CYTOKININS REGULATE CALCIUM BINDING TO A GLYCOPROTEIN FROM FUNGAL CELLS

H.B. LeJohn and Linda E. Cameron

Department of Microbiology, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada.

Received August 21,1973

SUMMARY

A glycoprotein that binds about 20 atoms of Ca per mole has been purified from the osmotic shock fluid of some unicellular coenocytic water-molds, Achlya spp. and Blastocladiella emersonii. The binding of calcium is allosterically regulated by N^6-(substituted)adenine derivatives, cytokinins. Pyrimidines, purine and pyrimidine nucleosides, auxins, and benzimidazole derivatives are ineffective in inhibiting calcium binding. Lysozyme partially inactivates the molecule so that a high affinity calcium binding site is destroyed. Trypsin and pronase inactivate the molecule so that Ca^{++} binding to both high and low affinity sites is affected. Cytokinins inhibit calcium binding to both sites.

Although cytokinins (plant growth regulators) have been studied intensively for more than two decades, there is no clear indication how these agents function to aid cell growth. Their biological manifestations include induction of mitosis, cell division and enlargement, alteration of morphogenesis, promotion of seed germination, stimulation of tumour formation in plants, and enhancement of protein and RNA synthesis (1). The effects of cytokinins, particularly the N^6-(substituted)adenine derivatives, on mammalian cells is less clear (2). One of the most potent cytokinin found to date is N^6-(Δ^2-isopentenyl)adenine and its riboside, and it has been detected in the tRNA of all species examined. But it is yet to be determined whether it or a derivative is a natural regulatory hormone (2).

We wish to present here data which implicates this and other cytokinins as regulators of Ca^{++} binding to a Ca^{++} sequestering glycoprotein present on the membrane surface of fungal cells.

MATERIALS AND METHODS

Organisms. The organisms used in these studies are Achlya sp. (1969) whose properties were described previously (3); A. ambisexualis CBS 100.50 obtained from the Centraalbureau voor Schimmelcultures, Baarn, Netherlands; and Blastocladiella emersonii.

Germination of Spores. The methods used for the growth, harvesting and germination of sporangiospores obtained from Achlya spp. are described in detail in other reports (4, 5, 6). Zoospores from Blastocladiella were collected and germinated as prescribed by Lovett (7).

Cold Osmotic Shock Treatment. The method of Heppel was followed with the modifications outlined elsewhere (8).

Equilibrium Binding. Binding of $^{45}Ca^{++}$ to purified and crude glycoprotein isolates from Achlya spp. and B. emersonii was determined by equilibrium dialysis using Visking dialysis sacs, and by the use of micro-dialysis chambers. Details are given in a separate report (8).

Protein Determination. Protein was determined by the colorimetric method of Lowry et al (9).

Chemicals. All biochemicals were obtained from Sigma Chemical Co. $^{45}Ca^{++}$ was purchased from Amersham/Searle.

RESULTS

Purification of Ca^{++} Binding Glycoprotein. Table 1 summarizes the procedure used to purify to homogeneity the Ca^{++} binding glycoprotein from Achlya sp. (1969). The Ca^{++} binding entities from the other fungi are not yet purified to homogeneity. Data on the isolation, purification and characterisation of the glycoprotein from Achlya sp. (1969) have been presented elsewhere (8).

The molecular weight of the Achlya glycoprotein was determined by SDS polyacrylamide gel electrophoresis and by Sephadex-G50 gel filtration to be $6,000 \pm 150$ and the material consisted of 65% carbohydrate, 35% protein with no phospholipid detectable.

Table 1. Purification of Ca^{++} binding glycoprotein from germinated sporangiospores of Achlya.

Fraction	Total protein* (mg)	Binding capacity (nmols Ca^{++})	Specific binding capacity (nmols Ca^{++} bound/mg protein)	Purification
1. Osmotic shock fluid	20.25	7515	371	1.0
2. Ultrafiltration**	6.07	3782	623	1.68
3. CM-cellulose and ultrafiltration	1.55	1539	993	2.68
4. Sephadex-G50	0.33	409.4	1240.6	3.34

The assay system employed during purification of the glycoprotein was equilibrium dialysis at 4° for 3 hr against 100 μM Ca^{++} in 1 mM Tris-acetate buffer, pH 7.

*Since the Ca^{++} binding entity is a glycoprotein, using protein as a standard unit for purification gives a false picture.

**An important step in the purification process. Over 65% of the proteins are removed some of which are anionic and do contaminate the CM-cellulose preparation if not filtered off.

Influence of Lysozyme, Trypsin and Pronase on Ca^{++} Binding.

The kinetics of $^{45}Ca^{++}$ binding by the purified glycoprotein is shown in the Scatchard plot of Fig. 1. The semi-reciprocal plot is biphasic with slopes (K_d's) corresponding to 5.4×10^{-6}M and 1.4×10^{-5}M. When the glycoprotein was treated with either trypsin or lysozyme before carrying out the equilibrium binding, the affinities were drastically altered. Lysozyme partially inactivated the glycoprotein changing the K_d values to 10^{-5}M and 3.6×10^{-5}M respectively. Trypsin, on the other hand, inactivated the high affinity binding sites for $^{45}Ca^{++}$ while decreasing the slope of the low affinity binding to 1.5×10^{-4}M.

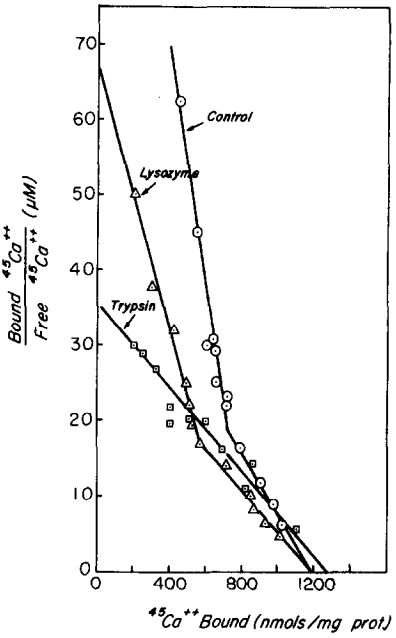

FIG. 1. Scatchard plot of the binding activity of Achlya glycoprotein untreated and treated with either lysozyme or trypsin.

Ca^{++} binding to both high and low affinity sites was drastically reduced by Mg^{++}, Mn^{++}, Sr^{++}, Ba^{++}, K^{+}, and Na^{+} (Table 2). The effect of Mg ions was particularly interesting since this was the only one of the elements found to regulate, in a cooperative manner, the transport of Ca^{++} on the membrane-localised Ca^{++} uptake system (10).

The Ca^{++} binding glycoprotein isolated from B. emersonii has similar properties as the Achlya glycoprotein as summarized in Table 2. Trypsin, pronase, and lysozyme partially inactivated this glycoprotein as well.

Cytokinins and Ca^{++} Binding. Both the Achlya and Blastocladiella glycoproteins interact with cytokinins. By means of equilibrium dialysis experiments, the Ca^{++} binding capacities of the glycoproteins were shown to be dramatically reduced when N^6-(substituted)adenine derivatives such as N^6-(Δ^2-isopentenyl)adenine, N^6-dimethyladenine, N^6-methyladenine, xanthine, hypoxanthine, N^6-(Trans-Y-hydroxymethyl-Y-methylallyl)adenine (zeatin), and adenine were present in the dialysing fluid. Interestingly, the compound,

Table 2. Influence of inorganic ions and ionic strength on the activity of Achlya and Blastocladiella Ca^{++} binding glycoproteins.

Assay system: The assay system used was equilibrium dialysis at 4^{o} for 4 hr against 1 mM Tris-acetate, pH 7 and 10 uM Ca^{++}

Treatment	Activity remaining as % of control value
Achlya:	
None	100
$MnCl_2$ (10^{-4}M)	8
$MgCl_2$ (10^{-4}M)	78
KCl (5×10^{-3}M)	4
NaCl (5×10^{-3}M)	3
$Sr(NO_3)_2$ (10^{-4}M)	56
$BaCl_2$ (10^{-4}M)	35
Pronase (1 mg/mg glycoprotein)	38
Trypsin (1 mg/mg glycoprotein)	32
Lysozyme (1 mg/mg glycoprotein)	45
Blastocladiella:	
None	100
Trypsin (1 mg/mg glycoprotein)	21
Pronase (1 mg/mg glycoprotein)	22
Lysozyme (1 mg/mg glycoprotein)	46

6-amino-3-(3-methyl-2-butenyl)purine (triacanthine) that is believed to be an inactive form of the natural growth factor, isopentenyladenine, and is activated by rearrangement of the hydrocarbon chain from the 3 to the 6 position, was very active. These results are summarised in Table 3. All nucleosides of purines and pyrimidines were inactive on the glycoproteins. Thus, where adenine, hypoxanthine, and xanthine inhibit, adenosine, inosine and xanthosine were ineffective. The degree of effectiveness of the various

Table 3. Influence of N^6-(substituted)adenine derivatives on the Ca^{++} binding activity of Achlya and Blastocladiella glycoprotein (CaBP).

Assay system: Ca^{++} binding activity was monitored by equilibrium dialysis at 4° for 4 hr against 1 mM Tris-acetate at the specified pH values in the presence of 50 μM Na^+ (determined to be the maximum amount of Na^+ present in the cytokinin solution added when NaOH was used to solubilize some of the cytokinins and purine derivatives). The concentration of Ca^{++} was held constant throughout at 100 μM. The experimental pH varied due to problems of solubility of some cytokinins at low pH values.

Agent and Source of CaBP	Concentration (μM)	pH	nmols Ca^{++} bound per mg protein
Unpurified CaBP (Achlya)			
(no addition)		7.0	332.3
		8.1	320.2
		9.0	628.6
(plus cytokinins)			
6-dimethylaminopurine	500	9.0	348.3 (44.6%)
6-methylaminopurine	500	9.0	202.2 (67.2%)
Isopentenyladenine	500	9.0	91.1 (85.5%)
Triacanthine	500	7.0	69.1 (79.2%)
Zeatin	400	8.1	152.1 (52.5%)
(other derivatives)			
Adenine	500	7.0	162.7 (51.0%)
Adenosine	500	7.0	319.4 (4.0%)
Hypoxanthine	500	9.0	406.6 (35.3%)
Inosine	500	7.0	332.3 (0.0%)
Purified CaBP (Achlya)			
(plus xanthine)	0	9.0	2153.0
	1		1611.0 (25.2%)
	10		1561.0 (27.5%)
	100		1102.0 (48.2%)
	500		575.0 (73.3%)
Unpurified CaBP (Blastocladiella)			
(no addition)		7.0	1555.0
		8.0	1496.6
		9.0	2941.1
Partially purified System			
(plus xanthine)	0	9.0	3941.0
	1		3542.0 (11.1%)
	5		2906.0 (26.3%)
	10		2701.0 (31.5%)
	100		1970.0 (50.0%)
	200		905.0 (78.0%)
	500		635.0 (83.9%)

The percentage values in brackets represent the calculated decrease in binding activity caused by the agent added.

Benzimidazole, pyrimidines, pyrimidine nucleosides, and indoleacetic acid were ineffective and are not included in the Table.

cytokinins in the Ca^{++} binding system matched closely the potency pattern obtained from standard tobacco callus bioassays (1). It is evident, then, that the cytokinins may not be functioning as chelators in the glycoprotein Ca^{++} binding system because only free bases of purines and cytokinins work. As chelators, purine nucleosides and a variety of other agents should inhibit equally well, but they are all ineffective.

DISCUSSION

In separate reports (5, 6, 8, 11) we have presented evidence in support of the thesis that the removal of a Ca^{++} binding glycoprotein from the periplasmic space of Achlya cells leads to a loss in the capacity of these cells to actively concentrate amino acids (5), nucleosides and sugars (11). The ability to take up Ca^{++} by facilitated diffusion is, however, retained by these cells (6). The accompanying report shows that these same cytokinins activate the intake of Ca^{++} by cells that have now been stripped of their Ca^{++} binding protein (10). The role of cytokinins in these fungal cells then appears to be two-fold: (a) stimulation of Ca^{++} release from a Ca-sequestering glycoprotein localised on the cytoplasmic membrane surface, and (b) enhancement of Ca^{++} intake into the cells. The glycoprotein may therefore be viewed as a reservoir from which Ca ions are leached, when upon physiological demand, excretion of cytokinins (hormones) signal the necessity for Ca^{++} in cellular metabolism. How a macromolecule as small as this glycoprotein interacts with such diverse compounds is an interesting feature for structural studies.

This study has opened the possibility of using these responsive fungal cells as tools for determining very quickly what biological agents may have potential cytokinin activity. A survey with this objective in mind is being pursued.

ACKNOWLEDGEMENTS

The technical assistance of Renate U. Meuser is greatly appreciated. The work was supported by grants from the National Research Council of Canada a predoctoral fellowship to L.E.C., and an operating grant #A4292 to H.B.L.

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