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SPECIFIC EFFECTS OF SPERMINE ON OUABAIN-SENSITIVE AND POTASSIUM-DEPENDENT PHOSPHATASE ACTIVITY OF KIDNEY PLASMA MEMBRANES

SPECIFICITY OF THE POTASSIUM SITES

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Summary

Specific inhibition of ouabain-sensitive and K^+-dependent *p*-nitrophenylphosphatase activity of rabbit kidney plasma membranes by spermine (*N,N'*-bis(3-aminopropyl)-1,4-butanediamine) was characterized kinetically.

1. Inhibition by spermine was competitive with K^+. The K_i for spermine was 31 μM in the presence of 1 mM Mg^{2+}.

2. Excess Mg^{2+} inhibited the ouabain-sensitive phosphatase activity in competition with K^+. The K_i for Mg^{2+} was 2.6 mM.

3. Increasing Mg^{2+} concentrations reduced the spermine inhibition. This could be observed at Mg^{2+} concentrations higher than that of K^+.

4. In the absence of inhibition by Mg^{2+}, spermine was noncompetitive with Mg^{2+} which was essential for the ouabain-sensitive phosphatase activity. This could be observed at Mg^{2+} concentrations lower than that of K^+.

5. Although Ca^{2+} was a strong inhibitor of the ouabain-sensitive phosphatase activity in the presence of K^+, it produced a small stimulation of the activity in the absence of K^+. Approximately 0.1 mM Ca^{2+} gave the maximum stimulation.

6. The observed Ca^{2+}- and Mg^{2+}-dependent phosphatase activity was inhibited strongly by ouabain and by spermine. The half-maximal inhibition concentrations of ouabain and spermine were 0.1 and 63 μM, respectively.

It is likely that Mg^{2+}, Ca^{2+} and spermine bind to the same site as does K^+.

Introduction

The hydrolysis of ATP by (Na^+ + K^+)-ATPase (ATP phosphohydrolase EC 3.6.1.3) is stepwise: a Na^+-dependent phosphorylation of the enzyme followed

by a K^+-dependent hydrolysis of the phosphorylated intermediate [1]. K^+-dependent and ouabain-sensitive *p*-nitrophenylphosphatase activity is possibly a different manifestation of the latter dephosphorylation step. Stimulation of the dephosphorylation step by K^+ was observed in the absence of free Mg^{2+}, while the K^+-dependent phosphatase activity requires free Mg^{2+} (ref. 2). This suggests that the Mg^{2+}-nitrophenylphosphate complex is a real substrate of the K^+-dependent phosphatase activity. Our recent observations [3] showed a link between Mg^{2+} and K^+. Inhibition of the K^+-dependent phosphatase activity by excess Mg^{2+} is dependent on the concentration of K^+. This link between Mg^{2+} and K^+ might play a role in the dephosphorylation step of the ATPase reaction. In a recent paper we reported that spermine inhibited the K^+-dependent phosphatase activity specifically [4].

The physiological roles of spermine and other aliphatic polyamines are involved in a number of cellular processes including those related to cell membranes [5,6]. A stabilizing effect of spermine on (Na^+ + K^+)-ATPase has been reported [7]. Polyamines have a regulatory effect on membrane-bound acetylcholinesterase [8]. Spermine mimics the action of insulin at a step subsequent to the insulin receptor site on the surface of cell membrane [9].

The present paper suggests by kinetical observations that spermine, Mg^{2+} and Ca^{2+} can be bound to the K^+-sites of the enzyme.

Experimental

Materials. The di-sodium salt of *p*-nitrophenylphosphate was purchased from Kyowa Hakko Co., Japan. The sodium salt was converted to the Tris salt as described previously [3,10]. Spermine (tetrahydrochloride) was purchased from Sigma Chemical Co., and used after its stock solutions were adjusted to pH 7.5 by 1 M Tris. All reagents were of the highest grade available. Water was deionized after distillation.

Membrane preparation. Plasma membranes were purified by the pH 9.0 treatment of microsomes from frozen rabbit kidneys as reported previously [3].

Analytical procedures. Nitrophenylphosphatase activity was measured in terms of the production of *p*-nitrophenol. Unless otherwise specified, K^+-dependent nitrophenylphosphatase activity was measured in the presence of 5 mM nitrophenylphosphate as the Tris salt, 1.0 mM $MgCl_2$, 0.5 mM KCl, 0.02 mM EDTA, 0.05% mercaptoethanol and 25 mM Tris · HCl, pH 7.4, with or without 0.2 mM ouabain in a final volume of 1.0 ml. The reaction mixture without the substrate was preincubated for 15 min at 2°C, and the reaction was allowed to proceed for 30 min at 37°C, immediately after the addition of 50 µl of 0.1 M nitrophenylphosphate. The reaction was stopped by the addition of 1 ml of cold 0.5 M NaOH immediately after cooling in an ice bath. The absorbance of nitrophenol liberated was read at 405 nm. The total liberation of nitrophenol was usually below 1% of the total nitrophenylphosphate present. Under the present experimental conditions (with 0.5 mM K^+ and 1 mM Mg^{2+}), K^+-dependent activity was abolished completely by the addition of 0.2 mM ouabain, which showed no effect on K^+-independent activity (see ref. 3). The K^+-dependent activity was calculated by subtracting the activity without K^+ in the

presence of ouabain from the total activity. A unit of enzyme is defined as the amount which releases 1 μmol of nitrophenol per min. Specific activity is expressed as units per mg protein.

Protein concentrations were measured by the method of Lowry and coworkers [11] using albumin as standard.

Results

Time course of K^+-dependent activity with spermine

The time course of the nitrophenylphosphatase activity in the absence of spermine at pH 7.5 was linear up to 60 min. In the presence of 0.05 mM spermine, which produced an incomplete inhibition of the K^+-dependent activity, the time course was approximately linear within 30 min, although it was slightly curved (Fig. 1).

Effect of spermine on K^+-sites. The two states of the enzyme with and without the inhibitory Mg^{2+}-binding show a different character in their saturation curves with K^+ (see ref. 3). Therefore, we investigated the effect of spermine on the K^+-sites both in the presence and absence of inhibitory Mg^{2+}. As reported previously [3], at K^+ concentrations lower than 1.0 mM, a double reciprocal plot of the velocity versus $[K^+]^2$ was linear (Fig. 2A). At K^+ concentrations higher than 1.0 mM, a double reciprocal plot of the velocity versus $[K^+]$ was linear (Fig. 2B). It was observed with both states of the enzyme that spermine was competitive with K^+ for the K^+-dependent activity. The competition between spermine and K^+ was reconfirmed by the Dixon plot of [1/velocity] versus [spermine] (Fig. 3). The K_i for spermine was calculated graphically, and the value was 31 μM in the presence of 1 mM Mg^{2+}, and was not dependent on the K^+ concentration.

Effect of spermine on Mg^{2+}-sites. In the presence of 0.5 mM K^+, spermine was simply noncompetitive with Mg^{2+} in the noninhibitory concentration range of Mg^{2+} below 0.5 mM (Fig. 4). The K_m for Mg^{2+} was calculated to be 0.37

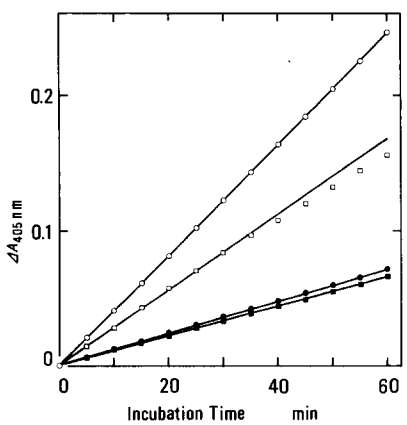

Fig. 1. The effect on spermine on time course of *p*-nitrophenylphosphatase activity. The enzyme activity was assayed in the presence (□, ■) or the absence (○, ●) of 0.05 mM spermine which gave a partial inhibition of the K^+-dependent activity. (○, □), without ouabain; and (●, ■), with 0.2 mM ouabain.

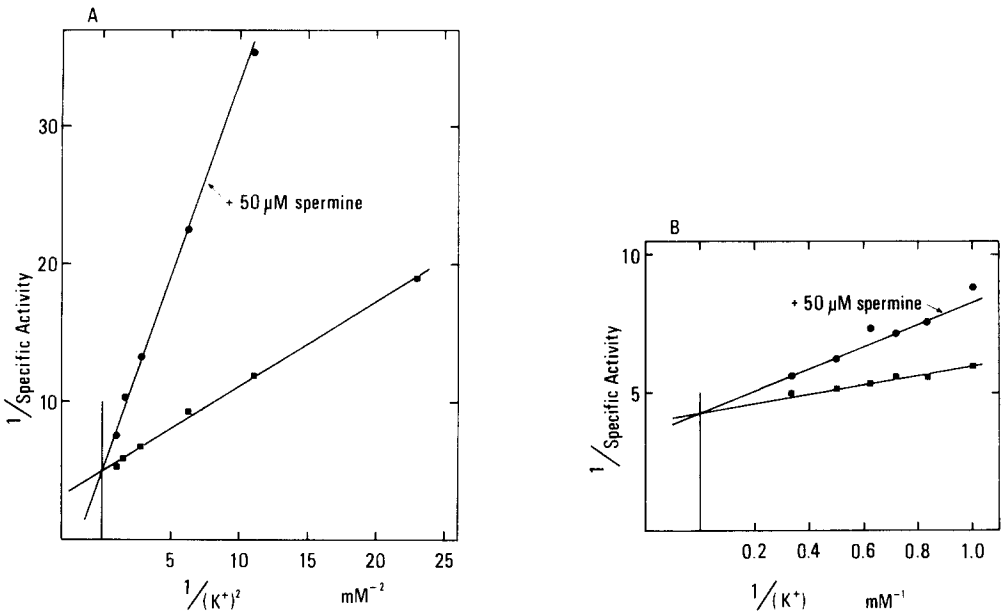

Fig. 2. Effect of spermine on activation by K^+ of K^+-dependent nitrophenylphosphatase activity. The kidney plasma membrane was preincubated in the assay media without the substrate for 15 min at 2°C. The enzyme reaction was started at 37°C after the addition of the substrate. (A) The concentration of K^+ was lower than that of Mg^{2+}, 1.0 mM. The rate of *p*-nitrophenol production is plotted against the square of the concentration of K^+ in the Lineweaver-Burk form. (B) The concentration of K^+ was higher than 1.0 mM. The rate of *p*-nitrophenol production is plotted against the concentration of K^+ in the Lineweaver-Burk form. (●), with spermine; (■), without spermine.

mM. By increasing the Mg^{2+} concentration from 1 mM to 10 mM, the spermine concentration required for 50% inhibiton was increased (Fig. 5). The values were approximately 0.05, 0.11 and 0.95 mM in the presence of 1, 3 and 10 mM Mg^{2+} with 0.5 mM K^+, respectively. These results suggest that inhibitory Mg^{2+} and spermine may bind to a common site.

Competition of Mg^{2+} at high concentrations with K^+

Our previous results [3] showed that the K_m value for K^+ was changed depending on the concentrations of Mg^{2+} at levels higher than that of K^+. In the present experiments we observed carefully the effect of the high Mg^{2+} concentrations, 1.0, 3.0 and 10 mM, on the K^+-activation curves (Fig. 6). Inhibitory Mg^{2+} was competitive with the square of K^+ concentrations. The Dixon plot of [1/velocity] versus [Mg^{2+}] at higher concentrations also showed the competition (data are not shown), and the K_i for Mg^{2+} was calculated to be 2.6 mM. Assuming that the Hill coefficient for K^+ activation, 2.0 (see Fig. 5 in ref. 3), can be used as the number of the K^+-sites although it can be actually used as the minimum number of the sites, Mg^{2+} as an inhibitor competes with one pair of K^+.

Activation by Ca^{2+}. When Ca^{2+} was added into the assay mixture in the absence of K^+, Ca^{2+} showed a small activation of the enzyme activity in the concentration range from 0.02 mM to 3 mM. The maximum activation was

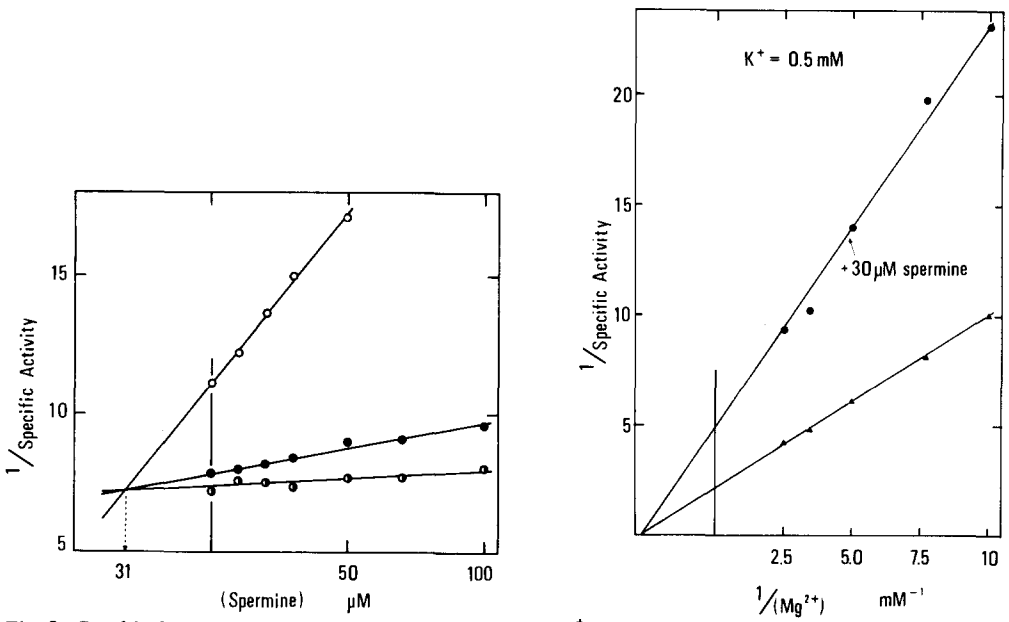

Fig. 3. Graphical determination of the K_i for spermine. K^+-dependent nitrophenylphosphatase activity was assayed as described in Experimental, in the presence of 0.5, 1.0 and 2.0 mM KCl with the indicated concentrations of spermine. The concentration of Mg^{2+} was 1.0 mM. Straight lines are obtained on plotting 1/velocity against spermine, and the K_i for spermine is calculated by the method of Dixon (Dixon, M. and Webb, E.C. (1964) Enzyme, pp. 327—329 Longmans, London). (○), K^+ = 0.5 mM; (●), K^+ = 1.0 mM; and (○), K^+ = 2.0 mM.

Fig. 4. Reciprocal plot of the velocity of K^+-dependent hydrolysis of nitrophenylphosphate as a function of Mg^{2+} concentration in the presence and absence of spermine. The catalytic activity was measured in the presence of 0.5 mM K^+. The Mg^{2+} concentrations were lower than that of K^+. (▲), no spermine; (●), 0.03 mM spermine.

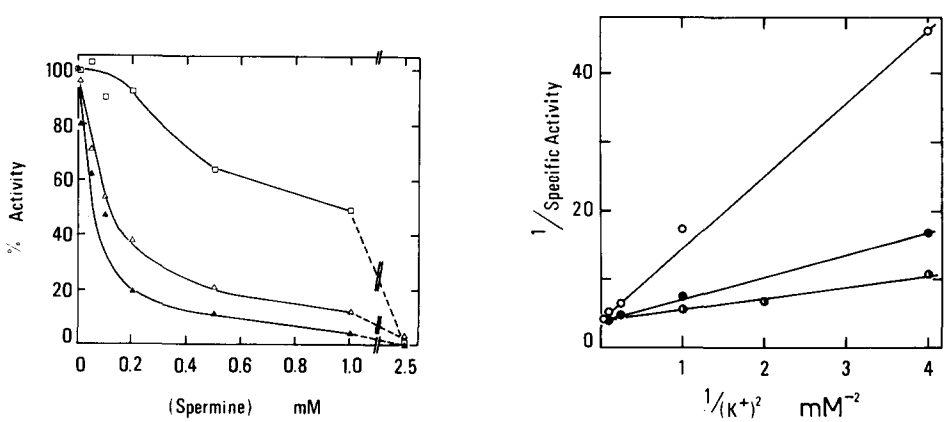

Fig. 5. The effect of high concentrations of Mg^{2+} on inhibition by spermine of K^+-dependent nitrophenylphosphatase activity. The concentration of K^+ was 0.5 mM. The Mg^{2+} concentrations were 1.0 mM (▲), 3 mM (△) and 10 mM (□) which were higher than that of K^+.

Fig. 6. The effects of high concentrations of Mg^{2+} on K^+-dependent nitrophenylphosphatase activity. The catalytic activity was measured as described in Experimental. The concentrations of Mg^{2+} were 1.0 (◑), 3.0 mM (●) and 10 mM (○). The data are shown as double reciprocal plots of the specific activity versus $[K^+]^2$.

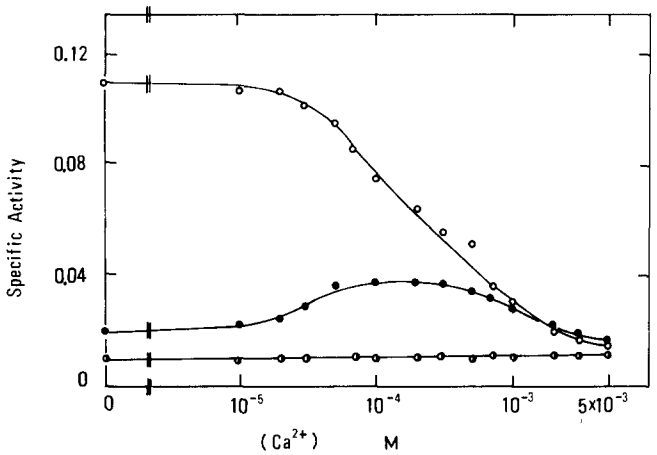

Fig. 7. Effect of Ca^{2+} on nitrophenylphosphatase activity in the presence and absence of 0.5 mM K^+. The Mg^{2+} concentration was 1.0 mM. The concentrations of other assay reagents were the same as those described in Experimental. (○), with K^+; (●), without K^+; and (◑), in the presence of 0.2 mM ouabain without K^+.

observed at 0.1 mM (Fig. 7). Ouabain insensitive nitrophenylphosphatase activity was not affected by Ca^{2+}.

In the presence of 0.5 mM K^+, Ca^{2+} was a strong and simply noncompetitive inhibitor as is well-known, and did not compete with K^+ (Fig. 8).

Inhibition of the (Ca^{2+} + Mg^{2+})-dependent phosphatase by ouabain. Ouabain inhibited the Ca^{2+}- and Mg^{2+}-dependent nitrophenylphosphatase activity (Fig. 9). In the presence of 1 mM Mg^{2+} and 0.1 mM Ca^{2+}, the ouabain concentra-

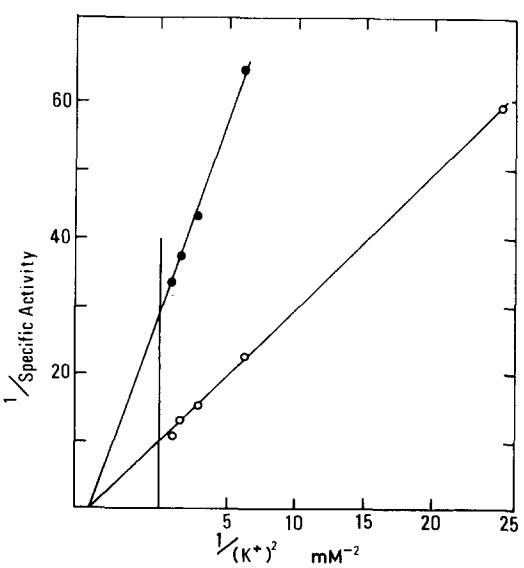

Fig. 8. Effect of Ca^{2+} on K^+-dependent nitrophenylphosphatase activity. The K^+-dependent activity was assayed in the presence of 1 mM Mg^{2+} and various concentrations of K^+ with (●) or without (○) 0.4 mM Ca^{2+}. The concentrations of other assay reagents were the same as those described in Experimental section.

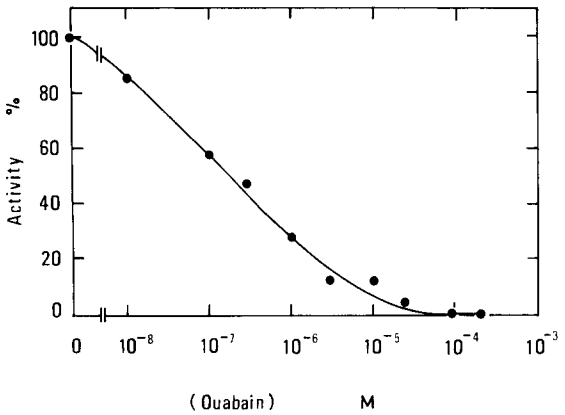

Fig. 9. Inhibition of ouabain-sensitive (Ca^{2+} + Mg^{2+})-dependent phosphatase activity by ouabain. Various concentrations of ouabain were added to the suspension of plasma membranes containing (final concentrations) 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 μM EDTA, 0.05% mercaptoethanol and 25 mM Tris · HCl, pH 7.5. After preincubation for 15 min at 2°C, *p*-nitrophenylphosphate was added at 5 mM, and the reaction was started at 37°C. The amount of liberated nitrophenol was determined after 30 min incubation. The ouabain-sensitive activity was calculated by subtraction of the activity with 0.2 mM ouabain from the total activity, and expressed as percentage remaining.

tion required for 50% inhibition was 0.1 μM, which was much smaller than that in the presence of 1 mM Mg^{2+} and 0.5 mM K^+, 8 μM (see ref. 3).

Inhibition of the (Ca^{2+} + Mg^{2+})-phosphatase by spermine

Spermine inhibited the Ca^{2+}- and Mg^{2+}-dependent nitrophenylphosphatase activity (Fig. 10). The spermine concentration required for 50% inhibition was 63 μM in the presence of 1 mM Mg^{2+} and 0.1 mM Ca^{2+}, which was roughly the same as in the presence of 1 mM Mg^{2+} and 0.5 mM K^+.

Inhibition of ouabain-sensitive Mg^{2+}-phosphatase activity by spermine. Ouabain-sensitive nitrophenylphosphatase activity supported by Mg^{2+} is observed in the absence of K^+ and Ca^{2+} in Fig. 7. This activity was significant,

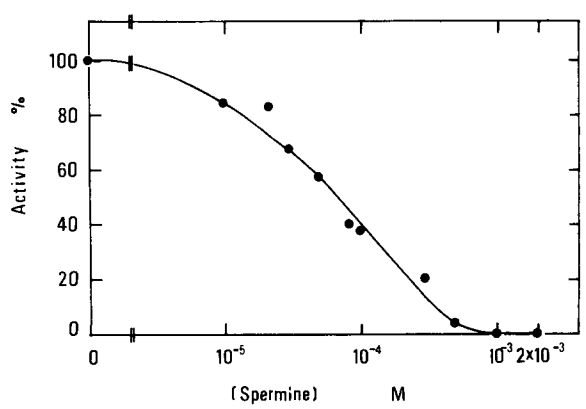

Fig. 10. Inhibition of ouabain-sensitive (Ca^{2+} + Mg^{2+})-dependent phosphatase activity by spermine. The experimental details were the same as described in Fig. 9 except that various concentrations of spermine were added. The ouabain-sensitive activity was calculated by subtraction of the activity with 0.2 mM ouabain from the total activity, and expressed as percentage remaining.

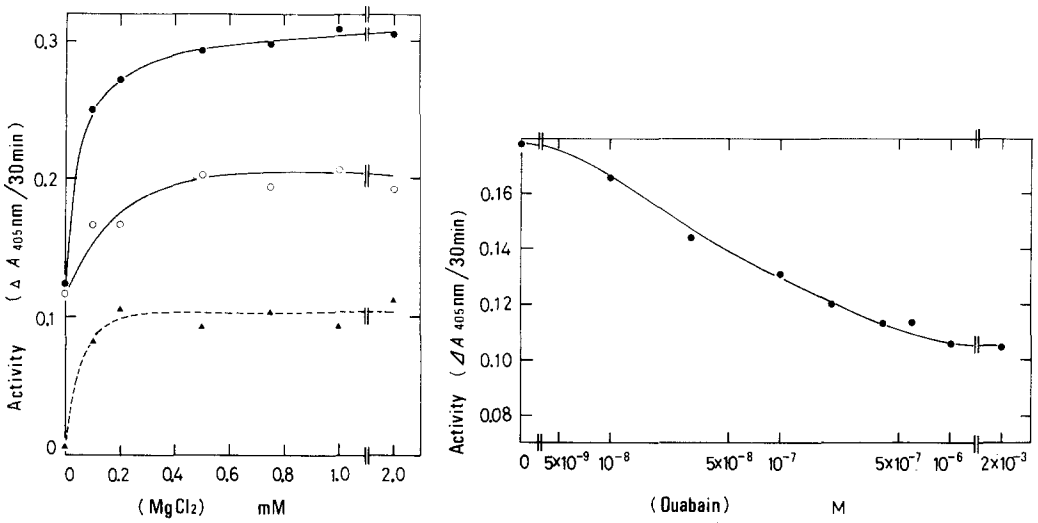

Fig. 11. The effect of Mg^{2+} on Mg^{2+}-dependent nitrophenylphosphatase activity. The enzyme activity was assayed with in the absence of K^+. The concentrations of other assay reagents were the same as those described in Experimental. (●), without ouabain; (○), with 0.2 mM ouabain; and (▲), ouabain-sensitive activity which was calculated by subtracting the activity with ouabain from the activity without ouabain.

Fig. 12. The effect of concentrations of ouabain on Mg^{2+}-dependent nitrophenylphosphatase activity. The Mg^{2+}-dependent activity was assayed in the presence of 1 mM Mg^{2+} and various concentrations of ouabain. Semi-log scale.

although the value was very small and negligible for the kinetical studies in the presence of K^+. The Mg^{2+} concentration required for 50% activation of the ouabain-sensitive activity was 0.08 mM (Fig. 11).

The maximum inhibition of the Mg^{2+}-dependent activity was obtained in the presence of 1 μM ouabain (Fig. 12). The midpoint of the ouabain saturation curve ($I_{5.0}$) was 0.04 μM.

Spermine at 0.5 mM inhibited the ouabain-sensitive portion of the Mg^{2+}-

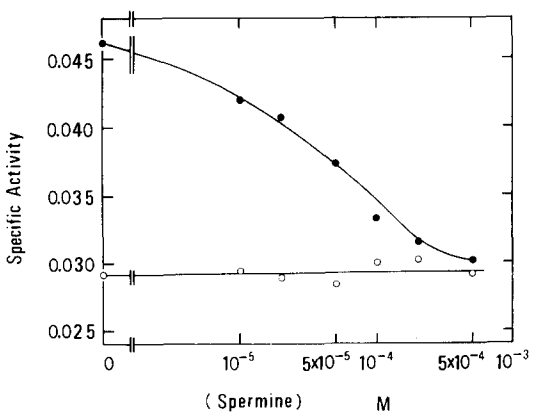

Fig. 13. The effect of concentrations of spermine on Mg^{2+}-dependent nitrophenylphosphatase activity. The Mg^{2+}-dependent activity was assayed in the presence of 1 mM Mg^{2+} and various concentrations of spermine. (●), without ouabain; and (○), with 0.2 mM ouabain. Semi-log scale.

dependent activity completely ($I_{0.5}$ = 50 μM). Spermine did not show any effects on the Mg^{2+}-dependent activity in the presence of 0.2 mM ouabain (Fig. 13). It is suggested that ouabain-sensitive and Mg^{2+}-dependent nitrophenylphosphatase activity is inhibited by spermine. The extremely high affinity of the enzyme for ouabain and Mg^{2+} as an activator may suggest that the ouabain-sensitive and Mg^{2+}-dependent activity is not due to any contaminating K^+ or other alkali cations. The inhibition by spermine and ouabain may suggest that Mg^{2+} activates very poorly the K^+-dependent nitrophenylphosphatase by substitution for K^+ like Ca^{2+}.

Discussion

The results would seem to permit three conclusions. (1) Spermine antagonizes the action of K^+ on ouabain-sensitive and K^+-dependent phosphatase activity in kidney plasma membranes. (2) Excess Mg^{2+} inhibits the phosphatase activity in competition with K^+. (3) Ca^{2+} seems to substitute for K^+, though only partially.

Excess Mn^{2+} substituted for excess Mg^{2+} as an inhibitor (data not shown). The K^+-sites of the K^+-dependent phosphatase accept not only alkali cations, but also bi- and poly-valent cations.

Robinson measured the affinity of (Na^+ + K^+)-ATPase for K^+ in terms of an inactivation by beryllium [12]. In their system for the ATPase activity, $MgCl_2$ did not appear to affect the dissociation constant for K^+. *p*-Nitrophenylphosphate [13] and acetyl phosphate [14—16] seem to produce the "E_2-P" in the reaction sequence of the ATPase. The "E_2-P" is a phosphorylated form of the ATPase incapable of participating ADP-ATP exchange. These suggest that the ouabain-sensitive and K^+-dependent phosphatase enters the reaction sequence of (Na^+ + K^+)-ATPase. However, the detailed relationship of the ouabain-sensitive phosphatase to the ATPase is still unclear. For example, (i) Israel and Titus [17] have suggested that the K^+-dependent acetyl phosphatase activity may represent a different entity than the (Na^+ + K^+)-ATPase. (ii) Sachs and co-workers [18] observed that the phosphorylation of (Na^+ + K^+)-ATPase by acetyl phosphate was stimulated by K^+ but not by Na^+, while Bond and co-workers [16] observed stimulation by Na^+. (iii) Sachs and co-workers [18] concluded that *p*-nitrophenylphosphate was an alternate substrate for the K^+-dependent acetyl phosphatase, while Inturrisi and Titus [19] observed the phosphorylation by *p*-nitrophenyl [^{32}P]phosphate only when the enzyme was inhibited by ouabain. The phosphorylation by acetyl [^{32}P]phosphate does not require ouabain.

The difference of Mg^{2+} concentrations in their experiments may be one of the reasons for these discrepancies.

K^+ inhibits ouabain binding to (Na^+ + K^+)-ATPase supported by (Mg^{2+} + P_i). It has been shown that K^+ competes with Mg^{2+} in this binding reaction [20]. On the contrary, recent reports show no competition of K^+ with Mg^{2+} (refs. 21 and 22). As mentioned above, at least two sets of Mg^{2+}-sites exist on the enzyme; one is the site essentially required for the activity, and another is the site for the inhibitory binding. We are studying whether the sites of Mg^{2+}

required for ouabain-binding are identical with one of the two sites for the K^+-dependent activity.

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References

1 Whittam, R. and Wheeler, K.P. (1970) Ann. Rev. Physiol. 32, 21—60
2 Kanazawa, T., Saito, M. and Tonomura, Y. (1970) J. Biochem. 67, 693—711
3 Tashima, Y. and Hasegawa, M. (1975) Arch. Biochem. Biophys. 171, 568—574
4 Tashima, Y. and Hasegawa, M. (1975) Biochem. Biophys. Res. Commun. 66, 1344—1348
5 Silver, S., Wendt, L., Bhattacharyya, P. and Beauchamp, R.S. (1970) Ann. N.Y. Acad. Sci. 171, 838—862
6 Herbst, E.J. and Bachrach, U. (1970) Ann. N.Y. Acad. Sci. 171, 691—1009
7 Nag, D. and Ghosh, J.J. (1973) J. Neurochem. 20, 1021—1027
8 Kossorotow, A., Walf, H.U. and Seiler, N. (1974) Biochem. J. 144, 21—27
9 Lockwood, D.H. and East, L.E. (1974) J. Biol. Chem. 249, 7717—7722
10 Post, R.L. and Sen, A.K. (1967) Methods in Enzymology, Vol. 10, p. 765, Academic Press, New York
11 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
12 Robinson, J.D. (1973) Arch. Biochem. Biophys., 156, 232—243
13 Robinson, J.D. (1972) Biochim. Biophys. Acta 274, 542—550
14 Dudding, W.F. and Winter, C.G. (1971) Biochim. Biophys. Acta 241, 650—660
15 Winter, C.G. (1972) Biochim. Biophys. Acta 266, 135—143
16 Bond, G.H., Bader, H. and Post, R.L. (1971) Biochim. Biophys. Acta 241, 57—67
17 Israel, Y. and Titus, E. (1967) Biochim. Biophys. Acta 139, 450—459
18 Sachs, G., Rose, J.D. and Hirschowitz, B.I. (1967) Arch. Biochem. Biophys. 119, 277—281
19 Inturrisi, C.E. and Titus, E. (1970) Mol. Pharmacol. 6, 99
20 Hansen, O. and Skou, J.C. (1973) Biochim. Biophys. Acta 311, 51—56
21 Schnurmans Stekhoven, H.M.A.H. and De Pont, J.J.H.H.M. (1976) Biochim. Biophys. Acta 419, 137—149
22 Hansen, O. (1976) Biochim. Biophys. Acta 433, 383—392