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Phosphorylation of ($Na^+ + K^+$)-ATPase; stimulation and inhibition by substituted and unsubstituted amines

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(1) In view of a previously established stimulation of steady-state phosphorylation of ($Na^+ + K^+$)-ATPase by imidazole and its inhibition by tris(hydroxymethyl)aminomethane, the effect of (structure, chemical composition and charge of) a number of primary, secondary and tertiary amines (including imidazole derivatives) has been investigated. (2) Primary amines are predominantly inhibitory and diamines are more inhibitory than monoamines. The strongest inhibition is exerted by ethylenediamine (I_{50} in 50 mM imidazole = 25 μM, vs. 60 mM for *n*-propylamine). Increasing the distance between the two amino groups from 3.7 to 8.7 Å increases the I_{50} 180-fold. The optimal distance of 3–4 Å indicates a similar distance between two ligand(presumably Na^+)-binding sites on the enzyme. (3) Screening or substitution of the central N-atom decreases inhibition by the nitrogen compound. Triple substitution by propyl or allyl groups leads to maximal activation, amounting to about 90% of the Na^+-activation level. Triethyl substitution gives suboptimal activation and tributyl substitution leads to inhibition. Substitution by polar or negatively charged carboxyl groups diminishes or even abolishes inhibition and also diminishes or abolishes activation. (4) Although occasionally positive charge is not required for inhibition, it is prerequisite for activation. Within certain families of compounds (e.g., ethylenediamine and imidazole derivatives) inhibition or activation increases with pK_a, hence with positive charge. (5) The above data are interpreted in terms of inhibition, which is competitive to Na^+, being governed by Coulomb interaction. Activation, on the other hand, is predominantly determined by lipophilic (van der Waals or $\pi-\pi$ electron) interactions, excluding water from the phosphorylation site, hence decreasing phosphoenzyme hydrolysis and increasing the phosphoenzyme level. The requirement of charge (though hidden by substitution) implies weak additional electrostatic interaction.

Introduction

In a previous article from our laboratory [1], we reported on the activation of the ATP-dependent phosphorylation of ($Na^+ + K^+$)-ATPase by imidazole and its inhibition by tris(hydroxymethyl)-aminomethane. These two compounds do not only

Abbreviations: CDTA, *trans*-1,2-diaminocyclohexanetetraacetic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine.

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TABLE I

AMINO COMPOUNDS TESTED IN THE ATP-DEPENDENT PHOSPHORYLATION OF $(Na^+ + K^+)$-ATPase

The commercial sources are indicated by their abbreviated names: Merck (Merck or Merck-Schuchard, Darmstadt or Hohenbrunn, F.R.G.); L.S. (Lancaster Synthesis, Morecambe, U.K.); Fluka (Fluka A.G., Buchs, Switzerland); Janssen (Janssen Chimica, Beerse, Belgium); Sigma (Sigma Chem. Co., St. Louis, MO, U.S.A.); C.D. (Chemical Dynamics, South Plainfield, NY, U.S.A.); Aldrich (Aldrich Chemie S.A., Brussels, Belgium). [a] Free base.

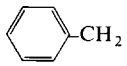
(1) Aliphatic primary, secondary and tertiary amines

General formula $R_1R_2R_3N$

Compound	R_1	R_2	R_3	commercial	
				form	source
n-Propylamine	$CH_3-(CH_2)_2$	H	H	– [a]	Fluka
Allylamine	$CH_2=CH-CH_2$	H	H	– [a]	Merck
Tris(hydroxymethyl)-aminomethane	$(CH_2OH)_3C$	H	H	– [a]	Janssen
Diallylamine	$CH_2=CH-CH_2$	$=R_1$	H	– [a]	Merck
N-Tris(hydroxymethyl-methylglycine	$(CH_2OH)_3C$	CH_2COOH	H	– [a]	Sigma
Trimethylamine	CH_3	$=R_1$	$=R_1$	– [a]	Merck
Triethylamine	CH_3-CH_2	$=R_1$	$=R_1$	– [a]	Merck
Triethanolamine	CH_2-CH_2OH	$=R_1$	$=R_1$	– [a]	Merck
Tripropylamine	$CH_3-(CH_2)_2$	$=R_1$	$=R_1$	– [a]	Merck
Triallylamine	$CH_2=CH-CH_2$	$=R_1$	$=R_1$	– [a]	Merck
Tributylamine	$CH_3-(CH_2)_3$	$=R_1$	$=R_1$	– [a]	Merck

(2) Aliphatic primary and secondary diamines

General formula $\begin{matrix} H & & H \\ R_1-N-(CH_2)_n-N- & & R_2 \end{matrix}$

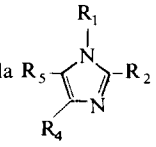
Compound	*n*	R_1	R_2	commercial	
				form	source
Hydrazine	0	H	H	HCl-salt	Fluka
Ethylenediamine	2	H	H	– [a]	Merck
N-Ethylethylenediamine	2	CH_3-CH_2	H	– [a]	L.S.
N-Benzylethylenediamine	2		H	– [a]	L.S.
N,N'-Di-*n*-propylethylene diamine	2	$CH_3-(CH_2)_2$	$=R_1$	– [a]	L.S.
1,3-Diaminopropane	3	H	H	– [a]	Aldrich
1,4-Diaminobutane	4	H	H	– [a]	Aldrich
1,5-Diaminopentane	5	H	H	– [a]	Merck
1,6-Diaminohexane	6	H	H	– [a]	Aldrich

differ in structure, the first being a tertiary and the second a primary amine, but also in pK_a value, the first with a pK_a of 7.0 and the second of 8.1.

We have made the suggestion [2] that activation of phosphorylation by protonated amines is caused by hydrogen-bond formation with an unprotonated histidyl-imidazole group on the enzyme, and that inhibition of the phosphorylation is caused by electrostatic interaction with carboxyl groups. The hydrogen-bond formation, and hence the ability to stimulate phosphorylation, should be dependent on amine and enzyme sharing a proton, whereas inhibition of phosphorylation would be related to a charge difference between the ligand and its

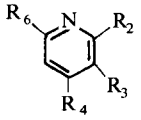
TABLE I (continued)

(3) Imidazoles

General formula 

Compound	R_1	R_2	R_4	R_5	commercial	
					form	source
Imidazole	H	H	H	H	– [a]	Merck
2-Methylimidazole	H	CH_3	H	H	– [a]	Sigma
2-Ethylimidazole	H	CH_3-CH_2	H	H	– [a]	Fluka
4-Methylimidazole	H	H	CH_3	H	– [a]	Fluka
4-Hydroxymethylimidazole	H	H	CH_2OH	H	HCl-salt	Aldrich
2,4-Dimethylimidazole	H	CH_3	CH_3	H	– [a]	Janssen
5-Chloro-1-methylimidazole	CH_3	H	H	Cl	– [a]	C.D.
Histamine	H	H	H_2N-$(CH_2)_2$	H	di-HCl-salt	Sigma
Histidine	H	H	$HOOC-\underset{\displaystyle NH_2}{\underset{\vert}{CH}}-CH_2$	H	– [a]	Merck

(4) Heterocyclic mono- and diamines

A. Pyridines: general formula 

Compound	R_2	R_3	R_4	R_6	commercial	
					form	source
3-Aminopyridine	H	NH_2	H	H	– [a]	Merck
4-Ethylpyridine	H	H	CH_3-CH_2	H	– [a]	Fluka
3,4-Dimethylpyridine	H	CH_3	CH_3	H	– [a]	Merck
2,4,6-Trimethylpyridine	CH_3	H	CH_3	CH_3	– [a]	Sigma
Pyrazine 					– [a]	Merck

ligating group on the enzyme, and thus to the ability of the amine to pick up a proton, rather than to share it.

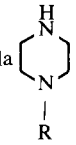
Either phenomenon should be dependent on the pK_a of the amine and the ligating group on the enzyme as well as on the pH of the medium in addition to structural or steric features. Therefore, we have tested as variable the degree of nitrogen substitution and pK_a of the substituted or unsubstituted amine in the phosphorylation process of $(Na^+ + K^+)$-ATPase. It turns out that in the range of pK_a values investigated (about 6–8) the effects of the amines on the ATP-dependent phosphorylation process are more strongly determined by steric and polarity factors than by the pK_a of the nitrogen group.

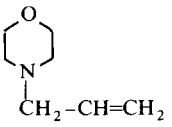
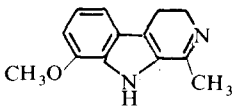
Materials and Methods

Enzyme preparation

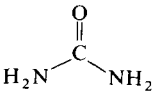
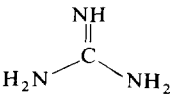
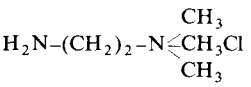
$(Na^+ + K^+)$-ATPase is purified from rabbit kidney outer medulla as described by Jørgensen

TABLE I (continued)

B. Piperazines: general formula 

	R	commercial	
		form	source
Piperazine	H	– [a]	Merck
1-Acetylpiperazine	CH_3-CO O	– [a]	Fluka
N-Allylmorpholine		– [a]	C.D.
Harmaline		– [a]	Sigma

(5) Miscellaneous amines

Compound	formula	commercial	
		form	source
Urea		– [a]	Merck
Guanidine		HCl-salt	Merck
Tetrapropylammonium chloride	$[CH_3-(CH_2)_2]_4NCl$	bromide	Merck
2-Aminoethyl trimethyl-ammonium chloride		HCl-salt	Fluka

[a] Free base.

[3], followed by removal of contaminating ATP, washing and storing of the preparation according to the procedure of Schoot et al. [4]. CDTA is omitted from the storage medium. Protein is determined by the Lowry method [23], following trichloroacetic acid precipitation, using bovine serum albumin as standard [3]. Specific activity of the preparations ranges between 940 and 1500 $\mu mol \cdot mg^{-1}$ protein per h.

Phosphorylation

Phosphorylation by ATP at 23°C and pH 7 is essentially according to the procedure, earlier described by us [5]: 40 μl of an aqueous solution of

$MgCl_2$ and ATP (final concentrations 100 and 20 μM, respectively) is mixed with 110 μl of a medium containing imidazole-HCl, (pH 7.0) (final concentration 50 mM), and enzyme (final concentration 0.1 mg protein/ml). After 3 s, the phosphorylation process is stopped by acid denaturation, followed by filtration and counting for ^{32}P of the acid-stable phosphoprotein [5]. The phosphorylation level in the basal assay medium of the composition as given above ranges from 0.60 to 2.0 $nmol \cdot mg^{-1}$ protein. ATP has been converted to its imidazole salt by passage of an aqueous solution of the disodium salt over a Dowex 50W X4 cation-exchange resin in the protonated form, followed by neutralization of the eluent with imidazole [6].

Effects on the 3 s phosphorylation level of amino compounds (primary, secondary and tertiary amines), including the inhibitory imidazole derivatives, are determined for the 5–150 mM concentration range in the presence of 50 mM imidazole-HCl (pH 7) and for the activating imidazole compounds in the absence of imidazole (except for 0.8 mM, derived from the enzyme suspension and ATP). The strongly inhibitory ethylenediamine derivatives and higher alkyldiamine homologues are assayed in the 0.02–20 mM concentration range with 50 mM imidazole-HCl (pH 7) present. The latter concentration provides for an average of about 50% (range 40–60%) of the maximal Na^+-activated phosphorylation level, hence providing a sufficient margin either for further stimulation or for inhibition by the additional amino compound. The phosphorylation obtained after 3 s (steady-state under stimulatory conditions, presteady-state under inhibitory conditions), is expressed as ratio of that in the presence of the additional amino compound over that in the presence of imidazole alone. I_{50} is the concentration (mM) of the amine giving a phosphorylation ratio of 0.5.

The amino compounds

The amino compounds that have been assayed and their commercial sources are ranked according to the systematic order of aliphatic mono- and diamines, imidazoles and other heterocyclic compounds, and miscellaneous amines in Table I, also defining the chemical structure of the various compounds.

Na^+ and K^+ contents are determined by flame photometry and range from less than 0.5 to 18.6 μM and from 0.4–4 μM per 50 mM of the amine, respectively. Amines containing inadmissable amounts of Na^+ (more than 35 μM) or K^+ (more than 3–4 μM) per 50 mM of the amine are recrystallized: *N*-tris(hydroxymethyl)methylglycine from H_2O, 2-methylimidazole from butanol-1, histamine dihydrogen chloride from methanol and 4-methanolimidazole-HCl from ethanol (fraction 1) and by addition of ethyl acetate to the mother-ley (fraction 2). The criterion for the Na^+-tolerance is that the amines are assayed in concentrations up to 150 mM, and Na^+ starts to stimulate above 100 μM only (3 × the criterion for 50 mM of the amine [1]). The tolerance for K^+ is set by the fact that K^+ starts to enhance dephosphorylation of the phosphoenzyme above 3 μM per 50 mM of the buffer [7].

HCl-salts of the amines (except for the neutral guanidinium chloride) are deprotonated by passage over Dowex-1 in the OH^- form, followed by freeze-drying and subsequent drying of the free base in vacuo over phosphorpentoxide or silica gel, except when the amine is a liquid (hydrazine) and it is kept as a solution. Since passage over Dowex-1 of 2-aminoethyltrimethylammonium chloride-HCl also removes Cl^- from the ammonium moiety, the compound is rechromatographed over Dowex-2 (Cl^- form, affinity ratio $Cl^-/OH^- = 1.5$). Tetrapropylammonium bromide is converted to the chloride salt by passage over Dowex-1 in the Cl^- form.

Subsequently, stock solutions (500 mM or less but saturating) are neutralized to pH 6.9–7.0 by addition of HCl. Since the pH electrode leaks K^+, pilot titrations are made using the electrode, but adding the determined amount of HCl to a separate solution of the amine that will not come in contact with the electrode. Because of hygroscopy of 2-aminoethyltrimethylammonium chloride, the HCl salt of it is neutralized with a solution of the free base. The acidic *N*-tris(hydroxymethyl)methylglycine is neutralized with the basic imidazole, and the imidazole in the assay medium corrected for its contribution (one-tenth of the *N*-tris(hydroxymethyl)methylglycine concentration).

Final concentrations of the amines in the stock solutions are determined by acidimetric and al-

kalimetric titration, by fluorometric means, using fluorescamine (excitation at 390, emission at 490 nm), dissolved in acetone, 0.2 M sodium borate as buffer system and standard solutions of the primary amines for calibration [8], or by spectrophotometry for chromophores as harmaline ($\varepsilon_{372} = 1.82 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ in 0.1 M HCl [9]).

Calculation of interatomic distances and conformational energies

Interatomic distances, in particular between the two nitrogens in the diamines at minimized energy conformations are calculated by computer programme, designated Chem-X, developed and distributed by Chemical Design, Oxford, U.K. Van der Waals energies for the different conformations (*syn*, *anti*, boat, twist boat and chair), consisting of potential, Coulomb and torsional energies are computed by the same programme and lead to energy differences between opposite conformations of 8–33 kcal/mol. Molecular mechanics computations give similar data. This means, according to the law of distribution (Maxwell-Boltzmann), a large preponderance of one conformation (*anti*, chair) over the other one (*syn*, boat or twist boat). In the results section only the N → N spans of the more relaxed conformations are given. It should be stressed that these distances hold for the diamines free in solution, not necessarily for their enzyme-bound configuration.

Results

Buffer activation and inhibition of phosphorylation

Fig. 1 shows the leading principle of the present investigation, demonstrating activating and inhibitory effects of imidazole and Tris on the (Na^+ + ATP)-dependent phosphorylation. In concentrations up to 50 mM, these buffers lower the $[Na^+]_{0.5}$ allosterically, i.e., at the opposite side of the membrane, while at concentrations above 50 mM they increase $[Na^+]_{0.5}$, conceivably via binding at the Na^+-activation site, i.e., at the same side of the membrane (see Discussion). Hence the enzyme displays a buffer activation and a buffer inhibition site, the latter with a lower affinity for the effector. Tris and imidazole differ in their affinities for the two sites, Tris having a lower affinity for the activator site and/or a higher affinity for the inhibition site, hence giving a smaller maximal decrease of $[Na^+]_{0.5}$ than imidazole.

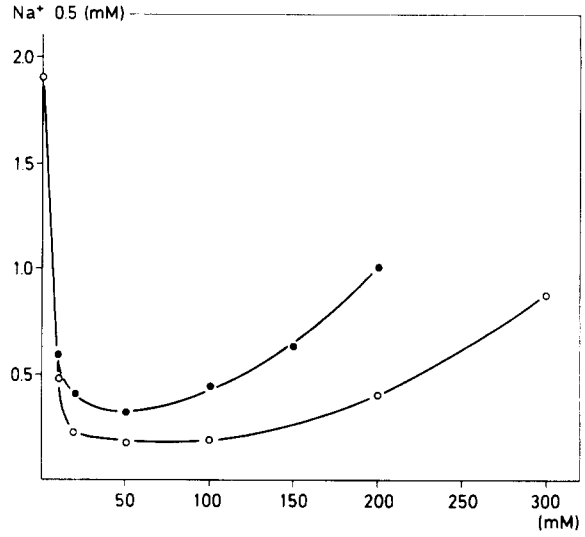


Fig. 1. Stimulation and inhibition of Na^+-activation in ATP-dependent phosphorylation of ($Na^+ + K^+$)-ATPase by tris(hydroxymethyl)aminomethane (●) and imidazole (○). $Na^+_{0.5}$ values are determined from 3-s phosphorylation levels as function of the Na^+ concentration (0.1–20 mM) in tris(hydroxymethyl)-aminomethane or imidazole of the indicated concentrations at pH 7 and 22 °C. 'Zero' buffer corresponds to 0.25 mM imidazole, contributed by the enzyme suspension.

The same principle of activation and inhibition of phosphorylation by one and the same com-

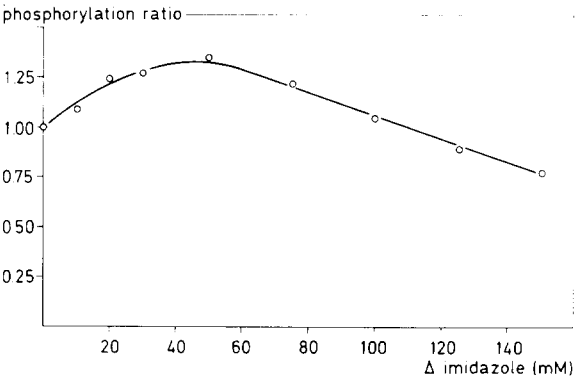


Fig. 2. Stimulation and inhibition of ATP-dependent phosphorylation by imidazole in the absence of added Na^+ (residual Na^+ and K^+ 5.7 and 2 μM per 50 mM of buffer, respectively). The phosphorylation level is expressed as ratio of the phosphorylation level at the given imidazole concentration (minus 50 mM) and that in 50 mM.

pound, viz., imidazole, but in the absence of Na^+ is shown in Fig. 2. At the same time, the figure demonstrates the general method of investigating these phenomena for other amino compounds as well. By taking phosphorylation in 50 mM imidazole as background (phosphorylation ratio = 1.0) one obtains a submaximal level, which amounts to 40–60% of the maximal Na^+ activated phosphorylation level. This allows ample room for a further stimulation (35% by an additional 50 mM imidazole in Fig. 2) as well as for inhibition (43% by a further 100 mM of this buffer in Fig. 2) to be recorded.

Activation and inhibition by primary amines

Table II summarizes the effects of several chemically distinct amines, subdivided according to their degree of substitution in primary, secondary and tertiary amines, and their degree of protonation or being positively charged by ranking them in order of increasing pK_a value. Some of them enhance the phosphorylation level in the

TABLE II

EFFECTS OF PRIMARY, SECONDARY AND TERTIARY MONO- AND DIAMINES ON PHOSPHORYLATION IN THE PRESENCE OF 50 mM IMIDAZOLE-HCl AT pH 7.0 AND 23° C

The phosphorylation level, obtained after 3 s, is expressed as ratio of that in imidazole alone. Indicated is the maximal ratio and the concentration (C_{max}) of the variable amine at which this maximum is reached. The compounds are ordered according to their pK_a values. The N → N distances of the diamines are given for the predominant anti- or chair-conformations. n.i., no inhibition.

Compound	pK_a	N → N distance (Å)	Maximal ratio of phosphorylation	C_{max} (mM)	I_{50} (mM)
(1) Primary amines					
3-Aminopyridine	6.0	3.7	1.15	20	>150
N-Benzylethylenediamine	6.5; 9.4	3.7	–	–	2
2-Aminoethyltrimethyl-ammonium chloride	7.1	3.7	–	–	0.8
N-Ethylethylenediamine	7.6; 10.8	3.7	–	–	0.8
Tris(hydroxymethyl)-aminomethane	8.1	–	–	–	50
Propylamine	10.5	–	1.14	10	60
(2) Secondary amines					
Piperazine	5.7; 9.8	2.9	–	–	0.6
N,N'-Di-*n*-propyl-ethylenediamine	7.5; 10.3	3.7	–	–	4.6
1-Acetylpiperazine	7.9	2.8	1.29	20	>150
N-Tris(hydroxymethyl)-methylglycine	8.2	–	–	–	n. i.
(3) Tertiary amines					
Pyrazine	1.1	2.7	–	–	n. i.
4-Ethylpyridine	6.0	–	1.23	20	56
3,4-Dimethyl-pyridine	6.5	–	1.05	10	50
N-Allylmorpholine	7.0	–	1.13	50–60	≫150
2,4,6,-Trimethyl-pyridine	7.5	–	1.80	50	134
Triethanolamine	7.8	–	1.11	50–60	≫150
Triallylamine	8.3	–	2.34	⩾60	n. i.
Harmaline	10	3.6	–	–	1.0

presence of 50 mM imidazole as indicated by the maximal phosphorylation ratio of more than 1.0. Others do not or rather inhibit the phosphorylation, as indicated by the mere presentation of I_{50} values. Nearly all stimulatory compounds display an inhibitory effect at concentrations above optimal, as shown for imidazole in Fig. 2 and indicated in Table II by C_{max} and I_{50} values.

Primary amines, such as 3-aminopyridine and propylamine show at low concentration a transient stimulation that is converted into an inhibition at higher concentrations (more than 10 mM, Fig. 3). Clearly, inhibition by the aliphatic amine is more pronounced than that by the aromatic, although both stimulations are the same. It should, however, be stressed that the closer C_{max} and I_{50} are, as in pertinent to propylamine, the lower the maximal phosphorylation ratio will be. When the nitrogen atom is substituted by a bulky branched chain hydroxyalkyl group, like in Tris (Table I.1), no stimulation is apparent and inhibition is predominant and virtually coincident with that caused by propylamine. The ethylenediamine derivatives

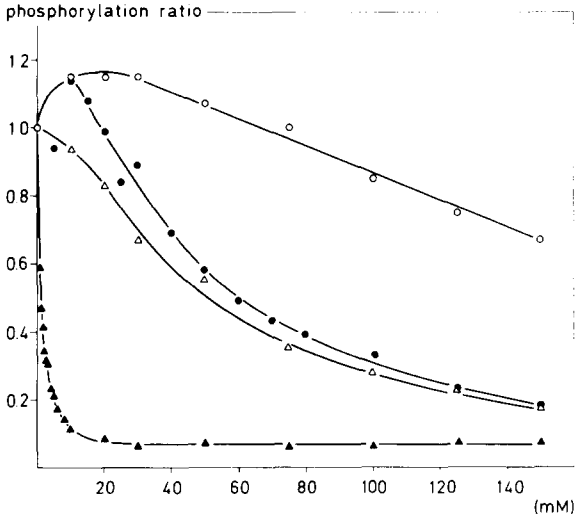


Fig. 3. Stimulation and inhibition of ATP-dependent phosphorylation by primary amines in the presence of 50 mM imidazole. The phosphorylation level is expressed as ratio of that in 50 mM imidazole without the indicated amines. Meaning of symbols: ○, 3-aminopyridine; ●, propylamine; △, tris(hydroxymethyl)aminomethane; and ▲, 2-aminoethyltrimethylammonium chloride. Residual Na^+ and K^+ concentrations range from 2.3–8.0 and from 0.7–2.3 μM per 50 mM amine, respectively.

N-benzylethylenediamine, 2-aminoethyltrimethylammonium chloride and *N*-ethylethylenediamine are strong inhibitors with I_{50} values of 0.8–2 mM (Fig. 3, Table II.1). These I_{50} values, multiplied by the fraction of double protonated or charge diamine, yields concentrations in the narrow range of 0.5–0.6 mM, suggesting that the double charged diamine is the inhibitory species. The K^+ content (1.5–2.3 μM per 50 mM diamine) cannot be the reason for a strong inhibitory potency, since the I_{50} for K^+ in 50 mM imidazole is about 12.5 μM [1]. In addition, the variation in K^+ content among the primary amines is small, and this content almost equal to that of the less inhibitory monoamines ($I_{50} \gtrsim 50$ mM).

The single-charged 3-aminopyridine, although containing the ethylenediamine structure with a N → N distance of 3.7 Å, is poorly inhibitory, possible due to the fact that it lacks the second point of attachment because of the poor electronegative character of the phenyl N, yet displaying the stimulatory properties of the tertiary pyridine derivatives (Table II.3). The primary amine moiety of the compound is at pH 7 only for 10% protonated as compared to the fully charged and more inhibitory Tris and propylamine.

From the above data on one can conclude that molecular structure and charge determine stimulation and inhibition by the primary amines of which inhibition is, generally speaking, predominant.

Secondary amines

Comparison of Tables II.1 and II.2 shows that secondary amines have much in common with the primary amines, although steric effects by the second substitution of the N-atom increase. For instance, the double-charged circular ethylenediaminepiperazine (chair conformation, N → N distance = 2.9 Å, Table II.2) is strongly inhibitory, considering that at pH 7 only 5% of the molecules is in the double-charged form and to be related to an I_{50} value of $0.05 \cdot 0.6 = 0.03$ mM, comparable to an I_{50} value of 0.025 mM for the open-chain ethylenediamine (Fig. 6). This contrasts to the single-charged acetylpiperazine with a similar N → N distance (2.8 Å, Table II.2). The shape of activation and inhibition by acetylpiperazine is similar to that of 3-aminopyridine (Fig. 3), but,

since the concentration of the charged form of acetylpiperazine is 9-times as high as that of 3-aminopyridine it must as a cation have less affinity to the inhibitory site. This is probably due to steric hindrance by the bulky acetyl substituent. A similar steric hindrance effect is observed on comparison of the secondary *N,N'*-di-*n*-propylethylethylenediamine (Table II.2) with the primary *N*-ethylethylenediamine (Table II.1). Despite a similar charge and N → N distance, the former is a 5.8-times weaker inhibitor on I_{50} basis.

Substitution of a hydrogen in the primary amino group of Tris by an acetyl group in Tricine (*N*-tris(hydroxymethyl)methylglycine) even abolishes inhibition and yields an inert buffer (Table II.2). Here also negative charge repulsion between carboxyl groups may come into play (see Discussion). Thus, conversion of a primary to a secondary amine in general decreases the fit to the inhibitory site although not necessarily converts it into a stimulatory compound. This presumption will be confirmed in the comparison of the allyl amines (see below).

Tertiary amines

The largest variety of responses, from steep inhibition (harmaline), via virtual ineffectiveness (*N*-allylmorpholine and triethanolamine) to almost complete activation (triallylamine) is found among the tertiary amines (Fig. 4, Table II.3). With a variation in K^+ content between 0.4 and 4.0 μM per 50 mM amine, it is obvious that this cannot have been the cause of the variability in responses.

Pyrazine, which is the fully unsaturated, but uncharged derivative of piperazine ($pK_a = 1.1$) with a N → N distance of 2.7 Å, is totally ineffective (Table II.3). On the other hand, harmaline, which also contains the ethylene diamine structure but has a single charge [9] and an almost equal N → N distance, is strongly inhibitory ($I_{50} = 1$ mM). This proves that a dual charge is not essential for tight fitting to the site of inhibition. However, the secondary amino group in the pyrrole ring of harmaline (Table I.4) may be able to form a hydrogen linkage in a second binding site to the enzyme.

Comparison of the pyridine derivatives shows that shielding of the N-atom by CH_3-groups in

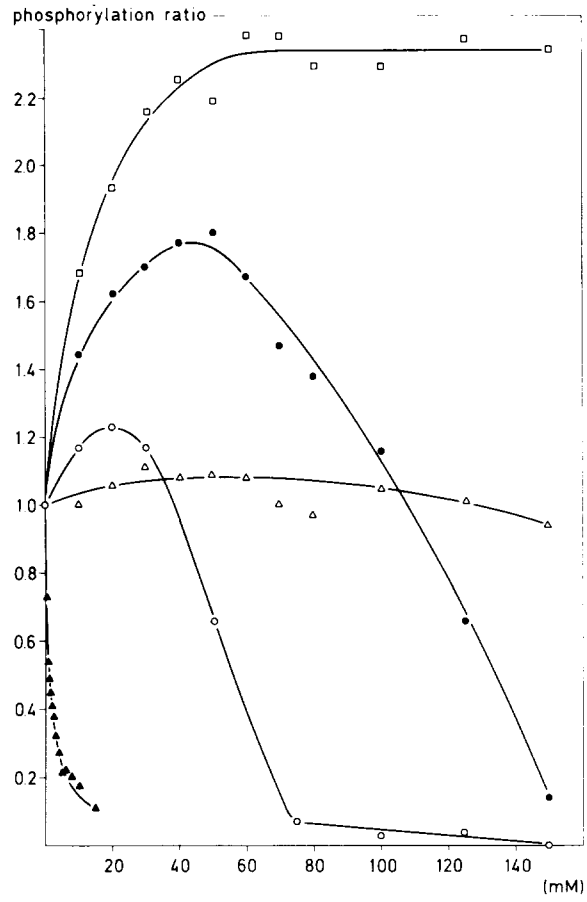


Fig. 4. Effects of tertiary amines on the phosphorylation ratio in the presence of 50 mM imidazole. The phosphorylation ratio is defined in the legend of Fig. 3. Meaning of symbols: ○, 4-ethylpyridine; ●, 2,4,6,-trimethylpyridine; △, triethanolamine; ▲, harmaline; and □, triallylamine. Residual Na^+ and K^+ concentrations range from 3.4–12.5 and from 0.4–4.0 μM per 50 mM amine, respectively.

2,4,6-trimethylpyridine decreases binding to the inhibition site and improves that to the stimulation site. On the other hand, mere alkylation of pyridine increases the fit to the inhibition site as is borne out by comparison of the I_{50} value for 3-aminopyridine (Table II.1) with that for 4-ethyl- and 3,4-dimethylpyridine (Table II.3). Their stimulations and C_{max} values are of the same order of magnitude (Figs. 3 and 4).

The C_{max} values of the remainder of tertiary amines all are 2.5 to 3 times as large, due rather to a decreased fit to the site of inhibition than to a low affinity to the site of stimulation. The reason

why triethanolamine is virtually ineffective, while triallylamine, having the same degree of substitution, is very effective (Fig. 4) could in part be the size and polarity of the alkyl group substituent. The latter might improve Van der Waals force interaction with lipids or hydrophobic amino-acid residues at the active site. This has been the subject of further investigation (see below).

Inhibitory imidazole derivatives

Since imidazole was the first nitrogen compound for which we detected a Na^+-like effect on the phosphorylation of $(Na^+ + K^+)$-ATPase [1], we have studied the effect of substitution and variation in pK_a (protonation) on stimulation and inhibition of phosphorylation. Our previous impression that a primary amino group promotes inhibition, led us to investigate the effect of amino-group substitution in imidazole. The primary amino group containing imidazole derivatives such as histidine and histamine are inhibitors of ATP-dependent phosphorylation with in the case of histidine a course of inhibition much like that given by Tris (Table III.1). Again the residual K^+ content can not explain the inhibition. However, omitting the carboxyl group from the molecule, which yields histamine and abolishes negative charge repulsion, increases the affinity for the inhibitor by a factor of 5. A similar effect may explain the difference in inhibitory potency between Tris and Tricine of which the latter contains a carboxyl group which may cancel the inhibitory effect of the secondary amino group.

Inhibition by 5-chloro-1-methylimidazole ($pK_a = 5.1$) shows that charge is not fully essential for a fit to the inhibitory site, since the compound is only for 1% positively charged at pH 7. Inhibition resembles a two-site titration (not shown), indicating that more than one site is involved. Absence of any activation can be considered as further proof for the thesis that charge is essential for activation.

TABLE III

INHIBITION AND STIMULATION OF PHOSPHORYLATION BY IMIDAZOLE DERIVATIVES AT pH 7.0 AND 23° C

Inhibition is determined in the presence of 50 mM imidazole-HCl and expressed as I_{50} value. Stimulation is determined in the absence of added imidazole-HCl (only 0.8 mM being derived from ATP and the enzyme preparation) and expressed as fraction of the phosphorylation level (EP_{max}) obtained in the presence of 100 mM Na^+ (1.82–2.05 nmol·mg^{-1} protein). As in Table II, the compounds are ordered according to their pK_a values.

Compound	pK_a	I_{50} (mM)	
(1) Inhibitory derivatives			
5-Chloro-1-methylimidazole	5.1	60	
Histidine	6.0; 9.2	44	
Histamine	6.0; 9.7	9	
Compound	**pK_a**	**$K_{0.5}$ (mM)**	**$EP_{max.}$ (rel)**
(2) Stimulatory derivatives			
4-Methanolimidazole	6.4	> 40	> 0.26
Imidazole	7.0	23	0.43
4-Methylimidazole	7.5	11	0.60
2-Methylimidazole	7.9	15.5	0.47
2-Ethylimidazole	8.0	8.5	0.26
2,4-Dimethylimidazole	8.4	11.5	0.38

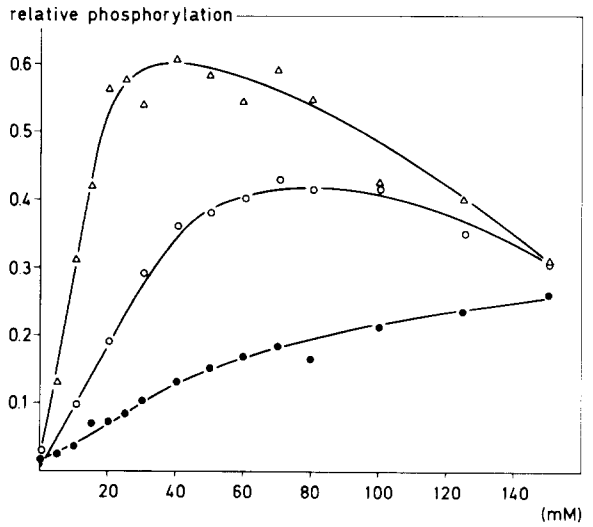


Fig. 5. Stimulation of phosphorylation by imidazole and its 4-CH_3- or CH_2OH- substituted derivatives. Phosphorylation occurs in the presence of the indicated concentration of buffer without extra imidazole. The phosphorylation level is related on the ordinate to the level obtained in the absence of the indicated derivative but presence of 100 mM Na^+. Meaning of symbols: ○, imidazole; ●, 4-methanolimidazole; and △, 4-methylimidazole. Contaminating Na^+ and K^+ concentrations range from 1.1–16 and from 0.3–2.7 μM per 50 mM amine, respectively.

Stimulatory imidazole derivatives

Imidazole and its 4-substituted derivatives follow in their pattern of stimulation the increase in pK_a value (Fig. 5, Table III.2), i.e., the $K_{0.5}$ values for 4-methanolimidazole, imidazole and 4-methylimidazole are about reciprocally proportional (approx. 4 : 2.3 : 1.1) to the molar fractions of protonated molecules (1 : 2.5 : 3.8). This indicates that the cation is the activating species. Methyl-group substitution in the 2-position has little effect, but ethyl-group substitution in that position significantly decreases the maximum level of stimulation, emphasizing the importance of that position for a proper fit to the activating site.

N → N distances in inhibition

Diamines of the ethylenediamine series invariably are strong inhibitors of phosphorylation, either when double-charged or when single-charged but containing the potency to form a hydrogen bond

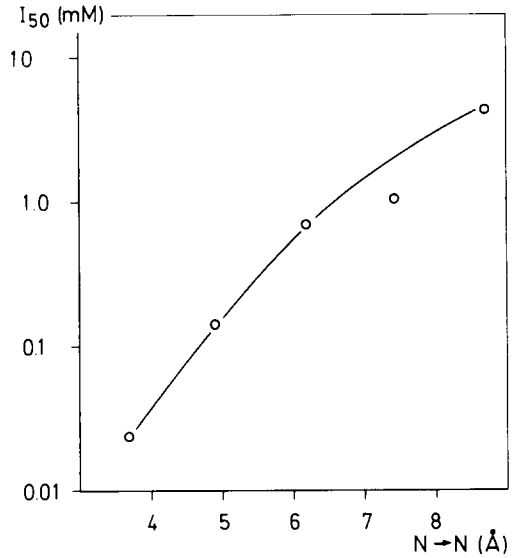


Fig. 6. Effect of charge separation on the inhibition of phosphorylation by primary diamines. Phosphorylation has been determined in the presence of 50 mM imidazole and increasing concentrations of ethylenediamine (N → N distance, 3.7 Å), 1,3-diaminopropane (N → N, 4.9 Å), 1.4-diaminobutane (N → N, 6.2 Å), 1,5-diaminopentane (N → N, 7.4 Å) and 1,6-diaminohexane (N → N, 8.7 Å). The half-maximally inhibitory concentrations are plotted as a function of the N → N distance in the relaxed anti-conformation. Contaminating Na^+ and K^+ concentrations range between 4.4 and 10.2 μM (Na^+) and from 2 to 4 μM (K^+) per 50 mM diamine.

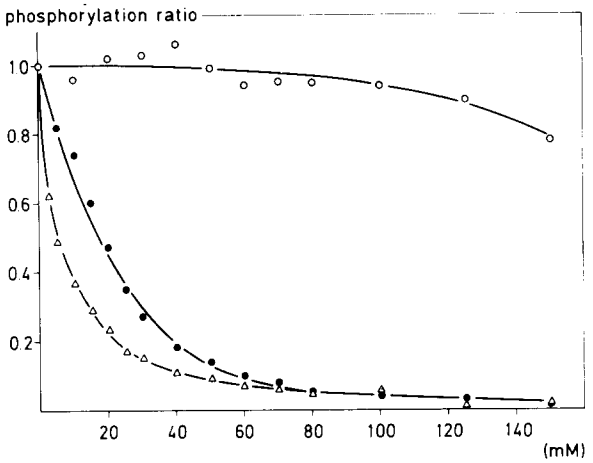


Fig. 7. Inhibition of ATP-dependent phosphorylation by small-chain diamines. Phosphorylation takes place in the presence of 50 mM imidazole and the level is expressed as ratio of that in the control (absence of diamines). Meaning of symbols: ○, urea (N → N distance, 2.3 Å); ●, guanidine (N → N, 2.3 Å); and △, hydrazine (N → N, 1.5 å). I_{50} is greater than 150 mM, 17, and 5 mM, respectively. The contaminating concentration of Na^+ ranges from less than 0.5 up to 2 μM, and K^+ is less than 0.6 μM per 50 mM of the diamine.

with the enzyme via the second nitrogen atom. This suggests that these diamines might staple on two activating sites. In stapling, obviously the size of the staple is of importance for a good fit, which apparently is the case when the nitrogen atoms are 3–4 Å apart (Table II). This rose the question as to the effect of increasing the diamine distance in the homologous series of diaminoalkanes. As to be expected, the I_{50} value increases with diamine distance, in fact 180-times, from 0.025 mM for ethylenediamine to 4.5 mM for 1,6-diaminohexane coincident with a 5 Å increase in charge separation (Fig. 6). The I_{50} value for the latter diamine is still not equal to that for a primary monoamine, which is in the range of 45–60 mM (Table II, Fig. 8A). Hence, the increase of I_{50} values shown in Fig. 6 may reflect the decreased probability for the diamine molecule to come into a position with a 3 Å N → N distance.

The distance has been reduced further to 2.3 Å in urea and guanidine, and even further to 1.5 Å in hydrazine. Urea is notorious for its formation of hydrogen bonds, but is uncharged, whereas guanidine and hydrazine contain only a single-charge on the secondary or one of the primary

amino groups, respectively. The uncharged urea is only slightly inhibitory (20% at 150 mM, Fig. 7), indicating that mere hydrogen bonding, if any, has not much influence in inhibition. On the contrary, the single-charged guanidine or hydrazine increase in inhibitory potency with decreasing size and increasing accessibility. Their I_{50} values of 17 and 5 mM, respectively, are one-third to one-tenth of those for the larger size single-charged primary amines, but approach that of the larger size dual-charged diamines (Fig. 6). Whether the strong inhibitory potency of guanidine and hydrazine is only due to their small size or in addition to a two site attachment (one electrostatic and the other by hydrogen bonding) remains an open question.

Shielding of the N-atom

Shielding of the N-atom by increased substitution might decrease the fit to the inhibitory site and increase that to the activating site. This effect was seen in substitution of the 2 and 6 position by methyl groups in pyridine (Fig. 4) and could underlie the stimulation by the tertiary triallylamine (Fig. 4) in contrast to the preponderating inhibition by the primary propylamine (Fig. 3). Indeed, substitution of the central nitrogen atom by allyl groups decreases the inhibition. The I_{50} value of diallylamine (150 mM) is 3.4-times as high as that of allylamine, but the optimal phosphorylation level is insignificantly higher (phosphorylation ratio 1.25 vs. 1.16, Fig. 8A). Further substitution of the nitrogen in triallylamine abolishes the inhibition and increases the optimal phosphorylation ratio to 1.6.

Substitution of the central nitrogen by propyl groups instead of allyl groups has little effect on the course of stimulation and inhibition (Fig. 8B). Triple substitution is favourable over quadruple substitution, possibly because tetrapropylamine exceeds the size of the optimal cavity filling space. Although the latter compound is an inhibitor of ouabain-sensitive Na^+ and K^+ transport through the red-cell membrane [10], it is certainly not an inhibitor of the phosphorylation step.

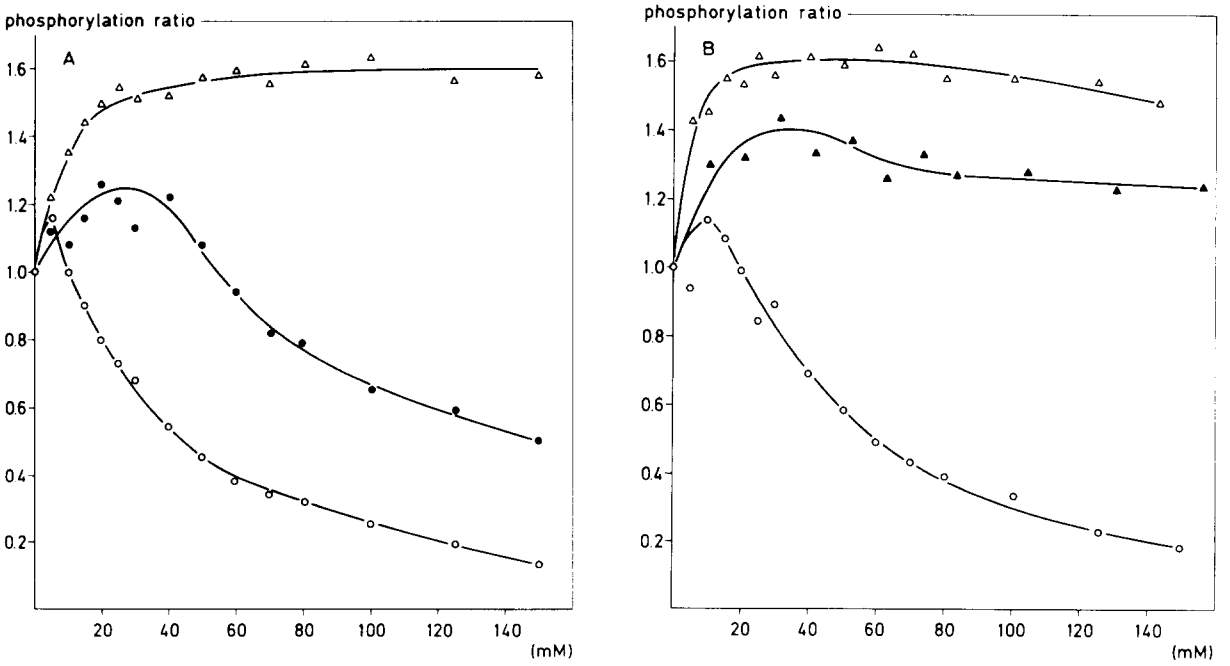


Fig. 8. Effect of increasing allyl (A) or propyl substitution (B) on phosphorylation. Phosphorylation occurs in the presence of 50 mM imidazole and the level is expressed as ratio of the control (cf. Fig. 3). Meaning of symbols in (A): ○, allylamine; ●, diallylamine; and △, triallylamine; and (B): ○, propylamine; △, tripropylamine; and ▲, tetrapropylamine. Contaminating concentration of Na^+ ranges from less than 0.6 up to 4.3 μM, and for K^+ from less than 0.6 up to 1.9 μM per 50 mM of the amine.

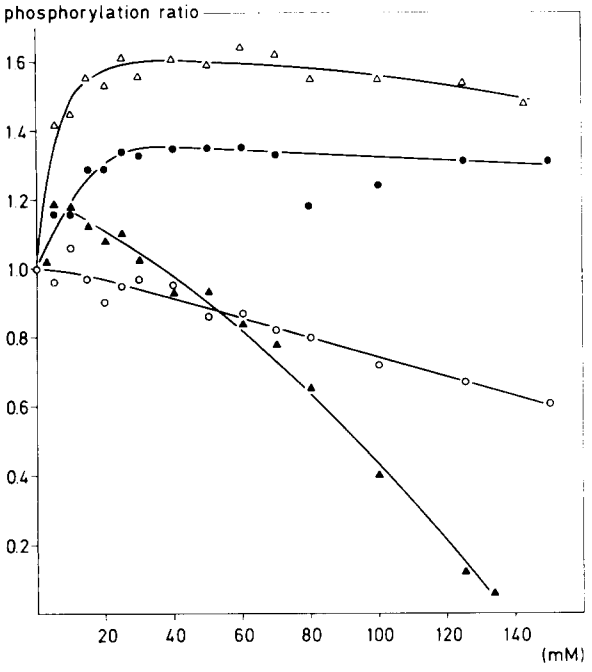


Fig. 9. Effect on phosphorylation of increasing the chain length in trialkyl substitution. Phosphorylation takes place in and is expressed as ratio of the level in 50 mM imidazole. Meaning of symbols: ○, trimethylamine; ●, triethylamine; △, tripropylamine; and ▲, tributylamine. Contaminating concentration of Na^+ is 3.3–18.6 μM and for K^+ 1.7–3.0 μM per 50 mM of the trialkylamine.

The alkyl chain length effect

Not all triple alkyl chain substitutions of the nitrogen atom lead to an optimal phosphorylation. Fig. 4 shows that triple hydroxyethyl substitution leads to an inert compound, whereas triple propyl or allyl substitution leads to a very effective stimulation of phosphorylation (Fig. 8). This hints us to the idea that the length of the alkyl chain has a profound influence on the effectiveness of stimulation. For this reason, we investigated the effect of increasing the chain length from methyl up to butyl in trialkyl substitution of the amino group.

Although trimethyl substitution is insufficient to give stimulation, triethyl substitution is the minimal condition in this series of homologues to provide for some intermediary stimulation and tripropyl substitution for a maximal effect (Fig. 9). A further increase of the alkyl chain length in tributyl amine gives, after a transient stimulation of the extent as given by aliphatic primary amines, mere inhibition. The I_{50} value of 93 mM is, however, higher than that of the aliphatic primary amines (44–60 mM, Figs. 3 and 8).

Effects in the absence of imidazole

When the additional amine would compete with the imidazole in the assay medium and would be less effective but stimulate on its own, one would have a situation in which the amine might inhibit in the presence but stimulate in the absence of imidazole. On the other hand, the additional amine might only activate in the presence of another amine like imidazole, provided that imidazole acts as an allosteric activator. Finally, imidazole might quench activation by the second amine and yield an inert buffer.

Candidates for each effect have been tested in the absence of imidazole, viz., the inhibitory Tris, the almost inert triethanolamine and the purely stimulatory triallylamine. In 50 mM concentrations they stimulate to 4, 17 and 90% of the maximal Na^+-activation level, respectively. In other words, these buffers keep the characteristics they had in the presence of imidazole also in the absence of imidazole.

Effects on the $E_2 \rightarrow E_1$ transition and on dephosphorylation

Effects on partial reactions preceding (the conformational $E_2 \rightarrow E_1$ transition) and following upon the primary phosphorylation step (the conformational $E_1 \sim P \rightarrow E_2P$ transition and the dephosphorylation step $E_2P + H_2O \xrightarrow{K^+} E_2 + P_i$) also affect the phosphorylation level. A number of amino compounds: Tris, ethylenediamine, triethanolamine, triallylamine, imidazole, 5-chloro-1-methylimidazole, and 2,4-dimethylimidazole have been tested on the $E_2 \rightarrow E_1$ conformational change at 20 °C and pH 7.0, using eosin as fluorescent probe for the E_1 conformation [11,12]. They all stimulate the transition with rate constants in the same order of magnitude as for Na^+ (Van Uem et al., unpublished data). Hence, within this series of buffers inhibition of phosphorylation cannot have been caused by a slow down of the preceding conformational transition.

On the other hand, enhancement of the phosphorylation level could have been caused by an inhibition of dephosphorylation, and a decrease of

this level by an activation of dephosphorylation. In a previous publication [7], we have shown that amino compounds of either category in phosphorylation, such as imidazole and Tris, reduce the dephosphorylation of E_2P and counteract activation by K^+ and Mg^{2+}. This and the fact that imidazole is more effective than Tris have recently been confirmed by Fukushima [13]. Therefore it appeared of interest to test the effect of triallylamine (40 mM) as optimal activator of phosphorylation and that of ethylenediamine (0.5 mM) as optimal inhibitor of phosphorylation on the dephosphorylation. Chasing with an excess of high (0.5 mM) or low (5 μM) concentration non-radioactive ATP in the presence or absence of ethylenediamine according to previously published methods [7,14] leads to identical conclusions. In triallylamine, the rate constant of dephosphorylation (23°C, pH 7.0) 0.026 s^{-1} (0.5 mM ATP), 0.074 s^{-1} (5 μM ATP) is 2–2.6 times lower than in imidazole (50 mM) under the same conditions. Ethylenediamine does not significantly change the rate constant of dephosphorylation in triallylamine, just as Tris did not in imidazole [7].

Hence, the conclusion appears justified that inhibition of dephosphorylation at least partly may have caused the high phosphorylation levels observed in the presence of certain amino-group-containing compounds such as triallylamine. On the other hand, the strong inhibition exerted by certain primary amines like ethylenediamine appears to be primarily directed to the phosphorylation step and not be caused by an increase of dephosphorylation.

Discussion

Stimulation and inhibition by amines of phosphorylation in $(Na^+ + K^+)$-ATPase is a common and fairly aspecific phenomenon. This does not mean, however, that no line of relative specificity and of underlying rules can be detected. We think it proper to summarize these.

(1) Aliphatic primary amines are predominantly inhibitory apart from a transient slight stimulation at low (10 mM) concentration. Inhibition parallels a decrease in affinity for Na^+, indicating involvement of the Na^+ activation sites. Among them the diamines are stronger inhibitors than the monoamines with an absolute culmination found for ethylenediamine and derivatives, displaying a $N \rightarrow N$ distance in the predominant anticonformation of 3.7 Å. For the apparent competitive ethylenediamine Na^+ antagonism see Ref. 15. Increasing the distance 2.3-fold by following the homologous series to 1,6-diaminohexane increases the I_{50} value 180-fold. This suggests that ethylenediamine may bridge activating sites which are 3–4 Å apart.

(2) Substitution or shielding of the N-atom leads to lower inhibition, i.e., a decreased fit to the inhibitory site. Inhibition is converted into stimulation, which becomes optimal in triple propyl or allyl group substitution of the central N-atom (95% of the Na^+-activated level). Second-best activations are by N-screened pyridine derivatives (e.g., 2,4,6-trimethylpyridine) and imidazole derivatives. Methyl group substitutions to the imidazole ring have little effect but ethyl group substitutions lead to lower activation, presumably by steric hindrance. Substitution by polar groups (acetyl, hydroxyethyl or the morpholine group), however, also tends to remove activation. A striking example is the almost ineffective tris-hydroxyethyl substituted triethanolamine (phosphorylation ratio 1.11) vs. the more apolar triethylamine (phosphorylation ratio 1.35). As another example may serve the absence of any stimulation at low (10 mM) concentration by the polar tris(hydroxymethyl)methyl group substituted Tris vs. the 10 mM stimulation by the more apolar propyl- or allylamine.

(3) Inhibition and activation each require a charged amine, indicating Coulomb interaction with a negative glutamic or aspartic acid residue, or hydrogen bonding. That interaction with a negatively charged carboxyl group is involved is suggested by the reduction or even abolishment of inhibition caused by the additional presence of a carboxyl group in the amino compound (*N*-tris(hydroxymethyl)methylglycine, histidine) as this substitution may cause charge repulsion. The order of inhibitory or activating potency that follows the degree of protonation of the compound (ethylenediamine derivatives in Table II.1, imidazole derivatives in Fig. 5, cf. Table III.2) is another indication for charge interaction being one of the underlying principles. Inhibition by the

uncharged 5-chloro-1-methylimidazole is an exception that may be caused by other forms of binding (e.g., via π–π electron interaction). Elongation of the *N*-trialkyl group substituents to C_3 decreases the Coulomb interaction while increasing the hydrophobic interactions leading to optimal activation by tripropyl- and triallylamine. Further elongation of the chain length in tributylamine again inhibits and may, via a detergent-like effect, interfere with the protein–lipid interaction essential for enzyme activity.

The above findings and rules may be explained on a more general basis as follows. The site of inhibition is less openly exposed than the site of activation. This explains why those buffers, which exert either action, activate at low but inhibit at high concentration. It also explains why inhibition by amines is strongly size dependent as well as dependent on the degree of shielding of the central nitrogen atom. If the site of inhibition is hidden and indeed turns out to be the Na^+-activation site this would thus mean that the Na^+-activation site is located more in the interior of the enzyme than the amino-buffer activation site. The site of inhibition being the Na^+-activation site, would also fit in which the idea (argument No. 3) that Coulomb interaction, rather than hydrogen bonding, causes inhibition. Na^+ undoubtedly will activate via negatively charged carboxyl groups. Further evidence comes from preliminary studies on the sidedness of inhibition which turns out to be at the cytoplasmic side (Van der Hijden et al., unpublished data), i.e., the side where Na^+ activates [16].

Amino-buffer activation, however, may bear on another principle. Apart from the feasibility of stimulation via speeding up of the transition from the non-phosphorylating E_2 to the phosphorylating E_1 conformation or the phosphorylation step per se, the amines may enhance the phosphoenzyme level via inhibition of dephosphorylation [7] and even Na^+ may do so [13]. The sidedness of activation by Na^+ is, however, opposite to that by amines, the latter of which enhance the phosphoenzyme level extracellularly (Van der Hijden et al., unpublished data). Hence, the lowering of $[Na^+]_{0.5}$ by Tris or imidazole, as observed in Fig. 1, is probably allosteric. Lipophilic interactions as exerted by tripropyl- and triallylamine conceivably hinder access of water to the site of hydrolysis and these amines are sufficiently screened by substitution to have little access to the Na^+-activation site. The latter is true also for amines that are substituted by polar groups like triethanolamine, but their polarity does not prevent water from access to the site of phosphoenzyme hydrolysis, hence no activation nor inhibition of phosphorylation takes place. The proposed polarity effect on dephosphorylation is also strongly supported by the absence of any inhibition of dephosphorylation in histidine buffer [13]. Meanwhile, the existence of inert though fully charged buffers (Tricine, triethanolamine) indicates the absence of a ionic strength effect in the concentration range used.

Similar effects of solvents on phosphoenzyme hydrolysis have been reported in the past. Organic solvents like acetone and butanol inhibit spontaneous as well as K^+-activated phosphoenzyme hydrolysis. They also stimulate rephosphorylation of E_2Rb and thus deocclude Rb^+ and convert E_2 into E_1 [17]. More recently, the phenomenon of lipophilic interaction of tetraalkylammonium derivatives with the red cell ($Na^+ + K^+$)-pump has been analyzed thermodynamically by Klein and Ellory [18] in terms of free energy of cavity formation (surface area term) and conformational change (hydrocarbon rotation term). The calculated values are in quantitative agreement with free energies of inhibition of K^+ transport, which may be related to the common inhibitory effect of amines on K^+-activated phosphoenzyme hydrolysis [7] or due to inhibition of phosphorylation by a detergent-like action as proposed for inhibition by the higher homologues of the trialkyl series. On the other hand, it would be curious if the stimulatory effect of the more polar imidazole and pyridine derivatives is due to lipophilic interactions only. They might interact, however, via π–π electron interaction with double bonds in the membrane.

Particularly promising for tracing of the Na^+-activation sites at the inner membrane face is inhibition by ethylenediamine because of its low dissociation constant (approx. 50 μM, Schuurmans Stekhoven et al., unpublished data) and the possibility of covalent coupling. Interaction with the Na^+-activation site is suggested by its competitive effect on Na^+ [15], which is shared by

harmaline [19] and spermine [20], being an ethylenediamine and a propanediamine derivative, respectively. These compounds also inhibit, like the other amines, the activation by K^+ on the outside of the membrane [19,20]. Hence ethylenediamine may prove to be a useful chemical in the elucidation of cation activation sites, just like fluorescein isothiocyanate has been proven useful in the elucidation of the ATP binding centre [21,22].

In conclusion: although inhibition and activation by the amines may parallel positive charge, size and chemical composition of the substituent groups is of even more striking influence.

Acknowledgement

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