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The role of calcium in aggregation and development of *Dictyostelium*

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Abstract. Changes in cytosolic Ca^{2+} play an important role in a wide array of cell types and the control of its concentration depends upon the interplay of many cellular constituents. Resting cells maintain cytosolic calcium ($[Ca^{2+}]_i$) at a low level in the face of steep gradients of extracellular and sequestered Ca^{2+}. Many different signals can provoke the opening of calcium channels in the plasma membrane or in intracellular compartments and cause rapid influx of Ca^{2+} into the cytosol and elevation of $[Ca^{2+}]_i$. After such stimulation Ca^{2+} ATPases located in the plasma membrane and in the membranes of intracellular stores rapidly return $[Ca^{2+}]_i$ to its basal level. Such responses to elevation of $[Ca^{2+}]_i$ are a part of an important signal transduction mechanism that uses calcium (often via the binding protein calmodulin) to mediate a variety of cellular actions responsive to outside influences.
Key words. ATPase; cAMP; calcium pools; cGMP; oscillation; signal transduction.

Determination of intracellular calcium

Two methods have been used to measure $[Ca^{2+}]_i$ in *Dictyostelium*: the calcium indicator fura-2 has been used with single cells, and the calcium-sensitive protein aequorin for analysis in cell suspensions or by imaging cell aggregates. Fura-2/AM was loaded by electroporation by Abe and colleagues[1-3] since the cell-permeable ester (without electroporation) does not lead to fura-2 accumulation within the cells[85]. The drawback of the procedure used by Abe et al. was that the cells were rounded and did not adhere to the coverslip by themselves[3]. As a consequence, their finding that cyclic AMP caused an increase in $[Ca^{2+}]_i$ in some of their cells should be considered with caution and may reflect damage to the control of $[Ca^{2+}]_i$ homeostasis in these cells.

Schlatterer et al.[85] overcame this problem by introducing fura-2 by scrape loading. A further improvement was the coupling of fura-2 to aminodextran in order to avoid sequestration. These authors could not detect global changes of $[Ca^{2+}]_i$ following 0.1–10 μM cyclic AMP stimulation, despite the fact that cyclic AMP clearly causes considerable Ca^{2+} influx in cell suspensions. An explanation for this discrepancy is that Ca^{2+} is rapidly taken up by intracellular organelles[32, 79]. Indeed, even after permeabilisation with 3 mg/ml saponin in the presence of 1 mM Ca^{2+} the initial increase in $[Ca^{2+}]_i$ was short-lived. Ca^{2+} sequestration may be modulated by GTP. After GTPγS was introduced into cells, addition of 10 μM cyclic AMP elicited transient $[Ca^{2+}]_i$ increases in 30% of the cells whereas control cells did not respond[87]. In hepatocytes it was found that GTP connects Ca^{2+} stores and thus provides for a greater releasable Ca^{2+} pool[41]. The presence of GTPγS may either keep phospholipase C in an activated state for longer and thus lead to a higher $InsP_3$ transient following cyclic AMP addition and/or increase Ca^{2+} pool size[29, 71]. Since the formation of large vacuoles containing Ca^{2+} was observed in the presence of GTPγS the second alternative seems likely.

The high concentrations of cyclic AMP that seem to be required to induce gene expression can apparently overwhelm the efficient Ca^{2+} sequestration in *Dictyostelium*. Schlatterer et al.[88] found that such high cyclic AMP doses induced receptor-mediated Ca^{2+} influx independent of Gα2 function and caused transient $[Ca^{2+}]_i$ increases that peaked about 20 s after stimulation in vegetative as well as aggregation-competent cells. The Ca^{2+} changes amounted to 100% increases over basal level, this being about 100 nM in vegetative cells and 50 nM in aggregative cells.

The measurement of Ca^{2+} with aequorin requires prior transformation of the *Dictyostelium* cells with the gene for the protein apoaequorin. Incubating the transformed cells with coelenterazine produces aequorin. In the presence of oxygen and Ca^{2+}, coelenterazine is oxidized to coelenteramide which emits blue light when bound to apoaequorin. Saran et al.[83] found that aggregative cell suspensions responded to repeated cyclic AMP stimulation of 50 nM concentration at 10 °C with tiny increases over basal level in about 12 s, whereas vegetative cells did not respond. The response time is consistent with the $InsP_3$-induced Ca^{2+} release observed in permeabilized cells[33]. The Ca^{2+} concentration of vegetative cells was estimated at 100 nM, which is in agreement with the fura-2 measurements. However, the basal level of aggregative cells was 264 nM, which far exceeds the value obtained with fura-2. The difference can be tentatively explained if one takes into consideration that aequorin reacts with Ca^{2+} more or less

irreversibly. Aggregative cells take up Ca^{2+} without being stimulated[17]. This flux may not produce a significant higher $[Ca^{2+}]_i$ level since the Ca^{2+} is sequestered. However, the steady flux is sensed by aequorin which is constantly turned over thus yielding a sustained luminescence. By contrast, fura-2 acts by equilibrium binding. It may, however, reduce the basal level and the height of Ca^{2+} elevations due to buffering at concentrations above 500 μM (ref. 6) a concentration that exceeds by far the one used by Schlatterer et al.[85].

Interestingly, Saran et al.[83] found that prestalk cells exhibited substantial increases in $[Ca^{2+}]_i$ upon repeated stimulation with low doses of cyclic AMP (140 nM), whereas prespore cells behaved more like aggregative cells. In agreement with this, Cubitt et al.[25] reported that elevated $[Ca^{2+}]_i$ levels were detected preferentially in those zones where prestalk cells are localized. Using imaging of aequorin luminescence they detected a rise of $[Ca^{2+}]_i$ over a period of 3 h, from 6 h after starvation to the mound stage. At that stage a prominent ring of cells displayed high $[Ca^{2+}]_i$ while later such zones of high $[Ca^{2+}]_i$ were detected in the prestalk zone and, to a minor extent, in the posterior part of the slug. These results hint at the possibility that elevation of $[Ca^{2+}]_i$ is a key component of the intracellular 'signal' controlling prestalk cell gene expression, whereas, during differentiation to aggregation competence, small, possibly local $[Ca^{2+}]_i$ changes are required for regulation of motility, orientation and signal transduction.

Ca^{2+}-ATPases and Ca^{2+} pumps

Plasma membrane pumps

Stimulation of cells with cyclic AMP induces a rapid influx of Ca^{2+} (ref. 17). This is frequently preceded by a brief phase of Ca^{2+} extrusion[33] and is followed by a more prolonged phase of extrusion[17]. These extrusion phenomena point to the existence of one or more plasma membrane pump(s), and Ca^{2+}-ATPase activities have been demonstrated in purified plasma membranes[13]. The pump studied by Milne and Coukell[68] and originally thought by them to be plasma-membrane localised was subsequently shown to be intracellular[69], and probably corresponds to the 'acidosomal' pump of Rooney and Gross[79].

Intracellular Ca^{2+} pumps

As *Dictyostelium* amoebae develop towards aggregation-competence they do not release all the Ca^{2+} that is taken up in response to successive cyclic AMP signals; instead some Ca^{2+} is presumably sequestered in internal stores[17] (see above), (fig. 1). Rooney and Gross[79] and Rooney et al.[78] have characterised a Ca^{2+}-sequestering activity in partially purified preparations of 'acidosomes' prepared from vegetative amoebae. Acidosomes received their name because they are rich in H^+-ATPase and are rapidly acidified when incubated with ATP (ref. 73). The acidosomal Ca^{2+} uptake is inhibited by vanadate, an inhibitor of those ion pumps that form a phosphorylated enzyme intermediate during their reaction cycle[75]. It is also sensitive to 2,5-di-*tert*-butyl-1,4-benzohydroquinone (BHQ), one of a group of recently identified inhibitors of mammalian sarcoplasmic and endoplasmic reticulum Ca^{2+}-ATPases (SERCA Ca^{2+}-ATPases)[35]. Sequestration and/or retention of vesicular Ca^{2+} by this activity is sensitive to dissipation of the acidosomal proton gradient by weak bases or by the vacuolar proton ATPase inhibitor bafilomycin and hence is at least partially dependent on the pH gradient across the vesicle membrane[79]. Ca^{2+} uptake in whole-cell lysates displays much the same properties and presumably reflects the same activity[79]. The Ca^{2+} uptake activity studied by Milne and Coukell[69] in filipin permeabilised cells is probably due to the same system and has been shown to be unchanged for at least the first 18 hours of normal development. A putative *Dictyostelium* Ca^{2+}-

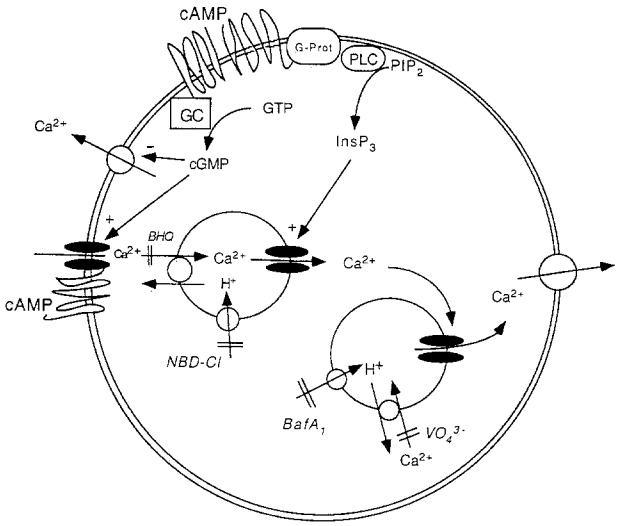

Figure 1. Hypothetical scheme for the regulation of Ca^{2+} fluxes in *Dictyostelium* amoebae. Binding of cyclic AMP to cell surface receptors (which span the membrane 7 times) activates phospholipase C (PLC) via the G-protein (Gα2). $InsP_3$ is formed which triggers Ca^{2+} release from the $InsP_3$-sensitive store. Alternatively $InsP_3$ could be formed by hydrolysis of $InsP_5$ or the sensitivity of the $InsP_3$ receptor to the resting level of $InsP_3$ could be increased by a cyclic AMP-induced phosphorylation of the $InsP_3$ receptor. The empty store elicits Ca^{2+} entry across the plasma membrane by an unknown mechanism.
During spike-like oscillations Ca^{2+} entry is enhanced by cyclic GMP formed by cyclic AMP-stimulation of guanylate cyclase (GC). The cyclic GMP could act either (as in visual transduction) by binding to the cytoplasmic side of the ion channel, or by inhibiting the Ca^{2+} ATPase that pumps Ca^{2+} out of the cell. The Ca^{2+} taken up by receptor stimulation is stored in the $InsP_3$-sensitive pool. During oscillation Ca^{2+} released from the $InsP_3$-sensitive pool may restimulate PLC and/or cause Ca^{2+} release from the ($InsP_3$-insensitive acidosomes) (CICR).
In addition to the $InsP_3$ pathway, βγ-subunits of a G-protein could be involved in the regulation of Ca^{2+} entry across the plasma membrane (indicated in the figure as direct contact of the cyclic AMP receptor with the Ca^{2+} channel). (Modified from Flaadt et al.[32])

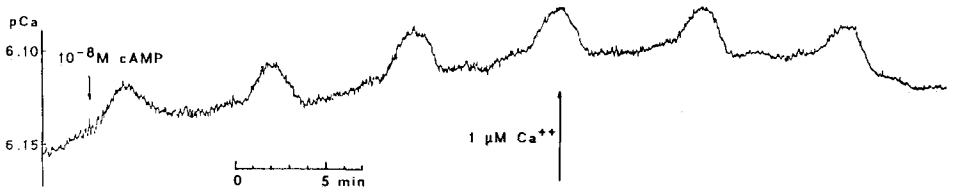

Figure 2. Cyclic AMP-induced oscillation of the extracellular Ca^{2+} concentration in mutant 32b. Note that oscillations begin with Ca^{2+} efflux, indicating $[Ca^{2+}]_i$ oscillations. The extracellular Ca^{2+} concentration was determined with a Ca^{2+} sensitive electrode[17] in a suspension of 1×10^8 cells per ml, 5 h after onset of differentiation (from Menz[66]) (Ca^{2+} efflux amounts to 40–50 pmol per 10^7 cells).

ATPase gene (PMCA1) that shows 46% homology with the mammalian plasma membrane Ca^{2+}-ATPases has recently been cloned (J. Moniakis and B. Coukell, pers. commun.). It displays a similar degree of homology with the PMC1 gene of *Saccharomyces cerevisae* that appears to code for a Ca^{2+}-ATPase located in the vacuole, not in the plasma membrane[26].

Acidosomes are probably fragments of the contractile vacuole complex involved in cellular osmotic regulation. The fact that these complexes have a high calmodulin content together with Ca^{2+} sequestering activity suggest that these structures are also active in cellular calcium regulation[104]. D. Traynor (pers. commun.) has obtained evidence that as well as the low affinity high capacity acidosomal Ca^{2+}-sequestering activity there exists a high affinity low capacity, ATP-dependent activity that is independent of a vesicular proton gradient. In addition, Malchow and his collaborators have identified a Ca^{2+} sequestering activity in permeabilised amoebae that is activated by cyclic AMP stimuli. This activity is insensitive to vanadate and hence appears not to involve a P-type ATPase; it is also reported to be sensitive to 2,5-di-(*tert*-butyl)-1,4-hydroquinone (BHQ) (and to 7-chloro-4-nitrobenz-2-oxa-1,3-diazole [NBD-Cl]) but not to bafilomycin A1 or thapsigargin[32].

Calcium oscillations

Oscillatory behaviour has been recognised in *Dictyostelium* ever since concentric rings and spirals were observed in preparations of amoebae aggregating on agar surfaces (see Wurster et al.[101] for review). Gerisch detected periodicity in the velocity of migration as well as in the orientation movements of the amoebae[36]. During starvation to aggregation competence amoebae display spike-shaped oscillations at 4–5.5 h (t_4–$t_{5.5}$) as determined by light scattering measurements[38]. These oscillations seem to be due in the most part to periodic cyclic AMP synthesis and release[37,94]. Afterwards oscillations continue in a sinusoidal form without clear cut changes of cyclic nucleotide concentrations[16].

Besides periodic changes of cyclic nucleotides, extracellular Ca^{2+} oscillations have been recorded in cell suspensions[16,17] whereas intracellular Ca^{2+} oscillations have not yet been reported, possibly due to the fact that Ca^{2+} is sequestered efficiently by intracellular organelles. An exception to this statement is the occurrence of semiperiodic Ca^{2+} spikes during later stages of development where $[Ca^{2+}]_i$ changes are larger[25]. Extracellular Ca^{2+} oscillations are also of both spike-shaped and sinusoidal character. However, most importantly, the transition between these two is not accompanied by a phase shift such as is observed in light-scattering measurements. This and the fact that sinusoidal Ca^{2+} oscillations underlie the spikes strengthens the notion that *Dictyostelium* possesses a basal form of Ca^{2+} oscillation during aggregation from t_4 onwards and that spikes occur as soon as cyclic AMP pulses are produced, providing an extracellular feed back loop that augments Ca^{2+} fluxes[101]. During spikes, phase shifts can be induced by micromolar Ca^{2+} concentrations (E. Jaworski and D. Malchow, unpublished). Since an external synchronizer of the sinusoidal periodicity has not been detected it may well be Ca^{2+} itself.

Extracellular Ca^{2+} oscillations usually begin with Ca^{2+} influx followed by a phase of release similar to that observed after cyclic AMP-induced Ca^{2+}-influx[17,99]. However, occasionally at low external $[Ca^{2+}]$, oscillations have been observed to start with Ca^{2+} release indicating that cytosolic Ca^{2+} concentration oscillates as well. Such oscillations were most prominent in a class of mutants selected for strong chemotactic migration by a method similar to that described by Segall et al.[90]. In this strain 10 nM cyclic AMP induced a periodic release of Ca^{2+} with a period length of 8 min (fig. 2) which is the same period length observed during Ca^{2+} influx oscillations[16].

The mechanism of $[Ca^{2+}]_i$ oscillation may be similar to that in higher eucaryotic cells[10]. It requires a positive and possibly a negative feedback loop, entry of Ca^{2+} at the plasma membrane and either periodic $InsP_3$-production due to stimulation of PLC activity by elevated $[Ca^{2+}]_i$ or Ca^{2+}-induced Ca^{2+} release (CICR), where Ca^{2+} released from the $InsP_3$- or ryanodine-sensitive store triggers a new cycle of release after the stores have been refilled via Ca^{2+} entry. Alternatively, oscillations arise due to an interplay between different Ca^{2+} pools[10,11] or between plasma membrane ion channels linked by activation as suggested for pacemaker

neurons of *Aplysia*[40] or by other messengers like sphingosine which releases Ca^{2+} from internal stores[102].

In *Dictyostelium* Ca^{2+} feedback-stimulation of PLC activity is known[14,15,24,51]. In addition, extracellular cyclic AMP induces transient increases of $InsP_3$. Therefore one would expect that $InsP_3$ levels almost certainly oscillate during spikes. Oscillations may therefore arise as specified by the two-pool model[9], since, as already mentioned, Ca^{2+} sequestering organelles[79] other than the $InsP_3$-sensitive pool[31,32] are known. Ca^{2+} uptake into these stores (or 'acidosomes') can be inhibited by bafilomycin A1, a V-type H^+-ATPase blocker, whereas the $InsP_3$-sensitive pool is not sensitive to bafilomycin but to the Ca^{2+} transport ATPase blocker BHQ and the V-type H^+-ATPase inhibitor NBD-Cl (ref. 32).

Light scattering spikes have been found to be transiently inhibited by any one of these compounds (Malchow, unpublished). This suggests that both Ca^{2+} stores participate in the generation of periodic Ca^{2+} elevations. It is not known whether Ca^{2+} or other messengers induce Ca^{2+}-release from acidosomes. However, both stores possess H^+-ATPase activity. Therefore, an interplay could also be achieved by H^+ fluxes. Indeed, extracellular pH oscillations have also been reported[60].

Besides Ca^{2+} and H^+ fluxes, periodic K^+ oscillations also occur[5] and such K^+ fluxes are subject to inhibition by elevated Ca^{2+} concentrations[4]. Ca^{2+} and K^+ may even be linked through a common pool, since Schlatterer et al.[86] found K^+ and Ca^{2+} containing mass-dense organelles in aggregative amoebae that are mainly located close to the plasma membrane. One would therefore expect that periodic changes of Ca^{2+} would be generated by an interconnected network of regulatory cycles.

The role of Ca^{2+} in chemotactic cell movement

Evidence for a Ca^{2+} requirement during chemotaxis

Despite the evidence for Ca^{2+} being required for the activity of various components of the signal transduction chain, the literature over the years has been contradictory in demonstrating a requirement for this ion during chemotaxis. Part of the problem has undoubtedly been the differing conditions used when trying to remove Ca^{2+} by chelators such as EGTA. For example, Mato et al.[64] found that cell aggregation and chemotaxis were 'independent of the presence of external Ca^{2+}' as judged by the effect of EGTA on cells on a washed agar substratum and this was confirmed by Saito[81] who found that EGTA at 2 mM (with the extracellular Ca^{2+} estimated at lower than 10^{-7} M) did not stop the amoebae aggregating at the same time and with the same sensitivity to extracellular cyclic AMP pulses as under plentiful Ca^{2+} supply. In contrast, Mason et al.[63] using amoebae aggregating on dialysis tubing found that they were inhibited by EGTA and that the extent of aggregation was a function of the free $[Ca^{2+}]$ over the range 10^{-6} to 10^{-4} M. This requirement for Ca^{2+} was confirmed by Malchow et al.[59] who found that amoebal suspensions at low population density (2×10^5 ml^{-1}) which were chemotactically moving to cyclic AMP-filled capillaries were inhibited by 1 mM EGTA and Ca^{2+} at 0.1–1 mM completely counteracted this inhibition.

The apparent discrepancy in the results may be understood from the work of Europe-Finner et al.[30] who studied the effects of leaching the Ca^{2+} stores out of amoebae by repeated (5-fold) washing with 7 mM EGTA. Under conditions of high population density, reduction of the level of intracellular Ca^{2+} seemed to be an essential prerequisite for demonstrating a requirement for this ion during chemotactic movement because the cells normally contain millimolar amounts of Ca^{2+} that rapidly equilibrate with the extracellular medium and negate the effects of added chelators. Previous experiments had not taken account of the intracellular Ca^{2+} stores; experiments successfully showing an inhibitory effect of added EGTA being those using low cell population densities where the effect of the cellular stores titrating out the added chelator would have been very small.

More recent experiments have focused on the question as to whether it is the orientation or locomotion aspects of chemotaxis that show the requirement for Ca^{2+}. Van Duijn and Van Haastert[96] used amoebae made permeable by electroporation and 'clamped' the intracellular Ca^{2+} concentration using 5.9 mM EGTA, the effect of the EGTA on the intracellular Ca^{2+} concentration being assayed indirectly via the activity of the Ca^{2+}-sensitive enzyme guanylate cyclase. Observing the results with individual amoebae on coverslips they found that, although lowered intracellular levels of Ca^{2+} effectively blocked cell locomotion to a capillary filled with cyclic AMP, these amoebae could, nevertheless, extend pseudopodia in the direction of the capillary. Orientation by pseudopodia formation seemed therefore to have a lowered requirement for Ca^{2+} compared to cell locomotion and the authors concluded that the two events were independently regulated. More recently Schlatterer and Malchow[87], and Unterweger and Schlatterer[95] further investigated the problem using the Ca^{2+} chelator BAPTA and derivatives, 'scrape-loaded' into amoebae. They found that the lowered intracellular Ca^{2+} levels brought about by this technique effectively inhibited not only cell locomotion but also the formation of pseudopodia and filopodia. This difference in results from those of Van Duijn and Van Haastert can be understood if the formation of pseudopodia and filopodia requires Ca^{2+} but at much lower concentrations than cell locomotion, the technique of electroporation used by Van Duijn and

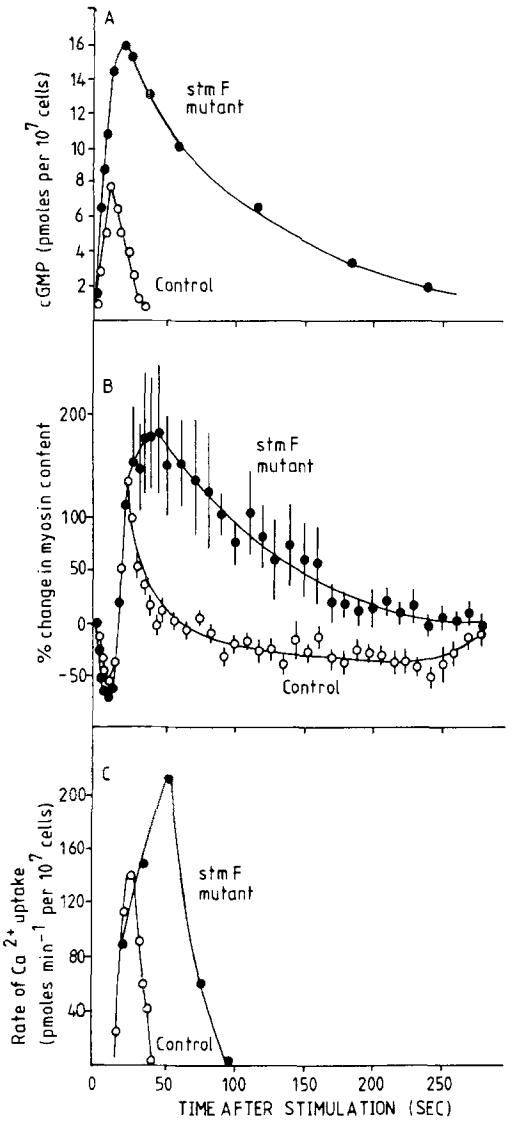

Figure 3. Effect of cyclic AMP stimulation of amoebae of parental strain XP55 and a streamer F mutant on *A*) the formation of cyclic GMP, *B*) association of myosin II with the Triton X-100 insoluble cytoskeleton; and *C*) the rate of calcium uptake from the medium. (From Liu and Newell[48].)

Van Haastert possibly producing incomplete buffering of the changes in intracellular Ca^{2+} brought about by the chemotactic stimulus.

The role of Ca^{2+} and cyclic GMP in the regulation of myosin interaction with the cytoskeleton

Evidence for Ca^{2+} and cyclic GMP being regulators of myosin interaction with the cytoskeleton during chemotaxis comes from work using the 'streamer F' mutants[72]. In these mutants, cyclic GMP (formed in response to extracellular pulses of cyclic AMP) is not destroyed rapidly as in the wild type cells but persists for approximately five-fold longer (fig. 3). The persistence of the induced peak of cyclic GMP results from a defect in the structural gene for the cyclic GMP-specific phosphodiesterase, and several independent isolates in the same complementation group were found to have the same phenotype[22,80,97]. The most obvious effect of this defective gene on the visible phenotype is that the amoebae remain in the elongated state during chemotaxis for approximately 5-fold longer than the parental strain XP55. This elongated period is not correlated with a longer period of cell movement but with the converse: amoebae show a marked drop in speed after stimulation with cyclic AMP, recovering in the wild type within 60–70 s, while in streamer F mutants recovery does not occur until about 300 s (ref. 89).

The correlation of the prolonged cyclic GMP response with the prolonged period of elongation of the streamer F cells suggested that this nucleotide might affect some event connected with the (Triton X-100-insoluble) cytoskeleton. When myosin was studied in the streamer mutants by Liu and Newell[46,50,72] it was found that its association with the cytoskeleton was dramatically different from the parental or wild type strains. After an initial small drop (seen in both mutants and parental strain) the myosin association with the cytoskeleton rapidly increased to a peak at about 25 s and in the parental and other wild type strains the association then rapidly declined. In the streamer mutants, however, this peak was persistent and only slowly declined to basal values. Such changes correlate well with the changes in cyclic GMP formation (fig. 3).

Further studies have suggested that the connection between cyclic GMP formation and movement of the myosin to the cytoskeleton may be connected with phosphorylation of the myosin heavy chain. Earlier studies by Berlot et al.[7,8] had shown that the myosin II heavy chain is phosphorylated (mainly on threonine residues) in response to a cyclic AMP stimulus. More recent studies by Liu and Newell[47] have indicated that phosphorylation of the myosin heavy chain is abnormal in the streamer F mutants and is considerably delayed compared to the parental strain, with a peak at 60 s rather than 25–30 s. It is noteworthy, however, that the timing of this phosphorylation correlates, not with the association of the myosin with the cytoskeleton, but rather with its dissociation. It has been found by several workers that myosin in the phosphorylated state is bent, which would greatly reduce its ability to associate in the form of thick filaments[18,43,44,74] and it was suggested that phosphorylation of the myosin heavy chain removes it from the cytoskeleton. In support of this notion, Liu and Newell[47] found that little of the myosin that was on the cytoskeleton was phosphorylated compared to that in the soluble cell fraction. A model was proposed in which (in the parental strain) the enzyme that phosphorylates the myosin (myosin heavy chain kinase) is transiently inhibited by a peak of cyclic GMP formed in response to a chemotactic cyclic AMP pulse.

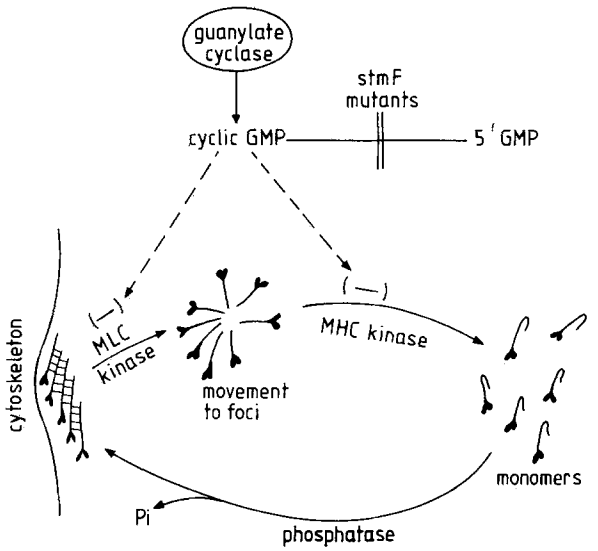

Figure 4. Model of the regulation of dissociation of myosin II from the cytoskeleton by cyclic GMP. The phosphorylated myosin II molecules are shown as bent monomers while the dephosphorylated molecules are in the form of parallel fibres that form thick filaments as a part of the cytoskeleton. The model proposes that the cyclic GMP directly or indirectly (possibly via Ca^{2+}/calmodulin) inhibits light chain and heavy chain phosphorylation, thereby inducing a shift in the equilibrium in favour of association of myosin on the cytoskeleton. In the absence of inhibitory cyclic GMP, phosphorylation of the myosin regulatory light chain by myosin light chain kinase promotes the actin-activated Mg^{2+}-ATPase and hence stimulates the movement of the myosin II molecules on cytoskeletal actin molecules to foci on the cytoskeleton on where myosin heavy chain kinase is present, the phosphorylation of the heavy chain then bringing about myosin dissociation. During the cyclic GMP peak induced by chemotactic cyclic AMP stimulation, the rate of myosin dissociation is decreased. Following the peak of cyclic GMP there is a transient increase in the rate of phosphorylation of the light and heavy chains that had accumulated on the cytoskeleton, and the normal equilibrium is re-established. In streamer F (*stmF*) mutants, the period of cyclic GMP formation in response to chemotactic cyclic AMP stimulation is prolonged (due to failure to hydrolyse the cyclic GMP), leading to a prolonged association of myosin II with the cytoskeleton and a delay in the transient increase in rate of phosphorylation of the myosin light and heavy chains (from Liu and Newell[49]).

In the unstimulated cell the addition of myosin to the cytoskeleton is in equilibrium with its removal. Transient inhibition of its removal by inhibition of myosin heavy chain kinase by cyclic GMP would have the effect of allowing more myosin to be added than removed during this time (fig. 4). After the cyclic GMP is hydrolysed the original state would be rapidly restored as phosphorylation restarted and removed the excess myosin from the cytoskeleton. In the streamer mutants, the extended period during which cyclic GMP is present would delay this renewed phosphorylation of the myosin and would extend the period during which myosin was associated with the cytoskeleton, as observed. As shown in figure 4, evidence has also recently been reported indicating that phosphorylation of the regulatory myosin light chain (and inhibition of this phosphorylation by cyclic GMP) are also involved in regulation of myosin interaction with the cytoskeleton via movement of the myosin to foci before liberation to the cytosol[49,50].

It is conceivable that cyclic GMP regulates myosin phosphorylation by means of its effects on a cyclic GMP-dependent protein kinase, and such an enzyme has been identified in *Dictyostelium*[98] although its substrate is unknown. Another possibility, however, is that the connection is more indirect via Ca^{2+} movements. Evidence in favour of this connection comes from the report of Menz et al.[67] with the streamer mutants showing that the rate of uptake of calcium from the medium in response to stimulation of amoebae with cyclic AMP was much greater in the streamer F mutants than their parental wild type strain with a peak occurring at about 50 s rather than the normal 25 s (fig. 3). It is of interest that Milne and Coukell[70] using a different calcium-uptake assay, did not observe any difference in calcium uptake between the streamer mutant NP368 and its parental strain. One reason for their different results is likely to be due to the different assays employed. Whereas the assay of Milne and Coukell measured the rate of uptake of $^{45}Ca^{2+}$ radioactivity into the cells, Menz et al. used a calcium electrode assay which measured calcium concentration changes in the medium around the cells. As the latter technique monitors the net effect of uptake and extrusion by the cells the different results may be explained if the effect of cyclic GMP is on the Ca^{2+} extrusion system rather than on the uptake system. However, another reason may be connected with the time of development of the cells used for study. Schaloske and Malchow (unpublished) have recently found that the prolonged Ca^{2+} uptake peak seen in the streamer F mutants was very much stronger between 4 and 6 h of development. This result might be significant as the cells used by Menz et al.[67] were at 5 h of development whereas those used by Milne and Coukell[70] were at approximately 9 h. The effect of cyclic GMP on Ca^{2+} uptake measured by the Ca^{2+} electrode assay has more recently been confirmed by Flaadt et al.[33] who stimulated the cells with the cell permeant derivative of cyclic GMP, Sp-8-Br-cyclic GMPS. With intact cells this compound increased the magnitude of the cyclic AMP Ca^{2+} uptake response by 1.5-fold. Evidence that this response was at the plasma membrane rather than via an effect on intracellular organelles (which are also stimulated to take up Ca^{2+} in response to cyclic AMP) comes from their finding that with filipin-permeabilised cells (which affects the plasma membrane but not organelle membranes) cyclic AMP still induced Ca^{2+} uptake but this response was not stimulated by Sp-8-Br-cyclic GMPS.

The correlation between the cyclic GMP, myosin and calcium uptake effects suggests that cyclic GMP might affect myosin via its effects on calcium uptake. Indeed

Yumura and Kitanishi-Yomura[103] have reported that in membrane-cytoskeletons of aggregating cells the release of myosin II was inhibited by Ca^{2+} and this effect was stimulated by calmodulin. The presence of increased calcium ions in the cell might inhibit myosin phosphorylation by inhibiting myosin heavy chain kinase. Such a kinase enzyme (which phosphorylated threonines on the myosin heavy chain) has been reported by Maruta et al.[62] to be inhibited by low concentrations of calcium in the presence of calmodulin in aggregation-competent cells. More recent reports by Ravid and Spudich[76] have described the isolation of a developmentally regulated myosin heavy chain kinase from amoebal membranes. Disruption of the gene for this enzyme caused cells to lose their ability to phosphorylate cytoskeletal myosin II, and this lead to abnormal cortical myosin accumulation (K. AbuElneel, M. Karchi, J. Judcovski and S. Ravid, pers. commun.). However, the enzyme (in the purified form) was not regulated by Ca^{2+}, Ca^{2+}/calmodulin, cyclic AMP or cyclic GMP. The corresponding cDNA clone[77] showed some similarity to members of the protein kinase C family but only a modest degree of homology with the DNA region that corresponds to the putative Ca^{2+}-binding domain that is conserved among protein kinase C subspecies[77]. While this evidence seems to conflict with the hypothesis that Ca^{2+} regulates myosin heavy chain kinase, it is notable that the purified kinase enzyme shows no obvious regulation by any of the second messengers tested. As several mammalian protein kinase C proteins do not demonstrate Ca^{2+}, phospholipid or diacylglycerol regulation after purification from cell extracts[77], the lack of Ca^{2+} or cyclic GMP regulation of the *Dictyostelium* heavy chain kinase could similarly have been lost during purification.

Ca^{2+} and the regulation of cell differentiation

A number of approaches have been explored in attempts to identify the role of Ca^{2+} in gene expression in the prestalk and prespore pathways, and in the choice between these two pathways of cell differentiation. These involve characterising Ca^{2+} levels in the prestalk and prespore cells and their precursors on the one hand, and trying to alter Ca^{2+} levels and pinpoint the effect on cell-type-specific gene expression on the other. Prestalk cells are known to have substantially larger Ca^{2+} stores than prespore cells[58,59] and recent evidence suggests that they also have higher basal cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$ and generate a larger and more prolonged $[Ca^{2+}]_i$ transient in response to cyclic AMP signals[1,83]. These findings hint that elevation of $[Ca^{2+}]_i$ may play an especially important role in prestalk gene expression. Consistent with this idea, Saran et al.[82] have obtained evidence that vegetative cells are heterogeneous in the extent of their Ca^{2+} stores, and that those cells with the higher content tend to develop into prestalk cells.

There is good evidence for a role of Ca^{2+}-dependent processes in sexual cell fusion in *D. discoideum* (see below). Although *Dictyostelium* calmodulin has been extensively characterised[61], calmodulin-binding proteins identified[100] and genes for calmodulin and for the A subunit of a Ca^{2+}/CaM-dependent protein phosphatase (calcineurin) have been cloned (ref. 39, and Mutzel, pers. commun.), there is little concrete evidence about the role of Ca^{2+} in gene expression during asexual development. Addition of EGTA to cell suspensions to chelate Ca^{2+} has little effect on the induction by cyclic AMP of early gene products[23], nor does it inhibit accumulation of prespore-cell gene transcripts[12]. These findings suggest that influx of Ca^{2+} from the medium in response to cyclic AMP signals is not required for developmental gene expression. However Fosnaugh and Loomis[34] have made the interesting observation that prespore gene expression can be induced either by cyclic AMP or by the presence of extracellular Ca^{2+}, suggesting a facultative role for Ca^{2+} in gene expression and both stalk cell and spore formation in cell monolayers are inhibited by the calcineurin inhibitors cyclosporin and FK506 (F. Horn and J. Gross, unpubl.).

A number of workers have observed an inhibitory effect of putative calcium antagonists on early and later gene expression in cell suspensions[12,23,84] but the effects of the various agents on the expression of DIF-dependent prestalk genes were not examined. Recent preliminary findings (K. Pinter and J. Gross, unpubl; A. Cubitt and R. Firtel, pers. commun.) suggest that the latter may be more dependent upon $[Ca^{2+}]_i$ than prespore cell gene expression. This would be consistent with the observation of higher $[Ca^{2+}]_i$ in prestalk than in prespore cells (see above). A further hint of a role of $[Ca^{2+}]_i$ in prestalk gene expression comes from the ability of thapsigargin to mimic the action of the stalk-cell inducing substance DIF-1 in inducing stalk cell formation[42]. However, since there is as yet no evidence for a thapsigargin-sensitive Ca^{2+}-ATPase in *Dictyostelium* it would be interesting to know whether BHQ, or Ca^{2+} ionophores, have the same effect on DIF-dependent processes.

Besides effects on transcription, Ca^{2+}/calmodulin may also regulate development by effects on translation. Evidence for such a possibility comes from the finding that one of the ribosomal proteins, L19, specifically binds Ca^{2+}/calmodulin, and that calmodulin antagonists and an anti-*D. discoideum* calmodulin monoclonal antibody inhibit protein synthesis in vitro[91,92].

The role of Ca^{2+} in spore germination

Asexual development in *Dictyostelium* leads to the formation of a heat and desiccation-resistant spore which

remains dormant until activated. Activation occurs by two general mechanisms: *1*) exogenous forces such as heat, or the presence of bacteria, dimethyl sulphoxide or urea[19,20,28] and *2*) an endogenous, diffusible, low molecular weight autoactivator that is produced autocatalytically by the spores themselves[21,27]. Following activation by either mechanism the spores are observed to swell and finally to rupture, each liberating an amoeba.

Evidence for an involvement of Ca^{2+} in the activation process comes from a series of experiments by Lydan and Cotter[53] showing that: *1*) $InsP_3$ added at 10 μM to saponin-permeabilized autoactivating spores induces precocious swelling (indicating an increased rate of activation in a dose-dependent manner); *2*) during the activation process there is a measurable efflux of Ca^{2+} from the spores over a period of about 2 h (as measured by a Ca^{2+} electrode in the surrounding medium) followed by a re-uptake during amoebal emergence; *3*) La^{3+} at 10 μM (which blocks Ca^{2+} movement through Ca^{2+} channels) delays the swelling of autoactivating spores by 1.5 h and (iv) TMB-8 (which blocks Ca^{2+} release from intracellular stores) at 50 μM decreases the rate of spore swelling by 50%, and at 100 μM completely inhibits the process.

Recent work of Cotter and colleagues has also provided evidence for the involvement of calmodulin in the mechanism of autoactivation. Compared to the spore swelling process, which is relatively unaffected by calmodulin inhibitors such as trifluoperazine (TFP) unless used at 5 μM or higher, the process of autoactivation is very sensitive, 1 μM effectively inhibiting the process[52]. Lydan et al.[52] have identified a 64,000 M_r calmodulin binding protein (CaMBP64) which they believe may be involved in the activation process, and they speculate that this protein may be used by the spore to repress germination. The protein is apparently heat labile and disappears from spores within 30 min of exposure to 45 °C. In their model, autoactivation occurs by the autoactivator inducing Ca^{2+} release within the spore which (via calmodulin) binds to and regulates calmodulin binding proteins such as CaMBP64.

Ca^{2+} requirement for gamete fusion during macrocyst formation

The process of cell fusion of sexual gametes that is involved in formation of the zygotic macrocysts can be divided into four observable stages: *1*) formation of gametes (small highly motile amoebae with dense nuclei), *2*) gamete fusion to produce predominantly binucleate cells, *3*) pronuclear fusion to form the zygote and *4*) macrocyst formation via a period of active phagocytosis in which surrounding amoebae are engulfed as a food source.

Although no role for Ca^{2+} has been found during the phagocytic macrocyst formation stage (this stage probably being regulated by adenosine[45], there is considerable evidence that Ca^{2+} is required for gamete cell fusion. Early studies of McConachie and O'Day[65] demonstrated a requirement for Ca^{2+} in the medium for the fusion of gametes. More recently Lydan and O'Day[55] used Lanthanum ions to block Ca^{2+} movements and show inhibition of cell fusion. When $InsP_3$ was added to cells (together with saponin) it was found to bring about an immediate precocious fusion of gametes, the maximal effect being seen at 10 μM. Chlortetracycline and TMB-8 (which are thought to inhibit the release of Ca^{2+} from intracellular stores) both inhibited the fusion process[56]. It was concluded that mobilization of Ca^{2+} from intracellular stores (replenished from outside the cell) is essential for gamete fusion.

The involvement of calmodulin in the fusion process has been deduced from work using the calmodulin inhibitors TFP and calmidazolium, both of which were found by Lydan and O'Day[55,56] to inhibit cell fusion in a dose-related (and reversible) manner. Studies employing a gel overlay technique with ^{35}S-labelled calmodulin have revealed 15 calcium-dependent calmodulin-binding proteins of which 6 were present only when the cells were undergoing sexual development[57]. Although the identities and functions of these proteins is unknown, it is of interest that one of them (91,000 M_r) was present only when the amoebae were developing into gametes and two others (38,000 M_r and 48,000 M_r) were present only during the cell fusion stage. The role of Ca^{2+}/calmodulin during the fusion stage is, so far, unknown but may involve a calmodulin-dependent kinase as such an enzyme (106,000 M_r) has been identified as present during the sexual stage[54].

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1 Abe, T., and Maeda, Y., The prestalk/prespore differentiation and polarized cell movement in *Dictyostelium discoideum* slugs – A possible involvement of the intracellular Ca^{2+}-concentration. Protopl. *151* (1989) 175–178.
2 Abe, T., and Maeda, Y., Cellular differentiation in submerged monolayers of *Dictyostelium discoideum*: Possible functions of cytoplasmic Ca^{2+} and DIF. Develop. Growth Differ. *33* (1991) 469–478.
3 Abe, T., Maeda,Y., and Iijima, T., Transient increase of the intracellular Ca^{2+} concentration during chemotactic signal transduction in *Dictyostelium discoideum* cells. Differentiation *39* (1988) 90–96.
4 Aeckerle, S., and Malchow, D., Calcium regulates cAMP-induced potassium ion efflux in *Dictyostelium discoideum*. Biochim. biophys. Acta *1012* (1989) 196–200.
5 Aeckerle, S., Wurster, B., and Malchow, D., Oscillations and cyclic AMP-induced changes of the K^+ concentration in *Dictyostelium discoideum*. EMBO J. *4* (1985) 39–43.
6 Al-Mohanna, F.A., and Hallert, M.B., The use of fura-2 to determine the relationship between cytoplasmic free Ca^{2+} and oxidase activation in rat neutrophils. Cell Calcium *9* (1988) 17–26.
7 Berlot, C. H., Chemoattractant-elicited increases in *Dictyostelium* myosin phosphorylation are due to changes in myosin

location and increases in kinase activity. J. biol. Chem. *262* (1987) 3918–3926.
8 Berlot, C. H., Spudich, J. A., and Devreotes, P. N., Chemoattractant-elicited increases in myosin phosphorylation in *Dictyostelium*. Cell *43* (1985) 307–14.
9 Berridge, M. J., Cytoplasmic calcium oscillations: A two pool model. Cell Calcium *12* (1991) 63–72.
10 Berridge, M. J., Inositol trisphosphate and calcium signalling. Nature *361* (1993) 315–324.
11 Berridge, M. J., and Irvine, R. F., Inositol phosphates and cell signalling. Nature *341* (1989) 197–204.
12 Blumberg, D. D., Comer, J. F., and Walton, E. M., Ca^{++} antagonists distinguish different requirements for cAMP-mediated gene expression in the cellular slime mold, *Dictyostelium discoideum*. Differentiation *41* (1989) 14–21.
13 Böhme, R., Bumann, J., Aeckerle, S., and Malchow, D., A high-affinity plasma membrane Ca^{2+}-ATPase in *Dictyostelium discoideum*: its relation to cAMP-induced Ca^{2+} fluxes. Biochim. biophys. Acta *904* (1987) 125–130.
14 Bominaar, A. A., Kesbeke, F., and Van Haastert, P. J. M., Phospholipase C in *Dictyostelium discoideum*. Cyclic AMP surface receptor and G-protein-regulated activity in vitro. Biochem. J. *297* (1994) 181–188.
15 Bominaar, A. A., and Van Haastert, P. J. M., Phospholipase C in *Dictyostelium discoideum*. Identification of stimulatory and inhibitory surface receptors and G-proteins. Biochem. J. *297* (1994) 189–197.
16 Bumann, J., Malchow, D., and Wurster, B., Oscillations of Ca^{++} concentration during the cell differentiation of *Dictyostelium discoideum*. Their relation to oscillations in cyclic AMP and other components. Differentiation *31* (1986) 85–91.
17 Bumann, J., Wurster, B., and Malchow, D., Attractant-induced changes and oscillations of the extracellular Ca^{++} concentration in suspensions of differentiating *Dictyostelium* cells. J. Cell Biol. *98* (1984) 173–178.
18 Côté, G. P., and McCrea, S. M., Selective removal of the carboxyl-terminal tail end of the *Dictyostelium* myosin II heavy chain by chymotrypsin. J. biol. Chem. *262* (1987) 13033–13038.
19 Cotter, D. A., and Raper, K. B., Spore germination in *Dictyostelium discoideum*. Proc. natl Acad. Sci. USA *56* (1966) 880–887.
20 Cotter, D. A., Sands, T. W., Virdy, K. J., North, M. J., Klein, G., and Satre, M., Patterning of development in *Dictyostelium discoideum*: factors regulating growth, differentiation, spore dormancy, and germination. Biochem. Cell Biol. *70* (1992) 892–919.
21 Cotter, D.A., and Glaves, M.L., Temporal control of autoactivator synthesis and secretion during spontaneous spore germination in *Dictyostelium discoideum*. Arch. Microbiol. *152* (1989) 44–51.
22 Coukell, M. B., and Cameron, A. M., Characterization of revertants of *stmF* mutants of *Dictyostelium discoideum*: evidence that *stmF* is the structural gene of the cGMP-specific phosphodiesterase. Devl Genet. *6* (1986) 163–177.
23 Coukell, M. B., and Cameron, A. M., Effects of suboptimal levels of extracellular calcium on the regulation of the cyclic AMP phosphodiesterase-inhibitor system and membrane differentiation in *Dictyostelium discoideum*. J. Cell Sci. *90* (1988) 691–700.
24 Cubitt, A. B., and Firtel, R.A., Characterization of phospholipase activity in *Dictyostelium discoideum*. Biochem. J. *283* (1992) 371–378.
25 Cubitt, A. B., Firtel, R. A., Fischer, G., Jaffe, L. F., and Miller, A. L., Patterns of free calcium in multicellular stages of *Dictyostelium* transformed with jellyfish apoaequorin. Development *121* (1995) 2291–2301.
26 Cunningham, K. W., and Fink, G. R., Calcineurin-dependent growth control in *Saccharomyces cerevisiae* mutants lacking *PMC1*, a homolog of plasma membrane Ca^{2+} ATPases. J. Cell biol. *124* (1994) 35–363.
27 Dahlberg, K. R., and Cotter, D. A., Activators of *Dictyostelium discoideum* spore germination released by bacteria. Microbios Lett. *9* (1978) 139–146.
28 Ennis, H. L., and Sussman, M., Mutants of *Dictyostelium discoideum* defective in spore germination. J. Bacteriol. *124* (1975) 62–64.
29 Europe-Finner, G. N., Gammon, B., Wood, C. A., and Newell, P. C., Inositol tris- and polyphosphate formation during chemotaxis of *Dictyostelium*. J. Cell Sci. *93* (1989) 585–592.
30 Europe-Finner, G. N., McClue, S. J., and Newell, P. C., Inhibition of aggregation in *Dictyostelium* by EGTA-induced depletion of calcium. FEMS Microbiol. Lett. *21* (1984) 21–25.
31 Europe-Finner, G. N., and Newell, P. C., Inositol 1,4,5-trisphosphate induces calcium release from a non-mitochondrial pool in amoebae of *Dictyostelium*. Biochim. biophys. Acta *887* (1986) 335–340.
32 Flaadt, H., Jaworski, E., and Malchow, D., Evidence for two intracellular calcium pools in *Dictyostelium*: the cAMP-induced calcium influx is directed into a NBD-Cl- and 2,5-di-(tert-butyl)-1,4-hydroquinone-sensitive pool. J. Cell Sci. *105* (1993) 1131–1135.
33 Flaadt, H., Jaworski, E., Schlatterer, C., and Malchow, D., Cyclic AMP- and Ins(1,4,5)P3-induced Ca^{2+} fluxes in permeabilised cells of *Dictyostelium discoideum*: cGMP regulates Ca^{2+} entry across the plasma membrane. J. Cell Sci. *105* (1993) 255– 261.
34 Fosnaugh, K. L., and Loomis, W. F., Coordinate regulation of the spore coat genes in *Dictyostelium discoideum*. Devl Genet. *12* (1991) 123–132.
35 Foskett, J. K., and Wong, D., Calcium oscillations in parotid acinar cells induced by microsomal Ca^{2+}-ATPase inhibitors. Am. J. Physiol. *262* (1992) 656–663.
36 Gerisch, G., Periodische Signale steuern die Musterbildung in Zellverbänden. Naturwissenschaften *58* (1971) 430–438.
37 Gerisch, G., and Wick, U., Intracellular oscillations and release of cyclic AMP from *Dictyostelium* cells. Biochem. biophys. Res. Commun. *65* (1975) 364–370.
38 Gerisch, G., and Hess, B., Cyclic AMP-controlled oscillations in suspended *Dictyostelium* cells: their relation to morphogenetic cell interactions. Proc. natl Acad. Sci. USA *71* (1974) 2118–2122.
39 Goldhagen, H., and Clarke, M., Identification of the single gene for calmodulin in *Dictyostelium discoideum*. Molec. Cell Biol. *6* (1986) 1851–1854.
40 Gorman, A. L. F., Hermann, A., and Thomas, M. V., Intracellular calcium and the control of neuronal pacemaker activity. Fed. Proc. *40* (1981) 2233–2239.
41 Hajnóczky, G., Lin, C., and Thomas, A. P., Luminal communication between intracellular calcium stores modulated by GTP and the cytoskeleton. J. biol. Chem. *269* (1994) 10280–10287.
42 Kubohara, Y., and Okamoto, K., Cytoplasmic Ca^{2+} and H^+ concentrations determine cell fate in *Dictyostelium discoideum*. FASEB J. *8* (1994) 869–874.
43 Kuczmarski, E. R., and Spudich, J. A., Regulation of myosin self-assembly phosphorylation of *Dictyostelium* heavy chain inhibits formation of thick filaments. Proc. natl Acad. Sci. USA *77* (1980) 7292–7296.
44 Kuczmarski, E. R., Tafuri, S. R., and Parysek, L. M., Effect of heavy chain phosphorylation on the polymerization and structure of *Dictyostelium* myosin filaments. J. Cell Biol. *105* (1987) 2989–2997.
45 Lewis, K. E., and O'Day, D. H., Cannibalistic sexual phagocytosis in *Dictyostelium discoideum* is modulated by adenosine via an A2-like receptor. Cellular Signalling *6* (1994) 217–222.
46 Liu, G., and Newell, P. C., Evidence that cyclic GMP regulates myosin interaction with the cytoskeleton during chemotaxis of *Dictyostelium*. J. Cell Sci. *90* (1988) 123–129.
47 Liu, G., and Newell, P. C., Evidence that cyclic GMP may regulate the association of myosin II heavy chain with the cytoskeleton by inhibiting its phosphorylation. J. Cell Sci. *98* (1991) 483–490.
48 Liu, G., and Newell, P. C., Role of cyclic GMP in signal transduction to cytoskeletal myosin. SEB Symposium: Cell Behaviour: Adhesion and Motility, vol. 47, pp. 283–295. Eds G. Jones, C. Wigley, and R. Warn, Company of Biologists Ltd., Cambridge, UK, 1993.

49 Liu, G., and Newell, P. C., Regulation of myosin regulatory light chain phosphorylation via cyclic GMP during chemotaxis of *Dictyostelium*. J. Cell Sci. *107* (1994) 1737–1743.

50 Liu, G., Kuwayama, H., Ishida, S., and Newell, P. C., The role of cyclic GMP in regulating myosin during chemotaxis of *Dictyostelium*: evidence from a mutant lacking the normal cyclic GMP response to cyclic AMP. J. Cell Sci. *106* (1993) 591–596.

51 Lundberg, G. A., and Newell, P. C., Membrane-associated phosphoinositidase C activity in *Dictyostelium discoideum*. FEBS Lett. *270* (1990) 181–183.

52 Lydan, M. A., Cotter, D. A., and O'Day, D. H., Calmodulin function and calmodulin-binding proteins during autoactivation and spore germination in *Dictyostelium discoideum*. Cellular Signalling *6* (1994) 751–762.

53 Lydan, M. A., and Cotter, D. A., The role of Ca^{2+} during spore germination in *Dictyostelium*: Autoactivation is mediated by the mobilization of Ca^{2+} via inositol (1,4,5)-trisphosphate while amoebal emergence requires entry of external Ca^{2+}. J. Cell Sci. *108* (1995) 1921–1930.

54 Lydan, M. A., and O'Day, D. H., Calmodulin-dependent phosphorylation and dephosphorylation during fertilization in *Dictyostelium discoideum*. Biochem. biophys. Res. Commun. *192* (1993) 1073–1078.

55 Lydan, M. A., and O'Day, D. H., Developmental effects of the major ions found in a groundwater sample on sexual cultures of *Dictyostelium discoideum*. Can. J. Microbiol. *34* (1988) 207–211.

56 Lydan, M., and O'Day, D. H., The role of intracellular Ca^{2+} during early sexual development in *Dictyostelium discoideum*: effects of $LaCl_3$, Ins(1,4,5)P_3, TMB-8, chlortetracycline and A23187 on cell fusion. J. Cell Sci. *90* (1988) 465–473.

57 Lydan, M. A., and O'Day, D. H., Calmodulin and calmodulin-binding proteins during cell fusion in *Dictyostelium discoideum*: developmental regulation by calcium ions. Expl Cell Res. *205* (1993) 134–141.

58 Maeda, Y., and Maeda, M., The calcium content of the cellular slime mold *Dictyostelium discoideum* during development and differentiation. Expl Cell Res. *82* (1973) 125–130.

59 Malchow, D., Böhme, R., and Gras, U., On the role of calcium in chemotaxis and oscillations of *Dictyostelium* cells. Biophys. Struct. Mech. *9* (1982) 131–136.

60 Malchow, D., Nanjundiah, V., and Gerisch, G., pH oscillations in cell suspensions of *Dictyostelium discoideum*: their relation to cyclic-AMP signals. J. Cell Sci. *30* (1978) 319–330.

61 Marshak, D. R., Clarke, M., Roberts, D. M., and Watterson, D. M., Structural and functional properties of calmodulin from the eukaryotic microorganism *Dictyostelium discoideum*. Biochemistry *23* (1984) 2891–2899.

62 Maruta, H., Baltes, W., Dieter, P., Marme, D., and Gerisch, G., Myosin heavy chain kinase inactivated by Ca^{2+}/calmodulin from aggregating cells of *Dictyostelium discoideum*. EMBO J. *2* (1983) 535–542.

63 Mason, J., Rasmussen, W. H., and Dibella, F., 3′:5′AMP and Ca^{2+} in slime mold aggregation. Expl Cell Res. *67* (1971) 156–160.

64 Mato, J. M., Krens, F. A., Van Haastert, P. J. M., and Konijn, T. M., 3′:5′-Cyclic AMP-dependent 3′:5′-cyclic GMP accumulation in *Dictyostelium discoideum*. Proc. natl Acad. Sci. USA *74* (1977) 2348–2351.

65 McConachie, D. R., and O'Day, D. H., The immediate induction of extensive cell fusion by Ca^{2+} addition in *Dictyostelium discoideum*. Biochem. Cell Biol. *64* (1986) 1281–1287.

66 Menz, S., Untersuchungen über die cAMP-stimulierte Ca^{2+}-Aufnahme bei *Dictyostelium discoideum*. Diplomarbeit, Konstanz 1988.

67 Menz, S., Bumann, J., Jaworski, E., and Malchow, D., Mutant analysis suggests that cyclic GMP mediates the cyclic AMP-induced Ca^{2+} uptake in *Dictyostelium*. J. Cell Sci. *99* (1991) 187–191.

68 Milne, J. L., and Coukell, M. B., Isolation and characterisation of a plasma membrane calcium pump from *Dictyostelium discoideum*. Biochem. J. *249* (1988) 223–230.

69 Milne, J. L., and Coukell, M. B., Identification of a high-affinity Ca^{2+} pump associated with endocytic vesicles in *Dictyostelium discoideum*. Expl Cell Res. *185* (1989) 21–32.

70 Milne, J. L., and Coukell, M. B., A Ca^{2+} transport system associated with the plasma membrane of *Dictyostelium discoideum* is activated by different chemoattractant receptors. J. Cell Biol. *112* (1991) 103–10.

71 Newell, P. C., Europe Finner, G. N., Liu, G., Gammon, B., and Wood, C. A., Signal transduction for chemotaxis in *Dictyostelium* amoebae. Seminars in Cell Biol. *1* (1990) 105–113.

72 Newell, P. C., Streamer F mutants and chemotaxis of *Dictyostelium*. BioEssays *14* (1992) 473–478.

73 Padh, H., Lavasa, M., and Steck, T. L., Characterization of a vacuolar proton ATPase in *Dictyostelium discoideum*. Biochem. biophys. Acta *982* (1989) 271–278.

74 Pasternak, C., Flicker, P. F., Ravid, S., and Spudich, J. A., Intermolecular versus intramolecular interactions of *Dictyostelium* myosin: possible regulation by heavy chain phosphorylation. J. Cell Biol. *109* (1989) 203–210.

75 Pederson, P. L., and Carafoli, E., Ion motive ATPases. I. Ubiquity, properties and significance to cell function. Trends Biochem. Sci. *12* (1987) 146–150.

76 Ravid, S., and Spudich, J. A., Myosin heavy chain kinase from developed *Dictyostelium* cells. Purification and characterization. J. biol. Chem. *264* (1989) 15144–15150.

77 Ravid, S., and Spudich, J. A., Membrane-bound *Dictyostelium* myosin heavy chain kinase: A developmentally regulated substrate-specific member of the protein kinase C family. Proc. natl Acad. Sci. USA. *89* (1992) 5877–5881.

78 Rooney, E. K., Gross, J. D., and Satre, M., Characterisation of an intracellular Ca^{2+} pump in *Dictyostelium*. Cell Calcium *16* (1994) 509–522.

79 Rooney, E. K., and Gross, J. D., ATP-driven Ca^{2+}/H^{+} antiport in acid vesicles from *Dictyostelium*. Proc. natl Acad. Sci. USA *89* (1992) 8025–8029.

80 Ross, F. M., and Newell, P. C., Streamers: Chemotactic mutants of *Dictyostelium discoideum* with altered cyclic GMP metabolism. J. gen. Microbiol. *127* (1981) 339–350.

81 Saito, M., Effect of extracellular Ca^{2+} on the morphogenesis of *Dictyostelium discoideum*. Expl Cell Res. *123* (1979) 79–86.

82 Saran, S., Azhar, M., Manogaran, S., Pande, G., and Nanjundiah, V., The level of sequestered calcium in vegetative amoebae of *Dictyostelium discoideum* can predict post-aggregative cell fate. Differentiation *57* (1994) 163–170.

83 Saran, S., Nakao, H., Tasaka, M., Iida, H., Tsuji, F.I., Nanjundiah, V., and Takeuchi, I., Intracellular free calcium level and its response to cAMP stimulation in developing *Dictyostelium* cells transformed with jellyfish apoaequorin cDNA. FEBS Lett. *337* (1994) 43–47.

84 Schaap, P., Van Lookeren Campagne, M. M., Van Driel, R., Spek, W., Van Haastert, P. J. M., and Pinas, J., Postaggregative differentiation induction by cyclic AMP in *Dictyostelium*: intracellular transduction pathway and requirement for additional stimuli. Devl. Biol. *118* (1986) 52–63.

85 Schlatterer, C., Knoll, G., and Malchow, D., Intracellular calcium during chemotaxis of *Dictyostelium discoideum*: a new fura-2 derivative avoids sequestration of the indicator and allows long-term calcium measurements. Eur. J. Cell Biol. *58* (1992) 172–181.

86 Schlatterer, C., Buravkov, S , Zierold, K., and Knoll, G., Calcium sequestering organelles of *Dictyostelium discoideum*: Changes in element content during early development as measured by electron probe x-ray microanalysis. Cell Calcium *16* (1994) 101–111.

87 Schlatterer, C., and Malchow, D., Intracellular guanosine-5′-O-(3-thiotriphosphate) blocks chemotactic motility of *Dictyostelium discoideum* amoebae. Cell Motil. Cytoskel. *25* (1993) 298–307.

88 Schlatterer, C., Gollnick, F., Schmidt, E., Meyer, R., and Knoll, G., Challenge with high concentrations of cyclic AMP induces transient changes in the cytosolic free calcium concentration in *Dictyostelium discoideum*. J. Cell Sci. *107* (1994) 2107–2115.

89 Segall, J. E., Behavioral responses of streamer F mutants of *Dictyostelium discoideum*: effects of cyclic GMP on cell motility. J. Cell Sci. *101* (1992) 589–597.
90 Segall, J. E., Fisher, P. R., and Gerisch, G., Selection of chemotaxis mutants of *D. discoideum*. J. Cell Biol. *104* (1987) 151–161.
91 Sonnemann, J., Bäuerle, A., Winckler, T., and Mutzel, R., A ribosomal calmodulin-binding protein from *Dictyostelium*. J. biol. Chem. *266* (1991) 23091–23096.
92 Sonnemann, J., Drugeon, G., Haenni, A.-L., and Mutzel, R., Elongation in a *Dictyostelium discoideum* in vitro translation system is affected by calmodulin antagonists. FEBS Lett. *329* (1993) 183–188.
93 Tirlapur, U. K., Gross, J. D., and Nanjundiah, V., Spatial variation of sequestered calcium in the multicellular stage of *Dictyostelium discoideum* as assayed by chlortetracycline fluorescence. Differentiation *48* (1991) 137–146.
94 Tomchik, K. J., and Devreotes, P. N., Adenosine 3′,5′-monophosphate waves in *Dictyostelium discoideum*: a demonstration by isotope dilution-fluorography. Science *212* (1981) 443–446.
95 Unterweger, N., and Schlatterer, C., Introduction of calcium buffers into the cytosol of *Dictyostelium discoideum* amoebae alters cell morphology and inhibits chemotaxis. Cell Calcium *17* (1995) 97–110.
96 Van Duijn, B., and Van Haastert, P. J. M., Independent control of locomotion and orientation during *Dictyostelium discoideum* chemotaxis. J. Cell Sci. *102* (1992) 763–768.
97 Van Haastert, P. J. M., van Lookeren Campagne, M. M., and Ross, F. M., Altered cGMP phosphodiesterase activity in chemotactic mutants of *Dictyostelium discoideum*. FEBS Lett. *147* (1982) 149–152.
98 Wanner, R., and Wurster, B., Cyclic GMP-activated protein kinase from *Dictyostelium discoideum*. Biochim. biophys. Acta *1053* (1990) 179–184.
99 Wick, U., Malchow, D., and Gerisch, G., Cyclic-AMP stimulated calcium influx into aggregating cells of *Dictyostelium discoideum*. Cell Biol. Int. Rep. *2* (1978) 71–79.
100 Winkler, T., Dammann, H., and Mutzel, R., Ca^{2+}/calmodulin-binding proteins in *Dictyostelium discoideum*. Res. Microbiol. *142* (1991) 509–519.
101 Wurster, B., Nanjundiah, V., and Malchow, D., Calcium oscillations in *Dictyostelium discoideum*, in: Calcium as an Intracellular Messenger in Eucaryotic Microbes, pp. 228–224. Ed. D. H. O'Day. American Society for Microbiology, Washington, D.C. 1990.
102 Yule, D. I., Essington, T. E., Shayman, I. A., and Williams, J. A., Sphingosine metabolism induces Ca^{2+} oscillations in rat pancreatic acinar cells. J. biol. Chem. *268* (1993) 12353–12358.
103 Yomura, S., and Kitanishi-Yomura, T., Release of myosin II from the membrane cytoskeleton of *Dictyostelium discoideum* mediated by heavy chain phosphorylation at the foci within the cortical actin network. J. Cell Biol. *117* (1992) 1231–1239.
104 Zhu, Q., Liu, T., and Clarke, M., Calmodulin and the contractile vacuole complex in mitotic cells of *Dictyostelium discoideum*. J. Cell Sci. *104* (1993) 1119–1127.