

## Modification of Fatty Acid Composition of the Phospholipids of Cultured Rat Ventricular Myocytes and the Rate of Phosphatidylinositol-4,5-Bisphosphate Hydrolysis

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J. M. J. LAMERS, D. H. W. DEKKERS, N. DE JONG, AND J. T. A. MEIJ. Modification of Fatty Acid Composition of the Phospholipids of Cultured Rat Ventricular Myocytes and the Rate of Phosphatidylinositol-4,5-Bisphosphate Hydrolysis. *Journal of Molecular and Cellular Cardiology* (1992) 24, 605-618. Cultured neonatal cardiac myocytes have been utilized as a model for the study of the role of fatty acids in the  $\alpha_1$ -adrenoceptor mediated phosphatidylinositol turnover. Experiments were started 24 h after seeding, when there was a confluent monolayer of beating cardiomyocytes. The cells were incubated for 3-4 days in sera containing culture medium with (1) no additives or (2) a mixture of 107  $\mu\text{M}$  18:0 and 18:1 $n$ -9, or (3) only 214  $\mu\text{M}$  18:2 $n$ -6 or (4) 214  $\mu\text{M}$  20:5 $n$ -3. No differences in the cellular content of the various phospholipid classes among the different groups of fatty acid treated cells were found. The predicted elevations of 18:1 $n$ -9, 18:2 $n$ -6 and 20:5 $n$ -3 associated with a partial depletion of 20:4 $n$ -6 were confirmed in all phospholipid classes, except for sphingomyelin. The mol % of 18:0, 18:2 $n$ -6, 20:4 $n$ -6 and 20:5 $n$ -3 in the phosphatidylinositol fraction were respectively 39, 4, 30 and 0.6 for the control treated cells, 34, 3, 15 and 0 for 18:0/18:1 $n$ -9 treated cells, 40, 17, 24 and 0.2 for the 18:2 $n$ -6 treated cells and 41, 3, 13 and 21 for the 20:5 $n$ -3 treated cells. Apart from the observed reductions in the basal rates, the phenylephrine (30  $\mu\text{M}$ ) stimulated production of inositolphosphates was reduced by 51% and 71%, respectively in the 18:2 $n$ -6 and 20:5 $n$ -3 treated cardiomyocytes. The basal rate of inositolphosphate formation was 37% increased in the 18:0/18:1 $n$ -9 treated cells. The [ $^3\text{H}$ ]-inositol incorporation into phosphatidylinositol 4,5-bisphosphate was only slightly reduced by 18:2 $n$ -6 and 20:5 $n$ -3 treatments (respectively 12 and 28% compared to control treated cells). Prolonged (30 min)  $\alpha_1$ -adrenergic stimulation did not affect the contents and fatty acid profiles of any class of phospholipid, not even phosphatidylinositol. In conclusion, variations in the polyunsaturated fatty acid composition of membrane phospholipids do affect the basal and the  $\alpha_1$ -adrenoceptor stimulated rate of phosphatidylinositol-4,5-bisphosphate hydrolysis. The reducing effects of 18:2 $n$ -6 and 20:5 $n$ -3 treatment on the rate of inositolphosphate production may be partially ascribed to altered levels of phosphatidylinositol 4,5-bisphosphate.

KEY WORDS: Cultured cardiomyocytes;  $\alpha_1$ -adrenergic receptors; Polyunsaturated fatty acids; Phospholipids; Phosphatidylinositol; Linoleic acid; Eicosapentaenoic acid; Phosphatidylinositol cycle; Fatty acid chain elongation; Triacylglycerides; Lipolysis; Protein kinase C.

### **<sup>1</sup>Introduction**

The fatty acyl composition of cell membranes can be markedly altered by dietary means, especially by changing the degree of polyunsaturation. For this reason it is important to

understand the consequences of such modification in terms of membrane phospholipid dynamics and function. Dietary  $n$ -3 polyunsaturated fatty acids ( $n$ -3 PUFA) have been shown to result in a reduction of the  $\alpha_1$ - but

<sup>1</sup>Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SPM, sphingomyelin; NEFA, non-esterified fatty acids; PIP, phosphatidylinositol-4-monophosphate; PIP<sub>2</sub>, phosphatidylinositol-4,5-bisphosphate; IP<sub>n</sub>, inositolphosphates; IP<sub>1</sub>, inositolmonophosphate; IP<sub>2</sub>, inositolbisphosphate; IP<sub>3</sub>, inositoltrisphosphate; 18:0, stearic acid; 21:0, heneicosanoic acid; 18:1, oleic acid; 18:2 $n$ -6, linoleic acid; 20:4 $n$ -6, arachidonic acid; 20:5 $n$ -3, eicosapentaenoic acid; 22:5 $n$ -3, docosapentaenoic acid; 22:6 $n$ -3, docosahexaenoic acid; PUFA, polyunsaturated fatty acids; DG, diacylglycerides; TG, triacylglycerides; TLC, thin-layer chromatography; BHT, butylated hydroxytoluene.

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not of the  $\beta$ -adrenoceptor mediated changes in cardiac inotropy (Reibel *et al.*, 1988) and so the question arises whether the difference in the  $\alpha_1$ -adrenergic response after incorporation of those PUFA into heart membranes is caused by alterations in the rate of phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>) breakdown by specific phospholipase C. A massive incorporation of eicosapentaenoic acid (20:5*n*-3) and docosahexaenoic acid (22:6*n*-3) at the expense of linoleic acid (18:2*n*-6) and arachidonic acid (20:4*n*-6) was observed in the cardiac total phospholipid fraction from rats fed with *n*-3 PUFA (Reibel *et al.*, 1988). Changes in platelet responsiveness to aggregating substances induced by intervention with dietary *n*-3 PUFA have usually been related to alterations in the production of eicosanoid precursor fatty acids and not merely to alterations in the fatty acid composition of membrane phospholipids (Hartog *et al.*, 1987; Lamers *et al.*, 1987 and Leaf and Weber, 1988). However, Medini *et al.* (1990) demonstrated that dietary 20:5*n*-3 modulated the pathway of inositol-1,4,5-trisphosphate (IP<sub>3</sub>) generation in platelets from rabbits independent of modification of thromboxane production. Likewise, Reibel *et al.* (1988) showed that changes in eicosanoid production induced by dietary fish oil were not likely involved in the attenuation of the myocardial  $\alpha_1$ -adrenoceptor response.

*In vivo* studies (Gudbjarnason *et al.*, 1978 and Montfoort *et al.*, 1986) have indicated that chronic noradrenaline stress produces changes in *n*-3 relative to the *n*-6 PUFA content of myocardial phospholipids, particularly that of the phosphatidylethanolamine fraction. It is not known whether this noradrenaline treatment effect is a direct  $\alpha_1$ - or  $\beta$ -adrenergic effect on the myocardial cells or an indirect  $\alpha_1$ - or  $\beta$ -adrenergic effect resulting from altered lipid metabolism in the liver. Nevertheless, the accelerated turnover of the myocardial (polyphospho)PI lipids, induced by  $\alpha_1$ -adrenoceptor stimulation, could be one of the factors involved in the *in vivo* effects of noradrenaline stress on myocardial phospholipids.

Several reports have described that the fatty acid composition of culture cardiomyocytes can be modified by addition of non-esterified fatty acids (NEFA) to the culture medium

(Grynberg *et al.*, 1988; Meij *et al.*, 1990; Nalbone *et al.*, 1990; Hallaq *et al.*, 1990). These cells, isolated from neonatal rat hearts, beat spontaneously with a regular rhythm and amplitude of contraction and can be incubated free from circulating agonists (Meij and Lamers, 1989a and b). They maintain their function for several days in culture medium, which allows time for incorporation of long-chain (polyunsaturated) fatty acids into the phospholipids of their cell membranes. Recently, we showed that 20:5*n*-3 as well as 18:2*n*-6 treatment of cultured heart cells had no effect on the basal and  $\alpha_1$ -adrenoceptor stimulated PIP<sub>2</sub> hydrolysis (Meij *et al.*, 1990). However, in this study only minor changes occurred in fatty acid composition of the phospholipids (e.g. the maximal *n*-3/*n*-6 PUFA molar ratio amounted 0.45). Nalbone *et al.* (1990) showed increases of the *n*-3/*n*-6 PUFA molar ratio up to 1.1 in cultured cardiomyocytes, very near to values reported in the study of Reibel *et al.* (1988).

In the present study, we investigated the effect of NEFA supplements to the serum in the primary culture of rat cardiomyocytes on the fatty acid composition of the various phospholipid classes, with particular attention to the PI fraction. The main object was to observe the influence of these changes on the rate of basal and  $\alpha_1$ -adrenoceptor mediated PIP<sub>2</sub> hydrolysis. We also evaluated the possible effects of long-term  $\alpha$ -adrenergic stimulation of the PI turnover on the fatty acid composition of the PI fraction as well as all the other phospholipid classes.

## Materials and Methods

### Chemicals

Growth medium: Nutrient mixture Ham F10 (Gibco, Scotland) supplemented with 10% v/v fetal calf serum, 10% v/v horse serum, 200 U/ml penicillin, 0.2 mg/ml streptomycin (all from Boehringer, Mannheim, FRG) and 135  $\mu$ g/ml CaCl<sub>2</sub>·2H<sub>2</sub>O; Petri dishes (TC 35/10 and TC 60/15) were from Greiner (Nürtingen, FRG); four-well multidishes were from Nunc (Denmark); trypsin (type III) was from Sigma as were the 18:1*n*-9, 18:2*n*-6 and 20:5*n*-3, the BF<sub>3</sub>/methanol and the lipid standards phosphatidylcholine (PC), phospho-

tidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SPM) and lysophosphatidylcholine; 18:0 was from BDH (Poole, England); standard fatty acid methylesters and the internal standard heneicosanoic acid (21:0) for the transmethyl-ation reaction were from Alltech Ass., Inc (Deerfield, USA); [2-<sup>3</sup>H]-myoinositol was from Amersham International PLC (Amersham, UK); [dipalmitoyl-1-<sup>14</sup>C]-PC and EN<sup>3</sup>HANCE spray were from NEN (Boston, USA); phenylephrine HCl was from Brocades (Delft, The Netherlands); Thin layer chromatography plates (Kieselgel 60 F-254) were from Merck (Darmstadt, FRG), Dowex 1-X8 (100-200 mesh, formate form) from BioRad Labs (California, USA) and Instagel was from Packard (Groningen, The Netherlands); Triacylglyceride and glycerol test kits (respectively, GPO-PAP and Precimat<sup>®</sup> glycerol) were from Boehringer

(Mannheim, FRG). All other chemicals were of analytical grade.

#### Primary heart cell culture

Cardiomyocytes were isolated from the ventricles of 2-4-day-old Wistar rats by trypsinization and grown according to Yagev *et al.* (1984). We applied a modified enrichment method of two successive periods of 30 and 90 min plating (Blondel *et al.* (1971); Meij and Lamers (1989a and b)). Of the final suspension containing  $9 \times 10^5$  myoblasts per ml, 2 ml were seeded per TC 35/10 Petri dish, 6 ml per TC 60/15 Petri dish and 0.6 ml per well in 4-well multidishes. After 24 h incubation (37°C, 5% CO<sub>2</sub>, 95% humidity) this resulted in a confluent monolayer of beating cardiomyocytes. About 24 h after seeding the growth medium was replaced by conditioned medium (Table 1).

TABLE 1 Total and non-esterified fatty acid content ( $\mu\text{M}$ ) of the conditioned media

Fatty acid	Control		18:0/18:1n-9		18:2n-6		20:5n-3	
	Total <sup>a</sup> $\mu\text{M}$	NEFA <sup>b</sup> $\mu\text{M}$	Total $\Delta\mu\text{M}^c$	NEFA $\Delta\mu\text{M}$	Total $\Delta\mu\text{M}$	NEFA $\Delta\mu\text{M}$	Total $\Delta\mu\text{M}$	NEFA $\Delta\mu\text{M}$
16:0	221	9.8	7	0.5	-2	-0.5	6	-0.2
18:0	226	5.9	80	104	-33	-0.8	-10	-0.2
18:1n-9	221	7.5	125	104	-3	-0.1	18	0.6
18:2n-6	410	5.9	-5	0	208	218	-11	-0.1
20:4n-6	19.4	0.2	0.7	0	3.2	0.1	2.7	0.1
20:5n-3	n.d. <sup>d</sup>	n.d.	n.d.	n.d.	n.d.	n.d.	201	215
22:5n-3	5.2	n.d.	0.1	n.d.	0.1	n.d.	0.6	n.d.
22:5n-6	6.5	n.d.	0.9	n.d.	1	n.d.	1.2	n.d.
$\Sigma\text{PUFA}^c$	441	6.1	-3	0	211	218	215	215

<sup>a</sup>Fatty acid content of the culture medium containing fatty acids esterified in phospholipids, cholesterol esters, TG and NEFA.

<sup>b</sup>NEFA stands for the non-esterified fatty acid fraction.

<sup>c</sup> $\Sigma\text{PUFA}$  is the sum of PUFA.

<sup>d</sup>n.d. means non-detectable.

<sup>e</sup> $\Delta\mu\text{M}$  is the change of total fatty acid or NEFA concentration versus control medium. The data represent the mean of duplicate measurements.

#### Cell incubations

At the beginning of each experiment the batch of dishes was divided into four sets, each set receiving one of the following conditioned media: (1) control medium; (2) 18:0/18:1n-9 rich medium which was supplemented with 18:0 and 18:1n-9, 107  $\mu\text{M}$  each; (3) 18:2n-6 rich

medium which was supplemented with 214  $\mu\text{M}$  18:2n-6; (4) 20:5n-3 rich medium which was supplemented with 214  $\mu\text{M}$  20:5n-3 (Table 1). The ethanolic fatty acid solutions were mixed under vigorous stirring with the concentrated calf and horse sera (containing excess of fatty acid poor albumin molecules) followed by

addition of the Ham F10 medium. Because the non-esterified fatty acids (NEFA) were dissolved in ethanol when added to the sera during preparation of each medium, control medium contained an equal concentration (0.3% v/v) of pure ethanol. Fresh conditioned media were given to each set after 65 h. Furthermore every medium was supplemented with either 88 nM unlabelled myoinositol or - to study [ $^3\text{H}$ ]-inositolphosphate formation - 88 nM [ $^2\text{-}^3\text{H}$ ]-myoinositol (2  $\mu\text{Ci/ml}$ ). The dishes were then incubated with those fresh media for at least 24 h (37°C, 5%  $\text{CO}_2$ , 95% humidity) before starting the cell stimulation. In the [ $^3\text{H}$ ]- $\text{IP}_n$  formation studies small dishes (type 35/10) were used and for the lipid and fatty acid analysis large dishes (type 60/15) were used. For the study of [ $^3\text{H}$ ]-inositol incorporation into PI, PIP and  $\text{PIP}_2$  4-well multidishes were used.

#### *Cell stimulation and assay of [ $^3\text{H}$ ]-inositolphosphates*

After 24 h preincubation of the cardiomyocytes with [ $^3\text{H}$ ]-myoinositol in the conditioned culture medium, the dishes were rinsed twice with 1 ml buffer (130 mM NaCl, 4.7 mM KCl, 1.3 mM  $\text{CaCl}_2$ , 20 mM  $\text{NaHCO}_3$ , 0.44 mM  $\text{NaH}_2\text{PO}_4$ , 1.1 mM  $\text{MgCl}_2$ , 0.2% w/v glucose, pH 7.4, equilibrated at 37°C with 5%  $\text{CO}_2$  at 97% humidity) and the cells were equilibrated in this buffer for 30 min. Then LiCl was added to a final concentration of 10 mM. After another 10 min of preincubation, phenylephrine at a final concentration of 30  $\mu\text{M}$ , or buffer was added to a final volume of 1 ml. Incubations were terminated after 30 min by rinsing the small dishes with 0.5 ml cold buffer followed by the addition of 0.5 ml cold methanol/12 M HCl (100:1, v/v). The cells were scraped using a rubber policeman and the suspension was collected in a glass tube. The dish was rinsed once more with 0.5 ml of methanol/12 M HCl, which was transferred to the same glass tube. Then 1 ml chloroform and 0.5 ml 2.5 M HCl were added to the tube. After centrifugation (10 min, 1550 g) the upper phase, containing the  $\text{IP}_n$ , was collected and the lower phase was washed once with 1.5 ml methanol/chloroform/0.6 M HCl (48:3:47, v/v/v). The upper phases were combined and 5 ml distilled water

was added. The  $\text{IP}_n$  were separated by anion exchange using 1 ml Dowex 1-X8 according to the method of Berridge *et al.* (1982). Briefly, free inositol and glycerophosphoinositides were eluted with 10 ml distilled water and 10 ml 5 mM disodiumtetraborate in 30 mM sodiumformate respectively. Inositolmonophosphate ( $\text{IP}$ ), inositolbisphosphate ( $\text{IP}_2$ ) and inositoltrisphosphate ( $\text{IP}_3$ ) were eluted all together using 10 ml 1.0 M ammonium formate in 0.1 M formic acid. One volume of the eluate was mixed with one volume of Instagel and counted for radioactivity by liquid scintillation (Tri-carb 2660, from Packard, Groningen, The Netherlands).

#### *Separation of lipids and quantification of fatty acid methyl esters*

For studying the incorporation of [ $^3\text{H}$ ]-inositol into PI, PIP and  $\text{PIP}_2$  of control and fatty acid treated cardiomyocytes, cells from 4-well multidishes were rinsed twice with 0.5 ml ice-cold Ham F10 medium without sera. Thereafter the cells were scraped off and extracted with the methanol/HCl procedure (Meij and Lamers, 1989b). The washed lower organic phase was evaporated to dryness with  $\text{N}_2$ , redissolved and spotted on TLC plates previously impregnated with 1% potassium-oxalate in methanol/ $\text{H}_2\text{O}$  (2:3, v/v) and activated for 30 min at 110°C. The plates were developed as previously described (Meij and Lamers, 1989b). After development the plates were dried and sprayed with  $\text{EN}^3\text{HANCE}$  to visualize the radioactive spots by fluorography (Kodak SB5). The different inositol containing spots, identified by co-elution of authentic standard (polyphospho)PI lipids, were scraped off and counted for radioactivity by liquid scintillation.

For studying the lipid composition of the treated unlabelled cells, the cell incubations containing unlabelled myoinositol were terminated after 30 min by rinsing the large dishes with 1.5 ml cold buffer, followed by the addition of 1.5 ml cold methanol. The cells were scraped using a rubber policeman and the suspension was collected in a glass tube. The dish was then rinsed again with 1.5 ml of methanol. After addition of 1.5 ml chloroform, the suspension was stored under nitrogen at -20°C overnight. For the estimation

of the recovery of total phospholipids, tracer amounts of [ $^{14}\text{C}$ ]-PC were added. The phases were then separated by adding chloroform and 0.1 M KCl to a final ratio of 2:2:1 (v/v/v) and centrifugation (10 min, 1550g), according to Bligh and Dyer (1959). The organic lower phases of the two extracts were combined.

Approximately 0.40–0.75 ml of the combined lipid extracts were used for the separation of total phospholipid and TG fractions from other lipids by thin layer chromatography, using activated plates and hexane/diethylether/acetic acid (60:40:1, v/v/v) as solvent system and 0.02% w/v butylated hydroxytoluene (BHT) as an antioxidant. The phospholipid spot remaining at the origin was scraped off and extracted twice with 1.5 ml methanol.

For the separation of various phospholipid classes, about 5 ml of the combined extracts were evaporated to dryness with  $\text{N}_2$  at 37°C and redissolved in a small volume of organic solvent. The samples were applied under  $\text{N}_2$  to an activated (30 min, 110°C) TLC plate. The plates were developed with the solvent chloroform/methanol/petroleum ether (bp 40–60°C)/acetic acid/boric acid (40:20:30:14:1.8, v/v/v/vw) containing 0.02% (w/v) BHT by a modified method ~ Gilfillan *et al.* (1983). The spots were visualized by spraying with Rhodamine 6G (0.01%). The individual phospholipids were identified by co-migration of standards. The plate was dried under  $\text{N}_2$  and the scraped spots were extracted twice with 1.5 ml methanol for fatty acid analysis.

After addition of 20 nmol of the internal standard 21:0 to the extracted phospholipid fractions, the methanol was evaporated under  $\text{N}_2$  and the phospholipids were transmethyl-esterified using  $\text{BF}_3$  as previously described by Morrison and Smith (1964). For the gas chromatographic separation of 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. The separated peaks were identified on the basis of the retention times as compared to standards (Montfoort *et al.*, 1986).

#### *Analysis of TG and glycerol*

TG was measured spectrophotometrically in the initial lipid extracts using the test kit. For

the study of glycerol release, the incubation media were collected just before the wash step at the end of the cell incubations. Glycerol was measured fluorimetrically according to the method of Laurell and Tibbling (1966).

#### *Statistical analysis*

All data are expressed as means  $\pm$  S.E.. Multiple comparisons were made between control and fatty acid treated cells by unpaired Student's *t*-test with  $P < 0.05$  being considered significant.

## Results

#### *Fatty acid composition of membrane phospholipids*

The effects on membrane composition were examined in cardiomyocytes isolated from neonatal rats that were incubated for 3–4 days in animal sera containing culture medium with (1) no additives or (2) a mixture of 107  $\mu\text{M}$  18:0 and 107  $\mu\text{M}$  18:1 $n$ -9, or (3) only 214  $\mu\text{M}$  18:2 $n$ -6 or (4) 214  $\mu\text{M}$  20:5 $n$ -3. Table 1 shows the total fatty acid and NEFA content in  $\mu\text{M}$  of the four conditioned media. Supplementation of the media with NEFA changed the total fatty acid content of the media only moderately (not more than 20% increase). The total pool of fatty acids is mainly composed of those esterified in TG, cholesterolesters and phospholipids which are all present in the lipoproteins of the added sera. However, it is important to note that the small NEFA pool constituting 2–3% of the total fatty acid pool, generally serves as the major extracellular source of fatty acid for cultured cells (Spector *et al.*, 1981). As can be seen in the NEFA fraction of the conditioned media, the 18:0, 18:1 $n$ -9, 18:2 $n$ -6 and 20:5 $n$ -3 contents have changed drastically due to the additions (Table 1). The results of the analysis of cardiomyocyte phospholipids under the influence of these various fatty acid supplements are shown in Table 2.

There was no detectable change due to fatty acid treatment of the cardiomyocytes in the relative or absolute amounts of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and sphingomyelin (SPM) fractions separated by one-dimensional thin-layer-chromatography (TLC). The composition is similar to that reported by Post *et al.* (1988) for cultures of neonatal

TABLE 2 Class composition of total phospholipids from control cardiomyocytes and cells treated with 18:0/18:1*n*-g, 18:2*n*-6 or 20:5*n*-3

Class of phospholipid	Control <i>n</i> = 6 <sup>a</sup>	18:0/18:1 <i>n</i> -g <i>n</i> = 2	18:2 <i>n</i> -6 <i>n</i> = 5	20:5 <i>n</i> -3 <i>n</i> = 4
<i>mol percentage (S.E.)</i>				
PC	45.9 (5.0)	48.8	44.4 (4.4)	52.0 (5.1)
PE	30.9 (3.3)	30.0	31.8 (4.8)	27.2 (3.6)
PS	4.0 (0.6)	4.4	5.3 (1.4)	4.9 (1.1)
PI	9.0 (1.2)	9.1	9.4 (1.0)	8.5 (0.8)
SPM	10.1 (2.3)	7.7	9.1 (1.2)	7.2 (1.7)

The composition is calculated from gaschromatographic analysis of the prepared methyl esters of the fatty acids constituting the respective phospholipid classes. No differences were found between the respective absolute phospholipid fatty acid contents (respectively  $191 \pm 10$ , 178,  $178 \pm 9$  and 202 nmol/mg protein) of control-, 18:0/18:1*n*-g-, 18:2*n*-6- and 20:5*n*-3 treated cardiomyocytes. Values are presented as means (S.E.).

<sup>a</sup>Number of determinations of the phospholipid class contents in each group of control or fatty acid treated cells.

cardiomyocytes except that in the present study PI and PS were determined separately. Since the amounts of the phospholipid classes were found to be the same in all four cell treatments, it was decided to express the results of the fatty acid analysis of the major phospholipid fractions PC and PE are shown in Table 3.

Supplementation of the culture medium with 18:0/18:1*n*-g mixture caused a marked increase in incorporation of these fatty acids in PC and predominantly a reduction in 16:0, 18:1*n*-7 and 20:4*n*-6 and the sum of PUFA decreased from 33.9 to 24.7 mol%. However, the *n*-3/*n*-6 PUFA molar ratio decreased from 0.17 to 0.11 resulting from a slight shift in incorporation of *n*-6 PUFA in favor of *n*-3 PUFA. Treatment of the cells with 18:2*n*-6 caused a dramatic increase of 18:2*n*-6 to 42.1 mol% of the total PC fatty acids and a decrease of incorporated 18:2*n*-6 metabolites, 20:4*n*-6 and 22:4*n*-6 (Table 3). There was still a marked increase of  $\Sigma$ PUFA from 33.9 to 49.9 mol%. The massive incorporation of 18:2*n*-6 into the fatty acids of PC, depressed the *n*-3/*n*-6 PUFA molar ratio to almost zero. Treatment of the cardiac cells with 20:5*n*-3 led to increased incorporation of this fatty acid and also of 22:5*n*-3 in PC. The increased incorporation of *n*-3 PUFA occurred at the expense of *n*-6 PUFA as the sum of 18:2*n*-6 and 20:4*n*-6 decreased from 26.5 to 12.6 mol%. Although  $\Sigma$ PUFA increased significantly from 33.9 to 41.5 mol% after 20:5*n*-3 treatment, the increase was much less compared to 18:2*n*-6 treatment. Thus it appeared that cardiomyocyte PC more readily

incorporated 18:2*n*-6 than 20:5*n*-3.

The general patterns of changes in phospholipids PE, PI and PS following fatty acid treatment of the cells were very similar to those seen in PC (Tables 3 and 4). There are, however, some interesting differences between the fatty acid composition of the various phospholipid classes per se and some phospholipid class-specific responses to fatty acid treatment. The preferential incorporation of 18:2*n*-6 compared with 20:4*n*-6, 20:5*n*-3 and 22:5*n*-3 was observed predominantly with PC, as the highest 18:2*n*-6 incorporation among the other phospholipid classes was certainly less than 22 mol%. This difference between PC and the other phospholipid classes PE, PI and PS can not be explained by the low PUFA contents of the non-PC phospholipid classes; on the contrary, the  $\Sigma$ PUFA of e.g. PE of control treated cells reaches almost 50 mol% (Table 3).

The results in Tables 3 and 4 show that PE, PI and even PS in control treated cells contain much more 20:4*n*-6 relative to 18:2*n*-6 (e.g. PI contains 30.0 relative to 4.4 mol%). Another remarkable finding is the relative homogeneity in fatty acid composition of the PI fraction in control treated cardiomyocytes. The PI fraction is mainly composed of molecular species with 18:0 on the *sn*-1 site and 20:4*n*-6 on the *sn*-2 site of the glycerol moiety. This homogeneity disappeared when the cardiomyocytes are exposed to the various types of fatty acid. It should also be noticed that in the PS fraction, in contrast to the other major phospholipid classes, there is a preferential incorporation of the 20:5*n*-3 metabolite 22:5*n*-3.

TABLE 3 Fatty acid composition (mol%) of phosphatidylcholine and phosphatidylethanolamine fractions from control cardiomyocytes and cells treated with 18:0/18:1n-g, 18:2n-6 or 20:5n-3

Fatty acids	Phosphatidylcholine				Phosphatidylethanolamine			
	Control	18:0/18:1n-g	18:2n-6	20:5n-3	Control	18:0/18:1n-g	18:2n-6	20:5n-3
	n = 6 <sup>a</sup>	n = 4	n = 6	n = 6	n = 6	n = 4	n = 6	n = 4
16:0	24.0 (0.9)	12.5 (0.8) <sup>d</sup>	18.5 (0.9) <sup>d</sup>	23.3 (0.7)	6.6 (0.4)	4.7 (0.6) <sup>d</sup>	5.5 (0.2) <sup>d</sup>	7.4 (0.40)
18:0	19.4 (0.7)	30.8 (1.4) <sup>d</sup>	19.0 (0.7)	19.9 (0.3)	22.0 (1.0)	34.3 (5.2) <sup>d</sup>	21.6 (0.5)	26.8 (1.0) <sup>d</sup>
18:1n-9	10.3 (0.3)	28.3 (0.1) <sup>d</sup>	5.9 (0.5) <sup>d</sup>	6.7 (0.4) <sup>d</sup>	5.3 (0.3)	20.8 (0.9) <sup>d</sup>	3.7 (0.4) <sup>d</sup>	3.8 (0.4) <sup>d</sup>
18:1n-7	6.5 (0.4)	n.d. <sup>d</sup>	2.6 (0.2) <sup>d</sup>	3.4 (0.3) <sup>d</sup>	2.1 (0.2)	n.d. <sup>d</sup>	1.8 (0.2)	1.8 (0.2)
18:2n-6	14.0 (0.7)	15.4 (0.5)	42.1 (1.7) <sup>d</sup>	6.4 (0.2) <sup>d</sup>	5.5 (0.7)	7.6 (1.6)	22.0 (1.9) <sup>d</sup>	2.9 (0.2) <sup>d</sup>
20:4n-6	12.5 (0.9)	5.4 (0.9) <sup>d</sup>	4.6 (0.6) <sup>d</sup>	6.2 (0.3) <sup>d</sup>	27.7 (1.4)	6.1 (4.5) <sup>d</sup>	22.2 (1.4) <sup>d</sup>	10.4 (0.8) <sup>d</sup>
20:5n-3	0.3 (0.1)	0.5 (0.1)	0.2 (0.1)	17.8 (0.7) <sup>d</sup>	0.7 (0.1)	1.5 (0.6) <sup>d</sup>	0.3 (0.1)	18.0 (1.6) <sup>d</sup>
22:5n-3	1.7 (0.3)	0.8 (0.1) <sup>d</sup>	0.4 (0.1) <sup>d</sup>	8.4 (0.2) <sup>d</sup>	3.8 (0.3)	2.1 (0.8) <sup>d</sup>	2.5 (0.4) <sup>d</sup>	13.0 (0.8) <sup>d</sup>
22:6n-3	2.0 (0.2)	0.7 (0.1) <sup>d</sup>	0.6 (0.1) <sup>d</sup>	1.6 (0.1) <sup>d</sup>	5.9 (0.4)	2.2 (1.1) <sup>d</sup>	3.6 (0.3) <sup>d</sup>	3.8 (0.1) <sup>d</sup>
others <sup>b</sup>	9.3 (1.0)	5.6 (0.3) <sup>d</sup>	6.1 (0.4) <sup>d</sup>	6.3 (0.5) <sup>d</sup>	20.6 (0.5)	20.9 (2.6) <sup>d</sup>	16.8 (0.9) <sup>d</sup>	12.1 (2.0) <sup>d</sup>
n-3/n-6 <sup>c</sup>	0.17 (0.01)	0.11 (0.01) <sup>d</sup>	0.03 (0.01) <sup>d</sup>	2.11 (0.07) <sup>d</sup>	0.29 (0.01)	0.26 (0.02)	0.14 (0.01) <sup>d</sup>	2.40 (0.16) <sup>d</sup>
ΣPUFA <sup>c</sup>	33.9 (1.1)	24.7 (1.3) <sup>d</sup>	49.9 (1.1) <sup>d</sup>	41.5 (0.7) <sup>d</sup>	49.2 (1.3)	33.2 (5.6) <sup>d</sup>	56.4 (0.6) <sup>d</sup>	49.7 (2.4)

<sup>a</sup>Number of determinations of the fatty acid content in each group of control or fatty acid treated cells.  
<sup>b</sup>Only the major fatty acids are listed and the sum of minor fatty acids (mainly 16:0dmsa, 18:3n-3, 20:3n-6, 22:0, 24:0 and 22:4n-6) are listed and termed "others". Dmsa is the abbreviation for dimethylacetate.  
<sup>c</sup>N-3/n-6 is the molar ratio of total % of n-3 PUFA over the total of n-6 PUFA.  
<sup>d</sup>P < 0.05 versus control cells. Values are presented as means (S.E.).  
<sup>e</sup>ΣPUFA is the sum of all PUFA.

TABLE 4 Fatty acid composition (mol %) of phosphatidylinositol and phosphatidylserine fractions from control cardiomyocytes and cells treated with 18:0/18:1n-g, 18:2n-6 or 20:5n-3

Fatty acids	Phosphatidylinositol				Phosphatidylserine			
	Control n = 6 <sup>a</sup>	18:0/18:1n-g n = 4	18:2n-6 n = 6	20:5n-3 n = 6	Control n = 6	18:0/18:1n-g n = 2	18:2n-6 n = 6	20:5n-3 n = 4
16:0	4.5 (0.3)	5.9 (2.0)	4.7 (0.6)	5.8 (0.2) <sup>d</sup>	4.9 (0.6)	2.5	6.5 (1.4)	6.1 (0.7)
18:0	39.2 (0.5)	34.0 (2.2) <sup>d</sup>	39.8 (0.7)	41.2 (1.6)	41.7 (1.7)	38.0	38.7 (1.6)	39.7 (1.1)
18:1n-9	6.4 (0.2)	18.3 (0.7) <sup>d</sup>	4.6 (0.7) <sup>d</sup>	4.1 (0.2) <sup>d</sup>	10.8 (1.0)	27.6	10.3 (0.5)	8.0 (1.0)
18:1n-7	2.3 (0.2)	n.d.	1.2 (0.1) <sup>d</sup>	0.9 (0.1) <sup>d</sup>	2.1 (0.1)	n.d.	2.3 (0.6)	1.6 (0.4)
18:2n-6	4.4 (1.0)	3.5 (0.9)	17.4 (1.4) <sup>d</sup>	3.3 (0.5)	5.1 (1.3)	1.7	14.9 (1.6) <sup>d</sup>	2.1 (0.1)
20:4n-6	30.0 (1.3)	15.5 (3.7) <sup>d</sup>	24.2 (1.7) <sup>d</sup>	12.9 (0.6) <sup>d</sup>	14.0 (0.9)	6.1	11.7 (0.9) <sup>d</sup>	4.4 (0.8) <sup>d</sup>
20:5n-3	0.6 (0.2)	n.d.	0.2 (0.0)	20.6 (1.0) <sup>d</sup>	1.1 (0.3)	n.d.	1.3 (0.2)	6.2 (1.1) <sup>d</sup>
22:5n-3	1.5 (0.2)	0.8 (0.5) <sup>d</sup>	0.8 (0.0) <sup>d</sup>	6.2 (0.4) <sup>d</sup>	3.9 (0.2)	3.8	2.1 (0.2) <sup>d</sup>	19.3 (1.0) <sup>d</sup>
22:6n-3	1.3 (0.1)	n.d.	1.2 (0.5)	1.0 (0.1)	6.4 (0.2)	1.7	2.8 (0.6) <sup>d</sup>	3.8 (0.4) <sup>d</sup>
others <sup>b</sup>	9.8 (0.6)	22.0 (3.7) <sup>d</sup>	6.7 (0.6) <sup>d</sup>	4.0 (0.5) <sup>d</sup>	10.0 (1.8)	18.7	9.4 (1.3)	8.8 (1.9)
n-3/n-6 <sup>c</sup>	0.12 (0.02)	0.44 (0.09) <sup>d</sup>	0.04 (0.01) <sup>d</sup>	1.72 (0.16) <sup>d</sup>	0.45 (0.04)	0.53	0.19 (0.05) <sup>d</sup>	3.52 (0.38) <sup>d</sup>
ΣPUFA <sup>c</sup>	42.0 (0.4)	30.2 (1.9) <sup>d</sup>	45.6 (1.6) <sup>d</sup>	44.4 (1.2)	36.5 (1.8)	16.0	37.4 (1.5)	39.1 (1.8)

<sup>a</sup>Number of determinations of the fatty acid content in each group of control or fatty acid treated cells.

<sup>b</sup>Only the major fatty acids are listed and the sum of minor fatty acids (mainly 16:0dma, 18:0dma, 18:3n-3, 22:0, 20:3n-6, 24:0 and 22:4n-6) are listed and termed "others". Dma is the abbreviation for dimethylacetal.

<sup>c</sup>N-3/n-6 is the molar ratio of total % of n-3 PUFA over the total of n-6 PUFA.

<sup>d</sup>P < 0.05 versus control cells.

<sup>e</sup>ΣPUFA is the sum of all PUFA. Values are presented as means (S.E.).



TABLE 5 Fatty acid composition (mol %) of the sphingomyelin fraction from control cardiomyocytes and cells treated with 18:0/18:1*n*-g, 18:2*n*-6 or 20:5*n*-3

Fatty acids	Control <i>n</i> = 4 <sup>a</sup>	18:0/18:1 <i>n</i> -g <i>n</i> = 2	18:2 <i>n</i> -6 <i>n</i> = 4	20:5 <i>n</i> -3 <i>n</i> = 3
16:0	26.6 (1.3)	22.1	28.5 (2.2)	36.3 (2.2) <sup>d</sup>
18:0	19.1 (1.5)	28.5	17.4 (1.7)	15.5 (3.3)
18:1 <i>n</i> -9	4.0 (0.5)	5.6	4.2 (0.9)	4.4 (1.0)
18:2 <i>n</i> -6	2.7 (0.9)	0.6	3.9 (1.3)	1.0 (0.0)
20:0	9.2 (0.6)	8.5	8.8 (0.6)	6.6 (0.1)
22:0	9.1 (0.7)	10.1	8.4 (1.2)	7.8 (1.0)
20:4 <i>n</i> -6	3.8 (0.8)	2.3	4.7 (1.2)	3.3 (0.2)
24:0	10.5 (1.0)	11.1	7.6 (0.5) <sup>d</sup>	8.2 (1.0)
20:5 <i>n</i> -3	0.8 (0.4)	n.d.	0.3 (0.0)	1.1 (0.1)
24:1 <i>n</i> -9	8.5 (0.7)	8.9	8.5 (0.8)	9.0 (1.0)
others <sup>b</sup>	5.8 (0.9)	2.8	7.6 (2.4)	6.8 (1.5)
<i>n</i> -3/ <i>n</i> -6 <sup>c</sup>	0.40 (0.11)	0.28	0.32 (0.13)	0.61 (0.05)
ΣPUFA <sup>c</sup>	9.6 (1.9)	3.6	11.8 (2.7)	7.6 (1.3)

<sup>a</sup>Number of determinations of the fatty acid content in each group of control or fatty acid treated cells.

<sup>b</sup>Only the major fatty acids are listed and the sum of minor fatty acids (mainly 16:0dma, 18:0dma, 18:1*n*-7, 18:3*n*-3) are listed and termed "others". Dma is the abbreviation for dimethylacetal.

<sup>c</sup>*N*-3/*n*-6 is the molar ratio of total % of *n*-3 PUFA over the total of *n*-6 PUFA.

<sup>d</sup>*P* < 0.05 versus control cells.

<sup>e</sup>ΣPUFA is the sum of all PUFA. Values are presented as means (S.E.).

SPM is a choline-containing phospholipid with only one fatty acid esterified per molecule. Long-chain (mono)unsaturates are reported to be mainly present. The results obtained from the cardiomyocyte are summarized in Table 5 and confirm this distinct SPM property as the 16:0, 18:0, 20:0, 22:0, 24:0 and 24:1*n*-9 contents vary between 8 and 27 mol% whereas the contents of 18:2*n*-6 and 20:5*n*-3 were both lower than 4 mol %. Therefore it is not surprising that 18:2*n*-6 or 20:5*n*-3 treatment of the cells did not lead to incorporation of these fatty acids into SPM. In contrast, slight changes in the 24:0 content of SPM in response to 18:2*n*-6 treatment and in the 16:0 and 20:0 content in response to 20:5*n*-3 treatment had occurred.

#### The rate of PIP<sub>2</sub> hydrolysis

The cardiomyocytes, pretreated with control medium and 18:0/18:1*n*-9, 18:2*n*-6 or 20:5*n*-3 rich medium, were tested for the basal and α<sub>1</sub>-adrenoceptor stimulated rate of PIP<sub>2</sub> hydrolysis. The [<sup>3</sup>H]-IP<sub>n</sub> production (Fig. 1) represents the sum of [<sup>3</sup>H]-IP, [<sup>3</sup>H]-IP<sub>2</sub> and [<sup>3</sup>H]-IP<sub>3</sub> formation. Previously we have shown that the major IP<sub>n</sub> formed is the mono-

phosphate, due to the rapid breakdown of IP<sub>2</sub> and IP<sub>3</sub> by phosphatases during cell incubation. The IP<sub>n</sub> formation was measured over a period of 30 min. Cardiomyocyte exposure to 18:0/18:1*n*-9 stimulates basal PIP<sub>2</sub> hydrolysis 37% whereas partial inhibition was found after exposure to 18:2*n*-6 and 20:5*n*-3 (35 and 49%, respectively). The maximal rate of PIP<sub>2</sub> hydrolysis that can be reached by adding 30 μM phenylephrine was not significantly affected by 18:0/18:1 *n*-g treatment but was also partially inhibited by treatment with the *n*-6 and *n*-3 PUFA (51% and 71%, respectively).

No selective depletion of any specific type of PUFA occurred in the PI fraction of the cardiomyocytes stimulated for 30 min with a maximal dosis of phenylephrine [Fig. 2(a) and (b)]. Similarly, the analysis of non-PI phospholipids showed no changes in fatty acid patterns due to α<sub>1</sub>-adrenergic stimulation (results not shown).

#### [<sup>3</sup>H]-Inositol incorporation in membrane (polyphospho)PI lipids

The [<sup>3</sup>H]-inositol incorporation into the membrane (polyphospho)PI lipids was measured during the final 24 h of the three

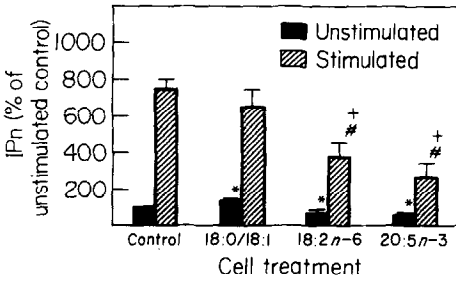


FIGURE 1. The basal rate and  $\alpha_1$ -adrenoceptor mediated phosphatidylinositol-4,5-bisphosphate breakdown of control cardiomyocytes and cells treated with 18:0/18:1n-9, 18:2n-6 or 20:5n-3. The incubation time was 30 min and the phenylephrine concentration for stimulation of the PI cycle was 30  $\mu$ M. The columns are means, bars are S.E., of [<sup>3</sup>H]-IP<sub>n</sub> formation expressed as % of the unstimulated control value in 7 experiments. \*  $P < 0.05$  vs unstimulated control treated cells; #  $P < 0.05$  vs stimulated control treated cells; +  $P < 0.05$  vs stimulated 18:0/18:1n-9 treated cells.

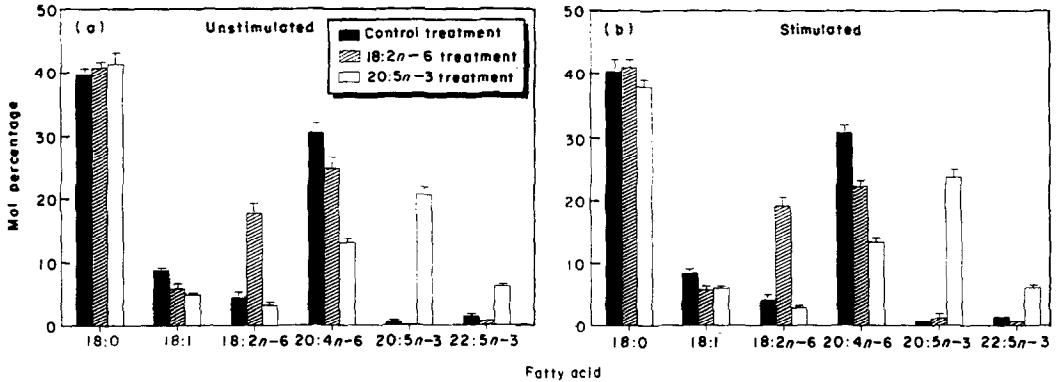


FIGURE 2. The effect of no stimulation (a) or prolonged stimulation of PI cycle (b) on the fatty acid composition of the PI fraction from control cardiomyocytes and cells treated with 18:0/18:1n-9, 18:2n-6 or 20:5n-3. The incubation time was 30 min and the phenylephrine concentration for the stimulation was 30  $\mu$ M. After incubating of the cells the phospholipids were extracted and PI separated by TLC. Only the major fatty acids are shown. The columns are means, bars are S.E. of mol% of the specific fatty acid of PI in 5 experiments.

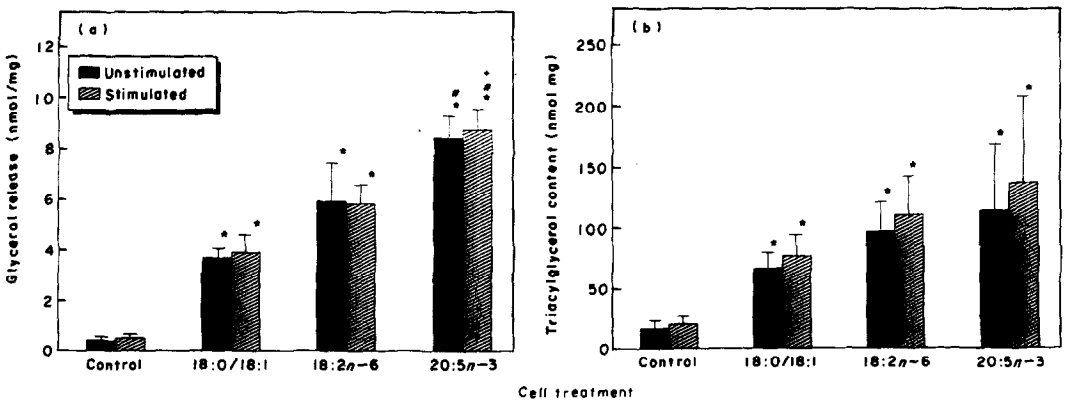


FIGURE 3. The rate of lipolysis (a) and the triacylglyceride content (b) of unstimulated and phenylephrine-stimulated cardiomyocytes, control cells and cells treated with 18:0/18:1n-9, 18:2n-6 or 20:5n-3. Glycerol release as a measure of lipolysis was determined over an incubation period of 30 min. The phenylephrine concentration for the stimulation of the PI cycle is 30  $\mu$ M. The columns are means, bars are S.E., of glycerol release and TG contents in respectively 4 and 5 experiments.

\*  $P < 0.05$  vs control treated cells; #  $P < 0.05$  vs corresponding values of 18:0/18:1n-9 treated cells; +  $P < 0.05$  vs corresponding value of 18:2n-6 treated cells.

TABLE 6 [<sup>3</sup>H]-inositol incorporation into (polyphospho)PI lipids of control cardiomyocytes and cells treated with 18:0/18:1*n*-g, 18:2*n*-6 and 20:5*n*-3

	Control		18:0/18:1 <i>n</i> -g		18:2 <i>n</i> -6		20:5 <i>n</i> -3	
PI, dpm <sup>b</sup>	39346	(509)	38467	(831)	35022	(579) <sup>a</sup>	31112	(409) <sup>a</sup>
PI, % <sup>c</sup>	93.2	(0.2)	93.9	(0.4)	92.8	(0.3)	93.4	(0.3)
PIP, dpm	1381	(70)	1263	(64)	1369	(94)	1078	(53) <sup>a</sup>
PIP, %	3.24	(0.19)	3.27	(0.16)	3.71	(0.20)	3.22	(0.16)
PIP <sub>2</sub> , dpm	1545	(50)	1407	(57)	1351	(66) <sup>a</sup>	1166	(36) <sup>a</sup>
PIP <sub>2</sub> , %	3.58	(0.13)	3.38	(0.10)	3.64	(0.12)	3.32	(0.10)
Sum, dpm	42062	(381)	41439	(896)	37387	(508) <sup>a</sup>	33273	(372) <sup>a</sup>
Sum, %	100		100		100		100	

During the final 24 h of control or fatty acid treatment of the cardiomyocytes 88 nM [2-<sup>3</sup>H]-myoinositol (2μCi/ml) was added. At the end of the preincubation of the cardiomyocytes with the conditioned media the (polyphospho)PI lipids were extracted, separated on TLC and counted by liquid scintillation. Sum means total (polyphospho)PI lipids (PI + PIP + PIP<sub>2</sub>). Incorporation is expressed as <sup>b</sup> dpm/4-well multidish and as <sup>c</sup> % of the total (polyphospho)PI lipid fraction. Values are presented as means (S.E.) of 16 experiments <sup>a</sup> *P* < 0.05 vs control cells.

days lasting incubation of cardiomyocytes with control or fatty acid supplemented medium has also been measured (Table 6). The relative distribution of [<sup>3</sup>H]-inositol into PI, PIP and PIP<sub>2</sub> remained unaffected by the fatty acid treatments. It should be noticed that PIP<sub>2</sub> constitutes only about 3.5% of the total (polyphospho)PI lipid fraction. The absolute [<sup>3</sup>H]-inositol incorporation in PI, PIP and PIP<sub>2</sub> was 30% reduced by 20:5*n*-3, but only 10% by 18:2*n*-6 treatment of the cardiomyocytes. No effect was observed when the cardiomyocytes were treated with 18:0/18:1*n*-g.

#### Cellular TG content and the rate of lipolysis

In order to explore the effect of fatty acid treatment of cardiomyocytes on neutral lipid metabolism, the cellular TG contents and rates of lipolysis were measured [Fig. 3(B)]. A large increase of cellular TG content and rate of lipolysis was seen after fatty acid treatment. The increases were not much dependent of the type of fatty acid treatment, although the glycerol release in 20:5*n*-3 treated cells was significantly higher than that in 18:0/18:1*n*-9 treated cells. Stimulation of the cells by phenylephrine (30 μM) did not affect the rate of lipolysis.

### Discussion

#### Fatty acid composition of membrane phospholipids

To delineate the mechanism(s) of the reported reduction of the α<sub>1</sub>-adrenergic inotropic

response by dietary *n*-3 vs. *n*-6 PUFA in the Langendorff perfused rat heart (Reibel *et al.*, 1988), we studied rat neonatal cardiomyocytes cultured in fatty acid enriched media. This preparation has several advantages: (i) it allows the examination of the α<sub>1</sub>-adrenoceptor mediated PI response in the cardiomyocyte free from other cell types in the myocardium (e.g. vascular smooth muscle and endothelial cells) known to possess an active PI turnover; (ii) cardiomyocytes survive in culture medium for several days allowing incorporation *in vitro* of specific fatty acids into cell membrane phospholipids and (iii) the myocyte polyphosphoinositide pool is more accessible for radioactive labelling with [<sup>3</sup>H]-myoinositol than in whole heart preparations.

As a consequence of supplementation of the culture medium with fatty acids the cardiomyocyte phospholipid composition varied widely, whereas its phospholipid class contents and composition were unchanged. In our experiments the total NEFA pool in the media varied between 30 μM (control treated cells) and 240 μM (fatty acid treated cells), concentrations which are less than twice the albumin concentration in the added sera. The albumin prevents fatty acid toxicity based on our previous observations that cardiac sarcolemmal membrane integrity is affected when NEFA to albumin molar ratio exceeds 5 (Lamers *et al.*, 1984). Furthermore, intracellular accumulation of NEFA was prevented by incorporation into TG. Indeed, the present study shows fatty acid treatment induced

accumulation of TG in the cardiomyocytes and this effect has been reported for other cultured cell preparations (Spector *et al.*, 1981). The rate of TG synthesis is primarily determined by the availability of initial substrates glycerol-3-phosphate and acylCoA, which are derived from stored glycogen (or extracellular glucose) and NEFA (Stam *et al.*, 1987), respectively. The TG accumulation likely was harmless as the cells continue to beat spontaneously at a constant rate (data not shown), although possible changes in the amplitude of contraction had not been measured. Hasin *et al.* (1982) cultured neonatal rat heart cells for up to 16 days in medium containing 20% animal sera supplemented with 50  $\mu$ M 18:0 or 18:2*n*-6 with no apparent loss of viability.

The predicted elevations of 18:1*n*-9, 18:2*n*-6 and 20:5*n*-3 associated with a partial depletion of 20:4*n*-6 were confirmed in all phospholipid classes except for SPM, of the respective groups of fatty acid treated cells. In the 20:5*n*-3 treatment group, the change in *n*-3/*n*-6 PUFA molar ratio of e.g. the major phospholipid class PC from 0.17 to 2.11 was even more pronounced than that reported by Reibel *et al.* (1988) for the total phospholipid fraction (from 0.31 to 1.14) after feeding rats with fish oil. In spite of the similar patterns of changes, each phospholipid class had a unique fatty acid composition and a distinct response to the fatty acid treatments. In particular, the PI fraction, which was very homogeneous in fatty acid composition in control treated cells, changed dramatically after the fatty acid treatments. The results of the control treated cells showed that 20:4*n*-6 is enriched (30.0%) in the PI fraction and that the other major fatty acid appeared to be 18:0 (39.2 mol%). These findings are in agreement with the generally accepted, but not experimentally proven, proposal in the literature that the *sn*-2 position of PIP<sub>2</sub> mainly is occupied by 20:4*n*-6, which is released in the form of DG after receptor-mediated stimulation of PIP<sub>2</sub> hydrolysis (Berridge, 1984). This homogeneity in the PI fraction was no longer apparent when the cells were treated with 18:0/18:1*n*-9, 18:2*n*-6 or 20:5*n*-3. In the 18:0/18:1*n*-9 treated cells 18:1*n*-9 was incorporated into PI to a large extent (from 6.5 to 18.3 mol%) in contrast to the 18:0

content of PI which actually decreased (from 39.2 to 34.0 mol%). In the 18:2*n*-6 treated cells 18:2*n*-6 is incorporated into PI to a large extent (from 4.4 to 17.4 mol%) and even more avidly into PC (from 14.0 to 42.1 mol%). In the 20:5*n*-3 treated cells, both 20:4*n*-6 and 20:5*n*-3 were enriched in the PI fraction (12.9 and 20.6%, respectively). In general it can be concluded that the main effect of all three fatty acid treatments on the various phospholipid classes was a partial replacement of the 20:4*n*-6.

No desaturation of 18:2*n*-6 occurred which finding is in agreement with the results reported by Hagve and Sprecher (1989). We were also able to show that neonatal rat heart myocytes have the capacity to elongate C20 fatty acids. This was not observed by Hagve and Sprecher (1989), who studied the metabolism of labelled 20:5*n*-3 in cardiomyocytes isolated, but not cultured, from the adult rat heart.

#### *Rates of basal and $\alpha_1$ -adrenoceptor mediated PIP<sub>2</sub> hydrolysis*

The rate of basal and  $\alpha_1$ -adrenoceptor mediated PI turnover was largely affected by the changes in fatty acid composition of PI and/or all other phospholipid classes. Apart from the observed reductions in the basal rates, the phenylephrine (30  $\mu$ M) stimulated production of inositolphosphates was reduced by 51% and 71%, respectively in the 18:2*n*-6 and 20:5*n*-3 treated cardiomyocytes. The basal rate of inositolphosphate formation was 37% increased in the 18:0/18:1*n*-9 treated cells. Under the present experimental conditions, the NEFA added to the culture medium were washed away prior to the activation of PIP<sub>2</sub> breakdown. Therefore it is unlikely, that a direct activation of protein kinase C by extracellularly derived PUFA is involved in the observed decrease in the rate of PIP<sub>2</sub> breakdown induced by *n*-6 or *n*-3 PUFA treatment of the cardiomyocytes (McPhail *et al.*, 1984 and Meij and Lamers, 1989b). Thus the effects on the PI cycle in this study can be attributed to the different composition of the membrane lipids. Since the changes of the various phospholipid classes induced by the fatty acid treatment were complex, it is not possible to identify one of them, e.g. a change

of  $n$ -3/ $n$ -6 PUFA molar ratio,  $\Sigma$ PUFA, or 20:5 $n$ -3 content of PI, to be causally related to the changes in basal and/or  $\alpha_1$ -adrenoceptor mediated rate of PIP<sub>2</sub> breakdown. Therefore no firm conclusions can be drawn from the results of the present study to explain the possible mechanism(s) of changes in the rate of PIP<sub>2</sub> breakdown. One possible mechanism would be that the fatty acids induce changes in the membrane content of PIP<sub>2</sub>. For this reason we measured the [<sup>3</sup>H]-inositol incorporation during the final 24 h of the three days lasting incubation of cardiomyocytes with control or fatty acid supplemented medium. The relative distribution of [<sup>3</sup>H]-inositol into PI, PIP and PIP<sub>2</sub> remained unaffected by the fatty acid treatments. The latter indicates that the equilibrium concentrations of these lipids are well maintained by active PI- and PIP-kinases and active PIP and PIP<sub>2</sub>-phosphatases. The absolute [<sup>3</sup>H]-inositol incorporation in PI, PIP and PIP<sub>2</sub> was 28% reduced by 20:5 $n$ -3, but only 12% by 18:2 $n$ -6 treatment of the cardiomyocytes. Therefore, a partial contribution of PUFA-induced reduction of the PIP<sub>2</sub> level to the attenuation of basal and  $\alpha_1$ -adrenoceptor mediated IP<sub>n</sub> production can not be excluded. At present it is not clear how PUFA cause a reduction of [<sup>3</sup>H]-inositol incorporation into the (polyphospho)PI lipids. They may interact with several enzymatic steps in the regeneration of PI from IP<sub>3</sub> and DG or with the transport of inositol across the plasma membrane.

Reibel *et al.* (1988) demonstrated that dietary  $n$ -3 PUFA result in a reduction of the  $\alpha_1$ -adrenergic inotropic response. The present data on 20:5 $n$ -3 treatment of cultured cardiomyocytes offer a possible mechanism of action of dietary  $n$ -3 PUFA via the IP<sub>3</sub> pathway and unlikely involving the eicosanoid pathway. Indeed, Reibel *et al.* (1988) showed already that changes in eicosanoid production were unrelated to the effects of dietary fish oil on  $\alpha_1$ -adrenergic stimulation. The reduced  $\alpha_1$ -adrenergic response caused by dietary  $n$ -3 PUFA could protect the myocardium against the pathophysiological consequences of cate-

cholamine release evoked by chronic adrenergic or ischemic stress (Gudbjarnason *et al.*, 1978 and Hartog *et al.*, 1987).

#### *Fatty acid composition of PI after prolonged $\alpha_1$ -adrenergic stimulation*

Previously we demonstrated that maximal stimulation by phenylephrine over a prolonged period (30 min) did not reduce the PI, PIP and PIP<sub>2</sub> contents of the cardiomyocyte membranes (Meij and Lamers, 1989b). This means that the cells have the capacity to rapidly resynthesize PI from CDP-diacylglyceride and inositol and subsequently to polyphosphorylate PI. A change in the PI fatty acid composition after prolonged stimulation of the PI cycle would be likely to occur, because the resynthesized PI will obtain its precursor fatty acids from a mixed pool of diacylglycerides (DG). In the present study no significant depletion of any of the PUFA occurred in the  $\alpha_1$ -adrenergic stimulated cardiomyocytes, not even in the PI fraction. We (Montfoort *et al.*, 1986) and others (Gudbjarnason *et al.*, 1978) have shown that chronic noradrenaline stress in rats resulted in an increase in  $n$ -3/ $n$ -6 PUFA molar ratio of the total phospholipid fraction. Based on the present results this stress effect may be purely of  $\beta$ -adrenergic nature. However, the latter hypothesis should be tested using a  $\beta$ -adrenergic agonist in the incubation of the cultured cardiomyocytes.

In conclusion, variations in the PUFA composition of membrane phospholipids do affect the basal and the  $\alpha_1$ -adrenoceptor stimulated rate of PIP<sub>2</sub> hydrolysis. The reducing effects of 18:2 $n$ -6 and 20:5 $n$ -3 treatment may be partially ascribed to lowering of PIP<sub>2</sub> levels.

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