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Regulation of Calcium Signaling by IplA in Electrotaxis of *Dictyostelium* Cells

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IplA에 의한 칼슘 신호 조절

2023 년 2 월 24 일

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이 논문을 이학석사학위 신청 논문으로 제출함

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ABBREVIATIONS

EF	Electric Field
PI3K	Phosphoinositide 3-kinase
TORC2	Target of rapamycin C2
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKB	Protein kinase B
IP_3	Inositol 1,4,5-trisphosphate
cAMP	Cyclic adenosine monophosphate
EGTA	Ethylene glycol-bistetraacetic acid
EDTA	Ethylene diamine-tetraacetic acid
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
IplA	Inositol 1,4,5-trisphosphate receptor-like protein
IP_3R	Inositol 1,4,5-trisphosphate receptor

ABSTRACT

Regulation of Calcium Signaling by IplA in Electrotaxis of *Dictyostelium* Cells

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Directional cell migration such as chemotaxis and electrotaxis is essential for various physiological phenomena, including development, immune response, wound healing and cancer metastasis. General molecular mechanism for directional cell migration has been studied using chemotaxis, in which the cells migrate towards the higher concentration of chemoattractant. In contrast, electrotaxis has not been studied yet. In the present study, I focused on investigating the molecular mechanism for directional cell migration in response to electric field. Several previous studies suggest that calcium ions have been involved in cell migration. It has been demonstrated that influx of extracellular calcium ions into the cells are required for electrotaxis. Inositol 1,4,5 triphosphate receptor-like protein A (IplA) is a known Inositol 1,4,5-trisphosphate receptor (IP_3R) calcium channel in *Dictyostelium,* a well-developed model organism for cell migration. IplA is required for the regulation of intracellular calcium signaling by chemotactic or electrotactic stimuli. Here, to investigate roles of IplA in electrotaxis and chemotaxis, I performed two parts of experiments. In part 1, I examined the phenotypes of *iplA* null cells and compared with

those of wild-type Ax2 cells. In part 2, I constructed a calcium sensor by subcloning calcium sensitive region of pGCaMP into *Dictyostelium*-specific plasmid, and then examined dynamic subcellular calcium response upon several chemotactic or electrotactic stimuli.

iplA null cells showed smaller cell size and reduced cell adhesion compared to wild-type cells. Migration study demonstrates that *iplA* null cells displayed increased migration speed in electrotaxis, chemotaxis, and random motility. Interestingly, the directedness of the vegetative *iplA* null cells was lost in electrotaxis, but not in chemotaxis. In contrast, the directionality of the aggregation-competent *iplA* null cells was similar to that of wild-type cells. These data indicate that IplA plays different roles depending on the developmental state of the cells in the regulation of directionality during electrotaxis. *iplA* null cells showed normal development.

Next, to understand dynamic roles of calcium in cell migration of *Dictyostelium discoideum*, I prepared a *Dictyostelium* specific calcium reporter plasmid (pDXA-GCaMP3) by subcloning the region of GCaMP in pGCaMP3 into the pDXA-3C using Gibson assembly kits. This plasmid was transformed into wild-type Ax2 and *iplA* null cells. *iplA* null cells showed no inducement of the calcium response upon cAMP stimulation. No calcium response was observed in Ax2 or *iplA* null cells in response to depolarization stimuli induced by high concentrations of potassium. When high concentration of extracellular calcium was uniformly applied into the media containing the cells, the intracellular calcium response of *iplA* null cells were delayed compared to Ax2 cells. These results indicate that live cell imaging for calcium was successfully performed in *Dictyostelium* through GCaMP and that calcium responses to various stimuli could be observed.

In conclusion, the present studies demonstrate that IplA plays an important role in the regulation of directionality during directional cell migration in response to external electric field. The roles of IplA in the control of the direction of cell movements are dependent upon the developmental state of the cells; IplA appears to play an important role in the vegetative state of the cells, but not in the developed aggregation-competent state. Live-cell imaging study using the calcium sensor plasmid demonstrates that IplA is required for the influx of calcium ions in response to extracellular calcium stimuli. Further studies on calcium response using the calcium sensor, which were developed in this study, would be helpful to understand functions of the calcium signaling in cell migration chemotaxis and electrotaxis.

국문초록

딕티오스텔리움 세포의 주전성 세포이동에서 IplA에 의한 칼슘 신호 조절

서 지 성

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주화성이동 및 주전성이동과 같은 방향성을 나타내는 세포이동은 발생, 면역 반응, 상처 치유 및 암 전이를 비롯한 다양한 생리 현상에 필수적이다. 방향성 세포이동에 대한 일반적인 분자 기작은 세포가 더 높은 농도의 화학유인 물질로 이동하는 주화성이동 분야에서 주로 연구되었다. 대조적으로, 주전성 이동은 아직 자세히 연구되지 않았다. 본 연구에서는 전기장에 반응해 일어나는, 방향성 세포이동에 대한 분자 기작을 조사하는 데 중점을 두었다. 이전의 여러 연구에서는 칼슘이 세포 이동에 관여한다고 제안되어 왔다. 그에 더해 주전성이동을 위해서는 세포외부에 있는 칼슘의 세포 내부로의 유입이 필요하다는 것이 입증되었다. 이노시톨 1,4,5 삼인산 수용체 유사 단백질 A(IplA)는 세포이동 연구에 사용되는 모델 생물인 딕티오스텔리움에서 알려진 유일한 이노시톨 1,4,5-삼인산 수용체(IP_3R)

칼슘이온통로이다. 기존 연구를 통해 IplA는 주화성 자극 또는 전기적 자극에 의한 세포내 칼슘 신호전달의 조절에 필요하다는 것이 밝혀졌다. 본 연구에서는 주전성이동과 주화성이동에서 IplA의 역할을 조사하기 위해 두 부분의 실험을 수행했다. 먼저, *iplA*가 제거된 세포의 표현형을 조사하고 야생형 Ax2 세포의 표현형과 비교했다. 다음으로 칼슘 표지자인 형광단백질 pGCaMP의 칼슘 민감성 영역을 딕티오스텔리움에 특이적으로 발현하는 플라스미드로 유전자 재조합을 하여 칼슘 표지자를 구성한 다음, 여러 가지 주화성 또는 전기적 자극에 대한 세포내 칼슘의 동적 반응을 조사했다.

iplA 제거 세포는 야생형 세포에 비해 더 작은 세포 크기와 감소된 세포-기질 부착을 보였다. 세포이동 연구는 *iplA* 제거 세포가 주전성, 주화성 및 무작위 운동에서 이동 속도가 증가함을 보였다. 흥미롭게도 식물성세포 상태인 *iplA* 제거 세포의 방향성은 주전성이동에서는 손실되었지만 주화성이동에서는 손실되지 않았다. 그에 반해, 응집 능력이 있는 세포 상태인 *iplA* 제거 세포의 방향성은 야생형 세포의 방향성과 유사했다. 이러한 결과는 IplA가 주전성이동 동안의 방향성 조절에서 세포의 발달 상태에 따라 다른 역할을 한다는 것을 의미한다. 추가적으로, *iplA* 제거 세포는 정상적인 발달 단계를 보였다.

다음으로 딕티오스텔리움의 세포 이동에서 동적인 칼슘조절을 이해하기 위해 깁슨 조립 키를 사용하여 pGCaMP3의 GCaMP 영역을 pDXA-3C로 유전자 재조합을 하여 딕티오스텔리움에서 발현하는 칼슘 표지 플라스미드(pDXA-GCaMP3)를 준비했다. 이 플라스미드는 야생형 Ax2 및 *iplA* 제거 세포에 형질전환 되었다. *iplA* 제거 세포는 cAMP 자극 시 칼슘

반응을 유도하지 않았다. 고농도의 세포외부 칼륨에 의해 유도된 탈분극 자극에 대한 반응으로 Ax2 또는 *iplA* 제거 세포에서 칼슘 반응이 관찰되지 않았다. 고농도의 세포외부 칼슘 자극에는, *iplA* 제거 세포의 세포내 칼슘 반응은 Ax2 세포에 비해 지연되었다. 이러한 결과는 GCaMP를 통해 딕티오스텔리움에서 살아있는 세포에서 칼슘 영상화가 성공적으로 수행되었으며, 다양한 자극에 대한 칼슘 반응을 관찰할 수 있음을 나타낸다.

결론적으로, 본 연구는 IplA가 외부 전기장에 반응하는 방향성 세포이동 동안 방향성 조절에 중요한 역할을 한다는 것을 입증한다. 세포의 이동 방향을 제어하는 IplA의 역할은 세포의 발달 상태에 따라 다르다. IplA는 식물성 세포에서 중요한 역할을 하는 것으로 보이지만, 발달을 거쳐 응집 가능한 상태의 세포에서는 그렇지 않다. 칼슘 표지자 플라스미드를 사용한 살아있는 세포에서의 칼슘영상화 연구는 IplA가 세포외부 칼슘 자극에 대한 반응으로 칼슘 이온의 유입에 필요함을 입증한다. 본 연구에서 개발한 칼슘 표지자를 이용한 칼슘 반응에 대한 추가적인 연구는 세포이동, 주화성이동 및 주전성이동에서 칼슘 신호전달의 기능을 이해하는데 도움이 될 것이다.

I. INTRODUCTION

I-1. Cell migration

Cell migration is a cellular process which is necessary for various physiological and pathological phenomena such as wound healing, cancer metastasis, immune response, and embryogenesis (Chung et al., 2001; Ridley et al., 2003). For the cells to migrate in response to external stimuli, the cells first should be polarized, F-actin polymerization at the front drying to pseudopod formation and myosin II assembly at the posterior and lateral regions of the migrating cells (Lee and Jeon, 2012). Cell migration is classified into many types, such as chemotaxis and electrotaxis, depending on the type of stimulus. Much of our understanding of the basic signaling pathways and molecules involved in directional cell migration is based on the studies of chemotaxis using *Dictyostelium discoideum*, a model organism for cell migration, and leukocytes (Artemenko et al., 2014; Kortholt and Haastert, 2008). *Dictyostelium* shows strong directional cell migration in response to chemoattractant, such as folic acid and cyclic adenosine monophosphate (cAMP). The molecular mechanisms of directional cell migration have been found to be primarily based on chemoattractant-directed cell migration. Cell migration toward a high concentration gradient of chemoattractant is mediated through multiple signaling pathways including phosphatidylinositol 3-kinase (PI3K), TORC2 complex, phospholipase A2, and phospholipase C (Kolsch et al., 2008). When the cells are stimulated with external chemoattractants, G-protein coupled receptors on the cell surface transduce the external signals into the internal signaling pathways driving asymmetrical polymerization of F-actin at the anterior and membrane protrusion from the cells, mediating directional cell migration towards a high gradient of chemotaxis (Artemenko et al., 2014; Kolsch et al., 2008).

I-2. Electrotaxis

Electrotaxis is a directional cell migration to the cathode or the anode in response to a direct current electric field (EF). Electrotaxis occurs in a wide range of species from bacteria to eukaryotes including social amoeba *Dictyostelium*, corneal epithelial cells, and osteoblasts from mammals (Shanley et al., 2006). Naturally occurring endogenous currents drive directional cell migration during development and wound healing. It has been demonstrated that misregulation of electrical signaling during embryonic development in mice results in abnormal development of skeletal and nervous system (Shanley et al., 2006; Zhao, 2009). Electrotaxis is involved in wound healing and membrane potential was proved to be related to these processes in *Dictyostelium* (Shanley et al., 2006). However, the detailed molecular mechanism underlying electrotaxis has not been characterized yet. It is generally accepted that the major signaling networks downstream of the receptor in electrotaxis are shared with that in chemotaxis (Zhao, 2009; Gao et al., 2015).

I-3. Calcium ion (Ca^{2+})

Extracellular calcium is important for cell-cell adhesion and cell-substrate adhesion (Lombardi et al., 2008). Intracellular calcium ion (Ca^{2+}) is bind to diverse actin-binding proteins such as α-actinin, severin, and gelsolin, and is involved in the control of the activity of the proteins. The activity of myosin light chain kinase (MLCK) is regulated through phosphorylation by calcium-dependent kinases (Crawford and Jacobson, 1998; Eddy et al., 2000; Huttenlocher et al., 1997; Leavesley et al., 1993; Lombardi et al., 2008; Walter et al., 1993). Ca^{2+} also serves as a secondary signal in many biochemical signaling pathways including chemotaxis. The concentration of Ca^{2+} is spatially and temporally regulated during cell migration. For example, eosinophils of newt show an intracellular Ca^{2+} concentration gradient from the front to the back when the cells were placed on the

gradient of chemoattractant (Brundage et al., 1993). Other highly motile cells, such as fish epithelial keratinocytes and human neutrophils, undergo a transient increase in intracellular Ca^{2+} concentration prior to contraction (Lombardi et al., 2008). The transient and rapid increase of intracellular Ca^{2+} concentration correlates with directional cell migration (Lombardi et al., 2008). *Dictyostelium* cells show a rapid influx of Ca^{2+} through the plasma membrane and the rapid influx is used in the signal relay during chemotaxis (Traynor et al., 2000). When *Dictyostelium* cells are treated with calcium chelator such as Ethylene glycol-bistetraacetic acid (EGTA) or Ethylene diamine-tetraacetic acid (EDTA), the formation of pseudopods and the movements are impaired. However, there are some results arguing that Ca^{2+} signaling is dispensable for chemotaxis. Intracellular Ca^{2+} levels are regulated through inositol 1,3,5-trisphosphate receptor-like gene (IplA) in response to chemoattractant stimulation (Lusche et al., 2012; Schaloske et al., 2005; Traynor et al., 2000). The Ca^{2+} signaling plays an essential role in electrotaxis as well as in chemotaxis. Neural crest cells, mouse embryonic fibroblasts, and human keratinocytes exhibit electrotaxis which is, in part, dependent on Ca^{2+} signaling (Shanley et al., 2006). *Dictyostelium* cells show a strong electrotaxis and an induction of a long-lasting increase intracellular Ca^{2+} in response to an external EF (Shanley et al., 2006).

I-4. IplA

IplA is a Ca^{2+} channel protein on the ER membrane in *Dictyostelium* with homology to the inositol triphosphate receptor ($InsP_3R$) of higher eukaryotes (Traynor et al., 2000). $InsP_3R$ respond to an inositol triphosphate (IP_3) and is involved in the regulation of intracellular calcium levels in response to diverse stimuli. It has a binding site for IP_3 and a short region constituting a tetrameric calcium channel (Taylor and Laude, 2002; Foskett et al., 2010). $InsP_3Rs$ are located in the membranes of ER and play an important role in regulating calcium homeostasis (Taylor et al., 2009). Disruption of the type 1 $InsP_3$

receptor gene in mice results in embryonic lethality (Traynor et al., 2000). Mutations in the *Drosophila* $InsP_3$ receptor are lethal for survival beyond the larval stage and inhibit cell growth and differentiation (Schaloske et al., 2005). Deletion of the *iplA* gene in *Dictyostelium* blocks Ca^{2+} influx through the plasma membrane induced by cAMP stimulation. However, there was no effect on cAMP-induced chemotaxis (Shanley et al., 2006). Another research showed that IplA plays an important role in cell migration upon physical stimuli in a vegetative state (Lombardi et al., 2008; Hashimura et al., 2022).

In this study, to understand the Ca^{2+} signaling in diverse cellular processes, I investigated the functions of IplA, which is a typical Ca^{2+} channel protein responding to IP_3. In the study of part 1, I prepared cells lacking IplA and examined the phenotypes of the *iplA* null cells cell morphology, adhesion, development, chemotaxis, and electrotaxis. In part 2, I developed a *Dictyostelium*-specific calcium sensor and reporter and examined the intracellular dynamics of Ca^{2+} in response to several external stimuli, such as folate, cAMP, and high concentration of Ca^{2+}.

II. MATERIALS AND METHODS

II-1. Strains and plasmid construction

Dictyostelium Ax2 strains were grown in HL5 axenic media or in association with *Klebsiella aerogenes* at 22 ℃. The knock-out strains and transformants were maintained in 10 μg/mL blasticidin or 10~20 μg/mL G418. *iplA null* cell (DBS0236260) was obtained from the DictyBase Stock Center. For expression of GCaMP in *Dictyostelium,* the region of GCaMP3 in pGCaMP3 was amplified by PCR and subcloned into the *Bam*HI site of the *Dictyostelium* expression vector pDXA-3C using the Gibson assembly kit.

II-2. Cell adhesion assay

Cell adhesion assay was performed as described previously (Kim et al., 2021). Log-phase growing cells on the plates were washed and resuspended at a density of 2×10^6 cells/mL in develop buffer, and then were placed on a 6-well culture plates followed by shaking the plates at 150 rpm for 1 h. Total cells and the attached cells after agitating and aspiration of the detached cells were counted. Cell adhesion was presented as a percentage of attached cells compared with total cells.

II-3. Random motility

Vegetative cells were prepared as described in the cell adhesion assay. Log-phase growing cells on the plates were washed and resuspended at a density of 5×10^5 cells/mL in develop buffer, and then were placed on a 30 mm culture plates containing 3 mL of develop buffer. Images were recorded for 20 min with an interval of 30 sec. Migrating cells were tracked and then the trajectory speed of the migrating cells was calculated using ImageJ software (NIH).

II-4. Electrotaxis

Electrotaxis experiments using vegetative cells was performed as described previously (Kim and Jeon, 2022). Fully grown cells on 24 well plates were washed three times with development buffer (DB; 5 mM Na_2HPO_4, 5 mM KH_2PO_4, 2 mM $MgCl_2$ and 1 mM $CaCl_2$) and then subjected to electrotaxis experiments. For experiments with aggregation-competent cells, exponentially growing cells were washed twice and resuspended at a density of 5×10^6 cells/mL in DB buffer. The cells were pulsed with 30 nM cAMP every 6 min for 6 h. All procedures were carried out at room temperature (~22 ℃). The prepared cells were seeded in an electrotactic chamber for 20 min and the unattached cells were washed off using DB buffer. A roof of cover glass was placed on the cells within a trough and sealed with silicone grease. Each chamber was then filled with sufficient DB. EF was applied at the indicated field strength through agar salt bridges. Cell migration was recorded at intervals of 1 min for 1 h using an inverted microscope (IX71; Olympus) with a camera (DS-Fil; Nikon) controlled by the NIS-Elements software (Nikon).

Time-lapse recordings of cell migration were analyzed using ImageJ (NIH) as previously described (Kim and Jeon, 2022). Directedness quantifies how directionally cells migrate in response to an EF. The directedness of the movement of the cells was measured as cosine θ, where θ is the angle between the direction of the field and a straight line connecting the start and end positions of the cell. Trajectory speed was assessed by dividing the total distance travelled by the cell by time. All data were obtained and analyzed from at least three independent experiments. The kinetics of directedness and trajectory speed were calculated by measuring the directedness and trajectory speed of cell migration every 1 min in a time-lapse recording and sequentially plotting the readings against time.

II-5. Development and chemotaxis analysis

Exponentially growing cells were harvested and washed twice with 12 mM Na/K phosphate buffer (pH 6.1) and plated on Na/K phosphate agar plates at a density of 3.5×10^7 cells/cm^2. The developmental morphology of the cells was examined by photographing the developing cells with a phase-contrast microscope. The chemotaxis towards cAMP. The aggregation competent cells were prepared by incubating the cells at a density of 5×10^6 cells/mL in Na/K phosphate buffer for 10 h. Cell migration was analyzed using Dunn Chemotaxis Chamber (Hawksley). The images of chemotaxing cells were taken at time-lapse intervals of 1 min for 50 min using an inverted microscope. The data were analyzed by using NIS-elements software (Nikon)

II-6. Fluorescence image acquisition and analysis

The vegetative cell or aggregation competent cells were allowed to adhere to the plate for 10 min. The cells were uniformly stimulated by quickly pipetting 200 μL of stimulants into the plate containing cells. The fluorescence images were taken at time-lapse intervals of 2 sec for 1 min by using NIS-elements software (Nikon) and Image J software (National Institutes of Health). The intensity of the fluorescence in the cytosol was measured, and the relative level of GCaMP was calculated by dividing the intensity before stimulation (E_o) with the intensity at each time point (E_t).

II-7. Statistical analysis

Statistical analysis was performed using Student's t-tests (two-tailed). All data were collected from at least three independent experiments and expressed as the means ± standard error of measurement (SEM). P value less than 0.05 was considered as statistically significant.

III. RESULTS

III-1. Domain structure of IplA

IplA is composed of 3177 amino acids and has two RYDR domains, which are IP_3 attachment site, at the N-terminal region, a RIH domain, which constitutes a Ca^{2+} channel, and five transmembrane regions at the C-terminus (Fig. 1A). IplA is the only IP_3 receptor found in *Dictyostelium*. The IP_3R family proteins are found other several species. Multiple alignments using the amino-acid sequences of the full-length IplA family proteins showed that they all had a common feature of having two RYDR domains and one RIH domain (Fig. 1B). Phylogenetic analysis showed that *D. discoideum* IplA had the highest homology with *C. elegans* IP_3R (Fig. 1C).

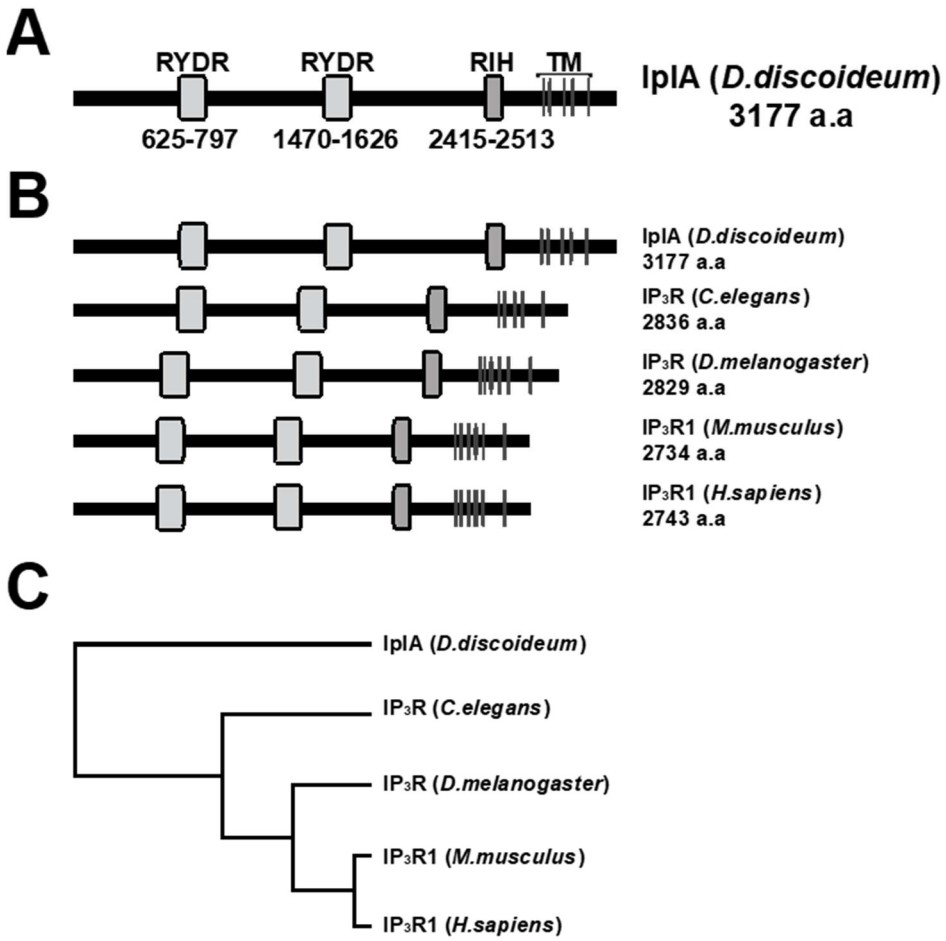

Fig. 1. IplA domain structure and phylogenetic analysis.

(A) Domain structure of *Dictyostelium* IplA. IplA contains two RYDR domains at the N-terminus and an RIH domain at the C-terminus. (B) Domain structure of IP_3 receptor family. (C) Phylogenetic tree of IP_3 receptor family. The full sequence amino acid of IplA and IP_3 family were aligned by MEGA7. IplA_*D.discoideum* (DDB_G0292564); IP_3R_*C.elecans* (Q9Y0A1); IP_3R_*D.melanogaster* (P29993-2); IP_3R1_*M.musculus* (P11881); IP_3R1_*H.sapiens* (Q14643).

III-2. Role of IplA in morphology and adhesion

To investigate the roles of IplA in the regulation of cell morphology and cell adhesion, the morphology and cell adhesion of *iplA* null cells were observed and compared to those of wild-type cells (Fig. 2). *iplA* null cells appeared slightly rounded and decreased size compared to wild-type cells. Quantification analysis showed that *iplA* null cells were smaller ($90 \pm 6\ \mu m^2$) than the wild type cells ($110 \pm 5\ \mu m^2$) (Fig. 1B). Examination of cell adhesion showed that *iplA* null cells had highly decreased cell adhesion strength (25 ± 1 %) compared to those of wild-type cells (70 ± 8 %) (Fig. 1C). The statistical analysis indicates that the difference in the cell size and cell adhesion between *iplA* null cells and wild-type cells was significantly meaningful. These results suggest that IplA plays an important role in the regulation of cell morphology and cell adhesion.

To examine the possible roles of IplA in cell migration, random motility of *iplA* null cells was compared to that of wild-type cells (Fig. 3). Vegetative cells were prepared, and random migration was recorded. *iplA* null cells showed approximately 2 times higher motility than wild-type cells. Quantifying the migration speed showed that the migration speeds of *iplA* null cells and wild-type cells were approximately 5.5 ± 0.6 μm/min and 3 ± 0.2 μm/min, respectively. Taken together with the results of cell adhesion, these results suggest that IplA plays a role in the regulation of migration speed by controlling cell adhesion.

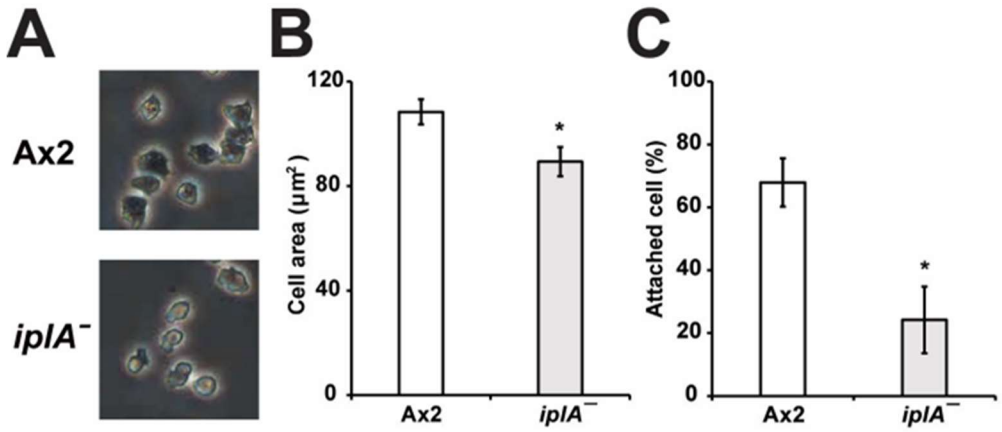

Fig. 2. Morphology and adhesion of *iplA* null cells.

(A) Morphology of *iplA* null cells. (B) Analysis of the cell area. The values (± SEM) of cell area from three independent experiments (* $p < 0.05$ compared to the control). (C) Cell-substrate adhesion. Adhesion of the cells to the substrate was expressed as a percentage of attached cells to total cells (* $p < 0.05$ compared to the control).

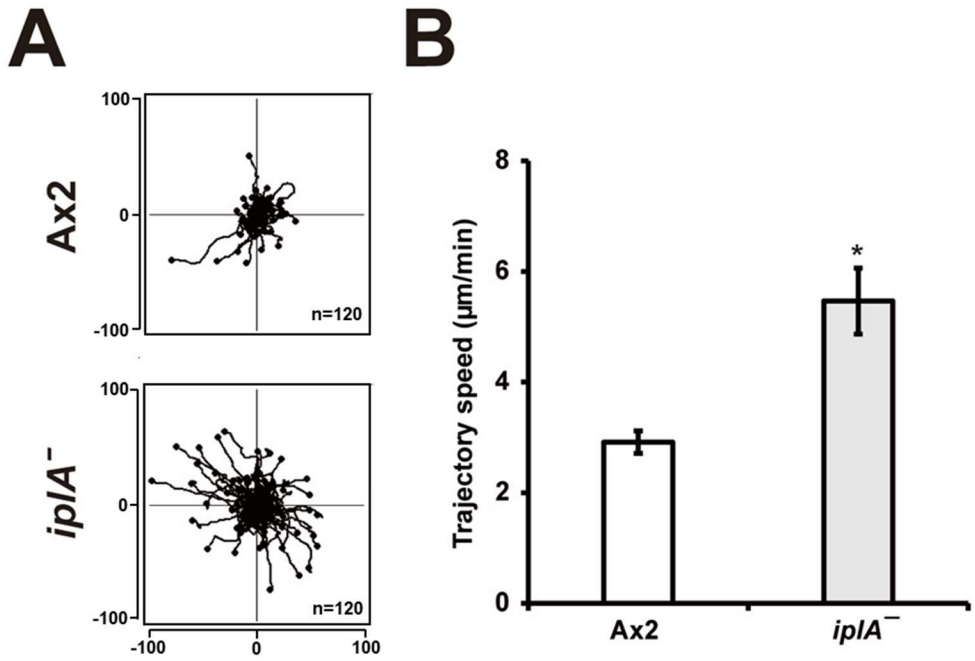

Fig. 3. Random motility of *iplA* null cells.

(A) Trajectory of random motility in *iplA* null cell. Plots show migration paths of the cells with the start position of each cell centered at point 0,0. (B) Quantification of migration speed of *iplA* null cell. Data are means ± S.E.M. from three independent experiments and statistical analysis was performed using the Student's t-test (* $p < 0.05$ compared to the control).

III-3. Role of IplA in electrotaxis

The previous random motility assay showed *iplA* null cells had increased migration speed compared to wild-type cells. To determine if IplA also plays some roles in electrotaxis, I examined the migration speed and the directedness of wild-type cells and *iplA* null cells in EF-directed cell migration. First, electrotaxis using the vegetative cells were examined (Fig. 4). Cell migration in the electrotaxsis chamber was recorded for 60 min at 1 min intervals. 10 V/cm of electric field was applied for 30 min (EF; 10 – 40 min for 60 min). The electric field was turned off at the first 10 min (Before) and the last 20 min (After) for 60 min recording to check random motility of the cells in the absence of electric field.

Upon exposure to EF, both wild-type Ax2 cells and *iplA* null cells showed similar pattern of an immediate response in trajectory speed. Trajectory speed gradually increased to the maximum level within 10 min and remained at the maximum level through the duration of the EF. Within 10 min of turning off the EF, the increased migration speed gradually decreased back to the basal level (Fig. 4). There was no distinct difference in the trajectory speed in EF-directed cell migration between the two strains, wild-type and *iplA* null cells (Fig. 4 B). However, there was a significant difference in the directedness between wild-type cells and *iplA* null cells. When the EF was applied, wild-type cells directionally moved to the cathode. Before applying EFs, cells moved randomly, indicated by the values of directedness, which was close to 0. When the EF was switched on, directedness highly increased to 0.4. In contrast, *iplA* null cells displayed complete loss of increase of directionality in an EF. Before turning on the EF, *iplA* null cells showed random motility, in which the directedness was close to 0. Even while the EF was turned on, the cells exhibited random motility (Fig. 4C). These results indicate that IplA is required for the control of the direction of movements, but not the migration speed, in electrotaxis using the vegetative cells.

Next, I examined EF-directed cell migration using the aggregation-competent cells, since chemotaxis assay usually uses aggregation-competent cells. Compared to the vegetative state, migration speed of both wild-type cells and *iplA* null cells highly increased, but the kinetics of the speed in response to an EF was similar to those using the vegetative cells (Fig. 5B). Interestingly, directionality of *iplA* null cells was normal compared to wild-type cells. *iplA* null cells showed transient increase of the directedness upon exposure to an EF as shown in wild-type cells (Fig. 5C). These results suggest that IplA is dispensable for EF-directed cell migration using the aggregation-competent cells.

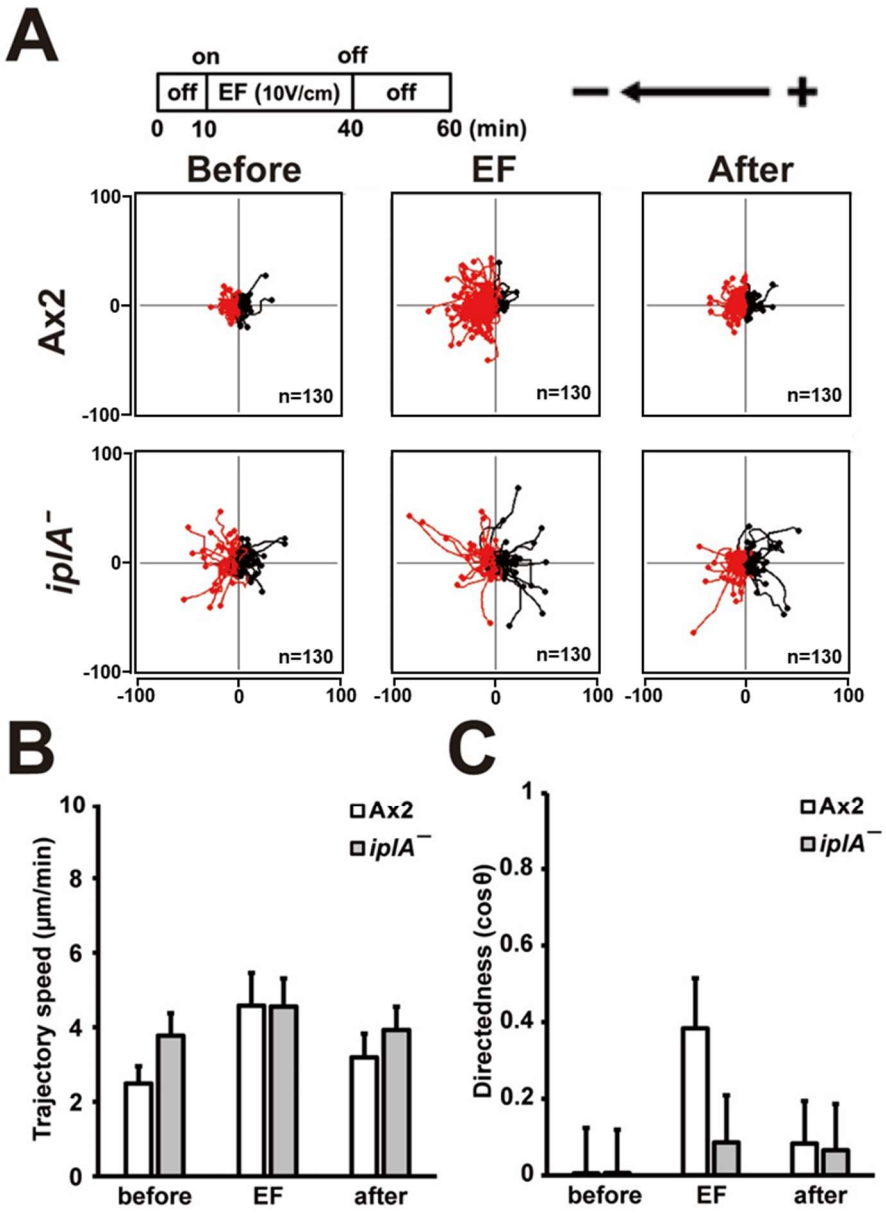

Fig. 4. Electrotaxis of *iplA* null cells in the vegetative state.

(A) Trajectories of wild-type and *iplA* null cells (Before 1~10 min, EF 31~40 min, After 41~50 min). (B) Quantitative analysis of the trajectory speed of *iplA* null cells. (C) Quantitative analysis of the directional migration of *iplA* null cells.

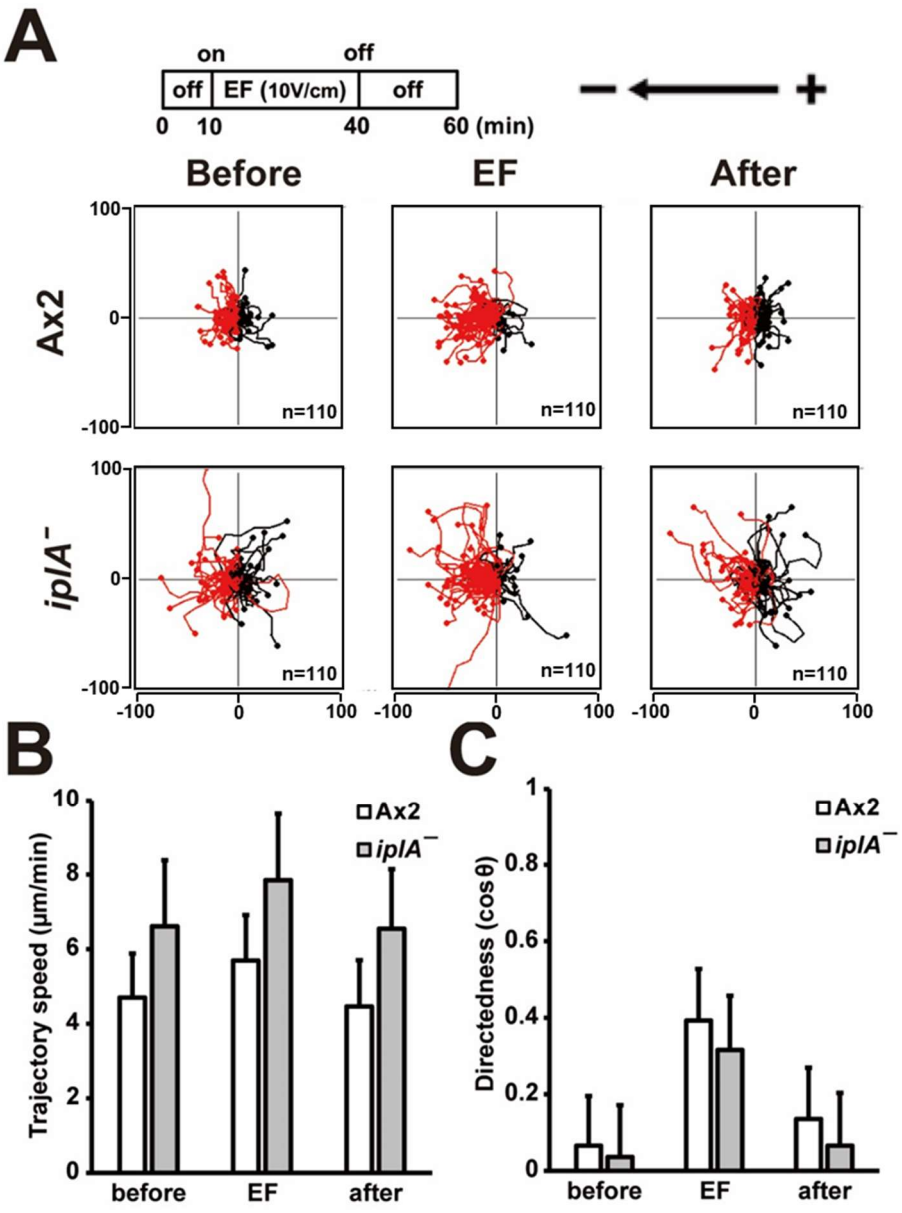

Fig. 5. Electrotaxis of *iplA* null cells in the developed aggregation-competent state.

(A) Trajectories of wild-type and *iplA* null cells. Aggregation competent cells were used in the experiment. (B) Quantitative analysis of the trajectory speed of *iplA* null cells. (C) Quantitative analysis of the directional migration of *iplA* null cells.

III-4. Role of IplA in chemotaxis and development

The previous random motility assay demonstrated that IplA were involved in the regulation of migration speed, possible through decreased cell adhesion to the substrate (Fig. 2 and 3). To determine the role of IplA in chemotaxis, I examined the phenotypes of *iplA* null cells in chemotaxis using a Dunn chamber and compared with those of wild-type cells. In this experiments, aggregation-competent cells were prepared by pulsing the cells with cAMP for 6 h in an interval of 6 min. Wild-type cells showed directional cell migration with the speed of approximately 5 μm/min and the directionality of approximately 0.6 (Fig. 6). *iplA* null cells showed increased migration speed of approximately 7 μm/min and slightly decreased directionality of 0.4, compared to wild-type cells (Fig. 6). *iplA* null cells progressed the normal developmental process similar to wild-type cells and showed slightly increased size of the spores in the fruiting bodies (Fig. 7).

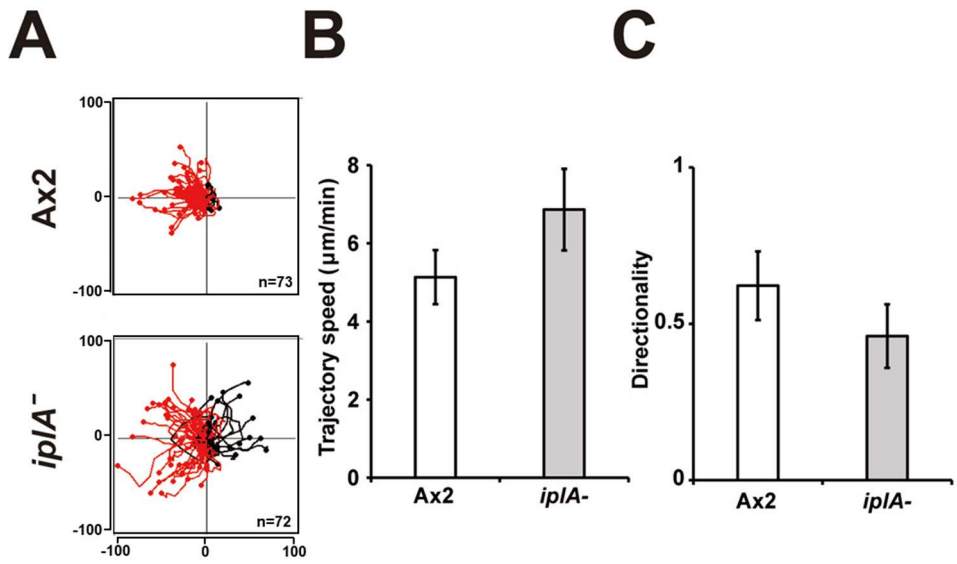

Fig. 6. Chemotaxis of *iplA* null cells.

(A) Trajectories of cells moving towards a gradient of cAMP chemoattractants. Aggregation-competent wild-type cells and *iplA* null cells were prepared and then placed in a gradient of cAMP in a Dunn chemotaxis chamber. Movements of the cells were recorded at intervals of 1 min for 50 min. Migration paths of the cells for 10 min (40~ 50 min) were tracked using ImageJ software and plotted with the start position of each cell centered at point 0.0. (B) Quantification of cell motility. Trajectories of cells during chemotaxis were traced and analyzed by using chemotaxis and migration tool in ImageJ software. Trajectory speed indicates the speed of the cell's movement along the total path. Directionality is a measure of how straight the cells move. Cells moving in a straight line have a directionality of 1.0. Error bars represent SD from three independent experiments.

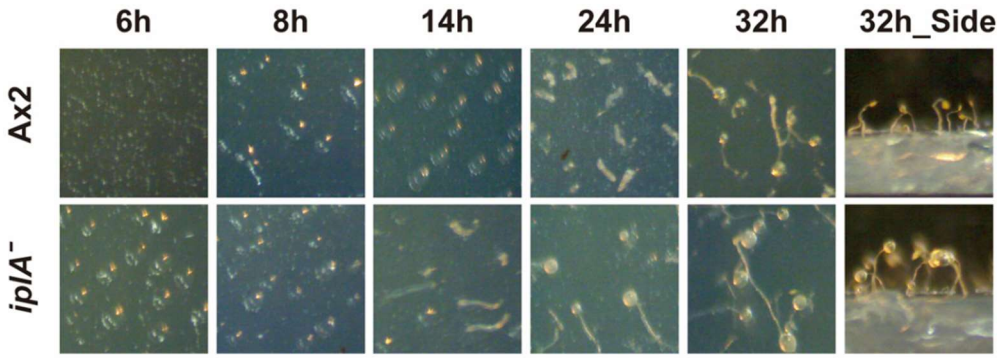

Fig. 7. Development of *iplA* null cells.

Development of *iplA* null cells of the cells on non-nutrient agar plates. Exponentially growing cells were washed and plated on non-nutrient agar plates. Photographs were taken at the indicated times after plating and representative developmental images at the developmental stages were shown.

III-5. Ca^{2+} sensor for live cell imaging in *Dictyostelium*

Vegetative *iplA* null cells had a severe defect in directionality during electrotaxis, suggesting that IplA plays an important role in the regulation of the direction of cell movements. To further understand the intracellular calcium dynamics in response to diverse external stimuli, I developed a *Dictyostelium*-specific Ca^{2+} reporter plasmid pDXA-GCaMP3 (Fig. 8B). The region of GCaMP in pGCaMP3, which is a Ca^{2+} reporter plasmid in mammalian cells (Tian et al., 2009), was amplified using the primers (table 1) and then subcloned into the *Bam*H1 site in the *Dictyostelium* expression vector pDXA-3C using the Gibson assembly kit (Fig. 8A).

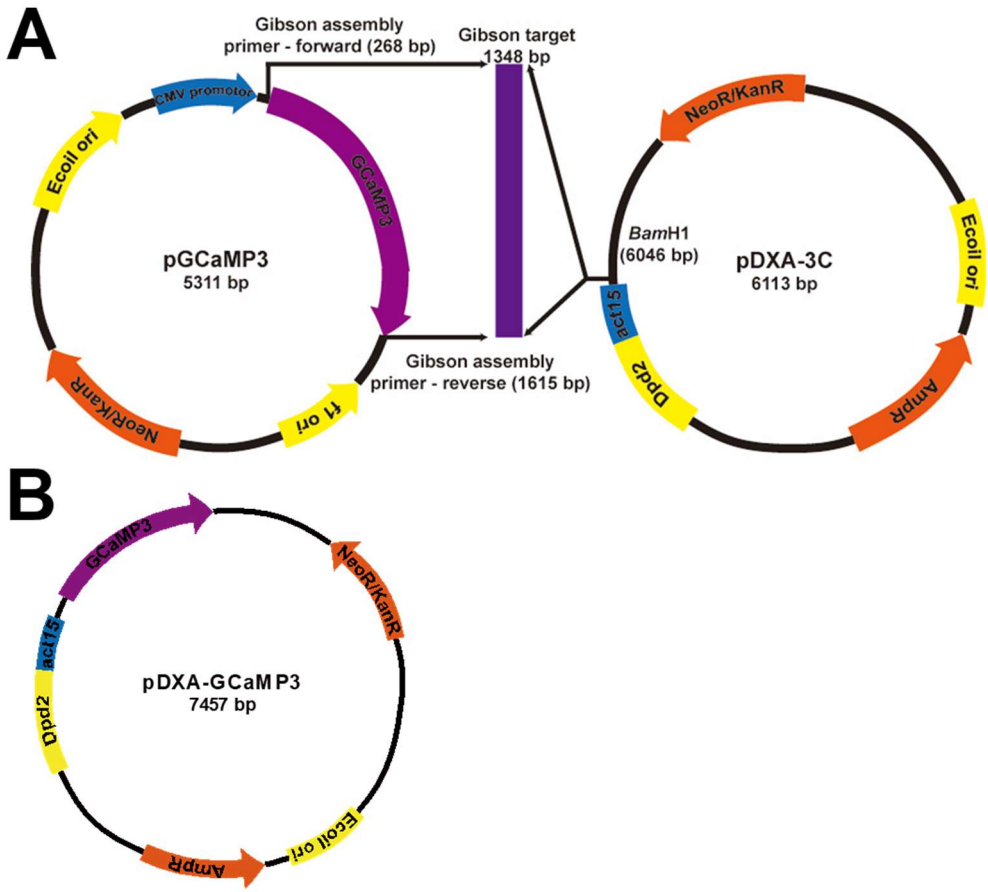

Fig.8. Schematic diagram for pDXA-GCaMP3.

(A) Subcloning the GCaMP region of pGCaMP3 into a *Dictyostelium*-specific expression vector pDXA-3C. (B) *Dictyostelium*-specific calcium sensor pDXA-GCaMP3.

Table 1. Primers used for GCaMP subcloning.

Primer	Sequence (5´ → 3´)
Gibson assembly – Forward	GGTACCGAGCTCGCTCATCATCA
Gibson assembly – Reverse	CTAATGCATCTCGAGTGGCTCACTTCG

III-6. Role of IplA in Ca^{2+} response

To investigate the dynamics of the intracellular Ca^{2+} levels, I performed uniform and global stimulation experiments using the *Dictyostelium*-specific Ca^{2+} sensor pDXA-GCaMP3. 100 μM of folate and cAMP chemoattractants were used to stimulate the cells of vegetative and aggregation-competent cells, respectively (Fig. 9). Both wild-type and *iplA* null cells showed no specific Ca^{2+} response to folate stimulation in the vegetative state. When the aggregation-competent cells were used, wild-type cells showed rapid and transient increase of the fluorescence, suggesting the increase of the intracelluar Ca^{2+} levels, whereas *iplA* null cells displayed constant intensity of the fluorescence (Fig. 9A and C). To quantify the induction of calcium levels, I measured the intensity of the fluorescence and graphed (Fig. 9B and D). Quantification of the fluorescence demonstrates transient increase with a peak at ~13 sec in response to cAMP chemoattractants (Fig. 9D). These results indicate that IplA is essential for the induction of Ca^{2+} level in response to cAMP stimulation in the aggregation-competent state, but not folate stimulation in the vegetative state.

To determine the intracellular Ca^{2+} response by depolarization stimulation, I performed the same global experiments as explained previously using high concentration of potassium (Fig. 10). There was no significant change in the fluorescence of both wild-type cells and *iplA* null cells when the cells were uniformly and globally treated with 50 mM potassium (Fig. 10A and C). Quantification of the fluorescence showed a gradual decrease of the intensity of the fluorescence, likely due to the spontaneous bleaching of the light (Fig. 10B and D). Even though this result showed no clear induction of the intracellular calcium, further confirmation experiments would be needed to determine if Ca^{2+} response is induced upon depolarization stimuli.

Ca^{2+} influx is induced through both the plasma membrane and the EF membrane. To determine if IplA plays some role in transporting the external Ca^{2+} into the intracellular, I investigated Ca^{2+} responses of the cells upon external Ca^{2+} stimulation (Fig. 11). The Ca^{2+} level of wild-type cells rapidly and transiently increased in response to the external 50 mM $CaCl_2$. In contrast, *iplA* null cells exhibited a delayed induction of Ca^{2+} in response to the external 50 mM $CaCl_2$ (Fig. 11A and C). Quantification of the fluorescence showed that wild-type cells had a maximum level of calcium at approximately ~5 sec, whereas *iplA* null cells showed approximately at 10~15 sec after stimulation (Fig. 11B and D). There was no distinct difference in the pattern of Ca^{2+} induction upon the external 50 mM $CaCl_2$ between the vegetative and the aggregation-competent states of the cells, though the aggregation-competent cells seemed to have a slightly late response. These results suggest that IplA plays some roles in the induction of intracellular Ca^{2+} in response to the external Ca^{2+} stimulation.

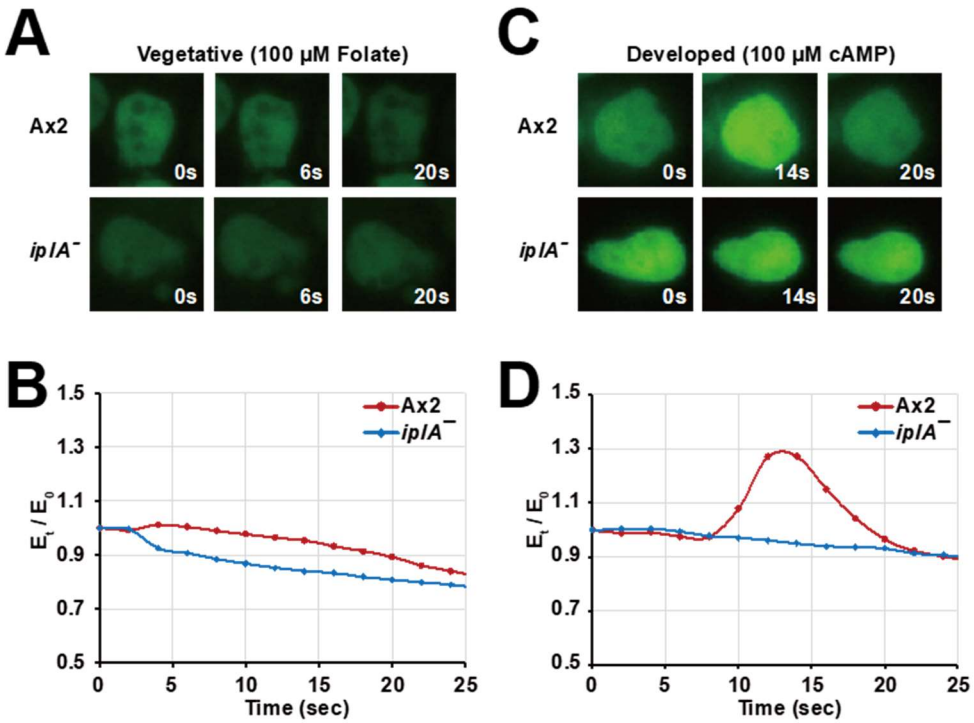

Fig. 9. Global Stimulation of GCaMPs by chemoattractants.

(A) Fluorescence of GCaMP proteins of vegetative cells after folate stimulation. (B) Quantification of the cytoplasmic fluorescence intensity of vegetative cells upon folate stimulation. (C) Fluorescence of GCaMP proteins of developed cells after cAMP stimulation. (D) Quantification of the cytoplasmic fluorescence intensity of developed cells upon cAMP stimulation.

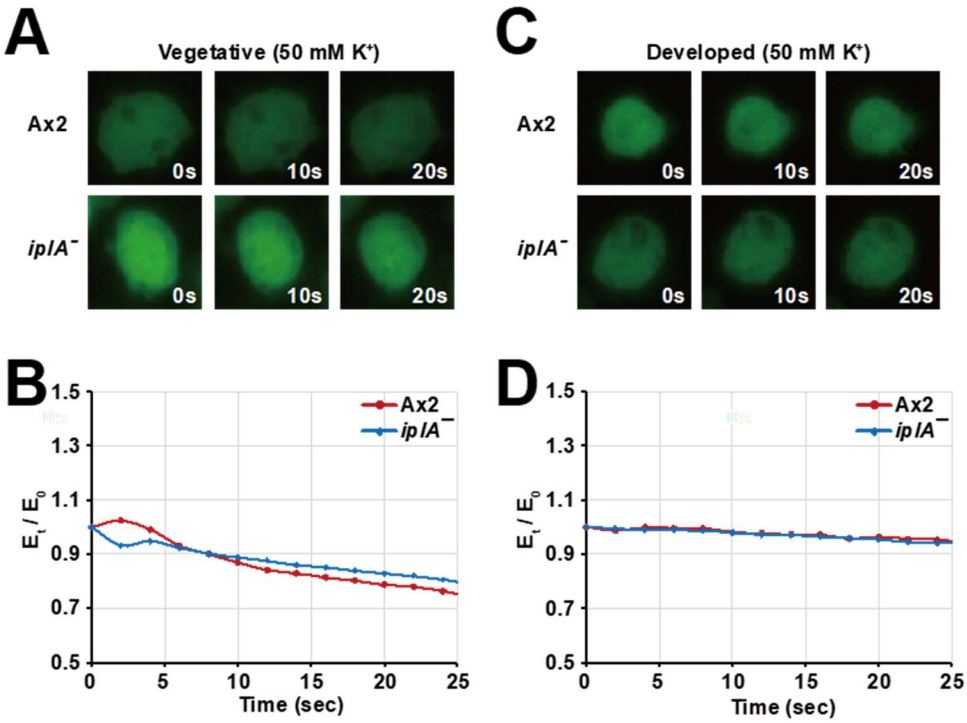

Fig. 10. Global Stimulation of GCaMPs by K^+

(A) Fluorescence of GCaMP proteins of vegetative cells after K^+ stimulation. (B) Quantification of the cytoplasmic fluorescence intensity of vegetative cells upon K^+ stimulation. (C) Fluorescence of GCaMP proteins of developed cells after K^+ stimulation. (D) Quantification of the cytoplasmic fluorescence intensity of developed cells upon K^+ stimulation.

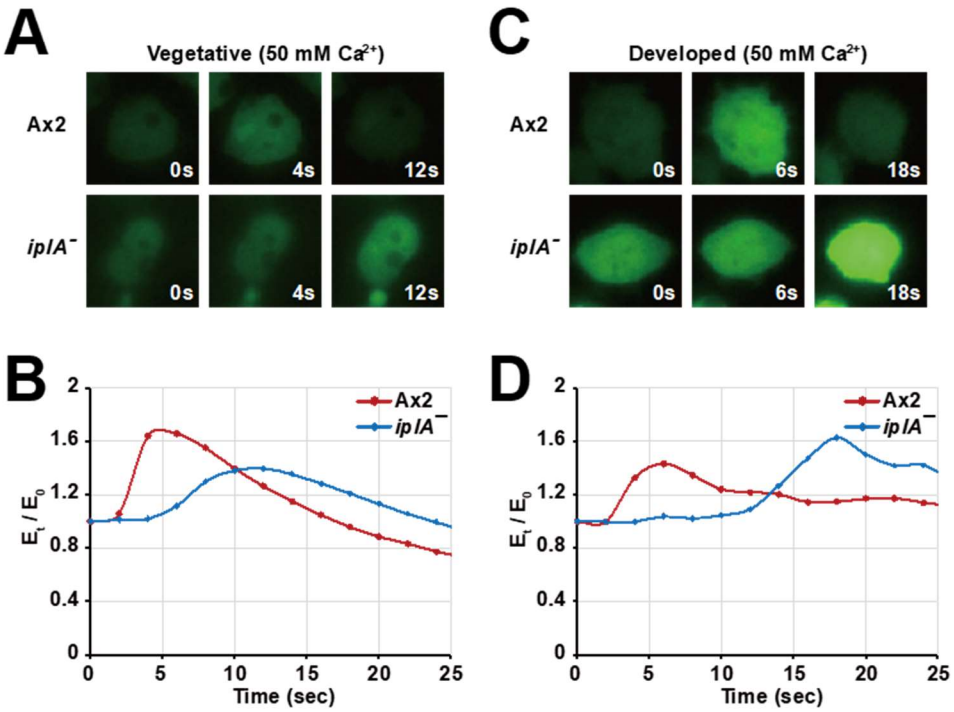

Fig. 11. Global Stimulation of GCaMPs by Ca^{2+}

(A) Fluorescence of GCaMP proteins of vegetative cell after Ca^{2+} stimulation. (B) Quantification of the cytoplasmic fluorescence intensity of vegetative cells upon Ca^{2+} stimulation. (C) Fluorescence of GCaMP proteins of developed cells after Ca^{2+} stimulation. (D) Quantification of the cytoplasmic fluorescence intensity of developed cells upon Ca^{2+} stimulation.

IV. DISCUSSION

Ca^{2+} signaling is important for directional cell migration and plays an auxiliary role in regulating cell orientation in chemotaxis (Lombardi et al., 2008). To study the roles of Ca^{2+} signaling in electrotaxis, I examined the phenotypes of *iplA* null cells in cellular processes including cell morphology, adhesion, development, chemotaxis, and electrotaxis. In addition, I developed a Ca^{2+} sensor for live-cell imaging to investigate cellular Ca^{2+} dynamics in response to diverse external stimuli.

IplA is required for an increase of intracellular Ca^{2+} level by extracellular Ca^{2+} influx and exflux from ER organelles in chemotaxis (Lusche et al., 2012). Cells lacking IplA showed decreased cell size and cell adhesion to the substrate, comparted to wild-type cells. *iplA* null cells displayed increased migration speed in all the migration experiments including random migration, chemotaxis, and electrotaxis. It is likely that elevated migration speed results from decreased adhesion of the cells to the substratum during migration. Interestingly, vegetative *iplA* null cells showed almost complete loss of directionality upon an EF stimulation while wild-type cells moved towards the cathode in response to an EF. Aggregation-competent *iplA* null cells showed directional cell migration to the cathode and the chemoattractants in electrotaxis and chemotaxis, respectively. These results indicate that IplA plays different roles in the regulation of directionality during cell movement depending on the developmental state; In the vegetative state, IplA plays a critical role for controlling the direction of cell movement in electrotaxis.

In the present study, I developed a *Dictyostelium* specific Ca^{2+} reporter plasmid (pDXA-GCaMP3) by subcloning the region of GCaMP in pGCaMP3, which is a Ca^{2+} reporter plasmid for mammalian cells (Tian et al., 2009), into the pDXA-3C using Gibson

assembly kits. This plasmid was transformed into wild-type and *iplA* null cells and the fluorescence of GCaMP was observed in response to diverse external stimuli. *iplA* null cells showed abolished Ca^{2+} response to cAMP stimulation, suggesting that IplA is required for Ca^{2+} induction in response to chemoattractant cAMP in chemotaxis. When the cells were treated with external high concentration of Ca^{2+}, *iplA* null cells exhibited a delayed intracellular Ca^{2+} induction comparted to wild-type cells. These results suggest that IplA is involved in the control of calcium influx from the outside. The *Dictyostelium* specific Ca^{2+} reporter, which was developed in this study, would contribute to understand Ca^{2+} signaling during cell migration as well as diverse cellular processes.

Cell morphology, adhesion, and cell migration are closely mediated through the regulation of the cytoskeleton. Ca^{2+} signaling is involved in the regulation of actin cytoskeleton affecting cell migration, cell adhesion as well as development (Giannone et al., 2002; Kamm and Stull, 2001; Lee et al., 1999). The most interesting phenotype of *iplA* null cells was the loss of directionality in electrotaxis. It has been previously reported that IplA is not required for electrotaxis using aggregation-competent cells (Shanley et al., 2006). In this study, I confirmed that *iplA* null cells at the developed aggregation-competent state showed normal directional cell migration in electrotaxis and chemotaxis. However, in the vegetative state, removal of IplA resulted in severe defect in the control of the direction during electrotaxis. This result demonstrates that IplA plays an important role in the directional sensing in response to an external EF in the vegetative state of the cells. Furthermore, it can be speculated that Ca^{2+} signaling may be involved in the regulation of directionality in electrotaxis using the vegetative cells. It has been recently reported that PI3K regulates cell mobility differently depending on the state of *Dictyostelium* cells (Kim and Jeon, 2022). Several signaling pathways including the PI3K pathway are involved in directional sensing and establishment of polarization in response to external stimuli (Chung et al., 2001). IplA is an IP_3 receptor and might be associated

with the PI3K signaling pathway. RasG is a key regulation of directionality during electrotaxis (Jeon et al., 2019). Further studies would be needed to determine if Ca^{2+} signaling is associated with the RasG/PI3K signaling pathways.

This study demonstrates that IplA is required for Ca^{2+} influx in response to cAMP chemoattractant stimulation. *iplA* null cells showed no response to cAMP, while wild-type cells immediately responded to cAMP stimulation and displayed increased levels of intracellular Ca^{2+}. This result is consistent with the previous findings that IplA is required for Ca^{2+} response by chemotactic stimulation (Schaloske et al., 2005; Traynor et al., 2000) and support that the *Dictyostelium*-specific Ca^{2+} reporter, which was developed in this study, would be used as a tool for further studies related to the Ca^{2+} signalings.

There are experimental difficulties in determining the direct Ca^{2+} response to electrical stimulation. In this study, I used an indirect method of inducting depolarization through extracellular potassium treatment (Gao et al., 2011). When high concentration of potassium was added into the plate containing the cells, there was no clear change in the intensity of the fluorescence of GCaMP in both wild-type cells and *iplA* null cells, suggesting that depolarization is unlikely to induce intracellular Ca^{2+} levels. When the cells were treated with high concentration of Ca^{2+}, *iplA* null cells showed a delayed Ca^{2+} induction compared to wild-type cells. This result indicates that IplA is involved in the influx of extracellular Ca^{2+}, suggesting that intracellular Ca^{2+} signaling is related to the phenotypes in cell morphology, adhesion, and cell migration. However, to explain the defects in the chemotactic migration of vegetative *iplA* null cells, further research is using a *Dictyostelium*-specific Ca^{2+} reporter is needed. This study will greatly contribute to understand the Ca^{2+} signaling in cell migration.

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