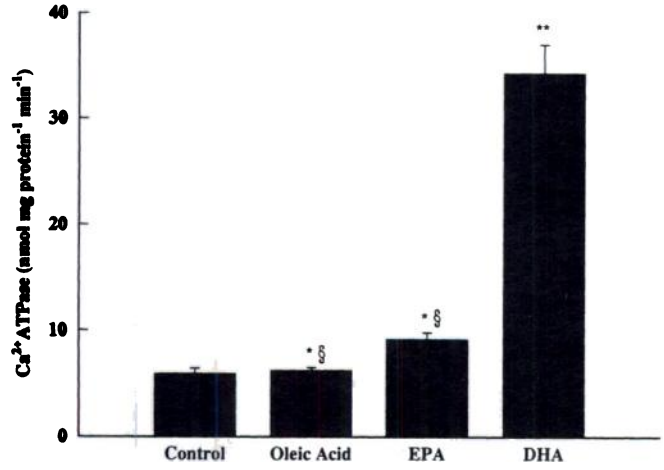

Fig. 6. Effects of UFAs on PM Ca^{2+}-ATPase activity of basolateral membranes of crypt cells. Basolateral membranes of colonic crypt cells (150 μg/ml) were preincubated for 1 min with 1 μM fatty acid to be tested and then the samples were subjected to the Ca^{2+}-ATPase assay, as described in Materials and Methods. Each *bar* represents the mean ± standard error of four paired experiments done in triplicate. Longer incubations (2–5 min) did not produce different results. *, Significantly different (p between 0.04 and 0.01) from control group; **, significantly different ($^*p < 0.0001$) from control group; §, significantly different ($p < 0.001$) from DHA-treated membranes.

TABLE 4

Summary of the main effects of DHA on PG and Ca^{2+} signaling pathways

Biological effects[a]	IC_{50}
	μM
Cyclooxygenase activity ↓	0.6
$[Ca^{2+}]_i$ ↓	1
PM Ca^{2+}-ATPase ↑	0.25

[a] ↓, Decrease; ↑, stimulation.

acid, a saturated fatty acid, did not absolutely affect the PM Ca^{2+}-ATPase activity (data not shown).

On this basis, we asked whether the DHA effects on Ca^{2+} efflux activation were mediated by calmodulin, the main activator of PM Ca^{2+}-ATPase, or cAMP (56). To this end, cells were incubated with 10 μM concentrations of the calmodulin inhibitor W-7 for 10 min and then stimulated with 1 μM DHA. We found that under these conditions DHA induced a fall in $[Ca^{2+}]_i$ similar to that observed in the control group (−47 ± 9.1% versus −45 ± 6.8% in controls, $p > 0.05$, mean ± standard error, six experiments). We also failed to demonstrate a role of cAMP in DHA-mediated Ca^{2+} efflux activation. However, pretreatment with 500 μM IBMX, an inhibitor of cyclic nucleotide phosphodiesterases, failed to potentiate the effects of DHA on $[Ca^{2+}]_i$ (−43 ± 5.2% in IBMX-treated cells versus −45 ± 5.4% in controls, mean ± standard error, $p > 0.05$, four experiments).

Lastly, we investigated whether the effects of DHA could be mediated by protein kinase C. UFAs are powerful activators of protein kinase C (24–27), which, in turn, has been shown to be a calmodulin-independent activator of PM Ca^{2+}-ATPase (57). We found that incubation of cell suspensions with 500 nM concentrations of the protein kinase C inhibitor staurosporine for 10 min before addition of 1 μM DHA did not affect the DHA-induced $[Ca^{2+}]_i$ fall (−52 ± 6.2% versus −54 ± 8.8% in paired controls, $p > 0.05$, mean ± standard error, six experiments). Similar results were obtained after incubation with 10 μM H-7 (data not shown).

Discussion

Effects on PGs. Preincubation with DHA produced a concentration-dependent inhibition of AA-mediated Cl^- secretion in intact mucosa. The AA pathway steps involved in Cl^- secretion and potentially targeted by DHA may be summarized as follows: 1) a potentially sensitive free fatty acid entry pathway (39); 2) cyclooxygenase activation, with consequent PG synthesis by mesenchymal cells (58); 3) binding of PGs to epithelial cell receptors and increases in cAMP levels secondary to stimulation of adenylate cyclase activity; and 4) triggering of Cl^- channel phosphorylation by protein kinase A, with consequent increases in apical membrane conductance (59). However, there is evidence that the effects of DHA on AA response primarily involve the cyclooxygenase activation step. First, the observation that the electrophysiological response to PGE_2 was unaltered in the presence of DHA clearly indicates that, contrary to what occurs in platelets (60), DHA does not affect receptor binding mechanisms; furthermore, the observation that DHA did not prevent the rise in cAMP after stimulation with PGE_2 (see Table 3) demonstrates that the inhibition of AA-mediated Cl^- secretion by DHA does not involve adenylate cyclase- or cyclic phosphodiesterase-dependent mechanisms.

However, it was also considered that the antisecretory effects of DHA might involve Cl^- exit, as determined from the observation that UFAs inhibited Cl^- channels in airway epithelium and the T_{84} colonic cell line (18, 19). To address this question, we assumed that, if this mechanism was involved in DHA inhibition, pretreatment with DHA would also affect the electrogenic Cl^- secretion stimulated by a direct activator of Cl^- channels, such as cAMP (61); however, it was found that, at concentrations inhibiting the response to AA, DHA did not alter the increase in chloride conductance elicited by cAMP. Because DHA inhibition requires a prolonged incubation, it is

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unlikely to be mediated by AA competition for a common entry pathway.

Finally, the conclusion that the DHA effects on the PG signaling pathway were mainly mediated by impairment of the enzyme activity is supported by *in vitro* cyclooxygenase assays, as shown in Fig. 3. Our finding, however, differs somewhat from previous studies (62–66), in that we found that its most distinctive feature was the dependence on incubation time; the inhibition began 15 min after addition of the fatty acid to the assay mixture and was maximal after 20 min. Therefore, this behavior suggests that the impairment of cyclooxygenase activity is mediated by some DHA metabolites and not by DHA itself. This observation gains more relevance from the pharmacological point of view, when it is considered that inhibition displays the same lag and dose dependence required for inhibition of the AA response in whole mucosa. It is, in fact, unlikely that DHA levels in intact cells reach a critical concentration only after 20 min of incubation, because the diffusion of long-chain free fatty acids into colonic mucosa that follows the facilitated diffusion step is already completed within 5 min (39).

Because the effects of EPA on rabbit colon mucosa (I_{SC} rise and stimulation of net Cl^- secretion) are entirely mediated by the activation of an indomethacin-sensitive pathway,[2] retroconversion to EPA is unlikely to be involved in the cyclooxygenase inhibition by DHA.

DHA is not a substrate for cyclooxygenase (63), but it is converted in human platelets and neutrophils to hydroxy acids (7-, 11-, and 14-HDHE) (67, 68). At present, the physiological role of these products, which are released in negligible amounts, is unknown. 14-HDHE has been shown to inhibit U46619-induced platelet aggregation (69), but it is uncertain whether this mechanism operates *in vivo*. However, it is remarkable that the maximal rate of production of these hydroxy acids occurs at times (12–15 min) (67) similar to those required to obtain *in vitro* inhibition of cyclooxygenase activity by DHA; on this basis, the role of HDHEs in DHA inhibition of PG synthesis should be assessed.

Apart from the uncertainties pertaining to the role of DHA metabolites, there is no doubt that DHA is a powerful inhibitor of PG synthesis in rabbit colon.

Effects on $[Ca^{2+}]_i$. Several lines of evidence indicate that the primary effect of DHA on $[Ca^{2+}]_i$ was mediated by changes in the Ca^{2+} efflux pathway. To address the question of whether the $[Ca^{2+}]_i$ decreased induced by 1 μM DHA arose from reduction in Ca^{2+} entry or increase in Ca^{2+} efflux, we examined the $[Ca^{2+}]_i$ response in cells maintained in Ca^{2+}-free medium. The rationale of these experiments was that, if DHA inhibited Ca^{2+} influx, no effects on $[Ca^{2+}]_i$ were expected under conditions in which basal Ca^{2+} influx was blocked. However, the observation that the stimulation of cells in Ca^{2+}-free medium did not prevent the DHA-induced $[Ca^{2+}]_i$ decrease clearly indicates that DHA triggers Ca^{2+} extrusion mechanisms.

In view of the effects of DHA on basal $[Ca^{2+}]_i$, we considered its potential role as a modulator of Ca^{2+} pathway activation. As shown in Fig. 4C, DHA did not affect the early $[Ca^{2+}]_i$ response to TG, whereas it fully suppressed the sustained component of the TG-induced $[Ca^{2+}]_i$ response. According to the proposal by Putney (70) of a capacitative model, this second phase of $[Ca^{2+}]_i$ response derives from increased Ca^{2+} permeability of the PM, induced by depletion of intracellular Ca^{2+} stores. On this basis, the sustained component of $[Ca^{2+}]_i$ response to TG could be considered the result of a dynamic balance between Ca^{2+} influx and efflux (71). In keeping with these observations, we found that the removal of extracellular Ca^{2+} blunted the sustained component of $[Ca^{2+}]_i$ response to TG (Fig. 4B); however, when the TG-induced influx was prevented by the absence of extracellular Ca^{2+}, DHA was still able to reduce $[Ca^{2+}]_i$ (Fig. 4D). The outcome of this protocol strongly indicates that the fall in $[Ca^{2+}]_i$ after DHA application was due to activation of the Ca^{2+} extrusion system and not to inhibition of Ca^{2+} influx.

Ca^{2+} extrusion is carried out by the PM Ca^{2+}-ATPase and $Na^{+\pm}/Ca^{2+}$ exchanger. The finding that free fatty acids stimulate the exchanger in cardiac sarcolemmal vesicles (72) led us to investigate whether the stimulatory effects of DHA on Ca^{2+} efflux could be mediated by exchanger activation. However, despite the inhibition of the exchanger produced by the replacement of external Na^+ by choline, DHA reduced $[Ca^{2+}]_i$ to the same extent as in paired controls (Fig. 5). Therefore, DHA seems to activate Ca^{2+} efflux through a mechanism other than the Na^+/Ca^{2+} exchanger.

The finding that DHA strongly stimulated PM Ca^{2+}-ATPase activity in basolateral membranes of crypt cells suggests that the effects of DHA on the Ca^{2+} pathway are primarily mediated by activation of the PM Ca^{2+} pump (Fig. 6). In addition, we found that the enzyme could also be stimulated, although to a lesser extent, by other UFAs. It is noteworthy that EPA, the other main component of fish oil, failed to affect $[Ca^{2+}]_i$ in intact cells at doses able to stimulate by 53% the Ca^{2+} pump in basolateral membranes; this finding implies that shifts in steady state $[Ca^{2+}]_i$ produced by PM Ca^{2+}-ATPase stimulation require a pumping rate well above that elicited by EPA. Alternatively, the possibility cannot be excluded that the *in vitro* assay may overestimate the size of the PM Ca^{2+}-ATPase stimulation by free fatty acids, compared with what occurs in intact cells. Lastly, the effects of free fatty acids on enzyme activity seem to be related to their degree of unsaturation, because palmitic acid, a saturated fatty acid, did not affect enzyme activity and the stimulatory effects of UFAs on the PM Ca^{2+} pump increased with increasing numbers of double bonds.

Because the lowering effects of DHA on $[Ca^{2+}]_i$ appeared to be independent of cAMP- or calmodulin- and protein kinase C-mediated mechanisms, it is conceivable that DHA triggers Ca^{2+} extrusion by direct activation of the PM Ca^{2+} pump. This conclusion is further supported by the observation that UFAs may activate PM Ca^{2+}-ATPase *in vitro* by calmodulin-independent mechanisms (73); in addition, membrane-embedded proteins, such as ion-transporting ATPases, are largely recognized to be highly sensitive to changes in membrane fluidity induced by polyunsaturated fatty acids (for review see Refs. 15 and 74). On this basis we may suppose that the effects of DHA on Ca^{2+} extrusion mechanisms are a simple consequence of membrane fluidity changes perturbing Ca^{2+}-ATPase activity (75). Nevertheless, we cannot exclude the involvement of more specific mechanisms if we consider that DHA was the sole fatty acid able to significantly stimulate Ca^{2+} efflux in rabbit colon.

The effects of DHA on Ca^{2+} extrusion mechanisms may have a remarkable pharmacological significance. The increased Ca^{2+}

[2] V. Calderaro, C. Parrillo, and F. Rossi. The effects of eicosapentaenoic acid on chloride secretion in rabbit colon. Manuscript in preparation.

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efflux rate during the sustained phase of the receptor-activated Ca^{2+} response (76) is mainly accomplished by triggering the PM Ca^{2+} pump, which thus appears to be the major mechanism of Ca^{2+} export from the cell (77). The Ca^{2+}-ATPase is therefore a "defense system" (78) that re-establishes basal $[Ca^{2+}]_i$ after a Ca^{2+} signaling event has occurred. Hence, a substance, like DHA, that accelerates the recovery of basal $[Ca^{2+}]_i$, may have a significant impact on cellular processes such as cell division and growth, secretion, inflammation, etc., occurring in the sustained phase of the $[Ca^{2+}]_i$ response.

In summary, the bulk of data arising from this study show that DHA strongly affects PG and Ca^{2+} signaling pathways, through separate mechanisms (see Table 4); it reduces PG synthesis through cyclooxygenase inhibition, probably mediated by one of its metabolites, and influences the $[Ca^{2+}]_i$ response by activation of PM Ca^{2+}-ATPase. Because DHA concentrations close to those achievable *in vivo* after fish oil feeding, ~10 μM (36, 79), appear to down-regulate the cellular response in situations where high levels of the second messengers Ca^{2+} and cAMP may be attained, it is tempting to speculate that DHA could be responsible, at least to a significant extent, for many of the aforementioned beneficial effects of fish oil. Therefore, careful examinations of the *in vivo* effects of low doses of DHA administered alone should be done to address this issue.

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