Eicosapentaenoic Acid Incorporation in Membrane Phospholipids Modulates Receptor-mediated Phospholipase C and Membrane Fluidity in Rat Ventricular Myocytes in Culture

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H. W. DE JONGE, D. H. W. DEKKERS, E. M. L. BASTIAANSE, K. BEZSTAROSTI, A. VAN DER LAARSE AND J. M. J. LAMERS. Eicosapentaenoic Acid Incorporation in Membrane Phospholipids Modulates Receptor-mediated Phospholipase C and Membrane Fluidity in Rat Ventricular Myocytes in Culture. *Journal of Molecular and Cellular Cardiology* (1996) **28**, 1097–1108. The influence of increased incorporation of linoleic acid (18:2n-6) and eicosapentaenoic acid (20:5n-3) in membrane phospholipids on receptor-mediated phospholipase Cβ (PLC-β) activity in cultured rat ventricular myocytes was investigated. For this purpose, cells were grown for 4 days in control, stearic acid (18:0)/oleic acid (18:1n-9), 18:2n-6 and 20:5n-3 enriched media, and subsequently assayed for the basal- and phenylephrine- or endothelin-1-induced total inositol phosphate formation. The various fatty acid treatments resulted in the expected alterations of fatty acid composition of membrane phospholipids. In 18:2n-6-treated cells, the incorporation of this 18:2n-6 in the phospholipids increased from 17.1 mol % in control cells to 38.9 mol %. In 20:5n-3-treated cells, incorporation of 20:5n-3 and docosapentaenoic acid (22:5n-3) in the phospholipids increased from 0.5 and 2.7 mol % in control cells to 23.2 and 9.7 mol %, respectively. When 20:5n-3-treated cells were stimulated with phenylephrine or endothelin-1, the inositolphosphate production decreased by 33.2% and increased by 43.4%, respectively, as compared to cells grown in control medium. No effects were seen in 18:2n-6-treated cells. When 18:0/18:1n-9-treated cells were stimulated with endothelin-1, inositolphosphate formation increased by 26.4%, whereas phenylephrine-stimulated inositolphosphate formation was not affected. In saponin-permeabilized cells, that were pre-treated with 20:5n-3, the formation of total inositolphosphates after stimulation with GTPγS, in the presence of Ca^{2+}, was inhibited 19.3%. This suggests that the 20:5n-3 effect on intact cardiomyocytes could be exerted either on the level of agonist–receptor, receptor–GTP-binding-protein coupling or GTP-binding-protein–PLC-β interaction. Investigation of the time course of saponin-induced permeabilization of the cardiomyocytes, measured by the release of lactate dehydrogenase, unmasked a slight decrease in the rate of permeabilization by 20:5n-3 pretreatment, indicating a protective effect. This led the authors to measure the cholesterol/phospholipid molar ratio, the double bond index of membrane phospholipids, and the membrane fluidity; the latter by using a diphenylhexatriene probe. In 20:5n-3-pretreated cells, a strong increase in the cholesterol/phospholipid molar ratio (from 0.23 to 0.39), a marked increase in the double bond index (from 1.76 to 2.33), and a slight decrease in fluidity (steady-state anisotropy r_{ss} of the diphenylhexatriene probe increased from 0.196 to 0.217) were observed. Thus, treatment of cardiomyocytes for 4 days with 20:5n-3, but not with 18:2n-6, causes alterations of receptor-mediated phospholipase Cβ activity. A causal relationship may exist between the 20:5n-3-induced alterations of the physicochemical properties in the bilayer and of the agonist-stimulated phosphatidylinositol cycle activity.

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KEY WORDS: Polyunsaturated fatty acids; Rat ventricular myocytes; Cell culture; Phosphatidylinositol cycles; Membrane phospholipids; Inositol phosphate production; Saponin permeabilization; Membrane cholesterol; Membrane fluidity; Contractile activity; Electrical activity.

Introduction

Polyunsaturated fatty acids (PUFAs) have structural roles in phospholipids of all cell membranes in the body, influencing membrane viscosity and permeability and, associated herewith, the function of membrane proteins. Moreover, different types of eicosanoids are formed from the fatty acids arachidonic acid (20:4n-6) and eicosapentaenoic acid (20:5n-3). In general, eicosanoids derived from 20:5n-3 are weak agonists compared to the corresponding compounds derived from 20:4n-6 (Harris, 1989; Leaf, 1994; Sassen *et al.*, 1994). Polyunsaturated fatty acids also have important functions in modulating triglyceride and cholesterol metabolism. Epidemiological studies have reported favourable effects in prevention of cardiovascular disease by the regular ingestion of fish, containing n-3 PUFAs, although the responsible mechanism remains obscure (Bang *et al.*, 1976; Kagawa *et al.*, 1982; Kromhout *et al.*, 1985; Lamers *et al.*, 1987; Leaf, 1994). Nevertheless, many experimental studies have provided evidence that n-3 PUFAs have diverse and often potent actions on e.g. platelet, endothelial cell, and vascular smooth muscle function, which may explain many of their salutary effects on cardiovascular diseases (Goodnight, 1991).

The phosphatidylinositol (PtdIns) cycle is a signalling pathway involved in receptor-mediated biological responses such as platelet aggregation, smooth muscle contraction, and formation of relaxing and contracting factors by endothelial cells. A common mechanism of n-3 and n-6 PUFAs in these biological responses may be by interference with the functioning of the PtdIns cycle. Previously, the authors reported that cultured cardiomyocytes, which were pretreated with linoleic acid (18:2n-6) or 20:5n-3, showed less phospholipase C-β activity in response to α_1-adrenoceptor stimulation (Lamers *et al.*, 1992). Recently, this was confirmed by Woodcock *et al.* (1995), who showed that feeding rats with n-3 and n-6 PUFAs caused a depression of total release of inositol phosphates (InsP$_n$) in left atrial tissue in the presence or absence of norepinephrine. In accordance, Reibel *et al.* (1988) demonstrated that dietary n-6 and n-3 PUFAs attenuated the α_1-adrenergic-mediated positive inotropy in perfused rat hearts. Furthermore, it was shown by Kang and Leaf (1995) that dietary n-6

or n-3 PUFAs could prevent and terminate isoproterenol-induced arrhythmias.

Modification of the fatty acyl composition of the membrane phospholipids can influence many steps of the PtdIns signalling cascade. It has been shown, for example, that incorporation of n-3 and n-6 PUFAs in the membrane phospholipids was associated with a decreased affinity of the α_1-adrenoceptors for their antagonist ligand [^{3}H]prazosin in heart muscle (Skúladóttir *et al.*, 1993). In isolated atrial cells, G$_k$ protein kinetics involved in the stimulation of inwardly rectifying K$^+$ current by muscarinic receptor agonist were demonstrated to be modulated by 20:4n-6-derived metabolites (Scherer and Breitwieser, 1990). Furthermore, the function of protein kinase C (PKC) isozymes may be affected by either free PUFAs or by alteration of the molecular species of 1,2-diacylglycerol [(1,2)DAG] due to changed fatty acid composition of the phospholipid source (Bordoni *et al.*, 1992; Graber *et al.*, 1994).

Obviously, we need to learn more about the effects of incorporation of n-3 and n-6 PUFAs in membrane phospholipids on structure and function of the sarcolemma, in terms of the various steps of the transmembrane signalling by the PtdIns cycle, and the physicochemical properties such as fluidity of the bilayer, cholesterol content and resistance to permeabilizing agents. In the present study, primary cultures of spontaneously beating, neonatal rat ventricular myocytes were used to investigate the influence of addition of n-6 and n-3 PUFAs to the culture medium on their spontaneous contractile activity and on the receptor-mediated activation of PLC-β. After permeabilization of the cells with saponin, the authors examined the effects of n-3 and n-6 PUFAs on PLC-β at the level of its GTP binding protein (G protein) and Ca^{2+} activation. As 20:5n-3 pretreatment of the cardiomyocytes appeared to decrease the rate of cell permeabilization by saponin, the membrane cholesterol contents and membrane fluidity were also measured.

Materials and Methods

Materials

Phenylephrine (PHE) was obtained from Sigma (St Louis, MO, USA) and endothelin-1 (ET-1) was from

Peninsula Laboratories (Belmont, CA, USA). The four-well Multidish and single culture dishes (diameter 35 mm) were from Nunc (Roskilde, Denmark), and the culture medium Ham F10 was from Gibco (Paisley, UK). Fetal calf serum, horse serum and penicillin/streptomycin were obtained from Boehringer (Mannheim, Germany). Trypsin (type III) was from Sigma. Myo[2-^{3}H]Ins (17–19 Ci/mmol) was obtained from Amersham International (Amersham, UK). TMA-DPH (1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene, p-toluenesulphonate) was obtained from Molecular Probes (Eugene, OR, USA). A_2C (2-(2-methoxyethoxy)ethyl 8-(cis-2-n-octylcyclopropyl)-octanoate) as well as the free fatty acids oleic acid (cis-9-octadecenoic acid, 18:1n-9), stearic acid (octadecanoic acid, 18:0), linoleic acid (cis-9,12-octadecadienoic acid, 18:2n-6) and cis-5,8,11,14, 17-eicosapentaenoic (20:5n-3) acid were from Sigma. Dowex AG 1-X8 (100–200 mesh formate form) was from BioRad Laboratories (Richmond, CA, USA). Free cholesterol was determined using a kit (Boehringer-Mannheim). All other chemicals were of analytical grade.

Cell culture

Primary cultures of neonatal ventricular myocytes were prepared from 2-day-old Wistar rats as described previously (Van Heugten $et\ al.$, 1994). For measurement of contraction frequency, cardiomyocytes were seeded on circular glass cover slips (diameter 25 mm) at 150 to 175×10^3 cells/cm^2. For measurements of PLC-β activity or lactate dehydrogenase (LDH) release, myocytes were seeded in wells with 1.8 cm^2 surface at 150 to 175×10^3 cells/cm^2. For the determination of cholesterol content and total phospholipids, the cardiomyocytes were seeded (150 to 175×10^3 cells/cm^2) in tissue culture dishes with 8 cm^2 surface. For measurement of membrane fluidity, cardiomyocytes were seeded on rectangular glass coverslips in similar tissue culture dishes (diameter 35 mm, 8 cm^2) at a density of 4×10^4 cells/cm^2. Cell cultures were grown at 37°C and 95% air/5% CO_2 in complete growth medium consisting of Ham F10 supplemented with 10% (v/v) fetal calf serum, 10% (v/v) horse serum, 100 U penicillin/ml and 100 μg streptomycin/ml.

The cultures were exposed to the following conditional growth media 24 h after seeding: (1) growth medium supplemented with the vehicle [0.3% (v/v) pure ethanol]; (2) growth medium supplemented with 18:0 and 18:1n-9 (107 μM

each); (3) growth medium supplemented with 18: 2n-6 (214 μM); and (4) growth medium supplemented with 20:5n-3 (214 μM), as described previously (Lamers $et\ al.$, 1992). After 2 days, the cultures were renewed with their concurring enriched medium. Treatment with fatty acids did not result in cell density or hypertrophic changes as was tested by assessment of the protein content and protein over DNA ratio, respectively. For instance, protein contents (mg) per mg DNA in the treated cells were 43.1 ± 1.7 (Control), 45.5 ± 0.7 (18:0/18:1n-9), 42.6 ± 2.6 (18:2n-6) and 43.8 ± 1.4 (20:5n-3). All experiments were performed 4–5 days after plating the cells.

Separation of the phospholipids and quantification of the fatty acid methyl esters

To examine the incorporation of supplemented fatty acids in the phospholipid fraction after 4–5 days, the cardiomyocytes were rinsed with cold buffer, scraped and extracted with organic solvents as described previously (Lamers $et\ al.$, 1992). Briefly, the phospholipid fractions were separated from the triglyceride, diacylglycerol, cholesterol and unesterified fatty acid fractions by thin layer chromatography. To separate the fatty acid methyl esters, a CP9000 capillary column chromatograph (Chrompack, Middelburg, The Netherlands), equipped with a CP-Sil 88-coated fused silica capillary column, was used (Lamers $et\ al.$, 1992).

Spontaneous contractions

After 4 days, the culture grown on a circular glass coverslip was rinsed once with incubation buffer I, containing 125 mM NaCl, 5.0 mM KCl, 2.5 mM CaCl$_2$, 1 mM MgSO$_4$, 1 mM KH$_2$PO$_4$, 10 mM NaHCO$_3$, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), and 5 mM glucose (pH 7.4, 37°C), and subsequently mounted on a teflon culture dish (Ince $et\ al.$, 1985). The dish was placed in a temperature-controlled micro-incubator (Ince $et\ al.$, 1983) and the contractions of the cardiomyocytes were visualized as described previously (Le $et\ al.$, 1993). The contracting cells were made visible on a videomonitor, which was equipped with an interactive system to convert the time-dependent grey levels in a particular cursor-defined area on the screen into a time-dependent voltage. This so-called contraction amplitude signal was counted in a heart rate counter to obtain the contraction

frequency signal expressed as beats/min. The ir-
regularity of beating equalled the variation co-
efficient of the contraction frequency.

Inositolphosphate production in intact and saponin-
permeabilized cardiomyocytes

Cardiomyocytes were labelled with $2 \mu Ci$ myo-[2-
^{3}H]Ins/ml for 48 h in conditioned growth medium.
After washing and pre-incubation (15 min, 37°C)
in incubation buffer II, containing 130 mM NaCl,
4.7 mM KCl, 1.3 mM $CaCl_2$, 0.44 mM NaH_2PO_4,
1.1 mM $MgSO_4$, 20 mM $NaHCO_3$, 11.1 mM glucose,
and 10 mM HEPES (pH 7.4, 37°C), the cells were
stimulated with $5 \mu M$ PHE or 5 nM ET-1 in the
presence of 10 mM LiCl. The incubations were
stopped by rapidly washing the cells with ice-cold
buffer. Water-soluble [^{3}H]Ins products were ex-
tracted by two subsequent extractions with 4%
$HClO_4$. The pooled $HClO_4$ fractions were neutralized
with 2 M KOH and 1 M K_2CO_3. The remainder, i.e.
water-insoluble products, was extracted sub-
sequently with ice-cold $CH_3OH:HCl$ (100:1 v/v) for
5 min at 4°C. The [^{3}H]inositol phosphates ([^{3}H]In-
sP_n) were separated from [^{3}H]Ins and [^{3}H]gly-
cerophosphoinositol by chromatography on Dowex
AG 1-X8 as described previously (Van Heugten *et
al.*, 1994).

The myo-[2-^{3}H]Ins pre-labelled cells were rinsed
with phosphate-buffered saline, at 37°C, and sub-
sequently treated with $100 \mu g$ saponin/ml in in-
tracellular buffer (10 mM NaCl; 110 mM KCl; 1 mM
KH_2PO_4; 4 mM $MgCl_2$; ethylene glycol-O,O′-bis(2-
aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA);
3 mM ATP; 8 mM creatine phosphate; 6 U/mol cre-
atine kinase; 20 mM HEPES; pH 7.0) for 5 min, by
37°C. Thereafter, the myocytes were rinsed three
times with intracellular buffer followed by in-
cubation for 15 min at 37°C in intracellular buffer
containing 10 mM LiCl, and about 96 nM free Ca^{2+}
ions (estimated according to SPECS, a software
program based on the formulas described by Fa-
biato, 1988) with or without $30 \mu M$ GTPγS. After
incubation, the collected buffer and the cells were
extracted for measurement of [^{3}H]InsP_n formation
as described previously (Van Heugten *et al.*, 1994).

Rate of permeabilization induced by saponin

The cardiomyocytes cultured with fatty acid en-
riched medium in 1.8 cm^2 wells, were washed to
remove the small amounts of LDH released upon

exchanging the growth medium for incubation buf-
fer, and pre-incubated with intracellular buffer.
After a 5-min pre-incubation period, a sample was
taken to measure the LDH release in the absence
of saponin. The buffer was subsequently removed
and replaced by intracellular buffer containing
$100 \mu g/ml$ saponin.

Samples of the incubation buffer were taken
after 1, 2, 5 and 10 min, and stored on ice for
determination of LDH release. Thereafter, the re-
mainder of the buffer was removed immediately
and a solution of 1% Triton in intracellular buffer
was added to release the rest of cellular LDH to
determine the total LDH content of the cells. The
LDH activity was assayed spectrophotometrically
in potassium phosphate buffer (95 mM, pH 7.0),
containing 7.6 mM sodium pyruvate and 0.2 mM
NADH, and was expressed as percentage of total
cellular LDH.

Membrane fluidity

Membrane fluidity was assessed by measuring the
fluorescence steady-state anisotropy (r_{ss}) of the
plasma membrane specific fluorophore 1-(4-tri-
methylammonium-phenyl)-6-phenyl-1,3,5-hexa-
triene *p*-toluenesulphonate (TMA-DPH), according
to Sumbilla and Lakowicz (1983) and modified by
Bastiaanse *et al.* (1994). The cardiomyocytes grown
on glass coverslips were washed twice and pre-
incubated with incubation buffer I for 30 min at
37°C. Thereafter, the cells were loaded with $10 \mu M$
TMA-DPH for 30 min at 37°C. The glass coverslips
were placed in a cuvette containing 2.5 ml buffer
I, and after a period of 10 min the fluorescence
steady-state anisotropy (r_{ss}) was measured ac-
cording to the following formula:

$$r_{ss} = (I_{vv} - I_{vh}*G)/(I_{vv} + 2I_{vh}*G) \qquad (1)$$

where I_{vv} and I_{vh} represent the fluorescence in-
tensities parallel and perpendicular to the excitation
plane (when set vertically), respectively. G is the
correction factor for the difference in the mono-
chromator's transmission efficiency for vertically
and horizontally polarized light, and equals I_{hv}/I_{hh}.
The measurements were performed on a spectro-
fluorometer (Perkin-Elmer LS-3), provided with a
polarization accessory (Perkin-Elmer 5212-3269),
at 37°C.

To validate the procedure, separate series of ex-
periments were conducted before and after the
membrane fluidity had been changed by addition
of the "fluidizer" 2-(2-methoxyethoxy)ethyl 8-(cis-

2-n-octylcyclopropyl)-octanoate (A$_2$C, 20 μM). After measuring r$_{ss}$ in the absence of A$_2$C, the glass coverslips were returned to their original pre-incubation loading dish, to which 20 μM A$_2$C was added, and subsequently incubated for 30 min at 37°C. Immediately thereafter, the r$_{ss}$ was measured again. To ensure a maximal fluorescent signal, the fluidity measurements were always carried out in cells seeded at an optimal density (4×10^4 cells/cm^2), which was not influenced by any fatty acid treatment.

Free cholesterol and total phospholipids

The myocytes cultured in fatty acid enriched medium grown in 8 cm^2 dishes were rinsed twice with a physiological salt solution. The cells were scraped in 2 ml methanol and transferred to a glass tube (fat free) containing 0.75 ml physiological salt solution. After addition of 1 ml CHCl$_3$, the mixture was stirred vigorously. The supernatant was separated from the pellet by centrifugation (5 min, 3000 r.p.m.). The supernatant was stored on ice, and the pellet was washed with 1.9 ml CHCl$_3$:CH$_3$OH:H$_2$O (5:10:4 v/v/v). Again the supernatant was separated from the pellet by centrifugation and added to the supernatant of the previous step. The pellet was discarded. Subsequently, 1.5 ml CHCl$_3$ and 1.5 ml H$_2$O were added to the supernatant fraction, and after vigorous mixing and centrifugation, the upper phase was separated from the lower phase. The latter was dried with a little scoop of Na$_2$SO$_4$, and was stored at -20°C for 12 h. Then the solution was transferred to a new glass tube, and the Na$_2$SO$_4$ pellet was washed with 1 ml CHCl$_3$. The wash fluid was combined with the solution. Subsequently, the organic phase was evaporated and the residue dissolved in 200 μl peroxide-free 2-propanol. Cholesterol was determined enzymatically using a kit (Boehringer, Mannheim, Germany). In order to determine the total phospholipid concentration, a sample of cell extract was destructed with H$_2$SO$_4$:HClO$_4$ (1:1 v/v) at 210°C for 5 h, until a clear solution was obtained. In this solution, the phosphate concentration was quantified according to Bartlett (1959), using a 1% instead of a 0.22% ammoniumheptamolybdat solution.

Statistics

Data are presented as mean values $\pm$ s.e.m. unless stated otherwise. Student's t-test was used for statistical comparison with corresponding control values, and analysis of variance was performed followed by the Student–Newman–Keul's test when appropriate. A P-value less than 0.05 was considered to indicate statistical significance.

Results

Irregularity in frequency of spontaneous contractions

A previous study (Hallaq *et al.*, 1992) using the same model of cultured ventricular myocytes has shown that incubation of the cells for 3–5 days with 5 μM 20:5n-3 prevented the arrhythmogenic effect of 0.1 mM ouabain. No effect was seen when the cells were pretreated with 5 μM 20:4n-6. In the present study, however, confluent monolayers of control cells in confluency always exhibit spontaneously irregular contractions. Figure 1(a) shows typical recordings from the control ventricular myocytes and those treated for 3 days with 18:0/18:1n-9, 18:2n-6 and 20:5n-3 rich medium. Pretreatment of cells with 18:2n-6 as well as with 20:5n-3 improved the regularity of the spontaneous contractions significantly in comparison to control pretreatment [Fig. 1(b)]. This effect, however, was not significant compared to 18:0/18:1n-9. So it appears that the fatty acid pretreatment, independently of the degree of polyunsaturation, induces a regular pattern of spontaneous contractions. As, in the model used, the cardiomyocytes are grown in a confluent monolayer, resulting in the spontaneous contractions, the particular area of monitoring by video could hardly be placed on a single cell, which would be necessary to assess shortening in velocity. Therefore, no exact measurements of the shortening velocity were performed.

Phospholipid fatty acid composition

Incubation of myocytes with media enriched with 18:0/18:1n-9, 18:2n-6 and 20:5n-3 changed the fatty acid composition of the phospholipids (Table 1). Incubation with a 18:0/18:1n-9 supplemented medium caused a markedly increased incorporation of 18:0 and 18:1n-9 fatty acids and a reduction in 16:0, 18:1n-7, 18:2n-6 and 20:4n-6 contents. As expected, the sum of PUFAs decreased from 47.0 to 38.5 mol%. Treatment with 18:2n-6 caused a dramatic increase of 18:2n-6 from 17.1 to 38.9 mol %, associated with a decrease of 18:1n-9 and 20:4n-6. Furthermore, as expected, the sum of PUFAs increased from 47.0 to 56.6 mol %. Treat-
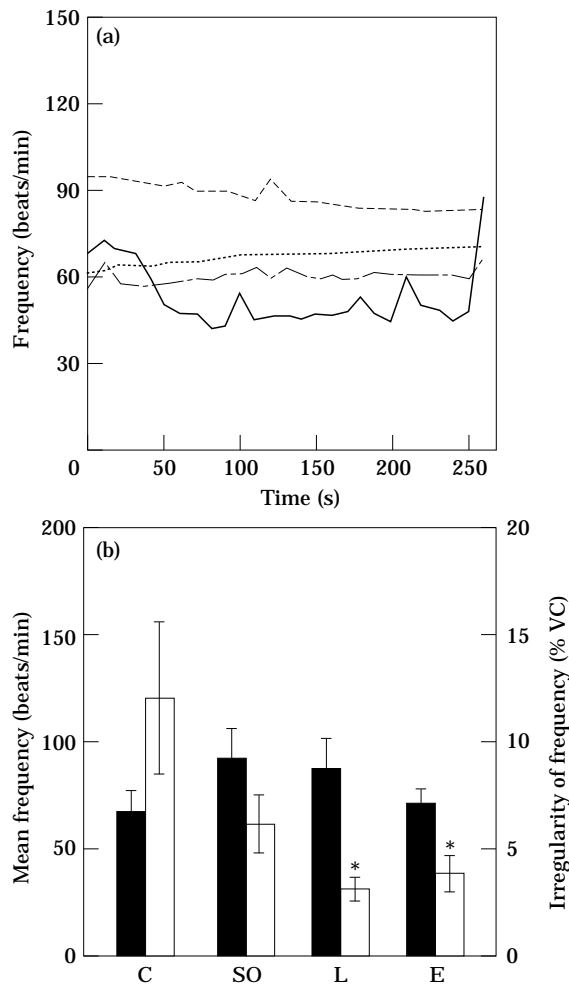
Figure 1 Frequency of spontaneous beating and regularity of beating frequency of ventricular myocytes grown in control medium (C, ---) or in 18:0/18:1 (SO, ---), 18:2n-6 (L, -·-·) or 20:5n-3 (E, ····) enriched medium. Cellular contractions were recorded using a video technique, and were counted to obtain contraction frequency and irregularity of frequency. (a) A representative experiment illustrating the frequency *vs* time for cardiomyocytes subjected to the various fatty acid treatments. (b) The mean frequency (solid bars) and irregularity of frequency (open bars), measured over a period of 5 min (% VC: variation coefficient) representing the mean data of four different cell batches. Bars represent mean values ± s.e.m. * $P < 0.05$ *vs* control cells.

ment with 20:5n-3 led to the increased in-corporation of this fatty acid (from 0.5 to 23.2 mol %) and its elongation product 22:5n-3 (from 2.7 to 9.7 mol %), but a decreased incorporation of 22:6n-3. The increased incorporation of n-3 PUFAs occurred mainly at the expense of n-6 fatty acids, as the sum of PUFAs remained almost constant. The double bond index (DBI; mean number of double bonds per mol fatty acid) is a parameter that is indicative for possible

changes in the fluidity of the membrane bilayer. As can be seen from Table 1, the treatment with 20:5n-3, in particular, caused a considerable increase of DBI from 1.76 (control cells) to 2.33. Compared to treatment with 18:0/18:1n-9 and 18:2n-6, the 20:5n-3-treated cells had much higher DBI. Moreover, in 18:2n-6-treated cells, a slight decrease from 1.76 to 1.60 was observed.

Agonist-induced [³H]InsP$_n$ response in intact cardiomyocytes

Previously, the authors have shown that stimulation of *myo*[2-³H]Ins-labelled neonatal rat ventricular myocytes with either PHE or ET-1 results in activation of PLC-β in a dose-dependent manner, both agonists reaching about the same maximal effect on the formation of total [³H]InsP$_n$ (Van Heugten *et al.*, 1993). To investigate the influence of an altered fatty acid composition of the plasma membrane phospholipids on the agonist-induced PLC activation, the cardiomyocytes grown in the control medium and media enriched with 18:0/18:1n-9, 18:2n-6 and 20:5n-3, were stimulated for 15 min with submaximal concentrations of PHE (5 μM) or ET-1 (5 nM). The formation of [³H]InsP$_n$ upon PHE and ET-1 stimulation, in cardiomyocytes that were cultured in control medium, amounted 11% and 9.5%, respectively, of total incorporated [³H]Ins, which was a 2.9 and 2.5 fold increase, respectively, compared to the basal [³H]InsP$_n$ production (3.8%) (Fig. 2). Pretreatment of cardiomyocytes with 18:0/18:1n-9 or 18:2n-6 enriched medium had no influence on the PHE stimulated [³H]InsP$_n$ formation. However, basal [³H]InsP$_n$ formation in the 18:0/18:1n-9-treated cells increased to 6.1% and the ET-1-stimulated [³H]InsP$_n$ formation to 11.6%. Phenylephrine stimulation of cardiomyocytes pre-exposed to 20:5n-3-rich medium resulted in significantly less [³H]InsP$_n$ formation (7.3% of total incorporated [³H]Ins) compared with control cardiomyocytes, whereas stimulation with ET-1 of 20:5n-3-treated cardiomyocytes caused a significantly greater [³H]InsP$_n$ formation (13.5% of total incorporated [³H]Ins).

Ca²⁺ and GTPγS-induced [³H]InsP$_n$ response in saponin-permeabilized cardiomyocytes

The finding that the agonist-induced [³H]InsP$_n$ formation depends upon the fatty acid composition of membrane phospholipids may be explained by

Table 1 Fatty acid composition (mol %) of the total phospholipid fraction of ventricular myocytes grown in control medium or in 18:0/18:1n-9 (SO), 18:2n-6 (L) or 20:5n-3 (E) enriched medium

Fatty acids	Control	SO	L	E
	($n=3$)	($n=3$)	($n=3$)	($n=4$)
16:0	13.83 ± 1.27	7.51 ± 0.25	12.55 ± 1.11§	14.80 ± 0.84§
18:0	23.31 ± 0.56	28.24 ± 0.51	21.08 ± 0.94§	23.03 ± 1.12§
18:1n-7	3.09 ± 0.06	1.68 ± 0.10	2.04 ± 0.07	2.59 ± 0.05
18:1n-9	9.61 ± 0.45	22.32 ± 0.52	5.25 ± 0.04	7.00 ± 0.22
18:2n-6	17.10 ± 1.43	14.43 ± 0.93§	38.87 ± 0.88	6.08 ± 0.48
20:4n-6	18.21 ± 1.16	15.39 ± 0.65§	11.46 ± 1.11	7.63 ± 0.58
20:5n-3	0.48 ± 0.07	1.12 ± 0.55§	0.20 ± 0.05	23.22 ± 1.31
22:5n-3	2.69 ± 0.27	2.33 ± 0.68§	1.53 ± 0.18	9.72 ± 0.44
22:6n-3	3.71 ± 0.34	2.38 ± 0.26	1.67 ± 0.14	1.33 ± 0.07
Others*	7.97 ± 0.06	4.59 ± 0.83	5.36 ± 0.22	4.60 ± 0.14
DBI†	1.76 ± 0.08	1.56 ± 0.07	1.60 ± 0.04	2.33 ± 0.05‖
ΣPUFA‡	47.05 ± 2.03	38.48 ± 0.80	56.50 ± 0.53	50.01 ± 0.64

Values represent mean values $\pm$ s.e.m.

 * Only the major fatty acids and the sum of minor fatty acids, termed "others" (mainly 16:0dma, 18:0dma, 18:3n-3, 20:3n-6, 22:0, 24:0 and 22:4n-6; dma; dimethylacetal) are listed.

 † double bond index (mean number of double bonds per mole fatty acid).

 ‡ ΣPUFA is the sum of all polyunsaturated fatty acids.

 § Values, not differing significantly from control values. All other values were significantly different from control.

 ‖ $P<0.05$ in comparison to C, SO and L treatments.

changes at the level of agonist–receptor binding, receptor–G-protein coupling and/or G-protein–PLC-β interaction. To study whether the G-protein–PLC-β interaction is affected by changes in the fatty acid composition of membrane phospholipids, the *myo*[2-³H]inositol-labelled cardiomyocytes (after exposure to the different fatty acids) were permeabilized by saponin and then stimulated for 15 min with non-hydrolysable GTPγS in the presence of Ca²⁺, or not stimulated. After permeabilization, about half of the [³H]InsP$_n$ formed was released in the incubation buffer. The basal [³H]InsP$_n$ production (in buffer plus cell extract) of the permeabilized cardiomyocytes was not affected by any of the fatty acid treatments (Table 2). GTPγS stimulation, in the presence of Ca²⁺, of the permeabilized cardiomyocytes pre-exposed to 20:5n-3 media resulted in significantly less (4.2%) [³H]InsP$_n$ formation compared to control cells (5.2%).

Resistance to saponin-induced permeabilization

As can be seen in Table 2, the fatty acid treatment dependent changes in [³H]InsP$_n$ formation released in the buffer do not completely reflect the fatty acid dependent changes in [³H]InsP$_n$ in the buffer plus cell extract, suggesting possible fatty acid treatment

dependent differences in the permeabilization. The data illustrated in Figure 3 show that there was a lagphase of LDH release up to 1 min of exposure to saponin unaffected by either fatty acid treatment. At 2 min, a significant decrease was observed only by 20:5n-3 treatment, and at 5 min, a small but not significant decrease was observed only by 20:5n-3 treatment. Figure 3, however, demonstrates that 5–10 min of treatment with saponin resulted in almost complete (70–80% of total LDH released) depletion of LDH, which was independent of the fatty acid supplementation. Therefore, at a time (2 min) of partial permeabilization, the cardiomyocytes grown in 20:5n-3 rich medium, revealed significantly less LDH release, that is 26% as compared to 36% in cells grown in control medium. A similar tendency of delayed LDH release was observed in the 18:2n-6-treated cells.

Cholesterol content and membrane fluidity

Saponin is believed to act mainly upon the cholesterol component of the sarcolemma (Dourmashkin *et al.*, 1962; Glauert *et al.*, 1962). Given the results of 20:5n-3-induced changes in cellular resistance to saponin permeabilization, the cholesterol content of whole cell extracts was determined. Cholesterol is expressed either as nmol/
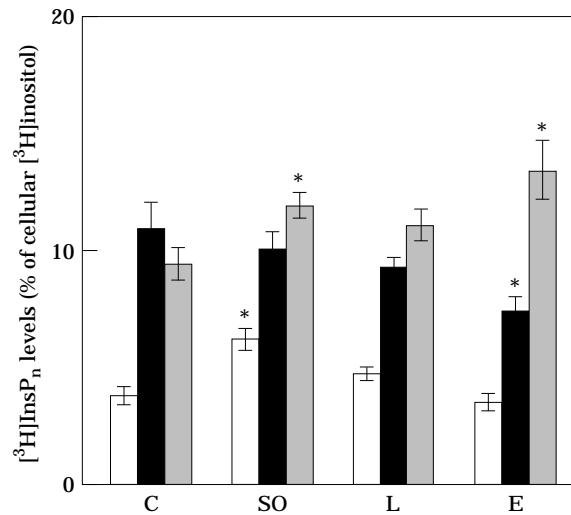
Figure 2 Agonist-stimulated [³H]InsP$_n$ production in intact ventricular myocytes grown in control medium (C), or 18:0/18:1 (SO), 18:2n-6 (L) or 20:5n-3 (E) enriched medium. Cardiomyocytes pre-incubated with *myo*-[2-³H]Ins were stimulated with either phenylephrine (PHE; 5 μM, solid bars) or endothelin-1 (ET-1; 5 nM, stippled bars) for 15 min at 37°C. Control cardiomyocytes, open bars). The cells were extracted and the [³H]InsP$_n$ formed was separated from the other [³H]Ins-labelled metabolites as described in Methods. Values are expressed as percentage of total incorporated [³H]Ins and represent the mean values ± S.E.M. of five and four experiments, with PHE and ET-1, respectively. *P<0.05, when comparing agonist-mediated [³H]InsP$_n$ responses in cells cultured in fatty acid enriched medium to the corresponding [³H]InsP$_n$ responses in cells pretreated with control medium.

mg protein or as nmol/nmol phospholipid (Fig. 4). Exposure of cardiomyocytes to 18:0/18:n-9 and 18:2n-6 resulted in a slight increase in cellular cholesterol content of 35 and 40 nmol/mg protein, respectively, as compared to 28 nmol/mg protein in control cells (Fig. 4). This cholesterol content
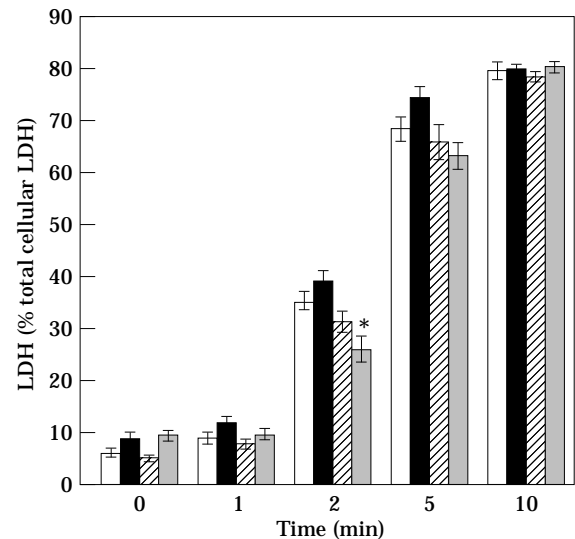
Figure 3 The rate of permeabilization by saponin (100 μg/ml) as assessed by release of lactate dehydrogenase (LDH) from ventricular myocytes grown in control medium (C, open bars) or in 18:0/18:1 (SO, solid bars), 18:2n-6 (L, striped bars) and 20:5n-3 (E, stippled bars) enriched medium. LDH activity was assayed spectrophotometrically. Values are expressed as percentage of total cellular LDH, and represent mean values ± S.E.M., in triplicate, of three different cell batches. * P<0.05 *vs* control.

was in the same range as that found for control cells in another report on the same model (Bastiaanse *et al.*, 1994, 1995). Cholesterol expressed per nmol phospholipid, however, did not change by these fatty acid treatments (Fig. 4). This is due to the fact that phospholipid content (in nmol/mg protein) also increased independently of the type of fatty acid treatment (see legends to Fig. 4). It was most remarkable to observe that 20:5n-3 pretreatment produced a three-fold increase in cellular cholesterol

Table 2 GTPγS stimulated total [³H]inositol phosphate ([³H]InsP$_n$) production in the presence of Ca^{2+} in saponin-permeabilized ventricular myocytes grown in control medium (C) or in 18:0/18:1n-9 (SO), 18:2n-6 (L), or 20:5n-3 (E) enriched medium

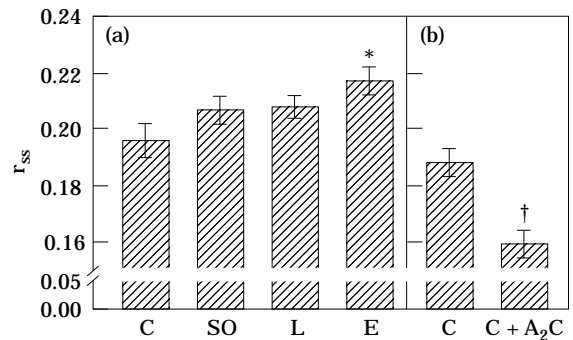|	[³H]InsP$_n$ production (% of total cellular [³H]Ins)			
	C	SO	L	E
Incubation buffer only				
−GTPγS	1.13 ± 0.08	0.87 ± 0.07*	0.86 ± 0.07*	1.01 ± 0.08
+GTPγS	3.10 ± 0.17	2.96 ± 0.23	2.46 ± 0.10*	2.52 ± 0.20*
Buffer plus cell extract				
−GTPγS	2.23 ± 0.10	1.81 ± 0.18	2.19 ± 0.32	2.11 ± 0.16
+GTPγS	5.19 ± 0.31	4.78 ± 0.36	4.53 ± 0.24	4.19 ± 0.29*

Values are expressed as percentage of total incorporated [³H]Ins and represent the mean values ± S.E.M. of eight measurements. * P<0.05 *vs* control.
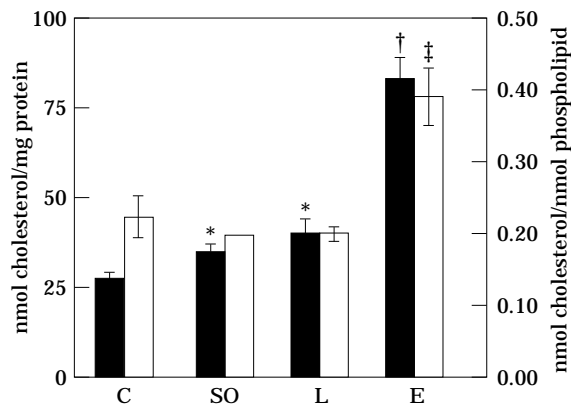
Figure 4 Cholesterol content of ventricular myocytes grown in control medium (C), or in 18:0/18:1 (SO), 18:2n-6 (L) or 20:5n-3 (E) enriched medium. Cholesterol was determined enzymatically in cellular extracts, and the total phospholipids in the extracts were determined by assessment of the phosphate concentration as described in Methods. Cholesterol is expressed either as nmol/mg protein (solid bars) or as nmol/nmol phospholipid (open bars). Phospholipid content (nmol/mg protein) did also change, however, independently of the fatty acid treatment: C, 134 ± 13; SO, 176 ± 15; L, 192 ± 18; E, 217 ± 27 ($P<0.05$, fatty acid treatment vs control). Values represent mean values $\pm$ s.e.m. of seven to eight determinations. * $P<0.02$ vs control; † $P<0.001$ vs C, SO, and L; ‡ $P<0.005$ vs C, SO and L.

Figure 5 Membrane fluidity of ventricular myocytes grown in control medium (C), or in 18:0/18:1 (SO), 18:2n-6 (L) or 20:5n-3 (E) enriched medium. (a) Ventricular myocytes grown in control medium (C) or in 18:0/18:1 (SO), 18:2n-6 (L) and 20:5n-3 (E) enriched medium were loaded with the fluorophore diphenylhexatriene, TMA-DPH for 30 min, at 37°C, whereafter the fluorescence steady-state anisotropy r_{ss} of the fluorophore TMA-DPH was assessed as described in detail in Methods. (b) To validate the experimental method, r_{ss} was measured before and after addition of the membrane mobility agent A_2C to TMA-DPH-loaded ventricular myocytes. Values are expressed as mean values $\pm$ s.e.m. of three experiments. * $P<0.05$ vs control.

Discussion

The aim of this study was to investigate the influence of increased n-6 and n-3 PUFA incorporation in the phospholipids of the cardiomyocyte membrane on the PHE- and ET-1-stimulated PtdIns cycle, as compared with cells grown in unsupplemented growth medium. Moreover, in comparison, similar experiments were performed with saturated/monosaturated fatty acid pretreated cells. The mixture of the supplemental free PUFAs with the concentrated horse and calf sera, the latter contain an excess of fatty-acid-poor albumin molecules, ensured that the toxicity of a high concentration of non-esterified fatty acids was prevented. This was also based upon earlier studies in which it was shown, in *in vitro* experiments, that cardiac sarcolemmal membrane kept its integrity if the non-esterified fatty acid to albumin molar ratio did not exceed 5 (Lamers *et al.*, 1984). Moreover, in the present study, the control cells usually exhibited irregularities in their contraction frequency, whereas treatment with n-6 and n-3 PUFAs improved significantly the regularity of spontaneous contractions. A tendency to such improvement was also seen after treatment with 18:0/18:1n-9.

Although the free fatty acid enriched incubation medium was always washed away carefully, followed by a period of stabilization before agonist stimulation of the cardiomyocytes, the additional

content expressed per mg protein and a 1.7-fold increase in cholesterol expressed per nmol phospholipid. The increase in cholesterol content per mg protein was much more dramatic compared with those seen with 18:0/18:1n-9 (1.2-fold) and 18:2n-6 (1.4-fold) treatment. Because of the large changes observed in membrane cholesterol and DBI due to 20:5n-3-treatment of the cardiomyocytes, the membrane fluidity was assessed by measuring the fluorescence steady-state anisotropy (r_{ss}) of TMA-DPH. Indeed, only the r_{ss} of cardiomyocytes grown in 20:5n-3 was significantly ($P<0.02$) affected, that is increased by 8% as compared to control cultures [Fig. 5(a)]. Apparently, the bilayer "stiffening" effect of an adaptively increased cholesterol content overcompensates for the fluidizing effect of increased degree of polyunsaturation of fatty acids in the phospholipids. To validate the method of fluidity measurement, a separate series of experiments was performed to determine whether the r_{ss} value was influenced by the membrane mobility agent A_2C [Fig. 5(b)]. Before and after exposure to 20 μM A_2C for 30 min, the r_{ss} of control cardiomyocytes was measured to revealed an increase of fluidity, i.e. a significant ($P<0.05$) decrease of r_{ss} by 15.4%.

influence of an altered composition of the non-esterified fatty acid pool which is performed by the normal turnover of cellular phospholipids can not be excluded. In neonatal cardiomyocytes, exogenous free n-3 PUFAs were found to exert their effect directly on Ca^{2+}-channels, as was assessed by measurement of the steady-state Ca^{2+} influx (Hallaq *et al.*, 1992) and of the cell contractions mediated by β-adrenergic stimulation of the cells (Kang and Leaf, 1995). The β-adrenergic-agonist-induced arrhythmias could be prevented by exogenous non-esterified 20:5n-3, but revived when the fatty acid was washed away with fatty-acid-free albumin (Kang and Leaf, 1995).

The various fatty acid treatments of the cardiomyocytes resulted in the expected alteration of fatty acid composition of the membrane phospholipids. In 18:2n-6-treated cells, incorporation of 18:2n-6 increased from 17.1 (control cells) to 38.9 mol %, while in the 20:5n-3-treated cells, incorporation of 20:5n-3 and its elongation product 22:5n-3 increased from 0.5 and 2.7 mol % (control cells), respectively, to 23.2 and 9.7 mol %, respectively. However, a marked increase in double bond index was observed in 20:5n-3-treated cells alone (2.33), in comparison with values of 1.76 in control, 1.60 in 18:2n-6-treated cells, and 1.56 in 18:0/18:1n-9-treated cells. As far as the PtdIns cycle is concerned, the most remarkable effects were observed in the 20:5n-3-treated ventricular myocytes. In those myocytes, the PHE- and ET-1-stimulated PLC-β response measured in intact cells was decreased (7.3% of total incorporated [^{3}H]Ins) and increased (13.5% of total incorporated [^{3}H]Ins), respectively, compared to ventricular myocytes grown in control medium (respectively 11 and 9.5% of total incorporated [^{3}H]Ins). In 18:2n-6-treated cells, no significant effect was observed on receptor-mediated PLC-β activity. Also, treatment with 18:0/18:1n-9 led to a slight increase of the ET-1-stimulated PLC-β response. Previously, the authors have shown that the effects of 20:5n-3 on agonist-induced [^{3}H]InsP$_n$ formation can not be ascribed to the alterations in the relative [^{3}H]Ins incorporation in phosphorylated PtdIns (Lamers *et al.*, 1992). Similar results were found by Bordoni *et al.* (1990) when cells were grown in 22:6n-3-enriched medium. The results, obtained with the saponin permeabilized cardiomyocytes, suggest that the effects of 20:5n-3 on receptor-mediated PLC-β of intact cardiomyocytes could be exerted at the level of either the agonist–receptor, receptor–G-protein coupling or G-protein–PLC-β interaction. In view of these findings, the observations of Skúladóttir *et al.* (1993) in adult cardiomyocytes are interesting.

Although in the latter study the changes of phospholipid fatty acid profile were obtained pharmacologically by repeated administration of hydrocortisone and/or epinephrine, the enhanced incorporation of 18:2n-6, 20:4n6 and 22:6n-3 in phosphatidylcholine and phosphatidylethanolamine paralleled the decreases in affinity of the α_1-adrenoceptors and coincided with down-regulation of the number of β-adrenergic binding sites. Next to the fact that the mechanisms responsible for α_1- and β-adrenoceptor regulation are different, it was speculated by Skúladóttir *et al.* (1993) that because adrenoceptors span the entire thickness of the membrane bilayer, a change in membrane phospholipid acyl chains could result in an increase in bilayer volume associated with a conformational change in the membrane receptor.

Although the results of the present study indicate that the level of agonist–receptor is an important step in the impoundment of alterations in the phospholipid fatty acid profile on the PtdIns-cycle, the effects are certainly not restricted to this level. The effect of polyunsaturated fatty acid incorporation in the phospholipids on the membrane dynamic characteristics, such as fluidity, membrane cholesterol in correlation to integrity might influence the process of activation and the maintenance in the PtdIns-cycle on one hand. On the other hand, the induced alterations in the molecular species of PtdIns(4,5)P$_2$ is also likely to be of importance.

The direct stimulation of the G-proteins of saponin-permeabilized cardiomyocytes with GTPγS showed an attenuation of total [^{3}H]InsP$_n$ accumulation in 20:5n-3-treated cells, as was measured in the cell extract plus incubation buffer. When the results on [^{3}H]InsP$_n$ formation in the buffer and cell extract were compared to those in the buffer alone, slight discrepancies show up concerning the effects of various fatty acid treatments. Thus, differences in permeabilization by saponin of the fatty-acid-treated cardiomyocytes could have occurred. However, investigation of the time course of saponin-induced permeabilization of cardiomyocytes, measured by the release of LDH, unmasked a membrane protecting effect of 20:5n-3-pretreatment of the cardiomyocytes. The membrane permeabilization by saponin is generally believed to be caused by complexation with cholesterol in the plasma membrane, leading to instability of the bilayer (Dourmashkin *et al.*, 1962; Glauert *et al.*, 1962). The inter-relationship between membrane stability, fluidity and cholesterol content of the bilayer, and DBI of the acyl groups in the phospholipids could be the basis of the unexpected finding of a membrane protective effect of 20:5n-3-

treatment of cardiomyocytes towards the permeabilizing agent, saponin. The massive increase of cholesterol induced by 20:5n-3-treatment may initially protect the membrane for the cholesterol clustering, destabilizing and subsequent permeabilizing action of saponin.

In addition, 20:5n-3-treatment-induced alterations in the plasma membrane fluidity and chemical composition, e.g. molecular species of phospholipids and/or cholesterol content, could mediate changes in the PtdIns-cycle activity was found to be most dramatic in n-3-PUFAs-treated atrial cells when comparing rats fed with n-6 PUFAs, mono-unsaturated or saturated fatty acids (Woodcock et al., 1995).

Only 20:5n-3 treatment resulted in a marked increase (1.7-fold) in cholesterol content expressed as cholesterol over phospholipid molar ratio. When cholesterol content was expressed per mg of protein, a slight increase in nmol cholesterol per mg cellular protein was observed in 18:0/18:1n-9- and in 18:2n-6-treated cells, but in the 20:5n-3-treated cells, the increase (three-fold) was much larger. In sarcolemma isolated from the hearts of pigs fed a dietary supplement of 20:5n-3/22:6n-3, an increase of the cholesterol/phospholipid ratio from 0.38 to 0.64 was also found by the present authors, although the phospholipid/protein ratio decreased from 0.94 to 0.69 (Lamers et al., 1987). However, in rat smooth muscle cells incubated with 20:5n-3 or 22:6n-3 bovine serum albumin complexes for 12 h, cholesterol content hardly changed (Dusserre et al., 1995). But in the latter study, only 8% and 9.5%, respectively, of these fatty acids were incorporated in the phospholipids, at the expense of n-6 PUFAs but also at the expense of 18:0 and 18:1n-9 fatty acid incorporation. It should be noticed that, in this model, the incorporation of 18:2n-6 and 20:4n-6 into the smooth muscle cell membrane phospholipids of the control fed animals was only 5% and 3.4% respectively, in comparison to the 17% and 18%, respectively, which have been found in neonatal cardiomyocytes.

The changes in cholesterol content led the authors to measure the membrane fluidity of the treated cardiomyocytes. Surprisingly, a decreased fluidity was found in the cells grown in 20:5n-3-enriched medium (Fig. 5). This finding suggests that the fluidizing effect of a high double bond index is overcompensated by a "stiffening" effect of increased cholesterol content. In agreement with this, the opposite effect was presented in a study on the same model of cultured neonatal cardiomyocytes (Bastiaanse et al., 1995). In the present study, it was suggested that the increase in sar-

colemmal fluidity that was observed after metabolic inhibition was caused by a decrease in sarcolemmal free cholesterol content.

In conclusion, in neonatal ventricular myocytes, the activity of the PtdIns cycle is affected by 20:5n-3 treatment of the cardiomyocytes and this effect could be exerted at either the level of agonist–receptor, receptor–G-protein coupling or G-protein–PLC-β interaction. It is hypothesized that a causal relationship may exist between the 20:5n-3-induced alteration of the physicochemical properties in the bilayer and the agonist-dependent effects on the PtdIns-cycle activity.

Acknowledgements

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