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Sodium and buffer cations inhibit dephosphorylation of $(Na^+ + K^+)$-ATPase *

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(1) Effects of various cations on the dephosphorylation of $(Na^+ + K^+)$-ATPase, phosphorylated by ATP in 50 mM imidazole buffer (pH 7.0) at 22°C without added Na^+, have been studied. (2) The dephosphorylation in imidazole buffer without added K^+ is extremely sensitive to K^+-activation (K_m $K^+ = 1$ μM), less sensitive to Mg^{2+}-activation (K_m $Mg^{2+} = 0.1$ mM) and Na^+-activation (K_m $Na^+ = 63$ mM). (3) Imidazole and Na^+ effectively inhibit K^+-activated dephosphorylation in linear competitive fashion (K_i imidazole 7.5 mM, K_i Na^+ 4.6 mM). The K_i for Na^+ is independent of the imidazole concentration, indicating different and non-interacting inhibitory sites for Na^+ and imidazole. (4) Imidazole inhibits Mg^{2+}-activated dephosphorylation just as effectively as K^+-activated dephosphorylation, as judged from the K_i values for imidazole in the two processes. (5) Tris buffer and choline chloride, like imidazole, inhibit dephosphorylation in the presence of residual K^+ (< 1 μM), but less effectively in terms of I_{50} values and extent of inhibition. Tris inhibits to the same extent as choline. This indicates different inhibitory sites for Tris or choline and for imidazole. (6) These findings indicate that high steady-state phosphorylation levels in Na^+-free imidazole buffer are due to the induction of a phosphorylating enzyme conformation and to the inhibition of $(K^+ + Mg^{2+})$-stimulated dephosphorylation.

Introduction

Recently [1] we have detected that the buffer cation imidazole-H^+ permits $(Na^+ + K^+)$-ATPase to be phosphorylated in the absence of Na^+ at low Mg^{2+} concentration (0.1 mM). This has allowed us for the first time to study the effect of Na^+ on the dephosphorylation step without disturbance by its strong activating action on the phosphorylation step. We have also been able to determine the effects of buffer cations on the dephosphorylation reaction in the absence of Na^+.

* This article is No. 59 in the series 'Studies on $(Na^+ + K^+)$-activated ATPase'.

Materials and Methods

Isolation of a purified preparation of $(Na^+ + K^+)$-ATPase from rabbit kidney outer medulla, removal of contaminating ATP, washing and storing of the preparation (in 10 mM imidazole-acetate (pH 7.0), 0.25 M sucrose and no CDTA) and determination of protein are described in a previous paper [1].

Phosphorylation by [γ-^{32}P]ATP (5 μM) at 22°C is carried out in 50 mM imidazole-acetate (pH 7.0) containing 0.1 mM Mg^{2+}, 0.1 mg/ml enzyme protein and no added Na^+ in a total volume of 0.1 ml.

Dephosphorylation is assayed by one of two tracer dilution methods.

Method 1: after 10 s phosphorylation as described above, we add 0.5 ml 2–50 mM imidazole-acetate (pH 7.0), containing 0.6 mM non-radioactive ATP and ligands (K^+, Mg^{2+}) in final concentrations as specified in the Results section. Dephosphorylation is stopped after 3 s by addition of 4 ml trichloroacetic acid (5% w/v)/H_3PO_4 (0.1 M).

Method 2: after 10 s phosphorylation we add 0.1 ml 50 mM imidazole-acetate (pH 7.0), containing 1 mM non-radioactive ATP; 10 s later we add 0.5 ml 8 or 50 mM imidazole-acetate (pH 7.0), containing 0.5 mM non-radioactive ATP and ligands (K^+, Na^+, final concentrations specified under Results). Dephosphorylation is stopped after 3 s by addition of trichloroacetic acid/H_3PO_4. The non-radioactive ATP (imidazole salt, final concentration 0.5 mM) contributes 2.7 mM to the final imidazole concentration.

Method 2 thus contain a 10-s dephosphorylation interval, which allows complete equilibration of residual enzyme bound [γ-^{32}P]ATP with non-radioactive ATP in solution. We apply this method when Na^+ is present during dephosphorylation, since in its presence phosphorylation by enzyme bound radioactive [γ-^{32}P]ATP would overrule the phosphoenzyme decay (see Results). In the absence of Na^+, methods 1 and 2 give similar results, and thus the more convenient method 1 has been applied.

Dephosphorylation is expressed as the decrease in acid-stable ^{32}P-enzyme level during the 3-s chase with non-radioactive ATP relative to the level at the start of this chase (10 s after start of phosphorylation in method 1, 20 s in method 2). Percentage hydrolysis is plotted as a function of the concentration of the activator or inhibitor. The $K_{0.5}$ of K_m value for the activator is defined as the concentration given a half-maximal increase in 3-s dephosphorylation in the presence or absence of inhibitor, respectively. I_{50} is defined as the inhibitor concentration giving half-maximal inhibition of dephosphorylation at a given activator concentration and K_i as the I_{50} value, extrapolated to zero activator concentration. The concentration of free Mg^{2+}, not complexed to ATP, is calculated by means of the equilibrium constants for the first ($K = 10^{6.25}$ M^{-1}) and second ($K = 10^{4.06}$ M^{-1}) protonation step of ATP^{4-} and for complexation of ATP^{4-} ($K = 10^{4.05}$ M^{-1}) and $ATPH^{3-}$ ($K = 10^{2.12}$ M^{-1}) with Mg^{2+}, reported by Sillén and Martell [2].

All further methods, such as determination of phosphoenzyme levels after acid denaturation, including determination of blanks [3], conversion of ATP to its imidazole salt [4] and determination of residual K^+ concentrations [1] have been published in earlier papers from our laboratory. Recrystallized K^+-free imidazole is used [1].

Results

Strategy of the dephosphorylation method

Dephosphorylation, after phosphorylation in 50 mM imidazole-acetate (pH 7.0) and 0.1 mM Mg^{2+}, is assayed according to method 1 or 2 (see Methods), illustrated in Fig. 1. Each method is based on a more than 600-fold tracer dilution by the addition of excess non-radioactive ATP and a 6- to 7-fold dilution of the Mg^{2+} concentration to a value of 3 μM for free Mg^{2+}. This free Mg^{2+} level does not affect the dephosphorylation rate (see section on Mg^{2+}-stimulated dephosphorylation). In method 1 the nonradioactive ATP and the dephosphorylation-effecting ligand are added simultaneously; in method 2 a 10-s interval is allowed for equilibration of residual enzyme-bound [γ-^{32}P]ATP with non-radioactive ATP in solution. When the ligand does not stimulate phosphorylation by residually bound [γ-^{32}P]ATP, we observe comparable dephosphorylation rates in methods 1 and 2 (Fig. 1).

On the other hand, when the ligand stimulates phosphorylation by residually bound [γ-^{32}P]ATP, as in the case of Na^+, method 1 leads to a transient increase in phosphorylation level above the steady-state phosphorylation before addition of the non-radioactive ATP (Fig. 2, upper line). This increase changes to a decrease above 10 mM Na^+, where stimulation of dephosphorylation starts to overrule stimulation of phosphorylation. Above 300 mM Na^+ dephosphorylation is inhibited, possibly by reversal of the $E_1P \rightharpoonup E_2P$ transition [5], E_1P being insensitive to Na^+-stimulated hydrolysis.

When a 10-s ATP-[γ-^{32}P]ATP exchange interval is introduced in method 2, the phosphorylation overshoot is abolished (Fig. 2, lower line). Na^+

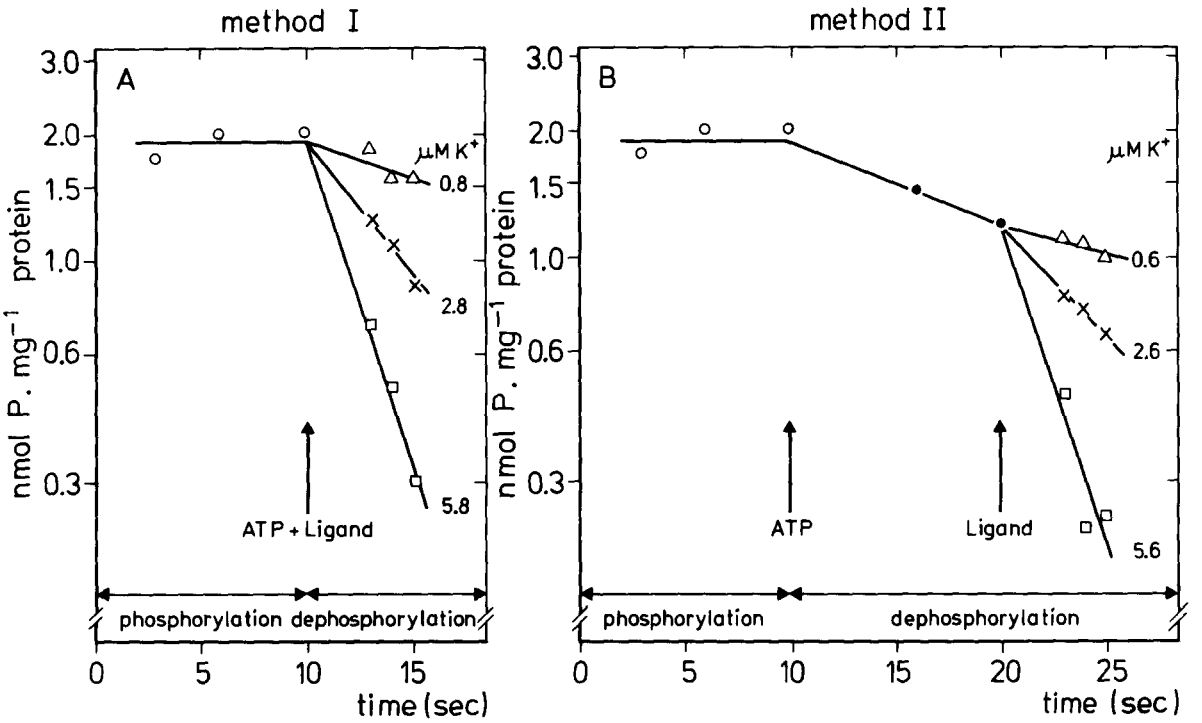


Fig. 1. Time-course of phosphorylation and dephosphorylation. Phosphorylation proceeds in 50 mM imidazole-acetate (pH 7.0) in the presence of 5 μM [γ-^{32}P]ATP and 0.1 mM Mg^{2+}, dephosphorylation is assayed by method 1 or 2. Method 1 (A): 5 vol. of 50 mM imidazole-acetate (pH 7.0), containing non-radioactive ATP (imidazole-salt, final concentration 0.5 mM) and K^+ (in the final concentrations indicated) are added at 10 s after the start of phosphorylation. Method 2 (B): same as (A), but K^+ added 10 s later than the other additions. The slightly lower dephosphorylation rate in the presence of 0.6 μM residual K^+ is due to its dilution upon addition of K^+-free buffer.

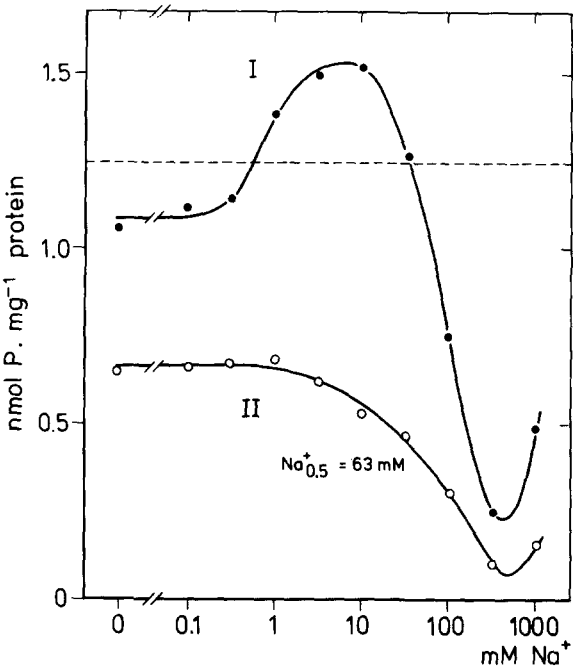
above 1 mM starts to enhance dephosphorylation with a K_m of 63 mM. At much higher Na^+ levels (above 300 mM) there is again inhibition of dephosphorylation.

In order to avoid the phosphorylation overshoot in the presence of Na^+ during dephosphorylation, we have applied method 2 in all experiments with Na^+. Method 1 is applied in all other experiments.

Na^+-inhibition of K^+-stimulated dephosphorylation

In the absence of Na^+ very low concentrations of K^+ stimulate dephosphorylation. Half-maximal stimulation in 50 mM imidazole-acetate (pH 7.0) occurs at 5.8 μM K^+ (Fig. 3A). This K^+ concentrations is 15-times lower than when phosphorylation has taken place in the presence of 100 mM Na^+ [6], indicating that Na^+ inhibits K^+-stimulated dephosphorylation. This inhibitory effect of Na^+ on K^+-stimulated dephosphorylation is evident from the shift to the right of the dose-response curve of percent dephosphorylation versus K^+ concentration upon addition of Na^+ together with K^+ (Fig. 3A). Up to 10 mM Na^+ is used, since this concentration gives only moderate enhancement of dephosphorylation by itself (about 20%, cf. Figs. 2 and 3A). Fig. 3B shows the K_m values for K^+ as a function of the Na^+ concentration at two different buffer concentrations. It demonstrates that the K_i value for Na^+ (4.4 mM in 53 mM imidazole), 4.8 mM in 23 mM imidazole) is virtually independent of the buffer concentration. This is different from the steady-state phosphorylation, in which buffer and Na^+ appear to cooperate (decreasing K_m value for Na^+ at increasing buffer concentration, Ref. 1).

Imidazole buffer also inhibits the K^+ effect, giving a 2.3-fold increase of $K^+_{0.5}$ upon a 2.3-fold increase of the Na^+-free imidazole concentration (Fig. 3B). Independence of the K_i value for Na^+



from the imidazole concentration indicates that these two inhibitors do not compete with each other and bind at separate non-interacting sites.

Imidazole-inhibition of K^+-stimulated dephosphorylation

Inhibition of the K^+-stimulated dephosphorylation by imidazole is shown by a shift of the dose-response curve to the right and by a more than 3-fold increase in the K_m for K^+ at increasing imidazole concentration from 13 to 53 mM

Fig. 2. Effect of Na^+ on the phosphoenzyme level during dephosphorylation. Phosphorylation and dephosphorylation occur according to method 1 (upper curve) or method 2 (lower curve) as described in Fig. 1, but with Na^+ added instead of K^+. Phosphoenzyme levels 3 s after addition of Na^+ are presented. The steady-state phosphorylation level before addition of non-radioactive ATP is given by the dashed line. Method 2 reveals a K_m value for Na^+ of 63 mM in the activation of dephosphorylation.

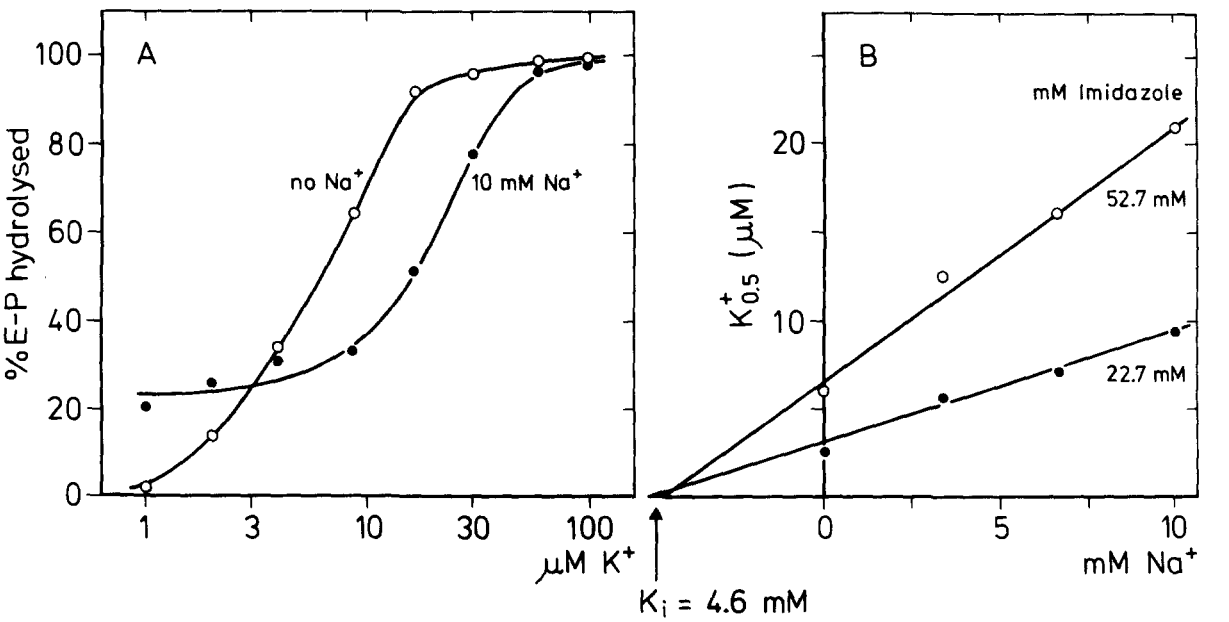


Fig. 3. Inhibition of K^+-activated dephosphorylation by Na^+. (A) Phosphorylation and dephosphorylation (method 2) as in Fig. 1B, but with Na^+ (0–10 mM) and K^+ (1–100 μM) present during dephosphorylation. Dephosphorylation for 3 s in the presence of 53 mM imidazole. (B) Dixon plot of $K_{0.5}$ for K^+ versus the Na^+ concentration at two (23 and 53 mM) imidazole concentrations (see Methods). Lower K^+ concentrations are applied at 23 mM imidazole than at 53 mM imidazole (1–16 μM vs. 1–100 μM) in view of the higher K^+-sensitivity at the lower imidazole concentration. $K^+_{0.5}$ is the K^+ concentration giving a half-maximal increase in percent EP hydrolysis (3 s) above that in the presence of residual K^+ (1 μM). The $K^+_{0.5}$ values in 23 and 53 mM imidazole (no Na^+) are 2.5 and 5.8 μM, respectively, and the K_i values for Na^+ (abscissa intercepts) 4.8 and 4.4 mM, respectively (average indicated).

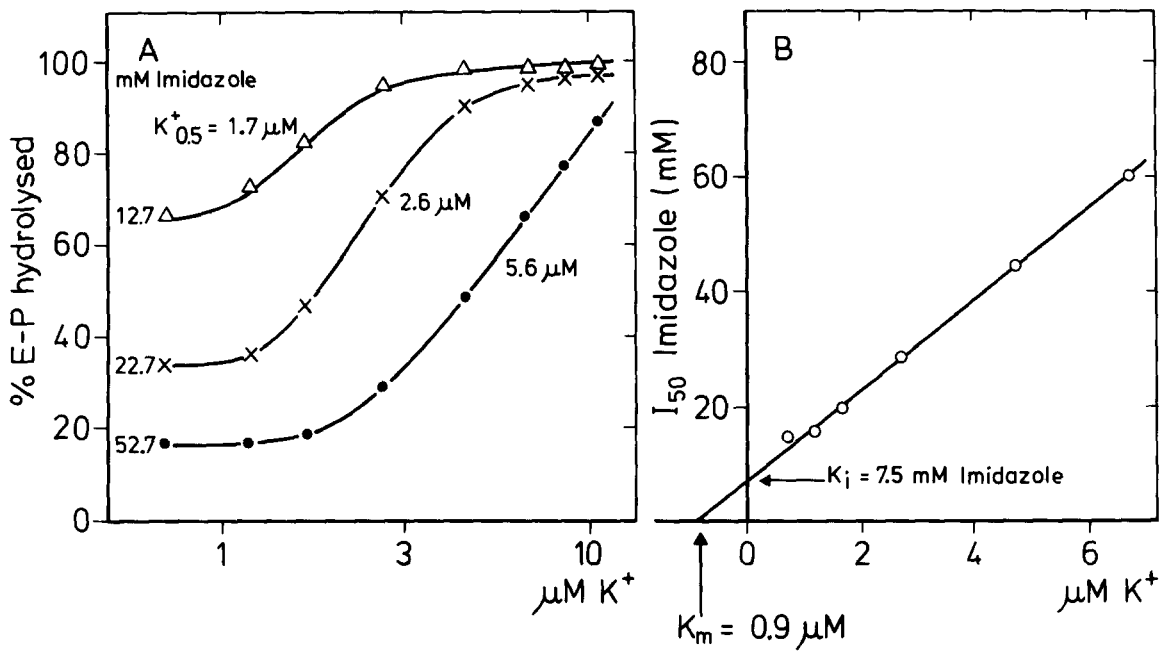


Fig. 4. Inhibition of K^+-activated dephosphorylation by imidazole. (A) Phosphorylation and dephosphorylation (method 1) as in Fig. 1A. Dephosphorylation for 3 s at 13–53 mM imidazole and 0.7–10.7 μM K^+. Imidazole concentrations below 53 mM are obtained by dilution. $K^+_{0.5}$ values are similar to those in Fig. 3B but without added Na^+. (B) Dixon plot of the I_{50} value for imidazole as a function of the K^+ concentration revealing a K_i value for imidazole of 7.5 mM and a K_m for K^+ of 0.9 μM. The I_{50} values are determined by Hill plot analysis of dephosphorylation as percent of the maximum as a function of five imidazole concentrations at several fixed K^+ concentrations. The minimum level at 53 mM imidazole, representing a back-ground level (cf. Fig. 6), has been deducted.

(Fig. 4A). The buffer cation appears to lack the K^+-like properties of Na^+, since only inhibition of dephosphorylation is evident upon increasing the buffer concentration. The half-maximally inhibiting concentration of imidazole increases in linear fashion with the K^+ concentration (Fig. 4B), revealing a K_i value for imidazole of 7.5 mM (3.4 mM for imidazole-H^+) and a K_m value for K^+ in the absence of imidazole of 0.9 μM. The latter value is strikingly similar to the minimal K_i value for K^+ in steady-state phosphorylation (0.7 μM, Ref. 1) and comes close to the K_d valules for K^+ binding (1.8–2.6 μM in the pH range of 6.7–7.8, Ref. 7).

Imidazole-inhibition of Mg^{2+}-stimulated dephosphorylation

Mg^{2+} concentrations above 0.1 mM stimulate dephosphorylation and this stimulation is also repressed by increasing imidazole concentrations (Fig. 5A). Plots of the K_m value for Mg^{2+} versus the imidazole concentration (Fig. 5B) and of I_{50} for imidazole versus the free Mg^{2+} concentration (Fig. 5C) show non-linear relations. These plots allow an estimation of the K_m and K_i values by extrapolation to zero imidazole and Mg^{2+} concentrations, respectively. This yields a K_m value for Mg^{2+} of 0.1 mM and a K_i value for imidazole of 11 mM. The latter value is comparable to the value of 14 mM in Fig. 4B at 0.7 μM residual K^+, considering that the data of the Mg^{2+} experiment are not corrected for contaminating K^+. Hence, we conclude that inhibition of Mg^{2+}- and K^+-stimulated dephosphorylation occurs via the same buffer cation binding site.

Inhibition of dephosphorylation by Tris buffer and choline

We have investigated whether inhibition of dephosphorylation is specific for Na^+ and imidazole-H^+ or that other buffers display the same behaviour. This has been done by comparing the

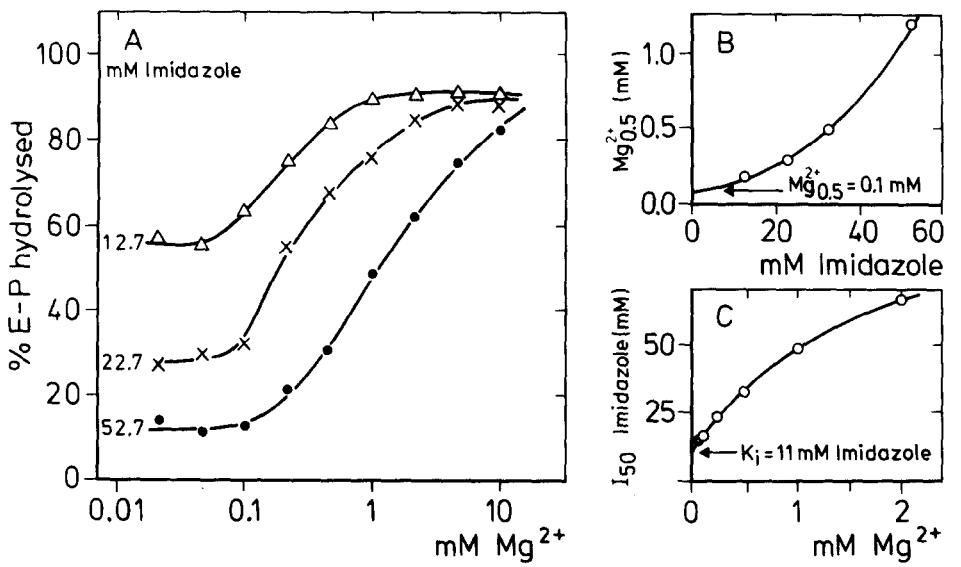


Fig. 5. Inhibition of Mg^{2+}-activated dephosphorylation by imidazole. (A) Phosphorylation and dephosphorylation (method 1) as in Fig. 1A, but with Mg^{2+} as the only ligand added. Dephosphorylation for 3 s at 13–53 mM imidazole and 0.02–10 mM free Mg^{2+}. Imidazole concentrations below 53 mM are obtained by dilution. $Mg^{2+}_{0.5}$ is defined as the concentration giving the half-maximal increase in percent dephosphorylation (3 s). (B) Dixon plot of $Mg^{2+}_{0.5}$ as a function of the imidazole concentration. A K_m value of 0.1 mM for free Mg^{2+} is estimated by extrapolation to zero imidazole concentration. (C) Dixon plot of I_{50} for imidazole as a function of the free Mg^{2+} concentration, revealing a K_i value for imidazole of 11 mM. The I_{50} values are determined as indicated in the legend of Fig. 4B, but at several fixed Mg^{2+} concentrations.

effect of Tris with that of imidazole, using choline chloride as ionic strength reference. Dephosphorylation is tested in 13 mM imidazole buffer at increasing ligand concentrations (Fig. 6). It is evident that imidazole inhibits dephosphorylation to a larger extent (90%) than Tris or choline chloride (65–70%). Half-maximal effects are obtained by addition of 4 mM imidazole (1.8 mM imidazole-H^+), 5 mM Tris (4.6 mM Tris-H^+) and 6 mM choline$^+$. Thus, although there is a slight (3.3-fold) difference in specificity as far as the half-maximally effective cation concentrations are concerned, the stronger inhibitory action of imidazole is mainly based on the extent to which rather than on the affinity by which it is exerted. The higher residual dephosphorylation rates in Tris and choline chloride than in imidazole are not caused by a simultaneous occurrence of a Tris- or choline-stimulated dephosphorylation. Addition of up to 150 mM Tris or choline chloride in 50 mM imidazole does not affect the imidazole inhibited dephosphorylation (not shown). This indicates that imidazole and Tris probably act at different sites.

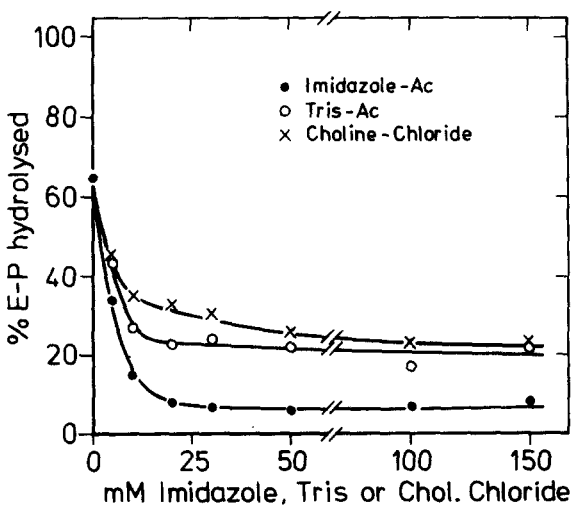


Fig. 6. Inhibition of dephosphorylation by imidazole, Tris and choline. Phosphorylation and dephosphorylation (method 1) as in Fig. 1A (no K^+ added), except that upon addition of non-radioactive ATP the imidazole concentration in the control is diluted to 12.7 mM. Dephosphorylation for 3 s at the indicated imidazole, Tris or choline concentrations, added on top of the 12.7 mM imidazole present in the control.

Discussion

Inhibition of dephosphorylation by Na^+ and amino buffers

By making use of our recent finding of the Na^+-replacing effect of imidazole buffer on the phosphorylation of $(Na^+ + K^+)$-ATPase, we have for the first time been able to study the effects of Na^+ and other cations (in the absence of Na^+) on the dephosphorylation reaction. The experiments reported here indicate that Na^+, amino-group containing buffers and choline$^+$ inhibit the K^+-stimulated dephosphorylation of $(Na^+ + K^+)$-ATPase. However, specificity differences seem to exist, so the inhibitory effects are not mainly due to an increase in ionic strength. In this light it is relevant to mention that choline$^+$ exerts a Na^+-like effect on the conformation of the enzyme [8,9], which is opposite to that of K^+. These effects have been assigned to intracellular sites of the membrane [8,10]. It is quite probable that the inhibitory effects of Na^+ and imidazole and the stimulation effect of K^+ on dephosphorylation involve conformational changes in the enzyme molecule. We consider it unlikely that these effects are exerted via intracellular sites for the following two reasons. First, in resealed red cell membranes dephosphorylation is stimulated by extracellular K^+ [11] and inhibited by low concentrations (5 mM) of extracellular Na^+ [12,13]. Secondly, imidazole enhances the affinity for Na^+ in phosphorylation [1], which takes place at intracellular sites [14], but it does not have an effect on the Na^+ affinity in the inhibition of K^+-stimulated hydrolysis. A definitive answer must await studies with the enzyme reconstituted into proteoliposomes.

The sites at which Na^+ inhibits dephosphorylation are probably different and independent from the buffer cation binding sites, since the K_i value for Na^+ is virtually independent of the imidazole concentration. Differences exist in the size of maximal inhibition between imidazole and Tris (80–100% by imidazole vs. 70% by Tris). The Dixon plot of I_{50} vs. the K^+ concentration reveals linear competitive inhibition rather than partial (hyperbolic) competitive inhibition. This suggests that activation by K^+ may be abolished completely at sufficiently high imidazole concentrations.

The variability in the maximal inhibition by imidazole may be due to a variable coupling of the hydrolysis to K^+-activation, with K^+-independent (spontaneous) hydrolysis being insensitive to the buffer. The lower maximal inhibitions exerted by Tris and choline may be due to their being partial competitive inhibitors or to their inability to complete the conformational change leading to inhibition within the 3-s dephosphorylation period. It has been proposed that Tris blocks the conformational transition to the K^+-sensitive phosphoenzyme, in addition to inhibiting its dephosphorylation [15]. It it unlikely that imidazole has such a blocking effect, since the phosphoenzyme remains K^+-sensitive (K_m app. $K^+ = 5.6$ μM) in the presence of high concentrations (53 mM = 7 K_i) of imidazole. The fact that Tris does not enhance the maximal inhibitory effect of imidazole argues against a different mechanism in the inhibition exerted by Tris. It may mean that Tris, like choline, cannot induce a further conformational change of the kind that has already come to its full effect in imidazole.

Imidazole also inhibits Mg^{2+}-activated dephosphorylation via a site of similar affinity for the buffer as determined from experiments with K^+ ($K_i = 11$ vs. 14 mM). This means that the same binding site for the buffer cation is involved. Dixon plots of K_m Mg^{2+} versus imidazole concentration and I_{50} versus Mg^{2+} concentration reveal that Mg^{2+} stimulates progressively less at higher imidazole concentrations. Similar plots for K^+ instead of Mg^{2+} are linear. The question whether this discrepancy is due to an interplay of Mg^{2+} with the residual K^+ present in our experiments must await studies with a series of K^+ additions.

These data lead to the conclusion that Na^+, imidazole and Tris inhibit dephosphorylation at different non-interacting sites but via a similar mechanism, viz. by increasing the K_m values for K^+. The Tris site is probably shared by the choline cation.

Impact of the inhibition on steady-state phosphorylation

It is clear that inhibition of dephosphorylation will lead to high steady-state phosphorylation levels, provided that the enzyme is in the phosphorylating E_1 conformation. So it is not surpris-

ing to find that imidazole, which induces such a conformation, induces high steady-state phosphorylation levels [1]. Tris, on the other hand, lowers the steady-state phosphorylation level obtained in imidazole medium, while it also inhibits dephosphorylation and promotes the $E_2 \rightarrow E_1$ transition [1]. Hence, Tris must inhibit the phosphorylation step. Na^+ exerts an action similar to that of imidazole, but in addition activates dephosphorylation in concentrations above 1 mM. Yet high steady-state phosphorylation levels in Na^+ are reached, because the phosphorylation rate is higher than the dephosphorylation rate. This is also evident from the overshoot in the phosphorylation level during dephosphorylation in the presence of Na^+, when using assay method 1. Imidazole lacks this overshoot effect, thus yields a lower phosphorylation rate than Na^+.

On the other hand, ligands which stimulate dephosphorylation will match this effect in steady-state phosphorylation. For example, the K_i value for K^+ in steady-state phosphorylation (0.7 μM), is strikingly similar to the K_m value for K^+ in dephosphorylation (0.9 μM). However, we cannot exclude an inhibitory effect of K^+ on the $E_2 \rightarrow E_1$ transition, since both dephosphorylation and binding of K^+ to the dephosphorylated enzyme lead to the same occluded E_2K complex [16,17]. The high K^+-sensitivity of dephosphorylation is also reflected in the relatively high ATPase activity (50% of the maximal Na^+-activated ATPase) in 50 mM imidazole buffer in the absence of added Na^+ but with 6 μM residual K^+ [18] present. Na^+ (0.5–5 mM) inhibits this ATPase activity like it does K^+-stimulated dephosphorylation.

Mg^{2+} also stimulates dephosphorylation, but its K_m value (0.1 mM) is one-third of the K_i value (0.3 mM) in imidazole-activated steady-state phosphorylation [1]. In addition the shapes of the plots of K_m for imidazole in steady-state phosphorylation and of I_{50} for imidazole in dephosphorylation as a function of the Mg^{2+} concentration are almost symmetric: upward concave in steady-state phosphorylation (Mg^{2+} being more effective at higher concentrations, Ref. 1), upward convex in dephosphorylation (Mg^{2+} being less effective at higher concentrations). It may be a reflection of an equally symmetry action of a ligand such as Na^+, which activates phosphorylation at the inner membrane sites [14] and inhibits at the outer membrane sites [12,13]. Nevertheless, it is safe to conclude that at least part of the Mg^{2+} inhibition of steady-state phosphorylation must be due to activation of dephosphorylation by Mg^{2+}.

In conclusion, a high steady-state phosphorylation level could be the resultant of (a) stimulation of the $E_2 \rightarrow E_1$ transition, (b) absence of inhibition of the phosphorylation step, (c) inhibition of the dephosphorylation step. Na^+ and imidazole-H^+ exercise all three effects. Na^+ is more active than imidazole-H^+ as cofactor in phosphorylation but equally active in effect (c). Tris exercises effects (a) and (c), but not effect (b).

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