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Pb^{2+} and imidazole-activated phosphorylation by ATP of $(Na^+ + K^+)$-ATPase

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In order to study whether Pb^{2+} and imidazole increase the ATP phosphorylation level of $(Na^+ + K^+)$-ATPase by the same mechanism, the effects of both compounds on phosphorylation and dephosphorylation reactions of the enzyme have been studied. Imidazole in the presence of Mg^{2+} increases steady-state phosphorylation of $(Na^+ + K^+)$-ATPase by decreasing, in a competitive way, the K^+-sensitivity of the formed phospho-enzyme (E-P · Mg). If Pb^{2+} is present during phosphorylation, the rate of phosphorylation increases and a K^+- and ADP-insensitive phosphointermediate (E-P · Pb) is formed. Pb^{2+} has no effect on the K^+-sensitivity of E-P · Mg and EDTA is unable to affect the K^+-insensitivity of E-P · Pb. These effects indicate that Pb^{2+} acts before or during phosphorylation with the enzyme. Binding of Na^+ to E-P · Pb does not restore K^+-sensitivity either. However, increasing Na^+ during phosphorylation in the presence of Pb^{2+} leads to formation of the K^+-sensitive intermediate (E-P · Mg), indicating that E-P · Pb is formed via a side path of the Albers-Post scheme. ATP and ADP decrease the dephosphorylation rate of both E-P · Mg and E-P · Pb. Above optimal concentration, Pb^{2+} also decreases the steady-state phosphorylation level both in the absence and in the presence of Na^+. This inhibitory effect of Pb^{2+} is antagonized by Mg^{2+}.

Introduction

The aim of studies on the mechanism of $(Na^+ + K^+)$-ATPase is to gain better insight in the relationship between structure and function of the enzyme. In these studies phosphorylation is an important tool which can lead to better insight into the coupling process between ATP hydrolysis and transport.

Recently, we reported on the effects of imidazole on the phosphorylation level obtained with [γ-^{32}P]ATP [1] and on the rate constant of dephosphorylation [2] of the phosphointermediate. The conclusion of these studies is that imidazole activates the steady-state phosphorylation, in the absence of Na^+, both by inducing a form of the enzyme (E_1) able to phosphorylate and by inhibiting the dephosphorylation reaction via a decrease of the K^+ sensitivity of the phosphoenzyme.

Siegel and co-workers have reported in a number of studies on the effects of Pb^{2+} on $(Na^+ + K^+)$-ATPase. Pb^{2+} inhibits the total ATPase [3] and the *p*-nitrophenylphosphate activity [4], lowers the ouabain binding level [5] and the rate of

Abbreviations: E-P-Mg, phosphointermediate of $(Na^+ + K^+)$-ATPase formed in the presence of Mg^{2+}; E-P-Pb, phosphointermediate of $(Na^+ + K^+)$-ATPase formed in the presence of Pb^{2+}.

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ADP–ATP exchange [6]. Pb^{2+}, on the other hand, increases in the absence of Na^+ the level of ATP phosphorylation [7]. On first sight there is some parallelism between the effects of imidazole and Pb^{2+} on the phosphorylation by ATP.

In the present study, we compare the effects of imidazole and Pb^{2+} on the steady-state phosphorylation level and on the dephosphorylation rate constant of the phosphoenzyme to see whether Pb^{2+}, like imidazole, acts on the K^+ sensitivity of the phosphoenzyme and thereby increases the steady-state phosphorylation.

Materials and Methods

$(Na^+ + K^+)$-ATPase is purified from rabbit kidney outer medulla according to Jørgensen [8] followed by removal of contaminating ATP and washing of the preparation as described by Schoot et al. [9]. The enzyme is stored at −20 °C in 0.25 M sucrose and 50 mM imidazole acetate (pH 7.0). Protein is determined by the Lowry method using bovine serum albumin as a standard [10]. Specific $(Na^+ + K^+)$-ATPase activity ranges from 800–1500 μmol ATP · mg^{-1} protein · h^{-1} hydrolysed.

Phosphorylation by ATP is carried out at room temperature or at 0 °C in a volume of 50–200 μl containing: 5 μM [γ-^{32}P]ATP (0.3 Ci/mmol), 50 mM imidazole acetate pH 7.0 and the different ligands as indicated in the text. Phosphorylation is started by adding $(Na^+ + K^+)$-ATPase in 50 mM imidazole acetate buffer to the reaction mixture (final concentration 0.1 mg/ml). After 3–60 s the reaction is stopped by adding 5% trichloroacetic acid in 0.1 M phosphoric acid. Further processing is carried out as described by Schuurmans Stekhoven et al. [11]. The phosphoenzyme generated in the absence of Pb^{2+} is called E-P · Mg, and in its presence E-P · Pb.

Dephosphorylation studies of the ^{32}P-phosphoenzyme, obtained after 10 s phosphorylation at room temperature, are performed either by diluting the phosphorylation medium six times with 0.5 mM ATP in 50 mM imidazole acetate (pH 7.0; method A) or by diluting only the radioactive ATP and the enzyme ten times, without changing the concentrations of the ligands present during phosphorylation (method B). Dephosphorylation is stopped after 3 s with 5% trichloroacetic acid/0.1 M H_3PO_4. In method A, a blank is prepared to which the stopping solution is added before the enzyme. This blank has a magnitude of 1–2% of the phosphorylation level. In method B, the dilution of the radioactivity yields a new radioactive steady-state level which is maximally 10% of the original level. All phosphorylation levels are corrected by the respective blank values. The acid-denatured phosphoenzyme is further processed as indicated in the previous section. Dephosphorylation is expressed as the decrease in acid-stable phosphoenzyme during 3 s of incubation (% hydrolysis).

The free Pb^{2+} concentrations have been calculated using the following dissociation constants: $Mg^{2+} + ATP^{4-} \rightleftharpoons MgATP^{2-}$, $K_1 = 1.0 \cdot 10^{-4}$ M [4]; $Pb^{2+} + ATP^{4-} \rightleftharpoons PbATP^{2-}$, $K_2 = 0.74 \cdot 10^{-4}$ M [4]; $H^+ + ATP^{4+} \rightleftharpoons HATP^{3-}$, $K_3 = 3.02 \cdot 10^{-7}$ M [12]. In all experiments in which an ATP concentration of 5 μM is used free Pb^{2+} nearly equals total Pb^{2+}.

K_m values in steady-state phosphorylation are defined as the concentration giving 50% of the phosphorylation level.

All further methods such as conversion of ATP to its imidazole salt and determination of residual K^+ in the enzyme preparation have been published previously [1,2].

Results

Activation and inhibition by Pb^{2+} of the phosphorylation level

Fig. 1A shows the combined effects of Mg^{2+} and Pb^{2+} on the ATP dependent phosphorylation in the presence of 50 mM imidazole without added Na^+. Mg^{2+} up to 0.1 mM increases [1], in the absence of Pb^{2+}, the steady-state phosphorylation level as activator of phosphorylation, but Mg^{2+} above 0.1 mM lowers this level, since it additionally activates the rate of dephosphorylation [2]. By increasing the Pb^{2+} concentration, the steady-state phosphorylation level is first increased and next decreased, both effects being dependent on the Mg^{2+} concentration. Mg^{2+} acts synergistically at suboptimal Pb^{2+} concentrations and antagonizes inhibition at higher Pb^{2+} concentrations. This inhibitory effect of Pb^{2+} is further analyzed in a Dixon-plot (Fig. 1B) and yields a K_i value for

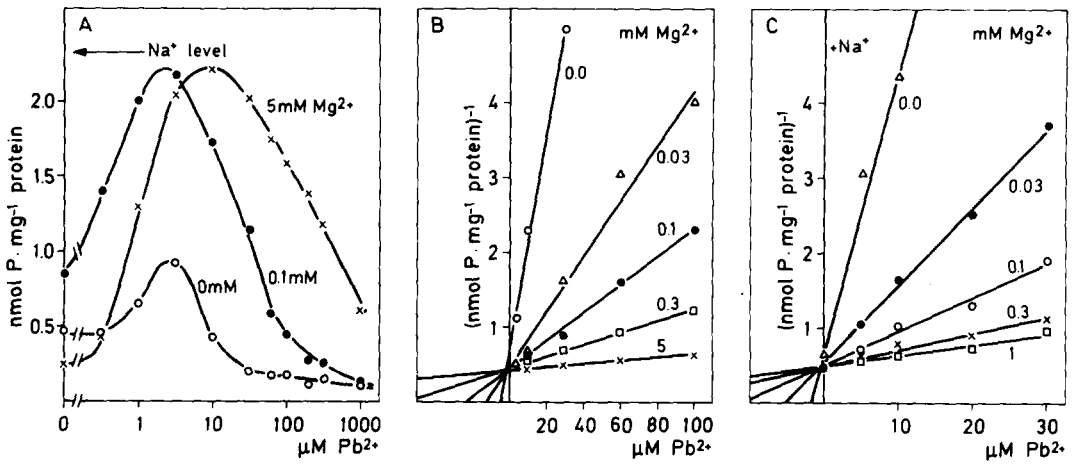

Fig. 1. (A) ATP-phosphorylation of $(Na^+ + K^+)$-ATPase as function of the Mg^{2+} and Pb^{2+} concentration. Phosphorylation levels after 3 s incubation at room temperature in 50 mM imidazole-acetate (pH 7.0), 5 μM [γ-^{32}P]ATP, 0.1 mg/ml protein and the indicated Pb^{2+} and Mg^{2+} concentrations. Representative of 3–4 experiments. (B) Dixon plot of the inhibitory effect of Pb^{2+} on the steady state phosphorylation at varying Mg^{2+} concentrations (data from A). (C) Dixon-plot of the effect of Pb^{2+} and Mg^{2+} on the inhibition of the steady-state phosphorylation in the presence of 100 mM Na^+, 5 μM [γ-^{32}P]ATP, 50 mM imidazole acetate pH 7.0 and 0.1 mg/ml enzyme. Representative of two experiments.

Pb^{2+} of 1.0 ± 0.1 μM. This inhibition is counteracted by Mg^{2+} in a linear competitive fashion between 0.0 and 0.3 mM Mg^{2+} yielding a K_m value for Mg^{2+} of 0.03 ± 0.02 mM. A similar inhibition by Pb^{2+} on the ATP-dependent phosphorylation is also seen in the presence of 100 mM Na^+. A Dixon plot of the inhibitory effect of Pb^{2+} in this case (Fig. 1C) gives a K_i value for Pb^{2+} of 0.8 ± 0.1 μM. Again the same Mg^{2+}–Pb^{2+} antagonism can be observed with a K_m value for Mg^{2+} of 0.04 ± 0.02 mM. With Na^+ present during phosphorylation, activation by Pb^{2+} does not occur, since the activation of phosphorylation by Na^+ is already optimal.

K^+-(in)sensitive phosphoenzyme

After phosphorylation in the presence of 50 mM imidazole and 0.1 mM Mg^{2+}, a K^+ sensitive phosphointermediate (E-P · Mg) is formed (Fig. 2A). The K_m for K^+ is 4 μM (see also Ref. 1). In the presence of 1 μM Pb^{2+}, without added Mg^{2+}, the phosphointermediate (E-P · Pb) is insensitive towards K^+ (Fig. 2A), indicating that Pb^{2+} does not act competitively on the K^+-site. The inhibitory effect of Pb^{2+} on the overall $(Na^+ + K^+)$-ATPase activity is not counteracted by K^+ either: the K_m for K^+ is 1.2 mM in the absence and 1.0 mM in the presence of 9 μM free Pb^{2+}. The latter Pb^{2+} concentration gives 50% inhibition of the $(Na^+ + K^+)$-ATPase activity (data not shown).

Increasing the Mg^{2+} concentration during phosphorylation in the presence of 3 μM Pb^{2+} does not change the K^+ insensitivity of the phosphoenzyme (Fig. 2B). However, increasing the Na^+ concentration above 0.3 mM in the presence of 3 μM Pb^{2+} and 0.1 mM Mg^{2+} changes the phosphoenzyme from a K^+-insensitive to a K^+-sensitive intermediate (Fig. 2C). In the absence of added K^+ the dephosphorylation rate constant is reduced between 1 and 10 mM Na^+, due to the fact that Na^+ counteracts the activating effect of residual K^+ [2]. The resulting k value is comparable with the dephosphorylation rate constant of the phosphoenzyme generated with Mg^{2+} (see also Table I). Above 10 mM Na^+, an increase in the dephosphorylation rate constant is observed due to a K^+-like effect of Na^+ [2].

Properties of the phosphointermediates formed with and without Pb^{2+}

The experiments described up until now have indicated that the properties of the phosphointermediate formed in the presence of Pb^{2+} are not affected by the additional presence of Mg^{2+}. Be-

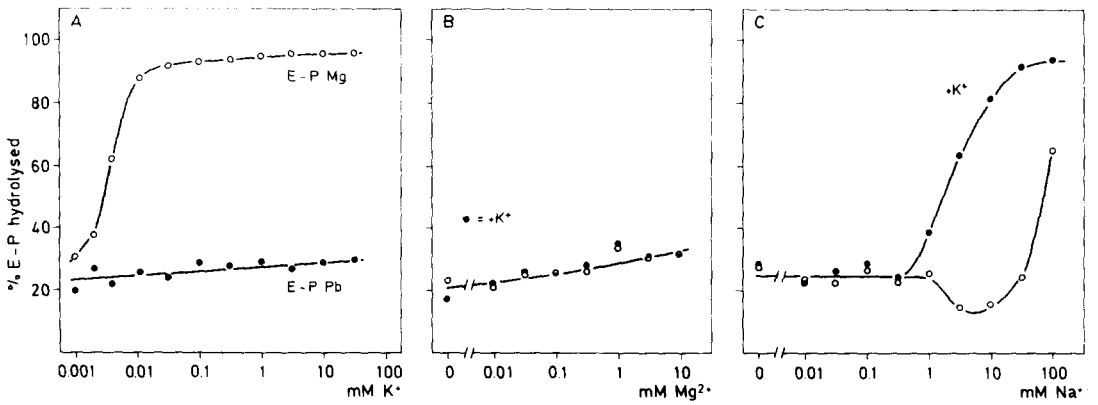

Fig. 2. (A) Effect of K^+ on the stability of the phosphoenzyme generated with Mg^{2+} or Pb^{2+}. ATP phosphorylation was performed for 10 s at room temperature in a medium containing 5 μM [γ-^{32}P]ATP, 0.1 mM Mg^{2+} (○) or 1 μM Pb^{2+} (●), 50 mM imidazole acetate pH 7.0 and 0.1 mg/ml enzyme, followed by 3 s dephosphorylation (method A) in medium containing 0.5 mM non-radioactive ATP and the K^+ concentrations as indicated (1 μM–30 mM K^+). The amount of E-^{32}P hydrolysed in 3 s is plotted as a function of the K^+ concentration in the dephosphorylation medium. E-P level is 1.0 (E-P·Mg) and 1.3 (E-Pb·Pb) nmol P·mg^{-1} protein. (B) Effect of the Mg^{2+} concentration present during phosphorylation in addition to 3 μM Pb^{2+} on the K^+ sensitivity of the phosphoenzyme. Phosphorylation was performed at room temperature for 10 s with 5 μM [γ-^{32}P]ATP, 3 μM Pb^{2+}, 50 mM imidazole-acetate (pH 7.0), 0.1 mg/ml enzyme and the indicated Mg^{2+} concentrations (0–10 mM) and followed by 3 s dephosphorylation (Method A) in a medium containing additionally 0.5 mM non-radioactive ATP plus (●) or minus 1 mM K^+ (○). E-P level was in the range 1.4–2.4 nmol P·mg^{-1} protein. (C) Effect of the Na^+ concentration present during phosphorylation with Mg^{2+} and Pb^{2+} on the K^+ sensitivity of the phosphoenzyme. Phosphorylation was performed at room temperature for 10 s with 5 μM [γ-^{32}P]ATP, 3 μM Pb^{2+}, 0.1 mM Mg^{2+}, 50 mM imidazole-acetate (pH 7.0), 0.1 mg/ml enzyme and the indicated Na^+ concentrations (0–100 mM) followed by 3 s dephosphorylation (method A) in a medium containing, in addition, 0.5 mM non-radioactive ATP plus (●) or minus 1 mM K^+ (○). E-P level was in the range 2.2–3.0 nmol P·mg^{-1} protein.

cause of the antagonistic effect of Mg^{2+} on the inhibition by Pb^{2+}, further experiments were carried out in the presence of 0.1 mM Mg^{2+}.

In order to study the properties of the phosphointermediates obtained with and without Pb^{2+}, they were prepared during 10 s incubation with 5 μM ATP, 50 mM imidazole, 0.1 mM Mg^{2+} and with or without 3 μM Pb^{2+}, after which the effect of different ligands on the dephosphorylation rate constant was studied. The two phosphointermediates are called E-P · Pb and E-P · Mg, respectively.

After phosphorylation in the absence of Pb^{2+}, the following properties of E-P · Mg are observed (Table I):

(i) An increase of the dephosphorylation rate constant by K^+, Mg^{2+} and low imidazole concentration (decreased K^+-imidazole antagonism), (lines 2, 3 and 4 in Table I; see also Ref. 2).

(ii) No effect of Pb^{2+} on the dephosphorylation rate constant. Upon combined addition of Pb^{2+} and K^+ the phosphointermediate remains K^+ sensitive (line 5 and 6).

(iii) No stimulatory effect of ADP on the hydrolysis of the phosphointermediate, so the intermediate is 'ADP-insensitive' (line 12).

(iv) In contrast, an inhibitory effect of both ATP and ADP on the dephosphorylation rate constant (line 11 and 12).

The effect of Na^+ on the dephosphorylation process cannot be studied by the technique used here, since in the presence of Na^+, the phosphorylation by the residually bound radioactive ATP is faster than the exchange rate of nonradioactive ATP leading to an increased phosphorylation level. The dephosphorylation rate will thus be underestimated [2]. The same problem of exchange for non-radioactive ATP and phosphorylation by [γ-^{32}P]ATP also holds for the experiments with Pb^{2+}. This may explain the lower dephosphorylation rate in the presence of Pb^{2+} with and without K^+

TABLE I

PROPERTIES OF THE PHOSPHOINTERMEDIATE AFTER ATP PHOSPHORYLATION IN THE ABSENCE AND PRESENCE OF Pb^{2+}

After phosphorylation of $(Na^+ + K^+)$-ATPase (0.1 mg/ml) with ATP[γ-^{32}P] 50 mM imidazole-acetate (pH 7.0), 0.1 mM Mg^{2+} in the absence (E-P·Mg) and presence (E-P·Pb) of 3 μM Pb^{2+} for 10 s at room temperature, dephosphorylation is started by adding 9 vol. to the phosphorylation medium and stopped after 3 s by acid denaturation (method B). The dephosphorylation medium contains no radioactive ATP but has the same composition as that of the phosphorylation medium with the exception of the specific ligand tested. Mean values are given with S.E. for $n = 2$–4. The phosphorylation levels for E-P·Mg and E-P·Pb are, respectively, 1.55 ± 0.22 and 2.56 ± 0.45 nmol P·mg^{-1} protein.

Dephosphorylation conditions (changes compared to the phosphorylation condition)	% hydrolysis in 3 s	
	E-P·Mg	E-P·Pb
(1) Control	46 ± 6	53 ± 1
(2) K^+ (1 mM)	94 ± 1	50 ± 3
(3) Mg^{2+} (5 mM)	84 ± 2	45 ± 1
(4) Imidazole (10 mM)	91 ± 1	49 ± 1
(5) Pb^{2+} (3 μM)	36 ± 2	50 ± 2
(6) Pb^{2+} (3 μM) + K^+ (1 mM)	84 ± 3	50 ± 5
(7) EDTA (1 mM)	32 ± 1	49 ± 2
(8) EDTA (1 mM) + K^+ (1 mM)	95 ± 1	41 ± 1
(9) Na^+ (100 mM)	–	54 ± 4
(10) Na^+ (100 mM) + K^+ (1 mM)	–	47 ± 2
(11) ATP (1 mM)	14 ± 2	24 ± 1
(12) ADP (1 mM)	23 ± 1	30 ± 4
(13) ATP (1 mM) + Mg^{2+} (5 mM)	74 ± 4	30 ± 2

(lines 5 and 6, Table I) compared to the dephosphorylation rate in the absence of Pb^{2+} (lines 1 and 2).

After phosphorylation in the presence of Pb^{2+} and Mg^{2+} a phosphointermediate (E-P · Pb) is generated which is:

(i) Insensitive to K^+, Mg^{2+}, Na^+ and lowering of the imidazole concentration (lines 2, 3, 4 and 9).

(ii) Still K^+ insensitive after chelating the Pb^{2+} by EDTA (lines 7 and 8).

(iii) Still K^+ insensitive after addition of Na^+ and K^+ (line 10).

(iv) 'Insensitive' to ADP (line 12).

(v) Again more slowly dephosphorylated in the presence of ATP and ADP (lines 11 and 12).

Effect of Pb^{2+} on the phosphorylation rate constant

In order to be able to measure the phosphorylation rate constant manually we lowered the temperature to 0 °C, since at room temperature phosphorylation already reaches a steady-state level after 3 s. Fig. 3 shows that in the presence of 5 mM Mg^{2+} a low steady-state phosphorylation level is reached after 60 s and the rate constant of phosphorylation is low (k 0.08 s^{-1}). Upon addition of 3 μM Pb^{2+}, both the phosphorylation rate constant (k 0.30 s^{-1}) and the phosphorylation level are increased. The rate constant of phosphorylation by ATP is not affected by lowering the Mg^{2+} concentration to 0.1 mM, either in the absence of Pb^{2+} or in its presence (the k values remain 0.09 s^{-1} and 0.29 s^{-1}, respectively). Increasing the Pb^{2+} concentration in the phosphorylation medium increases the ATP-dependent phosphorylation rate constant at 0 °C up to a maximum of 0.45 s^{-1} (Fig. 4C). The K_m value for this Pb^{2+} effect is 3 μM.

In the presence of 50 mM Tris and 0.1 mM Mg^{2+} (absence of Pb^{2+}) no ATP-dependent phosphorylation occurs. This is due to the inhibitory effect of Tris on the phosphorylation rate constant [2]. In the presence of Mg^{2+} and Pb^{2+}, however, a high steady-state phosphorylation level is also obtained when Tris is used as buffer. Thus, Tris apparently does not decrease the rate constant of phosphorylation, leading to the K^+-insensitive phosphointermediate (E-P · Pb).

Discussion

In the mechanistic studies on $(Na^+ + K^+)$-ATPase, the Albers-Post scheme [13,14] is the central framework for our understanding of the process. In Fig. 4 the basic principle of this scheme is given in reactions 1–5. The phosphorylation studies with Na^+ and imidazole fit well in this scheme. After phosphorylation of $(Na^+ + K^+)$-ATPase by ATP a phosphointermediate can be isolated when the rate constant of the E_2–E_1 transition (reaction 1) and that of the phosphorylation itself (reaction 3) are large as compared to the dephosphorylation rate constant (reaction 5).

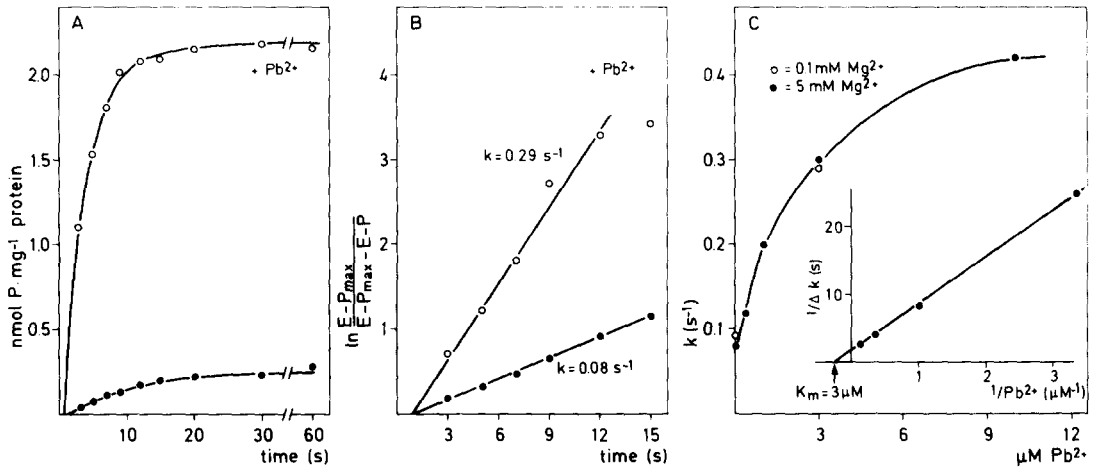

Fig. 3. Effect of Pb^{2+} on the phosphorylation rate constant of $(Na^+ + K^+)$-ATPase at 0 °C. The medium contained 5 μM [γ-^{32}P]ATP, 50 mM imidazole-acetate (pH 7.0), 5 mM Mg^{2+} (except open circles in part C), 0.1 mg/ml enzyme and the Pb^{2+} concentrations as indicated. (A) Time course of the level of phosphointermediate with (○) and without (●) 3 μM Pb^{2+}. (B) Logarithmic conversion of the data from part A (determination of pseudo first-order rate constant). (C) Rate constant of phosphorylation as a function of Pb^{2+} concentration in the presence of 5 mM Mg^{2+} (●) and 0.1 mM Mg^{2+} (○), respectively. inset: double reciprocal plot of the same results.

In the presence of Na^+ and absence of K^+, the rate constants of the E_2–E_1 transition and of phosphorylation are very high [15,16], whereas that of dephosphorylation is small. Moreover, Na^+ lowers the affinity of the enzyme for K^+ [2], so that the residual K^+ present in the enzyme preparation has no measurable effect on the dephosphorylation.

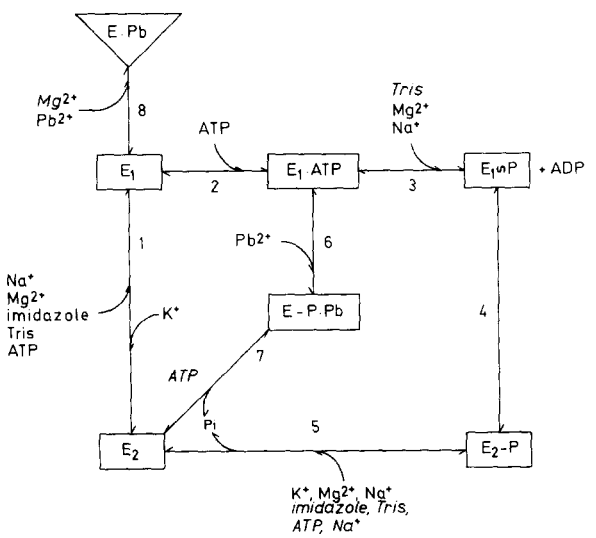

Fig. 4. Reaction scheme for $(Na^+ + K^+)$-ATPase including the effect of Pb^{2+}. Stimulatory (normal letters) and inhibitory (italics) ligands are given for each reaction step.

Imidazole, a promotor of the E_1 conformation [1], increases the steady-state phosphorylation level in the absence of Na^+ by decreasing the dephosphorylation rate constant via a reduction of the K^+-sensitivity of the phosphoenzyme [2]. Tris, which is also a promotor of E_1 [17], inhibits both the phosphorylation and dephosphorylation rate constant, so no phosphointermediate can be measured [2] except when the pH is lowered to below 7.0 [18]. With 5 mM Mg^{2+}, in the presence of imidazole, a low phosphoenzyme level is found due to the stimulatory effect of Mg^{2+} on the dephosphorylation rate [2].

In the presence of Pb^{2+}, a phosphointermediate is formed which does not fit well in the Albers-Post scheme. The phosphoenzyme is different from either $E_1 \sim P$ (ADP-sensitive) or E_2-P (K^+-sensitive) so that it cannot be one of either intermediate. We propose that it is a different type of intermediate, as indicated by the side path in the Albers-Post scheme, see Fig. 4. Pb^{2+} does not, like imidazole, enhance the steady-state phosphorylation level via an effect on the dephosphorylation, for Pb^{2+} has no effect on the phosphoenzyme generated with imidazole (E-P · Mg) and does not

change its K^+-sensitivity. But Pb^{2+} increases the rate of formation (reaction 6) of a phosphointermediate (E-P · Pb) which is insensitive towards K^+, Mg^{2+}, Na^+ and ADP (reaction 7). The dephosphorylation rate constant is, therefore, reduced simultaneously. The net result is an increase in the steady state phosphorylation as already reported by Siegel and Fogt [7]. Upon increasing the Na^+ concentration during phosphorylation in the presence of Pb^{2+}, the K^+-sensitivity is restored, indicating that the normal phosphointermediates [14] are formed again, via an enhancement of the rate of reaction 3. These results indicate that despite the presence of imidazole, no significant amount of E-P · Mg is formed when Pb^{2+} is also present. Thus, the rate constant of reaction 6 must be much higher than that of reaction 3, at least in the presence of Pb^{2+}. Direct measurements of the rate of phosphorylation (Fig. 3) suggest this to be the case.

In addition to the stimulatory effect of Pb^{2+}, an inhibitory effect can also be found with a lower intrinsic K_i value than the K_m for the stimulatory effect. Since the inhibitory effect is counteracted by Mg^{2+}, inhibition occurs at higher Pb^{2+} concentrations, the optimum shifts to the right at high Mg^{2+} concentrations. Mg^{2+} also seemingly antagonizes the stimulatory effect of Pb^{2+} (Fig. 1A). However, this is only due to the hydrolysis of E-P · Mg, being predominantly formed, at these high Mg^{2+} concentrations. This is an argument for the existence of two different Pb^{2+} binding sites. In the presence of Na^+, only an inhibitory effect is observed which is also counteracted by Mg^{2+}. Since Siegel and Fogt [7] used a high Mg^{2+} (5 mM) concentration during steady-state phosphorylation, only a small reduction by Pb^{2+} was observed in their experiments. Moreover, they used a much higher ATP concentration (1 mM) which complexes Pb^{2+}.

Siegel and co-workers [19] suggested that binding of Na^+ is necessary to make the phosphoenzyme sensitive towards K^+. They studied the effects of Na^+ and K^+ on the steady-state phosphorylation level in the presence of Pb^{2+} and found only a reduction of the phospho-level, when both cations were present. Their suggestion is very unlikely, since: (i) binding of Na^+ has no effect on the dephosphorylation rate constant of E-P · Pb and K^+ sensitivity is not restored; (ii) phosphorylation in the absence of Na^+ and Pb^{2+} already yields a K^+-sensitive intermediate [2]. We propose that these findings are due to the fact that during phosphorylation in the presence of Pb^{2+}, Na^+ restores K^+-sensitivity by increasing reaction 3 (Fig. 4).

The Mg^{2+}–K^+ antagonism in the steady-state phosphorylation as observed by Siegel et al. [19] is not an effect on the K^+ sensitivity of the phosphoenzyme. In our experiments (Fig. 2), the K^+ insensitivity of the phosphointermediate generated with different Mg^{2+} concentrations in the presence of Pb^{2+} does not change. Hence, for the Mg^{2+}–K^+ antagonism observed by them, another partial reaction of the steady-state phosphorylation must be involved. This could be the E_2–E_1 transition, since eosine binding experiments show such an antagonistic effect (unpublished observations).

In the presence of nucleotides, the dephosphorylation rate constant of both E-P · Mg and E-P · Pb is reduced. The effect of ATP is not simply the complexing of Mg^{2+} or Pb^{2+} since the dephosphorylation rate constant is only slightly reduced by EDTA. The same effect of ATP has been reported by Askari and Huang [20] on a phosphoenzyme generated from P_i and in dephosphorylation studies with $(K^+ + H^+)$-ATPase [21]. The interaction of nucleotides with the phosphointermediate correlates with the low affinity binding site for ATP in the overall reaction [20,22].

Mg^{2+} not only diminishes the ATP effect on E-P · Mg, as reported earlier for $(Na^+ + K^+)$-ATPase [22] as well as for $(K^+ + H^+)$-ATPase [21], but also stimulates the dephosphorylation itself. Also in the absence of ATP E-P · Mg is sensitive to Mg^{2+}. This ion has no effect on the E-P · Pb intermediate, even the ATP effect is not reduced. These effects indicate that during phosphorylation with Pb^{2+} a conformation of the phosphointermediate is obtained to which ATP normally can bind, but which is not reactive to cations.

The fact that E-P · Pb is totally insensitive towards K^+ and Mg^{2+} enables us to carry out experiments on the E_1–E_2 transition by means of Pb^{2+}-activated phosphorylation. Since E_1 can lead to a phosphorylated intermediate, whereas E_2 does

not, the amount of phosphorylated intermediate can be used as a measure for the E_1/E_2 ratio [23,24]. Pb^{2+} phosphorylation has the advantage above Mg^{2+} or Na^+ phosphorylation, since the rate of dephosphorylation, and so steady-state phosphorylation, is not affected by the ligands tested. Studies with this method are in progress.

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