Evidence that differentiation-inducing factor-1 controls chemotaxis and cell differentiation, at least in part, via mitochondria in *D. discoideum*

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**ABSTRACT**

Differentiation-inducing factor-1 [1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one (DIF-1)] is an important regulator of cell differentiation and chemotaxis in the development of the cellular slime mold *Dictyostelium discoideum*. However, the entire signaling pathways downstream of DIF-1 remain to be elucidated. To characterize DIF-1 and its potential receptor(s), we synthesized two fluorescent derivatives of DIF-1, boron-dipyrromethene (BODIPY)-conjugated DIF-1 (DIF-1-BODIPY) and nitrobenzoxadiazole (NBD)-conjugated DIF-1 (DIF-1-NBD), and investigated their biological activities and cellular localization. DIF-1-BODIPY (5 µM) and DIF-1 (2 nM) induced stalk cell differentiation in the DIF-deficient strain HM44 in the presence of cyclic adenosine monophosphate (cAMP), whereas DIF-1-NBD (5 µM) hardly induced stalk cell differentiation under the same conditions. Microscopic analyses revealed that the biologically active derivative, DIF-1-BODIPY, was incorporated by stalk cells at late stages of differentiation and was localized to mitochondria. The mitochondrial uncouplers carbonyl cyanide m-chlorophenylhydrazone (CCCP), at 25–50 nM, and dinitrophenol (DNP), at 2.5–5 µM, induced partial stalk cell differentiation in HM44 in the presence of cAMP. DIF-1-BODIPY (1–2 µM) and DIF-1 (10 nM), as well as CCCP and DNP, suppressed chemotaxis in the wild-type strain AX2 in shallow cAMP gradients. These results suggest that DIF-1-BODIPY and DIF-1 induce stalk cell differentiation and modulate chemotaxis, at least in part, by disturbing mitochondrial activity.

**KEY WORDS:** *Dictyostelium discoideum*, DIF-1, DIF-2, Mitochondria, Cell differentiation, Chemotaxis

**INTRODUCTION**

The vegetative amebae of the cellular slime mold *Dictyostelium discoideum* feed on bacteria. Starvation initiates morphogenesis: cells gather to form a slug-shaped multicellular aggregate and differentiate into two distinct types (prespore and prestalk), which eventually form a fruiting body consisting of spores and a multicellular stalk. Because of the simple pattern of its life cycle (cell differentiation and morphogenesis), *D. discoideum* is an excellent model in cell and developmental biology (Annesley and Fisher, 2009) (http://dictybase.org/).

Cyclic adenosine monophosphate (cAMP) and the chlorinated polyketides differentiation-inducing factor-1 [1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one (DIF-1)] and differentiation-inducing factor-2 [1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)pentan-1-one (DIF-2)] (Fig. 1A) play pivotal roles in the development of *D. discoideum*. While extracellular cAMP secreted by differentiating cells is essential for both prespore and prestalk cell differentiation, it also acts as a chemoattractant when cells gather to form the multicellular aggregate (Konijn et al., 1967; Bonner, 1970; Darmon et al., 1975; Kay, 1982). Initially, DIF-1 and DIF-2 were identified as inducers of stalk cell differentiation *in vitro* in the presence of cAMP (Town et al., 1976; Morris et al., 1987, 1988; Kay et al., 1989, 1999). The activity of DIF-1 is 2.5 times that of DIF-2 in *in vitro* assay with strains derived from V12M2, a wild-type strain (Kay et al., 1999; Masento et al., 1988). Differentiation-inducing factor-3 [1-(3-chloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one (DIF-3)] (Fig. 1A) is the first metabolite produced during the degradation of DIF-1 and has virtually no activity in the induction of stalk cell differentiation in *D. discoideum* (Morris et al., 1988; Kay et al., 1989).

DIF-1 might function, at least in part, via increases in cytosolic calcium or proton concentrations (Kubohara and Okamoto, 1994; Schap et al., 1996; Azhar et al., 1997; Kubohara et al., 2007; Lam et al., 2008). Several transcription factors, such as the basic-leucine zipper transcription factors, DimA and DimB, are involved in DIF-1 signaling (Thompson et al., 2004; Huang et al., 2006; Zhukovskaya et al., 2006; Keller and Thompson, 2008). In shallow cAMP gradients, DIF-1 inhibits chemotaxis via the phosphodiesterase GbpB, whereas DIF-2 stimulates chemotaxis via the phosphodiesterase RegA (Kuwayama and Kubohara, 2009, 2011). The mechanisms by which DIFs modulate chemotaxis differ, at least in part, from those they use to induce stalk cell differentiation (Kuwayama and Kubohara, 2009, 2011). Despite the importance of DIF-1 and DIF-2 in *D. discoideum* development, the entire signaling pathways they activate, including receptors, remain to be identified.

To elucidate the mechanisms underlying the effects of DIF-1 (and possibly DIF-2), we synthesized two fluorescent derivatives of DIF-1, boron-dipyrromethene (BODIPY)-conjugated DIF-1 (DIF-1-BODIPY) and nitrobenzoxadiazole (NBD)-conjugated DIF-1 (DIF-1-NBD) (Fig. 1B,C), and investigated their localization and function in *D. discoideum* cells. We show that DIF-1-BODIPY, but not DIF-1-NBD, is bioactive and appears to function similarly to...
DIF-1: this derivative induces stalk cell formation \textit{in vitro} in the presence of cAMP in HM44 (a DIF-deficient strain) (Kopachik et al., 1983) and suppresses chemotaxis of cells of the wild-type strain Ax2 in shallow cAMP gradients. We also show that DIF-1-BODIPY is undetectable inside the cells during an early stage of development but is localized to intracellular organelles, mainly mitochondria, during a later developmental stage. We examined the effects of DIF-1, DIF-1-BODIPY, and the mitochondrial uncouplers dinitrophenol (DNP) and carbonyl cyanide \textit{m}-chlorophenylhydrazone (CCCP), and the results suggest that DIF-1 (and DIF-1-BODIPY) induces stalk cell differentiation and modulates chemotaxis, at least in part, via mitochondria.

RESULTS

\textbf{Synthesis of fluorescent derivatives of DIF-1 and assay of stalk cell induction}

The synthetic schemes of DIF-1-BODIPY and DIF-1-NBD are shown in Fig. 1B,C. We also synthesized the control compound butyl-BODIPY (Bu-BODIPY) (Kubohara et al., 2013). The effects of DIF-1, DIF-2, and the fluorescent compounds on \textit{in vitro} stalk cell
differentiation in the DIF-deficient strain HM44 are shown in Fig. 2. Even in the presence of cAMP, HM44 cells cannot differentiate into stalk cells in vitro unless exogenous DIF is supplied; therefore, HM44 cells are suitable for the assay of stalk cell induction by DIF-like molecules (Kopachik et al., 1983; Kubohara et al., 1993; Kubohara and Okamoto, 1994). As expected, DIF-1 or DIF-2 (2 nM) induced stalk cell formation in HM44 in the presence of cAMP; DIF-1-BODIPY (0.1–5 µM) dose-dependently induced stalk cell formation in up to 60%–80% of the cells under the same conditions (Fig. 2). By contrast, neither Bu-BODIPY (5 µM) nor DIF-1-NBD (0.1–5 µM) induced any stalk cell formation (Fig. 2).

Cellular localization of DIF-1-BODIPY during in vitro stalk cell differentiation

We next compared the cellular localization of DIF-1-BODIPY and DIF-1-NBD in HM44 cells. After 1-h starvation (incubation), cells were ameboid and were hardly stained with DIF-1-BODIPY or DIF-1-NBD (Fig. 3A), whereas cells fixed with formalin after starvation were stained well with the bioactive derivative DIF-1-BODIPY, but not with the nonbioactive derivative DIF-1-NBD (Fig. 3B).

We then compared cellular localization of DIF-1-BODIPY and the nonbioactive control compound Bu-BODIPY during in vitro differentiation of HM44 cells. After 1-h starvation (incubation), cells were hardly stained with DIF-1-BODIPY (Fig. 4A). After 20-h incubation with cAMP and DIF-1-BODIPY, cells were still ameboid; some of them had formed aggregates, in which some cells were stained with DIF-1-BODIPY, and there was heterogeneity among the cells (Fig. 4C). At 28 h, most cells had differentiated into stalk cells and were stained with DIF-1-BODIPY to a variable extent (Fig. 4E). At 48 h, most cells had differentiated into stalk cells and were stained with DIF-1-BODIPY; the signal was stronger in cytoplasmic regions than in autophagic vacuoles (Fig. 4G). However, cells fixed with formalin were stained with DIF-1-BODIPY at each time point (Fig. 4B,D,F,G). These observations suggest that DIF-1-BODIPY (and possibly DIF-1) is unable to penetrate into the cells or is pumped out from the cells during the early phase, but not during later phases, of cell differentiation. By contrast, Bu-BODIPY neither induced stalk cell formation nor was detected in the cells at any time point, even if they were fixed with formalin (Fig. 4). Taken together, these results indicate that DIF-1-BODIPY can be used to probe cellular uptake and localization of DIF-1.

Target organelle of DIF-1-BODIPY

It is noteworthy that DIF-1, DIF-3 and their derivatives possess anti-tumor activities (Asahi et al., 1995; Kubohara, 1997, 1999; Gokan et al., 2005), and that DIF-3 derivatives are more active than DIF-1.
derivatives in suppressing tumor cell growth (Gokan et al., 2005; Kubohara, 1999). We have shown that the fluorescent derivative BODIPY-DIF-3 (Fig. 1A) localizes to mitochondria in mammalian cell lines (Kubohara et al., 2013, 2014). We thus compared localization of DIF-1-BODIPY and MitoTracker (a probe for mitochondria) in HM44 cells (Fig. 5). DIF-1-BODIPY co-localized to mitochondria stained with MitoTracker in formalin-fixed cells that had been starved for 1 h (Fig. 5A) or incubated for 21 h with cAMP; in the latter case, most cells had formed small aggregates (Fig. 5C). By contrast, Bu-BODIPY did not stain any organelles in formalin-fixed cells (Fig. 5B,D). These results strongly suggest that DIF-1-BODIPY (and possibly DIF-1) might function, at least in part, by affecting mitochondrial activity in D. discoideum.

Effects of CCCP and DNP on stalk cell differentiation

We have recently shown that DIF-1 and its derivatives act as mitochondrial uncouplers in mammalian cells (Kubohara et al., 2013, 2015). To determine whether DIF-1 (and DIF-1-BODIPY) induces stalk cell differentiation by affecting mitochondria in D. discoideum, we examined the effects of CCCP and DNP on stalk cell formation in HM44. As shown in Fig. 6, CCCP (25–50 nM) or DNP (2.5–5 µM) weakly but significantly induced stalk cell formation in the presence of cAMP; at higher concentrations, both uncouplers were toxic to the cells (data not shown). The stalk-inducing activities of CCCP and DNP did not exceed ~10% and ~20%, respectively (Fig. 6A); neither CCCP nor DNP showed additive effects with DIF-1 at a low concentration (0.4 nM). These results suggest that DIF-1 induces stalk cell differentiation partly by uncoupling mitochondrial activity but also via another pathway.

Effects of DIF-1-BODIPY on chemotactic cell movement

To verify whether DIF-1-BODIPY inhibits chemotaxis (similar to DIF-1) or stimulates chemotaxis (similar to DIF-2), we examined its effects on chemotactic movement of Ax2 cells toward cAMP (Fig. 7A). In shallow cAMP gradients, chemotaxis was suppressed by 10 nM DIF-1 and promoted by 10 nM DIF-2, as previously described (Kuwayama and Kubohara, 2009). Similar to DIF-1, DIF-1-BODIPY (1–2 µM) suppressed chemotaxis (Fig. 7A). To confirm that DIF-1-BODIPY can functionally mimic DIF-2, we next compared their effects on chemotaxis in gbpB− cells. Neither DIF-1 (10 nM) nor DIF-1-BODIPY (1–2 µM) affected chemotaxis in shallow cAMP gradients, whereas DIF-2 (used as a positive control) significantly promoted chemotaxis under the same conditions (Fig. 7A), indicating that DIF-1-BODIPY and DIF-1 exert their effects via a GbpB-dependent pathway (Kuwayama and Kubohara, 2009). Thus, DIF-1-BODIPY can functionally mimic DIF-1 in the regulation of chemotaxis.

Effects of CCCP and DNP on chemotactic cell movement

To demonstrate that DIF-1 might function by disturbing mitochondrial activity, we examined the effects of CCCP and DNP on chemotactic cell movement toward cAMP (Fig. 7B). As expected, CCCP (25–50 nM), DNP (5 µM) and DIF-1 (10 nM) significantly suppressed chemotaxis of Ax2 cells in shallow cAMP gradients but hardly affected chemotaxis of gbpB− cells (Fig. 7B). These results indicate that all three compounds suppress chemotaxis via a GbpB-dependent pathway and that DIF-1 (and possibly DIF-1-BODIPY) might suppress chemotaxis in shallow cAMP gradients by uncoupling mitochondrial activity.

Localization of DIF-1-BODIPY in aggregating Ax2 cells

Finally, we localized DIF-1-BODIPY in aggregating (chemotacting) Ax2 cells under submerged conditions without exogenous cAMP; under these conditions, cells formed streaming aggregates (Fig. S1). After 3-h incubation, we still observed single ameboid cells; living cells were not stained with DIF-1-BODIPY, although formalin-fixed cells were clearly stained (Fig. S1A). At 15 h, cells formed aggregates, in which a small fraction of the cells was clearly stained with DIF-1-BODIPY; formalin-fixed cells were strongly stained (Fig. S1B).

Fig. 3. Localization of DIF-1-BODIPY and DIF-1-NBD in living and formalin-fixed HM44 cells. (A) Cells were incubated in vitro for 1 h with 5 µM DIF-1-BODIPY or DIF-1-NBD. (B) Cells were incubated in vitro for 1 h with no additives, fixed with formalin, and stained for 0.5 h with 5 µM DIF-1-BODIPY or DIF-1-NBD. Cells were washed free of the additives and observed under phase-contrast and fluorescence microscopes. Scheme of the experiment is indicated above each panel.
Fig. 4. Localization of DIF-1-BODIPY and Bu-BODIPY in living and formalin-fixed HM44 cells. (A,B) Localization of DIF-1-BODIPY and Bu-BODIPY in undifferentiated cells. (A) Cells were incubated in vitro for 1 h with 5 µM DIF-1-BODIPY (DIF-1-BOD) or Bu-BODIPY (Bu-BOD). (B) Cells were incubated in vitro for 1 h without the additives, fixed with formalin, and stained for 0.5 h with 5 µM DIF-1-BODIPY or Bu-BODIPY. Cells were washed free of the additives and observed under phase-contrast and fluorescence microscopes. (C–F) Localization of DIF-1-BODIPY and Bu-BODIPY in differentiating cells. (C,E) Cells were incubated in vitro for (C) 20 h or (E) 28 h with 5 mM cAMP in the presence of 5 µM DIF-1-BODIPY or Bu-BODIPY. (D,F) Cells were incubated for the same time periods with 5 mM cAMP, fixed with formalin, and stained for 0.5 h with 5 µM DIF-1-BODIPY or Bu-BODIPY. Cells were washed and observed under phase-contrast and fluorescence microscopes. (G,H) Localization of DIF-1-BODIPY and Bu-BODIPY in stalk cells. (G) Cells were incubated in vitro for 48 h with 5 mM cAMP in the presence of 5 µM DIF-1-BODIPY or Bu-BODIPY. (H) Cells were incubated for the same time periods with 5 mM cAMP, fixed with formalin, and stained for 0.5 h with 5 µM DIF-1-BODIPY or Bu-BODIPY. Cells were washed and observed under phase-contrast and fluorescence microscopes. Scheme of the experiment is indicated above each panel.
Biological activities of DIF-1-BODIPY

In this study, we designed and synthesized the fluorescent DIF derivative DIF-1-BODIPY (Fig. 1B) and found that DIF-1-BODIPY (0.1–5 µM) induced stalk cell differentiation in the presence of cAMP in the DIF-deficient strain HM44 (Fig. 2). DIF-1-BODIPY (1–2 µM) also suppressed chemotaxis in shallow cAMP gradients in Ax2 cells (Fig. 7). Although we do not exclude the possibility that DIF-1-BODIPY at several micromolars might affect cellular functions nonspecifically, the present results indicate that DIF-1-BODIPY can mimic the effects of DIF-1 in *D. discoideum*.

Subcellular localization of DIF-1-BODIPY to mitochondria

The hydrophobic indices of DIF-1 [ClogP (CP), 4.21] and DIF-1-BODIPY (CP, 5.85) (Fig. 1A,B) indicate that both compounds are likely to penetrate the cell membrane. However, we found that DIF-
at least in part, from their uncoupling activity in the mitochondria of mouse liver; the anti-tumor activity of DIF derivatives might result, CCCP promote oxygen consumption in mitochondria isolated from 2015). Bioactive DIF derivatives and the mitochondrial uncoupler growth in some of the tumor cell lines tested (Kubohara et al., 2013, 2015). Akaishi et al., 2004; Kubohara et al., 2015; Oladimeji et al., 2015).

Gokan et al., 2005; Kubohara, 1999; Takahashi-Yanaga et al., 2003; Thompson, 2008; Luciani et al., 2009; Giusti et al., 2010). As we

1-BODIPY was absent in cells at early stages of development but gradually penetrated into or was taken up by cells differentiating to stalk cells (Fig. 4). DIF-1-BODIPY localized to mitochondria (Fig. 5). During early, but not late, stages of development, DIF-1-BODIPY (and thus DIF-1) might be pumped out of cells (Fig. 8). The molecular size of DIF-1-BODIPY is larger than that of DIF-1 and the hydrophobic indices of the two compounds are different (Fig. 1A,B). However, because Bu-BODIPY was not detected in any organelles (Figs 4 and 5), the mitochondrial localization of DIF-1-BODIPY was not caused by the BODIPY moiety but likely reflects localization of DIF-1.

DIFs and their derivatives possess anti-tumor activities when tested on mammalian tumor cells, and derivatives of DIF-3 are more potent anti-tumor agents than those of DIF-1 (Asahi et al., 1995; Gokan et al., 2005; Kubohara, 1999; Takahashi-Yanaga et al., 2003; Akaishi et al., 2004; Kubohara et al., 2015; Oladimeji et al., 2015). The fluorescent DIF-3 derivative BODIPY-DIF-3 penetrates the cell membrane, localizes to mitochondria, and suppresses cell growth in some of the tumor cell lines tested (Kubohara et al., 2013, 2015). Bioactive DIF derivatives and the mitochondrial uncoupler CCCP promote oxygen consumption in mitochondria isolated from mouse liver; the anti-tumor activity of DIF derivatives might result, at least in part, from their uncoupling activity in the mitochondria of tumor cells (Kubohara et al., 2013, 2015). In D. discoideum, DIF-1 can disturb mitochondrial membrane potential and respiration, suggesting that it might function as an uncoupler (Shaulsky and Loomis, 1995), although the effective concentration range of DIF-1 (0.1–1 µM) rather exceeded its putative physiological concentrations (at most ~0.1 µM; Kay, 1998). However, DIF-1 at 0.1 µM was later shown to affect mitochondrial membrane potential in Ax2 cells (Arnoult et al., 2001) and to promote mitochondrial oxygen consumption and induce ATP depletion in an autophagy mutant strain (Laporte et al., 2007; Giusti et al., 2009), suggesting that it can act as an uncoupler at physiological concentrations. In the present study, we have shown that the mitochondrial uncouplers CCCP and DNP induce partial stalk differentiation of HM44 cells (Fig. 6) and that the uncouplers and DIF-1 suppress chemotaxis in Ax2 cells in shallow cAMP gradients (Fig. 7B). Taken together, these data suggest that DIF-1 might function, at least in part, via mitochondria (possibly as an uncoupler) in D. discoideum. Unexpectedly, however, neither CCCP (25–50 nM) nor DNP (2.5–5 µM) showed an additive effect with 0.4 nM DIF-1 on stalk cell formation (Fig. 6). Although the cause of the absence of such effect is unknown, DIF-1 might function via multiple signaling cascades, only one of which may be mimicked by CCCP and DNP; DIF-1 at 0.4 nM might be sufficient to saturate this pathway. Alternatively, as CCCP >50 nM and DNP >5 µM were toxic to the cells (data not shown), their toxicity might cancel their stalk-inducing activity in the presence of DIF-1.

**Biological activity and cellular localization of DIF-1-NBD**

In the present study, we have also synthesized DIF-1-NBD, a fluorescent amide derivative of DIF-1 (Fig. 1C). DIF-1-NBD was expected to be a good probe for DIF-1 because some amide derivatives of DIF-1 are excellent inducers of stalk cell differentiation in HM44 cells (Kikuchi et al., 2008), and also because the molecular size of DIF-1-NBD is much smaller than that of DIF-1-BODIPY and its CP value (3.51) suggests that DIF-1-NBD can penetrate the cell membrane (Fig. 1A). Unfortunately, however, DIF-1-NBD (5 µM) neither induced stalk cell differentiation (Fig. 2) nor appeared to localize to any parts of HM44 cells (Fig. 3). Although we cannot exclude that the localization of DIF-1-BODIPY in mitochondria reflects its non-specific binding because of its high concentration, the absence of cell staining or activity of DIF-1-NBD (5 µM) and another non-bioactive compound Bu-BODIPY (5 µM) (Figs 2–4) indicates that it is likely that the biological activities and cellular localization of DIF-1-BODIPY (5 µM) reflect those of DIF-1 at nanomolar concentrations.

**Proposed scheme for DIF-1 function**

The functions of DIF-1 have been analyzed in many studies (Kubohara and Okamoto, 1994; Schaap et al., 1996; Azhar et al., 1997; Thompson et al., 2004; Huang et al., 2006; Zhukovskaya et al., 2008). As we have shown here the possible involvement of mitochondria in the effects of DIF-1, we mainly discuss the relationship between DIF signaling and mitochondria. We assume that DRI-D (putative DIF-1 receptor responsible for the induction of cell differentiation) (Fig. 8A) might mediate the induction of stalk cell differentiation by DIF-1 (at least in part via increases in intracellular calcium and/or proton concentrations) (Kubohara and Okamoto, 1994; Schaap et al., 1996; Azhar et al., 1997; Kubohara et al., 2007; Lam et al., 2008). In shallow cAMP gradients, DIF-1 suppresses chemotaxis via a GbpB-dependent pathway, whereas DIF-2 promotes...
chemotaxis via a RegA-dependent pathway (Kuwayama and Kubohara, 2009; Kuwayama et al., 2011); we assume here that DIF-1 functions via DR1-C (putative DIF-1 receptor responsible for modulation of chemotaxis) and that DIF-2 functions via DR2-C (putative DIF-2 receptor responsible for modulation of chemotaxis) (Fig. 8A). DIF-1 is likely to modulate chemotaxis by interfering with mitochondrial activity; if so, mitochondria might be the target organelles of DIF-1 that contain DR1-C (Fig. 8B). CCCP and DNP induce partial stalk cell differentiation (Fig. 6) and mitochondria affect intracellular calcium and proton concentrations (Swietach et al., 2013; de Marchi et al., 2014); therefore, DIF-1 might induce stalk cell formation, at least in part, by disturbing (uncoupling) mitochondrial activity (Fig. 8B,C).

MATERIALS AND METHODS

Cell lines and cell culture

The D. discoideum DIF-deficient strain HM44 (Kopachik et al., 1983) was used for in vitro stalk cell induction assay. The axenic strain Ax2 and the gbpB null strain gbpB' derived from Ax2 (Bosgraaf et al., 2002a,b; Goldberg et al., 2002) were used for chemotaxis assay. These strains were obtained from the National BioResource Project (NBRP Nenkin, Tsukuba, Japan). HM44 cells were grown in association with Klebsiella aerogenes on a modified SM agar plate (Inouye, 1988) at 21°C, whereas Ax2 and gbpB' cells were grown axenically at 21°C in HL-5 liquid medium (Sussman, 1987). Growing cells were collected by centrifugation (500×g, 3 min).

Reagents

DIF-1, DIF-2, and Bu-BODIPY (Fig. 1A) were synthesized as described previously (Gokan et al., 2005; Kubohara et al., 2013); they were dissolved in ethanol or dimethyl sulfoxide (DMSO) and stored at −20°C. Amino derivative of DIF-1 [6-amino-1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one (DIF-1-NH2)] (Fig. 1B) was synthesized as described previously (Kubohara et al., 2010). MitoTracker Red CMXRos (referred to as MitoTracker) (Ex=579 nm, Em=599 nm) and BODIPY FL, SE (succinimidyl ester) (Ex=505 nm, Em=513 nm) were purchased from Invitrogen. Hoechst 33342 (Ex=352 nm, Em=461 nm) solution (1 mg ml⁻¹ in H2O), CCCP, and DNP were obtained from Wako Pure Chemical Industries (Osaka, Japan). NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole) (Ex=470 nm, Em=530 nm) was from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

Synthesis of DIF-1-BODIPY

As depicted in Fig. 1B, DIF-1-NH2 (1.3 mg, 3.5 µmol) and triethylamine (10 µl) were added to a solution of BODIPY FL, SE (2.1 mg, 5.3 µmol) in N,N-dimethylformamide (0.5 ml) at room temperature (rt) in Fig. 1B in the dark. The reaction mixture was stirred for 12 h and then diluted with 0.2 M hydrochloric acid (5 ml) and extracted with ethyl acetate (10 ml) three times. The residue was subjected to recycle preparative high-performance liquid chromatography (HPLC) on a JAIGEL-GS-310 column (φ=20 mm×500 mm) (Japan Analytical Industry Co., Ltd., Tokyo, Japan). NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole) (Ex=470 nm, Em=530 nm) was from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

Fig. 7. Effects of DIF-1-BODIPY, CCCP, and DNP on chemotaxis toward cAMP. Ax2 and gbpB' cells were starved for 6 h, and cell droplets were placed on phosphate-buffered agar containing 3 mM caffeine (control) plus (A) DIF-1, DIF-2, or DIF-1-BODIPY (DIF-1-BOD) or (B) DIF-1, DIF-2, CCCP, or DNP. Cells were assayed for chemotaxis at the indicated concentrations of cAMP (10 cell droplets per concentration per plate were examined). Data are the mean±s.d. for triplicate sample plates. *P<0.05; **P<0.01 versus control cells (by one-tailed Welch’s t-test).
δ 10.75–10.92 (1H, br.s), 7.08 (1H, s), 6.88 (1H, d, J = 4.0 Hz), 6.30 (1H, d, J = 4.0 Hz), 6.11 (1H, s), 5.80–5.88 (1H, br.s), 3.98 (3H, s), 3.28 (2H, t, J = 7.4 Hz), 3.23 (2H, q, J = 6.9 Hz), 3.05 (2H, t, J = 7.6 Hz), 2.66 (2H, t, J = 7.4 Hz), 2.56 (3H, s), 2.24 (3H, s), 1.67 (2H, quint, J = 7.4 Hz), 1.47 (2H, quint, J = 7.4 Hz), 1.36 (2H, quint, J = 7.4 Hz), high resolution fast-atom bombardment mass spectrometry (HRFABMS) m/z 577.1645 [M+][F]

Synthesis of DIF-1-NBD

DIF-1-NBD was synthesized as follows (Fig. 1C). To a solution of 2,6-bis (benzyloxy)-4-methoxybenzoic acid (Kikuchi et al., 2008) (100 mg, 0.274 mmol) in dichloromethane (8.0 ml), 2-(trimethylsilyl)ethyl 2-aminoethylcarbamate (61.3 mg, 0.300 mmol), O-(7-aza-1H-benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HATU) (103 mg, 0.274 mmol) and N,N-diisopropylethylamine (71 µl, 0.407 mmol) were added at room temperature (rt in Fig. 1C). The reaction mixture was stirred for 1 h, poured into water and extracted with ethyl acetate three times. The combined organic layer was washed with water and dried over sodium sulfate and concentrated in vacuo. The residue was chromatographed over silica gel eluted with hexane–ethyl acetate (1:1) to give 2-(trimethylsilyl)ethyl 2-(2,6-bis(benzyloxy)-4-methoxyphenylamido)ethylcarbamate (106 mg, 0.192 mmol, 70% yield).
The latter (76.4 mg, 0.139 mmol) was stirred with 20% (w/w) palladium hydroxide on carbon (10.0 mg) in methanol (2.0 ml) at room temperature for 2 h under hydrogen atmosphere. After filtration through a celite pad, the filtrate was concentrated in vacuo. The residue was chromatographed over silica gel eluted with hexane–ethyl acetate (2:1) to afford 2-(trimethylsilyl)ethyl 2-(2,6-dihydroxy-4-methoxyphenylamido)ethylcarbamate (45.7 mg, 0.124 mmol, 89% yield).

To a solution of the latter (45.0 mg, 0.121 mmol) in N,N-dimethylformamide (1.5 ml), chloromethyl methyl ether (36 µl, 0.474 mmol) and potassium carbonate (51.1 mg, 0.370 mmol) were added at room temperature. The reaction mixture was stirred for 8 h, poured into water, and extracted with ethyl acetate three times. The combined organic layer was washed with water and brine, dried over sodium sulfate, and concentrated in vacuo. The solution was stirred for 2 h, poured into water, and extracted with ethyl acetate three times. The combined organic layer was washed with water and brine, dried over sodium sulfate, and concentrated in vacuo. The residue was chromatographed over silica gel eluted by hexane–ethyl acetate (2:1) to give 2-[(trimethylsilyl)ethyl] 2-(2-hydroxy-4-methoxy-6-(methoxymethoxy)phenylamido)ethylcarbamate (41.9 mg, 0.101 mmol, 84% yield).

To a solution of the latter (40.0 mg, 0.096 mmol) in tetrahydrofuran (THF) (1.5 ml), 1.0 M tetramethylammonium fluoride in THF (100 µl, 0.100 mmol) was added at room temperature. The reaction mixture was stirred for 1 h at 50°C, poured into water, and extracted with ethyl acetate three times. The combined organic layer was washed with water and brine, dried over sodium sulfate, and concentrated in vacuo. The residue was chromatographed over silica gel eluted with chloroform–methanol (4:1) to give N-(2-aminoethyl)-2-hydroxy-4-methoxy-6-(methoxymethoxy)benzamide (17.2 mg, 0.063 mmol, 66% yield).

To a solution of the latter (15.0 mg, 0.056 mmol) in THF (1.5 ml), NBD-F (20.5 µl, 0.112 mmol) and N,N-disopropylethylamine (40 µl, 0.230 mmol) were added at room temperature. The reaction mixture was stirred for 2 h, poured into water, and extracted with ethyl acetate three times. The combined organic layer was washed with water and brine, dried over sodium sulfate, and concentrated in vacuo. The residue was chromatographed over silica gel eluted with hexane–ethyl acetate (1:1) to give 2-hydroxy-4-methoxy-6-(methoxymethoxy)-N-(2-(7-nitrobenz-2-oxa-1,3-diazol-4-ylamino)ethyl) benzamide (13.8 mg, 0.032 mmol, 57% yield).

The latter (9.3 mg, 0.021 mmol) was dissolved in 5% (w/v) HCl in methanol (3.0 ml) at room temperature. The solution was stirred for 5 h and concentrated in vacuo. The residue was dissolved in chloroform (1.5 ml), and ethanol (30 µl) and sulfuric chloride (8.0 mg, 0.059 mmol) were added at room temperature. The reaction mixture was stirred for 2 h, poured into water, and extracted with ethyl acetate three times. The combined organic layer was washed with water and brine, dried over sodium sulfate, and concentrated in vacuo. The residue was chromatographed over silica gel eluted with hexane–ethyl acetate (1:1) to give DIF-1-NBD [6.4 mg, 0.014 mmol, 65% yield (two steps)]. Analytical data for DIF-1-NBD: 1H NMR (600 MHz, pyridine-d5) δ 11.58 (1H, s), 10.59 (1H, s), 8.61 (1H, d, J=8.4 Hz), 6.41 (1H, d, J=8.4 Hz), 3.89–3.98 (4H, m), 3.91 (1H, s), 13C NMR (150 MHz, pyridine-d5) δ 160.0, 159.5, 157.6 (2C), 144.4, 139.9, 137.5, 131.5, 120.4, 105.1 (2C), 103.6, 95.5, 60.8, 42.3, 38.8; HRFABMS m/z 750 [M+H]+ (456.0085 [M+H]+ (456.0114 calculated for C16H15N2O3S2Cl2).
...induces stalk cell differentiation in Dictyostelium discoideum. *FEBS Lett.* 322, 73-75.


