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Effect of hormones on phospholipid metabolism in human cultured fibroblasts

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The effect of hormones on phospholipid metabolism, pool size, ^{32}P labeling and changes in fatty acid of human adult fibroblasts was determined. Simultaneously the change in membrane fluidity of single cells was recorded via fluorescence recovery after photobleaching under the influence of hormones. From all substances tested (isoproterenol, phenylephrine, adrenalin, histamine, angiotensin II, dansylcadaverine, propranolol) only isoproterenol and adrenalin slightly decreased total amount of phosphatidylcholine (PC). The amount of the other phospholipids analyzed remained unchanged. The ^{32}P incorporation rate into phospholipids (PC, phosphatidylinositol (PI), phosphatidylethanolamine (PE)) was affected basicly different analyzing either PC, PI or PE. Histamine and propranolol provoked the highest incorporation of ^{32}P (240% increase in PI labeling). Isoproterenol and adrenalin decreased PC labeling (45% and 18%) whereas isoproterenol decreased ^{32}P incorporation into PI (18%), and adrenalin led to an incrase (37%). PE labeling showed no or a slight increase in ^{32}P incorporation applying the other agonists or antagonists. The fatty acid pattern of the respective phospholipids changed only to a minor extend. A decrease in hexadecanoic acid content of PI was found after administration of either isoproterenol, adrenalin or histamine. Parallel determination of membrane fluidity of single cells by fluorescence recovery after photobleaching showed an increase in the diffusion coefficient of a fluorescent lipid probe sticking in the membrane, following administration of isoproterenol and adrenalin, other substances tested exerted no effect. A relationship to changes in phospholipid metabolism became obvious. These results are discussed considering known mechanisms of receptor coupling and change in phospholipid metabolism and fluidity.

Introduction

Changes in fatty acid composition of plasma membrane phospholipids are reported to have effects on several membrane properties of animal cells e.g. chain length and saturation may influence enzyme activity [1,2], cell growth [3,4] or fusion [5]. These changes were mainly connected to changes in physical properties like phase transition and altered fluidity [6]. Moreover, changes in membrane behaviour are not only due to changes in fatty acid composition, but also to great extend to alterations in cholesterol [7] and phospholipid metabolism. Several metabolic pathways (e.g. methylation of phosphatidylethanolamine (PE) [8], activation of phospholipase A_2 [9], changes in phosphatidylinositol (PI) and poly-PI metabolism [10–12]) are supposed to be responsible for changing properties of the plasma membrane and thus tightly connected to receptor-mediated signal transmission by either cAMP or Ca^{2+} or unknown second messengers. Some membrane receptors, and enzymes obviously require changes in the lipid bilayer to be fully active [13,14].

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Because of some postulated common mechanisms influencing phospholipid metabolism in different cell types, e.g. via hormones [15], human fibroblast in culture was used as an experimental model to study basal and hormone stimulated phospholipid metabolism.

The aims of this study were: (i) to investigate the metabolic behaviour of a widely used experimental model after hormone administration and (ii) to elaborate basal values of phospholipid metabolism and (iii) to examine if hormone induced changes in phospholipid metabolism are correlated with changes in membrane fluidity.

Materials and Methods

Cell culture. Human adult foreskin fibroblasts, from three different healthy donors, between the 9th and 18th passage, were cultured in Basal Eagle medium, (Gibco No. 420 - 110) supplemented with 12% fetal calf serum (Boehringer) non essential amino acids (Seromed No. 0293), vitamins (Seromed No. 0273) and penicillin and streptomycin (10 U/10 μg per ml) in plastic dishes (Falcon No. 3003 F). Experiments were performed only with confluent monolayers and run in duplicate. Basal ^{32}P-incorporation rates were comparable within the three cell lines. Results are the mean $\pm$ S.D. of either two or three cell lines and the number of experiments (n) as indicated. Differences were calculated according to Student's t-test.

Chemicals. The chemicals were of analytical grade (except tetramethylammoniumhydroxide) and purchased from Merck, Darmstadt or Fluka, Ulm. DL-Isoproterenol, DL-propranolol, L-phenylephrine, monodansylcadaverine were purchased from Sigma, Munich; histamine, L-adrenalin from Serva, Heidelberg; and angiotensin II (Hypertensin Ciba) from Ciba, Wehr. [^{32}P]Orthophosphate, carrier-free (1 mCi/ml) was bought from Amersham International, Braunschweig. Fatty acid methylester as standard for gaschromatography were purchased from Supelco, Bellefonte. The lipid analog fluorophore 3,3′-dioctadecyloxacarbocyanine iodide (DiO_{18}) was synthesized according to Czikkely et al. [16]. 7-Nitrobenz-2-oxa-1,3-diazol-4-yldipalmitoylphoshatidylethanolamine (NBD-PE) was bought from Avanti Polar Lipids (No. 810 144), Birmingham, U.S.A.

Experimental design and analytical procedures. Confluent monolayers were washed two times with culture medium and incubated in 10 ml Basal Eagle medium (with 1 mM Na_2HPO_4) without fetal calf serum at 37°C in a fully water saturated atmosphere with defined gaseous phase (95% air/5% CO_2). After 2 h preincubation with [^{32}P]orthophosphate (spec. act. 0.5 mCi/mmol) the respective agonist/antagonist was added. The second incubation was terminated after 1 h by aspiration of medium, followed by two washings with ice-cold phosphate-buffered saline and addition of 3 ml 10% $HClO_4$ (v/v). Cells were scraped off and centrifuged (10 min, 2000 $\times$ g). Incorporation of ^{32}P into phospholipids was measured after organic solvent extraction of the cell pellet with chloroform/methanol/0.9% NaCl (2 : 1 : 0.6, v/v). The water phase was washed once with chloroform/methanol/0.9% NaCl (86 : 14 : 1, v/v) and the combined organic phase evaporated to dryness under nitrogen. Residues were redissolved in chloroform/4-methanol (10 : 1, v/v) and separated by thin-layer chromatography (TLC) on silica gel plates (Merck No. 5715). TLC plates were developed in chloroform/methanol/4-ethyl acetate/4-2-propanol/0.25% KCl in water (30 : 9 : 18 : 25 : 6, v/v) [17]. Spots were visualized with I_2 vapor and identified with phospholipid standards (purchased from Sigma, Munich). The phospholipids were scraped off and either transferred to scintillation vials and counted in a Beckman LS 1800 scintillation counter or eluted with chloroform/methanol (1 : 10, v/v), hydrolyzed, methylated [18] and extracted with hexane for gas chromatographic analysis. Inorganic phoshate determination of the scraped phospholipids was performed according to Rouser et al. [19].

Gas chromatographic analysis was exactly performed as described in Ref. 20. The fatty acid methyl ester were identified comparing the retention times with known standards. Heptadecanoic acid methyl ester was used as internal standard. The analyzed amount of fatty acids was corrected for response according to chain length and saturation degree.

Fluorescence recovery after photobleaching measurements. The apparatus is described in detail

elsewhere [21]. The fluorescence probe was either DiO_{18} or NBD-PE.

Since these substances are not incorporated spontaneously into the plasma membrane of the fibroblast following incubation procedures were applied: The monolayer was trypsinized and washed two times with Puck's saline (140 mM NaCl, 5 mM KCl, 4 mM $NaHCO_3$, 5 mM glucose, 10 mM Hepes). The cells from one dish, suspended in 5 ml Puck's saline with 50 μl dye solution (0.5 mg dye per ml ethanol) were incubated 30 min at 37°C. After centrifugation (10 min, $200 \times g$) and two washings cells were resuspended in culture medium and seeded on glass cover slides. 2 h later cells were tightly attached to be used in the experiment. The cover slide was mounted in a chamber which allows perfusion and variation of temperature under the microscope as described in [21]. Experiments introducing dye into the membrane of fibroblasts in the intact monolayer failed.

Results

Cellular phospholipid content of adult human fibroblasts determined as phospholipid P_i is shown in Table I. As shown for nearly all human tissues [22] phoshatidylcholine is the most abundant phospholipid. Interestingly, the amount of PE is much lower than that of PI, in contrast to nearly all other tissues. Lysophosphatidylcholine plus sphingomyeline, phosphatidylserine and phosphatidic acid amounted to 36 ± 8, 14 ± 7, and 4 ± 2 nmol/mg protein, respectively. The total amount of the different phospholipids tested seems to be very constant since addition of several hormones known to act via cAMP or Ca^{2+} did not change phospholipid content in a significant manner. Only isoproterenol (10^{-4} M), which decreased PC content, and phenylephrine (10^{-5} M), which increased PI content, had small but significant effects. Considering the fatty acid composition of the phospholipids studied (not differentiated between position 1 or two at the glycerol moiety), we found that PC, PI and PE consisted of 70, 84, 76% of saturated fatty acids, respectively. Moreover, the major fatty acid in PC was found to be palmitic acid, whereas PI and PE consisted to 62% and 46% of stearic acid, respectively. The most frequent occurring unsaturated fatty acid was found to be oleic acid in all three phospholipids, whereas arachidonic acid was negligible in PC and between 2 and 4% of total fatty acids in PI and PE. The distribution of the fatty acid pattern was similar to the fatty acids present in the fetal calf serum of the medium. This could likewise be shown for other cells in culture [23].

Effects of hormone treatment could be observed only in the palmitic acid content of PI, which decreased due to isoproterenol, adrenalin

TABLE I

AMOUNT OF MAIN PHOSPHOLIPIDS OF HUMAN ADULT FIBROBLASTS

Changes in total amount of main phospholipis of human adult confluent fibroblasts after administration (1 h) of different agonists/antagonists or hormones (10^{-4} M). Quantitation was performed after organic solvent extraction, TLC separation [17] and inorganic phosphate determination [19]. (Values are mean ± S.D.; in parentheses number of experiments).

Addition	P_i (nmol/mg protein)		
	PC	PI	PE
Control	60 ± 5 (16)	22 ± 4 (16)	14 ± 2 (16)
Isoproterenol	55 ± 4 (10) *	21 ± 4 (10)	13 ± 3 (10)
Adrenaline	58 ± 5 (10)	24 ± 5 (10)	14 ± 2 (10)
Phenylephrine (10^{-5} M)	58 ± 6 (8)	26 ± 3 (7)	15 ± 1 (7)
Histamine	58 ± 6 (10)	21 ± 4 (10)	14 ± 2 (10)
Angiotensin II (10^{-5} M)	57 ± 3 (4)	24 ± 2 (4)	15 ± 1 (4)
Propranolol	57 ± 3 (4)	24 ± 2 (4)	15 ± 1 (4)
Isoproterenol + propranolol	57 ± 3 (4)	24 ± 2 (4)	15 ± 1 (4)
Dansylcadaverine (10^{-5} M)	57 ± 4 (4)	26 ± 3 (4)	15 ± 1 (4)

* $P \leq 0.05$ compared to control.

and histamine; and a decrease in the oleic acid content of PE after adrenalin treatment (Table II).

To gain further insight into pospholipid metabolism we studied the time dependence and rates of ^{32}P incorporation into PC, PI and PE. As can be seen from Fig. 1 incorporation of ^{32}P into PC increased linearly after a lag preincubation phase of 2 h up to 4.5 h.

Highest incorporation rates were found in PI which increased linearly up to 4 h and then reaching a plateau after 4 h. PE labeling increased likewise linearly over 4 h, incorporation rate, however, was significantly lower than into PC and PI at every time measured.

Since incorporation rates into PC, PI and PE were linear after 2 h of incubation we have chosen for all further experiments a preincubation time with ^{32}P of 2 h and 15 min (Fig. 1). In a second incubation of 1 h the hormones indicated in Table III were added.

Basal incorporation rates were slightly higher into PI than into PC, but ten times lower into PE.

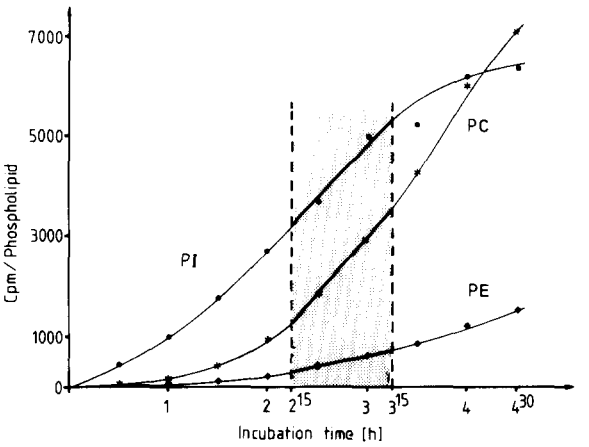

Fig. 1. Time-course of ^{32}P-label incorporation into main phospholipids of human adult confluent fibroblasts. Fibroblasts were incubated in Medium with 1 mM PO_4^{3-} and ^{32}P (carrier free, spec. act. 0.5 mCi/mmol) for the time indicated. Phospholipids were extracted, separated and counted in a liquid scintillation counter (points are the means of two dishes of one experiments). The shaded area represents the time the agonist/antagonist was applied.

TABLE II

CHANGES IN FATTY ACID COMPOSITION OF MAIN PHOSPHOLIPIDS IN HUMAN ADULT CONFLUENT FIBROBLASTS

After addition of different agonists (10^{-5} M, 1 h) phospholipids of fibroblasts were isolated, separated, hydrolyzed and methylated [18] and determined gaschromatographically as described in Ref. 20. Quantitation was performed by internal standardization (17:0 methyl ester). Values are the mean of 3–6 experiments.

Addition	Fatty acids (mol%)						
	14:0	16:0	16:1	18:0	18:1	18:2	20:4
PC							
Control	4.5	48.2	3.7	17.7	22.1	2.5	1.1
Isoproterenol	3.9	50.0	4.0	17.0	23.4	1.9 [b]	0.8 [b]
Adrenalin	4.1	49.6	4.3	17.6	22.9	1.8	0.9
Histamine	3.9	49.0	4.3	17.2	23.7	1.7	0.6 [b]
PI							
Control	2.5	19.3	2.7	62.3	13.8	1.4	2.4
Isoproterenol	2.3 [b]	14.9 *	1.5 [a]	64.3	16.6	1.5 [a]	6.0 [a]
Adrenalin	2.2	16.3 *	2.7	63.3	12.6	1.8 [a]	4.3
Histamine	1.4	14.1 *	– [c]	59.0	20.1 *	1.5	3.9
PE							
Control	4.2	25.8	5.0	45.8	21.2	2.9	4.0
Isoproterenol	3.4	20.5	– [c]	54.4	19.6	– [c]	2.1
Adrenalin	2.4	25.9	1.5 [b]	52.7	14.9 *	2.0	2.7
Histamine	1.9	19.9	– [c]	51.3	22.3	2.1	3.0 [b]

* $P \leqslant 0.05$.

[a] Analysed once.

[b] Analysed twice.

[c] Not detected.

TABLE III

^{32}P INCORPORATION INTO MAIN PHOSPHOLIPIDS OF HUMAN ADULT CONFLUENT FIBROBLASTS

After 2 h and 15 min preincubation with ^{32}P (spec. act. 0.5 mCi/mmol) the respective agonists (10^{-4} M) were added. 1 h later experiment was stopped, phospholipids extracted, separated [17] and counted in a liquid scintillation counter. (Values are mean ± S.D.; in parentheses number of experiments.)

Addition	nmol/100 mg protein per h		
	PC	PI	PE
Control	11.2 ± 1.5 (7)	15.3 ± 2.2 (6)	1.7 ± 0.1 (5)
Isproterenol	6.1 ± 1.0 (7) ↓↓↓	12.6 ± 2.5 (8) ↓	1.5 ± 0.3 (6)
Adrenalin	9.2 ± 1.8 (8) ↓	21.0 ± 3.4 (7) ↑↑	1.9 ± 0.2 (7) ↑
Phenylephrine (10^{-5} M)	11.8 ± 2.8 (4)	18.0 ± 3.5 (4)	2.0 ± 0.6 (4)
Histamine	13.4 ± 5.3 (7)	52.1 ± 15.1 (8) ↑↑↑	2.6 ± 0.5 (6) ↑↑
Angiotensin II	11.6 ± 3.1 (5)	12.5 ± 2.3 (5) ↓↓	2.2 ± 0.7 (6)
Propanolol	13.6 ± 3.2 (5)	52.1 ± 25.8 (5)	2.7 ± 1.1 (5)
Dansylcadaverine (10^{-5} M)	12.0 ± 1.8 (4)	19.4 ± 5.3 (4)	1.8 ± 0.3 (5)

↑ or ↓, $P \leq 0.05$.
↑↑ or ↓↓, $P \leq 0.01$.
↑↑↑ or ↓↓↓, $P \leq 0.001$ compared to control.

Isoproterenol and adrenalin significantly inhibit ^{32}P incorporation into PC by about 45% and 18%, respectively. The effect on PI labeling by isoproterenol was a decrease of 18%. In contrast to PC labeling adrenalin significantly increased ^{32}P incorporation into PI and PE by about 37% and 15%, respectively. The most pronounced effect on ^{32}P labeling was found with histamine, which increased incorporation into PI and PE by 240% and 54%, respectively.

Considering the effect of different agonists on phospholipid metbolism we tried to detect consequences for plasma membrane properties by determining the lateral diffusion coefficient of a lipid analog fluorophore. The measurements with different agonists were performed with DiO_{18} because the fluorescence intensity was stronger than with NBD-PE under the same labeling conditions. Moreover the plating efficiency after dye incubation was better with DiO_{18}. Strikingly the basal lateral diffusion coefficients were different, which could likewise be shown by Struck and Pagano [24].

Introducing dye into non-trypsinized fibroblasts led to a homogeneous fluorescence, but the dye molecules behaved immobile (Fig. 2).

The diffusion coefficient showed linear relationship with temperature in a semilogarithmic plot as earlier shown for erythrocytes [25] (data

TABLE IV

MEMBRANE FLUIDITY OF HUMAN ADULT FIBROBLAST

Lateral diffusion coefficient of a lipid analog 3,3′-dioctadecyloxacarbocyanine iodide (DiO_{18}) or 7-nitrobenz-2-oxa-1,3-diazol-4-yldipalmitoylphoshatidylethanolamine (NBD-PE) was measured by fluorescence recovery after photobleaching [42]. Human adult fibroblasts were trypsinized, incubated 30 min in Puck's saline with 1 vol% ethanol with dye (0.5 mg dye per ml ethanol), washed and seeded on cover slides. After attachment (2 h) they were mounted in a perfusion chamber. 5 min after perfusion of cells with the agonist (10^{-4} M) measurement started. Only control experiments were done with NBD-PE. (Values are mean ± S.D., four measurements per cell were performed, in parentheses number of experiments.)

Addition	D (10^{-8} cm^2/s)	Recovery (%)
Control (DiO_{18})	9.26 ± 1.41	79.3 ± 6.0 (52)
Control (NBD-PE)	5.08 ± 0.51	
Isoproterenol	10.63 ± 1.56 **	85.6 ± 3.9 (12) ***
Isoproterenol + +	10.23 ± 1.56 *	83.4 ± 3.5(10) *
Adrenalin	10.00 ± 1.51 *	82.9 ± 3.5 (17) *
Adrenalin +	9.13 ± 1.35	82.7 ± 6.8 (11)
Phenylephrine (10^{-5} M)	9.36 ± 1.30	76.6 ± 9.4 (10)
Histamine	9.26 ± 1.66	80.2 ± 7.0 (17)
Angiotensin II	10.00 ± 1.35	80.4 ± 4.8 (9)
Propranolol	9.99 ± 1.24 *	79.5 ± 8.2 (19)
Dansylcadaverine +	8.39 ± 1.91	81.5 ± 7.1 (5)
1% glutardialdehyde	2.93 ± 0.72 **	60.2 ± 5.5 (4) ***
10% glutardialdehyde	2.78 ± 0.64 ***	58.8 ± 7.9 (5) ***

* $P \leq 0.05$. ** $P \leq 0.01$. *** $P \leq 0.001$.
+ 1 h incubation with agonist.
+ + 2 h incubation with agonist.

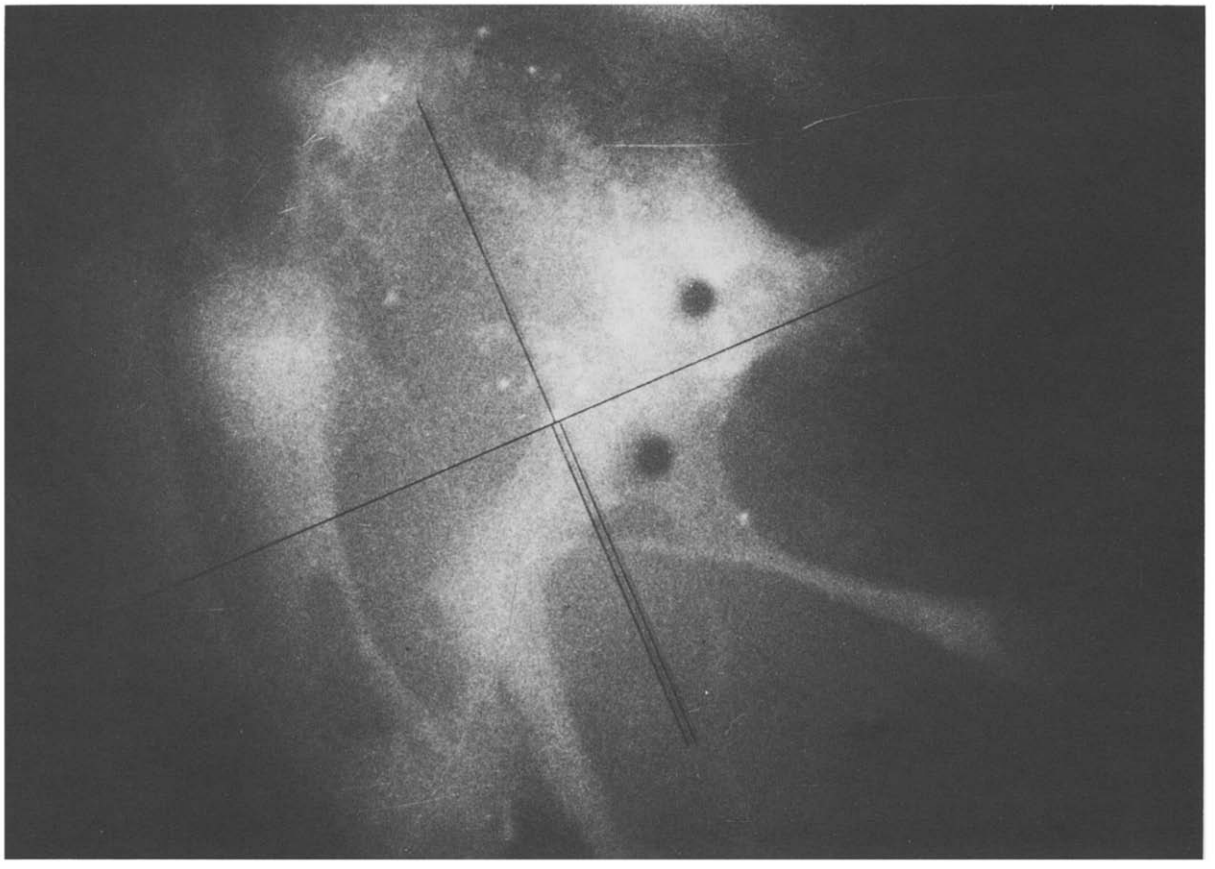

Fig. 2. Membrane fluidity depending on incubation procedure. Human adult fibroblasts were trypsinized, incubated 30 min in Puck's saline with 1 vol% ethanol with dye (3,3′-dioctadecyloxacarbocyanine iodide (DiO_{18}), 0.5 mg dye per ml ethanol), washed free of medium and seeded on cover slides. After attachment (2 h) they were mounted in a perfusion chamber. Omitting the trypsinization step led to an immobility of the fluorophore, perhaps due to sticking in the glycocalix.

not shown). Isoproterenol, five minutes after application, increased the lateral diffusion coefficient by about 15% compared to control (Table IV). This effect was persistent for 2 h (10%). Isoproterenol was the only agonist which decreased both the turnover rate and the pool size of PC (8%) (Tables I and III). On the other hand, phenylephrine which increased PI amount did not produce any effect on fluidity, whereas adrenalin slightly increased fluidity after 5 min. This effect disappeared after 1h.

Histamine which tremendously altered PI turnover, but not PI content, did not affect the lateral diffusion of the lipid probe. Dansylcadaverine which inhibits transglutaminase, responsable for receptor mediated endocytosis [26], either increased (after 5 min) or slightly decreased (after 1h) the membrane fluidity.

Changes in membrane fluidity (changed D) correlated with the fluorescence recovery of the dye after bleaching.

Discussion

Cultured human fibroblasts have frequently been used to investigate hormone effects [27,28] and genetic disease [29,30]. Since they contain a wide range of receptors [30–32] and of metabolic pathways they are a suitable model to study both phospholipid metabolism and changes of physical properties of the plasma membrane. Changes in fatty acid composition of membrane phospholipid

were reported to affect activities of certain membrane-bound enzymes [1], endocytosis [33] as well as lateral mobility of receptors [34]. Therefore it seems possible that changes in fluidity [35] may be a general link.

For these reasons we wanted to study if there exists a relation of hormone effects on biochemical and physical cellular events. Thus changes in precursor incorporation was shown to be connected to α_1-adrenergic [11,12] and β-adrenergic [15] receptor coupling. Moreover it was shown that effects of hormones acting via Ca^{2+} or unknown mechanisms are connected with alterations in phospholipid turnover (for review see Ref. 10).

Regarding the net amounts of the main phosholipids of human fibroblasts PC, PI and PE it became evident that compared to other tissues and organs [22] human fibroblasts contain a high PI content. Even mouse fibroblasts have a 6-times higher PE content than that of PI, whereas the PE content of human adult fibroblasts is 40% less than PI content. Addition of hormones, however, did not change the net amount of phospholipids, significantly. Remarkable constant phospholipid composition of human fibroblasts were reported upon addition of fatty acids [36,37] as well as in fibroblasts from achondrogenesis patients [30]. The only detected effect on net phospholipid content was a decrease in PC content through isoproterenol. This is in accordance with observations made in rat liver plasma membrane [38].

Likewise the fatty acid pattern of phospholipid studied did not show remarkable changes upon hormonal treatment. As was shown for other tissues and organs [22] the predominate fatty acid of PC was found to be palmitic acid, whereas PI and PE contained mainly stearic acid. Fatty acids were shown not to equilibrate with other extra- or intracellular sources of acyl groups, whereas fibroblasts triacylglycerol fatty acids interchange rapidly [39]. Since changes in phosholipid metabolism after hormone-receptor coupling [11, 12,15] are thought to occur only on distinct sites in the cell membrane these local changes in phospholipid content or fatty acid change might not be detected by net measurements. Therefore we studied ^{32}P incorporation into phospholipids which seems the most appropriate parameter to study hormone induced change in phospholipids [40].

Basal incorporation rates were similar into PC and PI, whereas PE labeling was 10-fold lower. Addition of different agonists exerted pronounced effects on PI labeling. Adrenalin and histamine increased PI labeling, whereas adrenalin decreased PC labeling. Adrenalin is known to act both via α- and β-adrenergic receptors. Compared to isoproterenol both α- and β-adrenergic effects became obvious.

Propranolol, a cationic amphiphilic drug and β-blocker has been shown to alter phospholipids distribution pattern in different ways [41]. It showed a remarkable effect only on PI labeling which was, however, not significant due to marked deviation and is supposed not to be receptor mediated.

Performing in addition the fluorescence recovery after photobleaching experiments under the same metabolic conditions we tried to find correlations to phospholipid metabolism.

The lateral diffusion coefficient of the lipid probe at constant temperature was increased in the presence of isoproterenol, adrenalin and dansylcadaverine. This would well agree in case of isoprotenerol and adrenalin to metabolic changes and to a postulated increase in lateral mobility of receptor-agonist complex. But considering the mode of action of dansylcadaverine it is surprising that it exerts an effect. Dansylcadaverine inhibits transglutaminases and reduces the endocytosis of receptor-agonist complexes [26]. One would expect a slightly more immobile membrane behaviour. The results show that changes in the fluidity observed in large regions of the cell surface (diameter bleach spot 7 μm) and metabolic behaviour can only hardly be correlated. Fluorescence recovery after photobleaching is a tool with certain limitations to answer the physical changes connected to signal transmission.

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