Fatty acids induce release of Ca^{2+} from acidosomal stores and activate capacitative Ca^{2+} entry in *Dictyostelium discoideum*

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cAMP-induced Ca^{2+} fluxes in *Dictyostelium discoideum* largely depend on phospholipase A_2 activity generating non-esterified fatty acids [Schaloske and Malchow (1997) Biochem. J. **327**, 233–238]. In the present study the effect of fatty acids on Ca^{2+} homoeostasis in *D. discoideum* was investigated. Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was analysed by digital imaging of single fura 2–dextran-loaded cells. Arachidonic acid and linoleic acid induced a transient increase in $[Ca^{2+}]_i$. The concentration of arachidonic acid determined the percentage of responding cells, with the mean height of the increase being dose-independent. In nominally Ca^{2+}-free medium or in the presence of bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (BAPTA), no $[Ca^{2+}]_i$ transient was detectable. In spite of this, we found that (1) arachidonic acid induced Ca^{2+} release from permeabilized cells and from vesicular fractions at concentrations that elicited Ca^{2+} influx in intact cells and (2) Ca^{2+} entry was inhibited by inhibitors of Ca^{2+}-transport ATPases and V-type H^+-ATPase, indicating that intracellular Ca^{2+} release precedes Ca^{2+} entry. Inhibition studies and mutant analysis point to the acidosomal Ca^{2+} stores as a target of fatty acids. Although fatty acids can substitute fully for cAMP with respect to Ca^{2+} influx in wild-type cells, experiments with a mutant strain revealed that cAMP also sensitizes the Ca^{2+}-entry mechanism: cAMP-induced Ca^{2+} influx was normal in a phospholipase C knockout mutant but influx was fairly insensitive to arachidonic acid in this strain. This defect could be overcome by higher doses of arachidonic acid which cause sufficient Ca^{2+} to be released from the stores to trigger extracellular Ca^{2+} entry.

INTRODUCTION

In the asexual life cycle of the cellular slime mould *Dictyostelium discoideum* regulation of Ca^{2+} homoeostasis plays a key role. During aggregation the chemotactic signalling cascade activated by the attractant cAMP induces transient changes in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) [1,2]. The basis for these changes is both release of Ca^{2+} from storage compartments and entry across the plasma membrane. The second messengers inositol trisphosphate (IP_3) and cGMP which are formed after cAMP stimulation are involved in liberation of Ca^{2+} from non-mitochondrial stores and influx of extracellular Ca^{2+} respectively (for a review, see [3]). However, the precise chain of events that co-ordinate release and entry is not fully understood.

The Ca^{2+}-storage compartments of *Dictyostelium* are intimately involved in the regulation of the agonist-induced Ca^{2+} fluxes. Two types of stores have been identified by Ca^{2+}-electrode measurements of both intact and permeabilized cells in suspension or fluorimetric Ca^{2+} measurements of partially purified vesicular fractions [4,5]. One type was shown to be IP_3-responsive; pharmacological characterization revealed sensitivity of its sequestering mechanism to low amounts of the sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase-type Ca^{2+}-ATPase blocker 2,5-di-(t-butyl)-1,4-hydroquinone (BHQ) and the V-type H^+-ATPase inhibitor 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (Nbd-Cl) [4]. The second type of storage compartment is of endosomal origin; it makes use of an H^+ gradient across its membrane to sequester Ca^{2+} [6]. These acidosomes are sensitive to the V-type H^+-ATPase inhibitors bafilomycin A_1 [6] and concanamycin A (CMA) [7], and to vanadate and high concentrations of BHQ [5]. The physiological agent that releases Ca^{2+} from the acidosomes is not known.

Receptor stimulation activates release of Ca^{2+} from the storage compartments and this is followed by Ca^{2+} uptake as measured with Ca^{2+}-sensitive electrodes in cell suspension [8]. The use of permeabilized cells revealed that the addition of cAMP led to uptake of Ca^{2+} into the IP_3-sensitive store [4]. The characteristics of this uptake were similar to those of Ca^{2+} entry across the plasma membrane of intact cells, both with respect to the amount of Ca^{2+} and the time course; this argued for a major role of the IP_3-sensitive store in agonist-induced Ca^{2+} entry and indicated capacitative store refilling [4]. However, how this storage compartment communicates with the plasma membrane and whether the acidosomes also participate in this process is not known. Candidates that could be involved in this regulation of Ca^{2+} homoeostasis are fatty acids; they have been shown to exert effects in a variety of other cell systems, by either inducing Ca^{2+} release from stores [9–11] or regulating extracellular Ca^{2+} entry [12]. Indeed, in differentiating *Dictyostelium* cells, cAMP-induced Ca^{2+} influx was inhibited by agents that affect phospholipase A_2 and mimicked by arachidonic acid and palmitic acid; activation of phospholipase A_2 seems to play a key role in chemotactic signal transduction leading to Ca^{2+} entry [13]. At present, the physiologically relevant fatty acid formed after cAMP stimulation is not known. The occurrence of arachidonic acid itself has not been reported and it is likely to be one or a combination of the various other fatty acids that are present [14–16].

In this study we aimed to analyse the mode of action of fatty acids ultimately leading to Ca^{2+} influx and thereby to obtain more information about the role of this second messenger in the

Abbreviations used: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; $[Ca^{2+}]_e$, extracellular Ca^{2+} concentration; IP_3, inositol trisphosphate; BHQ, 2,5-di-(t-butyl)-1,4-hydroquinone; Nbd-Cl, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole; CMA, concanamycin A; BAPTA, bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid; LY, Lucifer Yellow; PLC, phospholipase C.

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chain of events that make up the signalling cascade activated by cAMP in developing cells. First we addressed the question of whether the observed influx of Ca^{2+} is reflected by a change in $[Ca^{2+}]_i$, secondly whether influx is the result of a direct effect of fatty acids on the plasma membrane or due to an action on intracellular Ca^{2+}-storage compartments leading to store-operated Ca^{2+} entry, and finally which type of store is affected by the fatty acids. The effects of arachidonic acid and other polyunsaturated fatty acids on $[Ca^{2+}]_i$ of wild-type cells, on Ca^{2+} fluxes across the plasma membrane and on Ca^{2+} stores were investigated by $[Ca^{2+}]_i$ imaging of single cells and by Ca^{2+}-electrode recordings of suspensions of cells; pharmacological approaches and mutant analysis were used to obtain information on the signalling mechanism(s). We found that addition of arachidonic acid induced a transient increase in $[Ca^{2+}]_i$; fatty acids evoked release of Ca^{2+} from storage compartments and extracellular Ca^{2+} influx. The acidosomal Ca^{2+} store was affected by arachidonic acid, suggesting that fatty acids constitute the physiological trigger for release of Ca^{2+} from acidosomes.

MATERIALS AND METHODS

Materials

Arachidonic acid was from Fluka (Buchs, Switzerland) or ICT (Frankfurt, Germany); linoleic acid was purchased from ICT, and palmitic acid from Sigma (München, Germany). Bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (BAPTA), fura 2 and fura 2–dextran were obtained from MobiTec (Göttingen, Germany), and BHQ was from Aldrich (Steinheim, Germany). CMA was purchased from Fluka, and bafilomycin A_1 was from Biomol (Hamburg, Germany).

Culture of cells and induction of differentiation

Wild-type strain Ax2 was grown, with shaking, in liquid medium [17] supplemented with 5 μg/l vitamin B_{12} and 200 μg/l folic acid as described [18]. Mutant strain HD10 was grown in shaking culture in the above medium to which 100 μg/ml ampicillin and 20 μg/ml G418 were added. Mutant strain HGR8 was cultivated in medium supplemented with 7.5 mM methylene diphosphonate as described [19]. Differentiation was induced by washing cells free of medium three times in ice-cold Sørensen phosphate buffer [SP buffer: 17 mM $(KH_2/Na_2H)PO_4$, pH 6.0] as described [20]. Cells (2×10^7/ml) were shaken (150 rev./min) on a rotary shaker at 23 °C in the dark until use. Time x hours after induction of development is designated t_x.

Measurement of extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$)

$[Ca^{2+}]_e$ was determined as described [21]. Briefly, cells were washed in 5 mM Tricine/5 mM KCl, pH 7.0 (Tricine buffer) and adjusted to a density of 5×10^7 cells/ml; 2 ml of cell suspension was stirred and aerated in a cuvette. $[Ca^{2+}]_e$ was measured with a Ca^{2+}-sensitive electrode (ETH 1001; Möller, Zürich, Switzerland). Electrode recordings of Ca^{2+} flux across intracellular stores were made with cells permeabilized with filipin (15 μg/ml) in the presence of 1 mM $MgCl_2$ and 1 mM ATP [8].

Measurements of $[Ca^{2+}]_i$

Cells were loaded with the Ca^{2+} indicator fura 2–dextran by electroporation as described [2]; briefly, 50 μl of cell suspension (2×10^7 cells/ml) was centrifuged, resuspended in 20 μl of ice-cold fura 2–dextran solution (5 mg/ml in SP buffer + 1 mM Ca^{2+}) and pulsed once with an electroporator (GenePulser; Bio-Rad) at 850 V, 200 Ω and 3 μF. Then 80 μl of 5 mM $MgCl_2$ was added and the cells were kept on ice for 10 min. After a wash in 5 mM Hepes/5 mM KCl, pH 7.0 (H5 buffer), 10 μl aliquots of cell suspension were placed on glass coverslips and incubated in a humid chamber. Usually, cells were porated at t_2–t_3; $[Ca^{2+}]_i$ imaging was carried out at t_5–t_7. Resealing of cells after electroporation under conditions comparable with those of our study occurred within seconds when large pores permeable to FITC–BSA or dye-coupled dextrans were analysed [1]; pores permeable to [^{3}H]inositol were shown to reseal within 10–15 min when harsher electroporation conditions were applied with two pulses at 7000 V [22]. We used Lucifer Yellow (LY) to analyse resealing of small pores after electroporation: the fluorescence of cells porated with LY (1 mg/ml) was measured with a fluorimeter (model 650-10S; Perkin–Elmer; excitation wavelength 425 nm; emission wavelength 536 nm) and compared with the fluorescence of cells incubated with 1 mg/ml LY for 5 min on ice at different time points after electroporation. When LY was added directly after electroporation, the cells showed higher fluorescence than control cells (incubated with LY without prior electroporation) but below that of cells porated in the presence of LY. At 5–6 min after electroporation, cells were resealed, as incubation with LY no longer resulted in increased fluorescence compared with control cells.

At 10 min before $[Ca^{2+}]_i$ recordings, buffer covering the cells was replaced by 90 μl of fresh H5 buffer with or without Ca^{2+}. $[Ca^{2+}]_i$ imaging was performed with an AxiovertT100 (Zeiss, Jena, Germany). The cells were viewed with a 100 × Fluar objective (NA 1.3); 340 and 380 nm excitation was performed with a mercury arc lamp (HBO50W or HBO100W AttoArc system) and a rotating filter wheel as described [2]. Images of the cells were recorded with an ICCD camera (HL-A; Proxitronic, Bensheim, Germany); image digitization and calibration were as described previously [21]. Stimulation was achieved by adding a drop of solution (10 μl) to the buffer (90 μl).

Preparation of vesicle homogenate

Cells (30 ml of a 2×10^7 cells/ml suspension) were washed once in ice-cold 20 mM Hepes, pH 7.2, resuspended at 2×10^8/ml and lysed by passage through nucleopore filters. Then 3 % sucrose, 50 mM KCl, 1 mM $MgCl_2$, 20 μg/ml leupeptin, 1 μg/ml aprotinin and 2.5 mM dithiothreitol were added (final concentrations). After centrifugation for 5 min at 3000 ***g*** the supernatant was further fractionated by centrifugation for 20 min at 12000 ***g***. This supernatant was made 1 mM in BAPTA and centrifuged for 30 min at 40000 ***g***. The resulting sediment was washed once in the buffer specified above but in the absence of $MgCl_2$ and BAPTA and was then resuspended at about 1 mg/ml protein. In some experiments BAPTA was omitted, giving similar results.

Ca^{2+} uptake into vesicles and Ca^{2+}-release experiments

Ca^{2+} uptake was measured with fura 2 (335 and 380 nm excitation; 508 nm emission) with a double-wavelength fluorimeter (Sigma ZWS II; Sigma Instrumente, Berlin, Germany) in a total volume of 1 ml with the following additions: 10 mM Hepes, pH 7.2, 10 mM sodium oxalate, 50 mM KCl, 3 % sucrose, 6 μg/ml antimycin A, 6 μg/ml oligomycin A, 100 μM NaN_3, 2 mM $MgCl_2$ and 1.7 μM fura 2. ATP (1 mM) was added to activate uptake.

Measurement of V-type H^+- and Ca^{2+}-transport ATPase activity

V-type H^+-ATPase activity was determined essentially as described by Giglione and Gross [23] except that 100 μM nigericin

was used instead of gramicidin. The Nbd-Cl concentration was 60 μM and the reaction was performed at 37 °C. For Ca^{2+}-transport ATPase activity, 37 μM BHQ was used. Vesicles were preincubated at 4 °C for 10–20 min with the drug; controls received the same amount of ethanol.

RESULTS

Arachidonic acid evokes changes in $[Ca^{2+}]_i$ in single cells

We tested the effect of arachidonic acid on Ca^{2+} homoeostasis of differentiating wild-type *Dictyostelium* cells. Figure 1 shows that in the presence of extracellular Ca^{2+}, the addition of 10 μM arachidonic acid induced an immediate rapid transient increase in $[Ca^{2+}]_i$, which reached a peak value at 10 s and declined to basal levels again at 20 s. The increase in $[Ca^{2+}]_i$ was observed throughout the cytoplasm. The dose of arachidonic acid applied did not affect the height of the $[Ca^{2+}]_i$ increase but rather the percentage of cells that displayed the transient (Table 1). At 10 μM arachidonic acid, 87 % of the cells responded. Lowering the concentration led to a gradual decrease in the percentage of responding cells, and at 0.5 and 1 μM arachidonic acid a $[Ca^{2+}]_i$ increase was observed but only in a few cells. Independent of the concentration of arachidonic acid, the mean height of the $[Ca^{2+}]_i$ transient was in the range 30–40 nM. However, the actual size of the $[Ca^{2+}]_i$ increase in individual cells varied considerably, with values ranging from 10 to 100 nM. These findings imply that the dose of arachidonic acid necessary to evoke a response reflects a difference in the susceptibility of the individual cells to the fatty acid; the response itself is not graded but rather 'all or none'. The nature of this difference is not known.

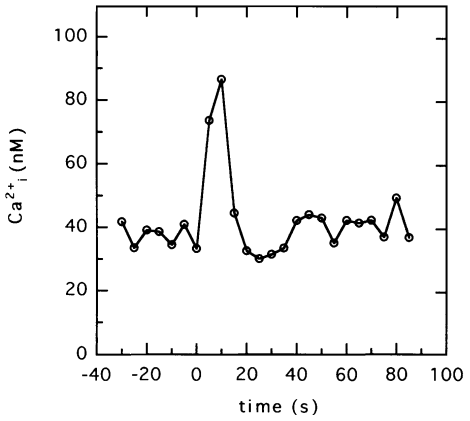

Figure 1 Stimulation of Ax2 cells with arachidonic acid elicits a $[Ca^{2+}]_i$ increase

$[Ca^{2+}]_i$ of a single developing cell was plotted against time. The cell was loaded with fura 2–dextran at t_2 by electroporation as outlined in the Materials and methods section. Stimulation was carried out at t_4; arachidonic acid (10 μM) was added at 0 s. The cell responded with an immediate $[Ca^{2+}]_i$ transient. The experiment was performed in buffer with 1 mM Ca^{2+}. One of 34 cells tested is shown.

Table 1 Effect of the dose of arachidonic acid on the $[Ca^{2+}]_i$ response of *Dictyostelium* cells

Cells were loaded with fura 2–dextran at t_2–t_4 by electroporation as described in the Materials and methods section. Stimulation with arachidonic acid (AA) was carried out at t_4–t_6. Values of $\Delta[Ca^{2+}]$ are means ± S.E.M. Basal $[Ca^{2+}]_i$ was 49 ± 1 nM. Values in parentheses are total numbers.

	$[Ca^{2+}]_i$ changes		
[AA] (μM)	$\Delta[Ca^{2+}]$ (nM)	$\Delta_{min}/\Delta_{max}$ (nM)	Response (% positive)
10	28 ± 5	10/100	87 (39)
5	33 ± 4	12/88	28 (117)
2	41 ± 5	15/81	17 (102)
1	30 ± 11	10/68	7 (67)
0.5	33 ± 6	10/84	5 (142)

A response comparable with that induced by arachidonic acid was observed when the cells were challenged with linoleic acid: addition of 5 or 10 μM linoleic acid to the cells led to an increase in $[Ca^{2+}]_i$ that had a similar time course and height (mean ± S.E.M. change in $[Ca^{2+}]_i$ was 40 ± 4 (n = 13) and 40 ± 6 nM (n = 17) for 5 and 10 μM linoleic acid respectively) to that obtained with arachidonic acid. Moreover, when measured with a Ca^{2+}-sensitive electrode in cell suspension, linoleic acid also evoked an influx of Ca^{2+} across the plasma membrane (results not shown) with a dose–response curve comparable with that reported for arachidonic acid [13]. This indicated that the effects on Ca^{2+} homoeostasis were not specific to arachidonic acid but could be generally induced by fatty acids.

Detection of the fatty acid-induced $[Ca^{2+}]_i$ increase was highly dependent on the Ca^{2+} content of the buffer. In the presence of 1 mM or 0.1 mM $[Ca^{2+}]_e$ most of the cells (87 %) displayed a $[Ca^{2+}]_i$ increase after addition of 10 μM arachidonic acid. When $[Ca^{2+}]_e$ was lowered to 10–50 μM, the cells responded less frequently; however, occasionally even at a $[Ca^{2+}]_e$ of 550 nM, transient $[Ca^{2+}]_i$ increases were observed. In nominally Ca^{2+}-free medium (150 nM Ca^{2+}) the response was absent. This indicates that the major component of the rapid $[Ca^{2+}]_i$ increase was the influx of extracellular Ca^{2+}. However, this finding does not exclude the possibility that the addition of fatty acids also evokes release of Ca^{2+} from intracellular storage compartments; the resulting elevation in $[Ca^{2+}]_i$ could be (i) spatially restricted, e.g. confined to the cell cortex, (ii) too fast to be reliably detected or (iii) too small to be detected.

Liberation of Ca^{2+} from internal stores

The increase in $[Ca^{2+}]_i$ could be due to a direct effect of the fatty acids on the plasma membrane leading to Ca^{2+} influx; alternatively, it could empty intracellular storage compartments thereby inducing a secondary influx of Ca^{2+}. To address this question, both permeabilized cells and partially purified vesicular fractions of the cells were analysed. First the Ca^{2+} concentration in the buffer was measured with a Ca^{2+}-sensitive electrode in a suspension of cells with permeabilized plasma membranes; under these experimental conditions, Ca^{2+} flux across Ca^{2+}-store membranes is monitored. Challenge with arachidonic acid (6 μM) led to a transient elevation of the Ca^{2+} concentration in the buffer, which reflected efflux of Ca^{2+} from internal storage compartments (Figure 2); Ca^{2+} release amounted to 327 ± 142 pmol/10^7 cells (mean ± S.E.M. from seven independent experiments). Subsequent addition of palmitic acid (6 μM) led to a further $[Ca^{2+}]_e$ elevation, which, however, was less pronounced. These data indicated that the fatty acids act on internal Ca^{2+} stores and induce release of Ca^{2+} from these compartments.

Analysis of Ca^{2+} stores with a spectrofluorimeter and the indicator dye fura 2 confirmed this result. In a partially purified vesicular fraction, we measured Ca^{2+} uptake into and Ca^{2+} release from stores with fura 2 added to the suspension. Fura 2 monitored the Ca^{2+} concentration in the buffer; pumping of Ca^{2+}

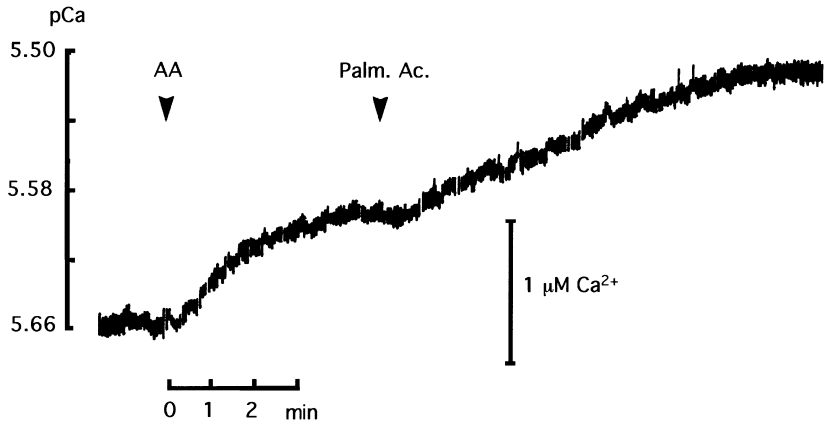

Figure 2 Arachidonic acid induces Ca^{2+} release from intracellular stores in permeabilized cells

Plasma membranes of cells at t_6 were permeabilized with filipin; the Ca^{2+} concentration in the buffer was recorded with a Ca^{2+} electrode in suspension. Addition of 6 μM arachidonic acid (AA) led to an increase in $[Ca^{2+}]$ that reflects Ca^{2+} release from stores (318 pmol/10^7 cells in 4 min). Palmitic acid (Palm. Ac.; 6 μM) produced a slower release (400 pmol/10^7 cells in 8 min). On the left, $[Ca^{2+}]$ is given as pCa; the change in $[Ca^{2+}]$ after addition of a calibration pulse of 1 μM Ca^{2+} is shown on the right. At the start of the experiment, $[Ca^{2+}]$ was 2.2 μM. The results of one of seven experiments are shown.

into storage compartments is reflected by a decrease in the Ca^{2+} concentration. Figure 3(A) shows Ca^{2+} uptake and release characteristics of vesicles isolated from Ax2 cells. Ca^{2+} sequestration was initiated by the addition of 1 mM ATP. When uptake had slowed down, the subsequent addition of low amounts of arachidonic acid evoked a rapid efflux of stored Ca^{2+}. This could have been due to a direct releasing effect on the store; alternatively arachidonic acid could have inhibited the sequestering pumps leading to leakage. However, after stored Ca^{2+} had been liberated by arachidonic acid, reuptake was observed when ATP was added (not shown); this suggests that inhibition of the pumps is unlikely to be the cause of the observed Ca^{2+}-releasing effect. Indeed, neither V-type H^+-ATPase nor Ca^{2+}-ATPase activity, which are both involved in Ca^{2+} sequestration [4,6], was reduced in the homogenate after addition of arachidonic acid (Table 2). Rather, a slight activation of H^+-ATPase pumping activity was observed. Therefore the action of arachidonic acid appears to be a direct one, affecting the Ca^{2+}-release mechanism of the stores.

A dose–response curve revealed that low concentrations of arachidonic acid were effective in releasing substantial amounts of Ca^{2+} (Figure 3B), to a similar extent to that found for permeabilized cells (Figure 2). From these data we conclude that the lack of detection of the corresponding $[Ca^{2+}]_i$ increase is due to either its short duration (less than 5 s) or spatial restriction, e.g. to the cell cortex.

Arachidonic acid acts on the acidosomal Ca^{2+} store

Next we analysed the type of Ca^{2+}-storage compartment affected by arachidonic acid. In suspensions of intact cells, $[Ca^{2+}]_e$ was recorded with the Ca^{2+}-sensitive electrode. Cells were incubated with known inhibitors of the IP_3-sensitive and/or the acidosomal Ca^{2+} stores; then they were challenged with either arachidonic acid or cAMP. CMA (5 μM), which completely blocks vacuolar H^+-ATPase activity of the acidosomes [7], was applied to empty this store. Alternatively, we preincubated cells with 100 μM BHQ, which has been shown to inhibit completely cAMP-induced Ca^{2+} uptake into the IP_3-sensitive stores of permeabilized cells [4] and is known to reduce pumping of Ca^{2+} into acidosomes by 50 % [5]. A combination of the two drugs was also tested. At the concentrations employed, both agents evoked influx of Ca^{2+} themselves, which started after 0–2 min (Figure 4A); after application of CMA it gradually ceased again after 5–15 min. In general, BHQ first caused a transient influx of Ca^{2+} which was followed by a steady influx which ceased after 20–30 min (in one case the transient influx component was absent).

Comparison of Ca^{2+} fluxes before and after addition of the drugs revealed that the arachidonic acid-induced Ca^{2+} entry was reduced after application of CMA or BHQ (Figures 4B and 4C). The degree of inhibition amounted to 50 % in both cases (Table 3). This is consistent with the inhibition profile of the acidosomal Ca^{2+} stores reported by Temesvari et al. [7] and Rooney et al. [5]. Concomitant addition of both drugs further increased the inhibition of Ca^{2+} influx to a value of 70 % (Table 3). Treatment of cells with another inhibitor of acidosomal H^+-pumping activity in *Dictyostelium* homogenates, bafilomycin A_1 [6], also reduced Ca^{2+} entry stimulated by 6 μM arachidonic acid, by 20 % (114 ± 11 pmol of $Ca^{2+}/10^7$ cells in the presence of 5 μM bafilomycin A_1 compared with 142 ± 10 pmol of $Ca^{2+}/10^7$ cells in the control; mean $\pm$ S.E.M. from three independent experiments).

An action of arachidonic acid on the acidosomes was further supported by analysis of the mutant strain HGR8. The capacity of this mutant to acidify endolysosomal compartments was reported to be reduced by 50 % compared with the wild-type [24]; Ca^{2+} flux across the acidosome membrane is coupled to the H^+ gradient [6]. When these mutant cells were stimulated with 6 μM arachidonic acid, a Ca^{2+} influx of only 93 ± 34 pmol/10^7 cells (mean $\pm$ S.D.; $n = 7$) was found. This value was reduced by 44 % compared with the wild-type (165 ± 40 pmol/10^7 cells; $n = 45$) and thus closely matches the reduction of its capacity to acidify endolysosomes [24]. Taken together, the data from the inhibition studies and the analysis of HGR8 indicate that the acidosomes are a target of the action of fatty acids; however, an additional effect on non-acidosomal stores is also possible.

In contrast with arachidonic acid activation, receptor-operated Ca^{2+} entry activated by cAMP was almost completely inhibited by pretreatment with 100 μM BHQ alone (Table 3). Preincubation with 5 μM CMA led to a reduction of uptake by 50 %. Again, 5 μM bafilomycin A_1 was less effective than CMA; it inhibited cAMP-induced Ca^{2+} influx by only 20 % (uptake of

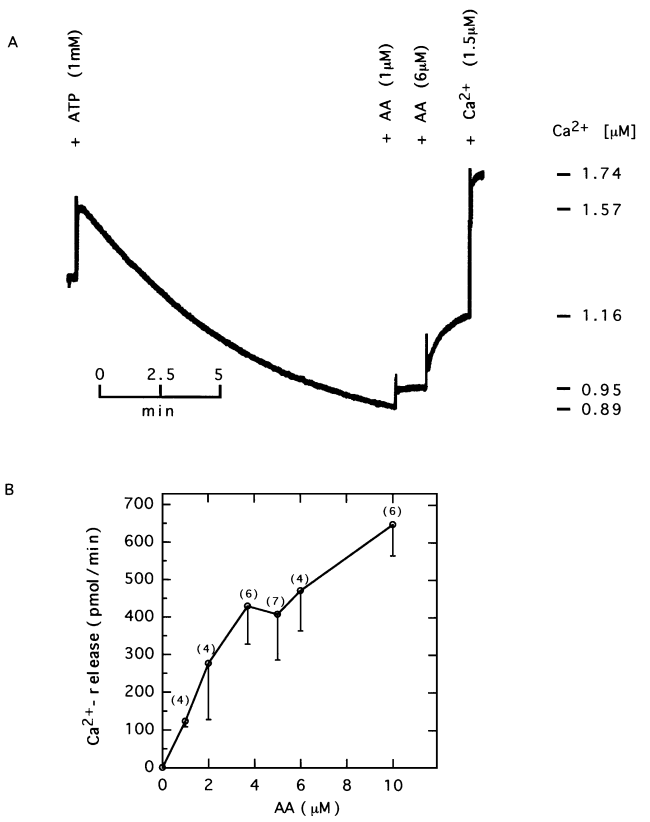

Figure 3 Arachidonic acid evokes release of Ca^{2+} from stores of *Dictyostelium* cells

Wild-type strain Ax2 was lysed at t_1 and fractionated as outlined in the Materials and methods section. The Ca^{2+} concentration in a suspension of a partially purified vesicular fraction was monitored with fura 2 and a two-wavelength fluorimeter. (**A**) The tracing represents the course of the Ca^{2+} concentration in the buffer over time; bars and numbers on the right give the Ca^{2+} concentration (μM). Addition of 1 mM ATP to the suspension led to a decrease in Ca^{2+} concentration which reflects Ca^{2+} uptake into storage compartments. Low amounts (1 and 6 μM) of arachidonic acid (AA) evoked rapid liberation of Ca^{2+} from the vesicles. The results of one of five experiments are shown. (**B**) Dose–response curve for the amount of Ca^{2+} released from vesicular fractions by increasing concentrations of arachidonic acid (AA). Values are means $\pm$ S.D. from three independent experiments; numbers in parentheses indicate the number of tests performed for each concentration of arachidonic acid.

Table 2 Effect of arachidonic acid (AA) on the activity of V-type H^+-ATPase and Ca^{2+}-ATPase

Homogenates were prepared from HD10 cells at t_4, and H^+- and Ca^{2+}-ATPase activities were determined as outlined in the Materials and methods section. Values are given for each experiment. Controls (100%) received an equal volume of ethanol.

	H^+-ATPase		Ca^{2+}-ATPase	
[AA] (μM)	Activity (%)	100% response (nmol of P_i/min per mg of protein)	Activity (%)	100% response (nmol of P_i/min per mg of protein)
20–25	153	6.4	–	–
	104	11.3	–	–
	114	6.9	–	–
	120	7.5	–	–
40	139	19.2	103	15.9
	109	13.8	99	11.7

150 ± 22 pmol of $Ca^{2+}/10^7$ cells compared with 187 ± 22 pmol of $Ca^{2+}/10^7$ cells in the control experiment; mean $\pm$ S.E.M. from three independent experiments). CMA may be more potent than bafilomycin A_1; differential sensitivity to the two drugs in tobacco cells has been reported [25]. From these results we presume that cAMP acts in two ways: (1) by activation of phospholipase A_2 causing liberation of fatty acids, which stimulate release of Ca^{2+} from storage compartments and (2) by exerting an additional effect on a component of the IP_3 pathway; otherwise 100 μM BHQ would not completely block the cAMP-evoked response.

Phospholipase C (PLC) potentiates the Ca^{2+} influx induced by arachidonic acid

We used intact HD10 cells to investigate whether the IP_3-signalling pathway was necessary to generate the arachidonic acid-induced effects on Ca^{2+} homoeostasis *in vivo*. This mutant has no functional PLC [26]. Analysis of $[Ca^{2+}]_e$ in cell suspensions with the Ca^{2+}-sensitive electrode revealed that cAMP-stimulated Ca^{2+} influx was about the same in HD10 and Ax2 cells and seems therefore to be independent of PLC activity (Table 4). In contrast, at 10 μM arachidonic acid, Ca^{2+} entry was reduced in strain HD10 compared with strain Ax2 (Table 4). An increase in the dose of arachidonic acid to 60 μM augmented influx of Ca^{2+} in HD10 cells. The reduced sensitivity of HD10 cells towards arachidonic acid was not due to a difference in the responsiveness of the storage compartments, as vesicular fractions derived from HD10 cells were comparable with those of Ax2 cells with respect to the release characteristics of stored Ca^{2+} (not shown). These results show that, in principle, fatty acids can substitute for cAMP with respect to Ca^{2+} influx. Triggering of Ca^{2+} entry by arachidonic acid seems to involve participation of the IP_3 pathway; otherwise it would be independent of the presence of a functional PLC. The results with HD10 cells also indicate that stimulation with cAMP induces an additional event that cannot be evoked by challenge with arachidonic acid alone (see the Discussion).

DISCUSSION

The aim of this study was to investigate the effect of fatty acids on Ca^{2+} homoeostasis in *Dictyostelium* cells. We found that arachidonic acid induced a transient $[Ca^{2+}]_i$ increase as the result of liberation of Ca^{2+} from storage compartments and influx of extracellular Ca^{2+}. This effect is not specific to arachidonic acid, but is induced by long-chain fatty acids in general, as (1) linoleic acid induced a $[Ca^{2+}]_i$ transient and entry of extracellular Ca^{2+} similar to that induced by arachidonic acid and (2) palmitic acid also led to release of Ca^{2+} from intracellular stores; moreover, the latter has also been shown to evoke influx of extracellular Ca^{2+} [13]. In *Dictyostelium*, the fatty acid that is formed after stimulation with the agonist cAMP is not yet known. Arachidonic acid, linoleic acid and and linolenic acid were detectable only when these compounds had been added to the growth medium [27]; otherwise, various other mono- and di-unsaturated long-chain fatty acids such as palmitoleic acid, oleic acid and 5,9- or 5,11-octadecadienoic acid [14–16] are present which could be the physiologically relevant agent(s). As arachidonic acid proved to be one of the most effective fatty acids tested, it was used in most of our experiments.

Cytosolic Ca^{2+} signals are generated by fluxes across both membranes of intracellular compartments and the plasma membrane. Arachidonic acid and other long-chain fatty acids have

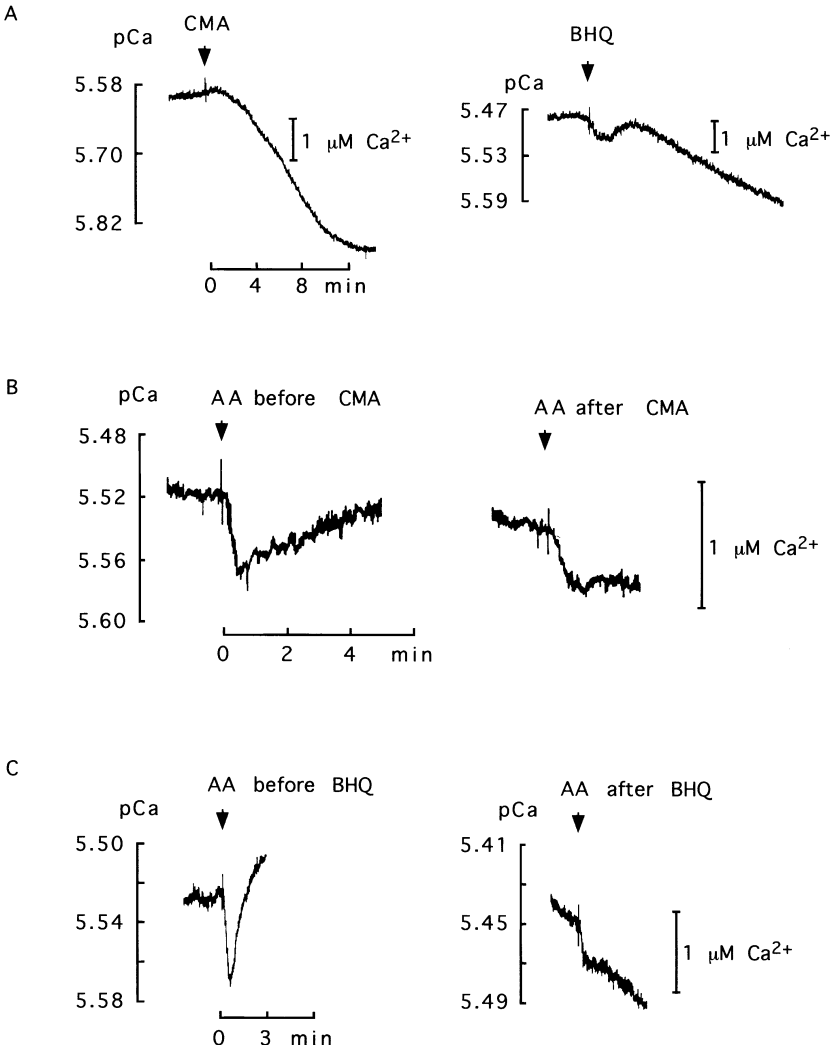

Figure 4 CMA and BHQ induce Ca^{2+} influx in intact cells

$[Ca^{2+}]_e$ in suspensions of cells was analysed with a Ca^{2+}-sensitive electrode as outlined in the Materials and methods section. (**A**) Addition of 5 μM CMA led to a decrease in $[Ca^{2+}]_e$, representing Ca^{2+} influx which gradually ceased. Cells were tested at t_6; at the start of the experiment, $[Ca^{2+}]_e$ was 2.5 μM. The results of one of four experiments are shown. Incubation with 100 μM BHQ first led to a transient Ca^{2+} influx; then slow steady Ca^{2+} entry occurred. The experiment was started at t_6 with $[Ca^{2+}]_e$ at 3.3 μM; the results of one of ten experiments are shown. Bars indicate $[Ca^{2+}]_e$ as pCa. The height of a calibration pulse of 1 μM Ca^{2+} is shown on the right. (B) Arachidonic acid (AA)-induced entry of Ca^{2+} was reduced after challenge with 5 μM CMA. The results of one of three experiments are shown; it was started at t_7. Influx was 130 pmol of $Ca^{2+}/10^7$ cells before and 70 pmol of $Ca^{2+}/10^7$ cells after CMA addition. The pCa bar and the calibration pulse are valid for both graphs. (**C**) 100 μM BHQ inhibited AA-evoked Ca^{2+} entry. The results of one of six experiments are shown. Ca^{2+} influx was 238 pmol/10^7 cells before addition of BHQ and 102 pmol/10^7 cells after challenge with BHQ for 12 min. The experiment was started at t_7. The height of the calibration pulse is valid for both graphs.

Table 3 Effect of CMA and/or BHQ on the arachidonic acid-induced influx of Ca^{2+}

Ca^{2+} influx was measured with a Ca^{2+}-sensitive electrode as outlined in the Materials and methods section. Cells were tested between t_4 and t_8 and were preincubated with 5 μM CMA or 100 μM BHQ for 5–15 min before the response to arachidonic acid (AA) or cAMP respectively was tested. In the case of CMA/BHQ, the cells were first incubated with CMA; after 20–30 min, BHQ was added to the same cell suspension. Data represent means ± S.E.M. (pmol of $Ca^{2+}/10^7$ cells). Control influx is the Ca^{2+} response on stimulation with AA or cAMP in the absence of inhibitors. cAMP-receptor stimulation was performed with a second batch of the same cell suspension, after performance of the experiment with arachidonic acid; as receptor-operated Ca^{2+} influx greatly increases in the course of differentiation [20], Ca^{2+} uptake after cAMP stimulation was larger than arachidonic acid-evoked uptake, which remains constant throughout development [13]. The number of experiments is indicated in parentheses.

	CMA		BHQ		CMA/BHQ	
Stimulant	Influx after incubation with CMA	Control influx	Influx after incubation with BHQ	Control influx	Influx after incubation with CMA/BHQ	Control influx
6 μM AA	55 ± 13 (3)	109 ± 28	87 ± 11 (6)	172 ± 42	33 ± 10 (3)	109 ± 28
1 μM cAMP	98 ± 20 (3)	195 ± 21	45 ± 54 (5)	329 ± 185	16 ± 12 (3)	195 ± 21

Table 4 Comparison of the cAMP- and arachidonic acid-induced Ca^{2+} influx in strains Ax2 and HD10

Arachidonic acid (AA)-induced Ca^{2+} influx in strain Ax2 and the PLC$^-$ strain HD10. cAMP-activated fluxes are shown as a reference. $[Ca^{2+}]_e$ measurements were performed at t_4–t_8 in cell suspensions with a Ca^{2+}-sensitive electrode as described in the Materials and methods section. Data represent means ± S.D. of Ca^{2+} influx. Influx of Ca^{2+} in Ax2 cells is saturated at 10 μM AA. The number of experiments is indicated in parentheses.

	Ca^{2+} influx (pmol/10^7 cells)	
Addition	Ax2	HD10
1 μM cAMP	202 ± 90 (44)	171 ± 82 (27)
10 μM AA	190 ± 58 (5)	44 ± 11 (3)
60 μM AA		113 ± 17 (4)

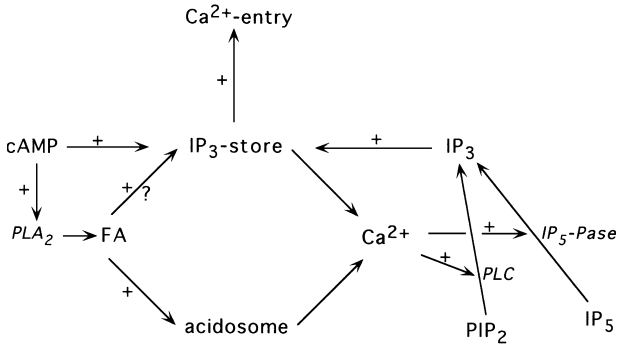

Figure 5 Hypothetical model for cAMP- or fatty acid-induced Ca^{2+} entry

cAMP binds to cAMP receptors which affect (i) phospholipase A_2 (PLA_2) and (ii) the IP_3 store. PLA_2 activation leads to fatty acid (FA) liberation which triggers Ca^{2+} release from acidosomes and possibly also the IP_3-sensitive store. The increased $[Ca^{2+}]_i$ stimulates PLC and at even higher Ca^{2+} concentrations also IP_5 phosphatase, further enhancing Ca^{2+} release via IP_3. cAMP activation of the IP_3 store could consist of IP_3-receptor sensitization then responding to small increments in IP_3 concentration. Subsequently, depletion of the IP_3-sensitive storage compartment initiates extracellular Ca^{2+} entry. PIP_2, Phosphatidylinositol bisphosphate.

been shown by the following to participate in these events: (1) arachidonic acid can induce extracellular Ca^{2+} entry in a non-capacitative fashion in e.g. secretory and smooth-muscle cells [28,29], and under conditions of stimulation with the physiological agonist, non-capacitative entry of Ca^{2+} is dependent on receptor occupancy rather than the filling status of the store, and influx precedes emptying of the store [28]; (2) arachidonic acid has been shown to activate influx of extracellular Ca^{2+}, which in turn leads to intracellular Ca^{2+} mobilization by Ca^{2+}-induced Ca^{2+} release in intestinal longitudinal muscle [30]; (3) internal Ca^{2+} storage compartments are also sensitive to arachidonic acid and other long-chain fatty acids that induce release of Ca^{2+} from PANC1 microsomes [9] or from stores of permeabilized chick embryo retina cells [11]. In human lymphoma cells, arachidonic acid activates capacitative Ca^{2+} entry [31], which is preceded by release of Ca^{2+} from stores and is critically dependent on the filling status of the stores (for a review, see [12,32,33]). Arachidonic acid influences several types of store such as mitochondria [34,35] and IP_3-insensitive [30] and IP_3-sensitive stores [36].

The mechanisms of action of arachidonic acid are manifold and their molecular basis is not understood in detail: it was suggested to act co-operatively with IP_3 in pancreatic islets [34], and in U937 cells activation of phospholipase A_2 generating arachidonic acid was proposed to be an internal effector system to propagate the IP_3-initiated signalling cascade [31]. Whereas a stimulatory effect of arachidonic acid together with tau proteins on PLC-γ purified from transfected HeLa cells was shown [37], in neutrophils an effect on PLC was not observed [38].

The potential targets and mode(s) of action of arachidonic acid within the cAMP signalling cascade in *D. discoideum* are depicted in the hypothetical model shown in Figure 5. During stimulation with cAMP, fatty acids are released as second messengers since drugs known to affect phospholipase A_2 inhibited Ca^{2+} entry [13]; moreover, cAMP exerts an additional effect on the IP_3-sensitive store (see below). Challenge of the cells with fatty acids alone can activate a shortcut pathway: addition of arachidonic acid or palmitic acid to intact cells leads to Ca^{2+} influx [13]. The basis for the cAMP- and fatty acid-induced influx of Ca^{2+} is its release from storage compartments: (1) in permeabilized cells, cAMP [8] as well as fatty acids (our study) caused liberation of Ca^{2+} and (2) preincubation of intact cells with the V-type H^+-ATPase blockers CMA and bafilomycin A_1 and the Ca^{2+}-ATPase inhibitor BHQ, which empty the acidosomal and IP_3-sensitive storage compartments, reduced both the cAMP- and the arachidonic acid-evoked Ca^{2+} influx. From these data we conclude that the rapid and substantial efflux of Ca^{2+} from the stores triggers influx of external Ca^{2+}.

Because of the effects of the inhibitors and the reduced Ca^{2+} influx observed in the mutant HGR8, we consider the IP_3-insensitive acidosomes to be one target of action of fatty acids and propose that fatty acids are the physiological agents causing release of Ca^{2+} from the acidosomes. Moreover, our findings also imply that the acidosomes comprise an integral part of the cAMP signal-transduction pathway leading to Ca^{2+} influx. An effect of fatty acids on the IP_3-sensitive store in addition to the acidosomes may also occur. Whether arachidonic acid also affects mitochondria as described in rat cerebellum for example [35] is at present not known.

Arachidonic acid may act directly on the store and induce release of Ca^{2+}, or it may lead to leakage of Ca^{2+} from the store either by influencing the sequestering enzymes or by unspecifically permeabilizing the vesicles and, in this case, also the plasma membrane. At the concentrations employed (5–10 μM), a permeabilizing action of arachidonic acid, similar to that of an ionophore, for example, is unlikely since, even at 100 μM, arachidonic acid-induced leakage of Ca^{2+} from liposomes was not observed [9]. In the vesicular fraction, inhibition of the sequestering pumps responsible for uptake of Ca^{2+} into the stores did not appear to be the cause of the observed release for the following reasons: (1) direct measurement of the activity of both V-type H^+-ATPase and the Ca^{2+}-ATPase revealed that the pumps were not inhibited by arachidonic acid and (2) reuptake of Ca^{2+} released by arachidonic acid occurred after addition of ATP. Therefore we consider a direct effect, possibly by an influence of the fatty acids on the components involved in release of Ca^{2+} from the store, to be probable.

In *Dictyostelium*, mediation of the arachidonic acid-evoked Ca^{2+} influx appeared to be coupled to PLC activity leading to IP_3 generation, possibly by a positive feedback mechanism. This mechanism was impeded in the PLC$^-$ mutant HD10: it showed reduced sensitivity to the fatty acid, and higher concentrations of arachidonic acid were required to evoke a substantial response. PLC has been shown to be Ca^{2+}-activated [39–41]. In wild-type cells, the fatty acid-induced release of Ca^{2+} from stores may activate PLC, leading to IP_3 production and thus to Ca^{2+} release from the IP_3-sensitive store; store depletion would augment influx of Ca^{2+}. In HD10 cells, such a mechanism cannot operate. Nevertheless, in HD10 cells, IP_3 is generated as well, by the

action of IP_5 phosphatase. This enzyme is also activated by Ca^{2+}, but requires higher concentrations [42]. Therefore increasing the amount of arachidonic acid could lead to a greater release of Ca^{2+} from stores sufficient to stimulate IP_5 phosphatase and consequently result in a positive feedback on IP_3 levels as in wild-type cells; indeed, we found that challenging HD10 cells with 60 μM arachidonic acid increased the size of the Ca^{2+} influx.

Surprisingly, Ca^{2+} influx in HD10 cells after the addition of cAMP was comparable with that of wild-type cells. Therefore, in contrast with stimulation by arachidonic acid, stimulation of Ca^{2+} entry by cAMP appears to be independent of PLC activity; indeed no significant IP_3 formation, above the rather high basal levels, occurred after addition of cAMP [26,42]. As the generation of the fatty acid as a second messenger after cAMP receptor stimulation is necessary to activate Ca^{2+} influx [13], the results obtained with HD10 cells reveal an additional event that is triggered by cAMP but not by the fatty acid; otherwise HD10 cells should be responsive to both cAMP and fatty acids, as observed in the wild-type. This additional event appears to be part of the IP_3-signalling cascade, since it is altered in HD10 cells. We propose that stimulation with cAMP evokes a 'sensitization' of the Ca^{2+}-entry mechanism. Such sensitization could be produced by increasing the susceptibility of the system to either fatty acids or IP_3, e.g. by modulation of the IP_3 receptor making it more sensitive to basal IP_3 concentrations. IP_3 levels in the *Dictyostelium* wild-type strain NC4 increase only slightly (30–40 %) after addition of cAMP [43] and may even be undetectable, as shown for wild-type Ax3 cells [26]. Therefore it is likely that an additional regulation of the IP_3 receptor is occurring, as reported for mammals, e.g. agonist-mediated control of IP_3 receptor responsiveness by Ca^{2+}-dependent phosphorylation and dephosphorylation in permeabilized 3T6 fibroblasts [44] or agonist-induced activation of the IP_3-activated Ca^{2+} channel at basal levels of IP_3 by a G-protein-dependent mechanism in permeabilized pancreatic acini [45]. In contrast, when challenged with the fatty acid alone, sensitization of the IP_3 receptor does not occur; therefore, in this case, an increase in IP_3 is necessary to activate Ca^{2+} release from the IP_3-sensitive store.

The mechanism by which the empty store induces influx of extracellular Ca^{2+} is obscure. For cAMP-mediated Ca^{2+} entry there is evidence that cGMP participates in this process, since a membrane-permeant analogue of cGMP has been shown to augment cAMP-induced Ca^{2+} influx across the plasma membrane [8] and influx is prolonged in streamer F mutants [46], in which hydrolysis of cGMP is slowed down [47]. Moreover, the $[Ca^{2+}]_i$ increase analysed in single cells peaked at 10–15 s, a point at which cAMP-induced formation of cGMP is also maximal [48], confirming that it is the rate of Ca^{2+} influx that determines $[Ca^{2+}]_i$ [46]. Arachidonic acid-activated release of Ca^{2+} from the stores could affect the level of cGMP; both inhibitory and stimulatory effects of Ca^{2+} on the production of cGMP in *Dictyostelium* have been described [49,50]. Future studies using arachidonic acid as a tool to empty the stores will help us to gain further insight into the coupling of the different types of storage compartments and the regulatory mechanisms underlying their communication with the extracellular space.

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