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Sidedness of the effect of amines on the steady-state phosphorylation level of reconstituted Na^+/K^+-ATPase

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The sidedness of the effects of several amines on the steady-state phosphorylation level of rabbit kidney Na^+/K^+-ATPase has been studied with the enzyme incorporated in phosphatidylcholine-cholesterol containing proteoliposomes. The presence of ouabain prevented phosphorylation of non-incorporated or rightside-out incorporated enzyme, so that only the inside-out incorporated Na^+/K^+-ATPase molecules were studied. Addition of either Na^+ or several amines to the extracellular side of the enzyme led to an enhancement of the steady-state phosphorylation level obtained with optimal concentrations of Na^+, Mg^{2+} and ATP at the cytosolic side. The series imidazole > Na^+ > triallylamine > Tris > ethylenediamine showed a decrease in affinity. Histidine, sorbitol and choline chloride had no effect at the extracellular side. This means that in addition to the well-known cytosolic ligands either Na^+ or a positively charged amine buffer has to be present extracellularly in order to obtain an optimal phosphorylation level. At the cytoplasmic side the tested amines exerted different effects. (i) Imidazole and triallylamine enhanced the steady-state phosphorylation level when the extracellular conditions were optimal (saturating amine concentration). (ii) Tris and ethylenediamine decreased the steady-state phosphorylation level and (iii) histidine had no effect. The cytoplasmic effects of the amine compounds correlate with those described by Schuurmans Stekhoven et al. (Biochim. Biophys. Acta 937 (1988) 161–171) for the unsided preparation. The extracellular effects, however, are apparently masked in experiments with fragmented enzyme preparations and are assumed to be potentiating effects which make the enzyme ready for phosphorylation upon a cytoplasmic trigger (e.g. Na^+).

Introduction

Na^+/K^+-ATPase as a member of the 'P'-type ATPase [1] forms a covalent phosphorylated intermediate as part of its reaction cycle. The phosphorylation reaction has extensively been studied [2]. It has become clear that the presence of Mg^{2+} and Na^+ [3,4] besides the substrate is a prerequisite for this reaction. In a recent study [5], however, the enhancement of the phosphorylation level by imidazole in the absence of Na^+ was reported. From this report it was clear that imidazole was able to substitute for Na^+ in the phosphorylation reaction of the Na^+/K^+-ATPase, but the mechanism of this phenomenon remained obscure. In a further study [6] some other amine compounds were found to enhance the phosphorylation reaction, whereas others were without effect or even inhibitory versus Na^+ or other stimulating compounds. This made the picture even more complex.

The assignment of the sites of action of those compounds could give some clues about a possible mechanism. Since the interaction of those charged compounds is probably mainly electrostatic it is difficult to derive information from binding studies. So far binding studies were not conclusive. An other approach to obtain more insight in this process is to study the sidedness of action of the amine compounds.

In a recent paper [7] we presented a study on the sided effects of physiologically important ions with respect to the phosphorylation reaction in reconstituted Na^+/K^+-ATPase. With this preparation it appeared to be possible to obtain information about the sidedness of

Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenedinitrotetraacetic acid; K_{cyt}, Na_{cyt} and Mg_{cyt} represent K^+, Na^+ and Mg^{2+} concentrations at the cytosolic side, respectively. K_{ext}, Na_{ext} and Mg_{ext} represent these concentrations at the extracellular side.

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action of the amine compounds. With this sided preparation the cytoplasmic and extracellular effects could be separated. By this separation stimulatory and inhibitory effects turned out to be located on different sides of the membrane and could be studied separately.

A model for the mechanism of action of the amine compounds is proposed. This model throws some light on observations in unsided preparations which are difficult to interpret.

Materials and Methods

Preparation of Na^+/K^+-ATPase

Na^+/K^+-ATPase from rabbit kidney outer medulla has been prepared according to the method described by Jørgensen [8]. About 340 mg microsomes (on protein base) were incubated for one hour at 20 °C in a medium containing 0.56 mg/ml sodium dodecyl sulfate and 25 mM imidazole-HCl (pH 7.4), 3 mM ATP and 2 mM EDTA (final protein concentration 1.45 mg protein per ml). After the extraction the microsomal suspension was centrifuged on a sucrose gradient (0–50%). The ATP of the pooled fractions of the gradient was removed by incubation at 37 °C in presence of Na^+, Mg^{2+} and K^+ and subsequent washing. The obtained membrane fragments, enriched in Na^+/K^+-ATPase, were stored in imidazole buffer (25 mM, pH 7.4) containing 10% sucrose. The specific Na^+/K^+-ATPase activity of the preparations ranged from 1.0 to 1.6 mmol P_i formed per mg protein per hour.

Preparation of liposomes

Liposomes have been prepared by a reversed phase evaporation method as described by Szoka and Papahadjopoulos [9]. Mixtures of cholesterol, phosphatidylcholine and phosphatidylserine (ratio indicated in text and legends) in chloroform were evaporated under a stream of nitrogen to remove the organic solvent. After repeated washing with diethylether, a 1:1 mixture of diethylether and buffer solution of different composition was added and the solution thoroughly mixed on a Vortex mixer, while the diethyl ether was again slowly evaporated by a stream of nitrogen. The final lipid content was between 20 and 50 mg/ml. After all ether had disappeared the liposomes formed were sonicated for 30 min in a Branson sonicator bath at maximal output.

Reconstitution procedure

Purified Na^+/K^+-ATPase (5 mg/ml) in 20 mM imidazole buffer (pH 7.2) was partially solubilized by incubation with cholate (final concentration 0.91% w/v) during 1 min at room temperature. This mixture was then added to a 10-fold volume of liposomes, giving a lipid to protein ratio of 40 to 100 (on weight basis). After thorough mixing, the preparation was frozen in liquid nitrogen or in a mixture of dry ice and acetone and subsequently thawed at room temperature. This freezing and thawing procedure was repeated twice. Thereafter the vesicle suspension was sonicated for 6 min in a Branson sonicator bath (maximal output). Detergent was removed from the proteoliposomes by centrifuging aliquots of the suspension over a 10-fold volume Sephadex G-25 (coarse) column (equilibrated with the appropriate buffer solution in a syringe). This centrifugation step lasted 5 min ($100 \times g$) and was repeated once. More than 99.9% of the cholate was removed by this procedure [10].

ATP hydrolysis

The ATP hydrolysis was determined as the release of $^{32}P_i$ from [γ-^{32}P]ATP [11]. To 10 μl proteoliposomes, containing compounds as indicated in the legends, 190 μl medium containing ouabain (0.2 mM), 1 mM Mg^{2+} and 1.0 μM labeled ATP were added at 20 °C and incubated for 10–30 s. For blank values the reconstituted Na^+/K^+-ATPase was denatured with trichloroacetic acid prior to incubation with the hydrolysis medium. The $^{32}P_i$ production was measured after stopping the reaction at a given time through addition of 0.4 ml 10% trichloroacetic acid followed by mixing with 0.4 ml 20% (w/v) aqueous charcoal suspension. The charcoal adsorbs the adenosine phosphates from the medium, but leaves P_i in solution. The suspension was mixed thoroughly repeatedly (three times) for 10 s every 5 min. Thereafter the charcoal was sedimented by centrifugation for 10 min at $2000 \times g$ at 0 °C. Aliquots (0.2–0.5 ml) were taken from the supernatant, mixed with 4.5 ml liquid scintillation fluid (Aqualuma Plus). Radioactivity was measured with a liquid scintillation counter.

Phosphorylation

Phosphorylation of the reconstituted Na^+/K^+-ATPase was carried out at 22 °C at pH 7.0. The ATP concentrations varied between 0.2 and 20 μM (The Radiochemical Centre, Amersham, U.K., specific radioactivity 3000 Ci/mol). The reaction was started by rapid mixing of 10 μl proteoliposomes (preincubated with 0.2 mM ouabain and 10 mM Mg^{2+}) with 90 μl of the medium containing ATP and the other ligands. The reaction was stopped after 3 s by addition of 3 ml 5% (w/v)trichloroacetic acid, containing 100 mM phosphoric acid. The denatured phosphoprotein was filtered on a 1.2 μm pore width Selectron filter (Schleicher and Schull, Dassel, F.R.G.), which was then washed three times with 3 ml of the stopping solution. Incorporated ^{32}P was determined by liquid scintillation counting. For blank values the proteolipesomes were mixed with the stopping solution prior to addition of the ATP solution.

Dephosphorylation

After phosphorylation during 10 s at room temperature 900 μl of the dephosphorylation medium was ad-

ded to the phosphorylation mixture (100 μl). The dephosphorylation mixture contained, apart from the buffer and cations, 1 mM unlabeled ATP in order to dilute the labeled ATP 1000-fold. Together with the 10-fold dilution of the volume of the medium the final dilution of the labeled ATP was 10000 in order to avoid further phosphorylation by the labeled ATP. After rapid mixing the dephosphorylation reaction was stopped (at the time indicated) by addition of 5 ml 5% (w/v) trichloroacetic acid containing 100 mM phosphoric acid. After stopping the reaction, the mixture was further treated as described in the phosphorylation procedure.

Materials

ATP and Tris were purchased from Boehringer, Mannheim, F.R.G. [γ-^{32}P]ATP and ^{86}Rb were obtained from Amersham, Buckinghamshire, U.K.; phosphatidylcholine (egg) and phosphatidylserine (bovine brain) were purchased from Avanti Polar Lipids, Birmingham, AL, U.S.A., and cholesterol, from Sigma, St. Louis, MO, U.S.A. All other chemicals were of reagent grade.

Results

Effect of extracellular amine buffers and Na^+ on the steady-state phosphorylation level of Na^+/K^+-ATPase containing proteoliposomes

Proteoliposomes, loaded with 100 mM Na^+, 10 mM Tris (pH 7.0) and 82 mM sorbitol, exhibited a maximal phosphorylation level under optimal cytoplasmic phosphorylation conditions (see below). This level could not be further increased by any change in the extracellular (intravesicular) composition, but could be decreased by lowering the extracellular (intravesicular) Na^+ concentration. Replacing the extracellular Na^+ by sorbitol under the above mentioned phosphorylation conditions at the cytoplasmic (extravesicular) side resulted in a 55% reduction of the steady-state phosphorylation level (Fig. 1). The enhancement of the phosphorylation level by intravesicular Na^+ was half maximal at about 1 mM. A further (25%) decrease of the steady-state phosphorylation level occurred when the 10 M intravesicular Tris was also replaced by sorbitol. In the absence of extracellular (intravesicular) Na^+ and Tris and in the presence of 300 mM intravesicular sorbitol only 20–30% of the above mentioned optimal phosphorylation level was obtained.

Increasing the intravesicular Tris concentration up to 100 mM stimulated the phosphorylation to the same level as obtained with intravesicular Na^+. The $K_{0.5}$ for Tris was about 6 mM (Fig. 1). Fig. 1 also shows that the effect of the extracellular ligands was not due to a change in ionic strength of the intravesicular medium. Increasing the ionic strength of the intravesicular medium by replacing extracellular sorbitol by choline chloride did not increase the steady-state phosphorylation level above the level with extracellular sorbitol alone.

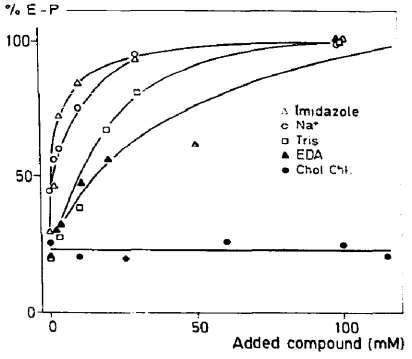

Fig. 1. Enhancement of the steady-state phosphorylation level by extracellular compounds. Na^+/K^+-ATPase reconstituted in liposomes (phosphatidylcholine 26, phosphatidylserine 2 and cholesterol 20 mg/ml) containing the compounds in concentrations as indicated in the figure (pH 7.2) were preincubated with ouabain (0.2 mM; 60 min at room temperature) in the presence of 10 mM Mg^{2+} and 150 mM Tris (pH 7.2). Sorbitol was added intravesicularly to maintain osmolarity. The proteoliposomes (protein content 0.5 mg/ml) were then phosphorylated with 1.0 μM labeled ATP in the presence of 100 mM Na^+, 3 mM Mg^{2+} and 51 mM Tris (pH 7.0). Further details of the phosphorylation reaction are described in Materials and Methods. The $K_{0.5}$ values for imidazole (△), Na^+ (○), Tris (□), ethylenediamine (EDA) (▲) and choline chloride (●) were obtained by Lineweaver-Burk and Scatchard analysis and given in the text. In the series with extracellular Na^+ 10 mM extracellular Tris was also present.

Other buffers tested exhibited a similar trans-effect on the steady-state phosphorylation level as Tris and Na^+. Imidazole and ethylenediamine (EDA) increased the phosphorylation level from the extracellular side with $K_{0.5}$ values of about 1 and 5 mM, respectively (Fig. 1). Triallylamine (TAA) also increased the phosphorylation level from the extracellular side, but unlike the other buffers tested above not according to the Michaelis-Menten formalism. After enhancement of the phosphorylation level by intravesicular triallylamine with a half maximal effect of 2–3 mM, a further increase of the intravesicular triallylamine concentration resulted in a considerable decrease of the phosphorylation level (Fig. 2), to be compared with the secondary inactivation also encountered in the open membrane system [12]. The maximal level of all the buffers tested however was the same as the maximal level obtained with intravesicular Na^+. The only buffer tested so far, which failed to increase the phosphorylation level from the extracellular side, was histidine (Fig. 2). Increasing the intravesicular histidine concentration up to 50 mM

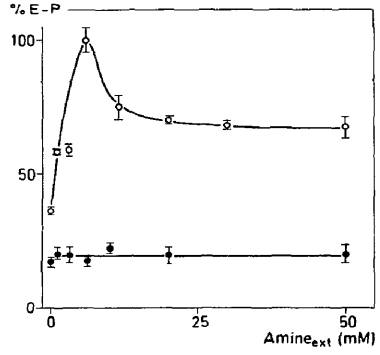

Fig. 2. The effect extracellular triallylamine (TAA) and histidine on the steady-state phosphorylation level. Proteoliposomes with the same lipid composition as described in Fig. 1, loaded with triallylamine (TAA) and histidine in the indicated concentrations were phosphorylated under the same conditions as described in Fig. 1. In the proteoliposomes containing triallylamine (○) 10 mM Tris (pH 7.0) was also present. The phosphorylation level of the proteoliposomes containing histidine are presented by (●). The symbols represent the mean ± S.D. of three experiments carried out in duplicate.

did not alter the steady-state phosphorylation level above the 20% obtained in the absence of intravesicular buffers.

The dephosphorylation rate of the phosphorylated intermediate in the presence of a low (10 mM) extracellular Tris concentration was not significantly different from that in the presence of a high (180 mM) extracellular Tris concentration: 0.25 ± 0.06 and 0.27 ± 0.07 s^{-1}, respectively. Furthermore the Na^+-dependent ATPase activity was linearly related to the phosphorylation level, independent from the type of amine or Na^+ extracellularly present (Fig. 3). This made the slow (1.66 ± 0.19 s^{-1}, at 20°C) turnover of the enzyme (P_i production per phosphorylated intermediate) independent of the phosphorylation level.

Effects of cytoplasmic (extravesicular) amine buffers on the steady-state phosphorylation level

When Na^+/K^+-ATPase, reconstituted in lipid vesicles loaded with 165 mM Tris (pH 7.0), was phosphorylated with labeled ATP the obtained steady-state phosphorylation level increased with the cytoplasmic Na^+ concentration [7]. The phosphorylation level in the presence of cytoplasmic Tris and in the absence of added Na^+ depended on the cytoplasmic ATP and Mg^{2+} concentrations [7]. At low free Mg^{2+} concentrations the level was high and diminished above 3 mM Mg^{2+}. In the presence of high (5 mM) Mg^{2+} and low (1

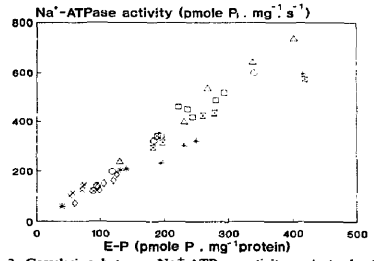

Fig. 3. Correlation between Na^+-ATPase activity and steady-state phosphorylation level. Proteliposomes as described in Fig. 1 containing Na^+ (△), Tris (○), imidazole (⧖), triallylamine (□), ethylenediamine (*), histidine (×) and choline chloride (◇) in the concentration ranges as shown in Figs. 1 and 2 were phosphorylated as described in Fig. 1 and Na^+-stimulated ATP hydrolysis was determined as described in Materials and Methods.

μM) ATP and in the absence of Na_{cyt} no influence on the phosphorylation level by variation of cytoplasmic Tris was observed (sorbitol being used to maintain isoosmolarity). Under these conditions the obtained phosphorylation level was low (only 2% of the maximal level with 100 mM Na_{cyt}). Imidazole and triallylamine in contrast to Tris markedly enhanced the steady-state phosphorylation level to 11 and 45% of the maximal

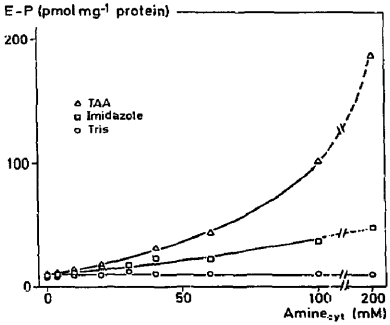

Fig. 4. Influence of cytoplasmic amine compounds on the steady-state phosphorylation level. Proteoliposomes as described in the legend of Fig. 1 loaded with 200 mM Tris (pH 7.0) were phosphorylated as described in Materials and Methods in the absence of Na_{cyt} and in the presence 1 μM ATP, 5 mM Mg^{2+} and the amino compounds as indicated; triallylamine (△), imidazole (□) and Tris (○). The phosphorylation level obtained with saturating Na_{cyt} was 610 pmol/mg protein. Sorbitol was added to the extravesicular medium to maintain osmolarity.

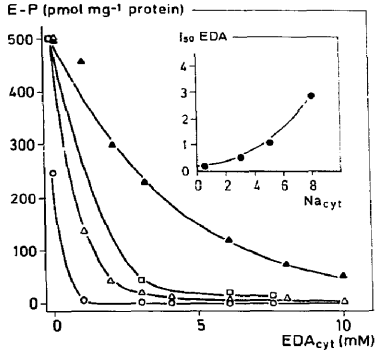

Fig. 5. Decrease of the steady-state phosphorylation level by cytoplasmic ethylenediamine (EDA) in the presence of different Na_{cyt} concentrations. Proteoliposomes as in Fig. 3 were phosphorylated as described in Fig. 3. The cytoplasmic medium contained ethylenediamine in the indicated concentrations and 0.5 (○), 3 (△), 5 (□) and 8 (▲) mM Na^+. The inset shows the dependency of the I_{50} value of ethylenediamine on the Na_{cyt} concentration.

level obtained with 100 mM cytoplasmic Na^+, respectively (Fig. 4). As with the effects of the amine buffers at the extracellular side, the influence on the phosphorylation level of these ligands at the cytoplasmic side was not due to ionic strength effects, as proven by choline chloride. Increasing the ionic strength along with the choline chloride concentration at the cytoplasmic side had no effect on the steady-state phosphorylation level (sorbitol being used to maintain isoosmolarity). Moreover histidine was also without effect on the steady-state phosphorylation level when added at the cytoplasmic side under the same conditions as choline chloride and the other buffers (not shown).

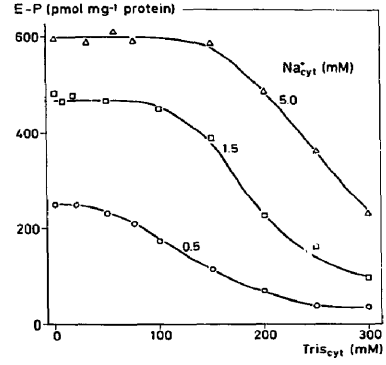

Fig. 6. The effect of cytoplasmic Tris on the steady-state phosphorylation level with different Na_{cyt} concentrations. Proteoliposomes (as in Fig. 4) loaded with 300 mM Tris (pH 7.0) were phosphorylated as described in Fig. 4. The cytoplasmic medium contained Na^+, 0.5 mM (○), 1.5 mM (□) or 5.0 mM (△) and Tris in the indicated concentrations. Sorbitol was added to the extravesicular medium to maintain the osmolarity of 540 mosM.

Ethylenediamine at the cytoplasmic side, in contrast to the former ligands, strongly reduced the phosphorylation level (Fig. 5)). The I_{50} of ethylenediamine was about 0.2 mM in absence of cytoplasmic Na^+ (derived by extrapolation of a plot of the I_{50} value of ethylenediamine as a function of cytoplasmic Na^+ (inset Fig. 5) and increased strongly with the Na^+ concentrations (Fig. 5). At low Mg^{2+} and high cytoplasmic ATP concentrations ethylenediamine also strongly reduced the phosphorylation level in the absence of added Na_{cyt} and also reduced the affinity for Na_{cyt}. The $K_{0.5}$ for Na^+ under these conditions was 1 mM in absence of ethylenediamine and increased to 2 and 20 mM in presence of 2 and 20 mM ethylenediamine, respectively. Cytoplasmic Tris had no effect on the phosphorylation level in the absence of Na_{cyt} but reduced the steady-state phosphorylation level in the presence of low cytoplasmic Na^+ concentrations (Fig. 6).

Discussion

Sidedness of the effect of amine compounds on the phosphorylation reaction of Na^+/K^+-ATPase

Recent work from several authors has demonstrated that amine compounds have an effect on the steady-state phosphorylation level of Na^+/K^+-ATPase [5,6,13–15]. Much has been speculated on the mechanism, but no general concept holds for the whole of these effects yet. More insight in the mechanism could be obtained by determination of the site of action of these amine compounds. Binding experiments of the amines to the enzyme could give some clues, but binding studies with ethylenediamine [12] have not elucidated this question so far.

Determination of the sidedness of action of these amines is an other possibility to get more insight in this matter. From the experiments with reconstituted Na^+/K^+-ATPase described in this paper it has become clear, that in addition to Na^+ all amine buffers tested at the extracellular side (except for histidine which has no effect at all) have a stimulatory effect on the steady-state phosphorylation level. At the cytoplasmic side, however, some amines inhibit and others stimulate steady-state phosphorylation. Amine compounds which are inhibitory in phosphorylation experiments with broken membrane sheets (ethylenediamine and Tris) appeared to be

also inhibitory at the cytoplasmic side, whereas compounds enhancing the phosphorylation reaction in open membrane preparations (imidazole and triallylamine) stimulate the phosphorylation reaction from the cytoplasmic side. Histidine, which has no effect on the phosphorylation of Na^+/K^+-ATPase containing membrane sheets and on the induction of conformational changes [16,17] was also inert in the phosphorylation of reconstituted Na^+/K^+-ATPase from both sides of the membrane. Thus the effect of the amines at the cytosolic side seems to be more important for the inhibitory effect in broken membrane sheets whereas the effect at the extracellular side may be the cause for the observed stimulatory effects observed in the unsided preparation.

An explanation for the inertness of histidine has been proposed by Schuurmans Stekhoven et al. [6]. According to these authors the interaction of the positively charged amines are mediated by Coulomb forces with negatively charged groups in the enzyme. The negatively charged carboxyl group of histidine, however, may cause charge repulsion with the enzyme. The experiment with reconstituted Na^+/K^+-ATPase described in this paper, showing that histidine fails to stimulate at the extracellular side and is without effect at the cytoplasmic side, suggested that this explanation holds for interactions at both sides of the membrane.

Mechanism of action of the amine compounds

The results of the experiments with the reconstituted Na^+/K^+-ATPase give more insight in the possible mechanisms by which the amine compounds exert their effect on the phosphorylation reaction of Na^+/K^+-ATPase. Since the steady-state phosphorylation level depends upon the equilibrium between formation and hydrolysis of the phosphorylated intermediate the effect can be explained by either stimulation or inhibition of one of these two reactions.

A. Extracellular effects

Since the extracellular effects, when present are all stimulatory the dephosphorylation reaction must be inhibited or the phosphorylation step must be enhanced. Both possibilities are considered below.

(i) Inhibition of the dephosphorylation by extracellular amine compounds. Two possible mechanisms for the inhibition of the dephosphorylation by amines at the extracellular side are proposed. The first one is based on an observation of Nøby et al. [18] that Tris inhibits the conformation change from E_1P to E_2P and by our observation [7] with reconstituted Na^+/K^+-ATPase that extracellular Tris and Na^+ seem to enhance the steady-state phosphorylation level possibly by inhibition of this conformational change. From the experiments described in this paper it has become clear that most other amines share this property with Tris. Alternatively the inhibition of the dephosphorylation step can be caused by screening off the K^+ sites by the extracellular amine compounds [13]. In this view the amine compounds prevent the residual K^+ from binding to its dephosphorylating site and so reduce the rate of the spontaneous dephosphorylation (i.e., in the absence of added K^+). This spontaneous dephosphorylation must here be seen as the reaction catalyzed by low concentrations of residual K^+. The influence of extracellular Na^+ on the dephosphorylation rate in an unsided preparation cannot be studied since it is impossible to exclude cytoplasmic effects. There is no concensus in the literature on this matter. Some authors reported a decrease in the dephosphorylation rate either by low concentrations of extracellular Na^+ [28] or by high Na^+ concentrations [14,29]. Other authors [13,30] observed an increase in the dephosphorylation rate at high Na^+ concentrations, whereas in one study [31] no influence on the dephosphorylation rate at different Na^+ concentrations was found. The influence on the dephosphorylation rate of extracellular buffers is also difficult to check, because of the low phosphorylation levels at low extracellular buffer concentrations and because of the impossibility of changing the extracellular (intravesicular) composition during the dephosphorylation reaction. The dephosphorylation rate of proteoliposomes containing high and low Tris concentrations appeared to be equal. This indicates that the increase of the phosphorylation level due to higher extracellular (intravesicular) Tris concentrations was not caused by accumulation of the phosphointermediate due to a slower breakdown. Furthermore the turnover number of the phosphorylated enzyme was independent of the ionic composition of the extracellular medium. This observation confirms the former observation that the dephosphorylation rate is not influenced by extracellular amines.

(ii) Stimulation of the phosphorylation reaction. Extracellular amine compounds and Na^+ can induce a conformation suited for phosphorylation (an E_1 like conformation), as already has been proposed before [5]. According to results of Rephaeli et al. [19], this conformation is not the conventional E_1 conformation as monitored by FITC fluorescence. In their experiments with reconstituted Na^+/K^+-ATPase extracellular Na^+ had no effect on the FITC fluorescence. In contrast to this observation, however, this conformation might be the same as the Na^+-form which can be monitored by eosin fluorescence [20] since ethylenediamine increases the eosin fluorescence although this compound decreases the steady-state phosphorylation level in unsided preparations [6]. Further support comes from experiments with FITC-labeled enzyme. An increase of the fluorescence of the labeled enzyme by ethylenediamine was observed recently [21]. In addition we have observed some increase in eosin fluorescence by extracellular ethylenediamine in reconstituted Na^+/K^+-ATPase (Van der Hijden, H.T.W.M., unpublished re-

sults). The conformation induced by extracellular amine compounds and Na_{ext} in this study is therefore supposed to be an E_1 or E_1-like conformation which needs a cytoplasmic trigger like high ATP concentrations, Na^+ or amine compounds to be phosphorylated [7].

B. Cytoplasmic effects

Amines at the cytoplasmic side of the membrane show a different behaviour. Some compounds like triallylamine and imidazole are stimulatory whereas others like ethylenediamine and Tris are inhibitory and competitive to Na^+. Histidine is inert with respect to the phosphorylation reaction. For the stimulating compounds a mechanism as proposed by Fukushima [14] can be valid. This author proposed that Na^+ and other positively charged compounds may bind to a carboxyl group of the enzyme responsible for the acceptance of a proton of a water molecule. The binding of the positively charged compound prevents the formation of the hydroxyl ion as a good nucleophile for dephosphorylation. This results in a retardation of the dephosphorylation rate and in an increase of the steady-state phosphorylation level. An analogous concept is proposed by Schuurmans Stekhoven et al. [6], who suggested that cytoplasmic amines prevent water necessary for the hydrolysis to enter the phosphate binding site. These concepts, however, are not as general applicable for the reconstituted Na^+/K^+-ATPase because triallylamine and imidazole fit well in it, but Tris and ethylenediamine exert opposite effects.

Another possibility is that the stimulatory amine substances trigger the phosphorylation reaction of Na^+/K^+-ATPase at the cytoplasmic side in a Na^+-like fashion [6]. The inhibitory amines, however, are also supposed to bind to the Na^+ site, which can be concluded from the competitive behaviour towards Na^+, but are not able to stimulate the phosphorylation reaction as Na^+ does. They inhibit phosphorylation by a direct action via this site or by preventing Na^+ binding to it.

Hypothetical model of the sided action of amine compounds on the phosphorylation reaction

Rejecting the concept in which extracellular amine compounds reduce the dephosphorylation rate one can derive the following model for the action of positively charged amine compounds on the phosphorylation reaction of Na^+/K^+-ATPase. Charged amines potentiate the Na^+/K^+-ATPase from the extracellular side, by induction of a E_1 or 'pre-E_1' conformational state. Na^+ and amines with a suitable geometry bring the enzyme into the genuine E_1 state from the cytoplasmic side. This conformation can be readily phosphorylated by ATP. High concentrations of ATP and low concentrations of Mg^{2+} also stimulate the phosphorylation reaction at the cytoplasmic side. Amines with a different geometry also bind to the cytoplasmic Na^+ binding site but inhibit phosphorylation by preventing Na^+ or Na^+-like cations to enter the Na^+ binding site. Apart from this effect they may inhibit the dephosphorylation rate either by preventing the formation of the hydroxyl ion [14] or by the prevention of water to enter the phosphorylation site [6].

With this model results obtained from experiments with broken membrane sheets, which are often confusing and difficult to interprete are more easily understood, notwithstanding the fact that the different lipid environment in both systems might have influenced the results. The stimulating effect followed by an inhibiting effect of amine compounds [6] can now tentatively be explained by a combination of effects on the two membrane sides: stimulation at the extracellular side and inhibition at the cytoplasmic side. At low amine concentrations the stimulation is predominant, whereas at higher concentrations the inhibition dominates.

Also the observation of Schuurmans Stekhoven et al. [5] that imidazole can stimulate phosphorylation in the absence of Na^+, whereas Tris is not able to exert this effect, fits in this model. Both amines are able to interact with the enzyme from the extracellular side. Imidazole in contrast to Tris can trigger the phosphorylation reaction at the cytoplasmic side.

The idea of the existence of different E_1 conformations as suggested by Schuurmans Stekhoven et al. [5] is confirmed by this study. In the light of the model the E_1-form which is not readily phosphorylating is the extracellularly potentiated form (pre-E_1 conformational state) whereas the phosphorylating form represents the classical E_1 conformation.

That the stimulating effect of amine compounds is a general Na^+-like effect [5] can be ruled out by this study. A Na^+-like effect is supposed to act at the cytoplasmic side, whereas the stimulating effects of the buffers are mainly extracellular. The cytoplasmic stimulatory effects are small compared to the extracellular effects. This paper shows (Figs. 1 and 2) that the presence of a charged amine or Na^+ at the extracellular side is a prerequisite for a maximal phosphorylation reaction of Na^+/K^+-ATPase. Therefore the observation that amine compounds increase the affinity for Na^+ in a trans fashion (at the opposite viz. the extracellular membrane side [6]), can be compared with the potentiation as proposed in the model. This however, cannot be the only effect of the extracellular amine because in presence of saturating Na^+ at the cytoplasmic side, only a very low steady-state phosphorylation level is obtained when insufficient amino compounds or Na^+ are present at the extracellular side. The inhibitory effects of ethylenediamine and Tris are located at the cytoplasmic side, which supports the idea [6] that the inhibition by the amines is exerted via the Na^+-binding site, or a site close to it.

Finally the results of this paper contribute arguments in the dispute whether free protons [14,15,22–27] or buffer substances influence the Na^+/K^+-ATPase with respect to the phosphorylation reaction in favour of the latter possibility and it is clear by now that protons as well as amine substances do exert effects on partial reactions of Na^+/K^+-ATPase.

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