Abstract

**Background:** Stimulation of *Dictyostelium discoideum* with cAMP evokes an elevation of the cytosolic free Ca$^{2+}$ concentration ([Ca$^{2+}$]). The [Ca$^{2+}$]-change is composed of liberation of stored Ca$^{2+}$ and extracellular Ca$^{2+}$-entry. The significance of the [Ca$^{2+}$]-transient for chemotaxis is under debate. Abolition of chemotactic orientation and migration by Ca$^{2+}$-buffers in the cytosol indicates that a [Ca$^{2+}$]-increase is required for chemotaxis. Yet, the *iplA*-mutant disrupted in a gene bearing similarity to IP$_3$-receptors of higher eukaryotes aggregates despite the absence of a cAMP-induced [Ca$^{2+}$]-transient which favours the view that [Ca$^{2+}$]-changes are insignificant for chemotaxis.

**Results:** We investigated Ca$^{2+}$-fluxes and the effect of their disturbance on chemotaxis and development of *iplA* cells. Differentiation was altered as compared to wild type amoebae and sensitive towards manipulation of the level of stored Ca$^{2+}$: Chemotaxis was impaired when [Ca$^{2+}$]-transients were suppressed by the presence of a Ca$^{2+}$-chelator in the cytosol of the cells. Analysis of ion fluxes revealed that capacitative Ca$^{2+}$-entry was fully operative in the mutant. In suspensions of intact and permeabilized cells cAMP elicited extracellular Ca$^{2+}$-influx and liberation of stored Ca$^{2+}$, respectively, yet to a lesser extent than in wild type. In suspensions of partially purified storage vesicles ATP-induced Ca$^{2+}$-uptake and Ca$^{2+}$-release activated by fatty acids or Ca$^{2+}$-ATPase inhibitors were similar to wild type. Mn$^{2+}$-quenching of fura2 fluorescence allows to study Ca$^{2+}$-influx indirectly and revealed that the responsiveness of mutant cells was shifted to higher concentrations: roughly 100 times more Mn$^{2+}$ was necessary to observe agonist-induced Mn$^{2+}$-influx. cAMP evoked a [Ca$^{2+}$]-elevation when stores were strongly loaded with Ca$^{2+}$, again with a similar shift in sensitivity in the mutant. In addition, basal [Ca$^{2+}$], was significantly lower in *iplA* than in wild type amoebae.

**Conclusion:** These results support the view that [Ca$^{2+}$]-transients are essential for chemotaxis and differentiation. Moreover, capacitative and agonist-activated ion fluxes are regulated by separate pathways that are mediated either by two types of channels in the plasma membrane or by distinct mechanisms coupling Ca$^{2+}$-release from stores to Ca$^{2+}$-entry in *Dictyostelium*. The *iplA* strain retains the capacitative Ca$^{2+}$-entry pathway and an impaired agonist-activated pathway that operates with reduced efficiency or at higher ionic pressure.
Background
Aggregation of Dictyostelium discoideum proceeds by an oriented migration of the amoebae towards a source of the attractant CAMP which is synthesized and released periodically by cells in the center of the aggregate. Stimulation with CAMP activates liberation of stored Ca$^{2+}$ and extracellular Ca$^{2+}$-entry [1] leading to a [Ca$^{2+}$]$_{i}$-transient [2-4]. Chemotaxis proceeds in the presence of extracellular EGTA but not in the presence of intracellular Ca$^{2+}$ buffers, so a [Ca$^{2+}$]$_{i}$-elevation is necessary and release of stored Ca$^{2+}$ is sufficient for oriented migration [5]. On the other hand, the view that a [Ca$^{2+}$]$_{i}$-increase is essential for chemotactic response and aggregation of Dictyostelium involves questions as to alteration of the Ca$^{2+}$-homeostasis. Capacitative Ca$^{2+}$-entry was used to determine the rates of ion fluxes into cells periodically by cells in the center of the aggregate.

The authors concluded that the [Ca$^{2+}$]$_{i}$-elevation were detected [6]. From these results the assumptions that the ipLA gene product is central to the regulation of [Ca$^{2+}$]$_{i}$ and that its presence and thus the presence of an agonist-activated [Ca$^{2+}$]$_{i}$-increase is not necessary for proper chemotaxis and development. However, agents that interfere with IP$_{3}$-receptor mediated signaling such as XestosponginC [7] were found to influence not only CAMP-induced Ca$^{2+}$-fluxes but also the chemotactic response and aggregation of Dictyostelium [8]. In this study we aimed to clarify these conflicting findings and analyzed both, capacitative and chemoattractant-induced Ca$^{2+}$-fluxes and the effect of their disturbance on chemotaxis and differentiation of the ipLA mutant. Mn$^{2+}$-influx was used to determine the rates of ion fluxes into cells with filled and emptied stores and related to Ca$^{2+}$-electrode recordings in cell suspensions. We found that ion fluxes, chemotaxis and differentiation were sensitive towards alteration of the Ca$^{2+}$-homeostasis. Capacitative Ca$^{2+}$-entry was normal in the mutant and upon stimulation with agonist Ca$^{2+}$- and Mn$^{2+}$-fluxes occurred, yet to a considerably reduced extent. Spontaneous motility and chemotactic performance of mutant amoebae was strongly impaired by the intracellular presence of a Ca$^{2+}$-chelator.

Results
Extracellular [Ca$^{2+}$] affects development and chemotaxis of wild type and ipLA-
As ipLA cells formed fruiting bodies, albeit somewhat smaller in size, it was concluded that chemotactic aggregation and differentiation was normal [6]. We analyzed development of the mutant in parallel with wild type at various conditions. When cells differentiated on H5-agar plates (control situation) we consistently found a delay in the onset of aggregation by 1–2 h in the mutant; the smaller size of fruiting bodies was due to breaking of aggregation strands yielding smaller mounds (Fig. 1). Next we asked whether the absence or presence of Ca$^{2+}$ affects development. Differentiation on EGTA-containing agar plates and thus the steady reduction of internal Ca$^{2+}$-levels dose dependently resulted in a delay of aggregation and a decrease in the size of aggregates and fruiting bodies in both strains. Doses of 5–10 mM EGTA in the agar did not significantly alter the time point of aggregation which is in accordance with previous data [9] showing requirement of additional multiple washing of amoebae with EGTA in order to affect aggregation. At concentrations of 15–20 mM EGTA however, aggregation occurred at later time points (Fig. 2), on average at 13 ± 3 h in wild type and at 19 ± 2 h in ipLA- cells (mean ± s.e.m. from 5 experiments); despite the daily variations in aggregation timing the mutant strain was delayed as compared to the wild type in each of the experiments performed. On the other hand, the presence of Ca$^{2+}$ in the agar and therefore the continuous loading of cells with Ca$^{2+}$ [10] resulted in stronger impairment of aggregation in the wild type. The delayed aggregation of wild type cells in the presence of Ca$^{2+}$ was not due to inhibition of chemotaxis (see below).

Now the formation of aggregates was observed consistently at earlier time points in the ipLA- strain than in wild type (Fig. 3; on average 7 ± 0.5 h vs. 15 ± 5 h until aggregation formation in ipLA and wild type amoebae, respectively, at 20 mM CaCl$_{2}$ in 6 independent experiments); indeed, under this condition differentiation of ipLA- cells resembled that of wild type observed in the control situation.

Then we analyzed the effect of treatment with either EGTA or Ca$^{2+}$ on basal cell motility. We found that under control conditions the general morphology of the cells as well as extension of pseudopods was practically identical in both strains (Fig. 4 A, B, see Additional file 1). Preincubation with 10 mM EGTA for 60 min led to strong rounding of wild type and mutant amoebae (Fig. 4 C, D) with reduced extension of small pseudopods [see Additional file 2]. By contrast, pretreatment with 10 mM CaCl$_{2}$ did not affect the morphology (Fig. 4 E, F) or the extension of pseudopods [see Additional file 3] in both strains. Next we tested chemotaxis of amoebae towards a CAMP-filled glass capillary. Under control conditions cells of both strains oriented and migrated towards the tip of the capillary (Fig. 5 A, B); the average chemotactic speed was not different between wild type and the mutant strain (10.7 ± 2.1 vs. 11.0 ± 0.7 µm/min; mean ± s.e.m. of 20 wild type and 43 mutant amoebae analyzed in 3 and 4 independent experiments, respectively). Incubation in 10 mM EGTA for 60 min abolished chemotaxis in most of the wild type and the ipLA- amoebae: small pseudopods were extended randomly and the cells did not approach the capillary tip.
(Fig. 5 C, D). Only rarely, cells of both strains exhibited an oriented but highly reduced migration towards the cAMP source (3% and 5% of 33 wild type and 19 mutant cells analyzed in 3 independent experiments, respectively). Thus the loss of Ca²⁺ from stores impairs both, orientation and migration also in the absence of the iplA gene prod-
uct. By contrast, when amoebae were incubated in 10 mM CaCl₂ for 60 min to load stores, they oriented and migrated towards the cAMP capillary (Fig. 5 E, F). The chemotactic speed of wild type cells (9.9 ± 1.2 µm/min; mean ± s.e.m. of 17 cells tested in 3 independent experiments) was comparable to that under control conditions whereas mutant amoebae chemotaxed significantly faster (13.6 ± 1.7 µm/min; mean ± s.e.m. of 15 mutant amoebae analyzed in 3 independent experiments) than wild type cells in the presence of 10 mM CaCl₂ (Mann-Whitney rank sum test, p = 0.041).

**Buffering of intracellular [Ca²⁺] impairs chemotaxis**

The observation that aggregation occurred in the mutant cell line although a cAMP-activated increase in [Ca²⁺], was not detectable resulted in the conclusion that [Ca²⁺], changes were not necessary to accomplish chemotaxis [6]. We used the mobile buffer approach originally described by Speksnijder et al. [11] which allows to analyze the requirement of a [Ca²⁺],-gradient for a given response. If in *Dictyostelium* a [Ca²⁺],-increase was necessary for chemotaxis, the presence of a Ca²⁺-chelator in the cytosol should impair orientation and/or migration. In a previous study, we had introduced the Ca²⁺-chelator BAPTA and its derivatives into the cytosol of wild type amoebae which indeed had inhibited chemotactic migration and reduced chemotactic orientation [5]. Here we used the Ca²⁺-indicator Fura2-dextran to clamp [Ca²⁺], and loaded the indicator into wild type and mutant cells in the absence of external CaCl₂. The treatment affected chemotactic performance of wild type as well as iplA- amoebae. Lack of extracellular CaCl₂ during the loading process induced strong rounding of the amoebae and loss of migration. Figure 6 shows that the capacity to orient chemotactically and to extend pseudopods towards the capillary tip was reduced by 58% in wild type (93 cells tested in 4 independent experiments). Inhibition was also evident in iplA- cells (Fig. 6) showing sensitivity of the mutant towards buffering of intracellular Ca²⁺-levels and eradication of [Ca²⁺],-changes: the fraction of pseudopods extended in direction of the cAMP-source was reduced by 75% (74 cells tested in 3 independent experiments). These results show that not only in wild type but also in the iplA cell line the ability to orient and to migrate in fact depends on an agonist-activated [Ca²⁺],-elevation.

**Analysis of Ca²⁺-fluxes**

Our findings that differentiation was sensitive towards depletion of Ca²⁺ or loading of the cells with Ca²⁺ and that chemotaxis was blocked by intracellular Ca²⁺-buffers led us to investigate Ca²⁺-fluxes in the mutant cell line. We used a Ca²⁺-sensitive electrode in cell suspensions to measure Ca²⁺-fluxes, an approach different from that of Traynor et al. [6] who had studied ion fluxes by ⁴⁵Ca²⁺-measurements. First we tested whether the coupling of stores to the plasma membrane, i.e. capacitative Ca²⁺-fluxes without prior stimulation with agonists were altered. In *Dictyostelium* induction of capacitative Ca²⁺-influx requires active intracellular Ca²⁺-pumps. Their
Figure 4
General morphology of wild type and iplA- cells under control conditions (A, B), in the presence of 10 mM EGTA for 60 min (C, D) or in the presence of 10 mM CaCl2 for 80 min (E, F). In H5-buffer or in the presence of 10 mM CaCl2 the morphology was not significantly different between wild type and mutant amoebae. However, in the presence of EGTA the cells of both strains were rounded. Photographs were taken at t5. Basal motility under these conditions can be viewed in the accompanying movies.
Chemotaxis of wild type and mutant amoebae at different experimental conditions. The tracks of individual cells (in red) migrating during chemotactic stimulation (position of the tip of the cAMP-filled capillary: green star) are shown. In H5-buffer (A, B) both cell types migrated in an oriented manner towards the capillary tip, albeit not always in a straight line. After preincubation with 10 mM EGTA for 60 min and in its continued presence during the chemotaxis assay (C, D) the cells remained stationary with random pseudopod extension. Preincubation of amoebae with 10 mM CaCl₂ (E, F) did not impair chemotaxis; rather, the cells of both strains migrated towards the capillary tip. Chemotaxis experiments were done at tₖ.
inhibition by either thapsigargin or 2,5-di-(t-butyl)-1,4-hydroquinone (BHQ) does not evoke influx; rather, stores have to be emptied by treatment with EGTA [12]. Capacitative Ca\(^{2+}\)-fluxes were studied early during differentiation (t\(_3\)–t\(_4\)). At this time Ca\(^{2+}\)-influx and Ca\(^{2+}\)-efflux are at an equilibrium which held true for both, wild type and mutant cells (not shown); in suspensions of cells at later stages of development influx strongly exceeds efflux [1]. In \(i plA^*\) and in wild type cells emptying of storage compartments via preincubation of amoebae with EGTA induced capacitative Ca\(^{2+}\)-entry (Fig. 7 A) which was blocked by addition of 1 mM NaN\(_3\) (Fig. 7 B). The characteristics of influx were comparable in wild type and mutant cells. These data show that capacitative Ca\(^{2+}\)-influx does not depend on the product of the \(i plA\) gene.

On the other hand, agonist-activated 45Ca\(^{2+}\)-entry had been reported to be absent in the mutant strain; in their study, Traynor et al. had stimulated cells with cAMP in the presence of 0.1 mM CaCl\(_2\) [6]. The use of a Ca\(^{2+}\)-sensitive electrode allows to measure much lower levels of extracellular Ca\(^{2+}\) to analyze Ca\(^{2+}\)-fluxes, in the range of approximately 1 \(\mu M\) Ca\(^{2+}\). Indeed, we found that under this condition reversible Ca\(^{2+}\)-entry occurred after addition of 1 \(\mu M\) cAMP (Fig. 8 A) that amounted to 10.2 ± 4.3 pmol Ca\(^{2+}\)/10\(^7\) cells (mean ± s.d. from 9 experiments). The level of influx represented roughly 5% of wild type influx (Fig. 8 B and [13,14]). Ca\(^{2+}\)-influx was delayed in the mutant and the time to reach the maximum was longer than in wild type cells [13,14]. In addition, challenge with arachidonic acid (AA) induced influx (Fig. 9 A). Again, the mutant was less sensitive and higher concentrations were required than those reported to evoke Ca\(^{2+}\)-entry in wild type cells (Fig. 8 B, 9 C and [13]). Neither 10 nor 20 \(\mu M\) AA were effective; in the wild type 10 \(\mu M\) AA activates influx of 190 ± 58 pmol Ca\(^{2+}\)/10\(^7\) cells [14]. At 60 \(\mu M\) AA entry occurred in the mutant strain which amounted to an average of 107 ± 21 pmol Ca\(^{2+}\)/10\(^7\) cells (mean ± s.d. from 7 experiments). Preincubation of cells with the SERCA-type Ca\(^{2+}\)-ATPase inhibitor BHQ reduced AA-induced influx by 82% (Fig. 9 B) to an average of 21 ± 18 pmol Ca\(^{2+}\)/10\(^7\) cells (mean ± s.d. from 3 experiments). These data show that Ca\(^{2+}\)-fluxes across the plasma membrane do occur in \(i plA^*\) cells as well but at a reduced level.

Next we tested whether the mutant strain was able to release stored Ca\(^{2+}\) when stimulated with cAMP or AA. Fluxes were measured in suspensions of cells with permeabilized plasma membranes; any change in [Ca\(^{2+}\)]\(_{cyt}\) thus reflects efflux of Ca\(^{2+}\) from storage compartments. Both, cAMP and arachidonic acid activated release of stored Ca\(^{2+}\) (Fig. 10 A). On average, addition of 1 \(\mu M\) cAMP released 7.3 ± 3.4 pmol Ca\(^{2+}\)/10\(^7\) cells and 16.3 ± 7.2 pmol Ca\(^{2+}\)/10\(^7\) cells were liberated after stimulation with 3 \(\mu M\) AA (mean ± s.d. from 10 and 3 experiments, respectively). The amount of Ca\(^{2+}\)-efflux from stores after cAMP stimulation was 61% of that found in wild type cells (Fig. 10 B and [15]) whereas release upon AA-challenge was in the range of 5–10% of wild type (see Fig. 2 in [14]). In addition to the Ca\(^{2+}\)-electrode recordings, we studied Ca\(^{2+}\)-fluxes in suspensions of partially purified storage compartments fluorimetrically. ATP induced Ca\(^{2+}\)-sequestration (Table 1) was of similar magnitude and rate as in wild type stores. This result indicates that the decreased release of Ca\(^{2+}\) from the stores measured with the Ca\(^{2+}\)-electrode is not due to a lack of storage capacity. Moreover, the addition of AA evoked release of Ca\(^{2+}\) from stores as did inhibition of Ca\(^{2+}\)-pump(s) by thapsigargin. XestosponginC that inhibits Ca\(^{2+}\)-uptake and activates Ca\(^{2+}\)-release in wild type [8] and the ionophore ionomycin also resulted in substantial Ca\(^{2+}\) release in the mutant cell line (Table 1). All values were in the same range as those for wild type.

**Mn\(^{2+}\)**-quenching experiments

The [Ca\(^{2+}\)]\(_{cyt}\)-recordings in suspensions of cells as described above detect the sum of Ca\(^{2+}\)-influx and efflux. Therefore, a complementary approach which monitors influx only was pursued, by using the Mn\(^{2+}\)-quenching technique in single intact amoebae. This method is based on the fact that many Ca\(^{2+}\)-channels are permeable to Mn\(^{2+}\) [16] and...
that the \( \text{Ca}^{2+} \)-indicator Fura2 binds \( \text{Mn}^{2+} \) with high affinity. Fluorescence of the indicator is quenched upon binding [17]. We compared quenching of Fura2-dextran fluorescence activated by addition of \( \text{Mn}^{2+} \) alone or in combination with 1 \( \mu \text{M} \) cAMP in wild type and mutant cells. Higher concentrations of \( \text{MnCl}_2 \) were required to quench Fura2-dextran fluorescence in \( \text{iplA}^- \)-amoebae (Fig. 11, Table 2). Reduction of fluorescence occurred at 1 \( \mu \text{M} \) \( \text{Mn}^{2+} \) and 1 \( \mu \text{M} \) \( \text{Mn}^{2+}/\text{cAMP} \) in wild type (Fig. 11 A, B; see also [12]) but not in the mutant (Fig. 11 C, D) where addition of 1 \( \mu \text{M} \) \( \text{NaN}_3 \) (one out of 5/4 determinations in 3/3 independent experiments). Measurements were done at \( t_5 \).

In principle, the \( \text{iplA} \) gene product could form a channel in the plasma membrane or in membranes of internal stores. The lack of the \( \text{iplA} \) gene product in the stores might impair their coupling to the plasma membrane. As we had observed capacitative \( \text{Ca}^{2+} \)-entry in the mutant we asked whether manipulation of the filling state of the stores altered ion fluxes. First we tested the effect of emptying of stores on \( \text{Mn}^{2+} \)-influx. When cells were preincubated with EGTA, the requirement for high doses of \( \text{Mn}^{2+} \) to quench fluorescence was abrogated. Now capacitative and also agonist-activated \( \text{Mn}^{2+} \)-influx occurred at concentrations of \( \text{MnCl}_2 \) comparable to those used under control conditions in wild type, in the range of 1–2 \( \mu \text{M} \) (Fig. 11 E, F). This result renders the possibility that the plasma membrane is altered in the mutant unlikely. Yet, the rate of \( \text{Mn}^{2+} \)-influx observed in EGTA-treated mutant amoebae was still less than in wild type cells with respect to both basal and cAMP-activated fluxes (53 and 58% of wild type [12], respectively).

**[Ca\(^{2+}\)]\text{e}-determination**

In wild type cells treatment with EGTA augments responsiveness and cAMP-elicted \([\text{Ca}\(^{2+}\)]\text{e}-\)transients are detected at low extracellular \([\text{Ca}\(^{2+}\)]\) [12]. However, an agonist-induced \([\text{Ca}\(^{2+}\)]\text{e}-\)elevation was not observed in \( \text{iplA}^- \) cells under these conditions. On the other hand, when stores were loaded in the continued presence of \( \text{CaCl}_2 \), we observed that differentiation of the mutant resembled the
wild type as described above. This led us to compare the 
[Ca\textsuperscript{2+}] response of wild type and mutant amoebae af
dertreatment with CaCl\textsubscript{2}. In wild type cells preincubation
with 1 mM CaCl\textsubscript{2} for 10–15 min and its continued
presence during the [Ca\textsuperscript{2+}]\textsubscript{i}-imaging experiment is
required to activate a [Ca\textsuperscript{2+}]\textsubscript{i}-transient after chal
lenge with cAMP (Fig. 12 A and see [12]); in nominally Ca\textsuperscript{2+}-
free medium or at very low extracellular [Ca\textsuperscript{2+}] a cAMP-acti
vated [Ca\textsuperscript{2+}]\textsubscript{i}-elevation is not observed [12]. In accordance
with the data of Traynor et al. [6] this condition (Fig. 12 B)
and even increasing the concentration of CaCl\textsubscript{2} to 20
mM resulted in no detectable [Ca\textsuperscript{2+}]\textsubscript{i}-increase in the
mutant strain (not shown). However, we found that the
basal [Ca\textsuperscript{2+}]\textsubscript{i} level in \textit{iplA} amoebae was significantly lower
than in wild type and amounted to an average of 36 ± 3
nM (mean ± s.e.m. of 8 determinations in 3 independent
experiments) as compared to 50 ± 2 nM in wild type
(mean ± s.e.m. of 29 determinations in 9 independent
experiments; Mann-Whitney rank sum test, \(p = 0.002\)).

For stronger loading of Ca\textsuperscript{2+}-stores, we preincubated cells
with 1 mM CaCl\textsubscript{2} for 4 h. After this treatment a [Ca\textsuperscript{2+}]\textsubscript{i}-elevation upon addition of cAMP was detected in 60% of
wild type amoebae even at low extracellular [Ca\textsuperscript{2+}] levels, i.e. when the buffer used to wash the cells had been sup
plemented with only 1 µM CaCl\textsubscript{2} (Fig. 12 C). Starting
from a basal level of 48 ± 4 nM, the height of the [Ca\textsuperscript{2+}]\textsubscript{i}-
transient amounted to 23 ± 2 nM (mean ± s.e.m. of 12
determinations in 8 independent experiments). Again,
these conditions were not effective in \textit{iplA} cells; rather, the
sensitivity of the mutant was shifted to higher Ca\textsuperscript{2+} con
centrations as had been found with Mn\textsuperscript{2+}-quenching
experiments. We preincubated \textit{iplA} amoebae with 20 mM
CaCl\textsubscript{2} for 3 h; after washing thoroughly, a cAMP-induced
[Ca\textsuperscript{2+}]\textsubscript{i}-elevation in the presence of 1 mM CaCl\textsubscript{2} was
observed (Fig. 12 D) in 28% of the cells; its average height
amounted to 67 ± 11 nM (mean ± s.e.m. of 15 determina
tions in 4 independent experiments) starting from a basal
level of 39 ± 2 nM. In the course of these experiments we
once observed a response also under standard condi
tions, i.e. at 1 mM [Ca\textsuperscript{2+}]\textsubscript{i} without prior incuba
tion in 20 mM CaCl\textsubscript{2}; the height of the increase amounted to 54 ± 6 nM
(mean ± s.e.m.). Yet, this was a rare event (once in 31
determinations).
Discussion

The role of cAMP-activated \([\text{Ca}^{2+}]\)-changes for chemotaxis has been questioned by Traynor et al. [6] who reported results obtained with the \(i\text{plA}^{-}\) mutant cell line favouring insignificance of \([\text{Ca}^{2+}]\) for the chemotactic response. The authors had observed formation of fruiting bodies even though neither 45Ca2+-fluxes nor an agonist induced \([\text{Ca}^{2+}]\)-elevation were detectable. The discrepancy between our view that a \([\text{Ca}^{2+}]\)-elevation is necessary for a proper chemotactic response [5] and the conclusion of Traynor et al. prompted us to analyze chemotaxis, differentiation and the \([\text{Ca}^{2+}]\)-regulation of the \(i\text{plA}^{-}\) mutant in detail. In particular, we tested not only basal and cAMP-activated ion fluxes but also capacitative Ca2+-entry which is induced by emptying internal Ca2+-stores via preincubation of amoebae with EGTA [12].

Aggregation and development of wild type and mutant cells on agar plates was sensitive towards continuous emptying or loading of Ca2+-stores. These effects are not necessarily caused by altering chemotactic migration. It is conceivable that other Ca2+-dependent processes were affected, e.g. that the timing or pattern of gene expression or the establishment of cell contacts was altered. Although incubation of mutant amoebae for 2 h with 20 mM CaCl2 or of wild type with 1 mM CaCl2 for 4–5 h or with 1 mM EGTA for 1 h [12] did not significantly increase or lower basal \([\text{Ca}^{2+}]\), it is possible that the continued presence of 10 mM EGTA or CaCl2 for many hours affects basal levels of \([\text{Ca}^{2+}]\), which in turn might mediate effects on gene expression as was shown for prolonged incubation of cells with BHQ [18]. During development *Dictyostelium* cells form Ca2+-dependent and EDTA/EGTA-sensitive cell contacts that are mediated by gp24 and DdCAD-1.
Basal and cAMP-induced Mn2+-influx. Influx was assayed by quenching of Fura2-dextran fluorescence. (A, B) The response of wild type amoebae is shown for comparison; 1 µM Mn2+ ± 1 µM cAMP was added. *iplA* cells in nominally Ca2+-free buffer were challenged with 100 µM Mn2+ ± 1 µM cAMP at t7 (closed symbols); when 1 µM Mn2+ was added (open symbols) no influx was detected (C, D). After preincubation with EGTA influx was observed at 1–2 µM Mn2+ ± 1 µM cAMP (E, F). Fluorescence intensity at 360 nm excitation is shown as mean ± s.e.m.
Figure 12
[Ca^{2+}]_i-recordings in cells preincubated with CaCl_2 in order to load stores. (A) The response of wild type upon stimulation with 1 µM cAMP (arrow) at standard conditions, i.e. after preincubation with 1 mM CaCl_2 for 10–15 min and stimulated in the presence of 1 mM CaCl_2 is shown. Values give mean ± s.e.m. of 7 cells. (B) When iplA^- cells were stimulated with 1 µM cAMP at standard conditions (as outlined in (A)), no response was observed. Mean ± s.e.m. of 6 cells is shown. (C) Wild type was preincubated with 1 mM CaCl_2 for 4–5 h; after washing cells were incubated in H5-buffer supplemented with 1 µM CaCl_2 and challenged with 1 µM cAMP (arrow). Values give mean ± s.e.m. of 16 cells. (D) iplA^- incubated for 3 h with 20 mM CaCl_2 were washed and subsequently [Ca^{2+}]_i-imaging was done in buffer containing 1 mM CaCl_2. Arrow indicates the time point when 1 µM cAMP was added. Mean ± s.e.m. of 10 cells is shown.
These results strengthen the view that Ca2+ has a necessary role in chemotaxis in wild type and in the mutant as well. When the cellular Ca2+-content falls below a critical value Ca2+-dependent cytoskeletal rearrangements [22,23] that are necessary for both, random pseudopod extension during spontaneous motility and oriented pseudopod formation after chemotactic stimulation no longer take place correctly. On the other hand, the presence of 10 mM CaCl2 induced no alteration of basal cell motility in wild type or mutant amoebae. Yet, during chemotactic stimulation the average speed of migration towards the capillary tip was higher in mutant than in wild type cells. In this respect it is of importance that the basal level of [Ca2+]i was significantly lower in the former. At standard conditions the reduced basal [Ca2+]i does not impair the capacity of the mutant to chemotax. Therefore, this particular mutant strain represents the "minimal solution” with respect to the concentration of cytosolic Ca2+-necessary to accomplish cytoskeletal rearrangements and extrusion of a pseudopod correctly. However, in the presence of 10 mM extracellular Ca2+ during cAMP-stimulation Ca2+-fluxes are enhanced allowing more efficient formation of pseudopods. We had shown previously that a small global elevation of [Ca2+]i activates the extension of pseudopods all over the cell’s circumference whereas a larger increase induces contraction of the amoebae [24]. In our view, the strongest evidence that a [Ca2+]i-transient is necessary for the extension of pseudopods rests upon the experiment where a Ca2+-chelator was introduced into the cytosol of the amoebae. This treatment led to rounding of the amoebae and a general reduction of pseudopod formation (see also [5]). Upon stimulation with a cAMP-filled capillary, the extension of oriented pseudopods was greatly reduced and migration towards the capillary tip was abolished. As Speksnijder et al. [11] had pointed out the fact that the presence of a chelator has an effect shows that a [Ca2+]i-gradient is essential for a given response. In summary, these data support the notion that an elevation of [Ca2+]i is required to extend pseudopods; suppression of the [Ca2+]i-elevation inhibits motility in general. Upon chemotactic challenge with cAMP this [Ca2+]i-gradient has to be established in a locally restricted fashion in order to allow local, oriented pseudopod formation (see [2,25,26]); otherwise pseudopods would be extended in all directions (see above, [24]). Our results imply that in iplA cells such a [Ca2+]i-gradient occurs as well, either nonrestricted allowing extension of pseudopods at random sites during spontaneous motility or restricted locally after chemotactic stimulation leading to oriented pseudopod formation. The fact that in the mutant cell line cAMP-activated [Ca2+]i-changes were practically undetectable under our standard condition argue for a [Ca2+]i-increase that is either smaller and/or more restricted to distinct domains within the cell than in wild type amoebae. Indeed, in only one out of roughly 30 determinations did we observe a CAMP-activated [Ca2+]i-transient under standard conditions. These results imply a crucial role but not an absolute necessity of the iplA gene product for the regulation of CAMP-induced [Ca2+]i-changes.

By using a Ca2+-sensitive electrode in cell suspensions, we analyzed which aspects of [Ca2+]i are controlled by the iplA gene product. Besides studying agonist-induced Ca2+-fluxes we also investigated capacitative Ca2+-entry and found that this type of influx was similar in mutant and wild type cell suspensions. We obtained equivalent results by testing Mn2+-quenching of Fura2-dextran fluorescence which showed that capacitative entry is independent of the iplA gene product.

On the other hand, using the Ca2+-sensitive electrode, we found that in the iplA mutant the agonist CAMP and also AA did activate Ca2+-entry into intact cells. The difference between the data published by Traynor et al. [6] and our results is most likely due to the experimental conditions: the magnitude of the Ca2+-fluxes that we observed was considerably lower than in wild type cells and detectable at low extracellular [Ca2+] only. The 45Ca2+-flux studies had been performed at 100 µM external CaCl2; so the fraction of 45Ca2+ entering the cells was presumably too low to be detected reliably. Moreover, we found CAMP- and AA-induced Ca2+-release from stores in cells with permeabilized plasma membranes. These data show that CAMP-induced Ca2+-release from stores in iplA cells is functional. However, much like the Ca2+-influx, agonist-
activated liberation from stores was smaller than in wild type amoebae. In line with these results are the findings using Mn\textsuperscript{2+}-quenching to assay ion fluxes in intact single cells: higher doses of Mn\textsuperscript{2+} were necessary to detect influx.

There are several interpretations for the results above. (i) There are two types of channels responsible for Ca\textsuperscript{2+}-influx: one type being activated by emptying of the stores and sustaining capacitative Ca\textsuperscript{2+}-entry which is unaffected in \textit{iplA} cells and the other one mediating agonist-induced Ca\textsuperscript{2+}-fluxes, the latter being under the control of the \textit{iplA} gene product. The view that there are two strictly separated ion channels seems unlikely as under conditions of emptied stores cAMP-activated Mn\textsuperscript{2+}-quenching occurred in the mutant as well. (ii) The same channel(s) mediate capacitative and agonist-activated fluxes but upon stimulation with agonists it cannot be addressed properly when \textit{iplA} is disrupted. This implies a role of the protein in the libration of Ca\textsuperscript{2+} from the stores which is a prerequisite for the triggering of Ca\textsuperscript{2+}-entry [12]. In the mutant this cannot proceed normally so subsequent activation of Ca\textsuperscript{2+}-influx and the generation of a full [Ca\textsuperscript{2+}]\textsubscript{i}-increase is impaired. The results of the experiments where stores were strongly loaded with Ca\textsuperscript{2+} prior to stimulation support this notion. In this situation release from stores should be augmented. Indeed, in both, wild type and mutant cells, cAMP-activated [Ca\textsuperscript{2+}]\textsubscript{i}-elevations occurred at an extracellular [Ca\textsuperscript{2+}] (see Fig. 12) where without pretreatment no increase was observed. Presumably, release of Ca\textsuperscript{2+} from the filled stores contributed to the observed [Ca\textsuperscript{2+}]\textsubscript{i}-increase to a greater extent than under standard conditions. The requirement for 20 fold higher concentrations of CaCl\textsubscript{2} during preincubation to elicit an agonist-induced [Ca\textsuperscript{2+}]\textsubscript{i}-elevation in \textit{iplA} cells are most likely due to the reduction in Ca\textsuperscript{2+}-entry which necessitates a higher concentration gradient across the plasma membrane to fill the stores efficiently.

An as yet unresolved issue is the mechanism that induces Ca\textsuperscript{2+}-entry upon liberation of Ca\textsuperscript{2+} from the stores. From our data we conclude that in \textit{Dictyostelium} these signals are different when the stores are emptied by EGTA or by agonist-activated signaling cascades. Otherwise one cannot explain normal capacitative Ca\textsuperscript{2+}- and Mn\textsuperscript{2+}-influx induced by EGTA-treatment and a requirement for 100 fold higher ion concentrations to induce Mn\textsuperscript{2+}-entry by cAMP. If indeed the \textit{iplA} gene product constitutes an IP\textsubscript{3}\textsuperscript{-}receptor like channel that is located on membranes of stores the physical coupling of the receptor to channels in the plasma membrane as a mechanism to activate extracellular Ca\textsuperscript{2+}-entry [27] should be missing in the mutant. On the other hand, emptying of stores by EGTA-treatment influences not only the IP\textsubscript{3}\textsuperscript{-}sensitive store but also other stores and thus exerts a much more general effect on the cells. Studies using microarrays should reveal whether the expression of other genes is affected by the absence of \textit{iplA} and thus might give a clue how [Ca\textsuperscript{2+}]\textsubscript{i} is regulated in the mutant although one type of Ca\textsuperscript{2+}-store is malfunctional.

**Conclusion**

Our results show that Ca\textsuperscript{2+} fluxes and regulation of Ca\textsuperscript{2+} homeostasis take place in the \textit{iplA} mutant and that chemotaxis and development of the mutant are sensitive to disturbance of the Ca\textsuperscript{2+} homeostasis. In wild type cells and in cells lacking the \textit{iplA} gene changes in [Ca\textsuperscript{2+}]\textsubscript{i} are necessary to orient and to migrate chemotactically; their abolition causes loss of chemotaxis towards a cAMP source. The \textit{iplA} gene product exerts a crucial role in the control of basal [Ca\textsuperscript{2+}]\textsubscript{i} and of agonist induced Ca\textsuperscript{2+}-fluxes. It is not required to activate capacitative Ca\textsuperscript{2+}-influx. Thus the mechanisms responsible for capacitative and agonist-activated Ca\textsuperscript{2+}-fluxes are different.

**Methods**

**Materials**

Fura2-dextran and Fura2 were purchased from MobiTec; cAMP was from Boehringer.

**Cell culture**

\textit{D. discoideum} wild type strain Ax2 and the \textit{iplA} cell lines HM1049 and HM1038 (kindly provided by Dr. D. Traynor) were cultured as described [14] in the absence or presence of 10 µg/ml Blasticidin S, respectively. There was no difference between the two mutant strains with respect to the assays performed; therefore, results of measurements with either HM1038 or HM1049 are shown. Cells were washed by repeated centrifugation and resuspension in cold Sørensen phosphate buffer (17 mM Na\textsuperscript{+}/K\textsuperscript{+}-phosphate, pH 6.0). Amoebae were shaken at 2 × 10\textsuperscript{7} cells/ml, 150 rpm and 23°C until use. The time, in hours, after induction of development is designated t\textsubscript{c}.

**[Ca\textsuperscript{2+}]\textsubscript{i}-electrode recordings**

[Ca\textsuperscript{2+}]\textsubscript{i} in cell suspensions was recorded as described elsewhere [14]. Cells at t\textsubscript{5}-t\textsubscript{6} were washed by repeated centrifugation and resuspended at 5 × 10\textsuperscript{7} cells/ml in 5 mM Tricine, 5 mM KCl, pH 7.0. Permeabilization was done by addition of filipin (15 µg/ml) to cell suspensions exactly as outlined in [15]. Capacitative Ca\textsuperscript{2+}-influx was analyzed in cells with emptied storage compartments [12]; amoebae at t\textsubscript{2}-t\textsubscript{4} were incubated with 5 mM EGTA for 30 min before washing in the above buffer.

**[Ca\textsuperscript{2+}]\textsubscript{i}-determination and Mn\textsuperscript{2+}-quenching experiments**

Cells were loaded with Fura2-dextran (5 mg/ml + 1 mM CaCl\textsubscript{2}) at t\textsubscript{4}-t\textsubscript{5}, as described [12]. Aliquots (2–5 µl) of washed cells in H5-buffer (5 mM Hepes, 5 mM KCl, pH 7.0) were placed on glass coverslips and incubated in a humid chamber. 10–15 min prior to the experiment, 85–
88 µl of H5-buffer + 1 mM CaCl₂ were added. In a series of experiments to load stores, wild type and iplA cells were incubated with 1 mM CaCl₂ for 4–5 h and with 20 mM CaCl₂ for 2–3 h, respectively. Then they were thoroughly washed exactly as described previously [12] and incubated either in H5-buffer supplemented with 1 µM CaCl₂ (wild type; free [Ca²⁺] in the solution was measured to be 2–2.5 µM, see also [12]) or in H5-buffer +1 mM CaCl₂ (iplA); final volume was 90 µl. Single cell [Ca²⁺] imaging was performed at t₇–t₈ as described [14]; stimulation was done by adding 10 µl of cAMP (10 µM). For Mn²⁺-quenching assays, washed cells were incubated in H5-buffer and challenged with Mn²⁺ or Mn²⁺/cAMP. In order to study fluxes in cells with partially emptied internal storage compartments cells were preincubated with EGTA (10 µl of H5-buffer plus 0.1 mM EGTA for 1–2 h). 10–15 min prior to the experiment this solution was carefully removed and 100 µl of H5-buffer was added. This was repeated three times; final volume was 90 µl. Fluorescence quenching was measured at 360 nm excitation; influx rates are given as decrease of fluorescence units/sec.

**Measurement of Ca²⁺-fluxes in partially purified storage compartments**

Analysis of vesicular Ca²⁺-fluxes was done as described [8]. In brief, 3 ml of cells at t₁–t₆ (2 x 10⁸ cells/ml) in 20 mM Hepes, pH 7.2, were lysed by passage through Nuclepore filters. A final concentration of 3 % sucrose, 50 mM KCl, 1 mM MgCl₂, 20 µg/ml leupeptin, 1 µg/µl aprotinin, 2.5 mM dithiothreitol and 1 µM microcystin were added; unbroken cells were removed by centrifugation at 3000 g for 5 min. The supernatant was centrifuged again at 12000 g for 20 min. The sediment (P) was resuspended in 1 ml of the above buffer. The rate of uptake and release was determined in the pellet and supernatant fraction by measuring the extravascular [Ca²⁺] with Fura2.

**Chemotaxis assays**

Cells were analyzed for chemotaxis towards a capillary filled with 0.1 mM CAMP [28]. 250 µl of 1 x 10⁵ cells/ml in H5-buffer were pipetted onto a glass coverslip and allowed to settle for 60 min. Chemotaxis was recorded on a video recorder for 30–45 min. Chemotaxis was also assayed in the presence of EGTA or CaCl₂; then amoebae were incubated in the respective agents for 60 min before they were challenged with CAMP. Images were digitized and the behaviour of the cells was analyzed using a computer program written for this purpose. For determination of cell velocity, a square area of interest (AI) of variable size (usually roughly 1/3 of the area of the cell) was placed at the perimeter of the cell in the first image digitized at the beginning of the assay. In the next image (images were digitized at a 2–4 sec time interval) the program analyzed an area larger than the AI (this area was defined by adding a given number of pixels on each side of the AI) for a pattern that resembled that of the AI; when such a pattern was found then the AI was placed on this new spot. The difference between the position of the AI in the first image to that in the second image was expressed as a vector of a given length. The changes in cell shape during migration were compensated by updating the pattern within the AI for every consecutive image analyzed. Calibration of the system allowed to convert the sum of the vector lengths to the distance in µm that the cells had migrated at the end of the experiment and to calculate the velocity of the amoebae. To test the effect of the intracellular presence of a Ca²⁺-buffer on chemotaxis, cells were loaded with Fura2-dextran (5 mg/ml in the loading solution) by electroporation in the absence of added external Ca²⁺. The amount of indicator present in the cytosol is in the range of 2–5% of the concentration present during electroporation [29]. 20 min after loading, cells were stimulated for 3–4 min by placing the cAMP-filled glass capillary at a distance of 10–20 µm of the cells and the number of cells that extended oriented pseudopods and thus elongated towards the capillary tip within this time period was counted. We had shown previously that loading of amoebae with FITC-dextran as a control does not alter chemotaxis as compared to untreated cells [5].

**Analysis of differentiation**

Time lapse recordings of the development of Ax2 and iplA cells on 1.5 % agar in H5-buffer (H5-agar) were done by placing 4 x 10⁶ cells each on one half of a petri dish (Ø 35 mm) at t₁. The two populations were separated from each other by a thin plastic disc that had been inserted in the melted agar during cooling. Only after removal of fluid and slight drying of the plate the disc was removed which resulted in a thin rim separating the strains. Differentiation was recorded by capturing an image of the plate every 30 min using a stereo microscope (Stemi 2000, Zeiss) equipped with a CCD camera (AVT Horn) under the control of the AxioVision software package (Zeiss). In addition, development was assessed at various levels of extracellular CaCl₂. Then H5-agar contained either 5–20 mM EGTA or 5–20 mM CaCl₂.

**List of abbreviations**

- Cytosolic free Ca²⁺ concentration: [Ca²⁺]ᵢ
- 2,5-di-(t-butyl)-1,4-hydroquinone: BHQ
- Arachidonic acid: AA
- Area of interest: AI

**Authors’ contributions**

RS recorded extracellular [Ca²⁺] in cell suspensions and participated in the design of the study. DFL participated in the recordings of extracellular [Ca²⁺] and the design of the...
study. KBR analyzed chemotaxis and differentiation of wild type and mutant cells. KH performed \([\text{Ca}^{2+}]\) measurements. DM analyzed fluxes in partially purified storage compartments and was involved in the design of the study. CS participated in \([\text{Ca}^{2+}]\) measurements, did Mn\(^{2+}\)-flux studies, participated in the design of the study and wrote the manuscript.

### Additional material

**Additional File 1**

Spontaneous cell motility of wild type and \(iplA\)-cells in H5-buffer. Images of cells at \(t_1\) were captured every 15 sec for 20 min. [Click here for file](http://www.biomedcentral.com/contentplementary/1471-2121-6-13-S1.mov)

**Additional File 2**

Basal cell motility after preincubation of wild type and mutant amoebaee with 10 mM EGTA for 60 min \((t_1-t_2)\). Images of cells at \(t_2\) were captured every 15 sec for 20 min in the continued presence of EGTA. Cells were rounded and extended smaller pseudopods than under control conditions. [Click here for file](http://www.biomedcentral.com/contentplementary/1471-2121-6-13-S2.mov)

**Additional File 3**

Cell motility of wild type and mutant cells after preincubation with 10 mM CaCl\(_2\) for 80 min \((t_1-t_3)\) is shown. Images of cells at \(t_3\) were captured every 15 sec for 20 min in the continued presence of CaCl\(_2\). The behaviour of treated cells was not different from control amoebaee. [Click here for file](http://www.biomedcentral.com/contentplementary/1471-2121-6-13-S3.mov)

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