Differentiation-inducing factor of *D. discoideum* raises intracellular calcium concentration and suppresses cell growth in rat pancreatic AR42J cells

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Abstract DIF (differentiation-inducing factor) is a putative morphogen that induces stalk cell differentiation in the lower eukaryote, *Dictyostelium discoideum*. In this study, we have examined the effects of DIF on growth and the intracellular calcium concentration ($[Ca^{2+}]_i$) in rat pancreatic acinar AR42J cells. Growth of AR42J cells was inhibited when DIF was present in the media, and approximately 50% growth inhibition was attained with 20 μM DIF. DIF was also found to raise $[Ca^{2+}]_i$ in a dose-dependent manner (1–40 μM), both in the presence and absence of extracellular Ca^{2+}. These results suggest that DIF elicits both calcium influx from the extracellular space and calcium release from intracellular pool(s), thereby inhibiting cell growth in AR42J.

Key words: Dictyostelium discoideum; DIF; Calcium; Cell growth

1. Introduction

Cell differentiation is one of the most conspicuous phenomena seen in the process of development in multicellular organisms. Normally, cells are programmed to differentiate into specific cell types at a proper ratio(s), communicating (interacting) with each other. The cell-to-cell interactions are often mediated by signal molecules and subsequent signal transduction systems. In contrast, cancer cells can be regarded as de-differentiated cells that have lost programmed properties or as cells that are unable to properly communicate with each other because of deficiencies in the signaling system. Therefore, the elucidation of the mechanism of cell differentiation involving signal transduction would provide important insights into cancer.

In the field of developmental biology, the cellular slime mold *Dictyostelium discoideum* has been widely used for the analysis of cell differentiation because of its simple pattern of development and cell differentiation. Upon starvation, vegetatively growing amoebae of this organism start morphogenesis so as to form a multicellular body consisting of only two cell types, spores and stalk cells. The signalling molecules, cyclic AMP and DIF (differentiation-inducing factor), have been shown to be involved in stalk cell differentiation [1–3]. DIF-1 (the most active form of DIF), 1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)-1-hexanone (Fig. 1A) [4], is now thought to be a morphogen in *D. discoideum* [5]. Yet, the precise mechanism of its signal transduction system has not been clarified.

Oka et al. [6] have isolated a factor, named differanisole A (DA), from conditioned medium of the soil microorganism *Chaetomium* (RB-001), which induces re-differentiation of mouse erythroleukemia cells into hemoglobin-producing cells. This molecule has the chemical structure 3,5-dichloro-2-hydroxy-4-methoxy-6-*n*-propyl-benzoic acid (Fig. 1B) [6], similar to that of DIF-1. Recently, it was shown that DA can induce stalk cell differentiation in *D. discoideum* [7], and also that DIF-1 can induce re-differentiation of the mouse erythroleukemia cells (Asahi, K., Sakurai, A., Takahashi, N., Kubohara, Y., Okamoto, K., Tanaka, Y., submitted for publication). These results raise the possibilities that (i) there exists a common mechanism in the induction processes of stalk cell differentiation in *D. discoideum* and of re-differentiation of tumor cells, (ii) DIF-1, as well as DA, may have therapeutic potential in cancer, and (iii) signal molecules which have the chemical structure common to DIF-1 and DA may play a crucial role(s) in cell differentiation in other species. However, the mechanism of DIF action in mammalian cells remained unsettled.

In this communication, we report the effects of DIF-1 on both growth and intracellular free calcium concentration ($[Ca^{2+}]_i$) of rat pancreatic acinar AR42J cells [8], and show that DIF-1 suppresses cell proliferation and raises $[Ca^{2+}]_i$ in this cell line.

2. Materials and methods

2.1. Chemicals

DIF-1 was purchased from Molecular Probes (USA), and stored at −20°C as a 10 mM solution in EtOH. EtOH (up to 0.4%, v/v) upon addition of DIF did not interfere the experiments. Unless otherwise mentioned, DIF means DIF-1 in this study. $LaCl_3$ was from Wako Pure Chemical (Japan).

2.2. Cell culture

AR42J cells were maintained at 37°C (5% CO_2) in growth medium (GM) comprising Dulbecco's modified Eagle's medium containing 4500 mg/l glucose, 1.5 g/l $NaHCO_3$, 25 mg/l penicillin, 50 mg/l streptomycin, and 10% (v/v) fetal bovine serum.

To examine the effects of DIF on cell growth, AR42J cells were collected from culture flasks by trypsin treatment, and then incubated in four wells (22-mm diameter) each containing 1 ml of GM. After several hours of incubation, at which point cells had adhered tightly to the bottom of the wells, original cell density ($3 \times 10^3 - 3 \times 10^4$ cells/cm^2) was counted using a phase-contrast microscope, and various amounts of DIF were added to the wells (final DIF concentrations were 0, 10, 20, and 40 μM). Cell morphology and cell growth were microscopically observed every 24 h, and the data given as the ratio of the cell number (cell density).

2.3. Fura-2 fluorometry: mass assay

AR42J cells were collected after trypsin treatment and suspended in GM (final 10^6 cells/ml) containing fura-2/AM at 1 μg/ml [9]. After incubation at 37°C (5% CO_2) for 30–60 min, the cells were washed with a calcium assay buffer (137.5 mM NaCl, 5 mM KCl, 0.8 mM $MgCl_2$, 5.5 mM glucose, 20 mM HEPES, 0.6 mM $NaHCO_3$, pH 7.2–7.4

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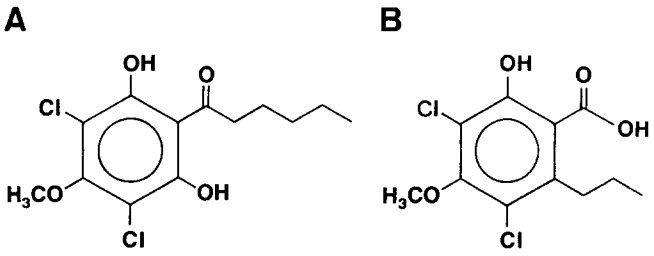

Fig. 1. Chemical structure of DIF-1 (A) and differanisole A (B).

adjusted by NaOH) and finally resuspended (10^6 cells/ml) in the same buffer supplemented with 2.5 mM $CaCl_2$ or 1 mM EGTA.

Fura-2 fluorometry was carried out at 28°C in a cuvette (2 ml of the cell suspension/cuvette) using a fluorescence spectrophotometer (F4010; Hitachi, Japan). Fluorescence intensities (emission at 510 nm) of fura-2 were monitored when excited at 340 nm and 380 nm alternately at 4-s intervals. The calcium concentration was calculated according to the formula [9]:

$$[Ca^{2+}]_i = K_d \times (F_{min}/F_{max}) \times (R - R_{min})/(R_{max} - R)$$

where R is the fluorescence ratio (510 nm emission by 340 nm excitation/510 nm emission by 380 nm excitation); R_{max} and R_{min} are the fluorescence ratios obtained by adding Triton X-100 (final 1%; R_{max}) followed by the addition of EGTA (final 15 mM; R_{min}); F_{max} and F_{min} are the fluorescence (510 mm) intensities excited by 380 nm when Triton X-100 was added (F_{max}) and then EGTA was added (F_{min}); and K_d is the dissociation constant (225 nM was used in this study).

2.4. Imaging analysis of $[Ca^{2+}]_i$: single cell assay

AR42J cells were collected after trypsin treatment, and suspended in GM. The cells were loaded onto a poly-L-lysine-coated cover glass, and incubated for several hours so that the cells adhered tightly to the cover glass. After treatment with fura-2/AM in a dish, the cover glass was set up in an assay chamber filled with the calcium assay buffer, and the assay buffer containing DIF was perfused. Imaging analyses based on fura-2 fluorometry were carried out using an image processing system (ARGUS-100/CA; Hamamatsu Photonics, Japan).

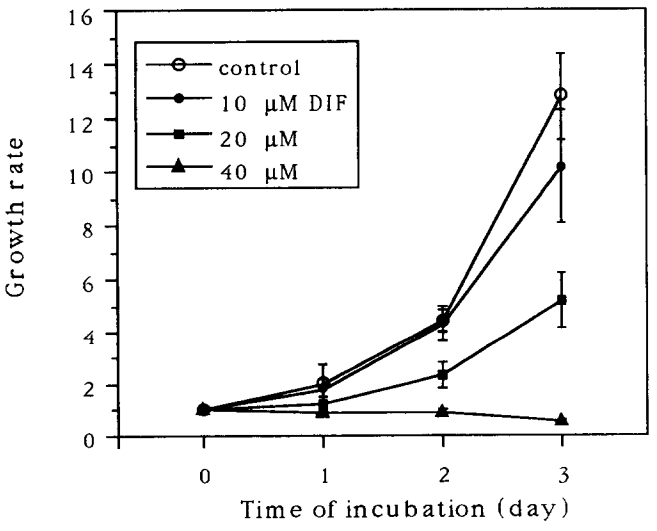

Fig. 2. Effects of DIF on the growth of AR42J cells. AR42J cells were incubated without (control) or with 10, 20, or 40 μM of DIF, and the cell number (density) was counted microscopically every day. The data are given as the mean values of the growth rate ± S.D. (n = 3).

3. Results

3.1. Effects of DIF on the growth of AR42J cells

We first examined if DIF affects the growth of rat pancreatic acinar AR42J cells. Addition of DIF to the growth media suppressed cell proliferation in a dose-dependent manner (Figs. 2 and 3). Although DIF at up to 1 μM did not significantly affect cell growth, cell proliferation was slightly inhibited by 2–10 μM DIF (Fig. 2). Approximately 50% growth inhibition was attained with 20 μM DIF (Fig. 2) whereas this concentration of DIF did not affect cell morphology (Fig. 3B). However, 40 μM of DIF was toxic to the cells, so that most of them died within 3 days (Figs. 2 and 3C).

3.2. Effects of DIF on intracellular calcium concentration in AR42J

Since many signal molecules increase intracellular free calcium concentration ($[Ca^{2+}]_i$) in their target cells, we examined if DIF affects $[Ca^{2+}]_i$ in AR42J cells.

In the presence of extracellular Ca^{2+}, DIF raised $[Ca^{2+}]_i$

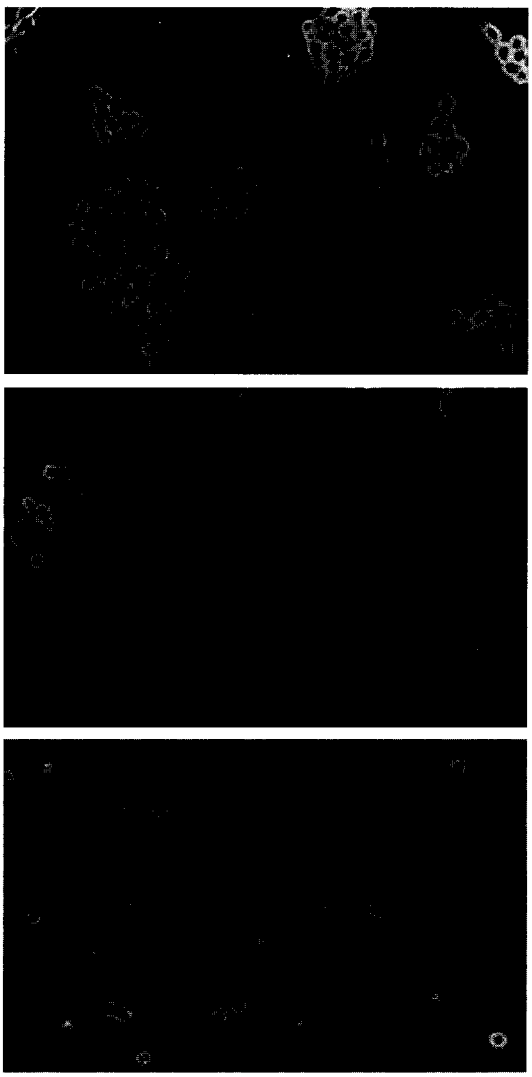

Fig. 3. Micrographs of DIF-treated cells. AR42J cells were incubated without (A) or with 20 μM (B) or 40 μM (C) DIF for 3 days, and observed by phase-contrast microscopy. Bar = 100 μm.

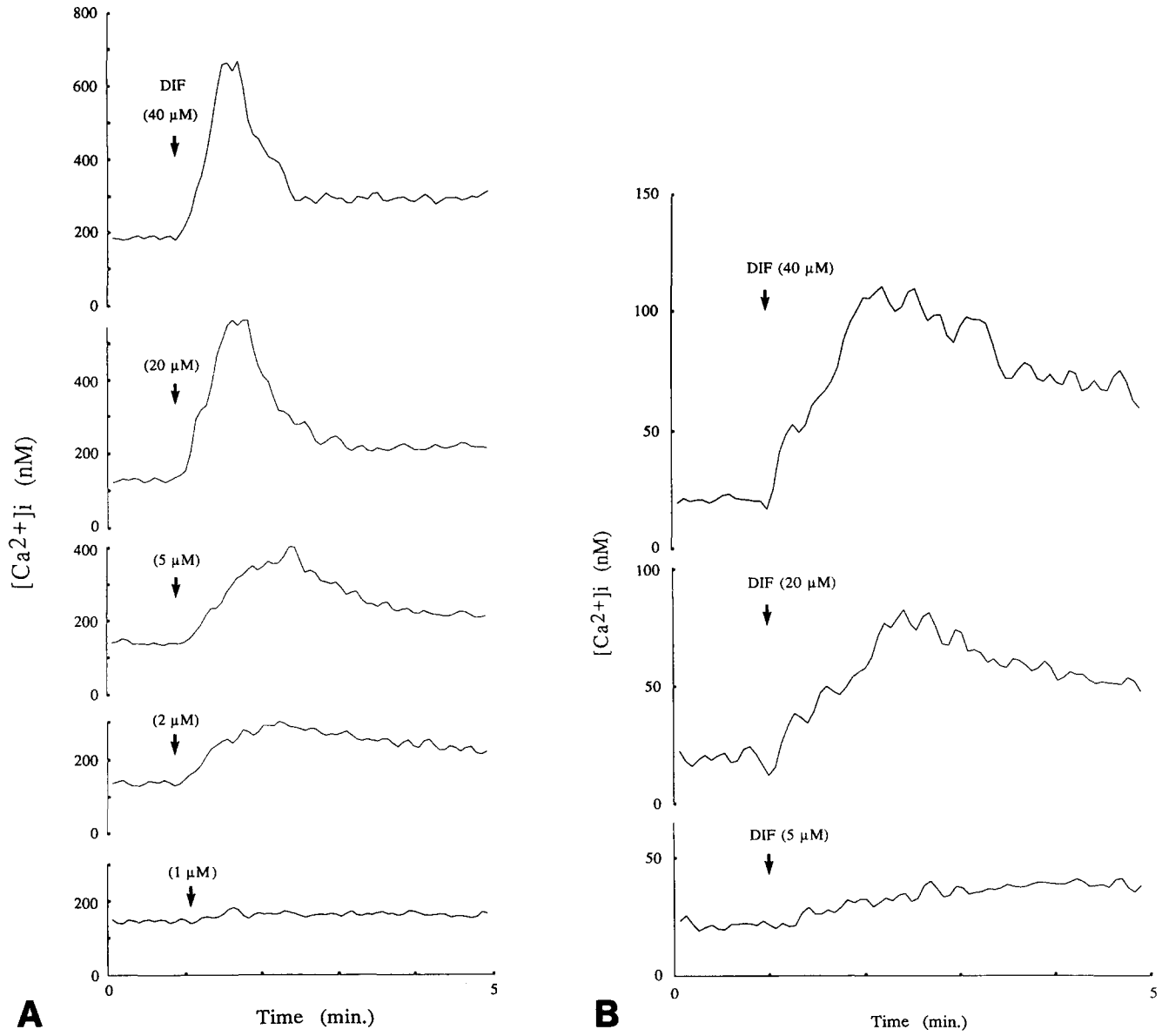

Fig. 4. Effects of DIF on $[Ca^{2+}]_i$. Fura-2-loaded cells were stimulated with various amounts of DIF in the presence of 2.5 mM Ca^{2+} (A) or 1 mM EGTA (B). Fluorescence changes were monitored for 5 min and $[Ca^{2+}]_i$ was calculated as described in section 2. Representative results are shown.

greatly, in a dose-dependent manner, over a concentration range of 1–40 μM (Fig. 4A). DIF at 20–40 μM raised $[Ca^{2+}]_i$ from the basal level (approx. 100 nM) up to 500–700 nM, and after the surge, $[Ca^{2+}]_i$ was maintained at a level higher than basal (Fig. 4A) for more than 15 min (not shown). In the absence of extracellular Ca^{2+}, DIF raised $[Ca^{2+}]_i$ to a lesser extent, again in a dose-dependent manner (Fig. 4B). Again, $[Ca^{2+}]_i$ was maintained at a higher level (Fig. 4B) for more than 15 min (not shown) after the DIF stimulation. It should be noted that the cells responded to DIF within 10 s and $[Ca^{2+}]_i$ reached a maximal level within 60–90 s (Fig. 4), indicating that the response is not mediated by gene transcription.

These results demonstrate that DIF elicits both calcium influx from the extracellular space and calcium release from some intracellular pool(s), and that the translocated calcium ions should be attributed mainly to the extracellular space.

To elucidate the mechanism of DIF action in AR42J cells, we examined the effects of La^{3+} (calcium channel blocker) [10], H7 (inhibitor of protein kinase C), and W7 (inhibitor of calmodulin-dependent protein kinase) [11] on DIF-induced calcium increase (DICI). La^{3+} (1 mM) disturbed but did not block the DICI (Fig. 5). H7 or W7 at 10 μM showed no marked effect on DICI (not shown).

3.3. *Image analysis of DICI: single cell $[Ca^{2+}]_i$ assay*

In order to confirm the effects of DIF on $[Ca^{2+}]_i$ in AR42J cells, we monitored the changes in $[Ca^{2+}]_i$ in single cells when stimulated with 20 μM DIF, using an imaging technique (Fig. 6). In the presence of extracellular Ca^{2+}, DIF raised $[Ca^{2+}]_i$ greatly in almost all the cells tested (Fig. 6A). In the absence of extracellular Ca^{2+}, a slight increase in $[Ca^{2+}]_i$ was observed when stimulated with 20 μM DIF (Fig. 6B).

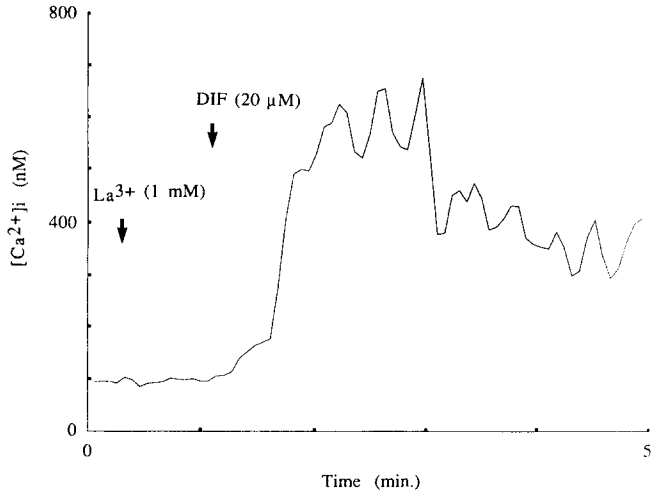

Fig. 5. Effects of La^{3+} on DIF-induced calcium increase. After the addition of 1 mM La^{3+}, fura-2-loaded cells were stimulated with 20 μM DIF in the presence of 2.5 mM Ca^{2+}. A representative result is shown.

4. Discussion

In *D. discoideum*, the mechanism of the DIF signal transduction system is unclarified, and thus the role(s) of intracellular calcium in cell differentiation is controversial. It has not been examined if and how DIF affects $[Ca^{2+}]_i$ in intact cells of this organism, because calcium indicators such as fura-2 can not be loaded to the cells without damaging them seriously or using specific techniques [12]. Very recently, however, it was shown that thapsigargin (Tg) induces stalk cell formation even in the absence of DIF [13]. Because Tg is a potent inhibitor of Ca^{2+}-ATPase present in endoplasmic and sarcoplasmic reticula, and is known to raise $[Ca^{2+}]_i$ in several cell lines (e.g. [14–17]), this result indirectly suggests that DIF may induce stalk cell differentiation by raising $[Ca^{2+}]_i$ in *D. discoideum* [13].

In this study, we have found that DIF raises $[Ca^{2+}]_i$ in the mammalian cell line, AR42J, in a dose-dependent manner at a concentration range of 1–40 μM (Fig. 4), and also that DIF at 1–40 μM suppresses cell proliferation (Figs. 2 and 3). Although the precise mechanism of DIF action remains unknown, it seems likely that DIF suppresses cell proliferation by raising $[Ca^{2+}]_i$ in this cell line.

Lastly, it should be noted that DIF also suppresses cell growth and raises $[Ca^{2+}]_i$ in human erythroleukemia cells (our unpublished observation), and that DIF induces the differentiation of mouse erythroleukemia cells into hemoglobin-producing cells (Asahi, K., Sakurai, A., Takahashi, N., Kubohara, Y., Okamoto, K., Tanaka, Y., submitted for publication). All these results indicate that (i) DIF may be utilized in the study of anti-tumor agents and of cell differentiation in general, (ii) *D. discoideum* may become a more important and useful model organism for the analysis of phenomena seen in mammals, and (iii) mammals may have their own (endogenous) factor(s) which has a functional and/or structural similarity to DIF.

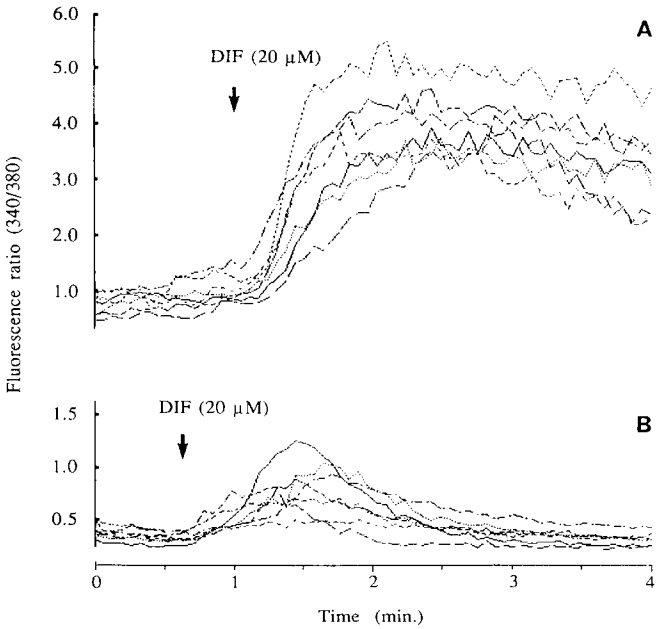

Fig. 6. Changes in $[Ca^{2+}]_i$ in single cells. DIF-induced calcium changes in single fura-2-loaded cells were analyzed by an image processor in the presence of 2.5 mM Ca^{2+} (A) or 1 mM EGTA (B). Representative results are shown, and the data are given as the change in the ratio of the fluorescence intensities.

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