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Developmental Decisions in *Dictyostelium discoideum*

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INTRODUCTION

Development in *Dictyostelium discoideum*, as in higher organisms, involves a controlled division of labor within a multicellular group (146). Multicellularity in *D. discoideum* is achieved by aggregation of previously separate cells rather than by successive division of a fertilized egg. The developmental process results in both cell differentiation (the production of cells with different characteristics as a result of differential gene expression) and morphogenesis (the generation by cell movement of the appropriate spatial organization of cell types). Development takes place during starvation, and there are only two major alternative cell fates. In addition to bifurcation of cell type, the developmental process includes a choice between alternative morphogenetic pathways: depending on environmental conditions, aggregates either construct fruiting bodies directly in a process known as culmination or migrate for a period; in the latter case the slug-shaped migrating structures, or "slugs," can again be induced to fruit by altered environmental conditions. This review will be concerned primarily with current understanding of the factors controlling the choice of morphogenetic pathway by cell assemblies and the choice of cell fate by individual cells. In considering these questions, I shall also review what is known of signal transduction mechanisms and the structure of cell aggregates. Other areas, such as the factors responsible for initiation of development, the mechanisms of cell movement and chemotaxis, and the forces involved in morphogenesis, are not covered.

In the vegetative phase of their life cycle, *D. discoideum* amoebae ingest bacteria by phagocytosis or, in the case of axenic derivates, take up nutrients by pinocytosis. Deprivation of certain amino acids may play a decisive role in signaling the onset of development (170). Several diffusible factors seem also to be involved (41, 83, 113, 177). Some hours after the onset of starvation, amoebae develop the components of a periodic intercellular signaling system that is based on the synthesis, release, and detection of cyclic AMP (74). As the cells move inward during aggregation toward "signaling centers" on a solid surface, they make contact and join into streams (147). These in turn collect into hemispherical mounds of up to 10^5 cells that become enclosed in a protein and cellulose slime sheath to form tight aggregates. A protruding tip then forms at the apex of each aggregate (Fig. 1). All subsequent movements are orchestrated by the tip. This behaves as an organizer, because it will take over some of the cells in its vicinity when grafted onto the side of another slug (217), and it inhibits formation of a new tip in its neighborhood (53, 154). It is likely, although not proven, that the tip is a

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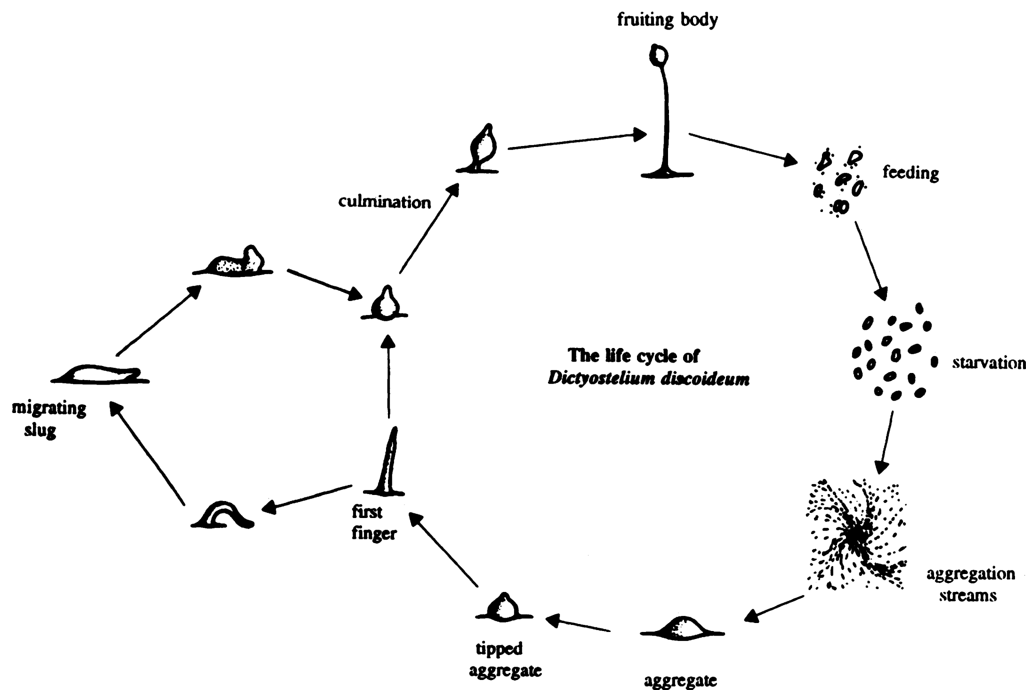


FIG. 1. Development of *D. discoideum*. Some hours after starvation, free-living amoebae aggregate into hemispherical mounds of cells. A tip appears at the apex of each mound and elongates vertically to produce a finger-like structure. This either transforms directly into a fruiting body, consisting of stalk cells and spores, or collapses onto the substratum and migrates for a period as a slug. Diagram courtesy of Lynne Davies.

pacemaker for relayed cyclic AMP signals during later development (55, 234, 242, 243, 267).

Once a tip has formed, the aggregate gradually elongates to give rise to an upright finger-like structure. The apex of aggregates and the anterior of the fingers and slugs that derive from them consist of prestalk cells, which are the precursors of the stalk cells of the mature fruiting body (217). The base of aggregates and the posterior of elongated structures is occupied largely by prespore cells, with a scattering of cells that are virtually indistinguishable from prestalk cells and are known as anterior-like cells (ALC) (49, 252, 284).

Studies of developmental gene regulation have revealed successive expression of sets of developmentally controlled genes, as well as bifurcation along two pathways corresponding to prestalk and prespore cells. Early developmental gene expression, as well as the entire pathway leading to spore formation, appears to be dependent on ongoing intracellular signaling via cyclic AMP. I shall therefore discuss the control of signaling and what is known of the signal transduction pathways involved before going on to consider the mechanisms of developmental choices.

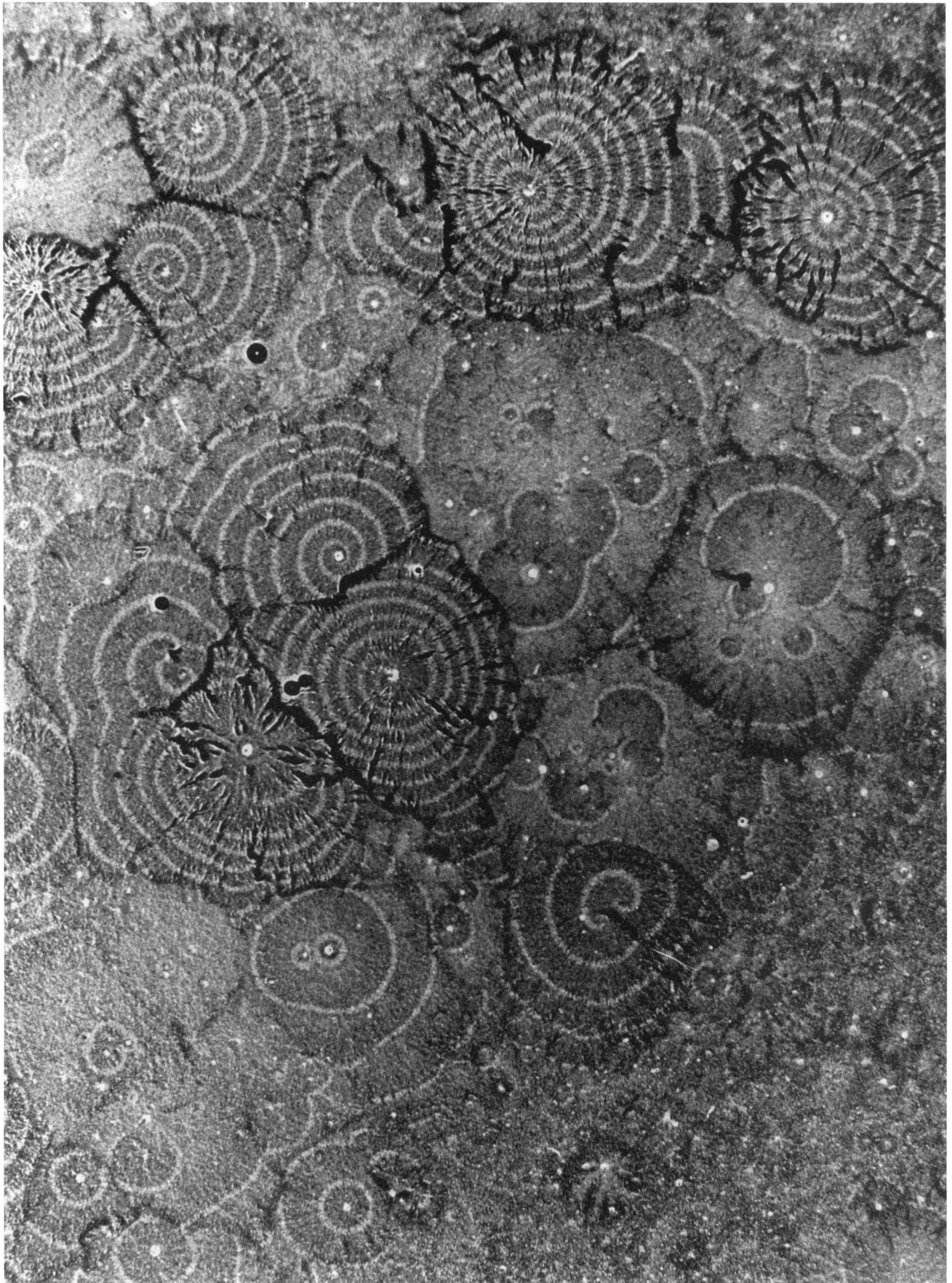
CYCLIC AMP SIGNALING

The components of the signaling system during aggregation, and probably also during later development, include adenylyl cyclase, cell surface cyclic AMP receptors, cyclic AMP phosphodiesterase and its inhibitor, and a variety of cellular components involved in signal transduction (74, 114, 128). Cells that have been stimulated with cyclic AMP make a directional movement step lasting about 1 min and themselves synthesize and release cyclic AMP (50). As a consequence, each successive signal is relayed outward through a field of aggregating amoebae and induces a wave of inward movement. Signaling manifests itself as propagating waves of chemotactic movement on a solid surface (Fig. 2) and as oscillations of light scattering in aerated cell suspensions (74) (see Fig. 3B). Waves of chemotactic movement on a solid surface can be visualized in high-magnification time-lapse films or by using low-magnification, low-angle dark-field optics that reveal differences in the light-scattering properties of moving, elongated cells and rounded, stationary cells (9, 91) (Fig. 2). Cyclic AMP waves have been visualized elegantly by isotope dilution fluorography (262).

Signal Generation

Wave propagation on agar and oscillations of light scattering in cell suspensions depend on two properties: the ability of certain cells to generate cyclic AMP signals autonomously, and the ability of the cell population as a whole to respond chemotactically and relay the signals to other cells. The basis of relay is rather better understood than that of autonomous oscillation. When cyclic AMP binds to developmentally controlled cell surface receptors, adenylyl cyclase is transiently activated, cyclic AMP accumulates for 1 to 2 min, and most of this cyclic AMP is rapidly released into the medium, where it can diffuse to, and stimulate, neighboring cells (50). These cells then make a movement step in the direction of the cyclic AMP source and relay the signal to more distal cells. Cyclic AMP in the vicinity of the excited cell is eventually destroyed by membrane-bound and secreted cyclic AMP phosphodiesterase.

Autonomous generation of cyclic AMP signals by individual cells is responsible for the formation of the concentric waves in the aggregation field illustrated in Fig. 2. Concentric waves are seen at the onset of aggregation and are short-lived and of variable, low frequency (91). They probably have their origin in rare cells (autonomous centers) which progress through a "limit cycle" in which adenylyl cyclase becomes activated



briefly above a threshold value, is then inhibited by some feedback mechanism, and subsequently recovers and again generates cyclic AMP (79). The time required to traverse such a limit cycle determines the interval between successive signals and hence the period of the resulting waves. It is presumably closely related to the cycle of adaptation and deadaptation of adenylyl cyclase activity discussed below.

Territories through which concentric waves are propagating are generally taken over later in aggregation by spiral waves (also seen in Fig. 2). These are generated by the continuous circulation of a relayed cyclic AMP signal around a central ring of cells rather than by the release of bursts of cyclic AMP by individual pacemaker cells. It is thought that the excitation travels around the ring in a length of time such that each cell is reexcited just when it has recovered from the previous excitation and can again relay the signal (52, 91). This time corresponds to the "relay refractory period" of the cells, that is, the minimum time for adaptation and deadaptation of adenylyl cyclase activity (52, 91). Signal relay loops are thought to arise early in aggregation, when a wave emerging from an autonomous center is interrupted by some irregularity in the field of propagating cells and, so to speak, catches its own tail (52).

Evolution of Signaling

There is some evidence that the cyclic AMP signaling system plays a vital role not only during aggregation but also during subsequent development (55, 234, 242, 267). It is therefore of interest to consider how signaling behavior evolves as development progresses. Relay refractory period, as measured by the interval between the arrival of successive turns of a spiral wave at a given point in an aggregation territory, has been shown to decline progressively during early aggregation. It then drops rather abruptly around the time when waves can no longer be seen in low-power images (88). An example of the evolution of spiral wave period is presented in Fig. 3A. The change in relay refractory period is possibly associated with the formation of tight intercellular contacts. In high-power time-lapse films, spiral wave propagation continues at constant high frequency until tip formation. Thereafter it is no longer visible, perhaps because of the slime sheath. However, recent studies with improved techniques have revealed that spiral or three-dimensional scroll waves continue to direct cell movements during tip formation and later development (242, 243). Cells overexpressing the Gα4 subunit remain as extended signaling loops for a prolonged period rather than forming tight aggregates, suggesting that overexpression of this subunit interferes in some way with the transition to the later mode of signaling; this interesting observation is consistent with the efficient synergy between the overexpressing cells and wild-type amoebae (94).

Signaling behavior also evolves in aerated cell suspensions. A series of spike-shaped oscillations is followed by regular sinusoidal oscillations of slightly shorter period (35, 75). These then give way to a highly variable number of very-long-period oscillations before light-scattering changes cease (Fig. 3B). Interestingly, whereas oscillations in cyclic AMP and cyclic GMP accompany spike-shaped oscillations, no changes in these cyclic nucleotides were detected during sinusoidal oscillations (35). The mechanism by which sinusoidal oscillations are synchronized remains a mystery.

TRANSDUCTION OF CYCLIC AMP SIGNALS

A diagram of some of the second-messenger pathways activated when extracellular cyclic AMP binds to cell surface receptors is presented in Fig. 4. The coupling of the surface receptors to adenylyl cyclase is of central importance; as I have already described, it is the periodic activation of this circuit and its adaptation and recovery that underlies signal generation and propagation (166). Cyclic AMP receptors are also coupled to guanylyl cyclase and phospholipase C (114, 151), as well as somehow controlling influx of Ca^{2+} through membrane channels (186). Inositol-1,4,5-trisphosphate is generated by the action of phospholipase C and appears to induce the release of Ca^{2+} from internal stores (59), as in higher eucaryotes. The other product of the action of phospholipase C, diacylglycerol, may activate protein kinase C (PKC) (150). During aggregation, the phospholipase C and guanylyl cyclase pathways are shut down, or adapt, within seconds of receipt of a cyclic AMP stimulus, whereas the adenylyl cyclase pathway adapts in 1 to 2 min. During later development, one or more of these pathways may no longer be shut off in response to the continuous presence of cyclic AMP, since later gene expression in cell suspensions can be induced by constant high levels of cyclic AMP (231, 267).

Receptors and G Proteins

During starvation cells express four cell surface cyclic AMP receptors (cAR1 to cAR4) at different times (228). These receptors are seven-transmembrane-domain proteins homologous to many mammalian receptors and to yeast pheromone receptors. Like these mammalian and yeast receptors, cAR1 and possibly the other cARs are linked to heterotrimeric G proteins (65, 98). Eight G-protein α subunits (Gα1 through Gα8) are expressed at various times during development (44). Disruption of cAR1 abolishes aggregation and the ability of chemotaxis toward cyclic AMP, as would be expected if it plays a central role in the chemotactic response (253). However, activation of adenylyl cyclase by exogenous cyclic AMP still occurs in this disruptant, although it is somewhat delayed and prolonged. Similarly, adenylyl cyclase can be activated in vegetative cells that express very little cAR1. This is surprising and indicates the existence of some as yet unidentified receptor for cyclic AMP in vegetative cells (214). cAR2 appears to be expressed preferentially in prestalk cells, and disruption blocks development at tip formation, although prestalk genes are expressed normally. *cAR2* may therefore be required for sorting of prestalk from prespore cells within aggregates (227). Elimination of the *cAR3* gene produces no obvious phenotype (228).

Disruption of Gα2 and of adenylyl cyclase each results in inability of cells to aggregate. Cells lacking Gα2 cannot activate adenylyl cyclase, guanylyl cyclase, or phospholipase C (136, 137, 200). They nevertheless display some cyclic AMP-induced responses such as ligand-induced receptor phosphorylation, calcium influx (186), and chemotaxis to folic acid (126).

FIG. 2. Dark-field photograph of aggregation "waves" on agar. Both concentric and spiral waves are visible. Bands of elongated amoebae moving toward the center of each territory in response to a cyclic AMP "signal" appear bright, whereas intervening bands of rounded-up and stationary amoebae are dark (9). The instability of autonomous periodic signaling giving rise to the concentric wave patterns is evident, as is invasion of the territory of the concentric waves by the higher-frequency spirals. Wave velocity is 200 to 400 μm/min. For further details, see the text and references 9 and 91. Magnification, approximately ×4. Photograph courtesy of P. C. Newell.

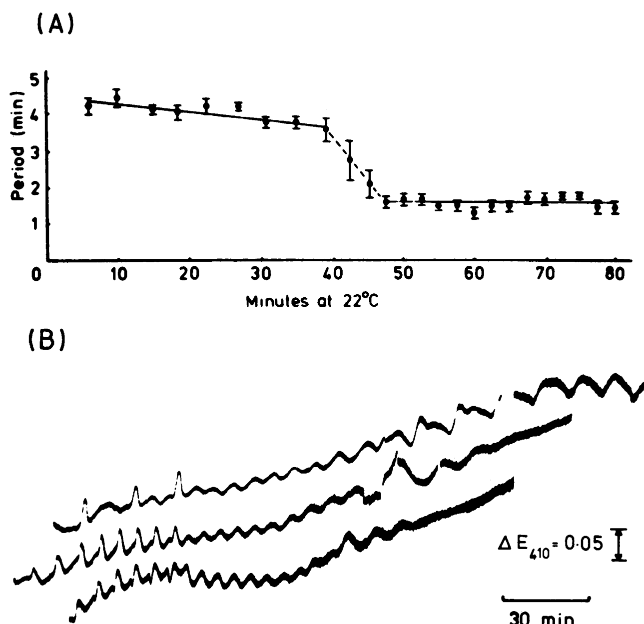


FIG. 3. (A) Intervals between successive chemotactic movement steps during aggregation. The center of a territory that was generating a spiral wave was filmed by high-power time-lapse photography. Bars represent the standard error of the mean values obtained in three separate analyses of the film. The abrupt drop in signaling period occurs when the cells appear to form tight contacts. Solid lines are regression lines. (B) Light-scattering oscillations in cell suspensions. Cells starved overnight at 7°C on nonnutrient agar were harvested and aerated in buffer, and the optical density at 21°C was recorded. An initial series of spike-shaped oscillations, with or without intercalated sinusoidal oscillations, is followed by a series of sinusoidal oscillations. The three sequences recorded on different days are aligned by using the last spike in each sequence as the reference. After some 8 to 10 regular sinusoidal oscillations, the optical density oscillations become highly irregular and variable in waveform. Analysis of simultaneous recordings on agar and in suspension shows that regular sinusoidal oscillations stop at the time when the abrupt drop in signaling period shown in panel A occurs. Panel A is reprinted from reference 88 with permission of the publisher. Data in panel B are from reference 207.

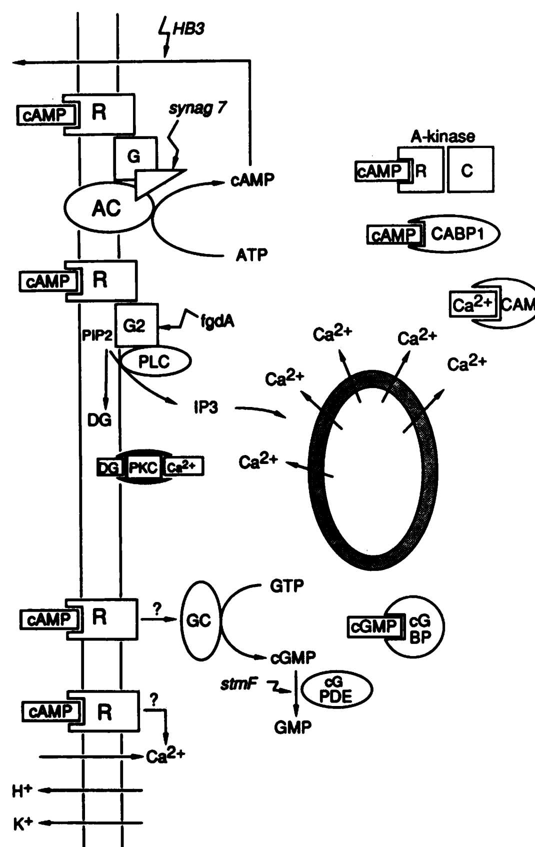


FIG. 4. Signal transduction pathways in *D. discoideum*. Tentative outline of the pathways activated by occupation of cell surface cyclic AMP receptors are shown. Abbreviations: cAMP, cyclic AMP; R, receptor; G, G protein; G2, G protein with Gα2 subunit; AC, adenylyl cyclase; GC, guanylyl cyclase; PLC, phospholipase C; IP3, inositol-1,4,5-triphosphate; cGBP, cyclic GMP-binding protein; cG PDE, cyclic GMP phosphodiesterase; CAM, calmodulin; cABP1, cyclic AMP-binding protein. The phospholipase C and guanylyl cyclase pathways are activated within seconds and adapt rapidly. The adenylyl cyclase pathway is activated some 30 s after stimulation and adapts by 90 to 120 s; virtually all the cyclic AMP produced is released from the cells where it propagates the cyclic AMP signal. Release is blocked in mutant HB3 (127). Some intracellular cyclic AMP also activates PKA (A-kinase). A cytosolic cyclic AMP-binding protein (CABP1) is also present; its function is unknown (84). The diacylglycerol (DG) level varies during development, but diacylglycerol may be generated by pathways other than phospholipase C (44). *ras* gene products and phosphotyrosine phosphatases also play roles in signal transduction (44). Mutants interfering with particular pathways are indicated. Reprinted from reference 209 with permission of the publisher. For further details, see reference 209 and the text.

Disruption of six of the seven other Gα proteins individually also does not eliminate these responses (186). Although intact cells lacking Gα2 are unable to activate adenylyl cyclase in response to binding of cyclic AMP to cell surface receptors, GTPγS is able to induce cyclic AMP production in extracts of these cells (126) and of all the other Gα-minus isolates. Lilly et al. (143) have pointed out that these findings could be explained if adenylyl cyclase were activated by the βγ subunits rather than the α subunit, since, in vitro, GTPγS could induce the release of βγ subunits from G proteins other than Gα2. βγ subunits are believed to activate the pheromone response in yeast cells (100) and phospholipase in mammalian rod outer segments (115). Consistent with this view, disruption of the single β subunit does result in a complete absence of cyclic AMP responsiveness (143).

Mutants with mutations in one of four genes that are specifically defective in activation of adenylyl cyclase by cyclic AMP are known (65). In lysates of one of these mutants, *synag 7*, adenylyl cyclase cannot be activated by GTPγS but activation can be restored by addition of a cytosolic protein from wild-type cells (246, 259, 272). The mutant appears, therefore, to be defective in this cytosolic protein. Clearly, much of interest remains to be learned of the mechanism by which adenylyl cyclase is activated.

Adaptation of the Relay Response

Cellular responses involving cell surface receptors may be said to adapt, or to desensitize, if they terminate while ligand continues to bind receptors (82, 131). Adaptation in *D. discoideum* is relative in that it is overcome by increasing the concentration of extracellular cyclic AMP, up to a maximum of 10 μM (50). Furthermore, cells that are desensitized with respect to their ability to synthesize cyclic AMP are still able to generate a chemotactic response to cyclic AMP (9). The

receptor is extensively phosphorylated in response to agonist binding (273), and adaptation and deadaptation have been ascribed to a cycle of such receptor phosphorylation and dephosphorylation (171, 273). However, in other well-studied systems, such as visual transduction, each step in the activation process is accompanied by a process that restores the system to the basal state (213); hence, adaptation in *D. discoideum* may involve the cyclic AMP receptors or later components in the sequence of steps leading to activation of adenylyl cyclase.

In a theoretical treatment of aggregation, adaptation has been ascribed to a cyclic AMP-induced rise in cytosolic calcium concentration acting at some step in adenylyl cyclase activation (187). There is evidence for a kinase that specifically phosphorylates the agonist-occupied form of the receptor (179), as in mammalian systems (82), and it is possible that an arrestin-like activity that would bind to the phosphorylated form of the receptor and uncouple it from G proteins (82) is also involved (98). However, no arrestin activity or gene has yet been reported. It has been argued that PKA is not required since adaptation occurs when adenylyl cyclase activation is inhibited (258). On the other hand, it does appear to be involved in some aspect of the control of adenylyl cyclase activity, since mutants in which PKA activity is constitutive (245) accumulate greatly reduced levels of cyclic AMP (4).

Exposure of intact wild-type or Gα2-defective cells to cyclic AMP followed by lysis of the cells appears to result in adaptation in that it substantially reduces the response of adenylyl cyclase to added GTPγS (259). Curiously, desensitization defined in this way does not occur in cAR1^{-} cells, even though adenylyl cyclase can be efficiently activated by GTPγS in lysates of such cells (214). This raises the interesting possibility that phosphorylated cAR1 in its hyperphosphorylated form somehow transmits an inhibitory signal to adenylyl cyclase (98, 214). A pertussis toxin-sensitive inhibitory G protein may be involved (246, 272).

The mechanism of the desensitization reaction has an important bearing on the nature of the cyclic AMP oscillator that underlies autonomous cyclic AMP signaling (16). A system involving delayed negative feedback inhibition of adenylyl cyclase is required, and Ca^{2+} ions could well play a role at some point in this sequence (16), as they do in vertebrate retinal light responses (175) (see below). It should be noted that most of what is known of the molecular basis of cell signaling applied to the aggregation stage. Signaling during later development is much harder to study (234, 242).

CYCLIC AMP-DEPENDENT GENE EXPRESSION

In addition to the role of cyclic AMP as a chemoattractant and as an agonist activating adenylyl cyclase, its binding to cell surface receptors regulates the expression of most or all of the genetic program of development. The contribution of the various second messengers in inducing different components of the genetic program is still poorly understood. During the aggregative stage, cyclic AMP pulses accelerate the appearance of proteins involved in the aggregation process, such as cyclic AMP phosphodiesterase, adenylyl cyclase, cyclic AMP receptors, and contact sites A (74). Other genes expressed early in development, such as discoidins I and II, are repressed by cyclic AMP pulses (65, 74). Continuous perfusion of cyclic AMP into suspensions of amoebae of strain NC4 and its axenic derivatives at the same average rate as provided by pulsatile application inhibits the appearance of these products and of the corresponding gene transcripts. This is presumably due to adaptation of the cyclic AMP response (74). In strain V12 and its derivatives, expression of these early developmental gene products is more responsive to continuous exposure to cyclic AMP (224), probably because of some modification of the adaptation mechanism.

Stages of Gene Expression

At about the time that cells make tight contacts in streams, accumulation of aggregation-stage products ceases and a large number of new gene transcripts and proteins accumulate (10, 19, 37, 263). Synthesis of these postaggregative products depends on continued cyclic AMP signaling. If aggregates are disaggregated or if cell suspensions are agitated rapidly, the majority of them do not appear, and instead aggregative-stage products continue to accumulate (11, 195, 256, 263); addition of cyclic AMP permits accumulation of the postaggregative products, and, remarkably, the cyclic AMP does not have to be applied in pulsatile fashion. Indeed, it has been claimed that continuous exposure to cyclic AMP is more effective (63, 142, 231, 256, 263), which is strange in view of the evidence that signaling is pulsatile throughout development (234, 242).

The postaggregative response of gene expression to binding of cyclic AMP to cell surface receptors differs in at least two respects from the aggregative response: it requires some 10- to 100-fold-higher concentrations of exogenous cyclic AMP, and it does not desensitize (231, 232). It may well be that surface receptors other than cAR1 are involved in this process. There is considerable evidence for a requirement for cell contact or conditioning factors in addition to cyclic AMP signaling for optimal postaggregative gene expression in strain NC4 and its derivatives (14, 40, 142, 178, 286). An important advance in understanding the mechanism of the switch from aggregative to postaggregative gene expression has been made recently by the cloning and disruption of a gene coding for a transcription factor required for the switch (239).

A number of cDNA clones complementary to developmentally regulated mRNA transcripts have been isolated (13, 149, 178, 209); some of these are enriched in prestalk or prespore cells. Since relatively high concentrations of cyclic AMP are required to induce postaggregative gene expression, it appeared at one time possible that the cyclic AMP leaks into the cells and acts directly on the cyclic AMP-dependent PK that is known to accumulate during development (see below) rather than acting on a cell surface cyclic AMP receptor. This idea has, however, been excluded by the demonstration that cyclic AMP does not penetrate cells and by showing that the ability of a variety of cyclic AMP derivatives to induce gene expression correlates with their affinities for the cyclic AMP receptor rather than for the regulatory subunit of PKA (80, 95, 129, 167, 202, 231, 233).

Role of PKA in Developmental Gene Expression

During aggregation the intracellular concentration of cyclic AMP oscillates in cells as adenylyl cyclase is periodically activated (50), and a marked rise in intracellular cyclic AMP levels has been demonstrated around the time of culmination (4, 183). In many kinds of cell, cyclic AMP acts as a second messenger, activating cyclic AMP-dependent PKA. PKA also exists in *D. discoideum*, although in this organism, unlike others, the regulatory subunit lacks a dimerization site and the inactive complex of one regulatory (R) and one catalytic (C) subunit is dissociated by binding of cyclic AMP to the R subunit, releasing a catalytically active C subunit (48, 164) (Fig. 4).

The genes coding for the regulatory and catalytic subunits of PKA have been cloned, and the latter gene has been disrupted by homologous recombination (168). The fact that this dis-

ruptant is viable indicates that PKA is not essential for vegetative multiplication of the amoebae, and, consistent with this, the enzyme is present at very low levels in wild-type vegetative cells. However, its concentration increases dramatically during development, and it seems to play important roles in gene expression throughout development. The evidence for this depends largely on the analysis of strains in which the genes coding for the R or C subunits have been genetically altered either to render activity constitutive (i.e., independent of cyclic AMP levels) or to eliminate it. Constitutive activity is observed in cells in which the R subunit is inactive (226, 245) or that overproduce the catalytic subunit (101, 169), whereas activity is eliminated either by disrupting the C subunit gene (168) or by overproducing a normal or dominant-negative form of the R subunit (64, 96, 244). In addition to this genetic manipulation, PKA can be artificially activated by application of the membrane-permeable analog, 8-bromo cyclic AMP (119, 160).

The most firmly established role of PKA is in the maturation of stalk cells and spores from their precursors. Thus, cells in which PKA activity is constitutive display precocious stalk and spore formation and are also able to form mature spores when incubated as monolayers under buffer in the presence of cAMP, conditions under which wild-type amoebae form prespore cells but are unable to mature into spores (101, 119, 169). Exposure of prestalk and prespore cells to 8-bromo cyclic AMP also induces maturation into stalk cells and spores (109, 119, 134, 139, 160). Moreover, cells harboring constructs in which a dominant-negative form of the R subunit is driven by prestalk-cell or prespore-cell-specific promoters are blocked in stalk and spore maturation (97, 102).

PKA also appears to be essential at earlier stages of development. Cells lacking PKA activity can undergo chemotaxis toward cyclic AMP (96) but are unable to aggregate (64, 168, 244) or to produce cyclic AMP in response to exogenous cyclic AMP (96). Their failure to aggregate is probably due to inability to accumulate the necessary aggregative stage gene products, since even when such mutant cells are pulsed artificially with cyclic AMP these products are not formed (244). When coaggregated with wild-type cells, they also fail to form gene products characteristic of prestalk or prespore cells (96). This defect could in principle be due to an inability to reach a stage at which they are competent to respond to cyclic AMP by accumulating postaggregative gene products. However, PKA does seem to be directly involved in postaggregative gene expression, since constructs in which a dominant-negative R subunit is driven by a prespore promoter have reduced levels of a number of prespore-cell-specific transcripts (102). An important question for the future concerns the possible involvement of PKA in the choice between prestalk and prespore pathways of differentiation.

The persuasive evidence for key roles of PKA in developmental gene expression would appear to be contradicted by evidence that activation of adenylyl cyclase is not required for aggregative or postaggregative gene expression. Activation of adenylyl cyclase consequent upon agonist binding to cell surface cyclic AMP receptors can be blocked genetically (232) or by caffeine treatment (28) or high osmolarity (202). In each case the cells remain able to synthesize developmental gene products, provided that cyclic AMP is supplied to activate cell surface receptors, and the same is even true for a mutant in which the only known developmental phase adenylyl cyclase gene has been inactivated by disruption (210, 211). These observations seem to imply that intracellular cyclic AMP is not required for aggregative and postaggregative gene expression. The apparent contradiction between these findings and the equally clear-cut evidence for a role of PKA has not yet been resolved. It is possible that there is some constitutive level of PKA activity in the absence of cyclic AMP or that there are additional forms of adenylyl cyclase.

ANATOMY OF AGGREGATES

All multicellular structures formed from the tipped aggregate stage onward are polarized, with an anterior zone of prestalk cells and a posterior zone made up largely of prespore cells. Prestalk and prespore cells can be distinguished by a difference in cell size and cell orientation (230). In larger aggregates a change in diameter at the prestalk prespore boundary can also be observed, the prestalk region being narrower (34). Prestalk cells stain more strongly with vital dyes, such as Nile blue and neutral red, that stain large intracellular acidic structures (autophagic vacuoles). The weaker staining of the prespore cells, at least at earlier stages such as the tipped aggregate, is probably due to a less acidic pH of the autophagic system (288). Later, in slugs, the autophagic vacuoles of prespore cells seem to be broken down, and the weaker staining of these cells may be due to a combination of higher pH and less extensive vacuolar network (163, 229, 288). Prestalk and prespore cells are also distinguished by differences in gene transcripts and developmental proteins (65, 74, 209).

There is disagreement about the time in development when the difference in vital staining pattern between prestalk and prespore regions first appears. Yamamoto and Takeuchi (288) report that tipped aggregates are clearly divided into a strongly staining tip and a weakly staining body, and this has also been the experience in my laboratory (72). Other workers, however, report that the differential pattern of staining emerges only at the slug stage (21, 23, 250). The contradictory observations may be due to differences in the salt content of the agar (288) or to differences in the intensity with which vegetative cells are stained with vital dyes. In any case, it is likely that vacuolar pH differs between the anterior and posterior cell types even when a differential staining pattern is not visible, since Bonner et al. (23) report the interesting finding that a pattern emerges in unpatterned slugs within 10 min of their immersion in mineral oil. The idea that cells of early aggregates already differ in pH is required in a model according to which such a difference is a cause, not simply a consequence, of differential gene expression in the two cell types (90).

Cell Sorting

It has been firmly established that early aggregates consist of a random mixture of prestalk and prespore cells, as defined by the expression of transcripts and gene products specific to the prespore and prestalk cells of slugs (55, 69, 257, 283). Prestalk cells subsequently sort out to the apex (and base) of the aggregate during tip formation, leaving the body of the aggregate occupied by a majority of prespore cells and a minority of ALC (Fig. 5). There is evidence that sorting out involves differential chemotaxis to cyclic AMP (176, 251, 267).

Heterogeneity of Cell Types

Recently evidence has accumulated that both the prespore (33, 93) and the prestalk cell populations are heterogeneous. Thus, deletions within the promoter of the prespore gene SP60 cause expression of this gene to be restricted to the more anterior part of the posterior zone (93), suggesting a gradient of prespore gene expression down the posterior zone. It has been shown that for the prestalk cell population, cells dissoci-

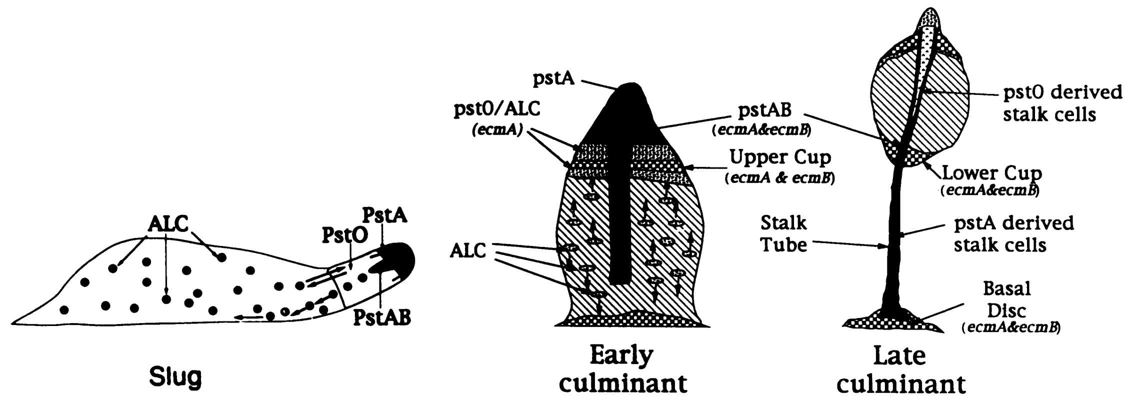


FIG. 5. Structure of the slug and culminants. Brackets indicate whether the *ecmA* and/or *ecmB* gene is expressed by given cell types. The posterior region is made up of prespore cells (not shown) with a minority of neutral red-staining ALC. In the slug, groups of pstAB move through the prespore region and are lost into the slime trail; these are thought to be replaced by a unidirectional respecification process in which prespore cells transform into ALC that migrate to the front and become pstA cells (250). At culmination the rear of the slug slides in under the anterior region, and the latter is thereby lifted into an apical position (216). The pstAB core cells migrate downward as a whole to contribute to the basal disc (250). Meanwhile, the pstA cells enter the stalk tube, express the *ecmB* gene, and mature into stalk cells, while ALC move from the posterior region to form the upper cup, lower cup, and part of the basal disc (118). The ALC are greatly enlarged in this diagram, kindly provided by J. G. Williams.

ated from the anteriormost 10% of the slug and incubated as monolayers form stalk cells in the absence of cyclic AMP, whereas the more posterior prestalk cells do not (139, 266). More recently, use of reporter gene constructs has confirmed and extended our understanding of this heterogeneity. This approach derived from the isolation of a mutant defective in synthesis of the stalk-cell-inducing substance, DIF-1 (see below). Exposure of the mutant to DIF-1 permitted the isolation of a set of prestalk-cell-specific gene transcripts and the cloning of the corresponding genes with their promoters. The products of two of these genes are components of the extracellular matrix, and the genes are referred to as *ecmA* and *ecmB* (282, 283). By linking the promoters of *ecmA* and *ecmB* to reporter genes, it has been shown that a central core of cells at the apex of the slug expresses both genes, and these cells are therefore referred to as prestalk AB (pstAB) cells. This core is surrounded by cells (pstA cells) that accumulate only the *ecmA* gene product (71, 118). In addition, distal to the pstA cells in the anterior region is a zone of cells (pstO cells) that express considerably lower levels of the *ecmA* gene product than do the pstA cells (Fig. 5), and below the pstA cells is a zone of cells (referred to as pstO cells) that express *ecmA* at a significantly lower level than do pstA cells (Fig. 5).

The evidence for a distinct category of pstO cells is of two kinds. Buhl and MacWilliams (34) have transplanted anterior prestalk cells carrying *lacZ* reporter genes to the back of the prestalk zone and vice versa. These elegant experiments demonstrate that both the anterior prestalk cells (comprising a mixture of pstA and pstAB cells) and the more posterior, pstO, cells return within ca. 1 h to their original positions when transplanted to the alternative region of the prestalk zone. (They subsequently disperse throughout the prestalk zone.) Either cell type, when transplanted to the prespore zone, sorts back to the prestalk zone and behaves initially as a pstO prestalk populations. These results demonstrate that pstO and pstA/AB cells have distinct sorting behavior and that this behavior is influenced by a period in the prespore zone. They also point to the importance of a combination of cell autonomous properties and positional information in determining the differentiation of cells.

Analysis of the *ecmA* promoter has provided additional support for the view that pstO cells (together with many of the ALC) constitute a distinct subclass of prestalk cells. Thus an upstream region of the *ecmA* promoter is sufficient for expression of *ecmA* in pstO cells and ALC but not in anterior prestalk cells (pstA cells), and the cells expressing *ecmA* from this promoter fragment go on to contribute substantially to the upper cup at culmination (56). A similar analysis has demonstrated that a separate region of the *ecmB* promoter is necessary and sufficient for expression of *ecmB* in upper cup cells but not in anterior prestalk cells or in cells entering the stalk tube (39).

ALC closely resemble prestalk cells. They have large, acidic autophagic vacuoles that stain strongly with neutral red, and they express almost the same set of prestalk-cell-specific genes (49). However, they differ in their sorting behavior in that they appear to be inhibited from sorting to the front of slugs by a substance secreted by anterior cells. By contrast, when prestalk cells are dissociated and examined for sorting in the same way as the ALC, most or all of them do sort (251). The ALC are approximately equal in number to the total prestalk cell population and are more concentrated on the ventral side of the prespore region. Numbers of ALC do not decline on prolonged migration (252). When posterior regions are isolated by surgery, the ALC sort to the anterior to re-form the anterior-posterior pattern, and the concentration of ALC in the posterior drops substantially. Over the next hours the proportion of ALC in the new posterior returns to its original value (possibly by conversion of prespore cells into ALC), and at the same time some of the prespore cells are respecified and contribute some 40% of the cells in the new prestalk zone (24, 252).

CHOICE BETWEEN MIGRATION AND CULMINATION

It is now well established that the decision to culminate rather than to begin or continue migration is a response to a reduction in the concentration of the unprotonated form of the weak base ammonia in aggregates. Ammonia is produced in large quantities by breakdown of amino acids during development. Culmination can be induced by exposure to an enzyme cocktail that removes ammonia (236), as well by low extracel-

lular pH, CO_2, and other agents that cause intracellular acidification. These agents almost certainly exert their effect by lowering the intracellular concentration of ammonia (103).

The importance of ammonia in inhibiting culmination is confirmed by studies with mutants known as sluggers, which remain as slugs for abnormally long periods. Those isolated to date have been assigned to 10 complementation groups, and some members of each group have been shown to be abnormally susceptible to inhibition by ammonia with respect to aggregation and/or the choice between migration and culmination (73, 196, 237). Culmination is not induced by exposure to even very high concentrations of DIF-1 (103), indicating that DIF-1 concentration is not rate limiting for culmination.

By preferential staining with methylene blue, it has been demonstrated that at the onset of culmination the core of pstAB cells migrate to the base of the culminant, eventually fusing with rearguard (ALC) cells to make up the inner zone of the basal disc (250). Similarly, the use of reporter gene constructs shows that pstA cells progressively enter the stalk tube and begin to express the *ecmB* gene (118). Meanwhile, some ALC migrate up to the anterior zone because, presumably, inhibition of their migration has been relieved, and they join with pstO cells to form the upper cup surrounding the eventual spore mass, while other ALC move downward to form the lower cup (56). During this process, the ALC also undergo a dramatic increase in *ecmB* gene expression (118).

It has been proposed that these cell movements and changes in gene expression, illustrated in Fig. 5, are themselves initiated by a drop in the effective concentration of ammonia (97). The key role of such a reduction in ammonia concentration in stimulating stalk cell maturation is supported by the observation that removal of this weak base with an enzyme mixture dramatically stimulates stalk cell formation when intact slugs are incubated in buffer in the presence of DIF-1 (276). Moreover, exposure of intact slugs migrating on an agar surface to an atmosphere of CO_2 causes rapid maturation of essentially all anterior cells, as well as ALC, into stalk cells (103). Exposure to high levels of DIF-1 has no such effect. This result reinforces the view that ammonia is the agent that prevents these cells from maturing into stalk cells.

How Does Ammonia Act?

Sussman and his coworkers have demonstrated that addition of millimolar concentrations of ammonia to suspensions of developing amoebae caused a rapid drop in levels of cellular cyclic AMP as well as of cyclic AMP in the medium (237). Intracellular cyclic AMP was reduced to a negligible level within 15 min of ammonia addition and remained there for at least a further 15 min. They also showed that the amount of cyclic AMP that accumulated within cells upon exposure to a saturating cyclic AMP stimulus was substantially reduced in the presence of ammonia, as was the amount of cyclic AMP released into the medium (280). The simplest interpretation is that ammonia interferes with the cyclic AMP relay response, that is, with activation of adenylyl cyclase in response to cyclic AMP binding to cell surface receptors (280).

The idea that ammonia interferes with cyclic AMP relay is consistent with a requirement for activation of cyclic AMP-dependent PK for stalk cell and spore maturation, both of which are inhibited by ammonia (see below). It also accounts for the marked rise in cellular cyclic AMP levels around the time of culmination (4, 183), given that this seems to be a response to lowering of ambient ammonia concentrations. To confirm the significance of this finding, it would be useful to examine changes in cyclic AMP levels during synchronous induction of culmination in order to determine exactly when the rise in cyclic AMP levels takes place. Perhaps the most convincing evidence that ammonia interferes with cyclic AMP relay is the observation that the cyclic AMP relay response is ammonia hypersensitive in a slugger mutant whose tendency to remain in the slug state is known to be due to ammonia hypersensitivity (280). On the other hand, Riley and Barclay observed premature accumulation of cell-associated cyclic AMP together with reduced levels of extracellular cyclic AMP when cells were incubated continuously with ammonia from the start of development, and they suggested that prolonged incubation with ammonia stimulates rather than inhibits cyclic AMP accumulation (218). The effect of ammonia on intracellular and extracellular cyclic AMP levels therefore deserves further study, but the balance of evidence at present suggests that ammonia inhibits culmination by preventing PKA activity from attaining some threshold value (97), probably by lowering intracellular cyclic AMP production.

A careful analysis of the *ecmB* promoter has provided some indication of how PKA may be working. Repression of *ecmB* gene expression in the majority of anterior slug cells (pstA cells) is dependent on a proximal upstream segment of the *ecmB* promoter (39). It has therefore been suggested that this promoter region binds a repressor whose activity is dependent on the presence of an extracellular inhibitor (39). This could conceivably be cyclic AMP, since exposure to sustained high concentrations of this substance has been observed to inhibit *ecmB* expression and stimulate *ecmA* expression (15). However, such a differential effect of cyclic AMP has not been confirmed by other workers (247, 286). It seems more probable that the inhibitor is ammonia and that repression by the DNA-binding protein is directly or indirectly relieved by the action of PKA (39).

It is likely that anterior cells are the main source of ammonia in the slug, since their autophagic apparatus is more highly developed (288) and they catabolize more of their proteins during development (87). How, then, can the cells in the central core of this zone be less strongly affected by ammonia than the pstA cells and hence able to express the *ecmB* gene during slug migration and, still more dramatically, during culmination? One suggestion, put forward by Inouye (107), invokes a lateral-inhibition model (43, 77) involving a local activator and long-range inhibitor displaying autocatalytic and cross-inhibitory dynamics. According to this proposal, pstAB cells produce, in addition to large amounts of ammonia that diffuses rapidly, a high local concentration of a slowly diffusing activator, possibly an organic acid. This local activator partially protects cells in the immediate vicinity from the effects of ammonia, and a reduction in the concentration of ammonia leads to a sustained increase in activator level and hence to transformation of the cells into mature stalk cells.

Inouye's proposal gains some support from the intriguing observation that the pH of the front of slugs drops by some 0.5 pH unit within minutes of the onset of culmination (215), as well as from the increased cellular content of several organic acids during development (125). In addition, there is an indication of a weak acid liberated by culminating stalk cells (107) and of a substance with acidic properties that promotes culmination of certain slugger mutants (255). Furthermore, prestalk cells are acidified to a markedly greater extent by exposure to a weak acid than are prespore cells, probably because of a lower activity of their plasma membrane proton pump (104, 105). This cell-autonomous difference between prestalk and prespore cells would serve to amplify any protective effect of a weak acid on the prestalk cells. Others have proposed that expression of *ecmB* in the anterior core of cells

is related to the (presumed) low cyclic AMP concentration in the centre of the scroll wave (149, 242). Alternatively, it may turn out that ammonia production is itself low in this core region.

Role of Acidic Compartments

The action of ammonia in maintaining the migratory slug phase of cell aggregates was early shown to depend on penetration by the uncharged form of the weak base (NH_3), since its effectiveness was a function of the pH of the medium (236). All effects of ammonia that have been studied in this respect are mimicked by other weak bases (47, 89), and it was therefore proposed that ammonia exerts its action by elevating intracellular pH (89). However, more recent evidence suggests that it acts not by raising cytosolic pH but by raising the pH of some component of the extensive acidic vesicle system.

The first piece of evidence for its action on an acidic compartment depends on the following argument. Equal concentration of all free bases should have the same effect on cytosolic pH because they all permeate freely across the plasma membrane in their unprotonated form and complex with cytosolic protons; on the other hand, their effectiveness in dissipating the pH gradient across acidic intracellular compartments varies by several orders of magnitude (212). This is because they accumulate to a very high concentration as charged species in acidic compartments and their rate of diffusion in this form out of such a compartment—the process responsible for dissipating the pH gradient—depends on their hydrophobicity (212). We have therefore compared the effectiveness of a series of weak bases in inhibiting aggregation and culmination, and we have observed the dramatic differences in effectiveness expected if they act on an intracellular acidic compartment (47). Second, we have shown that the relative effectiveness of these different weak bases in inhibiting aggregation and culmination is closely correlated with their ability to inhibit ATP-dependent acidification of a vesicle fraction in vitro. Third, we have demonstrated by in vivo nuclear magnetic resonance spectroscopy that concentrations of weak base that have powerful effects on aggregation and culmination cause a sustained elevation of the pH of intracellular acidic compartments but not of cytosolic pH (47). Fourth, mutants defective in vesicle acidification (20, 30) display a slugger phenotype, and their aggregation is hypersensitive to weak bases (46). Finally, we have shown that a transformant harboring an antisense construct of a vacuolar H^+-ATPase subunit under the control of the *discoidin* promoter also has a slugger phenotype (45).

As mentioned above, exposure of slugs to low pH, weak acids, and proton pump inhibitors causes either culmination or in situ stalk cell maturation, depending on dosage. There is good evidence that exposure to weak acids and proton pump inhibitors lowers cytosolic pH (8, 29, 103, 269; however, see references 116 and 122). It seems likely that these treatments reduce the intracellular concentration of free ammonia and so help to maintain the acidity of the intracellular acidic spaces. As discussed below, acidification of intracellular compartments may promote adenylyl cyclase activation by altering proton symport or antiport processes.

The identity of the acidic space that is important for the developmental effects of weak bases is not known. Cells of *D. discoideum* display very high rates of endocytosis as free-living amoebae (260). Those growing on bacteria contain acidic phagosomes or food vacuoles that are expelled some hours after the onset of starvation (229, 287). Axenically growing cells take up nutrients by fluid-phase pinocytosis via the classical coated-vesicle pathway (197). Probes taken up by pinocytosis first enter a highly acidic "preendosomal" compartment at pH 4.5 to 5.0 and then a substantially less acidic endolysosomal compartment at pH 5.8 to 6.0 (12). Thereafter they are efficiently returned to the extracellular medium by some kind of recycling or "postlysosomal" vesicle (12, 204). Exposure of cells to weak base, to the protonophore nigericin, or the vacuolar H^+-ATPase inhibitor bafilomycin all reduce or abolish acidification of endosomes (12). The finding that early *D. discoideum* endosomes are more acidic than later endocytic compartments contrasts with the situation in mammalian cells, as does the observation that there is no recycling pathway from early endosomes to the extracellular medium in *D. discoideum* (12, 204).

In addition to these acidic compartments, a major light vesicle fraction that is rich in vacuolar ATPase and rapidly acidified in an ATP-dependent manner in vitro has been identified (205, 206). These vesicles were originally thought to be responsible for acidifying components of the endocytic circuit; more recently, evidence has accumulated that they are fragments of contractile vacuoles and that the V-type ATPase may play a role in osmotic regulation (68, 99). Interestingly, the contractile vacuoles do not have highly acidic interiors in vivo (289). Use of a clathrin antisense construct has shown that formation of both contractile vacuoles and endosomes is clathrin dependent (197).

In axenically and bacterially grown cells, food vacuoles are replaced by autophagic vacuoles within a few hours of starvation. These vacuoles arise in both cell types but later decrease in number in prespore cells while accumulating further in prestalk cells. Autophagic vacuoles which are strongly stained by neutral red and other vital dyes are the major acid-phosphatase-positive granules in the cell, as befits their role as digestive organs (287).

MECHANISM OF CELL TYPE CHOICE

As mentioned above, it is now clear that early aggregates consist of a random mixture of prestalk and prespore cells and that prestalk cells sort out to the apex (and base) of the aggregate during tip formation, leaving the body of the aggregate occupied mainly by prespore cells (Fig. 6a and b). Moreover, the difference in cell type within aggregates can be traced back to heterogeneity among amoebae at the start of development.

Cell Cycle and Cell Fate

The tendency of any particular population of cells to occupy any particular cellular location in aggregates can be assessed by marking those cells in some way and mixing them with control cells. The location of the marked cells in the aggregates can then be determined during subsequent development. In this way it has been demonstrated that developmental choice is strongly influenced by the stage of the cell cycle at which they are starved to initiate development, as well as by previous growth conditions. Using cells whose cell cycle was synchronized by dilution from stationary phase and by mitotic wash-off, respectively, Weijer et al. (278) and McDonald and Durston (174) demonstrated that amoebae that are early in the cell cycle (i.e., just after mitosis) tend to differentiate into prestalk cells, whereas those late in the cycle differentiate into prespore cells (see also references 81 and 291). This preference seems to be remarkably strong, since there have been reports that cells that synchronized early in the cycle and develop on their own form slugs with two to four times as many prestalk cells as do

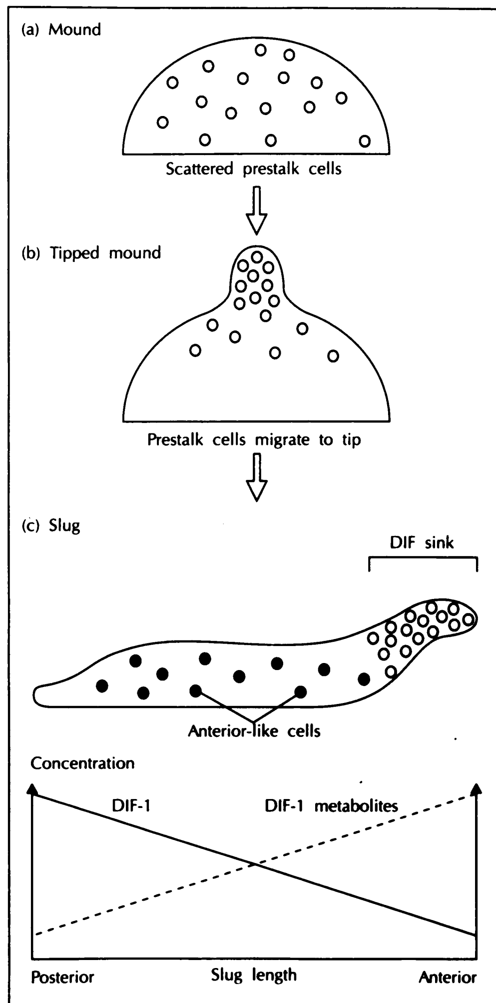


FIG. 6. Sorting out of cell types and possible DIF gradients. (a) Prestalk cells, when first recognized in aggregates, appear scattered among prespore cells. (b) They then sort out to the emerging tip. (c) When a slug is formed, ALC are scattered within the prespore zone and prestalk cells occupy the anterior region. The first enzyme in DIF-1 degradation, DIF-1 dechlorinase, is highly concentrated in the anterior zone. Kay has proposed that this zone is a DIF-1 sink and that DIF-1 and its metabolites are distributed in gradients as indicated (124). Reprinted from reference 120 with permission of the publisher.

those that synchronized late in the cycle (6, 274, 278). This behavior indicates that regulation of cell proportions may not be as effective as is sometimes suggested.

Cells starved early in the cell division cycle aggregate earlier (274, 278) and are much more active in initiating center formation (173). Moreover, cells that have developed for longer and are therefore further advanced appear to sort preferentially to the prestalk zone (172, 198). These results suggest that cell fate is related to rate of development, with cells that develop more rapidly tending to differentiate into prestalk cells (172, 274). However, rapid development and prestalk sorting can be dissociated, since in cells synchronized by a temperature shift (161) or grown in axenic medium with and without glucose (110), the cells that tend to become stalk cells develop less rapidly than those that preferentially form prespore cells.

Prestalk-sorting cells have been observed to move more rapidly in natural aggregates (55) and in response to cyclic AMP released from a micropipette (110). Their differences in chemotactic responsiveness may be related to a higher level of cyclic AMP receptors and other chemotaxis-associated proteins (110, 268). However, the origin of the preference for adopting one fate or the other remains unknown.

Although cells display a preference from the outset, their fate is not fixed, since anterior and posterior segments of aggregates isolated by microsurgery can regulate to give approximately normally proportioned fruiting bodies (217, 222, 223, 251) and differentiation in cell monolayers is influenced by DIF-1 and other factors.

Role of DIF-1

The stalk-cell-inducing substance DIF-1 was discovered by investigating the cell density dependence of stalk cell formation in monolayers of *D. discoideum* V12 incubated with cyclic AMP (265). DIF-1 is a chlorinated alkyl phenone (188), which accumulates around the time of tip formation (32, 248). DIF activity can be fractionated into five species, but DIF-1 represents 95% of the total activity (121). DIF is required for differentiation of prestalk and stalk cells in vitro and for the formation of a set of DIF-dependent messenger transcripts (117, 282). DIF-1 also antagonizes prespore gene expression (57, 70, 123, 135, 203), and a mutant that accumulates considerably less DIF-1 than normal forms tipless mounds that contain prespore but not prestalk cells (133).

Because weak acids and plasma membrane proton pump inhibitors can, under some circumstances, mimic the action of DIF-1, it was suggested that DIF-1 is itself a plasma membrane proton pump inhibitor that acts by acidifying the cytosol (89). This idea has been ruled out by the demonstration that DIF-1 neither inhibits pump activity in vitro (90, 153) nor causes any sustained acidification of the cytosol (103, 122). A later suggestion that DIF-1 promotes acidification of intracellular compartments (90) is also contradicted by the findings that weak acids synergize with DIF but do not substitute for it (275) and that it does not affect in vitro vesicle acidification (78). A developmentally regulated, high-affinity cytosolic DIF-binding activity has been detected, and the most likely idea at present is that DIF-1 acts like a steroid hormone, binding to and activating a nuclear transcription factor (111).

Since the choice between prestalk and prespore pathways in vitro is decisively influenced by DIF-1, and since DIF-1 is required for prestalk cell formation, the in vivo choice almost certainly depends on differences in the distribution of DIF-1 or in cellular responsiveness to DIF-1 (92, 120, 149). How one actually envisages the choice being made depends on an understanding of which cell type makes DIF-1 and how DIF-1 is distributed within aggregates and slugs. These questions are controversial.

Slug dissection experiments indicate that DIF levels are higher in the rear than in the front of slugs and have been taken as suggesting that DIF is produced by prespore cells (31, 149) or possibly by all the cells of the slug (120). However, when prestalk and prespore cells are fractionated by density gradient centrifugation and incubated as monolayers with cyclic AMP, a much higher rate of DIF production is found in a fraction expressing *ecmB* than in a fraction containing a mixture of prespore cells and pstA cells; this suggests that DIF is synthesized primarily by the pstAB cells of the anterior core (141). Similarly, agents such as weak acids and plasma membrane proton pump inhibitors that preferentially stimulate stalk cell formation in cell monolayers also increase DIF accumulation (140). Conversely, ammonia inhibits DIF accu-

mulation (72, 194), as might be anticipated since it has the opposite effect on cell type differentiation. Thus, the effective concentration of DIF-1 may actually be higher in the anterior zone, and much of the DIF detected in prespore fragments may in reality be in the slime sheath surrounding the slug or sequestered in some way within the cells of the posterior zone (193). The presence of a higher concentration of DIF-1 in the anterior of slugs would account in the most straightforward way for the conversion of prespore cells to prestalk cells during stalked migration of *D. mucoroides* (86). It would also fit with the interesting observation that cells of the mutant HM44 that are defective in DIF-1 synthesis are excluded from the prestalk zone in mixtures with wild-type cells (189), contrary to the initial report (133).

An alternative explanation of the conflicting results is suggested by the observation that the *ecmB* gene product is itself somewhat more concentrated in rear than in front fragments (124). This surprising finding is presumably due to expression of *ecmB* in ALC and suggests that cells expressing *ecmB* may indeed be the major source of DIF. In that case, one might envisage that the pstAB cells of the anterior core generate the DIF-1 that is responsible for keeping anterior cells in the prestalk state and that the DIF-1 in the posterior zone, although comparable in concentration, is largely prevented from inducing prestalk cell differentiation by other factors (see below).

The enzyme that dechlorinates and inactivates DIF-1, DIF-1 dechlorinase, is dependent on DIF-1 for its accumulation and has been unambiguously located to the prestalk zone of aggregates (124, 192). Kay (120) has suggested that this enzyme, together with products of DIF-1 degradation, plays a key role in the prestalk/prespore choice (Fig. 6). Kay et al. have also argued, because of the location of the DIF dechlorinase, that the front of the slug is a DIF-1 sink (124). An alternative possibility, if one discounts the evidence for higher DIF-1 levels in the back of slugs, is that the role of the DIF dechlorinase is to limit the spread of DIF-1 from the anterior to the posterior region.

Role of Ammonia

As already mentioned, large quantities of ammonia accumulate in aggregates as a result of protein catabolism and are responsible for inhibiting the culmination of aggregates. Ammonia has also been found to have a profound effect on cell differentiation in monolayers incubated in the presence of cyclic AMP: in dense monolayers, in which DIF-1 is generated endogenously, addition of millimolar concentrations of ammonia was found to inhibit stalk cell differentiation and stimulate spore formation in sporogenous mutants, i.e., mutants that generate spores as well as stalk cells under these conditions (89, 92, 194). Inhibition of stalk cell formation would be expected because of the inhibitory effect of ammonia on PKA activity at culmination. However, the stimulation of spore formation is not readily explained by inhibition of PKA alone, since current evidence indicates that spore formation is also dependent on PKA activity (120). Moreover, there is good evidence that under these conditions ammonia influences the choice between spore and stalk pathways at the level of the precursor cell types since it stimulates prespore gene expression and inhibits prestalk gene expression (27, 247). Such a differential effect could be due to inhibition of either DIF-dependent gene expression or DIF accumulation, or both, and there is some evidence for both. Thus, on the one hand ammonia and another weak base, methylamine, have been reported to inhibit DIF-dependent gene expression selectively in the presence of added DIF-1 (275; however, see reference 15), whereas on the other hand, ammonia has been found to produce a marked reduction in DIF accumulation in monolayers incubated with cyclic AMP (72, 194).

Elevation of extracellular pH has the same effect on cell type choice in monolayers as do weak bases (51, 89, 218). This is probably an indirect result of increasing the concentration of the uncharged form of ammonia, although there is some evidence that extracellular pH can also affect the choice of differentiation pathway at very low cell densities, at which ammonia accumulation should be negligible (201). Since extracellular pH has no significant effect on intracellular pH (122, 264), this latter finding may reflect an alteration of membrane potential (270) or of some other parameter.

Models of Cell Type Choice

Models of cell differentiation in *D. discoideum* have been reviewed extensively (42, 155, 158, 180, 190, 191, 220, 254, 277, 281). Any satisfactory model must account for (i) the initial "salt-and-pepper" distribution of prestalk and prespore cells in aggregates, (ii) the maintenance of the prestalk-prespore pattern in aggregates once sorting has taken place, and (iii) the ability of isolated anterior and posterior segments to regulate after surgical interventions that selectively remove one cell type.

Current models for the initial specification of prestalk and prespore cells are all of a "lateral inhibition" type, in which the first cell or cells to adopt a given cell fate inhibit neighbors from doing the same (85, 181, 249). This similarity follows from the fact, emphasized by Cox (43), that "short-range activation and long-range inhibition are necessary and sufficient conditions for the emergence of stable periodic structures in space, regardless of the physical mechanisms underlying the model." The various models all include a central role for DIF-1, and it has been suggested that adenosine, generated by hydrolysis of cyclic AMP, also promotes prestalk differentiation (230, 235). The view that DIF-1 is a key player in cell type choice is probably justified, although there is intriguing evidence that cells that have opted to express either prestalk-enriched or prespore-specific genes can form at low cell density, at which DIF-1 levels are negligible (81). Models of cell type choice generally include a component that biases the mechanism of choice to accommodate the association between cell cycle phase at starvation and cell fate.

In the most fully analyzed of these models (180), DIF-1 is not the stalk cell inducer itself but the precursor of such an inducer and lateral inhibition is provided by the fact that DIF-1 is used up by the cells that differentiate into prestalk cells. However, there is currently no evidence that any DIF-1 breakdown product is an efficient inducer of the stalk pathway.

Insall et al. (112) have proposed that the cells that become prestalk cells in early aggregates are those that are most sensitive to DIF; these are induced to make DIF-1 dechlorinase, and this enzyme then depletes the aggregate of DIF so that other cells cannot become prestalk cells; prestalk cells then sort to the newly forming tip of the aggregate. These authors account for the continued stability of the prestalk cells in spite of the apparently low concentration of DIF-1 in the anterior region (31) by supposing that prestalk cells become sensitized to DIF by an autocatalytic mechanism.

Loomis (148, 149) suggests a scheme in which the cells that first become prespore cells make the lateral inhibitor, in this case DIF-1; when this reaches threshold, no further prespore formation takes place and the remaining cells become prestalk cells. There is some evidence that prespore cells do indeed

make an inhibitor of prestalk to prespore cell conversion (106, 240), though current evidence does not favor the idea that this inhibitor is DIF-1 (108). In addition, as mentioned, the results of Kwong et al. (141) do not support the idea that DIF-1 is a prespore cell product. This model, like that of Insall et al. (112), also seems to experience difficulty in accounting for the stability of the prestalk state in anterior cells.

In view of the evidence for differential inhibition of the stalk pathway by ammonia, it has been suggested that ammonia and intracellular pH are important elements in cell type choice (88, 90). On this view, ammonia would be the lateral inhibitor of prestalk cell formation (92, 278). The original salt-and-pepper distribution of prestalk and prespore cells could derive from an initial pH heterogeneity among developing cells that influences their ability to synthesize and/or respond to DIF-1; those cells that make most DIF-1, or are most responsive to it, would become prestalk cells, and they would produce a (relatively) long-range inhibitor in the form of the weak base ammonia. Following sorting, the weak acid/weak base gradient discussed in connection with culmination could ensure that posterior cells are inhibited by ammonia from responding to DIF-1, while anterior cells would be able to respond. Regulation of slug fragments could be accounted for, since when posterior fragments are isolated by surgery the anterior of such fragments would be the first to experience a drop in inhibitor concentration; activator would be synthesized and the cells would respond to DIF-1 by converting to prestalk cells and synthesizing DIF-dechlorinase (123). In isolated prestalk fragments, inhibitor would accumulate at the rear and prestalk gene expression would be shut off.

The fact that exposure of migrating slugs to a high concentration of DIF-1 results only in a small increase in the size of the prestalk zone is consistent with the notion of an inhibitor of prestalk cell formation in the prespore zone (124, 275). Moreover, the existence of inhibitory and activatory gradients is also indicated by tip transplantation experiments (132, 154, 191). Cells taken from various positions down the slug display an anterior-posterior gradation in their ability to organize a new tip when grafted onto the side of intact slugs (53, 154). Similarly, isolated posterior sections regenerate a tip faster the more anterior the position of the cut (144). There is also an anterior-to-posterior gradient in tip-inhibiting ability shown by transplanting tips to different positions down the length of intact slugs (53, 145, 154).

Some suggestion that ammonia may actually influence cell type choice in intact aggregates comes from two sources. Durston and Vork (54) and MacWilliams and coworkers (17, 154, 157–159) have examined several mutants with reduced prestalk zones, including slugger mutants belonging to the slugger D and E complementation groups that are known to be hypersensitive to the effects of ammonia (73, 196, 237). They found that the formation of a reduced prestalk zone in mutant slugs is accompanied by a marked preference on the part of the mutant cells to form prespore cells when mixed with wild-type amoebae. Furthermore, in transplantation experiments these mutants were poor both in forming tips and in preventing wild-type transplants from doing so; in both respects, therefore, they behave as though they are hypersensitive to tip inhibition and have abnormally low levels of tip inhibitor. If one assumes that tip formation and prestalk cell differentiation are closely related, the fact that these mutants are hypersensitive to ammonia supports a role for ammonia as a rapidly diffusing inhibitor of prestalk cell differentiation. However, although slugs formed by these mutants have a reduced prestalk zone they have a roughly normal proportion of anterior-like cells (17, 159). This has led to the suggestion that cell type proportions are regulated by two distinct feedback loops, one controlling the prespore to ALC transition, the other controlling the ALC to prestalk transition, and that the slugger mutants are hypersensitive to the latter (17, 159). The other evidence that ammonia can influence cell type choice is work of Feit et al. (61), who showed that incubation of slugs in an atmosphere of ammonia for a matter of minutes causes a reduction in the size of the prestalk zone, as defined by neutral red staining. The reduction in the number of cells in the prestalk zone could be accounted for by an increase in the number of ALC in the prespore zone. These workers therefore suggest that ammonia is the substance that inhibits chemotaxis of ALC to the anterior zone. In agreement with this, exposure to ammonia is indeed reported to prevent sorting of ALC to form a new prestalk zone (60).

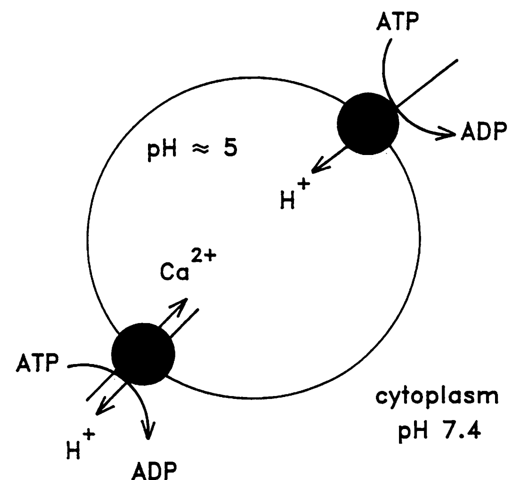


FIG. 7. Model of a calcium-sequestering acidic vesicle. The vesicle is acidified by a V-type H^+-ATPase to which is coupled a Ca^{2+}-ATPase, which functions as an ATP-dependent proton/calcium exchanger. Ca^{2+} sequestration is facilitated if the vesicle is acidic (219). Diagram courtesy of E. Rooney.

Improved understanding of the mechanism of DIF-1 synthesis and of the influence of ammonia on cell type choice may help distinguish between the various rival models or suggest new ones.

WEAK BASES AND CALCIUM FLUXES

In view of the evidence that the profound effects of ammonia on development result from raising the pH of an intracellular acidic compartment, it becomes of interest to enquire how processes taking place in an acidic compartment might influence events in the cytosol and nucleus. In many cell types the low pH of acidic compartments is important for recycling membrane proteins and for proteolytic processing and targeting of newly synthesized membrane proteins. It is also required for symport and antiport of various substances (182). In yeast and *Neurospora* cells, as well as in plant cells, Ca^{2+}/H^+ antiporters located in acidic vacuoles contribute to calcium homeostasis (130, 199). These antiporters are vanadate insensitive since they are exchangers whose calcium-sequestering activity is energized by the outward proton gradient generated by the vacuolar H^+-ATPase.

In *D. discoideum*, acidic compartments may play a role in the processing of lysosomal enzymes but they do not appear to be important in the proper targeting of precursor peptides (38). Moreover, one might expect a considerable delay before any

such effect could influence adenylyl cyclase activation, whereas ammonia exerts its effect on relay within 1 min of addition (280). A plausible mechanism by which alteration of the pH of an intracellular compartment might rapidly influence processes at the plasma membrane (such as adenylyl cyclase activation) involves influencing ionic movements. Binding of cyclic AMP to cell surface receptors is thought to lead to movement of several ionic species, any of which might mediate the action of weak bases. There is a rapid release of Ca^{2+} from stores (2, 59, 225) and prolonged Ca^{2+} influx from the extracellular medium (36, 278). There is also a rapid elevation of cytosolic pH, probably as a result of activation of the plasma membrane proton pump (7), and an efflux of potassium ions (5).

Ca^{2+} Sequestration

As mentioned above, one ionic species that might be responsible for the effects of the pH of an intracellular compartment on cellular processes is Ca^{2+} (2, 285). With this in mind, I have suggested previously that there might exist a calcium-sequestering activity that is localized in such an intracellular acidic compartment and that is stimulated by the pH gradient across the compartment boundary (90). According to this idea, dissipation of the gradient by a weak base would reduce sequestration activity and lead to larger and more prolonged Ca^{2+} transients in response to cyclic AMP stimulation. This could lead to less efficient cyclic AMP relay if the latter process was inhibited by Ca^{2+}.

It is clear now that one or more efficient calcium sequestration mechanisms do exist in *D. discoideum* and that a major component is indeed influenced by the pH of the compartment in which it is located. Efficient sequestration is indicated by the fact that the calcium influx induced by cyclic AMP binding leads to a barely detectable elevation in the level of cytosolic Ca^{2+} (see below) whereas it has been calculated that it would raise cytosolic Ca^{2+} to micromolar levels if it were not sequestered (36, 285).

Recently Rooney and Gross (219) demonstrated high-affinity ATP-dependent Ca^{2+} uptake both in whole-cell lysates and in a light acidic vesicle fraction (acidosomes) and showed that it was partially dependent on the presence of a transmembrane pH gradient, acid inside. We inferred that the membrane of the acidic vesicles contains an ATP-dependent Ca^{2+}/H^+ exchanger coupled to an ATP-dependent proton pump (Fig. 7) and that this activity makes a major contribution to cellular sequestration mechanisms. Milne and Coukell (184, 185) have demonstrated a similar activity in whole-cell lysates and in filipin-permeabilized cells. The Ca^{2+} that was sequestered was not released by caffeine or inositol-1,4,5-triphosphate, and the activity was unchanged during the first 15 h of development (184). However, these workers did not detect inhibition of Ca^{2+} uptake by protonophores (185).

It is probable that the compartment responsible for Ca^{2+} sequestration is the contractile vacuole, rather than an endosomal compartment, since current evidence indicates that the V-type H^+-ATPase-rich vesicles in the light-vesicle fraction are contractile vacuoles (99). These organelles have also been shown to contain a high concentration of calmodulin (289). Taken together, these results suggest that contractile vacuoles play a significant role in calcium homeostasis (290).

In addition to the "constitutive" Ca^{2+} sequestration mechanism just described, an interesting sequestration activity stimulated by binding of cyclic AMP to cell surface receptors has been found in permeabilized cells (66, 67). This activity, unlike the constitutive activity, appears to be vanadate and bafilomycin insensitive. However, it is inhibited by NBD-Cl, a finding that has been interpreted as pointing to the involvement of a V-type ATPase (67).

Role of Ca^{2+} in Signaling

Although a Ca^{2+}-sequestering activity exists that is influenced by the pH of acidic compartments, the evidence that weak-base effects on signaling involve Ca^{2+} levels is indirect. It has been shown that the amplitude of light-scattering oscillations is increased by ammonia (241) and decreased by ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) (76) and that exposure to ammonia speeds cell movement (22, 24–26, 62, 270). Such effects could reflect changes in cytosolic Ca^{2+} levels. The idea that cytosolic Ca^{2+} interferes with activation of adenylyl cyclase also receives support from the fact that the Ca^{2+} ionophore A23187 inhibits cyclic AMP relay (28) and the observation that sudden addition of Ca^{2+} to cells incubated in a medium lacking Ca^{2+} abolishes cyclic AMP oscillations (76). Moreover, a reduction in signal frequency, conceivably as a result of less efficient adenylyl cyclase activation, has been observed in the presence of a membrane-permeable analog of cGMP that appears to stimulate Ca^{2+} influx across the plasma membrane (67).

Also consistent with a role of intracellular Ca^{2+} in inhibiting adenylyl cyclase activation are the findings that the presence of Ca^{2+} in the medium can reduce the efficiency of cyclic AMP-induced adenylyl cyclase activation (271) and that exogenous Ca^{2+} can delay culmination (221). These effects saturate at extracellular Ca^{2+} concentrations that give rise to maximal rates of Ca^{2+} influx (5, 36) and hence could be due to elevation of cytosolic Ca^{2+}. Precedent for such an effect of cytosolic Ca^{2+} seems to be provided by the evidence that Ca^{2+} influx is responsible for reducing the activation of plasma membrane proton pump activity and of K^+ efflux in response to cyclic AMP stimulation (5, 165).

However, in evaluating these findings it should be noted that aggregation can proceed in the absence of exogenous Ca^{2+} (58, 221). Furthermore, Van Haastert reports that inhibition of cyclic AMP relay by exogenous Ca^{2+} is not prevented by ruthenium red or by lanthanum, both of which might be expected to prevent calcium influx (271). He therefore suggests that extracellular Ca^{2+} may interfere with cyclic AMP relay by an action at the cell surface. It should now be possible by the use of aequorin (225) to test whether weak bases do raise cytosolic Ca^{2+} levels and whether inhibition of the activity of Ca^{2+}-sequestering enzymes has the same effect on cyclic AMP-induced adenylyl cyclase activation as weak bases have.

Cytosolic Ca^{2+} Levels and Gene Expression

As already described, there is evidence that ammonia and other weak bases influence cell type choice, at least during cell differentiation in cell monolayers. It has been suggested that elevation of cytosolic Ca^{2+} levels by weak bases could account for this effect if prespore gene expression were selectively stimulated by such an elevation (90). Until recently it was unclear whether any elevation of cytosolic Ca^{2+} levels at all occurred in response to cyclic AMP stimulation. Abe et al. (2) reported a small Ca^{2+} transient in a proportion of aggregation-competent cells that had been loaded with fluorescent probes by electroporation. Others found that electroporation in the absence of extracellular Ca^{2+} damages cells, and they did not detect any elevation of cytosolic Ca^{2+} in response to exogenous cyclic AMP in cells scrape-loaded with fura-dextran (238). However, an important recent study involving cells loaded with the calcium-sensitive photoprotein aequorin (225)

supports the evidence for small cytosolic Ca^{2+} transients in response to cyclic AMP. Like Abe and Maeda (1), these researchers also observed that the resting level of cytosolic Ca^{2+} is somewhat higher in prestalk than in prespore cells and they detected larger and more prolonged Ca^{2+} transients in prestalk cells. This finding is not in agreement with the idea that the choice between prestalk and prespore pathways of gene expression is dictated simply by cytosolic Ca^{2+} levels, with higher levels giving rise to prespore gene expression (90, 261). However, it does not exclude the possibility that elevation of cytosolic Ca^{2+} concentration by ammonia could be one of the elements that promotes prespore cell differentiation, perhaps by inhibiting DIF production.

Although there is good evidence for a role of Ca^{2+}-dependent processes in sexual cell fusion in *D. discoideum* (152), evidence for their role in gene expression during development is fragmentary. Schaap et al. (232) examined the effect of a number of putative calcium antagonists on prespore gene expression in cell suspensions. They found, for example, that the calcium channel blocker TMB-8, which is believed to prevent mobilization of Ca^{2+} from intracellular stores, inhibited prespore gene expression, as did the calmodulin antagonist trifluoperazine. Blumberg et al. (18) also observed inhibition of prespore gene expression by TMB-8 as well as by another calmodulin antagonist, W7. However, in neither study were the effects of the various agents on DIF-dependent prestalk gene expression examined. In addition to these studies, a suggestion that elevation of the cytosolic Ca^{2+} level is involved in postaggregative gene expression comes from the observation that a cyclic AMP analog that does not stimulate inositol-1,4,5-triphosphate production is unable to activate transcription of two postaggregative genes (208). In this area, also, a clearer understanding of the role played by cytosolic Ca^{2+} in gene expression will be gained when methods of manipulating Ca^{2+}-sequestering enzymes are available.

CONCLUSIONS AND PROSPECT

I have focused on two main decisions faced during the development of *D. discoideum*: the choice between slug migration and fruiting, and the choice between prestalk and prespore pathways. One can be fairly confident that the former choice is controlled by the ambient level of ammonia and that the ammonia interferes with the activation of cyclic AMP-dependent PKA. It seems clear, moreover, that culmination is blocked when PKA activity is below some threshold level. There is also good reason to believe that ammonia exerts its effect by raising the pH of an acidic intracellular compartment. Future research should reveal whether the link between acidic vesicles and PKA activity is via effects of cytosolic Ca^{2+} on adenylyl cyclase activation. It should also help us to understand how protein phosphorylation by PKA brings about the dramatic transformation of prestalk and prespore cells into the mature cell types and which other components are involved.

Progress in this area will depend on further understanding of the mechanism linking cell surface binding of cyclic AMP to activation of adenylyl cyclase. This and all other aspects of the developmental process in *D. discoideum* promise to be advanced by the application of the procedure of restriction enzyme-mediated integration (138). In this procedure insertional mutants are generated by transformation with a restricted plasmid in the presence of the corresponding restriction enzyme. This enzyme appears to make cuts in the host DNA into which the plasmid can be inserted, and the hybrid structure is then resealed. Sequences flanking the insertion site can be recovered, and the mutated gene can be identified and studied. In this way it is expected that many genes that play important roles in development will be identified.

The mechanisms underlying cell type choice remain more obscure. We know that cells at the time of starvation already have a quite strong tendency to differentiate into one or the other of the two major cell types and that this depends in some unknown way on the stage of the cell cycle at the moment of starvation. We know also that prestalk cells and prespore cells are randomly interspersed within aggregates and that the spatial pattern of the two cell types arises by sorting out of prestalk from prespore cells. The stalk-cell-inducing factor DIF-1 clearly plays a central role in the stalk-versus-spore choice, and some kind of lateral inhibition mechanism presumably ensures that aggregates contain the appropriate ratio of cell types. Ammonia probably participates in the mechanism of cell type choice; it seems to inhibit DIF production and the expression of DIF-dependent genes and may be the substance responsible for lateral inhibition.

The mechanism of cell type choice will become clearer when more is understood of the pathways of signal transduction from cell surface receptors to gene expression. As pointed out by Loomis (149), it should also be facilitated by insights into the mechanism of synthesis and mode of action of DIF-1 that may be anticipated from the isolation and analysis of mutants obtained by restriction enzyme-mediated integration.

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