Influence of Cytokinins and Sulfhydryl Group-Reacting Agents on Calcium Transport in Fungi*

(Received for publication, July 2, 1973)

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SUMMARY

Achlya is a filamentous coenocytic fungus that grows only in the presence of Ca++. The Ca++ transport (uptake and binding) system of this organism was studied and shown to consist of two components: a low molecular weight Ca++ binding glycopeptide present within the cell wall matrix and a Ca++ uptake component associated with the cell membrane. Certain thiol group-reacting agents and cytokinin-active compounds enhanced Ca++ binding and uptake, respectively.

Ca++ transport by intact cells showed pH dependence with optima at pH 6.5 and 8.5 and temperature dependence with maximal transport occurring at 40° (without activation) and 15° (with activation). Ca++ transport proceeded by facilitated diffusion. The system is insensitive to metabolic inhibitors.

Compounds with known cytokinin activity stimulated the release of Ca++ bound to the cell wall glycopeptide, and these same compounds enhanced Ca++ intake into osmotically shocked cells (lacking the glycopeptide). The half-saturation values for Ca++ intake into cells and release from the glycopeptide are essentially identical.

This report is concerned with the dissection and partial characterization of the Ca++ uptake system of the organism, and the influence of plant growth substances, cytokinins, on its over-all activity.

MATERIALS AND METHODS

Organism, Maintenance and Culture

A homothallic species of Achlya was used in these studies. The methods described previously (1) for growing the organism and collecting and germinating the spores were followed with slight modifications. Spores were germinated at 11° for 18 hours. The germinated spores were collected by filtration using nylon cloth (61 μm mesh size) obtained from Henry Simon Ltd., Stockport, England, washed with sterile water, and resuspended in the pertinent experimental medium. The average length of the hyphae of germinated spores was 120 ± 20 μm and diameter of 5 μm.

Cell-Density-Protein Correlation

The growth and germinating conditions were standardized so that an accurate estimate of the cell number could be determined using a protein-optical density at 700 nm correlation. This relationship was derived by first determining the linear correlation between cell number and optical density at 700 nm and between cell number and protein content. A linear plot of optical density and protein content was used as the standard for estimating cell number. For any experiment, both the optical density and protein content were determined. Optical density was kept as uniform as possible in all experiments. Protein was determined by the method of Lowry et al. (3). Unless stated specifically, the optical density of intact germlings used in these experiments was maintained at 0.19 ± 0.01 A410 nm. Osmotically shocked germlings were used at a cell density of 0.32 ± 0.03 A410 nm.

Transport Assays

Intact Cells—Transport of "Ca++ was measured by the following procedure. Germlings were suspended in either Buffer A, composed of 10 mM Tris-acetate, pH 7, 2 mM KCl, 2 mM NaCl, and 10 mM glucose, or Buffer B, in which the concentration of Tris-acetate was reduced to 1 mM and all other constituents of Buffer A were unchanged. All cell suspensions were pre-equilibrated for 15 min before the start of an experiment. Reaction was started by addition of 1 ml of cells (approximately 2.5 x 10⁶ germlings) to the reaction solution and mixing rapidly. The final assay system, therefore, was one-half the concentration of Buffer A.

The cells were allowed to concentrate "Ca++ for 2½ min before the reaction was stopped by rapidly filtering the sample through presoaked Millipore HAWP filters in a 30-chamber Millipore manifold attached to vacuum pumps and washed within 10 s with 3 successive 5-ml volumes of unlabeled medium at room temperature. Washed filters were transferred to 10 ml of Bray’s (4) fluid in scintillation vials, dissolved, and counted in a Packard Tri-Carb liquid scintillation spectrometer.

Osmotically Shocked Cells—The osmotically shocked germlings...
were only 30 μm long and were obtained from a suspension of spores that had been permitted to germinate and grow for 120 min at 28°C. The protocol used for germination was as follows. Freshly discharged spores were collected aseptically at 5°C by centrifugation (3500 × g for 5 min) and resuspended in sterile growth medium composed of 0.5 g of yeast extract and 5 g of glucose per liter of distilled water. The density of spores, determined from hemocytometer count, was kept constant at about 10^6 cells per ml. Incubation was carried out in a Bellco spinner flask with constant stirring. More than 90% of the spores germinated after 120 min. The cells were recovered by filtration through 5 μm Millipore HAWP filters, washed with water, and then resuspended in sucrose solution composed of 0.5 M sucrose, 0.05 M Tris-HCl, and 0.01 M EDTA, pH 8. During sucrose treatment the density of the cells was increased to 10^7 per ml. The method of Heppel (5) was followed from this point on to obtain osmotically shocked cells and shock fluid. Osmotically shocked cells were then used in transport experiments in manner identical with that described for unshocked germlings.

**pH Studies**

Preincubation of the germlings was carried out in Buffer B. One milliliter of this suspension was transferred to 1 ml of the reaction solution containing 25 mM Tris-acetate or potassium phosphate at the specified pH for transport assay.

**Temperature Studies**

Pre-equilibration of the germlings and reaction solution was carried out at the temperature specified for 10 min before they were mixed and the initial reaction rate was studied.

**Evaluation of Initial Reaction Rate**

The quantity of radioactive material taken up as a function of time was determined as described under "Transport Assays." The period during which there was a linear relationship between uptake and time was taken to represent a measurement of initial reaction rate.

**Materials**

45Ca++ as CaCl₂ in aqueous solution was purchased from American-Searle, Arlington Heights, Ill. All other biochemicals and reagents were obtained from Sigma Chemical Co., St. Louis, Mo.

**RESULTS**

**Properties of Calcium Uptake Systems**

**Kinetics of Ca++ Uptake by Intact Cells**—Initial reaction rate studies of Ca++ uptake measured as a function of Ca++ concentration did not attain a constant value even at 1 mM as shown in rate-concentration and double reciprocal forms (Fig. 1). The double reciprocal plot was linear only when HgCl₂ was included in the assay, and, simultaneously, Ca++ uptake was stimulated about 4- to 5-fold.

**Activation of Ca++ Binding by Hg²⁺** When Ca++ uptake was studied as a function of HgCl₂ concentration (Fig. 2), Hg²⁺ activated Ca++ uptake at low HgCl₂ concentrations and inhibited it at high. The optimum for Hg²⁺ stimulation was 10 μM, and this value remained unchanged at different Ca++ concentrations. Kinetic analysis of the data showed that Ca++ and Hg²⁺ did not compete with each other for the same cellular site at low HgCl₂ concentrations, but did compete at high.

In these experiments, the age, cell number, and environmental conditions were standardized and kept constant.

**Activation by Other Thiol-reacting Agents**—I₂, Ag⁺, HgI₂, thimerosal, p-hydroxymercuribenzoate, and several organic mercurials stimulated Ca++ uptake. The organic mercurials and HgI₂ did not inhibit Ca++ binding when used at concentrations as high as 1 mM. This property was used successfully to distinguish between the Ca++ binding and uptake systems of the cell (see later). The effectiveness of the various thiol-reacting agents is shown in Table I.

**Dependence of Ca++ Uptake on pH and Temperature**—Studies of Ca++ uptake as a function of pH and temperature showed that Ca++ uptake depended upon temperature in a manner that suggested a protein carrier may be involved (Fig. 3). More important was the observation that thiol-reacting agents activated Ca++ uptake in a novel way. In the absence of mercurials, the
rate of Ca\textsuperscript{2+} uptake was unchanged from 5 to 20°C. Between 20 and 50°C, Ca\textsuperscript{2+} uptake was strongly dependent upon temperature with Q\textsubscript{10} greater than 2 as the temperature was raised from 22 to 31°C. This implied that a process requiring high activation energy may be involved.

In the presence of mercurials, Ca\textsuperscript{2+} uptake system responded better at low than at high temperatures. Optimal transport occurred at 15°C and above 25°C the transport rate was gr\textsuperscript{10} diminished. In no case was transport above 50°C considered significant.

Of interest was the observation that thimerosal-treated cells and HgI\textsubscript{2}-treated cells (data not shown) transported Ca\textsuperscript{2+} with optima at 15 and 40°C, the two temperatures at which the activated and nonactivated systems responded optimally. This provided strong support for the idea that two Ca\textsuperscript{2+} uptake components may be involved.

The rate of Ca\textsuperscript{2+} uptake was also dependent upon pH as shown in Fig. 4. In the absence of mercurials or other activators, Ca\textsuperscript{2+} uptake proceeded with optima at pH 6.5 and 8.5. With HgCl\textsubscript{2}, only one optimum at pH 6.5 was observed, and this rate was significantly enhanced. Other thiol-reacting agents affected Ca\textsuperscript{2+} uptake at different pH values in a similar manner.

**Release of Hg-activatable Ca\textsuperscript{2+}-binding Glycopeptide by Cold Osmotic Shock**

The Ca\textsuperscript{2+} taken up by cells treated with thiol-reacting agents was removable if cells were washed with cysteine and unlabeled Ca\textsuperscript{2+}. Cysteine alone was only partly effective. This indicated that the system on which Hg\textsuperscript{2+} acts is not within the cell. Some support for this conclusion was already available in early experiments where we had shown that chelating agents such as EDTA, ethylene glycol bis(\beta-aminoethyl ether)-N,N'-tetraacetic acid, and citrate removed Ca\textsuperscript{2+} bound to the cells without themselves being taken up (2).

An attempt was made to isolate the component to which Ca\textsuperscript{2+} was bound when thiol-reacting agents were present. Ca\textsuperscript{2+} bound to cells in the presence of Hg\textsuperscript{2+} was removable by cold osmotic shock as described under “Materials and Methods.” The Ca\textsuperscript{2+} was found to be bound to a glycopeptide moiety that was retarded when the osmotic shock fluid was ultrafiltered through EM-2 Diaflo filters under 70 p.s.i. of N\textsubscript{2}. This Ca\textsuperscript{2+}-binding entity has been purified and shown to be a low molecular weight glycopeptide that binds about 20 atoms of Ca\textsuperscript{2+}. Ca\textsuperscript{2+} binding by this glycopeptide is enhanced by thiol-reacting agents in vitro.

**Ca\textsuperscript{2+} Transport Properties of Osmotically Shocked Cells**

The Ca\textsuperscript{2+} uptake system of osmotically shocked cells was different from that of intact cells (Fig. 1). Whereas the rate-concentration plot for unshocked cells is nonsymptotic, that for shocked cells is normal Michaelian in form with an apparent K\textsubscript{m} of 40 \( \mu \text{M} \) (Fig. 5). At the relatively low concentration of 0.1 \( \mu \text{M} \), thiol-reacting agents inhibited this Ca\textsuperscript{2+} uptake activity (Fig. 5).

1 L. E. Cameron and H. B. LeJohn, manuscript in preparation.
were effective as strong Ca2+-releasing agents. A summary of the findings is given in Table II. Only cytokinins than 40 agents that were tested for their Ca2+-releasing ability.

The influence of pH and temperature was studied in the same way as performed for normal cells. The results have been included in Figs. 3 and 4 to provide an easy comparison. In shocked cells, the thiol-reacting agents no longer stimulated Ca2+ uptake at different pH values and temperature. As mentioned above, this thiol-reacting capacity was found to reside with the Ca2+-binding glycopeptide fraction of the shock fluid.

**Cytokinins as Agonists of Ca2+ Uptake by Intact Cells**

If cells naturally store Ca2+ in the cell wall complex using the glycopeptide as vehicle for this function, then a mechanism should exist that permits Ca2+ release when the physiological state of the cell demands this. A search for such a Ca2+-releasing agent led to the discovery that N\(^{6}\)-(substituted)-adenines (cytokinins) served this function. Cells fully stimulated by HgCl2 to take up optimal amounts of Ca2+ were exposed to varying concentrations of a variety of cytokinin-active compounds. Ca2+ was immediately released from the cells as a function of cytokinin concentration (see Fig. 6, a and b). The analysis was not confined to cytokinin-active compounds. Benzimidazole derivatives, adenine derivatives, nucleotides, nucleosides, and methylxanthines are among more than 40 agents that were tested for their Ca2+-releasing ability. A summary of the findings is given in Table II. Only cytokinins were effective as strong Ca2+-releasing agents.

**Cytokinins Stimulate Ca2+ Transport by Osmotically Shocked Cells**

If cytokinins stimulate Ca2+ release from the cell wall glycopeptide, they may also interact with the Ca2+ uptake system of the cell that was recognized in osmotically shocked cells (see Figs. 3 and 4). Osmotically shocked cells take up Ca2+ as a function of cytokinin concentration in a cooperative manner (Fig. 7). In the absence of HgCl2, the rate of Ca2+ uptake plotted as a function of 6ipAde concentration was sigmoid in form. When Hg2+ was added, this plot became hyperbolic and Ca2+ uptake was significantly enhanced at low cytokinin concentrations (Fig. 7). The anomaly of these results is that Ca2+ uptake as a function of Ca2+-binding glycopeptide is more effective when Hg2+ was present during Ca2+ transport by osmotically shocked cells. In the absence of cytokinin, Hg2+ inhibited Ca2+ uptake (Fig. 5). Therefore, besides antagonizing Mg2+ competition for Ca2+ binding sites, Hg2+ competes with Ca2+, and it is this inhibition which cytokinin prevents permitting Ca2+ to be transported optimally.

**DISCUSSION**

The influence of mercurials and other thiol-reacting agents can be interpreted in several ways, one of which is that they mimic a natural activator of Ca2+ transport. Alternatively, Hg2+ may desensitize a regulatory component of the transport system. This implies that cytokinins stimulate Ca2+ transport by functioning as allosteric modifiers. Of interest is the observation that the half-saturation value of cytokinin for Ca2+ release from

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**Table II**

Summary of activation (S\(_{a,c}\)) and inhibition (I\(_{a,c}\)) constants of various agents that influence Ca2+ release from germinated sporangiospores and Ca2+ uptake into osmotically shocked germlings of Achlya

<table>
<thead>
<tr>
<th>Agent</th>
<th>S(_{a,c})</th>
<th>I(_{a,c})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>x</td>
<td>1.6 x 10^-4</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>x</td>
<td>8.0 x 10^-5</td>
</tr>
<tr>
<td>Xanthine</td>
<td>5.0 x 10^-5</td>
<td>8.3 x 10^-5</td>
</tr>
<tr>
<td>Theophylline</td>
<td>x</td>
<td>2.5 x 10^-4</td>
</tr>
<tr>
<td>6-Benzyladenine</td>
<td>x</td>
<td>5.0 x 10^-6</td>
</tr>
<tr>
<td>6-Methylaminepurine</td>
<td>5.0 x 10^-5</td>
<td>4.0 x 10^-5</td>
</tr>
<tr>
<td>6-Dimethylaminopurine</td>
<td>2.5 x 10^-5</td>
<td>2.0 x 10^-5</td>
</tr>
<tr>
<td>2,6-Diaminopurine</td>
<td>x</td>
<td>7.5 x 10^-4</td>
</tr>
<tr>
<td>Zeatin(^\dagger)</td>
<td>x</td>
<td>5.0 x 10^-4</td>
</tr>
<tr>
<td>2-Aminobenzimidazole</td>
<td>x</td>
<td>1.0 x 10^-6</td>
</tr>
<tr>
<td>6-Isopentenyldadenine</td>
<td>4.6 x 10^-4</td>
<td>6.67 x 10^-6</td>
</tr>
</tbody>
</table>

\(^\dagger\) N6-(trans-4-hydroxy-3-methylbut-2-enyl) adenine.
Fig. 6. a, stimulation of 44Ca2+ release from germinated sporangiospores of *Achlya* by AMP (○), adenosine (□), adenine (△), hypoxanthine (●), and xanthine (■) in the presence of 10 μM HgCl2. — —, adenine-stimulated release of Ca2+ in the absence of Hg2+. b, stimulation of 44Ca2+ release from germinated sporangiospores of *Achlya* by cytokinins: triacanthine (6-aminomethylallylpurine) (○), zeatin N4-(trans-4-hydroxy-3-methylbut-2-enyl)adenine (△), 6ipAde (●), 6-methylaminopurine (□), and 6-dimethylaminopurine (●) in the presence of 10 μM HgCl2. In the experiments with zeatin and 6ipAde, the pH of the reaction was 7.3 rather than pH 7.0.

The possibility that cytokinins may be stimulating Ca2+ release by forming complexes with either Hg2+ or Ca2+ can be excluded because: (a) not all N6 (substituted)-adenines are effective; (b) all nucleotides, nucleosides, and pyrimidine compounds tested failed to release the bound Ca2+; (c) I2 replaced Hg2+ in stimulating Ca2+ uptake and the effect is reversible by cytokinins; and (d) cytokinins enhanced Ca2+ uptake by osmotically shocked cells in the absence of Hg2+.

These observations support the general conclusion that Ca2+ uptake in *Achlya* is affected primarily by compounds with established cytokinin activity in higher plants. As *Achlya* belongs to the class, Oomycetes, fungi that may be parasitic on higher plant forms, this cytokinin effect may have a physiological meaning.

The uptake of Ca2+ is not an active process because metabolic energy inhibitors have little or no effect on this phenomenon. Besides, all metabolites such as sugars, nucleosides, and amino acids that are actively concentrated by the organism (6) cannot be taken up by osmotically shocked cells whereas Ca2+ uptake continues. Ca2+ transport must proceed by facilitated diffusion since the uptake process is carrier-mediated (Figs. 3 and 4) depending upon pH and temperature. One of two components involved in the uptake of Ca2+ has been characterized and shown to have properties of a Ca2+ sequestrator (Footnote 1 and this report). The second component responsible for Ca2+ transport is yet to be isolated and characterized.

**Cell Growth and Polarization**—There is evidence which indicates that the outgrowth of a hypha from an ephemeral spore occurs by an obscure polarization mechanism (7). This problem of initiating and maintaining polarization in an actively growing cell is a problem that is central to all of developmental biology. These germinal studies of Ca2+ involvement in membrane activity of fungi, and the corresponding work on Ca2+ in development and differentiation of this organism (2, 8) may provide new clues to our understanding of the phenomenon of polarization in cell development.

**REFERENCES**


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