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 oleosolubrin (Fauvet 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 A₂ and 1,2-Diacylglycerol in Intact Sponge

<table>
<thead>
<tr>
<th></th>
<th>Extract</th>
<th>Extracellular</th>
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<tbody>
<tr>
<td>Phospholipase A₂ (U/mg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without anti-calectrin antibodies</td>
<td>2.7 ± 0.2</td>
<td>13.9 ± 0.9</td>
</tr>
<tr>
<td>With anti-calectrin antibodies</td>
<td>3.1 ± 0.2</td>
<td>47.3 ± 2.7</td>
</tr>
<tr>
<td>1,2-Diacylglycerol (nmol/mg)</td>
<td>131.9 ± 10.4</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

Phospholipase A₂ 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-calectrin 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 A₂ from Intact Cells

Dissociated G. cydonium cells released phospholipase A₂ 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 × 10⁶ 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).

Association of Calelectrin with the AF Particle

As summarized in Table 1, addition of anti-calectrin antibodies to the extracellular fraction from the sponge led to a 3.5-fold increase in phospholipase A₂. 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 M₉ 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-calectrin 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 1. Release of Phospholipase A₂ 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 (20000 × g at 2°C for 10 min), and the phospholipase A₂ (PLase A₂) 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 A₂ 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 A₂ (PLase A₂) was determined 5 min later.

The dependence of phospholipase A₂ 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 A₂ 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 A₂ by the calelectrin-related protein that is associated with the AF particle (see below) (data not shown).
light of earlier findings revealing that only below concentrations of 10 μM does phospholipase A2 follow the Michaelis-Menten kinetics (Lister et al., 1988). The results revealed the following kinetic data: Michaelis constant (Km) = 34.7 ± 3.9 μM, maximal reaction velocity (Vmax) = 353.7 pmol/min per mg of protein, and an inhibitor constant (Ki) = 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 E2 (approximately 3 pg/106 cells) and lower concentrations of prostaglandin D2 (1 pg/106 cells) and thromboxane D2 (0.2 pg/108 cells) (Table 2). These levels were almostidentical 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 B2 or prostaglandin D2 concentrations, but there was a substantial increase in the prostaglandin E2 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 E2 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-[m-(trifluoromethyl)phenyl]-2-pyrazoline; Otamiri et al., 1988) andETYA (5,8,11-eicosatetraynoic acid; Gut et al., 1988) had no influence on the concentration of prostaglandin E2 (Table 3).

incorporation of arachidonic acid into cellular and extracellular phospholipids

The incorporation of [14C]arachidonic acid into different
phospholipid species was determined both for the cellular as well as the extracellular compartment. After a pulse for 20 min with the precursor [14C]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 phosphatidylcholine and phosphatidylethanolamine (Figure 5A). The extracellular material contained arachidonate almost exclusively in the phosphatidylcholine and phosphatidyethanolamine 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/10⁶ cells (n = 5) after a 120 min period of chase.

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 A₂ into the extracellular medium in response to a lectin stimulus (Figure 2) and respond with an increased prostaglandin E₂ 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 A₂ as substrates to produce free arachidonic acid, which penetrates through the cell membrane and ultimately gives rise to the production of prostaglandin E₂. 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 A₂ substrate, 1-acetyl-2-[14C]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

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

<table>
<thead>
<tr>
<th>Lectin Concentration (µg/ml)</th>
<th>Released [14C]arachidonic Acid (dpm per 10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells Pretreated with AF for:</td>
</tr>
<tr>
<td></td>
<td>1 hr</td>
</tr>
<tr>
<td>Thromboxane B₂ 0</td>
<td>0.2 ± 0.02</td>
</tr>
<tr>
<td>Prostaglandin D₂ 0</td>
<td>0.2 ± 0.03</td>
</tr>
<tr>
<td>Prostaglandin E₂ 0</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>4.1 ± 0.6</td>
</tr>
</tbody>
</table>

Additional components:

- Anti-aggregation receptor, 10 µg/ml
- Indomethacin, 10 µM
- BW755C, 100 µM
- ETYA, 100 µM

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

<table>
<thead>
<tr>
<th>Additional Component</th>
<th>Released [14C]Arachidonic Acid (dpm per 10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells Pretreated with AF for:</td>
</tr>
<tr>
<td></td>
<td>1 hr</td>
</tr>
<tr>
<td>None</td>
<td>4310 ± 645</td>
</tr>
<tr>
<td>Anti-lectin antibodies</td>
<td>1240 ± 220</td>
</tr>
<tr>
<td>AF particle</td>
<td>1730 ± 250</td>
</tr>
<tr>
<td>AF particle plus anti-calelectrin antibodies</td>
<td>3040 ± 620</td>
</tr>
</tbody>
</table>

Cells that were preincubated for 1 or 10 hr in the standard incubation assay (3 µl) 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 µg of 1-acetyl-2-[14C]arachidonyl glycerophosphoethanolamine per 20 µg of extracellular protein. Where indicated, the extracellular material (20 µg) was preincubated for 10 min in the presence of 5 µg of anti-lectin antibodies, pretreated for 24 hr at 4°C with 2 µg of AF particle, or pretreated (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 [14C]arachidonic acid was determined as described (Errasfa et al., 1988). The results are the means (± SD) from five independent experiments.
Table 4. Distribution of Arachidonate in Nuclei and Nuclear Substructures

<table>
<thead>
<tr>
<th>Subcellular Structures (%)</th>
<th>Arachidonate in Subcellular Structures (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>100</td>
</tr>
<tr>
<td>Nuclear envelopes</td>
<td>17.8</td>
</tr>
<tr>
<td>Pore-complex laminae</td>
<td>0.71</td>
</tr>
<tr>
<td>Nuclear matrix</td>
<td>&lt;0.04</td>
</tr>
</tbody>
</table>

Sponge cubes were incubated in the presence of [14C]arachidonate 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 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 inhibitors of protein kinase C and phospholipase A2, strongly suppressed (Table 3). As mentioned below, one major extracellular component, the AF particle, is associated with calelectrin, an inhibitor of phospholipase A2. Hence, addition of an AF particle preparation to the reconstitution assay strongly inhibited the formation of arachidonic acid. The inhibition of phospholipase A2 could be abolished by antibodies directed against calelectrin (Table 3).

These data strongly suggest that free arachidonic acid is also produced in vivo in conditions in the extracellular space in sponges from phosphatidyethanolamine and/or phosphatidylcholine by phospholipase A2.

**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 [14C]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 [14C]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 phosphatidyethanolamine by phospholipase A2 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 inhibitors of protein kinase C and phospholipase A2, strongly suppressed (Table 3). As mentioned below, one major extracellular component, the AF particle, is associated with calelectrin, an inhibitor of phospholipase A2. Hence, addition of an AF particle preparation to the reconstitution assay strongly inhibited the formation of arachidonic acid. The inhibition of phospholipase A2 could be abolished by antibodies directed against calelectrin (Table 3).

These data strongly suggest that free arachidonic acid is also produced in vivo in conditions in the extracellular space in sponges from phosphatidyethanolamine and/or phosphatidylcholine by phospholipase A2.
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 E2 production was obtained at approximately 3 μM indomethacin. Reduction of the [3H]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

<table>
<thead>
<tr>
<th>Arachidonic Acid (μM)</th>
<th>ODC Activity (nmol/10^6 cells/hr)</th>
<th>[3H]thymidine incorporation (dpm x 10^-3/10^8 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μM</td>
<td>7.1 ± 0.7</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>10 μM</td>
<td>17.8 ± 1.3</td>
<td>11.8 ± 1.0</td>
</tr>
<tr>
<td>1 μM</td>
<td>12.3 ± 1.0</td>
<td>8.5 ± 0.7</td>
</tr>
<tr>
<td>0.1 μM</td>
<td>9.0 ± 0.8</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>10 μM plus:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 μM indomethacin</td>
<td>10.7 ± 0.1</td>
<td>4.1 ± 0.3</td>
</tr>
<tr>
<td>200 μM DFM0</td>
<td>5.3 ± 0.4</td>
<td>2.5 ± 0.2</td>
</tr>
</tbody>
</table>

Cells were preincubated for 10 hr in the presence of the cell binding fragment and then for 5 min in medium containing 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.

As a direct proof (not shown) that prostaglandin E2 displays a stimulatory effect on DNA synthesis in the sponge system, G. cydonium cells that had been pretreated with the cell binding fragment for 4 hr were incubated in the standard assay in the presence of exogenously added prostaglandin E2 (0.1 μg/ml) for 4 hr. Under these conditions, the incorporation rate of [3H]thymidine increased from 1.2 ± 0.1 (absence) to 7.5 ± 0.8 dpm x 10^-3/10^8 cells (presence of prostaglandin E2) (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 (6 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 A2 into the extracellular space—a process that initiates a second signaling pathway. In contrast, phospholipase A2 is apparently either spontaneously released from vertebrate cells (Pruzenski et al., 1988) or the stimuli are not yet known. The function of the extracellular sponge phospholipase A2 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 A2, caleletrin (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 caleletrin was observed during the aggregation phases—a finding that strongly indicates that caleletrin remained AF-associated. One plausible interpretation reconciling these data is that caleletrin displays its inhibitory function on phospholipase A2 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 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; solid circles) 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 [3H]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).
The imported material became associated with the nuclear envelope, 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 E2 to specific receptors in the nuclear membrane.

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 A2 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 E2.

At present we do not yet know in which cellular compartment prostaglandin E2 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 I2 synthetase (Smith et al., 1983). Hence it appears to be likely that prostaglandin E2 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 E2 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 E2 in this signal transduction chain. In view of earlier reports, it is possible that prostaglandin E2 stimulates c-fos expression (Kacich et al., 1988) followed by ornithine decarboxylase expression (Cosenza et al., 1988), which ultimately leads to DNA synthesis (Fusano 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-Omeoyl-2-[[3-3H]arachidonyl-sn-glycerol (67 mCi/mmole), 3-[[3-3H]]ornithine (50 mCi/mmole), [3H]thymidine (5.0 Ci/mmole), 1-acyl-2-[[3-3H]]arachidonyl glycerophosphoethanolamine (55 mCi/mmole), sn-1,2-diacylglycerol assay reagents system, [1-14C]arachidonic acid (50 mCi/mmole), and radioimmunoassay systems for thromboxane B2 and bicyclic prostaglandin E2 and D2 were from Amersham Int., Amersham (United Kingdom). Phospholipase A2 (from porcine pancreas; P6534), prostaglandin E2, and colloidal gold-labeled (10 nm) secondary anti-rabbit IgG from goat (G 3768) 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 × 10^9 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 (Grahn et al., 1989).

The G. cydonium lectin with an M, 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 Ca2+ and Mg2+-containing artificial seawater and Ca2+- and Mg2+-free seawater were given earlier (Rottmann et al., 1985).

Antibodies

Antibodies to calelectrin, purified from Torpedo marmorata (Fritsche et al., 1986), 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 (Saleh and Miret, 1987).

Antibodies to G. cydonium lectin were prepared as described (Conrad et al., 1985).

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 ± 5 × 10^5 cells per ml of Ca2+- and Mg2+-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 x 10^6 cells per ml in artificial seawater, 50 μg/ml of homologous glycoconjugates, 50 μg/ml of 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 x g for 30 min at 2°C. The supernatant (0.3 mg protein/ml) was collected and dialyzed against the phospholipase A2 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 A2 assay buffer and contained 1.7 mmoles 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, nucleoli were isolated according to Blobel and Potter (1966), nuclear envelopes were prepared from them (Kaulmann 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 A2 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-[1-14C]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, 1980), 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 A2 Inhibition Studies
For these studies the phospholipase A2 in the above mentioned assay was supplemented with 5 mM Ca^2+. In addition to 50 U of bovine pancreas phospholipase A2, 3-10 μM 1-acyl-2-[1-14C]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 x 2 x 2 mm^3) was incubated in 2 ml of artificial seawater for 20 min with 10 μM [14C]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, phosphatidylglycerol, phosphatidylinositol, phosphatidylycerine, ethanolamine plasmalogens, and choline plasmalogens.

Gel Electrophoresis and Protein blotting Procedure
One-dimensional NaDodSO4-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 (Schramm 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 (S-Sam-Lin and Schiele, 1969; Kissane and Robins, 1958). Eicosanoid concentrations (thromboxane B2, prostaglandin E2 and D2) were determined according to the manufacturer’s instructions (Amersham).

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References


