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PHOSPHOLIPASE C ACTIVITY IN PLASMA MEMBRANES ISOLATED FROM LAPINE SYNOVIAL CELLS IN MONOLAYER CULTURE

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Plasma membranes were isolated from lapine synovial cells grown in monolayer culture using discontinuous sucrose gradient centrifugation techniques. 5'nucleotidase was detected in great abundance while glucose-6 phosphate dehydrogenase and cytochrome oxidase were present at low to undetectable levels. Plasma membranes incubated at 37°C for 60 min with [^{3}H]-arachidonyl-phosphatidylinositol/phosphatidylserine synthesized [^{3}H]-diacylglycerides. Little if any [^{3}H]-diacylglyceride synthesis was measured when [^{3}H]-arachidonyl-phosphatidylcholine or [^{3}H]-arachidonyl-phosphatidyl-ethanolamine were used as substrates. These results are consistent with a plasma membrane-associated phosphatidylinositol-specific phospholipase C from lapine synovial cells in culture.

Three major classes of agents frequently used to treat arthritides are nonsteroidal anti-inflammatory agents, steroids, and antimalarial drugs. A common feature of these agents is their ability to alter arachidonic acid ($C_{20:4}$) metabolism in tissues (1-3). These drugs can variably inhibit prostaglandin synthesis (4) and secretion of proteinases such as collagenase and proteoglycanase that degrade cartilage extracellular matrix (5). Synovial cell $C_{20:4}$ metabolism has been extensively studied with respect to prostaglandin synthesis via the phospholipase A_2-cyclooxygenase pathways (4). Agents which stimulate prostaglandin E_2 synthesis in synovial cells may also stimulate collagenase secretion. This has been found with colchicine treatment of rabbit synovial monolayer cell cultures (6) and a factor (possibly interleukin I) secreted by mononuclear cells (7). However, indo-

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Abbreviations: $C_{20:4}$, arachidonic acid; BSS, balanced salt solution; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; G-6-PD, glucose-6 phosphate dehydrogenase.

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methacin inhbits PGE_2 synthesis without a concommitant effect on collagenase secretion (8). In another study, retinoic acid completely inhibited collagenase production, with PGE_2 synthesis being only moderately depressed (9). These findings raise the question whether prostaglandins modulate synovial cell metal-dependent neutral proteinase secretion.

An alternate pathway of $C_{20:4}$ metabolism has been reported in some tissues, but not in synovium. In this pathway, phospholipase C, specifically acting on PI, (most of which contains $C_{20:4}$) liberates a diacylglyceride (10). Elevated levels of membrane diacylglyceride or other PI metabolites have been associated with increased membrane fluidity and cell fusion,(11-16) and exocytosis of cellular products such as pancreatic amylase (17). When membrane fusion is chemically induced with polyethylene glycol, the resulting multi-nucleated cells secrete large amounts of collagenase (18). In rheumatoid synovial tissue, high levels of collagenase are secreted when multinucleated cells are present (18). This suggests that PI metabolites, cell fusion, and enzyme secretion may be related. The studies reported herein investigated whether phospholipase C activity exists in synovial cell plasma membranes.

METHODS AND MATERIALS

CULTURE OF SYNOVIAL CELLS. The infrapatellar fat pad was excised from 4 virgin female White New Zealand rabbits (1.5-2.5 kg). Synovial cells were isolated by sequential incubation at 37°C for 30 min each in BSS containing 2 mg/ml trypsin followed by 2 mg/ml Clostridial collagenase. The resulting cell suspension was centrifuged at 200 x G for 3 min and the cell pellet suspended in media and cultured in 20 flasks (175 cm^2) until confluent in an atmosphere of 10% CO_2/90% air. Media consisted of Dulbeccos modified Eagles medium (high glucose), 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml fungizone and 100 U/ml mycostatin. Cells were harvested by incubation with 0.25% trypsin in Hanks BSS (25 min., 37°C) and collected and washed by centrifugation at 200 x G (3 min 4°C) three times in BSS.

ISOLATION OF PLASMA MEMBRANES.
The washed cells (approximately 10^7/ml) were suspended (19) in 0.25 M sucrose, 0.001 M $CaCl_2$, and 0.01 M Tris-HCI pH 7.4 (sucrose buffer) and ultrasonically disrupted (75 watts, 30 sec/cycle, 6 cycles, 4°C). Samples were then centrifuged (400 x G, 4 min) and the pellet in the same sucrose buffer was again ultrasonically disrupted (3 cycles, 75 watts, 30 sec/cycle, 4°C) and recentrifuged as above. The supernatant of this and the previous centrifugation step were combined and recentrifuged this time at 700 x G (10 min, 4°C). The combined supernatant was centrifuged at 10,000 x G (15 min, 4°C) to isolate a particulate fraction. This fraction was suspended in 2 ml sucrose buffer and 0.7 ml volumes were applied to three 2 x ½ inch

pollyallomer centrifuge tubes (Beckman Instruments Inc., Palo Alta, CA, USA) over a discontinuous sucrose gradient containing 0.01 M Tris-HCl pH 7.4 and 0.001 M $CaCl_2$. The bottom layer in each tube was 0.83 ml of 60% sucrose (density 1.23 gm/ml). Middle layers were 1.33 ml of 45% sucrose (density 1.18 gm/ml) and 0.83 ml of 40% sucrose (density 1.16 gm/ml) respectively. The top layer contained 1.33 ml of 35% sucrose (density 1.14 gm/ml). Samples were centrifuged at 66,000 x G for 90 min at 4°C and the two interfaces enriched in plasma membranes (density 1.14/1.16 and 1.16/1.18) were collected using a Beckman tube slicer. Membranes were diluted with 2 volumes of sucrose buffer and collected by centrifugation at 100,000 x G for 45 min at 4°C. The membranes were resuspended in 2 ml of sucrose buffer and then reapplied over similar discontinuous sucrose gradients, recentrifuged, and collected by centrifugation at 100,000 x G for 45 min. Membranes were suspended in Geys BSS at an approximate protein concentration of 100 µg/ml.

PLASMA MEMBRANE ENZYME ACTIVITY.
Marker enzymes used for identification of endoplasmic reticulum, plasma membranes, and mitochondria were G-6-PD, 5'-nucleotidase, and cytochrome oxidase, respectively. The G-6-PD assay was performed as described by the Sigma kit (Sigma Chem Co., St. Louis, MO) except that 20 µl of sample was used and volumes of G-6-PD reagent and substrate were 0.5 ml and 1 ml respectively. The incubation time was for 60 min at room temperature. 5'nucleotidase assays were similarly modified to accomodate small amounts of membrane protein. 10 µl was incubated with 2.4 ml of substrates for 2.5 hours at 37°C. The incubation was halted by the addition of 0.5 ml 30% TCA and after centrifugation (1000 x G, 10 min) the supernatant was assayed for phosphate content. Differences between adenosine-5'monophosphate and Na β-glycerophosphate substrates were interpreted to reflect 5'-nucleotidase activity. Cytochrome oxidase assays (20) were performed by preparing a substrate of cytochrome C (Sigma) (2.1 mg/ml) in 0.3 M sodium phosphate buffer, pH 7.4. This solution was reduced by the addition of aliquots of 1.2 M sodium hydrosulfite. Then 20 µl of samples were added and incubated at room temperature for 20 min while following the decline in absorbance at 550 nm.

PREPARATION OF $[^3H]$-$C_{20:4}$ - PHOSPHOLIPID SUBSTRATES.
Synovial cells were harvested with trypsin as described above and then incubated in 2 ml Geys BSS with $[^3H]$-$C_{20:4}$ (10^{-5}M, 1 µCi/culture) for 1 hr. Metabolism of $[^3H]$-$C_{20:4}$ was terminated by the addition of 4 ml acetone and samples were centrifuged at 1000 x G, 10 min. The supernatant was subjected to vortex-evaporation under vacuum to remove acetone. Three successive extractions of the remaining aqueous phase with 4 ml diethylether resulted in collection of $[^3H]$-phospholipids. $[^3H]$-$C_{20:4}$ and diacylglycerides were removed from this preparation in the following manner: After evaporation of the ether, lipids were dissolved in 200 ml hexane:diethylether (19:1 v/v) and applied to 1.5 cm column of Silicar CC-7 (Mallinckrodt, Paris, KY, USA) in an Eppendorf pipet tip. $[^3H]$-Triacylglycerides, $[^3H]$-$C_{20:4}$, and diacylglycerides were eluted respectively with hexane:diethylether 19:1, 200 µl; 17.5:2.5, 400 µl; and 14:6, 600 µl (21). Phospholipids were then eluted with 600 µl of methanol. The methanol fraction was applied to 10 cm^2 plastic backed Silica Gel 60 thin layer chromatography (TLC) plates (EM Merck, Darmstedt, Germany) and developed in 2 dimensions (22). The first solvent contained chloroform:methanol:ammonium hydroxide (65:35:5.5). After vacuum drying a second solvent consisting of chloroform:acetone: methanol: acetic acid:water (30:40:10:10:5) was utilized. Phospholipids were eluted with methanol from areas corresponding to the R_f's of PC, PE and PI/PS. The latter two phospholipds were poorly separated and some PS contamination of PI was measured.

ASSAY OF PHOSPHOLIPASE ACTIVITY.
Plasma membranes (approximately 100 µg protein) in BSS were incubated with $[^3H]$-$C_{20:4}$-phospholipids and A23187 (10 µM) for 1 hr. The reaction was

terminated by adding 4 ml acetone, followed by centrifugation (1000 x G for 10 min). The supernatant was collected, acetone was evaporated as above, and lipids extracted into ether. Separation of [^{3}H]-phospholipids from the [^{3}H]-$C_{20:4}$ and [^{3}H]-diacylglycerides produced was achieved using the Silicar CC-7 column. The neutral lipid fractions were pooled and applied to a silica gel 60 TLC plate and developed using a solvent containing petroleum ether:diethylether:acetic acid (50:50:1) (23). [^{3}H]-Radioactivity in areas comigrating with diolein and $C_{20:4}$ were assessed by cutting out the areas identified by I_2 vapor staining followed by liquid scintilation spectrometry in Aquasol.

MATERIALS.
[5,6,8,9,11,12,13,15-^{3}H(N)] - $C_{20:4}$ (55 Ci/mMole) and Aquasol were purchased from New England Nuclear, Boston, MA, USA: Dulbecco's modified Eagle's medium (high glucose), penicillin-streptomycin, fungizone (Amphotericin B), mycostatin, and Gey's balanced salt solution (BSS) were from Grand Island Biological Co., (Grand Island, NY, USA); fetal bovine serum was procured from KC Biologicals Co., (Lenexa, KS); trypsin, and collagenase were purchased from Worthington Biochemicals (Freehold, NJ, USA); phospholipids, diolein, and $C_{20:4}$ were purchased from Sigma Chemical Co., (St. Louis, MO, USA); A23187 (sodium salt) was from Calbiochem-Behring Co. (Cleveland, OH, USA). All chemicals were analytical grade reagents.

RESULTS

ISOLATION OF PLASMA MEMBRANES. The 700-10,000 x G pellet fraction contained 10% of the G-6-PD activity, 31% of the 5'nucleotidase activity and 46% of the cytochrome oxidase activity (n=3). This fraction was applied to discontinuous sucrose gradients and sub-fractions collected. Table 1 shows the enzyme activities of the fractions not further purified from the first gradient (remaining endoplasmic reticulum and mitochondria) and of the final plasma membrane fraction isolated from the second sucrose gradient. Values for enzyme activities varied between experiments. This may be due in part, to the use of cell cultures initiated at different times. The upper fraction of the gradient contained residual G-6-PD and 5'nucleotidase activity, with relatively reduced levels of cytochrome oxidase when compared to the lower fraction. The middle fraction, containing the plasma membranes at density interfaces of 1.14/1.16 and 1.16/1.18 was repurified on a sucrose gradient and assayed for enzyme activity. 5'nucleotidase activity was present at high levels while G-6-PD and cytochrome oxidase activities were not (Table 1).

PHOSPHOLIPASE ACTIVITY

Metabolism of [^{3}H]-$C_{20:4}$ phospholipids to [^{3}H]-diacylglycerides is shown in Table 2. Metabolism of [^{3}H]-$C_{20:4}$ - PC and [^{3}H]-$C_{20:4}$ - PE was

TABLE 1

Synovial Cell Enzymes in Discontinuous Sucrose Gradients

Bottom fraction (mitochondria)

Expt	G-6-PD	5'Nucleotidase	Cytochrome Oxidase
1	4.6	51.2	4.0
2	7.0	9.0	5.2
3	6.2	8.9	10.7
4	ND	29.5	13.7
5	1.0	56.0	9.7

Top fraction (remaining endoplasmic reticulum)

Expt	G-6-PD	5'Nucleotidase	Cytochrome Oxidase
1	3.3	75.0	0.40
2	7.0	150.0	0.39
3	3.4	15.9	3.9
4	2.0	115.0	3.5
5	0.42	62.0	1.2

Repurified middle fraction

Expt	G-6-PD	5'Nucleotidase	Cytochrome Oxidase
1	0	121.0	0
2	0	150.0	0
3	3.1	50.9	0
4	0	132.0	ND
5	3.7	115.0	3.7

Enzyme activities expressed as: G-6-PD, A_{340}/μg protein/hr; 5'nucleotidase, μg phosphate x 10^3/μg protein/hr; cytochrome oxidase, A_{550} x 10^3/μg protein/20 min. ND, Not determined.

minimal. Only traces of [^{3}H]-diacylglycerides were formed from these substrates. By contrast, a portion of the [^{3}H]-PI/PS substrate was converted to [^{3}H]-diacylglycerides. These findings are consistent with the presence

TABLE 2

Phospholipase C Activity of Synovial Cell Plasma Membranes

Substrate	Incubation (min)	Experiment No. 1	2	3	4
		[^{3}H]-Diacylglyceride (dpm/100 μg protein)			
[^{3}H]-PI/PS	0	6	0	28	0
	60	2,547	11,527	3,714	3,646
[^{3}H]-PC	0	13	0	ND	ND
	60	32	0		
[^{3}H]-PE	0	31	0	ND	ND
	60	56	125		

Specific activities and amounts of substrates were: [^{3}H]-PI/PS 24,444 dpm/μg PO_4, 1.8 μg PO_4 as PI/PS added; [^{3}H]-PC 41,183 dpm/μg PO_4, 2 μg PO_4 as PC added; [^{3}H]PE 38,014 dpm/μg PO_4, 1.6 μg PO_4 as PE added. Experiments 1,2, and 3 utilized membranes isolated from primary synovial cell cultures, while the fourth experiment was from cell cultures sub-passaged once. ND, Not determined.

of a phosphatidylinositol-specific phospholipase C activity associated with plasma membranes.

DISCUSSION

This study indicated that cultured lapine synovial cells have a phospholipase-C activity at neutral pH which is associated with the plasma membrane fraction isolated using discontinuous sucrose gradient centrifugation. The phospholipase C activity was most likely specific for PI since PC and PE used as additional substrates did not result in marked [^{3}H]-diacylglyceride synthesis (24). It was unlikely that [^{3}H]-PS was utilized as a substrate for this membrane-bound phospholipase C activity since its conversion to [^{3}H]-diacylglycerides by a PS-specific phospholipase C activity has not been reported in any other tissue particulate fraction.

The [^{3}H]-labeled product was identified as a diacylglyceride by the following criteria, which are presented in detail elsewhere (25). Briefly, the [^{3}H]-labeled product 1) was synthesized in the presence of sodium meclofenamate (20 μM) and was not inhibited by the lipoxygenase inhibitor, nordihydroguiaretic acid (30 μM). 2) The [^{3}H]-compound after hydrolysis with KOH comigrated with $C_{20:4}$ on a TLC plate. 3) [^{3}H]-phospholipids synthesized by synovial cells from [^{3}H]-$C_{20:4}$ after digestion with Bacillus cereus phospholipase C comigrated on a TLC plate with a [^{14}C]-labeled diacylglyceride synthesized by synovial cells. 4) The synthesized [^{3}H]-diacylglyceride after conversion to [^{3}H]-phosphatidylcholine by sequential reaction with phosphorous oxychloride and choline chloride was incubated with bee venom phospholipase A_2. All of the [^{3}H] co-migrated with a $C_{20:4}$ standard on a TLC plate. We have concluded that the [^{3}H]-labeled compound contained sn-glycerol-1-acyl-2-$C_{20:4}$.

Enzyme activities of various subcellular fractions as well as phospholipase-C activities varied between experiments. Contributing to these differences may be individual variability between synovial tissue from outbred rabbits as well as differences associated with the phenotypic expression of synovial cells in each group of cultures. The cultures

contained variable amounts of cells with fibroblastic appearance, with multiple dendritic processes, and cells with a mononuclear cell appearance in a fashion described in other studies (26).

In other tissues, the rate of PI degradation by intact cells can be measured within a few minutes (10). However, once the cells are disrupted, phospholipase metabolism is much slower(27-29), either because of changes in the enzyme complex and its microenvironment or because exogenously added substrate is not as available to phospholipases as it is in intact cells. Thus, the use of a 60 min incubation time was not unreasonably long to assess phospholipase C activity by synovial cell plasma membranes.

The presence of a phospholipase C activity in synovial cell plasma membranes suggests that $C_{20:4}$-containing phospholipids in synovium may be metabolized by this phospholipase C as well as by phospholipase A_2 activities. The data were consistent with phospholipase C activity in the absence of detergents. In preliminary experiments, deoxycholate treatment of synovial cell plasma membranes did not substantially augment phospholipase C activity. Thus, the associations between phospholipase C and receptors that result in enzyme activity remained linked as they might exist in intact cells. In this situation, synovial plasma membranes may be useful for studies of physiological agonists which act through the phospholipase C pathway. Role(s) of the phospholipase C pathway in synovial tissue function as well as stimulators of this pathway remain to be investigated. Since the products of this pathway contain $C_{20:4}$, and an effect on $C_{20:4}$ metabolism appears to be a common characteristic of agents used in the therapy of rheumatic diseases, the phospholipase C pathway may be of considerable consequence in synovial pathophysiology.

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