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ORIGINAL ARTICLE

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Exposure to the n-3 polyunsaturated fatty acid docosahexaenoic acid impairs α_1-adrenoceptor-mediated contractile responses and inositol phosphate formation in rat cardiomyocytes

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Abstract The beneficial effects of n-3 polyunsaturated fatty acids of fish oil in the prevention of fatal arrhythmias in myocardial ischemia were suggested to be at least in part mediated by a modulation of dihydropyridine-sensitive L-type calcium channels. As cardiac α_1-adrenoceptor stimulation has been suggested to have no significant effect on L-type calcium channels, the aim of this study using cultured neonatal rat cardiomyocytes was to investigate whether chronic n-3 polyunsaturated fatty acid exposure may have an influence on α_1-adrenoceptor-induced positive inotropic effects and induction of arrhythmias. Pretreatment of the rat cardiomyocytes for 3 days in the presence of the n-3 polyunsaturated fish oil-derived fatty acid docosahexaenoic acid (60 μmol/l) markedly decreased α_1-adrenoceptor-stimulated increase in contraction velocity and induction of arrhythmias. The increase in contraction velocity of the cardiomyocytes induced by the β-adrenoceptor agonist isoprenaline was also markedly reduced by the n-3 fatty acid pretreatment. Basal contractile amplitude and spontaneous beating frequency of the cardiomyocytes were not significantly altered by the docosahexaenoic acid exposure. The pretreatment of the rat cardiomyocytes for 3 days in the presence of docosahexaenoic acid (60 μmol/l) decreased α_1-adrenoceptor-stimulated formation of the calcium-mobilizing second messenger IP_3 and its metabolites IP_2 and IP_1 by 55%. The depression of IP_3 formation by docosahexaenoic acid treatment was not mediated by a decreased uptake of myo-inositol into the cardiomyocytes nor by a decreased synthesis of phosphatidylinositol bisphosphate (PIP_2), the substrate of phospholipase C. The level of glycerol-3-phosphate, an important substrate of the phosphoinositide cycle, was unaltered by the docosahexaenoic acid pretreatment. Receptor binding studies revealed that the dissociation constant and maximal binding capacity of the α_1-adrenoceptor antagonist (^{3}H)prazosin was unchanged by the n-3 polyunsaturated fatty acid exposure. β-Adrenoceptor- and forskolin-stimulated adenylyl cyclase activities were not diminished by the docosahexaenoic acid pretreatment. Chronic exposure of the cardiomyocytes to the n-6 polyunsaturated fatty acid arachidonic acid (60 μmol/l) did neither significantly alter α_1-adrenoceptor-induced inositol phosphate formation nor α_1-adrenoceptor-stimulated increase in contraction velocity. The results presented show that chronic n-3 polyunsaturated fatty acid pretreatment of rat cardiomyocytes leads to a marked impairment of α_1-adrenoceptor-induced positive inotropic effects and induction of arrhythmias concomitant with a n-3 fatty acid-induced decrease in IP_3 formation. This derangement of the phosphoinositide pathway by chronic n-3 fatty acid exposure may, thus, contribute to the beneficial effects of fish oil-derived fatty acids in the prevention of fatal arrhythmias in myocardial ischemia.

Key words α_1-adrenoceptor · β-adrenoceptor · Inositol triphosphate · n-3 fatty acid · Cardiomyocytes

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Introduction

Dietary n-3 polyunsaturated fatty acids (PUFA's) became the target of extensive studies due to their possible beneficial influence on heart disease (Schmidt and Dyerberg 1994). Eicosapentaenoic acid (C20:5 n-3, EPA) and docosahexaenoic acid (C22:6 n-3, DHA), the major PUFA's in fish oil, have been shown to be readily incorporated in the phospholipids of many tissues including the cardiovascular system (Bordoni et al.

1990; Otten et al. 1993). Studies by McLennan et al. (1989, 1992) demonstrated in rats and monkeys a beneficial effect of dietary fish oils in the prevention of fatal ventricular fibrillation induced by ischemia. The mechanisms by which the fatty acids of fish oil may prevent fatal ventricular tachyarrhythmias are unknown.

Hallaq et al. (1990a, b) have previously reported that pretreatment of neonatal rat cardiomyocytes for 3–5 days in the presence of the n-3 fatty acids, EPA and DHA, but not of the n-6 PUFA arachidonic acid, completely prevented the toxic (arrhythmogenic) effects of the cardiac glycoside ouabain and of the L-type calcium channel agonist Bay K 8644 concomitant with a decrease in the level of cytosolic free calcium in response to ouabain and Bay K 8644. This effect was apparently not due to an alteration of ouabain-inhibited Na,K,ATPase activity (Hallaq et al. 1990a). The authors suggested that the prevention of arrhythmias in rat cardiomyocytes by n-3 PUFA exposure results from their modulatory effects on dihydropyridine-sensitive L-type calcium channels (Hallaq et al. 1990b). α_1-Adrenoceptor-mediated arrhythmias were previously suggested to play a considerable role in myocardial ischemia and reperfusion (Corr and Pogwizd 1988).

α_1-Adrenoceptor-mediated stimulation of the phosphoinositide pathway involves the receptor-mediated hydrolysis of the membrane phospholipid phosphatidylinositol bisphosphate (PIP_2) by a phosphatidylinositol (PI)-specific phospholipase C. PIP_2 is cleaved to diacylglycerol (DAG) which stimulates various isoforms of protein kinase C and inositol trisphosphate (IP_3) which releases Ca^{2+} from intracellular stores. Although no doubt exists about the occurrence of a significant elevation of IP_3 and Ca^{2+} due to α_1-adrenoceptor stimulation in the heart, the mechanisms by which activation of the phosphoinositide cycle mediates positive inotropic responses and induction of arrhythmias are not fully elucidated (De Jonge et al. 1995). As the changes in intracellular calcium concentration were reported to be too transient to explain the sustained positive inotropic effects, it is currently suggested that the positive inotropic effect of α_1-adrenoceptor stimulation is largely the result of an increase in myofibrillar Ca^{2+} sensitivity. Intracellular alkalinization may also contribute to the positive inotropic effect of α_1-adrenoceptor stimulation (Benfey 1993, De Jonge et al. 1995).

Cardiac α_1-adrenoceptor stimulation is suggested to have no significant effect on L-type calcium channels (Benfey 1993). The aim of this study was, therefore, to investigate first whether α_1-adrenoceptor-induced positive inotropic effects and induction of arrhythmias in rat cardiomyocytes may also be prevented by n-3 PUFA pretreatment, and second to determine the possible mechanisms by which n-3 PUFA's may alter α_1-adrenoceptor-mediated contractile effects in these cells.

Materials and methods

Materials. ATP, GTP, cyclic AMP, creatine kinase, iron-saturated transferrin, NAD and glycerol-3-phosphate dehydrogenase (EC: 1.1.1.8.) were from Boehringer (Mannheim, Germany). Recombinant human tumor necrosis factor α (TNF α), creatine phosphate, isoprenaline, forskolin, 3-isobutyl-1-methylxanthine, dexamethasone, bovine insulin, hydrazine, noradrenaline, timolol, prazosin, arachidonic acid and cis-4,7,10,13,16,19-docosahexaenoic acid (DHA) were from Sigma (Deisenhofen, Germany). Collagenase "Worthington" (125–250 U/ml, CLS II), fetal calf serum, horse serum and CMRL 1415 ATM medium were from Biochrom (Berlin, Germany). (^{3}H)myo-inositol (17.1 Ci/mmol), (^{3}H)prazosin (72.2 Ci/mmol) and (α-^{32}P)ATP were from DuPont (Dreieich, Germany). AG1X8 anion exchange columns (formate form) were from BIO-RAD Laboratories (Munich, Germany).

Cell culture techniques. Preparation and cultivation of cardiac muscle cells have been previously described in detail (Werdan and Erdmann 1989). In brief, the procedures are: preparation of 50–100 hearts from neonatal rats under sterile conditions, disaggregation of heart tissue at 37 °C with trypsin (0.12%)-collagenase (0.03%)-salt solution (Ca^{2+}, Mg^{2+}-free), seeding of the cells ($(1–2) \times 10^5$ cells/cm^2) in Nunclon plastic flasks (Nunclon Plastics, Roskilde, Denmark) in CMRL 1415 ATM medium supplemented with 10% fetal calf serum, 10% horse serum and 0.02 mg/ml gentamicin. After 24 h of culture, serum containing medium was replaced by a serum-free medium (CMRL 1415 ATM medium containing 4 µmol/l insulin, 0.1 µmol/l dexamethasone, 0.4 µmol/l iron-saturated transferrin and 0.4 µmol/l or 40 µmol/l bovine serum albumin) supplemented with 10 mmol/l HEPES, pH 7.4. The cells were cultured for 72 h with daily medium changes in the presence of the various concentrations of DHA dissolved in ethanol. The identical amount of ethanol was added to all samples. In some experiments the cells were cultured in the presence of various concentrations of TNFα for 72 h with daily medium changes.

For preparation of crude membranes (adenylyl cyclase activity), the cells were then washed three times with 5 ml ice-cold buffer A (20 mmol/l Tris/HCl, pH 8.0, 1 mmol/l EDTA, 1 mmol/l dithiothreitol). The cells were then removed from the plastic culture flasks by scraping and then homogenized (10 strokes). The homogenates were centrifuged at $40.000 \times g$ for 30 min at 4 °C. The pellets (crude membranes) were resuspended in buffer A to a concentration of 1–5 mg/ml. Crude membranes were snap frozen in liquid nitrogen and stored at − 70 °C. Cellular protein, membrane protein, and cytoplasmic protein was determined according to Lowry et al. (1951).

Determination of inositol phosphates and inositol phospholipids. Quantitation of inositol phosphate formation and inositol phospholipid synthesis have been previously described in detail (Reithmann and Werdan 1994). In brief, the cardiomyocyte monolayer was exposed for 2.5 h to an incubation buffer (1.0 ml) (135 mmol/l NaCl, 0.97 mmol/l $MgSO_4$, 1.3 mmol/l $CaCl_2$, 4.5 mmol/l KCl, 0.36 mmol/l NaH_2PO_4, 2.4 mmol/l Na_2HPO_4, 10 mmol/l HEPES, 7.5 mmol/l glucose) supplemented with 30 µCi/ml (^{3}H)myo-inositol (Kaku et al. 1991). After the labeling period, the cardiomyocyte monolayer was incubated for 15 min at 37 °C in the presence of LiCl (10 mmol/l)-containing incubation buffer and then incubated for 15 min at 37 °C in this buffer in the absence ("basal") or presence of 100 µmol/l noradrenaline + 10 µmol/l timolol ("α_1-adrenoceptor-stimulated"). Termination of the reaction was performed by washing the monolayer twice with ice-cold Ca^{2+}-sorbitol solution. In most of the experiments (only determination of inositol phosphate formation) 1 ml of 0.8 mol/l perchloric acid was then added to the cells (30 min at 4 °C). After neutralization with 2 mmol/l KOH containing 0.1 mol/l HEPES, the samples were applied to Dowex AG1X8 anion-exchange columns

according to the method of Berridge et al. (1983). Inositol was eluted with H_2O (16 ml), glycerophosphoinositol with 5 mmol/l disodium tetraborate plus 60 mmol/l sodium formate (8 ml), IP_1 with 0.1 mol/l formic acid plus 0.2 mol/l ammonium formate (8 ml), IP_2 with 0.1 mol/l formic acid plus 0.4 mol/l ammonium formate (8 ml) and IP_3 with 0.1 mmol/l formic acid plus 1.0 mol/l ammonium formate (8 ml).

In some of the experiments (determination of inositol phosphates and inositol phospholipids), the cells were removed from the plastic culture flasks by scraping subsequently after termination of the reaction with ice-cold Ca^{2+}-sorbitol solution. After the addition of 1.0 ml of acidified chloroform-methanol-HCl (500:1000:2), the lipids were extracted for 30 min at room temperature (Steinberg et al. 1989). Chloroform (310 µl) and water (310 µl) were then added to separate the phases (Scholz et al. 1988). 900 µl of the upper aqueous phase were removed and were neutralized with 0.85 mol/l KOH containing 0.1 mol/l HEPES prior to separation of inositol phosphates on Dowex AG1X8 anion exchange columns (formate form) according to the method of Berridge et al. (1983) (see above). 400 µl of the lower chloroform phase were removed for the determination of inositol phospholipids according to the method of Creba et al. (1983): The lipids were dried under a stream of N_2 at 4 °C and then dissolved in 0.5 ml of chloroform to which was added 0.1 ml of methanol and 0.1 ml of 1 mol/l NaOH in methanol/water (19:1, v/v). After 20 min at room temperature, 0.5 ml of chloroform, 0.3 ml of methanol and 0.3 ml of water were added and the samples were centrifuged. Of the upper aqueous phase 0.5 ml was removed and neutralized with 1 mol/l boric acid (400 µl). Samples were then diluted to 5 ml with the addition of ammonium formate and disodium tetraborate so as to give final concentrations of 0.18 mol/l and 5 mmol/l, respectively (Creba et al. 1983). Samples were applied to Dowex AG1X8 columns (formate form). PI was eluted with 5 mmol/l disodium tetraborate plus 0.18 mol/l ammonium formate (eluate from loading the column and 20 ml 5 mmol/l tetraborate/0.18 mol/l ammonium formate), PIP with 0.1 mol/l formic acid plus 0.3 mol/l ammonium formate (20 ml) and PIP_2 with 0.1 mol/l formic acid plus 0.75 mol/l ammonium formate (20 ml). In other series of experiments (after pretreatment in the presence or absence of myo-inositol) cells were pretreated with 10^6 cpm/ml $^{32}P_i$ for 70 min (Creba et al. 1983). Thereafter, termination of the reaction with ice-cold Ca^{2+}-sorbitol-solution, addition of acidified chloroform-methanol-HCl (500:1000:2) and determination of the phospholipids (^{32}P)PIP and (^{32}P)PIP_2 were performed as described above.

Each experiment shown herein was performed at least three times using different cardiomyocyte preparations with similar results being obtained. Representative experiments are shown with the individual experimental error.

α_1-Adrenoceptor binding assay. The conditions for binding of the α-adrenoceptor antagonist (^{3}H) prazosin to intact rat cardiomyocytes were as follows: a total volume of 4.1 ml incubated for 30 min at 37 °C in 25 cm^2 plastic culture flasks. Studies on time kinetics of (^{3}H)prazosin (0.3 nmol/l) binding revealed a maximum of specific (^{3}H)prazosin binding after 15 min remaining constant over a period of about 90 min. The concentration of (^{3}H)prazosin binding was 0.02–1,3 nmol/l in saturation experiments or 0.3 nmol/l in the other studies. Non-specific binding was determined in the presence of unlabelled 0.1 µmol/l prazosin and was subtracted from total binding. Non-specific binding was about 30% of total binding at a (^{3}H)prazosin concentration of 0.3 nmol/l. The equilibrium dissociation constants (K_D) for (^{3}H)prazosin were derived from linear Scatchard plots (Scatchard 1949).

Adenylyl cyclase assay. Adenylyl cyclase activity of cardiomyocyte membranes was determined in a reaction mixture containing 50 µmol/l (α-^{32}P)ATP, 5 mmol/l $MgCl_2$, 0.1 mmol/l EGTA, 1 mmol/l dithiothreitol, 0.1 mmol/l cyclic AMP, 1 mmol/l 3-isobutyl-1-methylxanthine, 5 mmol/l creatine phosphate, 0.4 ng/ml creatine kinase and the additions indicated in 50 mmol/l triethanolamine/HCl, pH 7.4, in a total volume of 100 µl. The reaction was started by addition of the membranes (100–200 µg) and performed for 20 min at 30 °C. Termination of the reaction and isolation of cyclic AMP formed were carried out as described (Jakobs et al. 1976).

Contractility experiments. Measurements of contraction amplitude and contraction velocity of rat cardiomyocytes was carried out using an electro-optical monitoring system previously described in detail (Werdan and Erdmann 1989; Reithmann and Werdan 1989). The contractility measurements were made on one cell per cardiomyocyte monolayer. Baseline amplitude of contraction was determined in spontaneously beating rat cardiomyocytes. For determination of agonist-stimulated increase in contraction amplitude and contraction velocity (dy/dt) plastic culture flasks with an attached monolayer of heart muscle cells were continuously superfused with serum-free, HEPES (10 mmol/l)-buffered incubation medium (lowered Ca^{2+} = 0.6 mmol/l or 0.9 mmol/l; 1 mmol/l ascorbic acid; temperature 37 °C) in the presence of 100 µmol/l noradrenaline plus 10 µmol/l timolol, 10 µmol/l isoprenaline or 10 µmol/l ouabain. Contraction amplitude and contraction velocity of electrically driven cells (external stimulation at 100 V, pulse duration 5 ms) were continuously registrated. After pretreatment for 3 days under the various conditions, the cardiomyocytes were superfused first with incubation medium without any additions for 5 min, then with 100 µmol/l noradrenaline plus 10 µmol/l timolol or with 10 µmol/l isoprenaline or with 10 µmol/l ouabain for 5 min.

Determination of cellular glycerol-3-phosphate level. For preparation of cytoplasmic fractions 600 µl of 0.6 mol/l perchloric acid were added to the cells (30 min at 4 °C). After neutralization with 5 mol/l potassium carbonate (K_2CO_3), cellular glycerol-3-phosphate level in rat cardiomyocyte cytoplasmic fractions was determined enzymatically with glycerol-3-phosphate dehydrogenase (GDH) according to Bergmeyer et al. (1983) using the following assay:

$$\text{Glycerol-3-phosphate} + NAD^+ \xrightarrow{GDH} \text{dihydroxyacetone phosphate} + NADH + H^+$$

The reaction mixture contained 0.189 mol/l hydrazine, 0.47 mol/l glycine, 2.7 mmol/l EDTA, 2.3 mmol/l NAD, 1.0 ml of deproteinized, neutralized sample and 1000 U/l of GDH suspension (0.02 ml) in a total volume of 2.12 ml. The change in adsorbance which occurs 20 min after addition of GDH suspension was determined at a wave length of 339 nm (NADH) using an Eppendorf (Hamburg, Germany) 1101 M photometer (light path 10 mm).

Statistical determination. Means are given as M ± SD. Statistical determinations were performed using the Student's *t*-test for unpaired observations with $P < 0.05$ taken as significant.

Results

α_1-Adrenoceptor-stimulated increase in contraction velocity of rat cardiomyocytes

Maximal α_1-adrenoceptor-induced increases in contraction velocity were induced by superfusion of rat cardiomyocytes with 100 µmol/l noradrenaline (in the presence of the β-adrenoceptor antagonist timolol (10 µmol/l) (Fig. 1). The α_1-adrenoceptor-mediated effects on contraction velocity of rat cardiomyocytes do not occur at high heart rates: In rat cardiomyocytes with a spontaneous beating rate > 250/min (335 ± 75)

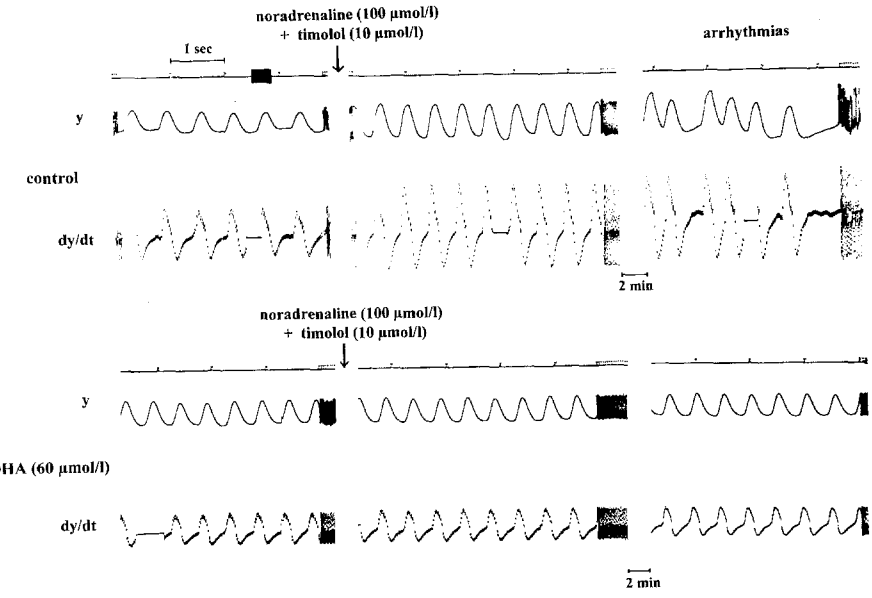

Fig. 1 Influence of DHA pretreatment on α_1-adrenoceptor-stimulated increase in contraction velocity and induction of arrhythmias. Rat cardiomyocytes were pretreated for 3 days in the absence (control) or presence of 60 µmol/l DHA. Thereafter, electrically driven cells were superfused for 5 min in the presence of 100 µmol/l noradrenaline + 10 µmol/l timolol. Contraction amplitude (y) and contraction velocity (dy/dt) are shown. Calibration line is indicated

α_1-adrenoceptor-stimulation by 100 µmol/l noradrenaline (in the presence of 10 µmol/l timolol) did not significantly increase contraction velocity (increase by 8 ± 10%). In contrast, in rat cardiomyocytes with a spontaneous beating frequency < 250/min (157 ± 45) superfusion of the cardiomyocyte monolayer with 100 µmol/l noradrenaline + 10 µmol/l timolol increased contraction velocity by 42 ± 27% (M ± SD, $n = 19$) ($P < 0.05$).

DHA pretreatment decreases α_1-adrenoceptor-induced contractile responses in rat cardiomyocytes

To study the effect of chronic treatment with the n-3 PUFA DHA on contractile responsiveness of rat cardiomyocytes, the cells were treated for up to 72 h in the presence of 60 µmol/l DHA. Baseline contraction amplitude of spontaneously beating rat cardiomyocytes was not significantly different in control cells (treated in the absence of DHA) and in cells pretreated for 72 h in the presence of 60 µmol/l DHA (Table 1). To investigate the influence of chronic DHA pretreatment on α_1-adrenoceptor-induced increase in contraction velocity, the rat cardiomyocytes were electrically stimulated at a stimulation rate slightly (about 20/min) above the spontaneous beating rate of the cells and were continuously superfused with 100 µmol/l noradrenaline and 10 µmol/l timolol for 5 min. Cardiomyocytes with a spontaneous beating rate > 250/min were excluded as α_1-adrenoceptor-induced increases in dy/dt were not observed at these high beating frequencies. Spontaneous beating rate was 157 ± 45 (M ± SD, $n = 19$) in control cells and 156 ± 36 (M ± SD, $n = 14$) in DHA-pretreated cells. Superfusion with noradrenaline (100 µmol/l) and timolol (10 µmol/l) increased contraction velocity of control cells by 42 ± 27% but had no significant effect on contraction velocity of DHA-pretreated cardiomyocytes (increase by 2 ± 9%) ($P < 0.05$) (Table 1, Fig. 1).

Table 1 Effect of DHA pretreatment on contractility of rat cardiomyocytes. Rat cardiomyocytes were pretreated for 3 days in the presence of DHA (60 µmol/l). Baseline contraction amplitude and basal contraction rate of spontaneously beating cardiomyocytes were determined. Electrically driven cardiomyocytes were superfused with noradrenaline (100 µmol/l) and timolol (10 µmol/l) (α_1-adrenoceptor-stimulated) or with isoprenaline (10 µmol/l) (β-adrenoceptor-stimulated) or with the cardiac glycoside ouabain (10 µmol/l) for 5 min, and the effect on contraction velocity (dy/dt) of the cardiomyocytes was determined as described in "Methods"

Pretreatment	Control	DHA (60 µmol/l)
Baseline contraction amplitude (µm)	3.27 ± 1.54 ($n = 29$)	3.92 ± 1.90 ($n = 28$)
Basal contraction rate (min^{-1})	157 ± 45 ($n = 19$)	156 ± 36 ($n = 14$)
α_1-adrenoceptor-induced increase in dy/dt (% of control)	142 ± 27 ($n = 19$)	102 ± 9* ($n = 14$)
α_1-adrenoceptor-mediated induction of arrhythmias	23/28 (82%)	2/15 (13%)
β-adrenoceptor-stimulated increase in dy/dt (% of control)	143 ± 20 ($n = 11$)	106 ± 10* ($n = 10$)
Ouabain-induced increase in dy/dt (% of control)	126 ± 5 ($n = 4$)	106 ± 8* ($n = 4$)

* Values were significantly different from control values, $P < 0.05$

In control cells α_1-adrenoceptor-stimulation by noradrenaline (100 µmol/l) and timolol (10 µmol/l)

induced persistent arrhythmias in 23 of 28 (82%) cardiomyocyte monolayers. In contrast, after pretreatment with DHA (60 μmol/l) for 3 days persistent arrhythmias only occurred in 2 of 15 (13%) cardiomyocyte monolayers (Table 1, Fig. 1).

In contrast to the effect of chronic n-3 PUFA treatment with DHA, the pretreatment with the n-6 PUFA arachidonic acid (60 μmol/l) did not significantly alter α_1-adrenoceptor-mediated contractile responses: Following chronic arachidonic acid (60 μmol/l) pretreatment for 3 days, superfusion of the cardiomyocytes with noradrenaline plus timolol induced an increase in contraction velocity by $35 \pm 4\%$ ($n = 4$). Spontaneous beating rate of arachidonic acid-pretreated cardiomyocytes was 160 ± 40 ($n = 4$). α_1-adrenoceptor-induced arrhythmias occurred in 4 of 4 monolayers of arachidonic acid-pretreated rat cardiomyocytes.

DHA pretreatment decreases β-adrenoceptor agonist-induced increase in contraction velocity of rat cardiomyocytes

The contractile response of the cardiomyocytes to β-adrenoceptor stimulation following DHA (60 μmol/l) pretreatment was studied by superfusion of the electrically driven rat cardiomyocytes for 5 min in the presence of the β-adrenoceptor agonist isoprenaline (10 μmol/l). In control cells (absence of DHA) the superfusion with the β-adrenoceptor agonist lead to an increase in contraction velocity of rat cardiomyocytes by $43 \pm 20\%$ ($M \pm SD$, $n = 11$), whereas in DHA-pretreated cells no significant increase in contraction velocity was observed (increase by $6 \pm 10\%$) ($n = 10$) ($P < 0.05$) (Table 1, Fig. 2). These data indicate that the β-adrenoceptor-mediated "positive inotropic" effect was also depressed by the n-3 PUFA pretreatment.

It has previously been reported that chronic DHA pretreatment of neonatal rat cardiomyocytes prevented the toxic (arrhythmogenic) effects of the cardiac glycoside ouabain (Hallaq et al. 1990a). In this study, the effect of ouabain on contraction velocity of rat cardiomyocytes was investigated: Ouabain increased contraction velocity of control cells by $26 \pm 5\%$ ($n = 4$) whereas in DHA (60 μmol/l)-pretreated cells ouabain had virtually no effect on contraction velocity (increase by $6 \pm 8\%$, $n = 4$) ($P < 0.05$) (Table 1).

DHA pretreatment decreases α_1-adrenoceptor-stimulated inositol phosphate formation in rat cardiomyocytes

To study a possible mechanism of the decrease in α_1-adrenoceptor-mediated contractile responsiveness, the rat cardiomyocytes were pretreated for up to 72 h in the absence or presence of the n-3 PUFA DHA or the n-6 PUFA arachidonic acid. The effects of the PUFA's were tested in a serum-free medium supplemented with 25 mg/l or 2500 mg/l albumin as cardiac sarcolemmal membrane integrity has been reported to be affected when non-esterified fatty acid to albumin ratio exceeds 5 (Lamers et al. 1984). The concentration of albumin present in culture medium (25 or 2500 mg/l) had no significant effect on inositol phosphate formation in the absence or presence of DHA (60 μmol/l). The α_1-adrenoceptor agonist exposure of the cells was carried out in the presence of LiCl (10 mmol/l) which has been shown to block the breakdown of IP_1 to inositol (Berridge et al. 1982). α_1-Adrenoceptor responsiveness of rat cardiomyocytes was tested by exposure

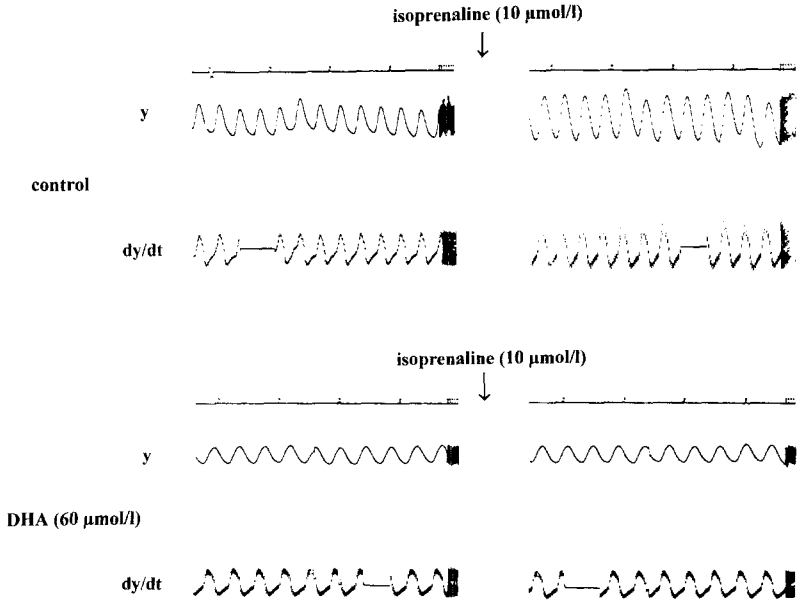

Fig. 2 Influence of DHA exposure on β-adrenoceptor-induced increase in contraction velocity of rat cardiomyocytes. After pretreatment for 3 days in the absence (control) or presence of DHA (60 μmol/l), electrically driven rat cardiomyocytes were superfused for 5 min in the presence of the β-adrenoceptor agonist isoprenaline (10 μmol/l). Contraction amplitude (y) and contraction velocity (dy/dt) are shown. Calibration line is indicated

of the cells for 15 min to 100 µmol/l noradrenaline in the presence of the β-adrenoceptor antagonist timolol (10 µmol/l). Following DHA (60 µmol/l) treatment for 3 days, α_1-adrenoceptor-induced inositol phosphate formation ($IP_1 + IP_2 + IP_3$) was decreased by $55 \pm 17\%$ ($M \pm SD$, $n = 6$ from 6 different cardiomyocyte preparations). As demonstrated in Fig. 3, only chronic pretreatment of the rat cardiomyocytes in the presence of DHA at concentrations ≥ 20 µmol/l decreased α_1-adrenoceptor-induced inositol phosphate formation. Determination of inositol phosphate formation in the presence of various noradrenaline concentrations (in the presence of 10 µmol/l timolol) after chronic DHA pretreatment revealed a marked decrease in the effect of noradrenaline at all concentrations studied and a slight decrease in basal inositol phosphate formation (in the absence of noradrenaline) (Fig. 4).

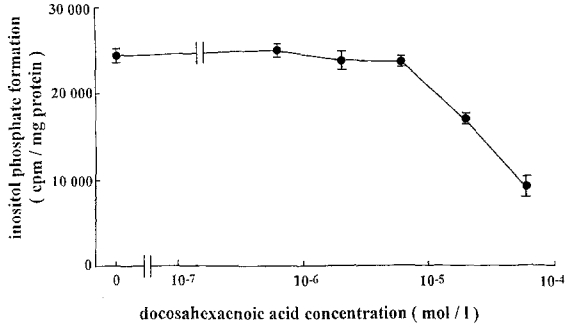

Fig. 3 Concentration-dependent effect of DHA pretreatment on α_1-adrenoceptor-stimulated inositol phosphate formatio17n i-n rat cardiomyocytes. Rat cardiomyocytes were pretreated for 3 days with various concentrations of DHA. Thereafter, α_1-adrenoceptor-stimulated inositol phosphate formation was determined in the presence of 100 µmol/l noradrenaline + 10 µmol/l timolol. Values are given as means ± SD, $n = 3$

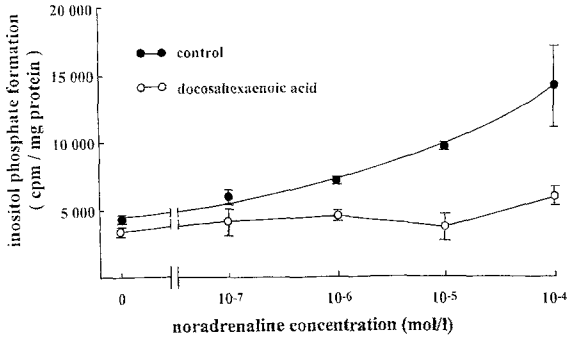

Fig. 4 Effect of DHA pretreatment on α_1-adrenoceptor-induced inositol phosphate formation. After pretreatment of rat cardiomyocytes for 3 days with 60 µmol/l DHA and an additional exposure to 10 mmol/l LiCl for 15 min (see "Methods"), cells were treated with various concentrations of noradrenaline in the presence of 10 µmol/l timolol and 10 mmol/l LiCl. Values are given as means ± SD, $n = 3$

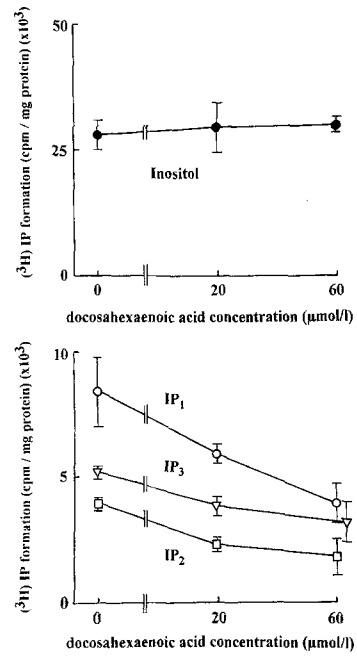

Fig. 5 Effect of DHA exposure on (^{3}H)inositol levels and α_1-adrenoceptor-stimulated formation of (^{3}H)IP_1, (^{3}H)IP_2 and (^{3}H)IP_3. Rat cardiomyocytes were pretreated for 3 days in the presence of 20 µmol/l or 60 µmol/l DHA or in the absence of DHA. Thereafter, the cells were incubated for 2.5 hours in an inositol-free salt solution supplemented with (^{3}H)myo-inositol as described in "Methods". Cells were then exposed to 10 mmol/l LiCl for 15 min and then to 100 µmol/l noradrenaline + 10 µmol/l timolol in the presence of 10 mmol/l LiCl for further 15 min and cellular levels of (^{3}H)inositol, (^{3}H)IP_1, (^{3}H)IP_2 and (^{3}H)IP_3 were determined. Values are means ± SD, $n = 3$

Pretreatment of the rat cardiomyocytes with DHA (60 µmol/l) for an incubation period of 6 h only slightly decreased inositol phosphate formation ($IP_1 + IP_2 + IP_3$) by $17 \pm 10\%$ ($n = 3$). In contrast to the DHA exposure for 3 days, exposure of the rat cardiomyocytes for 3 days to the n-6 PUFA arachidonic acid (60 µmol/l) had no significant effect on α_1-adrenoceptor-induced inositol phosphate ($IP_1 + IP_2 + IP_3$) formation (decrease by $12 \pm 10\%$, $n = 3$). Basal inositol phosphate formation was also unaltered by chronic arachidonic acid (60 µmol/l) pretreatment (data not shown).

To test the possibility that the DHA-induced decrease in inositol phosphate formation may be due to a decrease in the uptake of (^{3}H)myo-inositol into the cells or a decreased specific radioactivity of (^{3}H)myo-inositol, the cellular levels of (^{3}H)myo-inositol were determined in cardiomyocytes pretreated in the absence or presence of 20 and 60 µmol/l DHA. As shown in Fig. 5, the levels of (^{3}H)myo-inositol were not decreased by the DHA exposure whereas α_1-adrenoceptor-induced formation of IP_1, IP_2 and IP_3 was markedly reduced.

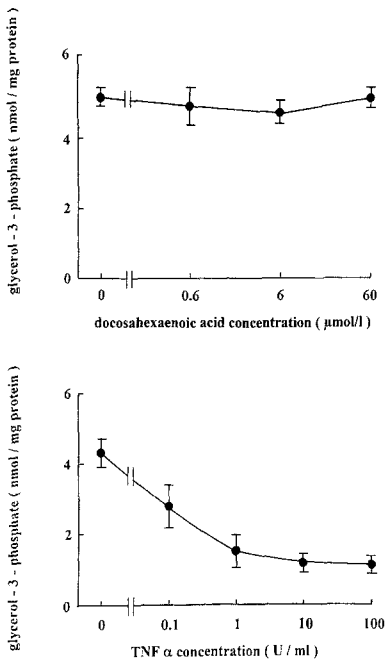

Fig. 6 Influence of pretreatment with DHA or TNFα on glycerol-3-phosphate levels in rat cardiomyocytes. After pretreatment of rat cardiomyocytes for 3 days in the presence of various concentrations of DHA or TNFα, respectively, cellular levels of glycerol-3-phosphate were determined as described in "Methods". Values are means ± SD, $n = 3$ from 3 different experiments (DHA) and $n = 5$ from 5 different experiments (TNFα), respectively

Effect of docosahexaenoic acid treatment on inositol phospholipid synthesis and glycerol-3-phosphate levels in rat cardiomyocytes

Recent studies had shown that the decrease in inositol phosphate formation induced by the cytokine TNFα was mediated by a TNFα-mediated decrease in the synthesis of PIP_2, the substrate of PI-specific phospholipase C (Reithmann et al. 1994). Therefore, the influence of chronic DHA pretreatment on the synthesis of PIP_2 and its precursors PI and PIP was studied in the rat cardiomyocytes. As shown in Table 2, DHA (60 μmol/l) pretreatment for 3 days had virtually no influence on the synthesis of PIP_2, PIP and PI in the rat cardiomyocytes. Glycerol-3-phosphate is an important substrate of lipogenesis. To compensate for the loss of diacylglycerol, additional phosphatidic acid has to be channeled constantly into the phosphoinositide cycle. Glycerol-3-phosphate which is derived form glycolysis and acyl-CoA are consumed for this process. Pretreatment of the rat cardiomyocytes with the cytokine TNFα decreased the cellular level of glycerol-3-phosphate by up to 73% whereas DHA exposure had no influence on the level of glycerol-3-phosphate (Fig. 6).

Table 2 Effect of DHA pretreatment on inositol phospholipid synthesis in rat cardiomyocytes. Following pretreatment for 72 h in the absence (control) or presence of 60 μmol/l DHA, 1000 μl of methanol/chloroform/HCl (1000:500:2) was added to the rat cardiomyocytes and inositol phospholipids were determined as described in "Methods"

Pretreatment	Control	DHA (60 μmol/l)
Inositol phospholipids (cpm/mg protein)		
PI	24265 ± 3642	22574 ± 5230
PIP	533 ± 82	547 ± 148
PIP_2	276 ± 35	234 ± 63

Values (PI, PIP, PIP_2) are given as means ± SD, $n = 3$

Effect of myo-inositol on PIP_2 synthesis and α_1-adrenoceptor-stimulated increase in contraction velocity and induction of arrhythmias in rat cardiomyocytes

It cannot be excluded that the treatment with the n-3 PUFA DHA may alter the cellular level of (non-labeled) myo-inositol. To study the possible influence of an altered myo-inositol level on PIP_2 synthesis and α_1-adrenoceptor responsiveness, the cells were cultured in an inositol-free medium in the absence or presence of 1 mg/ml myo-inositol. Thereafter, the synthesis of (^{32}P)PIP and (^{32}P)PIP_2 were determined. The myo-inositol (1 mg/ml) pretreatment had virtually no influence on the level of PIP (control: 5080 ± 2122 cpm/mg protein; 1 mg/ml myo-inositol-treated: 5335 ± 362, M ± SD, $n = 3$) nor on the level of PIP_2 (control: 955 ± 210; 1 mg/ml myo-inositol-treated: 862 ± 231; M ± SD, $n = 3$). The α_1-adrenoceptor-induced increase in contraction velocity was also not altered by the treatment in the presence of 1 mg/ml myo-inositol (increase by 41 ± 13%, $n = 5$) in comparison to control cells. Similarly, the α_1-adrenoceptor-stimulated induction of arrhythmias was unaltered by the pretreatment with 1 mg/ml myo-inositol (arrhythmias occurred in 5/6 monolayers of myo-inositol-pretreated cardiomyocytes).

Effect of DHA pretreatment on α_1-adrenoceptors in rat cardiomyocytes

To determine whether a decrease in the number or affinity of α_1-adrenoceptors may be one of the mechanisms of the DHA-induced decrease in inositol phosphate formation, binding of the α-adrenoceptor antagonist (^{3}H)prazosin to rat cardiomyocytes was studied. Saturation binding experiments under equilibrium conditions in rat cardiomyocytes revealed a single class of binding sites with a dissociation constant (K_D) of 0.25 (± 0.05) nmol/l (mean ± SD, $n = 3$ from three different cardiomyocyte preparations) and a maximal binding capacity of 41.5 ± 24.7 fmol/mg protein (mean ± SD,

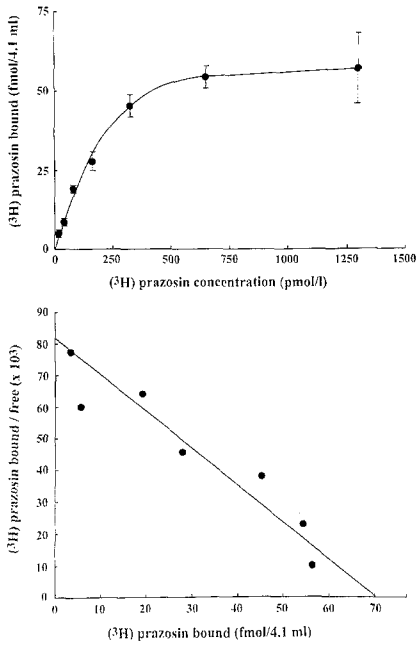

Fig. 7 Concentration-dependent binding of (^{3}H)prazosin to rat cardiomyocytes. Concentration-dependent, specific binding of the α_1-adrenoceptor antagonist (^{3}H)prazosin was determined in rat cardiomyocytes. Non-specific binding was determined in the presence of (unlabelled) 0.1 μmol/l prazosin and was subtracted from total binding. Values are given in triplicate from one experiment (*upper figure*). Scatchard plot analysis of these binding data revealed a dissociation constant (K_D) of 0.21 nmol/l ($r = -0.96$) (*lower figure*)

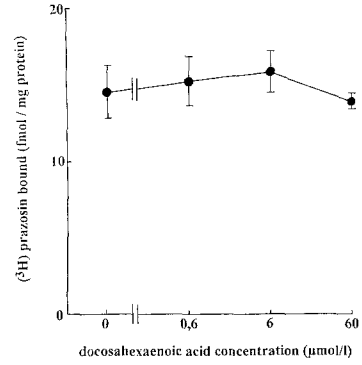

Fig. 8 Effect of pretreatment with DHA on (^{3}H)prazosin binding in rat cardiomyocytes. After pretreatment of rat cardiomyocytes in the presence of various concentrations of DHA, specific (^{3}H)prazosin binding at a concentration 0.34 nmol/l was determined. Non-specific binding was determined in the presence of 0.1 μmol/l prazosin and was subtracted from total binding. Values are given as means ± SD, $n = 3$

$n = 3$) representing about 6000 sites per cardiomyocyte. A representative experiment is shown in Fig. 7. Competition binding experiments indicated that specific (^{3}H)prazosin binding was not affected by the β-adrenoceptor antagonist (−) propranolol (100 nmol/l) and the α_2-adrenoceptor antagonist yohimbine (100 nmol/l), thus, classifying α-adrenoceptors of neonatal rat cardiomyocytes as α_1-adrenoceptors. Pretreatment of the rat cardiomyocytes for 3 days with various DHA concentrations did not significantly alter specific (^{3}H)prazosin binding at a concentration of 0.34 nmol/l (representing 1.5 fold K_D-value) (Fig. 8). Determination of concentration-dependent specific (^{3}H)prazosin binding after incubation of the cells for 3 days in the absence or presence of 60 μmol/l DHA revealed that K_D-values (control, 0.30 nmol/l; 60 μmol/l DHA, 0.24 nmol/l) and maximal binding capacities (control, 28.4 fmol/mg protein; 60 μmol/l DHA, 26.0 fmol/mg protein) of (^{3}H)prazosin were virtually unchanged (data not shown).

Effect of DHA pretreatment on adenylyl cyclase activity in rat cardiomyocytes

To study whether the attenuation of the isoprenaline-stimulated increase in contraction velocity may be due to a decreased adenylyl cyclase activity, the influence of chronic DHA pretreatment on adenylyl cyclase activity was determined. DHA (60 μmol/l) pretreatment for 3 days did not decrease but even slightly increased basal adenylyl cyclase activity (in the presence of GTP) and adenylyl cyclase activity in the presence of the β-adrenoceptor agonist isoprenaline and the direct adenylyl cyclase activator forskolin (Table 3).

Table 3 Effect of DHA pretreatment on adenylyl cyclase activity in membranes of rat cardiomyocytes. Following pretreatment of the rat cardiomyocytes in the absence (control) or presence of 60 μmol/l DHA, crude membranes were prepared as described in "Methods". Adenylyl cyclase activities were determined in the presence of 100 μmol/l GTP (basal), 100 μmol/l isoprenaline + 100 μmol/l GTP (isoprenaline-stimulated) or 100 μmol/l forskolin + 100 μmol/l GTP (forskolin-stimulated)

Pretreatment	Control	DHA (60 μmol/l)
Adenylyl cyclase activity (pmol cAMP × mg protein^{-1} × 20 min^{-1})		
Basal	26.0 ± 4.5	31.2 ± 2.3
Isoprenaline-stimulated	52.1 ± 4.1	61.3 ± 9.5
Forskolin-stimulated	92.6 ± 4.6	114.8 ± 11.2

Values are given as means ± SD, $n = 3$

Discussion

Several authors have reported that exposure of cultured neonatal rat cardiomyocytes to micromolar concentrations of the n-3 PUFA DHA for 3–5 days led to a marked enrichment of cardiomyocyte membranes in DHA and depletion in the n-6 PUFA arachidonic acid (Hallaq et al. 1990a; Bordoni et al. 1991; Lamers et al.

1992). The n-3/n-6 PUFA ratio was increased by the chronic DHA pretreatment in rat cardiomyocyte membranes (Grynberg et al. 1988).

Reibel et al. (1988) have previously shown that a 4 weeks feeding of rats with a fish oil supplemented diet markedly reduced the positive inotropic response to α_1-, but not to β-adrenoceptor stimulation. To our knowledge, the effect of chronic n-3 PUFA pretreatment on α_1-adrenoceptor-induced positive inotropic effects and induction of arrhythmias in rat cardiomyocytes has not been studied up to now.

In agreement with the data of other authors (Hallaq et al. 1990a; Fournier et al. 1994; Grynberg et al. 1995), the results of this study indicate that basal contractility (contraction amplitude) and the rate of spontaneous contractions of rat cardiomyocytes were not significantly altered by the n-3 PUFA treatment. The α_1-adrenoceptor-mediated increase in contraction velocity and induction of arrhythmias were significantly decreased (or even abolished) by the chronic DHA pretreatment whereas exposure of the cells to the n-6 PUFA arachidonic acid had virtually no effect on α_1-adrenoceptor-mediated increase in contraction velocity and induction of arrhythmias.

The findings of this study indicate that the prolonged DHA pretreatment of rat cardiomyocytes markedly decreases α_1-adrenoceptor-induced formation of IP_3. The production of IP_1 and IP_2 that apparently arises from the metabolism of IP_3 was also reduced by the n-3 PUFA pretreatment. In contrast, α_1-adrenoceptor-induced inositol phosphate formation was not significantly altered by exposure of the cells to the n-6 PUFA arachidonic acid. In agreement with these results, it has previously been reported by Lamers et al. (1992) that treatment of rat cardiomyocytes for 3–4 days with the n-3 PUFA EPA markedly decreased basal and α_1-adrenoceptor-stimulated PIP_2 hydrolysis. Similarly, Bordoni et al. (1990) have reported that treatment of rat cardiomyocytes in a medium containing DHA (60 µmol/l) decreased basal and α_1-adrenoceptor-stimulated inositol phosphate formation. Recently, Woodcock et al. (1995) have demonstrated that an eight weeks dietary supplementation enriched in n-3 PUFA's resulted in a reduced inositol phosphate response to α_1-adrenoceptor stimulation in isolated rat atria. The data suggest that the decrease in α_1-adrenoceptor-mediated IP_3 formation may be the mechanism by which n-3 PUFA's decrease α_1-adrenoceptor-induced positive inotropic effects and induction of arrhythmias.

The mechanism of the attenuation of inositol phosphate formation by n-3 PUFA pretreatment is at present unknown. Lamers et al. (1992) have suggested that the reducing effect of n-3 PUFA treatment on PIP_2 hydrolysis was partially due to an alteration in the level of PIP_2. In contrast to these data, the levels of PIP and PIP_2 were not significantly altered by the n-3 PUFA pretreatment in this study. We have recently demonstrated that the decrease in basal and α_1-adrenoceptor-mediated inositol phosphate formation in rat cardiomyocytes by chronic exposure to the cytokine TNFα was mediated by a decreased synthesis of PIP_2 and its precursors, PIP and PI (Reithmann et al. 1994). The TNFα-induced reduction in inositol phospholipid formation was apparently, at least in part, due to a marked decrease in the cellular level of glycerol-3-phosphate (Reithmann et al. 1994). Glycerol-3-phosphate which is derived form glycolysis is an important substrate of lipogenesis. It is consumed for the formation of phosphatic acid that has to be channeled constantly into the phosphoinositide cycle. In contrast to the mechanism by which chronic TNFα treatment of rat cardiomyocytes decreases inositol phosphate formation, the reduction of IP_3 production by chronic DHA exposure is apparently not mediated by a depletion of PIP_2, as the synthesis of PI, PIP and PIP_2 was not significantly altered by the DHA exposure. The level of glycerol-3-phosphate was also unchanged by pretreatment with the n-3 fatty acid while it was markedly reduced by TNFα.

Another possible explanation for the decreased inositol phosphate formation following DHA pretreatment would be an alteration of (^{3}H)myo-inositol uptake into the cardiomyocytes or an altered intracellular concentration of myo-inositol. This possible mechanism was excluded by the observation that (^{3}H)myo-inositol levels were unaffected by the n-3 fatty acid exposure. Furthermore, chronic treatment of the cells in the presence of a high concentration of myo-inositol had virtually no effect on PIP_2 synthesis and on α_1-adrenoceptor-induced increase in contraction velocity and arrhythmogenesis. Dissociation constant and maximal binding capacity of α_1-adrenoceptor antagonist binding in rat cardiomyocytes was unaltered by the chronic DHA pretreatment. It is, therefore, suggested that the effect of n-3 fatty acids on IP_3 formation in rat cardiomyocytes is not mediated by an alteration in the number or binding properties of α_1-adrenoceptors. The decrease in IP_3 formation by n-3 fatty acid exposure may rather be mediated by a decrease in the activity of the enzyme phospholipase C. Bordoni et al. (1991) have demonstrated that chronic DHA (60 µmol/l) treatment of cultured rat cardiomyocytes lead to increased cellular levels of diacylglycerol (DAG), and that this DAG was structurally different from DAG in control cells. The authors suggested that this structurally different DAG could be responsible for a different (increased) activation pattern of protein kinase C as DHA-enriched DAG induces a slower removal of DAG by DAG kinase and, thus, a longer persistance of the protein kinase C activity signal (Bordoni et al. 1991). As activation of protein kinase C has been reported to decrease phospholipase C activity in rat cardiomyocytes (Meji and Lamers 1989), a protein

kinase C-induced desensitization of phospholipase C might be a possible mechanism of the decreased IP_3 production in DHA-treated rat cardiomyocytes. At present, further studies are in progress to determine the mechanisms by which n-3 PUFA treatment decreases phospholipase C activity in rat cardiomyocytes.

The increase in contraction velocity in rat cardiomyocytes by the β-adrenoceptor agonist isoprenaline and the cardiac glycoside ouabain were also markedly reduced (or even abolished) by the n-3 PUFA pretreatment. β-Adrenoceptor-dependent and receptor-independent adenylyl cyclase activities were not decreased but even slightly increased the DHA exposure. Similarly, an increase in β-adrenoceptor-stimulated adenylyl cyclase activity has previously been found in fish oil-fed pigs (Lamers et al. 1987). Ouabain-inhibited Na, K, ATP-ase activity was recently shown to be unchanged by n-3 PUFA pretreatment in rat cardiomyocytes (Hallaq et al. 1990a).

It is possible that the DHA-induced impairment of the contractile responses to ouabain and α_1- and β-adrenoceptor agonists and the DHA-induced decrease in inositol phosphate formation are two distinct and independent effects induced by the n-3 fatty acid. On the other hand, a possible explanation for the decreased positive inotropic effects of the β-adrenoceptor agonist isoprenaline and ouabain in n-3 PUFA-treated rat cardiomyocytes may be the existence of a cross-talk between the derangement of the phosphoinositide pathway and various inotropic cascades. A possible candidate for a link between a derangement of the phosphoinositol cycle and a depression of various inotropic cascades might be a decrease in myofibrillar responsiveness based on a decreased IP_3 formation. Two lines of evidence support this hypothesis: First, exposure of rat cardiomyocytes to the cytokine TNFα was recently shown to decrease or even abolish the positive inotropic and arrhythmogenic effects of various inotropic agents (α_1- and β-adrenoceptor agonists, ouabain, Ca^{2+}) (Werdan et al. 1995) concomitant with a decrease in IP_3 formation while adenylyl cyclase activity was even increased by the TNFα pretreatment (Reithmann et al. 1991). Second, the phospholipase C inhibitor, 2-Nitro-4-carboxyphenyl-N,N-diphenyl-carbamate) (NCDC) was reported to inhibit the positive inotropic effect of ouabain in rabbit papillary muscle (Gotoh et al. 1993).

The beneficial effects of fish oil fatty acids in cardiac disease are suggested to be partially due to prevention of ventricular arrhythmias. In addition to the modulation of dihydropyridine-sensitive calcium channels, the derangement of the phosphoinositol pathway may, thus, be a mechanism by which n-3 PUFA's of fish oil may prevent fatal arrhythmias in ischemia and reperfusion.

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