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Role of Phospholipase A_2 in the Stimulation of Sponge Cell Proliferation by Homologous Lectin

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Summary

Using the Geodia cydonium system, we showed that after incubation of competent sponge cells in the presence of lectin, phospholipase A_2 was released from the cells. The substrates for this enzyme, phosphatidylethanolamine and phosphatidylcholine, were identified in the extracellular material of sponge tissue. In addition, the phospholipase A_2 inhibitor calelectrin was identified by immunobiochemical techniques; this molecule was associated with the aggregation factor. Reconstitution experiments strongly suggested that phospholipase A_2 catalyzed the release of arachidonic acid, which is then taken up by the cells. Intracellularly, arachidonic acid was metabolized primarily to prostaglandin E_2. Inhibition studies revealed that prostaglandin E_2 is involved in the ultimate increase of DNA synthesis. These findings suggest that the phospholipase A_2–arachidonic acid system is involved in the matrix-initiated signal transduction pathway in sponges.

Introduction

In the initial phase of reaggregation of sponge cells, the cells adhere to each other in a random fashion. In the second and third phase a sorting-out process occurs, during which the cells regain their ability to differentiate (Müller, 1982). These events are contingent on a multiplicity of precisely coordinated intracellular signal transduction pathways. Using the marine sponge Geodia cydonium we showed that the initial cell–cell contact is established by the interaction between the complex aggregation factor (AF) particle (Müller and Zahn, 1973) and the aggregation receptor (Müller, 1982). In a second phase, the cells produce a vast extracellular matrix composed primarily of collagen, glycoconjugates, and lectin (Müller et al., 1988). Simultaneously, a switch of the cell adhesion mechanisms occurs during aggregate formation, from a cell–cell to a cell–matrix interaction (Gramzow et al., 1988). In previous studies we showed that during the initial phase of cell–cell contact AF causes a rapid stimulation of the phosphatidylinositol pathway (Müller et al., 1987), resulting in an activation of protein kinase C (Müller et al., 1987) and a subsequent phosphorylation of DNA topoisomerase II (Rottmann et al., 1987). As one consequence of these processes, the cells undergo a phase of high DNA synthesis (Müller et al., 1987). However, at a later stage, AF loses its mitogenic activity; this function is then taken over by the matrix lectin (Schröder et al., 1988a). During this switch, the lectin receptor (anti-aggregation receptor; Müller et al., 1979) associates in the plasma membrane with the *ras* oncogene product (Schröder et al., 1988a).

In the present study, we focused our efforts on the intracellular transduction pathway that originates in the interaction of the lectin with its receptor and ultimately leads to a stimulation of DNA replication. Our findings indicate that phospholipase A_2 is rapidly released from the cells into the extracellular matrix, where it cleaves phosphatidylethanolamine and/or phosphatidylcholine as a substrate for the formation of arachidonic acid, which is then taken up by the cells. Evidence is presented that these events lead to enhanced formation of prostaglandin E_2 followed by a subsequent rise in ornithine decarboxylase level and increased DNA synthesis.

Results

Distribution of Phospholipase A_2 and 1,2-Diacylglycerol in Intact Sponge

G. cydonium contained phospholipase A_2 not only in the cellular compartment but also in the extracellular space (Table 1). In view of an earlier finding that phospholipase A_2 activity is inhibited by calelectrin (Fauvel et al., 1987), we preincubated both the cell extract and the extracellular material with antibodies against calelectrin prior to the determination of enzyme activity. After this treatment, the activity increased 3.4-fold in the extracellular material, but only 1.15-fold in the cellular extract (Table 1). In general, the phospholipase A_2 activity was 15.3-fold greater in the extracellular material than in the cellular extract.

The substrates of phospholipase A_2, phosphatidylcholine and phosphatidylethanolamine, are present in the extracellular material of the sponge (see below), while the reaction product of phospholipase C, diacylglycerol, was not detectable in that compartment (Table 1).

Table 1. Distribution of Phospholipase A_2 and 1,2-Diacylglycerol in Intact Sponge

	Extract	
	Cellular	Extracellular
Phospholipase A_2 (U/mg)		
Without anti-calelectrin antibodies	2.7 ± 0.2	13.9 ± 0.9
With anti-calelectrin antibodies	3.1 ± 0.2	47.3 ± 2.7
1,2-Diacylglycerol (nmol/mg)	131.8 ± 10.4	<0.1

Phospholipase A_2 activity and the amount of 1,2-diacylglycerol were determined as described in Experimental Procedures. Where indicated, the material (5 mg) was preincubated (for 12 hr at 2°C) with 3 mg of anti-calelectrin antibodies prior to determination of enzyme activity. The values given are based on milligrams of protein. The results are the means ± SD of five independent experiments.

Release of Phospholipase A_2 from Intact Cells

Dissociated G. cydonium cells released phospholipase A_2 into the extracellular medium after addition of homologous lectin (Figure 1). The extent of enzyme release was low if the cells had been in contact with the cell binding fragment of AF for only 1 hr, in contrast to cells that had been preincubated for 10 hr. The maximal release was measured after the short incubation period of 1 min and amounted to 0.2 U/ml for 1 hr preincubated cells (1 ml contains 8.3 × 10^6 cells) and 1.4 U/ml for 10 hr preincubated cells. The extent of enzyme release was dose dependent. Using 10 hr preincubated cells, the following enzyme activities were determined in the extracellular medium: addition of 5 μg/ml of lectin, 0.94 U/ml of enzyme released (1 min after addition of the lectin stimulus); 1 μg/ml, 1.42 U/ml; 0.3 μg/ml, 0.71 U/ml; and 0.1 μg/ml, 0.26 U/ml. In one control experiment in which an antibody-adsorbed lectin preparation was used instead of untreated lectin, it was established that the observed stimulation of enzyme release is indeed due to the homologous lectin (Figure 1).

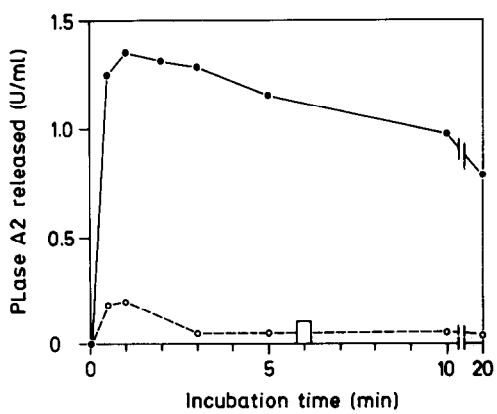

Figure 1. Release of Phospholipase A_2 from G. cydonium Cells after Addition of Homologous Lectin

Sponge cells preincubated with the cell binding fragment for 1 hr (open circles) or 10 hr (solid circles) were stimulated in the standard incubation assay with 1 μg/ml of lectin. After an incubation time of 0.5–20 min the cell suspension was centrifuged (2000 × g at 2°C for 10 min), and the phospholipase A_2 (PLase A_2) activity was determined in the supernatant. In one control assay, a lectin sample (1 μg/ml with respect to the lectin) that had been adsorbed with anti-lectin antibodies was added to 10 hr preincubated cells (open bar).

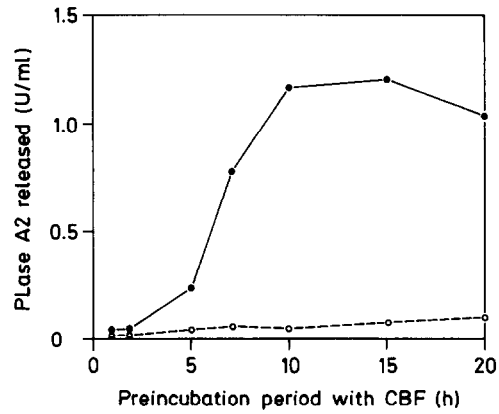

Figure 2. Release of Phospholipase A_2 from Cells after Addition of Lectin or Aggregation Factor

The cells were preincubated in the standard assay in the presence of the cell binding fragment (CBF). Then 1 μg/ml of lectin (solid circles) or 30 μg/ml of purified AF (open circles) was added to the cultures, and the activity of phospholipase A_2 (PLase A_2) was determined 5 min later.

The dependence of phospholipase A_2 release from cells preincubated for 1–20 hr with the cell binding fragment on the presence of the lectin stimulus is given in detail in Figure 2. It is striking that such a strong increase in the inducibility of phospholipase A_2 release by the lectin occurred after a 5 hr preincubation period with the cell binding fragment. The plateau was reached after a 10 hr period. Replacing lectin by the purified AF particle did not result in an increased release of enzyme from the cells (Figure 2). In control experiments in which antibodies were used against calelectrin, it was established that this low enzyme level is not due to inhibition of the phospholipase A_2 by the calelectrin-related protein that is associated with the AF particle (see below) (data not shown).

Association of Calelectrin with the AF Particle

As summarized in Table 1, addition of anti-calelectrin antibodies to the extracellular fraction from the sponge led to a 3.5-fold increase in phospholipase A_2. This finding was our first evidence that calelectrin is present extracellularly in this organism. Next, we studied whether calelectrin is associated with G. cydonium AF, which is known to be composed of a series of polypeptides among which is the cell binding fragment (Gramzow et al., 1986). Then it was shown that the AF particles also contained a protein with an M_r of 32,000 that was recognized in the immunoblot by antibodies against calelectrin (Figure 3, lane A). Applying the same amount of protein from the cellular extract for the gel electrophoresis analysis (Figure 3, lane C), only a faint band was visualized with the antibodies towards calelectrin. No immune complexes could be detected on the blot of AF-free extracellular material (Figure 3, lane B).

For further proof that the 32 kd polypeptide in the AF particle that reacted with anti-calelectrin is presumably calelectrin, inhibition studies were performed. Three different AF concentrations were added to increasing substrate concentrations (see Experimental Procedures). The highest substrate concentration of 10 μM was chosen in

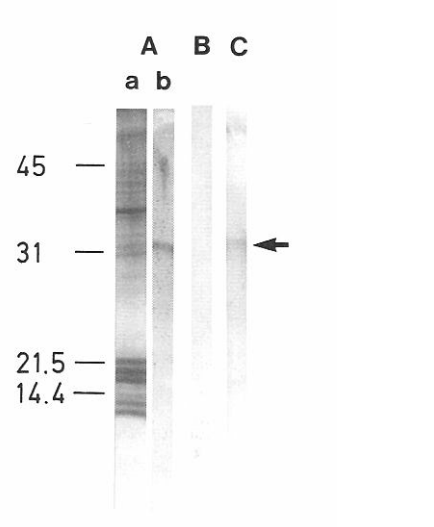

Figure 3. Identification of Calelectrin in the Purified AF Particle

The purified AF (50 μg of protein) was electrophoresed on a 15% polyacrylamide gel under denaturing conditions; proteins were either stained with Coomassie brilliant blue (lane Aa) or transferred to nitrocellulose sheets (lane Ab) and incubated first with anti-calelectrin rabbit antibodies and second with anti-rabbit IgG (peroxidase conjugated). In parallel experiments, 50 μg of extracellular material that had been freed from AF particles (B) and 50 μg of cellular extracts (C) were electrophoresed in the same way and subsequently blot-transferred and antibody treated. Details are given in Experimental Procedures. Molecular weight standards were bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). Arrow = p32 (protein with M_r of 32,000).

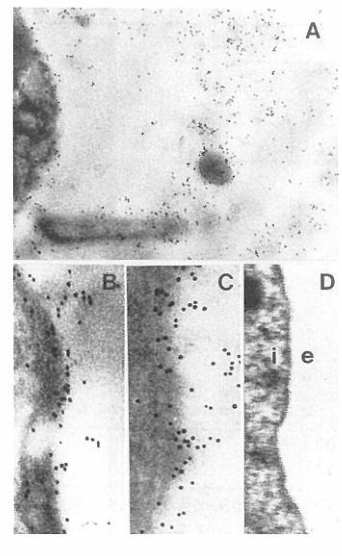

Figure 4. Association State of Calelectrin with the Cell Surface of G. cydonium Cells in Aggregates

(A) Cross section through a 5-hr-old aggregate (survey). Cross section through 2-hr- (B), 4-hr- (C), and 20-hr-old aggregates (D). The slices were immunostained with anti-calelectrin; the immune complex was visualized with colloidal gold-labeled anti-rabbit IgG as described in Experimental Procedures. e = extracellular space, i = intracellular space. For these studies the standard aggregation assay was supplemented with 50 μg/ml both of homologous glycoconjugates and of homologous collagen. Magnification: A = 10,600×, B–D = 31,800×.

light of earlier findings revealing that only below concentrations of 10 μM does phospholipase A_2 follow the Michaelis-Menten kinetics (Lister et al., 1988). The results revealed the following kinetic data: Michaelis constant (K_m) = 34.7 ± 3.9 μM, maximal reaction velocity (V_{max}) = 353.7 pmol/min per mg of protein, and an inhibitor constant (K_i) = 52.4 ± 2.9 μg/ml. Kinetic analysis using the Lineweaver-Burk method showed that the AF-caused inhibition was of the noncompetitive type. These data led us to believe that AF is associated with calelectrin or a calelectrin-like protein.

Change of the Association State of Calelectrin with the Cell Surface after Prolonged Reaggregation

To mimic natural conditions, the cells were incubated in the standard incubation assay with artificial seawater and intact AF and with the natural substrates for the cells to attach, collagen and glycoconjugates (Müller et al., 1988). In the initial phase, after addition of intact AF to the dissociated cells, calelectrin could be detected by immunoelectron microscopy at high density on the cell surface (Figures 4A, 4B, and 4C). At later stages (20 hr after incubation), the gold grains were completely absent (Figure 4D). This change (from the presence to the absence of calelectrin) on the cell surface of old aggregates correlates very well with earlier observations (Gramzow et al., 1988) that the association of AF with the cell membrane–bound aggregation receptor is no longer detectable after a 20 hr incubation period and supports the finding that calelectrin is associated with intact AF (see above).

Levels of Eicosanoids in Dissociated Cells

Among the different eicosanoids determined, dissociated G. cydonium cells contain predominantly prostaglandin E_2 (approximately 3 pg/10^8 cells) and lower concentrations of prostaglandin D_2 (1 pg/10^8 cells) and thromboxane D_2 (0.2 pg/10^8 cells) (Table 2). These levels were almost identical in cells incubated for only 1 hr or as long as 10 hr in the presence of the cell binding fragment. Addition of the homologous lectin resulted in no change in the thromboxane B_2 or prostaglandin D_2 concentrations, but there was a substantial increase in the prostaglandin E_2 concentration. The latter effect was observed only in those cell populations that had been preincubated for 10 hr with the cell binding fragment; addition of 1 μg/ml of lectin to the cell suspension caused a 6.6-fold increase in the prostaglandin E_2 level. This drastic change was prevented by coincubation of the lectin with the soluble lectin receptor (Müller et al., 1979) and by the cyclooxygenase inhibitor indomethacin (Otamiri et al., 1988), while the lipoxygenase inhibitors BW755C (3-amino-1–1-[m-(trifluoromethyl)phenyl]-2-pyrazoline; Otamiri et al., 1988) and ETYA (5,8,11,14-eicosatetraynoic acid; Gut et al., 1988) had no influence on the concentration of prostaglandin E_2 (Table 3).

Incorporation of Arachidonic Acid into Cellular and Extracellular Phospholipids

The incorporation of [^{14}C]arachidonic acid into different

Table 2. Alterations of the Levels of Selected Eicosanoids after Incubation of Dissociated Cells in the Presence of Homologous Lectin

	Lectin Concentration (μg/ml)	Concentration of Eicosanoids (pg per 10^6 cells)	
		Cells Pretreated with AF for: 1 hr	10 hr
Thromboxane B_2	0	0.2 ± 0.02	0.2 ± 0.03
	1	0.2 ± 0.03	0.3 ± 0.03
Prostaglandin D_2	0	0.9 ± 0.1	1.1 ± 0.1
	1	0.8 ± 0.1	1.4 ± 0.2
Prostaglandin E_2	0	3.2 ± 0.4	4.9 ± 0.6
	0.1	2.9 ± 0.3	8.3 ± 1.3
	1	3.7 ± 0.4	32.4 ± 5.0
	10	4.1 ± 0.6	27.0 ± 4.3
Additional components:			
Anti-aggregation receptor, 10 μg/ml	1	3.4 ± 0.5	5.3 ± 0.9
Indomethacin, 10 μM	1	2.7 ± 0.4	2.9 ± 0.5
BW755C, 100 μM	1	3.4 ± 0.4	30.0 ± 4.9
ETYA, 100 μM	1	2.8 ± 0.4	34.1 ± 5.0

Dissociated cells preincubated with the cell binding fragment (AF) for 1 or 10 hr in the standard incubation assay were stimulated with 0–10 μg/ml of lectin for 30 min in the presence of 50 nM arachidonic acid. Where indicated, the additional components were added together with the lectin to the cell system. Then the cells were collected by centrifugation (5000 × g at 2°C for 10 min), and a cell extract was prepared. The particle-free supernatant was prepared (100,000 × g at 2°C for 30 min), and the concentrations of the eicosanoids were determined. The results are the means (± SD) of five parallel experiments.

phospholipid species was determined both for the cellular as well as the extracellular compartment. After a pulse for 20 min with the precursor [^{14}C]arachidonic acid, the total incorporated arachidonate associated with individual phospholipids was determined after a 10, 60, and 120 min chase in the absence of the labeled precursor (Figure 5). The results revealed that arachidonate underwent a differential esterification into the different phospholipid classes in the cellular and extracellular compartments. In the cells, the highest incorporation was determined in the phosphatidylinositol fraction, followed by phospohatidylcholine and phosphatidylethanolamine (Figure 5A). The extracellular material contained arachidonate almost exclusively in the phosphatidylcholine and phosphatidylethanolamine fractions, and almost no incorporation was determined in the phosphatidylinositol fraction (Figure 5B). The total amount of arachidonate incorporation into phospholipids was 1.4 ± 0.4 nmol/10^6 cells (n = 5) after a 120 min period of chase.

Table 3. Reconstitution Assay to Demonstrate the Generation of Free Extracellular Arachidonic Acid

	Released [^{14}C]Arachidonic Acid (dpm per 10^6 cells)	
	Cells Pretreated with AF for:	
Additional Component	1 hr	10 hr
None	4310 ± 645	7380 ± 885
Anti-lectin antibodies	1240 ± 220	2090 ± 370
AF particle	1730 ± 250	2110 ± 380
AF particle plus anti-calelectrin antibodies	3940 ± 630	7570 ± 940

Cells that were preincubated for 1 or 10 hr in the standard incubation assay (3 ml) in the presence of 2 μg/ml cell binding fragment (AF) were incubated together with 20 μg/ml of extracellular homologous material (AF particle free) containing 1.7 μg/ml of lectin and supplemented with 10 μCi of 1-acyl-2-(1-^{14}C]arachidonyl-glycerophosphoethanolamine per 20 μg of extracellular protein. Where indicated, the extracellular material (20 μg) was preincubated (for 24 hr at 4°C) with 5 μg of anti-lectin antibodies, pretreated (for 5 min at 4°C) with 2 μg of AF particle, or pretreated (for 24 hr at 4°C) with 2 μg of AF particle together with 15 μg of anti-calelectrin. Incubation was performed at 30°C for 30 min. Then the released free [^{14}C]arachidonic acid was determined as described (Errasfa et al., 1988). The results are the means (± SD) from five independent experiments.

Reconstitution Assay for the Extracellular Generation of Arachidonic Acid

The data to this point indicated that cells preincubated for 10 hr in the presence of the cell binding fragment have the ability to release phospholipase A_2 into the extracellular medium in response to a lectin stimulus (Figure 2) and respond with an increased prostaglandin E_2 level (Table 2). Together with the finding that the sponge tissue contains phosphatidylcholine and phosphatidylethanolamine in the extracellular space (Figure 5), it is tempting to speculate that these compounds are used by the extracellular phospholipase A_2 as substrates to produce free arachidonic acid, which penetrates through the cell membrane and ultimately gives rise to the production of prostaglandin E_2. To test the latter assumption, a reconstitution assay was performed. Dissociated cells, preincubated in the standard assay for 1 or 10 hr in the presence of 2 μg/ml of cell binding fragment, were incubated with lectin-containing cell-free extracellular material that had been depleted of AF particles in order to remove the AF-bound calelectrin. In addition, the assay was supplemented with the radioactively labeled phospholipase A_2 substrate, 1-acyl-2-[1-^{14}C]arachidonyl glycerophosphoethanolamine (Table 3).

The results revealed that especially in those assays containing cells that had been preincubated with the cell binding fragment for 10 hr, almost 1% of the total amount of initially present enzyme substrate was converted to arachidonic acid and diacylglycerol. The fact that only half

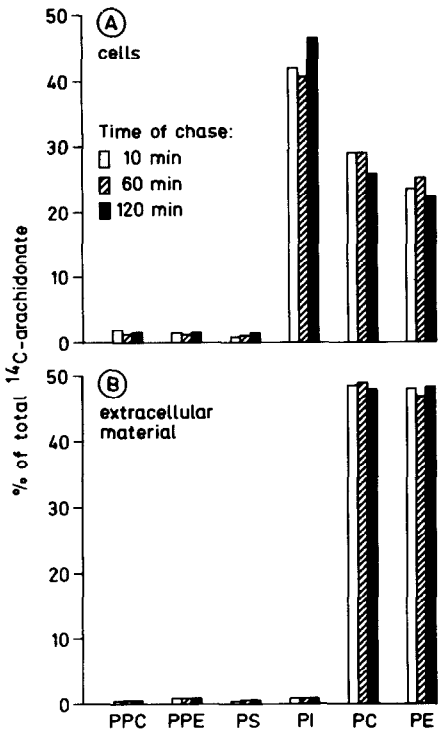

Figure 5. Incorporation of [^{14}C]Arachidonic Acid into Phospholipids

Sponge tissue cubes were incubated in the presence of [^{14}C]arachidonic acid for 20 min, followed by a 10, 60, or 120 min chase in the absence of the labeled precursor. Then the lipids were extracted from the cells (A) and from the extracellular material (B) essentially as described in Experimental Procedures. The values represent means from five independent experiments; the standard deviation was less than 30%. PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PPE, ethanolamine plasmalogens; PPC, choline plasmalogens.

as much arachidonic acid could be determined in those assays containing cells that had only 1 hr of contact with the cell binding fragment as in the assays with cells preincubated for 10 hr indicates that the cells differentially respond with phospholipase A_2 release to the lectin stimulus. To test this assumption, the extracellular homologous material was preincubated with anti-lectin antibodies prior to its addition to the reconstitution assay. Under these conditions, the extent of arachidonic acid release was strongly suppressed (Table 3). As mentioned below, one major extracellular component, the AF particle, is associated with calelectrin, an inhibitor of phospholipase A_2. Hence, addition of an AF particle preparation to the reconstitution assay strongly inhibited the formation of arachidonic acid. The inhibition of phospholipase A_2 could be abolished by antibodies directed against calelectrin (Table 3).

These data strongly suggest that free arachidonic acid is also produced in in vivo conditions in the extracellular space in sponges from phosphatidylethanolamine and/or phosphatidylcholine by phospholipase A_2.

Table 4. Distribution of Arachidonate in Nuclei and Nuclear Substructures

	Arachidonate in Subcellular Structures (%)
Cells	100
Nuclei	17.8
Nuclear envelopes	13.6
Pore-complex laminae	0.71
Nuclear matrix	<0.04

Sponge cubes were incubated in the presence of [^{14}C]arachidonic acid for 20 min, followed by a 120 min chase. Then, cells were isolated, and nuclei and the nuclear substructures were prepared as described in Experimental Procedures. The values given are in percent and are correlated with the incorporation rate measured in total cells (= 100%). The data are the means from five independent experiments.

Incorporation of Arachidonic Acid into Nuclei and Nuclear Substructures

For further clarification concerning the cellular compartment where arachidonic acid undergoes the incorporation into phospholipids, subcellular fractionation experiments were performed. Under the assay conditions summarized in Table 4, 1.3 ± 0.4 nmol of [^{14}C]arachidonate per 10^6 cells was incorporated into phospholipids; this value was set at 100%. A subsequent subcellular fractionation revealed that 17.8% of the total [^{14}C]arachidonate was recovered in the nuclear fraction: 75% of that was present in the nuclear envelope preparation, and a significant amount (4%) could also be identified in the pore-complex laminae, while the nuclear matrix fraction was obviously free of incorporated arachidonate.

Differential Stimulation of Mitogenesis by Free Arachidonic Acid

Our results suggested that arachidonic acid is produced extracellularly in sponge tissue from phosphatidylethanolamine by phospholipase A_2 hydrolysis. Moreover, the release of higher amounts of this enzyme occurs only after a 10 hr preincubation period in the presence of the cell binding fragment and a subsequent lectin stimulus. Therefore, we speculated that arachidonic acid is mitogenic to sponge cells, as already shown for vertebrate cells in earlier studies (Takuwa et al., 1988; Skouteris et al., 1988).

To test this possibility, sponge cells preincubated for 1 or 10 hr in the presence of the cell binding fragment were subsequently incubated with arachidonic acid (Figure 6). The mitogenicity was measured by determination of both ornithine decarboxylase activity and thymidine incorporation rate. The experiments revealed that after a 2 hr incubation period, the ornithine decarboxylase level increased 3.2-fold in cells preincubated with the cell binding fragment for 10 hr and 1.5-fold in 1 hr preincubated cells. The onset of the increase of thymidine incorporation was 3 hr after addition of arachidonic acid, and it reached a plateau 2 hr later; incorporation was stimulated 7.1-fold (10 hr preincubated cells) and 2.6-fold (1 hr preincubated cells) after a 5 hr incubation period.

The stimulatory effect of arachidonic acid was dose dependent (Table 5). At concentrations lower than 10 μM, the compounds caused a smaller stimulation. The cyclooxygenase inhibitor indomethacin, as well as the irreversible

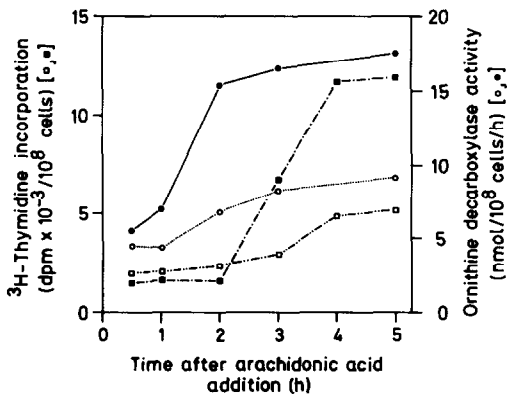

Figure 6. Time Course of Changes in Ornithine Decarboxylase Activity and Thymidine Incorporation Rate after Addition of Arachidonic Acid

Cells preincubated with the cell binding fragment for either 1 hr (open circles; open squares) or 10 hr (solid circles; solid squares) were incubated in the standard incubation assay (in the absence of the AF particles) in the presence of 1 μg/ml lectin and 10 μM arachidonic acid for the indicated periods of time. Then the cells were harvested and assayed for ornithine decarboxylase activity (open circles; solid circles), or the incorporation of [^{3}H]thymidine into acid-precipitable material was measured (open squares; solid squares) as described in Experimental Procedures. The means from five parallel experiments are given; the standard deviations were less than 8% (ornithine decarboxylase) and 10% (incorporation).

inhibitor of ornithine decarboxylase, α-difluoromethylornithine (Pegg et al., 1988), drastically suppressed the arachidonic acid mitogenicity. A dose-response curve was performed at an arachidonic acid concentration of 50 nM (Figure 7). A 50% inhibition of prostaglandin E_2 production was obtained at approximately 3 μM indomethacin. Reduction of the [^{3}H]thymidine incorporation by 50% was found at nearly the same inhibitor concentration (approximately 5 μM). These data strongly suggest that arachidonic acid has to be metabolized to eicosanoids prior to its modulating influence on DNA synthesis and that the ornithine decarboxylase activity is tightly coupled to the proliferation state of the cells.

Table 5. Effect of Arachidonic Acid on Sponge Cell Growth

Arachidonic Acid	ODC Activity (nmol/10^6 cells/hr)	[^{3}H]thymidine Incorporation (dpm x 10^{-3}/10^8 cells)
0 μM	7.1 ± 0.7	1.9 ± 0.2
10 μM	17.8 ± 1.3	11.8 ± 1.0
1 μM	12.3 ± 1.0	8.5 ± 0.7
0.1 μM	9.0 ± 0.8	2.7 ± 0.2
10 μM plus:		
10 μM indomethacin	10.7 ± 1.0	4.1 ± 0.3
200 μM DFMO	5.3 ± 0.4	2.5 ± 0.2

Cells were preincubated for 10 hr in the presence of the cell binding fragment and then for 5 hr with lectin and arachidonic acid as described in Figure 6. Then the cells were analyzed for ornithine decarboxylase (ODC) activity and thymidine incorporation. Where indicated, indomethacin or α-difluoromethylornithine (DFMO) was added with arachidonic acid. Further details are given in Experimental Procedures. Values are the means ± SD from three independent experiments.

As a direct proof (not shown) that prostaglandin E_2 displays a stimulatory effect on DNA synthesis in the sponge system, G. cydonium cells that had been pretreated with the cell binding fragment for 10 hr were incubated in the standard assay in the presence of exogenously added prostaglandin E_2 (0.1 μg/ml) for 4 hr. Under these conditions, the incorporation rate of [^{3}H]thymidine increased from 1.2 ± 0.1 (absence) to 7.5 ± 0.6 dpm x 10^{-3}/10^8 cells (presence of prostaglandin E_2) (n = 5).

Discussion

In the sponge model system, diacylglycerol plays a central role as second messenger. Using the sponge G. cydonium, we established that diacylglycerol is formed intracellularly in response to the extracellular AF stimulus and activates protein kinase C (Müller et al., 1987; Rottmann et al., 1987). This rapid process leads to cell proliferation during the early phase of cell–cell aggregation (until approximately 5 hr after the addition of AF). Then this protein kinase C–mediated intracellular signaling pathway is down-regulated (Gramzow et al., 1988; Schröder et al., 1989). In the later phase of cell aggregation (5 hr later), the AF loses its mitogenic activity and is replaced by the extracellularly localized lectin (Schröder et al., 1988a).

In the present report, experimental evidence is presented that the interaction between the lectin and the lectin receptor results in the release of phospholipase A_2 into the extracellular space—a process that initiates a second signaling pathway. In contrast, phospholipase A_2 is apparently either spontaneously released from vertebrate cells (Pruzanski et al., 1988) or the stimuli are not yet known. The function of the extracellular sponge phospholipase A_2 is very likely to hydrolyze phosphatidylethanolamine and hence to release arachidonic acid. This assumption is corroborated by the analytical data revealing that both phosphatidylethanolamine and phosphatidylcholine are present in the extracellular material and by the results showing that incubation of exogenously added 1-acyl-2-arachidonyl glycerophosphoethanolamine to the lipase-containing extracellular fraction gave rise to free arachidonic acid.

Interestingly, the extracellular compartment of the sponge also contains the inhibitor of phospholipase A_2, calelectrin (Fauvel et al., 1987). We found that this protein is associated with the sponge AF particle and noncompetitively inhibits the homologous enzyme. From a previous study it is known that the AF particle is associated with the cell membrane only during the early phase of reaggregation (Gramzow et al., 1988). At later stages, AF is localized in the bulky extracellular matrix, and there it is associated with the collagen fibrils. Essentially, the same translocation of calelectrin was observed during the aggregation phases—a finding that strongly indicates that calelectrin remained AF-associated. One plausible interpretation reconciling these data is that calelectrin displays its inhibitory function on phospholipase A_2 only during the early phase of reaggregation, while at later stages the enzyme in the vicinity of the cell membrane is functionally active and hence releases arachidonic acid.

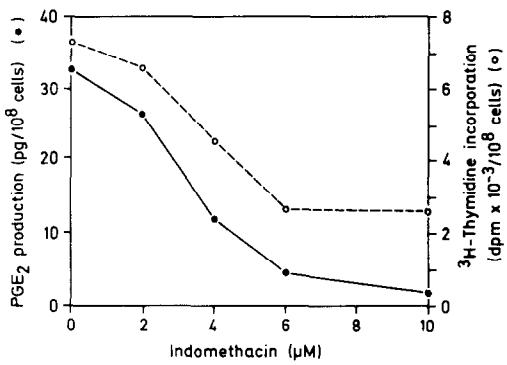

Figure 7. Dose Dependence of the Effect of Indomethacin on Lectin-Stimulated Prostaglandin E_2 Production and DNA Synthesis

Cells pretreated for 10 hr in the presence of the cell binding fragment were stimulated with 1 μg/ml of lectin for 30 min in the presence of 50 nM arachidonic acid and the indicated concentrations of indomethacin. Then the amount of released prostaglandin E_2 (PGE_2) (solid circles) as well as the incorporation rate of [^{3}H]thymidine (open circles) was determined as described in Experimental Procedures. The means from five parallel experiments are given; the standard deviations were less than 8% (prostaglandin E_2) and 10% (incorporation).

This assumption was corroborated by the finding that the cells in the initial phase of reaggregation show a low capacity to metabolize arachidonic acid to prostaglandin E_2 in the presence of homologous lectin. However, when the cells were preincubated for 10 hr with AF, they displayed a 7-fold increase of prostaglandin E_2 synthesis in response to the lectin stimulus. The conversion of the arachidonic acid to prostaglandin E_2 was inhibited by the cyclooygenase inhibitor indomethacin (Sung et al., 1988); no effect was observed with the lipoxygenase inhibitors DW755C (Otamiri et al., 1988) and ETYA (Gut et al., 1988). Our working hypothesis is that at the later stage of cell aggregation, lectin displays its mitogenic effect, as described earlier (Schröder et al., 1988a), via a dual influence on the cells. Binding of lectin to the lectin receptor causes an increased release of phospholipase A_2 in the extracellular space, resulting in an enhanced production of arachidonic acid, which is taken up by the cells and then ultimately metabolized to prostaglandin E_2.

At present we do not yet know in which cellular compartment prostaglandin E_2 is formed. Our data show that after incubation of the cells with arachidonic acid, 18% of the imported material became associated with the nuclei—more precisely, with the nuclear envelope, a component containing two enzymes in the arachidonate/icosanoid pathway, cyclooxygenase (Rollins and Smith, 1980) and prostaglandin I_2 synthetase (Smith et al., 1983). Hence it appears to be likely that prostaglandin E_2 is formed either in the cytosol or in the nuclear envelope compartment and then enters the nuclei, and perhaps there it displays its growth-inducing effect. At present it is not yet known if this mechanism involves the binding of prostaglandin E_2 to specific receptors in the nuclear membrane.

Our data show that after incubation of cells from 10-hr-old aggregates, they respond to lectin and arachidonic acid stimuli with a rapid increase in the ornithine decarboxylase level, followed by a strong rise in DNA synthesis. At present, one can only speculate about the role of prostaglandin E_2 in this signal transduction chain. In view of earlier reports, it is possible that prostaglandin E_2 stimulates c-*fos* expression (Kacich et al., 1988) followed by ornithine decarboxylase expression (Cosenza et al., 1988), which ultimately leads to DNA synthesis (Pisano and Greene, 1987). However, available experimental evidence for such a definitively programmed order of gene expression during the progression of cells out of G0 through G1 to S phase is fairly well established only in the latter step of this sequence.

Experimental Procedures

1-Stearoyl-2-[1-^{14}C]arachidonyl-*sn*-glycerol (57 mCi/mmol), DL-[1-^{14}C]-ornithine (50 mCi/mmol), [^{3}H]thymidine (5.0 Ci/mmol), 1-acyl-2-[1-^{14}C]-arachidonyl glycerophosphoethanolamine (55 mCi/mmol), *sn*-1,2-diacylglycerol assay reagents system, [1-^{14}C]arachidonic acid (65 mCi/mmol), and radioimmunoassay systems for thromboxane B_2 and bicyclic prostaglandin E_2 and D_2 were from Amersham Int., Amersham (United Kingdom). Phospholipase A_2 (from porcine pancreas; P6534), prostaglandin E_2, and colloidal gold-labeled (10 nm) secondary anti-rabbit IgG from goat (G 3766) were from Sigma Chemical Co., St. Louis, Mo.

Live specimens of G. cydonium were collected near Rovinj (Yugoslavia).

The G. cydonium AF particle was isolated and purified as described (Müller and Zahn, 1973; Conrad et al., 1984); it had a specific aggregation-promoting activity of 3.9×10^6 aggregation units per mg (1.6 mg/ml). The definition of aggregation units has been given previously (Müller and Zahn, 1973). The specific cell binding fragment was isolated from purified AF (Gramzow et al., 1986).

The G. cydonium lectin with an M_r of 36,500 was isolated and purified by affinity chromatography (Müller et al., 1983). Where indicated, 30 μg/ml of lectin solution was adsorbed with anti-lectin antibodies (5 mg/ml) for 24 hr at 2°C. Then the sample was centrifuged (100,000 × g at 5°C for 30 min), and the supernatant was used for the experiments.

The G. cydonium glycoconjugates and homologous collagen were prepared as described (Conrad et al., 1984; Diehl-Seifert et al., 1985).

The lectin receptor was isolated and purified as described (Müller et al., 1979).

Buffers

The descriptions of the compositions of Ca^{2+}- and Mg^{2+}-containing artificial seawater and Ca^{2+}- and Mg^{2+}-free seawater were given earlier (Rottmann et al., 1987).

Antibodies

Antibodies to calelectrin, purified from Torpedo marmorata (Fritsche et al., 1988), were raised in rabbits and were enriched by precipitation with ammonium sulfate (Müller et al., 1985). The antibodies cross-reacted with 67 kd, 32 kd, and 34 kd calelectrin (Saitoh and Miret, 1987).

Antibodies to G. cydonium lectin were prepared as described (Conrad et al., 1982).

Cells and Incubation Assay

A description of the preparation of viable sponge cells was given earlier (Müller and Zahn, 1973); mucoid cells, archaeocytes, and choanocyte clusters were prepared from the total cell populations (Rottmann et al., 1987). Six hours after dissociation, the cells were used for the experiments.

In the standard incubation assay (3 ml volume) (Müller and Zahn, 1973), a suspension of $25 \pm 5 \times 10^6$ cells per ml of Ca^{2+}- and Mg^{2+}-free seawater was placed into glass tubes and rolled at 35 rpm at 20°C. Unless stated otherwise, 2 μg/ml of cell binding fragment or 30 μg/ml of AF particle was added. The suspension was incubated for a period of 60 min. Then the assays were incubated for a period of up to 20 hr without moving.

Where indicated, 1 μg/ml of untreated lectin or adsorbed lectin was added to the cell suspension.

In the studies that determined, by immunocytological techniques, the distribution of calelectrin in aggregates as a function of incubation time, the standard incubation assay consisted of 25 ± 5 × 10^6 cells per ml in artificial seawater, 50 μg/ml of homologous glycoconjugates, 50 μg/ml of homologous collagen, and 30 μg/ml of AF particles. Incubation was performed for up to 20 hr. Aggregates were taken at the appropriate incubation times and worked up for immunoelectron microscopy inspection.

Cell Extract and Extracellular Material

Cells were extracted as follows: 0.5 g of packed cells was suspended in 1 ml of Ca^{2+}- and Mg^{2+}-free seawater and homogenized with 10 strokes of a Dounce homogenizer. The homogenate was centrifuged at 20,000 × g for 30 min at 2°C. The supernatant (0.3 mg protein/ml) was collected and dialyzed against the phospholipase A_2 assay buffer.

The cell-free extracellular material was isolated from sponge cubes essentially as described (Reuter et al., 1987). The material obtained was dialyzed against the phospholipase A_2 assay buffer and contained 1.7 mg/ml of protein; the DNA and RNA concentrations were less than 5 μg/ml. Where indicated, the AF particle was removed from the extracellular material by Sepharose 4B gel filtration as described (Müller and Zahn, 1973). The cell-free extracellular material contained 0.085 μg of lectin per μg of extracellular protein (Müller et al., 1983).

Preparation of Nuclear Substructures

The details of the preparation are given elsewhere (Schröder et al., 1988b). In brief, nuclei were isolated according to Blobel and Potter (1966), nuclear envelopes were prepared from them (Kaufmann et al., 1983), and subsequently pore-complex laminae were isolated (Schröder et al., 1988b). Nuclear matrices were prepared according to Comerford et al. (1986).

Phospholipase A_2 Assay

The assay was carried out in a final volume of 300 μl of 50 mM Tris-HCl buffer (pH 8.5), 5 mM Ca^{2+}, 10 μM 1-acyl-2-[1-^{14}C]arachidonyl glycerophosphoethanolamine, and the enzyme preparation. The incubation was performed at 30°C for 0–15 min. The reaction was stopped by addition of Dole's reagent (Dole and Meinertz, 1960), and the released free fatty acid was extracted and quantified (Horigome et al., 1987). One enzyme unit corresponds to 1 nmol of fatty acid released per min.

Phospholipase A_2 Inhibition Studies

For these studies the phospholipase A_2 in the above mentioned assay was supplemented with 5 mM Ca^{2+}, in addition to 50 U of bovine pancreas phospholipase A_2, 3–10 μM 1-acyl-2-[1-^{14}C]arachidonyl glycerophosphoethanolamine, and 0, 5, 10, or 20 μg of AF particle. After incubation at 30°C for 15 min, the released fatty acid was determined. The data were plotted according to Lineweaver and Burk (1934).

Determination of 1,2-Diacylglycerol

Diacylglycerol was determined according to Preiss et al. (1986) using a radioenzymatic assay system (Amersham).

Incorporation of Arachidonic Acid into Phospholipids

One-half gram of sponge tissue cubes (2 × 2 × 2 mm^3) was incubated in 2 ml of artificial seawater for 20 min with 10 μM [^{14}C]arachidonic acid. Then the medium was changed, and incubation proceeded for up to 120 min. At the time indicated, the tissue was transferred into Ca^{2+}- and Mg^{2+}-free seawater (2°C) containing trypsin (Müller and Zahn, 1973) to obtain single cells and the extracellular material. The single-cell suspension (0.5 ml) and the obtained extracellular material (0.5 ml) were supplemented with 2 ml of chloroform and 12 μl of concentrated HCl (Capriotti et al., 1988). The lipid extracts were resuspended in chloroform and analyzed by two-dimensional TLC, essentially as described (Capriotti et al., 1988), for the following lipid species: phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, ethanolamine plasmalogens, and choline plasmalogens.

Gel Electrophoresis and Protein Blotting Procedure

One-dimensional $NaDodSO_4$–polyacrylamide gel electrophoresis was performed as described by Laemmli (1970); the resolving gel contained 15% acrylamide. Either the gels were stained with Coomassie brilliant blue, or the proteins were transferred from the gels to nitrocellulose sheets according to the method of Towbin et al. (1979). To identify calelectrin, the blots were sequentially treated with anti-calelectrin and anti-rabbit IgG (peroxidase conjugated), essentially as described (Bachmann et al., 1986).

Immunoelectron Microscopy Studies

The detailed description was given earlier (Gramzow et al., 1988). In brief, the aggregates were fixed in 2% glutaraldehyde, dehydrated with dimethylformamide, and embedded in Lowicryl. After sectioning, the specimens were incubated with anti-calelectrin antibodies (1:500 dilution) and then with colloidal gold-labeled (10 nm) secondary anti-rabbit IgG (1:50 dilution). Finally, the material was counterstained, and observation was made with a Zeiss EM 9A electron microscope. Controls were performed with an IgG preparation from a nonimmunized rabbit, which gave no reaction with the sponge material.

Further Analytical Procedures

For protein determination, the Fluoram method was used (Weigele et al., 1973); the standard was bovine serum albumin. DNA and RNA were determined according to published procedures (I-San-Lin and Schjeide, 1969; Kissane and Robins, 1958).

Eicosanoid concentrations (thromboxane B_2, bicyclic prostaglandin E_2 and D_2) were determined according to the manufacturer's instructions (Amersham).

The activity of ornithine decarboxylase was measured as described (O'Brien and Diamonds, 1977) using DL-[1-^{14}C]ornithine as the substrate. Enzyme activities are expressed as nanomoles of CO_2 liberated in 60 min per 10^6 cells.

Incorporation studies were performed as described previously (Müller et al., 1987). In brief, after the indicated incubation period, cell suspensions were supplemented in the standard incubation assay with [^{3}H]thymidine (5 μCi/ml = 1 μM). After a further incubation period of 30 min at 20°C, the radioactivity in the acid-precipitable material was measured.

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