Biochimica et Biophysica Acta 815 (1985) 16–24
Elsevier

BBA 72553

Na^+-like effect of imidazole on the phosphorylation of ($Na^+ + K^+$)-ATPase *

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(Received November 16th, 1984)

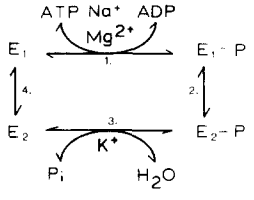
Key words: ($Na^+ + K^+$)-ATPase; Protein phosphorylation; Imidazole; (Rabbit kidney)

(1) A high basal level of phosphorylation (approx. 70% of the optimal Na^+-dependent phosphorylation level) is observed in 50 mM imidazole-HCl (pH 7.0), in the absence of added Na^+ and K^+ and the presence of 10–100 μM Mg^{2+}. In 50 mM Tris-HCl (pH 7.0) the basal level is only 5%, irrespective of the Mg^{2+} concentration. (2) Nevertheless, imidazole is a less effective activator of phosphorylation than Na^+ (K_m imidazole-H^+ 5.9 mM, K_m Na^+ 2 mM under comparable conditions). (3) Imidazole-activated phosphorylation is strongly pH dependent, being optimal at pH $\leqslant 7$ and minimal at pH $\geqslant 8$, while Na^+-activated phosphorylation is optimal at pH 7.4. This suggests that imidazole-H^+ is the activating species. (4) Imidazole facilitates Na^+-stimulated phoshorylation. The K_m for Na^+ decreases from 0.63 mM at 5 mM imidazole-HCl to 0.21 mM at 50 mM imidazole-HCl (pH 7; 0.1 mM Mg^{2+} in all cases). (5) Imidazole-activated phosphorylation is more sensitive to inhibition by K^+ (I_{50} = 12.5 μM) than Na^+-activated phosphorylation (I_{50} = 180 μM). (6) Mg^{2+} antagonizes activation by imidazole-H^+ and also inhibition by K^+. The K_i value for Mg^{2+} (approx. 0.3 mM) is the same for the two antagonistic effects. (7) Tris buffer (pH 7.0) inhibits imidazole-activated phosphorylation with an I_{50} value of 30 mM in 50 mM imidazole-HCl (pH 7.0) plus 0.1 mM Mg^{2+}. (8) We conclude that imidazole-H^+, but not Tris-H^+, can replace Na^+ as an activator of ATP-dependent phosphorylation, primarily by shifting the $E_2 \rightarrow E_1$ transition to the right, leading to a phosphorylating E_1 conformation which is different from that in Tris buffer.

Introduction

In the reaction mechanism of ($Na^+ + K^+$)-ATPase a ($Na^+ + Mg^{2+}$)-dependent phosphorylation by ATP is followed by a K^+-stimulated dephosphorylation of the phosphoenzyme (see for example, Ref. 1). The former step requires that the enzyme is in a particular conformation, designated E_1, which has a high affinity for the ligands participating in phosphorylation. The latter step requires that the enzyme is in a conformation E_2, which has a high affinity for the ligands participating in dephosphorylation. A conformational change of $E_1 \sim P$ to E_2-P(step 2) links steps 1 and 3, while a reverse conformational change ($E_2 \rightarrow E_1$, step 4) links steps 3 and 1 (Scheme I).

The requirement for Mg^{2+} is common to numerous phosphorylation and phosphatase reactions, suggesting that the actual substrate is a



* This article is No. 56 in the series 'Studies on ($Na^+ + K^+$)-activated ATPase'.

Mg-substrate complex. The other activator cations are thought to induce conformational changes in the enzyme, e.g. Na^+ induces a conformation E_1Na and K^+ a conformation E_2K, which can be converted into each other by addition of the opposite cation [2,3]. This made us wonder whether ionic compounds other than the alkali cation series can substitute for Na^+ or K^+ in inducing the E_1 or E_2 conformations. Striking examples have already been noticed several years ago: tetraphenyl boron and *m*-chlorocarbonylcyanide phenylhydrazone, uncouplers of oxidative phosphorylation and conductors of negative electric charge through phospholipid bilayers induce the E_1-conformation, while phlorizin retains the E_2-conformation of $(Na^+ + K^+)$-ATPase [4]. In addition, several solvent and buffer effects have been described on the induction or retention of either conformation [3,5–13], but none of these substances have been reported to be able to replace Na^+ in the phosphorylation reaction or K^+ in the dephosphorylation reaction.

Recently, we have detected that imidazole, which has been used as a buffer in most of our experiments, supports ATP-dependent phosphorylation to a high level in Na^+-free media [14]. Further studies of this effect of imidazole are reported in this paper.

Materials and Methods

Enzyme preparation

$(Na^+ + K^+)$-ATPase is purified from rabbit kidney outer medulla as described by Jørgensen [15], followed by removal of contaminating ATP, washing and storing of the preparation according to the procedure of Schoot et al. [16]. Protein is determined by the Lowry method, following trichloroacetic acid precipitation and using bovine serum albumin as standard [15].

Phosphorylation

Phosphorylation by ATP at 22°C in the presence or absence of Na^+ in 50 mM imidazole-HCl or 50 mM Tris-HCl medium (pH 7.0) containing 0.2 mM EDTA and uncomplexed Mg^{2+} concentrations, as specified under Results, is carried out as previously described by us [17]. ATP is converted to its imidazolyl- or Tris-salt by passage over a Dowex 50W-X4 exchange resin in the corresponding cation form [18]. Residual Na^+ and K^+ concentrations are determined with an Eppendorf flame photometer in the stock solutions and then calculated for the final dilutions used. In a typical assay medium the contributions of Na^+ and K^+ given by various components are for the enzyme protein (0.25 mg/ml) 2 and 2.4 μM, respectively, $MgCl_2$ (5 mM) 1.2 and 0.4 μM, imidazole-HCl (50 mM) 4.2 and 0.4 μM and Tris-HCl (50 mM) 0.1 and 0.05 μM, thus adding up to a total of 7.4 μM Na^+ and 3.2 μM K^+ in the imidazole medium and 3.3 μM Na^+ and 2.9 μM K^+ in Tris-medium. In some experiments ether-recrystallized imidazole has been used, which is virtually Na^+ and K^+ free, giving 0.14 μM Na^+ and 0.01 μM K^+ in 50 mM imidazole buffer.

Total $MgCl_2$ to be added in order to obtain the desired free Mg^{2+} concentration is calculated by means of the equilibrium constants reported by Sillén and Martell [19] for the first and second protonation step of $EDTA^{4-}$ and ATP^{4-} and for complexation of $EDTA^{4-}$, $EDTAH^{3-}$, ATP^{4-} and $ATPH^{3-}$ with Mg^{2+}.

Results

Mg^{2+}-Na^+ antagonism in phosphorylation by ATP

The effect of Mg^{2+} on phosphorylation of the enzyme by ATP in imidazole buffer has been determined. For the overall $(Na^+ + K^+)$-ATPase process [20] and binding of Na^+ to the enzyme [21] Mg^{2+} in millimolar concentrations is known to counteract the affinity for Na^+. We now find that Mg^{2+} has a similar effect on the phosphorylation by ATP in imidazole buffer (Fig. 1A). The half-maximally activating Na^+ concentration increases from 0.23 mM at 0.1 mM Mg^{2+} to 1.5 mM at 10 mM Mg^{2+} with a K_i value for Mg^{2+} of 2 mM (Fig. 1B). The K_m value for Na^+ extrapolated to $[Mg^{2+}] = 0$ is 0.24 mM, close to the value of 0.2 mM (25°C, pH 7.5) reported by Flashner and Robinson [22] at 50 μM Mg^{2+}, to the K_d for Na^+ binding (0.2 mM, 0°C, pH 7.5) in the absence of Mg^{2+} [21] and also to the K_m for Na^+ (0.26 mM, 37°C, pH 7.8) for $(Na^+ + K^+)$-ATPase activity extrapolated to $[K^+] = 0$ and $[MgATP] = 0$ [23]. This indicates that Mg^{2+} competes with Na^+ for the Na^+ binding site or affects the enzyme conformation such that Na^+ binding is decreased.

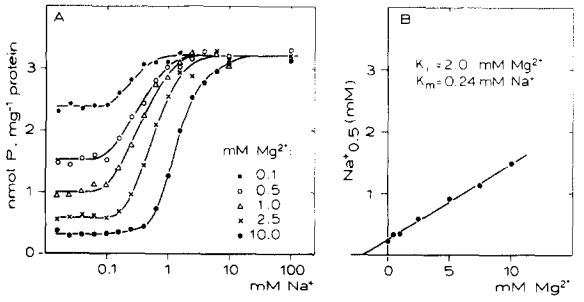


Fig. 1. Phosphorylation by ATP in imidazole-HCl as a function of the Na^+ and Mg^{2+} concentrations. (A) Levels of phosphorylation after 3 s incubation in 50 mM imidazole-HCl (pH 7.0) at 22°C in the presence of 20 μM ATP and the indicated Na^+ and Mg^{2+} concentrations: 0.13 mg/ml enzyme protein. At Na^+ concentrations below 0.1 mM the basal phosphorylation level is raised increasingly at decreasing Mg^{2+} concentration. (B) Dixon plot of half-maximally stimulating Na^+ concentrations as a function of the Mg^{2+} concentration. Na^+-stimulation is determined by subtracting the basal phosphorylation levels. The graph shows a K_i value of 2 mM for Mg^{2+} as abscissa intercept and a K_m value for Na^+ of 0.24 mM as ordinate intercept.

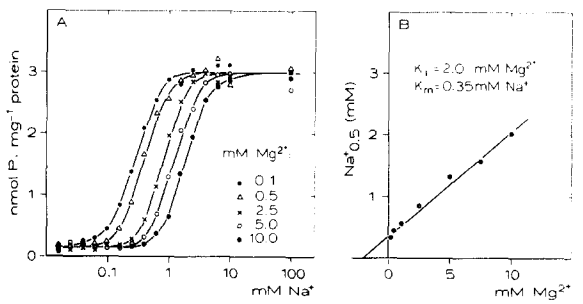


Fig. 2. Phosphorylation by ATP in Tris-HCl as a function of the Na^+ and Mg^{2+} concentrations. (A) Phosphorylation levels after 3 s incubation in 50 mM Tris-HCl (pH 7.0) at 22°C. Conditions are essentially the same as in Fig. 1A. Noticeable is the near-absence of basal phosphorylation in Tris at all Mg^{2+} levels. (B) Dixon plot of half-maximally stimulating Na^+ concentrations as a function of the Mg^{2+} concentration. The K_i for Mg^{2+} (= 2 mM) and K_m for Na^+ (= 0.35 mM) are determined as indicated in Fig. 1B.

Activation of the phosphorylation by imidazole

Fig. 1A demonstrates also another remarkable phenomenon. The basal phosphorylation level in imidazole buffer in the absence of added Na^+ increases to a level that is 75% of the maximal level in the presence of 100 mM Na^+, when the Mg^{2+} concentration is decreased to 0.1 mM Mg^{2+}. This effect is evidently due to the imidazole buffer, since in the presence of Tris no such effect is observed (Fig. 2A), even though in Tris at Mg^{2+} levels above 0.1 mM a similar Mg^{2+}-Na^+ antagonism as mentioned in the preceding section is encountered with a K_i value for Mg^{2+} of 2 mM. However, the K_m for Na^+ is 50% higher in Tris than in imidazole (Fig. 2B). Actually Tris inhibits the effect of imidazole, as will be shown later (Fig. 11).

The high basal phosphorylation level in imidazole is not caused by contaminating Na^+ as a result of an increased affinity for Na^+ in this buffer. We have investigated this by determining the K_m for Na^+ at four different imidazole concentrations (5–50 mM, pH 7.0) in the presence of low (0.1 mM) free Mg^{2+}. Contaminating Na^+ has been kept low (1.1–1.3 μM) by using imidazole recrystallized from ether and low enzyme protein levels (0.13 mg/ml). The basal phosphorylation level increases with the imidazole concentration (Fig. 3A), as expected when the buffer has an activating effect on phosphorylation. Meanwhile, the K_m value for Na^+ decreases from 0.63 mM in 5 mM imidazole to 0.21 mM in 50 mM imidazole (Fig. 3B). These K_m values for Na^+, which greatly exceed the micromolar residual Na^+ concentration, suggest that phosphorylation in the presence of imidazole is due to the buffer itself and not to residual Na^+.

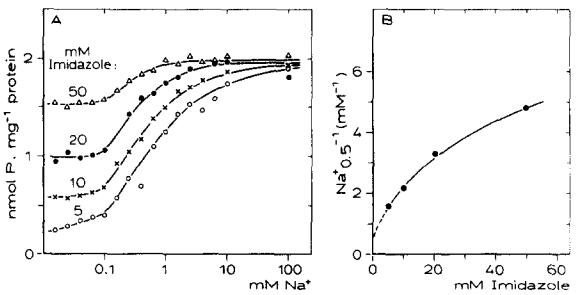


Fig. 3. Na^+ activation of phosphorylation as a function of the imidazole concentration. (A) Phosphorylation at 0.1 mM Mg^{2+} and pH 7.0 is determined as in Fig. 1A, but ether-recrystallized imidazole is used. Na^+ and imidazole concentrations are indicated in the figure. (B) Reciprocal plot of the half-maximally stimulating Na^+ concentration as a function of the imidazole concentration. The K_m value for Na^+ is determined as in Fig. 1B and decreases from 0.63 mM in 5 mM imidazole to 0.21 mM in 50 mM imidazole.

This conclusion should, however, be modified in the light of the following observation. Upon extrapolation of the imidazole concentration to zero, the K_m value for Na^+ approaches 2 mM (Fig. 3B). This value matches the K_m values for Na^+ of 1.9 mM found for the $E_2 \rightarrow E_1$ transition in histidine buffer, which buffer by itself does not induce conformational changes [3]. Hence, imidazole appears to support the effect of Na^+ on the transition to the proper enzyme conformation (E_1) for optimal phosphorylation capability. However, the high Na^+ requirement for the $E_2 \rightarrow E_1$ transition does not exclude the possibility that micromolar Na^+ could be saturating for the phosphorylation process and thus lead to the high phosphorylation level in imidazole buffer.

Activating and inhibitory effects of Mg^{2+}

Mg^{2+} in the millimolar range inhibits phosphorylation by increasing the K_m for Na^+, as shown in the first section. However, in the micromolar range it acts as an activator. Both effects can be seen in Fig. 4, which figure also shows that Na^+ counteracts the inhibition of phosphorylation by Mg^{2+} and increases its activating effect. In the nominal absence of Na^+, using ether-recrystallized imidazole, a clear activating effect of Mg^{2+} (K_m = 1 μM) can also be seen, followed by inhibition above 0.1 mM Mg^{2+} with half-maximal inhibition at 0.56 mM Mg^{2+}. In the presence of added Na^+ the half-maximally activating concentration of Mg^{2+} is shifted to below 1 μM and the half-maximally inhibitory concentration of Mg^{2+} to above the 0.56 mM value observed in the absence of Na^+. In other words: Mg^{2+} is a stronger inhibitor of the imidazole activation than it is of Na^+ activation. Conversely, it is a weaker activator in the presence of imidazole than of Na^+.

From these data it can be inferred that imidazole, unlike Tris, has a Na^+-like effect on phosphorylation and that millimolar Mg^{2+} antagonizes this effect as it does with Na^+. The inhibitory effect of Mg^{2+} on imidazole activation is studied further in the section on the effect of K^+.

Effect of pH on the imidazole activation

The effect of pH on phosphorylation in the presence and absence of Na^+ in imidazole and Tris media is shown in Fig. 5. In the presence of Na^+ the optimal pH is 7.5 in both media. In the nominal absence of Na^+ the phosphorylation in imidazole is optimal at pH 6–7 and then declines to virtually zero at pH 8. This pH profile resembles that of phosphorylation by inorganic phosphate [17], but that type of phosphorylation requires high Mg^{2+} (and P_i) concentrations. In Tris

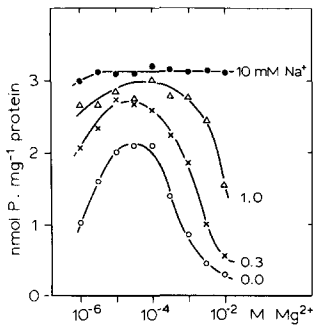


Fig. 4. Activation and inhibition of phosphorylation by Mg^{2+} as a function of the Na^+ concentration. Phosphorylation is determined as in Fig. 1A, except that ether-recrystallized imidazole is used and the Mg^{2+} concentration is extended downwards to 1 μM in order to demonstrate Mg^{2+} activation. Enzyme protein concentration is 0.17 mg/ml. The figure demonstrates decreasing half-maximally stimulating Mg^{2+} concentrations (left hand slopes) and increasing half-maximally inhibitory Mg^{2+} concentrations (right hand slopes) at increasing Na^+ concentrations.

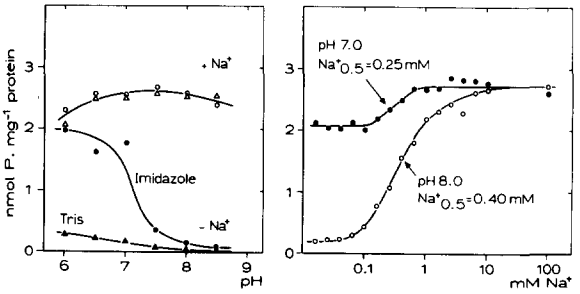


Fig. 5. (Left). Effect of pH on phosphorylation activated by imidazole and Na^+. Shown are 3-s phosphorylation levels in the presence of Tris (▲) or imidazole (●) (43 mM each) without added Na^+, and for comparison those induced by 100 mM Na^+ in Tris (△) or imidazole (○). Mg^{2+} is 0.17 mM, ATP 17 μM and enzyme 0.12 mg/ml.

Fig. 6. (Right). Effect of pH on the K_m value for Na^+. The half-maximally activating Na^+ concentrations are determined as indicated in Fig. 1B. Imidazole is 50 mM, Mg^{2+} 0.1 mM, ATP 20 μM and enzyme 0.08 mg/ml.

medium little phosphorylation occurs in the absence of Na^+, but here also phosphorylation decreases with increasing pH. In this respect, the action of imidazole differs from that of Na^+ in that it is more effective at low than at high pH. This is unlikely to be due to contaminating Na^+, as the K_m values for Na^+ (at 0.1 mM Mg^{2+}) at pH 7.0 and 8.0 do not strongly differ and are both high: 0.25 and 0.4 mM (Fig. 6).

The effect of pH is further illustrated in Fig. 7, where the phosphorylation level is plotted as a function of the protonated imidazole concentration. The latter parameter is used, since at low pH, where activation by imidazole is largest, this compound is mainly in the protonated form. The figure shows that the lesser effect of imidazole at high pH is probably due to inhibition occurring at above optimal imidazole concentrations, increasing at higher pH. The Scatchard plots in Fig. 7B confirm this behaviour and they show in addition that activation is positively cooperative for protonated imidazole. The location of the peak is solely dependent on the cooperativity index n, whereas the height of the peak also depends on the affinity for the activator [24]. So the shift of the peak to the right from pH 6.5 to 7.0 indicates an increase in n, and the concomitant increase of the height an increase of the affinity. The shift of the peak to the left at pH 7.5 is probably due to the increased inhibition at that pH. This inhibition is unlikely to be due to residual K^+, as recrystallized imidazole is used in this experiment.

Affinity for the substrate

In the preceding figures we find the maximal imidazole-stimulated phosphorylation level at neutral pH and low Mg^{2+} (0.1 mM) to be on the average 30% lower than the maximal Na^+-stimulated phosphorylation level. Several factors may contribute to this difference, such as the inhibition by high imidazole concentrations described above, the slower phosphorylation in the absence of Na^+, a higher K_m for ATP in the absence of Na^+ or a higher sensitivity of the phosphoenzyme to K^+ in the absence of Na^+. The difference cannot be due to insufficient phosphorylation time (3 s in these experiments), since 5 s phosphorylation gives identical results. The K_m for ATP in the nominal absence of Na^+ has been compared to that in the presence of a maximally stimulating Na^+ concentration (10 mM). In the absence and presence of Na^+ the K_m values for ATP are not significantly different (0.09 μM and 0.06 μM, respectively). Hence, a difference in affinity for ATP cannot explain the reduced phosphorylation levels in Na^+-free medium (Fig. 8). Moreover, we have used a saturating (20 μM) ATP concentration in the preceding phosphorylation experiments. In the

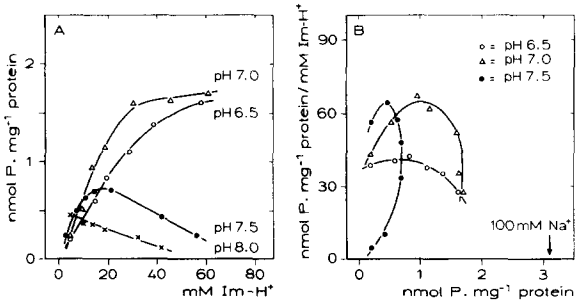


Fig. 7. Imidazole-activated phosphorylation as a function of the protonated imidazole concentration and the pH. (A) Profile for imidazole-H^+-activated phosphorylation (3-s levels) at the indicated pH. Ether-recrystallized imidazole is used, Mg^{2+} is 0.1 mM, ATP 20 μM and enzyme 0.25 mg/ml. Imidazole-H^+ concentrations are calculated from total imidazole concentration, pK imidazole and pH. (B) Scatchard plots of the data from A, indicating positive cooperativity for imidazole-H^+. Indicated is the phosphorylation level obtained at 100 mM Na^+ in 50 mM imidazole (pH 7.0).

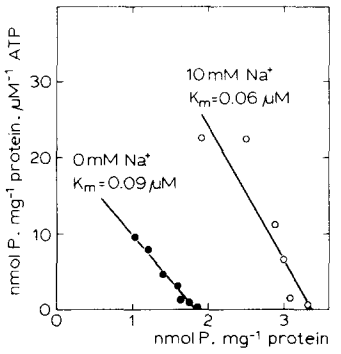


Fig. 8. K_m values for ATP in imidazole- and Na^+-activated phosphorylation. Scatchard plots of the phosphorylation in the absence ad presence of added Na^+ (10 mM) in 50 mM imidazole (pH 7.0) at 0.14–5 μM (initial concentrations) ATP, 0.1 mM Mg^{2+} and 0.03 mg/ml enzyme. The ATP concentrations at the ordinate are those remaining after 3-s phosphorylation. K_m in the absence of Na^+ is 0.09 μM, in the presence of 10 mM Na^+ it is 0.06 μM.

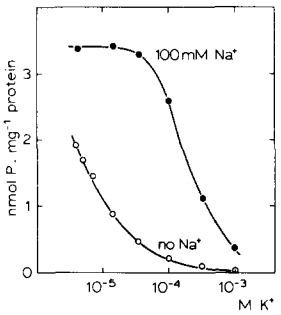


Fig. 9. Effect of K^+ on imidazole- and Na^+-activated phosphorylation. Phosphorylation is in 50 mM ether-recrystallized imidazole (pH 7.0) in the absence or presence of 100 mM Na^+ and in the presence of the indicated K^+ concentrations. Mg^{2+} is 0.1 mM, ATP 20 μM and enzyme 0.41 mg/ml. Half-maximal inhibition by K^+ in the absence of Na^+ is at 12.5 μM and in the presence of Na^+ 180 μM.

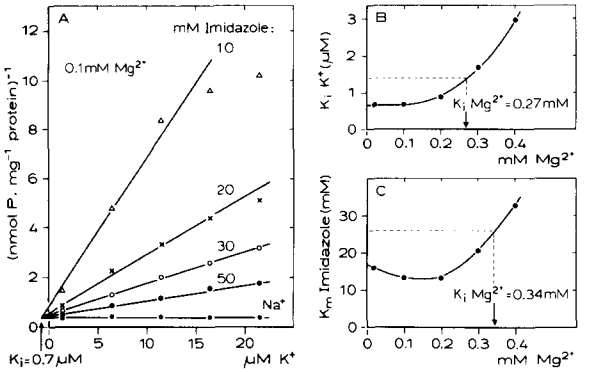


Fig. 10. Determination of K_i values for K^+ and Mg^{2+} and K_m value for imidazole in imidazole-activated phosphorylation. Phosphorylation is in 10–50 mM ether-recrystallized imidazole (pH 7.0) in the presence of 0.02–0.4 mM Mg^{2+}, 20 μM ATP, 0.14 mg/ml enzyme and the indicated K^+ concentrations. (A) Dixon plot of the data in the presence of 0.1 mM Mg^{2+}. The Na^+ line represents phosphorylation levels in the presence of 100 mM Na^+. The common intersection in the left-hand quadrant reveals a K_i value for K^+ of 0.7 μM. (B) K_i value for K^+ as a function of the Mg^{2+} concentration. The K_i is determined as indicated in (A) for five different Mg^{2+} concentrations. The K_i for Mg^{2+} (0.27 mM) is determined as the Mg^{2+} concentration, which doubles the minimal K_i value for K^+ (cf. Fig. 1B and 2B for the equivalent of this determination from a linear graph). (C) K_m value for imidazole as a function of the Mg^{2+} concentration. The K_m value is determined by Hill-plot analysis of the ordinate intercepts (K^+ = 0) of Dixon plots as shown in (A), taking the reciprocal of the common intersection as the maximal phosphorylation level. The Mg^{2+} concentration (K_i), which doubles the minimal K_m value for imidazole of 13 mM, is 0.34 mM.

next section we present evidence that inhibition by residual K^+ and uncharged imidazole is responsible.

Effect of K^+ on phosphorylation

We have investigated the sensitivity of the phosphoenzyme level to potassium in the absence and presence of Na^+, using ether-recrystallized imidazole. Fig. 9 shows that phosphorylation in Na^+-free imidazole (3.9 μM residual K^+, deriving mainly from the enzyme preparation) is far more sensitive to K^+ than Na^+-stimulated phosphorylation with 100 mM Na^+ present. Equal half-maximal phosphorylation levels are found at 180 μM K^+ in the presence of Na^+ and at only 5 μM K^+ in the absence of added Na^+. This is understandable, as K^+ will compete less easily with the physiological ligand (Na^+) than with the artificial one (imidazole).

Next, we have determined phosphorylation in imidazole as a function of the K^+ concentration at various sub-millimolar Mg^{2+} concentrations, analyzing the results by means of Dixon plots. The plots show in Fig. 10A represent data obtained in the presence of 0.1 mM free Mg^{2+}. They indicate that phosphorylation is competitively inhibited by K^+ and that saturating imidazole concentrations should provide the maximal phosphorylation level obtained with Na^+, if there were no inhibition by high imidazole concentrations (Fig. 7). It should be pointed out that at the imidazole concentrations used in Fig. 10A this inhibition is not yet evident. The plots also show deviations from linearity at high K^+ and low imidazole, which are more pronounced at higher Mg^{2+} concentrations (not shown). Preliminary experiments indicate that this is most likely due to ATP stimulating the $E_2(K) \rightarrow E_1(K)$ transition and thus raising the phosphorylation level. This effect increases with higher K^+ and Mg^{2+} concentrations and lower imidazole concentrations. Determination of the effect of K^+ in the 2–15 μM K^+ concentration range yields a K_i value for K^+ of 0.7 μM at 0.1 mM Mg^{2+}, as determined from the common intersection of the plots in Fig. 10A.

These effect of K^+ have been compared with those on the Na^+-stimulated phosphorylation in 50 mM Tris-HCl at the same Mg^{2+} concentration

(0.1 mM Mg^{2+}, 0.2–0.6 mM Na^+, pH 7.0). Under these conditions there is only little phosphorylation in the absence of added Na^+. The Dixon plots are now upwardly concave (not shown), indicating that hydrolysis of the K^+-sensitive phosphoenzyme (E_2P) is predominant. This suggests that the phosphoenzyme generated at low Na^+ concentrations is more K^+-sensitive than the phosphoenzyme generated in imidazole.

Fig. 10B shows the effect of Mg^{2+} on the inhibitory action of K^+ in imidazole-stimulated phosphorylation. The K_i for K^+ increases from 0.7 μM at 0.02 mM Mg^{2+} to 2.9 μM at 0.4 mM Mg^{2+}. The curve is upwardly concave, indicating positive cooperativity between Mg^{2+}-binding sites in this inhibition. The Mg^{2+} concentration doubling the minimal K_i value is determined at 0.27 mM. A similar effect of Mg^{2+} on the affinity for imidazole can be seen in Fig. 10C. The K_m value for imidazole increases from 13 mM at 0.1 mM Mg^{2+} to 33 mM at 0.4 mM Mg^{2+}. The Mg^{2+} concentration that doubles the minimal K_m value is 0.34 mM, close to that doubling the K_i for K^+ in this phosphorylation process.

These findings, in agreement with those of Fig. 4, indicate that the phosphorylation in imidazole is much more sensitive to inhibition by Mg^{2+} than the Na^+-stimulated phosphorylation. The ratio of K_i values for Mg^{2+} in the latter process over the former process is $2/0.34 \approx 6$. It is also clear that Na^+ is a more effective activator than protonated imidazole (ratio of K_m values is $5.9/2 \approx 3$, cf. Fig. 3B).

Inhibition by Tris

We have already shown that in Tris little spontaneous phosphorylation occurs, even at low Mg^{2+} concentrations (Figs. 2A and 5), although Tris has been reported to induce the E_1-conformation (K_m = 7 mM at pH 7.4 and 22°C, Ref. 3). In order to explore in more detail the discrepancy between imidazole and Tris in buffer-mediated phosphorylation, we have tested the effect of Tris on imidazole-stimulated phosphorylation. As shown in Fig. 11, Tris inhibits the imidazole-stimulated phosphorylation, maximally at 170 mM Tris and half-maximally at 30 mM. This effect is evidently not due to contaminating K^+, as 100 mM Tris contributes only 0.1 μM K^+, while half-maximal

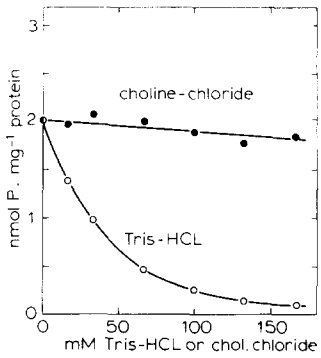


Fig. 11. Inhibition by Tris and choline chloride of imidazole-activated phosphorylation. Phosphorylation is in 50 mM imidazole (pH 7.0) in the presence of 0.1 mM Mg^{2+}, 20 μM ATP and 0.41 mg/ml enzyme. Half-maximal inhibition by Tris (pH 7.0) is at 30 mM.

inhibition of the imidazole-stimulated phosphorylation by K^+ under identical conditions occurs at 12.5 μM K^+ (Fig. 9). Neither can it be due to the ionic strength increase introduced by Tris, since the same concentration of choline chloride has only a minor inhibitory effect (10% at 170 mM, Fig. 11).

Discussion

Na^+ and imidazole-H^+ as activators of phosphorylation

The presented data demonstrate that Na^+ is not unique in promoting phosphorylation of (Na^+ + K^+)-ATPase, although it is the physiological and most effective cation for inducing the phosphorylating E_1 conformation. Li^+ has been known for some time to be able to substitute for Na^+ to a certain extent [25]. Now we show that even the structurally unrelated protonated imidazole ion causes the same effect and has several features in common with Na^+, like the stimulation by low Mg^{2+} concentrations and inhibition by high Mg^{2+} concentrations and the cooperative behaviour between the cation activating sites (cf. Ref. 26).

There are also important differences between the activation by Na^+ and by imidazole. Imidazole in concentrations above 50 mM inhibits phosphorylation at pH 7 and the inhibition increases progressively with increasing pH. This phenomenon is not seen in Na^+-stimulated phosphoryla-

tion, which has an optimal pH at 7.4. Since the fraction of uncharged imidazole in total imidazole ($pK = 6.9$) increases with the pH and becomes predominant above pH 7, it is possible that the uncharged imidazole inhibits phosphorylation, while the protonated imidazole activates it. The fact that imidazole gives higher stimulation of phosphorylation below pH 7 than above this pH does not rule out a role of the Bohr effect (cation-induced release of protons from the enzyme) in the induction of the phosphorylating E_1 conformation [12], since this effect may be masked by competitive inhibition of phosphorylation by the uncharged imidazole.

Another difference is that the imidazole-stimulated phosphorylation is rather strongly inhibited by Tris buffer (half-maximally at 30 mM and pH 7), whereas Tris buffer increases the K_m for Na^+ by only 50% at 50 mM and the same pH. This difference indicates a lower K_i for Tris in imidazole-H^+ activation than in Na^+ activation, which parallels the 6-times lower K_i value for Mg^{2+} in imidazole-activated phosphorylation compared to that in the Na^+-activated process. We are not certain at the moment whether this also indicates a different mechanism of activation for Na^+ and imidazole-H^+. However, the inhibition by Tris suggests that different enzyme conformations are comprised under the designation E_1 since Tris appears to induce the E_1 conformation (see next section) without appreciable phosphorylation.

The E_1 class of conformations

The existence of the E_1 conformation is currently based on evidence from intrinsic and extrinsic fluorescence [2,3,12,27], tryptic inactivation patterns [28,29] and nucleotide binding affinity [13]. Although Na^+, imidazole, Tris, Mg^{2+} and ATP all induce an E_1 conformation, it is clear from our data that essential differences exist in this family. The difference between the E_1Na and E_1Mg conformation shows up in all three of the above criteria for the E_1 conformation [13,30,31] and is also evident from the Mg^{2+}-Na^+ antagonism in our experiments. In addition, we have now shown that the E_1Tris conformation phosphorylates poorly, whereas the E_1Na and E_1imidazole conformations are strongly phosphorylating. Even the E_1MgATP complex is non-phosphorylating without a further conformational change induced by Na^+ or imidazole. This indicates that complex spatial conditions are required for the E_1 conformation to become phosphorylating.

Involvement of other partial reactions

There are other ways of explaining the difference between the effects of Tris and imidazole on phosphorylation than as a specific activation by imidazole. The phosphorylation level is determined as a steady state of phoshorylation and dephosphorylation, short circuited by the $E_2 \rightarrow E_1$ transition. Phosphorylation and $E_2 \rightarrow E_1$ transition may have different cation requirements. With this in mind we can then formulate five alternative explanations:

(1). Imidazole-H^+ directly activates ATP-dependent phosphorylation.

(2). Na^+ is the only cation (except for Li^+), which actually triggers phosphorylation in the E_1imidazole conformation but not in the E_1Tris conformation, even in a medium with a residual Na^+ concentration of only 1 μM (thus $K_m < 1$ μM).

(3). Imidazole inhibits dephosphorylation much more than Tris.

(4). Tris, but not imidazole, would promote dephosphorylation.

(5). Imidazole accelerates the $E_2 \rightarrow E_1$ transition much more than Tris does.

Preliminary experiments in our laboratory have shown that imidazole and Tris (50 mM, pH 7, 0.1 mM Mg^{2+}) give the same rates for the $E_2 \rightarrow E_1$ transition and lead to the same maximal E_1 level as given by Na^+, ruling out explanation 5. In addition, Tris (100 mM) does not affect dephosphorylation of the phosphoenzyme generated in imidazole medium under the same conditions of high basal phosphorylation, which rules out explanation 3 and 4. This conclusion is further supported by the results of ATPase determinations (50 mM buffer, pH 7.2, 37°C) in the absence of K^+: optimal Na^+-ATPase activity in Tris and imidazole is equal, ATPase activity in imidazole (no Na^+) is 50% of this rate, in Tris only 16%.

Thus the difference between Tris and imidazole, in terms of giving phosphorylation must be directly related to the phosphorylation step, leaving alternatives 1 and 2. Without evidence to the con-

trary, we presently favour alternative 1. However, neither alternative vitiates the conclusion that imidazole-H^+ appears to be able to substitute for Na^+ in ATP-dependent phosphorylation by induction of the phosphorylating E_1 conformation. Imidazole-H^+, compared to Na^+, has a lower affinity, requires higher Mg^{2+} concentrations and is antagonized by lower concentrations of Mg^{2+} and K^+.

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