Phytochemistry, Vol. 21, No. 3, pp. 547–550, 1982.
Printed in Great Britain.

0031-9422/82/030547-04$03.00/0

EFFECTS OF DIVALENT CATIONS ON PHOSPHATASE SECRETION IN CULTURED TOBACCO CELLS

KATSUJI UEKI

Laboratory of Applied Microbiology, Faculty of Agriculture, Yamagata University, Tsuruoka, Yamagata 997, Japan

(*Received* 30 *April* 1981)

Key Word Index—*Nicotiana tabacum*; Solanaceae; cultured tobacco cells; enzyme secretion; phosphatase; divalent cations.

Abstract—Some differences were found between Mg^{2+}- and Ca^{2+}-stimulated phosphatase secretion in cultured tobacco cells. The effect of Mg^{2+} ions was greater than that of Ca^{2+} ions, and Ca^{2+} ions at below 1 mM rather depressed the secretion. Upon the addition of Mg^{2+} ions plus Ca^{2+} ions, a synergistic stimulation of the secretion occurred. Different influences on the effects of Mg^{2+} and Ca^{2+} ions on the secretion were exerted by treating cells with metabolic inhibitors that reduced the level of cellular metabolic energy. Phosphate (Pi) and arsenate did not depress the secretion in the presence of Mg^{2+} ions, but did depress it in the presence of Ca^{2+} ions. These results strongly suggested that the secretion of phosphatase involved at least two different steps affected by divalent cations.

INTRODUCTION

In cultured tobacco XD-6 cells, the synthesis and release of phosphatase into the culture medium are enhanced by Pi deficiency [1–3]. The rate-limiting step of the enzyme release is the transport of the enzyme from the cytoplasm to the outside of the cell membrane (secretion in the narrow sense) [3]. Divalent cations enhance the enzyme release not only by stimulating the transport process, but also by liberating the enzyme retained exterior to the cell membrane, e.g. in the cell wall [4].

It is known that Ca^{2+} ions are required for tip growth in pollen tubes [5, 6]. Reiss and Herth [7] suggested that Ca^{2+} ions are required for the exocytosis of pollen wall materials. In animal secretory cells [8–10], Ca^{2+} ions specifically act as a mediator of the stimulus-secretion coupling event. This information as to the role of Ca^{2+} ions in plant and animal cells prompted me to investigate whether or not in XD-6 cells Ca^{2+} ions and other divalent cations acted on phosphatase transport in a common way.

In the present study, the effects of Mg^{2+} and Ca^{2+} ions on phosphatase transport in XD-6 cells were compared with each other.

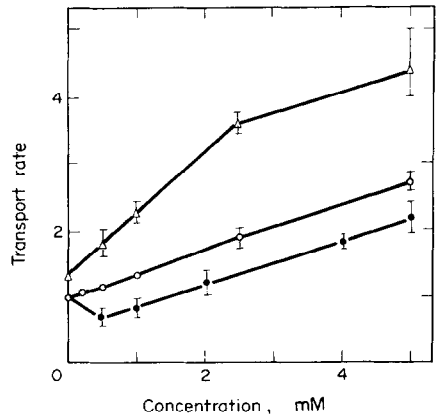

Fig. 1. Synergistic stimulation of phosphatase transport by Mg^{2+} and Ca^{2+} ions. Transport rates in the presence of $MgCl_2$ (○—○), $CaCl_2$ (●—●), or $MgCl_2$ (1 mM) plus $CaCl_2$ (△—△) are expressed as relative values taking the transport rate in the absence of divalent cations as 1. Averages of 2–4 replicate experiments are presented: the bars show the range of values in replicate experiments.

RESULTS AND DISCUSSION

As shown in Fig. 1, enzyme transport was more efficiently stimulated by Mg^{2+} ions than by Ca^{2+} ions, and it was slightly depressed by Ca^{2+} ions below 1 mM. The depression by Ca^{2+} ions did not occur, and transport of the enzyme was synergistically stimulated, when Ca^{2+} ions were added concomitantly with Mg^{2+} ions at low concentration. The effect of 1 mM Mg^{2+} ions plus 2.5 mM Ca^{2+} ions was equivalent to that of either 10 mM Mg^{2+} or 10 mM Ca^{2+} (Fig. 3). If Mg^{2+} and Ca^{2+} ions affected transport of the enzyme in a common way, the effect of Mg^{2+} ions plus Ca^{2+} ions should be at most equivalent to the sum of the individual effects of the cations. Thus, the synergistic effect of Mg^{2+}, ions plus Ca^{2+} ions suggested that these cations may have different roles in the transport process.

Different influences on the effects of Mg^{2+} and Ca^{2+} ions on the transport of the enzyme were exerted by treating cells with low concentrations of metabolic inhibitors which reduce the level of cellular metabolic energy. In 0.1 mM NaN_3-treated cells (Fig. 2), the maximum level of transport in the presence of Mg^{2+} ions was reduced to only about 1.8 times that of the control (without divalent cations) and the concen-

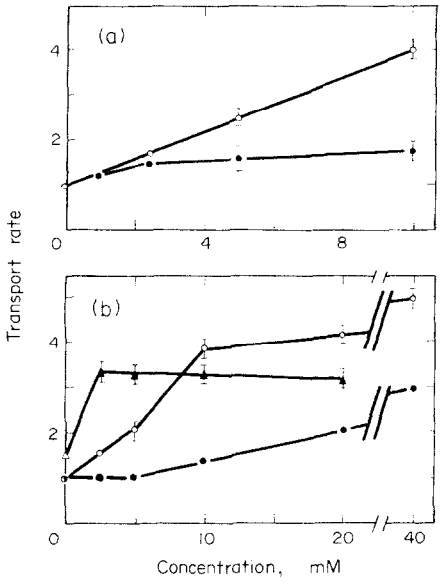

Fig. 2. Effects of Mg^{2+} and Ca^{2+} ions on phosphatase transport in NaN_3-treated cells. After 40 min pre-incubation with or without 0.1 mM NaN_3, various concns of $MgCl_2$ (a) or $CaCl_2$ (b) with or without NaN_3 were added and the transport rates measured. Averages of 2–4 replicate experiments are expressed as relative values taking the transport rate in the absence of divalent cations as 1: the bars show the range of values in replicate experiments. NaN_3 treatment had no effect in the absence of divalent cations. ○—○, without NaN_3 treatment; ●—●, with NaN_3 treatment; ▲—▲, with NaN_3 treatment in the presence of 2.5 mM $MgCl_2$.

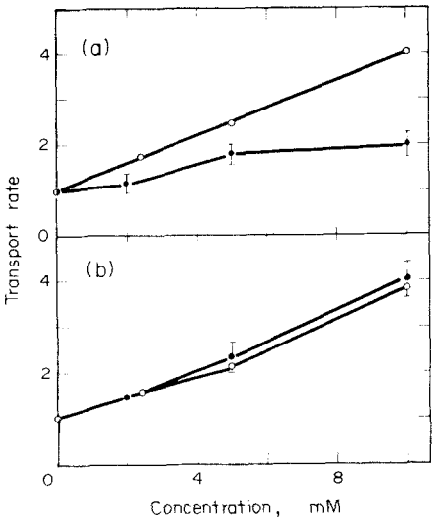

Fig. 3. Effects of Mg^{2+} and Ca^{2+} ions on phosphatase transport in L-ethionine-treated cells. Experimental procedure was the same as described in Fig. 2 except that 0.1 mM L-ethionine was substituted for 0.1 mM NaN_3. Averages of two replicate experiments are expressed as relative values taking the transport rate in the absence of divalent cations as 1: the bars show the range of values in replicate experiments. L-Ethionine treatment had no effect in the absence of divalent cations. (a) Effect of $MgCl_2$ with (●—●) or without (○—○) L-ethionine treatment. (b) Effect of $CaCl_2$ with (●—●) or without (○—○) L-ethionine treatment.

tration of Mg^{2+} ions, at which the maximum effect was achieved, shifted to a lower level, i.e. about 2.5 mM. On the other hand, Ca^{2+} ions at less than 5 mM did not affect transport in the NaN_3-treated cells, and were stimulatory at concentrations greater than 5 mM. The effect of Ca^{2+} ions did not reach the maximum level until 40 mM. The effects of various concentrations of Ca^{2+} ions in the presence of 2.5 mM Mg^{2+} ions on transport in the NaN_3-treated cells were also measured (Fig. 2). Although transport in the NaN_3-treated cells reached the maximum level in the presence of 2.5 mM Mg^{2+} ions alone, it was stimulated more by the concomitant addition of Ca^{2+} ions even at less than 5 mM. However, the maximum level achieved by Mg^{2+} ions plus Ca^{2+} ions with the azide-treatment was lower than that without the azide-treatment. L-Ethionine is known to reduce the cellular ATP level by trapping ATP as *S*-adenosylethionine [11], and in XD-6 cells the ATP level decreased to about 60% of the control upon treatment with 0.1 mM L-ethionine (Fig. 3). In the L-ethionine-treated cells (Fig. 3), the effects of Mg^{2+} ions on the enzyme transport was conspicuously depressed, but that of Ca^{2+} ions was not affected, when compared with those without the L-ethionine treatment. The maximum level of enzyme transport achieved by Mg^{2+} ions was reduced and the concentration, at which the maximum effect was achieved, shifted to a lower level, as in the NaN_3-treated cells.

These results from the treatment with NaN_3 or L-ethionine also supported the view that the transport process involved at least two different steps affected by divalent cations, a Mg^{2+}-stimulated step and a Ca^{2+}-stimulated step. In addition, the results also suggested a close relationship between the action of divalent cations and the energy-requiring step limiting enzyme transport. If the effect of divalent cations was directly dependent on the level of cellular metabolic energy, the maximum effects of the ions should have been reduced and should have been achieved at lower concentrations, when the supply of metabolic energy was reduced by treatment with metabolic inhibitors. The results indicated that the effect of Mg^{2+} ions was affected in such a manner by the treatment with metabolic inhibitors. Thus, it seemed that at least the Mg^{2+}-stimulated step was directly dependent on the level of metabolic energy.

In 2,4-dinitrophenol-treated cells, enzyme transport was depressed in the presence of Mg^{2+} ions, but not in the presence of Ca^{2+} ions (Table 1). This result was compatible with those from the treatments with NaN_3 and L-ethionine. A distinction between the effects of Mg^{2+} and Ca^{2+} ions was also made by treating cells with Pi and arsenate. Pi is known to inhibit enzyme transport even at 1 μM [3]. As shown in Fig. 4, Pi at 50 μM did not affect transport in the presence of 3 mM Mg^{2+} ions, but depressed it in the presence of 3 mM Ca^{2+} ions. The inhibitory effect of Pi was temporary as had been reported [3]. The time course of decrease in Pi concentration of the medium with

Table 1. Effects of Mg^{2+} and Ca^{2+} ions on phosphatase transport in DNP-treated cells

Addition	Transport rate (mU/ml/hr)
$MgCl_2$	2.80
$MgCl_2$ + DNP	1.62
$CaCl_2$	2.73
$CaCl_2$ + DNP	2.73

Experimental procedure was the same as described in Fig. 2, except that 0.1 mM DNP was substituted for 0.1 mM NaN_3. Averages of duplicate experiments are presented. Concentrations of $MgCl_2$ and $CaCl_2$ were adjusted to 5 mM.

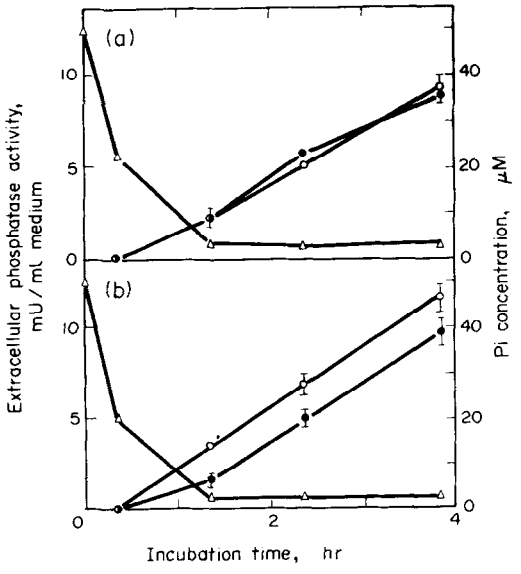

Fig. 4. Effect of Pi on phosphatase transport in the presence of Mg^{2+} or Ca^{2+} ions. Time course of the increase in extracellular phosphatase activity during incubations with (●—●) or without (○—○) 50 μM KH_2PO_4 in the presence of 3 mM $MgCl_3$ (a) or $CaCl_2$ (b) and the decrease in Pi concentration of the medium (△—△) are presented. The values are averages of duplicate experiments: the bars show the range of values in duplicate experiments.

Ca^{2+} ions did not significantly differ from that with Mg^{2+} ions. Thus, the different effects of Pi on enzyme transport seemed not to result from the difference in the degree of Pi absorption by the cells. The transport of the enzyme was also found to be depressed by arsenate only in the presence of Ca^{2+} ions (Table 2). The inhibitory effect of arsenate was not temporary, and the enzyme transport remained at a reduced rate for at least 6 hr of incubation. Arsenate is known to act as a metabolic inhibitor and thought to reduce the level of cellular metabolic energy. However, the effect of arsenate in this study was rather compatible with that of Pi. It seemed that arsenate at low concentration acted as an analogue of Pi.

Table 2. Effect of arsenate on phosphatase transport in the presence of Mg^{2+} or Ca^{2+} ions

Addition	Transport rate (mU/ml/hr)
$MgCl_2$	2.12
$MgCl_2$ + NaH_2AsO_4	2.29
$CaCl_2$	2.44
$CaCl_2$ + NaH_2AsO_4	1.33

Transport rates during the incubations with or without 50 μM NaH_2AsO_4 in the presence of 3 mM $MgCl_2$ or $CaCl_2$ are presented. The values are averages of duplicate experiments.

In conclusion, the present study suggested that the phosphatase transport process of XD-6 cells involved at least two different steps susceptible to divalent cations; a Mg^{2+}-stimulated step and Ca^{2+}-stimulated step. The Mg^{2+}-stimulated step seemed to be directly dependent on the level of cellular metabolic energy.

In the stimulus–secretion coupling event of animal cells [8–10], Ca^{2+} ions are thought to regulate the activation of the cytoskeleton system composed of microfilaments and microtubules, which occurs in response to specific stimuli and leads the release of secretory products outward from the cell membrane by consuming metabolic energy from ATP. It is also known in plant cells that Ca^{2+} ions play a role in secretory process. Reiss and Herth [7] confirmed microscopically that the calcium-ionophore A-23187 drastically disturbed the secretion of pollen wall materials. They suggested that the exocytosis of pollen wall materials was oriented by an intracellular gradient of Ca^{2+} ions [7]. Becerra and Lopez-Saez [12] also proposed that Mg^{2+} and Ca^{2+} ions participated in different manners in the secretory process in plant cells, by interpreting effects of these ions on cytokinesis in onion root meristems. Sticher *et al.* [13] recently showed that the peroxidase release in spinach cell suspension culture required Ca^{2+} ions. It is of interest whether in plant secretory systems Ca^{2+} ions play a similar role to that in animal systems.

EXPERIMENTAL

Cells. Tobacco cell line XD-6 was cultured without Pi, and was induced to synthesize and release phosphatase as described previously [2, 3].

Measurement of phosphatase transport. Phosphatase transport was measured according to the procedure described previously [4]. Cells (4.8×10^5) that had been washed with the basal medium (KNSV medium) were transferred to 100-ml concial flasks, suspended in 10 ml of the basal medium with or without inhibitors, and pre-incubated for 30–40 min at 25° on a reciprocal shaker. After the pre-incubation, 10 ml of the basal medium containing Mg^{2+} or Ca^{2+} ions with or without metabolic inhibitors was added to the flasks, and the incubation at 25° continued. The rate of phosphatase transport (transport rate) was defined as the increase in the extracellular phosphatase activity per hr (mU/ml/hr), and was calculated from the linear increase in the extracellular enzyme activity for at least 3 hr.

Enzyme assay. Phosphatase activity was measured at 30° using 5 mM *p*-nitrophenylphosphate as the substrate in

100 mM NaOAc buffer (pH 5.6) [2]. One unit of enzyme activity (1 U = 1000 mU) was defined as the activity hydrolysing 1 μmol substrate/min. The enzyme activity was not affected by divalent cations, Pi, arsenate and metabolic inhibitors at the concns used in the present study.

Analytical methods. ATP was measured using an ATP-Kit (Boeringer Mannheim Co.). Pi was measured by a modification [14] of the method of ref. [15].

Acknowledgement—I thank Dr. Sitiro Sato of the University of Tokyo for his helpful advice.

REFERENCES

1. Ueki, K. and Sato, S. (1971) *Physiol. Plant.* **24**, 506.
2. Ueki, K. and Sato, S. (1977) *Plant Cell Physiol.* **18**, 1253.
3. Ueki, K. (1978) *Plant Cell Physiol.* **19**, 385.
4. Ueki, K. (1979) *Plant Cell Physiol.* **20**, 789.
5. Brewbaker, J. L. and Kwack, B. H. (1963) *Am. J. Botany* **50**, 859.
6. Dickinson, D. B. (1967) *Physiol. Plant.* **20**, 118.
7. Reiss, H.-D. and Herth, W. (1979) *Planta* **145**, 225.
8. Douglas, W. W. (1968) *Br. J. Pharmacol.* **34**, 474.
9. Rusmussen, H. (1970) *Science* **170**, 404.
10. Foreman, J. C., Garland, L. G. and Mongar, J. L. (1976) *Calcium in Biological Systems* (Duncan, J. C., ed.) pp. 193–218. Cambridge University Press, Cambridge.
11. Polya, G. M. and Atkinson, M. R. (1969) *Aust. J. Biol. Sci.* **22**, 573.
12. Becerra, J. and López-Sáez, J. F. (1978) *Exp. Cell Res.* **111**, 301.
13. Sticher, L., Penel, C. and Greppin, H. (1981) *J. Cell Sci.* **48**, 345.
14. Nakamura, M. (1950) *J. Agric. Chem. Soc. Jpn* **24**, 1.
15. Allen, R. J. L. (1940) *Biochem. J.* **34**, 858.