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Phosphorylation of H^+/K^+-ATPase by inorganic phosphate. The role of K^+ and SCH 28080

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The effects of K^+ on the phosphorylation of H^+/K^+-ATPase with inorganic phosphate were studied using H^+/K^+-ATPase purified from porcine gastric mucosa. The phosphoenzyme formed by phosphorylation with P_i was identical with the phosphoenzyme formed with ATP. The maximal phosphorylation level obtained with P_i was equal to that obtained with ATP. The P_i phosphorylation reaction of H^+/K^+-ATPase was, like that of Na^+/K^+-ATPase, a relatively slow reaction. The rates of phosphorylation and dephosphorylation were both increased by low concentrations of K^+, which resulted in hardly any effect on the phosphorylation level. A decrease of the steady-state phosphorylation level was caused by higher concentrations of K^+ in a noncompetitive manner, whereas no further increase in the dephosphorylation rate was observed. The decreasing effect was caused by a slow binding of K^+ to the enzyme. All above-mentioned K^+ effects were abolished by the specific H^+/K^+-ATPase inhibitor SCH 28080 (2-methyl-8-[phenylmethoxy]imidazo-[1-2-a]pyrine-3-acetonitrile). Additionally, SCH 28080 caused a 2-fold increase in the affinity of H^+/K^+-ATPase for P_i. A model for the reaction cycle of H^+/K^+-ATPase fitting the data is postulated.

Introduction

H^+/K^+-ATPase is a member of the P-type transport ATPases, a class of membrane-bound ion-transporting ATPases that accept the terminal phosphate group of ATP to form a phosphorylated intermediate and then donate the phosphate group to water during the reaction sequence [1]. Walderhaug et al. [2] showed that during the phosphorylation reaction of H^+/K^+-ATPase the γ-phosphate group of ATP is bound to an aspartic acid residue of the enzyme. The dephosphorylation rate of the phosphorylated intermediate is stimulated by K^+ [3]. These and other experiments suggest that the mechanism of action of H^+/K^+-ATPase is in general according to the Post-Albers scheme.

One of the aspects of this scheme is that the E_2 form of these enzymes can be phosphorylated by inorganic phosphate. This has been shown to be the case both with Ca^{2+}-ATPase [4] and with Na^+/K^+-ATPase [5]. In the latter enzyme the same aspartic acid residue is phosphorylated both by ATP and P_i. Phosphorylation by either substrate mutually excludes phosphorylation by the other substrate [5]. For H^+/K^+-ATPase no comprehensive study on P_i phosphorylation has been presented. Jackson and Saccomani [6] published in abstract form that P_i-phosphorylation leads to a level of 2.8 nmol · mg^{-1} protein, which is double the level obtained by phosphorylation with ATP [7]. Helmich-De Jong et al. [8] found, however, that the phosphorylated intermediate formed by P_i phosphorylation had properties similar to those of the intermediate formed by ATP phosphorylation.

In a recent study [9] we observed that, after preincubation of H^+/K^+-ATPase with the inhibitor SCH 28080, the phosphorylation level at suboptimal P_i concentrations was increased, as is the case of Na^+/K^+-ATPase after preincubation with ouabain [10].

These in part contradictory and in part inexplicable results led us to study the P_i phosphorylation of H^+/K^+-ATPase in detail.

Abbreviations: P_i, inorganic phosphate; $EP_{(Pi)}$ and $EP_{(ATP)}$, the phosphointermediate formed by phosphorylation with inorganic phosphate and ATP, respectively; SCH 28080, 2-methyl-8-[phenylmethoxy]imidazo[1-2-a]pyrine-3-acetonitrile.

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Materials and Methods

Enzyme preparation. Isolation of H^+/K^+-ATPase-containing membrane sheets was carried out according to the procedure reported by Skrabanja et al. [11]. The isolated preparation was stored at −20 °C in 0.25 M sucrose. The specific activity of the enzyme preparations ranged from 50 to 100 μmol ATP hydrolysed per h per mg protein at 37 °C. The protein determination was done according to Lowry et al. [12] using bovine serum albumin as standard.

ATPase activity assay. K^+-stimulated ATPase activity was determined according to Schrijen et al. [13]. Gastric H^+/K^+-ATPase was incubated at 37 °C in the presence of 30 mM imidazole-HCl (pH 7.0), 5 mM Mg^{2+}, 0.1 mM ouabain, 5 mM ATP and 20 mM K^+ or 20 mM choline chloride for 20 min. The difference between the P_i production with and without K^+ present was taken as the H^+/K^+-ATPase activity. Stopping of the reaction and further treatment of the samples were carried out as described before [14].

Phosphorylation with ATP. Phosphorylation was carried out at 20 °C for various periods of time with 5 μM [γ-^{32}P]ATP (Amersham International, U.K.; specific radioactivity was adjusted with nonradiative ATP to 90 to 150 Ci/mol) in a buffer medium (100 μl) containing 50 mM imidazole-HCl (pH 6.0), 1 mM Mg^{2+} and 0.1 to 0.2 mg/ml H^+/K^+-ATPase protein. The reaction was started by rapid mixing of the medium containing the enzyme (90 μl) with 10 μl [γ-^{32}P]ATP and was stopped by addition of 3 ml 5% (w/v) trichloroacetic acid, containing 100 mM phosphoric acid. The denatured phosphoprotein was filtered on a 1.2 μm pore width selectron filter (Schleicher & Schüll, Dassel, F.R.G.), which was then washed three times with stopping solution. Incorporated ^{32}P was determined by liquid scintillation counting. For blank values the stopping solution was mixed with the enzyme prior to addition of [γ-^{32}P]ATP.

Phosphorylation with inorganic phosphate. Phosphorylation with [^{32}P]P_i (Amersham International; specific radioactivity adjusted with nonradioactive P_i to 50 to 200 Ci/mol) was carried out at 20 °C for various periods of time in a medium containing 50 mM imidazole-HCl (pH 6.0), 1 mM Mg^{2+} and 0.15 to 0.20 mg/ml H^+/K^+-ATPase protein. The reaction was started and stopped as described for phosphorylation with [γ-^{32}P]ATP. The denatured phosphoprotein was collected and washed and the incorporated ^{32}P was determined. For blank values the stopping solution was mixed with the enzyme prior to addition of ^{32}P.

Phosphorylation experiments with omeprazole. In these experiments H^+/K^+-ATPase was preincubated at 37 °C for 30 min in 50 mM imidazole (pH 6.0). Thereafter the mixture was cooled to 20 °C and labeled ATP or P_i was added to start the phosphorylation reaction.

Dephosphorylation of the phosphoenzyme formed with P_i and ATP. The dephosphorylation assay was carried out at 20 °C after phosphorylation of the enzyme with either [^{32}P]P_i or [γ-^{32}P]ATP. To 100 μl of the phosphorylation medium 900 μl dephosphorylation medium, containing 50 mM imidazole-HCl (pH 6.0), 1 mM Mg^{2+}, 0.1 mM nonradioactive ATP and 1 mM nonradioactive P_i was added.

Nonradioactive ATP (0.1 mM) was added in excess to prevent incorporation of ^{32}P (from [γ-^{32}P]ATP). Nonradioactive P_i was added in excess to prevent the incorporation of ^{32}P (from [^{32}P]P_i). The dephosphorylation reaction was stopped as described for phosphorylation with [γ-^{32}P]ATP. The denatured phosphoenzyme was collected and washed and the incorporated ^{32}P was determined by liquid scintillation analysis.

Hydroxylamine assay. The sensitivity of the phosphointermediates formed by P_i and ATP (as described above) to hydroxylamine was performed as described by Schuurmans Stekhoven et al. [15].

Determination of the K^+ concentration present in the phosphorylation assay mixture. The K^+ concentration present in the phosphorylation mixture, in absence of added K^+, was determined by flame photometry using an Eppendorf FCM 6343 apparatus.

Materials. ATP was purchased from Boehringer-Mannheim, F.R.G.; [γ-^{32}P]ATP and [^{32}P]P_i were obtained from Amersham International, U.K.; SCH 28080 (2-methyl-8-[phenylmethoxy]imidazo[1-2-a]pyrine-3-acetonitrile) and omeprazole were kindly provided by Dr. B. Wallmark, Hässle, Sweden; vanadate was purchased from Riedel de Haën, Seelze, Hannover, F.R.G.

Results

Properties of P_i phosphorylation of H^+/K^+-ATPase

The phosphorylation reaction of H^+/K^+-ATPase by P_i at 20 °C in the absence of added K^+ has a pseudo-first-order rate constant (k) of 0.09 s^{-1}. This reaction is rather slow as compared to ATP phosphorylation where a rate constant of 73 s^{-1} has been reported [16]. The rate of P_i phosphorylation is not significantly affected by pH values between 6 and 8. A maximal phosphorylation level was reached at a pH between 6 and 7. Since the rate of dephosphorylation increases considerably at higher pH values, all further experiments have been carried out at pH 6.0. The K_m for P_i, in the presence of 50 mM imidazole-HCl (pH 6.0) at a saturating Mg^{2+} concentration of 1 mM, is 47 ± 8 μM (Table I).

Vanadate, a known inhibitor of P-type transport ATPases [1,7,17], inhibits the reaction cycle of these ATPases by occupation of the ATP phosphorylation site [18]. This inhibitor also inhibited P_i phosphorylation in a competitive manner (Fig. 1) with a K_i of 0.3 μM. This value is in accordance with the K_d value for vanadate binding of 0.6 μM [7].

TABLE I

The effects of SCH 28080 on the K_m for P_i and the EP_{max}

H^+/K^+-ATPase (200 μg/ml) from several different preparations was phosphorylated with a range of P_i concentrations in the presence of 1 mM Mg^{2+}, 50 mM imidazole (pH 6.0) with and without 0.1 mM SCH 28080. The reaction was stopped after 60 s and the level of the phosphorylated intermediate (EP) was determined. From these values the K_m for P_i and the maximal phosphorylation level (EP_{max}) were obtained by Scatchard analysis. The ratios of the K_m and of the EP_{max} in the presence and absence of SCH 28080 were determined per experiment. These ratios were averaged and given as mean values ± S.E. of the mean (* $P < 0.05$).

	Control (a)	SCH 28080 (b)	Ratio b/a
(n):	(6)	(4)	(4)
K_m	47 ± 8 μM	17 ± 3 μM	0.41 ± 0.04 *
EP_{max}	604 ± 63 pmol/mg	689 ± 91 pmol/mg	1.12 ± 0.04 *

Omeprazole, a specific inhibitor of H^+/K^+-ATPase [19–21], inhibited the phosphorylation reactions with both P_i and ATP with IC_{50} values of 180 and 30 μM respectively (not shown).

Comparison of the phosphoenzymes formed by phosphorylation with P_i ($EP_{(Pi)}$) and ATP ($EP_{(ATP)}$)

The phosphoenzymes formed by phosphorylation with either P_i or ATP were both hydroxylamine-sensitive, indicating that the two phosphointermediates were mixed anhydrides. This suggests that both P_i and the phosphate group of ATP bind to an aspartic acid residue.

To examine whether $EP_{(Pi)}$ and $EP_{(ATP)}$ are indeed the same phosphoenzymes, the maximal phosphorylation level reached with either P_i (500 μM, 10-times the K_m) or ATP (at a saturating concentration of 5 μM) was determined for six different H^+/K^+-ATPase preparations. The ratio of the steady-state phosphorylation levels obtained with P_i and ATP was 1.03 ± 0.04 (mean ± S.E., $n = 6$).

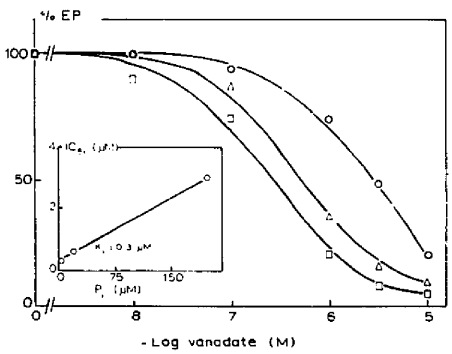

Fig. 1. The inhibitory effect of vanadate on P_i phosphorylation. H^+/K^+-ATPase (150 μg/ml) was incubated in the presence of 1 mM Mg^{2+}, 50 mM imidazole-HCl (pH 6.0) and varying concentrations of vanadate. Subsequently [^{32}P]P_i was added to a final concentration of 2 (□), 20 (△) or 200 μM (○). The reaction was stopped after 60 s and the level of phosphorylated intermediate (EP) was determined and expressed as percentage of the EP value in the absence of vanadate. Inset: The IC_{50} values obtained from these experiments are plotted against the P_i concentration.

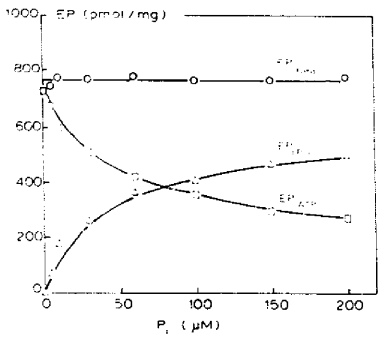

Fig. 2. Exclusion of ATP phosphorylation by P_i phosphorylation. H^+/K^+-ATPase (150 μg/ml) was preincubated for 60 s in presence of 1 mM Mg^{2+}, 50 mM imidazole-HCl (pH 6.0) with varying concentrations of [^{32}P]P_i (△) or P_i (□). Subsequently ATP (△) or [γ-^{32}P]ATP (□) was added to a final concentration of 5 μM. The reaction was stopped 4 s after addition of ATP and the level of labeled phosphorylated intermediate ($EP_{(ATP)}$ or $EP_{(Pi)}$) was determined. $EP_{(total)}$ (○) is the sum of $EP_{(Pi)}$ and $EP_{(ATP)}$.

To establish that phosphorylation by P_i forms the same phosphoenzyme as phosphorylation by ATP, a competition experiment as shown in Fig. 2 was carried out. H^+/K^+-ATPase was phosphorylated for 60 s with increasing concentrations of nonradioactive P_i in order to obtain a certain submaximal level of phosphorylated intermediate. Subsequently, radioactively labeled ATP at a saturating concentration was added and incubated for 4 s in order to phosphorylate the remaining unphosphorylated enzyme. In a parallel experiment the enzyme was first phosphorylated by labeled P_i followed by addition of non-radioactive ATP under otherwise identical conditions. Fig. 2 shows that the sum of the labeled phosphorylated intermediates in the two experiments was constant at each P_i concentration, indicating that P_i and ATP exclude each other from phosphorylation. This mutual exclusion makes it very likely that the $EP_{(Pi)}$ is the same phosphoenzyme as $EP_{(ATP)}$ with respect to the phosphate binding site.

Effects of K^+ on the dephosphorylation rate of EP and on the P_i phosphorylation rate and level

It has been shown that the phosphorylated intermediate formed by ATP is K^+-sensitive. If the phosphorylated intermediates were the same, the same K^+-dependence would be expected. In order to obtain comparable results with both substrates, the same dephosphorylation medium, containing both 0.1 mM ATP and 1 mM P_i, was used for the dephosphorylation step of the phosphointermediates formed by either [^{32}P]P_i or [γ-^{32}P]ATP.

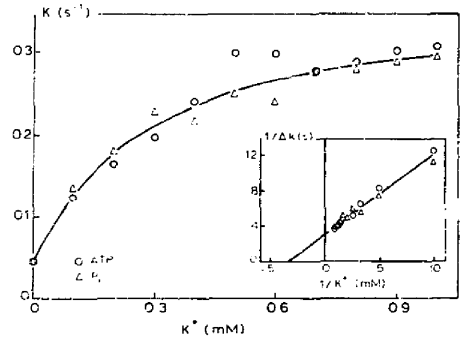

Fig. 3. K^+ dependence of the dephosphorylation rate of EP formed by either P_i or ATP phosphorylation. H^+/K^+-ATPase (150 μg/ml) was preincubated in the presence of 1 mM Mg^{2+}, 25 mM imidazole-HCl (pH 6.0) and 60 μM $^{32}P_i$ (Δ) or 5 μM [γ-^{32}P]ATP (○) during 60 and 10 s respectively. Subsequently dephosphorylation medium, containing unlabeled ATP (0.1 mM) and P_i (1 mM) instead of the labeled compounds and the indicated K^+ concentrations, was added. The reaction was stopped after 3 s and the level of phosphorylated intermediate (EP) was determined. The rate constants were calculated asuming first-order kinetics. In control experiments with the same low K^+ concentrations first-order kinetics were indeed measured during the first 10 s. Inset: the $K_{0.5}$ value of 0.3 mM was obtained by Lineweaver-Burk analysis.

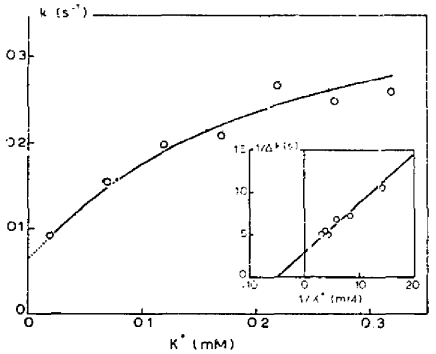

Fig. 4. K^+ dependence of the rate of formation of the phosphointermediate by P_i. H^+/K^+-ATPase (200 μg/ml) was preincubated in the presence of 1 mM Mg^{2+}, 50 mM imidazole-HCl (pH 6.0) and varying concentrations of K^+. Choline chloride was added to maintain constant salt concentrations of 0.3 mM. Subsequently [^{32}P]P_i was added to a final concentration of 60 μM. The reaction was stopped after various time intervals (3, 5, 7, 9 and 60 s) and the level of the phosphorylated intermediate (EP) was determined. The rate constants of these reactions were determined as the slope of the plot of $\ln(EP_{max}/EP_{max} - EP)$ and time (EP_{max} = EP at 60 s). The rate constant as a function of the K^+ concentration is shown. The assay mixture in absence of added K^+, including the enzyme, contained 19 μM K^+. The k value for 0 mM K^+ (k_0) was determined by extrapolation. Inset: double-reciprocal plot of k and the K^+ concentrations. k was obtained after subtraction of the k_0 from the observed k. $K_{0.5}$ = 0.2 mM.

In accordance with earlier results of Wallmark and Mårdh [3] the dephosphorylation reaction of $EP_{(ATP)}$ was stimulated by K^+. The pattern of stimulation of the dephosphorylation reaction of $EP_{(Pi)}$ is similar to that of $EP_{(ATP)}$ (Fig. 3). The $K_{0.5}$ for K^+ with respect to the increase of the dephosphorylation reaction rate is 0.3 mM for both phosphointermediates.

In contrast to ATP phosphorylation [3], the rate of P_i phosphorylation was also increased by low concentrations of K^+. The k for the phosphorylation reaction was increased by K^+ by a factor 2.5 with a $K_{0.5}$ of 0.2 mM (Fig. 4). In these calculations a correction has been made for the K^+ concentration of 19 μM (originating mainly from K^+ bound to the enzyme) present in the phosphorylation assay mixture in the absence of added K^+. The k for the phosphorylation reaction in absence of K^+ (0.06 s^{-1}) was obtained by extrapolation (Fig. 4). The steady-state phosphorylation level was hardly affected by K^+ concentrations up to 0.3 mM (not shown). A further increase of the K^+ concentration, however, reduced the steady-state phosphorylation level with a $K_{0.5}$ of 2.4 mM (Fig. 5).

The time-dependence of the formation of the phosphointermediate in the presence of K^+ is rather complex. By simultaneous addition of a high K^+ concentration (30 mM) and P_i (60 μM) a transient high phosphorylation level was reached within 3 s (Fig. 6). The transient was the result of the above-described effect of K^+ on the P_i phosphorylation rate and was followed by a slow decrease of the phosphorylation level to a lower steady-state value in 2 min. Preincubation of the enzyme with 30 mM K^+ shows that the latter effect was due to a slow binding of K^+, since in that case no transient was observed and the same steady-state phosphorylation level as that in the former experiment was reached within 3 s (Fig. 6).

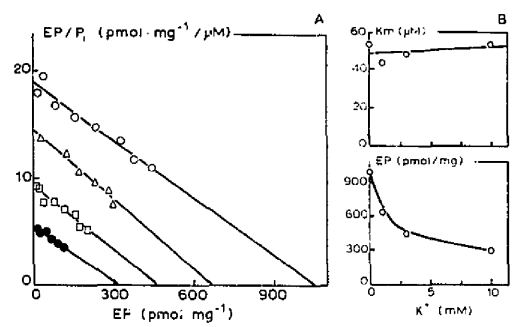

Fig. 5. Effect of K^+ on the K_m for P_i and the maximal phosphorylation level of P_i phosphorylation. (A) H^+/K^+-ATPase (150 μg/ml) was incubated in the presence of 1 mM Mg^{2+}, 50 mM imidazole-HCl (pH 6.0) with 0 (○), 1 (Δ), 3 (□) and 10 mM K^+ (●) for 30 min. Choline chloride was added to maintain constant ionic strength. Subsequently, varying [^{32}P]P_i concentrations were added. After 60 s the reaction was stopped and the level of phosphorylated intermediate (EP) was determined. (B) The values of K_m and EP_{max}, calculated from (A) are given as function of K^+.

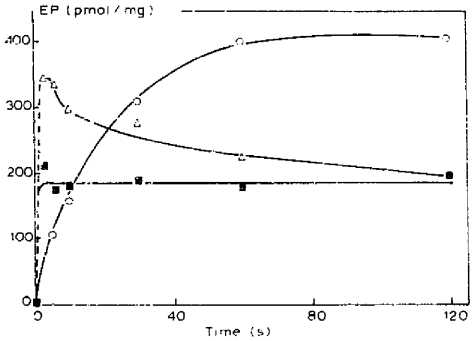

Fig. 6. Time-course for P_i phosphorylation in the presence and absence of 30 mM K^+. H^+/K^+-ATPase (200 μg/ml) was preincubated in the presence of 1 mM Mg^{2+}, 50 mM imidazole-HCl (pH 6.0), with (closed symbols) or without (open symbols) 30 mM K^+ for 30 min. Subsequently [^{32}P]P_i (to a final concentration of 60 μM) and 0 (■), 30 mM K^+ (△) or 30 mM choline chloride (○) were added. At the times indicated the reaction was stopped and the level of phosphorylated intermediate (EP) was determined.

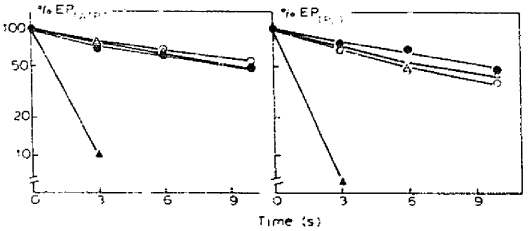

Fig. 7. Effect of SCH 28080 on the K^+-stimulated dephosphorylation rate of $EP_{(Pi)}$ and $EP_{(ATP)}$. (A) H^+/K^+-ATPase (150 μg/ml) was phosphorylated in the presence of 1 mM Mg^{2+} and 50 mM imidazole-HCl (pH 6.0) with 5 μM [γ-^{32}P]ATP during 10 s. Subsequently, SCH 28080 was added to a final concentration of 0 (open symbols) or 10 μM (closed symbols). Dephosphorylation medium containing 10 mM choline chloride (circles) or 10 mM K^+ (triangles) was added 10 s. after addition of SCH 28080. At the times indicated, the reaction was stopped and the level of phosphorylated intermediate $EP_{(ATP)}$ was measured. (B) H^+/K^+-ATPase (150 μg/ml) was preincubated in the presence of 1 mM Mg^{2+}, 50 mM imidazole-HCl (pH 6.0), 60 μM [^{32}P]P_i with (open symbols) or without (closed symbols) 0.1 mM SCH 28080 during 120 s. Subsequently, dephosphorylation medium containing 10 mM choline chloride (circles) or 10 mM K^+ (triangles) was added. The reaction was stopped after the indicated time intervals and the level of phosphorylated intermediate $EP_{(Pi)}$ was determined.

The effect of K^+ on the K_m for P_i and on the maximal phosphorylation level is given in Fig. 5. Since only the maximal phosphorylation level and not the K_m for P_i is influenced by K^+, the inhibitory effect of K^+ is noncompetitive.

Effects of SCH 28080 on the phosphorylation and dephosphorylation reactions

The effects of the specific H^+/K^+-ATPase inhibitor, SCH 28080, on the phosphorylation and the dephosphorylation reaction was studied. SCH 28080 inhibited the H^+/K^+-ATPase hydrolytic activity at pH 7.0 with an IC_{50} of 0.8 μM (cf. Ref. 22: 0.15 μM at pH 6.5, 1.5 μM at pH 7.5) and the phosphorylation reaction with ATP with an IC_{50} of 0.3 μM (cf. Ref. 23: 0.3 μM at pH 6.0 and 7.4).

The effect of SCH 28080 on the P_i phosphorylation, however, was opposite to that on the phosphorylation with ATP: at suboptimal P_i concentrations the steady-state phosphorylation level was markedly increased by increasing SCH 28080 concentrations. Table I shows that this increase is due mainly to an increase in the affinity of the SCH-28080-bound enzyme for P_i. A slight but significant increase in the maximal phosphorylation level of 12 ± 4% (Table I) was also observed.

Since it is suggested that SCH 28080 binds at the K^+-binding site [24] the inhibitor was expected to have an effect on the K^+-stimulated dephosphorylation reaction of both $EP_{(Pi)}$ and $EP_{(ATP)}$. This stimulation was in both cases totally abolished by 0.1 mM SCH 28080 (Fig. 7). No significant effect of SCH 28080 was found on the (basal) dephosphorylation rate in the absence of K^+.

In the presence of 0.1 mM SCH 28080 the effects of K^+ on the steady-state phosphorylation level and the phosphorylation rate were also abolished. As is shown in Fig. 8, the phosphorylation rate in the presence of 0.1 mM SCH 28080 was no longer affected by 10 mM K^+. The rate constants (0.06 s^{-1}) were equal to those in the absence of K^+ and SCH 28080.

SCH 28080 (0.1 mM) also abolished the reduction of the phosphorylation level by K^+. This occurred when

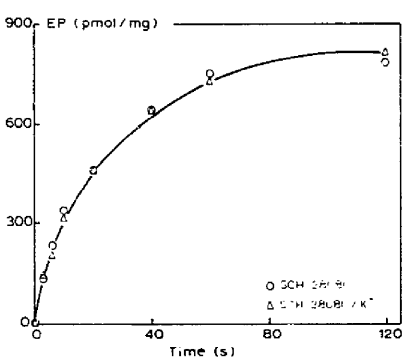

Fig. 8. Time-course of P_i phosphorylation with SCH 28080 and K^+. H^+/K^+-ATPase (150 μg/ml) was phosphorylated with 60 μM [^{32}P]P_i in the presence of 1 mM Mg^{2+}, 50 mM imidazole-HCl (pH 6.0), 0.1 mM SCH 28080 with (△) or without 10 mM K^+ (○). The reaction was stopped after the indicated time intervals and the level of the phosphorylated intermediate (EP) was determined.

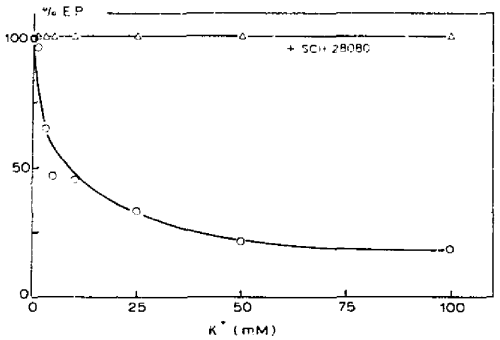

Fig. 9. Abolition by SCH 28080 of the effect of K^+ on the steady-state phosphorylation level. H^+/K^+-ATPase (150 μg/ml) was preincubated with 1 mM Mg^{2+}, 50 mM imidazole-HCl (pH 6.0) and varying K^+ concentrations for 30 min. Thereafter SCH 28080 (△) or vehicle (○) was added and the preincubation was continued for a further 30 min. Subsequently, [^{32}P]P_i was added to a final concentration of 60 μM. The reaction was stopped after 60 s and the level of the phosphorylated intermediate (EP) was determined.

K^+ was added either prior to SCH 28080 (Fig. 9) or together with the substrate (Fig. 8).

Discussion

In this study, phosphorylation of H^+/K^+-ATPase by inorganic phosphate has been studied. The most important observation is the fact that P_i and ATP phosphorylation are mutually exclusive. Although none of the experiments alone proves the identity of the phosphorylated intermediates, the sum of the experiments strongly supports this idea.

The kinetic experiments, in particular the effects of K^+ and SCH 28080 on the phosphorylation and dephosphorylation process, cannot easily be explained. We therefore give a scheme (Fig. 10) based on the Albers-Post scheme, which gives an explanation for the

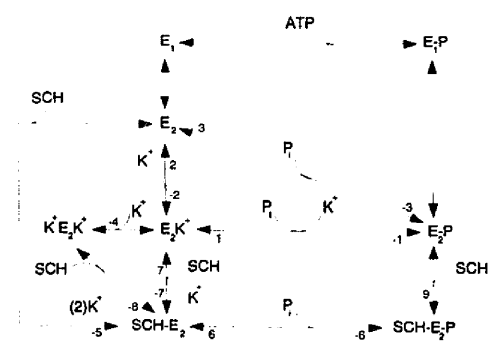

Fig. 10. Putative reaction mechanism of the catalytic activities of H^+/K^+-ATPase. The reactions depicted clockwise are indicated with a positive number; the reactions depicted anticlockwise are indicated with a negative number. Details are described in the text.

results. This does, however, not exclude the possibility that there are alternative explanations for our findings.

Effects of K^+ on the P_i phosphorylation and dephosphorylation

The K^+-stimulated dephosphorylation reaction is indicated as reaction 1 in Fig. 10. The binding of K^+ to the high-affinity K^+-binding site of the enzyme yielding E_2K^+ is represented by reaction −2. The subsequent P_i phosphorylation reaction is marked −1. This reaction has a $K_{0.5}$ for K^+ of 0.3 mM, which is in the same range as the $K_{0.5}$ for K^+ for the dephosphorylation reaction (reaction 1) (cf. Figs. 3 and 4). The increase in the exchange of ^{18}O between ^{18}O-labeled P_i and oxygen of the enzyme by K^+, as observed by Faller and Diaz [25], probably results from the higher rates of the phosphorylation and the dephosphorylation reactions caused by K^+. The rate-increasing effect of K^+ must be due to rapid binding of K^+, since the steady-state phosphorylation level was reached within 3 s (Fig. 6).

Extrapolation of the reaction rate as a function of the K^+ concentration yielded a rate constant for the P_i phosphorylation at zero K^+ of 0.06 s^{-1}. The rate constant for the dephosphorylation reaction in the absence of K^+ was in the same range. Therefore, one can conclude that there is a basal K^+-independent phosphorylation and dephosphorylation reaction. The basal P_i phosphorylation and dephosphorylation reactions are indicated −3 and 3, respectively. The rate constants of these reactions must be considerably smaller than these of the reactions in the presence of K^+ (−1 and 1), which approach values of 0.3 s^{-1} at infinite K^+ concentrations.

At higher K^+ concentrations the steady-state phosphorylation level is reduced ($K_{0.5}$ = 2.4 mM) in a non-competitive fashion. This effect can tentatively be explaned by binding of K^+ to a low-affinity binding site which, in the occupied state ($K^+E_2K^+$ in Fig. 10), withdraws the enzyme from phosphorylation with P_i. This reaction (reaction −4) is extremely slow, since the steady-state phosphorylation level is reached only after 2 min.

The initial rapid increase of the phosphorylation level shown in Fig. 6 is due to binding of K^+ to the high-affinity K^+-binding site causing a large portion of the enzyme being in the E_2K^+ form and thus an increase of the rate of reactions 1 and −1. Subsequently, the decrease of the phosphorylation level caused by the binding of K^+ to the low-affinity K^+ binding site (reaction −4) becomes apparent.

Preincubation with high K^+ long enough to give K^+ the opportunity to bind to the low-affinity K^+ binding site leads to an equilibrium between $K^+E_2K^+$ and E_2K^+. Therefore no transient is seen in the subsequent phosphorylation (Fig. 6).

SCH 28080 effects on the P_i phosphorylation and dephosphorylation reactions

SCH 28080 inhibits the K^+-stimulated ATPase activity, H^+ secretion, paranitrophenylphosphatase activity of H^+/K^+-ATPase by a competitive interaction with the K^+ site [22,26,27]. It also inhibits the ATP phosphorylation reaction [22,26,27]. In the present study, however, no inhibition but an unexpected stimulatory effect of SCH 28080 was observed on the steady phosphorylation level obtained with P_i, due to an increase in the affinity of the enzyme for P_i. A similar effect was observed for ouabain and Na^+/K^+-ATPase by Askari et al. [10]. These authors showed that ouabain stimulates the phosphorylation reaction of Na^+/K^+-ATPase with P_i. It is postulated that the binding of ouabain to Na^+/K^+-ATPase generates an E_2 form [28]. SCH 28080 bound to the enzyme might in a similar way also generate an E_2 form of the enzyme.

SCH 28080 abolishes the K^+ effects on the P_i phosphorylation rate constant, on the steady-state phosphorylation level and on the dephosphorylation reaction as can be expected from the fact that SCH 28080 binds to the K^+ binding site [24].

The P_i phosphorylation of the SCH-28080-bound enzyme is also represented in Fig. 10: preincubation of the enzyme with SCH 28080 yields SCH-E_2 (reaction -5); phosphorylation of this compound with P_i gives SCH-E_2-P (reaction -6). The rate constant of reaction -6 is much lower than that of reaction -1 and equal to the rate constant of reaction -3.

The abolishment by SCH 28080 of the rate-increasing effect of K^+ on the phosphorylation reaction is also explained in Fig. 10. Preincubation of H^+/K^+-ATPase with low K^+ concentrations results in the formation of E_2-K^+ (reaction -2). Addition of SCH 28080 replaces K^+ resulting in SCH-E_2 (reaction -7). Subsequent phosphorylation with P_i will also give SCH-E_2-P (reaction -6).

The lowering effect of K^+ on the steady-state phosphorylation level is abolished by SCH 28080 as well. This indicates that SCH 28080 influeces both the high- and the low-affinity K^+ binding site. By preincubating the enzyme with high K^+ concentrations, $K^+E_2K^+$ is formed (reactions -2 and -4, successively). By addition of SCH 28080 subsequently to K^+, the latter ion is removed from its binding site, resulting in the formation of SCH-E_2 (reaction -8); the phosphorylation with P_i proceeds as described by reaction -6. When the enzyme is preincubated with SCH 28080 in the absence of K^+ followed by addition of P_i reactions -5 and -6 take place successively. Simultaneous addition of K^+ with P_i does not influence this order of reactions, as is clear from the absence of the rate-stimulating effect of K^+.

SCH 28080 also abolishes the effect of K^+ on the dephosphorylation rate. This K^+ effect affects both the P_i and ATP phosphorylated enzyme. In the case of the ATP-phosphorylated enzyme it should be noted that SCH 28080 should be added after the phosphorylation reaction but prior to the addition of K^+. This is due to the slow binding of SCH 28080 [24] in comparison to that of K^+. This explains why Wallmark et al. [22] found no effect of SCH 28080 added simultaneously with K^+. If SCH 28080 is added to K^+ it will bind to the phosphorylated enzyme and give SCH-E_2-P (reaction 9) and will then be dephosphorylated in a K^+-independent way (reaction 6).

In the study of the dephosphorylation reaction of the P_i-phosphorylated enzyme, SCH 28080 was present during the phosphorylation reaction and so SCH-E_2-P was formed. The dephosphorylation reaction proceeds even in the presence of K^+ according to reaction 6.

Wallmark et al. [22] suggested, in contrast to Keeling et al. [24] and Mendlein and Sachs [29], that SCH 28080 only binds to the E_2 form and not to the E_2-P form. Our results indicate that this suggestion is incorrect. P_i phosphorylation can be carried out in the presence of SCH 28080. ATP phosphorylation is inhibited by SCH 28080, but after phosphorylation with ATP, SCH 28080 binds to the phosphorylated intermediate, as is shown by the fact that the intermediate becomes insensitive towards K^+. This is also the case when $EP_{(Pi)}$ is formed. SCH 28080 therefore can bind to both phosphorylated and unphosphorylated forms of the enzyme.

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