A Ca^{2+} Transport System Associated with the Plasma Membrane of *Dictyostelium discoideum* Is Activated by Different Chemoattractant Receptors

Jacqueline L. Milne and M. Barrie Coukell

Department of Biology, York University, North York, Canada M3J 1P3

Downloaded from http://rupress.org/jcb/article-pdf/112/1/103/1463870/103.pdf by guest on 04 October 2023

Abstract. Amebae of *Dictyostelium* exhibit a transient uptake of extracellular Ca^{2+} ∼5 s after activation of surface folate or cAMP receptors (Bumann, J., B. Wurster, and D. Malchow. 1984. *J. Cell Biol.* 98:173–178). To further characterize these Ca^{2+} entry systems, we analyzed $^{45}Ca^{2+}$ uptake by resting and activated amebae. Like the surface chemoreceptors, folate- and cAMP-induced Ca^{2+} uptake responses were developmentally regulated; the former response was evident in vegetative but not aggregation-competent cells, whereas the latter response displayed the opposite pattern of expression. In contrast, other characteristics of these Ca^{2+}-uptake pathways were remarkably similar. Both systems (*a*) exhibited comparable kinetic properties, (*b*) displayed a high specificity for Ca^{2+}, and (*c*) were inhibited effectively by Ruthenium Red, sodium azide, and carbonylcyanide *m*-chlorophenylhydrazone. These results, together with the finding that vegetative cells transformed with a plasmid expressing the surface cAMP receptor exhibit a cAMP-induced Ca^{2+} uptake, suggest that different chemoreceptors activate a single Ca^{2+} entry pathway. Additional pharmacological and ion competition studies indicated that receptor-mediated Ca^{2+} entry probably does not involve a Na^+/Ca^{2+} exchanger or voltage-activated channels. Chemoattractant binding appears to generate intracellular signals that induce activation and adaption of the Ca^{2+}-uptake response. Analysis of putative signaling mutants suggests that Ca^{2+} entry is not regulated by the guanine nucleotide-binding (G) protein subunits Gα1 or Gα2, or by G protein-mediated changes in intracellular cAMP or guanosine 3′,5′-cyclic monophosphate (cGMP).

In many higher eukaryotic cells, hormones and neurotransmitters induce changes in cytosolic free Ca^{2+} by promoting mobilization of sequestered intracellular Ca^{2+} and/or by stimulating entry of extracellular Ca^{2+} across the plasmalemma. Ca^{2+} mobilization responses can be mediated by increases in cytosolic Ca^{2+} (18) or by agonist-induced production of inositol 1,4,5-trisphosphate, which releases Ca^{2+} from the ER (4) and other cellular compartments (27, 48). Enhanced uptake of extracellular Ca^{2+} occurs following activation of voltage-regulated (40) or ligand-gated (3) Ca^{2+} channels, and likely serves a crucial role both in Ca^{2+} signaling events and in replenishing agonist-depleted stores (for review, see reference 22). Importantly, many of these Ca^{2+}-uptake systems, both voltage-dependent and voltage-independent, appear to be regulated by extracellular factors (for review, see reference 43). In addition, certain of these Ca^{2+} channels appear to be modulated directly by guanine nucleotide-binding protein (G protein)[1] subunits while others are regulated indirectly by second messengers such as cAMP, guanosine 3′,5′-cyclic monophosphate (cGMP), and inositol polyphosphates (43).

In the lower eukaryote, *Dictyostelium discoideum*, chemoattractant-induced changes in the concentration of free cytosolic Ca^{2+} are probably important in the regulation of certain cellular processes during development (see reference 35). Growing amebae feed on bacteria, and respond chemotactically to folate, a compound secreted by the bacteria (39). Upon starvation, these cells aggregate into multicellular structures, which undergo morphogenesis and differentiation to form fruiting bodies (for review, see reference 30). Cell aggregation and differentiation are regulated, in part, by endogenously generated waves of extracellular cAMP. Activation of the cAMP or folate chemoreceptors induce a number of cellular events including a rapid increase in intracellular cGMP (51), and an influx of extracellular Ca^{2+} (7, 16, 50). Relatively little is known about the properties or the nature of chemoattractant-mediated Ca^{2+} uptake systems in *Dictyostelium.* Moreover, it is unclear whether the folate and cAMP receptors couple with the same or different Ca^{2+} transport systems. To characterize further receptor-activated Ca^{2+} entry in this organism, we developed a $^{45}Ca^{2+}$ assay system to measure accurately uptake of

1. *Abbreviations used in this paper*: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; G protein, guanine nucleotide-binding protein.

© The Rockefeller University Press, 0021-9525/91/01/103/8 $2.00

low micromolar concentrations of Ca^{2+} into resting and chemoattractant-stimulated cells. Our results suggest that vegetative and aggregation-competent amebae of *Dictyostelium* possess a highly specific Ca^{2+} uptake system that is regulated by distinct chemoreceptors.

Materials and Methods

Materials

Materials used and their sources were as follows: $^{45}CaCl_2$ (14.7 mCi/mg Ca^{2+}; 1 Ci = 37 GBq) and [2,8-^{3}H]cAMP (27 Ci/mmol) (ICN Biomedicals, St. Laurent, Canada); NCS tissue solubilizer (Amersham Corp., Oakville, Canada); $CaCl_2$ standard (Orion Research Inc., Cambridge, MA); cAMP, DTT, methoxyverapamil (D-600), 3,4,5-trimethoxybenzoate 8-(diethylamino)octyl ester-HCl (TMB-8), nicardipine, and nifedipine (Sigma Chemical Co., St. Louis, MO); folate, $BaCl_2 \cdot 2H_2O$, $CoCl_2 \cdot 6H_2O$, and $MnCl_2 \cdot H_2O$, (BDH Inc., Toronto, Canada); $GdCl_3 \cdot 6H_2O$, and Ruthenium Red (Aldrich Chemical Co., Milwaukee, WI); sodium azide (J. T. Baker Chemical Co., Phillipsburg, NJ); DMSO (Fisher Scientific, Unionville, Canada); Geneticin (Gibco/BRL, Burlington, Canada). All other chemicals were of analytical grade and were obtained from the suppliers indicated in references 35, 36.

Strains and Culture Conditions

The following aggregation-competent, haploid strains of *D. discoideum* were used in this study: HC91 and HC6 (10), XP55 and NP368 (41), AX2 (49), and AX3 cells transformed with the plasmid pBS18B6, carrying a cloned cAMP receptor gene (cAR1 cells) or the plasmid pBS18, lacking the receptor sequence (BS18 cells) (26; provided by P. N. Devreotes [Johns Hopkins School of Medicine, Baltimore, MD]). Several other AX3 derivatives were used: GαlS, a transformant cell line overexpressing Gαl (28), JH131, a null mutant of the G1 α-subunit and JH130, a control transformant (provided by J. Hadwiger and R. A. Firtel [University of California, San Diego, CA]), and JH104, a null mutant of the G2 α-subunit (provided by J. Hadwiger, R. A. Firtel, and P. N. Devreotes). Two classes of aggregation-deficient strains were also used: a Synag mutant, HC347 (PD7-2-2) (32), and two Frigid A mutants, HC85 and HC112 (12). All cell lines, except AX2, cAR1, BS18, GαlS, and JH104 were grown in association with *Klebsiella aerogenes* on SM agar plates at 22°C as described (10). The other strains were grown axenically in liquid HL-5 medium as described (35), except that the growth medium for cAR1, BS18, and GαlS was supplemented with 20 μg of Geneticin/ml.

To obtain aggregation-competent amebae, vegetative cells were permitted to differentiate on nonnutrient agar at 7°C for 16 h (37). The plates were then transferred to 22°C for 1-2 h before the cells were harvested and used in Ca^{2+} uptake experiments. In some experiments, amebae of strains JH131, GαlS, JH104, HC85, and PD7-2-2 were treated with exogenous cAMP pulses as described (29).

Ca^{2+} Uptake Assay

Unless indicated otherwise, all Ca^{2+} uptake studies were performed with amebae of strain HC91. To prepare cells for uptake experiments, amebae at the desired developmental stage were harvested by centrifugation (700 *g*, 2 min, 22°C) in H buffer (20 mM Hepes/KOH, 5 mM KCl, pH 7.0) and washed twice in the same buffer. The amebae were then resuspended to a concentration of 1×10^8 cells/ml in H buffer and shaken (22°C) for 10 min at 250 rpm on a gyrotory shaker (model G76; New Brunswick Instruments, Edison, NJ).

In most experiments, Ca^{2+} uptake into resting amebae was initiated by adding 100 μl of cell suspension to a microcentrifuge tube containing 100 μl of an uptake medium (22°C) composed of 20 mM Hepes/KOH, 5 mM KCl, 100 μM $CaCl_2$, and ~0.5 μCi $^{45}CaCl_2$ (pH 7.0). To measure Ca^{2+} uptake into folate- or cAMP-stimulated cells, the uptake medium was supplemented with 40 μM folate or 2 μM cAMP, respectively. At the times indicated, $^{45}Ca^{2+}$ entry was terminated by the addition of 100 μl of ice-cold H buffer containing 775 mM $CaCl_2$. The cell suspensions were centrifuged immediately at 12,000 rpm for 4 s in an Eppendorf model 5414 centrifuge (Eppendorf Gerätebau, Hamburg, FRG), and the supernatants were discarded. The cell pellets were then resuspended in 1 ml of ice-cold H buffer containing 10 mM $CaCl_2$, recentrifuged, solubilized in NCS and counted as described (11). Nonspecific Ca^{2+} binding was determined by adding cells to uptake medium containing 225 mM $CaCl_2$. Chemoattractant-induced Ca^{2+} uptake at each timepoint was determined by subtracting the amount of Ca^{2+} taken up by resting cells from the amount accumulated by the stimulated cells. Protein was measured by the method of Lowry et al. (31) using BSA as a standard.

In certain experiments, Ca^{2+} uptake was followed in the presence of putative inhibitors of Ca^{2+} transport. Stock solutions of these compounds (10-40 mM) were prepared in DMSO (methoxyverapamil, nicardipine, nifedipine, and carbonylcyanide *m*-chlorophenylhydrazone [CCCP]) or H buffer (all other compounds), and stored at −20°C. The DMSO concentration in the assay system never exceeded 1%; this concentration had no effect on the Ca^{2+} uptake response.

cAMP-binding Assay

Amebae were harvested, washed twice by centrifugation in 10 mM Na_2HPO_4/KH_2PO_4 buffer (pH 6.5), and resuspended in the same buffer to give a concentration of 2×10^8 cells/ml. Binding of 100 nM [^{3}H]cAMP to the cells was determined in triplicate using the ammonium sulfate assay (45).

Results

Identification of Folate- and cAMP-stimulated Ca^{2+} Uptake

Fig. 1 *a* shows the kinetics of Ca^{2+} accumulation by suspensions of aggregation-competent amebae of strain HC91 in the presence or absence of cAMP. Amebae treated with cAMP initially took up Ca^{2+} at the same rate as nonstimulated cells. However, after a delay of 5.5 ± 0.4 s (mean ± SEM, n = 5), Ca^{2+} uptake by the stimulated cells increased sharply for 20-25 s, and then ceased. Similar results were obtained with amebae of strain AX2 grown axenically (data not shown). When the cAMP receptor was activated 10 s before the addition of Ca^{2+}, uptake proceeded immediately at a high rate. The time at which the response terminated, however, was unchanged, i.e., 25-30 s after addition of stimulus (Fig. 1 *b*). In growth-phase cells, folate stimulated Ca^{2+} uptake with kinetics very similar to those observed for the cAMP-mediated response of developing cells, except the delay preceding Ca^{2+} entry was 8.5 ± 0.3 s (mean ± SEM, n = 7) (data not shown).

The developmental regulation of folate- and cAMP-induced Ca^{2+} influx is illustrated in Fig. 2. When cells were starved on nonnutrient agar, folate-mediated uptake was maximal in vegetative cells and remained constant for ~4 h before declining steadily to undetectable levels by 10 h. Under similar conditions, cAMP-activated Ca^{2+} uptake was barely detectable during the first 4 h of development. The magnitude of this response increased dramatically between 4 and 9 h as the cells became fully aggregation competent, and then declined slightly over the next 5 h. The developmental regulation of folate-induced Ca^{2+} uptake was markedly different when cells were permitted to develop in suspension. Under these conditions, the response was maximal during the first 0.5 h of starvation, and then declined to low levels by 3.5 h.

Chemoattractants Alter the Kinetic Properties of Ca^{2+} Influx

The amount of Ca^{2+} accumulated by aggregation-competent amebae depended upon the concentration of the cAMP stimulus (Fig. 3 *a*). Stimuli of 1 nM failed to induce a detectable response while 10 μM cAMP elicited maximal uptake (302 ± 12 pmol Ca^{2+} transported/10^7 cells; mean ± SEM,

Downloaded from http://rupress.org/jcb/article-pdf/112/1/103/1463870/103.pdf by guest on 04 October 2023

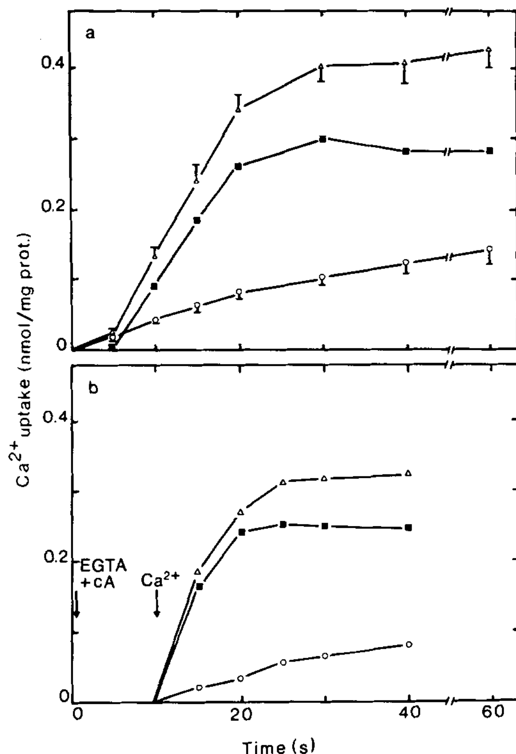

Figure 1. Time course of cAMP-induced Ca^{2+} uptake into amebae of strain HC91. (*a*) Basal or cAMP-stimulated Ca^{2+} uptake into aggregation-competent cells was assayed under standard conditions as described in Materials and Methods. (*b*) At zero time, aggregation-competent cells (1×10^7) were added to a modified uptake medium consisting of (final concentrations) 20 mM Hepes/KOH, 5 mM KCl, 20 μM EGTA (pH 7.0) and to an identical medium containing 1 μM cAMP. After 10 s, the free Ca^{2+} concentration was adjusted to 50 μM by the addition of $^{45}CaCl_2$. The amount of $^{45}Ca^{2+}$ added was calculated by means of a computer program based on Fabiato and Fabiato (19). At the times indicated, Ca^{2+} uptake was terminated and the samples were processed as described in Materials and Methods. Values are shown for Ca^{2+} uptake into resting (○) and cAMP-stimulated (△) cells, and for cAMP-induced uptake (■). Each point is the mean of data obtained in five to six (*a*) or two (*b*) independent experiments. In *a*, bars represent SEM.

$n = 3$). Half-maximal uptake occurred at 280 nM cAMP. The dose-response profile was not altered by the presence of 10 mM DTT, a compound that inhibits cyclic nucleotide phosphodiesterase activity in *Dictyostelium* (24). Also, a similar dose–response profile was obtained with axenically grown amebae of strain AX2 (data not shown). Concentrations of cAMP between 100 nM and 100 μM had no effect on the time of initiation or termination of Ca^{2+} uptake (Fig. 3 *b*); therefore, stimulus concentration appears to influence the rate, rather than the duration, of the response. To determine if cAMP alters the affinity of the uptake system for external Ca^{2+}, initial rates of Ca^{2+} entry into resting and cAMP-stimulated cells were determined at extracellular free Ca^{2+} concentrations ranging from 10 to 400 μM (Fig. 4 *a*). For nonstimulated cells, the initial rates of Ca^{2+} uptake increased slowly over the entire Ca^{2+} concentration range examined. In contrast, the rates of uptake into cAMP-stimulated cells increased sharply at external Ca^{2+} concentrations

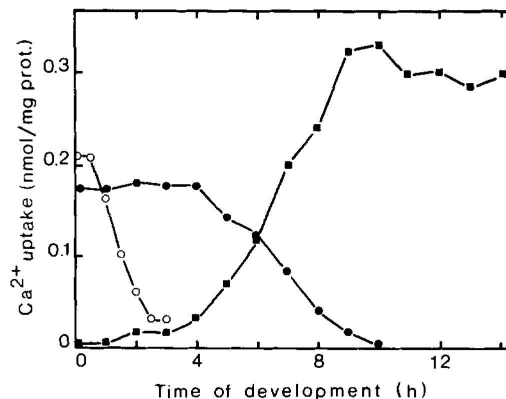

Figure 2. Developmental regulation of folate- and cAMP-stimulated Ca^{2+} uptake. Cells were permitted to develop at 22°C on phosphate-buffered agar as described by Coukell et al. (13). At the times indicated, cells were harvested, washed, and assayed for 30 s for folate- (●) and cAMP- (■) stimulated Ca^{2+} uptake as described in Materials and Methods, except that the stimulus was 100 μM. (○) Vegetative cells (1×10^8/ml) were shaken in H buffer for the times indicated and then assayed for 30 s for folate-induced Ca^{2+} uptake as described in Materials and Methods. Values shown are the means of data from two experiments.

up to 100 μM, and then remained constant at concentrations >200 μM. In Fig. 4 *b*, the same data are plotted according to Hanes (23). The results suggest that Ca^{2+} uptake follows Michaelis–Menten kinetics both in the presence and absence of a cAMP stimulus. However, addition of cAMP increases the V_{max} of Ca^{2+} transport approximately twofold and lowers the apparent K_m for Ca^{2+} approximately sixfold.

Ca^{2+} uptake by vegetative cells was influenced in a similar fashion by the addition of folate. Half-maximal and maximal levels of receptor-stimulated Ca^{2+} influx occurred at 135 nM and 10 μM folate, respectively (data not shown). The Ca^{2+} transport system(s) of resting and folate-stimulated vegetative cells also exhibited Michaelis–Menten kinet-

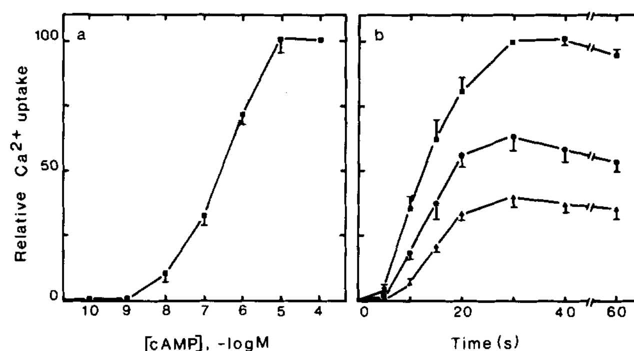

Figure 3. Effect of cAMP concentration on the magnitude (*a*) and time course (*b*) of cAMP-mediated Ca^{2+} uptake. Aggregation-competent cells were assayed for cAMP-dependent Ca^{2+} uptake as described in Materials and Methods except that in (*a*) uptake was followed for 30 s in the presence of 0.1 nM to 100 μM cAMP, and, in *b*, the assay system contained 100 nM (▲), 1 μM (●), or 100 μM (■). cAMP-induced Ca^{2+} uptake values are expressed relative to the 30 s timepoint value in the presence of 100 μM cAMP. Each point is the mean ± SEM of results obtained in three (*a*) or four (*b*) separate experiments.

Downloaded from http://rupress.org/jcb/article-pdf/112/1/103/1463870/103.pdf by guest on 04 October 2023

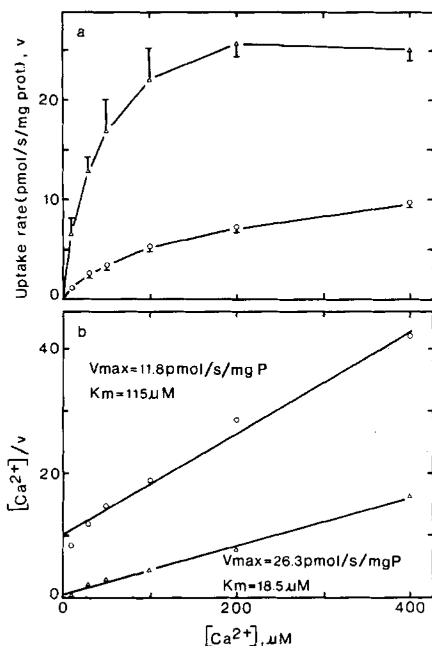

Figure 4. cAMP-induced changes in the kinetic properties of the Ca^{2+} transport system of aggregation-competent amebae. (*a*) Ca^{2+} uptake into resting (○) and cAMP-stimulated amebae (△) was measured under standard conditions except that the extracellular Ca^{2+} concentration was varied from 10 to 400 μM. To determine initial rates of Ca^{2+} entry, uptake was terminated 10, 15, and 20 s after the start of the reaction. Values are the means ± SEM of results from three independent experiments. (*b*) A Hanes plot of the data from *a*. The lines were fitted by regression analysis.

ics. Folate increased the V_{max} of uptake 1.5-fold (from 15.5 to 23.3 pmol Ca^{2+} transported/s per mg protein) and reduced the apparent K_m for external Ca^{2+} ~7.5-fold (from 125 to 16.9 μM) (data not shown).

A Single Ca^{2+} Entry Pathway Appears to Couple with Both the Folate and cAMP Receptors

The similar profiles of folate- and cAMP-stimulated Ca^{2+} uptake, together with the observations that the two chemoattractants induce comparable changes in the kinetic properties of Ca^{2+} influx, suggested that the different chemoreceptors might couple to the same Ca^{2+} transport system. To investigate this possibility, Ca^{2+} uptake was analyzed in growth-phase AX3 cells transformed with a plasmid carrying a gene encoding a surface cAMP receptor (cARl cells). These amebae expressed 8.9 ± 1.3-fold (mean ± SEM, n = 6; range 6.1–15.2-fold) higher levels of surface cAMP-binding sites than cells transformed with the same plasmid minus the receptor gene (BS18 cells). As observed with aggregation-competent wild-type cells, cAMP elicited a rapid influx of external Ca^{2+} into vegetative amebae expressing cARl (Fig. 5 *a*). With these cells, the cAMP-induced Ca^{2+} uptake began after a delay of 6.8 ± 0.9 s (mean ± SEM, n = 5) and continued for 15–20 s. However, unlike aggregation-competent wild-type cells, Ca^{2+} accumulation by the cARl transformants decreased after ~30 s (compare Figs. 1 and 5 *a*). This decline in cellular Ca^{2+} is not due to a loss of the uptake response over the course of the experiment, since cARl cells shaken in suspension for 1 h retained high levels of cAMP-induced Ca^{2+} uptake (data not shown). Growth-phase BS18 amebae failed to exhibit cAMP-mediated Ca^{2+} entry (Fig. 5 *b*), a finding consistent with the observation that these cells possessed low levels of surface cAMP-binding sites relative to cARl cells. Both BS18 and cARl vegetative amebae showed a low but reproducible Ca^{2+} uptake in response to folate (stimulated uptake was 75–100 pmol Ca^{2+}/mg protein 30 s after folate addition).

To compare further the Ca^{2+} uptake systems in vegetative and aggregation-competent cells, the effects of various putative inhibitors of Ca^{2+} transport were examined. The following Ca^{2+}-channel blockers, at the concentrations indicated, had little or no effect on cAMP-stimulated Ca^{2+} uptake in aggregation-competent cells: 3,4,5-trimethoxybenzoate 8-(diethylamino)octyl ester-HCl (TMB-8) (50 μM), verapamil (50 μM), diltiazem (100 μM), methoxyverapamil (100 μM), nifedipine (100 μM), and nicardipine (100 μM). In contrast, Ruthenium Red was an effective inhibitor of cAMP-induced Ca^{2+} uptake with an IC_{50} (i.e., concentration required to reduce Ca^{2+} uptake by 50%) of ~7.5 μM (Fig. 6). Similar dose-inhibition profiles and IC_{50} values (7–10 μM) were obtained when the effect of this compound was examined on the cAMP-stimulated Ca^{2+} uptake by growth-phase cARl cells and the folate-induced uptake by vegetative HC91 cells (Fig. 6). cAMP- and folate-stimulated Ca^{2+} uptake were also inhibited by sodium azide with IC_{50}s

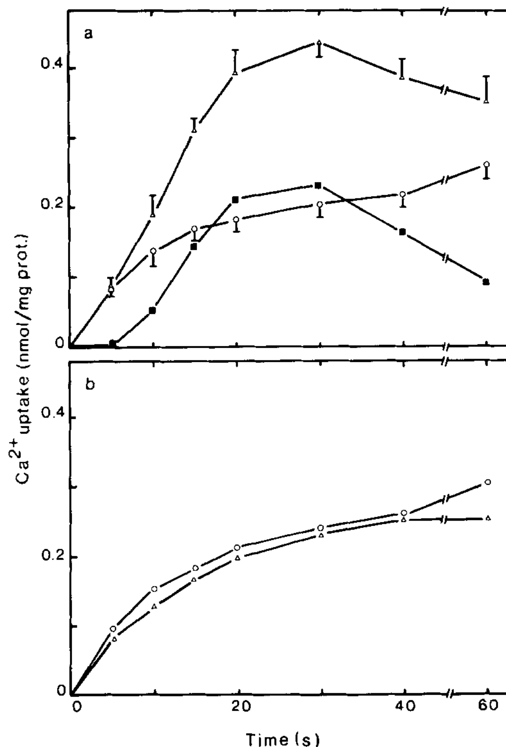

Figure 5. Time course of cAMP-stimulated Ca^{2+} entry into (*a*) cARl and (*b*) BS18 amebae. Growth-phase cells were assayed for Ca^{2+} uptake as described in Materials and Methods except that uptake was monitored in the presence (△) or absence (○) of 10 μM cAMP. Each point in *a* is the mean ± SEM of results from four experiments. (■) cAMP-stimulated Ca^{2+} uptake. In *b*, Ca^{2+} uptake values are the means of results obtained in two experiments.

Downloaded from http://rupress.org/jcb/article-pdf/112/1/103/1463870/103.pdf by guest on 04 October 2023

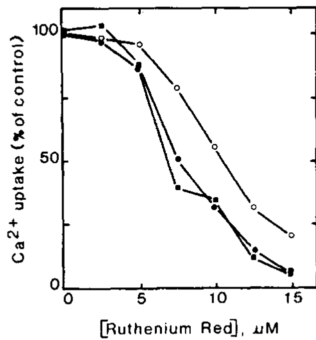

Figure 6. Effect of Ruthenium Red on chemoattractant-stimulated Ca^{2+} uptake. Folate-induced Ca^{2+} entry into vegetative (○) and cAMP-induced Ca^{2+} uptake into aggregation-competent (●) HC91 cells were assayed for 30 s as described in Materials and Methods except that the assay system contained 10 μM Ca^{2+} and various concentrations of Ruthenium Red. cAMP-induced Ca^{2+} entry into vegetative cAR1 cells (■) was monitored under identical conditions except that the cAMP stimulus was 10 μM. For each profile, results shown are the means of two separate experiments.

of ~9 and 14 μM, respectively, and by CCCP (IC_{50} = ~2 μM in both cases) (data not shown). Ion competition studies were performed to determine if various di- and trivalent cations could reduce folate- and cAMP-mediated Ca^{2+} entry. In the presence of 500 μM test ion and 10 μM extracellular Ca^{2+}, both Ca^{2+}-uptake systems were inhibited poorly by Co^{2+}, Mg^{2+}, Mn^{2+}, moderately by Cd^{2+} and Ba^{2+}, and strongly by La^{3+} and Gd^{3+} (Fig. 7). Further experiments revealed that La^{3+} and Gd^{3+} inhibited both folate- and cAMP-mediated Ca^{2+} uptake with IC_{50}s of 200–250 μM (data not shown). These inhibitor results, together with the finding that vegetative cAR1 (but not BS18) amebae expressed high levels of surface cAMP receptors and exhibited a cAMP-stimulated Ca^{2+} uptake similar to that of aggregation-competent wild-type cells, supports the idea that a single Ca^{2+}-uptake system might mediate folate- and cAMP-induced Ca^{2+} entry in *Dictyostelium*.

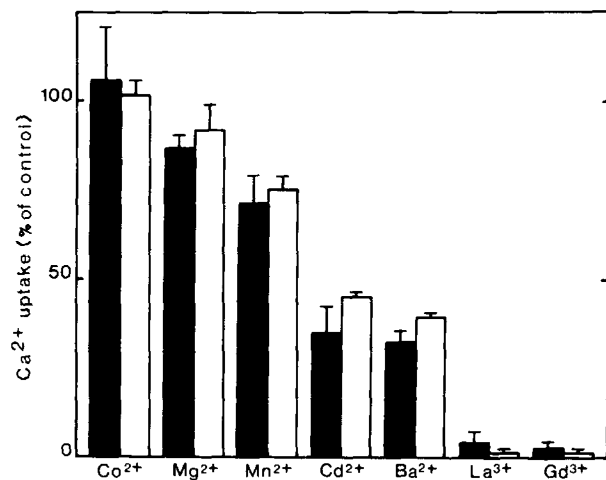

Figure 7. Ability of various cations to inhibit folate- and cAMP-mediated Ca^{2+} uptake. Folate-stimulated Ca^{2+} uptake into vegetative cells (*closed bars*) and cAMP-induced uptake into aggregation-competent cells (*open bars*) were measured for 30 s under standard conditions except that the assay system contained 10 μM Ca^{2+} and 500 μM test cation. Results are expressed relative to the folate- and cAMP-induced Ca^{2+} uptake of control cells not receiving test ions. Results shown are the means ± SEM of data obtained in three experiments.

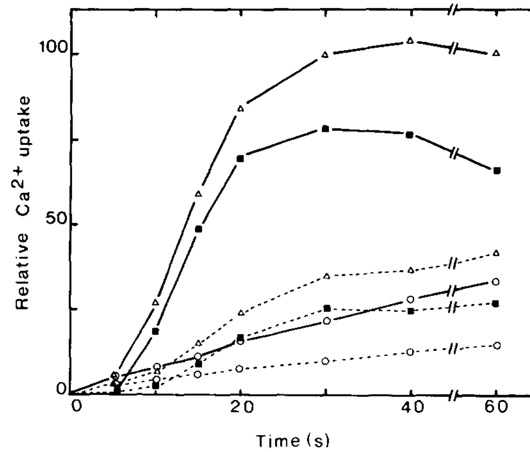

Figure 8. Effect of sodium azide on the cAMP-stimulated Ca^{2+} uptake of aggregation-competent cells. Ca^{2+} uptake into resting (○) or cAMP-stimulated (△) amebae was measured as described in Materials and Methods except that the assay system contained 10 μM Ca^{2+} (*solid lines*) or 10 μM Ca^{2+} and 15 μM sodium azide (*dashed lines*). (■) cAMP-stimulated Ca^{2+} uptake. Values are expressed relative to the level of Ca^{2+} uptake at 30 s by cAMP-stimulated cells not receiving sodium azide, and are the means of results obtained in two experiments.

Regulation of the Receptor-activated Ca^{2+} Transport System

The inability of high concentrations of classical voltage-gated Ca^{2+} channel blockers to inhibit cAMP-mediated Ca^{2+} uptake suggested that this transport system is not regulated directly by changes in membrane potential. This idea was supported further by experiments with sodium azide, a compound reported to rapidly depolarize the plasma membrane of *Dictyostelium* without altering appreciably cellular ATP levels (44). Addition of 100 μM sodium azide to the assay system reduced cAMP-stimulated Ca^{2+} uptake by ~90% (data not shown). To determine if membrane depolarization influences the kinetics of Ca^{2+} entry, Ca^{2+} uptake was measured in the presence of 15 μM sodium azide, a concentration that reduced Ca^{2+} transport by both resting and stimulated cells by 55–65%. Under these conditions, the times at which cAMP-stimulated Ca^{2+} uptake commenced and terminated were unchanged (Fig. 8).

To investigate other mechanisms possibly involved in coupling the chemoreceptors to Ca^{2+} uptake, a number of putative signal transduction mutants of *Dictyostelium* were analyzed. Recent evidence suggests that the folate and cAMP receptors are coupled to certain effector enzymes via G proteins (21, 25). Therefore, chemoattractant-activated Ca^{2+} transport was examined in the Frigid A mutants HC85 and HC112, which are defective in the gene encoding the G protein α-subunit, Gα2 (21). Growth-phase amebae of strains HC85 and HC112 exhibited a folate-stimulated Ca^{2+} uptake similar to their parental strains, HC6 and HC91, respectively. When these mutants were starved on nonnutrient agar at 22°C for 16 h, they failed to show a cAMP-induced Ca^{2+} uptake response, but continued to exhibit a strong folate-mediated uptake (data not shown). However, when the HC85 amebae were pulsed for ~9 h with 50 nM cAMP, they exhibited an 8.5 ± 0.9-fold (mean ± SEM, n = 3; range

Downloaded from http://rupress.org/jcb/article-pdf/112/1/103/1463870/103.pdf by guest on 04 October 2023

6.9–9.6-fold) increase in the levels of surface cAMP-binding sites and a small but reproducible cAMP-induced Ca^{2+} uptake (stimulated uptake was 50–90 pmol/mg protein 30 s after cAMP addition). Similar results were obtained using amebae of strain JH104, a Gα2-null mutant (data not shown). In other experiments, the following strains were found to exhibit normal kinetics of chemoreceptor-induced Ca^{2+} uptake upon activation with folate and/or cAMP: JH131, a mutant carrying a deletion of the G1 α-subunit gene; Gα1S, a transformant that expresses 10–20-fold higher levels of Gα1 than control cells (28); NP368, which possesses elevated cGMP pools (42); and PD7-2-2, which fails to exhibit adenylate cyclase activation in response to a cAMP stimulus (32). In each case, mutant or transformant cell lines were compared with their parent strain or to an appropriate control transformant. Chemoattractant-induced Ca^{2+} entry was also not influenced by 5 mM caffeine, a compound reported to block cAMP receptor-induced activation of the adenylate cyclase in *Dictyostelium* (6). Together, these observations suggest that the folate- and cAMP-stimulated Ca^{2+} uptake system(s) is not regulated directly by the G protein subunits Gα1 or Gα2, or by changes in the intracellular concentrations of cAMP or cGMP.

Discussion

To study receptor-activated Ca^{2+} entry in *Dictyostelium*, we developed a $^{45}Ca^{2+}$-uptake assay to measure Ca^{2+} accumulation by resting and chemoattractant-stimulated cells. The folate- and cAMP-induced Ca^{2+} uptake systems detected by this assay are probably the same systems identified previously by others (7, 50). Consistent with the findings of these investigators, responsive cells stimulated with folate or cAMP accumulated Ca^{2+} at the same rate as resting cells for 6–9 s, and then exhibited a dramatic influx of Ca^{2+}, which continued for 20–25 s. In contrast, Europe-Finner and Newell (16) reported that neither folate nor cAMP enhanced the rates of Ca^{2+} uptake into stimulated cells, although they increased the magnitude of Ca^{2+} uptake. Chemoattractant-induced increases in rates of Ca^{2+} entry likely were obscured in these experiments by the use of $LaCl_3$ (100 μM) to block $^{45}Ca^{2+}$ transport. Recent evidence (5; see Results) suggests that this concentration of $LaCl_3$ does not effectively inhibit Ca^{2+} influx in *Dictyostelium.*

Changes in the levels of folate- and cAMP-induced Ca^{2+} uptake during development suggest that these responses are regulated by specific chemoreceptors. For example, when amebae are starved on nonnutrient agar, the decrease in folate-induced Ca^{2+} uptake (Fig. 2) correlates closely with reported changes in the numbers of folate receptors on the surface of these cells and their chemotactic responsiveness to folate (14). Similarly, as the amebae become aggregation competent, there are parallel increases in cAMP-stimulated Ca^{2+} uptake (Fig. 2), the level of cell surface cAMP receptors (34), and chemotactic sensitivity of the cells to cAMP (47).

In agreement with Bumann et al. (7), we observed that maximal folate- and cAMP-induced Ca^{2+} uptake occur at a stimulus concentration of 10 μM. Moreover, the dose–response profiles obtained with folate in the two studies are comparable, with EC_{50} values of 135–200 nM. However, the cAMP-induced Ca^{2+} uptake system characterized in the present study appears considerably less responsive to low concentrations of cAMP (EC_{50} = 280 nM) than the transport system described earlier (EC_{50} = 5 nM). This difference is probably not due to the strains used or to degradation of the cAMP stimulus in our experiments because we obtained the same profile with the strain used in the earlier study (AX2), and with reaction mixtures containing a cyclic nucleotide phosphodiesterase inhibitor (DTT). Interestingly, a dose–response profile, very similar to the one presented here, was reported for the cAMP-induced efflux of K^+ from *Dictyostelium* cells, a process thought to be regulated by the influx of Ca^{2+} (compare Fig. 3 *a* to Fig. 1 in reference 1).

Analysis of Ca^{2+} uptake by vegetative and aggregation-competent cells suggests that the same Ca^{2+} transport system(s) is operative at both developmental stages. For example, at each stage, resting cells appear to possess a low-affinity system with comparable kinetic properties for Ca^{2+} transport. When the amebae are stimulated by folate (vegetative) or cAMP (aggregation-competent), both chemoattractants enhance dramatically (6–7.5-fold) the affinity of the Ca^{2+} transport system while exerting more modest increases (1.5–2-fold) on maximal rates of Ca^{2+} entry. At present, it is uncertain whether the chemoattractants activate the Ca^{2+} uptake system detectable in resting amebae or a second Ca^{2+} influx pathway. However, the observations that Ca^{2+} uptake into both folate- and cAMP-stimulated cells exhibits linear Michaelis–Menten kinetics support the former model. The idea that the folate and cAMP chemoreceptors couple with the same Ca^{2+} uptake system is also suggested by other results. For instance, Ca^{2+} uptake responses induced by both chemoattractants (*a*) exhibit similar time courses, (*b*) display comparable sensitivities to inhibition by Ruthenium Red (IC_{50} = 7–10 μM), sodium azide (IC_{50} = 9–14 μM) and CCCP (IC_{50} = ∼2 μM), and (*c*) show the same degree of specificity in the presence of competing multivalent cations (Fig. 7). Finally, growth-phase cAR1 receptor transformants (but not control transformants) express high levels of surface cAMP-binding sites and exhibit cAMP-mediated Ca^{2+} uptake with properties (e.g., time course and sensitivity to inhibition by Ruthenium Red) very similar to those of the cAMP-induced Ca^{2+} uptake system detectable in aggregation-competent wild-type cells (Figs. 1 *a*, 5 *a*, and 6).

What is the nature of the chemoattractant-induced Ca^{2+}-uptake system in *Dictyostelium*? It is unlikely that Ca^{2+} entry is mediated by a Na^+/Ca^{2+} exchanger. When cAMP-induced Ca^{2+} uptake was measured in a medium containing 5 mM Na^+, the reported intracellular Na^+ concentration in this organism (33), no reduction in rate of Ca^{2+} entry was observed (data not shown). In addition, the Ca^{2+} flux measurements of Bumann et al. (8) suggest that a H^+/Ca^{2+} exchanger is not involved. To determine if receptor-activated Ca^{2+} uptake in this organism occurs via plasma membrane channels, pharmacological experiments were performed. Treatment of the cells with sodium azide or CCCP, compounds reported to induce membrane depolarization (20, 44), did not change the time course of stimulated Ca^{2+} uptake (Fig. 8). This suggests that Ca^{2+} uptake does not involve voltage-gated channels. However, these agents did reduce the magnitude of Ca^{2+} uptake by both resting and stimulated cells, thus raising the possibility that changes in membrane potential might regulate transport indirectly by altering the electrochemical gradient. Sodium azide and

Downloaded from http://rupress.org/jcb/article-pdf/112/1/103/1463870/103.pdf by guest on 04 October 2023

CCCP inhibited folate- and cAMP-induced Ca^{2+} uptake over a very narrow concentration range (data not shown); this supports the idea that these agents depolarize the plasma membrane. Similar results were obtained with Ruthenium Red (Fig. 6). This compound is known to cause membrane depolarization in other systems, but it also blocks Ca^{2+} channels and interacts specifically with Ca^{2+}-binding proteins (9). Its mechanism of action in *Dictyostelium* remains to be determined. Unexpectedly, cAMP-induced Ca^{2+} uptake was insensitive to several classes of organic Ca^{2+} channel antagonists including the 1,4-dihydropyridines, (nifedipine, nicardipine), phenylalkylamines (verapamil, methoxyverapamil), and benzothiazepines (diltiazem). In addition, stimulated Ca^{2+} influx was inhibited poorly by cations (i.e., La^{3+}, Gd^{3+}, Co^{2+}, and Cd^{2+}) (Fig. 7) known to act as Ca^{2+} channel blockers in mammalian systems (38). Since different classes of Ca^{2+} channels vary widely in their biophysical and pharmacological properties (2), the inability of these chemicals and ions to block Ca^{2+} uptake does not eliminate the possibility that this transport system is a channel. Verification that Ca^{2+} channels are involved must await the appropriate electrophysiological experiments.

The kinetics of chemoattractant-mediated Ca^{2+} uptake suggest that receptor binding activates both the initiation and termination of Ca^{2+} transport. Receptor activation appears to stimulate the rate of Ca^{2+} entry (Figs. 1 and 3 *b*) by generating an intracellular signal(s) that increases the number of active channels (transporters?) and their affinity for Ca^{2+} (Fig. 4 *b*). Since the onset of stimulated Ca^{2+} uptake only occurs after a lag of >5 s, even in the presence of saturating stimulus (100 μM) (Fig. 3 *b*), the activation process might involve several biochemical steps or a slow process such as phosphorylation. This idea is supported by the observation that when cAMP receptors are activated 10 s before the addition of Ca^{2+}, ion influx occurs without a delay (Fig. 1 *b*). Receptor occupancy also appears to initiate an adaption process which limits the duration of Ca^{2+} transport. Stimulated Ca^{2+} uptake terminates ~30 s after receptor activation regardless of whether the amebae are treated with suboptimal or saturating levels of cAMP (Fig. 3 *b*). Chemoattractant-induced activation and adaption have been reported for several other responses in *Dictyostelium* (15, 46).

Recent evidence indicates that both the folate- and cAMP-mediated signal transduction pathways in *Dictyostelium* might be regulated by G proteins (for review see references 21, 25). Interestingly, the cloned cAMP receptor, when expressed in vegetative cells which possess very low levels of endogenous receptor, appears to couple to the chemoattractant-stimulated Ca^{2+} transport system (Fig. 5 *a*). This finding suggests that G proteins might also regulate receptor-mediated Ca^{2+} entry. At present, the biochemical components in this pathway are unknown; however, our results seem to eliminate a number of possibilities. First, the G protein α-subunit, Gα1, is unlikely involved since chemoattractant-induced Ca^{2+} uptake into a Gα1-null mutant and a Gα1-over-expressing cell line was similar to that of wild-type cells. Second, analysis of Frigid A mutants and a Gα2-disruption mutant suggests that Gα2 is not required for folate- or cAMP-induced Ca^{2+} entry. However, since cAMP-pulsed HC85 and JH104 cells exhibit a weak cAMP-stimulated Ca^{2+} uptake, it is possible that Gα2 is normally involved in the cAMP-activated process, and, in its absence, a pulse-induced "back-up" G-protein is able to couple the receptor to this Ca^{2+} entry system. Third, although G protein-linked signal transduction pathways appear to mediate activation of both adenylate and guanylate cyclases in *Dictyostelium* (25), our results with the signaling mutants NP368 and PD7-2-2 indicate that stimulated Ca^{2+} entry is not regulated by changes in either intracellular cGMP or cAMP. Noninvolvement of intracellular cAMP is also suggested by (*a*) pretreatment of cells with caffeine, a compound which inhibits adenylate cyclase activation (6), does not influence cAMP-induced Ca^{2+} uptake, (*b*) activation of adenylate cyclase by cAMP is slower (51) than the onset of cAMP-stimulated Ca^{2+} entry, and (*c*) folate does not induce production of cAMP in vegetative amebae (51). In *Dictyostelium*, G proteins also appear to couple the cAMP receptors to the production of inositol polyphosphates (17). It remains to be determined whether these intracellular messengers (or the diacylglycerol/protein kinase C system) are involved in the regulation of receptor-mediated Ca^{2+} entry systems in this organism.

We are grateful to Drs. Peter Devreotes, Maureen Pupillo, Richard Firtel, Jeff Hadwiger (Johns Hopkins University School of Medicine), and Akiko Kumagai (University of California, San Diego), for useful discussions and for providing strains used in this study, and to Ms. Anne Cameron for preparation of the figures.

J. L. Milne was a recipient of a Province of Ontario Graduate Scholarship. This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada.

Received for publication 18 June 1990 and in revised form 14 September 1990.

References

1. Aeckerle, S., and D. Malchow. 1989. Calcium regulates cAMP-induced potassium ion efflux in *Dictyostelium discoideum*. *Biochim. Biophys. Acta*. 1012:196–200.
2. Bean, B. P. 1989. Classes of calcium channels in vertebrate cells. *Annu. Rev. Physiol.* 51:367–384.
3. Berridge, M. J. 1982. Regulation of cell secretion: the integrated action of cyclic AMP and calcium. *Handb. Exp. Pharmacol.* 58:227–270.
4. Berridge, M. J., and R. F. Irvine. 1984. Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature (Lond.)*. 312:315–321.
5. Böhme, R., J. Bumann, S. Aeckerle, and D. Malchow. 1987. A high-affinity plasma membrane Ca^{2+}-ATPase in *Dictyostelium discoideum*: its relation to cAMP-induced Ca^{2+} fluxes. *Biochim. Biophys. Acta.* 904: 125–130.
6. Brenner, M., and S. D. Thoms. 1984. Caffeine blocks activation of cyclic AMP synthesis in *Dictyostelium discoideum*. *Dev. Biol.* 101:136–146.
7. Bumann, J., B. Wurster, and D. Malchow. 1984. Attractant-induced changes and oscillations of the extracellular Ca^{++} concentration in suspensions of differentiating *Dictyostelium* cells. *J. Cell Biol.* 98:173–178.
8. Bumann, J., D. Malchow, and B. Wurster. 1986. Oscillations of Ca^{++} concentration during the cell differentiation of *Dictyostelium discoideum*. Their relation to oscillations in cyclic AMP and other components. *Differentiation*. 31:85–91.
9. Charuk, J. H. M., C. A. Pirraglia, and R. A. F. Reithmeier. 1990. Interaction of ruthenium red with Ca^{2+}-binding proteins. *Anal. Biochem.* 188: 123–131.
10. Coukell, M. B. 1975. Parasexual genetic analysis of aggregation-deficient mutants of *Dictyostelium discoideum*. *Mol. Gen. Genet.* 142:119–135.
11. Coukell, M. B., and A. M. Cameron. 1987. Effects of calcium antagonists on cyclic AMP phosphodiesterase induction in *Dictyostelium discoideum*. *J. Cell Sci.* 88:379–388.
12. Coukell, M. B., S. Lappano, and A. M. Cameron. 1983. Isolation and characterization of cAMP unresponsive (frigid) aggregation-deficient mutants of *Dictyostelium discoideum*. *Dev. Genet.* 3:283–297.
13. Coukell, M. B., A. M. Cameron, C. M. Pitre, and J. D. Mee. 1984. Developmental regulation and properties of the cGMP-specific phosphodiesterase in *Dictyostelium discoideum*. *Dev. Biol.* 103:246–257.
14. De Wit, R. J. W., and T. F. Rinke De Wit. 1986. Developmental regulation of the folic acid chemosensory system in *Dictyostelium discoideum*. *Dev. Biol.* 118:385–391.

Downloaded from http://rupress.org/jcb/article-pdf/112/1/103/1463870/103.pdf by guest on 04 October 2023

15. Devreotes, P. N., and T. L. Steck. 1979. Cyclic 3',5'-AMP relay in *Dictyostelium discoideum*. II. Requirements for the initiation and termination of the response. *J. Cell Biol.* 80:300–309.
16. Europe-Finner, G. N., and P. C. Newell. 1985. Calcium transport in the cellular slime mould *Dictyostelium discoideum*. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 186:70–74.
17. Europe-Finner, G. N., and P. C. Newell. 1987. Cyclic AMP stimulates accumulation of inositol trisphosphate in *Dictyostelium*. *J. Cell Sci.* 87:221–229.
18. Fabiato, A., and F. Fabiato. 1975. Contractions induced by a calcium-triggered release of calcium from the sarcoplasmic reticulum of single skinned cardiac cells. *J. Physiol. (Lond.)*. 249:469–495.
19. Fabiato, A., and F. Fabiato. 1979. Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *J. Physiol. (Paris)*. 75:463–505.
20. Felle, H., and F. W. Bentrup. 1977. A study of the primary effect of the uncoupler carbonylcyanide m-chlorophenylhydrazone on membrane potential and conductance in *Riccia fluitans*. *Biochim. Biophys. Acta.* 464:179–187.
21. Firtel, R. A., P. J. M. Van Haastert, A. R. Kimmel, and P. N. Devreotes. 1989. G protein linked signal transduction pathways in development: *Dictyostelium* as an experimental system. *Cell.* 58:235–239.
22. Hallam, T. J., and T. J. Rink. 1989. Receptor-mediated Ca^{2+} entry: diversity of function and mechanism. *Trends Pharmacol. Sci.* 10:8–10.
23. Hanes, C. S. 1932. Studies on plant amylases. I. The effect of starch concentration upon the velocity of hydrolysis by the amylase of germinated barley. *Biochem. J.* 26:1406–1421.
24. Henderson, E. J. 1975. The cyclic adenosine 3':5'-monophosphate receptor of *Dictyostelium discoideum*. Binding characteristics of aggregation-competent cells and variation of binding levels during the life cycle. *J. Biol. Chem.* 250:4730–4736.
25. Janssens, P. M. W., and P. J. M. Van Haastert. 1987. Molecular basis of transmembrane signal transduction in *Dictyostelium discoideum*. *Microbiol. Rev.* 51:396–418.
26. Klein, P. S., T. J. Sun, C. L. Saxe III, A. R. Kimmel, R. L. Johnson, and P. N. Devreotes. 1988. A chemoattractant receptor controls development in *Dictyostelium discoideum*. *Science (Wash. DC)*. 241:1467–1472.
27. Krause, K.-H., and P. D. Lew. 1987. Subcellular distribution of Ca^{2+} pumping sites in human neutrophils. *J. Clin. Invest.* 80:107–116.
28. Kumagai, A., M. Pupillo, R. Gundersen, R. Miake-Lye, P. N. Devreotes, and R. A. Firtel. 1989. Regulation and function of $G\alpha$ protein subunits in *Dictyostelium*. *Cell.* 57:265–275.
29. Lo, E. K.-L., M. B. Coukell, A. S. Tsang, and J. L. Pickering. 1978. Physiological and biochemical characterization of aggregation-deficient mutants of *Dictyostelium discoideum*: detection and response to exogenous cyclic AMP. *Can. J. Microbiol.* 24:455–465.
30. Loomis, W. F., editor. 1982. The Development of *Dictyostelium discoideum*. Academic Press, New York. 551 pp.
31. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
32. Mann, S. K. O., C. Pinko, and R. A. Firtel. 1988. cAMP regulation of early gene expression in signal transduction mutants of *Dictyostelium*. *Dev. Biol.* 130:294–303.
33. Marin, F. T., and F. G. Rothman. 1980. Regulation of development in *Dictyostelium discoideum*. IV. Effects of ions on the rate of differentiation and cellular response to cyclic AMP. *J. Cell Biol.* 87:823–827.
34. Mato, J. M., and T. M. Konijn. 1975. Chemotaxis and binding of cyclic AMP in cellular slime molds. *Biochim. Biophys. Acta.* 385:173–179.
35. Milne, J. L., and M. B. Coukell. 1988. Isolation and characterization of a plasma membrane calcium pump from *Dictyostelium discoideum*. *Biochem. J.* 249:223–230.
36. Milne, J. L., and M. B. Coukell. 1989. Identification of a high-affinity Ca^{2+} pump associated with endocytotic vesicles in *Dictyostelium discoideum*. *Exp. Cell. Res.* 185:21–32.
37. Mullens, I. A., and P. C. Newell. 1978. cAMP binding to cell surface receptors of *Dictyostelium*. *Differentiation.* 10:171–176.
38. Nachshen, D. A. 1984. Selectivity of the Ca binding site in synaptosome Ca channels. Inhibition of Ca influx by multivalent metal cations. *J. Gen. Physiol.* 83:941–967.
39. Pan, P., E. M. Hall, and J. T. Bonner. 1972. Folic acid as second chemotactic substance in the cellular slime molds. *Nat. New Biol.* 237:181–182.
40. Reuter, H. 1983. Calcium channel modulation by neurotransmitters, enzymes and drugs. *Nature (Lond.)*. 301:569–574.
41. Ross, F. M., and P. C. Newell. 1979. Genetics of aggregation pattern mutations in the cellular slime mould *Dictyostelium discoideum*. *J. Gen. Microbiol.* 115:289–300.
42. Ross, F. M., and P. C. Newell. 1981. Streamers: chemotactic mutants of *Dicytostelium discoideum* with altered cyclic GMP metabolism. *J. Gen. Microbiol.* 127:339–350.
43. Schultz, G., W. Rosenthal, J. Hescheler, and W. Trautwein. 1990. Role of G proteins in calcium channel modulation. *Annu. Rev. Physiol.* 52:275–292.
44. Van Duijn, B., S. A. Vogelzang, D. L. Ypey, L. G. Van der Molen, P. J. M. Van Haastert. 1990. Normal chemotaxis in *Dictyostelium discoideum* cells with a depolarized plasma membrane potential. *J. Cell Sci.* 95:177–183.
45. Van Haastert, P. J. M., and E. Kien. 1983. Binding of cAMP derivatives to *Dictyostelium discoideum* cells. Activation mechanism of the cell surface cAMP receptor. *J. Biol. Chem.* 258:9636–9642.
46. Van Haastert, P. J. M., and P. R. Van der Heijden. 1983. Excitation, adaption, and deadaption of the cAMP-mediated cGMP response in *Dictyostelium discoideum*. *J. Cell Biol.* 96:347–353.
47. Varnum, B., and D. R. Soll. 1981. Chemoresponsiveness to cAMP and folic acid during growth, development, and dedifferentiation in *Dictyostelium discoideum*. *Differentiation.* 18:151–160.
48. Volpe, P., K.-H. Krause, S. Hashimoto, F. Zorzato, T. Pozzan, J. Meldolesi, and D. P. Lew. 1988. "Calciosome," a cytoplasmic organelle: the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} store of nonmuscle cells? *Proc. Natl. Acad. Sci. USA.* 85:1091–1095.
49. Watts, D. J., and J. M. Ashworth. 1970. Growth of myxamoebae of the cellular slime mould *Dictyostelium discoideum* in axenic culture. *Biochem. J.* 119:171–174.
50. Wick, U., D. Malchow, and G. Gerisch. 1978. Cyclic-AMP stimulated calcium influx into aggregating cells of *Dictyostelium discoideum*. *Cell Biol. Int. Rep.* 2:71–79.
51. Wurster, B., K. Schubiger, U. Wick, and G. Gerisch. 1977. Cyclic GMP in *Dictyostelium discoideum*. Oscillations and pulses in response to folic acid and cyclic AMP signals. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 76:141–144.

Downloaded from http://rupress.org/jcb/article-pdf/112/1/103/1463870/103.pdf by guest on 04 October 2023