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EFFECTS OF POLYAMINES ON PARTIAL REACTIONS OF MEMBRANE ($Na^+ + K^+$)-ATPase

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Summary

Spermine and spermidine inhibit the ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) reaction so that the effect increases as the ionic content due to Na^+ and K^+ in the reaction is reduced. Several other amines inhibit ($Na^+ + K^+$)-ATPase to varying degress and methylglyoxal-bis-(guanylhydrazone) was the most potent inhibitor among those tested.

The inhibition by polyamines of the ATPase is uncompetitive with respect to Mg^{2+} and ATP activation of the reaction. Various naturally occurring polyamines and other amines inhibited Na^+ activation of ($Na^+ + K^+$)-ATPase as well as Na^+-dependent phosphoenzyme formation in an apparently competitive manner with respect to Na^+. Likewise, K^+-activation of ($Na^+ + K^+$)-ATPase as well as K^+-*p*-nitrophenyl phosphatase was inhibited in an apparently competitive manner with respect to K^+. Both the cation charge and structure (e.g., aliphatic chain length) may contribute to the inhibitory effects of the amines; however, Na^+ sites appear to be more sensitive to cation charge than the aliphatic chain length of the amine, whereas the opposite appears to be true for K^+ sites.

The results do not indicate a specific effect of polyamines on ($Na^+ + K^+$)-ATPase or its partial reactions.

Introduction

Aliphatic polyamines (putrescine, spermidine, spermine) have been shown to influence a number of biochemical reactions involving membrane function.

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Abbreviation: MGBG, methylglyoxal-bis-(guanylhydrazone)dihydrochloride.

Thus, deMeis [1] reported that spermine and spermidine (at concentrations of 0.5—2.0 mM) activated Ca^{2+} uptake by muscle microsomes, while 4 mM spermine inhibited heart muscle microsomal ATPase by 50%. Working with a microsomal membrane ATPase of rat ventral prostate, we observed that 1 mM spermine inhibited the ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) by 15%, whereas spermidine at the same concentration had no significant effect [2]. More recently, Tashima and Hasegawa [3,4] suggested that polyamines exerted a specific inhibition of ouabain-sensitive, K^+-dependent *p*-nitrphenyl phosphatase activity, possibly by competition with K^+. Since this phosphatase activity, associated with ($Na^+ + K^+$)-ATPase preparations, may represent the terminal step of K^+-dependent dephosphorylation of the phosphoenzyme in ($Na^+ + K^+$)-ATPase [5—7], it was speculated that specific inhibition by spermine of the K^+-*p*-nitrophenyl phosphatase may regulate this latter step of the total ($Na^+ + K^+$)-ATPase reaction [3,4]. Membrane ($Na^+ + K^+$)-ATPase is generally considered to be the enzymic mechanism of cation transport across cell membrane [8—11]. Therefore, in view of our previous observation on the effect of spermine on the ($Na^+ + K^+$)-ATPase reaction [2] and of Tashima and Hasegawa [3,4] on K^+-*p*-nitrophenyl phosphatase, it seemed appropriate to examined in detail the effects of various polyamines on the kinetics and partial reactions of ($Na^+ + K^+$)-ATPase. We found that spermine and other related amines were not specific inhibitors of the K^+-*p*-nitrophenyl phosphatase; rather, they inhibit Na^+ and K^+ activation of the ATPase and Na^+-dependent phosphoenzyme formation as well as the K^+-*p*-nitrophenyl phosphatase. In all these reactions, the various amines appear to act competitively with respect to the monovalent cation involved. A preliminary account of this work has been given [12].

Materials

Spermine, spermidine, putrescine and cadaverine hydrochlorides, *p*-nitrophenyl phosphate (sodium salt), and fluorometric grade imidazole were obtained from Sigma Chemical Co., St. Louis, Mo. Methyl glyoxalbis-(guanylhydrazone)dihydrochloride (MGBG) and 1,6-hexanediamine were from Aldrich Chemical Co., Milwaukee, Wisc. Sodium dodceyl sulfate (SDS) was from Eastman Organic Chemicals, Rochester, N.Y. *p*-Nitrophenyl phosphate, EDTA and ATP (including [γ-^{32}P]ATP) were converted to the corresponding Tris salts prior to use. Details concerning this and other materials have been given previously [2,6,13,14]. Adult male Sprague-Dawley rats (ARS Sprague-Dawley, Madison, Wisc.) weighing 250—300 g were used throughout this study.

Methods

Preparation of ($Na^+ + K^+$)-ATPase. Rat brain microsomal membrane ($Na^+ + K^+$)-ATPase was prepared by the following modifications of previous procedures [6,13,15]. The initial homogenization of the tissue at 3°C was in 0.25 M sucrose/1 mM EDTA/10 mM imidazole-HCl (pH 8.0) (medium A). The microsomal fraction [6,13,15] was stored at —20°C for about 1 week before it was treated with SDS according to the procedure of Jorgensen [16]. Immediately

following incubation with SDS, the suspension was placed on ice and brought to a final concentration of 1.25 M sucrose/3 mM EDTA/15 mM imidazole-HCl (pH 8.0). After centrifugation at 24 000 × *g* for 140 min, the floated material was removed from the top of the tubes, washed 3 times with medium A and was stored in small aliquots at −20°C. The specific activity of this ($Na^+ + K^+$)-ATPase preparation was about 425 μmol P_i/mg per h; it remained unaltered over a period of several months.

Measurement of enzyme activities. Assay procedure for ($Na^+ + K^+$)-ATPase, K^+-*p*-nitrophenyl phosphatase and Na^+-dependent phosphoenzyme formation have been reported in detail in previous communications [15,17–19]. Any modifications from standard procedures are described in the text. Protein was assayed according to the procedure of Lowry et al. [20]. [γ-^{32}P]ATP was prepared as described before [17]. P_i was analyzed according to the method of Fiske and Subba Row [21] or by the measurement of ^{32}P released from [^{32}P]ATP [17].

Results

Effect of polyamines on ($Na^+ + K^+$)-ATPase. In previous work we have shown that 1 mM spermine gave a small inhibition of the rat ventral prostate membrane ($Na^+ + K^+$)-ATPase, whereas 1 mM spermidine was essentially without effect [2]. Using partially purified rat brain ($Na^+ + K^+$)-ATPase preparation, a similar result was obtained when the assay conditions for the ATPase reaction were optimal (Table I, column A). However, when suboptimal assay conditions were employed, i.e., by reducing the Na^+ in the reaction to 10 mM and K^+ to 1 mM thereby keeping the ratio of Na^+/K^+ essentially the same as for the optimal assay, it was observed that 2 mM spermine or spermidine significantly inhibited the ATPase reaction. Spermine was more active than spermidine while putrescine, cadaverine and 1,6-hexanediamine were without effect at 2 mM. For comparison, the effect of methylglyoxal-bis-(guanylhydrazone), an inhibitor of putrescine-stimulated *S*-adenosylmethionine decarboxylase, was also examined. It is clear from Table I that MGBG was a more potent inhibitor of ($Na^+ + K^+$)-ATPase than polyamines. Since MGBG carries approximately 6 positive charges (at neutral pH) as compared with 4 and 3 for spermine and spermidine, respecitvely, it seemed that the somewhat greater inhibitory effect of MGBG on the ($Na^+ + K^+$)-ATPase was related to its higher positive charge. To test this hypothesis, the ionic strength due to the various inhibitors was equalized. As shown in Table I (column C) spermine, spermidine, and MGBG were comparable in their effect under these experimental conditions. In addition, 1,6-hexanediamine appeared to be as potent an inhibitor as the above compounds, but, interestingly, putrescine and cadaverine remained poor inhibitors. Thus, factors other than charge alone may influence the effectiveness of the amine inhibitors. For example, aliphatic chain length [13] may be a contributing factor in making 1,6-hexanediamine more effective than putrescine and cadaverine. Further support of this comes from experiments where suboptimal Na^+/K^+ ratios (e.g., 110 mM Na^+ and 1 mM K^+ or 30 mM Na^+ and 0.3 mM K^+) were employed in the ($Na^+ + K^+$)-ATPase assay to study the effects of various polyamines. In this case, the inhibitions elicited by spermine,

TABLE I

EFFECT OF POLYAMINES AND RELATED COMPOUNDS ON $(Na^+ + K^+)$-ATPase UNDER VARYING ASSAY CONDITIONS

$(Na^+ + K^+)$-ATPase activity is μmol P_i/mg protein per h ± S.E. Mg^{2+}-ATPase activity was measured by including 0.10 mM ouabain in the total reaction medium; this was substrated from the activity in the presence of $Mg^{2+} + Na^+ + K^+$, was not altered by polyamines, and represented no more than 5% of the total activity. Reaction times were 15—20 min at 37°C. Column A gives the effects of various polyamines, present at 2 mM, under the following optimal assay conditions: 30 mM Tris-HCl (pH 7.45 at 37°C), 3 mM $MgCl_2$, 3 mM Tris-ATP, 110 mM NaCl, 10 mM KCl and 4.6 μg enzyme protein in a final volume of 2 ml. In column B, all conditions are the same as above, except that 10 mM NaCl and 1 mM KCl were present. Column C represents the same reaction medium as under B, except for the following concentrations: 4.5 mM putrescine, cadaverine or 1,6-hexanediamine, 1.15 mM spermine, 2 mM spermidine, and 0.50 mM MGBG; these concentrations correspond to a calculated ionic strength of 9 mM in the reaction, since at pH 7.45, these amines are known to be essentially fully protonated [25,26].

Polyamine	A $(Na^+ + K^+)$-ATPase		B $(Na^+ + K^+)$-ATPase		C $(Na^+ + K^+)$-ATPase	
	Activity	%I	Activity	%I	Activity	%I
Control	425 ± 4	—	225 ± 5	—	225 ± 5	—
Spermine	418 ± 5	2	163 ± 3	28	183 ± 3	19
Spermidine	416 ± 4	2	174 ± 3	23	175 ± 3	23
Putrescine	421 ± 3	1	220 ± 5	2	214 ± 4	5
Cadaverine	419 ± 4	1	222 ± 3	1	216 ± 8	4
1,6-Diaminohexane	419 ± 4	1	202 ± 3	10	180 ± 4	20
MGBG	368 ± 4	13	129 ± 3	43	185 ± 6	18

spermidine, putrescine, cadaverine and MGBG were of similar magnitude but 1,6-hexanediamine appeared to be the most potent inhibitor at 4 mM ionic strength (data not shown).

If any of the two polyamines were combined (each at 2 mM ionic strength) while maintaining the total ionic strength at 4 mM, the inhibitory effects were additive. Further, if prior to adding Na^+ and K^+ to start the reaction the enzyme was preincubated with polyamines alone, or with polyamines in the presence of Mg^{2+} and ATP, the inhibitions observed remained the same.

Effects of polyamines on the activation of $(Na^+ + K^+)$-ATPase by Mg^{2+} and ATP. Polyamines are known to substitute for Mg^{2+} in certain enzyme activities [22]. No such substitution of Mg^{2+} by spermine was detected in the $(Na^+ + K^+)$-ATPase assay. An analysis of the inhibitory effect of spermine on the kinetics of activation of $(Na^+ + K^+)$-ATPase by Mg^{2+} and ATP (maintained at equimolar concentrations) revealed that the inhibition was of an uncompetitive nature, characterized by a decrease in both the apparent V and the apparent K_m for Mg^{2+} (Fig. 1). This would be expected if spermine reacted with the ATPase following the binding of the substrate Mg^{2+}-ATP to the enzyme.

Effect of polyamines on the activation of $(Na^+ + K^+)$-ATPase by Na^+. The effect of 2.0 mM putrescine (as a representative polyamine) on the activation of $(Na^+ + K^+)$-ATPase by Na^+ was examined in the presence of 1.5 mM K^+ while varying Na^+ from 0.7 mM to 2.0 mM. It is apparent from Fig. 2 that putrescine inhibited the Na^+ activation of the ATPase. The data in Fig. 2 are further expressed in the inset by a modified Lineweaver-Burk plot [23,24] which

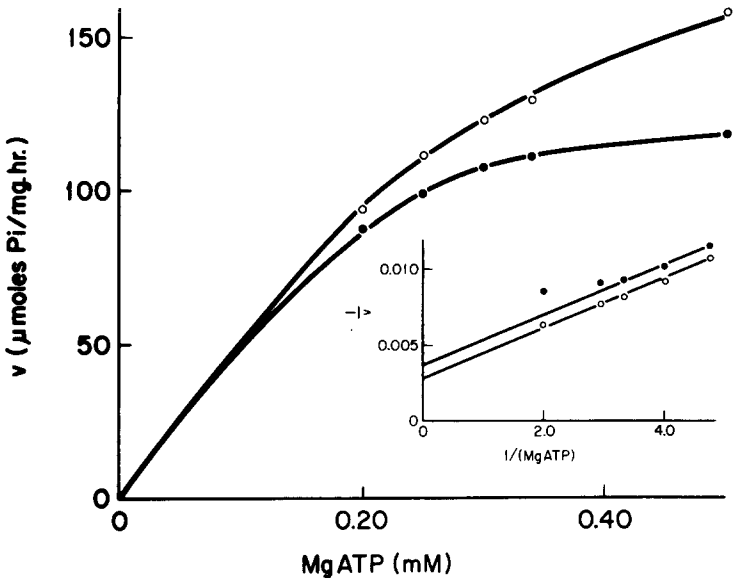

Fig. 1. The effect of spermine on the activation of $(Na^+ + K^+)$-ATPase by Mg^{2+} and ATP. Assay conditions were the same as in Table I, Column B, except that the concentrations of Mg^{2+} and ATP were varied from 0.2 mM to 0.50 mM while keeping the Mg^{2+}/ATP ratio equal to 1.0. The inset is a Lineweaver-Burk plot of the data. ○, control; ●, 1.0 mM spermine.

suggests that the inhibitory effect of putrescine on Na^+ activation of the $(Na^+ + K^+)$-ATPase is of a competitive nature as characterized by a decrease in the apparent K_m for Na^+ without a change in the apparent V. A similar result was obtained in the presence of spermine or MGBG (not shown).

Effect of polyamines on Na^+-dependent phosphoenzyme formation. Since polyamines inhibited Na^+ activation of the $(Na^+ + K^+)$-ATPase in an apparently competitive manner, it was of interest to examine whether a similar effect was observed on the Na^+-dependent phosphoenzyme formation in the ATPase reaction. The results given in Table II show that under optimal conditions, spermine, spermidine and MGBG only slightly inhibited the phosphoenzyme formation whereas putrescine, cadaverine and 1,6-hexadiamine were without appreciable effect (Column A). However, the inhibitory effect of these compounds was greatly enhanced when Na^+-dependent phosphoenzyme was formed in the presence of suboptimal Na^+ (1 mM) (Table II, Column B). This further substantiates the apparent competition between these amines and Na^+ in the ATPase reaction.

The data under Column C (Table II) show the effect on Na^+-dependent phosphoenzyme formation of maintaining a constant ionic strength (4 mM) of the various amines in the reaction. It is clear that whereas spermine, spermidine and MGBG were equally effective inhibitors, putrescine, cadaverine and 1,6-hexanediamine were considerably less so under these conditions. Furthermore, in this case, 1,6-hexanediamine was not more inhibitory than the other dibasic polyamines. This observation suggests the importance of ionic charge as opposed to structural characteristics (such as alphatic chain length), when these compounds interact at Na^+ sites on the enzyme.

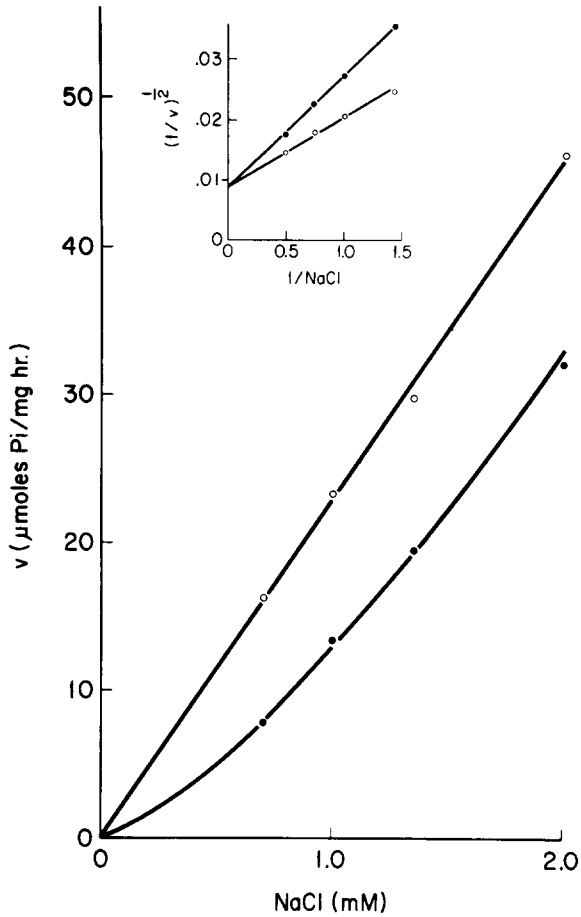

Fig. 2. The effect of putrescine on the activation of $(Na^+ + K^+)$-ATPase by Na^+. The assay conditions were the same as in Table I except that the enzyme concentration was 6.2 μg/ml, KCl was 1.5 mM, NaCl was varied from 0.50 to 2.0 mM and the incubation time was 30 min at 37°C. ○, control; ●, 2.0 mM putrescine. The inset is a modified Lineweaver-Burk plot of the data [23,24].

Since MGBG is even more highly charged than the aliphatic polyamines, it was of interest to examine further the nature of its inhibitory effect on Na^+-dependent phosphoenzyme formation. As is shown in Fig. 3 the inhibitory effect of MGBG decreases with increasing Na^+ concentration, and an analysis of the data by the modified Lineweaver-Burk plot [23,24] indicates the apparently competitive nature of the action of MGBG on Na^+-dependent phosphoenzyme formation. Spermine produced an identical result (data not given).

Effect of polyamines on K^+-p-nitrophenyl phosphatase and $(Na^+ + K^+)$-ATPase. Table III (Column A) shows that under optimal assay conditions spermine, spermidine and MGBG inhibited the reaction somewhat. However, the inhibitory effect was greatly enhanced when the concentration of K^+ in the reaction was reduced to 2 mM. In this case, putrescine, cadaverine and 1,6-hexanediamine exerted a lower, but significant, inhibition (Table III, Column B). Again, the efffect of maining a constant ionic strength of all the

TABLE II

EFFECT OF POLYAMINES ON Na^+-DEPENDENT PHOSPHOENZYME FORMATION UNDER DIFFERENT EXPERIMENTAL CONDITIONS

E-P represents the amount of [^{32}P] phosphoenzyme formed in a 5 s reaction at 0°C and is expressed as pmol Na^+-dependent phosphoenzyme formed/mg protein ± S.E.M. Column A shows the observed inhibitions at 2 mM polyamine at the following optimal assay conditions: 30 mM Tris-HCl (pH 7.4 at 0°C), 0.3 mM $MgCl_2$, 0.05 mM [^{32}P]ATP ($3 \cdot 10^4$ dpm/nmol ATP), 64 mM NaCl and 29 μg enzyme in a final volume of 1 ml. Column B, suboptimal 1 mM NaCl but otherwise identical conditions; column C, suboptimal NaCl and a polyamine ionic strength of 4 mM which is 2.0 mM putrescine, cadaverine, or 1,6-hexanediamine, 0.50 mM spermine, 0.90 mM spermidine and 0.22 mM MGBG. Non-specific labelling was estimated by omitting NaCl and substituting 16 mM KCl in the reaction; this value was subtracted from that obtained in the presence of Mg^{2+} + Na^+, and was less than 5% of the total.

Polyamine	A		B		C	
	E-P	%I	E-P	%I	E-P	%I
Control	791 ± 18	—	278 ± 17	—	278 ± 17	—
Spermine	693 ± 27	12	83 ± 9	70	193 ± 18	31
Spermidine	705 ± 17	11	117 ± 12	58	171 ± 10	38
Putrescine	772 ± 18	2	259 ± 10	7	259 ± 10	7
Cadaverine	794 ± 26	0	258 ± 15	7	258 ± 15	7
1,6-Diaminohexane	774 ± 26	2	258 ± 13	7	258 ± 13	7
MGBG	674 ± 22	15	94 ± 10	66	191 ± 17	31

inhibitors was examined and, as shown in Table III (Column C), spermine and spermidine remain the most potent inhibitors although their effect decreased. Interestingly, MGBG under these conditions showed only a small inhibitory effect. These data may be taken to indicate that unlike the Na^+ sites, the K^+ sites are not as sensitive to the cationic charge on the inhibitory molecules, but

TABLE III

EFFECT OF POLYAMINES ON K^+-*p*-NITROPHENYL PHOSPHATASE ACTIVITY UNDER DIFFERENT EXPERIMENTAL CONDITIONS

Activities are expressed as μmol *p*-nitrophenol/mg per h ± S.E.M. Column A expresses the percent inhibition observed at 2 mM polyamine when the following optimal assay conditions were used: 50 mM Tris-HCl (pH 7.45 at 37°C), 3 mM $MgCl_2$, 3 mM Tris-*p*-nitrophenyl phosphate, 10 mM KCl, 2.7 μg enzyme/ml, and an incubation time of 30 min at 37°C in a final volume of 2 ml. Column B represents the same conditions except that suboptimal 2 mM KCl and 4.1 μg enzyme/ml were used. Column C represents activities when the polyamines are present at 4 mM ionic strength (legend, Table II) and the KCl is suboptimal as above. The Mg^{2+}-stimulated component of the enzyme activity was measured by omitting KCl from the reaction. It was less than 5% of the total, was not affected by polyamines and was substracted from the values obtained in the presence of K^+. K^+-*p*NPPase, K^+-*p*-nitrophenyl phosphatase.

Polyamine	A K^+-*p*NPPase		B K^+-*p*NPPase		C K^+-*p*NPPase	
	Activity	%I	Activity	%I	Activity	%I
Control	41.4 ± 0.9	—	16.6 ± 0.4	—	16.6 ± 0.4	—
Spermine	29.4 ± 0.9	29	3.0 ± 0.2	82	8.5 ± 0.2	49
Spermidine	29.0 ± 0.9	30	3.7 ± 0.2	78	7.6 ± 0.4	54
Putrescine	40.0 ± 0.6	3	12.1 ± 0.4	27	12.1 ± 0.4	27
Cadaverine	37.8 ± 0.9	6	12.8 ± 0.4	23	12.8 ± 0.4	23
1,6-Hexanediamine	40.2 ± 0.8	3	13.4 ± 0.3	19	13.4 ± 0.3	19
MGBG	29.2 ± 0.5	29	4.5 ± 0.1	73	14.3 ± 0.5	14

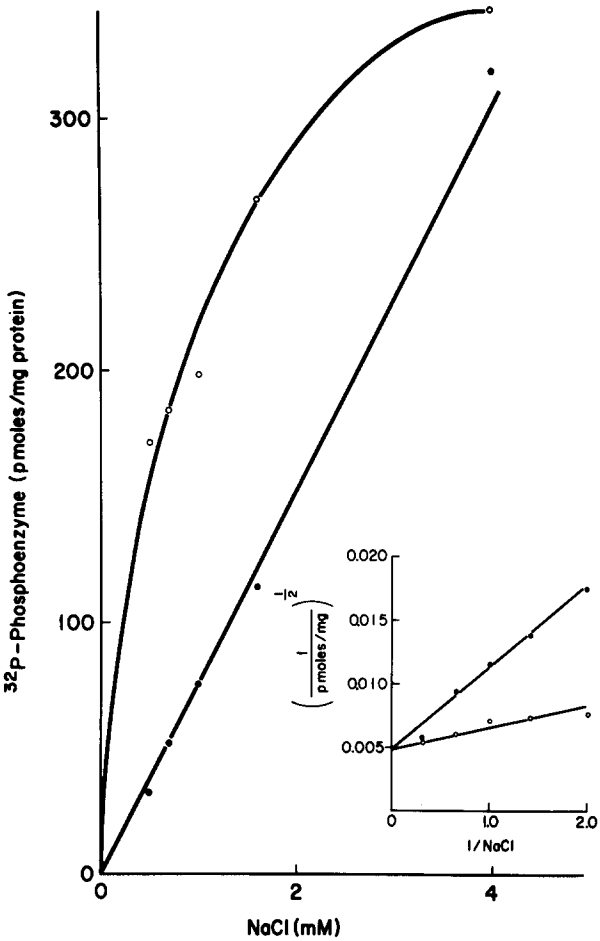

Fig. 3. The effect of methylglyoxal-bis-(guanylhydrazone) on the formation of phosphoenzyme in the presence of varying concentration of NaCl. The assay conditions were the same as for Table II. NaCl was varied from 0.50 to 4.0 mM. ○, control; ●, 1 mM MGBG. The inset is a modified Lineweaver-Burk plot of the data [23].

may be influenced to a greater extent by the molecular structure of the polyamines and other related compounds.

The effect of spermine on the kinetics of K^+ activation of *p*-nitrophenyl phosphatase activity is shown in Fig. 4. Spermine inhibits the K^+-dependent *p*-nitrophenyl phosphatase in an apparently competitive manner with respect to K^+. MGBG and spermidine gave a similar result. Since MGBG also appeared to act competitively with respect to K^+ in the K^+-phosphatase reaction, its effects on the K^+ activation of (Na^+ + K^+)-ATPase was examined by keeping all other conditions constant while varying K^+ in the reaction (Fig. 5). The results expressed in the inset in Fig. 5 clearly illustrates that MGBG inhibits (Na^+ + K^+)-ATPase activity competitively with respect to K^+. Again, a similar result was obtained when polyamines such as spermine and spermidine were tested (data not given).

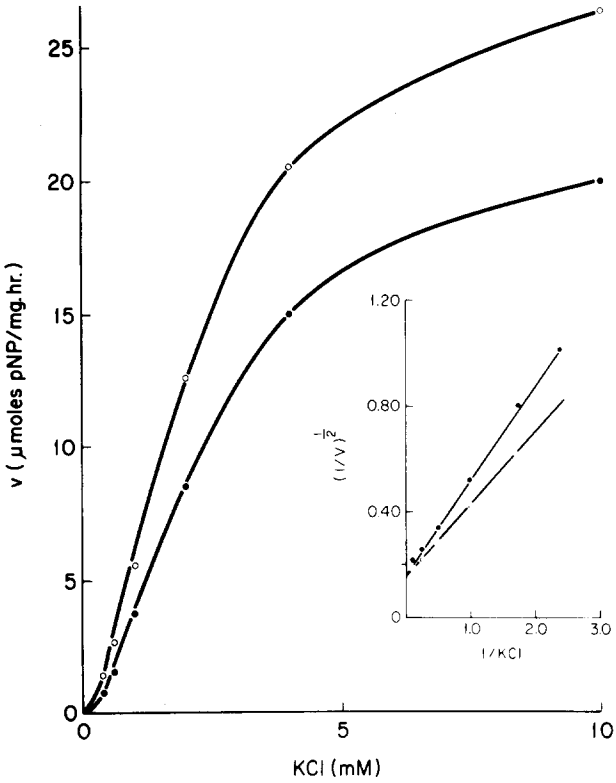

Fig. 4. The effect of spermine on the activation of *p*-nitrophenyl phosphatase activity by K^+. The assay conditions were the same as for Table I except that the enzyme concentration was 6.65 μg/ml and the incubation time was 45 min for the 0.40 and 0.60 mM KCl concentrations. KCl was varied from 0.4 to 10.0 mM. ○, controls, ●, 0.10 mM spermine. The inset represents a modified Lineweaver-Burk plot to linearize the data [23,24].

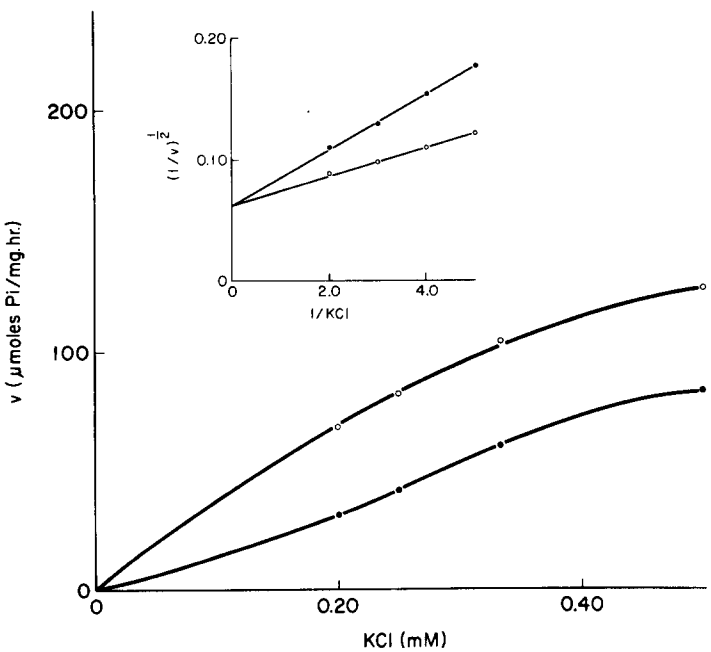

Fig. 5. The effect of methylglyoxal-bis-(guanylhydrazone) on the activation of $(Na^+ + K^+)$-ATPase by K^+. The assay conditions were the same as in Table III, except that the enzyme concentration was 7.4 μg/ml, NaCl was 30 mM, KCl was varied from 0.20 to 0.50 mM, and the incubation time was 20 min at 37°C. ○, control; ●, 1.0 mM MGBG. The inset is a modified Lineweaver-Burk plot to linearize the results [23,24].

Discussion

The foregoing results confirm the observation of Tashima and Hasegawa [3,4] that spermine and spermidine competitively inhibit the K^+-dependent *p*-nitrophenyl phosphatase activity of the ($Na^+ + K^+$)-ATPase system. However, our results do not lend support to the hypothesis [3,4] that inhibition by polyamines of the K^+-phosphatase is a specific regulatory mechanism in the operation of ($Na^+ + K^+$)-ATPase.

We have demonstrated that ($Na^+ + K^+$)-ATPase inhibition by polyamines is markedly dependent on the total cationic composition of the reaction medium. Besides spermine and spermidine, other aliphatic amines and MGBG (an even more highly charged compound which is not a membrane of the polyamines group) were also inhibitory to the ATPase reaction in varying degrees. The effects of polyamines on the kinetics of Na^+ activation of the ($Na^+ + K^+$)-ATPase reaction and Na^+-dependent phosphoenzyme formation suggest a comtitive relationship between these amines and Na^+ in the reaction. Our results suggest that inhibition of the overall ($Na^+ + K^+$)-ATPase reaction by various amines is dependent not only on the ionic charge of the molecule but also its structure with respect to aliphatic chain length. When the effects of various amines on the partial reactions of ($Na^+ + K^+$)-ATPase are examined, it becomes apparent that reactions involving Na^+ (i.e., Na^+-activation of ($Na^+ + K^+$)-ATPase or Na^+-dependent phosphoenzyme formation) are sensitive more to the ionic charge of the amine than to its structure. Thus, MGBG appeared as the most potent inhibitor among the various compounds tested on the Na^+-dependent phosphoenzyme formation. By contrast, the K^+-dependent reactions (e.g., K^+-*p*-nitrophenyl phosphatase) do not appear to be as sensitive to the total cationic charge of the inhibitor molecule. This seems to be substantiated by the observation that MGBG was less potent than spermine and spermidine in inhibiting the K^+-phosphatase reaction.

In conclusion, it appears that polyamines do not act at a single specific site on ($Na^+ + K^+$)-ATPase but rather, act by competing with the monovalent activating cations involved in the ATPase reaction.

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