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INOSITOL 1,4,5-TRISPHOSPHATE AND CALCIUM STIMULATE ACTIN POLYMERIZATION IN *DICTYOSTELIUM DISCOIDEUM*

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SUMMARY

The effect of chemoattractants such as cyclic AMP and folate on amoebae of the cellular slime mould *Dictyostelium discoideum* is to cause a series of rapid intracellular responses. One of the most rapid of these responses is the polymerization of actin associated with the cytoskeleton, an event correlated with pseudopodium formation, which ocurrs within 3–5 s of chemotactic receptor stimulation. We report that this response can be mimicked by addition of 5 μM-inositol 1,4,5-trisphosphate (IP_3) or by addition of 100 μM-Ca^{2+} to saponin-permeabilized amoebae.

The data suggest that cytoskeletal actin polymerization occurs in normal cells as a result of IP_3 formation in response to cell surface receptor stimulation and the consequent release of Ca^{2+} from internal stores.

INTRODUCTION

The cellular slime moulds provide an amenable eukaryotic system for studying cellular chemotaxis and the transduction of extracellular signals into intracellular actions. During the food-seeking phase in the species *Dictyostelium discoideum*, the amoebae respond chemotactically to folate and during the subsequent starvation phase (that eventually leads to the production of motile multicellular aggregates) they respond to cyclic AMP. The folate and cyclic AMP signals are detected by separate cell surface receptors, yet seem to induce an array of intracellular molecular reactions common to both stimuli.

Two of the best-documented responses are the polymerization of actin associated with the cytoskeleton, which peaks 3–5 s after stimulation with folate or cyclic AMP (McRobbie & Newell, 1983, 1984*a*, 1985*a*,*b*), and the transient accumulation of cyclic GMP that peaks 9–12 s after stimulation (Mato *et al.* 1977; Wurster *et al.* 1977; Ross & Newell, 1981). Both of these responses have been implicated in chemotactic movement, although from work with streamer mutants there is evidence that they operate *via* independent signalling pathways, with cyclic GMP affecting cell movement at a point after the initial polymerization of actin (McRobbie & Newell, 1984*b*).

Recent evidence has suggested that release of Ca^{2+} from intracellular stores may be the rapid intracellular response that lies on the signal transduction pathway between

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activation of the cell surface receptor and the formation of cyclic GMP, and probably the actin response (Europe-Finner & Newell, 1984; Europe-Finner *et al.* 1984, 1985). Stimulation of Ca^{2+} uptake from the external medium has also been observed (Wick, Malchow & Gerisch, 1978; Bumann *et al.* 1984). Recent data, however, indicate that such increased uptake is not the primary response, as it does not occur within the first 10 s after chemotactic stimulation when the actin and cyclic GMP responses are observed (Europe-Finner & Newell, 1985*a*).

In mammalian cells the link in the signalling pathway between binding of hormones to cell surface receptors and release of internally sequestered Ca^{2+} has been shown to involve activation of a plasma membrane-bound phosphodiesterase, which cleaves phosphatidyl inositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, the IP_3 produced stimulating release of Ca^{2+} mainly from the endoplasmic reticulum (Berridge, 1984; Joseph, 1984; Berridge & Irvine, 1984; Burgess *et al.* 1984; Joseph *et al.* 1984).

In a preliminary study we showed that, in *D. discoideum*, low concentrations of IP_3 applied externally to permeabilized amoebae induced the rapid formation of cyclic GMP (Europe-Finner & Newell, 1985*b*). In this report we show that IP_3 can also stimulate the polymerization of actin associated with the cytoskeleton and we compare the characteristics of this response with that of cyclic GMP induction.

MATERIALS AND METHODS

Materials

Inositol 1,4,5-trisphosphate (potassium salt) and the cyclic GMP radio-immunoassay kit were obtained from Amersham Int. Plc. Coomassie Brilliant Blue R, ethidium bromide, Trypan Blue and saponin were obtained from Sigma.

Harvesting of amoebae

D. discoideum strain NC4 was grown in association with *Klebsiella aerogenes* (strain OXF1) on SM nutrient agar (Sussman, 1966). Amoebae were prepared by growth as lawns on SM agar under conditions permitting uniform clearing of the bacteria by the feeding amoebae. Amoebae were harvested from the bacterial growth plates in PB (17 mM-Na/K phosphate buffer, pH 6·1) and washed free of bacteria by centrifugation at 190 ***g*** for 2 min. After three such washes the cells were resuspended in PB at $10^7\,ml^{-1}$ and either used immediately for experiments involving folate stimulation or saponin treatment, or incubated at 22°C with aeration in a rotary incubator at 170 rev. min^{-1} for 5 h for those experiments involving stimulation by cyclic AMP or requiring aggregation-competent cells.

Permeabilization of amoebae

To permeabilize the cells, samples were resuspended in PB at 1×10^7 to $5\times10^7\,ml^{-1}$ and shaken at 22°C in a rotary incubator at 170 rev. min^{-1} for 30 min in the presence (or, for controls, in the absence) of 1 mg ml^{-1} saponin. Both controls and saponin-treated amoebae were then washed three times in PB and resuspended in PB at $10^8\,ml^{-1}$ or 2×10^7 cells ml^{-1} for actin or cyclic GMP responses, respectively. The concentration of saponin used was chosen as that showing no significant cell lysis and giving reproducible permeability as judged by uptake of Trypan Blue (0·1 %), seen with a phase-contrast microscope, and uptake of ethidium bromide (100 μg ml^{-1}), seen under ultraviolet light with an epifluorescence microscope (Europe-Finner & Newell, 1985*b*).

Stimulation of amoebae with IP_3 for cyclic GMP formation

Samples of 100 μl containing 2×10^7 permeabilized (or untreated) cells ml^{-1} were dispensed into a series of microcentrifuge tubes shaken on an IKA-Vibrax platform shaker at 1400 rev. min^{-1}. Amoebae were stimulated with 20 μl of IP_3 to give a final concentration of 5 μM and the reaction was terminated at selected times from 0–31 s by addition of 100 μl of 3·5 % (v/v) $HClO_4$. Assay of cyclic GMP used a radio-immunoassay kit from Amersham with sample preparation as described by Van Haastert *et al.* (1981). Controls showed that IP_3 did not interfere with the binding properties of the serum used in the radioimmunoassay.

Stimulation of amoebae with IP_3, cyclic AMP or folate, and isolation of cytoskeletal actin

Cytoskeletal proteins were isolated as proteins insoluble in Triton X-100, using the method of McRobbie & Newell (1984). Typically, 150-μl samples of permeabilized (or untreated) cells at 10^8 ml^{-1} were distributed to microcentrifuge tubes shaken on an IKA Vibrax platform shaker at 1400 rev. min^{-1}. Amoebae were stimulated in individual tubes by addition of 10 μl of IP_3, cyclic AMP or folate to give a final concentration of 5 μM, 50 nM or 50 μM, respectively, and the reaction was stopped at measured times from 0–30 s by addition of 150 μl of Triton stock solution (containing 2 % Triton X-100, 20 mM-KCl, 20 mM-imidazole, 20 mM-EGTA, 4 mM-sodium azide, pH 7·0). After separation and washing of the cytoskeletons by centrifugation at 8000 *g* for 4 min (twice) the protein pellet was prepared for one-dimensional gel electrophoresis by heating to 80 °C for 10 min in 50 μl of sample buffer (containing 2 % sodium dodecyl sulphate, 10 mM-dithiothreitol and 10 % glycerol).

Stimulation of amoebae with Ca^{2+} and isolation of cytoskeletal actin

Samples of 150 μl of permeabilized amoebae at 10^8 ml^{-1} were distributed to microcentrifuge tubes shaken on an IKA Vibrax platform shaker at 1400 rev. min^{-1}. Amoebae were stimulated in individual tubes by addition of 10 μl of 1·6 mM-$CaCl_2$ to give a final concentration of 100 μM. The reaction was stopped at measured times by addition of 150 μl of Triton stock solution and cytoskeletons were prepared as described above.

Electrophoresis of cytoskeletal proteins

One-dimensional electrophoresis was carried out in 10 % (w/v) polyacrylamide slab gels with 4·5 % stacking gels according to the method of Laemmli (1970). Proteins were stained with Coomassie Brilliant Blue R, destained in methanol/water/acetic acid (2:7:1, by vol.) and the resulting stained protein bands were scanned on a Joyce-Loebel scanning densitometer. Changes in actin content were quantified by cutting out, weighing and comparing the appropriate peaks from the scanning traces obtained.

RESULTS

Cytoskeletal actin in unstimulated, permeabilized amoebae

Before attempting to investigate the effects of IP_3 on cytoskeletal actin, preliminary experiments were carried out to determine the stability of the unstimulated cytoskeletal actin in saponin-permeabilized amoebae. It was found that with 10^8 permeabilized amoebae per ml a slow but steady polymerization of cytoskeletal actin occurred during the initial 6 min of incubation following permeabilization and resuspension. After 6 min, the rate of polymerization rapidly increased (Fig. 1). At higher cell densities the rapid rise occurred progressively earlier. In view of the results with Ca^{2+} stimulation described in detail below, this change in cytoskeletal

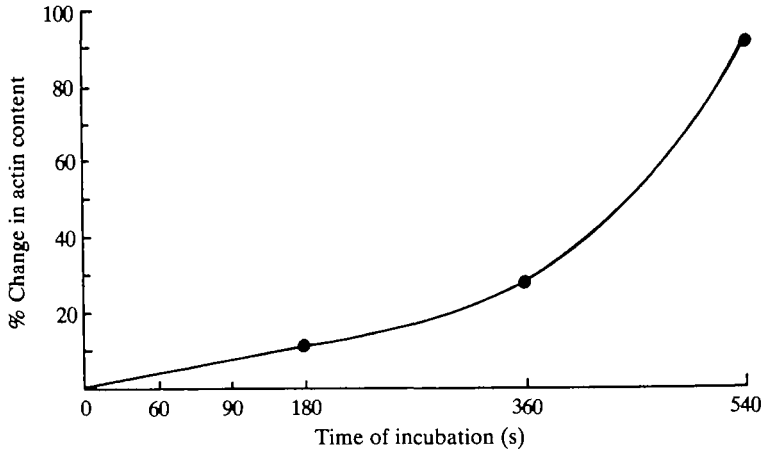

Fig. 1. Time course of changes in actin content of the cytoskeleton of *D. discoideum* in unstimulated saponin-permeabilized vegetative (0 h) amoebae. The results are for a typical experiment with each point the mean of assays in triplicate.

actin in unstimulated cells can probably be attributed to a slow release of Ca^{2+} from intracellular stores in the permeabilized cells.

As a consequence of these results, subsequent experiments were performed with cell densities not exceeding 10^8 ml^{-1} and measurements made within the first 5 min of incubation. The actin content of cytoskeletons determined in unstimulated cells under these conditions immediately before and after determination of IP_3- or Ca^{2+}-stimulated samples showed that the unstimulated actin polymerization under these conditions was no more than 10–15 % of the starting value during the course of the 2–3 min period of any experiment.

Changes in cytoskeletal actin following stimulation with IP_3

When saponin-permeabilized amoebae were stimulated with 5 μM-IP_3, the actin associated with their cytoskeletons increased rapidly over the initial 15 s (Fig. 2). When this effect is compared with that normally produced by the chemoattractants folate or cyclic AMP in non-permeabilized amoebae (Figs 3, 4) it is apparent that while the magnitude of the response is similar, IP_3 causes only the increase in actin association with the cytoskeleton but not the subsequent sharp decrease that is a characteristic of the response to chemoattractants. The time course also differs, with IP_3 producing its effects more slowly over 15 s compared to 3–5 s for folate and cyclic AMP.

For comparison with the effects of folate and cyclic AMP on normal non-permeabilized amoebae, the effect of IP_3 on such non-permeabilized amoebae was also tested. The result was surprising (Fig. 5). It had been anticipated from work with mammalian cells that permeabilization of the amoebae would be essential for the effects of IP_3 to be seen. However, in intact amoebae IP_3 produced an increase in the actin associated with the cytoskeleton very similar to that produced with

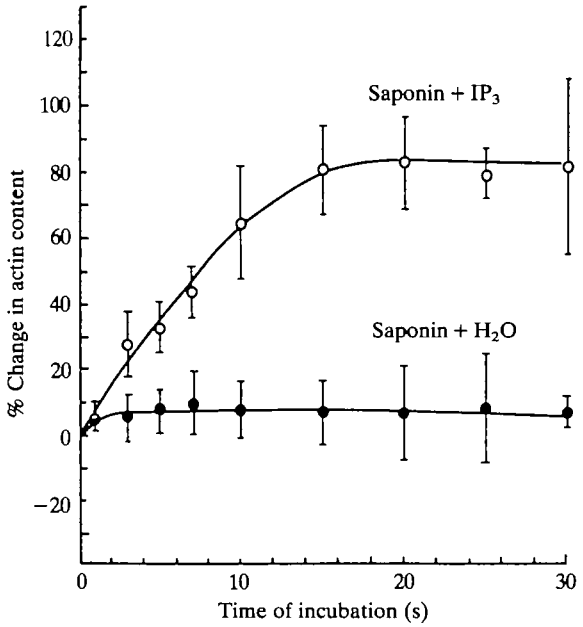

Fig. 2. Time course of changes in actin content of the cytoskeletons of *D. discoideum* in saponin-permeabilized vegetative (0 h) amoebae following stimulation with 5 μM-IP_3 (○——○) or water (●——●). Results are means of five separate experiments. Bars represent the S.E.M.

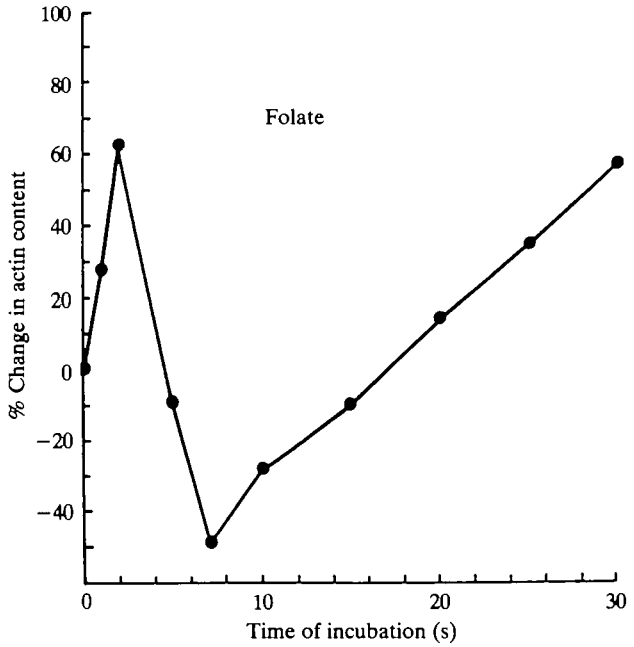

Fig. 3. Time course of changes in actin content of the cytoskeletons of *D. discoideum* in 0 h amoebae following stimulation with 50 μM-folate. Results are the means of two experiments.

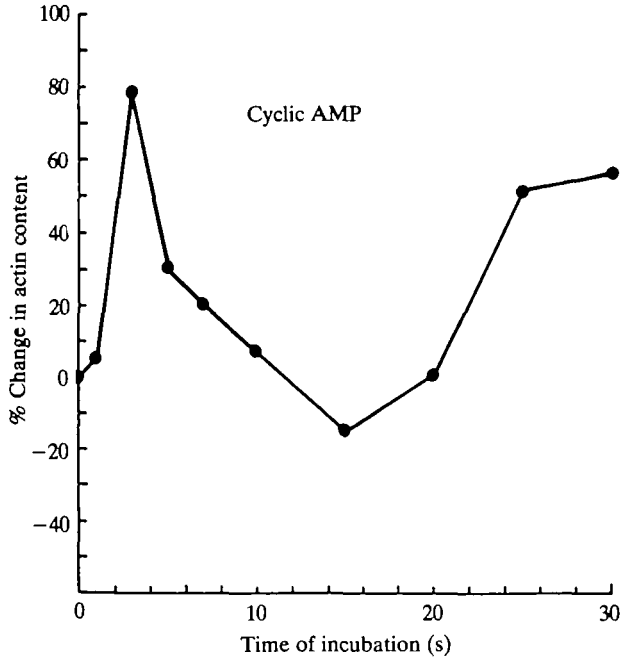

Fig. 4. Time course of changes in actin content of the cytoskeletons of *D. discoideum* in 5 h amoebae following stimulation with 50 nM-cAMP. Results are means of two experiments.

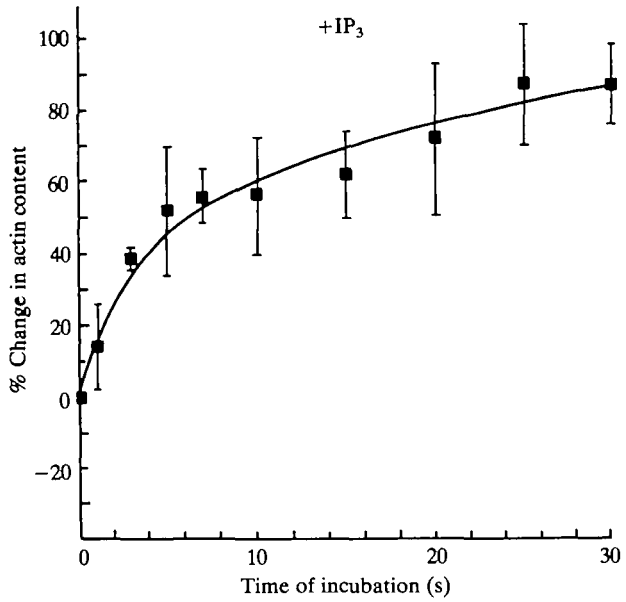

Fig. 5. Time course of changes in actin content of the cytoskeletons in 0 h amoebae (that had not been permeabilized) following stimulation with 5 μM-IP_3. Results are means of five experiments. Bars represent S.E.M. Controls treated with water showed no significant change in actin content (data not shown).

permeabilized amoebae. This effect was observed whether the amoebae used were taken directly from the growth phase or after 5 h of development in starving suspensions. To confirm that the effect of the IP_3 was indeed due to its action on intact amoebae, rather than an effect after cell lysis during preparation of the cytoskeletons, an experiment was performed in which IP_3 was added to amoebae simultaneously with the Triton X-100 for cell lysis. The results confirmed that IP_3 had no effect on actin polymerization if added at this time.

Comparison of IP_3 effects on actin polymerization and cyclic GMP formation

Using conditions identical to those used for experiments on actin polymerization, IP_3 induced a rapid accumulation of cyclic GMP (Fig. 6). The magnitude of the response in permeabilized cells was similar to that produced by the chemoattractant cyclic AMP in normal non-permeabilized amoebae but showed a peak at approximately 15 s, rather than the more rapid response shown after cyclic AMP stimulation, which always peaked at 9–12 s. In contrast to the potent effects of IP_3 on the actin response in non-permeabilized amoebae, IP_3 had a measurable but much reduced effect in stimulating cyclic GMP formation in non-permeabilized cells (Fig. 6).

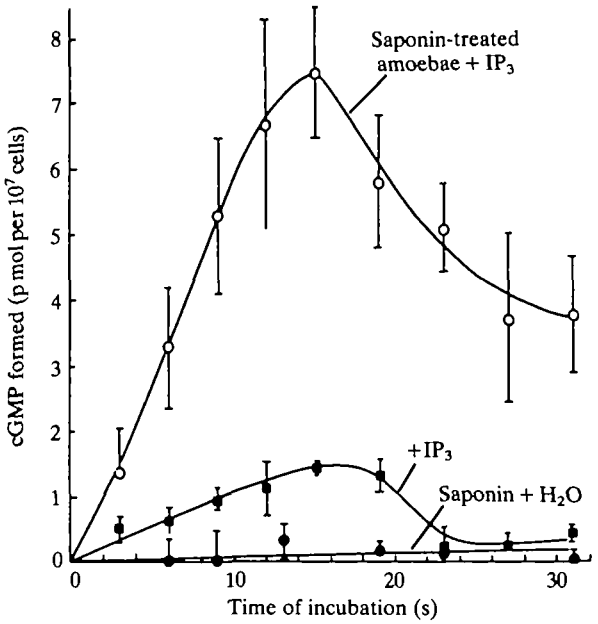

Fig. 6. Accumulation of cyclic GMP in 0 h saponin-permeabilized (○——○) and non-permeabilized (■——■) amoebae of *D. discoideum* following stimulation with 5 μM-IP_3. Saponin-permeabilized amoebae stimulated with water (●——●) were also included as controls. The basal values for cyclic GMP concentration (which vary between different batches of amoebae in the range 0·2 to 1·5 pmol per 10^7 cells) have been deducted from all values shown. Results are means of six experiments for IP_3-treated cells and three experiments for water controls. Bars represent the S.E.M.

Stimulation of actin polymerization by calcium

The effect of IP_3 in mammalian cells is to release Ca^{2+} from internal stores such as the endoplasmic reticulum (Berridge, 1984). If IP_3 operated in a similar manner in *D. discoideum* it seemed reasonable to suppose that addition of Ca^{2+} to permeabilized amoebae would have an action on actin polymerization similar to that of IP_3. This was found to be correct. Addition of 100 μM-Ca^{2+} produced a rapid cyto-

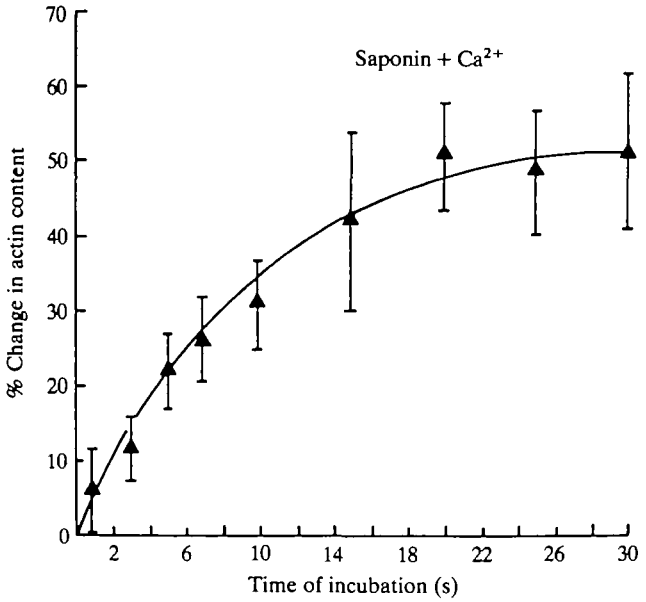

Fig. 7. Time course of changes in actin content of the cytoskeletons of *D. discoideum* in saponin-permeabilized amoebae following stimulation with 100 μM-$CaCl_2$. Results are means of five separate experiments. Bars represent the S.E.M.

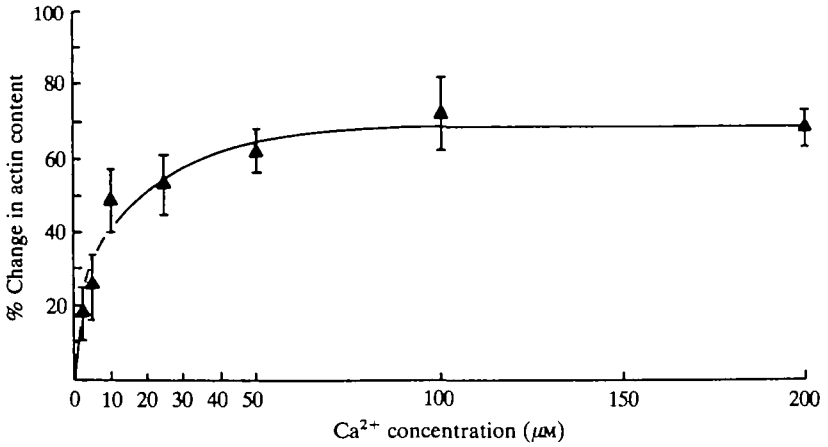

Fig. 8. Dose–response curve for Ca^{2+} stimulation of cytoskeletal actin changes in 0 h saponin-permeabilized *D. discoideum* amoebae. Results are means of six separate experiments. Bars represent the S.E.M.

skeletal accumulation of actin (Fig. 7). The magnitude of the response showed some variability between different batches of cells but the kinetics of the response and the mean extent of actin polymerization from five experiments were similar to those produced by IP_3 (compare Figs 2 and 7). A dose–response curve (Fig. 8) revealed that the response to Ca^{2+} showed a plateau between 50 μM and 100 μM, with a half-maximal response at 5–10 μM. Similar studies using other ions (Na, K, Mg and Mn) showed no significant responses.

DISCUSSION

Our finding, that IP_3 is able to stimulate both cytoskeletal actin polymerization and the formation of cyclic GMP to an extent similar to that produced by normal chemoattractants, suggests that *D. discoideum*, like many mammalian cells, uses IP_3 as a link in the transduction of signals emanating from the cell surface membrane receptors.

A notable difference in the effects of IP_3 on the formation of cyclic GMP and the cytoskeletal accumulation of actin is that in the case of cyclic GMP formation a rise and fall is elicited, which is similar to that produced by chemoattractants, whereas for actin accumulation only a rise to a plateau value is produced (compare Figs 2 and 6). It is interesting in this connection that Wurster & Butz (1983) reported that the level of intracellular cyclic GMP adapts to a continuous application of cyclic AMP by forming a peak at 20 s and then decreasing to 50 %, even in the presence of a continuous supply of 5 nM-cyclic AMP per s. As we find that bypassing the cell surface cyclic AMP receptors by stimulation with IP_3 shows a similar degree of adaptation for cyclic GMP formation (but not for actin polymerization) it seems likely from our data that such adaptation of the cyclic GMP response is not mediated through the cell surface cyclic AMP receptors. Instead, we suggest that the decrease in the cyclic GMP after the initial rise may be due to the mechanism of regulation of the cyclic-GMP-specific phosphodiesterase that rapidly breaks down the cyclic GMP after it is formed. Van Haastert & Van Lookeren Campagne (1984) have shown that this enzyme is strongly activated by its substrate, cyclic GMP, and as a consequence a rise in cyclic GMP concentration would rapidly activate destruction of the additional cyclic GMP formed.

The finding that IP_3 does not bring about a rapid decrease in cytoskeletal actin after the initial peak of accumulation at 3–5 s, as normally seen after stimulation of amoebae with cyclic AMP or folate, suggests that the normal decrease is not triggered automatically by such a cytoplasmic mechanism as for the cyclic GMP response, but requires further receptor-controlled regulation which is bypassed by IP_3. This actin depolymerization, which is commonly seen as a trough in the cytoskeletal actin curve that dips below the initial baseline (see Fig. 3), and which has been correlated with the rounding up or 'cringe' response in chemotactically responding amoebae (McRobbie & Newell, 1985*b*), seems, therefore, to require the rapid termination and destruction of the IP_3 signal and, or, another counteractive signal from the cell surface chemotactic receptors.

A surprising finding in this study was the ability of IP_3 to induce the actin accumulation response in non-permeabilized control amoebae. A possible explanation for such a result could have been that the IP_3 (which contained less than 2% phosphate-containing impurities as analysed by Amersham) was contaminated with a chemoattractant such as cyclic AMP or folate. However, against this explanation is the observation just referred to that IP_3 does not show the rapid loss of actin from the cytoskeleton after the inital peak that is a characteristic of all chemoattractants tested with *Dictyostelium* species (McRobbie & Newell, 1984*a*). Additionally, IP_3 had its effects equally on 0 h vegetative and 5 h starved cells, whereas folate shows its maximal effect at 0 h and little activity at 5 h, and cyclic AMP shows scarcely measurable activity at 0 h and maximal activity after 5–8 h. Moreover, although IP_3 did have a small effect on cyclic GMP formation in non-permeabilized amoebae, this effect was very much increased after saponin treatment, implying that (at least for this response) entry to the cell by the stimulant was rate-limiting. In a previous study it was found that amoebae that have been harvested and subjected to the necessary rigours of being pelleted and resuspended are to a slight extent permeable to the test compound ethidium bromide (Europe-Finner & Newell, 1985*b*), and we suggest that the effects of IP_3 on control cells was due to this slight permeability under the conditions used. The fact that with such non-permeabilized cells the effect of IP_3 on actin polymerization is much greater than it is on cyclic GMP formation possibly indicates that the sensitivities of the two responses are different, such that cyclic GMP formation requires a higher Ca^{2+} concentration than does actin accumulation before the response is triggered. A detailed comparative study of the effects of Ca^{2+} on actin polymerization and cyclic GMP formation is in progress that may confirm or refute this possibility.

The finding that Ca^{2+} can mimic the effect of IP_3 on actin polymerization further supports the hypothesis that *D. discoideum* amoebae regulate their actin polymerization during chemotaxis by liberation of Ca^{2+} from internal stores, as a consequence of the IP_3 produced in response to cell surface receptor occupation by cyclic AMP. A more direct test of the effect of IP_3 on cytosolic Ca^{2+} levels is currently under way.

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