The Ca\textsuperscript{2+} - CaM-Dependent Phosphoprotein Phosphatase Calcineurin: Its Role in Chemotaxis of Axenic Cells and Analysis of Possible Substrates in \textit{Dictyostelium discoideum}

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science Graduate Department of Zoology University of Toronto

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The Ca\textsuperscript{2+}/CaM-Dependent Phosphoprotein Phosphatase Calcineurin: Its Role in Chemotaxis of Axenic Cells and Analysis of Possible Substrates in \textit{Dictyostelium discoideum}.


Abstract

Characteristics affecting the chemotactic response were examined in two strains of axenic amoebae derived from wild-type NC-4, the classic axenic strain AX-3 and JH10, a thymidine auxotroph derived from AX-3. Parameters leading to a maximal response to the two major chemoattractants, cAMP and folic acid (FA) were determined. Using the radial bioassay, both AX-3 and JH10 amoebae exhibited maximal chemotaxis to cAMP when harvested in mid-log growth (5-9 \times 10^6 cells/mL for AX-3, 7-12 \times 10^6 cells/mL for JH10), starved for 2 hours and exposed to 1 \mu M extracellular cAMP. Chemotaxis to FA was optimal in unstarved amoebae and declined sharply as starvation time increased. Migration rate was found to peak at 40 \mu M FA. The role of the Ca\textsuperscript{2+}/CaM dependent phosphatase calcineurin (CN) during chemotaxis to both cAMP and FA was investigated pharmacologically by use of the specific CN inhibitor deltamethrin. Permethrin, similar in structure to deltamethrin but non-inhibitory to CN was employed as a negative control.

Chemotactic mobility to cAMP was not significantly affected by deltamethrin or permethrin. Conversely, chemotaxis to FA was increased by 82% in the presence of deltamethrin while permethrin showed no significant effects. Cell extracts of amoebae chemotactically competent to cAMP were immunoblotted with antibodies against phosphoserine in an attempt to identify possible substrates of CN. Sixteen proteins ranging in apparent molecular weight between 42.6-185.6 kD were identified based on increased levels of phosphoserine incorporation in the presence of deltamethrin. These data are discussed in relation to current knowledge and ongoing investigations.
Acknowledgements

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List of Abbreviations

ABP - actin-binding protein
AC - adenyl cyclase
ADF - 19 kDa actin depolymerizing factor
CaM - calmodulin
cAMP - cyclic 3', 5' adenosine monophosphate
cAR - cAMP cell surface receptor
CBP - CREB-binding protein
cGMP - cyclic 3',5' guanosine monophosphate
CMF - conditioned media factor
CRAC - cytosolic regulator of adenylyl cyclase
CRE - cAMP-responsive element
CREB - cAMP-responsive element binding protein
CysA - cyclosporin A
CyP - cyclophilin
DAG - 1,2-sn-diacyglycerol
DEL - deltamethrin
ePD - extracellular cyclic nucleotide phosphodiesterase
ERK2 - mitogen-activated kinase 2
FA - folic acid
FAR - folate cell surface receptor
FD - folate deaminase
FBP - folic acid binding protein
FK506 - tacrolimus
FKBP12 - 12 kD FK-binding protein
GC - guanylyl cyclase
GTP - guanosine triphosphate
IP$_3$ - inositol trisphosphate
kD - kilodaltons
IPD - late aggregation specific phosphodiesterase
mPD - membrane-bound phosphodiesterase
PIP - phosphoinositide phosphate
PIP$_2$ - phosphoinositide bisphosphate
PIP$_3$ - phosphoinositide trisphosphate
PKA/cAPK - cAMP-dependent protein kinase
PKC - protein kinase C
PLC - phosphoinositide-specific phospholipase C
PP1 - protein phosphatase 1
PP2A - protein phosphatase 2A
PP2B or CN - calcineurin
PP2C - Mg$^{2+}$-dependent protein phosphatase
PSF - prestarvation factor
PTP1B - protein tyrosine phosphatase 1B
PVDF - polyvinylidine difluoridine
R.I. - relative intensity
R$_f$ - relative mobility
SDS - sodium dodecyl sulphate
TCA - trichloroacetic acid
TEMED - N, N, N', N'-tetramethyl ethylenediamine
Introduction

1) The Asexual Life Cycle of Dictyostelium discoideum

Amoebae of the genus *Dictyostelium* belong to the family Acrosporomycetes and live in decaying leaf matter and soil detritus of deciduous forests where they multiply vegetatively by phagocytosing bacteria (Bonner, 1967). Due to the haploid nature of the organism and the simplicity of maintaining populations in the laboratory, *Dictyostelium* has become a popular model system for the study of several processes, including cell-cell adhesion, differentiation and chemotaxis (for review see Spudich, 1987; Kimmel, 1988). The asexual cycle is the most intensively studied phase of the life cycle. Upon conditions of starvation (i.e. depletion of the bacteria) and in the presence of light a sequence of characterized, synchronous events begins (Sussman, 1987; Fig. 1). Within a population of amoebae certain cells begin to secrete cAMP in a pulsatile manner (Gerisch, 1987). Contact of cAMP with membrane-bound receptors of surrounding cells lead to up-regulation of transcription of several genes involved in signalling, including an additional class of cAMP receptor, adhesion molecules, cyclic nucleotide phosphodiesterases and their inhibitors, actin-binding proteins and other proteins involved in the transduction of the extracellular signal intracellularly (Gerisch, 1987; Kessin, 1988). Notable among proteins activated by cAMP is a membrane-bound adenylyl cyclase, leading to the synthesis and secretion of more cAMP by the stimulated cells. In this way the initial signal is propagated as an extracellular wave (Gerisch, 1987). Amoebae exhibit positive chemotaxis up the gradient of cAMP initially forming concentric rings of migrating cells. As development progresses certain adhesion molecules are expressed (e.g. contact sites A; Gerisch, 1987). Migration patterns then change to streams of mutually cohesive amoebae (Siu et al., 1988). Cell differentiation and the spatial distribution of cell types within the early aggregate are regulated by cell-cell contacts and diffusible factors secreted by the
cells themselves. Additional factors, such as cell cycle phase at time of aggregation, also
determine the fate of individual amoebae within the aggregate (Janssens and van Haastert,
1988; Sharpe and Watts, 1985). Formation of a tip in the aggregate is associated with the
beginning of postaggregative development. By the time chemotaxis is complete up to \(10^5\)
cells have aggregated to form a multicellular aggregate which appears as a mound with a
distinct tip. Within the mound cAMP regulates development and terminal differentiation
via the action of the cAMP-dependent kinase, cAPK (reviewed in Williams et al., 1993).
Differentiation within the mound leads to the formation of the pseudoplasmodium which
can undergo a migration phase. Morphogenetic events within the pseudoplasmodium
result in the formation of at least four different cell types (Gerisch, 1987): prestalk cells,
which comprise approximately twenty percent of the slug (Kessin, 1988) located in the
anterior of the slug and rich in acidic lysosomes (Sternfeld and David, 1981, 1982); and
prespore cells, which express high levels of UDP galactose polysaccharide transferase and
accumulate spore coat material in preparation for spore coat production (Devine et al.,
1983) are the two predominant cell types. In addition there exist at least two other types
of cells in the slug. Anterior-like cells are characterized by their similarity to prestalk cells
but are located in the prespore region of the aggregate (Sternfeld and David, 1981, 1982)
and show lower levels of prestalk-specific proteins. Additionally, a protein in high
abundance in prespore cells, PSP59 is also expressed in these anterior-like cells (Devine
and Loomis, 1985). A fourth type of cells has been identified in the slug, those which are
labelled by neither prestalk-specific antibodies nor prespore-specific antibodies (Gomer et
al., 1986). Following the brief migratory period and under continued conditions of
starvation in the presence of overhead light amoebae undergo culmination, a further
process of morphogenesis (Fig. 1). At the apex of the culmination process a tube of
cellulose is produced within the slug by central prestalk cells. The remaining prestalk cells
migrate chemotactically through this tube.
Figure 1. Asexual life cycle of *Dictyostelium discoideum*. Electron micrograph courtesy of M.J. Grimson and R.L. Blanton, Texas Tech University, Lubbock, TX.
(Williams et al., 1993) terminally differentiate into stalk cells and die (Kessin, 1988). Prespore cells differentiate into a spore mass surrounded by a slime sheath to protect against dehydration. A basal disc derived from anterior-like cells of the prespore region forms at the base of the stalk and functions in support of the structure (Gerisch, 1987).

Axenic culturing of *D. discoideum* originated over forty years ago; however cell yields and growth rate were poor and a complex liquid medium containing an undefined bacterial component was required, making this method unattractive for studies of development and differentiation at the cellular level (Sussman and Sussman, 1967). Through serial subculture of NC-4, a strain capable of growth in liquid media without bacteria as the food source was obtained (Sussman and Sussman, 1967). In the presence of Wilson's Liver concentrate and fetal calf serum a stationary phase yield of $1 \times 10^7$ cells/mL, corresponding to that of the wild type in the presence of bacteria was obtained (Sussman and Sussman, 1967). This strain was named AX-1. A second axenic strain, AX-2, was obtained through serial subculture of AX-1 in the absence of both fetal calf serum and liver concentrate (Watts and Ashworth, 1970). The mutant strain AX-3 was raised independently of the prior two axenic strains. AX-3 was selected for rapid growth in Sussman's complex media following treatment of haploid NC-4 cells with N-methyl-N'-nitro-N-nitrosoguanidine (Loomis, 1971). Today AX-3 is the most commonly used axenic strain of *Dictyostelium*.

Chemotaxis in higher eukaryotes is seen in immune responses to both tissue injury/death and to bacterial invasion. Macrophages track invading bacteria by chemotactic migration up gradients of peptides released as waste products by the bacteria (Dexter and Spooncer, 1987). Similarly, neutrophils chemotactically migrate toward wound sites along extracellular fibronectin and vitronectin trails found in connective tissue stroma and at sites of tissue injury (Hendey et al., 1992). Due to the ease in obtaining large quantities of material rapidly and the relative simplicity of genetic study, *Dictyostelium discoideum* is the best characterized model chemotactic system and several
parallels have been shown in the cell motility systems employed in these different organisms.

II) Chemotaxis in Dictyostelium discoideum

Chemotaxis is the directed movement of a cell in response to an extracellular gradient of chemoattractant. In the cellular slime mould Dictyostelium discoideum this process is essential in many stages of its life cycle. Free-living vegetative amoebeae are chemotactic to folic acid and pterine compounds excreted as waste by the bacteria on which the amoebeae feed (Pan, 1972). In response to conditions of starvation, amoebeae aggregate by chemotaxis toward 3'-5' cyclic adenosine monophosphate (cAMP) secreted by the amoebeae themselves (Konijn et al., 1967). Chemotactic cell movement has also been suggested to play an important role in patterning and cell-type differentiation post-aggregatively (Mutzel et al., 1987; Williams et al., 1993). While folic acid and cAMP are the two major chemotactic compounds in Dictyostelium discoideum, other chemoattractants have been identified in other species (van Haastert, 1994; Sordano et al., 1993; Jalink et al., 1993). Four separate aggregative chemotactic compounds are currently known: cAMP for Dictyostelium discoideum and three related species (Konijn et al., 1967), a dipeptide called glorin produced by Polysphondylium violaceum (Shimomura et al., 1982), a pterin derivative secreted by D. lacteum (van Haastert et al., 1982) and a folate derivative found in D. minutum (DeWit and Konijn, 1983). Focus here will be primarily on chemotaxis by Dictyostelium discoideum to cAMP, with some comparative comments on chemotaxis to folic acid.

Chemotactic movement has been identified in several stages of the Dictyostelium life cycle (Fig. 1). Chemotactic movement is important in pre-aggregative stages as well as post-aggregatively in cell sorting and terminal differentiation (Williams et al., 1993) and evidence is accumulating that both cAMP and folic acid are chemoattractants in the post-
aggregative structure (Williams et al., 1993; Hadwiger et al., 1994). The behavioural response to an extracellular gradient has been intensively studied. Upon initial detection of an extracellular gradient of cAMP, cells initially exhibit the "cringe" response (Futrelle et al., 1982). Pseudopods are retracted and contraction of myosin leads to a rounded immotile amoeba. The "cringe" persists for 25-30s, after which polymerization of actin and its association with the cytoskeleton result in extension of pseudopods in the direction of the increasing gradient (McRobbie, 1986; Condeelis et al., 1992). During this period of immobility amoebae orient the migratory machinery in the direction of the gradient and then migrate in the direction of increasing chemoattractant (Wessels et al., 1992; Chandrasekhar et al., 1995). Post-orientation, all characteristics of migration are wave-mediated; the larger the wave, the greater the speed of migration (Wessels et al., 1992; Chandrasekhar et al., 1992). Further evidence for the importance of the extracellular signal in continued regulation of the chemotactic response comes from work showing mutant cells when transferred to a population of migrating normal cells can exhibit migration normal in all aspects, while the reverse is true for normal cells transferred to a population of aberrantly-migrating amoebae (Chandrasekhar et al., 1995). Motility once begun is essentially blind, remaining unidirectional for approximately 120-150 seconds (Wessels et al., 1992). An adaptive mechanism results in decreased cell velocity until at the peak of extracellular chemoattractant no net movement is observed (van Haastert, 1994). During this "rebound" period (Wessels et al., 1992) cells extend small pseudopods randomly in all directions and show no net movement, preventing migration down the "back" of the chemoattractant wave and away from aggregation centres (Wessels et al., 1992). Whether sensing of the gradient is spatial, temporal or spatiotemporal is a question not yet fully answered (Vicker, 1994).
III) Chemotactic Signal Transduction in Dictyostelium discoideum

Transmembrane signal transduction is characterized largely by the components of the signalling system: ligand, cell-surface receptor, intracellular membrane-bound GTP-binding proteins and associated effector enzymes (Bominaar et al., 1991). In Dictyostelium discoideum, signal transduction has been extensively studied. While the ligands and their corresponding receptors may be different, the overall mechanism appears to be very similar in higher eukaryotes (Janssens and van Haastert, 1987). The extracellular ligand binds to the cell-surface receptor, inducing a conformational change in the receptor. This results in dissociation of the heterotrimeric G-protein, which itself splits into two cytosolic effector polypeptides: the GTP-bound α-subunit and the βγ heterodimer. Both subunits are capable of activating membrane-bound or cytosolic effector proteins.

i) Signal regulation and extracellular gradient formation

Inherent in the process of directed cell movement up a gradient is establishment of a gradient. Mechanisms for gradient production have evolved in Dictyostelium discoideum for chemotaxis to either cAMP or folic acid. In both cases, signal-regulating enzymes exist as both extracellular and membrane-bound enzymes (Orlow et al., 1981; van Ophem and van Driel, 1985) and these isoforms are responsible for performing different functions at separate stages in the developmental process.

Three developmentally-regulated isozymes have been identified in Dictyostelium, products of a single PD gene. Expression of the gene is regulated by three distinct promoters activated at different life stages (Faure et al., 1990). It is likely that post-translational modification and/or compartmentalization, in addition to temporal expression result in differential substrate affinities displayed by these enzymes (Dicou and Brachet,
The cAMP signal is degraded by an excreted cyclic nucleotide phosphodiesterase (abbreviated as ePD). ePD is expressed and is present in low levels, presumably to maintain low basal levels of cAMP and prevent precocious initiation of the aggregation development program (Franke and Kessin, 1981, 1992). An increase in extracellular cAMP occurs during starvation. cAMP in the extracellular medium leads to increased expression of the aggregation-stage, membrane-bound PD (mPD) and concomitant decrease in activity of the ePD (Pannbacker and Bravard, 1972; Franke and Kessin, 1992). At the same time, levels of the glycoprotein inhibitor, PDI are inhibited in the presence of cAMP (Faure et al., 1990). The third *Dictyostelium* PD is expressed 10-12 hours post-starvation and only in prestalk cells (Rutherford and Brown, 1983; Franke and Kessin, 1992). Initial expression of ePD is independent of cAMP, Gα2, Gβ and AC (Faure et al., 1990) but under conditions leading to aggregation becomes cAMP-dependent, exhibiting a two-stage mechanism of regulation at the gene level (Wu et al., 1995b). Beyond translation, several additional levels of regulation affect localization and substrate affinity of the enzyme. For example, the PDI inhibits only ePD and has no effect at physiological concentrations on purified mPD (Dicou and Brachet, 1980; Orlow et al., 1981) or crude membrane preparations of mPD (Malchow et al., 1972). cAPK-regulated expression of PDI is cAMP-independent, since it is unaffected in *aca* cells (Wu et al., 1995b). Increased ePD activity upon cAMP stimulation can be partially attributed to the inhibitory effects of cAMP on PDI (van Ophem and van Driel, 1985). Pulsatile exposure of vegetative amoebae to folic acid can result in increased ePD activity (Malchow et al., 1972), and a candidate for the specific induction of ePD/PDI expression is Ca\(^2^+\) (Rutherford and Brown, 1983; Coukell and Cameron, 1988; Wu et al., 1995b) since levels of PD activity closely correspond to levels of G-protein independent Ca\(^2^+\) influx (Caterina et al., 1994; Milne et al., 1995). These isoforms are independently regulated (van Ophem and van Driel, 1985; Wu et al., 1995a) and the membrane-bound form is regulated in parallel with the discoidin adhesion sites and cAMP-binding receptors (Janssens and van...
proteins whose resolution is suspected to be under the control of cAPK (Firtel and Chapman, 1990; Williams et al., 1993). Certain workers have reported data that suggests possible multimerization of the PD catalytic enzyme in response to varying environmental conditions (Orlow et al., 1981; Franke and Kessin, 1981). The ePD has been reported in both low and high-molecular weight forms. The low-molecular weight enzyme separates into four distinct bands by two-dimensional electrophoresis differing in degrees of glycosylation or carboxy-terminal processing (Franke and Kessin, 1992). Candidate sites for posttranslational modifications are in the regions between the stage-specific promoter and the coding region for the enzyme, as all three enzymes are derived from mRNAs of differing lengths (Faure et al., 1990) and all three possess the same 3' UTR. The high-molecular weight form of the ePD, between 150-200 000 M_r, is a complex of several proteins including the PD catalytic peptide and several other proteins of as yet unknown function (Franke and Kessin, 1992). Mammalian isoforms of cNPD have been reported to be Ca^{2+}/CaM regulated (Sharma and Kalra, 1994), although whether the enzyme is directly bound by Ca^{2+}/CaM or indirectly stimulated by cAPK is currently unknown. While the peptide comprising the catalytic activity of the Dictyostelium PD is not thought to possess CaM-binding or other Ca^{2+}-regulated regions (Franke and Kessin, 1992) the members of the multiprotein high molecular weight ePD complex are currently unidentified, so regulation by Ca^{2+} cannot be ruled out. The possibility of similar regulation in Dictyostelium exists, and would suggest another cross-talk point in the cAMP signalling and Ca^{2+} signalling pathways.

A number of similarities exist between the folic acid-degrading and the cAMP-degrading systems employed by Dictyostelium. Both cNPD and folic acid deaminase (FD) exist in extracellular and membrane-bound forms (Greiner et al., 1992). The activity of both extracellular forms increases in response to cAMP and folic acid when applied continuously or in a pulsatile manner (Malchow et al., 1972; Bernstein et al., 1981; Orlow et al., 1981). FD has been identified as a large glycoprotein, running as a 138 kDa band
on SDS-PAGE in either extracellular or membrane-bound cell preparations (Greiner et al., 1992). Additionally, it has been postulated that both the membrane-bound FD activity and the binding sites for the folate-mediated chemotactic response are resident on the same protein (Greiner et al., 1992) while these activities are performed by separate proteins in response to cAMP (Klein et al., 1988; Franke and Kessin, 1992). No glycoprotein inhibitor analogous to that identified for extracellular phosphodiesterase (Franke and Kessin, 1981) has been identified for FD.

ii) The cAMP-binding receptors

CAMP acts as both a first messenger in inducing chemotaxis and as a second messenger in the developmental response to conditions of starvation. This dual role of cAMP leads to a high level of complexity in its signalling pathways, beginning with the cell-surface receptor. The cAMP receptor spans the membrane seven times, characteristic of a large class of GTPase binding receptors (Klein et al., 1988). Four different isoforms of the cAMP receptor (cAR) are presently known in *Dictyostelium discoideum* (Chen et al., 1996). Expression of these receptors at both the mRNA level and the protein level, is tightly regulated and occurs at different stages of the developmental programme (Louis et al., 1994; Devreotes, 1994; Yu and Saxe, 1996). All four receptors can exist in different conformations, R and D, that run with different apparent molecular weights on SDS-PAGE gels dependent on phosphorylation state of the receptor (Devreotes and Sherring, 1985; Klein et al., 1985; Theibert et al., 1984). cAR1 is the earliest expressed and highest affinity receptor and cells without a functional cAR1 will not enter the developmental programme, instead remaining as individual amoebae (Klein et al., 1988; Johnson et al., 1992). cAR3 is expressed in the unicellular stage before aggregation commences (Saxe et al., 1991; Johnson et al., 1993) and cannot be detected on cAR2-expressing prestalk cells once aggregation is underway (Yu and Saxe, 1996). Formation of the multicellular
pseudoplasmodium and subsequent differentiation of prestalk cells leads to expression of cAR2 after 15 hours (Saxe et al., 1991, 1993) and cAR4 at a later stage in these cells (Yu and Saxe, 1996; Louis et al., 1994). While cAR1, cAR2 and cAR3 are all individually capable of producing all cAMP-mediated responses, dose-response curves vary by receptor. Differential receptor expression therefore seems to be designed to switch cellular sensitivity and cell type response to extracellular signals (Chen et al., 1996). An additional level of regulation of receptor response to the extracellular signal is present in the shifting between kinetic forms of the receptor (van Haastert, 1994). Cell-surface cAMP-binding sites are heterogeneous (van Haastert and De Wit, 1984) showing A sites coupled to adenylyl cyclase and involved in cAMP-mediated cAMP release and B sites coupled to guanylyl cyclase and involved in production of cGMP (van Haastert et al., 1986). B sites are further subdivided into three classes: B\textsuperscript{f}, with a cAMP release rate of 2.5s, which is rapidly converted to B\textsuperscript{s}. Conversion of B\textsuperscript{s} to B\textsuperscript{r} is G-protein mediated (van Haastert et al., 1986) and is the key step in activation of the downstream G-proteins, whereas inhibition of this conversion is involved in desensitization of the receptor (van Haastert et al., 1986).

Activation of the receptor also lead to the initiation of events responsible for down-regulation of the receptor-mediated signal (van Haastert et al., 1992). Several components of a receptor down-regulation mechanism have been proposed: receptor phosphorylation, loss of ligand binding without loss of protein, redistribution and/or degradation of the receptor and decreased levels of receptor protein mRNA (van Haastert et al., 1992). Experiments have shown that activation of adenylyl cyclase and intracellular activation of cAPK are required for final degradation of the receptor, but not for receptor phosphorylation, loss of ligand binding or loss of cAR1 mRNA (van Haastert, 1994).
iii) The folate-binding receptors (FARs)

Vegetative amoebae are sensitive to extracellular gradients of folic acid, secreted by the bacteria on which they feed as a waste product (Bonner et al., 1970; Pan et al., 1972). As aggregation competence is gained (i.e. under conditions of starvation) folate sensitivity decreases, concurrent with disappearance of transcripts of Ga4, the Gα subunit coupled to folic acid receptors (Hadwiger et al., 1991; Hadwiger and Firtel, 1992; Hadwiger et al., 1994). It has been postulated that receptor-mediated sensitivity to folate returns in the multicellular forms of Dictyostelium in regions of the slug corresponding to anterior-like cells (Tillinghast and Newell, 1984; Hadwiger et al., 1994). A second class of folate receptors was identified in these cells identifying a previously unsuspected and presently uncharacterized role for folates in mound-stage morphogenesis and cell differentiation (Tillinghast and Newell, 1984).

A number of similarities exist between the folic acid cell-surface receptor and the cAMP receptor. Folate binds to two receptor types on the cell surface, A and B types (de Wit and van Haastert, 1985). The A types are currently believed to initiate intracellular production of cAMP to be used as a second messenger. B sites are involved in initiation of the chemotactic response (de Wit and Bulgakov, 1986a, 1986b) through activation of guanylyl cyclase and in intracellular cAMP production in preaggregative cells (de Wit et al., 1986). Conditions of starvation decrease signalling measured through the B sites, leaving the A sites coupled to adenylyl cyclase as the only active folate-binding receptors (de Wit et al., 1986). Binding of folate to the B receptors instigates a cycle of shifts in affinity of the receptor for its ligand from a low-affinity, fast-dissociating site termed Bf, through two higher-affinity slower-dissociating sites, B⁸ and B⁸⁺⁺ respectively (de Wit and Bulgakov, 1986b; Segall et al., 1988). Similar kinetic and affinity shifts have been reported for the cell surface cAMP receptors, regardless of isotype (van Haastert et al., 1986). Stimulation of amoebae with folate (or cAMP) produces a number of similar
biochemical and physical changes related to the chemotactic response, including increased actin association with the cytoskeleton (McRobbie and Newell, 1983; Dharmawardhane et al., 1989) production of intracellular cGMP (Mato and Konijn, 1977; Wurster et al., 1977), calcium release (Europe-Finner and Newell, 1986), phosphorylation of myosin heavy and light chains (Liu and Newell, 1991, 1993, 1994; Liu et al., 1993; Newell and Liu, 1992), cell volume (Futrelle et al., 1982) rates of movement (Browning et al., 1995) and light scattering measurements of cell suspensions, probably related to actin filament restructuring (Dharmawardhane et al., 1989; Segall et al., 1992). These responses will be discussed in detail in the following sections. While it has been hypothesized that different kinetic forms of the receptors are responsible for transducing different intracellular effects based on affinities for G-proteins (van Haastert et al., 1986; van Haastert, 1994) concrete evidence remains forthcoming (Segall et al., 1988). Molecular identification and cloning of the FARs has lagged behind similar attempts made with the cARs, possibly because of the difficulty in isolating membrane-bound folic acid-binding proteins such as the FAR forms from folate deaminase and folate C9-N10-cleaving enzyme, additional membrane proteins present in Dictyostelium that display significant affinity to folic acid (de Wit and Konijn, 1983; de Wit et al., 1985). FARs are associated with the insoluble cytoskeleton (Tillinghast and Newell, 1984) and it has also been suggested that the folate-binding activities involved in transduction of the chemotactic signal and FD activity are resident on the same membrane-bound protein (Greiner et al., 1992).

iv) GTP-binding proteins transduce signals intracellularly

Transduction of the cAR signal intracellularly is accomplished through heterotrimeric GTP-binding proteins. The specificity of G-protein-receptor and G-protein-effector interaction determines whether a response will be pleiotypic, leading to broad-range effects, or stimulate only a single effector leading to a single response (Neer.
Eight different α-subunits are currently known in *Dictyostelium* (Wu and Devreotes, 1991; Lilly et al., 1993). A single β-subunit has been identified (Lilly et al., 1993) while the γ-subunit has not yet been cloned (Chen et al., 1996). Heterotrimeric G-proteins have been implicated in multiple cellular processes in *Dictyostelium*, including biomembrane fusion, fertilization, phagocytosis, morphogenesis, zygote differentiation and chemotaxis (Browning et al., 1993; Browning and O’Day, 1995; Kesbeke et al., 1990; Dharmawardhane et al., 1994; Hadwiger et al., 1994). The Gα2 and Gβ subunits are required for most, but not all cAR-mediated responses and in the absence of either subunit cAMP-stimulated effects on cGMP, cAMP and IP3 production as well as actin polymerization and myosin phosphorylation are lost (Kumagai et al., 1991; Wu et al., 1995c). Evidence that the βγ subunit is the critical component of all cAMP-mediated intracellular effects comes from experiments using gβ- cell lines, in which chemotaxis to both folic acid and cAMP is completely abolished (Wu et al., 1995b, 1995c). The βγ heterodimer is necessary for activation of adenyl cyclase (Wu et al., 1995b, 1995c); this will be discussed in detail in a later section. Deletion of Gα2 has no effect on folic-acid mediated chemotaxis (Kesbeke et al., 1990); however this response is abolished in mutant lines lacking Gα4 while cAMP-mediated chemotaxis is unaffected (Hadwiger et al., 1994; Dharmawardhane et al., 1994) lending credence to the belief that chemotactic responses to cAMP and folic acid are mediated by different G-proteins (Kesbeke et al., 1990). Roles for many of the remaining Gα subunits have yet to be determined, with the exception of Gα1. The Gα1 subunit has been implicated in regulating prestalk morphogenesis, possibly by negative regulation of prestalk AB cell development (Dharmawardhane et al., 1994). Deletion or mutation of the other Gα subunits by homologous recombination leads to phenotypes nearly indistinguishable from the wild type (Kumagai et al., 1991; Dharmawardhane et al., 1994; Wu et al., 1994). Broad tissue distribution is not characteristic of these subunits; they are in fact often restricted to only a subtype of cells (Dharmawardhane et al., 1994; Wu et al., 1994). Functional redundancy is a possible
explanation for the number of G\(\alpha\) subunits observed. The specific developmental role of G\(\alpha_1\) suggests that discrete developmental processes may be specifically regulated by separate G\(\alpha\) subunits.

v) Adenyl cyclase (AC)

Aggregation of single amoebae, patterning and morphogenesis in the multicellular pseudoplasmodium are all events mediated by cAMP. Pulsatile secretion of endogenous cAMP occurs through stimulation of the A subclass of cell-surface cAMP binding sites (Theibert and Devreotes, 1986). Similar activation of adenyl cyclase has been observed for folic acid-mediated signalling (de Wit et al., 1986). G-protein-transduced signals are critical to these events (Small et al., 1987; Kumagai et al., 1991; Wu et al., 1995b).

Biochemical changes associated with these G-protein activated processes include production of cAMP (Keskeke et al., 1990), cGMP (Mato and Konijn, 1977; Wurster et al., 1977), inositol(1,4,5) trisphosphate (IP\(_3\); Europe-Finner and Newell, 1987b), 1, 2 sn-diacylglycerol (DAG; Bominaar and van Haastert, 1993), altered fluxes in Ca\(^{2+}\), K\(^-\) and H\(^-\) (Abe et al., 1988; Ackerle et al., 1985; Aerts et al., 1987) polymerization of actin in association with the cytoskeleton (McRobbie and Newell, 1983; Dharawardhane et al., 1989) and activation of protein kinase A (cAPK; Mutzel et al., 1987; Simon et al., 1989).

Binding of cAMP at the A site results in activation of the aggregation isoform of adenyl cyclase (ACA) via a \(\beta\gamma\)-mediated signal and concomitant synthesis of cAMP (Tang and Gilman, 1991; Pitt et al., 1992, 1993). A second AC isoform is expressed only in the process of spore germination (ACG) and is not present in preaggregative or early postaggregative cells (Pitt et al., 1993). A third AC isoform, ACX, has been postulated in Dictyostelium, based on the activation of cAPK in ACA null cells (Endl et al., 1996).

Detection of this isoform has thus far eluded researchers. Whether the A sites are kinetic forms of cAR1 is currently under some question, as activation of ACA has been observed
in cAR1- cells (Pupillo et al., 1992), suggesting the possible existence of a fifth cAR as yet unidentified. Since cAMP serves as the ligand responsible for inducing its own synthesis, the potential for a positive feedback loop leading to runaway production of cAMP exists and Dictyostelium therefore possesses several regulatory mechanisms to prevent excess cAMP secretion. One recently identified mechanism unique to Dictyostelium is CRAC, the cytosolic regulatory protein of adenylyl cyclase (Insall et al., 1994; Lilly and Devreotes, 1994, 1995). In order to activate AC, CRAC is recruited to the membrane from the cytoplasm. The mechanism by which CRAC is activated is as yet unknown, although brief pretreatment of resting cells with cAMP or GTPγS, a non-hydrolyzable Gα-binding analogue both result in CRAC enrichment in the membrane fraction (Lilly and Devreotes, 1995). CRAC binds to the membrane via a pleckstrin-homology domain (PH; Insall et al., 1994) and can activate AC only in association with the freed βγ subunit. PH domains, involved in either protein-protein binding or protein-phospholipid interaction at the cell membrane (Cohen et al., 1995), have been identified in other membrane-localized proteins involved in signalling (Pitcher et al., 1995; Lomasney et al., 1996) including phospholipase Cδ-1. A second possible ACA regulatory protein, ERK2 has been recently suggested (Segall et al., 1995). ERK2 is a member of the MAP kinase family (Boulton et al., 1991) and disruption of the ERK2 gene in Dictyostelium leads to dramatically reduced cAMP-induced cAMP release while not affecting cGMP production (Segall et al., 1995). Indirect effects on development resulting from decreased intracellular cAMP (e.g. inactive cAPK [Williams et al., 1993]) are possible results.

ACA is not directly involved in the chemotactic response. Pharmacological perturbation of cAMP-dependent cAMP synthesis does not effect cAMP-dependent cGMP synthesis or chemotaxis (Brenner and Thoms, 1984). In addition, mutant cell lines devoid of ACA show normal levels of intracellular cGMP and cytoskeletal alteration (Pitt et al., 1993) and so ACA will not be further discussed here.
vi) Guanylyl cyclase (GC)

Several lines of evidence implicate guanylyl cyclase (GC) in the chemotactic response (Valkema and van Haastert, 1994). GC activity is coupled to stimulation of the B-sites of both the cAMP and the folic acid receptors through their respective heterotrimeric G-proteins (Kesbeke et al., 1990; Kumagai et al., 1991; Schulkes et al., 1992; Hadwiger et al., 1994), and stimulation of these receptors leads to increased intracellular levels of cGMP peaking within ten seconds of activation (Wurster et al., 1977; Mato et al., 1977; Schulkes et al., 1992). The cGMP remains primarily intracellular, and is eventually hydrolyzed by the intracellular cNPD (Faure et al., 1990). Its intracellular roles include activation of phospholipase C (Newell et al., 1990; Lundberg and Newell, 1990) and release of Ca²⁺ from non-mitochondrial stores (Europe-Finner and Newell, 1986). A cGMP-dependent protein kinase has been identified in Dictyostelium (Wanner and Wurster, 1990). Additional cGMP-binding proteins have been reported, including DNA-binding proteins and nuclear-localized proteins (Parissenti and Coukell, 1989; Coukell et al., 1995) suggesting nuclear and possibly transcriptional roles for cGMP.

The roles of cGMP in chemotaxis have been greatly elucidated by use of a group of complementation mutants referred to as streamer F mutants (stmF; Ross and Newell, 1981; Coukell and Cameron, 1986). These mutants are deficient in the aggregation-stage cyclic nucleotide phosphodiesterase (mPD). This enzyme shows a higher affinity for cGMP, and its absence results in elevated [cGMP]ₙ and several distinct physical and biochemical abnormalities. Characteristic of this phenotype is a prolonged elongation of amoebae in response to cAMP pulses at the receptor (Newell et al., 1990). In response to cAMP at the surface, wild-type amoebae normally elongate and exhibit chemotactic movement in the direction of the increasing gradient. This motion continues for 60-100s at which time adaptation to the response results in restoration of the resting cell shape, and
the cell resumes random movement until the reception of the next pulse of cAMP (van Haastert, 1991; Chandrasekhar et al., 1992). In stmF mutants, the motile phase is significantly longer; movement continues for over 500 seconds (Liu and Newell, 1991). Degradation of cGMP in these cells occurs via activity of the vegetative cNPD that slowly returns cGMP to prestimulus levels (Coukell and Cameron, 1986; Newell and Liu, 1992). The prolonged migration results from a defective adaptive mechanism (Newell and Liu, 1992; Segall, 1992). StmF mutants in the process of chemotactic migration possess the ability to receive and respond to the next pulse of chemoattractant without the desensitization and refractory phase seen in wild-type cells. It was initially assumed that increased association of actin with the cytoskeleton was the cause of aberrant chemotaxis seen in stmF mutants; this was not shown to be the case (Liu and Newell, 1991, 1993, 1994). In fact association of myosin II with the cytoskeleton was found to be prolonged, leading to the elongate phenotype. Myosin II has been postulated to be involved in polarity and normal cell elongation and not force generation involved in motility (Newell et al., 1990; Newell and Liu, 1992; Segall, 1992). In wild-type cells, myosin heavy chain (MHC) is phosphorylated in response to stimulation of the cells with cAMP, peaking at approximately 30-40s and decreasing to basal levels near 160s post-stimulus (Liu and Newell, 1991). This corresponds to dissociation of MHC from the cytoskeleton when phosphorylated and subsequent reassociation upon dephosphorylation. In stmF mutants the initial increase in MHC associated with the cytoskeleton closely matches that observed for the wild-type. This association persists up to five times longer than in the non-mutant cells however, in correspondence with the persistence of cGMP (Newell et al., 1990). It was found by Liu and co-workers that phosphorylation of MHC, an event that was proposed to lead to its dissociation from the cytoskeleton (Berlot et al., 1985, 1987) was inhibited in the presence of high [cGMP]i (Liu and Newell, 1991). MHC kinase (MHCK) itself was found to be inhibited by calmodulin, leading to increased levels of unphosphorylated MHC in the cytoskeleton in the presence of Ca2+ (Liu and Newell,
Models for the regulation of MHC phosphorylation by cGMP have been proposed in which MHCK is transiently inhibited by cGMP. Current evidence suggests this to be an indirect inhibition rather than a direct effect of cGMP on the MHCK. Second messengers, including cGMP have not been shown to have any direct effect on MHCK in vitro (Cote and Bukiejko, 1987; Ravid and Spudich, 1989). Models suggesting an indirect effect of cGMP are strengthened by the fact that in stmF mutants uptake of Ca^{2+} is prolonged in response to cAMP stimulation (Menz et al., 1991). The presence of a CaM-inhibited MHCK also lends support to this model (Maruta et al., 1983); however, this enzyme has not yet been shown to be responsible for phosphorylation of cytoskeletal MHC. A MHCK gene has been cloned (Ravid and Spudich, 1992). The purified enzyme was found to contain a PKC structural motif distinct from all known PKCs. Additionally this enzyme was not regulated by calcium, phospholipid or diacylglycerol. It is currently accepted that during localized actin-myosin contraction of the cytoskeleton, MHC molecules move toward actin foci on the cell membrane, where they are phosphorylated by MHCK, inducing disassembly and release into the cytosol (Yumura and Kitanishi-Yumura, 1992).

The light chains of myosin have also been implicated in the chemotactic process through study of the stmF mutants and other cGMP-deficient cell lines (Liu and Newell, 1994). Two myosin light chains (MLCs) have been identified: an 18 kDa regulatory peptide that can be phosphorylated, and a 16 kDa chain essential to chemotaxis (Clarke and Spudich, 1974; Kuczma and Spudich, 1980). The RLC kinase (MLCK) is unique in that it is not a Ca^{2+}/CaM-binding enzyme. It does however contain consensus sites for phosphorylation by cGPKs and cAPKs (Tan and Spudich, 1990a, b, 1991), defining yet another level of regulation of myosin association with the cytoskeleton by cGMP. The RLC is specifically involved in inhibition of the actin-activated Mg^{2+}-ATPase (Uyeda and Spudich, 1993) and this inhibition is relieved by phosphorylation. Clearly cGMP is involved in myosin regulation. Further clarification of the roles of GC in chemotaxis awaits molecular characterization of the enzyme in Dictyostelium. Membrane-localizing
and membrane-spanning domains have been identified in other isoforms, as have regions of homology between GC and several protein tyrosine kinases (Singh et al., 1988).

In addition an inositol metabolic pathway is activated by pulsatile cAMP. This pathway has been suggested to be related to intracellular production of cGMP, and evidence is accumulating in favour of a role for inositol polyphosphates in regulating polymerization of actin filaments in pseudopod formation, either by a direct mechanism, or indirectly by sequestering of actin binding/capping proteins (reviewed in Schleicher and Noegel, 1992; Welch et al., 1997 and discussed in an upcoming section). Evidence for the involvement of the inositol pathway in cGMP production comes from work showing that application of 5 μM IP$_3$(1,4,5) or 60 μM Ca$^{2+}$ to saponin-permeabilized amoebae resulted in production of cGMP at similar levels as observed in cAMP stimulated whole amoebae (Europe-Finner and Newell, 1987a). However, Ca$^{2+}$ has since been shown to inhibit GC activity in vivo in electropeneabilized amoebae (van Duijn and van Haastert, 1992; Valkema and van Haastert, 1992). Additional data from fgdA mutants has shown that this mutant can respond to cAMP stimulation with normal levels of cGMP production, but is completely deficient in IP$_3$(1,4,5) production (Bominaar et al., 1991). Furthermore, mutants completely lacking in PLC activity (plc$^-$ cells) show normal levels of cAMP, cGMP and IP$_3$(1,4,5) production, as well as normal gene expression, chemotaxis and development (Drayer et al., 1994). PLC is membrane-localized via PH domains (Cifuentes et al., 1993; Lomasney et al., 1996), regulated in Dictyostelium by Ca$^{2+}$ (Lundberg and Newell, 1990; Drayer and van Haastert, 1992; Bominaar et al., 1994) and dually GTP-protein regulated (stimulated by Go2 and inhibited by Go1; Bominaar and van Haastert, 1993, 1994). These facts taken together suggest an important role for PLC in chemotaxis and development. It is likely that in chemotaxis, PI$P_2$ metabolism is more important as a source of diacylglycerol and polyphosphoinositides than as a mediator of [Ca$^{2+}$]. DAG, the second product of IP$_3$(1,4,5) production from PI$P_2$ has been shown to activate PKC (Nishizuka, 1988; Berridge and Irvine, 1989), although not all isoforms
present in Dictyostelium (Luderus et al., 1989; Tan and Spudich, 1990a, b, 1991). More importantly, DAG has since been suggested to play an important role in actin nucleation in the cell cortex (Kuwayama et al., 1993; Shariff and Luna, 1992). PLC is therefore probably important in chemotaxis of wild-type cells in Dictyostelium. The presence of multiple inositol kinases, polyphosphate kinases and phosphatases present an alternate route of IP₃ production (Bominaar and van Haastert, 1993, 1994) and the complexity of this back-up mechanism is further evidence in support of the importance of PLC in Dictyostelium.

IV) The Importance of Calcium as a Signal Transducing Agent

Calcium in its ionic form is the most common signal transduction element. Paradoxically, high [Ca²⁺], due to its ability to precipitate phosphate is fatal to the cell (Clapham, 1995). Since Ca²⁺ cannot be metabolized cells have developed other means of keeping [Ca²⁺] low, such as membrane-bound ion exchangers which remove Ca²⁺ from the cytoplasm, often exchanging it with extracellular K⁺ (Coukell et al., 1995) and intracellular Ca²⁺ stores including mitochondria and endoplasmic reticula. Ca²⁺ influx in Dictyostelium can be induced by activation of the chemotactic receptor. Both cARs and FARs activate the same Ca²⁺ transporter (Milne and Devreotes, 1993). This ion transporter is unique to Dictyostelium (Milne and Coukell, 1991) is voltage-independent (Milne and Coukell, 1991; Milne and Devreotes, 1993) and does not involve Na⁺ or H⁺ exchange (Milne and Coukell, 1991; Newell et al., 1995). Although induced by ligand binding at the chemotactic receptor, Ca²⁺ influx is G-protein independent (Milne and Devreotes, 1993; Caterina et al., 1994; Milne et al., 1995), revealing a new method of signalling in Dictyostelium. These methods can effectively maintain [Ca²⁺] levels 20 000 times lower than the 2 mM extracellular concentration (Clapham, 1995).
Ca$^{2+}$ as a second messenger is involved in many processes in *Dictyostelium*. While calcium-dependent signal transduction is not essential to sexual phagocytosis (Lewis et al., 1994), the other common members of the calcium-regulated signal transduction pathways have been identified as being essential in cell and pronuclear fusion during fertilization (Lewis et al., 1994; Lewis and O'Day, 1994; Lydan and O'Day, 1993). Prior research has indicated a role in fertilization for PKC (Gunther et al., 1995). Ca$^{2+}$/CaM dependent dephosphorylation of a 31 kDa protein has also been detected in 22 hr sexual cultures, suggesting a role for CaM in pronuclear fusion (Lydan and O'Day, 1993). Ca$^{2+}$/CaM has been implicated in cell cycle transition (Means, 1994; Takuwa et al., 1995), notable in *Dictyostelium* since it has been suggested that cell cycle location at time of initiation of starvation determines fate of cells in the postaggregative slug (Sharpe and Watts, 1985). Spore germination is calcium-dependent (Lydan et al., 1994; Lydan and Cotter, 1995), as is gamete fusion and macrocyst formation (McConachie and O'Day, 1986).

Intracellular Ca$^{2+}$ concentrations can be increased rapidly in two ways. Firstly, membrane-located mammalian Ca$^{2+}$ channels can open, allowing entry from the high concentrations in the external milieu (approx. 2 mM) to enter the cell, where free Ca$^{2+}$ exists in a much lower concentration (approx. 100 nM; Clapham, 1995). This process is G-protein independent (Caterina et al., 1994; Milne et al., 1995). A second means of increasing intracellular Ca$^{2+}$ involves release from certain non-mitochondrial intracellular stores via the metabolism of PIP$_2$ to produce IP$_3$ (O'Day, 1990). Calmodulin (CaM), a cytoplasmic Ca$^{2+}$-binding protein, binds free intracellular Ca$^{2+}$ and can then activate proteins in the signal transduction pathways. The involvement of Ca$^{2+}$ in the cytoskeletal response to chemotactic stimulation has been controversial. Several authors have reported normal chemotaxis in the absence of extracellular calcium (Mato et al., 1977; Saito, 1979). Other groups have argued that orientation in the gradient is Ca$^{2+}$-independent but locomotion requires Ca$^{2+}$ (van Duijn and van Haastert, 1992), while others have more
recently argued no aspect of chemotaxis can proceed in the absence of calcium (Schlatterer and Malchow, 1993; Unterweger and Schlatterer, 1995; Yumura et al., 1996). Conflicting data arose based on ineffective methods of calcium measurement or incomplete chelation from intracellular sources. Removal of calcium from the cytoplasm can be quickly compensated for in principle by release of calcium from intracellular stores, which can sequester calcium in concentrations as high as 2 mM (Clapham, 1995). This contradiction was resolved by Schlatterer and colleagues who accounted for the intracellular Ca$^{2+}$ release by use of low-affinity Ca$^{2+}$-chelating agents (BAPTA and its derivatives) intracellularly (Schlatterer and Malchow, 1993; Unterweger and Schlatterer, 1995). These results were confirmed by a separate group of investigators using a calcium indicator, fura-2 coupled to bovine serum albumin (fura-2/BSA). The fura-2/BSA complex is not easily endocytosed by Dictyostelium allowing for accurate, relatively long-term (up to 12 hours) measurement of spatial distribution of Ca$^{2+}$ (Yumura et al., 1996).

V) The Cytoskeletal Response

Rapid reorganization of the actin cytoskeleton in response to receptor stimulation is critical to the chemotactic process. Activation of the chemotactic receptor results in a multitude of intracellular changes aimed at initiating a cytoskeletal response. These responses are common to all chemotactic signals. Intracellular Ca$^{2+}$ increases transiently, as does (cGMP)$_i$ in chemotactically competent cells, resulting in decreased phosphorylation of myosin II. The involvement of inositol polyphosphates, specifically PIP(4,5)$_2$ is beyond question. Both PIP(4,5)$_2$ and its hydrolysis products, IP$_3$ and DAG have been demonstrated to play a role in chemotaxis in Dictyostelium and other cell types. Additionally roles for other phosphoinositides such as PI(4)P and PI(3,4,5)P$_3$ have recently been suggested (Hartwig et al., 1995; Barkalow et al., 1996). Cytoplasmic GTPases such as rho, rac and cdc42 have recently been shown to affect assembly of the
actin cytoskeleton in chemotactically responding cells (Nobes and Hall, 1995; Tapon and Hall, 1997). While polymerization of actin filaments alone is sufficient to generate motile force in some pathogenic bacteria (Theriot et al., 1994), singular actin filaments bend easily and do not alone possess the flexural rigidity to displace the plasma membrane in the formation of pseudopods (Condeelis, 1993). The currently accepted hypothesis for pseudopod formation in response to stimulation at the cell surface receptor involves hydrostatic pressure generated by actomyosin contraction in the rear of the cell (Tan and Spudich, 1991), localized depolymerization of the cortical actin cytoskeleton leading to membrane displacement and support of the displaced membrane by polymerized and crosslinked actin filaments (reviewed by Condeelis, 1990, 1993). Polymerization of actin to form filopodia or pseudopodia involves i) release of monomeric actin from monomer-sequestering proteins in the cytoplasm (the two most common of which are profilins and thymosin β4); ii) increased F-actin by de novo filament formation or uncapping of and addition to pre-existing filaments, and; iii) cross-linking of filaments, which provides rigidity and the strength required for actin bundles and arrays to maintain their elongate morphology. Key players in cytoskeletal remodelling responses in Dictyostelium are the discoidin proteins. Discoidin proteins are expressed several generations prior to starvation of amoebae, and are required for the effective streaming of amoebae to aggregation centres and maintenance of the elongate "streaming" phenotype (Alexander et al., 1992). Amoebae lacking the discoidin proteins (discoidinless mutants) form more dispersed pseudopodial regions at the cell periphery, possess abnormal microtubule-organizing centres and are generally blunt and rounded in appearance (Alexander et al., 1992). In spite of this these amoebae display instantaneous velocities and directional persistence in migration comparable to that of normal amoebae (Alexander et al., 1992) indicating that the elongate phenotype seen in aggregation streams is not required for effective chemotactic movement. Discoidins may therefore be necessary for altering cell shape allowing cell-cell contact and binding of cohesive amoebae in aggregation.
Of the two major vertebrate monomeric-actin sequestering proteins, profilins and thymosin β-4, only profilins have been found in Dictyostelium (Hauwitz et al., 1991). Two profilin genes, differentially expressed during development, are present. Both isoforms bind similarly to actin and show limited similarity in the N- and C-terminal domains with essentially the same actin-binding domain (Hauwitz et al., 1991). While profilins bind monomeric actin with high affinity, their primary roles are probably more related to nucleotide exchange increasing affinity of actin for polymerization into the cytoskeleton and "shuttling" of monomeric G-actin to barbed end of the filament (Schafer and Cooper, 1995). Thymosin β-4 binds G-actin with moderate affinity similar to that of profilin, but shows a preference for ADP-actin (Schafer and Cooper, 1995). The similar affinities of thymosin β-4 and profilin for actin suggest a mechanism of profilin-mediated monomer addition to the microfilament in which thymosin β-4 is displaced by profilin which translocates to the barbed end of the filament, catalytically exchanges bound ADP for ATP, and is removed by PI(4,5)P_2 upon addition of the G-actin molecule to the F-actin filament (Condeelis, 1993; Schafer and Cooper, 1995; Welch et al., 1997). Proteins functioning as G-actin sequesterers have not yet been conclusively identified in Dictyostelium. Profilins show affinity for PI(4,5)P_2 implying a mechanism of signal regulation; however, the rate of F-actin polymerization upon stimulation is far in excess of that accounted for by profilin-mediated addition, suggesting other mechanisms of increased association of cytoskeletal actin must be occurring (Condeelis, 1993). Additionally the in vivo levels of profilin are not enough alone to maintain the vast cytoplasmic pool of unpolymerized actin. While profilin is undoubtedly involved in both F-actin increase and G-actin sequestration, other mechanisms must be operating.

The profilin-mediated mechanism of increased F-actin is not sufficient to account for increased F-actin observed in vivo. An F-actin nucleating activity must also be present and the fact that the majority of de novo nucleation can be blocked by cytochalasin suggests that new barbed ends themselves are the main nucleating activity in signal-
stimulated cells (Condeelis et al., 1988). Increased nucleation is the result of the activity of gelsolin, an 80 kDa, Ca\(^{2+}\) and pH-regulated barbed-end capping and filament-severing protein found in nearly all cell types studied (Schleicher et al., 1995; Schafer and Cooper, 1995) and other members of the gelsolin family of proteins possessing some or all of the binding and severing characteristics of gelsolin (reviewed in Schleicher et al., 1995; Schafer and Cooper, 1995). Upon stimulation, gelsolin transiently associates with the sides of actin filaments, severing the filaments and capping the newly-generated barbed end; this capping can be released by high PI(4,5)P\(_2\) (Schafer and Cooper, 1995). These findings are in agreement with those of Shariff and Luna (1992) who found a Ca\(^{2+}\)-independent, DAG-stimulated nucleating activity in the cortical cytoskeleton of Dictyostelium. A nucleating activity has been indirectly attributed to gelsolin as the number of barbed ends are increased 10-fold (Barkalow et al., 1996). In concert with the increased number of nucleation sites, barbed ends of existing filaments are uncapped in response to stimulation. Capping protein (also referred to as CapZ) is a heterodimeric, Ca\(^{2+}\)-independent protein that can cap F-actin filaments, preventing actin flux at the barbed end (Hug et al., 1995). A nucleation activity previously attributed to this dimer in vitro has proven to be un reproducible in vivo (Hug et al., 1995). An additional signal-mediated capping activity has been identified in Dictyostelium in association with a 70 kDa Ca\(^{2+}\)-independent protein referred to as aginactin (agonist-inhibited actin binding protein; Sauterer et al., 1991) and dissociation of this protein leading to uncapped barbed ends has been proposed to make up the difference in barbed ends released by gelsolin and polymerization mediated by profilin (Sauterer et al., 1991; Condeelis, 1993). While the direct mechanism of regulation of this protein remains obscure, fgdA mutants show no aginactin-mediated uncapping, identifying Go2 and probably the PLC pathway as the mediator of aginactin activity (Sauterer et al., 1991). Aginactin association with the actin cytoskeleton shows a reciprocal time course to increased actin polymerization (Eddy et al., 1993). Interestingly, this protein exhibits significant sequence similarity (73%) to heat-
shock cognate protein hsc70 from several species (Eddy et al., 1993). The time course of
dissociation of aginactin with the cytoskeleton suggests it may be the primary cause of the
global increase in actin polymerization observed at 5s post-stimulation, referred to as the
"freeze" response (Dharmawardhane et al., 1989; Eddy et al., 1993).

Pseudopod extension is driven by arrangement of actin filaments in an orthogonal
array held in place by several crosslinking proteins, notably actin binding protein-120
(ABP-120; Condeelis et al., 1988; Bresnick and Condeelis, 1993), ABP-280 (non-muscle
filamin; Gorlin and Hartwig, 1993) and α-actinin (Critchley, 1993). ABP-120 is present in
high amounts in association with cortical actin, specifically in regions of pseudopod
formation in Dictyostelium (Condeelis et al., 1988). All three possess significant
homology in the actin-binding domain but otherwise differ in structure, and all three
associate in vivo as homodimeric proteins. ABP-120 associates in an antiparallel
conformation in vivo as a 240 kDa homodimer crosslinking adjacent microfilaments via
the N-terminally located actin-binding domain (Condeelis et al., 1988; Bresnick and
Condeelis, 1993). Based on immunolocalization it is currently believed that ABP-120 is
the crosslinking protein most important in construction of pseudopods in the chemotactic
response (Condeelis et al., 1988, Dharmawardhane et al., 1989). Clarification of the
question by standard methods such as mutation or deletion have given inconclusive data,
probably because the activities can be compensated for by α-actinin. ABP-280 (filamin) is
a 280 kDa vertebrate phosphoprotein which promotes orthogonal branching by single
actin filament binding sites present at the N-terminal ends of each chain which are
arranged in a "Y" conformation (Gorlin and Hartwig, 1993). ABP-280 also promotes
association of actin filaments with the plasma membrane. α-actinin functions primarily as
a filament-bundling protein (Critchley, 1993) with the ability to link filaments to
intracellular structures. While ABP-280 has not been identified in Dictyostelium, its
activities may reside in a peptide of comparable mass, ABP-240 (Condeelis et al., 1988).
Binding of α-actinin to cytoskeletal proteins involved in assembly of the focal adhesion
complex, specifically vinculin and β1 integrins has been reported (Critchley, 1993 and references therein), suggesting a role for α-actinin in cytoskeletal attachment to the focal adhesion complex. α-actinin is also Ca$^{2+}$-regulated as determined by the presence of EF-hand Ca$^{2+}$-binding motifs within its primary structure (Critchley, 1993; Noegel, 1996). The EF-hand domains, specifically the first, have been shown to be critical in the crosslinking capacity of α-actinin (Janssen et al., 1995). Two final facts lend evidence to the hypothesis that ABP-120 is the major filament crosslinker in vivo. Cells deficient in ABP-120 exhibit reduced chemotactic velocity and impaired pseudopod formation (Brink et al., 1990; Cox et al., 1992), while mutants of α-actinin show no detectable phenotype (Schleicher et al., 1988). Secondly, the fact that α-actinin crosslinks filaments in a nearly parallel alignment, while ABP-120 promotes orthogonal, nearly right-angled binding, an arrangement more energetically favourable for maintenance of pseudopods (Janssen et al., 1995) suggest that ABP-120 is the likely candidate for microfilament crosslinking in the pseudopod production process.

Upon pseudopod extension, adhesion to the substratum via the assembly of focal adhesion complexes results in traction, and retrograde flow of actin filaments from the cortex into the cytoplasm provides motile force. Side-binding of actin filaments to integral and peripheral membrane proteins converts signal-induced actin polymerization into cell movement via retrograde flow of actin filaments from the cell cortex internally (Welch et al., 1997). Filaments alongside the plasma membrane bind ponticulin, the major actin-binding integral membrane protein in Dictyostelium (Wuestehube and Luna, 1987; Luna, 1993; Shutt et al., 1995). After the cell passes the tail is retracted in an actomyosin II mediated contractile event (Liu and Newell, 1993). Signal-regulated members of the focal adhesion complex include vinculin and talin (Fukami et al., 1994; Kreitmeier et al., 1995) both critical in linking the cytoskeleton to the plasma membrane.

The final step in actin cycling into the cytoskeleton is the depolymerization of filaments leading to the retraction of pseudopods and recycling of the monomeric protein
back to the barbed ends. While monomer dissociation from the pointed end of the filament can occur in vitro by a treadmilling process (Alberts et al., 1994; Welch et al., 1997) observations of dissociation rates in vivo show rates much more rapid than passive dissociation (Welch et al., 1997). This process is probably dually regulated by capping/severing proteins as well as monomer-sequestering proteins. Recent evidence suggesting interactions between cofilin and phosphoinositides renders a possible signalling role for cofilin (Aizawa et al., 1995; Welch et al., 1997). The question of monomer-sequestration in Dictyostelium has not yet been clearly answered. Candidate proteins with high in vitro G-actin binding activity including profilin (Haugwitz et al., 1991) ABP-50 (EF-1α; Demma et al., 1990; Yang et al., 1990) and cofilin (Aizawa, 1995; 1996) suggested to play such a role in higher vertebrates have been located in Dictyostelium, but have since been assigned other primary functions. An intensive literature search failed to uncover reports of any of the major monomeric actin binding proteins including thymosin β-4, 19 kDa actin depolymerizing factor (ADF), destrin or actophorin. Two hypotheses exist to cover these facts. The three monomer-binding proteins could conceivably work in concert to maintain the cytoplasmic pool of unpolymerized actin, relying on differential monomer-binding affinities and subcellular localizations to regulate polymerization in response to signals. Alternatively, additional monomer-binding activities responsible for the rapid rates of G-actin dissociation from the cytoskeleton remain to be discovered. Discoidins have been proposed to be involved in monomer sequestration due to their near-uniform cytoplasmic distribution (Alexander et al., 1992). This question remains as yet unanswered in Dictyostelium.

VI) Protein phosphorylation

Protein phosphorylation has been implicated in the regulation of nearly all cellular functions in one way or another. Phosphorylation of proteins on serine, threonine or
tyrosine residues acts as an on/off switch for catalytically active proteins and can also target proteins (e.g. GTPases; reviewed by Devreotes, 1994) to various subcellular locales. A review of the activities and identities of protein kinases is beyond the scope of this review; however two important Dictyostelium-specific examples warrant mention. cAPK has been suggested to be the central mediator in differentiation and terminal morphogenesis of both prestalk and prespore cells via different mechanisms (Williams et al., 1993). PKC has been implicated in several processes associated with the chemotactic response (reviewed by Nishizuka, 1988; Berridge, 1993) and members of this family of enzymes have been shown to regulate the association of myosin II with thick filaments involved in cytoplasmic streaming and translocation of the cell mass in amoeboid movement (Tan and Spudich, 1991; Liu and Newell, 1994). Critical to the process of reversible protein phosphorylation is removal of added phosphate. Several phosphoprotein phosphatases have been identified to perform these roles under a wide variety of intracellular conditions. Most PPs were originally thought to be involved only in attenuation of receptor-mediated phosphorylation responses (Pallen et al., 1992); this however has been shown not to be case with the discovery of basal levels of activity of PTP1B, a PP specific to tyrosine-phosphorylated proteins (Fischer et al., 1991) and CN, a Ca\(^{2+}\)/CaM regulated serine-threonine PP (Clipstone and Crabtree, 1992) directly involved in interleukin-2 (IL2) induced gene transcription (Liu et al., 1991).

VII) Calcineurin

Calcineurin is a phosphoprotein phosphatase that preferentially dephosphorylates serine or threonine residues, although some tyrosine dephosphorylation has been demonstrated in vitro under nonphysiological conditions (Chan et al., 1986). It was originally identified as a calmodulin-binding protein that could inhibit the activity of calmodulin-stimulated cyclic nucleotide phosphodiesterase (Klee and Krinks, 1978),
although this inhibition was later determined to be the result of removal of CaM from the cytosol (Klee and Cohen, 1988). The name originated from the supposition that the enzyme was predominantly located in mammalian nervous tissue (Mumby and Walker, 1993). CN has since, however, been found to have a much broader distribution, distinct isoforms having been found in nearly all mammalian tissue types, as well as lower eukaryotes (McKeon, 1991; Kincaid, 1993; Dammann et al., 1996; Lydan et al., manuscript in preparation). While most serine/threonine protein phosphatases are recognized to be involved in signal attenuation (Pallen et al., 1992) CN is one of the few that has been identified as an active participant in several signal transduction pathways.

CN has been implicated in transcription of early activation T-cell genes (Pallen et al., 1992; Schreiber, 1992) by facilitating nuclear import of the cytoplasmic transcription factor NF-AT4 (Shibasaki et al., 1996). CN has also been postulated to play a role in neurotransmitter receptor activity (Liu et al., 1994; Villafranca et al., 1996) and cell cycle progression (Mumby and Walker, 1993; Means, 1994). CN has been suggested to be part of a phosphatase cascade, activating protein phosphatase 1 (PP1) by dephosphorylation and subsequent inactivation of the PP1-specific inhibitor protein inhibitor-1 (Guerini et al., 1990). CN has been proposed to be involved in neutrophil chemokinesis by regulating integrin-mediated adhesion to vitronectin in the process of tail retraction (Hendey et al., 1992) and in the regulation of integrin recycling to the leading edge in neutrophils (Lawson and Maxfield, 1995). Interestingly, while CN was shown to have no effect on integrin-mediated binding to fibronectin in neutrophils, it was found to be critical in the α5β1 integrin-fibronectin binding response of Chinese hamster ovary (CHO) cells (Pomies et al., 1995). CN has also been implicated in synaptic vesicle recycling in response to nerve terminal depolarization (Liu et al., 1994). Inactivation of CN in growing nerve cells resulted in asymmetric retraction of growth cone filopodia (Chang et al., 1995). CN activity has also been detected in agonist-stimulated, Ca²⁺-regulated production of cAMP in rat adrenal glomerulosa cells (Baukal et al., 1994).
Mammalian CN is a heterodimer consisting of 59-61 kD catalytic subunit (CNA) and a 19 kD regulatory subunit (Kissinger et al., 1995). Although the regulatory subunit is similar in size and sequence identity to CaM (35% sequence identity: Kissinger et al., 1995), both bind CNA at separate sites and both are required for full activity of the enzyme (Stemmer and Klee, 1994). Multiple isoforms of CNA exist in higher eukaryotes, while CNB is encoded by a single gene (Guerini et al., 1990). CNA also contains in its C-terminal region an autoinhibitory domain that masks the catalytic site in the absence of CaM (Kissinger et al., 1995). Two divalent metal ions, Fe$^{2+}$ and Zn$^{2+}$ are believed to be required for activation of the catalytic subunit (Kissinger et al., 1995) although Mn$^{2+}$ can be substituted. *Dictyostelium* CNA is much larger than the isoforms of any of its higher eukaryotic counterparts. A single-copy gene encodes a 643 amino-acid polypeptide with a predicted molecular weight of 71.3 kD (Dammann et al., 1996; Lydan et al., Genbank accession number DDU22397) however western blots of whole cell extracts have detected an 80 kD protein (Dammann et al., 1996). While CNB has not yet been found in *Dictyostelium* nor the gene cloned, it presumably exists, as the CNB-binding site is conserved and required for full activity of the enzyme in vivo (Dammann et al., 1996). Four methionines have been identified in the N-terminal region of the peptide corresponding to possible transcription initiation sites (Dammann et al., 1996; Lydan et al., manuscript in preparation). Expression of the cDNA in *E. coli*, a standard expression system, results in initiation of transcription from the second methionine, and additionally a 58 kD peptide from the fourth methionine start site, while the first and third are poorly recognized in *E. coli* (Dammann et al., 1996). As none of the start sites possess any significant difference in similarity to the consensus *E. coli* transcription initiation site, the reason for the differential transcription is not known (Dammann et al., 1996). N-terminal acetylation of the protein at serine 2 could also contribute to the difference in predicted and observed molecular weight (Dammann et al., 1996). All sequence reported include the putative regulatory subunit (CNB) binding site and a Ca$^{2+}$/CaM binding site in the C-
terminal regions of the sequence (Kincaid, 1993; Dammann et al., 1996; Lydan et al., manuscript in preparation). The large additional N-terminal sequence beyond the transcription initiation site is not required for catalytic activity of the enzyme, as the 58 kD peptide is catalytically active with similar activity (Dammann et al., 1996). This region is rich in serine, threonine, asparagine and glutamine residues. The significance of this is not known. A similar enrichment of asparagines and glutamines has been reported in the N-terminal domain of cAPK (Anjard et al., 1993). It has been speculated that these regions could be involved in compartmentalization of these enzymes to specific regions of the cell cortex. Interestingly, an A kinase anchor protein (AKAP79) has been reported with significant affinity to cAPK and CNA in bovine brain (Coghlan et al., 1995). Binding of CNA to AKAP79 inactivates the phosphatase (Coghlan et al., 1995). Whether similar spatial regulation exists in Dictyostelium remains to be seen.

VIII) Inhibition of Calcineurin

Along with the autoinhibitory properties of CNA imparted by the autoinhibitory region and the trivial inactivation of the enzyme in the absence of either CNB or Ca²⁺/CaM, several mechanisms of inactivation of CN have been reported. CNA can be inactivated by free superoxide ions, probably by ionic modification of the valency state of the metal ions, specifically Fe²⁺-Fe³⁺ transition at the catalytic core of the enzyme and this inactivation can be prevented by the enzyme superoxide dismutase (Wang et al., 1996). AKAP79 preferentially binds CNA in the inactive conformation (Coghlan et al., 1995) and could conceivably reduce CN activity by removing the enzyme from possible interaction with Ca²⁺/CaM, although Ca²⁺/CaM alone is not enough to dissociate CNA from the anchor (Coghlan et al., 1995). Several conserved serine, threonine and tyrosine residues exist in the sequence of the CNA subunit and both CaM-dependent kinase II (CaMKII) and PKC have been reported to phosphorylate CNA in vitro (Martensen et al., 1989;
Calab et al., 1990; Liu et al., 1994;) suggesting phosphorylation as a means of regulating CN activity. Additional posttranslational modifications of the protein include possible acetylation at the N-terminus (Dammann et al., 1996) and methylation (Billingsley et al., 1985) the function of which is currently unknown. Mammalian CNB is known to be N-terminally myristoylated indicating a possible membrane localization of this subunit (Klee and Cohen, 1988).

The discovery by Liu and colleagues (1991) that the immunosuppressants cyclosporin A (CyS) and FK-506 strongly inhibited the phosphatase activity of CN opened the gateway to CN research. Although CyS contains the leucine-rich domain believed to be involved in binding to CN (Fliri et al., 1993) it was also found that neither the drugs nor the receptors alone conferred phosphatase inhibition; rather, the drug-receptor complex was required. The gain-of-function model for the inhibition of CN was supported by the fact that major conformational changes accompany the binding of both CyS and FK-506 to their receptors cyclophilin A (CyPA) and 12 kDa FK-binding protein (FKBP12) respectively (Wiederrecht et al., 1993). Determination of the crystal structure of the enzyme complexed to the FK506-FKBP12 complex has shown inhibition of CN function is non-competitive, due to partial blockage of the CNB binding site (Kissinger et al., 1995). Researchers using paranitrophenyl phosphate as a substrate have in fact reported increased CN activity in complex with CyS-CyPA (Liu et al., 1991) in support of non-competitive inhibition. While a similar mechanism of inhibition is observed for complexes of CyPA-CyS, this complex binds to a distinct but overlapping site (Kissinger et al., 1995). At immunosuppressive doses only a fraction of the total intracellular CyPA and FKBP12 are bound by the drugs (Wiederrecht et al., 1993). This fact argues against the loss-of-function model, as a high percentage of the receptors remain unaffected by the addition of the drug, suggesting that the receptor itself is not enough to negatively affect CN action.
Recently a new class of CN inhibitors has been identified. Synthetic pyrethroids are commonly employed as insecticides and include cypermethrin (CPR), deltamethrin (DEL) and fenvalerate (FEN). Previous investigations of these organic compounds have concentrated on their effects on the gating kinetics of the Na\(^+\) channel (for review see Narahashi, 1989). It was noticed however that a significant portion of synaptosomal stimulation could not be accounted for solely by increased Na\(^+\) influx (Enan and Matsumura, 1992). Since protein phosphorylation-dephosphorylation processes were known to be involved in synaptic transmission (Schulman and Greengard, 1978) attention was turned toward the activities of enzymes involved in these processes. The insecticides were found to strongly inhibit the phosphatase activity of CN (IC\(_{50}\) 0.03 nM, 0.03 nM, 20 nM respectively; Enan and Matsumura, 1992). It was questioned whether the insecticides were working by inhibition of CN activity or by enhancement of the activity of PKC (Enan and Matsumura, 1993); the pyrethroids were found however to strongly and specifically inhibit the activity of purified CN (Enan and Matsumura, 1992). Structurally similar insecticides such permethrin (PER) were observed to have no significant effect on CN activity. These drugs, referred to as type II synthetic pyrethroids, are differentiated from type I synthetic pyrethroids such as permethrin (PER) by the presence of a cyano group in the α-position (Narahashi, 1989).

CyPA and FKBP12 are among the most abundant proteins within the eukaryotic cell, comprising between 0.2% and 0.4% of the total cytosolic protein (Wiederrecht et al., 1993). A cyclophilin analogue has been reported in Dictyostelium (Barisic et al., 1991). For this reason use of CyS and FK-506 as inhibitors of CN activity has been very popular. Use of pharmacological inhibitors can also provide useful information in regard to the roles and activities of catalytic proteins. Very little work has been done on the mechanism of CN inhibition by pyrethroids (Enan and Matsumura, 1992). The role for these drugs as inhibitors of CN has made it possible now to examine in detail the role of CN in eukaryotic signal transduction processes.
The radial bioassay method of quantifying rates of cell migration was originally employed as a method of examining the rate of amoebal migration during sexual development (O'Day, 1979). This method was further modified by Browning and colleagues to investigate the role of tyrosine phosphorylation in chemotaxis of the wild-type cell line V12 (Browning et al., 1995). In neither case was the axenic cell line AX-3 examined. The axenic mutants are presently one of the most commonly used cell lines for mutant analysis. To date, no research has been done on the chemotactic response in axenic cell lines. Since the cn' mutants were generated in the thymidine auxotroph strain JH10 which was derived from AX-3 (Lydan et al., manuscript in preparation) it was important to define the parameters of the bioassay that lead to maximal rates of migration for both AX-3 and JH10 amoebae. Lydan and colleagues (manuscript in preparation) observed that the rates of migration of cn' amoebae were 54% higher than JH10 amoebae. Since CN null mutants are completely deficient in CN activity it is impossible to study stage-specific cellular processes such as differentiation or post-aggregative chemotaxis within the multicellular structure. For such questions a pharmacological approach may be more useful. For this reason studies involving the pharmacological inhibition of CN were performed using the type II synthetic pyrethroid deltamethrin, a potent specific inhibitor of CN activity (Enan and Matsumura, 1992) and permethrin, a type I synthetic pyrethroid that has shown no effect on activity of purified CN (Enan and Matsumura, 1992). To further understand mutant strain chemotaxis, the rates of chemotactic migration obtained for the axenic cell lines will be compared to their parental progenitor NC-4. Establishment of optimal parameters for chemotaxis by axenic amoebae will allow for examination of the role of CN in chemotaxis at various developmental stages, a comparison not allowed by the use of the cn' mutant line. Pharmacological inhibition of chemotaxis to both folic acid, the primary chemoattractant of vegetative amoebae and cAMP the chemoattractant in
later stages of development will be examined. Overall, these studies should complement and extend those obtained from examination of the cn\(^{-}\) cell line since inhibition of CN at specific developmental stages is impossible in the null mutant.

Finally, the presence of substrates for CN associated with the process of chemotaxis were examined. This was done by probing chemotactically competent cell extracts with antibodies to phosphoserine and phosphothreonine. In the presence of inhibitors of CN activity it is expected that serine/threonine phosphorylation will increase, thereby identifying possible targets for CN action.
Methods

i) Culture Methods

AX-3 cells were grown in liquid HL-5C media (5g bacto-yeast extract, 2.5g bacto tryptone, 2.5g casein-digested peptone, 5g proteose peptone, 10g D-glucose, 1.2g KH₂PO₄, 0.35g Na₂HPO₄, 500 µg/mL streptomycin sulfate in 1L distilled water, pH 6.5). Amoebae were harvested at desired cell density and resuspended for conditions of starvation in 15 mM Tris, pH 6.5 at a final cell density of 5 X 10⁷ cells/mL. Flasks were shaken on a rotary shaker at 110 rpm, 21°C. As a thymidine auxotroph, JH10 cells were grown in the same media supplemented with 100µg/mL thymidine under the same growth conditions. NC-4 amoebae were grown in association with E. coli strain B/r on SM agar plates, (5% D-glucose (ICN), 5% proteose peptone #3 (Difco), 0.5% yeast extract (Difco), 1.8% Bacto-agar (Difco)) until amoebae had just cleared the bacterial lawn. In order to increase synchronicity of spore germination and amoebal release, spores were heat-activated at 45°C for 30 minutes. An inoculating loopful of bacteria was added to the suspended spore volume and vortexed to break up the bacterial agglomerates. 100 µL aliquots were spread on plates, inverted and incubated at 21°C. Lawn clearance was generally observed between 40-44 hours of incubation.

ii) Quantification of Chemotaxis

Chemotaxis was assayed using the radial bioassay, originally to examine chemotaxis to cAMP by O'Day (1979) and modified for chemotaxis to both cAMP and FA by Browning et al. (1995). 1.0% agarose (Bio-Rad) was dissolved in 15 mM Tris, pH 6.5 and combined with the drugs under investigation in a 13 X 100 mm glass culture tube to a final volume of 2 mL. The agarose was then pipetted into 35 X 10 mm plastic culture
plates (Nunclon) in 1 mL volumes resulting in duplicate plates for each experimental condition. Cells were spotted at regular intervals in the central region of the agarose surface to obtain the flattest surface possible and allowed to air dry. At least ten cell drops were placed on every plate. Approximately 7 X 10^3 cells were present in each cell drop. When the excess moisture had dissolved into the agarose the plates were capped and initial diameters were measured using a dissecting microscope (magnification 60X) and a metric-ruled slide. Dot diameters between 0.5 - 1.0 mm were regularly obtained. Each trial was allowed to proceed for 3 hours after which the plates were uncapped. Cell migration was halted by a brief incubation of the plates at 37°C to kill amoebae. Final diameters were measured as above and net change in diameter was recorded and standard deviation determined at the end of the experiment. Rates of migration were determined by dividing net change in halo diameter by time of assay minus the lag period in which no cell movement was observed. This time course was usually 150 minutes.

iii) Pharmacology

Deltamethrin (Bio-Can) was dissolved in dimethylsulfoxide (Caledon Laboratories) to final stock concentrations of 10 mM. Experimental concentrations did not exceed 20 μM in 1% DMSO, as a cloudy white precipitate was visible in the agarose above this concentration. Adenosine 3'-5' cyclic monophosphate (cAMP) (Sigma) was dissolved in distilled water and diluted from 1 mM stocks (frozen at -20°C) immediately before use. Folic acid (ICN Biochemicals) was dissolved in 0.5M Na₂CO₃ (Sigma) as per Merck (1979). Stocks were prepared immediately before use and beakers were wrapped in foil to prevent light inactivation.
iv) **Time-lapse videomicrography**

Video movies were filmed using a Panasonic CCTV video camera (courtesy of Rob Baker), Panasonic WV-5470 black and white video monitor (courtesy of Glenn Morris) and RCA RC-15 time-lapse video recorder (courtesy of Nick Collins). Cells were harvested at mid-log growth, starved and dotted onto agarose plates as described previously. Plates were capped to prevent dehydration, inverted on the stage of a Zeiss 9901 stereomicroscope and examined under bright-field optics with a 16X objective. Dots were oriented in recording area to obtain maximum coverage of the leading edge of the responding amoebae. Observations were made at the leading edge to reduce possible "gradient noise" arising from secreted waves of phosphodiesterase passing in opposing directions. Leading edge recordings were made for 30 minute recordings of all trials (i.e. zero cAMP, 1 µM cAMP, 1% DMSO, 10 µM DEL, 20 µM DEL). Inversion of plates on the microscope stage had no measurable effect on response as measured by average change in halo diameter. For calculations of rate of cell migration, the view screen was measured using the graded area of a corpuscle counting chamber. A distance of 1 cm on-screen corresponded to an actual distance of 65.7 µm. Amoebae were measured from the leading edge at the point of initial placement and final location after set migration periods. Wherever distinguishable individual amoebae were measured; individuals were not visible in zero chemoattractant or 1 µM cAMP recordings. In all calculations of migration at least three amoebae were tracked and their displacements averaged.

Images of leading edge amoebae were obtained by direct screen capture. The video recorder was connected directly to a thermal printer and images chosen by examination of individual frames at similar trial intervals.
v) **One-dimensional PAGE**

SDS gels were poured according to Laemmli (1970). Briefly, 12.5% acrylamide gels were made from 3.1 mL 40% stock acrylamide, 2.5 mL 1.5M Tris, pH 8.8, 4.25 mL ddH₂O and 0.1 mL 10% SDS. This solution was degassed for 15-30 minutes and the polymerization reaction was catalyzed with the addition of 50 μL 1% ammonium persulfate and 5 μL N, N, N', N'-tetramethyl ethylenediamine (TEMED). Stacking gels were prepared according to the recipe for a 4% gel, substituting 0.5 M Tris buffer, pH 6.8 and allowed to polymerize for 30-45 minutes. Polymerization for longer time periods resulted in severe dehydration and well shrinkage, decreasing usable gel space. Samples were adjusted to desired protein concentration and boiled in 2X SDS sample buffer (12.5% 0.5 M Tris, pH 6.8, 0.01% bromophenol blue, 20% glycerol, 2% SDS and 5% 2-β-mercaptoethanol) for five minutes (see Appendix A). Five μg protein was loaded per lane and gels were run at 200V for 40-45 minutes, or until the dye front had migrated off the bottom of the gel. For protein visualization gels were stained in 0.1% Coomassie Brilliant Blue and destained with 40% methanol, 10% acetic acid usually overnight. Gels were dried on filter paper and stored.

vi) **Indirect Immunodetection**

Protein transfer was modified from Towbin et al. (1979). Briefly, gels were equilibrated in transfer buffer (25mM Tris, 192mM glycine, 20% methanol) at room temperature for 15-20 minutes. Scotch-Brite filter pads and filter paper were soaked in transfer buffer and assembled in the Bio-Rad miniblot transfer apparatus as per manual (Bio-Rad). PVDF membranes were activated in 100% methanol, rehydrated in ddH₂O and equilibrated for 10-15 minutes in transfer buffer prior to assembly of blot apparatus. To
ensure contact between the gel and the membrane in all areas. Gel-membrane assembly was performed submerged in transfer buffer. Protein was transferred at 100V constant voltage for 90 minutes. Icepacks were changed at 45 minutes as required (i.e. to maintain amperage of 250 mA or below). For qualitative examination of levels of protein transfer, membranes were stained prior to blocking with 1% amido black for two minutes and destained with 40% methanol, 10% acetic acid. Unstained blots were blocked in 1% BSA in TBS (Tris-buffered saline, pH 7.5) for 2 hours at room temperature or overnight at 4°C. Incubations with titres of 1:2000 for the primary antibody and 1:70000 for the secondary antibody were in the same solution and washes after the primary and secondary probes were in 1% BSA-TBS with added 0.05% Tween 20, 4 times for 10 minutes per wash in several volumes per blot. Preincubation controls were performed by gentle shaking of usual titres of the primary antibody with 25 mM phospho-D-serine (Sigma) for one hour prior to continuation with the probing protocol (see Appendix A). The secondary antibody was detected by chemiluminescence (Boehringer-Mannheim) using Kodak X-O-Mat film. Because molecular weight markers were strongly recognized by the antiphosphoserine antibody, relative mobilities were calculated from the exposed film. Relative mobilities (Rf) were determined using Gibco high-range prestained markers and a curve fit to the plot of migration distances of marker proteins using Macintosh DeltaGraph Professional 1.0. Complementary experiments on metabolic cell labelling with 32P-orthophosphate were also attempted (see Appendix B).

vii) Densitometry

Immunoblots were analyzed densitometrically using NIH Image v. 1.57. A background intensity reading was taken in an area corresponding to no visible signal and
was found to be 53; however relative intensity readings of 62 or below were undetectable. R.I. values of less than 62 were therefore taken as the baseline.

viii) Photography

Halo images were obtained using a Sony video camera module X575 connected to a dissecting microscope. Images were photographed at 60X magnification and edited in Northern Exposure v. 1.0. Leading edge images were obtained by connection of the time-lapse video recorder directly to a Mitsubishi thermal printer. Screen capture images were chosen at approximately equivalent trial durations.

ix) Statistics

Statistical comparisons of rates of cell migration were performed using the Mann-Whitney U test for nonparametric determination of variances between two means (Zar. 1984). P-values of 0.05 or greater were not considered significant.
Results

I) Characterization of the chemotactic response in axenic cell lines

i) Growth characteristics

Previous investigations using the radial bioassay employed parental strains in sexual crosses (NC-4 × V12; O’Day, 1979) or bacterially-grown V12 on agar plates (Browning et al., 1995). To this point analysis of axenic cells had not been performed. It was therefore necessary to determine the characteristics that would give an optimal chemotactic response in the two strains examined, AX-3 and JH10. As the parental progenitor of these two axenic strains (Williams et al., 1974) NC-4 was included for comparative purposes.

Growth rates for AX-3 and JH10 were determined as described in Methods (Fig. 2). From initial inoculant densities of 1 × 10^5 cells/mL, cell numbers were counted at four-hour intervals in duplicate 250 mL Erlenmeyer flasks shaken at 110 rpm, 21°C in the presence of light until no further increase in cell number was observed. AX-3 cell number did not increase for 24 hours, after which rapid increase in cell number proceeded for 24-32 hours, peaking at a cell density of 1.2 × 10^7 cells/mL. Significant cell lysis was evident above this density, and so no further readings were made. Active growth proceeded for JH10 on a longer time scale. Rapid increase in cell number began at 30 hours and proceeded at a slower rate. JH10 amoebae reached a higher density of 1.75 × 10^7 cells/mL at 84 hours growth after which no further measurements were made. NC-4 amoebae were grown on bacteria on SM agar plates just until clearance of the bacterial lawn, approximately 40-44 hours. Amoebal growth on agar plates made acquisition of cells at synchronous stages of development comparatively difficult, as even minor differences in rates of clearance of the bacterial lawn result in different levels of starvation, leading to different levels of development. For that reason, NC-4 was included initially as a comparative control but was not analyzed in depth in later experiments.
Figure 2. Growth rates of AX-3 (●) and JH10 (○) amoebae in liquid culture. Growth media and conditions were as described in Methods. Cell densities were measured at 4-hour intervals in duplicate flasks and counted until stationary cell densities or significant cell lysis was observed.
Figure 3. Halo formation in AX-3 migration to cAMP. Halo images were obtained photographically at the conclusion of chemotactic trials as outlined in Methods. Halo images are presented at 60X magnification. A) zero chemoattractant; B) 1 μM cAMP; C) 1 μM cAMP + 1% DMSO; D) 1 μM cAMP + 10 μM DEL; E) 1 μM cAMP − 20 μM DEL. Scale bar represents 0.2 mm.
ii) The Radial Chemotaxis Bioassay

Chemotaxis was measured using the radial chemotaxis bioassay developed by O'Day (1979) and modified by Browning et al. (1995). Drop diameters were measured at the beginning and end of an experiment and net change was calculated by subtracting initial diameter from final diameter. In all experiments at least ten drops were placed on each plate and each separate experiment was replicated a minimum of five times. Results were statistically confirmed where required. Assays in the absence of chemoattractant resulted in decreased drop diameter after the three-hour assay period (Fig. 3a). Decreased halo diameter corresponded to aggregative behaviour. Chemotactic movement was differentiated from trivial, random outward migration by the formation of a distinct halo of cells outside the perimeter of the initial drop (Fig. 3b-e) visible three hours after the beginning of a trial. Migration rate values were obtained by dividing net change in halo diameter by time of assay corrected for a lag period in which no cell movement was observed. Lag period was determined to be thirty minutes by videomicroscopic examination and was invariant under all experimental conditions employed.

iii) Chemotaxis is Chemoattractant, Starvation and Growth-Phase Dependent

Chemotactic response required the presence of chemoattractant in the agarose (Fig. 4, 5). Responses to a range of chemoattractant concentrations by AX-3 amoebae were measured. For chemotaxis to cAMP, concentrations of 0 μM, 0.1 μM, 1 μM, 5 μM, 10 μM and 20 μM were tested (Fig. 4). The largest halos, indicative of highest rate of migration, were observed at a concentration of 1 μM cAMP using mid-log growth amoebae. Positive (i.e. outward) migration was not observed for 0 or 0.1 μM cAMP. Positive migration was observed for 1, 5, 10 and 20 μM trials; however, compared to 1μM cAMP, halo diameters were generally smaller at 5 and 20 μM, corresponding to a reduced rate of migration while migration at 10 μM was indistinguishable from that at 1 μM. One μM cAMP was therefore employed in all further experiments. Folic acid chemotaxis was assayed using extracellular folate concentrations of 0, 10, 20, 40 and 80
Figure 4. Chemotactic responses of AX-3 amoebae to cAMP. Migration rates in the presence of varying levels of extracellular cAMP were determined. Experiment was replicated five times using duplicate plates for each trial and at least ten cell drops per plate in each experiment. A representative set of data is presented. Amoebae were starved as described in Methods and assayed for chemotactic mobility as described. Migration rate was determined by dividing average change in halo diameter by time course of the assay to express migration in $\mu$m/min.
Figure 5. Chemotactic responses of AX-3 amoebae to folic acid. Migration rates in the presence of varying levels of extracellular folic acid were determined as outlined in Methods. Experiment was replicated six times using duplicate plates in each trial. At least ten cell drops were placed on each plate. A representative data set is shown. Amoebae were harvested in mid-log growth and assayed for chemotactic mobility with no starvation period. Migration rate was determined by dividing average change in halo diameter by time course of assay to express migration in μm/min.
Chemoattractant concentration (pM FA)

Migration rate (μm/min)

- 0 μM
- 10 μM
- 20 μM
- 40 μM
- 60 μM
- 80 μM
µM folic acid (Fig. 5). Outward cell movement was not observed in the absence of chemoattractant. Cell movement for in the absence of chemoattractant (Fig. 5) was not accompanied by formation of a distinct halo of cells indicating that outward cell movement in this experiment was not chemotactic. Halo formation, indicative of directed cell movement, was observed at all chemoattractant concentrations, peaking at 40 µM and decreasing at higher concentrations. This trend of responses to variations in extracellular folic acid closely resembles that reported by Browning and colleagues (1995) for V12 amoebae, confirming the accuracy of this assay.

Not surprisingly, the duration of the starvation period also affected chemotactic response (Table 1). Chemotaxis to folic acid was maximal when assayed without a starvation period in both AX-3 amoebae (7.73 +/- 1.0 µm/min) and JH10 amoebae (2.33 +/- 0.45 µm/min). A 2 hour starvation period resulted in reduced migration to folic acid of at least 50% for both strains (3.39 +/- 1.5 µm/min for AX-3, 0.55 +/- 0.79 µm/min for JH10). Longer periods of starvation resulted in negative migration (Table 1) consistent with the aggregation of amoebae within the site of the original drop placement. Response to cAMP was low but present at 0 hours, showing positive rates of migration of 1.38 +/- 0.096 µm/min in AX-3 amoebae and 0.61 +/- 0.06 µm/min in JH10. A 2 hour starvation period greatly increased response to cAMP. AX-3 migration rates increased to 8.47 +/- 0.59 µm/min. JH10 halo diameters showed a similar level of increase, peaking at 7.4 +/- 0.67 µm/min. AX-3 and JH10 amoebae both showed decreased cell movement in response to increased starvation periods; however the drop-off in motility was much more evident in AX-3 amoebae than in JH10 (Table 1).

Amoebae of both AX-3 and JH10 were found to exhibit chemotactic responses to cAMP dependent whether cells were actively growing or not (Fig. 6). Responses were examined at the onset of rapid increase in cell number during active growth and at high cell density where active growth levels were decreasing. Chemotaxis was not measured prior to the onset of rapid cell division or after it had ceased. "Early" was defined for AX-3 cells as cell density of 2-5 X 10^6 cells/mL, "mid" was between 5 X 10^6 and 9 X 10^6 cells/mL and "late" corresponded to densities of 9 X 10^6 cells/mL to 1.1 X 10^7 cells/mL. JH10 culture phases were designated as 2-7 X 10^6 cells/mL for "early". 7 - 12 X 10^6
Table 1. Effects of starvation time on migration rate

<table>
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<tr>
<th>Starvation period (Hr)</th>
<th>Migration rate (μm/min) - AX-3</th>
<th>Migration rate (μm/min) - JH10</th>
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<tr>
<td></td>
<td>1 μM cAMP 40 μM FA</td>
<td>1 μM cAMP 40 μM FA</td>
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<tr>
<td>0</td>
<td>1.38 +/- 0.96 7.73 +/- 1.04</td>
<td>0.61 +/- 0.06 2.33 +/- 0.45</td>
</tr>
<tr>
<td>2</td>
<td>8.47 +/- 0.59 3.39 +/- 1.46</td>
<td>7.4 +/- 0.67 0.55 +/- 0.79</td>
</tr>
<tr>
<td>4</td>
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<td>6.61 +/- 0.45 *</td>
</tr>
<tr>
<td>6</td>
<td>0.50 +/- 0.41 *</td>
<td>4.55 +/- 0.85 *</td>
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</table>

Amoebae were harvested from liquid cultures and starved for the time indicated as described in Methods. Migration rate was determined by dividing net change in halo diameter by time of assay. 1 μM cAMP was employed as the chemoattractant in all cAMP experiments and 40 μM folic acid (FA) in all folic acid trials. Negative migration is indicated by "*".
Figure 6. Growth-phase dependence of chemotactic response to cAMP. Migration rates in early ("low") growth, rapid cell division ("mid") and high ("high") cell density of AX-3 amoebae (black bars) and JH10 amoebae (striped bars) in liquid HL-5C culture media were determined. A representative data set is shown. Experiment was repeated five times using duplicate plates in each trial and at least ten cell drops per plate. Growth phases were defined as described in Results. AX-3 amoebae were harvested at growth densities of $3.3 \times 10^6$ cells/mL, $7.0 \times 10^6$ cells/mL and $1.1 \times 10^7$ cells/mL for low, mid and high trials respectively. Corresponding cell densities for JH10 were $4.0 \times 10^6$ cell/mL, $1.1 \times 10^7$ cells/mL and $1.6 \times 10^7$ cells/mL. Migration rates were determined from average changes in halo diameters as described in Methods.
cells/ml for "mid" and 1.2 - 1.7 X 10^7 cells/mL for "late". AX-3 amoebae were harvested at an average cell density of 3.3 X 10^6 cells/mL in the "early" growth trials. 7.0 X 10^6 cells/mL for "mid" and 1.1 X 10^7 cells/mL in "late" phase. Corresponding cell densities for JH10 were 4.0 X 10^6 cells/mL. 1.1 X 10^7 cells/mL and 1.6 X 10^7 cells/mL (Fig. 6). Because of the close parallels observed in responses of amoebae to chemoattractant concentration and duration of starvation period, and because folic acid-directed chemotactic responses were generally much smaller than those to cAMP, growth-phase dependent responses to folic acid were not measured.

II) Effects of deltamethrin

i) Effects of deltamethrin on rates of cell migration

The effects of the type II synthetic pyrethroid deltamethrin (DEL) were determined. DEL is a potent inhibitor of CN activity resulting in markedly increased phosphorylation levels in rat brain synaptosomes at subnanomolar levels of application (Enan and Matsumura, 1992). Permethrin, a type I synthetic pyrethroid differing in structure from deltamethrin only in lacking a cyano sidechain (Enan and Matsumura, 1992) was employed as a pharmacological control since it has been shown to have no effect on CN activity (Enan and Matsumura, 1992). Amoebae in the presence of DEL showed no significant variation in halo diameter size in migration to cAMP (Fig. 7). Attempts were made to observe the effects of the drug at higher concentrations to resolve the question of dose-dependence: however 30 μM DEL was observed to precipitate out of the agarose forming a cloudy white precipitate and preventing homogenous distribution of the drug, making accurate assessment of its effects impossible. At this dosage the concentration of the solvent employed (1% DMSO), although seemingly high, showed no significant effect on migration rates obtained in control trials (Fig. 7, compare 1 μM cAMP bars to 1% DMSO bars) except in migration of JH10 amoebae to cAMP where migration rate was reduced from 4.87 +/- 1.18 μm/min in the absence of 1% DMSO to 3.0 +/- 0.847 μm/min.
Figure 7. Effects of deltamethrin on chemotaxis to cAMP. Changes in the average change in halo diameters in the presence of indicated concentrations of deltamethrin were determined. Experiment was replicated five times using duplicate plates in each trial. At least ten cell drops were placed on each plate. A representative data set is shown. 1 μM cAMP was present in all plates except the “zero chemoattractant” trial. 1% DMSO was present in all experiments except the “zero chemoattractant” and “1 μM cAMP”. AX-3 (black bars) and JH10 (striped bars) amoebae were starved to chemotactic competence to cAMP as described in Methods. Migration rates were calculated from average changes in halo diameters as described in Methods.
Figure 8. Effects of permethrin on chemotaxis to cAMP. Changes in the average halo diameter in the presence of the indicated concentrations of permethrin were determined in AX-3 amoebae. The experiment was replicated at least five times using duplicate plates in each trial and at least ten cell drops per plate. 1 μM cAMP and 1% DMSO were present in all plates. Amoebae were starved to chemotactic competence to cAMP as described in Methods. Migration rates were calculated from average changes in halo diameters as described in Methods.
In contrast, migration rates were significantly higher in the presence of deltamethrin in chemotaxis to folic acid (Fig. 9). AX-3 amoebae showed large increases in halo diameter and corresponding migration rate at 10 μM deltamethrin (Fig. 9 - from 2.93 +/- 0.56 μm/min to 4.27 +/- 0.71 μm/min. p=0.0005). This effect was not dose-dependent at the concentrations used as migration rates at 20 μM deltamethrin did not differ significantly from the rate in the presence of 10 μM deltamethrin (3.8 +/- 0.63 μm/min. p=0.18). JH10 showed a similar increase in migration rate in the presence of 10 μM deltamethrin (Fig. 9 - from 2.67 +/- 0.447 μm/min to 4.87 +/- 0.893 μm/min. p=0.0002). Migration rate at 20 μM deltamethrin was not significantly different from that at the lower concentration (4.47 +/- 0.55 μm/min. p=0.16).

Migration rates of AX-3 amoebae were also determined in the presence of the type I pyrethroid permethrin. No significant difference was observed between the effects of 10 μM deltamethrin or 10 μM permethrin (Fig. 7 3.83 +/- 1.37 μm/min vs. Fig. 8 3.51 +/- 1.23 μm/min. p=0.47) on migration rate to cAMP. No significant differences in effects were observed at higher concentrations of permethrin. Chemotactic migration rate was significantly higher to folic acid in the presence of 10 μM deltamethrin than in the presence of 10 μM permethrin (Fig. 9 4.27 +/- 0.71 μm/min vs. Fig. 10 2.47 +/- 0.63 μm/min. p=0.0002).

Migration rates determined for NC-4 amoebae under identical conditions are included for comparative purposes in Fig. 11. Chemotaxis to cAMP by NC-4 amoebae in the absence of DEL was calculated to be 18.8 +/- 1.54 μm/min. No significant difference was observed in the presence of 10 μM DEL (18.9 +/- 2.25 μm/min. p = 0.832). 20 μM DEL was found to result in a significant difference in migration rate (22.6 +/- 2.53 μm/min. p = 0.014) as was 30 μM DEL (21.2 +/- 1.95 μm/min. p = 0.012). The migration rate of vegetative amoebae to folic acid was found to be 3.27 +/- 0.37 μm/min. In the presence of 10 μM DEL migration rate increased significantly to 5.93 +/- 1.39 μm/min. p = 0.0007. Both 20 μM DEL (6.93 +/- 0.56 μm/min. p > 0.0001) and 30 μM DEL (7.26 +/- 0.8 μm/min. p = 0.0001) also resulted in significantly increased migration rates.
Figure 9. Effects of deltamethrin on chemotaxis to folic acid. Changes in the average change in halo diameters in the presence of indicated concentrations of deltamethrin were determined. AX-3 (black bars) and JH10 (striped bars) amoebae were harvested in vegetative growth as described in Methods. The experiment was replicated five times using duplicate plates in each trial and at least twelve cell drops on each plate. 40 μM folic acid was used as the chemoattractant in all plates except the “zero chemoattractant” condition. 1% DMSO was present in all experiments except the “zero chemoattractant” and “40 μM folic acid” trials. Migration rates were calculated from average changes in halo diameters as described in Methods.
Figure 10. Effects of permethrin on chemotaxis to folic acid. Changes in the average change in halo diameter in the presence of the indicated concentrations of permethrin were determined for AX-3 amoebae. The experiment was repeated six times using duplicate plates in each trial and at least ten cell drops per plate. 40 μM folic acid and 1% DMSO were present in all experiments. Amoebae were harvested in vegetative growth and migration rates were calculated from average change in halo diameter as described in Methods.
Figure 11. Effects of deltamethrin on chemotactic response of NC-4 amoebae to cAMP. Amoebae were harvested from SM agar plates at clearance of the bacterial lawn and starved to obtain chemotactic competence as described in Methods. Migration rates were calculated from average changes in halo diameters for chemotaxis to cAMP (black bars) and folic acid (FA) (striped bars) as described in Methods. The experiment was repeated five times using duplicate plates in each trial and at least ten cell drops per plate. No chemoattractant was present in the “zero chemoattractant” trials. 1% DMSO was present in all experiments except the “zero chemoattractant” and “max. cmt.” trials. Maximum chemoattractant concentrations as determined (1 µM cAMP, 40 µM FA) were employed for bars labelled "max. cmt" and all subsequent experiments.
Omax. cmt 1% DMSO 10 μM DEL 20 μM DEL 30 μM DEL

Migration rate (μm/min)

0 max. cmt 1% DMSO 10 μM DEL 20 μM DEL 30 μM DEL
ii) **Comparison of rates obtained from video recordings and halo measurements**

Calculations of chemotactic rate were made indirectly by measurement of the changes in halo diameter (Fig. 3) and by direct observation of migrating amoebae using video microscopy (Fig. 12). In Figure 3, a magnification sequence of drops produced in the radial bioassay is presented. Under the higher magnification used for time-lapse videomicrography images individual amoebae were visible at the periphery of cell drops (Fig. 12). Calculations were made from plates by dividing net change in halo diameter by time of assay corrected for a thirty minute lag period as described in Methods. Migration rates from video recordings were calculated as a vectoral displacement by determining linear migration from initial position on the monitor screen, converting to actual distance migrated and divided by time of filming to obtain an individual speed in μm/min (Fig. 13).

Significant differences were observed between migration rates obtained from calculations of vectoral displacement and average changes in halo diameter. The migration rate of AX-3 amoebae to cAMP was calculated from video recordings to be 28.5 +/- 3.6 μm/min which is much higher than rates published in the literature. Migration rates calculated from changes in halo diameters ranged between 3.1 +/- 1.2 μm/min (Fig. 7) to 8.5 +/- 0.6 μm/min (Fig. 6) in agreement with literature values of 9.0 μm/min (Browning et al., 1995).

**III) Deltamethrin increases phosphorylation of several proteins in Dictyostelium**

In an attempt to identify potential substrate proteins dephosphorylated by CN, AX-3 amoebae were starved to obtain chemotactic competence in 15mM Tris, pH 6.5 in the presence of CN inhibitors. One μM cAMP was added to the starving cells at 105 minutes of starvation and incubated for 15 minutes. After the 2 hour starvation period, amoebae were pelleted in a microfuge for 30 seconds and resuspended in 2X SDS sample buffer to stop all metabolic reactions. Protein levels in each sample were determined (Appendix A. Fig. A1) and samples were loaded onto a 12.5% polyacrylamide gel (Appendix A. Fig. A2a). Commercial monoclonal antibodies raised against phosphoserine detected as many
Figure 12. Video images of cAMP-competent leading edge AX-3 amoebae. Amoebae were made chemotactically competent to cAMP as described in Methods. Video recordings of leading edge amoebae were made under various experimental conditions as described. Individual images were obtained as described in Methods. A) zero chemoattractant; B) 1 μM cAMP; C) 1 μM cAMP + 1% DMSO; D) 1 μM cAMP + 10 μM DEL; E) 1 μM cAMP + 20 μM DEL. Scale bar equals 65 μm.
Figure 13. Cell migration rates of AX-3 amoebae to cAMP from video recordings (black bars) and halo diameters (white bars). Calculated rates of cell migration were compared as determined from determination of linear displacement from video recordings and from changes in average halo diameter obtained from plates.
as twenty-one distinct proteins in whole cell extracts (Fig. 14). Blots were 
densitometrically scanned and relative intensities recorded (Table 2). Using a curve 
fitting function of Macintosh Deltagraph Professional a table of relative mobilities (Rf) 
was generated and these were matched with relative intensities (Table 3). Cells are 
chemotactically responsive to both chemoattractants examined at 2 hours of starvation 
(Table 1). Possible targets for the action of DEL were therefore identified by 
examination of changes in relative signal intensity between lanes containing 
chemotactically-competent cell extracts in the presence of 20 μM DEL alone (lane 2), 1 
μM cAMP alone (lane 3) or both 20 μM DEL and 1 μM cAMP (lane 4). Phosphoserine-
labelled bands in lane 4 that showed an increase in R.I. of less than 20 percent were not 
considered significant in this analysis. Sixteen bands ranging in estimated molecular 
weight between 185.6 kD and 42.6 kD show significant increase in phosphoserine 
incorporation in the presence of DEL and cAMP (Table 4). Increases in phosphoserine 
incorporation in lane 4 were similar when compared to phosphoserine signal intensities in 
lanes 2 and 3 with four exceptions. Three band (bands M, P, Q) showed significantly 
increased phosphoserine incorporation between lane 2 and 4, but not between lanes 3 and 
4 (Table 2). Band O showed an increased band intensity between lanes 3 and 4 but not 
between lanes 2 and 4.
Figure 14. Immunolocalization of phosphoserine-containing proteins. Cell extracts of AX-3 amoebae were probed with a monoclonal antibody against phosphoserine under control conditions and in the presence of the CN inhibitor 20 μM deltamethrin as described in Methods. Lane 1 - 1% DMSO. Lane 2 - 20 μM DEL. Lane 3 - 1% DMSO + 1 μM cAMP. Lane 4 - 20 μM DEL + 1 μM cAMP.
Table 2. Serine phosphorylation of Dictyostelium proteins

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<tr>
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Serine-phosphorylated proteins were identified using a monoclonal antibody against phosphoserine and detected by chemiluminescence as described in Methods. Relative intensity (R.I.) was determined by densitometry as described in Methods. R.I. values below 62 were not visible and were taken as the baseline. Lanes correspond to lanes as labelled in Figure 14.
Table 3. Serine phosphorylation in chemotactically competent AX-3 amoebae

<table>
<thead>
<tr>
<th>Band</th>
<th>Lane 3 Rf (mm)</th>
<th>Est. mw</th>
<th>R.I.</th>
<th>Lane 4 Rf (mm)</th>
<th>Est. mw</th>
<th>R.I.</th>
</tr>
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<tr>
<td>A</td>
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<td>56</td>
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<td>153</td>
</tr>
<tr>
<td>B</td>
<td>4.5</td>
<td>158.88</td>
<td>57</td>
<td>4.5</td>
<td>158.88</td>
<td>155</td>
</tr>
<tr>
<td>C</td>
<td>5.8</td>
<td>132.27</td>
<td>64</td>
<td>5.8</td>
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<td>177</td>
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<tr>
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<td>120.83</td>
<td>59</td>
<td>6.2</td>
<td>125.52</td>
<td>165</td>
</tr>
<tr>
<td>E</td>
<td>not visible</td>
<td>---</td>
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<td>99.13</td>
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<td>79.20</td>
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<td>150</td>
</tr>
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<td>H</td>
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<tr>
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<td>77</td>
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<td>99</td>
</tr>
<tr>
<td>L</td>
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<td>162</td>
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<td>220</td>
</tr>
<tr>
<td>M</td>
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<tr>
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<tr>
<td>O</td>
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<td>18.5</td>
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<td>149</td>
</tr>
<tr>
<td>P</td>
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<td>27.60</td>
<td>239</td>
<td>31.5</td>
<td>29.16</td>
<td>243</td>
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</tbody>
</table>

Amoebae were harvested and starved to obtain chemotactic competence to cAMP as described in Methods. Amoebae were starved in the presence of 20 μM deltamethrin (lane 4, Fig. 14) and serine phosphorylation levels as determined immunologically were compared to control proteins in the absence of deltamethrin (lane 3, Fig. 14). Migration distances (Rf) were measured and corresponding molecular weights (Est. mW) determined using molecular weight marker proteins (Gibco) and a curve constructed in Macintosh Deltagraph Professional 1.0 at a polynomial function of 4.
Table 4. Possible CN substrates in chemotactically competent AX-3 amoebae

<table>
<thead>
<tr>
<th>Band</th>
<th>R.I.</th>
<th>Lane 4</th>
<th>% increase</th>
<th>Lane 4 Rf (mm)</th>
<th>Est. mw</th>
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</thead>
<tbody>
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</tr>
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</tr>
<tr>
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<td>172.0</td>
<td>4.5</td>
<td>158.88</td>
</tr>
<tr>
<td>C</td>
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<td>177</td>
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<tr>
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<tr>
<td>G</td>
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<td>21.0</td>
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Serine phosphorylation levels were determined as described in Methods and in Figure 14. Relative intensity (R.I.) of phosphoserine signals in amoebae chemotactically competent to cAMP were compared between amoebae in the presence of 20 μM DEL alone (lane 2), 1 μM cAMP alone (lane 3) or 20 μM DEL and 1 μM cAMP (lane 4). Migration distances were determined as described in Methods and estimated molecular weights (Est. mw) calculated from a curve generated in Macintosh Deltagraph Professional 1.0. Band numbers correspond to those as labelled in Figure 14. Unless otherwise indicated proteins displayed similar increases in phosphoserine incorporation in lane 4 in comparison to either lane 2 or lane 3. Asterisks (*) indicate proteins that showed a significant increase in phosphoserine incorporation between lanes 2 and 4 only. Band O (#) showed significantly increased phosphoserine incorporation between lanes 3 and 4 only.
Discussion

Phosphoprotein phosphatases act in concert with kinases to regulate intracellular responses. Historically, dephosphorylation was perceived as a step important only in returning the intracellular phosphorylation balance to normal in preparation for stimulation by the next kinase cascade. The discovery of basal levels of phosphatase activity (Pallen et al., 1992), as well as the multitude of regulatory subunits and binding proteins associated with several phosphatases, not to mention the high number of distinct phosphatases themselves, argue against this hypothesis (Hunter, 1995). As the only known phosphatase directly regulated by an intracellular second messenger, CN is a prime candidate to be involved in Ca\(^{2+}\)-dependent processes of signal transduction. The hunt for CN substrates has been most successful in cell types whose function is regulated by rapid Ca\(^{2+}\) flux, such as neutrophil chemokinesis (Hendey et al., 1992) and the elongating growth cone of the growing neuron (Liu et al., 1994). Ca\(^{2+}\) flux is also present in cAMP-mediated signalling through cAMP cell-surface receptors (Milne and Devreotes, 1993). This fact, coupled with the history of investigation of Ca\(^{2+}\)-regulated events in signal transduction in this laboratory, led to the investigation of the role of CN in chemotactic signalling in *Dictyostelium*. Previous reports have identified several of the classic components of eukaryotic transmembrane signalling including membrane-bound adenosine receptors (Lewis and O'Day, 1994), GTP-binding proteins (Lewis et al., 1994) and PKC (Gunther et al., 1995). Additionally, and quite by surprise, a 31-kD protein was found in developing sexual cultures to be dephosphorylated over time in the presence of Ca\(^{2+}\)/CaM, suggesting the activity of the Ca\(^{2+}\)/CaM-dependent phosphatase CN in sexual development in *Dictyostelium* (Lydan and O'Day, 1993). It was therefore of interest to examine CN and its possible involvement in the process of chemotaxis since chemotaxis is a key component in events of both sexual and asexual development. While the present work was underway,
the catalytic subunit was cloned, sequenced and the gene knocked out by homologous recombination (Lydan et al., 1995; Dammann et al., 1996) producing the cn- cell line in the thymidine auxotrophic cell line JH10.

The chemotactic responses to cAMP and folic acid in axenic strains and their original parental counterpart NC-4 were characterized. This was important since the radial chemotaxis bioassay was to serve as the primary chemotactic evaluation method for future research in this laboratory on null mutants for calmodulin-binding proteins (e.g. Lydan et al., in preparation). To date, no studies had specifically addressed the chemotactic responses of JH10 or its parental cell line AX-3. Furthermore, a comparative analysis of chemotaxis of diverse cell strains to the two major chemoattractants, cAMP and folic acid, which serve different purposes in cellular slime mould development should increase our understanding of this fundamental process. For these reasons, chemotaxis in both vegetative amoebae and in amoebae in the early stages of aggregation competence was examined. Chemotactic mobility as measured by the radial bioassay was significantly higher in NC-4 amoebae than in either of the axenic strains. The reason for this difference remains to be resolved but it may reflect primary defects in the chemotactic ability of the mutant axenic strains. While axenically-grown amoebae are arguably the most popular cell types currently in use by Dictyostelium researchers, the axenic mutation itself is as yet uncharacterized. If these strains are to be used for continued studies then more insight into their basic biology and possible defects is essential. It has been speculated that the axenic mutation may involve expression of the discoidin family of proteins (Burdine and Clarke, 1995). Expression of the cytoplasmic discoidin proteins is altered in axenic amoebae but since discoidin proteins have not been observed in association with the plasma membrane it seems unlikely that discoidins would have a direct involvement in substratum-membrane binding. Additionally discoidinless mutants show instantaneous velocities and directional persistence in migration comparable to normal axenic cells,
casting doubt upon the involvement of discoidins in chemotactic movement. Differences in mobilities between the AX-3 cell line and the thymidine auxotroph JH10 should be examined with some care (Fig. 7, 9) since JH10 growth in liquid culture is significantly slower than AX-3 under the conditions examined (Fig. 2). Differences in growth characteristics could conceivably result from altered levels of prestarvation factor (PSF) and/or conditioned media factor (CMF) two developmental morphogens secreted in response to different conditions of population density (Burdine and Clarke, 1995; Yuen et al., 1995). For example, the level of cAR expression on the cell surface has been directly linked to the level of CMF in the extracellular milieu (van Haastert et al., 1996). An altered response to cAMP could therefore be an artifact of thymidine addition and not due to actual differences in migratory abilities of the two cell types. Examination of chemotactic responses by JH10 under varying levels of thymidine addition would help to resolve this question. This hypothesis could also be tested by addition of CMF to growth media of lag phase or early log phase amoebae, growth conditions under which CMF levels are probably low, and determination of the resultant rate of cell migration in comparison to that under normal exposure to CMF. PSF, necessary for the transition of bacterially-grown amoebae from the vegetative state to a state of aggregation competence (Burdine and Clarke, 1995) could be tested in a similar manner. Presumably increased levels of PSF, concurrent with disappearance of the bacterial food source, lead to induction of expression of cellular factors necessary to obtain cAMP-response competence.

Measurements of the rates of cell migration in the presence of the type II synthetic pyrethroid deltamethrin (DEL) gave results that on the surface appear contradictory. This compound has displayed high affinity for CN with an IC_{50} in the subnanomolar range (0.03 nM; Enan and Matsumura, 1992). Chemotaxis to cAMP as measured by the radial bioassay was unaffected by the addition of DEL (Fig. 7) or by the addition of the non-
inhibitory permethrin (PER; Fig. 8). Conversely migration rates to folic acid were seen to significantly increase in the presence of DEL (Fig. 9) but not in the presence of PER (Fig. 10). This increase was similar to that observed in CN null mutant cell lines where rate of migration increased by over 50% (Lydan et al., in preparation). In support of these data expression of CN mRNA and the catalytic subunit is high in vegetative cells which are chemotactically responsive to FA and drops to undetectable levels in early starvation at the same time that cells are developing chemotactic competence to cAMP (Dammann et al., 1996). Probing of cell extracts starved for 2 hours with a commercially-obtained antibody raised against the rabbit CNA detected no catalytic subunit at this stage of development (M.A. Lydan, pers. comm.). Since this antibody may not recognize a Dictyostelium antigen, probing of vegetative cell extracts and those starved for extended periods (i.e. at least 8 hours) as well as purified Dictyostelium CN will be necessary to draw any final conclusions about these results.

When attempts were made examine the migratory ability of individual amoebae using video microscopy a dose-dependent decrease in the rate of chemotaxis was observed (Fig. 13) in the presence of DEL. This data must be interpreted with care. The calculated rate of cell migration for chemotaxis to cAMP from video microscopic data was 28.5 +/- 3.7 μm/min, nearly three times higher than other values reported for chemotaxis to cAMP (Browning et al., 1995). Since estimates of cell migration only measured linear displacement from an initial starting point, deviations from a straight-line path would not be measured, leading to an estimation of migration rate lower than the actual migration observed. Confining calculations of migration to amoebae at the leading edge leaves open the possibility of obtaining data skewed by the inclusion of amoebae exhibiting faster than normal motility. The potentially inhibitory effects of the intense, continuous light essential for videomicroscopy on cell movement might also be involved in decreasing the rate of chemotaxis and should be further analyzed. For these reasons the values for rate of
chemotaxis obtained by examination of average change in halo diameter under the standard experimental setup of Browning et al. (1995) provide a more accurate estimate of the effect of CN inhibition on cell migration since these result from measurements of populational migrations and not single amoebae. and are less likely to be distorted by abnormally high rates of cell movement in any single cells. The radial bioassay has proven useful in examining populational responses to chemotactic stimulation and net responses to pharmacological inhibitors of several components of the chemotactic process and has generated estimations of cell migration rates in agreement with those obtained using other methods of assessment (Browning et al., 1995). The radial bioassay cannot however reliably distinguish individual morphological characteristics of migrating amoebae such as directional persistence as the precise direction of gradient formation cannot be ascertained. The radial bioassay is also not best suited for assessment of shape changes of individual amoebae in response to chemotactic stimulation. The radial chemotactic bioassay is an excellent method for examination of populational responses of amoebae to chemotactic stimulation.

Rates of chemotactic migration to cAMP in cn- mutants were found to be approximately 50% higher than those of corresponding wild-type axenic amoebae (Lydan et al., in preparation). Pharmacological data presented here does not support this, as no significant effect was seen in the presence of DEL (Fig. 7). The possible reasons for these contradictory findings are twofold. The inhibitor may not be 100% effective. Since complete removal of the gene results in 50% increase in the rate of migration the drug-induced effects should be somewhat less, and more variable. Future work should include analysis of the effect of DEL on CN with an aim toward clarifying its level of effectiveness. An increased rate of chemotaxis to folic acid was observed in the presence of DEL (Fig. 9). This suggests a possible role for CN in regulation of some component of the chemotactic machinery common to both folic acid and cAMP at an early stage. The
chemotactic response of the null mutant to FA was not attempted. These experiments are necessary and will add further insight into the mechanisms of regulation of chemotactic cell movement. Positive identification of the common members of the chemotactic processes to both cAMP and folic acid is required to address this question. An approach to resolving this question would involve comparison of serine-threonine phosphorylation profiles of proteins from DEL-treated and untreated vegetative cells to identify possible CN substrates.

Despite the apparent lack of effect on chemotaxis to cAMP as measured by the radial bioassay, chemotaxis to FA was found to be strongly increased in the presence of DEL suggesting a role for CN in mediation of the chemotactic response. Additionally chemotaxis to cAMP was shown in CN null mutant cell lines to be increased by approximately 50% indicating a possible involvement for CN in chemotaxis in Dictyostelium. Chemotactically competent amoebae were therefore examined for possible CN substrates. Monoclonal antiphosphoserine antibodies recognize several proteins in Dictyostelium (Fig. 14). At least twenty-one distinct bands show phosphoserine labelling in cAMP-stimulated amoebae. Densitometric analysis of these bands compared to bands generated in the absence of DEL resulted in the identification of sixteen proteins showing significant differences in level of phosphoserine in the presence of DEL. This is by no means an exhaustive roster of CN substrates. CN also removes phosphate from threonine residues, and these would not be visible under these assay conditions. Identification of phosphothreonine-containing proteins was attempted under similar conditions using a monoclonal antibody raised against phosphothreonine; poor quality of the antibody resulted in no visible signals for phosphothreonine (not shown). Positive identification of CN substrates in Dictyostelium will await further experimentation; however several targets for CN activity have been described in other eukaryotic systems and it is possible that these could be common targets in multiple signalling systems. Three known CN targets
are dynamin I, a protein involved in vesicle recycling in the rat brain synapse; tau, a microtubule-associated protein and GAP-43 (growth-associated protein 43), a protein involved in reorganization of cortical F-actin in the neural growth cone (Chang et al., 1996). Interestingly, phosphorylation of GAP-43 decreases the concentration of PIP$_2$ in the immediate vicinity. Decreased PIP$_2$ would result in increased gelsolin-mediated actin filament severing. This is postulated to result in growth cone retraction; however filament severing is hypothesized to be a major contributor to the rapid increase in number of cortical actin nucleation sites as a precursor to pseudopodial extension according to the cortical expansion model (Condeelis et al., 1993). Increased phospho-GAP-43 would lead to increased filament severing increasing nucleation sites and the number of actin filaments present in the cortical cytoplasm in response to receptor stimulation. GAP-43 has been reported to range in apparent molecular weight between 46 and 50 kD. One candidate band in this CN activity assay (band N) exhibits an estimated molecular weight of 48.6 kD (Table 4) in agreement with reported sizes of GAP-43. Relative intensity of band N increases moderately (by 20.8%) in CN-inhibited cell extracts (R.I. 162 in lane 3 - 196 in lane 4; Table 3, 4; Fig. 14). GAP-43 has not been reported in Dictyostelium, so this remains conjectural.

CN has been directly implicated in migration of neutrophils on vitronectin (Hendey et al., 1992) in recycling of integrins from the tail region of migrating neutrophils back to the leading edge. Inhibition of CN activity by the immunosuppressant inhibitors FK506 and CyS resulted in prolonged attachment of the tail to the substrate leading to decreased cell migration (Hendey et al., 1992). Integrins in the tail region are recycled to the front of the migrating neutrophil by enclosure in vesicles which are then shuttled to the front where they are reinserted in the membrane. Dynamin I is a protein shown to be involved in vesicle recycling (Liu et al., 1994) and it is possible that CN inhibition of integrin recycling could function via blockage of the vesicle cycle. Integrins have not been identified in
**Dictyostelium.** The precise mechanism by which Dictyostelium pseudopods attach to the substratum is presently unclear. A phosphoprotein of apparent molecular weight similar to mammalian dynamin I was identified by significant increase in serine phosphorylation in the presence of DEL (Fig. 14. lane 4, band E) suggesting that dynamin I may be present in Dictyostelium. A third well-characterized CN substrate is the microtubule-associated protein tau (Ferreira et al., 1993). Tau proteins associate with microtubules in growing neurons and mediate assembly, leading to neurite outgrowth. Tau is serine-and threonine-phosphorylated, and hyperphosphorylated tau cannot associate with microtubules. CN can dephosphorylate all six phosphoserines of tau (Fleming and Johnson, 1995) and tau phosphorylation has been shown to be both Ca$^{2+}$ and cAMP-regulated. If present in Dictyostelium, tau may be among the phosphorylated proteins seen here.

The involvement of CN in transcription of immediate early genes from activated T-cell receptors suggests the presence of CN substrates in the transcriptional machinery. CN was in fact recently shown to be involved in nuclear shuttling of the cytoplasmic transcription factor NF-AT4. CN has also been shown to be involved in association of the cAMP response binding element (CREB) to its binding protein (CBP) and the association of this complex with the cAMP-responsive element (CRE) in the promoter region of cAMP-responsive genes (Hunter, 1995). cAMP-responsive genes are well-documented in Dictyostelium and many are vital components of signalling pathways including the mPD, cAR1, discoidin I and gp80 (Desbarats et al., 1992). CN involvement in cAMP-induced gene transcription has been shown in higher eukaryotes via the dephosphorylation of CREB leading to the dissociation of the CREB-CBP complex (reviewed by Hunter, 1995). CREB is phosphorylated primarily by cAPK. Interestingly, data has been provided suggesting a subcellular localization of the regulatory subunit of cAPK and CN in the bovine brain (Coghlan et al., 1995) via the A-kinase anchor protein AKAP79. mRNA expression of both the regulatory subunit of cAPK and CN show similar time courses,
peaking in stages of late aggregation (Simon et al., 1992; Dammann et al., 1996). This evidence taken together suggests that CN may be involved in transcription of cAMP-induced genes in *Dictyostelium*. As cAPK is proposed to be the key mediator in events of terminal cell differentiation in multicellular stages (Reymond et al., 1995) this suggests a role for CN in morphogenesis and terminal differentiation in multicellular development and culmination. While CN appears to modulate chemotaxis, inhibition may be having more severe long-term effects on the potential of cells to differentiate. A future avenue of investigation might be examination of CN inhibition in morphogenesis and terminal differentiation of prestalk and prespore cells. Examination of CN activity at later stages of development might prove more interesting in identifying possible substrates for this enzyme in cAPK-mediated differentiation processes. Identification of an AKAP homologue in *Dictyostelium* would lend strong support to these hypotheses. Additional, parallel investigations into the role of CN in vegetative growth may also be rewarding in identification of targets of CN in chemotaxis. While this work was in progress the developmental time course of expression of both the mRNA and the CN catalytic subunit (CNA) was reported (Dammann et al., 1996). Both the mRNA and the CNA were found to be present in vegetative cells. However both dropped below detectable levels in early starvation of amoebae (from 0-8 hours starvation) and increased thereafter, peaking at culmination (Dammann et al., 1996). Examination of chemotaxis to FA and to amoebae starved for extended periods in the cn+ cell line are immediate follow-ups to this research. Examination of phosphoserine and phosphothreonine patterns in the presence and absence of DEL in both axenic cell lines and the cn- mutant strain under these conditions would be relevant in the search for roles for CN in *Dictyostelium* chemotaxis.
SUMMARY

The process of chemotaxis in axenic strains of *Dictyostelium discoideum* was examined and parameters leading to maximum chemotactic response in these cell lines were determined for use in future investigations. The axenic cell lines AX-3 and JH10, a thymidine auxotroph derived from AX-3 both showed chemotactic motilities significantly slower than the wild-type NC-4 from which they were derived. Both axenic cell lines showed maximal chemotaxis to cAMP when grown to a mid-log cell density between 6-9 X 10^6 cells/mL, starved for 2 hours and examined via the radial bioassay in the presence of 1 µM extracellular cAMP. Chemotaxis to folic acid was maximal in mid-log amoebae; however migration rate was highest in unstarved cells. 40 µM folic acid was found to produce the optimal extracellular concentration.

In the presence of the type II synthetic pyrethroid deltamethrin, a specific CN inhibitor cAMP-mediated chemotaxis showed no significant deviation from control amoebae. Permethrin, a compound similar in structure to DEL but with no CN-inhibitory properties also did not affect chemotaxis to cAMP. Conversely, DEL resulted in a significant increase in the migration rate of vegetative amoebae to FA while no effect was seen in cells exposed to PER.

Amoebae made chemotactically responsive to cAMP were examined for possible CN substrates. Monoclonal antibodies raised against phosphoserine identified sixteen possible proteins that showed increased incorporation of phosphoserine in the presence of DEL.
Appendix A.

Protein Determination, Gel Loading and Confirmation of Antibody Specificity

i) Protein Determination and Gel Loading

A modified Bradford assay was employed in the determination of protein, according to the methods as outlined for the Bio-Rad microassay procedure (Bio-Rad) using bovine serum albumin as the standard at 595 nm. Serial dilutions were prepared and added to the diluted copper reagent. Samples were allowed to mix and OD\textsubscript{595} measured 5 minutes after. For consistency of gel loading, optical densities of samples under various experimental conditions were obtained and protein levels determined by interpolation on the curve constructed (Fig. A1). Volumes loaded per well were adjusted to 5 μg protein per well and gels were run as described in Methods (Fig A2).

ii) Determination of antibody specificity

Specificity of the antibody was tested by preincubation of monoclonal primary antibody raised against phosphoserine (Sigma). Antibody was diluted in normal probing solution as described in Methods in the presence of 25 mM phospho-D-serine (Sigma) for sixty minutes. Control preincubated serum was then used to probe an experimental blot as described in Methods (Fig A3a). Faint signals from the marker protein lane, as well as a very faint band present in all lanes was visible. Specificity of the primary antibody was ascertained by a primary-alone probe in which no signal was observed (Fig A3b). A similar control was performed for the secondary antibody. Again no signal was observed on the blot probed with secondary antibody in the absence of primary (Fig. A3c).
Figure A1. Protein Absorbance Curve. A standard curve of protein absorbance at OD595 was constructed according to the procedure outlined for the Bio-Rad microassay and described in Methods. Bovine serum albumin was employed as the standard. Dilutions of the stock solution were made, mixed with the assay reagent and protein levels read after five minutes.
Figure A2. Representative gels and blots. a) Polyacrylamide gels were run in the presence of SDS as described in Methods. Protein levels of each sample were determined prior to loading. 5 mg protein was loaded per lane. Corresponding sample volumes were determined based on OD$_{595}$ reading of sample and interpolation from standard curve constructed (Fig. A1). b) Gels were blotted as described in Methods. Visible levels of protein remained in the gel even after 90 minutes transfer when stained with Coomassie Brilliant Blue. Marker proteins were nearly quantitatively transferred. c) Post-blot, successful transfer of protein was confirmed by staining of polyvinylidene difluoridine (PVDF) membrane with 1% amido black as described in Methods. All cell extracts were starved for 2 hours as described in Methods. Lane 1 - 1% DMSO. Lane 2 - 20 μM DEL. Lane 3 - 1% DMSO + 1 μM cAMP. Lane 4 - 20 μM DEL + 1 μM cAMP. M - prestained molecular weight markers.
Figure A3. Immunolocalization controls. Several control blots were performed to confirm specificity of both the primary antibody and the signal generated. a) Primary antibody was added to standard probe buffer as described in Methods containing 25 mM phospho-D-serine. Antibody was preincubated for one hour and blots were then probed. In parallel non-preincubated blots primary antibody was shaken in standard probe buffer minus phospho-D-serine at room temperature for an equivalent length of time. Signals were detected by chemiluminescence as described in Methods. b) Primary antibody alone was used to probe blot. Chemiluminescent detection was employed to detect signal in absence of secondary antibody. c) Secondary antibody alone was used to probe blot. Chemiluminescent detection in the absence of primary antibody was employed. Lane 1 - 1% DMSO. Lane 2 - 20 μM DEL. Lane 3 - 1% DMSO + 1 μM cAMP. Lane 4 - 20 μM DEL + 1 μM cAMP. M - molecular weight marker proteins.
Appendix B.

**Metabolic whole-cell labelling with $^{32}$P-orthophosphate**

i) **Introduction**

Antibodies raised against phosphoserine show several proteins serine phosphorylated in chemotactically competent AX-3 amoebae. It is possible however that certain serine-phosphorylated proteins are not recognized by the antibody due to tertiary or quaternary characteristics preventing antibody recognition of the phosphoamino acid epitope. Attempts were therefore made to confirm and perhaps extend the data obtained from the immunochemical study.

ii) **Methods**

**$^{32}$P-orthophosphate incorporation assay**

Vegetative AX-3 amoebae were harvested in mid-log growth, washed and resuspended in fresh HL-5C media to a density of $2 \times 10^7$ cells/mL. 50 μCi/mL $^{32}$P-orthophosphate (Amersham) was added to the cell suspension. At various time intervals as indicated (Fig. B1) 100 μL aliquots were removed. Cells were pelleted at 3000 rpm for 45 seconds in a microfuge and total radioactivity was determined in supernatant and cell pellet fractions by liquid scintillation counting. To avoid overloading of the counter with high specific activity (measured in disintegrations per minute[dpm]) levels, supernatant samples were diluted by a factor of 10 prior to scintillation counting. Amount of $^{32}$P-orthophosphate incorporated after 30 minutes was 4.9% of total radiation in the sample.
Figure B1. Incorporation of $^{32}$P-orthophosphate. AX-3 amoebae were harvested and washed at mid-log cell densities as for all chemotaxis assays as described in Methods. Amoebae were resuspended in fresh HL-5C growth media and 100 mCi/mL $^{32}$P-orthophosphate was added. Total activity was measured by liquid scintillation counting in the cell pellet (P) and supernatant (S) and plotted as a function of total incorporation of label as outlined in Appendix B.
and did not fluctuate significantly over a 4 hour labelling period (Fig. B1). A 30 minute labelling period was therefore used in all subsequent experiments.

**Whole-cell labelling**

To allow for maximum orthophosphate uptake amoebae were labelled for 30 minutes vegetatively prior to any starvation procedure was begun. Cells were harvested as described previously and resuspended to $2 \times 10^7$ cells/mL in 15 mM Tris, pH 6.5.

**In vivo dephosphorylation**

An in vivo dephosphorylation protocol was adapted from the in vivo phosphorylation procedure of Berlot et al. (1985). After a two-hour starvation period under experimental conditions exactly as described for immunochemical analyses, 1$\mu$M cAMP was added to the cell suspension. At various time points thereafter, 100 $\mu$L samples were withdrawn from a cell suspension shaking at 110 rpm and added to 200 $\mu$L of a reaction mixture containing 0.2% Triton X-100, 2mM MgCl$_2$, 7.5 mM Tris, pH 7.5 and 20 $\mu$M deltamethrin. Proteins were precipitated with 4% trichloroacetic acid (TCA) (Sigma). DNA was removed using 2.5 mg/mL DNase I (Ausubel et al., 1995) (Sigma). TCA/DNase reactions were for 30 minutes at 0$^\circ$ in the presence of protease inhibitors 50 $\mu$g/mL antipain, 50 $\mu$g/mL leupeptin and 1% phenylmethylsulphonylfluoride (PMSF), after which protein was pelleted at 500g, 4$^\circ$ for 20 minutes and washed once in 1mL 15 mM Tris, pH 6.5. Samples were diluted in 2X SDS running buffer and boiled for 5 minutes. Gels were run as usual, dried down on filter paper and exposed to film with an intensifying screen.
iii) Results

Orthophosphate labelling of whole cells resulted in strong label incorporation in several bands (Fig. B2a). Molecular weights cannot be estimated, as marker proteins were not run on these gels. Three bands in particular appeared to show differential orthophosphate incorporation under different experimental conditions (labelled 1, 2, 3, Fig. B2a). Band 1 is of high molecular weight and appears to be present only in the presence of 1% DMSO after starvation (lane 1). Addition of 20 μM DEL to vegetative labelled cells and subsequent starvation resulted in disappearance of this signal (lanes 2, 3) and it did not reappear in the presence of 1 μM cAMP (lane 4). Bands 2 and 3 both appear to increase in signal intensity in the presence of 20 μM DEL in amoebae labelled and starved to chemotactic competence (lane 4).

Nucleic acids and phospholipids are strongly radiolabelled and form a heavy, dark band of label near the top of the gel, obscuring any possible high molecular weight data points in that region. Attempts were therefore made to remove nucleic acids from the final sample by the inclusion of 2.5 mg/mL DNase I (Ausubel et al., 1995). TCA protein precipitation was also attempted in order to remove cellular proteins from phospholipids. Unfortunately, attempts to remove nucleic acids via the inclusion of DNase I and TCA protein precipitation were unsuccessful (Fig. B2b). Near-total proteolysis occurred during the TCA/DNA precipitation-digestion step. While some trends of differential migration rate and label incorporation are suggested (Fig. B2a) this experiment was generally unsuccessful in corroborating the results obtained with antiphosphoserine (see Results) and so further attempts to resolve these questions were abandoned.
Figure B2. Metabolic labelling of whole-cell extracts. Amoebae were metabolically labelled as described in Appendix B. A) Amoebae were treated with inhibitors and with or without cAMP as described for immunoblots. 12% gel was run at 200V for 45 minutes, dried on filter paper and exposed to film with an intensifying screen for 12 hours. Bands 1, 2, 3 are described in Appendix B. B) Metabolic labelling and gel conditions were as previously described. Protein was trichloroacetic acid-precipitated in the presence of DNase I and protease inhibitors as described in Appendix B. Gel was exposed to film for 144 hours. Lane 1 - 1% DMSO. Lane 2 - 20 μM DEL. Lane 3 - 1% DMSO + 1 μM cAMP. Lane 4 - 20 μM DEL + 1 μM cAMP.
iv) **Discussion**

Several bands show differences in orthophosphate incorporation in the presence of the CN inhibitor deltamethrin (bands 1, 2, 3, Fig. B2a). These bands unfortunately could not be identified as molecular weight markers were not included on the gel. Several attempts were made to remove radiolabelled nucleic acids and phospholipids which contribute to the heavy, dark signal at the top of the gel (Fig. B2a). These attempts were unsuccessful (Fig. B2b). As a phagocytic organism, *Dictyostelium* is rich in lysosomes (Sussman, 1987) and the corresponding high levels of proteases must be counteracted in order for whole cell samples to be obtained. Nearly complete proteolysis occurred in the TCA protein precipitation step, even at 0°C in the presence of antipain, leupeptin and PMSF (Fig. B2b). Supplies of $^{32}$P-orthophosphate were exhausted and these experiments were not pursued further.
References


Dictyostelium: Pharmacological Analyses and the Presence of a Substrate Protein
Suggest Protein Kinase C Functions During Gamete Fusion. Exp. Cell Res. 220:
325-31.


multicellular development in Dictyostelium. Genes Dev. 6: 38-49.

receptors and identifies a signaling pathway that is essential for multicellular

Stossel, T.P. 1995. Thrombin Receptor Ligation and Activated Rac Uncap Actin
Filament Barbed Ends Through Phosphoinositide Synthesis in Permeabilized

Dictyostelium discoideum contains two profilin isoforms that differ in structure


Theibert, A. and Devreotes, P.N. 1986. Surface Receptor-mediated Activation of


Tillinghast Jr., H.S. and Newell, P.C. 1984. Retention of folate receptors on the

Towbin, H., Staehelin, T. and Gordon, J. 1979. Electrophoretic transfer of proteins from
dilayslaidamide gels to nitrocellulose sheets: Procedure and some applications.

Unterweger, N. and Schlatterer, C. 1995. Introduction of calcium buffers into the cytosol
of Dictyostelium discoideum amoebae alters cell morphology and inhibits


cyclase by intracellular calcium ions in Dictyostelium cells. Biochem. Biophys.


