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Principles of selectivity of sodium and potassium binding sites of the Na^+/K^+-ATPase. A corollary hypothesis

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The mechanisms whereby the sodium and potassium binding sites of heart sacrolemmal Na^+/K^+-ATPase (EC 3.6.1.3) distinguished between monovalent cations were investigated using methods of enzyme kinetics. The properties of the sodium binding sites were studied in the presence of 2,4,6-trinitrobenzenesulfonic acid in concentrations completely inhibiting the action of potassium on the enzyme. To test the selectivity of potassium binding sites, K^+-*p*-nitrophenylphosphatase activity was employed as a model. The results suggest that the selectivity of Na^+- and K^+-binding sites of Na^+/K^+-ATPase may be due to two independent mechanisms: (i) The principle of key and lock (formation of coordination bounds); (ii) Optimal difference between solvatation energy (in the specific binding site) and hydration enthalpy of the respective cation.

Introduction

Na^+/K^+-ATPase (EC 3.6.1.3) utilizes the energy released by hydrolysis of one molecule of ATP for outward transport of three sodium ions and inward transport of two potassium ions across the cell membrane [1,2]. Detailed knowledge has accumulated concerning both the structure and the chemical composition of the active site of the enzyme. Nevertheless, data on the structure and molecular properties of the Na^+/K^+-ATPase binding sites for cationic ligands are still rather scarce, though high selectivity of these sites is based on a very strict differentiation between sodium and potassium ions. The selectivity of potassium binding sites can be studied using K^+-*p*-nitrophenylphosphatase as a model. The activity of the latter enzyme is present in all preparation of Na^+/K^+-ATPase [3]. The selectivity of sodium binding sites can be adequately investigated in Na^+-ATPase i.e., the sodium stimulated part [4] of the total Na^+/K^+-ATPase activity present in membrane preparations after blocking the potassium binding sites by 2,4,6-trinitrobenzenesulfonic acid [5].

The aim of the present study was to extend our present understanding of principles of selectivity of sodium and potassium binding sites of the Na^+/K^+-ATPase, and to present a hypothesis concerning the mechanism underlying the process of differentiation between Na^+ and K^+ ions during their interaction with the enzyme molecule.

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Materials and Methods

Rat heart membrane preparation enriched in sacrolemma was prepared by the method of hypotonic shock combined with NaI treatment. The membranes formed loosely closed vesicles with predominantly right-side-out-orientation. The contamination of the sacrolemmal fraction by debris of other intracellular organels did not exceed 3% [6].

For the determination of the Na^+-ATPase activity the membrane fraction was treated with 2,4,6,-trinitrobenzenesulfonic acid (TNBS, 0.1 $\mu mol \cdot mg^{-1}$ membrane protein) for 30 min at room temperature under intermittent shaking. After TNBS treatment the fraction was spunned down at $1500 \times g$, washed twice with 10 $mmol \cdot l^{-1}$ Tris-HCl buffer (pH 7.0), and resuspended in the same buffer.

Na^+/K^+-ATPase activity was determined as the difference in the amount of phosphate liberated during splitting of ATP (2 $mmol \cdot l^{-1}$) in the presence of 100 $mmol \cdot l^{-1}$ NaCl, 10 $mmol \cdot l^{-1}$ KCl and 2 $mmol \cdot l^{-1}$ $MgCl_2$, and in the presence of 2 $mmol \cdot l^{-1}$ $MgCl_2$ only.

Na^+-ATPase activity was determined as the difference in phosphate produced by splitting of ATP (2 $mmol \cdot l^{-1}$) in the presence or absence of 100 $mmol \cdot l^{-1}$ NaCl, respectively.

K^+-*p*-nitrophenylphosphatase (K^+-pNPPase) activity was estimated from the amount of *p*-nitrophenol (pNP) liberated during splitting of *p*-nitrophenyl phosphate (pNPP, 2 $mmol \cdot l^{-1}$) in the presence or absence of 5 $mmol \cdot l^{-1}$ KCl, respectively.

Enzyme reactions were run in 1 ml of incubation medium containing 50 $mmol \cdot l^{-1}$ imidazole-HCl buffer (pH 7.0) and 50–90 μg membrane protein (at 37 °C, usually for 10 min). The reactions were started by adding substrate, and they were stopped by ice-cold trichloroacetic acid (0.73 $mol \cdot l^{-1}$), or by NaOH (0.1 $mol \cdot l^{-1}$) in the case of K^+-pNPPase.

The amount of orthophosphate liberated from ATP was estimated using the method of Taussky and Shorr [7]. The amount of *p*-nitrophenol formed from *p*-nitrophenyl phosphate was established according to De Pont et al. [8]. The protein content in the membrane fraction was determined by the method of Lowry et al. [9].

Parameters of enzyme kinetics were computed by non-linear regression applying the gradient procedure, by the following equation:

$$V = V_{max} \frac{c^n}{c^n + [K_{0.5}^{Me^+}]^n}$$

where V is the initial rate of enzyme reaction at concentration c of monovalent ion; V_{max} is the maximal initial velocity of enzyme reaction at $c \to \infty$; $K_{0.5}^{Me^+}$ is the apparent Michaelis constant for the stimulation of enzyme activity by cationic ligand; n is the cooperativity constant obtained from the Hill equation [10]. The statistical significance of the values obtained for the parameters of enzyme kinetics was characterized by 90% confidence intervals [11].

The chemicals applied were purchased from Sigma (U.S.A.) or LACHEMA (C.S.S.R.), and were of analytical grade.

Results

Modification with 2,4,6-trinitrobenzenesulfonic acid (10 $\mu mol \cdot mg^{-1}$ membrane protein) of essential amino groups in the potassium binding sites of the Na^+/K^+-ATPase in the membrane fraction investigated was followed by complete loss of the enzyme capability to be stimulated by potassium ions [5,12]. The TNBS concentration used has no effect on the sodium stimulated ATP splitting (Fig. 1). The fraction treated in this way was suitable for the investigation of stimulation of ATP hydrolysis by Na^+/K^+-ATPase in the sodium binding site since any interactions of cations with the potassium binding site were excluded. Li^+, Na^+, K^+ and Rb^+ ions were tested (5–100 $mmol \cdot l^{-1}$), and only sodium ions were able to stimulate the ATPase activity in a non-cooperative mode (Fig. 2). Stimulation of the enzyme activity by Li^+, K^+ and Rb^+ ions exhibited typical cooperativity with cooperativity constants of 1.753 ± 0.003, 1.702 ± 0.004 and 2.403 ± 0.003, respectively. The kinetic parameters of stimulation were $K_{0.5}^{Li^+} = 24.40 \pm 0.35$, $K_{0.5}^{Na^+} = 24.80 \pm 0.49$, $K_{0.5}^{K^+} = 33.90 \pm 0.23$ and $K_{0.5}^{Rb^+} = 37.19 \pm 0.65$ $mmol \cdot$

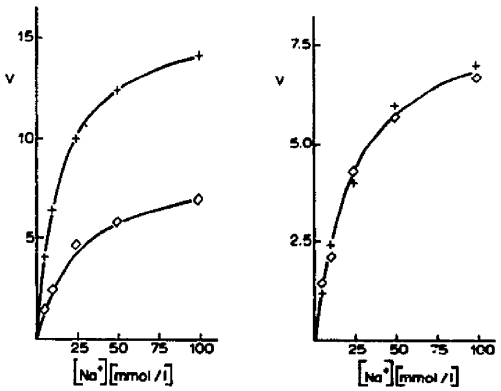

Fig. 1. Stimulation of Na^+/K^+-ATPase or Na^+-ATPase activity by sodium ions. Left panel, control fraction; Right panel, fraction treated with TNBS (0.1 mmol·mg^{-1}). Initial velocities of enzyme reactions (V) in the presence (+, Na^+/K^+-ATPase) or absence (◇, Na^+-ATPase) of 10 mmol·l^{-1} KCl. Kinetic parameters: control fraction, Na^+/K^+-ATPase $V_{max}^{Na^+} = 16.20 \pm 0.21$, $K_{0.5}^{Na^+} = 15.21 \pm 0.13$; Na^+-ATPase $V_{max}^{Na^+} = 8.63 \pm 0.31$, $K_{0.5}^{Na^+} = 24.51 \pm 0.52$; TNBS treated fraction, Na^+/K^+-ATPase $V_{max}^{Na^+} = 8.62 \pm 0.47$, $K_{0.5}^{Na^+} = 25.23 \pm 0.56$; Na^+-ATPase $V_{max}^{Na^+} = 8.58 \pm 0.27$, $K_{0.5}^{Na^+} = 24.80 \pm 0.65$. V and V_{max} are expressed in μmol·h^{-1}·mg^{-1} membrane protein, $K_{0.5}$ is given in mmol·l^{-1}.

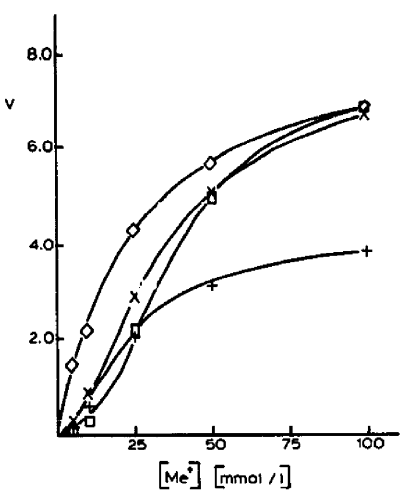

Fig. 2. Stimulation of ATP hydrolysis by monovalent cations interacting with the sodium binding site of Na^+/K^+-ATPase upon blockade of the potassium binding sites by TNBS. Initial velocities (V, μmol P_i·h^{-1}·mg^{-1} membrane protein) were estimated in the presence of 5–100 μmol·l^{-1} Li^+(+), Na^+(◇), K^+(x) or Rb^+(□) ions in 1 ml of reaction medium containing 2 mmol·l^{-1} $MgCl_2$, 50 mmol·l^{-1} imidazole-HCl buffer (pH 7.0) and 50 μg membrane protein.

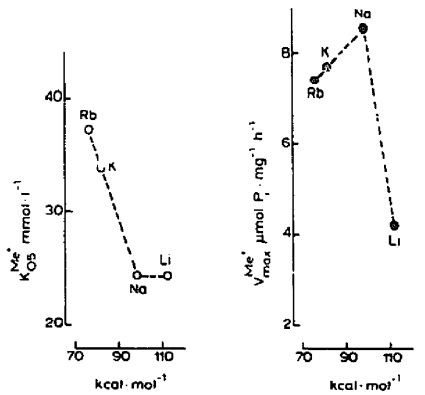

Fig. 3. Relationship between the kinetic parameters of stimulation of Na^+-ATPase by monovalent cations and their respective hydration enthalpies taken from Gažo [20].

l^{-1} and $V_{max}^{Li^+} = 4.17 \pm 0.25$, $V_{max}^{Na^+} = 8.58 \pm 0.27$, $V_{max}^{K^+} = 7.72 \pm 0.35$ and $V_{max}^{Rb^+} = 7.44 \pm 0.42$ μmol P_i·h^{-1}·mg^{-1}; these values were plotted against hydration enthalpies of the respective cations (Fig. 3). For Rb^+, K^+ and Na^+ (Fig. 3) a linear decrease of $K_{0.5}^{Me^+}$ values as well as a linear increase of $V_{max}^{Me^+}$ values could be observed with respect to their hydration enthalpies. For Li^+ a characteristic break was observed in both relations.

The properties of K^+-binding sites of the Na^+/K^+-ATPase were investigated using K^+-pNPPase as a model. The activity of the latter enzyme in our membrane preparation was stimu-

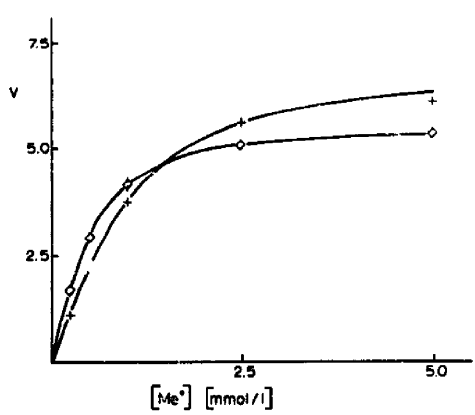

Fig. 4. Stimulation of pNPPase activity by potassium and rubidium ions. +, potassium; ◇, rubidium. Initial velocities (V) are expressed in μmol P_i·h^{-1}·mg^{-1} membrane protein.

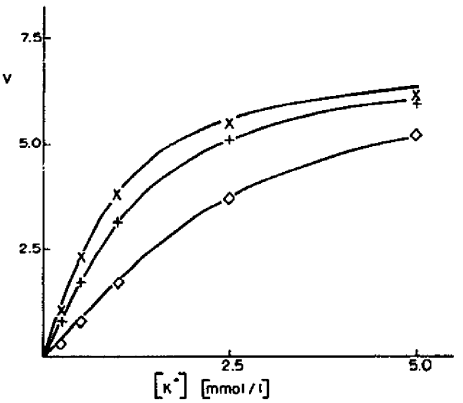

Fig. 5. Inhibition of K^+-pNPPase activity by sodium and lithium ions. x, control; +, inhibition by Li^+ (5 mmol·l^{-1}); ◇, inhibition by Na^+ (5 mmol·l^{-1}).

lated by both K^+ and Rb^+ ions in a cooperative mode (Fig. 4). The respective kinetic constants were: n 1.358 ± 0.008 and 1.423 ± 0.007, $K_{0.5}^{Me^+}$ 0.85 ± 0.05 and 0.44 ± 0.03 mmol·l^{-1}, and $V_{max}^{Me^+}$ 6.88 ± 0.07 and 5.35 ± 0.06 μmol $P_i \cdot h^{-1} \cdot mg^{-1}$ for potassium and rubidium, respectively. Sodium and lithium did not stimulate the activity of K^+-pNPPase (not shown); on the contrary, these ions inhibited stimulation of the enzyme activity by potassium (Fig. 5). This effect was characterized by an increase in $K_{0.5}^+$ value from 0.85 ± 0.05 to

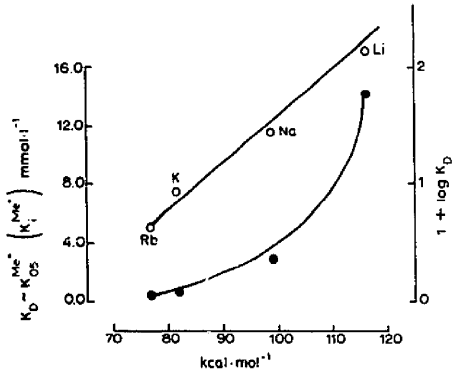

Fig. 6. Relationship between the apparent affinities of K^+-pNPPase for monovalent cations and their hydration enthalpies. Values of $K_{0.5}$ for enzyme stimulation by K^+ and Rb^+ as well as values of K_i for its inhibition by Na^+ and Li^+ ions were taken for apparent affinities. Hydration enthalpies of the cations were taken from a textbook by Gažo [20].

1.15 ± 0.04 mmol·l^{-1} for lithium (5 mmol·l^{-1}), and to 2.23 ± 0.05 mmol·l^{-1} for sodium (5 mmol·l^{-1}). The $V_{max}^{K^+}$ value for stimulation of pNPPase activity by potassium ions as well as the respective constant of cooperativity remained unchanged. This clearly points to a competitive mode of inhibition. Inhibition constants $K_i^{Me^+}$ for Li^+ and Na^+ ions of 14.29 ± 0.42 and 3.08 ± 0.10 mmol·l^{-1} were calculated from changes of $K_{0.5}^{Me^+}$ values. The inhibition constants $K_i^{Li^+}$ and $K_i^{Na^+}$ as well as the values of $K_{0.5}^{K^+}$ and $K_{0.5}^{Rb^+}$ for stimulation of pNPPase activity by the latter two cations were plotted against hydration enthalpies of the above cations (Fig. 6). Fig. 6 shows that with increasing hydration enthalpies of monovalent cations their $K_{0.5}^{Me^+}$ or $K_i^{Me^+}$ values also increase. In semilogarithmic plot the latter relationship is a straight line with a correlation coefficient of 0.992 ($P < 0.01$).

Discussion

The existence of highly specific sites with particularly appropriate physico-chemical properties for binding of sodium and/or potassium is a prerequisite for the binding site to be able to sufficiently distinguish between these two cations during their interaction with the Na^+/K^+-ATPase molecule. Two types of interaction may be considered in this respect: (i) ionic interactions with anionic functional groups of membrane proteins and phospholipids; (ii) coordination interactions with nucleophilic groups of lipoprotein complex in the membranes.

Because of small differences in the net charges of diverse alcaline ions [13], ionic interactions may hardly meet the criteria required for the selectivity of their interaction. On the other hand, if during the interaction alcaline ions would act as acceptors of free electron pairs of functional groups of the binding sites, an appropriate geometric arrangement of the latter corresponding to the structure of hybrid orbitals of the respective cation, might provide an adequate selection based on principles of a key and lock [14]. Accordingly, the geometric structure of a selective potassium binding site would correspond to the configuration of hybrid orbitals of potassium with coordination number 8, at which sodium ions do not occur [15]. This notion is supported by the fact that Rb^+

ions, which form bonds at coordination number 8, may replace K^+ ions in stimulating the K^+-pNPPase activity (Fig. 4), but Li^+ and Na^+ ions, forming bonds with respective coordination numbers 4 and 6 only [15], fail to exert such a stimulatory effect. On the contrary the latter ions act as competitive inhibitors of stimulation by potassium of the pNPPase activity (Fig. 5). This points to the fact that Li^+ and Na^+ ions may somehow interact with the potassium binding site. Hence, in the given case Li^+ and Na^+ ions may act as similar but unproper keys which do not evoke the anticipated natural response.

Nevertheless, an appropriate geometric structural arrangement of the binding site itself may not always grant a sufficient selectivity. As an example the binding site for sodium can be considered: potassium and other ions may also occur with coordination numbers 4 and 6 (typical for sodium ions). This is ilustrated in Fig. 2: although differently from Na^+ ions, Li^+, K^+ or Rb^+ ions all stimulate the hydrolysis of ATP by Na^+/K^+-ATPase in its sodium binding site. The differences in stimulatory effects observed with the latter three cations may be explained by the hypothesis of Nagata and Aida [13] implicating the interaction energy of ions with the binding site as an essential criterion for their selection by the sodium channel. Applying the hypothesis of Nagata and Aida to the sodium pump, the energy released during the interaction of a monovalent cation with the respective cation binding site of the Na^+/K^+-ATPase has to cover the demands of two independent processes: (i) the interconversion of Na^+/K^+-ATPase molecule between E_1 and E_2 states – a process shown to be independent of energy derived from ATP splitting [16,17]; (ii) dehydration of the respective monovalent ion – a process always preceding the binding and/or transport [14,18]. In terms of this hypothesis K^+ and Rb^+ ions will interact with the sodium binding site of Na^+/K^+-ATPase with lower affinities than do Na^+ ions (the apparent Michaelis constants increase in the order Na^+, K^+, Rb^+, see Fig. 3), since the interaction energies of K^+ and Rb^+ with the sodium binding site are lower than that of sodium [13]. On the contrary, in this respect Li^+ ions with an interaction energy exceeding that of sodium are not limited in their binding to the sodium binding site. Nevertheless, the resulting more stable Li^+-enzyme complex is also more slowly hydrolyzed after termination of the reaction cycle of the Na^+-ATPase. This results in lower V_{max} values of ATPase activation by lithium in comparison to that for sodium ions (Fig. 3). Accordingly, the magnitude of the energy profit originating from the difference between energy liberated by cation interaction with the Na^+-binding site and that utilized for its dehydration also plays a determining role in selectivity of the sodium binding site of Na^+/K^+-ATPase. From the data reported by Nagata and Aida [13] it also appears that in parallel with the enhancement of hydration enthalpies of monovalent ions ($Rb^+ < K^+ < Na^+ < Li^+$) the difference between the interaction energies of the above cations with organic ligands and their hydration enthalpies also increase. Therefore the affinities of these ions to the sodium binding site of Na^+/K^+-ATPase, characterized by $K_{0.5}$ follow the same sequence as their hydration enthalpies (Fig. 3). The affinities of alkaline ions to the K^+-binding site of K^+-pNPPase (the selectivity of which is essentially granted by its geometric arrangment) follow the opposite sequence (Fig. 6). This means that the affinity of binding of a cation into K^+-binding site is the lower the more energy is required for its dehydration. Assuming that the K^+-binding site of Na^+/K^+-ATPase will operate on selectivity principles similar to those of K^+-pNPPase [19], the discussed opposite sequence of binding affinities may represent the active principle for a proper selection of cation to be bound to Na^+/K^+-ATPase during the respective steps of its reaction cascade.

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