The role of intracellular Ca\textsuperscript{2+} during early sexual development in \textit{Dictyostelium discoideum}: effects of LaCl\textsubscript{3}, Ins(1,4,5)P\textsubscript{3}, TMB-8, chlortetracycline and A23187 on cell fusion

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Summary

The agents LaCl\textsubscript{3}, Ins(1,4,5)P\textsubscript{3}, TMB-8, chlortetracycline (CTC) and A23187 were used to study the requirement for internal calcium mobilization during gamete cell fusion in \textit{Dictyostelium discoideum}. The inhibition of the influx of calcium (LaCl\textsubscript{3}) prevented cell fusion in a dose-dependent manner. At the intracellular level, Ins(1,4,5)P\textsubscript{3}, an endogenous regulator of calcium release from intracellular stores, stimulated cell fusion within one hour following its addition. Treatment with agents that prevent the release of calcium from intracellular stores (TMB-8, CTC) also inhibited cell fusion in a dose-dependent manner. However, the non-specific augmentation of cytosolic calcium levels through the use of the ionophore A23187 inhibited cell fusion, and the amount of inhibition was directly related to the drug concentration. Studies on cell morphology and growth plus results from reversibility experiments involving the ability to form macrocysts reveal that these effects are not due to non-specific drug toxicity. In total, these results suggest that the mobilization of calcium both from the extracellular environment and from intracellular stores is important and is probably regulated during gamete cell fusion in \textit{D. discoideum}.

Key words: calcium, cell fusion, calcium antagonists, \textit{Dictyostelium}.

Introduction

Sexual development in heterothallic \textit{Dictyostelium discoideum} begins with the event of gamete fusion to form zygotes and finishes with the late events of aggregation, phagocytosis and wall formation, which produce the dormant macrocyst (O'Day, 1979; O'Day & Lewis, 1981; Newell, 1982; Szabo \textit{et al.} 1982; Raper, 1984; O'Day \textit{et al.} 1987).

During sexual development, extracellular calcium is required for both gamete cell fusion and zygote differentiation but not for gamete formation (Chagla \textit{et al.} 1980; Szabo \textit{et al.} 1982; O'Day \textit{et al.} 1987). Evidence for an intracellular role for calcium during early sexual development is indicated by the requirement of functional calmodulin. Calmodulin is a positive regulator of both gamete cell and pronuclear fusion but is a negative regulator of the calcium-independent event of gamete cell formation (Lydan & O'Day, 1988a).

Calcium regulates cellular events in eukaryotic cells by acting as a second messenger through changes in its intracellular concentration (Hidaka & Tanaka, 1985; Hollenberg, 1986; Putney, 1986). Examples of some cellular functions in which calcium plays a regulatory role are: exocytosis of secretory granules, actin polymerization, the sperm acrosome reaction, and cortical granule exocytosis during fertilization (Fraser, 1984; Stapelton \textit{et al.} 1985; Gomperts, 1986; Newell \textit{et al.} 1987).

Studies of the role of intracellular calcium have been facilitated by the use of a number of chemical agents that affect the mobilization of this divalent cation. Calcium entry from the extracellular environment \textit{via} receptor-mediated calcium channels can be blocked by La\textsuperscript{3+} (Europe-Finner & Newell, 1985; Putney, 1986). At the intracellular level, calcium-dependent events that require the release of Ca\textsuperscript{2+} from membrane-bound stores can be monitored by the use of inositol-1,4,5-...
triphosphatc (Ins(1,4,5)P$_3$), TMB-8, and chlortetracycline (CTC). Ins(1,4,5)P$_3$ is a natural regulator that is produced by a cell following a stimulus such as hormone-receptor binding (Rasmussen et al. 1986). In D. discoideum, the binding of cyclic AMP or folate to its cell surface receptor results in the internal production of Ins(1,4,5)P$_3$, which then promotes the release of calcium from non-mitochondrial stores (Europe-Finner & Newell, 1986; Newell et al. 1987). TMB-8 has been shown to block release of Ca$^{2+}$ from intracellular membrane-bound locations (Europe-Finner & Newell, 1984). CTC is a fluorescent derivative of the antibiotic tetracycline, which chelates calcium found in association with membranes and blocks calcium release from these structures (Morin, 1984; Stapleton et al. 1985). In contrast, the calcium ionophore A23187 promotes transport of Ca$^{2+}$ across bilayer membrane causing an increase in intracellular levels of calcium (Jensen & Rasmussen, 1977; Steinhardt & Epel, 1974). Thus, a series of diverse but relatively specific agents can be used to alter the intracellular mobilization of Ca$^{2+}$, and, hence, to probe the importance of this divalent cation in specific cellular processes.

In the present study La$^{3+}$, Ins(1,4,5)P$_3$, TMB-8, CTC and A23187 were used to determine whether intracellular Ca$^{2+}$ mobilization is required during gamete cell fusion in D. discoideum. The effects of the different concentrations of these agents upon the developmental appearance of specific cell types and upon cell fusion were determined. To confirm that the agents were not working in a non-specific, deleterious manner the morphology of the cell types and cellular growth were closely monitored. In addition, the effects and reversibility of the drug treatments upon macrocyst formation were also assessed.

**Materials and methods**

**Culture methods**

Stock fruiting body cultures of D. discoideum strains NC4 and V12 were maintained separately on SM agar with Escherichia coli B/r as a food source. Mixed mating type cultures were made by suspending 2.5x10$^7$ spores ml$^{-1}$ of each strain in samples of Asp-1PP (Lydan & O'Day, 1988b), heat shocking the spores for 30 min at 45°C, mixing the spores in equal numbers and suspending the heat-shocked spores to 2.5x10$^7$ spores ml$^{-1}$ in Asp-1PP supplemented with 1 mM-Ca$^{2+}$ in black taped Erlenmeyer flasks. E. coli B/r was added as a food source and the cultures were shaken at 150 revs min$^{-1}$ at 22(±1)°C. The culture methods used were the same as those described by Szabo et al. (1982) and Lydan & O'Day (1988b).

**Quantification of cell types and percentage cell fusion**

At 20 h after heat shock, samples (250μl) of vortexed cell culture were placed on gelatin-subbed slides in a humidity chamber for 8-10 min to allow the cells to adhere to the slide. The amoebae were fixed for 3-4 min by placing the slide in 75:25 (v/v) methanol/acetic acid fixative and post-fixed for 2 min in phosphate-buffered saline (PBS). The slides were stained for 1 min in 0.4 mg ml$^{-1}$ Hoechst 33258 (Sigma Chemical Co, St Louis, MO) in PBS. They were then rinsed once for 2 min and twice for 1 min in PBS after which a small sample of 9:1 (v/v) glycerol:PBS was added, followed by a coverslip.

Stained slides were observed by phase-contrast and fluorescence microscopy and 400 cells per slide were scored in a systematic manner for cell and nuclear morphology (Szabo et al. 1982; O'Day et al. 1987). Gametes have a small cytoplasmic volume and a compact nucleus that fluoresces brightly following Hoechst staining (Szabo et al. 1982; O'Day et al. 1987). Binucleate cells contain two nuclei and represent the fusion product of two gametes (Szabo et al. 1982; O'Day et al. 1987). Other cell types that have the ending -nucleate, such as trinucleate, refer to cells containing the number of nuclei described by the prefix. Zygote giant cells are typically round in shape, have a large cytoplasmic volume and a large, diffuse nucleus (Szabo et al. 1982). Each experiment was repeated at least three times with two replicate slides from each of two replicate flasks.

Cell fusion percentages were calculated from the cell type percentages using the following formula derived from those of Szabo et al. (1982) and McConachie & O'Day (1986):

$$\% \text{ Cell fusion} = \frac{\sum b + \sum t + \sum p + \sum s + \sum o + \sum d}{100 - (h + i + h + t + h + s + o + n + d) + y} \cdot$$

where $b$ = % binucleates, $i$ = % trinucleates, $t$ = % tetranucleates, $p$ = % pentanucleates, $h$ = % heptanucleates, $s$ = % septanucleates, $o$ = % octanucleates, $n$ = % nonanucleates, $d$ = % decanucleates and $y$ = 2h + 3i + 4t + 5p + 6h + 7s + 8o + 9n + 10d.

Using a dissecting microscope, the microscopy of macrocycts was determined at 5-7 days to determine if sexual development was completed in a normal manner (O'Day & Rivera, 1987). Photographs were taken on Kodak Plus-X Pan film and printed on Kodak Polychromat H Rapid RC photographic paper (Eastman Kodak, Rochester, NY).

**Effect of selected agents on cell fusion**

LaCl$_3$ and Ins(1,4,5)P$_3$ were suspended to 50 mM and 1 mM, respectively, in double distilled water and TMB-8 was suspended in 100 mM in absolute ethanol. CTC and A23187 were suspended to 125 mM and 5-25 mM, respectively, in 100% dimethyl sulphoxide (DMSO). All chemicals were purchased from Sigma Chemical Co. (St Louis, MO) and were of the highest purity possible. LaCl$_3$, TMB-8, CTC and A23187 were added at various concentrations at 10 h of development and samples of the culture were removed 10 h later, at 20 h, for staining as detailed above. Ins(1,4,5)P$_3$ was added at various concentrations at 10 h and samples of the culture were removed at three successive hourly intervals for staining. The total time of treatment used for Ins(1,4,5)P$_3$ was 3 h since Ins(1,4,5)P$_3$, being an endogenous regulator of calcium levels, is readily metabolized by the cells. Saponin, an agent used to enhance the uptake of Ins(1,4,5)P$_3$, was added to all Ins(1,4,5)P$_3$-treated cultures at 0-01 mg ml$^{-1}$.
which was the highest concentration of saponin that was found to have no effect on developmental kinetics (data not shown). The solvent used for each agent was added at the same concentration to all flasks regardless of agent concentration. The developmental ages of 10 and 20 h correspond to peak gamete cell levels prior to fusion and peak cell fusion levels, respectively, in untreated cultures (Lydan & O'Day, 1988).

Agent reversibility studies
At 20 h, samples of treated cultures at the maximum agent concentration used were spun down, washed three times in agent-free Asp-LPP, and resuspended to 3 × 10⁶ to 6 × 10⁶ cells ml⁻¹ in 10 ml of Asp-LPP supplemented with 1 mM-Ca²⁺ in 50 ml black taped Erlenmeyer flasks. Similar samples were treated as above, but in addition to the Ca²⁺, the Asp-LPP was supplemented with the original concentration of the agent to ensure that the reversal seen was not due to the reversal treatment. Macrocysts were scored at 5–7 days to determine whether the effect of the agent was reversed by removal.

Results

Effects of La³⁺
Lanthanum ions inhibit Ca²⁺ uptake from the extracellular medium by blocking receptor-mediated Ca²⁺ channels in the cell membrane. LaCl₃ affected the appearance of specific cell types and inhibited cell fusion in a dose-dependent manner in sexual cultures of D. discoideum (Fig. 1). Inhibition of binucleate formation was linear over the concentration range used with about 2% binucleates appearing in cultures treated with 100 μM-LaCl₃ (Fig. 1A). This represents a greater than 90% decrease from control levels. The effect of LaCl₃ on zygote giant cell numbers appeared to be biphasic over the concentration range used, with moderate levels (50 μM) resulting in a 3.5-fold increase and high levels (100 μM) resulted in a decrease of about 50% as compared to controls (Fig. 1A). Gametes increased in a linear fashion from less than 2% of the cell population to about 12% in cultures treated with 100 μM-LaCl₃ (Fig. 1A). This represents a sixfold increase. Total cell fusion decreased linearly, being inhibited by over 90% at the maximum concentration (Fig. 1B). Cell morphology was not altered by the treatment with LaCl₃ (Fig. 2). Although the cells appeared normal, the numbers of readily detectable fusion products such as binucleates decreased over the concentration range used (Fig. 2).

Fig. 1. The effect of LaCl₃ on cell type percentages (A) and total cell fusion (B). LaCl₃ was added to 10-h cultures and binucleate (□), gamete (○) and zygote giant cell (◇) percentages were determined at 20 h. The cell type percentages were used to calculate the total cell fusion (△). Cell type percentages and total cell fusion were determined as described in Materials and methods. The error bars represent the standard error.

Fig. 2. Cell morphology in 20-h cultures treated with various concentrations of LaCl₃ after 10 h. Mixed mating type cultures had LaCl₃ added to 0 (A), 10 (B), 50 (C), or 100 μM (D) at 10 h. Cells were fixed, stained and photographed at 20 h as described in Materials and methods. Bar, 40 μm. b, binucleate; g, gamete.
Fig. 3. The effect of Ins(1,4,5)P$_3$ on sexually developing cultures of D. discoideum. Ins(1,4,5)P$_3$ was added to 10-h cultures at 0.01 μM (X), 0.1 μM (●), or 1 μM (▲) and binucleate (A) and total cell fusion (B) percentages were determined at 20 h for both cultures treated with Ins(1,4,5)P$_3$ and control (■) cultures. Cells were fixed and stained with Hoechst 33258 and cell type percentages and total cell fusion were determined as described in Materials and methods. The error bars represent the standard error.

Effects of Ins(1,4,5)P$_3$

Inositol triphosphate is an endogenous regulator, or second messenger, which stimulates Ca$^{2+}$ release from non-mitochondrial cytoplasmic stores. When added to 10-h mated cultures Ins(1,4,5)P$_3$ was found to have an immediate effect on cell fusion (Fig. 3). A concentration of 0.01 μM-Ins(1,4,5)P$_3$ was found to stimulate binucleate formation by about 30% over control cultures an hour after its addition (Fig. 3A). Total cell fusion after 1 h of Ins(1,4,5)P$_3$ treatment was also about 30% higher in cultures treated with 0.01 μM-Ins(1,4,5)P$_3$ as compared to control cultures (Fig. 3B). After 2 h, both 0.01 μM and 0.1 μM-Ins(1,4,5)P$_3$ enhanced binucleate levels by about 20% over control cultures (Fig. 3A). In cultures treated with either 0.01 μM or 0.1 μM-Ins(1,4,5)P$_3$ total cell fusion was about 15% higher than in control cultures (Fig. 3B). After 3 h, Ins(1,4,5)P$_3$ had no stimulatory effect on binucleate levels of total cell fusion, suggesting that Ins(1,4,5)P$_3$ induces precocious fusion of the gametes present during the time period studied rather than increasing the number of fusion-competent cells (Fig. 3). Ins(1,4,5)P$_3$ at 1 μM showed little effect on binucleate formation (Fig. 3A).

Fig. 4. The effect of TMB-8 on cell type (A) and total cell fusion percentages (B). Cells were treated with either 0, 25, 50 or 75 μM-TMB-8 at 10 h and cells were fixed, and stained with Hoechst 33258 at 20 h to determine the binucleate (□), gamete (○), zygote giant cell (▼) and total cell fusion (△) percentages. Cell type and total cell fusion percentages were determined as described in Materials and methods. The error bars represent the standard error.

Effects of TMB-8

TMB-8 blocks the release of Ca$^{2+}$ from intracellular stores and thus operates in a manner opposite to that of Ins(1,4,5)P$_3$. TMB-8 inhibited cell fusion and the presence of binucleates and zygotes in a dose-dependent manner (Fig. 4). Binucleates decreased from about 13% of the cell population in control cultures to about 3% in cultures treated with 75 μM-TMB-8, representing a decrease of about fourfold (Fig. 4A). Zygote giant cells decreased from about 1% in control cultures to 0% in cultures treated with 75 μM-TMB-8, while gametes increased threefold (Fig. 4A). Total cell fusion in control cultures was about 30%, which decreased to about 6.5% in cultures treated with 75 μM-TMB-8 (Fig. 4B). Thus, 75 μM-TMB-8 inhibited cell fusion by about 80%. A comparison of control levels of cell types and percentage fusions in Fig. 1 and 4 reveals that 0.1% ethanol, which was
required to solubilize the TMB-8, had significant inhibitory effects. The basis of this inhibition is unknown but, in spite of it, TMB-8 still significantly inhibits cell fusion in D. discoideum. Cell morphology was not affected by treatment with TMB-8 or by ethanol, and cells treated with TMB-8 appeared to adhere to the slides and extend pseudopods normally (Fig. 5).

Effects of CTC
Chlortetracycline chelates membrane-bound Ca\(^{2+}\), preventing its release into the soluble cytoplasm from such potential storage sites. In keeping with this, CTC inhibited cell fusion and the appearance of specific cell types in a dose-dependent manner (Fig. 6). Binucleates decreased drastically from a level of about 27% of the population in control cultures to about 2.5% in cultures treated with 100 \(\mu\)M-CTC, representing a decrease of about 11-fold (Fig. 6A). Zygote giant cell numbers showed a biphasic response, finally decreasing to 0% in cultures treated with 100 \(\mu\)M-CTC (Fig. 6A). Gametes increased rapidly from 1.5%, then slowly to peak at about 19% of the cell population in cultures containing 100 \(\mu\)M-CTC (Fig. 6A). The kinetics of inhibition of total cell fusion were similar to those seen for binucleates (Fig. 6B). Total cell fusion values for 100 \(\mu\)M-CTC-treated cultures represented an inhibition of about 90% as compared to control cultures (Fig. 6B).

Effects of A23187
The calcium ionophore A23187 non-specifically increases cytoplasmic Ca\(^{2+}\) levels by promoting the movement of this divalent cation across cell membranes. A23187 inhibited cell fusion and the appearance of specific cell types in a dose-dependent manner (Fig. 7). Binucleate formation was inhibited by about 90% by treatment with 1 \(\mu\)M-A23187 (Fig. 7A). Similarly, zygote giant cell numbers were abruptly decreased to 0% in cultures treated with concentrations of 0.5 \(\mu\)M or higher (Fig. 7A). Coincident with the decrease in binucleates and zygote giant cells, gamete cell numbers increased in a dose-dependent manner from about 1.5% of the cell population in control cultures to about 14.5% in cultures treated with 1 \(\mu\)M-A23187 (Fig. 7A). The total cell fusion decreased from 53% in control cultures to about 7% in cultures treated...
Fig. 7. The effect of A23187 on cell type (A) and total cell fusion percentages (B) in sexually developing cultures of \textit{D. discoideum}. Binucleate (□), gamete (○) and zygote giant cell (◇) percentages were determined at 20 h and were used to calculate the total cell fusion (Δ). Cells were fixed and stained with the fluorescent stain Hoechst 33258 as described in Materials and methods. The error bars represent the standard error.

with 1 \textmu M-A23187 representing an inhibition of about 90\% (Fig. 7B).

A23187 can increase intracellular calcium through either the cell membrane or intracellular stores (Jensen & Rasmussen, 1977). In order to separate the two functions, we varied extracellular calcium levels. Cultures grown in the absence of calcium were treated with A23187 at 10 h to determine whether the effect of A23187 was due primarily to an influx of extracellular Ca$^{2+}$ or to a direct effect on intracellular stores of Ca$^{2+}$. In Ca$^{2+}$-free medium, A23187 at 1 \textmu M still inhibited the fusion of gamete cells to binucleates by about fivefold (Fig. 8). Binucleates made up about 2.5\% of the cell population in Ca$^{2+}$-free medium and about 0.5\% in cultures supplemented with 1 \textmu M-A23187 (Fig. 8). Cell fusion also decreased from about 5\% to about 1.5\%, a decrease of about threefold, as a result of the addition of A23187 to Ca$^{2+}$-free cultures (Fig. 8). Cell fusion was possible in the absence of Mg$^{2+}$ in the medium.

Fig. 8. The effect of 1-0 \textmu M-A23187 on sexually developing cultures of \textit{D. discoideum} in the presence and absence of 1-0 \textmu M-Ca$^{2+}$. Binucleate, gamete and total cell fusion percentages were determined in 20-h cultures following the addition of either A23187 or DMSO at 10 h. Four treatments were performed: control or Ca$^{2+}$ - A23187 (■), Ca$^{2+}$ + A23187 (□), Ca$^{2+}$-free - A23187 (▲) or Ca$^{2+}$-free + A23187 (◇). Cells were fixed and stained and cell type percentages were determined as described in Materials and methods. The error bars represent the standard error.

Table 1. The effect of LaCl$_3$, TMB-8, CTC, A23187 and Ins(1,4,5)P$_3$ on cell growth and macrocyst formation

<table>
<thead>
<tr>
<th>Agent</th>
<th>Cell number (cells ml$^{-1}$)</th>
<th>Macrocyts (relative no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LaCl$_3$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.1 \times 10^4</td>
<td>++</td>
</tr>
<tr>
<td>10</td>
<td>2.3 \times 10^4</td>
<td>++</td>
</tr>
<tr>
<td>50</td>
<td>2.1 \times 10^5</td>
<td>+</td>
</tr>
<tr>
<td>100</td>
<td>2.8 \times 10^5</td>
<td>-</td>
</tr>
<tr>
<td>TMB-8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.3 \times 10^5</td>
<td>++</td>
</tr>
<tr>
<td>25</td>
<td>2.1 \times 10^6</td>
<td>++</td>
</tr>
<tr>
<td>50</td>
<td>2.5 \times 10^6</td>
<td>++</td>
</tr>
<tr>
<td>75</td>
<td>2.8 \times 10^6</td>
<td>-</td>
</tr>
<tr>
<td>CTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.1 \times 10^4</td>
<td>++</td>
</tr>
<tr>
<td>10</td>
<td>2.4 \times 10^6</td>
<td>++</td>
</tr>
<tr>
<td>100</td>
<td>3.7 \times 10^5</td>
<td>-</td>
</tr>
<tr>
<td>A23187</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.5 \times 10^4</td>
<td>++</td>
</tr>
<tr>
<td>0.1</td>
<td>2.7 \times 10^4</td>
<td>+</td>
</tr>
<tr>
<td>0.5</td>
<td>2.6 \times 10^5</td>
<td>+/</td>
</tr>
<tr>
<td>1.0</td>
<td>3.0 \times 10^5</td>
<td>+/</td>
</tr>
<tr>
<td>Ins(1,4,5)P$_3$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.0 \times 10^5</td>
<td>++</td>
</tr>
<tr>
<td>0.01</td>
<td>1.2 \times 10^5</td>
<td>++</td>
</tr>
<tr>
<td>0.1</td>
<td>1.0 \times 10^5</td>
<td>+/</td>
</tr>
<tr>
<td>1.0</td>
<td>1.1 \times 10^5</td>
<td>+/</td>
</tr>
</tbody>
</table>

LaCl$_3$, TMB-8, CTC and A23187 were added to mixed mating type cultures at 10 h of development, samples of the cultures were removed at 20 h and the number of cells ml$^{-1}$ was determined using a haemocytometer. Ins(1,4,5)P$_3$ was added at 10 h and samples of Ins(1,4,5)P$_3$-treated cultures were removed after 1 h to determine the number of cells ml$^{-1}$. Macrocysts were scored at 5-7 days for relative numbers as described in Materials and methods.
Table 2. Reversibility of the effect of LaCl₃, TMB-8, CTC and A23187 on macrocyst formation

<table>
<thead>
<tr>
<th>Agent</th>
<th>Treated macrocyst numbers</th>
<th>Reversed macrocyst numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>100 μM-LaCl₃</td>
<td>+</td>
<td>+ + + + + + +</td>
</tr>
<tr>
<td>75 μM-TMB-8</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>100 μM-CTC</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>1 μM-A23187</td>
<td>+ / –</td>
<td>+ / + + +</td>
</tr>
</tbody>
</table>

The agents were added to mixed mating type cultures at 10 h of development and macrocysts were scored at 3–7 days for relative numbers as described in Materials and methods.

Effect of agents on cell and macrocyst numbers

LaCl₃, Ins(1,4,5)P₃, TMB-8, CTC and A23187 were clearly not toxic, as evidenced by the cell numbers produced in treated versus control cultures (Table 1). LaCl₃, TMB-8, CTC and A23187 all exhibited a general dose-dependent increase in cell number, presumably due to the decreased level of cell fusion in the presence of these drugs (Table 1). Ins(1,4,5)P₃ appeared to cause an increase in cell number at low (0–0.1 μM) concentrations, followed by a decrease back to the control level (Table 1).

Macrocyst formation was also inhibited in a dose-dependent manner by LaCl₃, TMB-8, CTC and A23187 (Table 1). The maximum concentration of agents used in all treatments resulted in the complete or nearly complete inhibition of macrocyst formation (Table 1). Ins(1,4,5)P₃ did not significantly alter macrocyst levels.

Reversibility studies

As seen by the production of macrocysts in control and drug-treated cultures that had been washed and resuspended in agent-free medium, the effects of LaCl₃, TMB-8, CTC and A23187 were readily reversible (Table 2). Reversal ranged from the ability to produce macrocysts at control levels, as was the case for TMB-8 or CTC, to a marked enhancement of macrocyst formation, as was seen in reversed cultures with LaCl₃- or A23187-treated cells (Table 2). Since Ins(1,4,5)P₃ was not inhibitory, reversal experiments were not carried out.

Discussion

Sexual development in D. discoideum begins with the appearance of tiny, amoeboid gametes, which fuse to form mainly binucleate cells (Szabo et al. 1982; O’Day et al. 1987). Pronuclear fusion converts the binucleates into zygotes, which serve as the foci for continued development to the macrocyst stage (O’Day, 1979; Szabo et al. 1982; McConachie & O’Day, 1987). Both gamete fusion and zygote differentiation are dependent on the presence of extracellular calcium in D. discoideum (Chagla et al. 1980; Szabo et al. 1982; O’Day et al. 1987). At least some of the functions of calcium during these sexual processes are clearly mediated at the cell surface. The present work extends our understanding of the role of Ca²⁺ in sexual development of D. discoideum.

The use of LaCl₃ shows that calcium uptake from the extracellular medium is also required for cell fusion to occur in sexual cultures of D. discoideum. La³⁺ effectively inhibits cell fusion at a concentration that has been shown to diminish markedly Ca²⁺ uptake into assexual amoebae of D. discoideum (Europe-Finner & Newell, 1985). The effect of La³⁺ seen here could be due to a decreased rate of influx of Ca²⁺ affecting the fusion event directly or to an inability to re-establish intracellular stores following release of Ca²⁺ from those stores (Putney, 1986; Newell et al. 1987).

Intracellular Ca²⁺ has been shown to play a role during gamete cell fusion in D. discoideum through the calcium-binding regulatory protein calmodulin (Lydan & O’Day, 1988α). In assexual amoebae, the production and action of Ins(1,4,5)P₃ triggers the release of Ca²⁺ from intracellular stores (Newell et al. 1987). Here we have shown that in sexually developing cultures specific concentrations of Ins(1,4,5)P₃ enhance cell fusion. This argues that Ins(1,4,5)P₃-induced calcium release may regulate cell fusion in D. discoideum. A certain exogenous level of Ins(1,4,5)P₃ may supplement cytoplasmic concentrations, and thus provide a sufficient intracellular Ins(1,4,5)P₃ level to stimulate the events leading to cell fusion. Below that specific level there would be insufficient Ins(1,4,5)P₃, while above the stimulatory level exogenous Ins(1,4,5)P₃ might cause, for example, the activation of the Ca²⁺ pump of the plasma membrane to reestablish normal Ca²⁺ levels (Schatzmann, 1986).

The use of other chemical agents emphasizes the importance of intracellular Ca²⁺ stores in D. discoideum. In support of the results found with Ins(1,4,5)P₃, studies with TMB-8 and CTC show that mobilization of calcium from intracellular stores is essential for cell fusion during sexual development. In previous research, both TMB-8 and CTC have been shown to stop the release of Ca²⁺ from intracellular stores (Europe-Finner & Newell, 1984; Stapleton et al. 1985). In support of this, the direct observation of greater numbers and sizes of fluorescent granules in cells from cultures treated with CTC as compared to cells from control cultures post-stained with CTC supports this sequestering mode of action (Lydan & O’Day, unpublished). In D. discoideum, it has been suggested that the immobilization of Ca²⁺ inhibits cyclic AMP-dependent cell aggregation by decreasing both the polymeriz-
ation of actin and the intracellular production of cyclic GMP in response to an extracellular cyclic AMP stimulus (Europe-Finner & Newell, 1980, 1984; Mato et al. 1979; McRobbie & Newell, 1983). The cytoskeleton plays critical roles in myoblast fusion and thus may have importance during cell fusion in D. discoideum (Wakelam, 1985). The primary effect of TMB-8 appears to be due to an inhibition of calcium mobilization, rather than a decrease in respiration, since cultures treated with TMB-8 have similar kinetics to those treated with CTC, a second agent that acts to inhibit calcium release, and the cells treated with TMB-8 did not appear to be inhibited metabolically (Europe-Finner et al. 1985).

The importance of regulating the levels of intracellular Ca$^{2+}$ during sexual cell fusion in D. discoideum is further emphasized by the results with the calcium ionophore A23187. Previous studies, analysing the effect of A23187 on cell fusion in myoblasts, have found that fusion occurs precociously when A23187 is added to cultures before the acquisition of fusion competence (Wakelam, 1985). After the onset of myoblast fusion, A23187 addition has no effect (Wakelam, 1985). In this study, A23187 was added to cultures at the onset of cell fusion and caused a marked decrease in cell fusion. Thus, unlike myoblast fusion, the fusion of D. discoideum gametes is sensitive to A23187. The effect of the ionophore was primarily on intracellular stores, as evidenced by the continued inhibition of cell fusion even when extracellular calcium was limiting. The results seen were not due to A23187-related toxicity, as evidenced by cell morphology in treated cultures and by reversibility experiments. While the work with Ins(1,4,5)P$_3$, TMB-8 and CTC reveals that the release of calcium from intracellular stores is essential for gamete cell fusion, the results with A23187 would argue that the quantity released and the mode or timing of release are also critical (Rasmussen et al. 1986; Newell et al. 1987). The use of chemical means, either to increase or decrease the level of intracellular calcium have the same net effect, an inhibition of cell fusion. These results suggest that a simple release of calcium from intracellular stores is not a sufficient stimulus for cell fusion, rather it appears that calcium release must be co-ordinated with other cellular systems, probably through the phosphoinositide cycle.

In summary, the results presented here suggest that regulation of the level of intracellular calcium is important for cell fusion during sexual development in D. discoideum. During this event, the cytosolic levels of Ca$^{2+}$ are apparently regulated by both influx from the extracellular environment and release from intracellular stores. Investigations into the role of the mobilization of calcium have shown that calmodulin mediates at least some of its critical functions during biomembrane fusion (Lydan & O’Day, 1988a). Our future work will focus upon the means by which surface interactions, such as gamete cell contact, generate the intracellular signals that affect Ca$^{2+}$ mobilization and the calcium-dependent responses that follow during sexual development in D. discoideum.

We thank Steve Jaunzems for his expert photographic assistance. This study was funded by an operating grant from the Natural Sciences and Engineering Research Council of Canada.

References


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