Calmidazolium leads to an increase in the cytosolic Ca^{2+} concentration in *Dictyostelium discoideum* by induction of Ca^{2+} release from intracellular stores and influx of extracellular Ca^{2+}

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The Ca^{2+} stores of *Dictyostelium discoideum* amoebae take part in control of homoeostasis of the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and the cyclic-AMP-induced $[Ca^{2+}]_i$-signalling cascade. In order to characterize regulatory mechanisms of these stores, we incubated cells with the calmodulin antagonist calmidazolium. Measurement of permeabilized and intact cells in suspension with a Ca^{2+}-sensitive electrode revealed that calmidazolium induced Ca^{2+} release from intracellular stores, influx of Ca^{2+} across the plasma membrane and subsequent efflux. In single fura-2-loaded cells calmidazolium evoked rapid and global transient elevations of $[Ca^{2+}]_i$. Other calmodulin antagonists (trifluoperazine, chlorpromazine, fendiline and W7) also induced transient elevations of $[Ca^{2+}]_i$, which were, however, slower and observed in fewer cells. The calmidazolium-induced influx of extracellular Ca^{2+} was inhibited by preincubation with 2,5-di-(t-butyl)-1,4-hydroquinone (BHQ) and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl), both known to interact with pumps of the inositol 1,4,5-trisphosphate (IP_3)-sensitive store, and by the V-type H^+-ATPase inhibitor bafilomycin A_1, which affects the acidosomal Ca^{2+} store. Incubation with pump inhibitors did not itself induce changes in $[Ca^{2+}]_i$. We conclude that the effects of calmidazolium are, at least in part, mediated by its calmodulin-antagonizing properties, that it acts by inducing Ca^{2+} release from filled storage compartments, and that its target of action is both the IP_3-sensitive store and the acidosome; emptying of these stores leads to influx of extracellular Ca^{2+}.

INTRODUCTION

Chemotaxis and differentiation of the cellular slime mould *Dictyostelium discoideum* both depend on the precise regulation of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). Within a few hours after the induction of development, the cells establish a signalling system that is based on the attractant cyclic AMP (cAMP) [1] and that they use to form multicellular aggregates of up to 10^5 cells. The chemotactic signal cascade starts with the binding of cAMP to the cell-surface receptor, which induces activation of phospholipase C. The formation of inositol 1,4,5-trisphosphate (IP_3) leads to the release of Ca^{2+} from non-mitochondrial pools; additionally extracellular Ca^{2+} is taken up (for review see [2]) by a mechanism involving the action of cGMP [3,4].

Measurement of aequorin-transfected cells at low temperature revealed small $[Ca^{2+}]_i$ changes after chemotactic cAMP stimulation [5]; under physiological conditions this agonist-induced increase in $[Ca^{2+}]_i$ is restricted spatially and/or temporally [5–7]. In contrast, a global and transient $[Ca^{2+}]_i$ elevation is observed after addition of cAMP doses that induce differential gene expression [8]; the underlying signal-transduction cascade is unknown. Intracellular Ca^{2+}-storage compartments take part in the regulation of the cAMP-induced $[Ca^{2+}]_i$ changes due to the rapid release and uptake of Ca^{2+}. There is evidence for the presence of at least two types of Ca^{2+} stores in *Dictyostelium* cells, characterized pharmacologically by their different sensitivities towards inhibitors of H^+- or Ca^{2+}-ATPases: (1) a pool of endosomal origin ('acidosomes') that makes use of a pH gradient to sequester Ca^{2+} and that releases Ca^{2+} after incubation with the V-type H^+-pump inhibitor bafilomycin A_1 [9,10]; (2) a compartment that is sensitive to the SERCA-type Ca^{2+}-pump inhibitor 2,5-di-(t-butyl)-1,4-hydroquinone (BHQ) and the H^+-pump inhibitor 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) represents the IP_3-releasable store [11]. By using electron-microscopic X-ray microanalysis, organelles that continuously accumulate high amounts of Ca in the course of development have been identified [12]. The relationship of these compartments to the pharmacologically characterized stores is still to be established.

In many other cell types, both communication between different Ca^{2+} stores and a feedback regulation of Ca^{2+} influx across the plasma membrane by the filling status of the stores have been shown (for reviews see [13,14]); as yet the signalling mechanism from the store to the plasma membrane leading to Ca^{2+} influx is poorly understood [15]. In *Dictyostelium* it is unclear whether and how the Ca^{2+} stores are connected, whether there is a link between the pools and the plasma membrane, and how the components responsible for release and uptake of Ca^{2+} from and into the pools are regulated.

One compound involved in both maintenance and agonist-induced changes of the $[Ca^{2+}]_i$ homoeostasis in *Dictyostelium* might be calmodulin. In other cell types calmodulin has been shown to control Ca^{2+}-pumping activity [16,17]. On the other hand, it was proposed to be involved in the release of Ca^{2+} from

Abbreviations used: BAPTA, 1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid; BHQ, 2,5-di-(t-butyl)-1,4-hydroquinone; cAMP, cyclic AMP; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; $[Ca^{2+}]_e$, extracellular free Ca^{2+} concentration; IP_3, inositol 1,4,5-trisphosphate; NBD-Cl, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; R340/380, ratio of emission of fura-2 at 340 and 380 nm excitation; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulphonamide.

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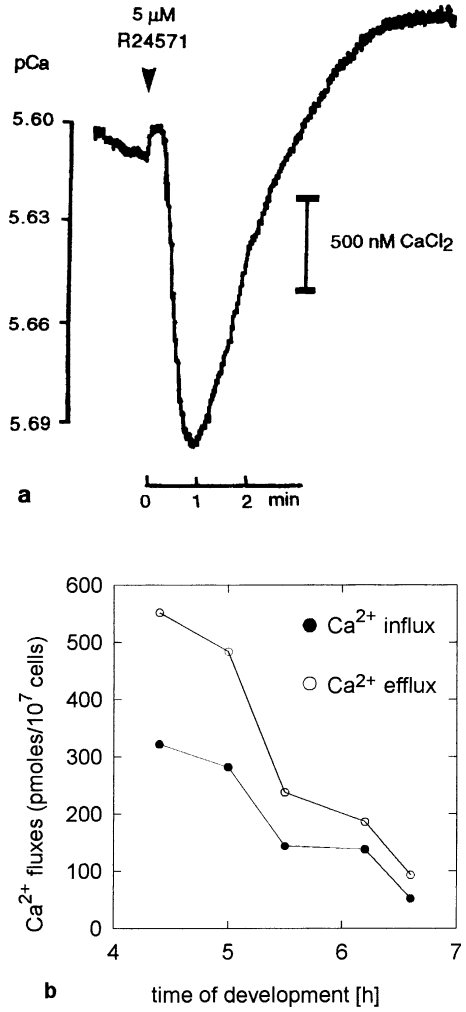

Figure 1 R24571 evokes Ca^{2+} fluxes across the plasma membrane

$[Ca^{2+}]_e$ was measured with a Ca^{2+} electrode as described in the Materials and methods section. (**a**) The time point of addition of R24571 to the cell suspension (at t_5) is marked by an arrow. R24571 induced rapid changes in $[Ca^{2+}]_e$, reflecting uptake and subsequent efflux of Ca^{2+} from the cells. (**b**) The stage of differentiation affected the magnitude of both the R24571-induced influx and efflux of Ca^{2+}. The developmental time is plotted versus the amounts of Ca^{2+} (pmol/10^7 cells) taken up or released after the addition of R24571 (5 μM). Results of one out of three experiments are shown.

the IP_3-sensitive pool by the action of calmodulin-dependent protein kinase and phosphatase [18] and to take part in oscillations of $[Ca^{2+}]_i$ [19]. In the present study we therefore examined the effect of the calmodulin antagonist calmidazolium (R24571) on differentiating *Dictyostelium* amoebae. Analysis of its effect on Ca^{2+} fluxes showed that calmidazolium induced a transient increase in $[Ca^{2+}]_i$ which was the result of liberation of Ca^{2+} from storage compartments and influx of extracellular Ca^{2+}. Other known calmodulin antagonists also induced transient changes in $[Ca^{2+}]_i$, but at a decreased potency.

MATERIALS AND METHODS

Materials

Fura-2 and 1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (BAPTA) were from Molecular Probes (Eugene, OR, U.S.A.); calmidazolium (R24571), chlorpromazine, trifluoperazine, fendiline, thapsigargin, NBD-Cl, filipin complex and poly-L-lysine were from Sigma (Munich, Germany); *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W7) and EGTA were from Fluka (Buchs, Switzerland); BHQ was from Aldrich (Steinheim, Germany); bafilomycin A_1 was generously given by Dr. K. Altendorf (Osnabrück, Germany); U73122 and U73343 were from Biomol (Hamburg, Germany).

Culture of cells

Wild-type strain Ax2 was grown in liquid medium [20] supplemented with vitamin B_{12} (24 μg/l) and folic acid (98.5 μg/l) and shaken at 150 rev./min, 23 °C. Differentiation was induced by washing exponentially growing amoebae three times with ice-cold Sørensen phosphate buffer [SP-buffer: 17 mM (KH_2/Na_2H)-PO_4, pH 6.0]. The cell density was adjusted to 2×10^7 cells/ml and the cells were shaken until use at 23 °C. The time (h) after induction of development is designated t_x.

Measurement of the extracellular Ca^{2+} concentration

The extracellular concentration of Ca^{2+} was determined as described [21]. In brief, 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. Extracellular Ca^{2+} was measured with a Ca^{2+}-sensitive electrode (ETH 1001; Möller, Zürich, Switzerland). For the measurement of Ca^{2+} fluxes across intracellular storage compartments with the Ca^{2+} electrode, the amoebae were permeabilized with filipin (15 μg/ml) in the presence of 1 mM $MgCl_2$ and 1 mM ATP as described [4].

$[Ca^{2+}]_i$ measurements

Scrape-loading of amoebae with the indicator fura-2 was performed as described [6]. The loaded cells were kept in 5 mM Hepes/5 mM KCl, pH 7.0 (H5-buffer), on ice until use. Then 100 μl of cell suspension (maximum density 5×10^6 cells/ml) was pipetted on to a glass coverslip, and the cells were allowed to settle for 10 min before transfer to the stage of an inverted microscope (Zeiss IM). Amoebae were stimulated by addition of a drop (usually 10 μl) of solution to the buffer (100 μl) covering the cells. The cells were viewed with a 100× Ultrafluar [NA (numerical aperture) = 1.25] objective (Zeiss, Oberkochen, Germany). The fluorescence microscope was equipped with a 50 W mercury lamp and 340 and 380 nm excitation filters; the 380 nm filter was paired with a 30% neutral-density filter. The filters were housed in a rotating filter wheel. Each excitation filter was kept in the light path for 500 ms; the time for changing the filters was 126 ms. Images were recorded with a low-light SIT camera (Heimann, Wiesbaden, Germany).

$[Ca^{2+}]_i$ imaging

Images recorded with the camera were digitized (512 × 512 pixels; 256 gray values) using a frame grabber and processor card (model DT 2851/DT 2858; Data Translation, Bietigheim-Bissingen, Germany). The frame grabber was triggered by a 5 V signal from the filter wheel which was generated as soon as the filter was positioned in the light path. After a delay time of 120 ms, images were captured and stored on the hard disk of a personal computer. After subtraction of background images, corresponding pixels of the 340 and 380 nm images were divided. The resulting ratio image (R340/380) was displayed in false colours. For calibration, images of fura-2 solutions with defined

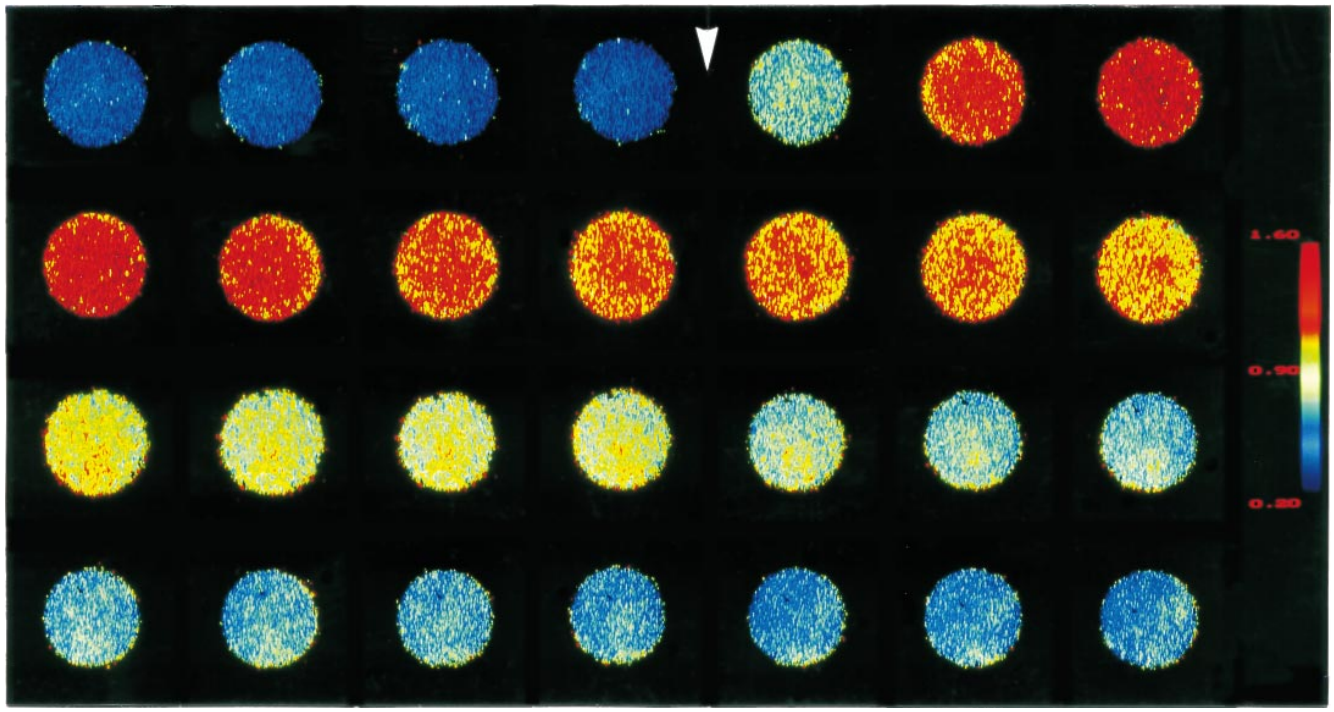

Figure 2 R24571 induces an increase in $[Ca^{2+}]_i$

Cells were analysed by light microscopy as described in the Materials and methods section. False colour images of R340/380 (to be read as in a book, starting top left and ending bottom right) of a single fura-2-loaded cell at t_5 reveal an overall increase in $[Ca^{2+}]_i$ after the addition of R24571 (marked by an arrowhead); 10 μl of R24571 (100 μM) was added to the medium (100 μl; final concentration 9 μM). The time between consecutive ratio images was 5 s. The experiment was carried out in the presence of 1 mM Ca^{2+}.

free Ca^{2+} concentrations [6] in microslide tubes (Vogel, Giessen, Germany) were digitized and processed.

Photometric measurements

For the determination of R340/380, images were recorded on a video recorder (Panasonic, Osaka, Japan). The light intensity of fluorescence images displayed on a video screen was measured with a photodiode housed in a Nikon camera. Signals were digitized and the ratios were calculated. Microscopic calibration was done as described [6].

RESULTS

Ca^{2+} fluxes across the plasma membrane

Figure 1 shows the time course of the extracellular Ca^{2+} concentration measured with a Ca^{2+}-sensitive electrode when intact cells (at developmental time t_4) were incubated with calmidazolium. Stimulation of the amoebae with R24571 first induced a small efflux of Ca^{2+}, followed by an influx and then a second efflux of Ca^{2+}. The fact that this second efflux exceeded the previous influx indicated that R24571 also exerted an effect on Ca^{2+}-storage compartments (see below). Subsequent addition of the chemoattractant cAMP evoked only a small influx of Ca^{2+}; only 10–12 min later the cells again exhibited the characteristic response towards cAMP, with the full magnitude of influx as described [21] (results not shown).

The changes in $[Ca^{2+}]_e$ induced by R24571, as well as the proportion of influx versus efflux of Ca^{2+}, depended on the developmental stage of the cells (Figure 1b). During the early aggregation phase (t_4–t_5) the response of the cells was maximal; afterwards it declined. When stimulated at early time points of differentiation, the amount of Ca^{2+} that was taken up was always smaller than the amount that was released into the medium. After t_6 Ca^{2+} influx and efflux were in the same range. This change in responsiveness during differentiation might be the result of a lower amount of Ca^{2+} released from the storage compartment(s). This could be due to a decrease in size of the affected store(s) and/or to the acquisition of an additional, R24571-insensitive, compartment into which Ca^{2+} was also taken up, thus resulting in a smaller release of Ca^{2+} into the extracellular space. An increase in the overall Ca^{2+}-storage capacity and efficiency is indicated by the fact that in the course of differentiation extracellular Ca^{2+} that is taken up after cAMP stimulation is no longer released back into the medium [21], together with the observation that organelles accumulate large amounts of Ca^{2+} during development [12].

Measurement of $[Ca^{2+}]_i$ with the indicator fura-2

The direct measurement of $[Ca^{2+}]_i$ in cells that were challenged with R24571 revealed a rapid overall increase in $[Ca^{2+}]_i$ that started within few seconds after the application of the drug (Figure 2). The elevation was transient, with a maximum after 15–20 s, and in this cell declined within the measurement time to a value that was slightly above the basal level. Usually $[Ca^{2+}]_i$ returned to pre-stimulation levels. The average increase in $[Ca^{2+}]_i$ is given in Table 1. The size of the R24571-induced $[Ca^{2+}]_i$ elevation was dependent on the concentration of extracellular Ca^{2+}: in nominally Ca^{2+}-free medium it was smaller than in the presence of 1 mM Ca^{2+}. When cells were incubated with 1 mM EGTA or BAPTA no response was observed (results not shown).

Table 1 Effect of addition of R24571 and large amounts of $CaCl_2$ on $[Ca^{2+}]_i$ dependent on the extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$)

Fura-2-loaded cells (at t_5) were analysed by light microscopy as outlined in the Materials and methods section; 10 μl of R24571 (100 μM) or 10 μl of $CaCl_2$ (100 mM) or R24571 followed by $CaCl_2$ (same amounts as above) was added to the medium (100 μl). The average increase ($\pm$S.E.M.) in $[Ca^{2+}]_i$ is shown. The $[Ca^{2+}]_i$ change given in the last line refers to the change after addition of $CaCl_2$. The number in parentheses indicates the number of cells tested. Abbreviation: nd, not determined.

		$[Ca^{2+}]_i$ change (nM)	
Addition	$[Ca^{2+}]_e$...	1 mM	Nominally Ca^{2+}-free
R24571		135 $\pm$ 19 (16)	27 $\pm$ 5 (4)
$CaCl_2$		nd	47 $\pm$ 8 (7)
R24571, $CaCl_2$		nd	295 $\pm$ 38 (4)

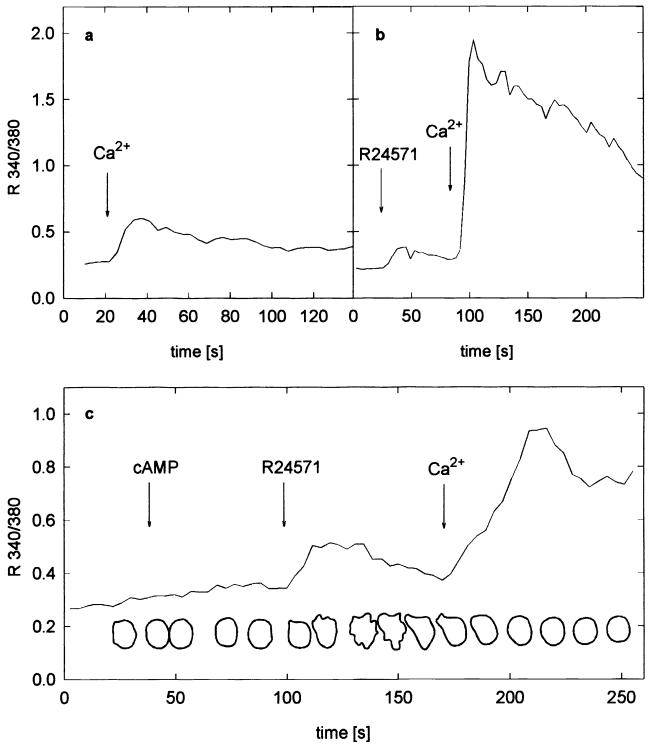

Figure 3 $[Ca^{2+}]_i$ increases after addition of large amounts of $CaCl_2$

Photometric measurement of the time course of R340/380 in single aggregation-competent (at t_5) cells loaded with fura-2. The experiments were carried out in nominally Ca^{2+}-free buffer. The time course of R340/380 of the whole cell is plotted. (**a**) The arrow indicates the time point of addition of $CaCl_2$ (final concentration 9 mM) to the cell that leads to an elevation of $[Ca^{2+}]_i$. (**b**) Addition of R24571 (final concentration 9 μM) to the cell evoked a small change in $[Ca^{2+}]_i$; subsequent addition of the same amount of $CaCl_2$ as in (**a**) led to a greater and longer-lasting elevation of $[Ca^{2+}]_i$ than that depicted in (**a**). (**c**) A similar experiment to that in (**b**), together with drawings of the cell's circumference demonstrating pseudopod extension or contraction depending on the size of the $[Ca^{2+}]_i$ change. Addition of cAMP (final concentration 90 μM) does not lead to a $[Ca^{2+}]_i$ increase in the absence of extracellular Ca^{2+} [8] or a change in morphology; the small global elevation of $[Ca^{2+}]_i$ by R24571 caused an overall extension of pseudopods, whereas the cell contracted during the larger $[Ca^{2+}]_i$ change induced by subsequent addition of $CaCl_2$ (final concentration 9 mM).

The presence of R24571 also had an effect on $[Ca^{2+}]_i$ changes induced by addition of large amounts of Ca^{2+} (to give a final $[Ca^{2+}]$ of 9 mM) to the cells (Table 1, Figure 3). Addition of

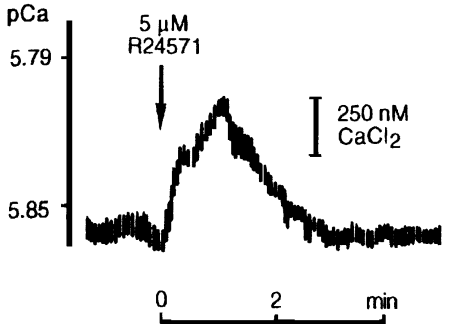

Figure 4 Ca^{2+} is liberated from storage compartments by R24571

The plasma membrane of cells (at t_5) was permeabilized as outlined in the Materials and methods section, and Ca^{2+} fluxes across intracellular vesicle membranes were measured with a Ca^{2+} electrode. Results of one out of two experiments are shown. Addition of R24571 led to a reversible efflux of Ca^{2+} from the stores.

$CaCl_2$ to aggregation-competent cells led to a transient global increase in $[Ca^{2+}]_i$ (Figure 3a). This increase was much higher and lasted longer when the cells had been preincubated with R24571 (Figure 3b), indicating that the drug potentiated the Ca^{2+} influx across the plasma membrane. Figure 3(c) shows that $[Ca^{2+}]_i$ elevations induced by R24571 or large amounts of Ca^{2+} resulted in shape changes of the cell. This behaviour was observed frequently; a rather small global increase in $[Ca^{2+}]_i$ evoked the extrusion of pseudopods over the whole cell's circumference; on the other hand, larger changes in $[Ca^{2+}]_i$ led to a contraction of the cell. These shape changes are consistent with a Ca^{2+}-dependent rearrangement of the cytoskeleton [22,23].

The R24571-mediated $[Ca^{2+}]_i$ change could be the result of an immediate direct action at the plasma membrane, or of an interaction with intracellular pools followed by Ca^{2+} influx. The addition of R24571 to cells in nominally Ca^{2+}-free medium led to a small, but significant, global elevation of $[Ca^{2+}]_i$ (Table 1, Figures 3b and 3c). However, to induce high $[Ca^{2+}]_i$ changes by R24571 (Table 1, Figure 2) it was necessary to preincubate the cells for at least 10 min in buffer containing Ca^{2+} (1 mM). Neither the incubation of cells in nominally Ca^{2+}-free buffer, followed by concomitant addition of R24571 and Ca^{2+} (to give a final $[Ca^{2+}]$ of 1 mM), nor the addition of Ca^{2+} (final $[Ca^{2+}]$ of 1 mM) about 30 s before stimulation with R24571, was sufficient to evoke an increase in $[Ca^{2+}]_i$ that was comparable with that shown in Figure 2. Therefore we conclude that calmidazolium does not act directly at the plasma membrane, but rather acts by emptying a filled Ca^{2+}-storage compartment which in turn induces an influx of Ca^{2+} across the plasma membrane.

Ca^{2+} fluxes across membranes of intracellular stores

Permeabilized cells in suspension were measured with the Ca^{2+}-sensitive electrode to assess whether R24571 acted on Ca^{2+}-storage compartments. Under these conditions any change in the Ca^{2+} concentration in the medium results from fluxes across the membranes of these compartments. Incubation of permeabilized cells with 5 μM calmidazolium indeed led to an increase in the Ca^{2+} concentration in the cell suspension, reflecting an efflux from Ca^{2+} stores (Figure 4).

In order to elucidate which type of store is affected by R24571, we measured the Ca^{2+} influx across the plasma membrane in the presence of specific inhibitors (Table 2). U73122, a compound that has been shown to block phospholipase C activity and/or the IP_3-sensitive store [24,25], almost completely abolished the

Table 2 Influence of inhibitors on the R24571-induced influx of Ca^{2+} across the plasma membrane, as measured in cell suspensions (at t_4–$t_{6.6}$) with the Ca^{2+} electrode

The amount of Ca^{2+} influx evoked by R24571 (5 μM) in control cells was set to 100%. The effect of preincubation (5 min) with the different inhibitors on R24571-induced Ca^{2+} influx is expressed as percentage (mean ± S.D.) inhibition of Ca^{2+} influx. The number in parentheses gives the number of experiments.

Addition	Concentration (μM)	Ca^{2+} influx of control cells (pmol/10^7 cells)	Inhibition (%)
U73122	5	233–375	93 ± 9.4 (3)
U73343	5	264–384	0 (2)
BHQ	100	232–584	75 ± 7.5 (3)
NBD-Cl	50	109–384	100 (2)
Bafilomycin A_1	20	275–330	38 ± 1 (2)

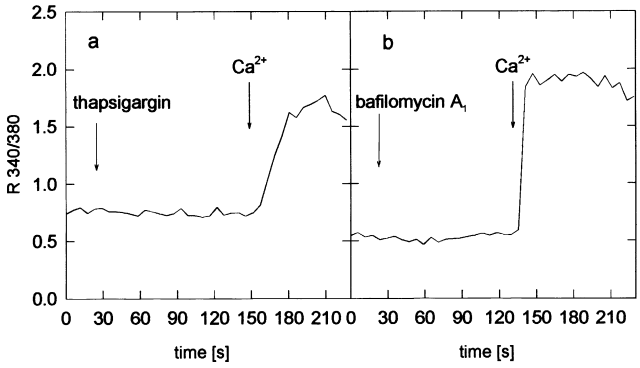

Figure 5 Influence of pump inhibitors on $[Ca^{2+}]_i$

Single fura-2-loaded cells at $t_{4.5}$ were analysed by $[Ca^{2+}]_i$ imaging. The time course of R340/380 is plotted. The experiments were carried out in buffer containing 1 mM $CaCl_2$. Addition of thapsigargin (**a**) or bafilomycin A_1 (**b**) is marked by an arrow. Incubation with these drugs (at a final concentration of 4.5 μM and 9 μM respectively) did not evoke transient changes in $[Ca^{2+}]_i$. The subsequent addition of Ca^{2+} at a final concentration of 9 mM led to high $[Ca^{2+}]_i$ elevations.

R24571-mediated Ca^{2+} influx; in contrast, the closely related but inactive analogue, U73343, was not effective. Therefore an influence of calmidazolium on the IP_3-sensitive store seems to be necessary for induction of influx across the plasma membrane. The Ca^{2+}-and H^+-pump inhibitors BHQ and NBD-Cl respectively, which have been shown to interact with the IP_3-sensitive store [11], likewise inhibited the Ca^{2+} influx. Preincubation with the H^+-pump inhibitor bafilomycin A_1, which affects Ca^{2+} uptake into acidosomes [9], also led to an inhibition of the R24571-induced Ca^{2+} influx, which was, however, less pronounced. From these data we conclude that, besides the IP_3-sensitive store, the acidosomes also are targets of action of R24571.

Effect of pump inhibitors on $[Ca^{2+}]_i$

The use of pump inhibitors also allowed us to distinguish whether R24571 acts by inhibiting the uptake or by inducing a release of Ca^{2+} from the storage compartments, or both. We tested the effect of addition of thapsigargin, BHQ or bafilomycin A_1 on $[Ca^{2+}]_i$. Addition of thapsigargin or bafilomycin A_1 (Figure 5) or BHQ (results not shown) alone did not induce global transient $[Ca^{2+}]_i$ changes comparable with those evoked by R24571. Yet the subsequent addition of large amounts of Ca^{2+}

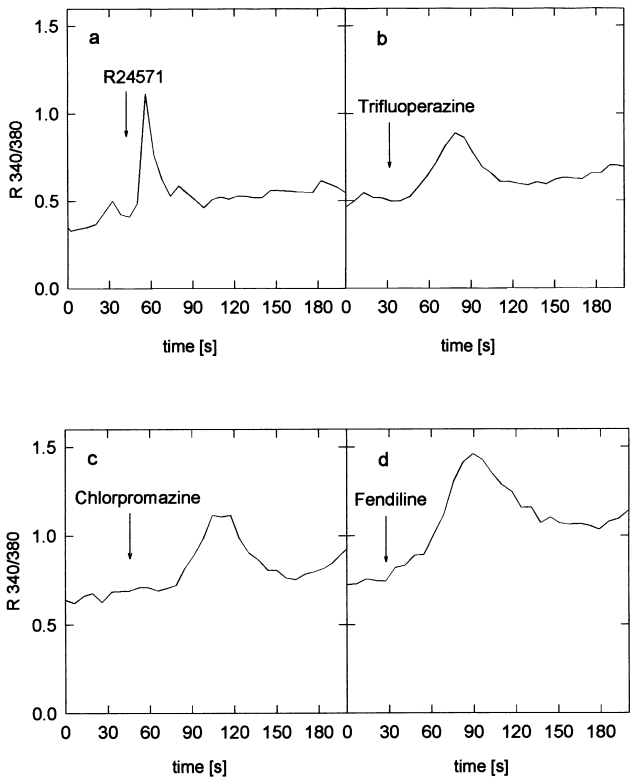

Figure 6 Transient $[Ca^{2+}]_i$ changes induced by other calmodulin antagonists

The time course of R340/380 of single fura-2-loaded cells analysed by $[Ca^{2+}]_i$ imaging at $t_{4.5}$ is plotted. The experiments were performed in buffer containing 1 mM Ca^{2+}. The arrows indicate the time point of addition of the drugs. (**a**) R24571 (final concentration 9 μM) induced a rapid $[Ca^{2+}]_i$ increase. (**b**)–(**d**) Addition of trifluoperazine, chlorpromazine and fendiline (final concentrations 1.8 μM, 1.8 μM and 34 μM respectively) also induced transient, yet slower, changes in $[Ca^{2+}]_i$.

(final concentration 9 mM) similarly led to large elevations of $[Ca^{2+}]_i$, which indicates that these drugs indeed had an inhibitory effect on the Ca^{2+}-pumping activity of the cells. Since R24571 could affect more than one Ca^{2+} pump and thereby induce $[Ca^{2+}]_i$ changes, we incubated the cells either with combinations of two or with all three of the above-mentioned pump inhibitors; however, these conditions also did not lead to a global transient elevation of $[Ca^{2+}]_i$ (results not shown). From these data we consider it improbable that inhibition of Ca^{2+} pumping is the sole mode of action of R24571.

Comparison of the effect of R24571 and other calmodulin antagonists on $[Ca^{2+}]_i$

To find out whether the effects of calmidazolium are calmodulin-mediated, we tested the $[Ca^{2+}]_i$ response of the cells to other well-established calmodulin antagonists, trifluoperazine, chlorpromazine, fendiline and W7. These compounds also induced transient changes in $[Ca^{2+}]_i$ (Figure 6); however, these $[Ca^{2+}]_i$ elevations proceeded more slowly as compared with the immediate and rapid increases evoked by R24571. Quantitative evaluation revealed that the percentage of cells responding with transient $[Ca^{2+}]_i$ changes was also decreased (Table 3). Calmidazolium at a concentration of 5–10 μM induced $[Ca^{2+}]_i$ transients in all of the cells tested; 2 μM of the compound led to 22% of responding cells. It is important to note that under these conditions the cells were fully viable, displaying normal cyto-

Table 3 Effect of calmodulin antagonists on $[Ca^{2+}]_i$

The percentage of fura-2-loaded cells (at t_4) responding with a transient increase in $[Ca^{2+}]_i$ was determined. In each experiment, 10 μl of substance was added to the medium covering the cells (100 μl). The numbers in parentheses give the positive/total number of cells tested. Cell viability was judged by the shape of the cells, the presence of cytoplasmic streaming and the extension of pseudopods.

Substance	Concentration (final) (μM)	Percentage of responding cells	Cell viability
R24571	5–10	100	Fully viable
R24571	2	22 (2/9)	Fully viable
Trifluoperazine	2–5	11 (2/18)	Occasional cell death
Chlorpromazine	2–5	19 (3/16)	Occasional cell death
Fendiline	34	25 (3/12)	Occasional cell death
W7	10–100	4 (1/26)	Occasional cell death

plasmic streaming and extension of pseudopods. In contrast, incubation with the other calmodulin antagonists induced occasional cell death (stopping cytoplasmic streaming, and considerable swelling of the cell) at the concentrations employed (which, under the experimental conditions of low cell density, were comparatively high, yet necessary to evoke an effect on $[Ca^{2+}]_i$); this was accompanied by slow and steady increases in $[Ca^{2+}]_i$ and finally in loss of fura-2 fluorescence (results not shown). The percentage of cells responding with transient $[Ca^{2+}]_i$ changes was 4 % for W7, 11 % and 19 % for trifluoperazine and chlorpromazine, respectively, and 25 % for fendiline. The rank order of the percentage of cells responding to calmidazolium, trifluoperazine, chlorpromazine and W7 is in agreement with the rank order of potency of these drugs to inhibit bovine heart calmodulin-dependent phosphodiesterase [26].

DISCUSSION

In this study we investigated the effect of the calmodulin antagonist calmidazolium on developing *Dictyostelium* cells, in order to elucidate mechanisms of regulation of the $[Ca^{2+}]_i$ homoeostasis in this organism. We found that incubation of the amoebae with R24571 induced both a rapid release of Ca^{2+} from intracellular storage compartments and an influx of Ca^{2+} across the plasma membrane which led to an instant global transient increase in $[Ca^{2+}]_i$.

From comparison of the results obtained with R24571 and other calmodulin antagonists, we conclude that interference with calmodulin-dependent reactions is involved in the effect of R24571 on $[Ca^{2+}]_i$ homoeostasis. Trifluoperazine, chlorpromazine, fendiline and W7 also induced transient $[Ca^{2+}]_i$ changes; yet the percentage of responding cells was lower, and these $[Ca^{2+}]_i$ increases were not as rapid as those induced by calmidazolium. These differences could be explained by the relative rank order of potency of these drugs to inhibit calmodulin-dependent processes; however, an action of calmidazolium that is independent of calmodulin cannot be excluded, and could take part in mediating the rapid changes in $[Ca^{2+}]_i$. To speculate on the mode of action of R24571, it is therefore necessary to consider both calmodulin-dependent and -independent effects of the drug.

Calmidazolium could act by inhibiting calmodulin-dependent Ca^{2+}-pumping activities of the cells. The plasma-membrane Ca^{2+} pump in other cell systems is regulated by calmodulin [16]. Until now, in *Dictyostelium* amoebae regulation of Ca^{2+}-ATPases by calmodulin has not been observed, in purified plasma-membrane vesicles [27], endocytotic vesicles [28] or acidosomes [10]. However, Rooney et al. [10] pointed out that the failure to detect an involvement of calmodulin could have been due to limited proteolysis of the pump during the purification procedure, leading to irreversible activation of the enzyme. From these data an inhibitory action of R24571 (or of the other calmodulin antagonists tested) on Ca^{2+} pumps cannot be excluded. Indeed, in homogenates of *Dictyostelium* cells calmidazolium inhibits $^{45}Ca^{2+}$ uptake into vesicles by inhibiting Ca^{2+} pumping; however, it also acts by directly activating Ca^{2+} release (M. Gröner and D. Malchow, personal communication). Such a combined action of inhibition of Ca^{2+} sequestration with the concomitant activation of Ca^{2+} release from storage compartments could be responsible for the observed effect of calmidazolium on $[Ca^{2+}]_i$. An influence of calmidazolium on the IP_3-sensitive store is reasonable: U73122, which, either directly [25] or indirectly (via inhibition of IP_3 production [24]), affects the IP_3-sensitive store, almost completely suppressed the R24571-mediated influx of Ca^{2+} across the plasma membrane.

The releasing action of calmidazolium could be due to antagonism of the Ca^{2+}/calmodulin-dependent phosphatase calcineurin. In fibroblasts, Ca^{2+} efflux from the IP_3-sensitive store is regulated by calcineurin: the IP_3-induced Ca^{2+} release is inhibited by high Ca^{2+} concentrations, and this inhibition was prevented by cyclosporin A and FK506 [18]. From their results the authors concluded that the IP_3 receptor is inactivated by dephosphorylation via calcineurin. *In vitro* 10 μM R24571 blocked both the calmodulin-stimulated and the calmodulin-independent, Ni^{2+}-stimulated, phosphatase activity of bovine brain calcineurin [29]; inhibition by W7 required roughly 100-fold higher concentrations. Likewise, both the calmodulin-dependent and -independent activity of recombinant catalytic calcineurin A from *Dictyostelium* was blocked by calmidazolium and W7 at similar concentrations to those outlined above (S. Hellstern and R. Mutzel, personal communication). If in *Dictyostelium discoideum* the release of Ca^{2+} by IP_3 was also regulated by phosphorylation and dephosphorylation via calcineurin, the presence of R24571 could lead to a greater portion of phosphorylated receptors susceptible to IP_3. The basal IP_3 concentration of about 3 μM [30] is already sufficient to induce substantial release of Ca^{2+} from the store when added to permeabilized cells [4].

Our experiments with inhibitors of Ca^{2+} pumps further strengthen the view that R24571 indeed affects the IP_3-sensitive pool. BHQ, which has been shown to inhibit agonist-induced Ca^{2+} fluxes across the membrane of the IP_3-sensitive store [11], decreased the R24571-mediated influx of extracellular Ca^{2+} by 75 %. However, bafilomycin A_1, which leads to an emptying of the acidosomal Ca^{2+} store by 50 % [9], also inhibited R24571-induced Ca^{2+}-influx, although to a lower degree, by 38 %. NBD-Cl, which affects both the IP_3-sensitive store [11] and the acidosomes [9], completely abolished the Ca^{2+} influx. From these data we conclude that both types of stores are affected.

From (1) the above-mentioned inhibition of the R24571-mediated extracellular Ca^{2+} influx by emptying of the stores via pump inhibitors and (2) the fact that it was necessary to preincubate the cells with Ca^{2+} in order to induce large $[Ca^{2+}]_i$ changes, we conclude that the target store(s) has to be in a filled state to be influenced and emptied by R24571. This process in turn leads to the opening of the plasma membrane and influx of Ca^{2+}. In many other higher eukaryotes, Ca^{2+} influx across the plasma membrane is regulated by the filling state of the stores; capacitative Ca^{2+} entry by activation of Ca^{2+}-repletion currents such as I_{CRAC}, I_{DAC} or I_{DC} can be induced by incubation with pump inhibitors and leads to global $[Ca^{2+}]_i$ increases (for reviews see [14,31]). However, in *Dictyostelium* cells depleting the stores

by inhibition of uptake with thapsigargin and/or BHQ and/or bafilomycin A_1 did not result in global $[Ca^{2+}]_i$ changes (although spatially restricted $[Ca^{2+}]_i$ changes could have taken place), whereas depletion by induction of release with R24571 did. One difference between the action of pump inhibitors and R24571 is the rate of liberation of Ca^{2+} from the stores: whereas BHQ evoked a rather slow efflux of $^{45}Ca^{2+}$ from intact preloaded cells, which was half-maximal at roughly 3 min [11], the Ca^{2+} release induced by R24571 in permeabilized cells was half-maximal already at roughly 30 s, as measured with the Ca^{2+}-sensitive electrode (see Figure 4). In *Dictyostelium* it might be necessary to deplete Ca^{2+}-storage compartments rapidly in order to evoke full capacitative Ca^{2+} entry. Treatment with pump inhibitors could result in only a comparably small amount of extracellular Ca^{2+} uptake, not sufficient to induce global $[Ca^{2+}]_i$ elevations. As yet, both the signalling pathway leading to opening of the plasma membrane and the mechanism of Ca^{2+} entry are unknown.

In *Dictyostelium* amoebae the use of R24571 will provide a tool to examine the consequence of transient changes in $[Ca^{2+}]_i$ homoeostasis on different parameters both short-term, such as rearrangements of the cytoskeleton leading to altered cell shape and motility, and also long-term effects on the development of the cells and the patterning of multicellular structures.

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